

New Strategies for Mutant Rescue of the Tumor Suppressor p53 through Covalent Warheads

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List of Abbreviations

2xYT	2x yeast extract tryptone broth
ACN	acetonitrile
APL	acute promyelocytic leukemia
APS	ammonium persulfate
AUC	area under the curve
ATM	ataxia telangiectasia mutated
ATO	arsenic trioxide
ATR	ataxia telangiectasia and Rad3-related
BAX	BCL2-associated X, apoptosis regulator BAX
bp	base pairs
BSA	bovine serum albumin
CBP	CREB-binding protein
CDK	cyclin-dependent kinase
CL	cysteine light, C124/182/229/275/277S
CovLib	covalent fragment library
CRG	covalent reactive group
CTD	C-terminal regulatory domain
CV	column volume
DBD	DNA-binding domain
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DSF	differential scanning fluorimetry
DTT	dithiothreitol
ϵ	molar extinction coefficient

<i>E.coli</i>	<i>Escherichia coli</i>
EDG	electron-donating group
EDTA	ethylenediaminetetraacetic acid
ESI	electrospray ionization
ESP	electrostatic potential
EWG	electron-withdrawing group
FAM	carboxyfluorescein
FBDD	fragment-based drug discovery
FDA	U.S. Food and Drug Administration
FL	full-length
FP	fluorescence polarization
GADD45 α	growth arrest and DNA-damage inducible 45 alpha
GSH	glutathione
ΔG_u	Gibbs free energy of unfolding
HEFLib	Halogen-Enriched Fragment Library
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLT	6x His-tag lipoyl domain TEV protease cleavage site
HPLC	high-performance liquid chromatography
HSAB	hard and soft acids and bases
HSQC	heteronuclear single quantum coherence
HTS	high-throughput screening
IPTG	isopropyl 1-thio- β -D-galactopyranoside
ITC	isothermal titration calorimetry
JNK3	c-Jun N-terminal kinase 3
K _d	dissociation constant
KLD	kinase-ligase-Dpnl
KPi	buffer made up of KH ₂ PO ₄ /K ₂ HPO ₄ in water

LB	lysogeny broth
LG	leaving group
logP	logarithm of the partition coefficient
MAPK	mitogen-activated kinase
MDM2	murine double minute 2 homolog; also known as HDM2
MDM4	murine double minute 4 homolog; also known as MDMX, HDM4, or HDMX
MOPS	3-(<i>N</i> -morpholino)propanesulfonic acid
MQ	methylene quinuclidinone
MS	mass spectrometry
MWCO	molecular weight cut-off
N/A	not available
N/D	not determined
NMR	nuclear magnetic resonance
NOXA	superoxide-generating NADPH oxidase
PAI1	plasminogen activator inhibitor 1
PAINS	Pan-Assay Interference Compounds
PAT	antimony tartrate
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDB	Protein Data Bank
PEG	polyethylene glycol
PROTAC	proteolysis-targeting chimera
PRR	proline-rich region
PUMA	Bcl2-binding component 3
RNase	ribonuclease
ROS	reactive oxygen species

rpm	revolutions per minutes
PVDF	polyvinylidene difluoride
SAR	structure-activity relationship
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEC	size exclusion chromatography
SMARTS	SMILES Arbitrary Target Specification
SMILES	Simplified Molecular Input Line Entry Specification
S _N Ar	nucleophilic aromatic substitution
SOC	super optimal broth with catabolite repression
SSG	sodium stibogluconate
STD	saturation transfer difference
TAD	N-terminal transactivation domain
TAE	TRIS-acetate-EDTA
TCI	targeted covalent inhibitor
TEMED	<i>N,N,N',N'</i> -tetramethyl ethylenediamine
TET	tetramerization domain
TEV	tobacco etch virus
T _m	melting temperature
ΔT _m	T _m shift
TOF	time-of-flight
TIRS	2-amino-2-hydroxymethylpropane-1,3-diol
t _{1/2}	half-life
UHPLC	ultra-high-performance liquid chromatography
V _{max}	most positive electrostatic potential on the σ-hole when mapping the electrostatic potential onto the isodensity surface
wt	wild-type

1 General Introduction

1.1 The Tumor Suppressor p53

1.1.1 The p53 Signaling Pathway

p53 is a tumor suppressor protein with a molecular weight of 43.653 kDa and a length of 393 amino acids. It was first discovered in 1973 [1]. The cellular tumor antigen p53, also known as the “guardian of the genome,” plays a pivotal role in the cell cycle [2-5]. p53 is activated in response to DNA damage, oncogenic or other cellular stress signals via various activation pathways to protect from cancer development [5-8]. The activation of p53 regulates the transcription of numerous genes involved in cell cycle arrest, apoptosis, DNA repair, and cellular senescence [6, 7, 9]. In addition to the aforementioned canonical functions, recent studies have demonstrated the intricate and interconnected nature of the p53 pathway [1]. It has been discovered that p53 is also involved in various processes, including autophagy, metabolic homeostasis, protection against oxidative stress, antiangiogenesis, immune microenvironment, ferroptosis, and regulation of stemness of cells [4, 10-12].

In unstressed cells, p53 protein levels are kept low [13]. Therefore, in a tightly controlled feedback loop, p53 induces the expression of its key negative regulators murine double minute 2 homolog (MDM2; also known as HDM2) and murine double minute 4 homolog (MDM4; also known as MDMX, HDM4, or HDMX) [5, 14]. The MDM2 protein represents an E3 ubiquitin ligase, leading to proteasomal degradation of p53 [12, 15]. In contrast, the homologous MDM4 protein has an inactive C-terminal ubiquitin ligase domain. However, MDM4 can heterodimerize with MDM2, stimulating the ubiquitin ligase activity of MDM2 [16-19]. Furthermore, MDM2 and MDM4 bind to the N-terminal transactivation domain of p53, thereby inhibiting its interaction with transcription factors [19-21]. MDM2 and MDM4 compete with p53 coactivators, such as p300, for binding to the N-terminal transactivation domain [5].

Under stress conditions, the interaction between p53 and its key negative regulators MDM2 and MDM4 is disrupted, resulting in increased levels of the p53 protein [1, 2, 6]. Subsequently, p53 is activated and stabilized through various posttranslational modifications, including phosphorylations and acetylations [1, 12, 22-24].

The most extensively studied pathway of p53 activation is that induced by DNA damage [25, 26]. This process involves several protein kinases, including DNA-dependent protein kinase, ataxia telangiectasia mutated (ATM), and Chk2 kinases [2, 27, 28]. These protein kinases phosphorylate p53 at its N-terminal transactivation domain, which interrupts the interaction between p53 and its negative regulators MDM2 and MDM4 [29, 30]. A second activation pathway of p53 is initiated by the increased expression of oncogenes [7, 31]. This pathway is independent of the p53 activation through DNA damage response [2, 32]. Oncogenes, such as Myc or Ras, lead to the transcription of the p14^{ARF} gene or stabilize the p14^{ARF} protein [2, 33]. p14^{ARF} binds to MDM2 and thus also inhibits the p53-MDM2 interaction [34-36]. Furthermore, UV light and chemotherapeutic drugs activate p53 through a pathway involving the kinases ataxia telangiectasia and Rad3-related (ATR) and casein kinase II [2, 24]. In addition to these three activation pathways, several other partially understood pathways activate p53 [5].

The activated and stabilized p53 protein forms tetramers and then binds to specific DNA sequences to regulate the transcription of numerous target genes [2, 12, 37]. Some of these target genes promote cell cycle arrest, such as p21, which inhibits cyclin-dependent kinases (CDKs) and induces G1 arrest [7, 38]. Additionally, p53 induces the expression of 14-3-3 σ [39, 40], growth arrest and DNA damage-inducible 45 (GADD45) [41], and Reprimo [42], which are involved in a G2 block. Furthermore, p53 activates multiple signaling pathways that regulate cellular senescence [43]. p53 target genes, such as plasminogen activator inhibitor 1 (PAI1) [44] and p21 [45], can initiate permanent cell cycle arrest, resulting in senescence [1]. In addition, p53 downstream genes are also involved in genetic stability and protecting the genome by coordinating several DNA repair mechanisms [2, 46]. In response to DNA damage, p53 induces apoptosis [2, 6, 7, 47-49]. Therefore, p53 regulates the transcription of multiple downstream targets, such as BCL2-associated X, apoptosis regulator BAX (BAX) [50, 51], Bcl2-binding component 3 (PUMA) [52, 53], and superoxide-generating NADPH oxidase (NOXA) [54], which mediate programmed cell death. Moreover, p53 plays a role in the expression of certain genes that help to restrict the growth of tumors by blocking the formation of new blood vessels, known as angiogenesis [2, 7].

Despite these classical functions, p53 affects the transcription of many other genes involved in various complex downstream signaling pathways and biological processes [1, 12]. In addition, p53 also represses the expression of various genes [2, 7].

Furthermore, p53 also has non-transcriptional functions, including the regulation of mitochondrial apoptosis [1, 6, 49]. Figure 1.1 provides an overview of the complex and highly interconnected p53 network.

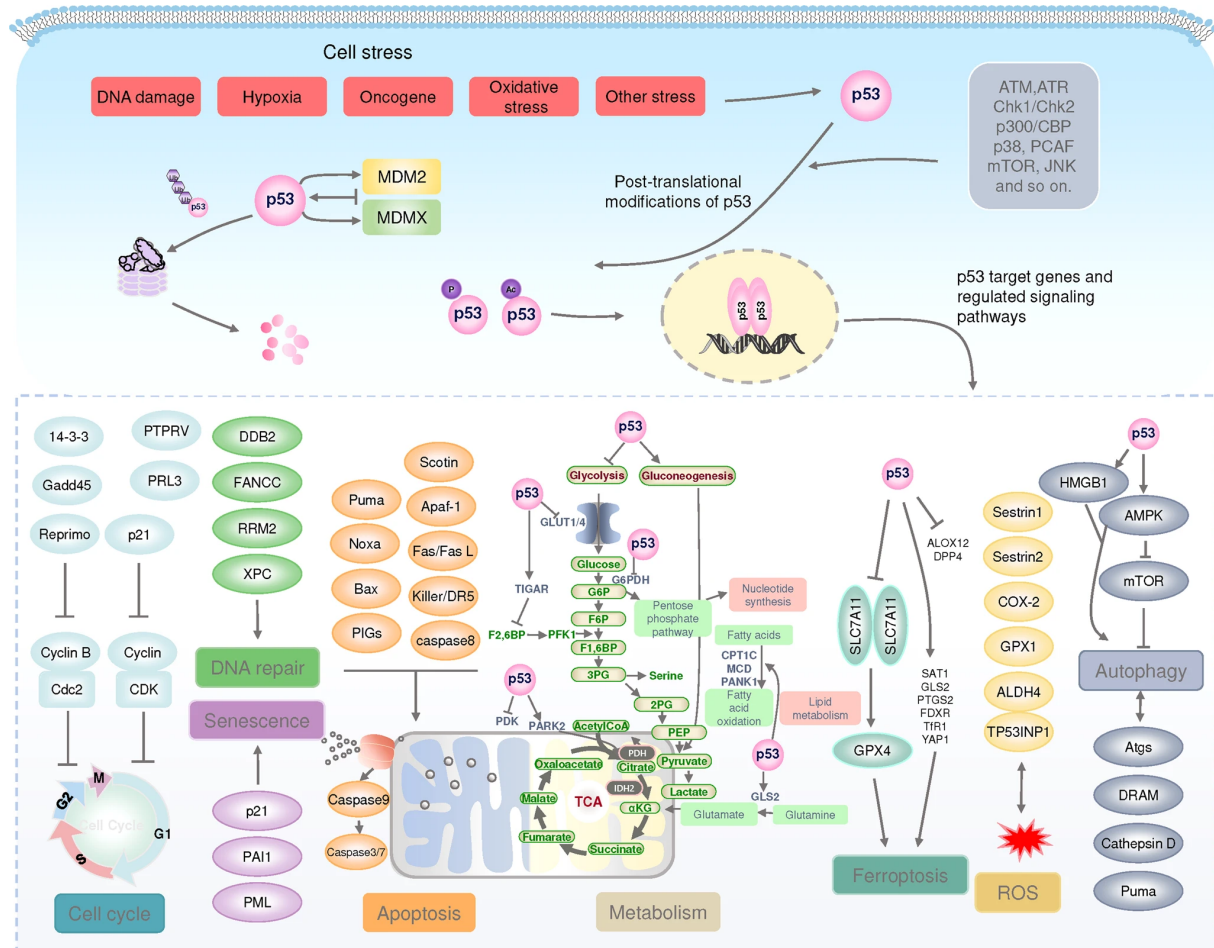


Figure 1.1: Overview of the p53 signaling pathway. Under unstressed conditions the p53 levels are kept low by its two negative regulators MDM2 and MDM4. In response to several stress signals, p53 is activated and stabilized. Activation of p53 leads to the transcription of genes involved in cell cycle arrest, apoptosis, DNA repair, senescence, and a multitude of other biological processes. The figure was taken from Wang et al. [1] under the CC BY 4.0 license (Chapter 8.16).

1.1.2 The Domain Structure of p53

The complex and diverse functions of p53 are reflected in its domain structure [1, 5, 12]. p53 forms a homotetramer consisting of four monomers, each containing 393 amino acids [12]. Each p53 monomer has a modular structure that includes an N-terminal transactivation domain (TAD), a proline-rich region (PRR), a DNA-binding domain (DBD), linked by a flexible linker to a tetramerization domain (TET), and a C-terminal regulatory domain (CTD) [5, 8, 12, 55]. Approximately 40% of the full-length (FL) protein consists of large regions of intrinsic disorder, in addition to the folded

DNA-binding and tetramerization domains [1, 5, 56]. This modular structure is common in signaling proteins, allowing conformational adaptability for interactions with a range of binding partners and modulation of binding affinities through posttranslational modification sites [12, 57, 58]. Figure 1.2 illustrates the domain structure of FL p53.



Figure 1.2: Domain structure of p53 with the N-terminal transactivation domain (TAD), the proline-region (PRR), the DNA-binding domain (DBD), which is linked by a flexible linker to the tetramerization domain (TET), and the C-terminal regulatory domain (CTD).

The N-terminal region of p53 is intrinsically unfolded and involves the amino acid residues 1-93 [5]. It consists of an acidic TAD and the PRR [59]. The TAD is divided into two subdomains, TAD1 (residues 1-40) and TAD2 (residues 40-61) [5, 60, 61]. The TAD serves as a binding site for components of the transcription machinery [62, 63] and various coactivators, such as p300 [64, 65] and CREB-binding protein (CBP) [66, 67], and also allows interaction with the negative regulators MDM2 [15] and MDM4 [1, 5, 68]. The TAD is intrinsically disordered, but it has some conserved hydrophobic residues that adopt an α -helical conformation upon binding to partner proteins [15, 69-71]. Moreover, phosphorylation of the TAD is involved in the p53 activation process by shifting the affinity for the different proteins that compete for p53 binding [1, 5]. In general, p53 is an effective transcription factor due to the flexible structure of the TAD combined with posttranslational modifications [1, 58]. The PRR (residues 62-93) acts as a linker between the TAD and the DBD [5, 59]. It contains five PXXP motifs that can bind to the Src homology 3 domains and play a role in signal transduction [72-74]. Some parts of the PRR may adopt a polyproline-helix-like structure [5]. However, the exact role and function of this region are still poorly characterized [1, 5].

The DBD or core domain of p53 (p53C) (residues 94-292) consists of an immunoglobulin-like β -sandwich formed by two anti-parallel β -sheets, representing the basis for the extended DNA-binding surface [5, 8, 12]. The DNA-binding surface is formed by a loop-sheet-helix motif and two large loops (L2 and L3). The loop-sheet-helix motif includes loop L1, β -strands S2 and S2', parts of β -strand S10, and the C-terminal helix H2 [5, 12]. This motif binds to the DNA major groove, while the two large loops L2 and L3 bind to the DNA minor groove [5, 8, 75]. The loops L2 and L3 are stabilized by a zinc ion, which is tetrahedrally coordinated by Cys176, His179,

Cys238, and Cys242 [5, 8, 12, 76]. The loss of the zinc ion significantly reduces the thermodynamic stability, increases aggregation tendency, and leads to a loss of DNA-binding specificity [5, 77-79]. The human p53 core domain has low overall stability and a melting temperature (T_m) only slightly above the body temperature (42-44 °C), determining the stability of full-length p53 [5, 77, 80, 81]. Furthermore, p53 is also kinetically unstable. It quickly unfolds at body temperature within a half-life ($t_{1/2}$) of 9 min [82]. The DBD is involved in binding to specific DNA-response elements and mediates the interaction with various proteins [1, 8]. p53 binds to its response elements as a dimer of primary dimers in a highly cooperative manner with a 4:1 stoichiometry [5, 83]. There are several key residues involved in DNA-binding. Arg248 in the loop L3 binds to the minor groove of the DNA and contacts the DNA backbone directly or through water-mediated contacts [5, 8]. The residues Ser241 and Arg273 directly bind the phosphate backbone [5]. Moreover, Arg273 forms a salt bridge with Asp281, which makes a water-mediated contact with the DNA. In the major groove of the DNA, Arg280 contacts a conserved guanine base. Furthermore, Lys120, Ala276, and Cys277 create sequence-specific contacts in the major groove [5, 8, 84].

The TET (residues 325-355) is attached to the DBD by a flexible linker region [5, 8, 85]. The TET controls the oligomerization state of p53 [8, 73]. The monomeric TET consists of a short β -strand that is connected to an α -helix by a sharp turn [5]. The primary p53 dimers are stabilized via an antiparallel intermolecular β -sheet and antiparallel helix-packing interactions [5, 8]. The primary dimers are loosely assembled and allow the interaction of the core domain with regulatory proteins [5]. Two dimers assemble to a tetramer almost perpendicularly via hydrophobic contacts in their helix interfaces [8]. The TET contains a leucine-rich nuclear export signal that is masked in tetrameric p53 and requires dissociation of the tetramers for exposure [5, 8].

The CTD (residues 356-393) follows the TET and is intrinsically disordered [8]. The CTD has multiple regulatory functions, including activation, stabilization, degradation, and cellular localization of p53 [1, 12]. Furthermore, the CTD binds DNA non-specifically, enabling p53 to slide along the DNA to search for its response elements [86-89]. The CTD is also subject to various posttranslational modifications, such as acetylation, phosphorylation, and methylation [1, 12, 90]. Additionally, the CTD interacts with a myriad of regulatory proteins [5, 12]. The CTD switches from disorder to order when binding to partner proteins and adopts a different conformation depending on the binding partner or posttranslational modification pattern [1, 8].

1.1.3 p53 Cancer Mutations

Nearly 50% of all human cancer types contain a p53 mutation [12]. In a subset of tumors that retain the wild-type (wt) protein, p53 activity is reduced due to interference with associated pathways, such as the upregulation of the negative regulators MDM2 and MDM4 [91]. The majority (95%) of somatic p53 mutations are located in the DBD, and most of these oncogenic mutations are missense mutations (75%) [6, 69, 73, 77, 92]. In addition to the loss of function, many of these mutants have a dominant-negative effect on wt p53 by forming mixed tetramers [5, 73, 93]. Moreover, some of the p53 mutants acquire other transcriptional regulatory functions, resulting in a gain-of-function phenotype [4-6]. The missense core domain mutants can be categorized as DNA contact mutants, which eliminate a crucial DNA contact, and structural mutants, which induce structural alterations destabilizing the protein [55, 69, 73].

Table 1.1 and Figure 1.3 show the relative distribution of p53 missense point mutations based on the R20 version of the IARC/NCI *TP53* Database (somatic count after filtering missense point mutations N= 21,399) [94]. The six most frequently mutated hot spot residues in p53 are Arg248, Arg273, Arg175, Gly245, Arg282, and Arg249 [69, 73, 77]. These residues are located in or near the DNA-binding surface and often contain CpG dinucleotides [73, 77].

Table 1.1: Relative mutation frequency of the p53 missense mutations in human cancers.

Residue	Relative mutation frequency [%]	Structural region/ Cancer mutant class
R248	8.7	DNA contact
R273	8.5	DNA contact
R175	6.2	Zinc region, structural
G245	3.9	DNA-binding region, structural
R282	3.3	DNA-binding region, structural
R249	3.2	DNA-binding region, structural
Y220	2.2	β -sandwich, structural
H179	2.0	Zinc region, structural
C176	1.8	Zinc region, structural

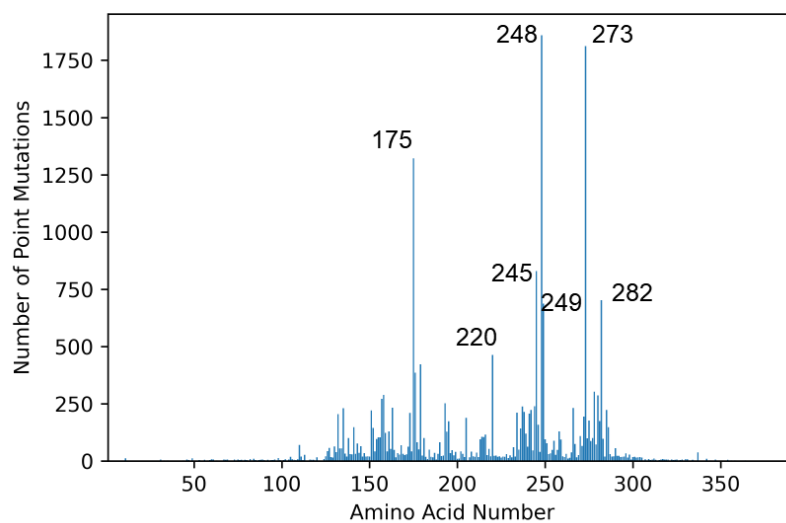


Figure 1.3: Relative frequency of p53 missense mutation in human cancers. The amino acid numbers of the most frequent p53 mutations are highlighted.

The hot spot mutants R248Q/W and R273C/H are classical DNA contact mutants. Arg248 and Arg273 are residues that interact with the phosphate backbone of the DNA. Thus, their mutation leads to the loss of an essential DNA contact [69, 73].

The low overall stability of the DBD makes p53 susceptible to the introduction of destabilizing mutations [73]. All structural mutants thermodynamically destabilize the protein. However, they have a different impact on the DNA-binding affinity in their folded states [69, 73].

The L3 loop, anchored to the minor groove of the DNA via Arg248, contains two structural cancer hotspot mutations: G245S and R249S. The G245S mutation results in small structural alterations in the L3 loop, affecting residues in the core-core domain interface. This structural mutation causes a modest destabilization and decrease in DNA-binding affinity [69, 73, 77]. The R249S cancer mutation is associated with exposure to aflatoxin B1 [95, 96]. Arg249 plays a key role in maintaining the hairpin conformation of the L3 loop. It is involved in a hydrogen bond network with Gly245 and Met246 and in a salt bridge with Glu171, thereby positioning the DNA contact residue Arg248. Due to the mutation, these stabilizing interactions are lost, inducing high flexibility and favoring alternative conformations of the L3 loop. The large structural changes reduce the stability of this mutant and severely affect its DNA-binding [8, 55, 73].

The most common structural mutant is the hot spot mutant R175H [77, 94]. Arg175 is located in the L2 loop and is adjacent to the zinc-binding site. The introduction of a bulky residue may directly interfere with the zinc coordination. The mutation has the same effect as zinc ion removal from wt p53, such as strongly reduced stability, increased aggregation tendency, and loss of DNA-binding specificity. The mutation can be matched with the mutation of residues that are directly involved in zinc binding, such as C176F, H179R, and C242F [73, 77].

The R282W mutation is a structural mutation located in the periphery of the DNA-binding surface. This mutation disrupts the loop-sheet-helix motif, which largely destabilizes the core domain, resulting in a temperature-sensitive phenotype [73, 77].

In addition to the hot spot mutants, there are structural mutations in the central β -sandwich of the core domain that are distant from the DNA-binding region. These mutants are highly destabilizing but maintain the DNA-binding affinity at lower temperatures. The β -sandwich mutations V143A and F270L involve substitutions from large to small hydrophobic buried residues. The mutations create an internal hydrophobic cavity in the core of the β -sandwich without disturbing the surrounding structure [55, 73].

The β -sandwich mutant Y220C is the ninth most common p53 mutant with estimated 130,000 annual cancer cases and the most common mutant outside of the DNA-binding region [73, 97-99]. In the wt protein, the Tyr220 is located at the start of the loop that connects β -strand S7 and S8 at the far end of the β -sandwich. The benzene moiety of Tyr220 is part of the hydrophobic core, whereas the hydroxyl group points towards the solvent [69, 73]. The mutation from the tyrosine to the cysteine creates a solvent-accessible hydrophobic crevice [69, 73, 100]. The hydrophobic Y220C pocket is filled with water molecules at specific positions, forming a hydrogen bond network. The mutation connects two small clefts (subsite 1 and subsite 2, Figure 1.5 C, D) that are already present in the wt p53 [55, 69, 73]. The Y220C mutant is strongly thermodynamically destabilized, and the T_m is reduced by about 8 °C, leading to rapid denaturation and aggregation at body temperature [73, 101]. However, the overall structure of the core domain remains intact [69]. Furthermore, the mutation-induced Y220C cleft is far from the DNA-binding site and the dimerization interface and is not involved in essential protein contacts. Therefore, the crevice represents a possible binding pocket for stabilizing small molecules [69, 73, 100].

The TET usually harbors a low frequency of somatic cancer mutations [97]. However, approximately 20% of p53 germline mutations found in Li-Fraumeni and related syndromes are associated with a mutation of Arg337 in the TET [5, 8, 97, 102]. The mutation prevents an intermonomer salt bridge at the primary dimerization interface [5, 103].

1.1.4 p53 Rescue Strategies and Drug Discovery

In most human cancers, the tumor suppressor p53 is inactivated either through mutation or through disruption of its associated signaling pathways [12, 104]. Therefore, p53 is an attractive target for cancer therapy. There is a great interest in developing compounds that reactivate p53 mutants and restore p53 function [6, 12, 97, 105]. Nevertheless, the p53 protein is a challenging target for drug discovery and has long been considered undruggable [13, 104]. However, in recent years, several fruitful approaches have been developed to recover the p53 function. Nonetheless, none of these has yet received U.S. Food and Drug Administration (FDA) approval [1, 12, 104].

In cancer cells that retain the wt p53, targeting the p53-MDM2/MDM4 interaction is a promising strategy for developing anticancer drugs [12, 106]. Therefore, small molecules were developed that imitate the TAD of p53 and bind to the hydrophobic pockets of MDM2 and MDM4 [5, 14]. The first specific MDM2 inhibitors, discovered in 2004, were *cis*-imidazoline analogs called Nutlins [107]. Subsequently, drug discovery efforts have focused on targeting the interaction between MDM2 and p53, leading to numerous compounds currently under investigation in clinical trials [104, 107, 108]. Furthermore, dual MDM2/MDM4 inhibitors and peptide antagonists are under development [106, 107, 109]. A dual inhibitor in clinical trials is the stapled peptide ALRN-6924, which imitates the α -helical structure of the TAD and binds to MDM2 and MDM4 [107, 110]. In addition, an alternative approach for rescuing wt p53 involves MDM2-based proteolysis-targeting chimeras (PROTACs) [1, 104].

Approximately one-third of all p53 cancer mutants are structural mutants that are temperature-sensitive and thermodynamically destabilized, leading to rapid denaturation and aggregation at body temperature [12, 77, 111, 112]. Nonetheless, many of these mutants exhibit transcriptional activity and adopt an wt-like conformation at low temperatures [93, 113]. Therefore, there is a potential to recover the function of these temperature-sensitive mutants through stabilizing molecules [98, 100, 101, 114].

Stabilizing small molecules can act in a generic manner (generic drugs) or selectively for specific p53 mutations (mutant-specific drugs) [12, 104].

One rescue strategy comprises the reactivation of mutant p53 by covalent reactive, cysteine modifying molecules. PRIMA-1 and its clinically more advanced methylated analog APR-246 (PRIMA-1^{Met}, eprenetapopt) induce apoptosis and upregulation of p53 target genes in cancer cell lines of structural or DNA contact p53 mutants [115, 116]. APR-246 was the first mutant p53-reactivating small molecule to enter clinical trials [104]. PRIMA-1 and APR-246 represent prodrugs with methylene quinuclidinone (MQ) as the bioactive compound. MQ, containing an α,β -unsaturated ketone, reacts in an 1,4-Michael addition with thiol groups of cysteines at the surface of the p53 DBD [117-119]. However, the observed cell effects of PRIMA-1 and APR-246 are multifactorial, as the reaction with glutathione, the inhibition of glutaredoxin and thioredoxin systems, and the associated increase in reactive oxygen species (ROS) levels also appear to be responsible for the p53 independent antitumor effect [120-122].

Arsenic trioxide (ATO) is an approved drug for the treatment of acute promyelocytic leukemia (APL) [123, 124]. ATO is a thiol-reactive compound that has been detected as a rescuing agent for destabilized structural p53 mutants [1, 104, 125]. X-ray crystallography has revealed that ATO covalently binds to a cryptic pocket defined by the residues Cys124, Cys135, Cys141, and Met133. Thus, ATO increases the thermostability of structural p53 mutants, promotes protein folding, and restores transcriptional activity [126].

Similar cellular effects were observed with the two cysteine alkylating compounds, STIMA-1 and MIRA-1, which have Michael acceptor properties [127-129]. In addition, other thiol-reactive agents, such as 3-benzoylacrylic acid [130], the 2-sulfonylpyrimidine PK11007 [131], and the curcumin analog HO-3867 [132], have been identified to covalently modify cysteine residues in the p53 DBD.

The discovery of these cysteine-reactive agents demonstrates that covalent modifications of cysteines in p53 can achieve general stabilization of the protein and restore mutant p53 conformation, providing a rescue strategy through simultaneous reactivation of destabilized p53 mutants [104, 131].

Another agent that restores mutant p53 conformation is sodium stibogluconate (SSG), a derivative of the antiparasitic agent potassium antimony tartrate (PAT). Both compounds non-covalently rescue temperature-sensitive p53 mutants by releasing antimony ions that bind to the same binding pocket as ATO [1, 104, 133].

In addition, the hot spot mutant R175H, which is deficient in zinc-binding, can be reactivated by the thiosemicarbazone ZMC1 (NSC319726) [134]. ZMC1, a zinc metallochaperone, acts as an ionophore that transfers zinc ions across the cell membrane, thereby increasing the cellular Zn^{2+} concentration. Modulating the cellular zinc levels helps to coordinate the Zn^{2+} at the corresponding binding site and rescues the structure and function of the R175H mutant [1, 12, 135, 136]. Furthermore, ZMC1 displays a zinc-independent mechanism, which presumably involves the chelation of redox-active copper and iron, resulting in increased levels of ROS [12, 136].

The β -sandwich mutant Y220C is strongly temperature-sensitive and represents a prime target for the design of stabilizing drugs [12, 112]. So far, several small molecules have been discovered addressing the Y220C cavity, thereby increasing the T_m and stabilizing the mutant protein (Figure 1.4). Some have also demonstrated cellular activity [137]. Initial efforts to target the Y220C crevice have identified the *N*-ethylcarbazole derivative PK083, which binds to the mutational cleft and stabilizes the Y220C mutant by 0.8 °C (at 125 μM PK083) [100]. Trifluorination of the *N*-ethyl anchor of PK083 leads to multipolar interactions and fluorine-sulfur contacts in the binding pocket and enhances the stabilization to 1.2 °C (at 125 μM PK083-4) [138]. The series of Y220C stabilizing molecules also includes the 2,4-diiodophenol-based compound PK784, identified by screening of a halogen-enriched fragment library. PK784 forms a halogen bond with Leu145 in the Y220C binding pocket and stabilizes the protein by 0.55 °C (at 250 μM PK784) (Figure 1.5 A, B, E) [98]. Optimization of the lead compound PK784 resulted in PK5196, which incorporates an acetylene linker to extend the ligand into subsite 2 of the mutation-induced binding pocket. PK5196 increases the T_m of the Y220C mutant by 3.61 °C (at 250 μM PK5196) [98]. Screening of a fragment library identified the pyrazole derivative PK7088 as a cleft binder, stabilizing the Y220C protein by 1.0 °C (at 350 μM PK7088). The pyrrole side chain of compound PK7088 points into the transiently open subsite 3 of the Y220C binding pocket, created by a flip of Cys220 (Figure 1.5 F) [139]. To target the different subsites of the Y220C binding pocket, the diiodophenol scaffold was combined with the pyrrole moiety, resulting in the aminobenzothiazole derivative MB710, which stabilizes the

β -sandwich mutant by 2.0 °C (at 250 μ M MB710) [114]. Fluorination of the pyrrole side chain of MB710 yielded compound JC744. The aminobenzothiazole JC744 displays fluorine-protein interaction in subsite 3 and stabilizes the Y220C mutant by 2.7 °C (at 200 μ M JC744). JC744 is the first ligand identified that binds to the Y220C pocket with nanomolar affinity [101]. Furthermore, PMV Pharma has developed PC14586, an orally available small molecule reactivator that targets the Y220C mutational cleft and stabilizes the Y220C mutant [140]. PC14586 is currently in phase 1/2 clinical trials in patients with advanced solid tumors with a p53-Y220C mutation (NCT04585750) [141, 142]. Moreover, Guiley and Shokat [143] recently identified the first covalent reactive compounds addressing selectively the mutation-induced Cys220 in the hydrophobic pocket of the Y220C mutant. Through structure-based design with warhead attachment to a previously identified non-reactive carbazole-based cleft binder [144, 145], Cys220-specific acrylamide warhead-containing compounds (e.g., KG13) were developed, increasing the melting temperature of the Y220C mutant up to wt level [143].

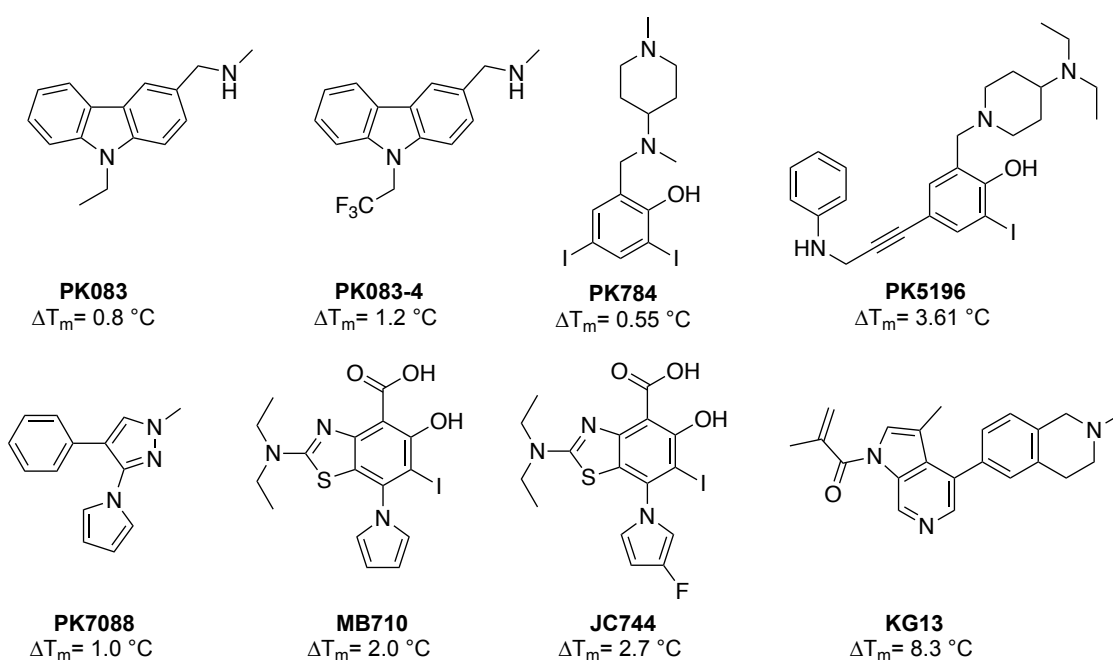


Figure 1.4: Overview of the series of p53-Y220C cleft binders and their corresponding T_m shifts (ΔT_m).

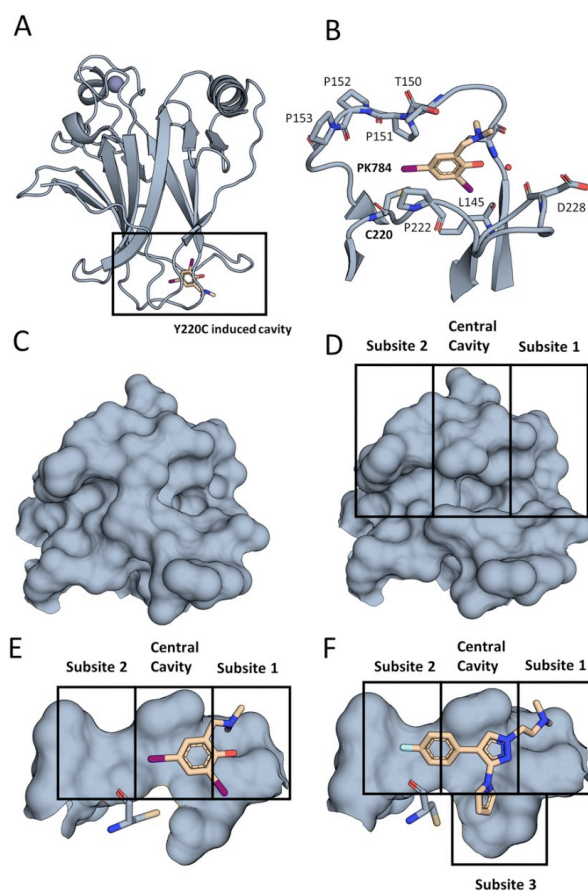


Figure 1.5: The mutation-induced Y220C crevice. **(A)** Overview of the DBD of p53-Y220C in complex with PK784 (Protein Data Bank (PDB) code: 4AGL [98]). **(B)** The 2,4-diiodophenol derivative PK784 forms a halogen bond with Leu145 in the Y220C binding pocket. The additional iodine points towards subsite 2 and serves as a vector for ligand growing. **(C)** Surface view of wt p53 around Tyr220 (PDB code: 1UOL [146]). **(D)** Surface view of p53-Y220C around Cys220. The mutation creates a druggable cavity, connecting the pre-existing subsites 1 and 2 (PDB code: 4AGL [98]). **(E)** PK784 occupies the central cavity and subsite 1, while subsite 2 and the lower part of the crevice are not addressed. **(F)** PK7242, a more soluble analog of PK7088, targets subsite 3 of the Y220C binding pocket, formed by displacement of Cys220 (PDB code: 3ZME [139]). Figure was reproduced from [99] with the permission of the Royal Society of Chemistry (Chapter 8.16).

In addition to the compounds described, there are numerous other methods to address mutant p53 [104]. These approaches include the depletion of mutant p53 through interventions in the chaperone machinery [147], the use of p53 mutation-specific short interfering RNA [148], the blocking of survival pathways associated with mutant p53 [104, 149], the aggregation inhibition (ReACp53 [150], ADH-6 [151]) [136], and synthetic lethality-based approaches to inhibit mutant p53 [13, 104, 152]. In addition, nonsense mutated p53 can be rescued by compounds that achieve a readthrough of the premature stop codon [153, 154]. Additional approaches to restore p53 function include gene therapy, p53-specific vaccines, peptides and antibody drugs, and T cell receptor-T cell therapies [1, 13, 104].

2 Objective of the Thesis

The tumor suppressor gene *TP53* is most frequently mutated in human cancers [1, 55, 155]. Mutations of p53 are related to rapid tumor progression, worsening of cancer prognosis, and chemoresistance [73, 155, 156]. Therefore, p53 is an attractive target for cancer therapy, and there is a great interest in finding drugs that can rescue p53. However, p53 has long been considered undruggable [13]. Despite extensive efforts in p53 research and drug discovery, no drug has yet received FDA approval, and only a limited number are in clinical trials [104, 155]. Therefore, this thesis aimed to discover novel small molecules for mutant p53 rescue using fragment-based approaches.

In the first part of the study, the diversity-optimized Halogen-Enriched Fragment Library (HEFLib) was evaluated with the strongly destabilized p53-Y220C mutant and its challenging binding site. The HEFLib was screened using differential scanning fluorimetry (DSF) as the primary screening method to find hits for the temperature-sensitive cancer mutant. The aim was the identification of new stabilizing small molecules with unconventional binding modes in the Y220C pocket based on halogen bonding.

In the second part of this thesis, a covalent fragment library (CovLib) was designed, including fragments with diverse warheads and a wide range of reactivities. The CovLib was evaluated with p53 and different DNA contact and structural mutants. The objective was to identify covalent reactive fragments that stabilize p53 and its mutants by modifying surface-exposed cysteine residues, thereby achieving a generic and mutation-independent rescue of p53. Therefore, the CovLib was screened using DSF and intact protein mass spectrometry (MS) as the primary biophysical screening methods. The hits were validated via $^1\text{H}/^{15}\text{N}$ -heteronuclear single quantum coherence nuclear magnetic resonance ($^1\text{H}/^{15}\text{N}$ -HSQC-NMR). Moreover, the influence of the hits on the DNA-binding affinity of full-length p53 was investigated employing a fluorescence polarization (FP) assay. Finally, protein crystallization was performed to elucidate the binding mode of the identified fragment hits.

The results of the CovLib screen revealed a fragment with a vinyl sulfone warhead, achieving a general stabilization of p53. Furthermore, fragments reacting via nucleophilic aromatic substitution were identified, stabilizing the temperature-sensitive cancer mutant Y220C and arylating the mutational cysteine 220 in the hydrophobic pocket. Therefore, in the third part of the thesis, extensive structure-activity relationship

(SAR) studies were performed to optimize the highly reactive CovLib hits. The primary goal of the SAR screen was to identify fragments with a more balanced reactivity profile that target the Y220C binding pocket and achieve enhanced stabilization of the cancer mutant. Thus, the compounds were screened using DSF as the primary biophysical method and intact protein mass spectrometry to confirm the covalent modification. The binding mode of the SAR fragments was determined by X-ray crystallography.

All aspects of the project are focused on discovering new hits for p53 and its mutants using fragment-based approaches, specifically for the thermosensitive p53-Y220C mutant with halogen bonding in the pocket or covalent modification of the Cys220 residue.

3 Results and Discussion

3.1 Mutagenesis

3.1.1 The DNA Contact Mutant R273H

Arg273 is the second most cancer-associated residue, affected by estimated 8.5% of all p53 missense point mutations, resulting in approximately 800,000 annual cancer cases [94]. The DNA contact Arg273 has two predominant substitutions: R273H and R273C [77]. The cancer hot spot mutant R273H is one of the five most cancer-associated mutations [69]. The mutation of Arg273 is a classical contact mutation. Arg273 makes direct contact with the phosphate backbone of the DNA at the center of a half site, and the R273H mutation removes an essential DNA contact. However, the conformation of neighboring residues and the overall structure of the core domain are preserved [55, 73]. Therefore, the mutation has only little effect on the thermodynamic stability of the protein compared to wt p53 [77]. The loss of a crucial DNA contact leads to a reduced DNA-binding affinity and especially binding selectivity [81, 157]. The remaining FL R273H DNA-binding affinity is insufficient to bind to p53 DNA response elements and transcribe p53 target genes normally [73, 93]. In addition, the R273H mutant may exhibit gain-of-function activity that promotes tumor invasion and migration and induces cancer stemness [4, 158, 159]. Two second-site suppressor mutations, S240R [160] and T284R [161], have been reported, introducing a new DNA contact near the mutant residue 273 [73].

3.1.1.1 Polymerase Chain Reaction (PCR) Mutagenesis

The R273H point mutation was introduced by polymerase chain reaction (PCR) using the Q5[®] Site-Directed Mutagenesis Kit (New England Biolabs, Ipswich, MA, USA) according to the protocol described in Section 4.7.4. Primers were designed with the NEBaseChanger[®] tool [162], and their corresponding sequences, annealing temperatures, and PCR programs are detailed in Chapter 4.7.4. The T-p53C or the xT-p53C plasmids served as templates for the PCR mutagenesis. The vector represented a pET24a(+)-HLT vector with a 6x His-tag and a tobacco etch virus (TEV) protease cleavage site. The pET24a(+)-HLT vector included the gene for the core domain of the p53 protein with the amino acid residues 94-312 and the four stabilizing point mutations M133L, V203A, N239Y, and N268D [163]. This quadruple

thermostable mutant of the p53 core domain (T-p53C) has wt characteristics and is more suitable for biophysical measurements and structural characterization [146]. In the superstable mutant, the overall structure of the wt protein is conserved except for the mutant side chains, which provide rigidity and stability and raise the melting temperature of the protein by 6 °C [5, 73, 81]. The point mutation was introduced in both the T-p53C construct used for biophysical experiments and the xT-p53C construct with a deletion of the GGS overhang after the TEV cleavage site employed for crystallization. To introduce the DNA contact mutation, Arg273 was replaced with a histidine by mutating the CGT base triplet encoding for Arg273 to CAT. The triplet CAT was selected as it is the most commonly used codon for histidine in *Escherichia coli* (*E.coli*) [164]. The generation of a PCR product was confirmed by agarose gel electrophoresis. The agarose gels for the T-p53C-R273H and xT-p53C-R273H PCR products (Figure 3.1) showed a signal at approximately 6000 base pairs (bp), corresponding to the expected size of the linearized plasmid fragments of 6240 bp for T-p53C-R273H and 6231 bp for xT-p53C-R273H. However, only a faint signal at 6000 bp was visible on the gel of the T-p53C-R273H PCR product. Additional impurities were detectable on both gels, which did not interfere with the subsequent kinase-ligase-DpnI (KLD) enzyme mix treatment, heat shock transformation, Miniprep, and Maxiprep.

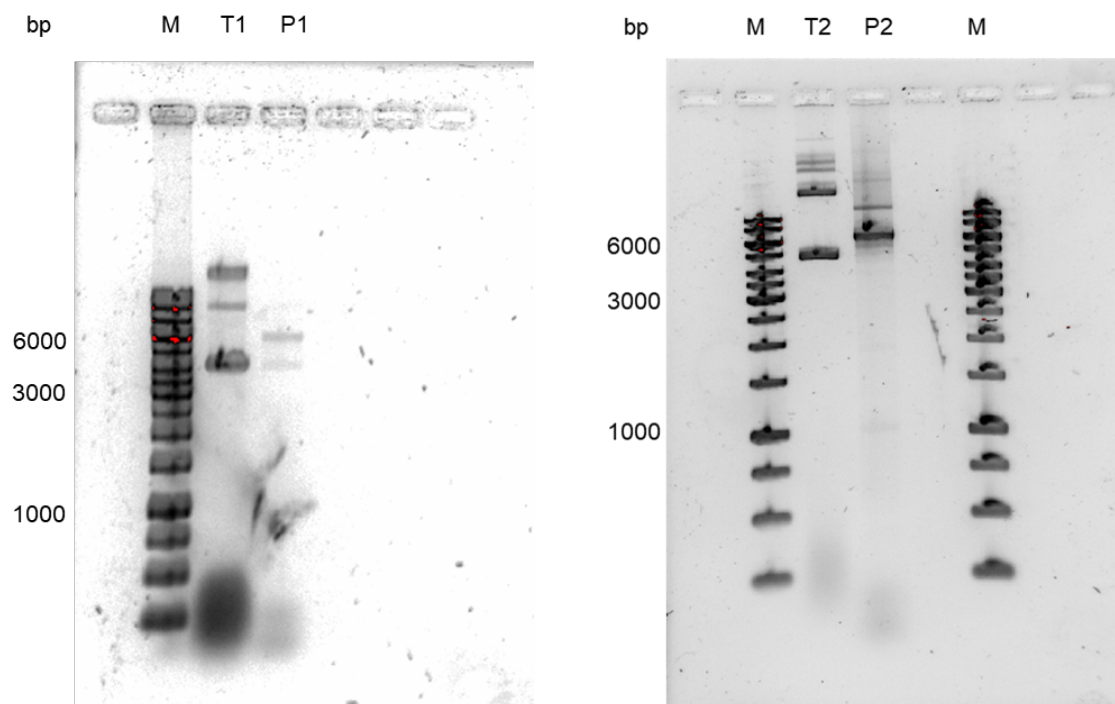


Figure 3.1: Agarose gel of the T-p53C-R273H (left side) and xT-p53C-R273H PCR (right side) products. M= marker (1 kb DNA ladder), T1= template T-p53C, T2= template xT-p53C, P1= PCR product of T-p53C-R273H with a size of 6240 bp, P2= PCR product of xT-p53C-R273H with a size of 6231 bp.

The final sequence analysis of the Maxipreps (Figure 3.2, Figure 3.3) confirmed the successful mutagenesis with the introduction of the arginine-to-histidine mutation into both plasmids. The plasmid concentrations were 270 $\mu\text{g/mL}$ for T-p53C-R273H and 280 $\mu\text{g/mL}$ for xT-p53C-R273H.

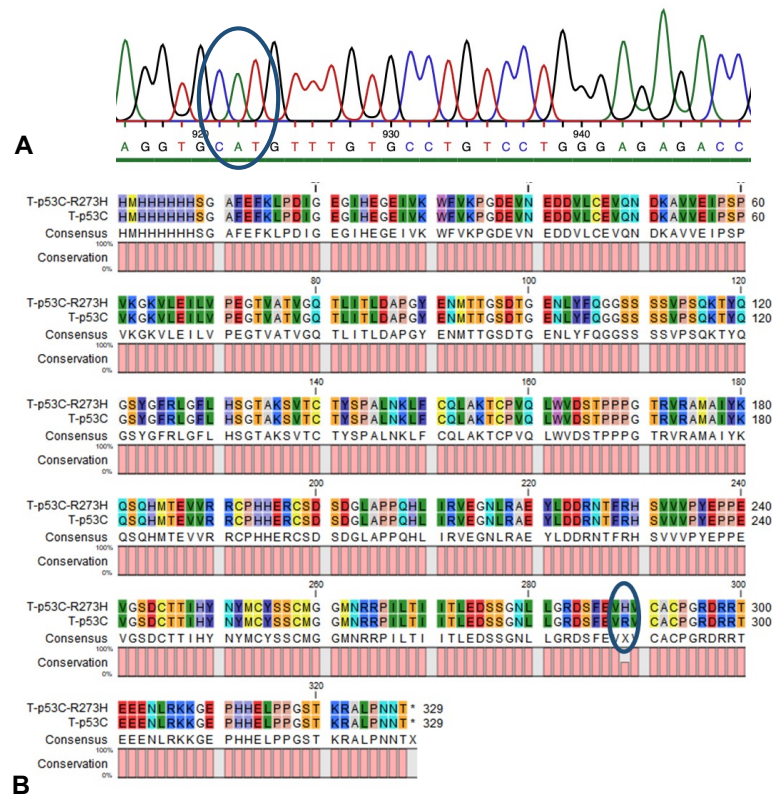


Figure 3.2: Sequencing results of the T-p53C-R273H Maxiprep. **(A)** Sequence analysis of the T-p53C-R273H Maxiprep with the point mutation from CGT to CAT. **(B)** The amino acid sequence of the T-p53C-R273H mutant in comparison to the T-p53C template sequence with the introduction of the point mutation from arginine to histidine. The figure was prepared with CLC Sequence Viewer (Qiagen Bioinformatics, Aarhus, Denmark).

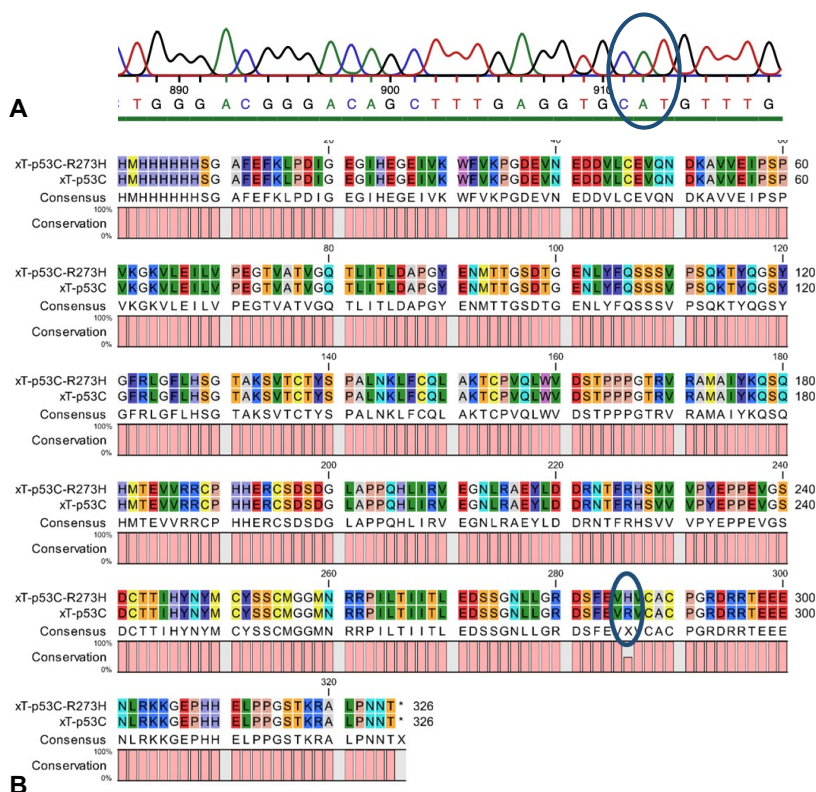


Figure 3.3: Sequencing results of the xT-p53C-R273H Maxiprep. **(A)** Sequence analysis of the xT-p53C-R273H Maxiprep with the point mutation from CGT to CAT. **(B)** The amino acid sequence of the xT-p53C-R273H mutant in comparison to the xT-p53C template sequence with the introduction of the point mutation from arginine to histidine. The figure was prepared with CLC Sequence Viewer (Qiagen Bioinformatics, Aarhus, Denmark).

3.1.2 The Structural Mutant R282W in the C-Terminal Helix

The hot spot mutant R282W is a structural mutant in the DNA-binding region of p53 [77]. The residue Arg282 has one predominant mutation: R282W. It accounts for 3.3% of all p53 missense point mutations, corresponding to approximately 300,000 annual cancer cases [94]. Arg282 is located in the C-terminal helix (H2) in the periphery of the DNA-binding region. It is involved in several interactions that maintain the structural integrity of the DNA-binding loop-sheet-helix-motif [73]. Arg282 stabilizes the motif via hydrophobic interactions and a hydrogen bond network and packs the helix H2 against the S2-S2' hairpin and loop L1 [55]. The mutation of arginine to tryptophan results in the loss of several stabilizing interactions. Trp282 disrupts the neighboring L1 loop due to steric hindrance, resulting in disorder in this region, which also involves the DNA contact Lys120. Furthermore, Trp282 is less embedded in the core of the protein and more solvent-exposed than the arginine residue. These structural changes lead to a major thermodynamic destabilization of the p53-R282W protein [55, 69, 73, 77]. Nevertheless, the entire structure of the DNA-binding region, including the helix H2, is

preserved, and the DNA-binding activity of the R282W mutant is maintained at lower temperatures [77]. Furthermore, the R282W mutant is associated with gain-of-function activity of mutant p53 [165].

3.1.2.1 PCR Mutagenesis

The structural mutants T- and xT-p53C-R282W were generated by PCR mutagenesis using the T-p53C or xT-p53C constructs as templates. For the mutation of arginine 282 to tryptophan, the base triplet CGG encoding arginine was mutated to TGG [164]. The PCR protocol, the sequence of the primers, and the respective annealing temperature are presented in Chapter 4.7.4. The success of the PCR mutagenesis was monitored by agarose gel electrophoresis. As depicted in Figure 3.4, both mutants exhibited a detectable signal at about 6000 bp on the agarose gel, which corresponds to the expected size of the linearized plasmid. Furthermore, for both mutants, an additional sharp signal was evident at approximately 500 bp. These impurities represented non-specific PCR products resulting from random primer annealing. Therefore, a preparative agarose gel electrophoresis was conducted following the procedure outlined in Chapter 4.7.6. The appropriate signals at 6000 bp were excised from the gel and subsequently extracted. Thereafter, the KLD-reaction, heat shock transformation, Miniprep, and Maxiprep were performed.

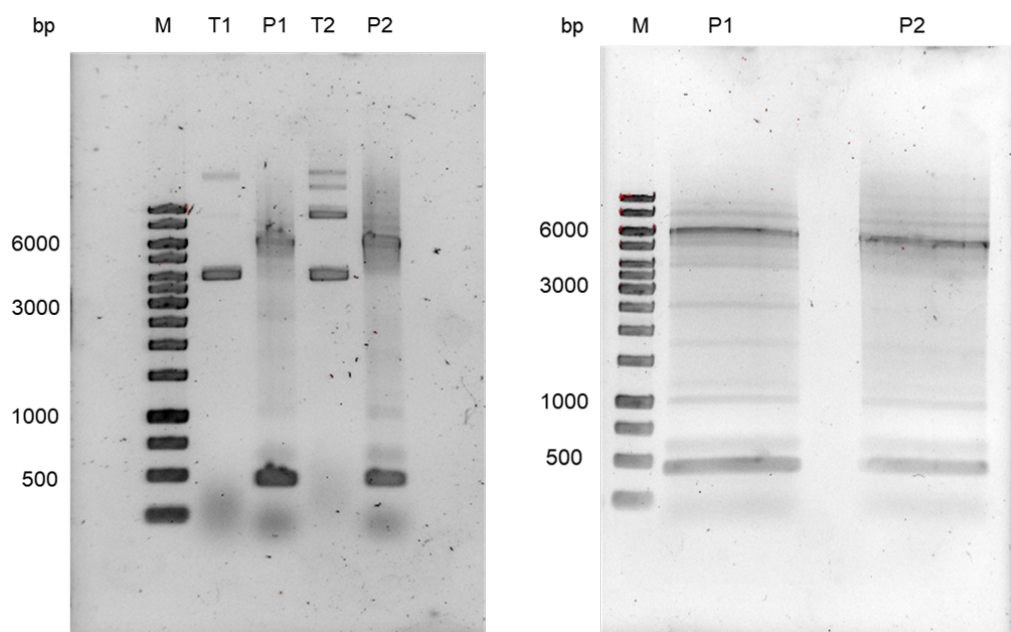


Figure 3.4: Agarose gel of the T-/xT-p53C-R282W PCR products (left side) and the preparative gel (right side). M= marker (1 kb DNA ladder), T1= template T-p53C, T2= template xT-p53C, P1= PCR product of T-p53C-R282W with a size of 6240 bp, P2= PCR product of xT-p53C-R282W with a size of 6231 bp.

Finally, the successful mutagenesis was confirmed by sequence analysis of the Maxipreps (Figure 3.5, Figure 3.6). Both constructs contain the desired arginine to tryptophan point mutation. The concentrations of the Maxiprep were 250 µg/mL for T-p53C-R282W and 680 µg/mL for xT-p53C-R282W.

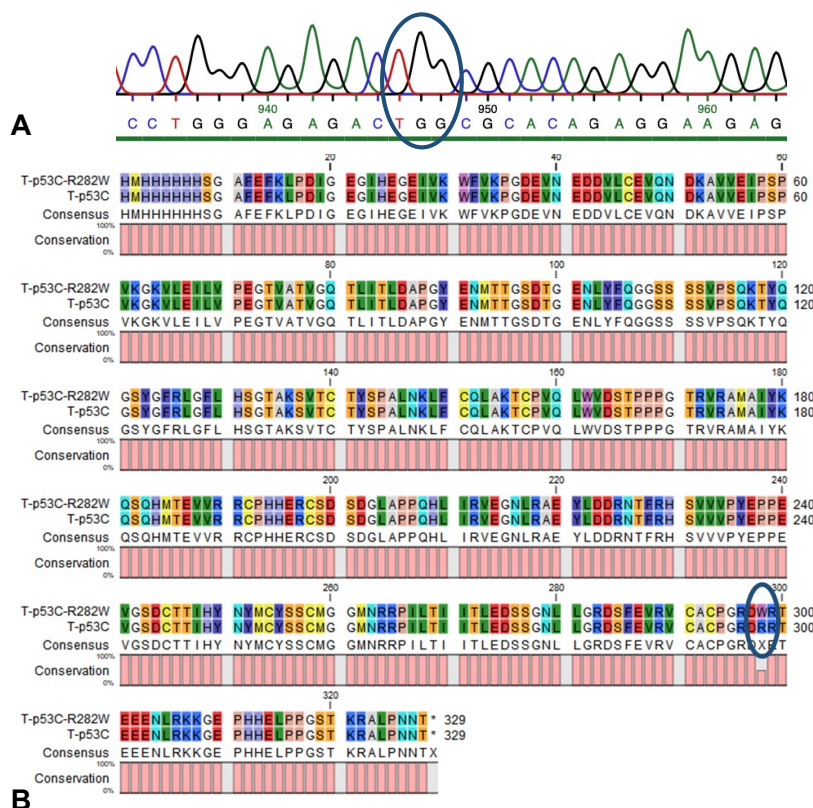


Figure 3.5: Sequencing results of the T-p53C-R282W Maxiprep. **(A)** Sequence analysis of the T-p53C-R282W Maxiprep with the introduction of the point mutation from CGG to TGG. **(B)** The amino acid sequence of the T-p53C-R282W mutant in comparison to the T-p53C template sequence with the introduction of the point mutation from arginine to tryptophan. The figure was prepared with CLC Sequence Viewer (Qiagen Bioinformatics, Aarhus, Denmark).

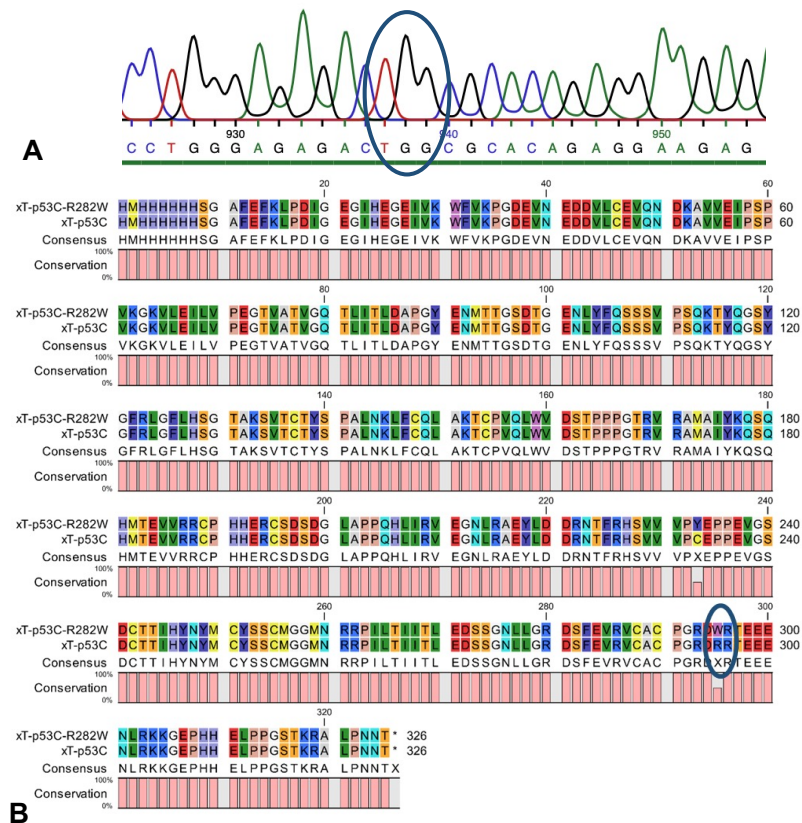


Figure 3.6: Sequencing results of the xT-p53C-R282W Maxiprep. **(A)** Sequence analysis of the xT-p53C-R282W Maxiprep with the introduction of the point mutation from CGG to TGG. **(B)** The amino acid sequence of the xT-p53C-R282W mutant in comparison to the xT-p53C template sequence with the introduction of the point mutation from arginine to tryptophan. The figure was prepared with CLC Sequence Viewer (Qiagen Bioinformatics, Aarhus, Denmark).

3.2 Protein Expression and Purification

3.2.1 Expression and Purification of T-p53C, T-p53C-Y220C, T-p53C-R273H, T-p53C-R282W, T-p53C-C124/182/277S, and T-p53C-C124/182/229/275/277S

T-p53C and its mutants were expressed and purified according to the same protocol described in Section 4.8.2. Therefore, the expression and purification of T-p53C-Y220C is exemplified in this chapter.

The plasmid of the T-p53C-Y220C was transformed into *E.coli* BL21(DE3)pLysS cells (Novagen, Merck, Darmstadt, Germany). The vector was a pET24a(+)_HLT vector, with the stabilized core domain of p53 as insert, T-p53C-Y220C (94-312, M113L/V203A/N239Y/N268D/Y220C). For biophysical experiments and especially for X-ray crystallography, high yields and a high purity of the protein are desirable. The purification procedure consisted of three separate purification steps.

First, the T-p53C-Y220C protein was purified using a nickel affinity chromatography step. The fractions with the T-p53C-Y220C protein were pooled, and TEV protease was added to cleave off the His-tag. The second purification step also involved an affinity chromatography step using a heparin column. The arginine residues of the DNA-binding protein p53 interacted with the negatively charged sulphate groups of the heparin. Thus, the T-p53C-Y220C protein was separated from the His-tag and the His-tagged TEV protease.

To remove the remaining impurities of the sample, a final size exclusion chromatography (SEC) step was performed. The protein eluted from the column after approximately 170 mL. The corresponding UV absorption spectrum of the T-p53C-Y220C SEC run is depicted in Figure 3.7 A. The UV peak of the SEC chromatography step was fractionated, and samples were taken for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The SDS-PAGE in Figure 3.7 B showed the successful expression of the T-p53C-Y220C protein with a signal between 26 kDa and 34 kDa, representing the T-p53C-Y220C protein with a molecular weight of 24.696 kDa. It should be noted that the apparent mass of the p53 proteins in SDS-PAGEs is overestimated due to their high number of prolines, slowing down their migration on the gels. The name p53 was derived from its molecular weight of 53 kDa, which was observed by SDS-PAGE analysis [166]. Moreover, the gel

revealed that the fractions 2.A5 to 2.A6 contained an additional signal over the protein signal. Therefore, to ensure high purity for the biophysical assays and crystallization, these fractions were discarded. Finally, the fractions 1.E8 to 2.A4, containing the pure T-p53C-Y220C, were pooled. The overall yield of the T-p53C-Y220C expression was approximately 23 mg per liter of culture. Each expression of p53 and its mutants achieved high purity and a high yield of up to 80 mg per liter of culture for the T-p53C-R273H expression.

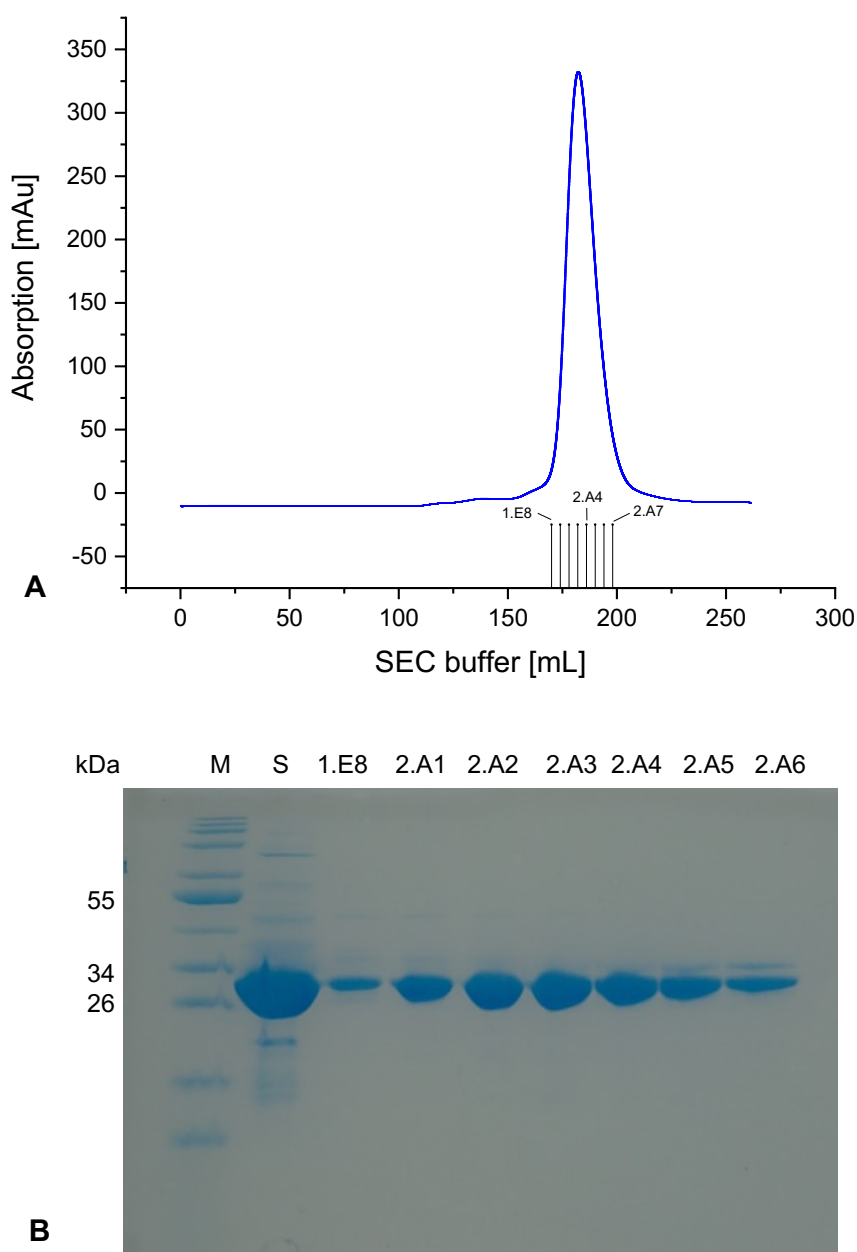


Figure 3.7: SEC run of T-p53C-Y220C. **(A)** UV chromatogram of the T-p53C-Y220C SEC run, displaying the protein peak after approximately 170 mL and fractions containing the T-p53C-Y220C protein. **(B)** The SDS-PAGE after the respective SEC run. M= marker, S= concentrated T-p53C-Y220C after the heparin column, 1.E8-2.A4= pooled fractions containing the pure T-p53C-Y220C protein with a size of 24.696 kDa, 2.A5-2.A6= discarded fractions with an additional signal over the protein signal.

The SDS-PAGEs of T-p53C, T-p53C-R273H, T-p53C-R282W, T-p53C-C124/182/277S, and T-p53C-Y220C-C124/182/229/275/277S (T-p53C-Y220C-cysteine light (CL)) are presented in Figure 3.8 to Figure 3.12. For crystallization experiments, constructs with a deletion of the GGS overhang after the TEV-cleavage site, herein referred to as xT-p53C, were used. The expression and purification of ^{15}N -labeled T-p53C-Y220C was performed to conduct $^1\text{H}/^{15}\text{N}$ -HSQC-NMR experiments. During the expression, ^{15}N -labeled NH_4Cl was used as the sole nitrogen source. The SDS-PAGEs of xT-p53C and its mutants and of the ^{15}N -labeled T-p53C-Y220C protein are displayed in the supplementary data of this thesis (Section 8.2).

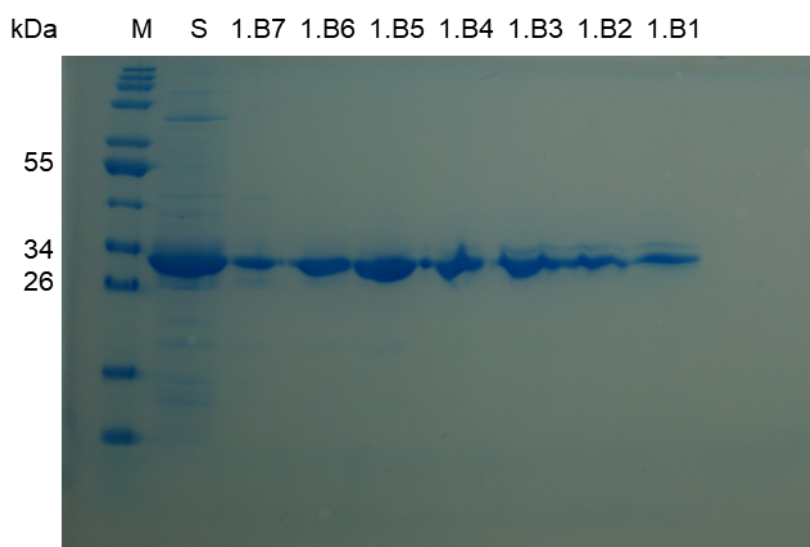


Figure 3.8: SDS-PAGE after the SEC run of T-p53C. M= marker, S= concentrated T-p53C after the heparin column, 1.B7-1.B3= pooled fractions containing the pure T-p53C protein with a size of 24.756 kDa, 1.B2-1.B1= discarded fractions with an additional signal over the protein signal.

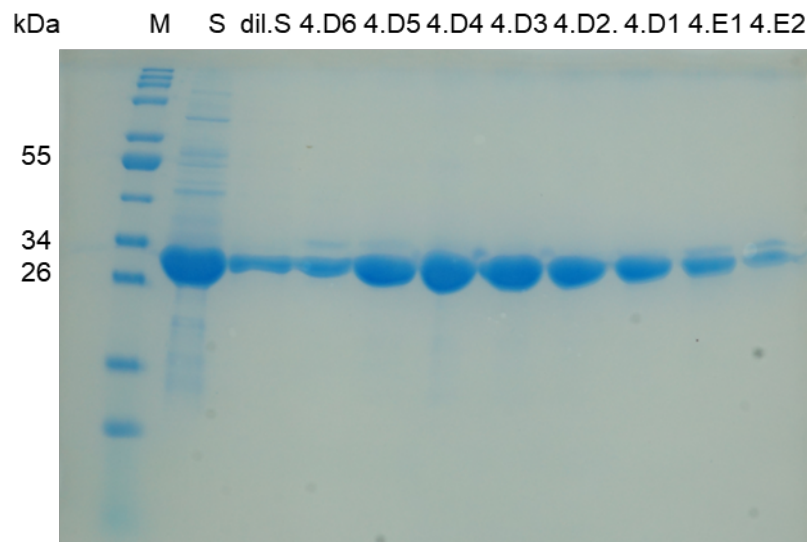


Figure 3.9: SDS-PAGE after the SEC run of T-p53C-R273H. M= marker, S= concentrated T-p53C-R273H after the heparin column, dil. S= diluted sample with the factor 1:5, 4.D5-4.D1= pooled fractions containing the pure T-p53C-R273H protein with a size of 24.737 kDa, 4.D6, 4.E1-4.E2= discarded fractions with an additional signal over the protein signal.

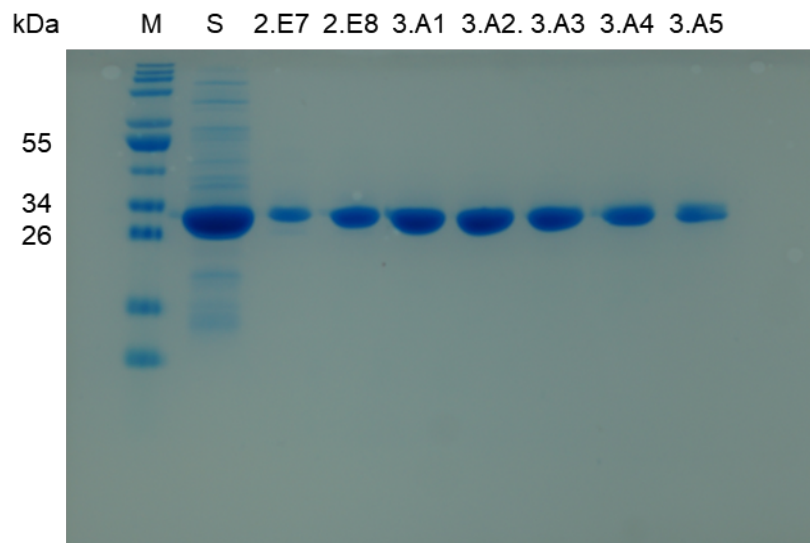


Figure 3.10: SDS-PAGE after the SEC run of T-p53C-R282W. M= marker, S= concentrated T-p53C-R282W after the heparin column, 2.E7-3.A5= pooled fractions containing the pure T-p53C-R282W protein with a size of 24.786 kDa.

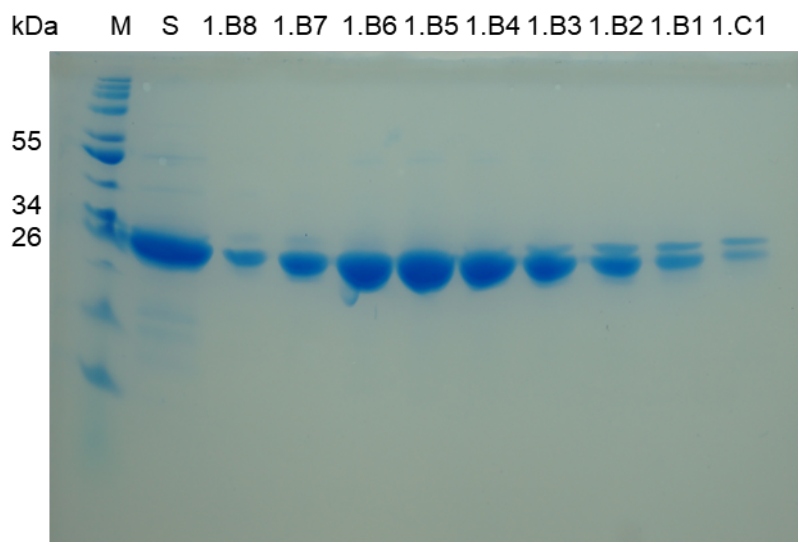


Figure 3.11: SDS-PAGE after the SEC run of T-p53C-C124/182/277S. M= marker, S= concentrated T-p53C-C124/182/277S after the heparin column, 1.B8-1.B3= pooled fractions containing the pure T-p53C-C124/182/277S protein with a size of 24.708 kDa, 1.B2-1.C1= discarded fractions with an additional signal over the protein signal.

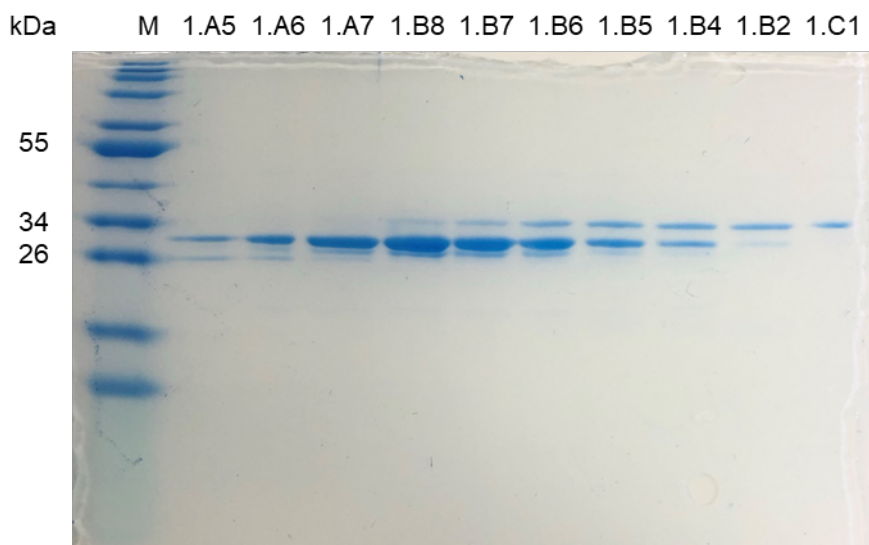


Figure 3.12: SDS-PAGE after the SEC run of T-p53C-Y220C-C124/182/229/275/277S. M= marker, 1.A5-1.B7= pooled fractions containing the pure T-p53C-Y220C-C124/182/229/275/277S protein with a size of 24.616 kDa, 1.B6-1.C1= discarded fractions with an additional signal over the protein signal.

3.2.1.1 Intact Protein Mass Spectrometry

The mass of the purified T-p53C-Y220C protein was confirmed using ultra-high-performance liquid chromatography electrospray ionization mass spectrometry (UHPLC-ESI-MS). In the deconvoluted MS spectrum of T-p53C-Y220C (Figure 3.13), a peak was identified with a mass of 24,695.68 Da. This reflects a mass difference of -0.32 Da or -13.0 ppm between the theoretical mass of T-p53C-Y220C (24,696.00 Da) and the experimentally determined mass (Table 3.1). The intact protein mass spectrometry results confirmed the successful expression and purification of the T-p53C-Y220C protein.

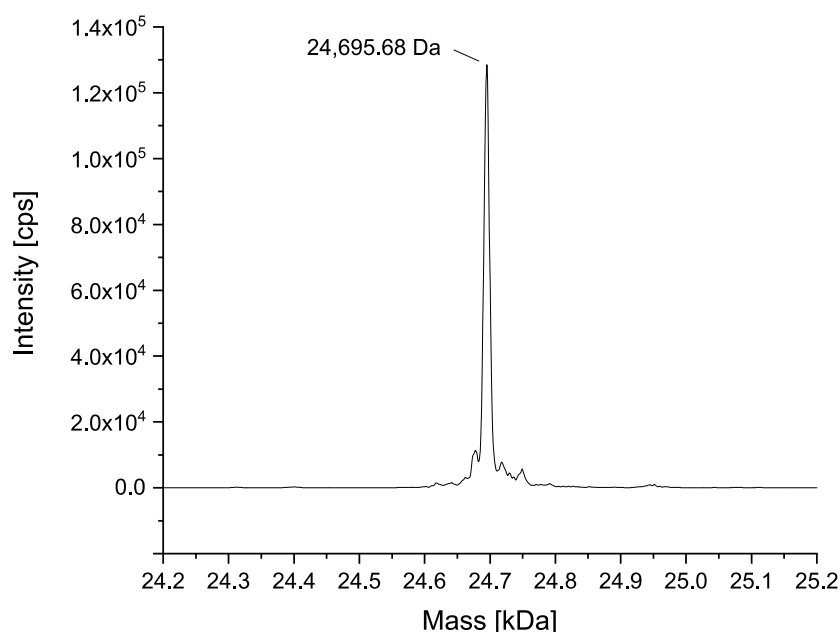


Figure 3.13: Deconvoluted MS spectrum of the intact T-p53C-Y220C protein. Theoretical mass: 24,696.00 Da, experimental mass: 24,695.68 Da.

The deconvoluted MS spectra of the T-p53C (A), T-p53C-R273H (B), T-p53C-R282W (C), T-p53C-C124/182/277S (D), and T-p53C-Y220C-C124/182/229/275/277S (E) proteins are depicted in Figure 3.14. Each spectrum showed a single distinct protein peak. The experimental detected masses, reported in Table 3.1, align well with the theoretical average molecular weights of the respective proteins. In conclusion, the expression and purification of T-p53C and its mutants were successful.

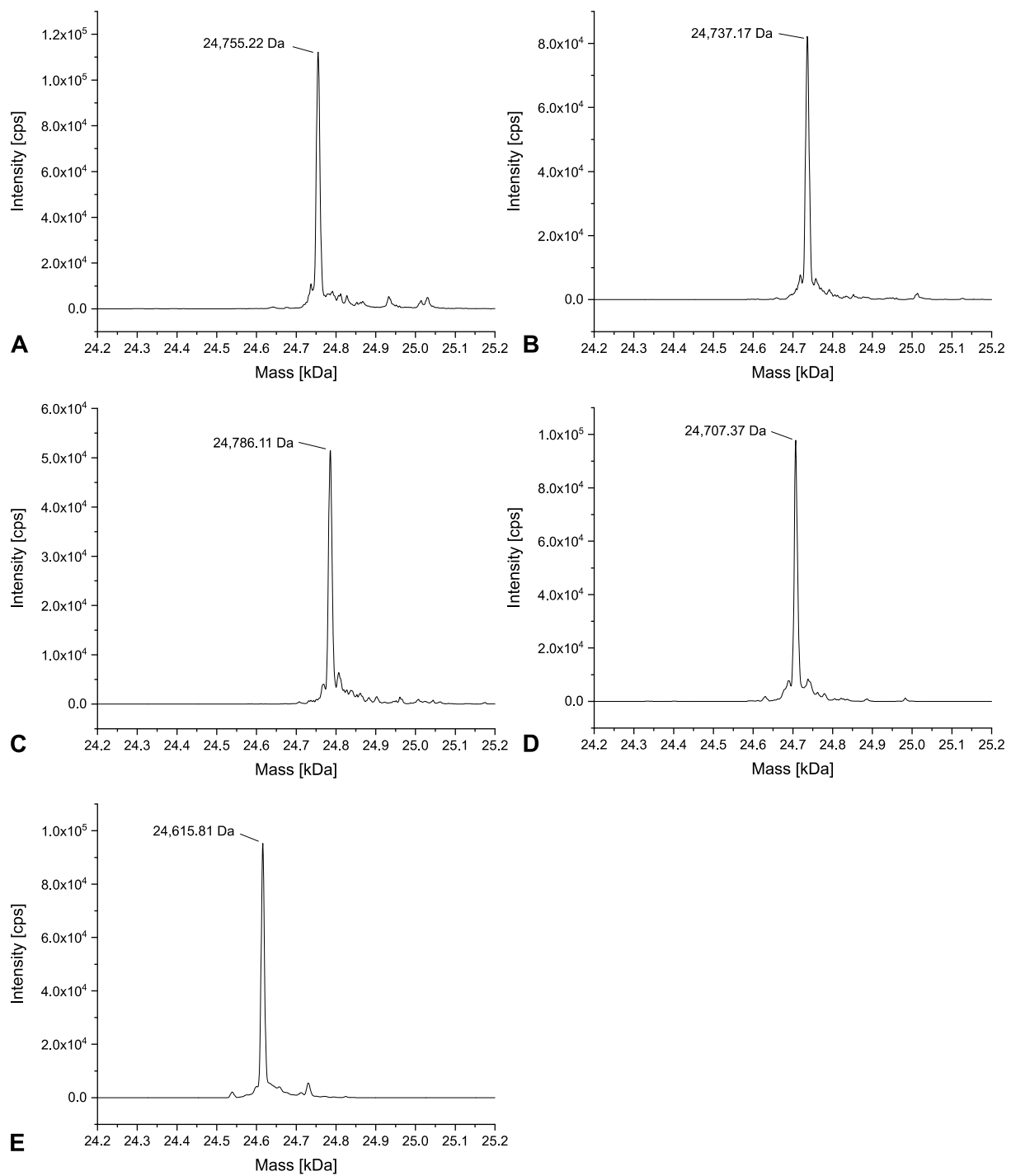


Figure 3.14: Deconvoluted MS spectra of T-p53C (A), T-p53C-R273H (B), T-p53C-R282W (C), T-p53C-C124/182/277S (D), T-p53C-Y220C-C124/182/229/275/277S (T-p53C-Y220C-CL) (E).

Table 3.1: Results of the intact protein mass spectrometry of T-p53C and its mutants. Theoretical masses were calculated with PeakView 2.2.0 software (Sciex).

Protein	Theoretical mass [Da]	Experimental mass [Da]	Δm [Da]	Δm [ppm]
T-p53C-Y220C	24,696.00	24,695.68	-0.32	-13.0
T-p53C	24,756.03	24,755.22	-0.81	-32.7
T-p53C-R273H	24,736.99	24,737.17	0.18	7.3
T-p53C-R282W	24,786.06	24,786.11	0.05	2.0
T-p53C-C124/182/277S	24,707.84	24,707.37	-0.47	-19.0
T-p53C-Y220C- C124/182/229/275/277S	24,615.68	24,615.81	0.13	5.3

3.2.2 Expression and Purification of FL-T-p53

FL-T-p53 was expressed and purified for DNA-binding studies. In contrast to the core domain constructs, which lack the tetramerization domain, the full-length p53 protein can form a dimer of primary dimers and binds to the DNA as a tetramer [5, 167].

The full-length p53 protein construct includes the amino acids 1-393 with the stabilizing mutations M133L, V203A, N239Y, and N268D. The vector used was a pET24a(+)-HLT vector with a 6x His-tag and a TEV protease cleavage site.

The FL-T-p53 expression was carried out similarly to the expression of the core domain constructs with only minor deviations from the previously described protocol. To prevent non-specific proteolytic cleavage of the expressed full-length p53 protein and to increase the overall yield, an ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) was added to the lysis buffer. The purification protocol for His-tagged FL-T-p53 involved an affinity chromatography step using a nickel column, followed by an overnight TEV digest, and a heparin column as a second affinity chromatography step.

The final size exclusion chromatography was performed using a HiLoad™ 26/60 Superdex 200 pg column (Cytiva Sweden AB, Uppsala, Sweden) due to the protein's larger size and dimer-forming capability. The UV chromatogram of the SEC run is shown in Figure 3.15 A. The FL-T-p53 protein eluted after approximately 120 mL. Before the main peak containing the protein, a shoulder was observed, and these fractions were discarded. After the main peak, there were a few small peaks containing larger impurities. The SDS-PAGE (Figure 3.15 B) showed a clear signal at 55 kDa,

representing the FL-T-p53 protein with a size of 43.858 kDa. A small amount of residual additional impurities below and above the protein signal were detectable in the pooled fractions. The purity of the protein was sufficient for biophysical testing, and the small quantity of impurities could be tolerated. The overall yield of the expression and purification of FL-T-p53 was 4 mg of protein per liter of culture, which is rather moderate compared to the expression of the core domain constructs.

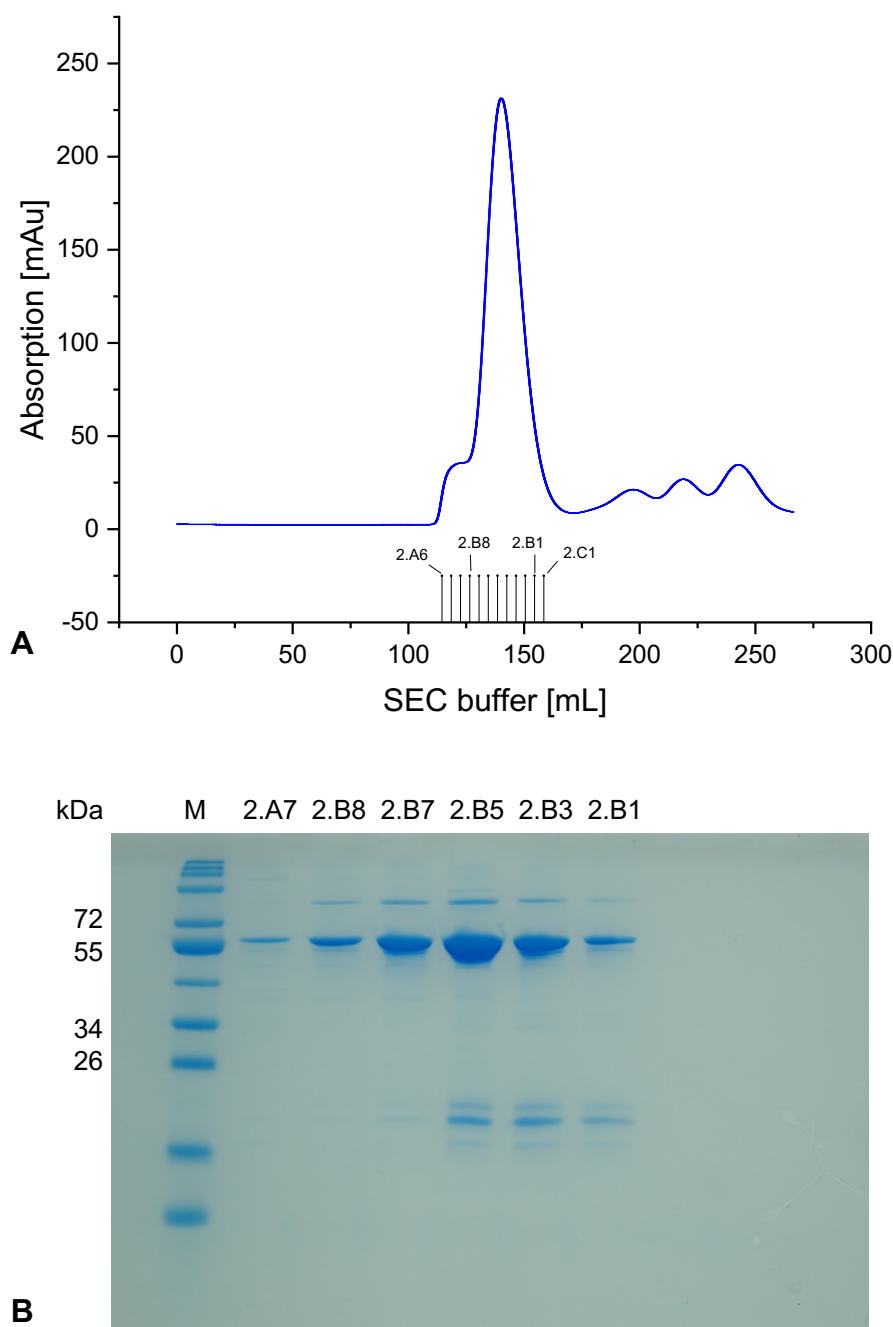


Figure 3.15: SEC run of FL-T-p53. **(A)** UV chromatogram of the FL-T-p53 SEC run, displaying the protein peak after approximately 120 mL and fractions containing the purified protein. **(B)** The SDS-PAGE after the respective SEC run. M= marker, 2.A7= discarded fraction, 2.B8-2.B1= pooled fractions containing the purified FL-T-p53 protein with a size of 43.858 kDa.

3.2.2.1 Intact Protein Mass Spectrometry

The mass of FL-T-p53 was verified using intact protein mass spectrometry. Figure 3.16 shows the deconvoluted MS spectrum of the intact FL-T-p53 protein with a detected mass of 43,857.32 Da. The determined experimental mass is in good accordance with the theoretical average mass of 43,858.44 Da. The mass defect between the experimental and calculated masses is -1.12 Da or -25.5 ppm. The intact protein MS result showed that the FL-T-p53 protein expression and purification were successful.

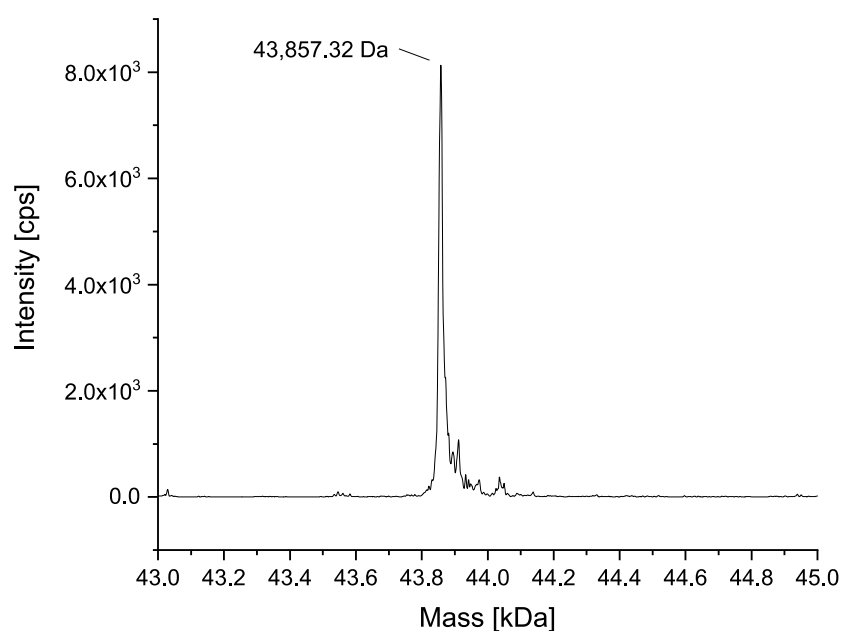


Figure 3.16: Deconvoluted MS spectrum of the intact FL-T-p53 protein. Theoretical mass: 43,858.44 Da, experimental mass: 43,857.32 Da.

3.3 Screening of the Halogen-Enriched Fragment Library (HEFLib) with T-p53C-Y220C

Parts of this Section 3.3 were reproduced from J. Stahlecker, T. Klett, M. Schwer, S. Jaag, M. Dammann, L. N. Ernst, M. B. Braun, M. O. Zimmermann, M. Kramer, M. Lämmerhofer, T. Stehle, M. Coles and F. M. Boeckler, Revisiting a challenging p53 binding site: a diversity-optimized HEFLib reveals diverse binding modes in T-p53C-Y220C, *RSC Med. Chem.*, 2022, **13**, 1575 DOI: 10.1039/D2MD00246A [99] with the permission of the Royal Society of Chemistry.

Author contribution: Conceptualization was performed by FMB. JS and MC carried out and analyzed $^1\text{H}/^{15}\text{N}$ -HSQC experiments. MD and MK performed the STD experiments, JS and MD analyzed them. TK performed and analyzed the DSF studies. MS performed the GSH assays. SJ and ML performed and analyzed the ESI-MS experiments. Mutagenesis was done by LE. Crystallographic data reduction and refinement was performed by JS, MB, and TS. FMB, JS, TK, and MS wrote the final manuscript.

3.3.1 Introduction: Halogen Bonding and HEFLib

Halogen bonds represent highly directional, non-covalent interactions of the type $\text{R-X}\cdots\text{Y-R}'$, whereby the halogen X functions as a Lewis acid and Y represents a Lewis base or π -electron system [168, 169]. Halogen bonds are mediated by the σ -hole, which is a positively charged region of the halogen along the R-X bond axis. This is due to the anisotropy of the charge distribution on the halogen [168, 170]. The atoms Cl, Br, and I have an $s^2p_x^2p_y^2p_z^1$ configuration (where z is oriented along the R-X axis) with a deficiency of electrons along the z-axis, the so-called σ -hole [169]. The magnitude of the σ -hole is described by the V_{max} value as the maximum electrostatic potential (ESP) value on the electron isodensity surface [171, 172]. The σ -hole allows the interaction of the halogen with nucleophiles, such as the backbone carbonyl oxygen [173], nitrogen or oxygen atoms of electron rich side chains (e.g., aspartate, glutamate) [174], serine, histidine [175], and the sulfur in methionine side chains [176, 177]. The deficiency of the electron density is compensated by an electron-rich belt around the halogen, which enables the interaction with electrophiles [168, 169]. One factor that influences the strength of a halogen bond is the type of halogen involved. Fluorine shows no positively charged σ -hole due to its considerable electronegativity.

As the size of the halogen atom increases from chlorine to iodine, the electronegativity decreases, while the size of the σ -hole increases [178-180]. Other parameters that determine the strength of the interaction are the distance between the halogen and the Lewis base, which is a consequence on the prevailing electrostatic nature of the interaction, as well as the σ -hole angle $C_{ar}-X\cdots Y$ (ideally 180°) [179]. The smaller the σ -hole angle, the smaller the range of the tolerated distance [168]. A tuning of the halogen bond is achieved by the incorporation of electron-withdrawing substituents into a preexisting scaffold [171, 181]. Another way of increasing the strength of a halogen bond is the replacement of the halogen itself by a higher-order halogen ($Cl < Br < I$). In addition, the strength of the bonding can be increased by an exchange of the halogen-bearing scaffold [182, 183]. Although the halogen bond is a weak interaction, it can be a useful tool in medicinal chemistry to increase the affinity, specificity, and permeability of compounds and to modulate protein-ligand interactions [168, 184].

Fragment-based drug discovery (FBDD) has gained attention in the early stages of the drug discovery process [185, 186]. In comparison to the classical high-throughput screening (HTS), smaller libraries with a reduced number of compounds are used to efficiently cover a vast amount of chemical space. This allows the identification of weakly binding compounds with diverse possible binding modes [187-189]. In drug discovery, halogen bonding is increasingly used to exploit the unconventional binding mode for increased selectivity and affinity of a hit or lead fragment [190-193]. A fragment-based approach can be utilized to incorporate halogen bonds early in the drug discovery process [194]. The geometric demands of a halogen bond as a high directional molecular interaction provide a considerable challenge when introduced at a late stage of the drug discovery process with already established key interactions between ligand and target [192, 194, 195].

The initial concept of a halogen-enriched fragment library was first applied to identify p53-Y220C mutant stabilizers. The 2,4-diiodophenol derivative PK784 was detected as a cavity binder, forming a halogen bond with the backbone carbonyl of Leu145 [98]. The extension of this concept was the design of a diversity-optimized HEFLib [194]. The library is characterized by diverse binding motifs, including an extensive number of halogen atoms, to identify unconventional binding modes mainly driven by halogen bonds [194, 196]. The applicability of the diversity-optimized HEFLib is not focused on a specific target and leads to unconventional binding modes for various protein

targets [195, 196]. However, the number of iodine-containing compounds in the HEFLib is limited due to availability constraints and restrictive cost filtering. As a result, only 14 of the 191 compounds contain a strongly tuned iodine [194]. Tuning effects have a significant influence on the σ -hole strength and were considered in the selection process of the diversity-optimized HEFLib [168, 171, 181, 194]. Nevertheless, a halogen atom attached to an electron-deficient heteroarene decorated with electron-withdrawing groups (EWGs) can also act as a leaving group (LG) in nucleophilic aromatic substitution (S_NAr) reactions. Therefore, a stronger halogen bond tuning could in principle result in covalent reactive fragments with reduced chemical stability [196, 197].

The diversity-optimized HEFLib was tested with T-p53C-Y220C to identify new hits, including unconventional binding modes for the challenging binding site. The HEFLib was screened using DSF and saturation transfer difference nuclear magnetic resonance (STD NMR) as primary screening techniques. The hits were validated by $^1H/^{15}N$ -HSQC-NMR. Cleft binders, covalent reactive compounds targeting surface-exposed cysteines, and fragments binding to an uncharacterized binding site of the protein were identified. The chemical stability of the fragment hits was analyzed by glutathione (GSH) reactivity assay. The covalent modification of the T-p53C-Y220C protein was confirmed by UHPLC-ESI-MS. Finally, a crystal structure of fragment 1151 targeting the Y220C binding pocket was obtained.

It should be noted that this thesis focuses on the DSF hits and intact protein MS results of the HEFLib screen. I performed the DSF measurements and was involved in the sample preparation and evaluation of the UHPLC-ESI-MS experiments.

3.3.2 DSF Screen of the HEFLib with T-p53C-Y220C

3.3.2.1 Primary DSF Screen

The DSF was used as the primary screening method for the HEFLib. DSF measures the temperature-dependent unfolding of the protein by monitoring an increment in the fluorescence of a dye that binds to the hydrophobic parts of a protein, which become solvent-exposed as the protein denatures [198]. The temperature at which the protein unfolds is determined by its stability, which is directly related to the Gibbs free energy of unfolding (ΔG_u) [198, 199]. ΔG_u is temperature-dependent; as the temperature increases, ΔG_u decreases. It becomes zero when the concentrations of folded and unfolded protein are equivalent, and the temperature at this point is defined as the melting temperature of the protein (T_m). Upon ligand binding to the protein, the free energy contribution of the ligand-protein interaction usually leads to an increase in ΔG_u , which in turn can lead to an increase in T_m [198]. It was found that there is a close correlation between ligand binding and stabilization of the protein [192, 198, 200, 201].

The Y220C mutation is a structural temperature-sensitive mutation, therefore the thermal stabilization and increase in melting temperature of the protein was a primary readout. The influence of the fragments on the T_m was directly measured by DSF. The results of the DSF screen of the HEFLib compounds with T-p53C-Y220C at a protein-to-compound ratio of 1:125 after 30 min of incubation are shown in Table 3.2. A compound was considered a hit if the shift in melting temperature (ΔT_m) was at least 0.50 °C [130], corresponding to a significant change in the T_m ($p < 0.05$). Compounds that lowered the melting temperature of T-p53C-Y220C were not considered, as the Y220C mutant is already thermosensitive and should not be further destabilized.

Table 3.2: ΔT_m values and standard deviations (SDs) of the DSF screen of T-p53C-Y220C (8 μ M) with compounds of the HEFLib (1 mM) after 30 min of incubation. Measurements were performed at least in triplicate. Hits with a minimum ΔT_m of 0.50 $^{\circ}$ C are highlighted in bold.

Barcode	Simplified Molecular Input Line Entry Specification (SMILES)	ΔT_m [$^{\circ}$ C]	SD [$^{\circ}$ C]
B02289594	<chem>Clc1c(ccc(c1)Cl)C(N)CCO</chem>	0.05	0.07
B02289595	<chem>Clc1ncnc2c1[nH]cc2</chem>	0.00	0.12
B02289596	<chem>Brc1c(n[o]c1C)C</chem>	0.05	0.12
B02289597	<chem>Clc1nc(ccc1)C(=O)O</chem>	0.05	0.14
B02289599	<chem>B(O)(O)c1c(nc(cc1)Cl)F</chem>	-0.55	0.12
B02289600	<chem>lc1c(nccc1)N</chem>	0.10	0.12
B02289601	<chem>Brc1[nH]cc(n1)[N+](=O)[O-]</chem>	0.15	0.19
B02289602	<chem>Clc1cnc(cc1)C(=O)O</chem>	-0.05	0.14
B02289603	<chem>Brc1c(c(ccc1)N)O</chem>	0.15	0.12
B02289604	<chem>Clc1cncc(c1)C(=O)O</chem>	0.05	0.12
B02289605	<chem>Brc1n[nH]c2c1cc(cc2)N</chem>	-0.50	0.07
B02289606	<chem>Clc1ncc(cc1)CO</chem>	-0.05	0.07
B02289607	<chem>lc1c(cncc1)N</chem>	0.00	0.14
B02289608	<chem>lc1c(nc(nc1)N)OC</chem>	-0.50	0.19
B02289609	<chem>Brc1n[o]c(c1)CN.Cl</chem>	0.15	0.07
B02289610	<chem>Brc1c2[n](nc1)C=CC=N2</chem>	0.15	0.07
B02289611	<chem>Clc1nc(cnc1)C(=O)O</chem>	0.10	0.19
B02289612	<chem>Brc1cnc(c(c1)C(=O)O)N</chem>	0.05	0.14
B02289613	<chem>Clc1nc(c[o]1)C(=O)OCC</chem>	0.05	0.12
B02289614	<chem>Brc1c(nccc1)C(=O)O</chem>	0.20	0.07
B02289615	<chem>Clc1c(cc(cc1)C(=O)O)O</chem>	-0.05	0.19
B02289616	<chem>Clc1nc(ccc1N)C</chem>	0.05	0.07
B02289617	<chem>Brc1[n]2c(nc1)C=NC=C2</chem>	-0.05	0.07
B02289618	<chem>Brc1c(ncnc1O)O</chem>	-0.10	0.12
B02289619	<chem>Brc1c(nccc1)C</chem>	0.25	0.12
B02289620	<chem>Brc1n[n](cc1)C</chem>	0.25	0.00
B02289621	<chem>Clc1ncnc(c1Cl)N</chem>	0.10	0.21
B02289809	<chem>Brc1cncnc1</chem>	0.10	0.07
B02290087	<chem>Brc1cnc(nc1)N2CCNCCC2</chem>	0.25	0.14
B02290088	<chem>Clc1c(ccc(c1)OC)CN</chem>	0.10	0.25
B02290089	<chem>FC(F)(F)c1cnc(c(c1)Cl)N(N)C</chem>	-0.20	0.12
B02290090	<chem>Clc1c(c(cc(c1)Cl)CN)N</chem>	0.20	0.12
B02290091	<chem>[s]1nc(c(c1Cl)Cl)C(=O)O</chem>	-0.10	0.12
B02290112	<chem>lc1cc(c(cc1)OC)CNCCO</chem>	-0.10	0.19
B02290113	<chem>Brc1c[n](nc1)c2[nH]nnn2</chem>	-0.20	0.07
B02290114	<chem>Brc1c[n](nc1)C(C)C(=O)O</chem>	-0.15	0.12
B02290115	<chem>Brc1c[nH]c(c1)C(=O)N2CCOCC2</chem>	0.05	0.14
B02290116	<chem>Brc1c([n](nc1)C)C(=O)O</chem>	-0.05	0.14
B02290117	<chem>Clc1c[n](nc1)CCCN.Cl</chem>	-0.10	0.07
B02290118	<chem>Clc1n[nH]c(n1)C(=O)N</chem>	0.00	0.12
B02290119	<chem>Brc1n[nH]c(n1)CCC(=O)O</chem>	0.00	0.12
B02290120	<chem>Brc1n[n](c(n1)NN)C</chem>	0.00	0.12
B02290121	<chem>Clc1ncc(cc1)CN2CCNCCC2.Cl.Cl</chem>	-0.10	0.07
B02290122	<chem>[s]1c(cc(c1)Br)CN2CCNCCC2.Cl.Cl</chem>	-0.20	0.14
B02290123	<chem>Clc1c([n](nc1[N+](=O)[O-])CC(=O)O)C</chem>	0.05	0.07

B02290401	<chem>Brc1ccc(cc1)C(=O)C32CN4CN(CN(C4)C3)C2</chem>	0.00	0.00
B02290402	<chem>[S](=O)(=O)(CCO)c1n[nH]c(n1)Cl</chem>	0.00	0.00
B02290403	<chem>Brc1n[n](c(n1)C(=O)N)COC</chem>	0.05	0.19
B02290404	<chem>Brc1n[n](c(n1)O)COC</chem>	-0.10	0.31
B02290407	<chem>[s]1c(ncc1Br)C2OCCO2</chem>	-0.05	0.07
B02290408	<chem>Clc1cnc(cc1)NC(=O)C(=O)O</chem>	0.05	0.21
B02290459	<chem>lc1c([nH]nc1C)C</chem>	-0.05	0.12
B02290460	<chem>Clc1cnc(c(c1)[N+](=O)[O-])O</chem>	0.30	0.07
B02290461	<chem>[s]1c(ncc1Br)N.Br</chem>	0.15	0.14
B02290462	<chem>Brc1[n]2c(nn1)CNCC2.Cl</chem>	-0.55	0.44
B02290463	<chem>Brc1c[nH]nc1C(=O)O</chem>	0.15	0.07
B02290464	<chem>Brc1c([nH]nc1)C#N</chem>	0.20	0.07
B02290465	<chem>ClC1=CC(=O)N(C(=O)N1C)C</chem>	-0.15	0.12
B02290466	<chem>Clc1[o]c(cc1)C(=O)O</chem>	0.00	0.07
B02290467	<chem>Clc1c2c(ccc1)C(=O)C(=O)N2</chem>	0.15	0.07
B02290468	<chem>Clc1cnc2nn[nH]c2c1</chem>	-0.50	0.07
B02290469	<chem>Brc1[n]2c(nc1)C=CC=N2</chem>	0.00	0.21
B02290470	<chem>Clc1nnc(cc1C(=O)O)Cl</chem>	0.00	0.07
B02290471	<chem>Clc1c(c(ccc1O)Cl)N.Cl</chem>	-0.50	0.25
B02290472	<chem>Clc1c(ncnc1C(=O)O)N</chem>	-0.15	0.00
B02290473	<chem>lc1c(cncc1)F</chem>	0.10	0.12
B02290474	<chem>Clc1nc2nc([nH]c2cc1)O</chem>	-0.10	0.12
B02290475	<chem>Brc1cnc(c(c1)CO)N</chem>	0.10	0.00
B02290476	<chem>Clc1nc2nc[nH]c2c(n1)N</chem>	-0.05	0.07
B02290477	<chem>[s]1cnc(c1)Cl</chem>	0.05	0.07
B02290481	<chem>Clc1c[n](nc1)CC(C)C(=O)O</chem>	0.05	0.00
B02290482	<chem>Brc1cnc2[nH]nnc2c1</chem>	0.05	0.12
B02290483	<chem>Clc1c(cccc1)C(N2CCN(CC2)C)CN</chem>	0.25	0.19
B02290521	<chem>lc1c(n[o]c1C)N</chem>	-0.05	0.07
B02290522	<chem>[S](=O)(=O)(N)c1c([n](nc1C)C)Cl</chem>	0.30	0.12
B02290523	<chem>Brc1cnc(c(c1)C(=O)O)O</chem>	-0.25	0.19
B02290524	<chem>ClC1=CC(=O)N(C(=O)N1)C</chem>	0.10	0.07
B02290525	<chem>Fc1c(c(ccc1C)Cl)CN</chem>	0.05	0.07
B02290658	<chem>lc1c(c(ncc1)F)C(=O)O</chem>	0.00	0.00
B02290659	<chem>Clc1c(nccc1Cl)C(=O)O</chem>	0.00	0.00
B02290660	<chem>[S](=O)(=O)([n]1nccc1Br)N(C)C</chem>	0.10	0.07
B02290661	<chem>Clc1ncnc2[nH]cc(c21)C(=O)C</chem>	0.05	0.14
B02291100	<chem>Brc1c([o]cc1O)O</chem>	-0.10	0.07
B02291101	<chem>Brc1c(nc(nc1O)O)N</chem>	-0.35	0.39
B02291102	<chem>Clc1nc(nc(c1Cl)C)N</chem>	-0.65	0.25
B02291150	<chem>Brc1c([n](nc1)CC)C(=O)N</chem>	0.30	0.24
B02291151	<chem>FC(F)(F)c1[nH]nc(c1Br)N</chem>	0.80	0.07
B02291152	<chem>Clc1c[n](nc1C)CCN</chem>	0.00	0.12
B02291153	<chem>Clc1cc(c(cc1)O)\C=C\C32CN4CN(CN(C4)C3)C2</chem>	0.15	0.00
B02291154	<chem>Clc1c[n](nc1C(=O)O)C[n]2nccc2</chem>	-0.10	0.07
B02291155	<chem>Brc1c([n](nc1)CC)CNC</chem>	0.00	0.00
B02291156	<chem>Brc1c[n](nc1N)CC(=O)O</chem>	-0.05	0.07
B02291212	<chem>Brc1c2c(ccc1)CNC2.Cl</chem>	-0.10	0.07
B02291213	<chem>[S](=O)(=O)(C)c1c(ccc(c1)[S](=O)(=O)C)Cl</chem>	0.00	0.07
B02291214	<chem>FC(F)(F)c1n[n](c(c1CO)Cl)C</chem>	0.15	0.14
B02291215	<chem>[s]1nc(c(c1C(=O)O)Cl)Cl</chem>	0.00	0.19

B02291216	Clc1[nH]c2c(n1)N(C(=O)N(C2=O)C)C	-0.25	0.07
B02291217	Clc1c(c2n[o]nc2cc1)N	-0.45	0.19
B02291218	Clc1c([n](nc1C(=O)O)C)C	-0.20	0.12
B02291219	FC(F)(F)c1cnc(c(c1)Cl)N	-0.10	0.14
B02291220	Brc1n[nH]c(n1)Cl	0.00	0.07
B02291221	Clc1c[nH]c(c1)C(=O)O	-0.10	0.21
B02291222	Clc1nc2c(cc1C(=O)N)CCC2	0.20	0.12
B02291223	[S](=O)(=O)(C)c1nc(c(c1)Cl)C(=O)O	2.00	0.07
B02291224	Clc1ncccn1	-0.15	0.07
B02291225	Clc1c[n](nc1)CC(OCC)OCC	-0.40	0.21
B02291226	S1CCC(c2c1ccc(c2)Cl)N	-0.50	0.14
B02291227	Clc1nc(ccc1C(=O)N)C	0.20	0.12
B02291228	[s]1c(nc(c1)C(=O)O)Cl	-0.20	0.14
B02291229	ClC1=CC=Cc2[n]1nnn2	-0.40	0.24
B02291230	Clc1ncnc(c1N)NC2CC2	-0.05	0.14
B02291231	Brc1n[nH]c(n1)O	-0.35	0.12
B02291232	Brc1cnc(cc1)N	0.10	0.14
B02291233	S1c2[n](c(c(n2)Cl)CO)C=C1	0.25	0.14
B02291234	ClC1=C(C(=O)N(C(=O)N1C)C)C#N	-0.45	0.07
B02291235	Clc1cc2c(cc1CO)OCO2	0.25	0.07
B02291236	[S](=O)(=O)(N2CCNCC2)c1[s]c(cc1)Cl.Cl	-0.45	0.07
B02291237	[s]1nc(nc1Cl)C(C)C	-0.05	0.28
B02291238	lc1c(ncc(c1)F)N	-0.55	0.32
B02291239	Clc1nccc(c1)C(=O)O	0.10	0.07
B02291240	Clc1nccnc1	0.30	0.14
B02291241	[s]1c(ccc1CN2CCNCC2)Cl	-0.70	0.12
B02291242	Clc1c2c(cc(c1)CN)OCCCO2.Cl	-0.30	0.25
B02291243	Clc1c([n](nc1C)CC(=O)O)C	-0.25	0.24
B02291244	Clc1cc(c(c(c1)N)O)[N+](=O)[O-]	-0.15	0.07
B02291245	Brc1cnc(cc1)C(=O)O	0.05	0.12
B02291246	Clc1nc(cc(n1)OC)OC	0.15	0.07
B02291247	Clc1c(cccc1)[C@@H](O)C(=O)O	0.05	0.12
B02291248	Brc1c(nc(nc1)O)O	0.15	0.14
B02291249	Clc1ncc(cc1)CC(=O)O	0.30	0.07
B02291250	lc1c([n](nc1C)CCO)N	0.05	0.12
B02291251	Clc1ncc(nc1)C(=O)O	0.20	0.00
B02291252	Brc1n[n](c(c1C(=O)NCC(=O)O)N)C	-0.10	0.12
B02291253	[S]1(=O)(=O)CCC(=O)c2c1ccc(c2)Cl	0.20	0.07
B02291254	Clc1ncnc2[nH]ccc21	-0.25	0.07
B02291255	Clc1c2c(ccc1)NC(=O)C2=O	0.30	0.07
B02291256	Clc1nc(cnc1)[n]2nccc2	0.00	0.07
B02291257	Clc1c(ccc(c1)C#N)O	0.20	0.00
B02291258	Clc1ncccc1C(=O)O	0.10	0.14
B02291259	Clc1c(ccc(c1)N)C(=O)O	-0.25	0.07
B02291260	Clc1c(cc(cc1)N)C(=O)O	-0.20	0.00
B02291261	Brc1cncc(c1)C(=O)O	-0.05	0.00
B02291262	lc1c(nccc1)F	-0.15	0.14
B02291263	[S](=O)(=O)(N)c1c(ccc(c1)C(=O)O)Cl	-0.15	0.07
B02291264	Brc1c[nH]c(c1)C(=O)O	0.15	0.07
B02291265	Clc1n[nH]c2c1cccc2	-0.15	0.14
B02291266	Brc1[nH]c2c(n1)N(C(=O)N(C2=O)C)C	-0.15	0.07

B02291267	Clc1ncc(cn1)Cl	-0.20	0.12
B02291268	Ic1c(c(cc(c1)CO)OC)O	0.10	0.07
B02291269	Clc1ccncc1.Cl	-0.20	0.00
B02291270	Clc1ccc(cc1)C(N)CC	0.00	0.07
B02291271	Clc1nccc(c1)C#N	-0.10	0.07
B02291272	Brc1n[o]c(n1)C2CC2	0.25	0.19
B02294481	Clc1c2c(cc(c1)NC)OCO2.Cl	0.10	0.07
B02294482	FC(F)(F)c1cnc(nc1)Cl	0.50	0.07
B02294483	Brc1c([nH]nc1C)C(=O)O	-0.10	0.12
B02294484	Brc1c([nH]nc1[N+](=O)[O-])C(=O)O	-0.30	0.07
B02294485	Clc1ccc(cc1)O	0.00	0.07
B02294486	Clc1[n]2c(nc1)C=CC=C2	-0.05	0.07
B02297394	Clc1cnc(c(c1)OC)OC	-0.15	0.12
B02297395	Brc1c(nc(cc1)N)C(=O)O	-0.35	0.07
B02297396	Brc1cnc(c(c1)C(=O)O)OC	-0.15	0.19
B02297397	Fc1c(cc(c(c1)C2NCCNC2)Cl)Cl	0.00	0.00
B02297398	[s]1c(c(cc1)Cl)C(=O)O	-0.40	0.12
B02297399	B(O)(O)c1c(cnc1)Br	-0.15	0.00
B02297400	Clc1ccc(cc1)C(=O)N2CCOCC2	0.00	0.21
B02297401	Brc1c(cccc1O)O	0.10	0.07
B02297402	Clc1nc(cnc1C)C	-0.55	0.42
B02297403	Brc1c(cnc1)C(=O)O	0.25	0.19
B02297404	Brc1cnc(cc1)N2CCN(CC2)C	0.20	0.07
B02297405	Clc1c(ncc(c1C)Cl)N	0.20	0.07
B02297406	Brc1cnc(cc1N)C.Cl	-0.25	0.24
B02297407	[s]1c(c(cc1C(=O)OC)N)Cl	-0.25	0.21
B02297408	[s]1c(ccc1C2NCCNC2)Br	0.10	0.25
B02297409	Brc1c2nc[nH]c2ccc1N	-0.10	0.12
B02297410	Clc1ncnc(c1)N2CCC(CC2)O	-0.20	0.25
B02297411	Clc1cncc(c1C(=O)O)Cl	-0.15	0.21
B02297412	Clc1nc(nc(c1NC=O)Cl)N	0.00	0.07
B02297413	Brc1c(cc(c(c1)C(=O)O)O)O	0.15	0.00
B02297414	[s]1nc(c(n1)N2CCOCC2)Cl	0.00	0.21
B02297415	Brc1c[n](nc1N)C	-0.05	0.07
B02297416	Clc1c(nc(cc1)NC(=O)C)C(=O)O	-0.35	0.28
B02297417	Clc1cc(ccc1)C(O)C(=O)O	-0.25	0.07
B02297418	Clc1c(ncc(c1)Cl)C(=O)O	0.05	0.19
B02297419	Clc1cnc(c(c1)C(=O)O)O	0.05	0.07
B02297420	Ic1c(nc(nc1C)O)O	-0.05	0.14
B02297421	Clc1cc[n+](cc1)[O-]	0.30	0.12
B02297422	Brc1cnc(nc1)N2CCNCC2	-0.05	0.19
B02297423	Clc1c(ncc(c1)C(=O)O)OC	-0.20	0.00
B02297424	Brc1c(cnc1)N	0.00	0.21
B02297425	Clc1cnc(nc1)O.Cl	-0.20	0.07
B02297426	Brc1cnccc1C(=O)O	-0.15	0.12
B02297427	Clc1cnc(cc1O)O	0.00	0.12
B02297428	Clc1nc(ccc1C(=O)O)C	-0.15	0.12
B02297429	Clc1cnc(c(c1)C(=O)O)OC	-0.20	0.12
B02297655	Brc1c(ncc(c1)C(=O)O)N	0.00	0.12
B02297765	Fc1c(cc(cc1)Cl)[S](=O)(=O)CC(=O)C	0.00	0.12
B02297766	Ic1c([n](nc1C)C)C(=O)NN	0.05	0.12

In total, three hits were identified by DSF: compounds 1151, 1223, and 4482 (Figure 3.17). For readability reasons, the compound identification numbers are reduced to the last four digits of the HEFLib barcodes. The determined ΔT_m values of the identified fragment hits, in addition to the STD NMR, $^1\text{H}/^{15}\text{N}$ -HSQC-NMR, and GSH results are depicted in Table 3.3.

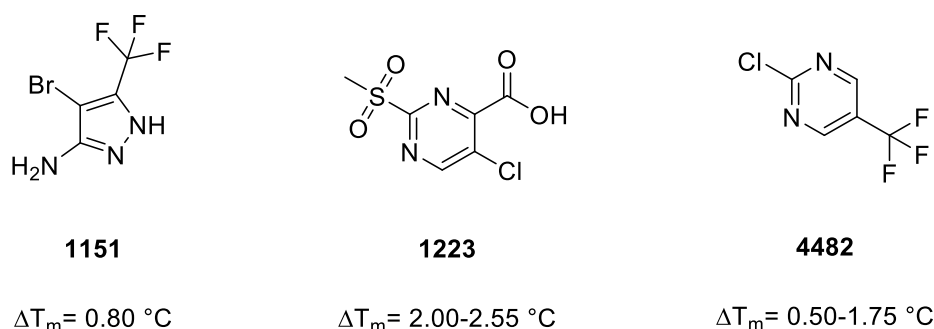


Figure 3.17: Overview of the HEFLib hits identified by the primary DSF screen. The identification numbers of the compounds are reduced to the last four digits of the HEFLib barcodes for readability.

Table 3.3: All hits of the HEFLib identified by the DSF screen. A fragment was considered as a DSF hit if the ΔT_m was $\geq 0.50\text{ }^\circ\text{C}$. For the STD experiment, a protein-to-compound ratio of 1:50 was applied. STD NMR data of the NMR inactive fragment 1151 are not available (N/A). Additional $^1\text{H}/^{15}\text{N}$ -HSQC-NMR studies were performed with the DSF hits, and significant peak shifts were evident. Furthermore, the chemical stability of the compounds was evaluated by GSH stability assay.

Compound	$\Delta T_m\text{ [}^\circ\text{C]}$	STD	HSQC	$t_{1/2}$ GSH [h]
1151	0.80	N/A	Yes	>100
1223	2.00-2.55	Yes	Yes	0.81
4482	0.50-1.75	No	Yes	2.80

3.3.2.2 Fragment Hit 1151

As shown in Figure 3.18, HEFLib compound 1151 was identified as a hit using DSF. The compound significantly stabilized T-p53C-Y220C by about $0.80 \pm 0.07\text{ }^\circ\text{C}$ at a protein-to-compound ratio of 1:125. Fragment 1151 is NMR inactive due to the lack of a C-H bond. Thus, no STD screening could be conducted. In addition, the reactivity of the fragment hit 1151 was investigated by GSH stability studies, revealing a $t_{1/2}$ of >100 h, indicating that the fragment is chemically stable. As a fragment with a half-life of >20 h was defined to be sufficiently stable for non-covalent fragment screening [196], this fragment meets the stability requirements. Moreover, the isothermal titration calorimetry (ITC) experiment of compound 1151 with T-p53C-Y220C did not yield a

binding curve. Therefore, fragment 1151 exhibited a binding affinity lower than the detection limit of about 1 mM in the ITC analysis. However, despite having a high dissociation constant (K_D) value (>1 mM), fragment 1151 significantly increased the melting temperature of the T-p53C-Y220C protein by about 0.80 °C. Higher concentrations of compound 1151 could not be studied by DSF due to the strong decrease in fluorescence intensity at compound concentrations >1 mM. Reduced fluorescence signal intensity can result from interactions of the compound with the optical properties of the fluorescent dye SYPRO orange, leading, for example, to partial fluorescence quenching [114, 198].

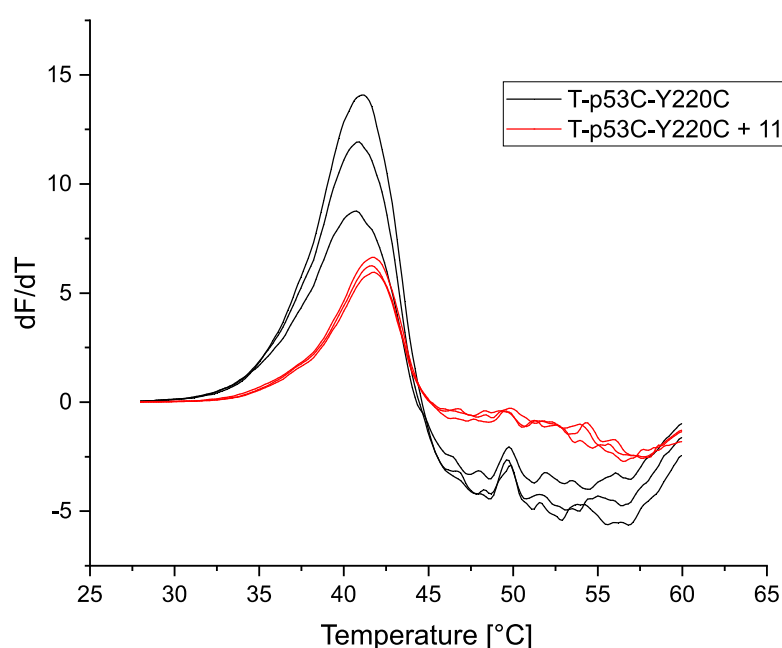


Figure 3.18: DSF measurements of T-p53C-Y220C with 1151. The first derivatives of the melting curves of T-p53C-Y220C (8 μ M) and T-p53C-Y220C (8 μ M) with compound 1151 (1 mM) after 30 min of incubation at 20 °C are shown.

3.3.2.3 Fragment Hit 1223

The compound 1223 was later integrated into the HEFLib and corresponds to the covalent reactive and p53 arylating compound PK11000. The 2-sulfonylpyrimidine PK11000 modifies especially the surface-exposed cysteines 182 and 277 of p53, resulting in a time-dependent increase in the melting temperature by up to 2.50 °C and achieving a general stabilization of the protein [131]. Hence, PK11000 was included in the screening process as a positive control for a fragment that undergoes a chemical reaction with p53 to establish a covalent bond.

3.3.2.4 Fragment Hit 4482

The 2-chloropyrimidine 4482 is a reactive fragment with a half-life of 2.80 h in the GSH stability assay. This reactivity enables covalent binding to nucleophiles, mainly cysteines, in p53 [196]. Covalent fragments exhibit a time-dependent reaction behavior, with increasing target occupancy as the incubation time increases [144]. Thus, time-dependent DSF measurements were performed in addition to the primary DSF screen with a 30-minute incubation period. The results of the DSF analysis indicated a gradual increase in the melting temperature of T-p53C-Y220C. Specifically, the ΔT_m values increased from 0.50 °C after 30 min, to 0.90 °C after 4 h, and up to 1.75 °C after 24 h of incubation at 20 °C (Figure 3.19, Table 3.4). However, the maximum stabilizing effect of compound 4482 (ΔT_m by up to 1.75 °C) was less pronounced compared to the 2-sulfonylpyrimidine PK11000 (ΔT_m by up to 2.50 °C).

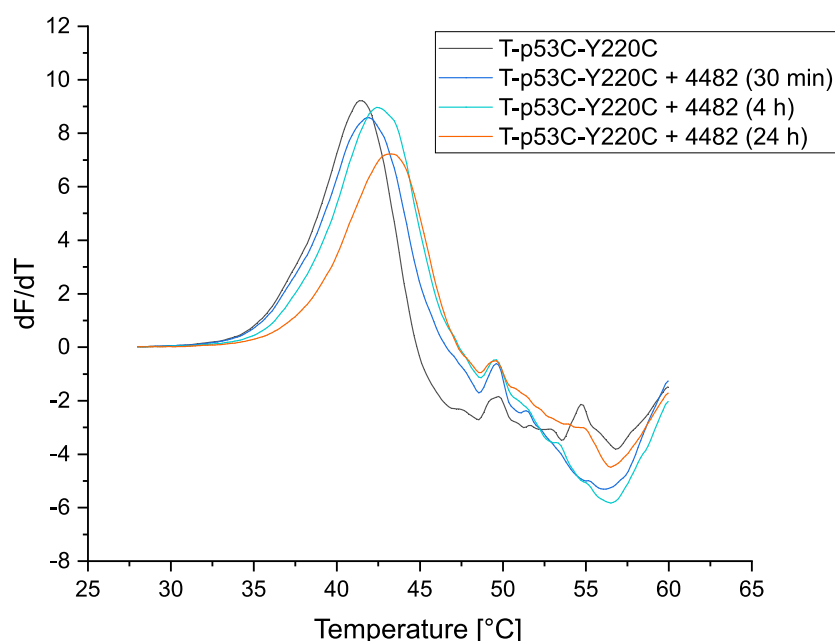


Figure 3.19: DSF measurements of T-p53C-Y220C with 4482. The first derivatives of the melting curves of T-p53C-Y220C (8 μ M) and T-p53C-Y220C (8 μ M) with compound 4482 (1 mM) after 30 min, 4 h, and 24 h of incubation at 20 °C are presented.

Table 3.4: $\Delta T_m \pm$ SD of T-p53C-Y220C (8 μ M) with 1 mM 4482 after 30 min, 4 h, and 24 h incubation time at 20 °C.

	30 min	4 h	24 h
$\Delta T_m \pm$ SD [°C]	0.50 ± 0.07	0.90 ± 0.07	1.75 ± 0.24

3.3.3 Intact Protein Mass Spectrometry of T-p53C-Y220C with 4482

The covalent reaction mechanism of the HEFLib hit 4482 and the modification of T-p53C-Y220C were confirmed by UHPLC-ESI-MS. Compound 4482 is an electron-deficient heteroarene with an electron-withdrawing CF₃ moiety and a chlorine atom as the LG. It reacts with proteinogenic nucleophiles via an S_NAr reaction. One molecule of 4482 covalently bound to a cysteine corresponds to an increase in protein mass of 146 Da, as shown in Figure 3.20.

In the deconvoluted MS spectrum (Figure 3.20), multiple arylations of T-p53C-Y220C were detected after 4 h of incubation with 4482 (protein-to-compound ratio of 1:125). Up to three molecules were attached to the protein, with the two times modified protein species as the major peak. No unmodified protein peak was visible in the deconvoluted MS spectrum.

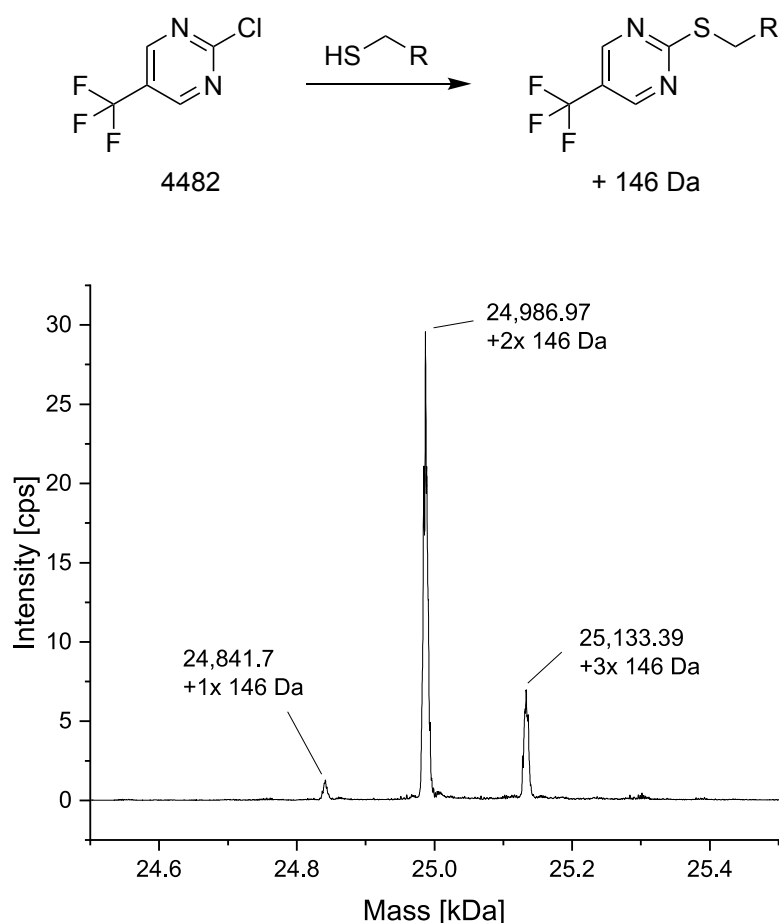


Figure 3.20: S_NAr reaction mechanism of fragment 4482 and deconvoluted MS spectrum of T-p53C-Y220C (50 μM) with 4482 (6.25 mM) after 4 h of incubation at 20 °C (theoretical mass of unmodified T-p53C-Y220C: 24,696.00 Da).

Furthermore, the triple cysteine control mutant T-p53C-C124/182/277S, which has mutations of the solvent-exposed cysteines 124, 182, and 277 to serine residues, was also tested with compound 4482 using UHPLC-ESI-MS [117, 118, 131]. The corresponding deconvoluted MS spectrum after 4 h of incubation at a protein-to-compound ratio of 1:125 is depicted in Figure 3.21. The MS spectrum showed unmodified protein and a main peak for the single arylated protein species. In comparison, the MS spectrum of the T-p53C-Y220C protein with compound 4482 showed up to three times modified protein. Therefore, it can be assumed that covalent binding of fragment 4482 to the cysteines 124, 182, and 277 occurred. However, fragment 4482 did not selectively label the three cysteines in p53. Another residue in p53 was also arylated, as evidenced by the presence of a peak for the protein with one covalently attached molecule in the deconvoluted MS spectrum of the triple cysteine control mutant.

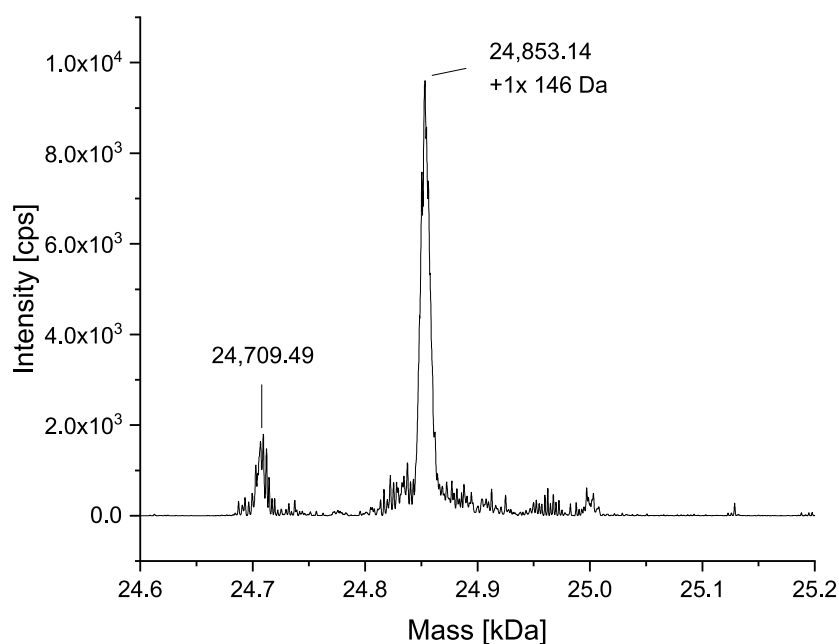


Figure 3.21: Deconvoluted MS spectrum of T-p53C-C124/182/277S (50 μ M) with 4482 (6.25 mM) after 4 h of incubation at 20 $^{\circ}$ C. The highest signal corresponded to the single arylated protein species. Native T-p53C-C124/182/277S was also detected (24,709.49 Da).

The identified fragment hit 4482 has a scaffold similar to the known p53 stabilizer PK11000, and both compounds react via S_NAr with nucleophiles. The compounds 4482 and PK11000 are pyrimidine derivatives with a leaving group at position 2 and EWGs to enhance reactivity. PK11000 has a methyl sulfinic acid as a leaving group and a chlorine in the para position to the LG, which acts as an EWG. In contrast, the chlorine in 4482 serves as the leaving group. In comparison to 4482, PK11000

exhibited higher selectivity for specific cysteine residues in p53, resulting in a maximum of two modified cysteines in the intact protein MS experiment [131]. Moreover, Bauer et al. [131] discovered that the fragment 2-chloro-5-(fluoromethyl)-pyrimidine did not stabilize p53. In contrast, we observed a significant increase in the melting temperature of T-p53C-Y220C with the trifluoro derivative 4482. This indicates that a strong EWG, such as the CF₃ group, in the para position to the leaving group is important for the reaction of 2-chloropyrimidines with cysteines in p53. In conclusion, with the discovery of 4482 and the published fragment PK11000, a general stabilization of p53 was achieved, demonstrating the potential of mutation-independent rescue of p53 by covalent modification of specific cysteine residues.

3.3.4 Additional Results

The DSF hit 1151 was identified as a Y220C cavity binder by ¹H/¹⁵N-HSQC-NMR, with multiple peak shifts common to already published cleft binders (Figure 3.22 1). Finally, a crystal structure of compound 1151 in complex with xT-p53C-Y220C was solved (Figure 3.22 2). The crystal structure (PDB: 8A92) indicated two binding poses, especially in chain A. In chain B, the occupancy was low, and a single pose was built. Similar to the pyrrole ring of compound MB710 [114], the CF₃ moiety of 1151 pointed towards the cleft, displacing the Cys220 and opening the subsite 3 of the binding pocket. Presumably, the binding of fragment 1151 in the pocket was mainly carried by hydrophobic interactions of the CF₃ group. In the crystal structure, the bromine of 1151 pointed towards the subsite 2 of the Y220C pocket or towards the backbone oxygen of Leu145, forming a halogen bond with an angle of 153.4° and a distance of 3.0 Å. In the second pose, the bromine formed an additional orthogonal hydrogen bond with the backbone nitrogen of Thr230. Calculation of the adduct-formation energy (ΔE) at the MP2/TZVPP level of theory and the V_{max} at an isodensity level of 0.02 au revealed that the CF₃ moiety is the main contributor for the tuning of the halogen bond, with an increase in the V_{max} from 0.1597 to 0.1737 au, which is in a similar order of magnitude as iodobenzene with 0.182 au [182]. The amino group of 1151 had only a small effect on the tuning of the halogen bond and could be used as a growing vector or to enhance the tuning through conversion to a tertiary amine [202].

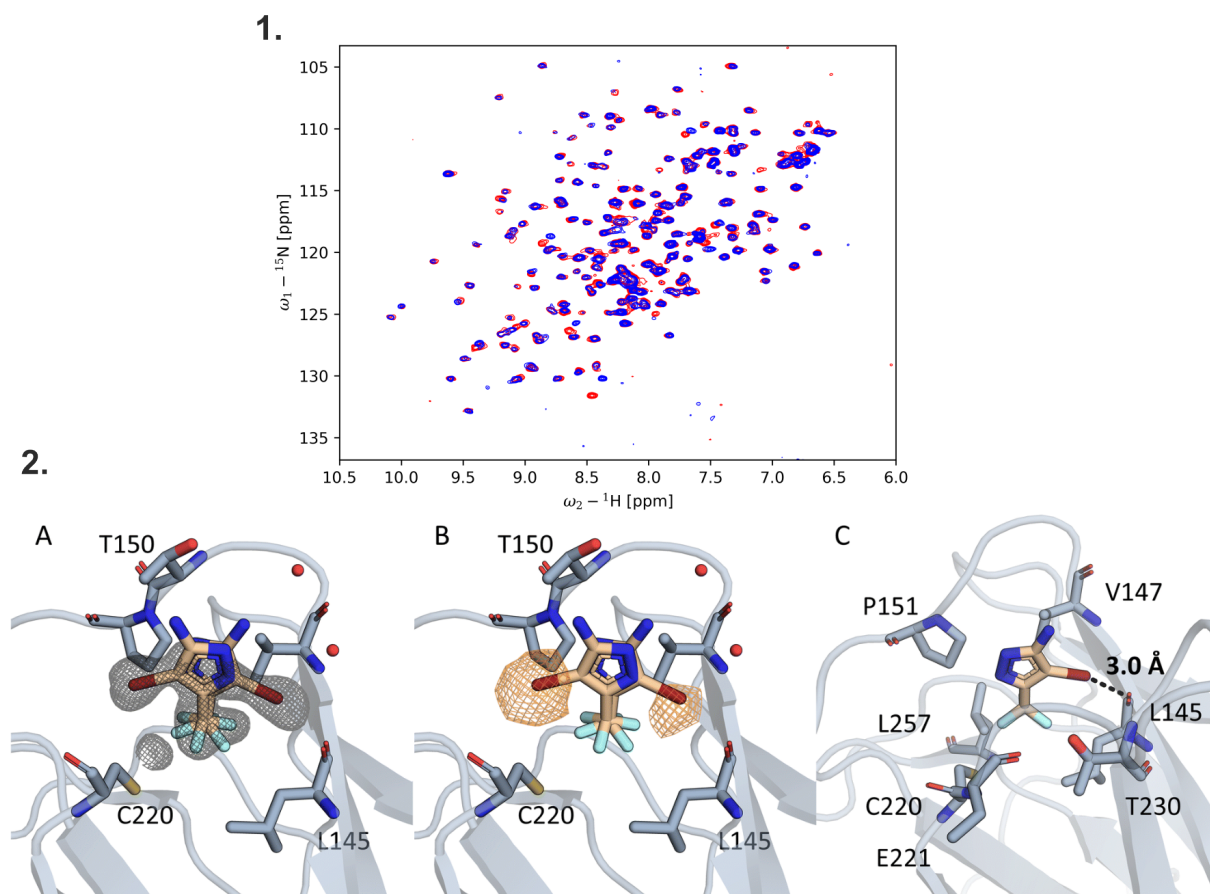


Figure 3.22: Additional results of the HEFLib screen. (1.) $^1\text{H}/^{15}\text{N}$ -HSQC-NMR spectra of T-p53C-Y220C (red) and T-p53C-Y220C (65 μM) with 2 mM 1151 (blue). (2.) (A) Around compound 1151, the electron density is shown in gray as an unbiased omit map with a contour of 3σ . Two different binding poses were visible in chain A, with the CF_3 moiety pointing towards subsite 3 in both cases. (B) The map of the unbiased anomalous difference is plotted in orange at 4.5σ . The bromine adopts two different conformations. (C) The bromine forms a halogen bond with the backbone carbonyl of Leu145, with an angle of 153.4° and a distance of 3.0 Å.

3.4 Covalent Fragment Library (CovLib)

Parts of this Section 3.4 were published or submitted in:

Klett T., Schwer M., Ernst L.N., Engelhardt M.U., Jaag S.J., Masberg B., Knappe C., Lämmerhofer M., Gehringer M., Boeckler F.M., *Evaluation of a Covalent Library of Diverse Warheads (CovLib) Binding to JNK3, USP7, or p53*. Drug Des. Devel. Ther., 2024. **18**: p. 2653-2679. [203]

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Author contribution: FMB envisioned the research. TK, LE, MS, and FMB conceptualized the experiments and designed the study. MS performed the Turbidimetric Solubility Assay, DTNB Assay, and Glutathione Assay. TK and LE prepared the proteins by heterologous expression and performed the DSF studies. SJ, BM, CK, and ML performed and analyzed the UHPLC-ESI-MS experiments. ME performed all computational investigation including the customized fragment filtering process. TK, MS, FMB, and MG selected the final compounds. TK, LE, MS, and FMB wrote the manuscript. ME, SJ, BM, CK, ML, and MG critically reviewed the manuscript.

Klett T., Stahlecker J., Jaag S., Masberg B., Knappe C., Lämmerhofer M., Coles M., Stehle T., Boeckler F. M., *Covalent Fragments Acting as Tyrosine Mimics for Mutant p53-Y220C Rescue by Nucleophilic Aromatic Substitution*.

Submitted

Author contribution: Conceptualization was performed by FMB. TK prepared the protein and performed and analyzed the DSF studies. TK prepared the MS samples. SJ, BM, CK, and ML performed and the UHPLC-ESI-MS experiments. SJ, BM, CK, TK, and ML analyzed the UHPLC-ESI-MS experiments. Mutagenesis was done by TK. JS and MC carried out and analyzed ¹H/¹⁵N-HSQC experiments. Crystallographic data reduction and refinement was performed by JS and TS. FPA experiments were performed by TK. TK, JS, and FMB wrote the final manuscript.

3.4.1 Introduction: Targeted Covalent Inhibitors (TCI)

The so-called targeted covalent inhibitors (TCIs) contain a covalent reactive group (CRG, also called “warhead”), addressing a typically non-catalytic amino acid at the protein of interest [144, 204, 205]. TCIs have gained increasing attention in drug discovery processes in recent years [144, 204-209]. Several TCIs have received FDA approval, such as covalent protein kinase inhibitors (e.g., afatinib, osimertinib, neratinib) [210-212] or the KRAS-G12C inhibitor sotorasib [213, 214].

The development of intrinsically highly reactive covalent drugs has historically been considered challenging due to potential off-target reactions and idiosyncratic toxicity [205, 215, 216]. However, the covalent binding mechanism offers numerous advantages over the classical non-covalent mechanism, such as improved selectivity if properly implemented, (yet time-dependent) potency, and a long duration of action with a possible decoupling of pharmacodynamics from pharmacokinetics [144, 205, 217, 218]. A TCI design that provides a balanced reactivity profile can reduce toxicity and benefit from the covalent mode of action [144, 219].

TCIs with their electrophilic CRGs target nucleophilic amino acid residues, mainly the thiol group of cysteines, which is highly polarizable and a soft nucleophile [145, 220, 221]. Most TCIs contain an attenuated Michael acceptor, typically an acrylamide derivative, as the warhead targeting non-catalytic cysteine residues [222, 223]. Cysteines are amino acids that are rarely found in the proteome [222]. The highly nucleophilic thiolate is formed by deprotonation of the cysteine side chain, which reacts rapidly with electrophiles [144, 224]. The thiol group of cysteines ($pK_a \approx 8.6$) can be more easily deprotonated to its nucleophilic species in contrast to the side chains of lysine and tyrosine ($pK_a \approx 10-10.5$) or serine and threonine ($pK_a \approx 13-15$) [145]. The pK_a is also strongly dependent on the protein environment and can cover a wide range, resulting in varying nucleophilicities and reactivities. In particular, the pK_a of catalytic cysteines is often strongly decreased, resulting in increased nucleophilicity [224, 225].

The binding of the TCI to the target is generally a two-step process involving an initial non-covalent, reversible binding, followed by the (irreversible) formation of a covalent bond between the TCI and the targeted amino acid residue. This process is best described by the second-order rate constant k_{inact}/K_I [226-229]. The first step is characterized by the binding constant K_I . K_I describes the ligand concentration required to achieve a half-maximal rate of covalent bond formation [144]. The second step is

described by the kinetic constant k_{inact} , which indicates the maximum rate of the covalent modification [144, 228, 230, 231]. Therefore, the binding of the TCI to the target is a time-dependent process, and increased incubation time results in increased occupancy and thus potency [144, 213, 228]. For highly reactive compounds, no non-covalent equilibrium may exist, and binding is one step. The binding is then described by the constant k_{chem} [228].

The TCI discovery process involves several strategies. The most popular approach is structure-based design. In this approach, the warhead is attached to an optimized non-covalent ligand at an optimal orientation to the amino acid of interest in the binding pocket [144, 209, 232, 233]. Alternative TCI design techniques include re-design of off-target hits [234, 235], screening of DNA-encoded libraries with covalent inhibitors [236-238] or covalent fragment libraries [197, 209, 232, 239, 240], and virtual screening with covalent docking [145, 241-243].

3.4.2 Design of the Covalent Fragment Library (CovLib)

As revealed by the HEFLib screen, stronger tuning and an increase in V_{max} can result in covalent reactive fragments. The HEFLib compound 4482 achieves general stabilization of the tumor suppressor p53 by covalently modifying surface-exposed cysteines instead of forming σ -hole interactions as intended [99, 196]. Moreover, the covalent reaction mechanism is receiving increasing attention in FBDD. There are several successful covalent FBDD stories for challenging and previously undruggable targets, such as the oncogenic G12C mutant of KRAS [209, 213, 214, 244]. In contrast to the structure-based approach, covalent FBDD does not necessitate the availability of a non-covalent ligand binding near a targetable reactive amino acid residue [209, 232]. Therefore, this strategy is also known as the “electrophile first approach” [204]. Furthermore, the advantages of fragment-based approaches can be exploited by covering a broad chemical space, thereby identifying new binding modes and “cryptic” pockets, as well as addressing new targets [187-189, 209]. Thus, the idea of developing a covalent fragment library (CovLib) was initiated.

For covalent fragment libraries, in addition to the standard FBDD criteria (“rule of three”) [245], further characteristics of the warhead such as reactivity, stability, size, and positioning must be considered [231-233]. The desired warhead properties also depend on the nucleophilicity of the amino acid to be targeted [144, 246] and the protein environment that affects the nucleophilicity [247, 248]. Moreover, the selection

should comprise fragments with a wide range of reactivities and different warheads to maximize diversity in library development [209, 232].

Initially, fragments from six different warhead classes (acrylamide, propiolamide, α -cyanoacrylamide, epoxide, electron-deficient (hetero)arene with leaving group (S_NAr), vinyl sulfone) should be integrated into our CovLib to achieve valuable hit rates with structurally diverse protein targets and to represent different parts of the wide chemical space with the generation of different binding modes, interaction patterns, and reaction geometries. First, fragments with a broad range of reactivities should be included in the CovLib to determine the minimum level of reactivity required to identify hits, as well as to provide a better understanding of the distinct characteristics of the different warhead types. In addition to the various electrophilic groups and reactivities, the solubility and the logarithm of the partition coefficient (logP) of the fragments, which should not be greater than 2, were always considered in the selection process.

For the selection of suitable ligands for the CovLib, a data set of commercially available compounds from Aldrich Market Select (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) was used as a starting point. To begin, the data set was filtered according to the following criteria: removal of all compounds with more than 22 and less than six heavy atoms and a logP >2. For structural filtering of the remaining compounds, SMILES Arbitrary Target Specifications (SMARTS) were generated for the desired warhead classes. The compounds filtered in this way were grouped into clusters with similar structures. Finally, the desired fragments were manually selected according to the estimated reactivities and the structural and steric properties. Price and available quantities of the fragments were also considered.

Due to financial limitations and availability constraints, a set of 20 fragments with four different warhead classes was finally ordered. The CovLib includes five α -cyanoacrylamides/acrylates (CA), three epoxides (EO), four vinyl sulfones (VS), and eight electron-deficient (hetero)arenes with leaving group (S_NAr/SN) warheads.

3.4.2.1 Fragments of the CovLib with an α -Cyanoacrylamide/acrylate (CA) Warhead

α -Cyanoacrylamides are more reactive derivatives of the well-studied acrylamide warhead, potentially resulting in higher hit rates. α -Cyanoacrylamides are dually activated Michael acceptors, reacting in a reversible covalent hetero-Michael addition

with cysteine residues [144, 249, 250]. The electrophilic group displays a high reactivity towards thiol groups, however, due to the α -CH acidity and the destabilization of the neutral covalent adduct, the reverse reaction occurs rapidly and is thermodynamically preferred [144, 251]. Reversible covalent electrophilic groups retain the advantages of irreversible TCIs, such as long duration of action and improved selectivity if properly implemented, but due to the reversible reaction, disadvantages such as permanent modification of off-targets and thus the toxicological risk are reduced [144, 250]. The reactivity of the α -cyanoacrylamide warheads can be modulated by the attachment of electron-withdrawing heteroarenes at the α -position instead of the amide group [249, 252] and by the addition of steric groups at the β -position [249, 250, 253].

Figure 3.23 shows the fragments of the CovLib with an α -cyanoacrylamide/acrylate warhead. Compound CA004 represents an α -cyano- α,β -unsaturated ester (cyanoacrylate) instead of an amide. Acyclic α,β -unsaturated esters show higher reactivity with GSH than acrylamides [249]. All compounds except CA005 are disubstituted at the β -position. Fragments CA001, CA002, and CA003 are *N*-cyclic fragments and represent structure-activity relationship compounds.

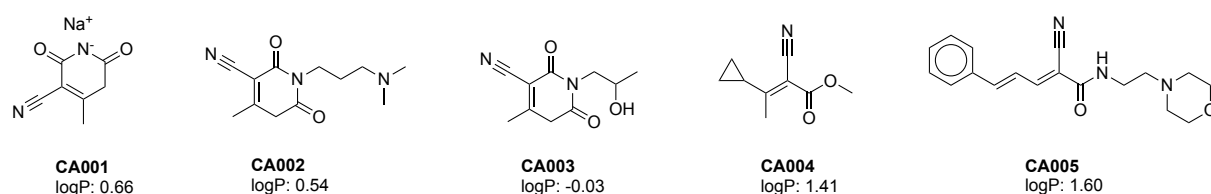


Figure 3.23: Compounds of the CovLib with an α -cyanoacrylamide/acrylate warhead and their logP values.

3.4.2.2 Fragments of the CovLib with an Epoxide (EO) Warhead

Epoxides are often used as warheads against various proteases [254] and glycosidases [255, 256]. In addition, epoxides that form a covalent bond with non-catalytic cysteines are also found in approved drugs, such as the antibiotic fosfomycin [257] or the proteasome inhibitor carfilzomib [258, 259]. The antibiotic fosfomycin is used for bacterial urinary tract infections and covalently targets Cys115 of the bacterial UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA) [260].

Epoxides, representing small-size warheads, react with nucleophiles via the S_N2 mechanism, resulting in ring opening [144, 261]. The reactivity of the epoxides is caused by the large ring strain. Epoxides can be attacked by nucleophiles at two

possible adjacent positions. The attack is preferred at the less sterically hindered position in the absence of acid catalysis and oxygen protonation [144, 262]. The major disadvantages of epoxides are their toxicity and DNA alkylating potential [255]. Moreover, epoxides are rapidly hydrolyzed by epoxide hydrolases. Hydrolysis can also produce toxic metabolites [263]. In addition to cysteines, methionine [264], histidine [265], and lysine [266] residues can also be alkylated by epoxides warheads. Other covalent reactive three-membered heterocycles, such as aziridines [267], are less established than epoxides.

The fragments of the CovLib with an epoxide warhead are depicted in Figure 3.24. Epoxides with different substitution patterns should be selected for the CovLib. Specifically, epoxides EO001 and EO003 are substituted at the α - and β -positions, while EO001 and EO002 are di-substituted at the α -position. EO002 has no substituent at the β -position. Additionally, in compound EO003, the epoxide is part of a bi-cycle. Epoxides EO001 and EO002 have a structural similarity to the antifungal drug fluconazole, and EO003 is structurally similar to the nucleoside thymidine.

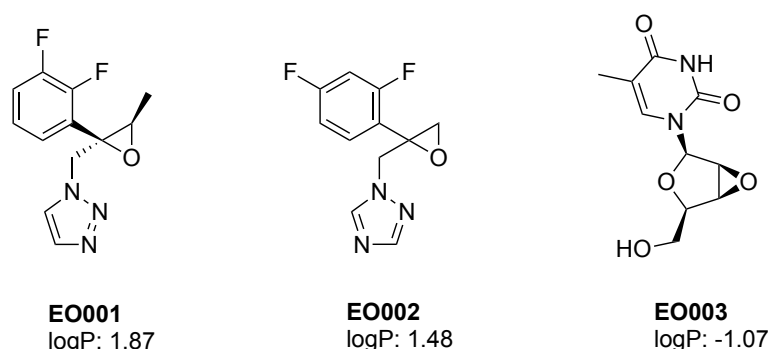


Figure 3.24: Compounds of the CovLib with an epoxide warhead and their logP values.

3.4.2.3 Fragments of the CovLib with a Vinyl Sulfone (VS) Warhead

Vinyl sulfones as CRGs have gained attention in recent years as inhibitors of cysteine proteases [268-270]. Vinyl sulfones participate in 1,4-addition reactions with nucleophiles [271, 272]. Similar to α -cyanoacrylamides, vinyl sulfones are also derivatives of the commonly used acrylamides. Both have a higher reactivity than the well-studied acrylamide warhead. As with other Michael-type CRGs, the reactivity of vinyl sulfones is dependent on the substituents at the α - and β -positions [144].

Besides reacting with thiol side chains of cysteines, vinyl sulfones are also used to address the ϵ -amino group of lysine. Due to the electron-withdrawing properties of the

sulfonyl group, the β -position of vinyl sulfones is less polarizable, making it a harder electrophile according to the hard and soft acids and bases (HSAB) concept [273]. Therefore, it reacts preferentially with hard nucleophiles. The ε -amino side chain of lysine represents a harder nucleophile than the thiol group of cysteines and can be addressed by vinyl sulfones [144, 145, 274]. Therefore, vinyl sulfones exhibit a higher reactivity with lysines compared to the corresponding α,β -unsaturated acrylamides [144, 275]. However, lysine has a much higher pK_a (≈ 10.5) than cysteine ($pK_a \approx 8.6$) and is therefore mainly present in its protonated form at physiological pH. The nucleophilic deprotonated lysine is only present when the pK_a is lowered by the protein environment [144, 145].

Four fragments with vinyl sulfones as CRGs were selected for the CovLib (Figure 3.25). Fragment VS001 is the only acyclic vinyl sulfone and has an α -cyano group, favoring a reversible covalent reaction. Compound VS002 is the only fragment bearing a vinyl sulfonamide warhead. VS001 and VS002 represent dually activated vinyl sulfones, and the thiol addition is expected to be reversible. Fragments VS003 and VS004 represent SAR compounds with different substituents at the β -position. Compounds VS001, VS002, and VS004 have a leaving group at the β -position and may undergo addition-elimination followed by adduction of a second equivalent of thiol(ate) (i.e., thiol exchange) [144].

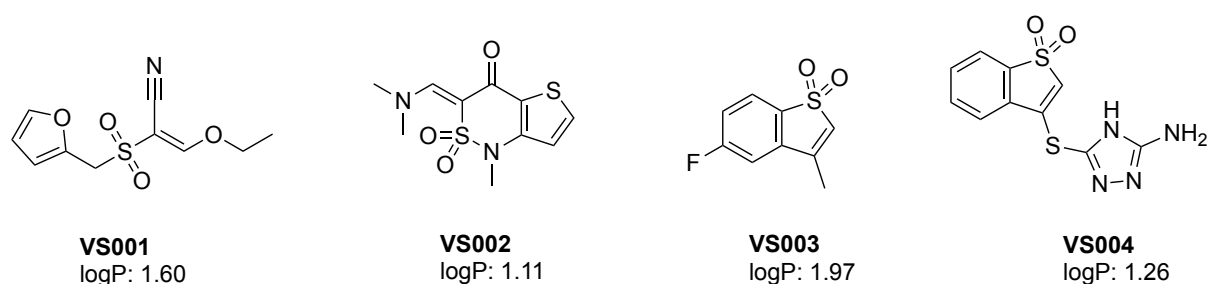


Figure 3.25: Compounds of the CovLib with a vinyl sulfone warhead and their logP values.

3.4.2.3.1 Fragments of the CovLib with an S_NAr Warhead

Nucleophiles, such as the thiol group of cysteines, react with electron-deficient (hetero)arenes via an S_NAr reaction with displacement of a leaving group. The S_NAr reaction requires a strongly deactivated (hetero)arene and the presence of a suitable LG. The reaction proceeds according to a two-step addition-elimination mechanism, with the nucleophile attack being the rate-determining step. Electron-withdrawing substituents in the ortho or para position to the LG cause resonance stabilization of the intermediately formed anion, which is also known as Meisenheimer- or σ -complex [276-278]. However, recent studies imply that many S_NAr reactions occur in a concerted manner through Meisenheimer-like transition states [144, 276, 279].

The reactivity of the activated (hetero)arenes can cover a wide range. It rises with increasing electron deficiency and strongly depends on the (hetero)arene, the electron-withdrawing substituents, and the LG. S_NAr reactions occur preferentially on π -electron-deficient heteroarenes, such as pyridines. -M substituents in ortho or para positions lead to a delocalization of the negative charge of the σ -complex and therefore to high reactivity. In common, nucleophile attack is the rate-determining step in S_NAr reactions. Hence, the reactivity of halogens as LGs increases in the following order: F >> Cl $\approx$ Br > I. Suitable LGs are characterized by a strong polarization of the C ^{δ +}-X ^{δ -} bond as the most important factor and a good stabilization of the intermediate σ -complex [144, 276, 277].

Although S_NAr reactions have not yet played a major role in TCI development, these warheads offer many advantages, such as modification of the reactivity by variations in EWGs and LGs, structural rigidity, and established synthesis routes. Furthermore, many heteroarenes are already present in non-covalent ligands. Consequently, a covalent compound can be designed by introducing an electron-deficient heteroarene or LG near an addressable nucleophile in the binding pocket [144, 197]. S_NAr warheads mainly target the thiol group of cysteines, but recently a highly reactive dichlorotriazine-containing inhibitor of the glutathione S-transferase Pi was found to establish a covalent bond with a tyrosine residue [280].

A set of eight S_NAr-type electrophiles was purchased (Figure 3.26). The library includes five-membered (pyrazole, triazole) and six-membered (pyridine, pyrazine) heterocycles with different EWGs (-CN, -NO₂, -X) and LGs (-X, -SO₂R). It should be noted that compound SN008 has two possible LGs with the halogen and the

sulfonamide group. Furthermore, it should also be considered that the activated nitrile group attached to an electron-deficient heterocycle can participate in a nucleophilic addition of cysteine residues and represents a reversible covalent warhead. Nitrile groups react in a reversible manner with cysteine residues to thioimidates via a Pinner-type addition [144, 197, 281].

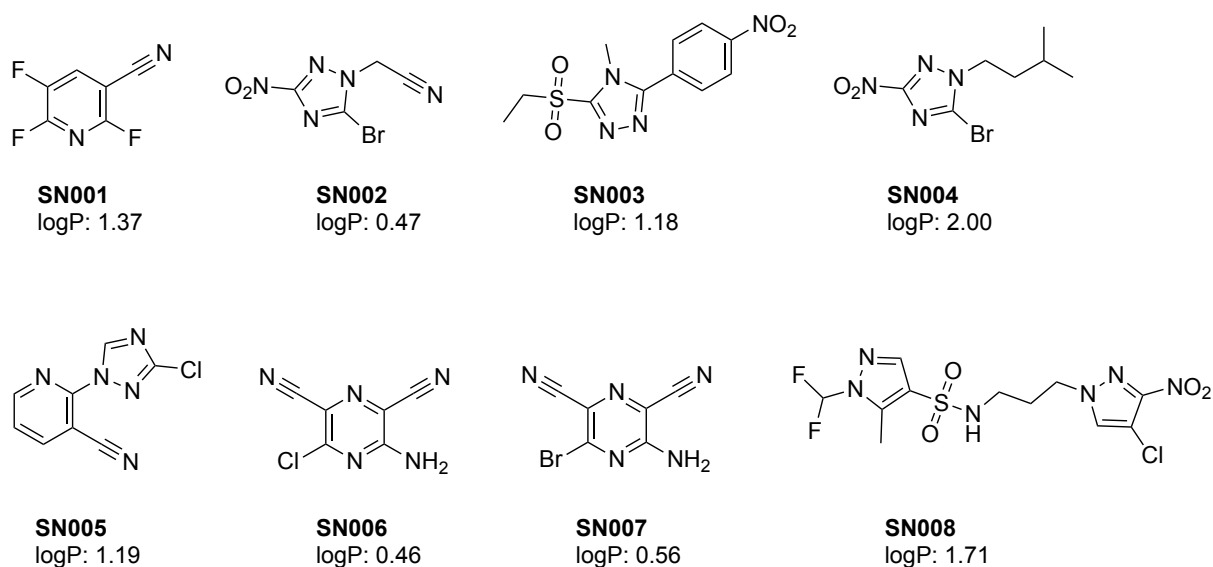


Figure 3.26: Fragments of the CovLib with an S_NAr warhead and their logP values.

3.4.3 Reactivity Characterization of the CovLib

These experiments were performed by Martin Schwer.

The intrinsic reactivity of the CovLib compounds was investigated using a high-performance liquid chromatography (HPLC)-based GSH reactivity assay. Additional measurements in phosphate-buffered saline (PBS) buffer without GSH were also performed to determine the aqueous stability of the fragments. Afatinib, an approved covalent EGFR tyrosine kinase inhibitor for the treatment of non-small cell lung cancer, was used as a reference for the GSH assay [282].

Table 3.5 shows the result of the GSH reactivity assay. The compounds with a reversible covalent α -cyanoacrylamide/acrylate warhead were chemically stable (CA001, CA002, CA003) or showed low reactivities (CA004 ($t_{1/2}$ = 27 h), CA005 ($t_{1/2}$ = 59 h)). It should be noted that CA001 was not soluble in acetonitrile (ACN) at 3.33 mM and was added as a suspension. The compound was completely dissolved in the ACN/PBS mixture, but the exact concentration remains imprecise. Interestingly, all of the tested α -cyanoacrylamides/acrylates were less reactive than expected. This can be explained by the steric hindrance at the β -position (or δ -position in the case of CA005) and the rapid reversibility of the reaction, which makes it difficult to detect adducts.

All compounds with an epoxide warhead were unreactive under assay conditions. However, the identity of compound EO002 was not confirmed, and HPLC and NMR data indicated fragment degradation. EO001 and EO003 are substituted at both ring-carbons, which explains their low reactivity due to steric hindrance.

The HPLC chromatogram of the dually activated vinyl sulfone VS001 showed several overlapping peaks, indicating degradation on the column caused by the acidic aqueous eluent. Consequently, the compound could not be evaluated by GSH assay. Nevertheless, it was classified as a reactive fragment. Due to solubility issues of the dually activated vinyl sulfonamide VS002, the supernatant of the 3.33 mM ACN stock was evaluated. The fragment showed a $t_{1/2}$ >100 h in the GSH assay. However, it should be considered that the used concentration of the compound in the GSH assay was significantly lower. Fragment VS003 also showed a $t_{1/2}$ >100 h in the GSH assay. In contrast, fragment VS004, differing in the β -substituent, showed a notable reactivity with a $t_{1/2}$ of 0.82 h.

The S_NAr reactive five-membered heterocycles SN003, SN004, SN005, and SN008 showed no reactivity in the GSH assay with $t_{1/2} > 100$ h. However, fragment SN002 was the only five-membered heteroarene that showed an intermediate GSH reactivity with a $t_{1/2}$ of 11 h. In contrast, the pyridine derivative SN001 and pyrazine derivatives SN006 and SN007 were highly reactive fragments with a calculated $t_{1/2} < 0.33$ h, since no remaining fragment could be detected from the second measurement point after 20 min in the GSH assay. It should be noted that fragment SN001 showed an intermediate aqueous stability with a $t_{1/2}$ of 15 h in PBS.

Table 3.5: Results of the reactivity assessment of the CovLib fragments using GSH assay and the aqueous stability in PBS buffer without GSH.

CovLib Compound	$t_{1/2}$ GSH $\pm$ SD [h]	$t_{1/2}$ PBS [h]
Afatinib	1.1 ± 0.0	>100
CA001	>100 ^a	>100 ^a
CA002	>100	>100
CA003	>100	>100
CA004	27 ± 0	25
CA005	59 ± 10	>100
EO001	>100	>100
EO002	n.a. ^b	n.a. ^b
EO003	>100	>100
VS001	n.a. ^c	n.a. ^c
VS002	>100 ^d	>100 ^d
VS003	>100	>100
VS004	0.82 ± 0.03	>100
SN001	<<0.33	15
SN002	11 ± 1	>100
SN003	>100	>100
SN004	>100	>100
SN005	>100	>100
SN006	<<0.33	>100
SN007	<<0.33	>100
SN008	>100	>100

^a3.33 mM ACN stock not soluble, the stock was added to the aqueous buffer as a homogenous suspension and was dissolved in the buffer/organic solvent mixture. ^bThe identity and purity of the compound could not be confirmed. ^cThe compound was not quantifiable by HPLC. ^d3.33 mM ACN stock not soluble. Not soluble in ACN buffer mixture at the required concentration. For the reactivity measurement, the supernatant of the 3.33 mM ACN stock was added to the aqueous buffer, containing detectable amounts of the compound.

3.4.4 Overview of the Biophysical Screening of the CovLib with T-p53C and Its Mutant

Reactive fragments, such as the 2-sulfonylpyrimidine PK11000 [131], the HEFLib compound 4482 [99], and the methylene quinuclidinone PRIMA-1 [116-118, 283], achieve a generic stabilization of p53 through modification of surface-exposed cysteine residues and thus represent a simultaneous rescue strategy for destabilized p53 cancer mutants [99, 130, 131]. Furthermore, PRIMA-1 also rescues different DNA contact mutants [115]. Therefore, the binding of the CovLib fragments to T-p53C and different structural and DNA contact mutants was investigated. The core domain of p53 contains ten cysteines, whereas the cysteines 124, 182, 229, 277, and 275 are fully or partially solvent-exposed and thus amenable to covalent modification (Figure 3.27) [130, 143]. The thiol groups of cysteines 277 and 182 have the highest solvent accessibility [284]. Cysteines 176, 238, and 242 are involved in zinc coordination and are therefore not susceptible to modification. However, the specific covalent modification of cysteines in p53 is a challenge [285]. Arylations of the surface-exposed Cys277 and Cys182 were found for mild arylating 2-sulfonylpyrimidine-containing compounds [131, 284, 285]. MQ, the bioactive compound of PRIMA-1 and its methylated analog APR-246, is a Michael acceptor and modifies multiple cysteines on the surface of the p53 core domain [117-119]. The crystal structures of the DNA contact mutants R273H/C and the structural mutant R282W with MQ confirmed the modification of cysteines 124, 182, 229, 273 (in the R273C mutant), 275, and 277. When complexed with DNA, only the alkylation of the cysteines 124, 229, and 277 was detected. Modification of these cysteine residues by MQ results in the stabilization of the p53-DNA complex. Alkylation of Cys277 stabilizes the protein-DNA interface, whereas modification of Cys124 and Cys229 stabilizes the interfaces between dimers. Likely, the alteration of Cys182 and Cys275 by MQ is not compatible with DNA-binding [118].

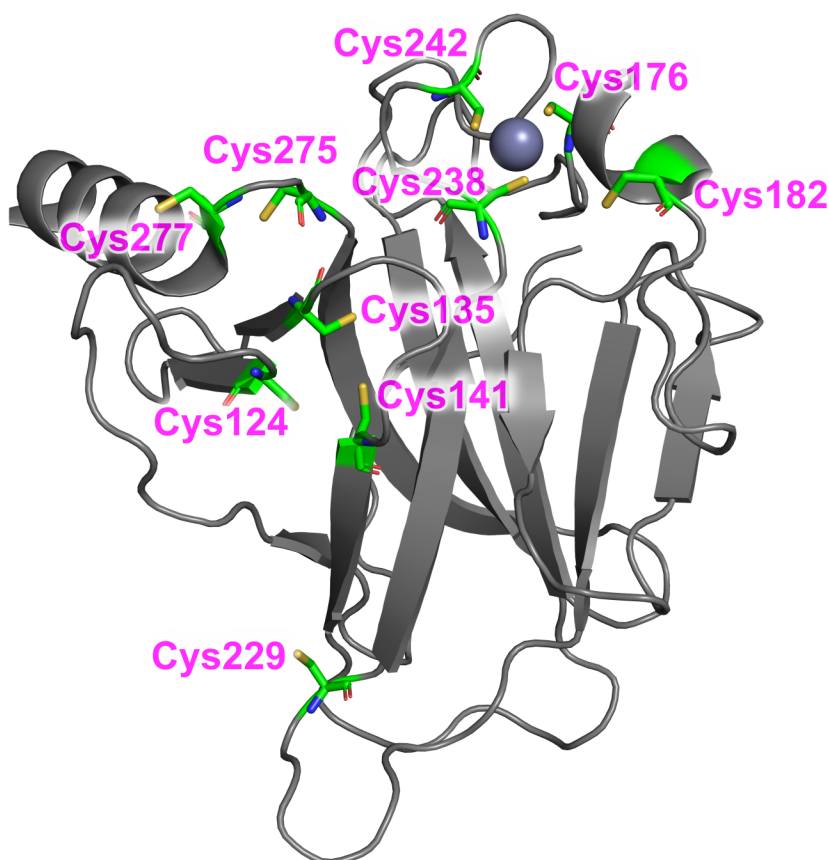


Figure 3.27: Structure of T-p53C (PDB code: 1UOL [146]). All cysteines in the core domain of p53 are highlighted. Cysteines 124, 182, 229, 275, and 277 are fully or partially solvent-exposed. Cysteines 176, 238, and 242 are involved in zinc-coordination.

The biophysical screening procedure of the CovLib with T-p53C and its mutants is shown in Figure 3.28. Two orthogonal methods were applied for primary screening. All 20 fragments of the CovLib were screened with T-p53C using UHPLC-ESI-MS. Intact protein mass spectrometry is a principal screening method in covalent FBDD [209, 239, 240, 286-288]. With MS, covalent fragment binding can be measured directly by the increase in the native protein mass due to the attached fragments. DSF was included in the analysis of the CovLib to cross-validate the MS results. DSF is an efficient and fast primary screening method for ligand identification [198, 289, 290]. Furthermore, DSF is a key method for the discovery of hits for the tumor suppressor p53. Due to the large number of thermodynamically destabilized structural mutants, the thermal stabilization of p53 in the DSF experiment is a main indicator for a fragment hit [12, 77, 98, 100, 101, 114, 130]. The CovLib was screened by DSF with T-p53C and various structural and DNA contact mutants.

The fragment hits identified using DSF and intact protein MS were further investigated by $^1\text{H}/^{15}\text{N}$ -HSQC-NMR as a secondary screening technique. NMR screening of covalent ligands offers advantages due to the covalent binding mechanism and resulting slower exchange, which leads to prominent peak shifts [209, 291].

Moreover, the influence of the identified fragment hits on the DNA-binding affinity of p53 was investigated by a fluorescence polarization assay using the fluorescein-labeled GADD45 α DNA p53 response element [81, 131, 292, 293]. Since there are surface-exposed cysteines in the DNA-binding region of p53, covalent modification of these residues may affect the DNA-binding capacity of p53 [118, 130, 294].

Finally, the best hits were validated by X-ray crystallography. X-ray crystallography is widely applied in FBDD and provides detailed information about the covalently modified amino acid residues and the binding mode. Furthermore, the obtained structural information serves as a starting point for the subsequent merging, linking, and growing of the identified fragment hits [209, 232].

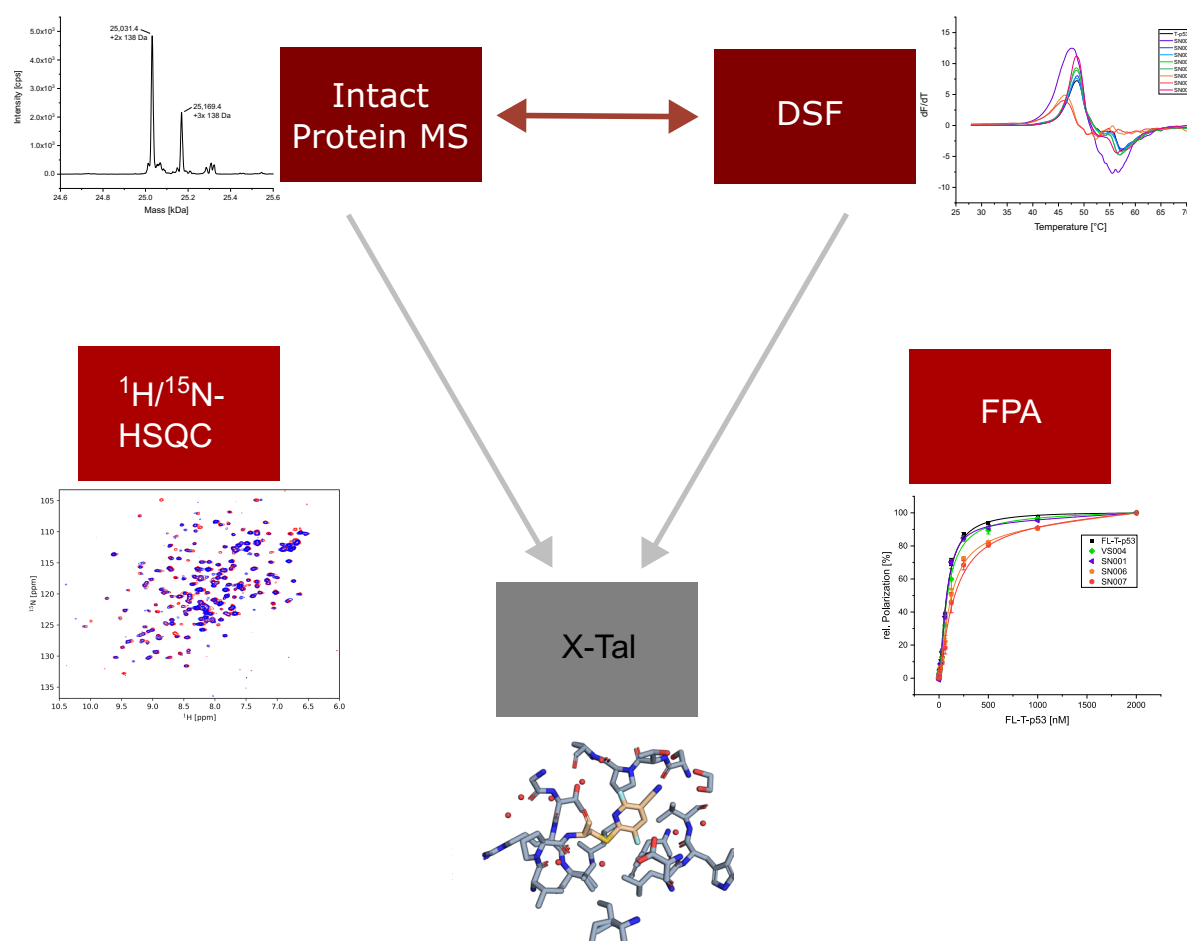


Figure 3.28: Overview of the biophysical screening methods used for the CovLib screen with T-p53C and its mutants.

3.4.5 Primary Screening of the CovLib with T-p53C Using Intact Protein Mass Spectrometry

The CovLib was screened with T-p53C using UHPLC-ESI-MS. T-p53C was incubated with the fragments (molar ratio 1:100) for 4 h at 20 °C before MS analysis [131]. In total, the covalent modification of p53 was confirmed for six fragments. The fragment hits included two vinyl sulfones (VS001, VS004) and four S_NAr reactive compounds (SN001, SN002, SN006, and SN007). The deconvoluted MS spectra of the reactive fragments indicated multiple modifications of T-p53C.

In the deconvoluted MS spectra of T-p53C with the CovLib compounds with either epoxide or α -cyanoacrylamide/acrylates as CRGs, only native T-p53C was detectable. However, covalent adduct formation with α -cyanoacrylamides cannot be observed by protein mass spectrometry due to the rapid reversibility of binding. This is particularly the case if the target protein denatures, which results in the loss of stabilization of the covalent complex [249, 250]. The MS results were consistent with the reactivity of the fragments determined in the GSH assay.

The deconvoluted MS spectra of the reactive CovLib hits with a vinyl sulfone as a CRG are depicted in Figure 3.29. In the deconvoluted spectrum of fragment VS001 with T-p53C (Figure 3.29 A), different peaks with an increase in the native protein mass were detected, indicating a covalent binding of fragment VS001 to T-p53C. However, the number of VS001 molecules attached to T-p53C (up to six modifications) exceeded the number of solvent-accessible cysteines in T-p53C (five partially or fully solvent-accessible Cys). This suggests binding to buried cysteines or other amino acids besides cysteines. According to the HSAB concept [273], vinyl sulfones are also known to target the ϵ -amino group of lysine [144, 145, 274]. Furthermore, addition-elimination of lysine to strongly activated Michael acceptors with β -LG group is also not uncommon [295]. The mass difference (Δm) between each peak was approximately 195 Da. However, the observed mass difference was not consistent with the increase in the mass of T-p53C by hetero-Michael addition of VS001 (241 Da). Thus, there was a mass difference of 46 Da between the experimentally detected adducts and the mass of the expected adduct formed by the addition of VS001. Determining the protein-bound structure of VS001 is challenging due to its hydrolytic instability and Pan-Assay Interference Compounds (PAINS) nature [296].

Nevertheless, the addition of cysteine and the removal of an ethanolate from VS001 results in a mass increase of 195 Da.

The spectrum of VS004 (Figure 3.29 B) showed multiple labeling of T-p53C. Both the nucleophilic addition to the α,β -unsaturated vinyl sulfone (280 Da) and the product of subsequent elimination of the heteroaryl thiolate leaving group (i.e., thiol exchange) (164 Da) were found. The protein species with two VS004 molecules attached and loss of the heteroaryl thiolate LG gave the highest signal.

For the highly reactive S_NAr -type electrophiles SN001, SN006, and SN007, multiple arylations of T-p53C were detected after 4 h of incubation at a protein-to-compound ratio of 1:100 (Figure 3.30 A, C, D). In the deconvoluted MS spectra, the protein species with two attached molecules represented the highest peak. Moreover, small peaks corresponding to T-p53C labeled with three molecules of SN001, SN006, or SN007 were also observed. Furthermore, in the spectrum of SN007, peaks with lower intensities indicating the four- and five-times modified T-p53C were detected. It is conceivable that the augmented overall intensity of the spectrum of T-p53C with SN007 compared to those with SN001 and SN006 contributed to the detection of these additional peaks.

Compound SN002, another S_NAr -type electrophile with intermediate reactivity, was also identified as a hit by intact protein mass spectrometry. In the deconvoluted MS spectrum, the single modified protein species gave the highest signal (Figure 3.30 B). However, native T-p53C was also detected. This can be explained by the lower reactivity and longer $t_{1/2}$ of SN002 in the GSH assay, compared to the other MS hits. The incubation time of 4 h before the MS experiment might be too short for the complete reaction of the fragment. Therefore, extending the incubation time might also result in an increased modification of T-p53C.

In summary, the MS experiments discovered the fragments VS001, VS004, SN001, SN002, SN006, and SN007 that covalently modify T-p53C. The CovLib hits represent (highly) reactive compounds that multiply and non-specifically label T-p53C.

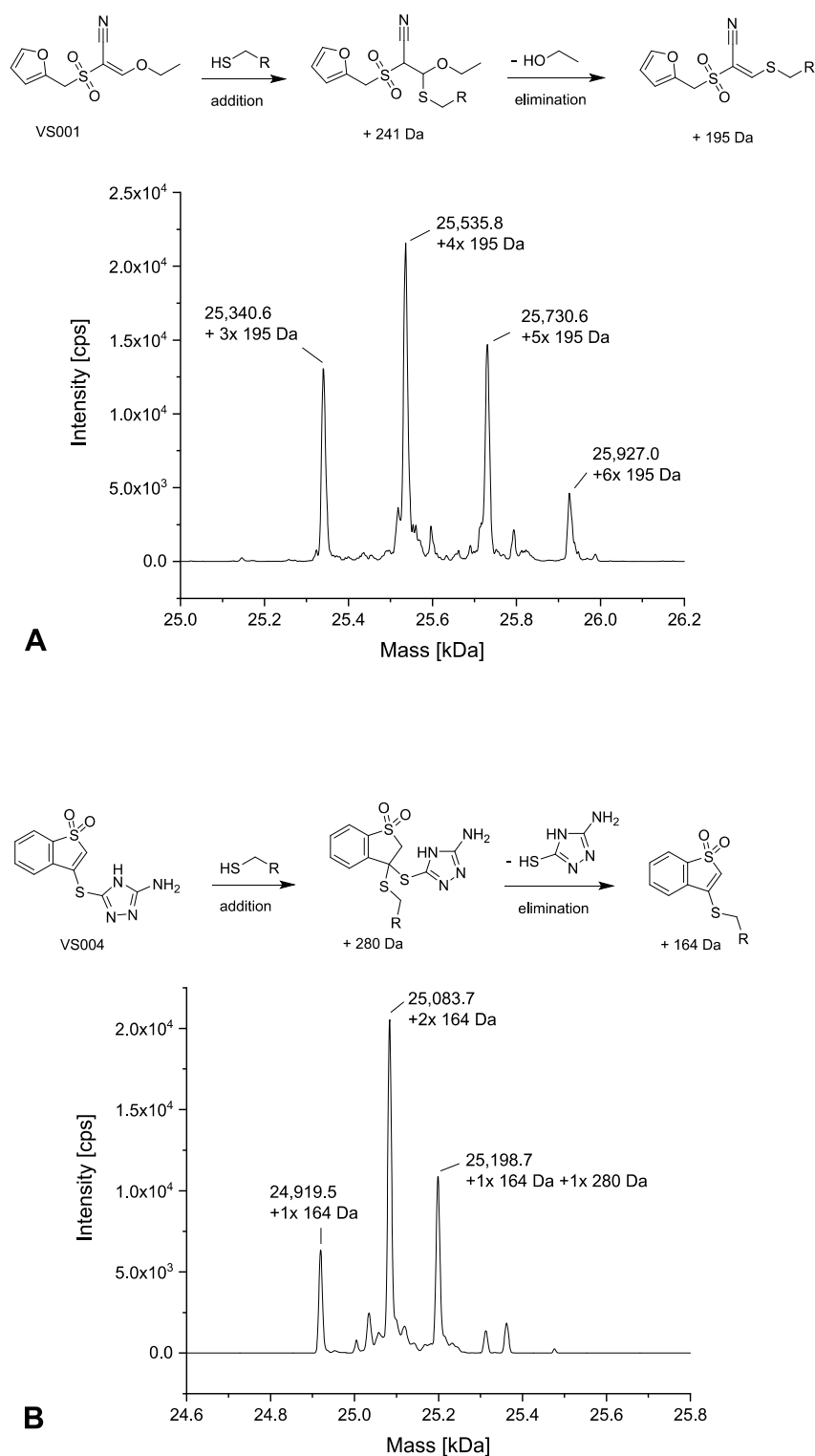


Figure 3.29: Deconvoluted MS spectra of T-p53C with the identified CovLib hits with a vinyl sulfone as CRG: VS001 (A) and VS004 (measured without dithiothreitol (DTT)) (B) with T-p53C (protein-to-compound ratio 1:100, theoretical mass of unmodified T-p53C: 24,756.03 Da) after 4 h of incubation at 20 °C.

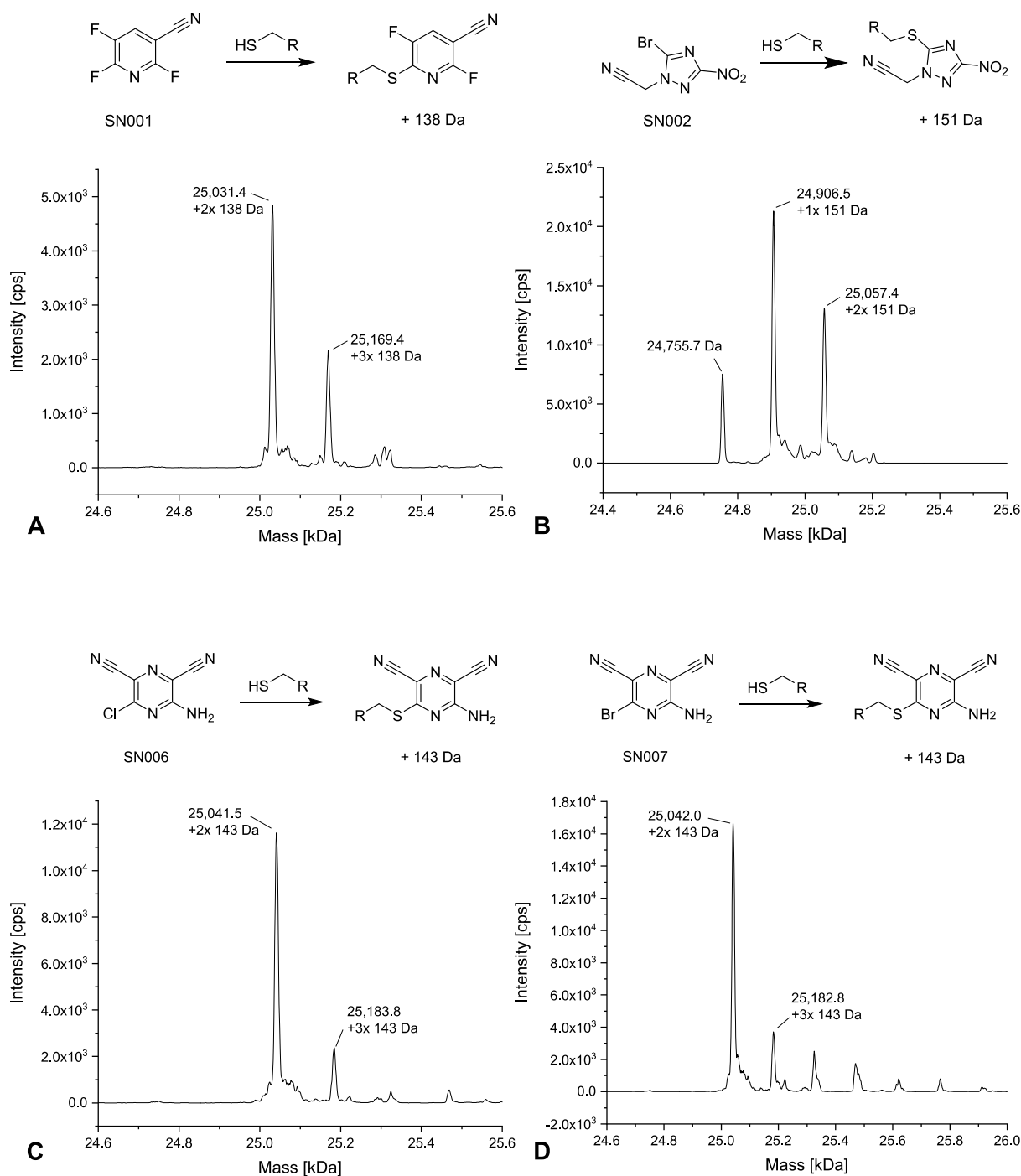


Figure 3.30: Deconvoluted MS spectra of T-p53C with the identified S_NAr -type CovLib hits: SN001 (A), SN002 (measured without DTT) (B), SN006 (C), and SN007 (D) with T-p53C (protein-to-compound ratio 1:100, theoretical mass of unmodified T-p53C: 24,756.03 Da) after 4 h of incubation at 20 °C.

3.4.6 Primary Screening of the CovLib with T-p53C Using DSF

To cross-validate the identified MS hits, the CovLib fragments were screened with T-p53C using DSF. The primary DSF screen was conducted at a compound concentration of 1 mM, corresponding to a protein-to-compound ratio of 1:125 [131]. An initial incubation time of 30 min was chosen for the CovLib analysis. Figure 3.31 illustrates the first derivatives of the melting curves of T-p53C after incubation with 1 mM CovLib compounds for 30 min. Furthermore, incubation times of 4 h and 24 h were also investigated due to the time-dependency of the covalent bond formation (Figure 8.6, Figure 8.7). The corresponding ΔT_m values are presented in Table 3.6.

The tested compounds with a reversible covalent α -cyanoacrylamide/acrylate and epoxide warhead had only a negligible impact on the thermal stability of T-p53C. Even after 24 h of incubation, no substantial stabilization of T-p53C was detected. Overall, none of the CovLib fragments containing epoxide or α -cyanoacrylamide/acrylate warheads produced any hits in the MS and DSF experiments with T-p53C. Furthermore, these findings are consistent with the GSH reactivity characterization.

The reactivity studies suggested that the vinyl sulfones either represent stable compounds (VS002, VS003) or are reactive (VS004). The reactivity characterization of VS001 was associated with several problems due to its PAINS characteristic associated with high reactivity and hydrolytic instability. However, in the MS experiment, VS001 and VS004 were identified as hits. In the DSF experiment, VS001 increased the T_m of T-p53C slightly by 0.60 °C after 30 min of incubation.

Fragment VS002 could not be screened by DSF due to extremely low fluorescence intensity. Therefore, no protein melting curves were obtained. The compound could have interacted with the fluorescent dye SYPRO Orange, causing partial fluorescence quenching and reduced signal intensity [114, 198]. As anticipated, due to its relatively low thiol reactivity, VS003 did not markedly affect the T_m of T-p53C.

The vinyl sulfone VS004 exhibited a large increase in the melting temperature of T-p53C. The T_m was increased by up to 5.06 °C (1 mM, 30 min). In the melting curves of T-p53C with compound VS004, two transitions appeared. The first derivative (Figure 3.31 C) showed a first minor shoulder and a major peak, possibly indicating differently modified protein species with different melting points or contributions from non-covalently bound fragments with distinct melting points [264, 289, 297]. In the MS

spectrum, the hetero-Michael addition and the subsequent elimination of the heteroaryl thiolate LG were detected, which could explain the occurrence of two transitions.

Fragments SN003, SN004, SN005, and SN008, which would react via S_NAr , are chemically stable in the GSH experiment. Consistent with the MS results, the DSF experiment revealed no change in the T_m of T-p53C, suggesting that these compounds did not interact with T-p53C.

Fragments SN001, SN006, and SN007 showed a very short $t_{1/2}$ in the GSH assay (<0.33 h) and multiple modifications of T-p53C in the intact protein MS analysis. Nevertheless, incubation with SN001 only slightly destabilized T-p53C by about -0.57 °C (1 mM, 30 min). This suggests that the arylation of cysteine residues in T-p53C by fragment SN001 has only small effects on the stability of the protein.

In contrast, fragment SN006 and its bromine analog SN007 substantially decreased the melting temperature of T-p53C by about -2.31 °C and -2.45 °C, respectively, after 30 min of incubation. The decrease in the T_m can be attributed to the high reactivity of the fragments and multiple arylations of T-p53C, resulting in a substantial loss of correct protein folding and stability [12, 284]. Furthermore, it was shown that the destabilization of the proteins by some ligands indicates binding in the unfolded state of the proteins, which results in a reduction of the T_m [298, 299]. Nevertheless, the negative shifts do not additionally exclude binding in the native state [289].

The MS screening detected various covalent modifications of T-p53C by fragment SN002. However, the DSF data did not indicate an increase in the T_m or fragment binding after 30 min of incubation ($\Delta T_m = 0.10$ °C). Fragment SN002 exhibited an intermediate $t_{1/2}$ of 11 h in the GSH assay. Therefore, additional incubation periods of 4 h and 24 h were evaluated. The DSF results in Table 3.6 showed that even after 24 h of incubation, the melting temperature of T-p53C was only slightly affected ($\Delta T_m = 0.40$ °C). Hence, fragment SN002 did not appear as a hit in the DSF screen, despite its proven covalent binding to T-p53C.

The CovLib compounds were also studied after 4 h and 24 h incubation periods. For the hits SN001 and VS004, there was no discernible impact of incubation time on the shift in the melting temperature of T-p53C. However, the destabilization of T-p53C increased with prolonged incubation time for the compounds SN006 and SN007. After 24 h incubation time, no melting curves of T-p53C were detectable. High fluorescence

intensity was measured even at low temperatures, indicating denaturation, aggregation, or misfolding [289].

In summary, only hits identified by intact protein MS were also DSF hits. The DSF experiment did not reveal any additional hits. The vinyl sulfone VS004 showed a substantial stabilization of T-p53C with an increase in the melting temperature by up to 5.06 °C after 30 min of incubation. Fragment SN001 slightly decreased the T_m of T-p53C, while SN006 and SN007 caused a pronounced destabilization of the protein.

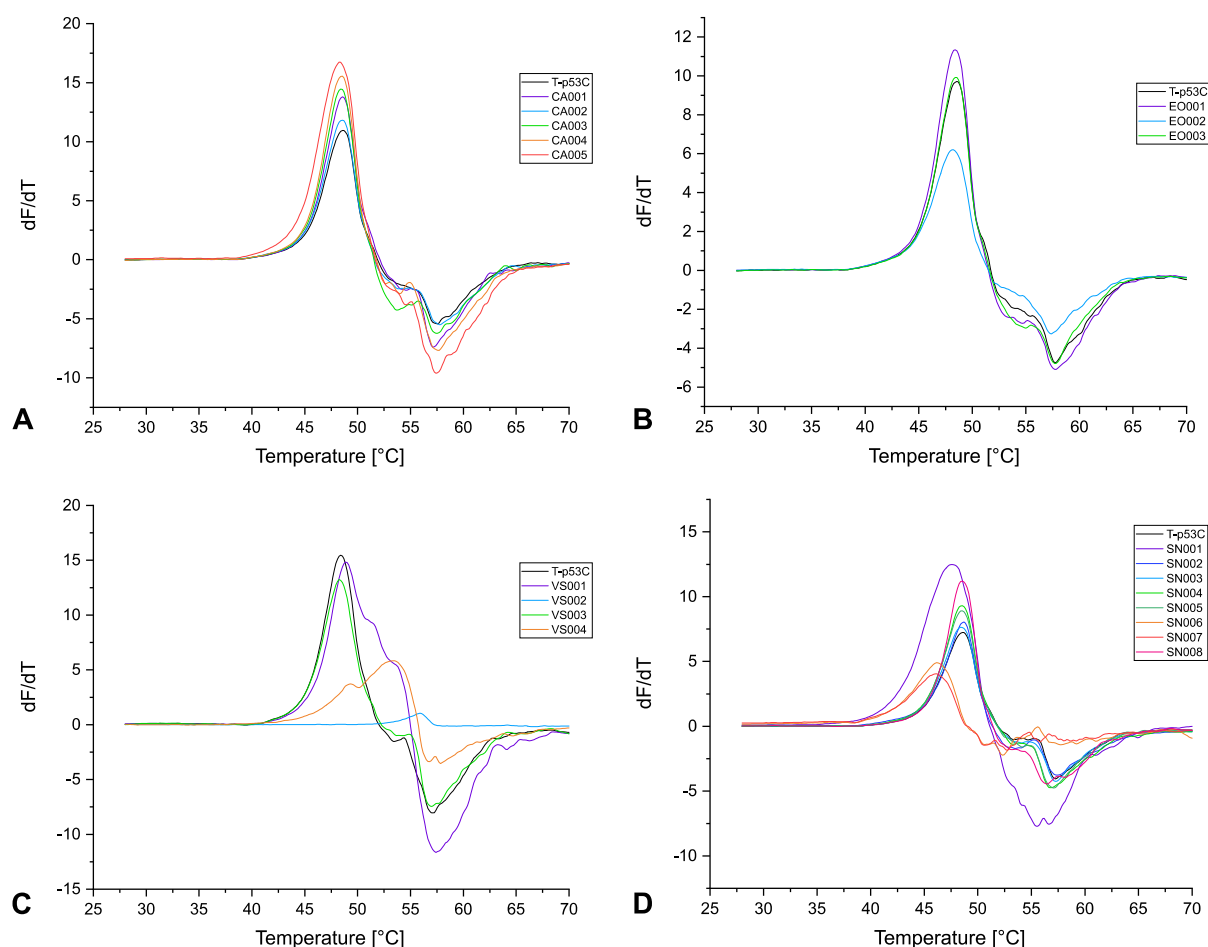


Figure 3.31: DSF screen of the CovLib with T-p53C. The first derivatives of the melting curves of α -cyanoacrylamides/acrylates (A), epoxides (B), vinyl sulfones (C), and S_NAr (D) fragments (1 mM) with T-p53C (8 μ M protein) after 30 min of incubation at 20 °C are presented.

Table 3.6: $\Delta T_m \pm SD$ of T-p53C (8 μ M protein) incubated with 1 mM CovLib compound (protein-to-compound ratio 1:125) for 30 min, 4 h, and 24 h at 20 °C. Measurements were performed at least in triplicate. Fragments with a ΔT_m of at least 0.50 °C [99, 130] and the identified MS hits are highlighted in bold.

CovLib Compound	30 min $\Delta T_m \pm SD$ [°C]	4 h $\Delta T_m \pm SD$ [°C]	24 h $\Delta T_m \pm SD$ [°C]
CA001	-0.05 $\pm$ 0.09	-0.15 $\pm$ 0.12	0 $\pm$ 0.16
CA002	-0.10 $\pm$ 0.12	-0.05 $\pm$ 0.12	0.20 $\pm$ 0.16
CA003	-0.20 $\pm$ 0.09	-0.10 $\pm$ 0.09	-0.07 $\pm$ 0.16
CA004	-0.15 $\pm$ 0.12	-0.10 $\pm$ 0.09	0.07 $\pm$ 0.12
CA005	-0.25 $\pm$ 0.19	-0.10 $\pm$ 0.09	0.13 $\pm$ 0.16
EO001	-0.20 $\pm$ 0.12	-0.10 $\pm$ 0.09	0 $\pm$ 0.16
EO002	-0.35 $\pm$ 0.12	-0.10 $\pm$ 0.09	-0.13 $\pm$ 0.12
EO003	-0.10 $\pm$ 0.09	0.05 $\pm$ 0.09	0 $\pm$ 0.16
VS001	0.60 $\pm$ 0.12	0.45 $\pm$ 0.12	0.30 $\pm$ 0.07
VS002	N/A ^a	N/A ^a	N/A ^a
VS003	-0.05 $\pm$ 0.09	-0.10 $\pm$ 0.09	0.07 $\pm$ 0.12
VS004	5.06 $\pm$ 0.09^b	5.30 $\pm$ 0.14^b	5.33 $\pm$ 0.17^b
SN001	-0.57 $\pm$ 0.10	-0.50 $\pm$ 0.13	-0.40 $\pm$ 0.17
SN002	0.10 $\pm$ 0.09	0.20 $\pm$ 0.09	0.40 $\pm$ 0.16
SN003	-0.15 $\pm$ 0	-0.05 $\pm$ 0.12	0 $\pm$ 0.16
SN004	-0.05 $\pm$ 0.09	-0.05 $\pm$ 0.12	0.07 $\pm$ 0.12
SN005	0 $\pm$ 0.15	-0.10 $\pm$ 0.09	-0.13 $\pm$ 0.12
SN006	-2.31 $\pm$ 0.09	-2.90 $\pm$ 0.21	N/A ^a
SN007	-2.45 $\pm$ 0.12	-2.90 $\pm$ 0.20	N/A ^a
SN008	-0.05 $\pm$ 0.23	-0.15 $\pm$ 0.12	0.07 $\pm$ 0.12

^aNo evaluable melting curves and first derivatives with clearly defined melting points were obtained.

^bMelting curves with two transitions. First derivatives of the melting curves showed a first small shoulder.

3.4.7 DSF Screen of the CovLib with c-Jun N-Terminal Kinase 3 (JNK3)

To screen the CovLib with targets other than p53, the c-Jun N-terminal kinase 3 (JNK3) was selected as an additional target. The serine-threonine kinase JNK3 belongs to the mitogen-activated kinases (MAPK) family and is involved in various signal transduction, cell differentiation, and inflammatory processes [300-303]. Whereas the other members of the highly conserved JNK family (JNK1 and JNK2) are ubiquitously expressed, the expression of JNK3 is primarily restricted to the central neuronal system, testis, and cardiac myocytes [304, 305]. Thus, the tissue contribution of JNK3 in combination with knockout mice experiments demonstrated that JNK3 is a promising target for the treatment of neurodegenerative disorders, such as Parkinson's, Huntington's, and Alzheimer's diseases [306-310]. All members of the JNK3 family have a highly conserved and unique non-catalytic cysteine (Cys154, JNK3 numbering) that has been covalently addressed by a variety of acrylamide-containing protein kinase inhibitors [235, 311, 312]. In addition to Cys154 in the ATP-binding pocket, Cys283 in the C-terminal domain of JNK3 was identified as another reactive residue [313-315].

The CovLib was screened with JNK3 using DSF. The first derivatives of the melting curves after 30 min of incubation and the resulting ΔT_m values of JNK3 are shown in Figure 3.32 and Table 3.7. Typically, ligand binding in the ATP-binding pocket significantly increases the T_m of kinases, with a correlation between ligand binding and stabilization within a structural series [192, 201]. The α -cyanoacrylamides CA003 and CA005 were the only fragments tested that slightly stabilized JNK3 by about 0.50 °C and 1.10 °C, respectively. However, due to the large shifts in the melting temperature normally observed for kinase inhibitors [192, 201, 316], the compounds were not further characterized.

The other compounds that affected the melting temperature of JNK3 were the same as the hits identified in the screening with T-p53C. However, fragments VS001, VS004, SN001, SN006, and SN007 all strongly destabilized JNK3. Although VS004 led to a high stabilization of T-p53C, it declined the T_m of JNK3 ($\Delta T_m = -2.40$ °C), probably due to multiple modifications of the protein. Fragment SN001 only slightly decreased the T_m of T-p53C, whereas JNK3 was substantially destabilized. The first derivative of the melting curve of JNK3 indicated high destabilization by fragment SN001. However, no clearly defined melting point was determinable. No melting curves for JNK3 were

obtained with fragments SN006 and the bromine analog SN007. High fluorescence intensity was measured even at low temperatures, indicating denaturation, aggregation, or misfolding [289]. This suggests that the high reactivity of the fragments leads to multiple arylations, which results in a strong loss of protein stability, in the case of JNK3 even to denaturation.

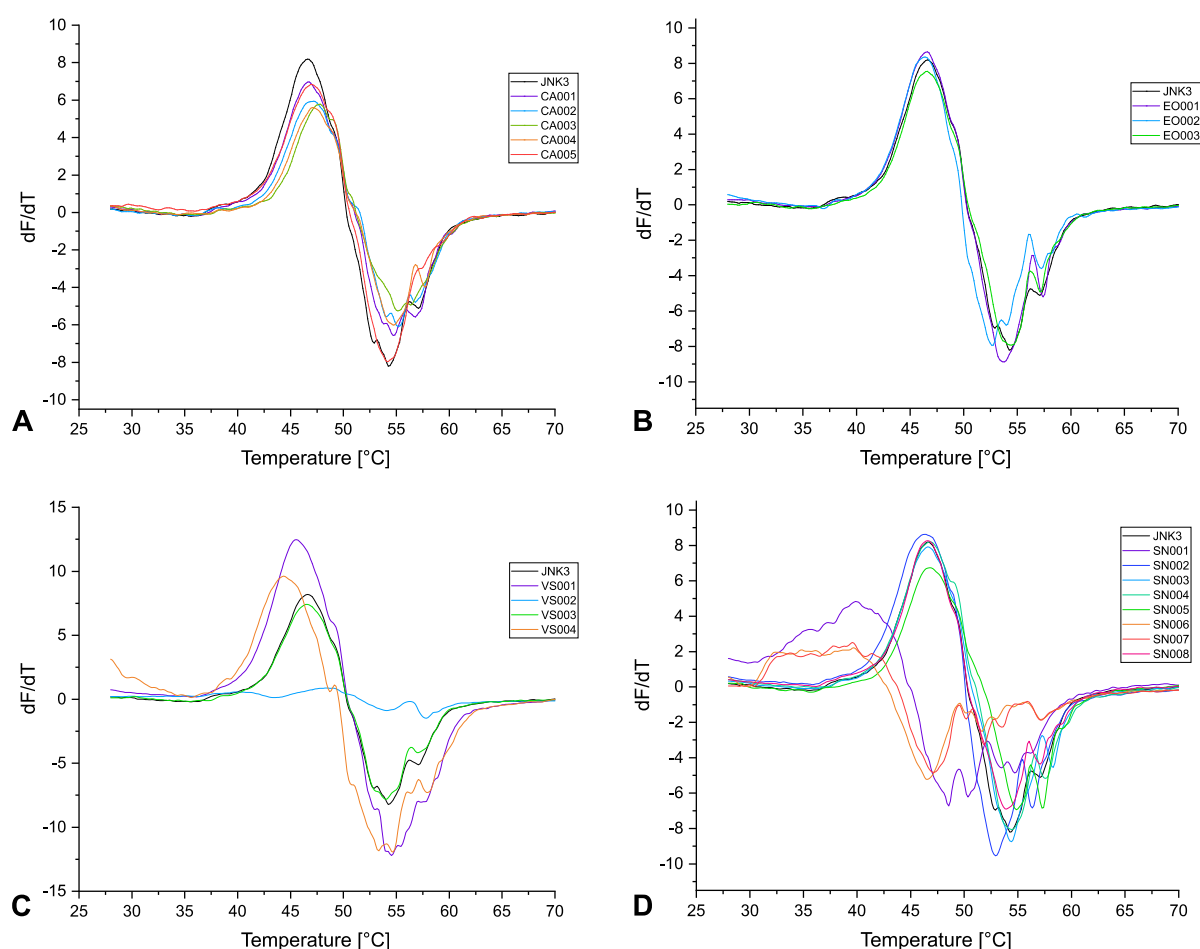


Figure 3.32: DSF screen of the CovLib with JNK3. The first derivatives of the melting curves of α -cyanoacrylamides/acrylates (A), epoxides (B), vinyl sulfones (C), and S_NAr (D) fragments (1 mM) with JNK3 (8 μ M protein) after 30 min of incubation at 20 °C are shown.

Table 3.7: $\Delta T_m \pm SD$ of JNK3 (8 μM protein) incubated with 1 mM CovLib compound (protein-to-compound ratio 1:125) for 30 min at 20 °C. Measurements were performed at least in triplicate. Fragments with a ΔT_m of at least 0.50 °C [99, 130] and the previously identified hits for p53 are highlighted in bold.

CovLib Compound	30 min $\Delta T_m \pm SD$ [°C]
CA001	0.10 $\pm$ 0.09
CA002	0.35 $\pm$ 0.36
CA003	1.10 $\pm$ 0.12
CA004	0.45 $\pm$ 0.12
CA005	0.50 $\pm$ 0.19
EO001	-0.10 $\pm$ 0.12
EO002	-0.30 $\pm$ 0.12
EO003	0 $\pm$ 0.12
VS001	-1.10 $\pm$ 0.31
VS002	N/A ^a
VS003	-0.10 $\pm$ 0.12
VS004	-2.40 $\pm$ 0.19
SN001	N/A ^a
SN002	-0.35 $\pm$ 0.09
SN003	-0.15 $\pm$ 0.19
SN004	0.05 $\pm$ 0.12
SN005	0.10 $\pm$ 0.09
SN006	N/A ^a
SN007	N/A ^a
SN008	-0.25 $\pm$ 0.24

^aNo evaluable melting curves and first derivatives with clearly defined melting points were obtained.

3.4.8 DSF Screen of the CovLib with T-p53C and Its Mutants

The effect of the CovLib on the T_m of various p53 mutants was tested. Besides T-p53C, the DNA contact mutant R273H, the structural mutant R282W in the DNA-binding helix H2, and the β -sandwich mutant Y220C were also examined. Additionally, the triple cysteine mutant T-p53C-C124/182/277S was analyzed as a control with the hits of the primary screening. Cysteines 124, 182, and 277 in p53 were previously characterized as residues that were covalently modified by mild alkylating fragments [88, 89, 208]. Table 3.8 shows the ΔT_m values after 30 min of incubation at 20 °C at a protein-to-compound ratio of 1:125. The corresponding first derivatives of the melting curves are depicted in Figure 8.8 to Figure 8.11.

Overall, no new hits were identified by the DSF screen with the p53 mutants. The dually activated vinyl sulfone with β -LG VS001, which has PAINS properties, stabilized all mutants tested. However, VS001 considerably increased the T_m of the R282W mutant by 4.92 °C.

VS004 exhibited a large increase in the melting temperature of T-p53C and each p53 mutant tested. In particular, VS004 stabilized the structural mutant R282W in the C-terminal helix H2, exhibiting a ΔT_m value of 5.96 °C. Notably, the T_m of the triple cysteine mutant was substantially increased ($\Delta T_m = 4.30$ °C), suggesting that other residues besides Cys124, Cys182, or Cys277 were covalently modified, thereby enhancing the thermal stability of the protein. Two transitions were observed in the melting curves of the p53 variants (T-p53C, T-p53C-R273H, and T-p53C-R282W) incubated with VS004, particularly in the melting curves of the triple cysteine mutant T-p53C-C124/182/277S. The first derivatives showed two peaks, which could be indicative of differently modified protein species with different melting points or contributions from non-covalently bound fragments with distinct melting points [264, 289, 297].

Interestingly, for the S_NAr -type electrophiles SN001, SN006, and SN007, different influences on the T_m values were found dependent on the mutation tested. Compound SN001 resulted in a slight decrease in the melting temperature of the p53 proteins. However, it uniquely stabilized the temperature-sensitive Y220C mutant with a large increase of 3.22 °C. Fragment SN002 did not have a considerable stabilizing effect on the different p53 mutations, as previously studied with T-p53C.

Compound SN006 and its bromine analog SN007 decreased the T_m values of most proteins, especially that of T-p53C and T-p53C-R273H. Moreover, the first derivatives of the melting curves of T-p53C-R282W with SN006 and SN007 indicated a high destabilization. Nonetheless, the curves were not appropriate for accurate determination of the T_m due to the high fluorescence intensity at low temperatures. This indicates possible denaturation, aggregation, or misfolding [289]. Furthermore, these highly reactive pyrazine derivatives caused a decrease in the T_m values of the triple cysteine control mutant, implying labeling of additional surface-exposed residues other than Cys124, Cys182, or Cys277. However, SN006 and SN007 moderately raised the T_m of the Y220C mutant by up to 1.26 °C and 1.21 °C, respectively. In addition, both compounds exhibited a relative $\Delta\Delta T_m$ in comparison to T-p53C only slightly below the level of SN001 stabilization.

Notably, SN001, SN006, and SN007 stabilized exclusively T-p53C-Y220C, suggesting that these compounds arylated the cancer-specific Cys220, leading to an increase in the melting temperature.

Table 3.8: $\Delta T_m \pm SD$ of T-p53C and its mutants (8 μ M protein) incubated with 1 mM CovLib compound (protein-to-compound ratio 1:125) for 30 min at 20 °C. Measurements were performed at least in triplicate. Fragments with a ΔT_m of at least 0.50 °C [99, 130] and the identified MS hits are highlighted in bold.

CovLib Compound	T-p53C $\Delta T_m \pm SD$ [°C]	T-p53C- Y220C $\Delta T_m \pm SD$ [°C]	T-p53C- R273H $\Delta T_m \pm SD$ [°C]	T-p53C- R282W $\Delta T_m \pm SD$ [°C]	T-p53C- C124/182/277S $\Delta T_m \pm SD$ [°C]
CA001	-0.05 $\pm$ 0.09	-0.10 $\pm$ 0.09	0.05 $\pm$ 0.17	0.10 $\pm$ 0.27	N/D ^c
CA002	-0.10 $\pm$ 0.12	-0.15 $\pm$ 0.12	0 $\pm$ 0.15	0 $\pm$ 0.21	N/D ^c
CA003	-0.20 $\pm$ 0.09	-0.50 $\pm$ 0.19	-0.10 $\pm$ 0.17	-0.30 $\pm$ 0.15	N/D ^c
CA004	-0.15 $\pm$ 0.12	0.05 $\pm$ 0.09	0 $\pm$ 0.15	-0.15 $\pm$ 0.15	N/D ^c
CA005	-0.25 $\pm$ 0.19	0.10 $\pm$ 0.19	-0.40 $\pm$ 0.17	-0.20 $\pm$ 0.23	N/D ^c
EO001	-0.20 $\pm$ 0.12	0.05 $\pm$ 0.17	0.10 $\pm$ 0.23	-0.15 $\pm$ 0.15	N/D ^c
EO002	-0.35 $\pm$ 0.12	-0.05 $\pm$ 0.12	-0.20 $\pm$ 0.17	-0.20 $\pm$ 0.17	N/D ^c
EO003	-0.10 $\pm$ 0.09	0.10 $\pm$ 0.12	0.15 $\pm$ 0.15	-0.10 $\pm$ 0.17	N/D ^c
VS001	0.60 $\pm$ 0.12	1.33 $\pm$ 0.15	1.10 $\pm$ 0.17	4.92 $\pm$ 0.09	0.40 $\pm$ 0.09
VS002	N/A ^a	N/A ^a	N/A ^a	N/A ^a	N/D ^c
VS003	-0.05 $\pm$ 0.09	-0.10 $\pm$ 0.21	-0.05 $\pm$ 0.17	-0.10 $\pm$ 0.12	N/D ^c
VS004	5.06 $\pm$ 0.09^b	3.00 $\pm$ 0.16	5.37 $\pm$ 0.12^b	5.96 $\pm$ 0.10^b	0.83 $\pm$ 0.10 and 4.30 $\pm$ 0.14
SN001	-0.57 $\pm$ 0.10	3.22 $\pm$ 0.10	-0.40 $\pm$ 0.12	-1.05 $\pm$ 0.15	-0.50 $\pm$ 0.12
SN002	0.10 $\pm$ 0.09	-0.45 $\pm$ 0.15	-0.10 $\pm$ 0.17	-0.30 $\pm$ 0.12	-0.20 $\pm$ 0.09
SN003	-0.15 $\pm$ 0	0.05 $\pm$ 0.17	0 $\pm$ 0.15	-0.05 $\pm$ 0.09	N/D ^c
SN004	-0.05 $\pm$ 0.09	-0.55 $\pm$ 0.17	-0.25 $\pm$ 0.17	-0.40 $\pm$ 0.12	N/D ^c
SN005	0 $\pm$ 0.15	0.15 $\pm$ 0.30	0 $\pm$ 0.15	-0.20 $\pm$ 0.09	N/D ^c
SN006	-2.31 $\pm$ 0.09	1.26 $\pm$ 0.12	-1.98 $\pm$ 0.09	N/A ^a	-0.85 $\pm$ 0.09
SN007	-2.45 $\pm$ 0.12	1.21 $\pm$ 0.11	-2.21 $\pm$ 0.10	N/A ^a	-1.18 $\pm$ 0.11
SN008	-0.05 $\pm$ 0.23	0.20 $\pm$ 0.23	0.15 $\pm$ 0.15	0.10 $\pm$ 0.09	N/D ^c

^aNo evaluable melting curves and first derivatives with clearly defined melting points were obtained.

^bMelting curves with two transitions. First derivatives of the melting curves showed a first small shoulder.

^cNot determined (N/D). Only the identified fragment hits were investigated with T-p53C-C124/182/277S.

3.4.9 Concentration-Dependent DSF Measurements

Concentration-dependent DSF measurements with T-p53C-Y220C and T-p53C after 30 min of incubation were performed with the previously identified CovLib hits VS004, SN001, SN002, SN006, and SN007. Compound VS001 was excluded from further investigation due to its PAINS characteristic.

For the concentration-dependent measurements, only the measured values from the corresponding runs for the 1 mM compound concentration and 30 min incubation time were considered, not the mean value of all measurements as in the primary screening in Section 3.4.8.

3.4.9.1 Concentration-Dependent DSF Measurements with VS004

For covalent fragments, such as 3-benzoylacrylic acid, stabilization of the protein typically increases up to a maximum with increasing compound concentrations [130]. This trend was also observed for VS004. With increasing compound concentration, the stabilization of T-p53C-Y220C increased up to a maximum stabilization (Figure 3.33 B, Table 3.9). From a compound concentration of 250 μM ($\Delta T_m = 2.90\text{ }^\circ\text{C}$) onwards, the stabilization of T-p53C-Y220C tends to reach its maximum value. Up to a lowest effective concentration of 62.5 μM VS004 (protein-to-compound ratio 1:7.81), a distinct stabilization of T-p53C-Y220C was evident ($\Delta T_m = 0.75\text{ }^\circ\text{C}$).

Concentration-dependent measurements of T-p53C incubated with VS004 showed higher overall stabilization values compared to T-p53C-Y220C. A large stabilization of T-p53C was evident from 250 μM to 1 mM (Figure 3.33 A). The highest increase in the T_m ($\Delta T_m = 6.33\text{ }^\circ\text{C}$) was observed at 250 μM VS004. A further decrease in VS004 concentration resulted in a pronounced decline in the stabilization of T-p53C. At 125 μM VS004, the main peak corresponded to a ΔT_m value of 0.53 $^\circ\text{C}$ (Table 3.9).

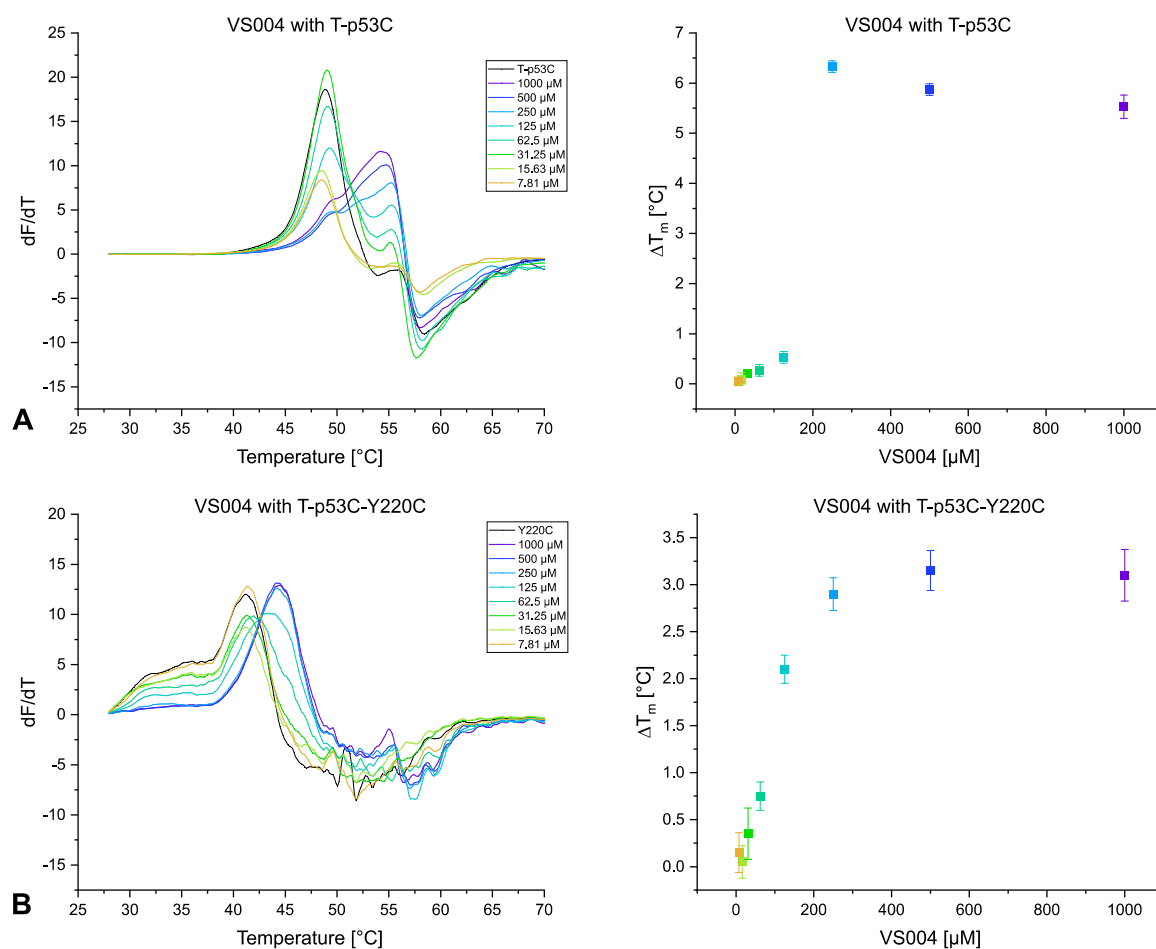


Figure 3.33: Concentration-dependent DSF measurements with VS004. The first derivatives of the melting curves and resulting ΔT_m values of T-p53C (**A**) and T-p53C-Y220C (**B**) (8 μ M protein) with various concentrations of compound VS004 after 30 min of incubation at 20 °C are shown.

Table 3.9: $\Delta T_m \pm$ SD of T-p53C and T-p53C-Y220C (8 μ M protein) incubated with different concentrations of compound VS004 for 30 min at 20 °C. Measurements were performed at least in triplicate.

VS004	T-p53C	T-p53C-Y220C
[μ M]	$\Delta T_m \pm$ SD [°C]	$\Delta T_m \pm$ SD [°C]
1000	5.53 ± 0.23	3.10 ± 0.27
500	5.87 ± 0.12	3.15 ± 0.21
250	6.33 ± 0.12	2.90 ± 0.17
125	0.53 ± 0.12	2.10 ± 0.15
62.5	0.27 ± 0.12	0.75 ± 0.15
31.25	0.20 ± 0	0.35 ± 0.27
15.63	0.10 ± 0.12	0.05 ± 0.17
7.81	0.05 ± 0.09	0.15 ± 0.21

3.4.9.2 Concentration-Dependent DSF Measurements with SN001

For the S_NAr reactive pyridine derivative SN001, no classical concentration-dependent behavior was observed in the ΔT_m values of T-p53C-Y220C and T-p53C. It was evident that the stabilization of T-p53C-Y220C increased as the concentration of SN001 decreased (Figure 3.34 B, Table 3.10). The highest stabilization of T-p53C-Y220C was achieved at 250 μM SN001, with a ΔT_m of 4.45 $^{\circ}C$, corresponding to a protein-to-compound ratio of 1:31.25. A stabilizing effect of T-p53C-Y220C ($\Delta T_m = 1.00$ $^{\circ}C$) was detected up to a lowest effective concentration of 31.25 μM SN001 (protein-to-compound ratio 1:3.91).

In contrast to the Y220C mutant, a destabilization of T-p53C was observed at an SN001 concentration of 1 mM (Figure 3.34 A). However, as the compound concentration diminished, the destabilization also decreased, and at 250 μM SN001 even a stabilization of T-p53C was detectable ($\Delta T_m = 1.00$ $^{\circ}C$). The characteristic concentration-dependent behavior of fragment SN001 could be explained by its high reactivity. At high compound concentrations, there may be multiple arylations of the protein, with the arylation of some residues being destabilizing. At lower concentrations, there may be more selective arylation of specific cysteine residues (predominantly Cys220), resulting in an overall higher level of stabilization. Therefore, the concentration-dependent DSF measurements also illustrated that compound SN001 particularly stabilized the T-p53C-Y220C mutant. This suggests that Cys220 was arylated, which increased the melting temperature of this thermolabile cancer mutant.

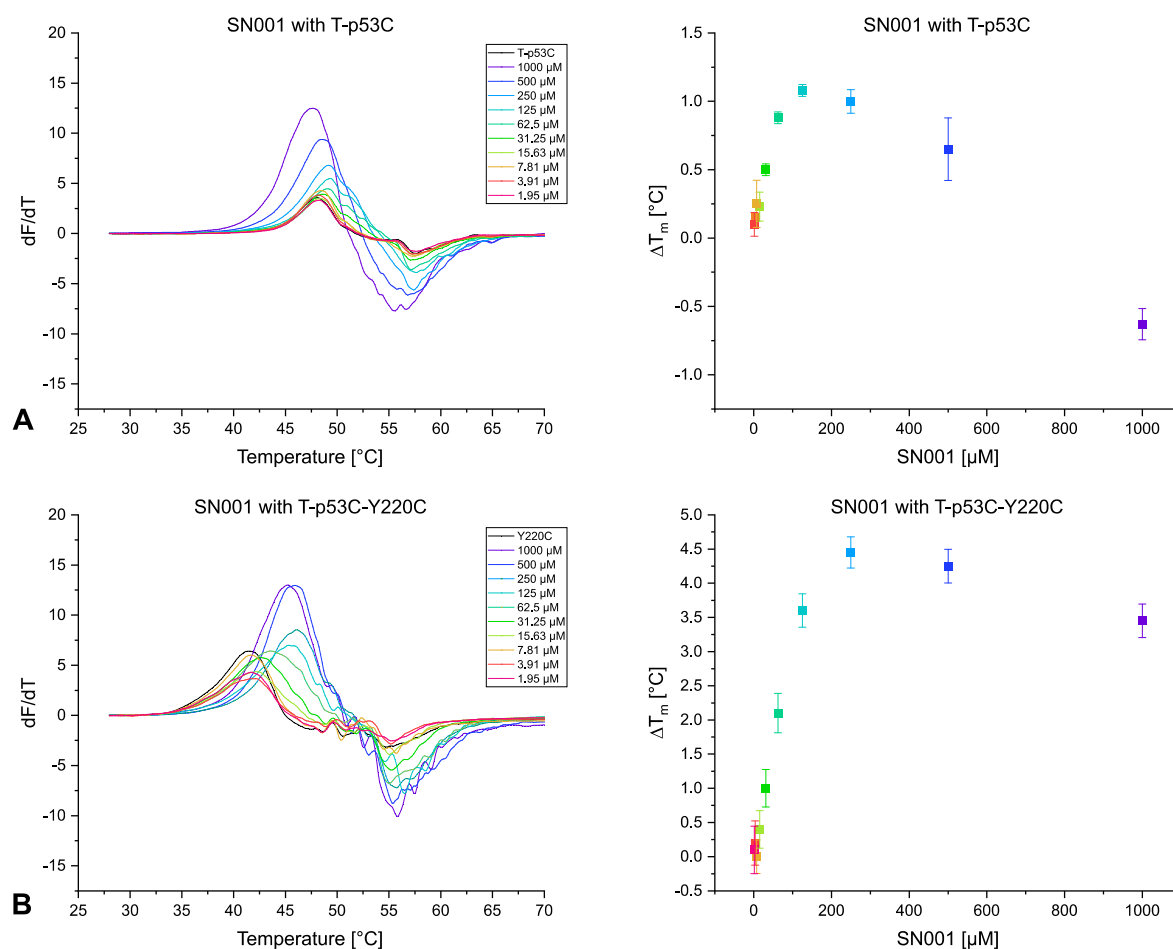


Figure 3.34: Concentration-dependent DSF measurements with SN001. The first derivatives of the melting curves and resulting ΔT_m values of T-p53C (A) and T-p53C-Y220C (B) (8 μM protein) with various concentrations of compound SN001 after 30 min of incubation at 20 °C are depicted.

Table 3.10: $\Delta T_m \pm$ SD of T-p53C and T-p53C-Y220C (8 μM protein) incubated with different concentrations of compound SN001 for 30 min at 20 °C. Measurements were performed at least in triplicate.

SN001	T-p53C	T-p53C-Y220C
[μM]	$\Delta T_m \pm$ SD [°C]	$\Delta T_m \pm$ SD [°C]
1000	-0.63 ± 0.11	3.45 ± 0.24
500	0.65 ± 0.23	4.25 ± 0.24
250	1.00 ± 0.09	4.45 ± 0.23
125	1.08 ± 0.04	3.60 ± 0.24
62.5	0.88 ± 0.04	2.10 ± 0.29
31.25	0.50 ± 0.04	1.00 ± 0.27
15.63	0.23 ± 0.11	0.40 ± 0.27
7.81	0.25 ± 0.17	0 ± 0.24

3.4.9.3 Concentration-Dependent DSF Measurements with SN002

The CovLib compound SN002 was identified as a hit by intact protein MS, leading to covalent modification of p53, but it did not stabilize p53 in the primary DSF screen. However, different concentrations of SN002 were also investigated with T-p53C, with compound concentrations up to 5 mM tested. Figure 3.35 and Table 3.11 show that even variations in the concentrations of SN002 had no substantial effect on the T_m of T-p53C.

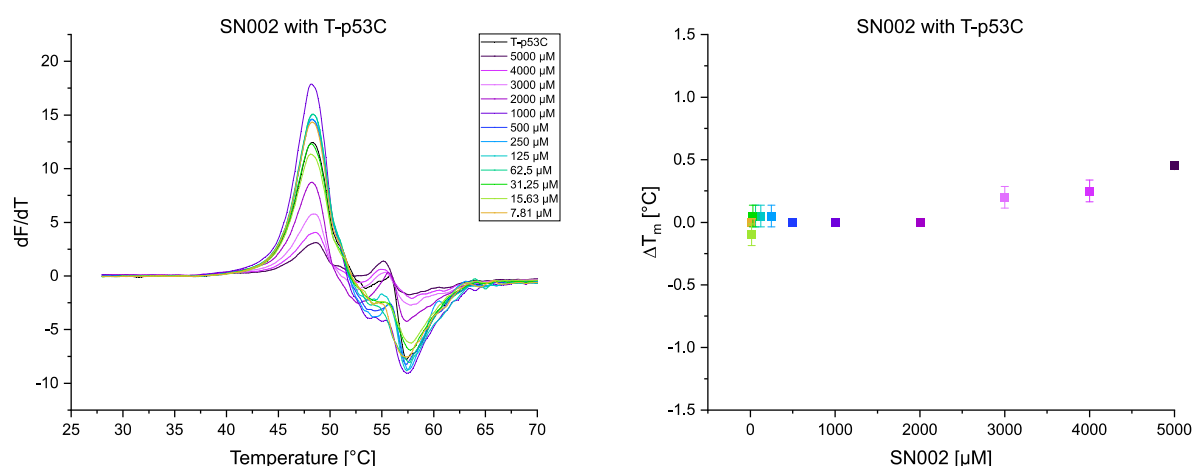


Figure 3.35: Concentration-dependent DSF measurements with SN002. The first derivatives of the melting curves and resulting ΔT_m values of T-p53C (8 μ M protein) with various concentrations of compound SN002 after 30 min of incubation at 20 °C are presented.

Table 3.11: $\Delta T_m \pm$ SD of T-p53C (8 μ M protein) incubated with different concentrations of compound SN002 for 30 min at 20 °C. Measurements were performed at least in triplicate.

SN002 [μ M]	T-p53C $\Delta T_m \pm$ SD [°C]
5000	0.45 ± 0
4000	0.25 ± 0.09
3000	0.20 ± 0.09
2000	0 ± 0
1000	0 ± 0
500	0 ± 0
250	0.05 ± 0.09
125	0.05 ± 0.09
62.5	0.05 ± 0.09
31.25	0.05 ± 0.09

3.4.9.4 Concentration-Dependent DSF Measurements with SN006 and SN007

The two S_NAr reactive compounds SN006 and SN007 differ only in their LGs (chlorine or bromine) and, thus, their interactions once attached to the proteinogenic nucleophiles should be identical. However, the frequency and statistics of modifying specific residues of the protein could differ based on their individual reactivity. The stabilization profile of both fragments proved to behave quite comparable. The two pyrazine derivatives SN006 and SN007 exhibited similar concentration-dependent effects as the fragment SN001. Again, the stabilization of T-p53C-Y220C increased with decreasing compound concentrations (Figure 3.36 B, Figure 3.37 B). The highest stabilization occurred at 500 μ M SN006 ($\Delta T_m = 1.82$ °C, Table 3.12) and 250 μ M SN007 ($\Delta T_m = 2.03$ °C, Table 3.13), respectively. However, the stabilizing effect on T-p53C-Y220C was less evident even at these optimal concentrations compared to the pyridine derivative SN001.

Conversely to T-p53C-Y220C, high concentrations of SN006 and SN007 strongly destabilized T-p53C, with a ΔT_m of -2.30 °C at 1 mM SN006 (Figure 3.36 A) and a ΔT_m of -2.45 °C at 1 mM SN007 (Figure 3.37 A). At lower concentrations than 125 μ M SN006 and SN007, even a slightly increased melting temperature of T-p53C was detected. At 62.5 μ M SN006 and SN007 or below, the relative $\Delta\Delta T_m$ between T-p53C and T-p53C-Y220C (Table 3.12, Table 3.13) becomes very small or shows even higher stability of T-p53C, suggesting that Cys220 does not play significant role in the stabilization at these low concentrations.

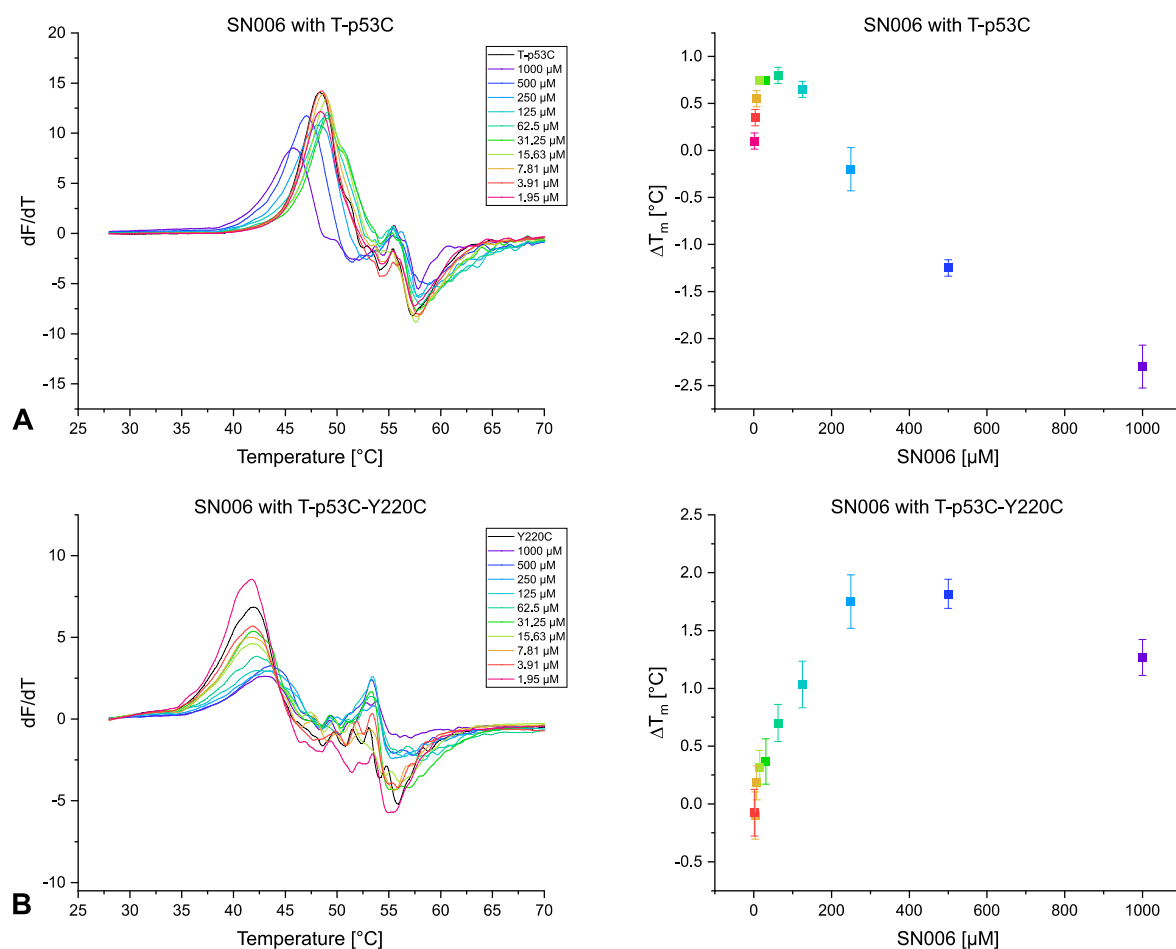


Figure 3.36: Concentration-dependent DSF measurements with SN006. The first derivatives of the melting curves and resulting ΔT_m values of T-p53C (A) and T-p53C-Y220C (B) (8 μ M protein) with various concentrations of compound SN006 after 30 min of incubation at 20 °C are shown.

Table 3.12: $\Delta T_m \pm$ SD of T-p53C and T-p53C-Y220C (8 μ M protein) incubated with different concentrations of compound SN006 for 30 min at 20 °C. Measurements were performed at least in triplicate.

SN006	T-p53C	T-p53C-Y220C
[μ M]	$\Delta T_m \pm$ SD [°C]	$\Delta T_m \pm$ SD [°C]
1000	-2.30 ± 0.23	1.27 ± 0.15
500	-1.25 ± 0.09	1.82 ± 0.13
250	-0.20 ± 0.23	1.75 ± 0.23
125	0.65 ± 0.09	1.03 ± 0.20
62.5	0.80 ± 0.09	0.70 ± 0.16
31.25	0.75 ± 0	0.37 ± 0.20
15.63	0.75 ± 0	0.32 ± 0.15
7.81	0.55 ± 0.09	0.18 ± 0.15

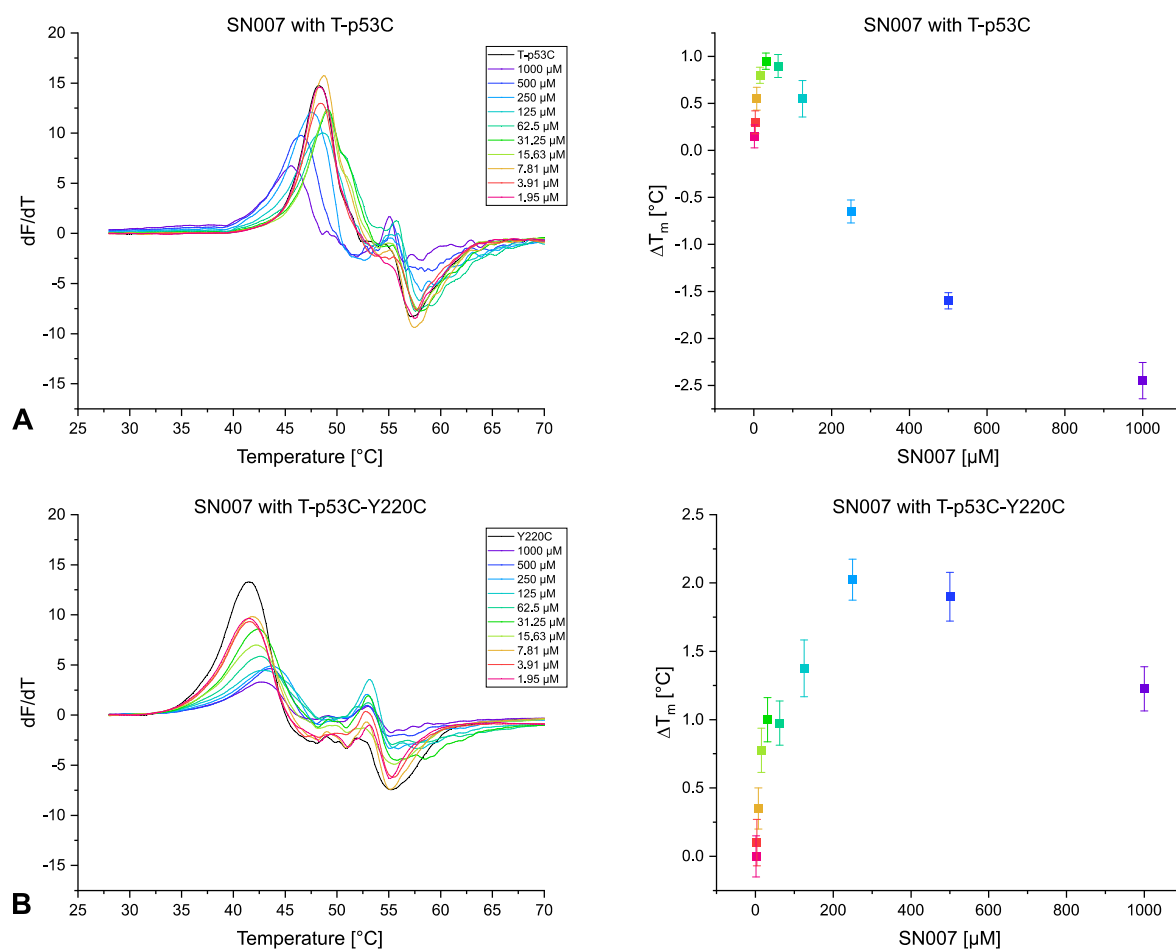


Figure 3.37: Concentration-dependent DSF measurements with SN007. The first derivatives of the melting curves and resulting ΔT_m values of T-p53C (A) and T-p53C-Y220C (B) (8 μ M protein) with various concentrations of compound SN007 after 30 min of incubation at 20 °C are shown.

Table 3.13: $\Delta T_m \pm$ SD of T-p53C and T-p53C-Y220C (8 μ M protein) incubated with different concentrations of compound SN007 for 30 min at 20 °C. Measurements were performed at least in triplicate.

SN007 [μM]	T-p53C $\Delta T_m \pm$ SD [°C]	T-p53C-Y220C $\Delta T_m \pm$ SD [°C]
1000	-2.45 ± 0.19	1.23 ± 0.16
500	-1.60 ± 0.09	1.90 ± 0.18
250	-0.65 ± 0.12	2.03 ± 0.15
125	0.55 ± 0.19	1.38 ± 0.21
62.5	0.90 ± 0.12	0.98 ± 0.16
31.25	0.95 ± 0.09	1.00 ± 0.16
15.63	0.80 ± 0.09	0.78 ± 0.16
7.81	0.55 ± 0.12	0.35 ± 0.15

3.4.10 Time-Dependent DSF Measurements

Covalent fragments show a time-dependent reaction behavior. The target occupancy increases with prolongation of the incubation period [144]. Hence, time-dependent effects of the compounds on the T_m of T-p53C and T-p53C-Y220C were investigated. As depicted in Table 3.5, the identified CovLib hits VS004, SN001, SN006, and SN007 are reactive or highly reactive fragments and have a $t_{1/2}$ of <1 h with GSH. Fragment SN002 is the only fragment exhibiting intermediate reactivity ($t_{1/2}$ GSH= 11 h).

For the time-dependent measurements, only the measured values from the corresponding runs were considered, not the mean value of all measurements as in the primary screening in Section 3.4.6 and Section 3.4.8.

3.4.10.1 Time-Dependent DSF Measurements with VS004

The vinyl sulfone with β -LG VS004 is a reactive fragment. In the GSH experiment, it exhibited a $t_{1/2}$ of 0.82 h. Time-dependent DSF measurements with T-p53C and 1 mM VS004 confirmed the rapid reaction with the target (Figure 3.38, Table 3.14). After 30 min of incubation, no substantial time-dependent effects were detected upon incubation with T-p53C.

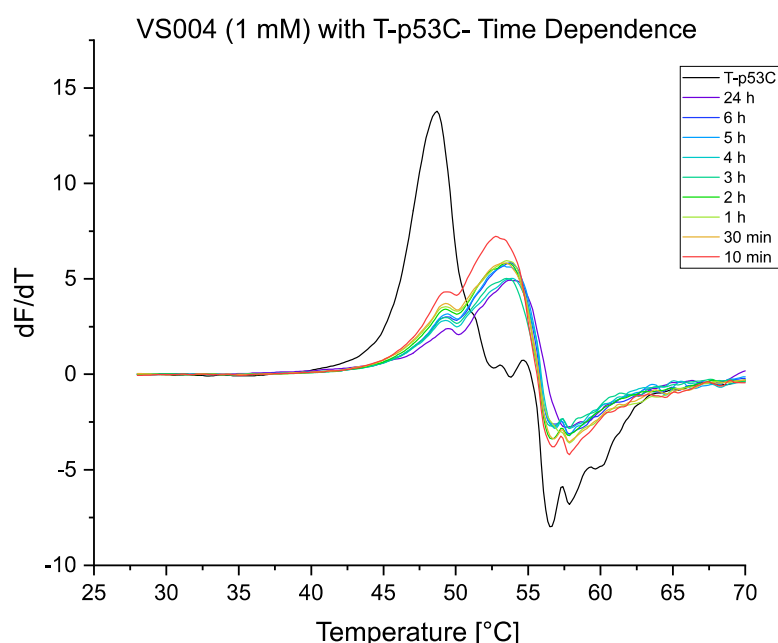


Figure 3.38: Time-dependent DSF measurements with VS004. The first derivatives of the melting curves of T-p53C (8 μ M) with VS004 (1 mM) after varying incubation periods at 20 °C are shown.

Table 3.14: $\Delta T_m \pm$ SD of T-p53C (8 μ M) incubated with VS004 (1 mM) after different periods of time at 20 °C. Measurements were performed in triplicate.

Incubation Time	VS004 (1 mM) with T-p53C
	$\Delta T_m \pm$ SD [°C]
24 h	5.20 ± 0.23
6 h	4.90 ± 0.31
5 h	4.90 ± 0.27
4 h	5.25 ± 0.19
3 h	4.95 ± 0.39
2 h	4.80 ± 0.12
1 h	5.05 ± 0.27
30 min	4.95 ± 0.12
10 min	4.35 ± 0.36

3.4.10.2 Time-Dependent DSF Measurements with SN001

The pyridine derivative SN001 (1 mM) was analyzed with T-p53C (Figure 3.39 A) and T-p53C-Y220C (Figure 3.39 B) after different incubation periods. Additionally, the time-dependent impact of SN001 at a concentration of 250 μ M on the stabilization of T-p53C-Y220C was also tested, as this concentration showed the highest increase in the T_m in the concentration-dependent assessments (Figure 3.39 C). In general, no time-dependent effects on the stabilization of either T-p53C or T-p53C-Y220C (Figure 3.39, Table 3.15) were detected. There were no substantial changes observed in the destabilization of T-p53C or the stabilization of T-p53C-Y220C with increasing incubation time. The S_NAr -type electrophile SN001 markedly stabilized T-p53C-Y220C after just 10 min at both 1 mM ($\Delta T_m = 3.05$ °C) and 250 μ M SN001 ($\Delta T_m = 4.25$ °C). GSH reactivity studies revealed that fragment SN001 is a highly reactive fragment with a $t_{1/2} \ll 0.33$ h. Thus, it reacts immediately with nucleophiles, explaining the lack of time-dependent effects.

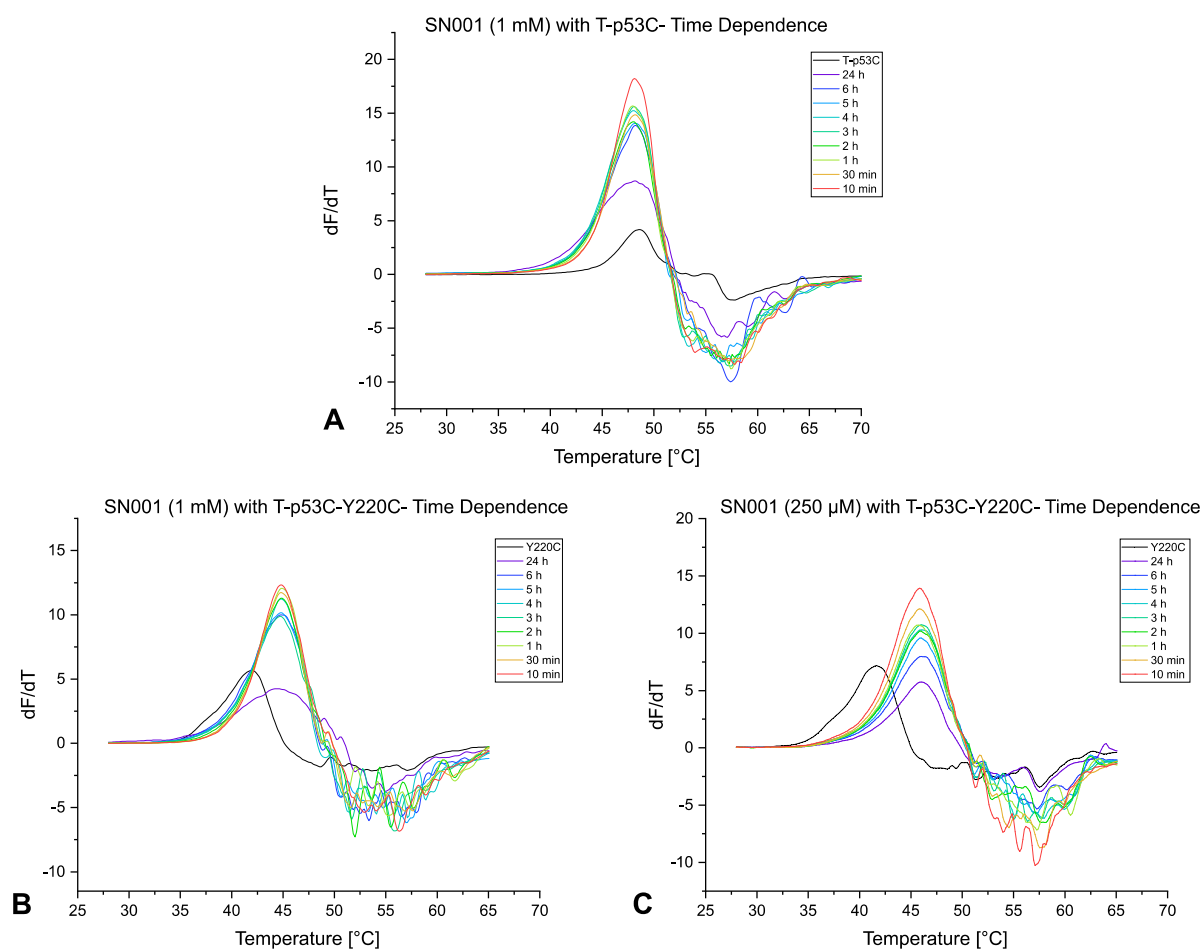


Figure 3.39: Time-dependent DSF measurements with SN001. The first derivatives of the melting curves of T-p53C (8 μ M) with SN001 (1 mM) (A), and T-p53C-Y220C (8 μ M) with 1 mM (B) and 250 μ M (C) SN001 after varying incubation periods at 20 $^{\circ}$ C are shown.

Table 3.15: $\Delta T_m \pm$ SD of T-p53C (8 μ M) incubated with SN001 (1 mM) and T-p53C-Y220C with SN001 (1 mM and 250 μ M) after different periods of time at 20 $^{\circ}$ C. Measurements were performed in triplicate.

Incubation Time	SN001 (1 mM) with T-p53C $\Delta T_m \pm$ SD [$^{\circ}$ C]	SN001 (1 mM) with T-p53C-Y220C $\Delta T_m \pm$ SD [$^{\circ}$ C]	SN001 (250 μ M) with T-p53C-Y220C $\Delta T_m \pm$ SD [$^{\circ}$ C]
24 h	-0.40 ± 0.17	2.95 ± 0.17	4.40 ± 0.09
6 h	-0.40 ± 0.17	3.00 ± 0	4.40 ± 0.17
5 h	-0.30 ± 0.12	2.95 ± 0.09	4.35 ± 0.12
4 h	-0.60 ± 0.12	3.05 ± 0.09	4.40 ± 0.09
3 h	-0.50 ± 0.12	3.05 ± 0.09	4.35 ± 0.12
2 h	-0.60 ± 0.12	3.05 ± 0.09	4.40 ± 0.09
1 h	-0.55 ± 0.09	3.05 ± 0.09	4.25 ± 0.17
30 min	-0.45 ± 0.19	3.05 ± 0.09	4.30 ± 0.12
10 min	-0.35 ± 0.12	3.05 ± 0.09	4.25 ± 0.09

3.4.10.3 Time-Dependent DSF Measurements with SN002

The triazole derivative SN002 is the only CovLib fragment that showed an intermediate level of reactivity in the GSH experiment ($t_{1/2} = 11$ h). To ascertain the impact of an elongated incubation period on target engagement and thermal stabilization, a series of incubation periods were evaluated (Figure 3.40, Table 3.16). Time-dependent DSF measurements revealed that even after an incubation time of 24 h, no substantial stabilization of T-p53C was achieved ($\Delta T_m = 0.40$ °C). Although the MS experiment confirmed the covalent modification of T-p53C, compound SN002 was not further investigated as the primary objective was the thermal stabilization of T-p53C and its mutants.

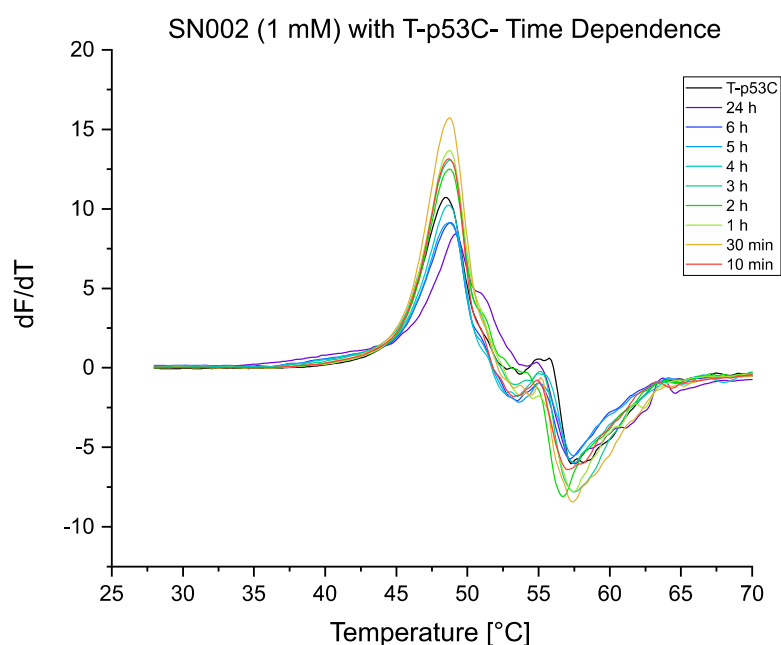


Figure 3.40: Time-dependent DSF measurements with SN002. The first derivatives of the melting curves of T-p53C (8 μM) with SN002 (1 mM) after varying incubation periods at 20 °C are shown.

Table 3.16: $\Delta T_m \pm SD$ of T-p53C (8 μ M) incubated with SN002 (1 mM) after different periods of time at 20 °C. Measurements were performed in triplicate.

Incubation Time	SN002 (1 mM) with T-p53C $\Delta T_m \pm SD$ [°C]
24 h	0.40 ± 0.12
6 h	0.30 ± 0.12
5 h	0.25 ± 0.12
4 h	0.20 ± 0.09
3 h	0.25 ± 0.12
2 h	0.25 ± 0.12
1 h	0.20 ± 0.09
30 min	0.20 ± 0.09
10 min	0.20 ± 0.09

3.4.10.4 Time-Dependent Measurements with SN006 and SN007

The two pyrazine derivatives SN006 and SN007 are also highly reactive fragments with a $t_{1/2} \ll 0.33$ h in the GSH assay. Both fragments were tested with T-p53C-Y220C at concentrations of 1 mM and 250 μ M. As shown in Figure 3.41 A and Figure 3.41 C, the stabilization of T-p53C-Y220C decreased with prolonged incubation time at 1 mM SN006 and SN007. After 24 h incubation of T-p53C-Y220C with 1 mM SN006 and SN007, no melting curves of the protein were retained. High initial fluorescence intensities were detected, possibly due to protein denaturation or aggregation effects [289]. The highly reactive compounds likely cause multiple arylations of T-p53C-Y220C at a concentration of 1 mM.

Concentration-dependent DSF measurements showed that incubation with 250 μ M SN006 and SN007 resulted in a higher overall stabilization of T-p53C-Y220C than that observed at 1 mM compound concentration. Time-dependent measurements also revealed differences between the two tested compound concentrations. In contrast to 1 mM SN006/7, at 250 μ M SN006 and SN007, the T_m of T-p53C-Y220C increased quite substantially as the incubation time increased (Figure 3.41 B, D; Table 3.17). While speculations about the molecular basis for these opposite trends at 1 mM and 250 μ M SN006 and SN007 could be made, they do not appear to be beneficial from a ligand optimization perspective.

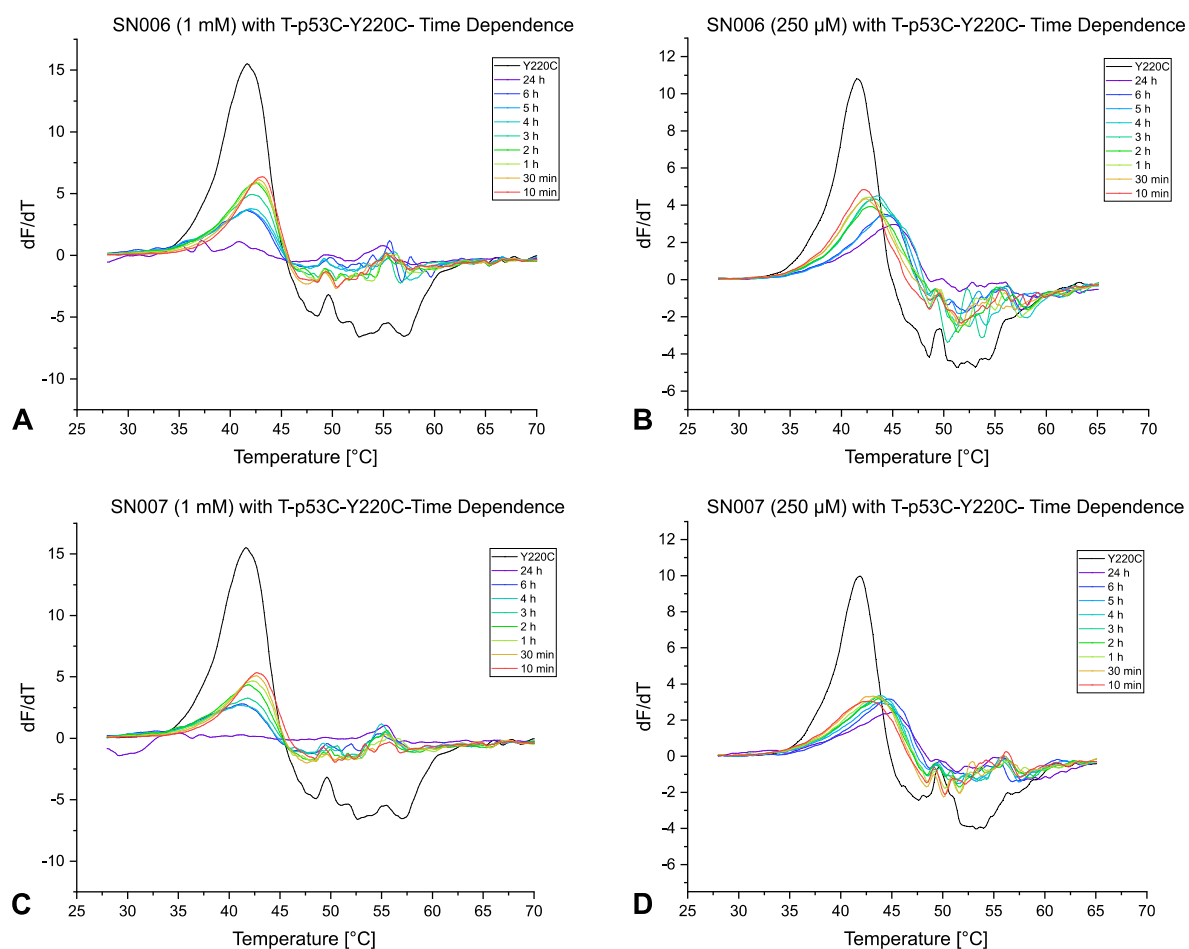


Figure 3.41: Time-dependent DSF measurements with SN006 and SN007. The first derivatives of the melting curves of T-p53C-Y220C (8 μM) with 1 mM SN006 (A), 250 μM SN006 (B), 1 mM SN007 (C), and 250 μM SN007 (D) after various incubation periods at 20 $^{\circ}\text{C}$ are shown.

Table 3.17: $\Delta T_m \pm$ SD of T-p53C-Y220C (8 μ M) incubated with SN006 (1 mM and 250 μ M) or SN007 (1 mM and 250 μ M) after different periods of time at 20 °C. Measurements were performed in triplicate.

Incubation Time	SN006 (1 mM) with T-p53C-Y220C $\Delta T_m \pm$ SD [°C]	SN006 (250 μ M) with T-p53C-Y220C $\Delta T_m \pm$ SD [°C]	SN007 (1 mM) with T-p53C-Y220C $\Delta T_m \pm$ SD [°C]	SN007 (250 μ M) with T-p53C-Y220C $\Delta T_m \pm$ SD [°C]
24 h	N/A ^a	3.30 $\pm$ 0.32	N/A ^a	3.50 $\pm$ 0.23
6 h	-0.05 $\pm$ 0.12	2.95 $\pm$ 0.30	-0.40 $\pm$ 0.09	3.45 $\pm$ 0.12
5 h	0 $\pm$ 0.36	2.90 $\pm$ 0.45	-0.35 $\pm$ 0.24	2.40 $\pm$ 0.19
4 h	0.25 $\pm$ 0.19	2.15 $\pm$ 0.09	-0.50 $\pm$ 0.24	2.40 $\pm$ 0.12
3 h	0.25 $\pm$ 0.32	2.00 $\pm$ 0.15	0.15 $\pm$ 0.12	2.25 $\pm$ 0.12
2 h	0.85 $\pm$ 0.32	1.80 $\pm$ 0.11	-0.05 $\pm$ 0.39	2.30 $\pm$ 0.09
1 h	1.10 $\pm$ 0.17	1.75 $\pm$ 0.09	0.75 $\pm$ 0.12	2.15 $\pm$ 0.31
30 min	1.25 $\pm$ 0.17	1.55 $\pm$ 0.15	1.05 $\pm$ 0.12	1.78 $\pm$ 0.14
10 min	1.50 $\pm$ 0.12	1.55 $\pm$ 0.09	1.20 $\pm$ 0.12	1.65 $\pm$ 0.12

^aNo evaluable melting curves and first derivatives with clearly defined melting points were obtained.

3.4.11 $^1\text{H}/^{15}\text{N}$ -HSQC-NMR Experiments with ^{15}N -Labeled T-p53C-Y220C

$^1\text{H}/^{15}\text{N}$ -HSQC-NMR experiments were performed to validate the hits identified by MS and DSF analysis and to obtain information about the binding site of the compounds. Additionally, the potential binding of the fragments SN001, SN006, and SN007 in the Y220C pocket was examined. Since the identified fragments have a covalent binding mechanism, no fast exchange with an average resonance was observed, but rather a slow exchange with two different resonances for the bound and the unbound protein. In titration experiments involving a slow exchange, only the intensities of the peaks are modulated, with a decrease in the intensity of the peak corresponding to the unbound protein and an increase in the signal intensity of the bound peak [317-320]. Therefore, no titration experiments to trace peak shifts could be performed. The slow exchange complicates the localization of the shifted peaks and requires a reassignment of the NMR spectrum of the bound protein for an accurate analysis [318, 319].

However, prominent peak shifts were evident in the $^1\text{H}/^{15}\text{N}$ -HSQC-NMR spectrum of compound SN001 with T-p53C-Y220C (Figure 3.42). Typical shifts in common with already published Y220C cleft binders were identified, as shown in Figure 3.42 B [99, 100]. This provided further evidence that fragment SN001 covalently targets Cys220 in the mutational cleft. In addition, distinct peak shifts similar to the already known p53

arylated 2-sulfonylpyrimidine PK11000 were apparent (Figure 3.42 C), indicating a slow exchange and covalent modification of cysteine residues [131].

In the $^1\text{H}/^{15}\text{N}$ -HSQC-NMR spectra of fragments VS004, SN006, and SN007, only minor changes were visible (Figure 3.43).

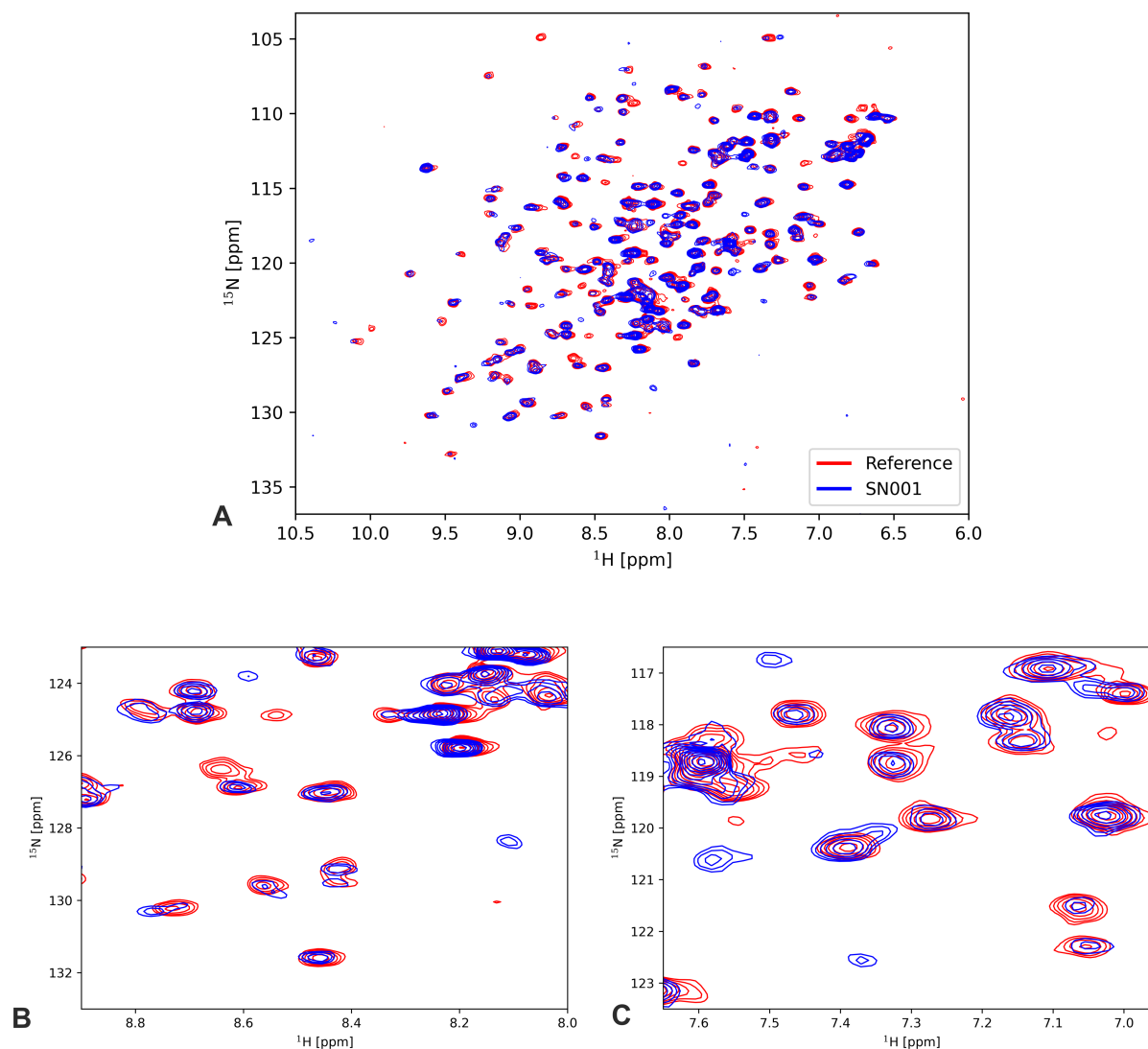


Figure 3.42: $^1\text{H}/^{15}\text{N}$ -HSQC-NMR experiments with ^{15}N -labeled T-p53C-Y220C and SN001. (A) $^1\text{H}/^{15}\text{N}$ -HSQC-NMR spectra of T-p53C-Y220C (70 μM) (red) and of T-p53C-Y220C with 1 mM SN001 (blue). Prominent peak shifts were observed, indicating binding into the Y220C pocket [99, 100] (B) as well as slow exchange with covalent binding analogous to compound PK11000 [131] (C).

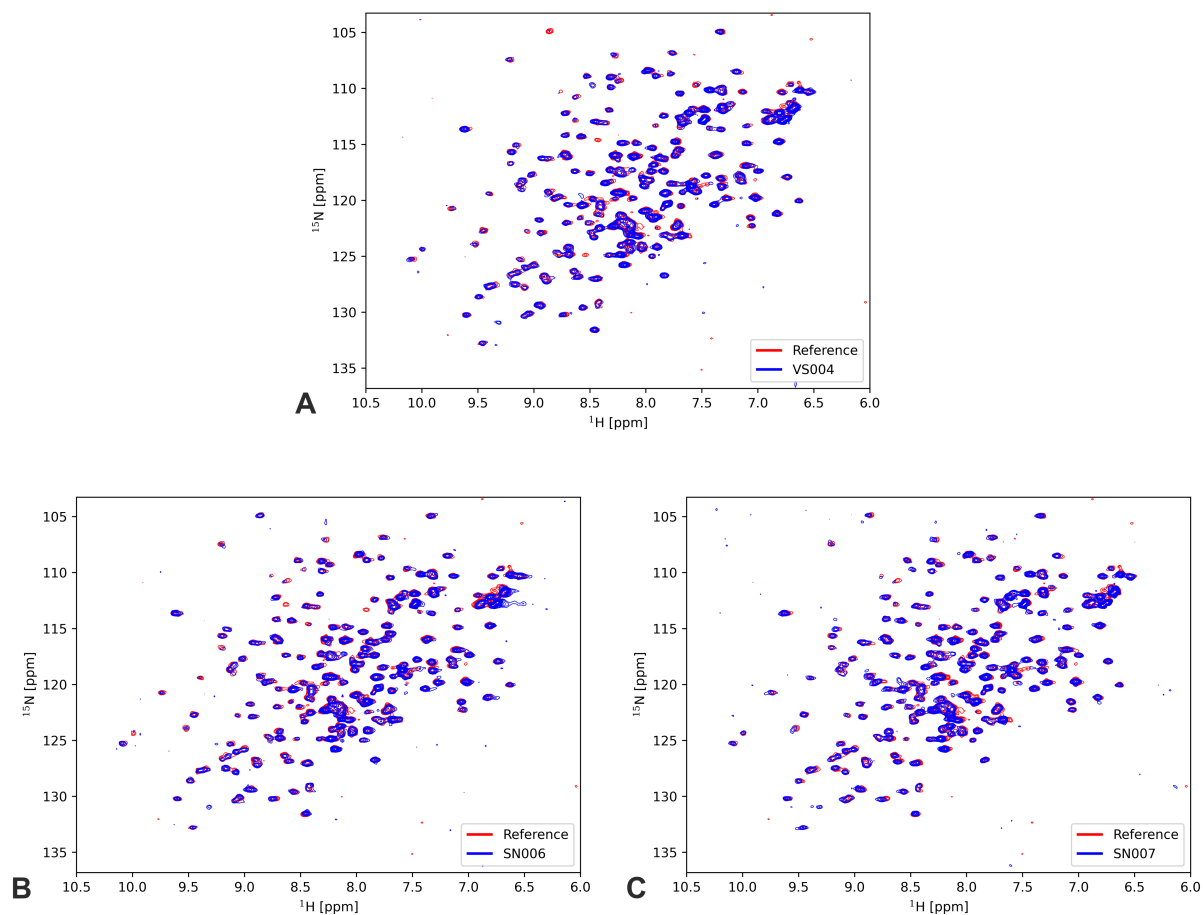


Figure 3.43: $^1\text{H}/^{15}\text{N}$ -HSQC-NMR experiments with ^{15}N -labeled T-p53C-Y220C and VS004, SN006, and SN007. $^1\text{H}/^{15}\text{N}$ -HSQC-NMR spectra of T-p53C-Y220C (70 μM) (red) and of T-p53C-Y220C with the addition of ligand (blue): 1 mM VS004 (A), 500 μM SN006 (B), and 500 μM SN007 (C). Due to solubility issues, only 500 μM SN006 and SN007 were added.

3.4.12 Intact Protein Mass Spectrometry of T-p53C-Y220C with SN001

The assumption that SN001 is covalently bound to Cys220 in the binding pocket was substantiated by UHPLC-ESI-MS measurements with T-p53C-Y220C. In the deconvoluted MS spectrum of T-p53C (molar ratio 1:100), the double modified protein represented the highest signal (Figure 3.30 A). In contrast, in the spectrum of the Y220C mutant (molar ratio 1:100), containing the additional Cys220, the triple arylated species exhibited the highest signal (Figure 3.44 A). This indicates a covalent modification of the mutational Cys220. Analysis of extracted ion chromatograms (XICs) for each combination of protein with compound showed that up to seven times arylated protein was detectable (Figure 3.44 B). However, even at the high molar ratio of 1:100 the five (3.79%), six (1.55%), and seven (1.52%) times modified protein species represented only a very small fraction. As there are only five surface-exposed cysteines and one additional cysteine in the binding pocket of T-p53C-Y220C, a weak level of covalent binding to other nucleophilic amino acids or buried cysteines is possible at a molar ratio of 1:100 and a considerable excess of SN001.

In addition, MS experiments of T-p53C-Y220C incubated with SN001 at different molar ratios were performed. The analysis of XICs illustrated that with decreasing SN001 concentration, protein species with fewer covalently attached molecules were more abundant (Figure 3.44 C). In contrast, the results of DSF experiments indicated increasing stabilization as the concentration of SN001 diminished. Indeed, this supports the hypothesis that with high concentrations of the reactive fragment SN001, many surface-exposed cysteines are arylated, with some negative effect on the melting temperature of the protein. With decreasing concentration of the compound, presumably the Cys220 in the binding pocket and potentially specific surface-exposed cysteines are more selectively arylated, which has a positive effect on the stability of the protein.

At a protein-to-compound ratio of 1:25, single or double modified protein displayed the largest peak area with 34.01% and 32.93%, respectively. Furthermore, unmodified T-p53C-Y220C (14.01%) was detectable. This molar ratio is comparable to the concentration used in the DSF screen that achieved the highest stabilization (molar ratio 1:31.25, 250 μ M SN001).

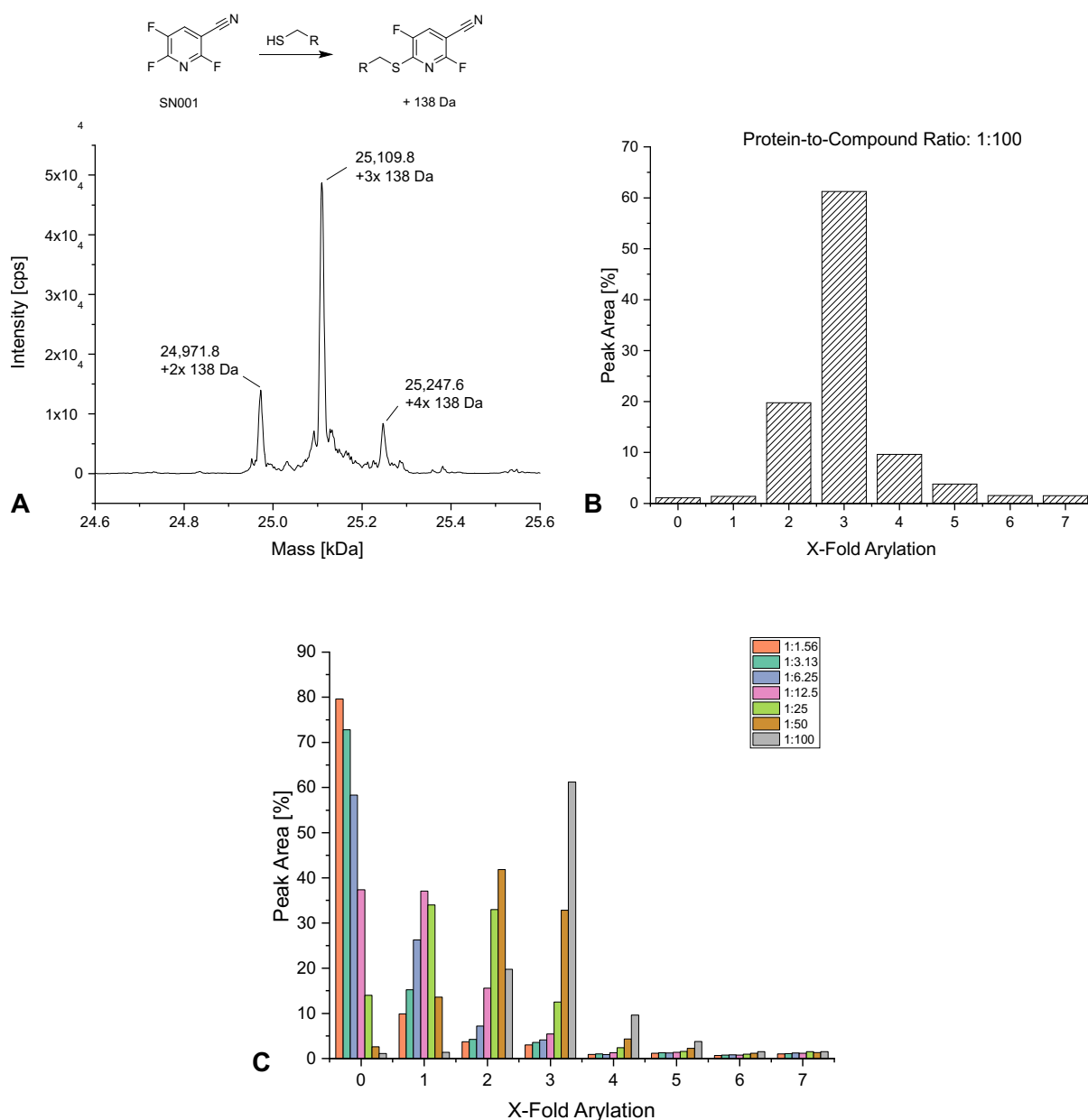


Figure 3.44: Intact protein MS of T-p53C-Y220C with SN001. **(A)** Deconvoluted MS spectrum of T-p53C-Y220C (theoretical mass of unmodified T-p53C-Y220C: 24,696.00 Da) with SN001 (molar ratio 1:100) after 4 h of incubation at 20 °C. **(B)** Relative quantification of T-p53C-Y220C with SN001 (molar ratio 1:100) after 4 h of incubation at 20 °C for each combination of protein plus compound (0-7 molecules). Peak areas were calculated using the +20 charge state of each species. **(C)** Relative quantification for each combination of protein plus compound of T-p53C-Y220C incubated with different concentrations of SN001 (molar ratios 1:100-1:1.56) for 4 h at 20 °C.

3.4.13 Evaluation of the Hits SN001, SN006, and SN007 with T-p53C-Y220C-CL

The CovLib screening results indicated that the S_NAr reactive compounds SN001, SN006, and SN007 arylated Cys220 in the hydrophobic pocket of the p53-Y220C mutant, in addition to the arylation of surface-exposed cysteines. To evaluate the possible labeling of Cys220 in the pocket and investigate the impact of this covalent modification on the T_m , a cysteine light T-p53C-Y220C construct (T-p53C-Y220C-CL) [143] was tested as a control with the compounds. The T-p53C-Y220C-CL mutant still includes the Cys220, whereas the partially or fully solvent-exposed cysteines 124, 182, 229, 275, and 277 were mutated to less nucleophilic serine residues [143]. The CovLib compounds SN001, SN006, and SN007 were analyzed with T-p53C-Y220C-CL using DSF and intact protein MS.

The results of the DSF and MS measurements of the pyridine derivative SN001 are depicted in Figure 3.45 A, Table 3.18, and Figure 3.46. The T_m of T-p53C-Y220C-CL was increased by 2.90 °C after 30 min of incubation with 1 mM SN001. This raise correlates with the increase observed for the T-p53C-Y220C mutant ($\Delta T_m = 3.22$ °C, 1 mM). No substantial increment in the T_m of T-p53C-Y220C-CL was detected with declining SN001 concentration ($\Delta T_m = 3.05$ °C, 250 μ M). In contrast, the stabilization of T-p53C-Y220C raised ($\Delta T_m = 4.45$ °C, 250 μ M) with decreasing compound concentration. However, the increase in the T_m with diminishing concentration of SN001 was also observed for T-p53C, indicating that the modification of other cysteines than Cys220 is responsible for this effect.

In the deconvoluted MS spectrum of T-p53C-Y220C-CL with SN001 (Figure 3.46 A) after 24 h of incubation at a molar ratio of 1:100, signals were detected for both the native protein and the protein species with one SN001 molecule attached via S_NAr . As the molar ratio decreased, the intensity of the peak corresponding to the native protein increased (Figure 3.46 B). The presence of a peak for the native protein and another peak for T-p53C-Y220C-CL arylated with one SN001 molecule indicates that the CovLib fragment SN001 covalently modifies the remaining Cys220. The DSF and the MS studies with the Y220C cysteine light mutant support the hypothesis that the stabilization of T-p53C-Y220C results from the covalent modification of Cys220 in the hydrophobic pocket.

Furthermore, the DSF results of T-p53C-Y220C-CL with fragments SN006 and SN007 showed a stabilizing effect of the CovLib compounds (Figure 3.45 B, Table 3.18). After 30 min of incubation with 1 mM SN006, the T_m of T-p53C-Y220C-CL was increased by 2.95 °C, and with SN007, it increased by 2.70 °C. The T_m shifts of T-p53C-Y220C-CL were larger compared to the Y220C mutant ($\Delta T_m = 1.26$ °C (SN006), $\Delta T_m = 1.21$ °C (SN007)). However, the fragments destabilized T-p53C at 1 mM SN006 and SN007 ($\Delta T_m = -2.31$ °C (SN006), $\Delta T_m = -2.45$ °C (SN007)). This suggests that the covalent modification of surface-exposed cysteines has a destabilizing effect on the protein, leading to reduced stabilization of T-p53C-Y220C compared to T-p53C-Y220C-CL. Interestingly, incubation with 250 μ M SN006 for 30 min did not affect the T_m of the Y220C cysteine light protein. However, the corresponding first derivative of the melting curve presented a broad peak. After 4 h of incubation, the T_m was increased by 2.55 °C. This suggests that after 30 min of incubation at a concentration of 250 μ M, SN006 was still reacting with the target, and the target occupancy and stabilization increased with extended incubation time. In contrast, after 30 min of incubation with 250 μ M SN007, the protein was stabilized by up to 3.00 °C. However, after 24 h of incubation with 1 mM SN007, the signal intensity of the melting curve was low, and the stabilization of T-p53C-Y220C-CL was decreased, indicating destabilization through multiple arylations of the protein.

The intact protein MS results of the reactive pyrazine derivatives SN006 and SN007 showed peaks corresponding to T-p53C-Y220C-CL with more than one molecule attached. This indicates binding to amino acids besides Cys220, such as the buried cysteines 135 and 141 [130] or surface-exposed amino acids other than cysteines. In the deconvoluted MS spectra of T-p53C-Y220C-CL with SN006 at a molar ratio of 1:100 after 24 h of incubation, single and double modified protein species were detected (Figure 3.47 A). In the deconvoluted MS spectra with SN007, an additional peak corresponding to the triple arylated T-p53C-Y220C-CL was evident. The presence of this extra peak indicates that fragment SN007 is more reactive, resulting in more frequent arylation of T-p53C-Y220C-CL. Accordingly, this also accounts for the different DSF results of SN006 and SN007. Overall, the MS data showed that SN006 and SN007 are highly reactive compounds that arylate other amino acids besides Cys220 in T-p53C-Y220C-CL.

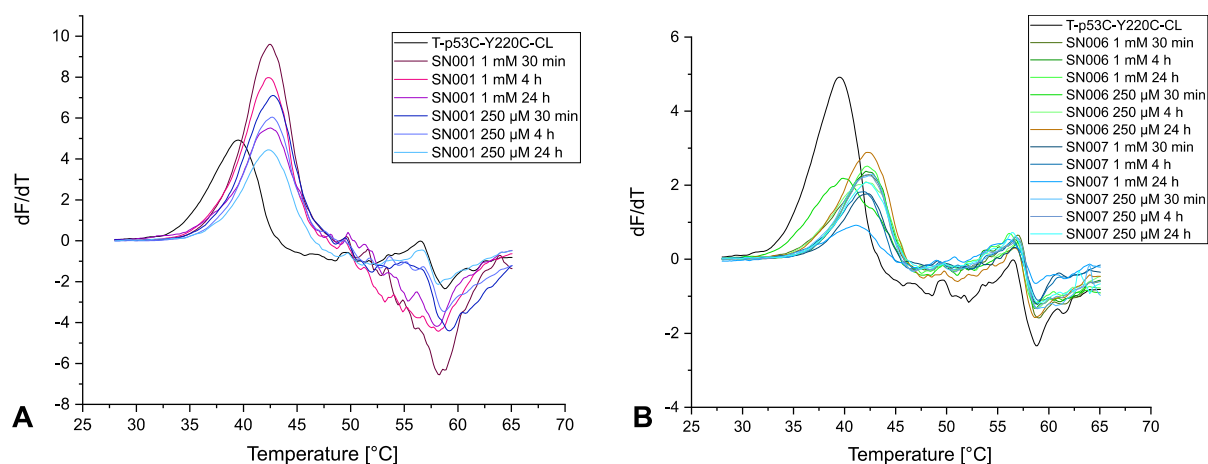


Figure 3.45: DSF measurements of T-p53C-Y220C-CL with SN001, SN006, and SN007. The first derivatives of the melting curves of T-p53C-Y220C-CL (8 μ M) with SN001 (**A**), SN006, and SN007 (**B**) (1 mM, 250 μ M) after varying incubation periods at 20 $^{\circ}$ C are shown.

Table 3.18: $\Delta T_m \pm$ SD of T-p53C-Y220C-CL (8 μ M) incubated with SN001, SN006, and SN007 (1 mM and 250 μ M) after different periods of time at 20 $^{\circ}$ C. Measurements were performed at least in triplicate.

T-p53C-Y220C-CL						
$\Delta T_m \pm$ SD [$^{\circ}$ C]						
	1 mM 30 min	1 mM 4 h	1 mM 24 h	250 μ M 30 min	250 μ M 4 h	250 μ M 24 h
SN001	2.90 ± 0.09	2.80 ± 0.17	3.08 ± 0.12	3.05 ± 0.09	3.15 ± 0	2.95 ± 0.04
SN006	2.95 ± 0.12	2.60 ± 0.09	3.05 ± 0.06	0.15 ± 0	2.55 ± 0	2.63 ± 0.06
SN007	2.70 ± 0.24	2.40 ± 0	1.68 ± 0.11	3.00 ± 0	2.55 ± 0	2.60 ± 0.06

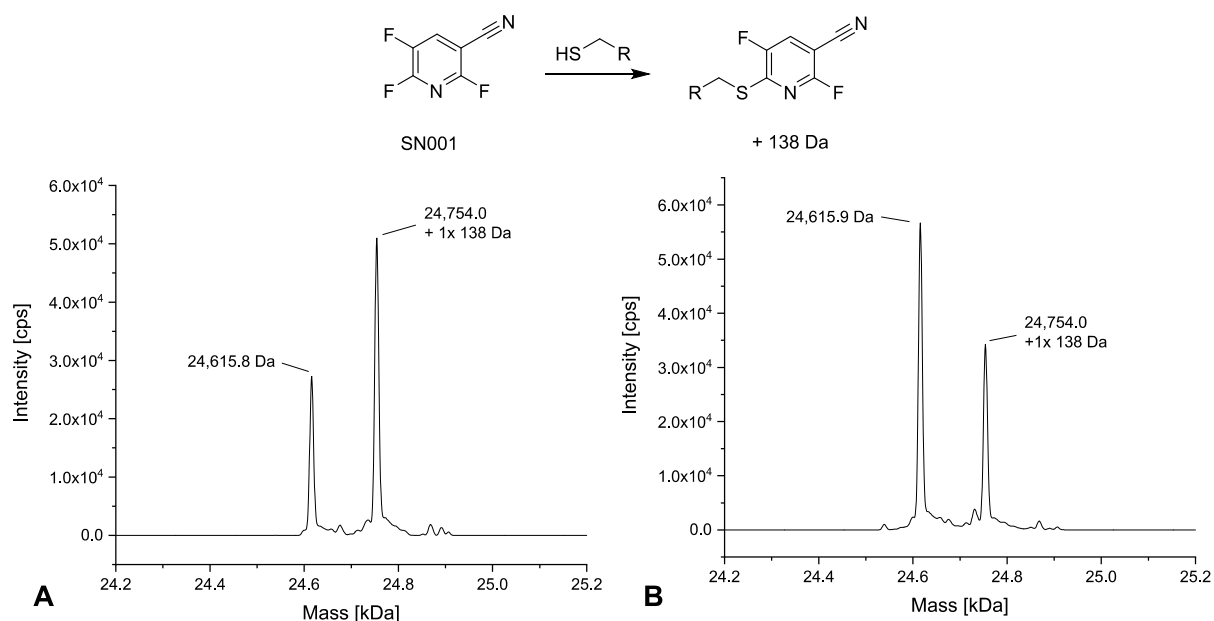


Figure 3.46: Deconvoluted MS spectra of T-p53C-Y220C-CL with SN001 at a molar ratio of 1:100 (**A**) and 1:31.25 (**B**) after 24 h of incubation at 20 °C (theoretical mass of unmodified T-p53C-Y220C-CL: 24,615.68 Da).

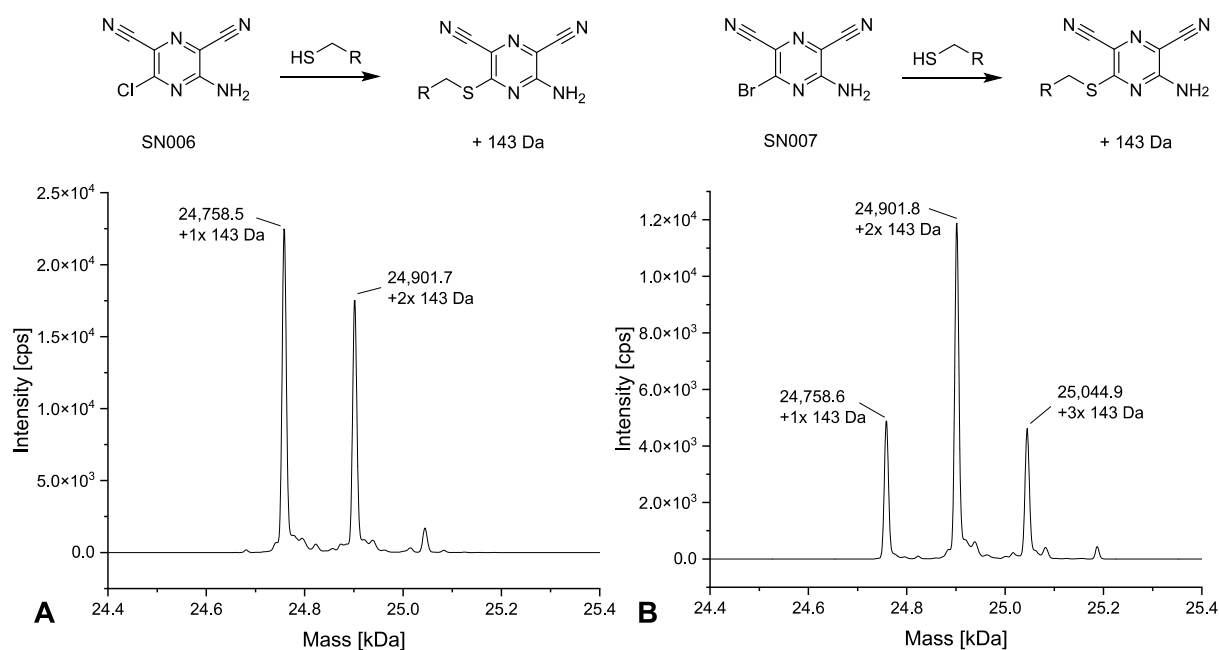


Figure 3.47: Deconvoluted MS spectra of T-p53C-Y220C-CL with SN006 (**A**) and SN007 (**B**) at a molar ratio of 1:100 after 24 h of incubation at 20 °C (theoretical mass of unmodified T-p53C-Y220C-CL: 24,615.68 Da).

3.4.14 Fluorescence Polarization Assay of FL-T-p53 with GADD45 α

p53 regulates the transcription of many genes through binding to its DNA response elements. FL p53 has a TET and binds as a dimer of primary dimers to the DNA consensus sequence in a highly cooperative manner [5, 76, 83, 84]. p53 recognizes a specific double-stranded DNA consensus sequence with two copies of a 10-base pair containing half-site motif: 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3'. The half-site motifs are separated by 0-13 bp, whereby the spacer usually does not exceed two base pairs in length [7, 321, 322]. The p53 binding site in the GADD45 α gene conforms to the consensus sequence in 19 out of 20 base pairs [323]. The protein encoded by the GADD45 α gene is involved in G2/M and G1/S cell cycle arrest, DNA repair, cell survival, apoptosis, and senescence [324-328].

The influence of the identified CovLib hits VS004, SN001, SN006, and SN007 on the DNA-binding affinity of FL-T-p53 was investigated by FP assay using the fluorescein-labeled non-apoptotic GADD45 α DNA p53 response element [81, 130, 131, 292, 293]. The theoretical basis of the FP assay is that the polarization of the emitted light from a fluorescence-labeled molecule, excited with linearly polarized light, is reciprocally proportional to the rate of molecular rotation. The free labeled molecule in solution has a high tumbling rate during the fluorescence lifetime, resulting in a largely depolarized emitted light. When the labeled molecule binds to a large protein, the tumbling rate of the resulting complex decreases significantly. This decrease is reflected in a stronger polarization of the emitted light. The unbound and bound states of the small, labeled molecule have different polarization values: a low value for the unbound state and a high value for the bound state. The FP assay can directly measure the binding of a labeled molecule, i.e., peptide, or nucleic acid, to a protein because the observed polarization depends on the fraction of bound ligand [329-331].

The incubation of FL-T-p53 with SN001 or VS004 at a molar ratio of 1:200 for 2 h only slightly affected the DNA-binding of FL-T-p53 with a K_d for GADD45 α DNA from 81 nM for FL-T-p53 to 83 nM with SN001 and 89 nM with VS004 (Figure 3.48, Table 3.19). However, SN006 and SN007 reduced the DNA-binding of FL-T-p53 with an increase in the K_d to 127 nM for SN006 and 146 nM for SN007, corresponding to a 1.6-fold and 1.8-fold decreased affinity of FL-T-p53 to GADD45 α .

Cys275 and Cys277 are located at the DNA-binding surface of p53, and Cys277 is involved in specific interactions at the major groove with the bound DNA sequence [75,

76, 84]. Hence, alteration of these residues could reduce DNA-binding capability of p53 [118, 130, 294]. Nevertheless, it was demonstrated that covalent modification of Cys277 by the 2-sulfonylpyrimidine PK11000 did not affect the DNA-binding of FL-T-p53 towards GADD45 α [131]. In addition, modification of cysteines 124, 229, and 277 by MQ resulted in stabilization of p53 and its DNA, first by stabilizing the protein-DNA interface (Cys277) and second by stabilizing the interfaces between dimers (Cys124/Cys229) [118]. However, modification of Cys275 by MQ was only observed in the DNA-free structure, and alkylation of this residue by MQ is probably incompatible with DNA-binding of p53.

Incubation with SN006 and SN007 resulted in a reduction of the binding activity of FL-T-p53 to the GADD45 α response element. Based on the literature data, this reduction could be attributed to the arylation of Cys277 (and possibly Cys275). In contrast, after incubation of FL-T-p53 with SN001 and VS004 at a molar ratio of 1:200, binding to GADD45 α was conserved, although modification of cysteine residues and, in particular, modification of Cys277 in p53 is likely. This suggests that normal transcriptional activity of p53 could be rescued by the thermal stabilization in cellular assays.

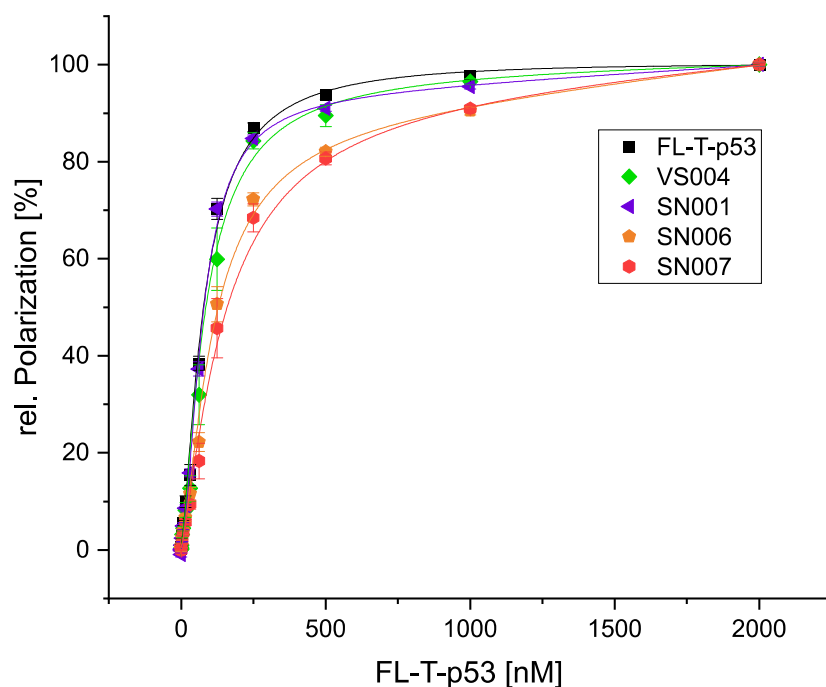


Figure 3.48: FP assay of FL-T-p53 incubated with VS004, SN001, SN006, and SN007 with GADD45 α . The relative fluorescence polarization data [%] of FL-T-p53 with 20 nM 5'-carboxyfluorescein-labeled GADD45 α DNA response element and 5% dimethyl sulfoxide (DMSO) are shown. FL-T-p53 was incubated with compounds VS004, SN001, SN006, and SN007 at a protein-to-compound ratio of 1:200 for 2 h at 20 °C [131]. FP data were recorded every 10 min for 1 h, and data were averaged. Measurements were performed two times in quadruplicate.

Table 3.19: Resulting K_d values $\pm$ SD of the direct titrations of FL-T-p53 with or without the addition of CovLib compounds (molar ratio 1:200) with GADD45 α DNA. Measurements were performed two times in quadruplicate.

	FL-T-p53	VS004	SN001	SN006	SN007
$K_d \pm SD$ [nM]	81 ± 5	89 ± 6	83 ± 6	127 ± 14	146 ± 15

3.4.15 Determination of the Binding Mode of SN001 and SN006 Using X-Ray Crystallography

Fragments SN001 and SN006 were soaked into xT-p53C-Y220C crystals to confirm that Cys220 in the hydrophobic pocket is arylated. In addition, we also investigated whether different soaking concentrations of SN001 have a noticeable effect.

Two crystal structures were obtained for compound SN001, soaked with 5 mM and 40 mM compound, respectively. As illustrated in Figure 3.49, both structures indicated that fragment SN001 reacted with cysteine residues in p53 via an S_NAr reaction. The fluorine atom in the para position to the electron-withdrawing nitrile group acted as the LG. As shown in Figure 3.50, compared to the wt p53, compound SN001 adopted a similar conformation (Tyr-like conformation) in the central cavity as the originally present tyrosine in the wt protein.

The 5 mM-soaked crystal structure displayed well-defined density for arylation at Cys220 (Figure 3.49 A) and diffuse density around Cys277. Consistent with the high rate of modification observed in the intact protein MS experiments (Figure 3.44), in the 40 mM-soaked structure, the protein was arylated at residues Cys124, Cys182, Cys220, Cys229, and Cys277. Furthermore, density was found around Tyr103 and His233, which could be attributed to the compound. Although the structure of the protein soaked with 40 mM compound was heavily modified, fragment SN001 bound similarly into the pocket, adopting the same Tyr-like conformation (Figure 3.49 B). It occupied most of the space in the central cavity, with the ortho-fluorine pointing towards subsite 2 and the meta-fluorine pointing into the hydrophobic subsite 3. The CN moiety pointed towards subsite 1 and Thr150.

In the 40 mM-soaked structure, the arylation of Cys229 by fragment SN001 led to a flip of the side chain. As shown in Figure 3.49 C and Figure 3.49 D, this space was previously occupied by Trp146, which was also flipped to accommodate the arylation. The interaction of Trp146 to Gln144 was disrupted, resulting in less defined density for Gln144. Additionally, Arg110 was slightly shifted. However, as the compound was close to crystal contacts, the exact interaction must be interpreted carefully.

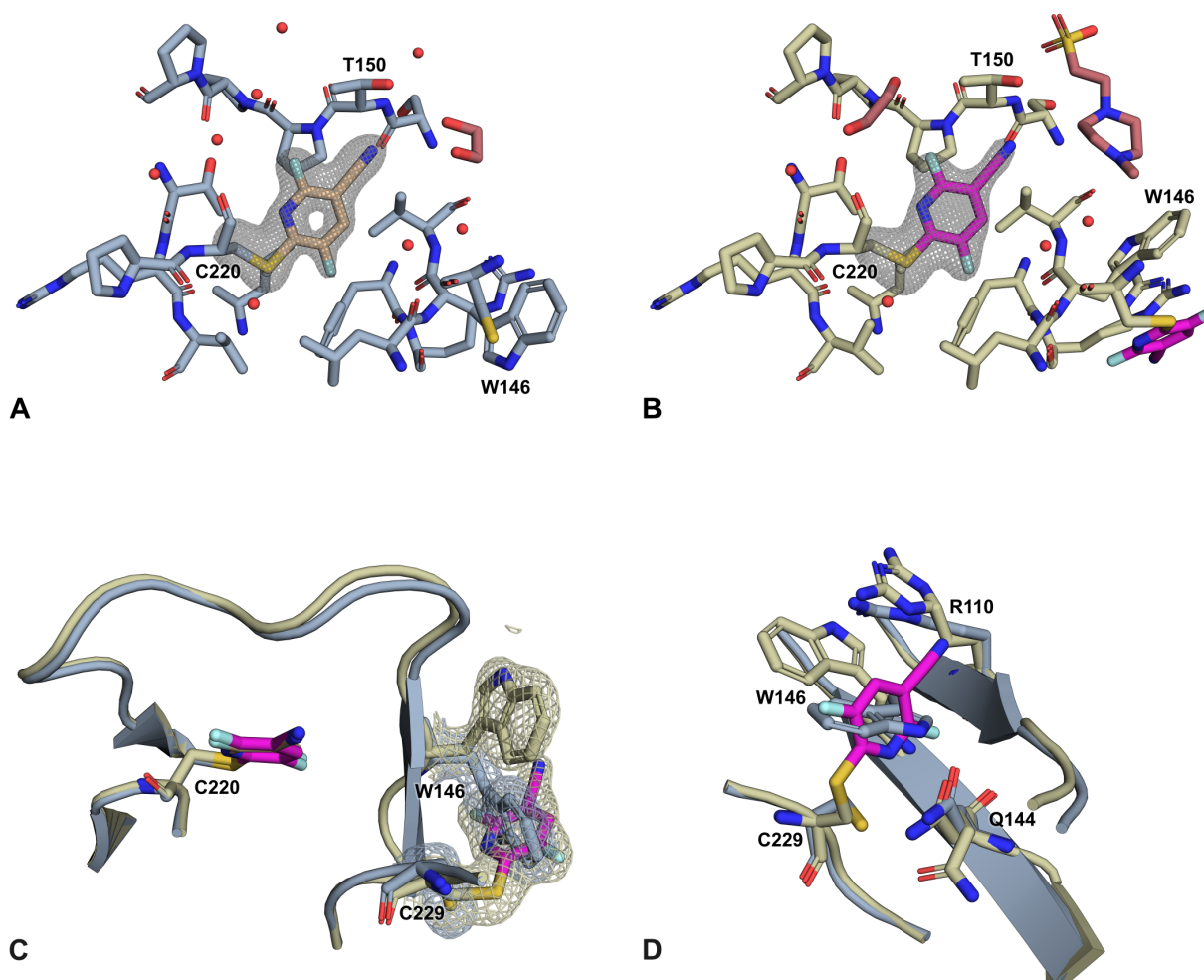


Figure 3.49: Comparison of the binding modes of SN001 soaked at 5 mM (blue, PDB: 8RBA, chain A) and 40 mM (green, PDB: 8RBB, chain B). **(A)** The Y220C induced pocket of the 5 mM-soaked structure is depicted. The ortho-fluorine points towards subsite 2, while the meta-fluorine points towards subsite 3. The unbiased omit map is displayed at a contour level of 3σ . **(B)** Multiple arylations of the protein do not affect the binding pose of SN001 in the Y220C pocket. The unbiased omit map is contoured at 3σ . **(C)** The arylation of Cys229 leads to a flip of Cys229 and Trp146. The 2mFo-DFc map, contoured at 1σ , is shown in blue for 8RBA and green for 8RBB. **(D)** Upon arylation of Cys229 and flip of Trp146, the interaction with Gln144 is interrupted, and Arg110 has to move slightly to accommodate the space filled by Trp146.

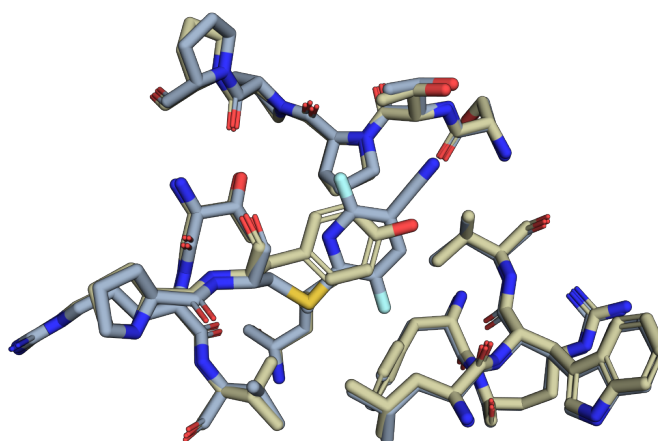


Figure 3.50: Comparison of the wt tyrosine 220 (green, 1UOL [146]) and the binding pose of SN001 (blue, 8RBA, chain A). The pyridine ring of SN001 occupies a central cavity space similar to the orientation of the phenol ring of Tyr220 in the wt protein.

As displayed in Figure 3.51, the pyrazine derivative SN006 bound into the Y220C induced cavity analogous to SN001. It adopted the same Tyr-like conformation and mimicked the wt Tyr220. The nitrile groups pointed towards subsite 3 (similar to the meta-fluorine in compound SN001) and towards Thr150. In the 5 mM-soaked structure, density around Cys229 was observed, suggesting an intermediate between bound and unbound protein, which may explain why the side chain of Trp146 was not resolved. Additional modifications were found at Cys182, and some unexplained density was detected around Cys277.

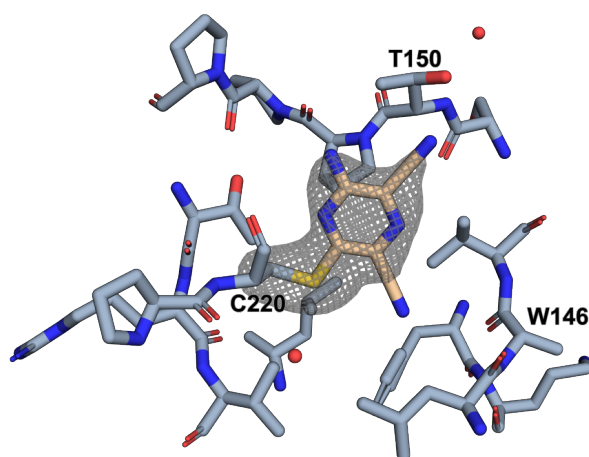


Figure 3.51: Overview of the binding mode of SN006 (soaked at 5 mM, PDB: 8RBC) into the Y220C induced crevice. The compound binds similarly to SN001 in a Tyr-like conformation, with the nitrile group pointing towards subsite 3. The unbiased omit map is shown at a contour level of 3 σ .

3.5 Structure-Activity Relationship Screen of the Identified CovLib Hits

The screening of the CovLib with T-p53C and different p53 mutants identified the reactive fragments VS004, SN001, SN006, and SN007 as hits. The crystal structure of T-p53C-Y220C soaked with compounds SN001 and SN006 confirmed the covalent modification of Cys220 in the mutation-induced binding pocket. Furthermore, compound SN001 remarkably stabilized T-p53C-Y220C by up to 4.45 °C at a protein-to-compound ratio of 1:31.25. In addition, fragment VS004 achieved mutation-independent general stabilization of p53 by arylating surface-exposed cysteines.

However, these fragment hits exhibited high reactivity, resulting in multiple arylations of p53 and multiple binding sites. Hence, the goal was to decrease the reactivity of the compounds and enhance their selectivity for specific cysteines in p53, particularly for Cys220 in the pocket. Therefore, a large set of SAR compounds of the identified hits with variations in halogenation patterns, EWGs, LGs, electron-donating groups (EDG), and (hetero)arenes was investigated. The SAR compounds were evaluated using DSF as the primary screening method. The identified hits were confirmed to covalently modify p53 by intact protein mass spectrometry.

3.5.1 SN001 SAR Compound Screen

The tested SN001 SAR compounds are depicted in Figure 3.52. A set of fragments with varying numbers of different halogen atoms attached to the pyridine ring were selected. Compounds SN001-1 to SN001-4 have fewer electron-withdrawing fluorines compared to SN001. Fragments SN001-17 to SN001-23 have varying numbers of electron-withdrawing chlorines, and fragments SN001-24 to SN001-29 have bromines instead of the original present fluorines. Fragment SN001-32 has a mixture of an electron-withdrawing bromine and chlorine. Different compounds with variations in the EWG were ordered to examine the impact of the nitrile group. Compounds SN001-33 and SN001-5 lack the electron-withdrawing nitrile group. Fragments SN001-6 to SN001-11 contain an electron-withdrawing NO₂ group, while fragments SN001-12 to SN001-16 possess an electron-withdrawing CF₃ group, replacing the initial nitrile group. To investigate the influence of the pyridine ring system, the benzonitriles SN001-30 and SN001-31 were tested.

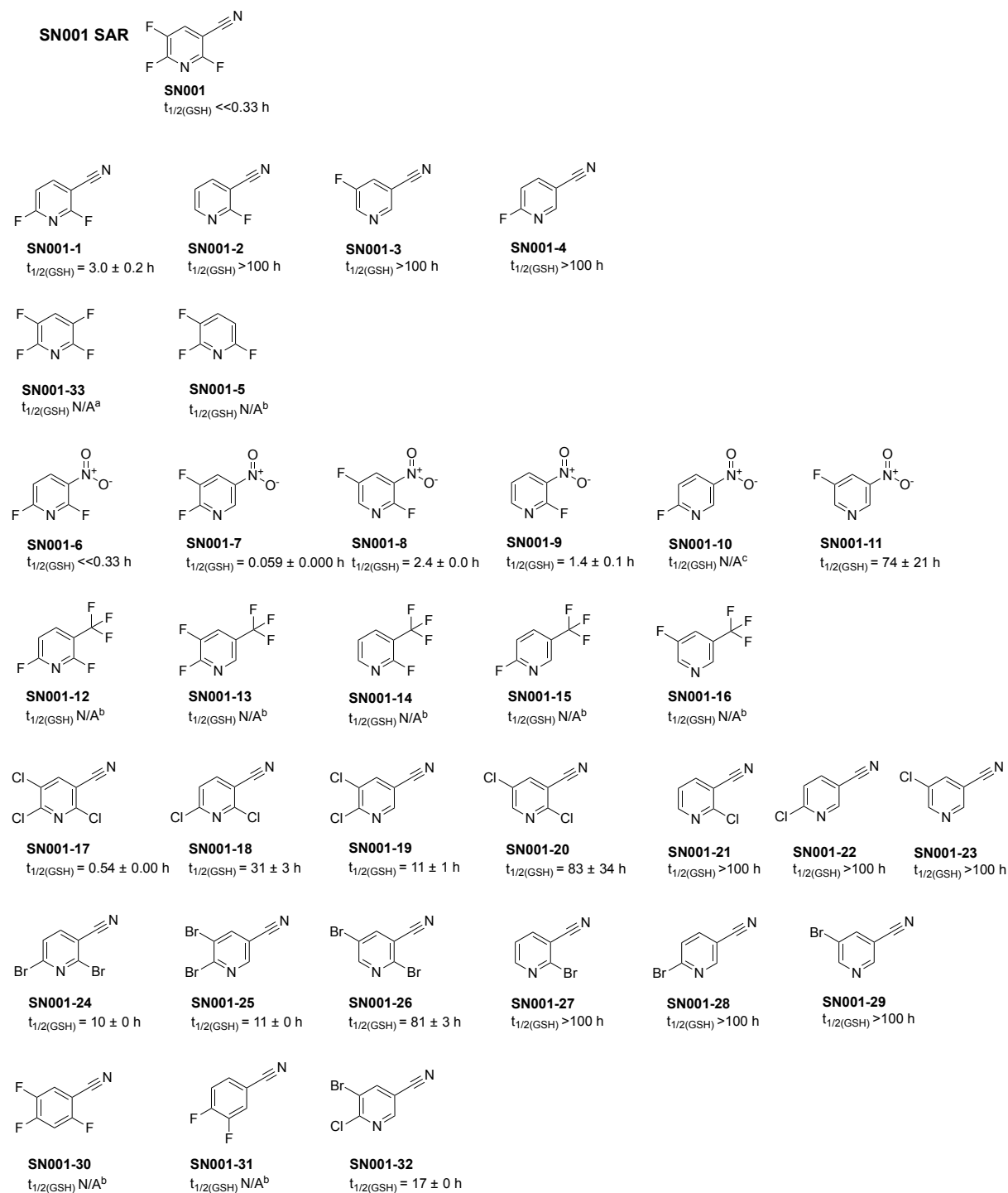


Figure 3.52: Overview of the tested SN001 SAR compounds and their $t_{1/2} \pm \text{SD}$ determined via GSH assay. The GSH assay was performed by M. Schwer. ^aThe compound was not soluble in ACN buffer mixture at the required concentration. ^b $t_{1/2}$ of the fragments in PBS buffer appeared to be similar or lower than in GSH-containing PBS buffer. A possible explanation is the precipitation of the fragments during the measurement process. ^cThe purity of the compound could not be confirmed by HPLC-UV.

3.5.1.1 DSF Screen of SN001 SAR Compounds with T-p53C and Its Mutants

The SN001 SAR compounds were screened using DSF with T-p53C and its mutants. Due to the expected reduced reactivity of certain SAR compounds, the DSF screen was also performed with prolonged incubation times. The results after 30 min, 4 h, and 24 h incubation time of p53 and its mutants with 1 mM SN001-SAR compounds are depicted in Table 3.20, Table 3.21, and Table 3.22, respectively. The corresponding first derivatives of the melting curves are shown in Chapter 8.7. Moreover, the SN001 SAR compounds were also tested at a concentration of 250 μ M with T-p53C (Table 8.6) and T-p53C-Y220C (Table 8.7). Table 3.23 and Table 3.24 list the results of the identified hits tested with T-p53C and T-p53C-Y220C at 1 mM and 250 μ M compound concentrations.

The SN001 SAR compound screen revealed that reducing the number of electron-withdrawing halogen atoms attached to the heteroarene decreases the reactivity of the compounds and the stabilization of p53 and its mutants. Fragment SN001-1 lacks an electron-withdrawing fluorine atom in the ortho position to the LG compared to SN001, making the heteroaromatic system less electron-deficient. In relation to the highly reactive CovLib compound SN001 ($t_{1/2}$ GSH $\ll$ 0.33 h), the reactivity of the SAR compound SN001-1 was reduced with a $t_{1/2}$ of 3.0 h in the GSH assay. Therefore, in contrast to SN001, the stabilization of T-p53C-Y220C gradually increased with increasing incubation time. The T_m of T-p53C-Y220C shifted by 0.83 $^{\circ}$ C after 30 min, to 1.25 $^{\circ}$ C after 4 h, and 2.45 $^{\circ}$ C after 24 h of incubation with 1 mM SN001-1. Moreover, a 24-hour incubation period was required to stabilize T-p53C-Y220C at 250 μ M SN001-1. The maximum stabilization of 1.35 $^{\circ}$ C at 250 μ M was substantially lower compared to 1 mM SN001-1 (2.45 $^{\circ}$ C). Thus, in comparison to the highly reactive compound SN001, the SAR compound SN001-1 showed a typical reaction behavior with a decrease in stabilization of T-p53C-Y220C with decreasing concentration of SN001-1. However, SN001-1 only stabilized T-p53C-Y220C by 2.45 $^{\circ}$ C (1 mM, 24 h), which is less pronounced than the 4.45 $^{\circ}$ C increase in melting temperature achieved by SN001 (250 μ M, 30 min). Compound SN001-1 lacks a fluorine atom at position 5 of the pyridine ring, pointing into the hydrophobic subsite 3. However, the SAR compound with two fluorines at positions 5 and 6 of the pyridine ring was not available for purchase due to price restrictions. Additionally, compound SN001-1 stabilized T-p53C and each tested mutant after 24 h of incubation, resulting in a generic, mutation-independent stabilization of p53. Hence, the removal of a fluorine in the meta position

to the electron-withdrawing nitrile group did not result in increased selectivity for the Y220C pocket or stabilization of T-p53C-Y220C.

The SAR fragments SN001-2, SN001-3, and SN001-4, each with a single fluorine as the LG at different positions at the pyridine ring and without any additional EWGs except for the CN moiety, were unreactive ($t_{1/2}$ GSH > 100 h) and did not influence the T_m of p53 and its mutants even after 24 h of incubation. In addition, compounds lacking the electron-withdrawing CN group (SN001-5, SN001-33) did not substantially affect the melting temperature of T-p53C-Y220C and other mutants.

The introduction of the stronger electron-withdrawing NO₂ group instead of the CN moiety increases the electron deficiency in the aromatic system [276]. However, no evaluable melting curves and first derivatives with clearly defined melting points were retained for all p53 variants after incubation with the reactive compounds SN001-6, SN001-7, and SN001-8, containing the NO₂ group and two fluorine substituents. High fluorescence intensity was measured at low temperatures, indicating denaturation, aggregation, or misfolding of the proteins [289]. Only an evaluable melting curve of T-p53C-Y220C was measured after 30 min of incubation with 250 μ M SN001-8 (ΔT_m = -0.85 $^{\circ}$ C, broad peak). The SAR compound SN001-10, which contains a fluorine atom in the para position to the nitro group, was the only compound tested in this series that showed an analyzable melting curve and an increase in the T_m after 24 h of incubation at a compound concentration of 1 mM for all p53 proteins assayed. However, this compound mainly stabilized the T-p53C-R282W mutant (ΔT_m = 3.40 $^{\circ}$ C, 1 mM, 24 h). Therefore, SN001-10 resulted in a generic stabilization of p53 rather than specifically targeting the Y220C mutant. Moreover, it is important to note that the purity of SN001-10 was not confirmed by HPLC-UV. Consequently, its DSF results should be interpreted with caution. After incubating for 24 h with compound SN001-11 (1 mM), which contains a meta-fluorine, no first derivatives of the melting curves with clearly defined melting points were obtained for any of the tested p53 variants. Compound SN001-9, which includes a fluorine atom in the ortho position to the EWG, slightly stabilized p53 and its mutants after 4 h of incubation. Nevertheless, high fluorescence intensities were measured at low temperatures, and no evaluable melting curves were retained for all p53 variants tested after 24 h of incubation with 1 mM SN001-9. The proteins may have denatured and aggregated due to prolonged incubation with the reactive compound ($t_{1/2}$ GSH = 1.4 h). However, T-p53C-Y220C was stabilized by SN001-9 after 24 h of incubation at a compound concentration of 250 μ M, increasing

the T_m by up to 1.90 °C. In contrast, no analyzable melting curves of T-p53C were measured after 24 h of incubation at 250 μ M SN001-9. In summary, none of the tested compounds with an NO₂ moiety as EWG appeared to be suitable for extensively stabilizing T-p53C-Y220C.

The SAR compounds SN001-12 to SN001-16 have a weaker electron-withdrawing CF₃ group instead of the initial CN moiety [276, 277]. The analog compound of SN001 with three fluorines and a CF₃ moiety as EWG was not available for purchase. In general, all tested compounds with a CF₃ moiety as EWG did not affect the melting temperature of T-p53C-Y220C and other p53 variants even after 24 h of incubation. In conclusion, the DSF experiments with different EWGs demonstrated that the CN group in the para position to the LG plays a crucial role in the stabilization of T-p53C-Y220C.

The SAR compounds SN001-17 to SN001-23 feature chlorine substituents at the pyridine ring, which are weaker electron withdrawers than the original fluorines [277]. The replacement of the three fluorine atoms in SN001 with chlorines results in compound SN001-17. This compound exhibited lower reactivity in the GSH assay than the CovLib compound SN001 ($t_{1/2}$ GSH <<0.33 h), but it is still reactive with a $t_{1/2}$ of 0.54 h. Fragment SN001-17 showed a concentration-dependent stabilization of T-p53C-Y220C, which was analogous to SN001. Additionally, time-dependent effects were observed. The stabilization of Y220C increased with decreased concentration and prolonged incubation time (Table 3.24). At a concentration of 1 mM SN001-17, the stabilization of T-p53C-Y220C increased from 2.93 °C after 30 min, to 3.77 °C after 4 h, and to 4.42 °C after 24 h of incubation. Furthermore, SN001-17 substantially raised the melting temperature of the temperature-sensitive T-p53C-Y220C mutant by up to 5.19 °C after 24 h of incubation and 250 μ M compound concentration. Compared to the increase in the T_m of T-p53C-Y220C by up to 4.45 °C with the fluorine compound SN001 (250 μ M, 30 min), SN001-17 provided a higher maximum stabilization of about 5.19 °C (250 μ M, 24 h). Like SN001, compound SN001-17 exclusively stabilized T-p53C-Y220C, indicating a covalent modification of Cys220 in the mutation-induced crevice. The melting temperature of T-p53C was only slightly affected by fragment SN001-17. After 30 min of incubation with 1 mM SN001-17, the T_m of T-p53C was decreased by -0.90 °C. After 24 h of incubation, the ΔT_m values of T-p53C were -0.40 °C at 1 mM and 0.35 °C at 250 μ M SN001-17. However, SN001-17 (1 mM) destabilized the R273H mutant by -1.50 °C after 24 h of incubation. Furthermore, incubation with 1 mM SN001-17 resulted in unanalyzable melting curves with high

initial fluorescence intensities for the R282W mutant, which could be attributed to protein denaturation and aggregation effects [289].

In analogy to the SAR compounds SN001-1 to SN001-4, the chlorine compounds also exhibited a declining stabilization as the number of electron-withdrawing chlorine atoms attached to the heteroarene and the reactivities decreased (SN001-18 to SN001-23). Only compound SN001-19 (1 mM) with chlorine atoms in the meta and para positions to the EWG slightly increased the melting temperature of T-p53C-Y220C by up to 0.85 °C after 24 h of incubation. Fragment SN001-19 also had the shortest $t_{1/2}$ of 11 h in the GSH assay compared to the other compounds with two chlorine substituents.

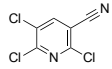
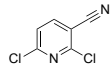
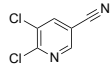
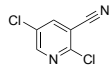
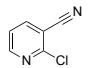
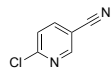
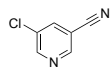
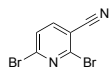
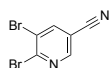
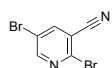
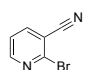
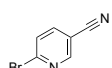
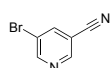
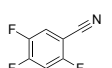
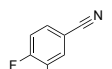
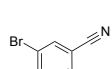
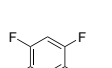
The bromine analog of SN001 and SN001-17 was not commercially available. The compounds with two or one bromine substituents at the pyridine ring showed little or no effect on the T_m of T-p53C-Y220C (SN001-24 to SN001-29). Only SN001-24 ($\Delta T_m = 0.70$ °C, 1 mM) and SN001-25 ($\Delta T_m = 0.73$ °C, 1 mM) with ortho- and para-bromine, and meta- and para-bromine, respectively, showed a slight increase in the T_m for T-p53C-Y220C after 24 h of incubation. Interestingly, compound SN001-24, which contains bromine atoms in the ortho and para positions to the EWG, increased the T_m of the Y220C mutant, whereas the chlorine analog SN001-18 did not affect the T_m . However, SN001-24 also showed a higher reactivity in the GSH assay with a $t_{1/2}$ of 10 h compared to its chlorine analog SN001-18 ($t_{1/2}$ GSH = 31 h). Moreover, fragment SN001-32 with meta-bromine and para-chlorine did not substantially affect the T_m of T-p53C-Y220C.

Additionally, the benzonitrile derivatives SN001-30 and SN001-31 did not increase the T_m of T-p53C or its mutants. This indicates that the electron-deficient pyridine ring is essential for reactivity and stabilization.

In summary, the SN001 SAR DSF screen revealed that the electron-withdrawing nitrile group and the pyridine ring were crucial for a substantial stabilization of the Y220C mutant and thus a fragment hit. Moreover, the DSF screen showed that reducing the number of halogens attached to the heteroarene resulted in a decreased stabilization of T-p53C-Y220C. In addition, an interesting new hit was identified with the 2,5,6-trichloro-3-pyridinecarbonitrile SN001-17, which substantially stabilized T-p53C-Y220C by up to 5.19 °C.

Table 3.20: $\Delta T_m \pm SD$ of T-p53C and its mutants (8 μM) incubated with 1 mM SN001 SAR compounds for 30 min at 20 °C. Measurements were performed at least in triplicate. Fragments with a ΔT_m of at least 0.50 °C are highlighted in bold [99, 130].

Compound	T-p53C	T-p53C- Y220C	T-p53C- R273H	T-p53C- R282W	T-p53C- C124/182/ 277S
	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]
SN001-1 	0.35 $\pm$ 0.27	0.83 $\pm$ 0.09	0.45 $\pm$ 0.40	0.45 $\pm$ 0.19	0.25 $\pm$ 0.09
SN001-2 	0 $\pm$ 0.12	-0.10 $\pm$ 0.24	-0.05 $\pm$ 0.09	-0.15 $\pm$ 0.12	N/D ^b
SN001-3 	0.20 $\pm$ 0.09	-0.10 $\pm$ 0.19	-0.05 $\pm$ 0.09	-0.05 $\pm$ 0.12	N/D ^b
SN001-4 	-0.05 $\pm$ 0.12	-0.15 $\pm$ 0.12	-0.05 $\pm$ 0.09	-0.05 $\pm$ 0.12	N/D ^b
SN001-5 	0.05 $\pm$ 0.17	-0.10 $\pm$ 0.12	0 $\pm$ 0	-0.10 $\pm$ 0.09	N/D ^b
SN001-6 	N/A ^a	N/A ^a	N/A ^a	N/A ^a	N/D ^b
SN001-7 	N/A ^a	N/A ^a	N/A ^a	N/A ^a	N/D ^b
SN001-8 	N/A ^a	N/A ^a	N/A ^a	N/A ^a	N/D ^b
SN001-9 	0.30 $\pm$ 0.12	-0.15 $\pm$ 0.19	0.35 $\pm$ 0.09	0 $\pm$ 0.19	0.05 $\pm$ 0.12
SN001-10 	-0.15 $\pm$ 0.12	0.15 $\pm$ 0.12	-0.15 $\pm$ 0	-0.05 $\pm$ 0.12	-0.15 $\pm$ 0.12
SN001-11 	-0.05 $\pm$ 0.12	0 $\pm$ 0.12	-0.10 $\pm$ 0.09	-0.10 $\pm$ 0.09	N/D ^b
SN001-12 	-0.05 $\pm$ 0.19	-0.25 $\pm$ 0.24	-0.05 $\pm$ 0.09	-0.15 $\pm$ 0.19	N/D ^b
SN001-13 	-0.05 $\pm$ 0.12	-0.05 $\pm$ 0.09	0 $\pm$ 0	0 $\pm$ 0.19	N/D ^b
SN001-14 	-0.10 $\pm$ 0.09	-0.25 $\pm$ 0.12	-0.10 $\pm$ 0.09	-0.15 $\pm$ 0.12	N/D ^b
SN001-15 	-0.05 $\pm$ 0.12	-0.25 $\pm$ 0.24	-0.10 $\pm$ 0.09	-0.15 $\pm$ 0.12	N/D ^b
SN001-16 	-0.05 $\pm$ 0.12	-0.20 $\pm$ 0.17	0 $\pm$ 0	-0.05 $\pm$ 0.19	N/D ^b

SN001-17		-0.90 ± 0	2.93 ± 0.17	-1.55 ± 0.09	N/A ^a	-0.80 ± 0
SN001-18		-0.05 ± 0.19	-0.05 ± 0.09	-0.10 ± 0.09	-0.15 ± 0.12	N/D ^b
SN001-19		0 ± 0.14	0.20 ± 0.12	-0.15 ± 0	-0.10 ± 0.09	-0.10 ± 0.12
SN001-20		-0.20 ± 0.12	-0.15 ± 0.24	-0.20 ± 0.07	-0.25 ± 0.09	N/D ^b
SN001-21		-0.15 ± 0.12	-0.40 ± 0.19	-0.10 ± 0.09	-0.10 ± 0.09	N/D ^b
SN001-22		0 ± 0.12	-0.20 ± 0.17	-0.15 ± 0	-0.10 ± 0.09	N/D ^b
SN001-23		-0.10 ± 0.09	-0.25 ± 0.19	-0.15 ± 0	-0.15 ± 0.12	N/D ^b
SN001-24		0.15 ± 0.26	0.15 ± 0.12	-0.30 ± 0	-0.25 ± 0.09	-0.10 ± 0.12
SN001-25		0.15 ± 0	0.35 ± 0.09	0 ± 0	0.20 ± 0.17	-0.10 ± 0.09
SN001-26		-0.30 ± 0	-0.45 ± 0.12	-0.15 ± 0	-0.30 ± 0	N/D ^b
SN001-27		-0.25 ± 0.09	-0.35 ± 0.17	-0.15 ± 0	-0.20 ± 0.09	N/D ^b
SN001-28		-0.20 ± 0.09	-0.30 ± 0.12	-0.10 ± 0.09	-0.15 ± 0.15	N/D ^b
SN001-29		-0.15 ± 0	-0.10 ± 0.10	-0.10 ± 0.07	0 ± 0	N/D ^b
SN001-30		-0.05 ± 0.12	-0.05 ± 0.09	-0.15 ± 0	-0.05 ± 0.09	N/D ^b
SN001-31		-0.15 ± 0.33	0 ± 0.12	-0.15 ± 0	-0.20 ± 0.09	N/D ^b
SN001-32		0.15 ± 0.15	0.10 ± 0.17	0.05 ± 0.09	-0.10 ± 0.09	N/D ^b
SN001-33		0.15 ± 0.26	0 ± 0.15	0.25 ± 0.12	0.05 ± 0.09	N/D ^b

^aNo evaluable melting curves and first derivatives with clearly defined melting points were obtained.

^bOnly the identified fragment hits were investigated with T-p53C-C124/182/277S.

Table 3.21: $\Delta T_m \pm SD$ of T-p53C and its mutants (8 μM) incubated with 1 mM SN001 SAR compounds for 4 h at 20 °C. Measurements were performed at least in triplicate. Fragments with a ΔT_m of at least 0.50 °C are highlighted in bold [99, 130].

Compound	T-p53C	T-p53C-Y220C	T-p53C-R273H	T-p53C-R282W	T-p53C-C124/182/277S
	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]
SN001-1	0.50 $\pm$ 0.09	1.25 $\pm$ 0.09	0.85 $\pm$ 0.17	1.50 $\pm$ 0.15	0.35 $\pm$ 0.09
SN001-2	0.10 $\pm$ 0.12	0 $\pm$ 0.11	-0.10 $\pm$ 0.12	0.15 $\pm$ 0	N/D ^c
SN001-3	0.05 $\pm$ 0.09	0.08 $\pm$ 0	0 $\pm$ 0.12	0.10 $\pm$ 0.09	N/D ^c
SN001-4	0.05 $\pm$ 0.09	-0.05 $\pm$ 0.04	-0.10 $\pm$ 0.12	0.10 $\pm$ 0.09	N/D ^c
SN001-5	0 $\pm$ 0.12	0.10 $\pm$ 0.04	-0.05 $\pm$ 0.09	0.15 $\pm$ 0.15	N/D ^c
SN001-6	N/A ^a	N/A ^a	N/A ^a	N/A ^a	N/D ^c
SN001-7	N/A ^a	N/A ^a	N/A ^a	N/A ^a	N/D ^c
SN001-8	N/A ^a	N/A ^a	N/A ^a	N/A ^a	N/D ^c
SN001-9	0.45 $\pm$ 0.12 ^b	1.08 $\pm$ 0.10^b	0.90 $\pm$ 0.12^b	1.45 $\pm$ 0.17	0.35 $\pm$ 0.09
SN001-10	0.20 $\pm$ 0.09	0.15 $\pm$ 0.06	0.10 $\pm$ 0.09	0.30 $\pm$ 0	-0.05 $\pm$ 0.12
SN001-11	0.25 $\pm$ 0.12	0 $\pm$ 0.06	-0.60 $\pm$ 0.12	0 $\pm$ 0	N/D ^c
SN001-12	-0.05 $\pm$ 0.12	0.10 $\pm$ 0.04	-0.05 $\pm$ 0.09	0.15 $\pm$ 0	N/D ^c
SN001-13	0.05 $\pm$ 0.09	0.05 $\pm$ 0.04	0.10 $\pm$ 0.09	0.15 $\pm$ 0	N/D ^c
SN001-14	0.05 $\pm$ 0.17	0.15 $\pm$ 0.06	-0.05 $\pm$ 0.09	0.05 $\pm$ 0.09	N/D ^c
SN001-15	0.10 $\pm$ 0.12	0.13 $\pm$ 0.04	0 $\pm$ 0.12	0.05 $\pm$ 0.09	N/D ^c
SN001-16	0 $\pm$ 0.12	0.10 $\pm$ 0.09	0 $\pm$ 0.12	0.15 $\pm$ 0	N/D ^c
SN001-17	-0.45 $\pm$ 0.26	3.77 $\pm$ 0.03	-1.40 $\pm$ 0.09	N/A ^a	-0.80 $\pm$ 0.19
SN001-18	0 $\pm$ 0.12	0.13 $\pm$ 0.10	-0.15 $\pm$ 0.12	0 $\pm$ 0	N/D ^c
SN001-19	0.10 $\pm$ 0.19	0.30 $\pm$ 0.08	0 $\pm$ 0.12	0.15 $\pm$ 0	-0.10 $\pm$ 0.09
SN001-20	0 $\pm$ 0.12	0.08 $\pm$ 0.06	-0.20 $\pm$ 0.09	-0.15 $\pm$ 0	N/D ^c
SN001-21	-0.15 $\pm$ 0.12	0.08 $\pm$ 0.06	-0.05 $\pm$ 0.09	0 $\pm$ 0	N/D ^c
SN001-22	-0.10 $\pm$ 0.17	0.03 $\pm$ 0.06	-0.05 $\pm$ 0.09	0.05 $\pm$ 0.09	N/D ^c
SN001-23	-0.20 $\pm$ 0.12	0.03 $\pm$ 0.04	-0.05 $\pm$ 0.09	-0.05 $\pm$ 0.07	N/D ^c
SN001-24	-0.05 $\pm$ 0.19	0.10 $\pm$ 0.09	-0.10 $\pm$ 0.12	0 $\pm$ 0.26	-0.30 $\pm$ 0.12
SN001-25	0.25 $\pm$ 0.12	0.45 $\pm$ 0.15	0.20 $\pm$ 0.09	0.40 $\pm$ 0.09	0.10 $\pm$ 0.19
SN001-26	-0.20 $\pm$ 0.24	-0.05 $\pm$ 0.09	-0.15 $\pm$ 0	-0.30 $\pm$ 0	N/D ^c
SN001-27	-0.15 $\pm$ 0.12	-0.05 $\pm$ 0.09	0.05 $\pm$ 0.09	-0.15 $\pm$ 0.15	N/D ^c
SN001-28	-0.10 $\pm$ 0.17	0 $\pm$ 0	0.05 $\pm$ 0.09	-0.05 $\pm$ 0.17	N/D ^c
SN001-29	-0.05 $\pm$ 0.12	0 $\pm$ 0	0.05 $\pm$ 0.12	-0.05 $\pm$ 0.09	N/D ^c
SN001-30	0 $\pm$ 0.12	N/D	0.10 $\pm$ 0.09	-0.20 $\pm$ 0.09	N/D ^c
SN001-31	-0.10 $\pm$ 0.12	N/D	0 $\pm$ 0	-0.25 $\pm$ 0.09	N/D ^c
SN001-32	0.05 $\pm$ 0.09	0.05 $\pm$ 0.19	0.05 $\pm$ 0.17	0.05 $\pm$ 0.09	N/D ^c
SN001-33	0.05 $\pm$ 0.17	-0.30 $\pm$ 0.19	-0.05 $\pm$ 0.12	0 $\pm$ 0	N/D ^c

^aNo evaluable melting curves and first derivatives with clearly defined melting points were obtained.

^bFirst derivatives of the melting curves displayed broad peaks. ^cOnly the identified fragment hits were investigated with T-p53C-C124/182/277S.

Table 3.22: $\Delta T_m \pm SD$ of T-p53C and its mutants (8 μM) incubated with 1 mM SN001 SAR compounds for 24 h at 20 °C. Measurements were performed at least in triplicate. Fragments with a ΔT_m of at least 0.50 °C are highlighted in bold [99, 130].

Compound	T-p53C	T-p53C-Y220C	T-p53C-R273H	T-p53C-R282W	T-p53C-C124/182/277S
	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]
SN001-1	0.95 $\pm$ 0.12 and 2.85 $\pm$ 0.12^a	2.45 $\pm$ 0.09	1.20 $\pm$ 0	2.00 $\pm$ 0.09	0.55 $\pm$ 0.09
SN001-2	0.30 $\pm$ 0.12	0 $\pm$ 0	0.10 $\pm$ 0.09	0.10 $\pm$ 0.09	N/D ^d
SN001-3	0.05 $\pm$ 0.12	0 $\pm$ 0	0.05 $\pm$ 0.09	0.10 $\pm$ 0.09	N/D ^d
SN001-4	0 $\pm$ 0.12	0.10 $\pm$ 0.09	0.15 $\pm$ 0	0.10 $\pm$ 0.09	N/D ^d
SN001-5	0.05 $\pm$ 0.12	0.05 $\pm$ 0.09	0.05 $\pm$ 0.09	0.15 $\pm$ 0	N/D ^d
SN001-6	N/A ^b	N/A ^b	N/A ^b	N/A ^b	N/D ^d
SN001-7	N/A ^b	N/A ^b	N/A ^b	N/A ^b	N/D ^d
SN001-8	N/A ^b	N/A ^b	N/A ^b	N/A ^b	N/D ^d
SN001-9	N/A ^b	N/A ^b	N/A ^b	N/A ^b	N/A ^b
SN001-10	0.50 $\pm$ 0.39^c	0.85 $\pm$ 0.31	1.20 $\pm$ 0^c	3.40 $\pm$ 0.43	0.50 $\pm$ 0.09
SN001-11	N/A ^b	N/A ^b	N/A ^b	N/A ^b	N/D ^d
SN001-12	-0.05 $\pm$ 0.09	-0.10 $\pm$ 0.09	0.10 $\pm$ 0.09	0 $\pm$ 0	N/D ^d
SN001-13	0.05 $\pm$ 0.12	-0.05 $\pm$ 0.09	0.15 $\pm$ 0	0 $\pm$ 0.15	N/D ^d
SN001-14	0.10 $\pm$ 0.09	0.05 $\pm$ 0.09	0.15 $\pm$ 0	-0.05 $\pm$ 0.09	N/D ^d
SN001-15	0.05 $\pm$ 0.12	0.05 $\pm$ 0.09	0.15 $\pm$ 0	-0.05 $\pm$ 0.09	N/D ^d
SN001-16	0.05 $\pm$ 0.19	-0.05 $\pm$ 0.23	0.10 $\pm$ 0.09	-0.10 $\pm$ 0.09	N/D ^d
SN001-17	-0.40 $\pm$ 0.17	4.42 $\pm$ 0.11	-1.50 $\pm$ 0	N/A ^b	-0.25 $\pm$ 0
SN001-18	0 $\pm$ 0.12	0.30 $\pm$ 0	0 $\pm$ 0	0.20 $\pm$ 0	N/D ^d
SN001-19	0.45 $\pm$ 0.12	0.85 $\pm$ 0.09	0.35 $\pm$ 0.09	0.45 $\pm$ 0.15	0.05 $\pm$ 0.09
SN001-20	-0.15 $\pm$ 0.12	0 $\pm$ 0.15	0 $\pm$ 0	-0.10 $\pm$ 0.09	N/D ^d
SN001-21	-0.05 $\pm$ 0.09	0 $\pm$ 0	0.05 $\pm$ 0.09	0 $\pm$ 0	N/D ^d
SN001-22	-0.05 $\pm$ 0.09	0 $\pm$ 0	0.05 $\pm$ 0.09	0 $\pm$ 0	N/D ^d
SN001-23	0 $\pm$ 0.12	-0.20 $\pm$ 0.23	0 $\pm$ 0	0 $\pm$ 0	N/D ^d
SN001-24	0.05 $\pm$ 0.27	0.70 $\pm$ 0.12	-0.15 $\pm$ 0	0.15 $\pm$ 0.15	-0.25 $\pm$ 0.09
SN001-25	0.30 $\pm$ 0.12	0.73 $\pm$ 0.11	0.10 $\pm$ 0.09	0.65 $\pm$ 0.17	0.10 $\pm$ 0.09
SN001-26	-0.35 $\pm$ 0.12	-0.05 $\pm$ 0.14	-0.20 $\pm$ 0.09	-0.15 $\pm$ 0	N/D ^d
SN001-27	-0.20 $\pm$ 0.12	0 $\pm$ 0.12	-0.10 $\pm$ 0.09	-0.05 $\pm$ 0.09	N/D ^d
SN001-28	-0.10 $\pm$ 0.17	0.03 $\pm$ 0.14	0 $\pm$ 0	0 $\pm$ 0.15	N/D ^d
SN001-29	-0.10 $\pm$ 0.17	0.08 $\pm$ 0.08	0 $\pm$ 0	0 $\pm$ 0.21	N/D ^d
SN001-30	0.10 $\pm$ 0.09	0.05 $\pm$ 0.15	-0.05 $\pm$ 0.09	0 $\pm$ 0	N/D ^d
SN001-31	0.25 $\pm$ 0.09	0 $\pm$ 0.09	-0.10 $\pm$ 0.09	-0.05 $\pm$ 0.09	N/D ^d
SN001-32	0.10 $\pm$ 0.17	0.30 $\pm$ 0.19	0.05 $\pm$ 0.09	0.25 $\pm$ 0.31	N/D ^d
SN001-33	0.30 $\pm$ 0.26	0 $\pm$ 0.19	0.05 $\pm$ 0.09	0 $\pm$ 0	N/D ^d

^aMelting curves with two transitions. First derivatives of the melting curves showed two peaks. ^bNo evaluable melting curves and first derivatives with clearly defined melting points were obtained. ^cFirst derivatives of the melting curves displayed broad peaks. ^dOnly the identified fragment hits were investigated with T-p53C-C124/182/277S.

Table 3.23: $\Delta T_m \pm$ SD of T-p53C (8 μ M protein) incubated with 1 mM or 250 μ M SN001 SAR hit compounds for 30 min, 4 h, and 24 h at 20 °C. Measurements were performed at least in triplicate.

T-p53C						
$\Delta T_m \pm$ SD [°C]						
	1 mM 30 min	1 mM 4 h	1 mM 24 h	250 μM 30 min	250 μM 4 h	250 μM 24 h
SN001-1	0.35 $\pm$ 0.27	0.50 $\pm$ 0.09	0.95 $\pm$ 0.12 and 2.85 $\pm$ 0.12 ^a	0.15 $\pm$ 0	0.35 $\pm$ 0.17	0.40 $\pm$ 0.09
SN001-9	0.30 $\pm$ 0.12	0.45 $\pm$ 0.10 ^c	N/A ^b	0 $\pm$ 0	0.35 $\pm$ 0.17	N/A ^b
SN001-10	-0.15 $\pm$ 0.12	0.20 $\pm$ 0.09	0.50 $\pm$ 0.39 ^c	0 $\pm$ 0	0.10 $\pm$ 0.19	0.10 $\pm$ 0.09
SN001-17	-0.90 $\pm$ 0	-0.45 $\pm$ 0.26	-0.40 $\pm$ 0.17	0.10 $\pm$ 0.17	0.50 $\pm$ 0.09	0.35 $\pm$ 0.09
SN001-19	0 $\pm$ 0.14	0.10 $\pm$ 0.19	0.45 $\pm$ 0.12	0 $\pm$ 0	0.15 $\pm$ 0.19	0.05 $\pm$ 0.09
SN001-24	0.15 $\pm$ 0.26	-0.05 $\pm$ 0.19	0.05 $\pm$ 0.27	0.05 $\pm$ 0.09	0 $\pm$ 0	0.20 $\pm$ 0.17
SN001-25	0.15 $\pm$ 0	0.25 $\pm$ 0.12	0.30 $\pm$ 0.12	0.20 $\pm$ 0.09	0.10 $\pm$ 0.09	0.40 $\pm$ 0.12

^aMelting curves with two transitions. First derivatives of the melting curves showed two peaks. ^bNo evaluable melting curves and first derivatives with clearly defined melting points were obtained. ^cFirst derivatives of the melting curves displayed broad peaks.

Table 3.24: $\Delta T_m \pm$ SD of T-p53C-Y220C (8 μ M protein) incubated with 1 mM or 250 μ M SN001 SAR hit compounds for 30 min, 4 h, and 24 h at 20 °C. Measurements were performed at least in triplicate.

T-p53C-Y220C						
$\Delta T_m \pm$ SD [°C]						
	1 mM 30 min	1 mM 4 h	1 mM 24 h	250 μM 30 min	250 μM 4 h	250 μM 24 h
SN001-1	0.83 $\pm$ 0.09	1.25 $\pm$ 0.09	2.45 $\pm$ 0.09	0.30 $\pm$ 0.19	0.25 $\pm$ 0.17	1.35 $\pm$ 0.32
SN001-9	-0.15 $\pm$ 0.19	1.08 $\pm$ 0.10 ^a	N/A ^b	0.30 $\pm$ 0.12	0.20 $\pm$ 0.09	1.90 $\pm$ 0.12
SN001-10	0.15 $\pm$ 0.12	0.15 $\pm$ 0.06	0.85 $\pm$ 0.31	0.30 $\pm$ 0.12	0.10 $\pm$ 0.09	0.15 $\pm$ 0.12
SN001-17	2.93 $\pm$ 0.17	3.77 $\pm$ 0.03	4.42 $\pm$ 0.11	0.55 $\pm$ 0.09	4.25 $\pm$ 0.09	5.19 $\pm$ 0.06
SN001-19	0.20 $\pm$ 0.12	0.30 $\pm$ 0.08	0.85 $\pm$ 0.09	0.25 $\pm$ 0.17	0.10 $\pm$ 0.09	0.30 $\pm$ 0.12
SN001-24	0.15 $\pm$ 0.12	0.10 $\pm$ 0.09	0.70 $\pm$ 0.12	0.35 $\pm$ 0.17	0.40 $\pm$ 0.24	0.45 $\pm$ 0.24
SN001-25	0.35 $\pm$ 0.09	0.45 $\pm$ 0.15	0.73 $\pm$ 0.11	0.50 $\pm$ 0.17	0.45 $\pm$ 0.12	0.95 $\pm$ 0.12

^aFirst derivatives of the melting curves displayed broad peaks. ^bNo evaluable melting curves and first derivatives with clearly defined melting points were obtained.

3.5.1.2 Intact Protein Mass Spectrometry of T-p53C-Y220C with SN001 SAR Hits

The covalent modification of T-p53C-Y220C by the SN001 SAR hits identified by DSF was verified using UHPLC-ESI-MS. To account for the expected reduced reactivity of certain SAR compounds, the incubation time prior to MS analysis was extended to 24 h compared to 4 h for the primary CovLib MS screen.

In the deconvoluted MS spectra of T-p53C-Y220C after 24 h of incubation with fragment SN001-1, multiple modifications were evident (Figure 3.53). As shown in Figure 3.53 A, at a molar ratio of 1:100, the triple and double modified T-p53C-Y220C represented the most intense signal. In addition, a signal was detected for the protein with four SN001-1 molecules attached. This suggests that cysteines in p53 were non-specifically arylated, as previously indicated by the general stabilization of different p53 mutants in the DSF experiment by SN001-1. At a molar ratio of 1:31.25 (Figure 3.53 B), the number of modifications decreased and a small peak corresponding to the native protein was also observed.

The MS data of the nitro compounds SN001-9 and SN001-10 in Figure 3.54 showed that compound SN001-10 with a fluorine in the para position to the nitro group resulted in less modification of T-p53C-Y220C than compound SN001-9 with the fluorine in the ortho position. In the spectrum of T-p53C-Y220C with SN001-9 (molar ratio 1:100) (Figure 3.54 A), peaks corresponding to the single and double arylated protein were detected. In contrast, unmodified T-p53C-Y220C represented the highest signal in the deconvoluted MS spectrum of T-p53C-Y220C with SN001-10 (molar ratio 1:100) (Figure 3.54 B). The reduced number of arylations of the protein after incubation with fragment SN001-10 observed in the MS experiment may explain the DSF results. In the DSF screen, SN001-10 was the only fragment with an electron-withdrawing NO₂ moiety that resulted in detectable melting curves for all p53 mutants tested after incubation for 24 h at a compound concentration of 1 mM. However, due to the unconfirmed purity of SN001-10 by HPLC-UV, the intact protein MS results of SN001-10 must be interpreted with caution.

The deconvoluted MS spectra of T-p53C-Y220C and T-p53C-C124/182/277S with fragment SN001-17 are depicted in Figure 3.55. Incubation with the reactive fragment SN001-17 resulted in multiple arylations of T-p53C-Y220C. At a molar ratio of 1:100, up to triple modified protein was detected, with the double arylated protein species as the highest signal (Figure 3.55 A). Even at a protein-to-compound ratio of 1:31.25

(Figure 3.55 B), which corresponds to the molar ratio achieving the highest stabilization of T-p53C-Y220C in the DSF experiment (equal to an SN001-17 concentration of 250 μ M), up to three times arylated protein was evident, with the most intense signal for the double modified species. In the spectra of the triple cysteine mutant, native T-p53C-C124/182/277S and the single arylated species were detected. At a molar ratio of 1:100 (Figure 3.55 C), the single arylated protein represented the highest signal, while at a ratio of 1:31.25 (Figure 3.55 D), native T-p53C-C124/182/277S was the highest peak. The number of modifications decreased compared to the measurements with T-p53C-Y220C, indicating a covalent binding to the now absent cysteines 124, 182, 277, and 220. Since the deconvoluted MS spectra of T-p53C-C124/182/277S still showed a single modified protein species, other residues besides the mutated cysteines were labeled. This is consistent with the change in the T_m of T-p53C-C124/182/277S of -0.80 $^{\circ}$ C (1 mM, 30 min, 4 h) observed in the DSF screen. Based on the negative ΔT_m value, this covalent modification appears to destabilize the triple cysteine mutant.

Figure 3.56 shows the deconvoluted MS spectra of T-p53C-Y220C incubated with compounds SN001-18 and SN001-19 (molar ratio 1:100). Despite the lack of stabilization of p53 and its mutants in the DSF experiment, SN001-18 was examined using intact protein MS and identified as a hit that covalently modifies T-p53C-Y220C (Figure 3.56 A). In the deconvoluted MS spectra of T-p53C-Y220C with compounds SN001-18 and SN001-19, peaks were detected corresponding to the native protein and the single and double arylated protein species. The reduction of the number of chlorine atoms in compounds SN001-18 and SN001-19 resulted in lower reactivities and less arylations of T-p53C-Y220C compared to fragment SN001-17. In the DSF experiment, the diminished number of halogens also resulted in reduced stabilization of T-p53C-Y220C.

Analogous MS results were obtained with the bromine SAR compound SN001-24 (Figure 3.57 A). Peaks corresponding to the native protein and the single and double arylated protein species were detected. Interestingly, in the deconvoluted MS spectrum of T-p53C-Y220C with SN001-25 (molar ratio 1:100) shown in Figure 3.57 C, native T-p53C-Y220C represented the main peak, and only the protein labeled with one molecule of SN001-25 was observed. However, a peak corresponding to the single arylated protein was still detected in the deconvoluted MS spectra of the triple cysteine mutant with compounds SN001-24 and SN001-25 (Figure 3.57 B, D). The remaining

partially surface-exposed cysteines 229 and 275 of the T-p53C-C124/182/277S protein are the residues potentially arylated by compounds SN001-24 and SN001-25 [118, 143].

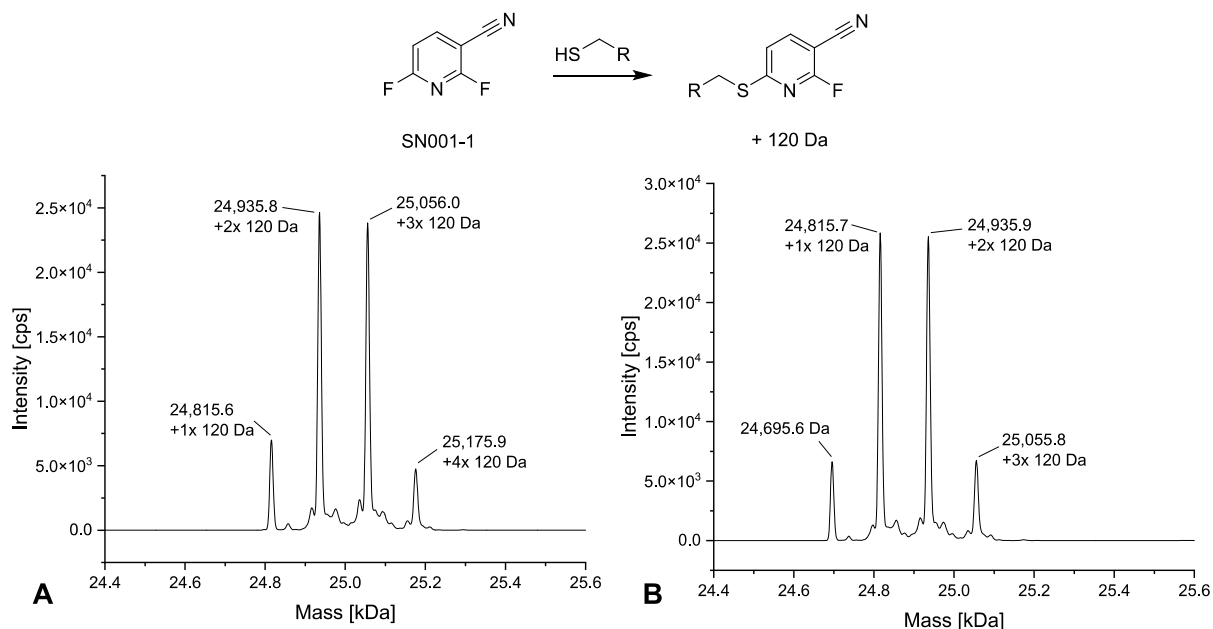


Figure 3.53: Deconvoluted MS spectra of T-p53C-Y220C with SN001-1 at a molar ratio of 1:100 (**A**) and 1:31.25 (**B**) after 24 h of incubation at 20 °C (theoretical mass of unmodified T-p53C-Y220C: 24,696.00 Da).

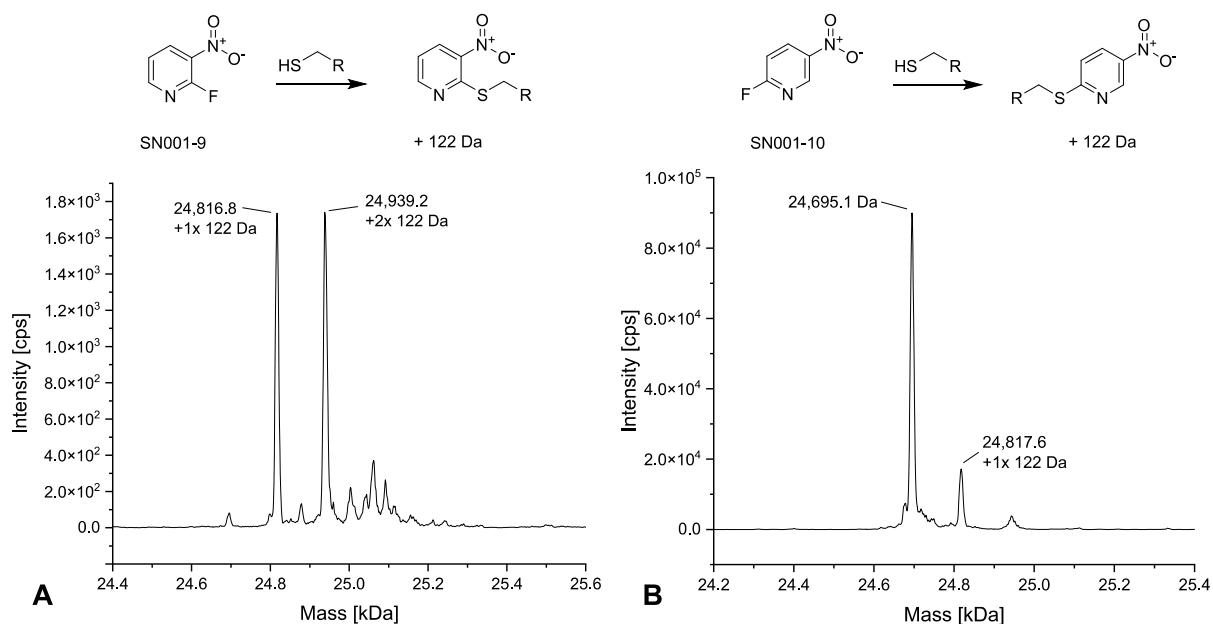


Figure 3.54: Deconvoluted MS spectra of T-p53C-Y220C with SN001-9 (**A**) and SN001-10 (**B**) at a molar ratio of 1:100 after 24 h of incubation at 20 °C (theoretical mass of unmodified T-p53C-Y220C: 24,696.00 Da).

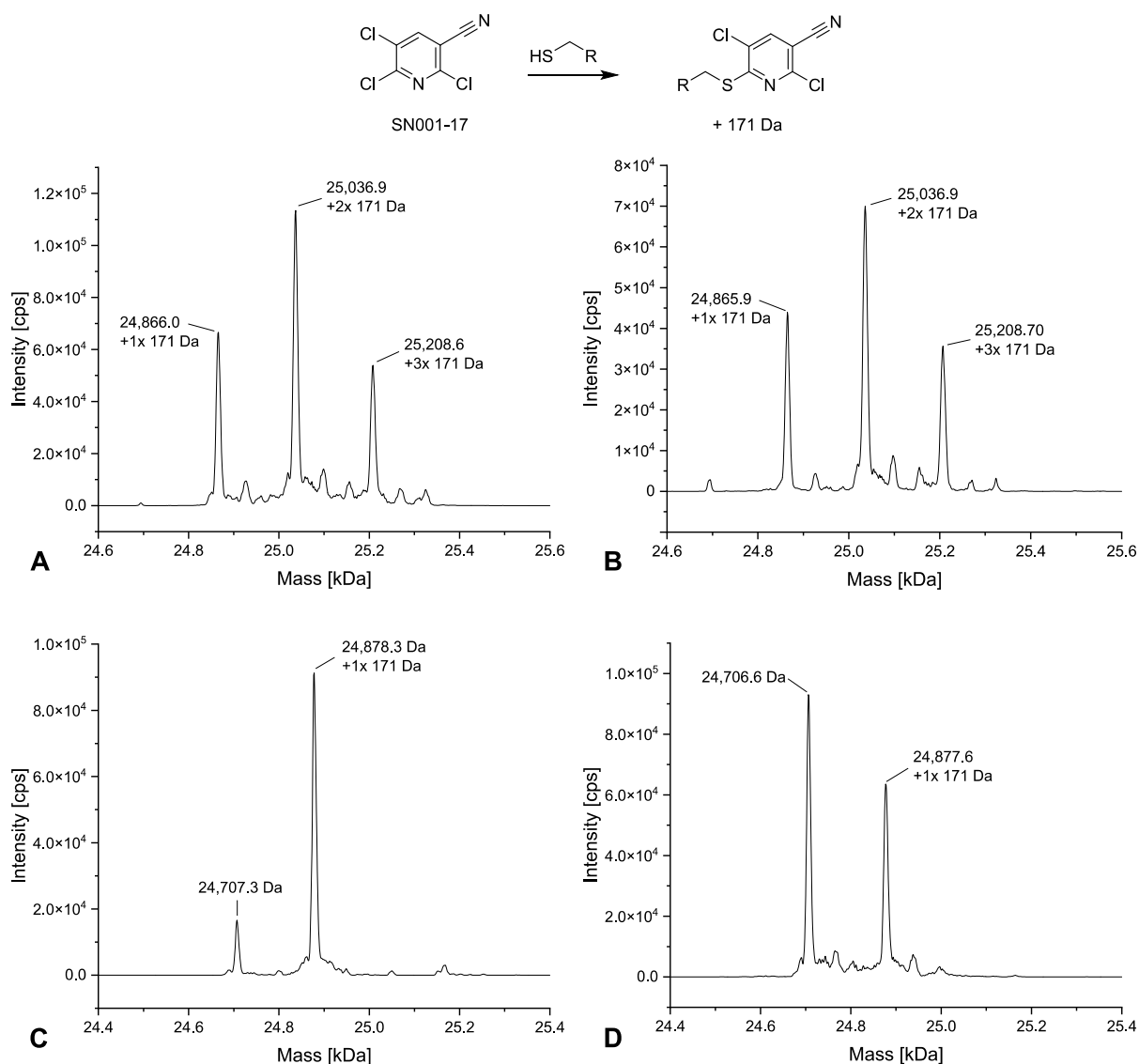


Figure 3.55: Intact protein MS of T-p53C-Y220C and T-p53C-Y220C-C124/182/277S with SN001-17. The deconvoluted MS spectra of T-p53C-Y220C (theoretical mass of unmodified T-p53C-Y220C: 24,696.00 Da) with SN001-17 at a molar ratio of 1:100 (**A**), 1:31.25 (**B**), and of T-p53C-C124/182/277S (theoretical mass of unmodified T-p53C-C124/182/277S: 24,707.84 Da) with SN001-17 at a molar ratio of 1:100 (**C**) and 1:31.25 (**D**) after 24 h of incubation at 20 °C are shown.

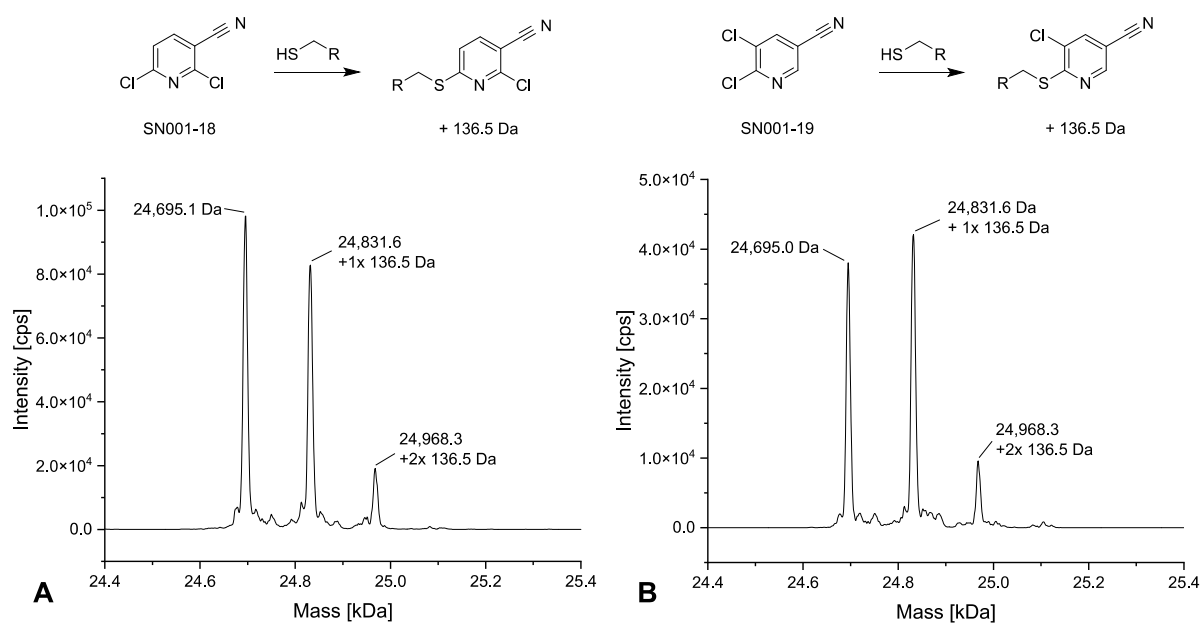


Figure 3.56: Deconvoluted MS spectra of T-p53C-Y220C with SN001-18 (**A**) and SN001-19 (**B**) at a molar ratio of 1:100 after 24 h of incubation at 20 °C (theoretical mass of unmodified T-p53C-Y220C: 24,696.00 Da).

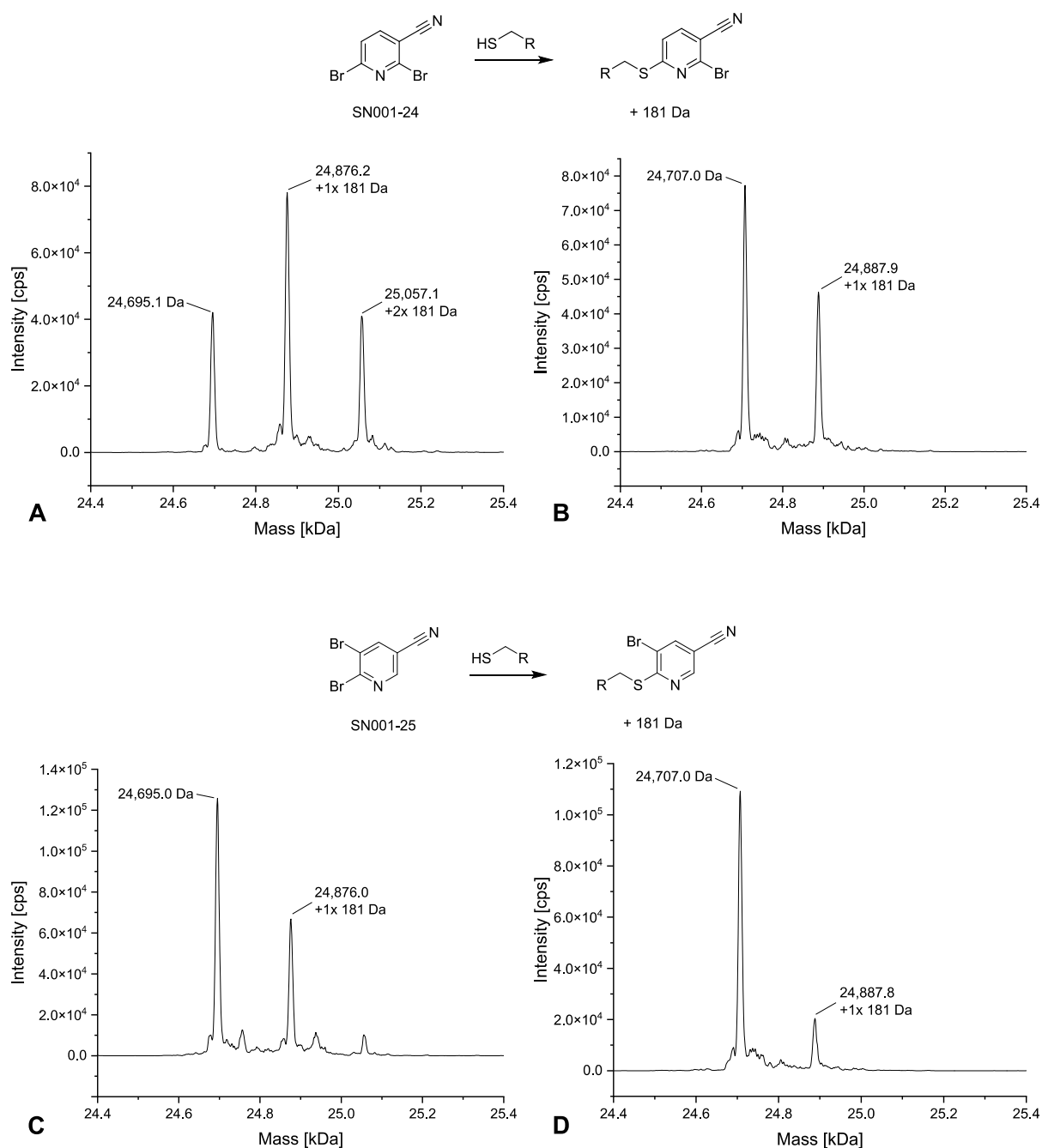


Figure 3.57: Intact protein MS of T-p53C-Y220C and T-p53C-Y220C-C124/182/277S with SN001-24 and SN001-25. The deconvoluted MS spectra of T-p53C-Y220C (theoretical mass of unmodified T-p53C-Y220C: 24,696.00 Da) with SN001-24 (**A**), SN001-25 (**C**), and of T-p53C-C124/182/277S (theoretical mass of unmodified T-p53C-C124/182/277S: 24,707.84 Da) with SN001-24 (**B**), SN001-25 (**D**) at a molar ratio of 1:100 after 24 h of incubation at 20 °C are shown.

3.5.1.3 DSF Studies of the Y220C Stabilizing Hit SN001-17

For all concentration-dependent and time-dependent DSF measurements in Chapter 3.5, only the measured values from the corresponding runs were considered, not the mean value of all measurements as in the primary DSF screen.

The screening of the SAR compounds using DSF and intact protein MS identified compound SN001-17 as a fragment hit, specifically stabilizing the cancer mutant T-p53C-Y220C by covalent modification. The results of the concentration- and time-dependent measurements of T-p53C-Y220C incubated with SN001-17 are depicted in Figure 3.58 and Table 3.25. The 2,5,6-trichloropyridine derivative SN001-17 showed a concentration-dependent reaction behavior, but due to the reduced reactivity compared to SN001, time-dependent effects were also discovered. After 30 min of incubation (Figure 3.58 A), a distinct stabilization of T-p53C-Y220C by up to 3.10 °C was only detectable at a compound concentration of 1 mM. A broad peak with two maxima was obtained at 500 µM SN001-17, possibly indicating differently modified protein species with different melting points or contributions from non-covalently bound fragments with distinct melting points [264, 289, 297]. This suggests that the compound was still reacting with the target. After 30 min incubation time at 250 µM compound concentration, only a slight increase in the melting temperature of the protein ($\Delta T_m = 0.50$ °C) was evident. In comparison, the 2,5,6-trifluoropyridine derivative SN001 stabilized T-p53C-Y220C by up to 4.45 °C at 250 µM after 30 min of incubation (Table 3.10). However, in contrast to the original compound SN001, the stabilization of the Y220C mutant by the fragment SN001-17 raised with the incubation time (Figure 3.58 B). The highest stabilization of T-p53C-Y220C was achieved after 24 h of incubation and 250 µM compound concentration at a protein-to-compound ratio of 1:31.25. Fragment SN001-17 extensively stabilized the temperature-sensitive cancer mutant T-p53C-Y220C by up to 5.10 °C. This increase in the T_m was higher than the maximum increase by the original fragment SN001 of 4.45 °C at 250 µM after 30 min or 4.40 °C at 250 µM after 24 h of incubation. Even at a concentration of 1 mM, the maximum stabilization of T-p53C-Y220C by about 4.68 °C (24 h) was higher than the stabilization achieved with fragment SN001 by about 3.45 °C (1 mM, 30 min) or 2.95 °C (1 mM, 24 h). Furthermore, SN001-17 stabilized the p53-Y220C protein after 24 h of incubation up to a lowest effective concentration of 62.5 µM, corresponding to a protein-to-compound ratio of 1:7.81 ($\Delta T_m = 1.20$ °C).

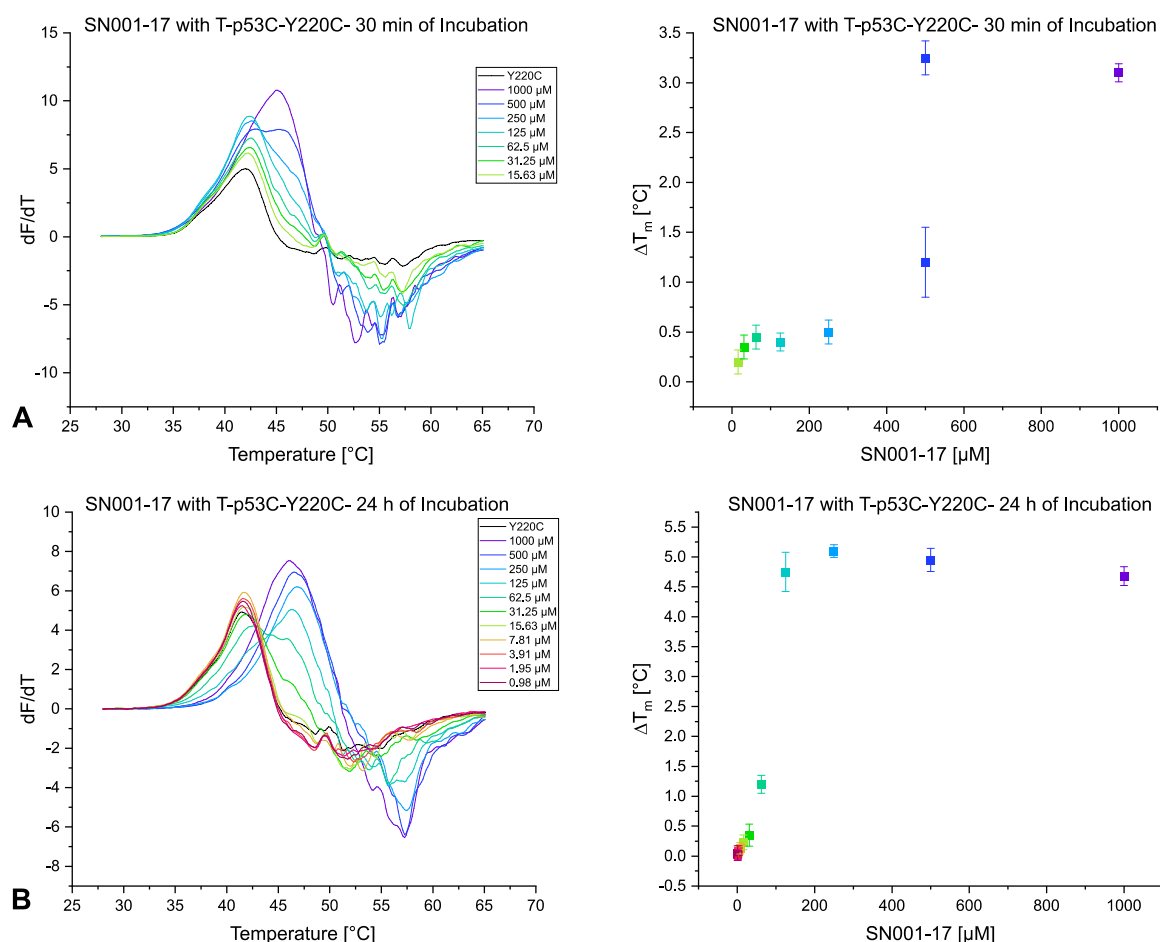


Figure 3.58: DSF measurements of T-p53C-Y220C with SN001-17. The first derivatives of the melting curves and resulting ΔT_m values of T-p53C-Y220C (8 μ M protein) with various concentrations of compound SN001-17 after 30 min (**A**) and 24 h (**B**) of incubation at 20 °C are shown.

Table 3.25: $\Delta T_m \pm$ SD of T-p53C-Y220C (8 μ M) incubated with different concentrations of compound SN001-17 for 30 min and 24 h at 20 °C. Measurements were performed at least in triplicate.

SN001-17 [μ M]	T-p53C-Y220C 30 min $\Delta T_m \pm$ SD [°C]	T-p53C-Y220C 24 h $\Delta T_m \pm$ SD [°C]
1000	3.10 ± 0.09	4.68 ± 0.16
500	1.20 ± 0.36 and 3.25 ± 0.17	4.95 ± 0.19
250	0.50 ± 0.12	5.10 ± 0.11
125	0.40 ± 0.09	4.75 ± 0.33
62.5	0.45 ± 0.12	1.20 ± 0.15
31.25	0.35 ± 0.12	0.35 ± 0.18
15.63	0.20 ± 0.12	0.23 ± 0.12
7.81	N/D	0.13 ± 0.10
3.91	N/D	0.08 ± 0.10
1.95	N/D	0.03 ± 0.09
0.98	N/D	0.05 ± 0.12

3.5.1.4 Evaluation of the Hits SN001-1 and SN001-17 with T-p53C-Y220C-CL

The identified hits SN001-1 and SN001-17 were examined with T-p53C-Y220C-CL to investigate the effect of a covalent modification of Cys220 on the melting temperature.

The DSF results of the Y220C cysteine light mutant incubated with fragment SN001-1 are shown in Figure 3.59 and Table 3.26. The 2,6-difluoro-3-pyridinecarbonitrile SN001-1 stabilized T-p53C-Y220C-CL only at a concentration of 1 mM after 24 h of incubation. Compound SN001-1 provided substantially less stabilization of T-p53C-Y220C-CL, with a ΔT_m of 1.10 °C (1 mM, 24 h), compared to the first 2,5,6-trifluoropyridine hit SN001, which resulted in a ΔT_m of 3.08 °C (1 mM, 24 h). These findings reinforce the conclusion that the removal of the fluorine atom in the meta position to the electron-withdrawing nitrile group did not result in increased stabilization of T-p53C-Y220C-CL and a more selective binding into the Y220C pocket.

The results of the DSF screen with compound SN001-17 showed a substantial stabilization of T-p53C-Y220C-CL (Figure 3.59, Table 3.26), indicating modification of cysteine 220 in the mutation-induced crevice. After incubation with 1 mM SN001-17 for 30 min, the melting temperature of the Y220C cysteine light mutant was increased by 4.57 °C. However, the first derivative of the melting curve displayed a broad curve with an initial shoulder, indicating that the compound was still reacting with the target and had not occupied the target completely. After 24 h of incubation with compound SN001-17 (1 mM), T-p53C-Y220C-CL was stabilized by 4.95 °C. This stabilization was considerably higher compared to the ΔT_m of 3.08 °C (1 mM, 24 h) of T-p53C-Y220C-CL incubated with the original trifluoro compound SN001. In addition, fragment SN001-17 showed a time-dependent effect at a compound concentration of 250 μ M. After 30 min of incubation at 250 μ M, no stabilization of T-p53C-Y220C-CL was evident. However, as illustrated in Figure 3.59, the first derivative of the T-p53C-Y220C-CL melting curve displayed a broad curve with a second plateau. After 4 h of incubation, no melting point was measured. The first derivative was a broad curve with multiple peaks. After 24 h of incubation at 250 μ M SN001-17, a stabilization of T-p53C-Y220C-CL by about 5.20 °C was measured. No substantial difference was observed between the stabilization of the Y220C cysteine light protein at concentrations of 1 mM SN001-17 (ΔT_m = 4.95 °C) and 250 μ M SN001-17 (ΔT_m = 5.20 °C) after 24 h of incubation. However, as previously noted for T-p53C-Y220C, the 2,5,6-trichloropyridine derivative SN001-17 showed a higher

overall stabilization of T-p53C-Y220C-CL ($\Delta T_m = 5.20$ °C, 250 μ M, 24 h) compared to the original 2,5,6-trifluoropyridine derivative SN001 ($\Delta T_m = 2.95$ °C, 250 μ M, 24 h). Furthermore, the increase in the T_m of T-p53C-Y220C-CL by 5.20 °C with SN001-17 (250 μ M, 24 h) is consistent with the maximum stabilization of 5.19 °C (250 μ M, 24 h) observed for T-p53C-Y220C. This suggests that the covalent modification of Cys220 in the hydrophobic pocket is responsible for the stabilization of T-p53C-Y220C and T-p53C-Y220C-CL.

The deconvoluted MS spectra of fragment T-p53C-Y220C-CL incubated with SN001-17 are depicted in Figure 3.60. At a molar ratio of 1:100 (Figure 3.60 A) and 1:31.25 (Figure 3.60 B), only the native Y220C cysteine light protein and the single arylated protein were recognizable, indicating a covalent modification of the remaining Cys220. As the concentration of fragment SN001-17 decreased, the intensity of the peak corresponding to the single arylated protein also diminished.

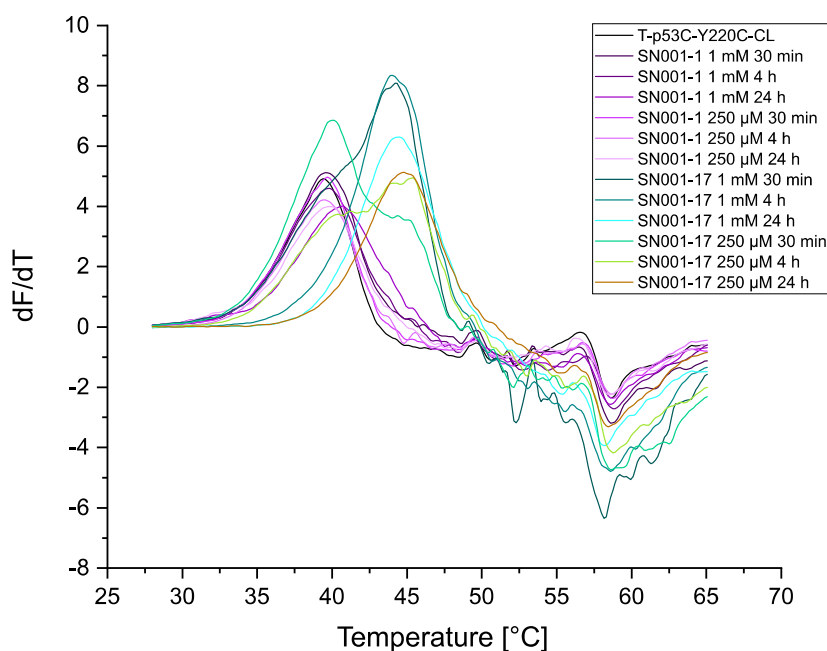
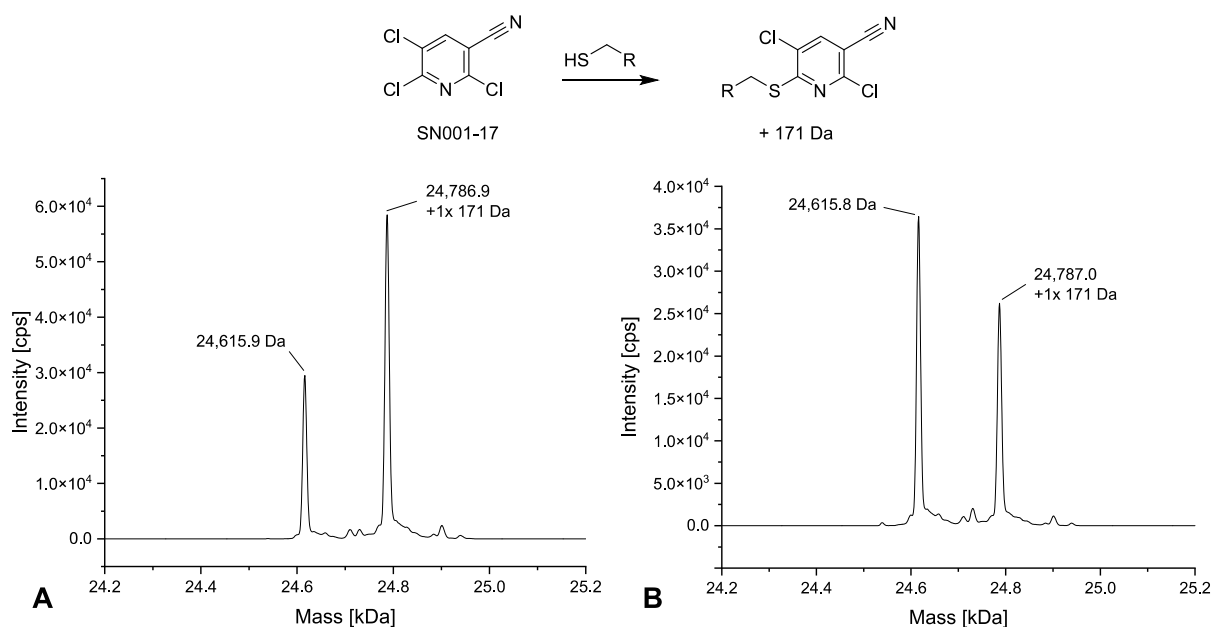


Figure 3.59: DSF measurements of T-p53C-Y220C-CL with SN001-1 and SN001-17. The first derivatives of the melting curves of T-p53C-Y220C-CL (8 μ M) with SN001-1 and SN001-17 (1 mM, 250 μ M) after varying incubation periods at 20 °C are shown.

Table 3.26: $\Delta T_m \pm SD$ of T-p53C-Y220C-CL (8 μM) incubated with SN001-1 and SN001-17 (1 mM and 250 μM) after different periods times at 20 °C. Measurements were performed at least in triplicate.

T-p53C-Y220C-CL						
$\Delta T_m \pm SD$ [°C]						
	1 mM 30 min	1 mM 4 h	1 mM 24 h	250 μM 30 min	250 μM 4 h	250 μM 24 h
SN001-1	0.10 ± 0.12	0.25 ± 0.09	1.10 ± 0.17	-0.05 ± 0.09	0.10 ± 0.23	0.05 ± 0.27
SN001-17	4.57 ± 0.07^a	4.35 ± 0	4.95 ± 0.12	0.25 ± 0.17^a	N/A ^b	5.20 ± 0.12

^aFirst derivatives of the melting curves displayed broad curves with additional shoulders. ^bNo evaluable melting curves and first derivatives with clearly defined melting points were obtained. First derivatives showed no distinct peak.

**Figure 3.60:** Deconvoluted MS spectra of T-p53C-Y220C-CL with SN001-17 at a molar ratio of 1:100 (**A**) and 1:31.25 (**B**) after 24 h of incubation at 20 °C (theoretical mass of unmodified T-p53C-Y220C-CL: 24,615.68 Da).

3.5.1.5 Determination of the Binding Mode of SN001-17 Using X-Ray Crystallography

The crystal structure of xT-p53C-Y220C soaked with 5 mM SN001-17 for two days confirmed the covalent modification of Cys220. As shown in Figure 3.61, the crystal structure displayed well-defined density for arylation at Cys220. The 5 mM-soaked structure indicated that the 2,5,6-trichloropyridine derivative SN001-17 reacted with cysteine residues in p53 via an S_NAr reaction, with the chlorine atom in the para position to the electron-withdrawing nitrile group acting as the LG. The SAR compound SN001-17 showed the same binding pose in the pocket as the original compound SN001, adopting the same Tyr-like conformation in the mutational cleft. Similar to SN001, SN001-17 occupied most of the space in the central cavity, mimicking the wt tyrosine residue lost by the mutation. The electron-withdrawing nitrile group pointed towards subsite 1 and Thr150. The ortho-chlorine (similar to ortho-fluorine in SN001) pointed towards subsite 2, and the meta-chlorine (similar to meta-fluorine in SN001) pointed into the hydrophobic subsite 3. Presumably, the meta-fluorine in SN001 and the meta-chlorine in SN001-17 contributed to the binding in the Y220C pocket via hydrophobic contacts. Compared to the meta-fluorine, the meta-chlorine occupied a larger space in the hydrophobic subsite 3, which may lead to the higher stabilization of the cancer mutant Y220C with fragment SN001-17 by up to 5.19 °C.

After soaking for two days with 5 mM SN001-17, in addition to Cys220, the cysteines 182, 229, and 277 were also arylated in the crystal structure. Moreover, the side chain of Trp146 was flipped upon modification of Cys229 by fragment SN001-17 to accommodate the arylation. This side chain flip was also observed in the structure of xT-p53C-Y220C soaked with SN001 at 40 mM (Figure 3.49).

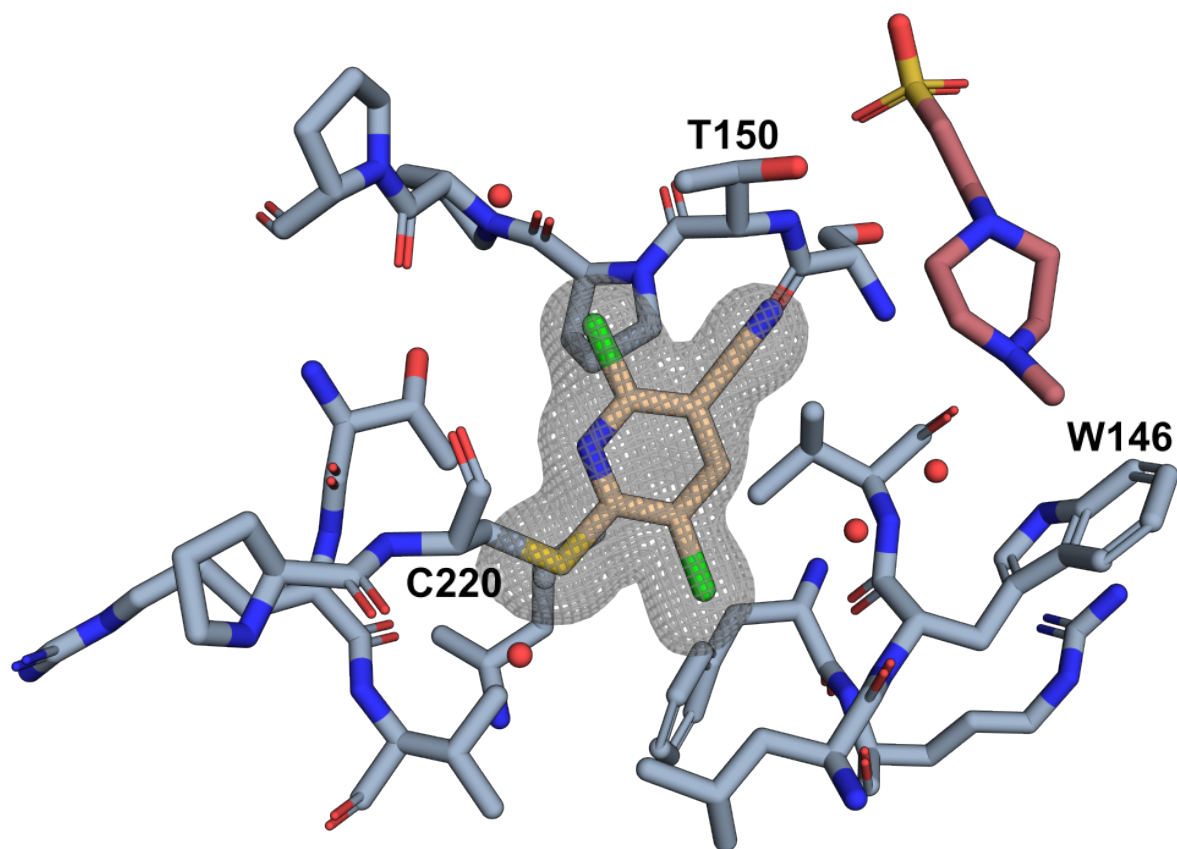


Figure 3.61: Overview of the binding mode of SN001-17 (soaked at 5 mM for two days) into the Y220C induced crevice. The compound binds similarly to SN001 in a Tyr-like conformation, with the nitrile group pointing towards subsite 1 and Thr150. The ortho-chlorine points towards subsite 2, and the meta-chlorine points into the hydrophobic subsite 3. The unbiased omit map is shown at a contour level of 3 σ .

3.5.2 SN006/7 SAR Compound Screen

Figure 3.62 shows the tested SN006/7 SAR compounds. Fragments SN006/7-1 and SN006/7-6 lack a second electron-withdrawing nitrile group in the para or ortho position to the LG compared to the original compound SN006. In compounds SN006/7-2 to SN006/7-5, the electron-donating NH₂ group was also removed. The pyrazine derivatives SN006/7-2 to SN006/7-5 contain either chlorine or bromine as a LG in the ortho or para position to the nitrile group.

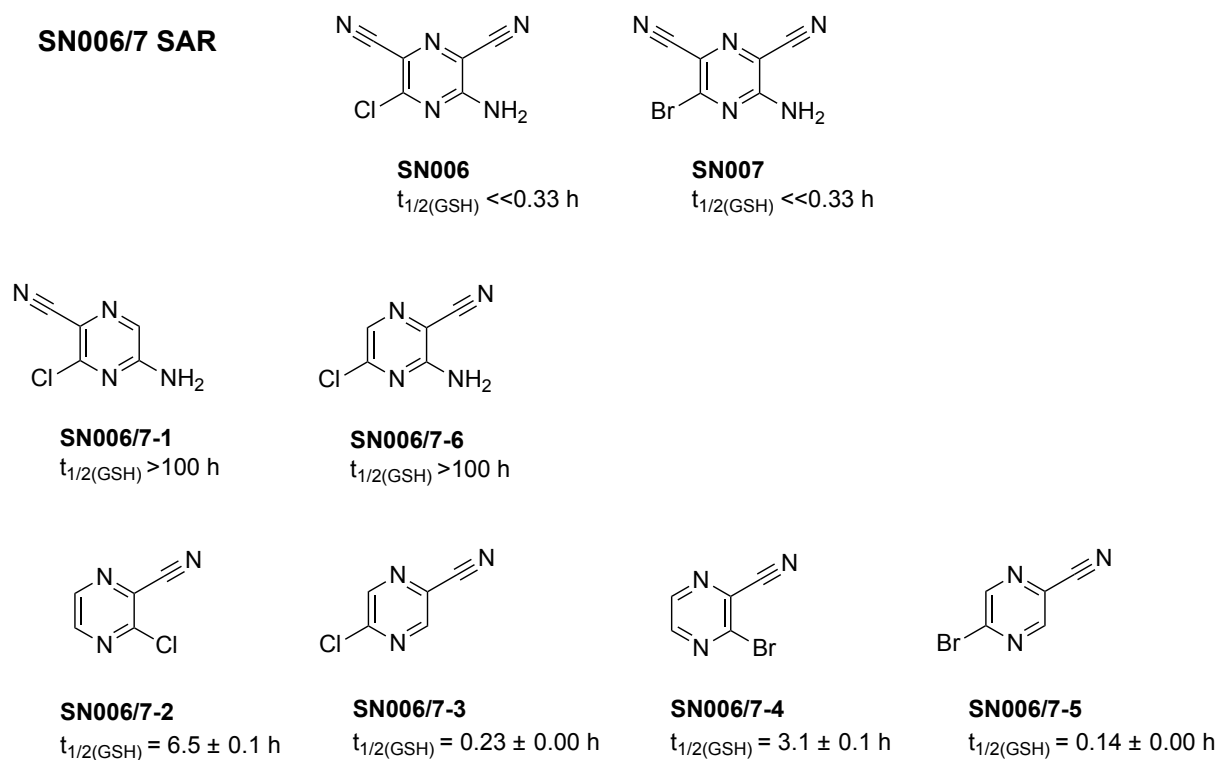


Figure 3.62: Overview of the tested SN006/7 SAR compounds and their $t_{1/2} \pm \text{SD}$ determined via GSH assay. The GSH assay was performed by M. Schwer.

3.5.2.1 DSF Screen of SN006/7 SAR Compounds with T-p53C and Its Mutants

The CovLib compounds SN006 and SN007 are highly reactive fragments with two electron-withdrawing nitrile groups, covalently modifying Cys220 in the hydrophobic pocket. The DSF screen results of the tested SN006/7 SAR compounds (1 mM) after 30 min, 4 h, and 24 h incubation time are depicted in Table 3.27, Table 3.28, and Table 3.29.

The tested fragments SN006/7-1 and SN006/7-6 had no considerable effect on the melting temperatures of T-p53C and its mutants even after 24 h of incubation. Hence, the compounds lacking the additional electron-withdrawing nitrile group in the para or ortho position to the LG are chemically stable ($t_{1/2}$ GSH >100 h) and not reactive enough to stabilize T-p53C-Y220C by covalent modification.

The pyrazine derivatives SN006/7-2 to SN006/7-5 were identified as hits by DSF screen. SN006/7-2 and SN006/7-4 have a halogen in the ortho position to the EWG. As depicted in Table 3.27, no stabilization of T-p53C and its mutants was detected after 30 min of incubation. However, with increasing incubation time and target occupancy, the stabilizing effect increased. After 24 h of incubation, both compounds stabilized p53 and its mutants except for the triple cysteine mutant. Nevertheless, it is unclear from the DSF screen results whether a covalent binding to Cys220 in the binding pocket of T-p53C-Y220C occurred, as all mutants were stabilized. The melting curves of T-p53C supplemented with both compounds after 24 h of incubation were biphasic. The first derivatives showed two maxima (Figure 8.36 A), corresponding to different arylated protein species with different melting points or non-covalently bound fragments with distinct melting points [264, 289, 297]. A reversible covalent Pinner-type addition of proteinogenic nucleophiles to the nitrile group of the compounds [144, 197] is also conceivable, which could lead to differently modified protein species. Additionally, the compounds were tested at a concentration of 250 μ M with T-p53C and T-p53C-Y220C. Table 3.30 and Table 3.31 show that as the compound concentrations decreased, the stabilization of T-p53C and T-p53C-Y220C also decreased. The highest increase in the melting temperatures was achieved with 1 mM SN006/7-2 and SN006/7-4 after a 24-hour incubation period.

After only 30 min of incubation, fragments SN006/7-3 and SN006/7-5, which have a chlorine or bromine para to the EWG, stabilized T-p53C-Y220C by 2.75 °C and 1.65 °C, respectively. Fragments SN006/7-3 ($t_{1/2}$ GSH= 0.23 h) and SN006/7-5

($t_{1/2}$ GSH= 0.14 h) also exhibited a higher reactivity in the GSH assay compared to compounds SN006/7-2 ($t_{1/2}$ GSH= 6.5 h) and SN006/7-4 ($t_{1/2}$ GSH= 3.1 h) with the ortho-LG. This higher reactivity is consistent with the increasing stabilization of the σ -complex by EWGs in the order meta $\ll$ ortho $<$ para [276]. S_NAr substitutions that involve an -M substituent in the para position are expected to occur at higher rates than those with ortho activation. The reason for this is that the inductive effect of the substituent located ortho to the LG is outweighed by the highest resonance stabilization of -M substituent para to the LG [276, 332].

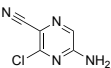
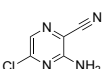
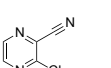
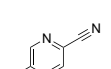
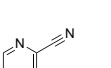
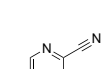
Table 3.27 shows that after 30 min of incubation, SN006/7-3 and SN006/7-5 exhibited a particularly pronounced stabilizing effect on the Y220C mutant, indicating a covalent modification of the mutation-induced cysteine in the crevice. Similar to the original compounds SN006 and SN007, the R282W mutant showed no evaluable melting curves and first derivatives with clearly defined peaks after incubation with SN006/7-3 and SN006/7-5 (Figure 8.34 B, Figure 8.35 E, Figure 8.37 B). It can be assumed that SN006/7-3 caused a considerable destabilization of T-p53C-R282W after 30 min of incubation, although the first derivative of the melting curve is not suitable for determining the melting point, displaying a broad peak (Figure 8.34 B). After 24 h of incubation, compound SN006/7-3 with a chlorine atom para to the EWG strongly increased the melting temperature of T-p53C-Y220C by up to 3.62 °C (1 mM, 24 h). In addition, SN006/7-3 also stabilized T-p53C. After 24 h of incubation, the first derivatives of the melting curves of T-p53C showed two maxima, with a ΔT_m of 0.70 °C and 2.55 °C, respectively. Moreover, the compound slightly stabilized T-p53C-C124/182/277S by 0.55 °C (1 mM, 24 h), indicating a covalent modification of additional cysteines besides Cys124, Cys182, and Cys277. The bromine analog SN006/7-5 stabilized T-p53C-Y220C less effectively (ΔT_m = 1.65 °C, 1 mM, 24 h) than SN006/7-3. However, after 24 h of incubation, both T-p53C (ΔT_m = -0.60 °C and 0.30 °C) and T-p53C-R273H (ΔT_m = -1.48 °C) were destabilized. The destabilization can be attributed to the higher reactivity of SN006/7-5 ($t_{1/2}$ GSH= 0.14 h) determined via GSH assay compared to SN006/7-3 ($t_{1/2}$ GSH= 0.23 h), leading to hyperarylation and impairments with normal protein folding [12].

SN006/7-3 and SN006/7-5 were also examined at a concentration of 250 μ M with T-p53C and T-p53C-Y220C (Table 3.30, Table 3.31). Table 3.31 indicates that the overall stabilization of T-p53C-Y220C was reduced at 250 μ M SN006/7-3. After 24 h of incubation, a stabilization of 2.87 °C was measured, which was lower than the

stabilization observed at a compound concentration of 1 mM ($\Delta T_m = 3.62\text{ }^{\circ}\text{C}$). In contrast, the stabilization of T-p53C-Y220C increased as the concentration of the reactive fragment SN006/7-5 decreased. This concentration-dependent reaction behavior has already been observed for the highly reactive CovLib compounds SN006 and SN007. The stabilization of T-p53C-Y220C by $2.65\text{ }^{\circ}\text{C}$ after 24 h of incubation with $250\text{ }\mu\text{M}$ SN006/7-5 was comparable to the ΔT_m value of $2.87\text{ }^{\circ}\text{C}$ measured with SN006/7-3 at the same concentration.

In summary, compared to the original compounds SN006 and SN007, evaluable melting curves of T-p53C-Y220C and T-p53C were detectable with all tested SAR compounds after 24 h of incubation. Furthermore, except for SN006/7-5 (24 h, 1 mM), the compounds did not destabilize T-p53C. In contrast, SN006 ($\Delta T_m = -2.30\text{ }^{\circ}\text{C}$) and SN007 ($\Delta T_m = -2.45\text{ }^{\circ}\text{C}$) largely destabilized T-p53C at a compound concentration of 1 mM after 30 min incubation time. In addition, except for SN006/7-5, the stabilization of T-p53C-Y220C decreased with decreasing concentrations of the identified SAR hits. Removal of one CN group resulted in unreactive compounds (SN006/7-1, SN006/7-6), showing no influence on the T_m of the tested proteins. Compounds with ortho-EWG were also less reactive and not more selective for the Y220C pocket (SN006/7-2, SN006/7-4) than compounds with para-EWG (SN006/7-3, SN006/7-5). Compound SN006/7-3 was identified as the best hit, exhibiting the most prominent stabilization of the Y220C mutant. The stabilization of T-p53C-Y220C by about $3.62\text{ }^{\circ}\text{C}$ (1 mM, 24 h) was also more pronounced than the maximum stabilization observed with the original compounds SN006 ($\Delta T_m = 1.82\text{ }^{\circ}\text{C}$, $500\text{ }\mu\text{M}$, 30 min) and SN007 ($\Delta T_m = 2.03\text{ }^{\circ}\text{C}$, $250\text{ }\mu\text{M}$, 30 min).

Table 3.27: $\Delta T_m \pm SD$ of T-p53C and its mutants (8 μ M) incubated with 1 mM SN006/7 SAR compounds for **30 min** at 20 °C. Measurements were performed at least in triplicate. Fragments with a ΔT_m of at least 0.50 °C are highlighted in bold [99, 130].

Compound	T-p53C	T-p53C- Y220C	T-p53C- R273H	T-p53C- R282W	T-p53C- C124/182/ 277S
	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]
SN006/7- 1 	-0.05 ± 0.09	-0.15 ± 0.12	-0.10 ± 0.09	-0.20 ± 0.09	N/D ^b
SN006/7- 6 	0.35 ± 0.09	0.15 ± 0	N/D	N/D	N/D ^b
SN006/7- 2 	0.10 ± 0.09	0.05 ± 0.12	0 ± 0	-0.05 ± 0.09	0.20 ± 0.12
SN006/7- 3 	0.70 ± 0.09	2.75 ± 0.11	-0.45 ± 0.15	N/A ^a	0.35 ± 0.12
SN006/7- 4 	0.30 ± 0	0.10 ± 0.09	0.10 ± 0.09	0 ± 0.15	0.10 ± 0.09
SN006/7- 5 	0.30 ± 0.15	1.65 ± 0.09	-0.70 ± 0.09	N/A ^a	0.30 ± 0.12

^aNo evaluable melting curves and first derivatives with clearly defined melting points were obtained.

^bOnly the identified fragment hits were investigated with T-p53C-C124/182/277S.

Table 3.28: $\Delta T_m \pm SD$ of T-p53C and its mutants (8 μ M) incubated with 1 mM SN006/7 SAR compounds for **4 h** at 20 °C. Measurements were performed at least in triplicate. Fragments with a ΔT_m of at least 0.50 °C are highlighted in bold [99, 130].

Compound	T-p53C	T-p53C-Y220C	T-p53C-R273H	T-p53C-R282W	T-p53C-C124/182/277S
	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]
SN006/7-1	-0.20 $\pm$ 0.19	0 $\pm$ 0	0 $\pm$ 0	-0.10 $\pm$ 0.09	N/D ^c
SN006/7-6	0.15 $\pm$ 0.12	N/D	N/D	N/D	N/D ^c
SN006/7-2	0.20 $\pm$ 0.09	0.50 $\pm$ 0.09	0.30 $\pm$ 0	0.45 $\pm$ 0	0.15 $\pm$ 0.12
SN006/7-3	0.45 $\pm$ 0.12 ^a	3.53 $\pm$ 0.15	-0.05 $\pm$ 0.09	N/A ^b	0.50 $\pm$ 0.09
SN006/7-4	0.35 $\pm$ 0.09 ^a	0.75 $\pm$ 0.23	0.65 $\pm$ 0.09	0.85 $\pm$ 0.23	0.20 $\pm$ 0.09
SN006/7-5	0.25 $\pm$ 0.12	1.75 $\pm$ 0.09	-0.65 $\pm$ 0.17	N/A ^b	0.25 $\pm$ 0.12

^aFirst derivatives of the melting curves displayed broad peaks. ^bNo evaluable melting curves and first derivatives with clearly defined melting points were obtained. ^cOnly the identified fragment hits were investigated with T-p53C-C124/182/277S.

Table 3.29: $\Delta T_m \pm SD$ of T-p53C and its mutants (8 μ M) incubated with 1 mM SN006/7 SAR compounds for **24 h** at 20 °C. Measurements were performed at least in triplicate. Fragments with a ΔT_m of at least 0.50 °C in bold [99, 130].

Compound	T-p53C	T-p53C-Y220C	T-p53C-R273H	T-p53C-R282W	T-p53C-C124/182/277S
	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]
SN006/7-1	-0.20 $\pm$ 0.12	0.05 $\pm$ 0.10	-0.15 $\pm$ 0	0.05 $\pm$ 0.09	N/D ^b
SN006/7-6	0.20 $\pm$ 0.12	0.15 $\pm$ 0	N/D	N/D	N/D ^b
SN006/7-2	0.60 $\pm$ 0.12 and 2.50 $\pm$ 0.12	1.38 $\pm$ 0.10	0.90 $\pm$ 0	1.70 $\pm$ 0.17	0.30 $\pm$ 0
SN006/7-3	0.70 $\pm$ 0.12 and 2.55 $\pm$ 0.12	3.62 $\pm$ 0.11	0.23 $\pm$ 0.23	N/A ^a	0.55 $\pm$ 0.09
SN006/7-4	0.65 $\pm$ 0.12 and 2.55 $\pm$ 0.12	2.00 $\pm$ 0.11	1.00 $\pm$ 0.09	1.85 $\pm$ 0.09	0.30 $\pm$ 0
SN006/7-5	-0.60 $\pm$ 0.12 and 0.30 $\pm$ 0.12	1.65 $\pm$ 0.30	-1.48 $\pm$ 0.28	N/A ^a	0.30 $\pm$ 0

^aNo evaluable melting curves and first derivatives with clearly defined melting points were obtained.

^bOnly the identified fragment hits were investigated with T-p53C-C124/182/277S.

Table 3.30: $\Delta T_m \pm$ SD of T-p53C (8 μ M protein) incubated with 1 mM or 250 μ M SN006/7 SAR hit compounds for 30 min, 4 h, and 24 h at 20 °C. Measurements were performed at least in triplicate.

T-p53C						
$\Delta T_m \pm$ SD [°C]						
	1 mM 30 min	1 mM 4 h	1 mM 24 h	250 μM 30 min	250 μM 4 h	250 μM 24 h
SN006/7-2	0.10 ± 0.09	0.20 ± 0.09	0.60 ± 0.12 and 2.50 ± 0.12	0.05 ± 0.09	0.05 ± 0.09	0.25 ± 0.12
SN006/7-3	0.70 ± 0.09	0.45 ± 0.12^a	0.70 ± 0.12 and 2.55 ± 0.12	0.50 ± 0.17^a	0.90 ± 0 and 2.60 ± 0.09	0.90 ± 0.12 and 2.85 ± 0.19
SN006/7-4	0.30 ± 0	0.35 ± 0.09^a	0.65 ± 0.12 and 2.55 ± 0.12	0.15 ± 0.17	0.20 ± 0.09	0.50 ± 0.09
SN006/7-5	0.30 ± 0.15	0.25 ± 0.12	-0.60 ± 0.12 and 0.30 ± 0.12	0.50 ± 0.09^a	0.80 ± 0.09 and 2.50 ± 0.09	0.80 ± 0.09 and 2.65 ± 0.12

^aFirst derivatives of the melting curves displayed broad peaks.**Table 3.31:** $\Delta T_m \pm$ SD of T-p53C-Y220C (8 μ M protein) incubated with 1 mM or 250 μ M SN006/7 SAR hit compounds for 30 min, 4 h, and 24 h at 20 °C. Measurements were performed at least in triplicate.

T-p53C-Y220C						
$\Delta T_m \pm$ SD [°C]						
	1 mM 30 min	1 mM 4 h	1 mM 24 h	250 μM 30 min	250 μM 4 h	250 μM 24 h
SN006/7-2	0.05 ± 0.12	0.50 ± 0.09	1.38 ± 0.10	0.10 ± 0.12	0.10 ± 0.12	0.70 ± 0.09
SN006/7-3	2.75 ± 0.11	3.53 ± 0.15	3.62 ± 0.11	0.88 ± 0.19	2.10 ± 0.12	2.87 ± 0.14
SN006/7-4	0.10 ± 0.09	0.75 ± 0.23	2.00 ± 0.11	0.05 ± 0.31	0.25 ± 0.12	1.00 ± 0.09
SN006/7-5	1.65 ± 0.09	1.75 ± 0.09	1.65 ± 0.30	1.30 ± 0.12	2.20 ± 0.12	2.65 ± 0.17

3.5.2.2 Intact Protein Mass Spectrometry of T-p53C-Y220C with SN006/7 SAR Hits

For all identified SN006/7 SAR DSF hits, a covalent modification of the protein was detected by intact protein MS. After 24 h of incubation, all DSF hits multiply arylated T-p53C-Y220C at a protein-to-compound ratio of 1:100, as shown in Figure 3.63.

The deconvoluted MS spectra of compounds SN006/7-2 (Figure 3.63 A) and SN006/7-4 (Figure 3.63 C) showed peaks corresponding to the triple arylated protein. The highest peaks were observed for the protein species labeled with two molecules of SN006/7-2 or SN006/7-4. Additionally, in the deconvoluted MS spectrum of T-p53C-Y220C incubated with SN006/7-2, there was a prominent signal for the protein species with one molecule of SN006/7-2 attached via S_NAr . The presence of a large signal for the single arylated protein may explain the slightly lower stabilization of T-p53C-Y220C in the DSF experiment by compound SN006/7-2 ($\Delta T_m = 1.38\text{ }^\circ\text{C}$, 1 mM, 24 h) compared to the bromine analog SN006/7-4 ($\Delta T_m = 2.00\text{ }^\circ\text{C}$, 1 mM, 24 h). Moreover, the 3-bromopyrazine derivative SN006/7-4 ($t_{1/2}$ GSH = 3.1 h) also had a shorter $t_{1/2}$ in GSH assay than the 3-chloropyrazine derivative SN006/7-2 ($t_{1/2}$ GSH = 6.5 h).

In the deconvoluted MS spectra of SN006/7-3 (Figure 3.63 B) and SN006/7-5 (Figure 3.63 D), triple arylated protein species were recognizable as minimum modification. The protein species with one or two attached molecules were not discernible in the spectra of the compounds with the nitrile group in the para position to the halogen. Hence, fragments SN006/7-3 and SN006/7-5 caused more modifications of T-p53C-Y220C than fragments SN006/7-2 and SN006/7-4. This is consistent with the expected higher reactivity induced by the higher stabilization of the substituted anion by π -accepting EWGs in the para position than in the ortho position [276]. Moreover, the spectrum of T-p53C-Y220C incubated with SN006/7-3 (Figure 3.63 B) showed peaks where SN006/7-3 was attached to T-p53C-Y220C three, four, or five times via S_NAr . Additionally, signals were visible corresponding to T-p53C-Y220C modified with SN006/7-3 via both S_NAr and Pinner-type addition to the nitrile. In contrast, the deconvoluted MS spectrum of the bromine analog SN006/7-5 (Figure 3.63 D) only displayed peaks corresponding to the three and four times arylated T-p53C-Y220C. No reversible covalent Pinner-type addition to the nitrile was detected. The reduced stabilization of T-p53C-Y220C by fragment SN006/7-5 compared to the

chlorine analog SN006/7-3 may be attributed to the differences in the deconvoluted MS spectra of T-p53C-Y220C with the compounds.

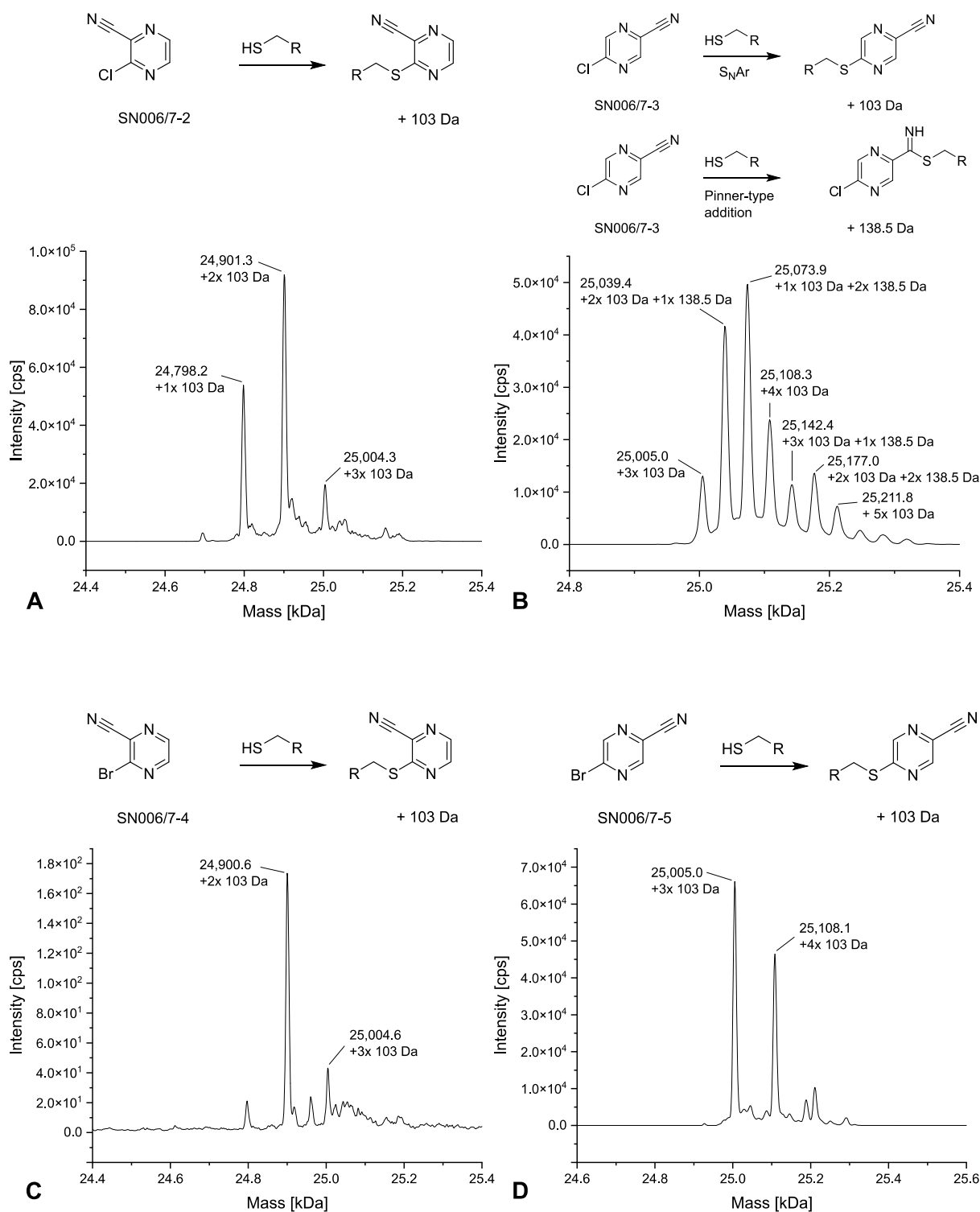


Figure 3.63: Deconvoluted MS spectra of T-p53C-Y220C with the identified SN006/7 SAR hits: SN006/7-2 (**A**), SN006/7-3 (**B**), SN006/7-4 (**C**), and SN006/7-5 (**D**) at a protein-to-compound ratio of 1:100 after 24 h of incubation at 20 °C (theoretical mass of unmodified T-p53C-Y220C: 24,696.00 Da).

3.5.2.3 DSF Studies of the Y220C Stabilizing Hit SN006/7-3

The Y220C stabilizing hit SN006/7-3 was further studied using DSF. The results of the concentration-dependent and time-dependent DSF measurements with T-p53C-Y220C are depicted in Figure 3.64, Table 3.32, Figure 3.65, and Table 3.33. The concentration-dependent DSF measurement with compound SN006/7-3 after 30 min of incubation (Figure 3.64 A) showed that the highest stabilization of T-p53C-Y220C was achieved with the highest tested compound concentration (1 mM, $\Delta T_m = 2.65\text{ }^{\circ}\text{C}$). In contrast to the original hits SN006 and SN007, a high compound concentration did not lead to a reduced stabilization of the protein. However, as the concentration of SN006/7-3 decreased, so did the ΔT_m values. After 30 min of incubation, up to a lowest effective compound concentration of 125 μM (protein-to-compound ratio 1:15.63), a slight stabilization was detectable ($\Delta T_m = 0.70\text{ }^{\circ}\text{C}$). For the initially identified highly reactive fragments SN006 and SN007, no evaluable melting curves of the T-p53C-Y220C protein could be measured after 24 h incubation time at a compound concentration of 1 mM. In contrast, the SAR compound SN006/7-3 showed a characteristic concentration-dependent stabilization of T-p53C-Y220C (Figure 3.64 B). Compared to the DSF measurement after 30 min, the overall ΔT_m values increased with prolonged incubation time. After 24 h of incubation, the highest stabilization of T-p53C-Y220C by up to $3.55\text{ }^{\circ}\text{C}$ was achieved at 1 mM SN006/7-3. Up to a lowest effective fragment concentration of 31.25 μM , which is equivalent to a protein-to-compound ratio of 1:3.91, there was a considerable increase in the melting temperature of T-p53C-Y220C, with a ΔT_m of $0.98\text{ }^{\circ}\text{C}$.

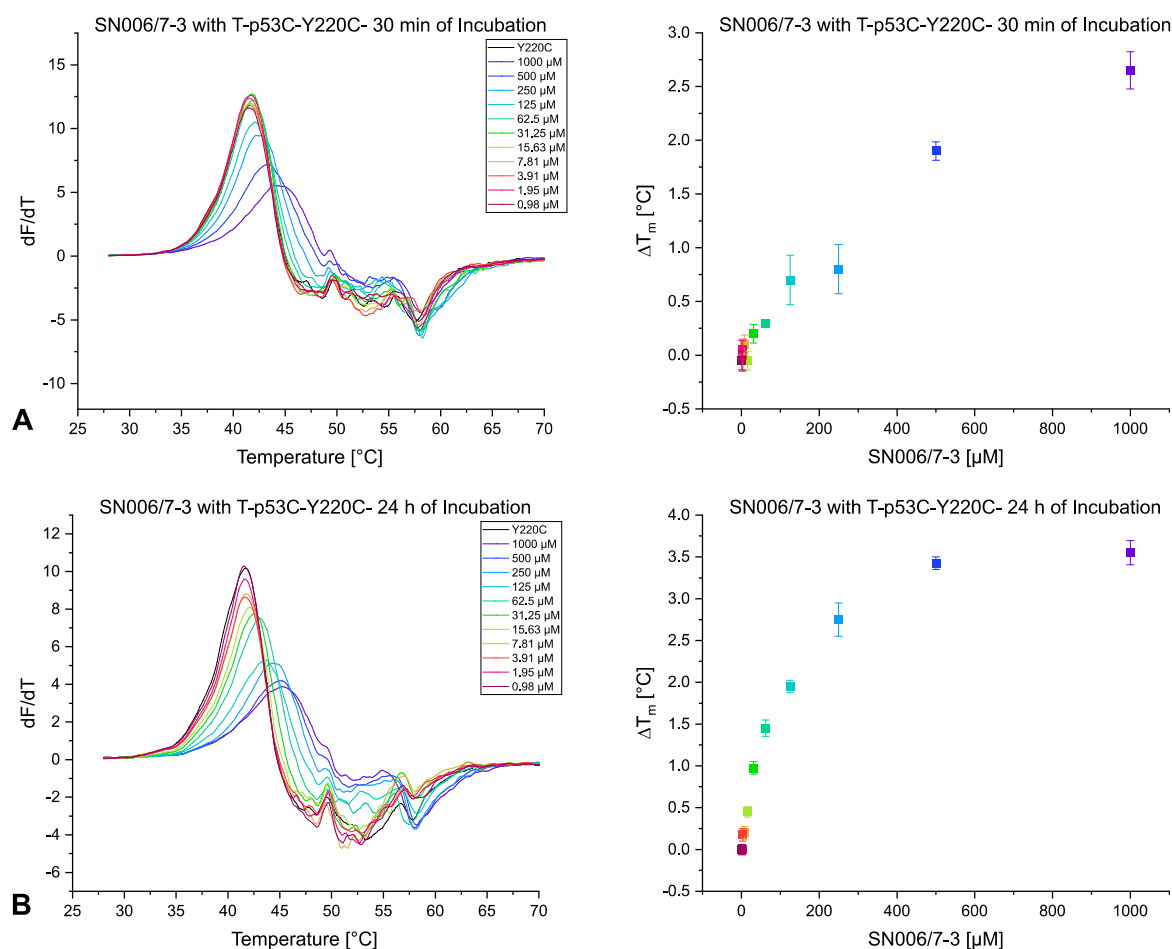


Figure 3.64: Concentration-dependent DSF measurements of T-p53C-Y220C with SN006/7-3. The first derivatives of the melting curves and resulting ΔT_m values of T-p53C-Y220C (8 μM protein) with various concentrations of compound SN006/7-3 after 30 min (A) and 24 h (B) of incubation at 20 °C are shown.

Table 3.32: $\Delta T_m \pm SD$ of T-p53C-Y220C (8 μM) incubated with different concentrations of compound SN006/7-3 for 30 min and 24 h at 20 °C. Measurements were performed at least in triplicate.

SN006/7-3 [μM]	T-p53C-Y220C 30 min $\Delta T_m \pm SD$ [°C]	T-p53C-Y220C 24 h $\Delta T_m \pm SD$ [°C]
1000	2.65 ± 0.17	3.55 ± 0.14
500	1.90 ± 0.09	3.43 ± 0.08
250	0.80 ± 0.23	2.75 ± 0.20
125	0.70 ± 0.23	1.95 ± 0.08
62.5	0.30 ± 0	1.45 ± 0.10
31.25	0.20 ± 0.09	0.98 ± 0.08
15.63	-0.05 ± 0.09	0.45 ± 0.06
7.81	0.10 ± 0.09	0.20 ± 0.08
3.91	0 ± 0.15	0.18 ± 0.08
1.95	0.05 ± 0.09	0 ± 0.06
0.98	-0.05 ± 0.09	0 ± 0.06

The detailed time-dependent DSF measurements were performed with 1 mM SN006/7-3, representing the concentration leading to the highest stabilization of T-p53C-Y220C. Figure 3.65 and Table 3.33 show that a substantial increase in the melting temperature of T-p53C-Y220C was achieved after only 10 min of incubation ($\Delta T_m = 2.30$ °C). This is consistent with the short $t_{1/2}$ of 0.23 h for fragment SN006/7-3 in the GSH assay. After about 2 h of incubation at 20 °C, the increase in the ΔT_m values plateaued, and the temperature-sensitive cancer mutant T-p53C-Y220C was maximally stabilized.

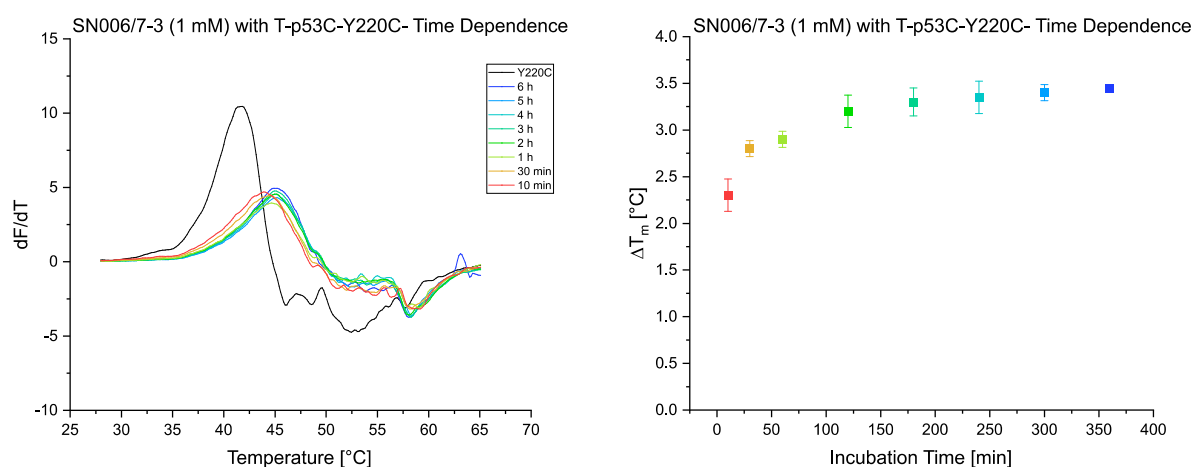


Figure 3.65: Time-dependent DSF measurements of T-p53C-Y220C with SN006/7-3. The first derivatives of the melting curves and resulting ΔT_m values of T-p53C-Y220C (8 μ M protein) with 1 mM SN006/7-3 after various incubation periods at 20 °C are presented.

Table 3.33: $\Delta T_m \pm$ SD of T-p53C-Y220C (8 μ M) incubated with SN006/7-3 (1 mM) after different periods of time at 20 °C. Measurements were performed in triplicate.

Incubation Time	SN006/7-3 (1 mM) with T-p53C-Y220C $\Delta T_m \pm$ SD [°C]
6 h	3.45 ± 0
5 h	3.40 ± 0.09
4 h	3.35 ± 0.17
3 h	3.30 ± 0.15
2 h	3.20 ± 0.17
1 h	2.90 ± 0.09
30 min	2.80 ± 0.09
10 min	2.30 ± 0.17

3.5.2.4 Evaluation of the Hit SN006/7-3 with T-p53C-Y220C-CL

The identified hit SN006/7-3 was investigated with T-p53C-Y220C-CL using DSF and UHPLC-ESI-MS. The DSF results are depicted in Figure 3.66 and Table 3.34, and the deconvoluted MS spectrum is shown in Figure 3.67. The 5-chloropyrazine-2-carbonitrile SN006/7-3 stabilized T-p53C-Y220C-CL by up to 2.40 °C (1 mM, 24 h). At a compound concentration of 250 μ M, the stabilization decreased, consistent with previous findings for T-p53C-Y220C. However, the stabilization of T-p53C-Y220C-CL was lower compared to T-p53C-Y220C (ΔT_m = 3.62 °C, 1 mM, 24 h), indicating that the labeling of cysteines other than Cys220 also has a stabilizing effect. Additionally, compared to the identified Cys220 arylating pyridine derivatives SN001 (ΔT_m = 3.08 °C, 1 mM, 24 h) and SN001-17 (ΔT_m = 4.95 °C, 1 mM, 24 h), the pyrazine derivative SN006/7-3 caused a smaller increase in the melting temperature of the Y220C cysteine light mutant. Furthermore, the original compound SN006 also induced a higher increase in the T_m of T-p53C-Y220C-CL (ΔT_m = 3.05 °C, 1 mM, 24 h). However, the deconvoluted MS spectrum of T-p53C-Y220-CL with SN006 showed peaks with more than one fragment attached to the protein (Figure 3.47 A). As depicted in Figure 3.67, the deconvoluted MS spectrum of the Y220C cysteine light mutant incubated with the SAR compound SN006/7-3 also displayed two peaks. One signal corresponded to the protein species with one molecule attached via S_NAr , while the higher signal corresponded to protein with one molecule of SN006/7-3 attached via reversible covalent Pinner-type addition to the nitrile. Hence, it is unclear from the MS data whether SN006/7-3 exclusively labels the remaining cysteine 220 in the pocket of the Y220C cysteine light mutant and which reaction mechanism is responsible for stabilizing T-p53C-Y220C-CL.

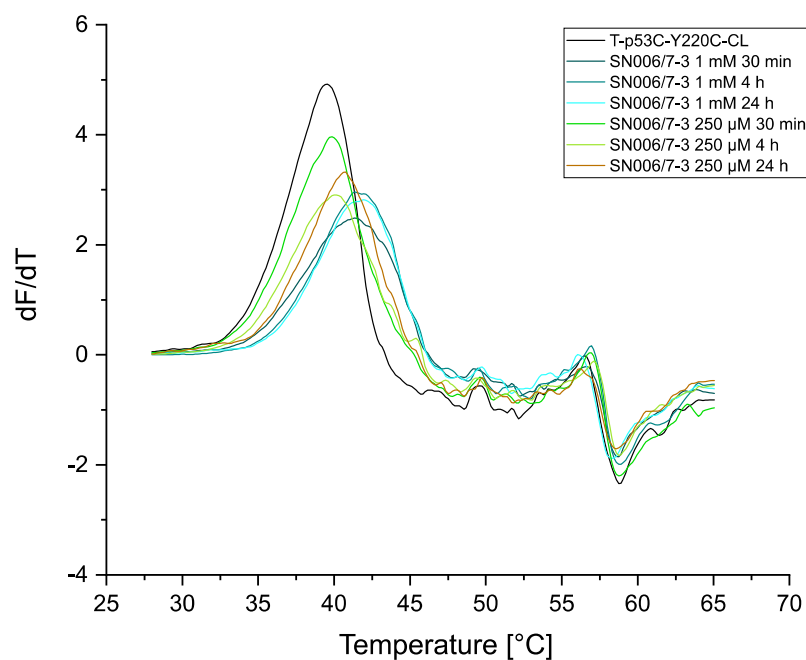


Figure 3.66: DSF measurements of T-p53C-Y220C-CL with SN006/7-3. The first derivatives of the melting curves of T-p53C-Y220C-CL (8 μ M) with SN006/7-3 (1 mM, 250 μ M) after varying incubation periods at 20 °C are shown.

Table 3.34: $\Delta T_m \pm$ SD of T-p53C-Y220C-CL (8 μ M) incubated with SN006/7-3 (1 mM and 250 μ M) after different periods of time at 20 °C. Measurements were performed at least in triplicate.

T-p53C-Y220C-CL						
$\Delta T_m \pm$ SD [°C]						
	1 mM 30 min	1 mM 4 h	1 mM 24 h	250 μ M 30 min	250 μ M 4 h	250 μ M 24 h
SN006/7-3	1.50 ± 0.10	1.80 ± 0	2.40 ± 0.12	0.20 ± 0.09	0.55 ± 0.17	1.25 ± 0

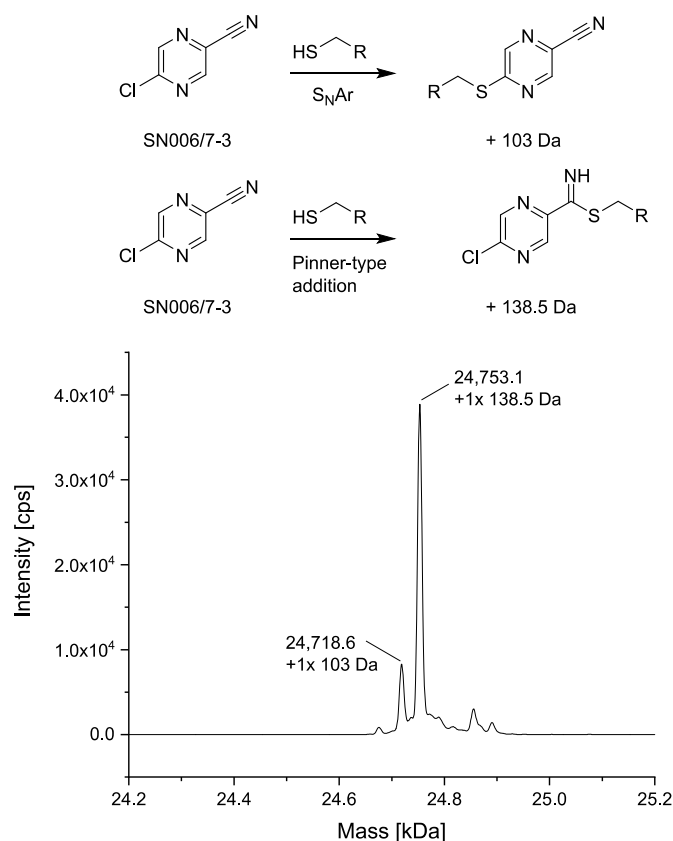


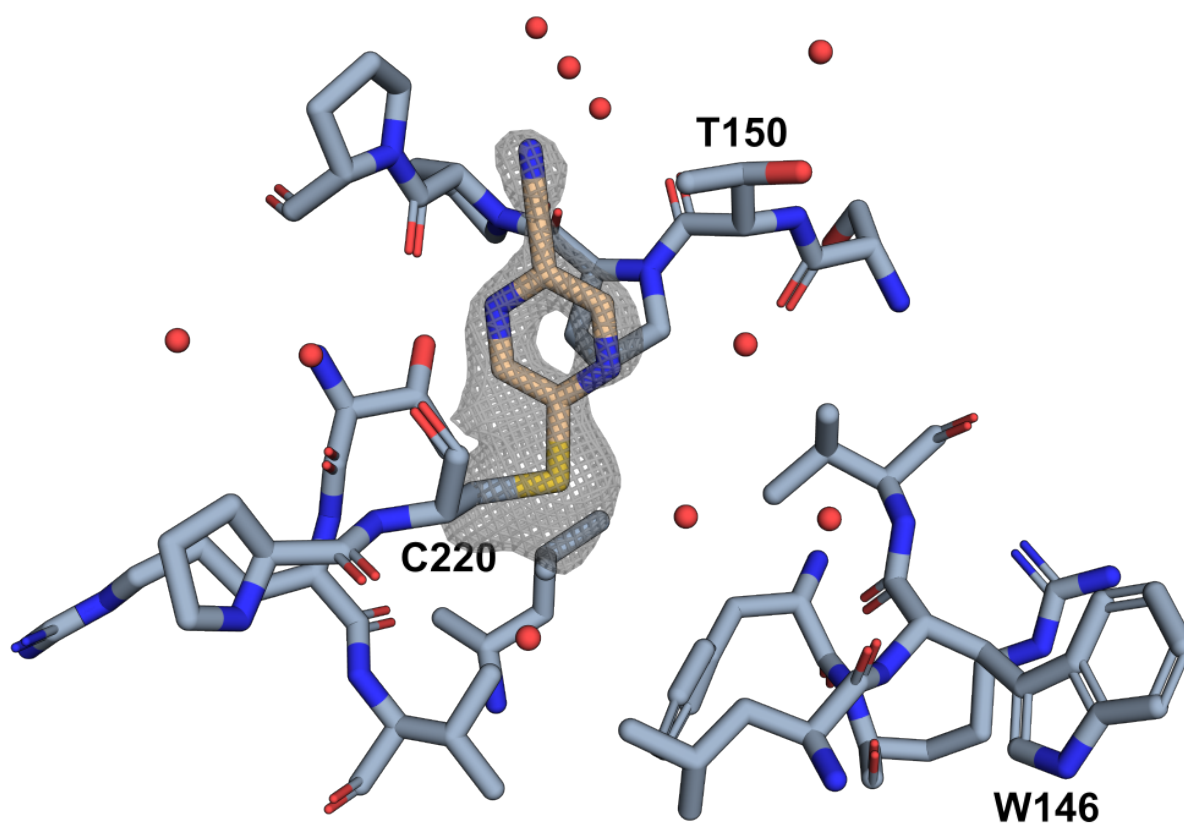
Figure 3.67: Deconvoluted MS spectrum of T-p53C-Y220C-CL with SN006/7-3 at a molar ratio of 1:100 after 24 h of incubation at 20 °C (theoretical mass of unmodified T-p53C-Y220C-CL: 24,615.68 Da).

3.5.2.5 Determination of the Binding Mode of SN006/7-3 Using X-Ray Crystallography

The X-ray structure of xT-p53C-Y220C soaked with 5 mM SN006/7-3 confirmed that this pyrazine derivative is another Cys220 arylating compound, displaying well-defined density in the binding pocket of the Y220C mutant, as shown in Figure 3.68. Additional modifications were found at Cys182 and Cys277 after soaking for one day with 5 mM SN006/7-3. In the X-ray structure, only the modification of cysteine residues by SN006/7-3 via an S_NAr reaction was detected. The reversible covalent Pinner-type addition of proteinogenic cysteines to the nitrile group of the compound observed in the MS experiment was not visible, possibly due to the reversibility of the reaction. The 5 mM-soaked structure indicated that pyrazine derivative SN006/7-3 reacted with Cys220 via an S_NAr reaction, with the chlorine atom acting as the LG.

Interestingly, the crystal structure revealed an alternative binding pose of SN006/7-3 in the Y220C cleft compared to the further identified Cys220 arylating compounds SN001, SN006, and SN001-17. The pyridine derivatives SN001 and SN001-17 adopted a Tyr-like conformation, occupying the central cavity and mimicking the wt

Figure 3.68: Overview of the binding mode of SN006/7-3 (soaked at 5 mM) into the Y220C induced crevice. The pyrazine SN006/7-3 reacted with Cys220 via an S_NAr reaction. The compound adopts an alternative conformation, occupying subsite 2 of the binding pocket. The unbiased omit map is shown at a contour level of 3σ .



3.5.3 VS004 SAR Compound Screen

A generic stabilization of p53 was achieved with the CovLib compound VS004, a vinyl sulfone with β -LG. Nevertheless, this compound is reactive and modifies several cysteines in p53. Therefore, we wanted to modulate the reactivity through different substituents at the α -position and β -position of the vinyl sulfone structure [144]. However, only a limited number of SAR compounds were commercially available. In total, two SAR compounds, VS004-1 and VS004-2, with different β -LGs were tested. The structures of the tested VS004 SAR compounds are shown in Figure 3.69. Moreover, the CovLib fragment VS003, which contains an electron-donating methyl group at the β -position, already represents an SAR compound of VS004.

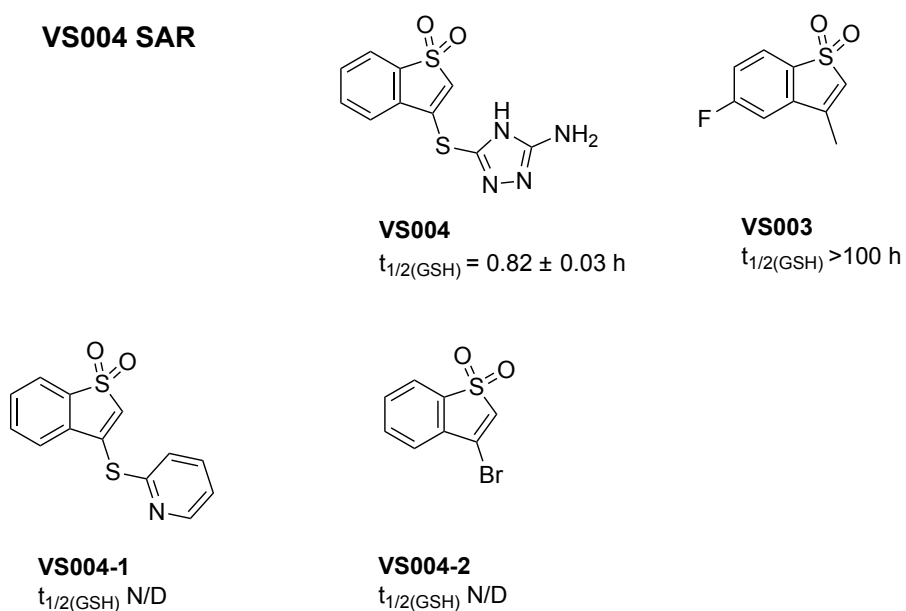


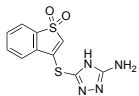
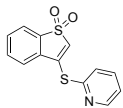
Figure 3.69: Overview of the tested VS004 SAR compounds.

3.5.3.1 DSF Screen of VS004 SAR Compounds with T-p53C and Its Mutants

Table 3.35 displays the results of the DSF screen of the VS004 SAR compounds with T-p53C and different mutants after 30 min of incubation at 20 °C. The DSF measurements showed that the SAR compounds VS004-1 and VS004-2 strongly stabilized p53 and its mutants after only 30 min of incubation. The ΔT_m values were comparable to those of the original hit VS004. Moreover, like VS004, the SAR compounds effectively stabilized the structural mutant R282W in the C-terminal helix, resulting in an increase in the T_m by up to 6.55 °C with VS004-1 and 5.75 °C with VS004-2. Similar to VS004, the first derivatives of the melting curves of T-p53C, T-p53C-R273H, and T-p53C-C124/182/277S showed an additional first shoulder in front of the main peak (Figure 8.40). The first derivatives of the melting curves of T-p53C-R273H incubated with VS004-2 even displayed two distinct peaks. In addition, the melting curves of T-p53C-R282W incubated with VS004 SAR compounds showed fluorescence signals with low intensities, and the first derivatives displayed broad, small curves. The melting temperature of the triple cysteine mutant T-p53C-C124/182/277S was substantially increased by VS004-1 ($\Delta T_m = 4.80$ °C) and VS004-2 ($\Delta T_m = 3.90$ °C). This suggests that the vinyl sulfones also stabilized the protein by modifying cysteines other than Cys124, Cys182, and Cys277. In addition, since the stabilization of the Y220C mutant was least pronounced, no binding of the vinyl sulfones VS004-1 and VS004-2 into the hydrophobic pocket could be assumed. In conclusion, fragments VS004-1 and VS004-2 with different LGs at the β -position achieved similar stabilization of p53 and its mutants as VS004. These SAR fragments are not more selective or less reactive than VS004.

Furthermore, the introduction of an electron-donating methyl group (VS003) resulted in a stable fragment with a $t_{1/2} > 100$ h [233], as already observed in the CovLib screen. The CovLib compound VS003 did not react with cysteines in p53 and did not affect the melting temperature of T-p53C even after 24 h of incubation (Table 3.6).

Table 3.35: $\Delta T_m \pm SD$ of p53 variants (8 μ M) incubated with 1 mM VS004 SAR compounds for **30 min** at 20 °C. Measurements were performed at least in triplicate. Fragments with a ΔT_m of at least 0.50 °C are highlighted in bold [99, 130].

Compound	T-p53C	T-p53C-Y220C	T-p53C-R273H	T-p53C-R282W	T-p53C-C124/182/277S
	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]
VS004 	5.06 ± 0.09^a	3.00 ± 0.16	5.37 ± 0.12^a	5.96 ± 0.10^a	0.83 ± 0.10 and 4.30 ± 0.14
VS003 	-0.05 ± 0.09	-0.10 ± 0.21	-0.05 ± 0.17	-0.10 ± 0.12	N/D ^c
VS004-1 	5.40 ± 0.26^a	3.33 ± 0.11	5.80 ± 0.09^a	6.55 ± 0.09^b	4.80 ± 0^a
VS004-2 	4.55 ± 0.38^a	3.33 ± 0.15	1.10 ± 0.09 and 4.85 ± 0.35	5.75 ± 0.17^b	3.90 ± 0.26^a

^aMelting curves with two transitions. First derivatives of the melting curves showed a first small shoulder.

^bLow fluorescence intensity of the melting curves. First derivatives of the melting curves displayed a broad curve. ^cOnly the identified fragment hits were investigated with T-p53C-C124/182/277S.

3.5.3.2 Intact Protein Mass Spectrometry of T-p53C with VS004 SAR Compounds

The covalent modification of T-p53C by compounds VS004-1 and VS004-2 was confirmed by intact protein mass spectrometry (Figure 3.70).

In the MS spectrum of T-p53C incubated with VS004-1 for 24 h (molar ratio 1:100) (Figure 3.70 A), peaks for the native protein and for several protein-compound adducts were detected. The most intense signal corresponded to the protein species with two VS004-1 molecules attached and loss of the heteroaryl thiolate LG (i.e., thiol exchange), as previously observed for compound VS004. Additionally, a signal for T-p53C labeled with up to four VS004-1 molecules with the elimination of the heteroaryl thiolate LG was visible. Furthermore, a small peak was observed, where the protein was modified by both the addition to the α,β -unsaturated vinyl sulfone (275 Da) and the product of subsequent elimination of the heteroaryl thiolate leaving group (164 Da). In addition, two peaks with mass differences of 410 Da and 576 Da were detected. These mass increases cannot be attributed to a specific reaction of the compound with the protein. However, there is an approximate difference of 164 Da between the peaks,

which corresponds to a change in mass due to the addition of a cysteine with subsequent elimination of the LG. Overall, these peaks highlighted that VS004-1 is a highly reactive compound that generated multiple adducts with proteinogenic nucleophiles in the MS experiment.

In the MS spectrum of VS004-2 (Figure 3.70 B), the highest signal also corresponded to the dual modified T-p53C, resulting from addition and loss of the LG. Additionally, a signal was detected for the protein labeled by triple addition and subsequent elimination of the LG. In contrast to VS004, only protein species with the attachment of VS004-2 and the subsequent elimination of the leaving group were detected in the deconvoluted spectrum. The addition to the α,β -unsaturated vinyl sulfone was not discernable. The elimination of the leaving group is probably a (slower) follow-up reaction after the addition. Therefore, the extended incubation time of 24 h before MS analysis of VS004-2, compared to 4 h in the experiment with VS004, could result in an increase in the amount of the “thiol exchange product.”

In summary, the MS data emphasize that the SAR compounds VS004-1 and VS004-2 are reactive fragments that non-selectively covalently modify T-p53C, exhibiting comparable behavior to the original CovLib compound VS004.

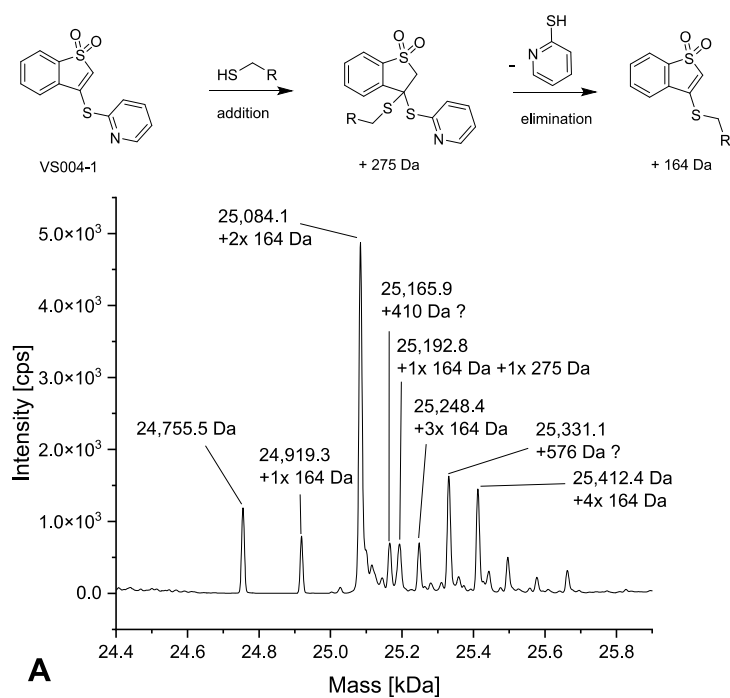
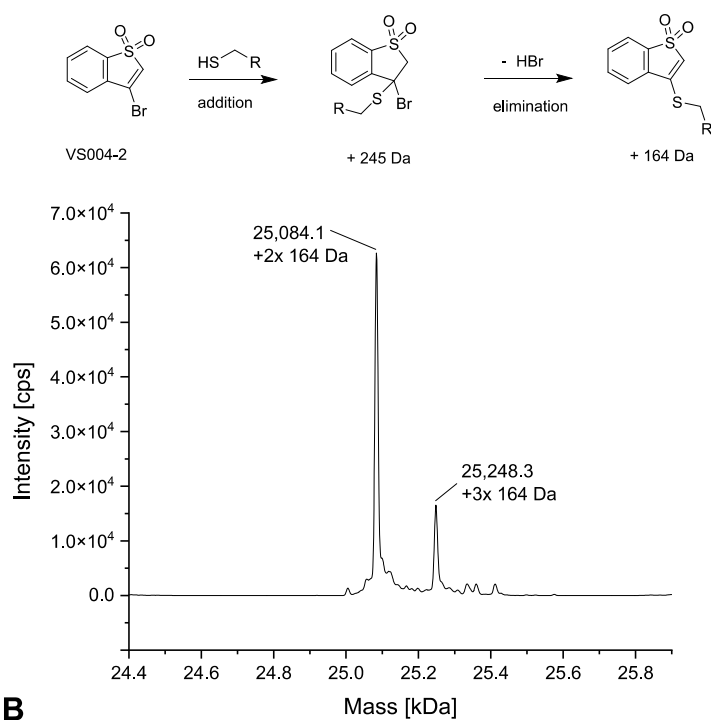
**A****B**

Figure 3.70: Deconvoluted MS spectra of VS004-1 (**A**) and VS004-2 (**B**) with T-p53C (measured without DTT) at a molar ratio of 1:100 after 24 h of incubation at 20 °C (theoretical mass of unmodified T-p53C: 24,756.03 Da).

3.5.4 Effects of the SAR Compounds on the DNA-Binding Capacity of FL-T-p53

The effect of the identified SAR hits on the DNA-binding affinity of FL-T-p53 to GADD45 α was investigated using FPA. To account for the expected reduced reactivity of certain SAR compounds, the incubation time before the FP measurements was extended to 24 h. The results of the identified SN001 SAR hit compounds are depicted in Figure 3.71 and Table 3.36. It was observed that all SN001 SAR hits had only a minor effect on the DNA-binding of FL-T-p53 to GADD45 α . According to the CovLib hit SN001, the DNA-binding of FL-T-p53 to GADD45 α was conserved after incubation with its SAR compounds.

The two CovLib hits SN006 and SN007 negatively affected DNA-binding, whereas all identified SAR hits did not substantially affect the binding of FL-T-p53 to GADD45 α even after 24 h of incubation, as shown in Figure 3.72 and Table 3.37.

The vinyl sulfones VS004-1 and VS004-2 led to multiple modifications of T-p53C in the MS experiment. However, the DNA-binding ability was unaffected despite the labeling of cysteines in p53 (Figure 3.73, Table 3.38).

The SAR compounds may have modified Cys277 of p53, which is located in the DNA-binding region of p53 and exposed on the surface. However, as previously demonstrated for compounds PK11000 [131] and MQ [118], the DNA-binding ability remained unaffected despite the potential covalent modification of Cys277. The FP assay results indicated that the normal DNA-binding activity of FL-T-p53 for the GADD45 α response element was maintained after incubation with all SAR compounds tested. Furthermore, normal transcriptional activity of p53 could be restored by the thermal stabilization in cellular systems.

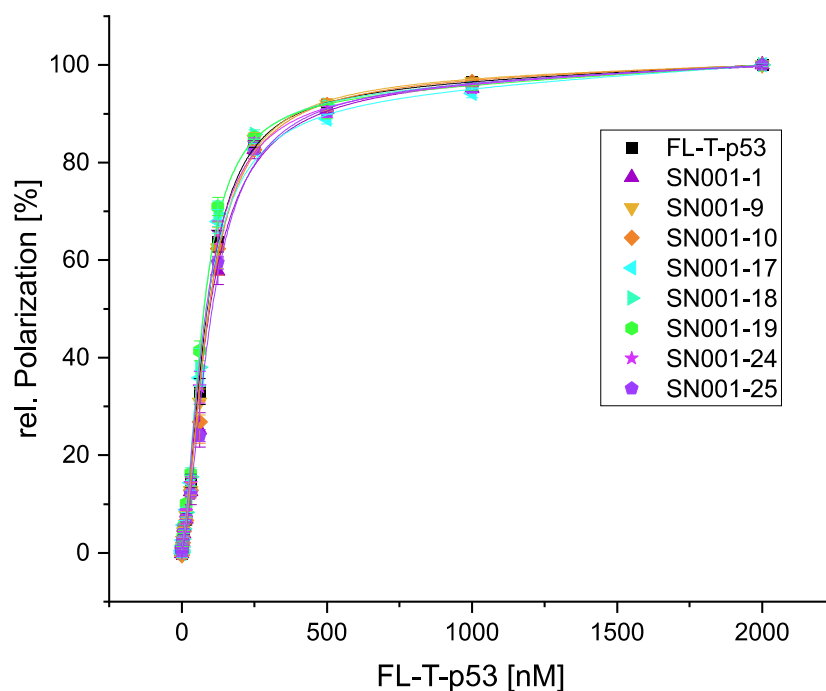


Figure 3.71: FP assay of FL-T-p53 incubated with SN001 SAR hits with GADD45 α . The relative fluorescence polarization data [%] of FL-T-p53 with 20 nM 5'-carboxyfluorescein-labeled GADD45 α DNA response element and 5% DMSO are shown. FL-T-p53 was incubated with SN001 SAR hit compounds at a protein-to-compound ratio of 1:200 for 24 h at 20 °C [131]. FP data were recorded every 10 min for 1 h, and data were averaged. Measurements were performed at least one time in quadruplicate.

Table 3.36: Resulting K_d values $\pm$ SD of the direct titrations of FL-T-p53 with or without the addition of SN001 SAR compounds (molar ratio 1:200) with GADD45 α DNA. Measurements were performed at least one time in quadruplicate.

FL-T-p53 Incubated with	$K_d \pm SD$ [nM]
DMSO	83 ± 4
SN001-1	80 ± 5
SN001-9	92 ± 9
SN001-10	91 ± 7
SN001-17	79 ± 8
SN001-18	71 ± 5
SN001-19	73 ± 5
SN001-24	80 ± 8
SN001-25	101 ± 10

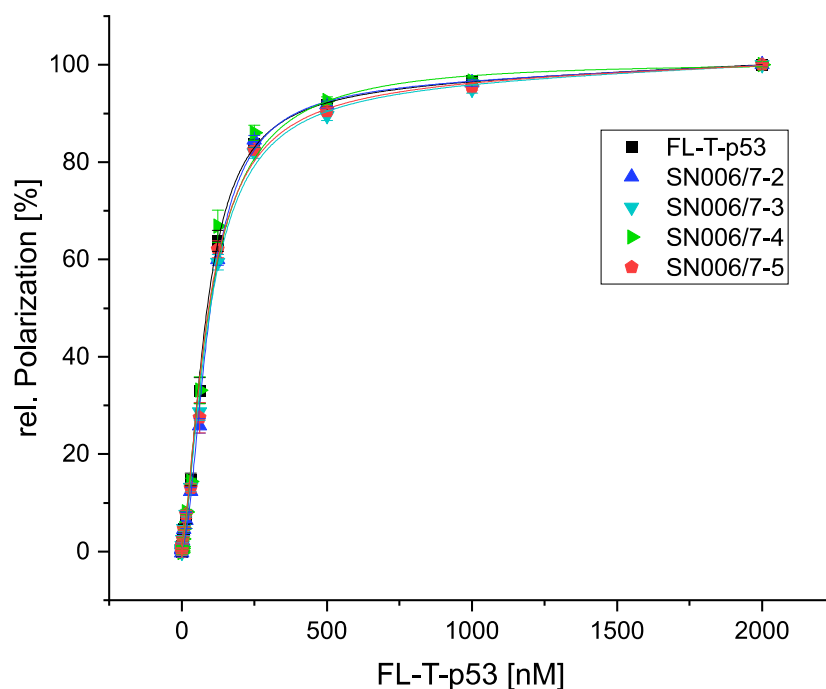


Figure 3.72: FP assay of FL-T-p53 incubated with SN006/7 SAR hits with GADD45 α . The relative fluorescence polarization data [%] of FL-T-p53 with 20 nM 5'-carboxyfluorescein-labeled GADD45 α DNA response element and 5% DMSO are shown. FL-T-p53 was incubated with SN006/7 SAR hit compounds at a protein-to-compound ratio of 1:200 for 24 h at 20 °C [131]. FP data were recorded every 10 min for 1 h, and data were averaged. Measurements were performed at least one time in quadruplicate.

Table 3.37: Resulting K_d values $\pm$ SD of the direct titrations of FL-T-p53 with or without the addition of SN006/7 SAR compounds (molar ratio 1:200) with GADD45 α DNA. Measurements were performed at least one time in quadruplicate.

	FL-T-p53	SN006/7-2	SN006/7-3	SN006/7-4	SN006/7-5
$K_d \pm SD$ [nM]	83 ± 4	96 ± 5	95 ± 9	89 ± 7	93 ± 6

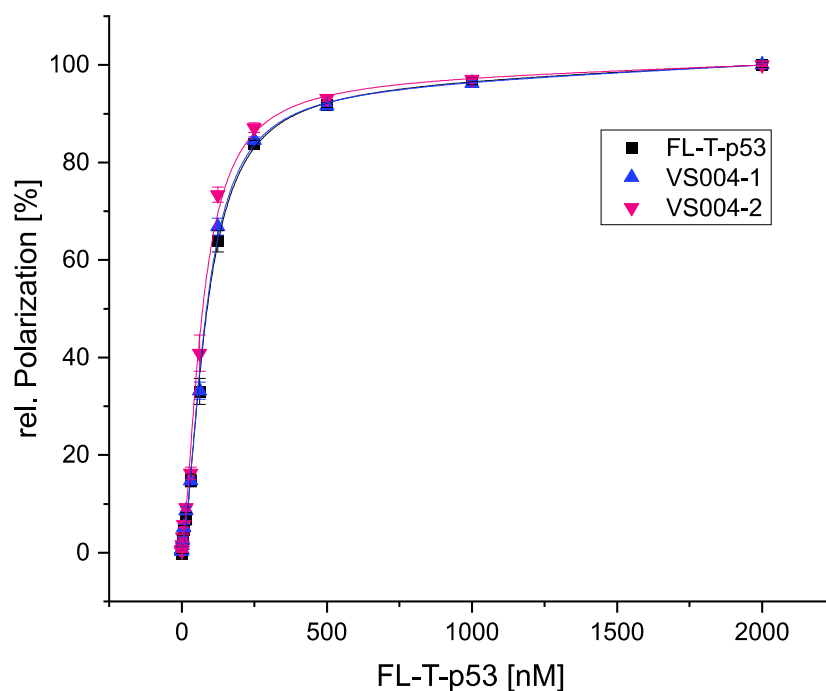


Figure 3.73: FP assay of FL-T-p53 incubated with VS004 SAR compounds with GADD45 α . The relative fluorescence polarization data [%] of FL-T-p53 with 20 nM 5'-carboxyfluorescein-labeled GADD45 α DNA response element and 5% DMSO are shown. FL-T-p53 was incubated with VS004 SAR compounds at a protein-to-compound ratio of 1:200 for 24 h at 20 °C [131]. FP data were recorded every 10 min for 1 h, and data were averaged. Measurements were performed at least one time in quadruplicate.

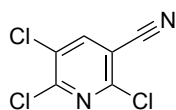
Table 3.38: Resulting K_d values $\pm$ SD of the direct titrations of FL-T-p53 with or without the addition of VS004 SAR compounds (molar ratio 1:200) with GADD45 α DNA. Measurements were performed at least one time in quadruplicate.

	FL-T-p53	VS004-1	VS004-2
$K_d \pm SD$ [nM]	83 ± 4	82 ± 5	71 ± 4

3.6 Structure-Activity Relationship Screen of the Identified Hits SN001-17 and SN006/7-3

The SAR screen revealed two additional S_NAr reactive compounds, SN001-17 and SN006/7-3, arylating Cys220 in the hydrophobic pocket and stabilizing T-p53C-Y220C. SN001-17, a 2,5,6-trichloro-3-pyridinecarbonitrile, substantially increased the T_m of T-p53-Y220C by up to 5.19 °C. In addition, the X-ray structure of xT-p53C-Y220C soaked with the pyrazine derivative SN006/7-3 showed an alternative binding pose, with SN006/7-3 occupying subsite 2 of the Y220C pocket. However, both fragments are still reactive, modifying several cysteines in p53. Therefore, additional SAR fragments were ordered for the recently discovered hits, which contain electron-donating groups at the heteroarenes (Figure 3.74). Since the previously identified cleft binders SN001, SN001-17, SN006/7, and SN006/7-3 are pyridine or pyrazine derivatives, additional six-membered heterocycles such as pyrimidines and pyridazines were tested.

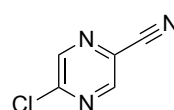
SN001-17 SAR



SN001-17

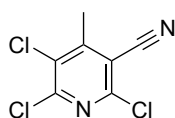
$t_{1/2}(\text{GSH}) = 0.54 \pm 0.00 \text{ h}$

SN006/7-3 SAR



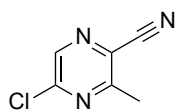
SN006/7-3

$t_{1/2}(\text{GSH}) = 0.23 \pm 0.00 \text{ h}$



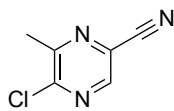
SN001-34

$t_{1/2}(\text{GSH}) = 1.8 \pm 0.1 \text{ h}$



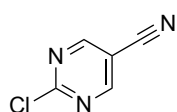
SN006/7-7

$t_{1/2}(\text{GSH}) = 0.45 \pm 0.00 \text{ h}$



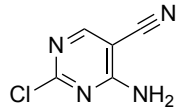
SN006/7-8

$t_{1/2}(\text{GSH}) = 0.28 \pm 0.00 \text{ h}$



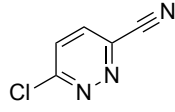
SN009

$t_{1/2}(\text{GSH}) \text{ N/A}^a$



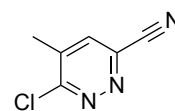
SN010

$t_{1/2}(\text{GSH}) \text{ N/A}^a$



SN011

$t_{1/2}(\text{GSH}) = 3.0 \pm 0.1 \text{ h}$



SN012

$t_{1/2}(\text{GSH}) = 7.7 \pm 0.4 \text{ h}$

Figure 3.74: Overview of the tested SN001-17 and SN006/7-3 SAR compounds and their $t_{1/2} \pm \text{SD}$ determined via GSH assay. The GSH assay was performed by M. Schwer. ^aThe purity of the compounds could not be confirmed by HPLC-UV.

3.6.1 DSF Screen of SN001-17 and SN006/7-3 SAR Compounds with T-p53C and Its Mutants

The ΔT_m values of T-p53C and its mutants after 30 min, 4 h, and 24 h of incubation with SN001-17 and SN006/7-3 SAR compounds (1 mM) are depicted in Table 3.39, Table 3.40, and Table 3.41.

Compared to fragment SN001-17 ($t_{1/2}$ GSH= 0.54 h), compound SN001-34 contains an additional electron-donating methyl group at position 4 of the pyridine ring and had a longer $t_{1/2}$ of 1.8 h in the GSH assay. As shown in Table 3.42, incubation with fragment SN001-34 did not substantially affect the T_m of T-p53C. Similar to the original compound SN001-17, SN001-34 only increased the melting temperature of the Y220C mutant, suggesting a covalent modification of Cys220 in the binding pocket. However, due to the additional electron-donating methyl group, the reactivity decreased, resulting in a pronounced time-dependent increase in the T_m values of T-p53C-Y220C. After 30 min of incubation, there was no considerable shift in the melting temperature of the Y220C mutant even at 1 mM SN001-34 (ΔT_m = 0.30 °C). However, after 24 h incubation time, the melting temperature of T-p53C-Y220C increased by up to 2.45 °C (1 mM). As depicted in Table 3.43, the stabilization of T-p53C-Y220C was observed to be similar at both 1 mM and 250 μ M compound concentrations. In contrast to SN001-17, the melting temperature did not increase as the concentration of SN001-34 decreased. In addition, the R282W mutant was not destabilized to the same extent, and evaluable melting curves were still detectable after 24 h of incubation (ΔT_m = -1.45 °C). However, the maximum stabilization of T-p53C-Y220C with SN001-34 by up to 2.55 °C (250 μ M, 24 h) was substantially lower than the ΔT_m value of 5.19 °C achieved with SN001-17 (250 μ M, 24 h).

Compounds SN006/7-7 and SN006/7-8 have an additional electron-donating methyl group in the meta or ortho position to the LG compared to the pyrazine derivative SN006/7-3. In relation to SN006/7-3 ($t_{1/2}$ GSH= 0.23 h), the GSH reactivity of SN006/7-7 ($t_{1/2}$ GSH= 0.45 h) slightly diminished, whereas that of SN006/7-8 ($t_{1/2}$ GSH= 0.28 h) was only minimally affected. Both compounds particularly stabilized T-p53C-Y220C, indicating cleft binding. After 30 min of incubation, SN006/7-8 already stabilized all tested mutants, while SN006/7-7 only slightly increased the T_m of T-p53C (Table 3.39). Additionally, the triple cysteine mutant was stabilized by SN006/7-8, indicating labeling of additional cysteines besides Cys124, Cys182, and Cys277.

However, SN006/7-8 had a smaller stabilizing effect on T-p53C-Y220C, with a ΔT_m of 1.71 °C after 30 min, compared to SN006/7-3, which lacks the methyl group, with a ΔT_m of 2.75 °C. Time-dependent effects were observed for both SN006/7-7 and SN006/7-8. After 4 h of incubation, T-p53C-Y220C was especially stabilized by SN006/7-7 (ΔT_m = 1.28 °C, 1 mM) and SN006/7-8 (ΔT_m = 4.43 °C, 1 mM). The first derivatives of the melting curves of T-p53C incubated with SN006/7-8 for 4 h or 24 h showed two distinct peaks similar to compound SN006/7-3, indicating different modified protein species with different melting points or non-covalently bound fragments with distinct melting points [264, 289, 297]. A reversible covalent Pinner-type addition of proteinogenic nucleophiles to the nitrile group of the compounds [144, 197] could also lead to differently modified protein species. Similar to the original compounds SN006, SN007, SN006/7-3, and SN006/7-5, the R282W mutant did not show any evaluable melting curves and first derivatives with clearly defined peaks after 24 h of incubation with compounds SN006/7-7 and SN006/7-8 (Figure 8.46). However, unlike the original compounds, melting curves of T-p53C-R282W could still be measured after 30 min and 4 h of incubation with SN006/7-7 and SN006/7-8 (1 mM). After 30 min of incubation with 1 mM SN006/7-8, even a stabilization of the R282W mutant was observed (ΔT_m = 1.15 °C). Interestingly, compound SN006/7-8 substantially increased the melting temperature of the Y220C mutant by up to 5.02 °C (1 mM) after 24 h of incubation (Table 3.42). The introduction of a methyl group in the ortho position to the LG increased the stabilizing effect of this scaffold compared to SN006/7-3 (ΔT_m = 3.62 °C, 1 mM, 24 h) without a methyl group and SN006/7-7 (ΔT_m = 2.08 °C, 1 mM, 24 h) with a methyl group in the meta position. Through the introduction of the methyl group, the electron deficiency of the heteroarene is reduced compared to fragment SN006/7-3. However, increased stabilization by fragment SN006/7-8 suggests that the methyl group at position 6 of the pyrazine ring induces favorable interactions in the Y220C pocket. Compound SN006/7-7, which contains the methyl group in the meta position relative to the LG, resulted in a substantially lower stabilization of T-p53C-Y220C compared to fragments SN006/7-8 and SN006/7-3. Additionally, the compounds were tested at a concentration of 250 μ M with T-p53C and T-p53C-Y220C (Table 3.42, Table 3.43). As the compound concentration decreased, the stabilization of T-p53C and T-p53C-Y220C also diminished. The highest increment in the melting temperatures was achieved with 1 mM SN006/7-7 and SN006/7-8 after a 24-hour incubation period. However, even at a compound

concentration of 250 μM , SN006/7-8 showed a higher stabilization of T-p53C-Y220C ($\Delta T_m = 4.45\text{ }^\circ\text{C}$, 24 h) compared to SN006/7-7 ($\Delta T_m = 1.10\text{ }^\circ\text{C}$, 24 h) and SN006/7-3 ($\Delta T_m = 2.87\text{ }^\circ\text{C}$, 24 h).

The two 2-chloropyrimidines SN009 and SN010 caused a thermal stabilization of p53 and its mutants in the DSF experiment. However, no evaluable melting curves were obtained for the R282W mutant with compound SN009 after 4 h and 24 h of incubation. Notably, SN010, which has an electron-donating NH_2 group in the ortho position to the CN moiety, stabilized p53 after 24 h of incubation in contrast to its pyrazine analog SN006/7-6 (Table 3.29). The DSF data showed that the stabilization of T-p53C by SN009 and its pyrazine analogs SN006/7-3 and SN006/7-8 was similar. However, SN006/7-8 ($\Delta T_m = 5.02\text{ }^\circ\text{C}$, 1 mM, 24 h) and SN006/7-3 ($\Delta T_m = 3.62\text{ }^\circ\text{C}$, 1 mM, 24 h) led to a substantially higher stabilization of the Y220C mutant compared to the pyrimidine derivative SN009 ($\Delta T_m = 2.05\text{ }^\circ\text{C}$, 1 mM, 24 h). A pronounced stabilization of T-p53C-Y220C and binding into the Y220C pocket cannot be deduced from the DSF data for the 2-chloropyrimidines SN009 and SN010. This discovery aligns with previous findings on pyrimidines substituted at position 2, such as the 2-sulfonylpyrimidine PK11000 [131] or the 2-chloropyrimidine 4482 [99]. These compounds achieved a general stabilization of p53, but they did not label Cys220 in the mutational cleft. Nevertheless, it is important to note that the purity of SN009 and SN010 was not confirmed by HPLC-UV. Consequently, the results should be interpreted with reserve.

After 24 h of incubation, the pyridazine derivatives SN011 and SN012 moderately stabilized all tested mutants within a similar range. Compared to the pyrazine analog SN006/7-3 ($\Delta T_m = 3.62\text{ }^\circ\text{C}$, 1 mM, 24 h) and the 2-chloropyrimidine SN009 ($\Delta T_m = 2.05\text{ }^\circ\text{C}$, 1 mM, 24 h), the stabilization of T-p53C-Y220C by the pyridazine derivative SN011 was less pronounced, with a maximum of $1.10\text{ }^\circ\text{C}$ (1 mM, 24 h).

Overall, the results showed that the introduction of methyl groups reduced the reactivities of the heteroarenes (SN001-34, SN006/7-7) and, in the case of SN006/7-8, increased the stabilization of the oncogenic mutant T-p53C-Y220C. Moreover, the DSF results indicated that altering the heteroarene did not result in high a stabilization of the cancer mutant Y220C and selective binding into the mutational cleft.

Table 3.39: $\Delta T_m \pm SD$ of T-p53C and its mutants (8 μM) incubated with 1 mM SN001-17 and SN006/7-3 SAR compounds for **30 min** at 20 °C. Measurements were performed at least in triplicate. Fragments with a ΔT_m of at least 0.50 °C are highlighted in bold [99, 130].

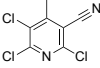
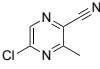
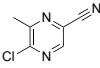
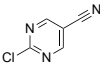
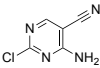
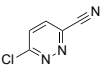
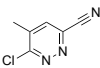
Compound		T-p53C	T-p53C- Y220C	T-p53C- R273H	T-p53C- R282W	T-p53C- C124/182/ 277S
		$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]
SN001-34		0 ± 0.15	0.30 ± 0.17	-0.35 ± 0.12	-0.65 ± 0.09	-0.15 ± 0
SN006/7-7		0.75 ± 0.15	0.47 ± 0.08	0.25 ± 0.12	0.20 ± 0.09	0 ± 0
SN006/7-8		0.90 ± 0	1.71 ± 0.10	0.80 ± 0.09	1.15 ± 0.09	0.60 ± 0
SN009		0.95 ± 0.09	1.60 ± 0.17	0.75 ± 0.12	0.20 ± 0.09	0.70 ± 0.09
SN010		0.30 ± 0.15	0.15 ± 0.19	0.20 ± 0.09	0.15 ± 0	0.15 ± 0
SN011		0.10 ± 0.09	0.10 ± 0.17	0.10 ± 0.12	0.15 ± 0	0.20 ± 0.09
SN012		0 ± 0	0.05 ± 0.17	0.05 ± 0.09	-0.10 ± 0.09	0 ± 0

Table 3.40: $\Delta T_m \pm SD$ of T-p53C and its mutants (8 μ M) incubated with 1 mM SN001-17 and SN006/7-3 SAR compounds for 4 h at 20 °C. Measurements were performed at least in triplicate. Fragments with a ΔT_m of at least 0.50 °C are highlighted in bold [99, 130].

Compound	T-p53C	T-p53C- Y220C	T-p53C- R273H	T-p53C- R282W	T-p53C- C124/182/ 277S
	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]
SN001-34	-0.15 $\pm$ 0	0.80 $\pm$ 0	-0.45 $\pm$ 0.12	-1.35 $\pm$ 0	0.10 $\pm$ 0.19
SN006/7-7	0.65 $\pm$ 0.09	1.28 $\pm$ 0.10	0.50 $\pm$ 0.27	0.25 $\pm$ 0.17	0.25 $\pm$ 0.12
SN006/7-8	0.90 $\pm$ 0.12 and 2.70 $\pm$ 0.12	4.43 $\pm$ 0.18	1.10 $\pm$ 0.27	0.65 $\pm$ 0.17	0.55 $\pm$ 0.12
SN009	0.85 $\pm$ 0.12 and 2.90 $\pm$ 0.09	2.15 $\pm$ 0.24	1.45 $\pm$ 0.12	N/A ^a	0.65 $\pm$ 0.09
SN010	0.20 $\pm$ 0.09	0.35 $\pm$ 0.12	0.60 $\pm$ 0.12	1.05 $\pm$ 0	0.35 $\pm$ 0.17
SN011	0.05 $\pm$ 0.09	0.40 $\pm$ 0.09	0.20 $\pm$ 0.17	0.70 $\pm$ 0.23	0.35 $\pm$ 0.09
SN012	0 $\pm$ 0.12	0.35 $\pm$ 0.19	0 $\pm$ 0.12	0.10 $\pm$ 0.17	0.15 $\pm$ 0.24

^aNo evaluable melting curves and first derivatives with clearly defined melting points were obtained.

Table 3.41: $\Delta T_m \pm SD$ of T-p53C and its mutants (8 μ M) incubated with 1 mM SN001-17 and SN006/7-3 SAR compounds for 24 h at 20 °C. Measurements were performed at least in triplicate. Fragments with a ΔT_m of at least 0.50 °C are highlighted in bold [99, 130].

Compound	T-p53C	T-p53C- Y220C	T-p53C- R273H	T-p53C- R282W	T-p53C- C124/182/ 277S
	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]
SN001-34	0 $\pm$ 0	2.45 $\pm$ 0.17	-0.75 $\pm$ 0.12	-1.45 $\pm$ 0.35	-0.55 $\pm$ 0.17
SN006/7-7	0.87 $\pm$ 0.09^a	2.08 $\pm$ 0.12	0.20 $\pm$ 0.17	N/A ^b	0.45 $\pm$ 0.12
SN006/7-8	1.03 $\pm$ 0.10 and 2.90 $\pm$ 0.17	5.02 $\pm$ 0.10	1.15 $\pm$ 0.12	N/A ^b	0.70 $\pm$ 0.09
SN009	1.05 $\pm$ 0.08 and 3.23 $\pm$ 0.12	2.05 $\pm$ 0.19	1.30 $\pm$ 0.12	N/A ^b	0.80 $\pm$ 0.12
SN010	0.82 $\pm$ 0.08^a	0.65 $\pm$ 0.23	1.20 $\pm$ 0.12	2.00 $\pm$ 0.09	0.85 $\pm$ 0.09
SN011	0.75 $\pm$ 0.08	1.10 $\pm$ 0.17	1.10 $\pm$ 0.09	1.70 $\pm$ 0.09	0.70 $\pm$ 0.09
SN012	0.55 $\pm$ 0.09	0.55 $\pm$ 0.19	0.40 $\pm$ 0.12	1.55 $\pm$ 0.09	0.55 $\pm$ 0.17

^aMelting curves with two transitions. First derivatives showed an additional second shoulder. ^bNo evaluable melting curves and first derivatives with clearly defined melting points were obtained.

Table 3.42: $\Delta T_m \pm SD$ of T-p53C (8 μM protein) incubated with 1 mM or 250 μM SN001-17 and SN006/7-3 SAR compounds for 30 min, 4 h, and 24 h at 20 °C. Measurements were performed at least in triplicate.

T-p53C						
$\Delta T_m \pm SD$ [°C]						
	1 mM 30 min	1 mM 4 h	1 mM 24 h	250 μM 30 min	250 μM 4 h	250 μM 24 h
SN001-34	0 ± 0.15	-0.15 ± 0	0 ± 0	0 ± 0	-0.10 ± 0.09	-0.15 ± 0.15
SN006/7-7	0.75 ± 0.15	0.65 ± 0.09	0.87 ± 0.09^a	0.65 ± 0.09	0.60 ± 0.12	0.80 ± 0.10
SN006/7-8	0.90 ± 0	0.90 ± 0.12 and 2.70 ± 0.12	1.03 ± 0.10 and 2.90 ± 0.17	0.90 ± 0	0.90 ± 0.12 and 2.70 ± 0.12	1.00 ± 0.09 and 2.90 ± 0.19
SN009	0.95 ± 0.09	0.85 ± 0.12 and 2.90 ± 0.09	1.05 ± 0.08 and 3.23 ± 0.12	1.10 ± 0.09 and 3.05 ± 0.17	1.00 ± 0.12 and 2.75 ± 0.09	1.00 ± 0.09 and 2.93 ± 0.12
SN010	0.30 ± 0.15	0.20 ± 0.09	0.82 ± 0.08^a	0.25 ± 0.23	0 ± 0.12	0.57 ± 0.09
SN011	0.10 ± 0.09	0.05 ± 0.09	0.75 ± 0.08	0.25 ± 0.17	0.05 ± 0.17	0.52 ± 0.08
SN012	0 ± 0	0 ± 0.12	0.55 ± 0.09	0.10 ± 0.17	0.05 ± 0.09	0.17 ± 0.10

^aMelting curves with two transitions. First derivatives of the melting curves showed an additional second shoulder.

Table 3.43: $\Delta T_m \pm SD$ of T-p53C-Y220C (8 μM protein) incubated with 1 mM or 250 μM SN001-17 and SN006/7-3 SAR compounds for 30 min, 4 h, and 24 h at 20 °C. Measurements were performed at least in triplicate.

T-p53C-Y220C						
$\Delta T_m \pm SD$ [°C]						
	1 mM 30 min	1 mM 4 h	1 mM 24 h	250 μM 30 min	250 μM 4 h	250 μM 24 h
SN001-34	0.30 ± 0.17	0.80 ± 0	2.45 ± 0.17	0.35 ± 0.15	0.75 ± 0.09	2.55 ± 0
SN006/7-7	0.47 ± 0.08	1.28 ± 0.10	2.08 ± 0.12	0.23 ± 0.09	0.45 ± 0.24	1.10 ± 0.11
SN006/7-8	1.71 ± 0.10	4.43 ± 0.18	5.02 ± 0.10	0.80 ± 0.07	1.85 ± 0.12	4.45 ± 0.15
SN009	1.60 ± 0.17	2.15 ± 0.24	2.05 ± 0.19	1.30 ± 0.24	1.85 ± 0.12	1.55 ± 0.35
SN010	0.15 ± 0.19	0.35 ± 0.12	0.65 ± 0.23	0 ± 0.15	0.15 ± 0.12	0.45 ± 0.19
SN011	0.10 ± 0.17	0.40 ± 0.09	1.10 ± 0.17	0 ± 0.19	0.45 ± 0.19	0.80 ± 0.17
SN012	0.05 ± 0.17	0.35 ± 0.19	0.55 ± 0.19	0 ± 0.15	0.10 ± 0.17	0.40 ± 0.19

3.6.2 Intact Protein Mass Spectrometry of T-p53C-Y220C with SN001-17 and SN006/7-3 SAR Compounds

Figure 3.75 and Figure 3.76 show the deconvoluted MS spectra of T-p53C-Y220C incubated with SN001-17 and SN006/7-3 SAR compounds for 24 h at a molar ratio of 1:100.

The MS results confirmed the covalent modification of T-p53C-Y220C by fragment SN001-34. Additionally, the deconvoluted MS spectrum in Figure 3.75 A also revealed the reduced reactivity of compound SN001-34 with cysteine residues in p53 compared to the original fragment SN001-17. In contrast to SN001-17, native T-p53C-Y220C was still detected after 24 h of incubation with SN001-34 (molar ratio 1:100). Moreover, there were peaks corresponding to the protein species with one and two attached molecules of SN001-34. T-p53C-Y220C labeled with one molecule of SN001-34 represented the most intense signal, while in the deconvoluted MS spectrum of SN001-17, it was the double modified protein (Figure 3.55 A).

The deconvoluted MS spectra of T-p53C-Y220C incubated with the pyrazine derivatives SN006/7-7 and SN006/7-8 are depicted in Figure 3.75 B and Figure 3.75 C, respectively. Besides the electron-donating methyl group in the meta or ortho position to the LG, both compounds are still reactive, resulting in multiple arylations of T-p53C-Y220C. In both deconvoluted MS spectra, the protein species with three attached molecules of SN006/7-7 or SN006/7-8 represented the highest signal, and up to four times arylated protein was detected. However, the number of modifications was reduced compared to the original fragment SN006/7-3 (Figure 3.63 B). Furthermore, the deconvoluted MS spectrum of SN006/7-3 showed numerous peaks indicative of the protein modified by Pinner-type addition to the nitrile group. In contrast, only peaks for the protein arylated via S_NAr were detectable in the spectra with the methylated SAR compounds SN006/7-7 and SN006/7-8. Compound SN006/7-7, which has the methyl group in the meta position to the LG, resulted in a substantially lower stabilization of T-p53C-Y220C compared to fragments SN006/7-8 and SN006/7-3. However, in the deconvoluted MS spectrum of SN006/7-7 with T-p53C-Y220C, an additional small peak corresponding to the double arylated protein was evident.

Intact protein MS confirmed the covalent modification of T-p53C-Y220C by the 2-chloropyrimidines SN009 and SN010 (Figure 3.76 A, Figure 3.76 B). Despite the introduction of an amino group in the meta position to the LG, compound SN010 is reactive, leading to multiple arylations of T-p53C-Y220C. Interestingly, the deconvoluted MS spectrum of T-p53C-Y220C with SN009 still showed native protein, whereas in the spectrum with SN010, only peaks for the single or double arylated protein were evident. Nevertheless, the lack of confirmation of the purity of these compounds by HPLC-UV necessitates a cautious interpretation of the results.

The DSF experiment demonstrated that the pyridazine derivatives SN011 and SN012 only slightly stabilized p53 and its mutants. The maximum stabilization of T-p53C-Y220C observed was 1.10 °C with SN011 (1 mM, 24 h) and 0.55 °C with SN012 (1 mM, 24 h), respectively. However, the UHPLC-ESI-MS analysis revealed multiple and non-specific modifications of T-p53C-Y220C by both compounds, as shown in Figure 3.76 C and Figure 3.76 D. Therefore, the tested pyridazine derivatives are not suitable for the selective arylation of Cys220 and other cysteines in p53 or for substantial stabilization of p53 and its mutants.

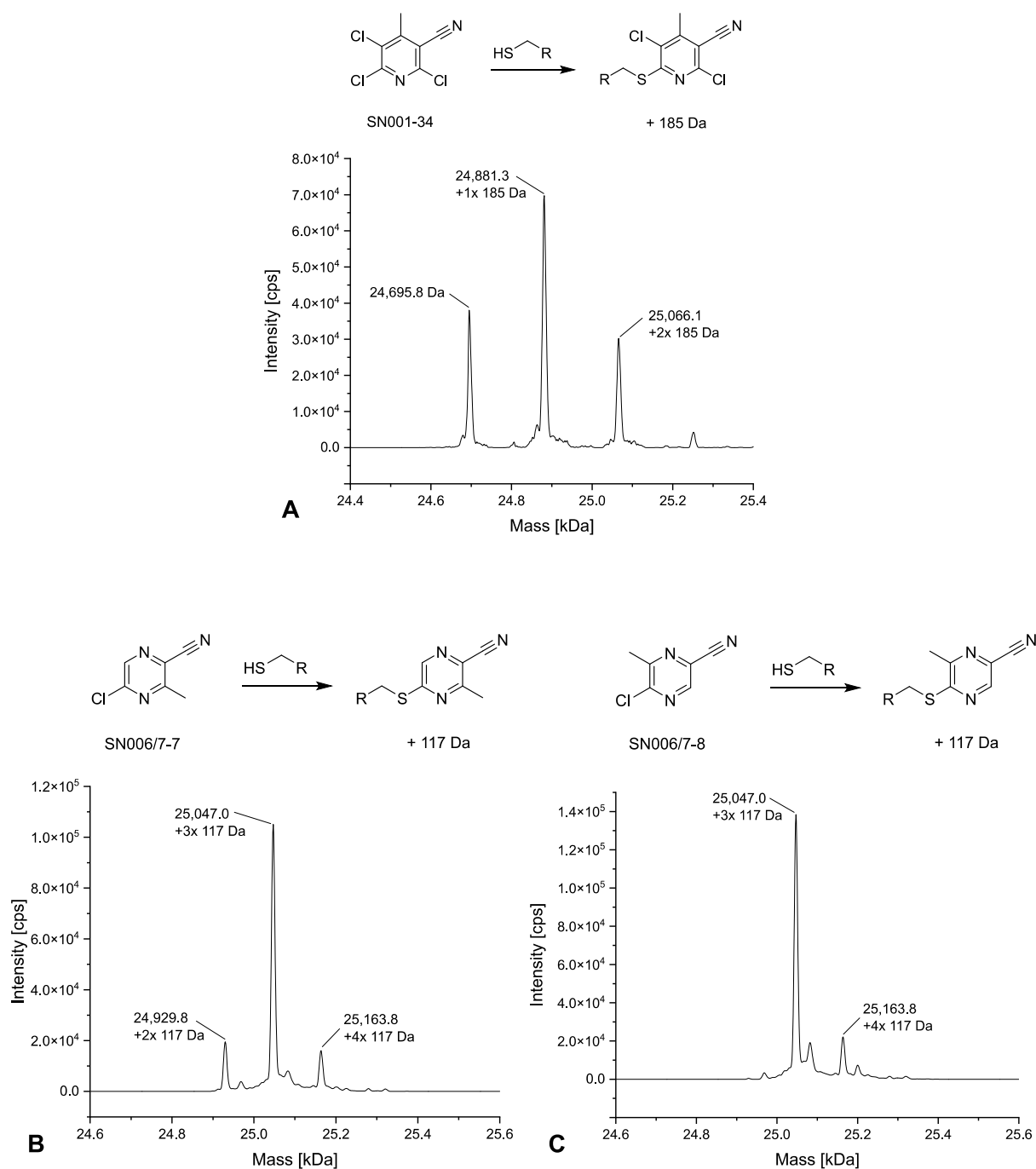


Figure 3.75: Deconvoluted MS spectra of T-p53C-Y220C with SN001-34 (A), SN006/7-7 (B), and SN006/7-8 (C) at a protein-to-compound ratio of 1:100 after 24 h of incubation at 20 °C (theoretical mass of unmodified T-p53C-Y220C: 24,696.00 Da).

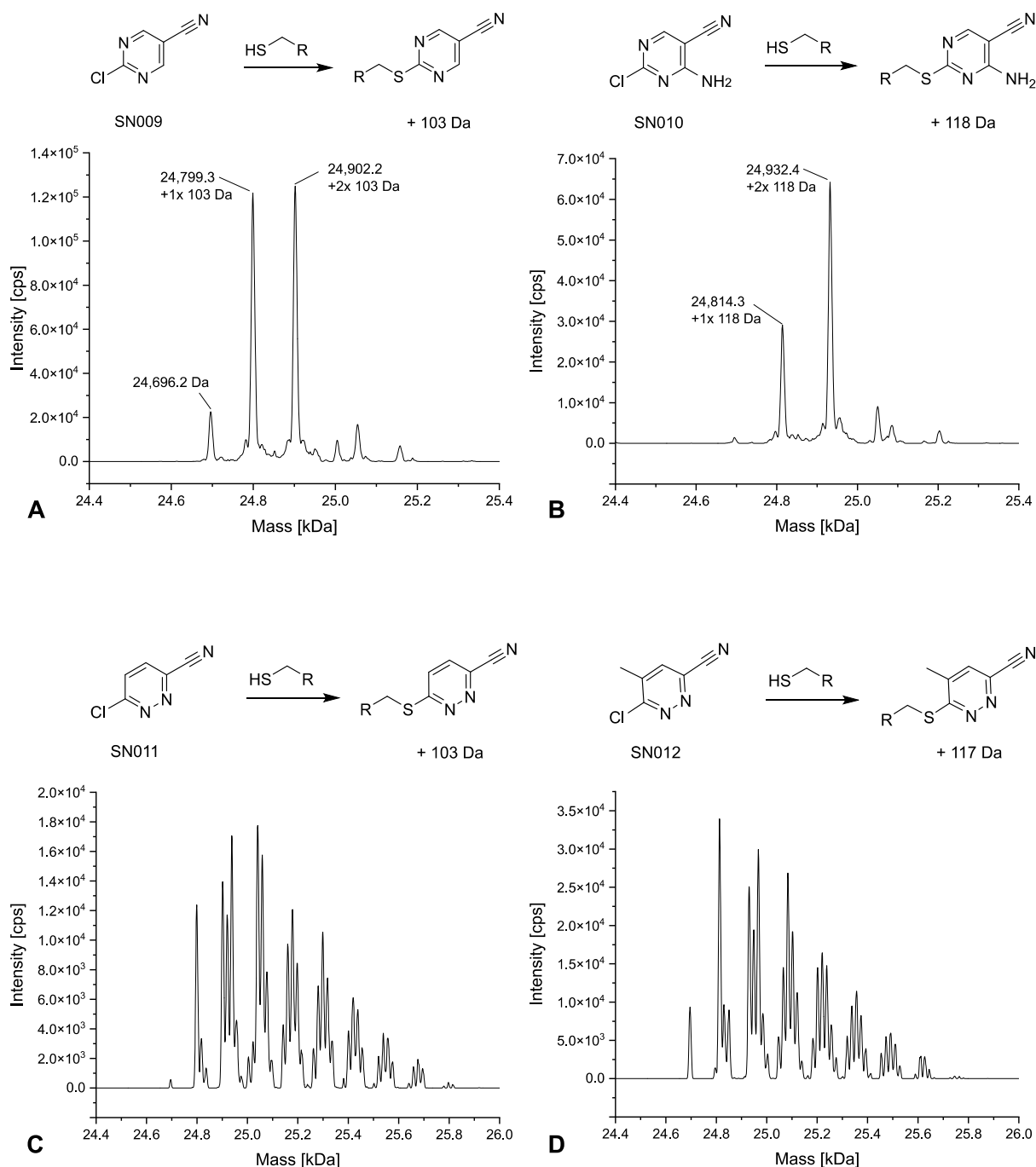


Figure 3.76: Deconvoluted MS spectra of T-p53C-Y220C with SN009 (A), SN010 (B), SN011 (C), and SN012 (D) at a protein-to-compound ratio of 1:100 after 24 h of incubation at 20 °C (theoretical mass of unmodified T-p53C-Y220C: 24,696.00 Da).

3.6.3 DSF Studies of the Y220C Stabilizing Hits SN006/7-7 and SN006/7-8

For all concentration-dependent and time-dependent DSF measurements in this chapter, only the measured values from the corresponding runs were considered, not the mean value of all measurements as in the primary DSF screen.

The concentration-dependent and time-dependent DSF measurements of T-p53C-Y220C incubated with fragment SN006/7-7 are depicted in Figure 3.77, Table 3.44, Figure 3.78, and Table 3.45. As shown in Figure 3.77 A and Table 3.44, after 30 min of incubation with varying concentrations of compound SN006/7-7, there were almost no discernible stabilizing effects on T-p53C-Y220C. Only at a concentration of 1 mM SN006/7-7, a slight stabilization of the cancer mutant by about 0.55 °C was evident. However, after 24 h incubation time (Figure 3.77 B, Table 3.44), a considerable increase in the T_m of T-p53C-Y220C by up to 2.15 °C was apparent at a concentration of 1 mM SN006/7-7. The stabilization of T-p53C-Y220C declined as the compound concentration decreased. An increase in the melting temperature of T-p53C-Y220C was detected ($\Delta T_m = 0.85$ °C) up to a minimal effective concentration of 125 μ M (molar ratio of 1:15.36).

The stabilizing effect of the 5-chloro-3-methylpyrazine SN006/7-7 was found to be time-dependent, as shown in Figure 3.78 and Table 3.45. A decent stabilization of T-p53C-Y220C was visible from about 3 h to 4 h of incubation. However, the highest stabilization was achieved after a 24-hour incubation period at 1 mM SN006/7-7, with a ΔT_m of 2.15 °C. Compared to SN006/7-3 ($t_{1/2}$ GSH= 0.23 h), T-p53C-Y220C showed a substantial increase in the T_m only after a longer incubation time with SN006/7-7 ($t_{1/2}$ GSH= 0.45 h), indicating a more pronounced time dependency of the stabilization. Nevertheless, the stabilization of the Y220C mutant was still reduced after a 24-hour incubation period with SN006/7-7 compared to SN006/7-3. Additionally, a substantial increase in the melting temperature was only detectable up to a lowest effective concentration of 125 μ M for SN006/7-7, whereas for SN006/7-3, it was detectable up to 31.25 μ M. The introduction of the methyl group at position 3 of the pyrazine ring, ortho to the electron-withdrawing nitrile group, reduced the electron deficiency of the heteroarene and therefore decreased the reactivity of the fragment. However, also the overall stabilization of the cancer mutant T-p53C-Y220C was reduced compared to the original compound SN006/7-3.

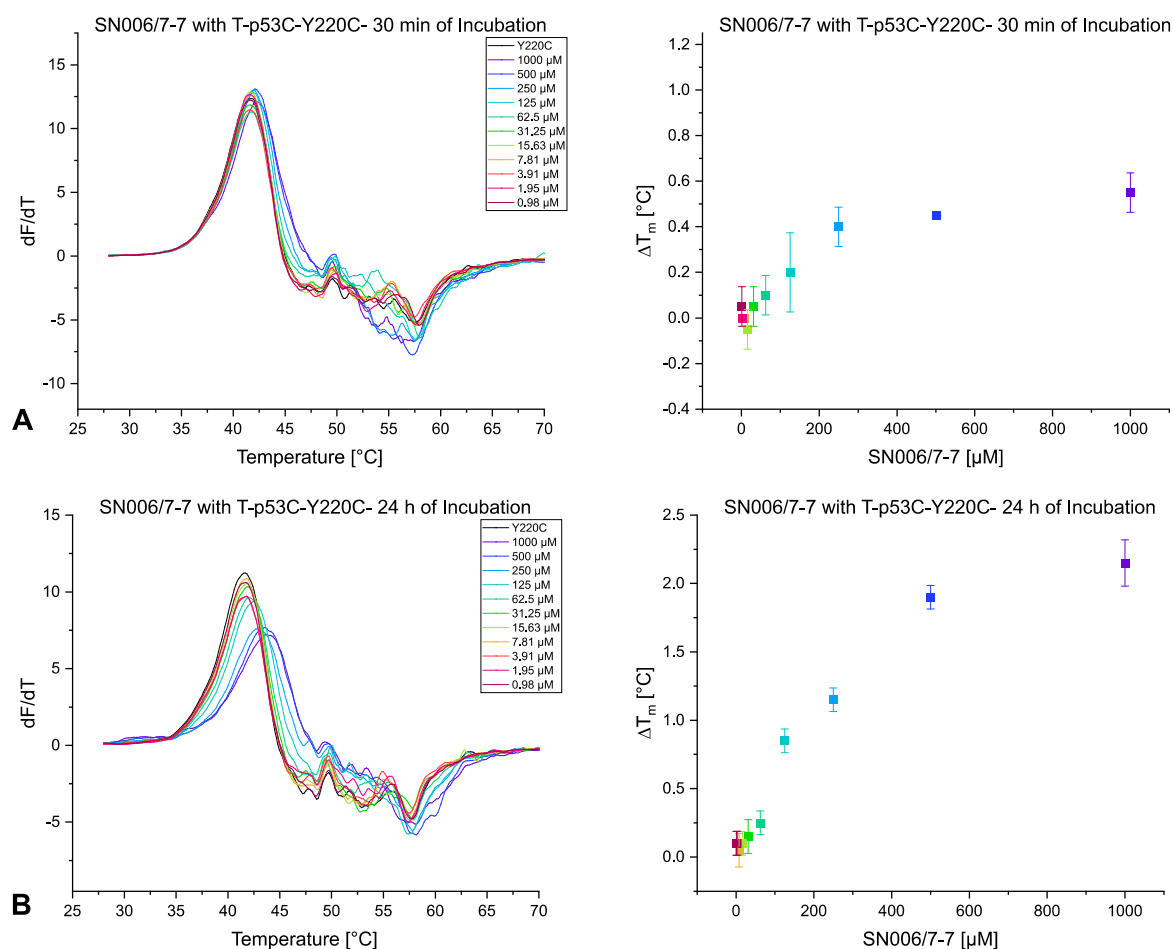


Figure 3.77: Concentration-dependent DSF measurements of T-p53C-Y220C with SN006/7-7. The first derivatives of the melting curves and resulting ΔT_m values of T-p53C-Y220C (8 μ M protein) with various concentrations of compound SN006/7-7 after 30 min (A) and 24 h (B) of incubation at 20 °C are shown.

Table 3.44: $\Delta T_m \pm$ SD of T-p53C-Y220C (8 μ M) incubated with different concentrations of compound SN006/7-7 for 30 min and 24 h at 20 °C. Measurements were performed at least in triplicate.

SN006/7-7 [μM]	T-p53C-Y220C 30 min $\Delta T_m \pm$ SD [°C]	T-p53C-Y220C 24 h $\Delta T_m \pm$ SD [°C]
1000	0.55 ± 0.09	2.15 ± 0.17
500	0.45 ± 0.09	1.90 ± 0.09
250	0.40 ± 0.09	1.15 ± 0.09
125	0.20 ± 0.17	0.85 ± 0.09
62.5	0.10 ± 0.09	0.25 ± 0.09
31.25	0.05 ± 0.09	0.15 ± 0.12
15.63	-0.05 ± 0.09	0.10 ± 0.09
7.81	0 ± 0	0.05 ± 0.12
3.91	0 ± 0	0.10 ± 0.09
1.95	0 ± 0	0.10 ± 0.09
0.98	0.05 ± 0.09	0.10 ± 0.09

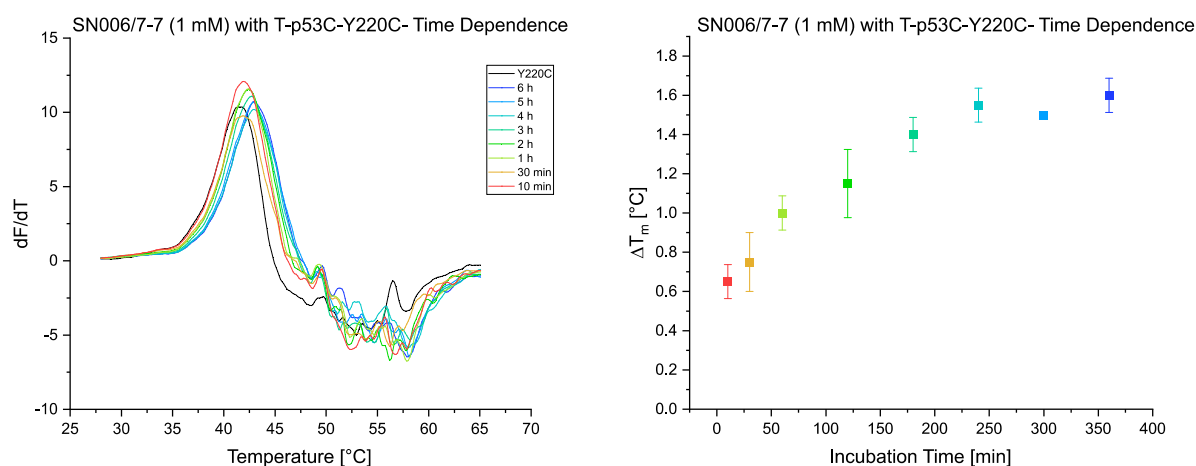


Figure 3.78: Time-dependent DSF measurements of T-p53C-Y220C with SN006/7-7. The first derivatives of the melting curves and resulting ΔT_m values of T-p53C-Y220C (8 μ M protein) with 1 mM SN006/7-7 after various incubation periods at 20 °C are shown.

Table 3.45: $\Delta T_m \pm$ SD of T-p53C-Y220C (8 μ M) incubated with SN006/7-7 (1 mM) after different periods of time at 20 °C. Measurements were performed in triplicate.

Incubation Time	SN006/7-7 (1 mM) with T-p53C-Y220C $\Delta T_m \pm$ SD [°C]
6 h	1.60 ± 0.09
5 h	1.50 ± 0
4 h	1.55 ± 0.09
3 h	1.40 ± 0.09
2 h	1.15 ± 0.17
1 h	1.00 ± 0.09
30 min	0.75 ± 0.15
10 min	0.65 ± 0.09

The concentration-dependent and time-dependent DSF measurements of T-p53C-Y220C incubated with compound SN006/7-8 are shown in Figure 3.79, Table 3.46, Figure 3.80, and Table 3.47. In contrast to fragment SN006/7-7, the 5-chloro-6-methylpyrazine SN006/7-8 showed a distinct stabilization of the Y220C mutant up to a lowest effective concentration of 250 μM after only 30 min of incubation (Figure 3.79 A, Table 3.46). Nevertheless, the maximum stabilization achieved by fragment SN006/7-8 ($\Delta T_m = 1.83\text{ }^{\circ}\text{C}$, 1 mM) after 30 min was lower than that of the original fragment SN006/7-3 ($\Delta T_m = 2.65\text{ }^{\circ}\text{C}$, 1 mM). Additionally, compound SN006/7-8 only stabilized up to 250 μM , while SN006/7-3 stabilized up to 125 μM compound concentration after 30 min of incubation. After 24 h incubation time, the stabilization of T-p53C-Y220C by compound SN006/7-8 with 4.98 $^{\circ}\text{C}$ (1 mM) was much more pronounced than by fragments SN006/7-3 ($\Delta T_m = 3.55\text{ }^{\circ}\text{C}$, 1 mM) and SN006/7-7 ($\Delta T_m = 2.15\text{ }^{\circ}\text{C}$, 1 mM). Even at concentrations of 500 μM and 250 μM , SN006/7-8 achieved considerably higher ΔT_m values than SN006/7-3 and SN006/7-7. Similar to SN006/7-3, T-p53C-Y220C was stabilized up to a lowest effective compound concentration of 31.25 μM after 24 h of incubation, corresponding to a molar ratio of 1:3.91. Overall, the introduction of a methyl group in the ortho position to the LG substantially enhanced the stabilization of T-p53C-Y220C compared to SN006/7-3 without a methyl group and SN006/7-7 with a methyl group in the meta position to the LG.

As shown in Figure 3.80 and Table 3.47, SN006/7-8 stabilized T-p53C-Y220C by up to 1.43 $^{\circ}\text{C}$ after only 10 min of incubation. The ΔT_m values of T-p53C-Y220C supplemented with 1 mM SN006/7-8 increased with increasing incubation time, tending towards a maximum after 3 h of incubation. This time dependence is comparable to that of its constitutional isomer SN006/7-7 (Figure 3.78), whereas SN006/7-3 (Figure 3.65) achieved a higher stabilization of T-p53C-Y220C and thus target occupancy at shorter incubation times.

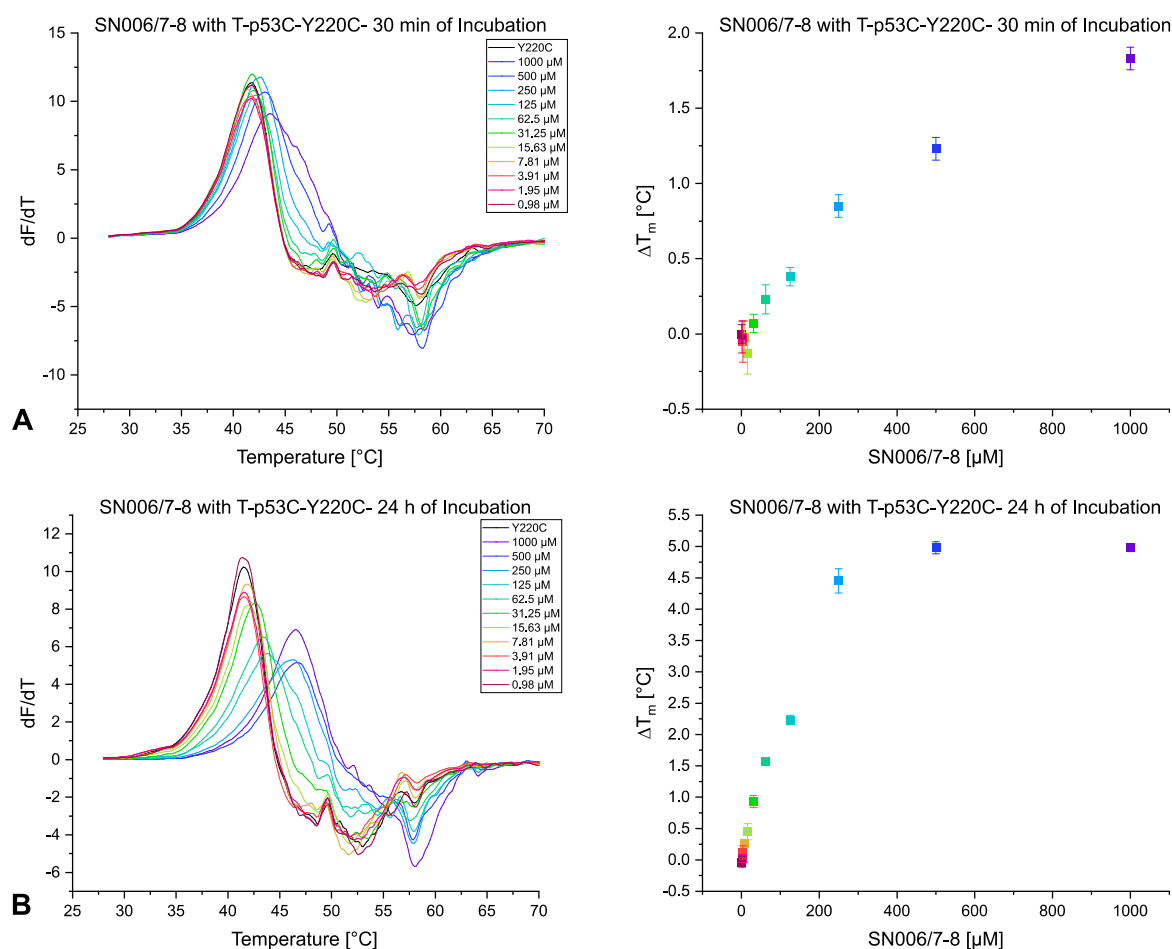


Figure 3.79: Concentration-dependent DSF measurements of T-p53C-Y220C with SN006/7-8. The first derivatives of the melting curves and resulting ΔT_m values of T-p53C-Y220C (8 μM protein) with various concentrations of compound SN006/7-8 after 30 min (A) and 24 h (B) of incubation at 20 °C are shown.

Table 3.46: $\Delta T_m \pm SD$ of T-p53C-Y220C (8 μM) incubated with different concentrations of compound SN006/7-8 for 30 min and 24 h at 20 °C. Measurements were performed at least in triplicate.

SN006/7-8 [μM]	T-p53C-Y220C 30 min $\Delta T_m \pm SD$ [°C]	T-p53C-Y220C 24 h $\Delta T_m \pm SD$ [°C]
1000	1.83 ± 0.08	4.98 ± 0.06
500	1.23 ± 0.08	4.98 ± 0.10
250	0.85 ± 0.08	4.45 ± 0.19
125	0.38 ± 0.06	2.23 ± 0.07
62.5	0.23 ± 0.10	1.57 ± 0.06
31.25	0.07 ± 0.06	0.93 ± 0.10
15.63	-0.13 ± 0.14	0.45 ± 0.13
7.81	-0.02 ± 0.11	0.27 ± 0.04
3.91	-0.05 ± 0.14	0.12 ± 0.11
1.95	-0.02 ± 0.11	0.02 ± 0.06
0.98	0 ± 0.06	-0.03 ± 0.09

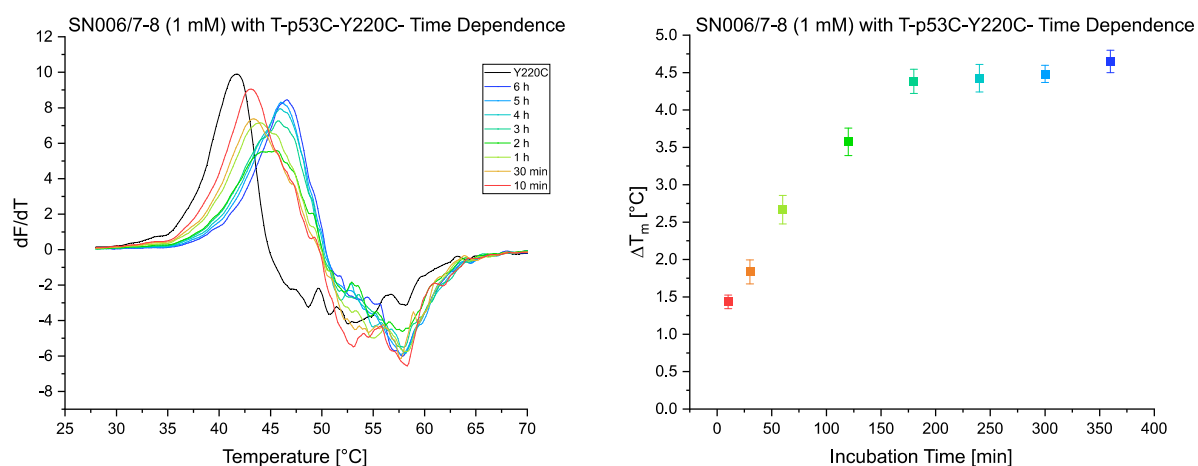


Figure 3.80: Time-dependent DSF measurements of T-p53C-Y220C with SN006/7-8. The first derivatives of the melting curves and resulting ΔT_m values of T-p53C-Y220C (8 μ M protein) with 1 mM SN006/7-8 after various incubation periods at 20 °C are presented.

Table 3.47: $\Delta T_m \pm$ SD of T-p53C-Y220C (8 μ M) incubated with SN006/7-8 (1 mM) after different periods of time at 20 °C. Measurements were performed in triplicate.

Incubation Time	SN006/7-8 (1 mM) with T-p53C-Y220C $\Delta T_m \pm$ SD [°C]
6 h	4.65 ± 0.15
5 h	4.48 ± 0.12
4 h	4.43 ± 0.18
3 h	4.38 ± 0.16
2 h	3.58 ± 0.18
1 h	2.67 ± 0.19
30 min	1.83 ± 0.16
10 min	1.43 ± 0.09

3.6.4 Evaluation of the Hits SN001-34, SN006/7-7, and SN006/7-8 with T-p53C-Y220C-CL

The identified T-p53C-Y220C stabilizing hits SN001-34, SN006/7-7, and SN006/7-8 were further investigated with the Y220C cysteine light mutant. The results of the DSF screen and intact protein MS are shown in Figure 3.81, Table 3.48, and Figure 3.82.

Fragment SN001-34 considerably stabilized the Y220C cysteine light protein by up to 3.43 °C (1 mM) after 24 h of incubation, indicating a covalent modification of the remaining Cys220. SN001-34 increased the T_m of T-p53C-Y220C-CL after 24 h incubation time at both compound concentrations of 1 mM ($\Delta T_m = 3.43$ °C) and 250 μ M ($\Delta T_m = 3.60$ °C). Similar to T-p53C-Y220C, the stabilization of T-p53C-Y220C-CL increased with enhanced incubation time. Compared to the original fragment SN001-17, the time dependence was more pronounced, indicating a reduction in the reactivity of compound SN001-34. After 30 min of incubation, a slight increase in T_m was observed only at a concentration of 1 mM SN001-34 ($\Delta T_m = 0.65$ °C). After 4 h of incubation, the first derivative of the melting curve of T-p53C-Y220C-CL incubated with 250 μ M SN001-34 displayed a broad peak. Interestingly, the T_m of the Y220C cysteine light protein increased more substantially with compound SN001-34 than the T_m of the T-p53C-Y220C protein ($\Delta T_m = 2.55$ °C, 250 μ M, 24 h). However, the 2,5,6-trichloro-4-methylnicotinotirile SN001-34 ($\Delta T_m = 3.60$ °C, 250 μ M, 24 h) showed a lower overall stabilization of T-p53C-Y220C-CL compared to the trichloro compound SN001-17 ($\Delta T_m = 5.20$ °C, 250 μ M, 24 h), but a higher stabilization compared to the original trifluoro hit SN001 ($\Delta T_m = 2.95$ °C, 250 μ M, 24 h). The deconvoluted MS spectrum of T-p53C-Y220C-CL incubated with fragment SN001-34 (Figure 3.82 A) showed a signal for the native protein and a more intense signal for the single arylated protein species, which was similar to the spectra of fragment SN001-17 (Figure 3.60). This indicates that the stabilization of the Y220C cysteine light protein in the DSF experiment is due to the covalent binding of fragment SN001-34 to the remaining Cys220 in the crevice. In summary, compound SN001-34 with an additional methyl group may covalently modify Cys220 in the pocket and has reduced reactivity. However, the thermal stabilization of T-p53C-Y220C and T-p53C-Y220C-CL was lower compared to the 2,5,6-trichloro pyridine derivative SN001-17 without a methyl group.

Fragment SN006/7-7, which has a methyl group in the meta position to the LG, stabilized T-p53C-Y220C-CL by up to 1.50 °C (1 mM, 24 h). The stabilization was

dependent on concentration and time, as previously observed for T-p53C-Y220C. However, the stabilization of T-p53C-Y220C-CL by fragment SN006/7-7 was lower compared to T-p53C-Y220C ($\Delta T_m = 2.08\text{ }^\circ\text{C}$, 1 mM, 24 h), indicating that the covalent binding of the fragment to the mutated surface-exposed cysteines also has a stabilizing effect. In addition, compared to the identified Cys220 arylating compound SN006/7-3 without a methyl group ($\Delta T_m = 2.40\text{ }^\circ\text{C}$, 1 mM, 24 h), the stabilization of the Y220C cysteine light mutant by compound SN006/7-7 was reduced. However, the deconvoluted MS spectrum of T-p53C-Y220C-CL with the original fragment SN006/7-3 showed a peak for the protein with one molecule of SN006/7-3 attached via S_NAr and a higher signal for the protein labeled with one molecule of SN006/7-3 via an addition to the nitrile (Figure 3.67). In contrast, in the deconvoluted MS spectrum of the Y220C cysteine light mutant with SN006/7-7 (Figure 3.82 B), the highest peak corresponded to the protein with one molecule attached via S_NAr -type reaction, indicating arylation of the remaining Cys220. Furthermore, a small peak for the native protein was also visible.

The 5-chloro-6-methylpyrazine-2-carbonitrile SN006/7-8 increased the T_m of the Y220C cysteine light mutant by up to $3.68\text{ }^\circ\text{C}$ (1 mM, 24 h). As previously noted for T-p53C-Y220C, incubation with the fragment SN006/7-8, which has a methyl group in the ortho position to the LG, resulted in a considerable higher stabilization of T-p53C-Y220C-CL compared to its constitutional isomer SN006/7-7 ($\Delta T_m = 1.50\text{ }^\circ\text{C}$, 1 mM, 24 h) and the 5-chloro-pyrazine-2-carbonitrile SN006/7-3 ($\Delta T_m = 2.40\text{ }^\circ\text{C}$, 1 mM, 24 h). In addition, a comparably high stabilization of T-p53C-Y220C-CL was measured after 24 h of incubation with 1 mM ($\Delta T_m = 3.68\text{ }^\circ\text{C}$) and 250 μM ($\Delta T_m = 3.58\text{ }^\circ\text{C}$) SN006/7-8. Furthermore, as already observed for the Y220C mutant, the stabilization of T-p53C-Y220C-CL was time-dependent. Similar to SN006/7-3 and SN006/7-7, the stabilization of T-p53C-Y220C-CL by fragment SN006/7-8 was lower than that of T-p53C-Y220C ($\Delta T_m = 5.02\text{ }^\circ\text{C}$, 1 mM, 24 h), indicating that the modification of cysteines besides Cys220 also has a stabilizing effect. Among the tested compounds with a pyrazine heterocycle, SN006/7-8 exhibited the highest stabilization of the Y220C cysteine light mutant, with a ΔT_m of $3.68\text{ }^\circ\text{C}$ (1 mM, 24 h). Compared to the discovered cysteine 220 arylating hits with a pyridine heterocycle, fragment SN006/7-8 stabilized T-p53C-Y220C-CL more than SN001 ($\Delta T_m = 3.08\text{ }^\circ\text{C}$, 1 mM, 24 h), comparably to SN001-34 ($\Delta T_m = 3.43\text{ }^\circ\text{C}$, 1 mM, 24 h), but less than the most stabilizing hit SN001-17 ($\Delta T_m = 4.95\text{ }^\circ\text{C}$, 1 mM, 24 h). The deconvoluted MS spectra of T-p53C-Y220C-CL

incubated with SN006/7-8 at molar ratios of 1:100 and 1:31.25 are shown in Figure 3.82 C and Figure 3.82 D. At a molar ratio of 1:100, only one peak was visible, corresponding to the single arylated protein. At a ratio of 1:31.25, an additional small peak was present for the native Y220C cysteine light protein. Only the protein species arylated with one molecule of SN006/7-8 was detected, suggesting that the mutation-induced cysteine 220 was modified. In summary, the DSF and intact protein MS results with the Y220C cysteine light mutant indicated that SN006/7-8, which contains a methyl group in the ortho position to the LG, provided a higher thermal stabilization compared to SN006/7-3 and SN006/7-7. These findings may be attributed to a more selective binding of SN006/7-8 into the Y220C pocket.

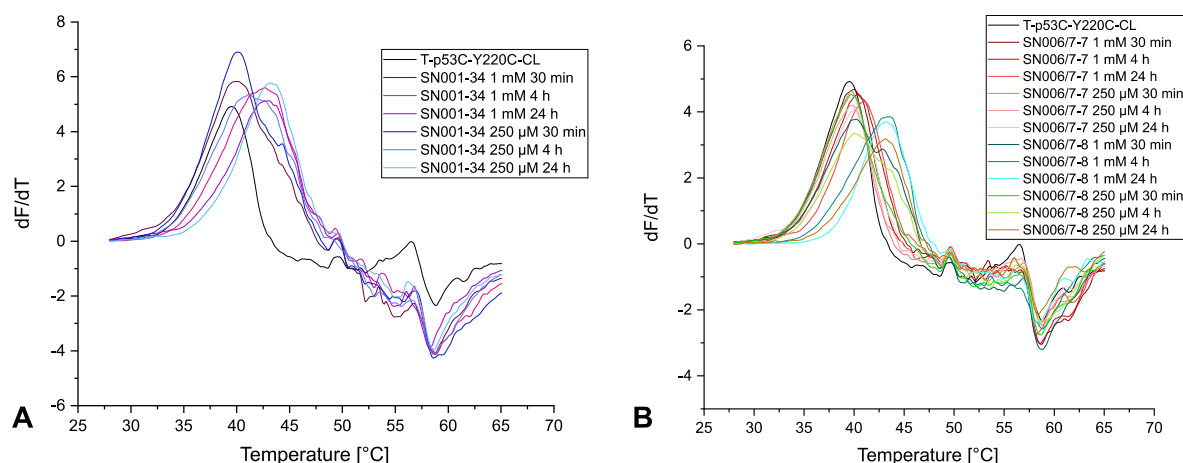


Figure 3.81: DSF measurements of T-p53C-Y220C-CL with SN001-34, SN006/7-7, and SN006/7-8. The first derivatives of the melting curves of T-p53C-Y220C-CL (8 μ M) with SN001-34 (**A**), SN006/7-7, and SN006/7-8 (**B**) (1 mM, 250 μ M) after varying incubation periods at 20 $^{\circ}$ C are shown.

Table 3.48: $\Delta T_m \pm$ SD of T-p53C-Y220C-CL (8 μ M) incubated with SN001-34, SN006/7-7, and SN006/7-8 (1 mM and 250 μ M) after different periods of time at 20 $^{\circ}$ C. Measurements were performed at least in triplicate.

T-p53C-Y220C-CL						
$\Delta T_m \pm$ SD [$^{\circ}$ C]						
	1 mM 30 min	1 mM 4 h	1 mM 24 h	250 μ M 30 min	250 μ M 4 h	250 μ M 24 h
SN001-34	0.65 ± 0.09	3.15 ± 0	3.43 ± 0.16	0.40 ± 0.09	1.65 ± 0^a	3.60 ± 0.08
SN006/7-7	0.40 ± 0.32	0.75 ± 0	1.50 ± 0.10	0 ± 0	0.20 ± 0.09	0.63 ± 0.08
SN006/7-8	0.40 ± 0.09^b	4.10 ± 0.09	3.68 ± 0.21	0.10 ± 0.09	0.50 ± 0.09^a	3.58 ± 0.10

^aFirst derivatives of the melting curves displayed a broad curve. ^bMelting curves with two transitions. First derivatives of the melting curves showed a second shoulder.

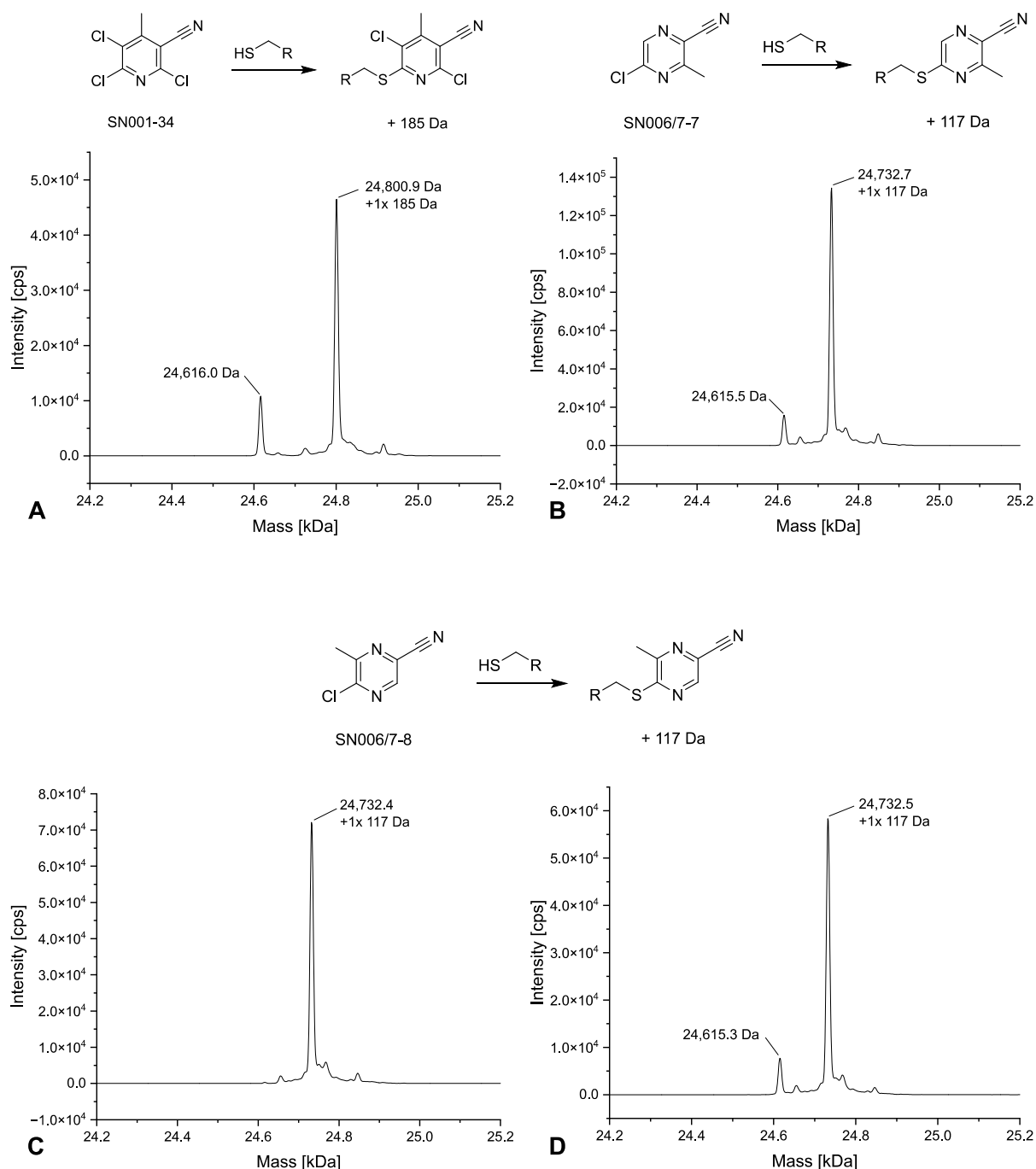


Figure 3.82: Intact protein MS of T-p53C-Y220C-CL with SN001-34, SN006/7-7, and SN006/7-8. The deconvoluted MS spectra of T-p53C-Y220C-CL (theoretical mass of unmodified T-p53C-Y220C-CL: 24,615.68 Da) with SN001-34 (**A**), SN006/7-7 (**B**) at a molar ratio of 1:100, and SN006/7-8 at a molar ratio of 1:100 (**C**) and 1:31.25 (**D**) after 24 h of incubation at 20 °C are shown.

3.6.5 Determination of the Binding Mode of SN006/7-8 Using X-Ray Crystallography

The covalent modification of Cys220 by compound SN006/7-8 was confirmed using X-ray crystallography, as depicted in Figure 3.83. Compared to fragment SN006/7-3, pointing into subsite 2 of the hydrophobic pocket, fragment SN006/7-8 with the additional methyl group adopted the same Tyr-like conformation as the pyridine derivatives SN001 and SN001-17 and the pyrazine derivative SN006. Fragment SN006/7-8 reacted with Cys220 via an S_NAr reaction, with the chlorine atom acting as the LG. It occupied most of the space in the central cavity, mimicking the wt tyrosine residue. The electron-withdrawing CN moiety pointed towards subsite 1 and Thr150. The methyl group at position 6 of the pyrazine ring pointed into the hydrophobic subsite 3. Favorable hydrophobic interactions of the methyl group in subsite 3 could lead to the enhanced stabilization of T-p53C-Y220C compared to SN006/7-3 and SN006/7-7. It is noteworthy that the non-covalent cleft binders PK7088 [139] and MB710 [114] also exhibited favorable hydrophobic interactions in subsite 3, formed by a flip of the Cys220, upon the introduction of a pyrrole side chain.

In addition, other residues that were arylated in the 5 mM-soaked structure included Cys182 and Cys277.

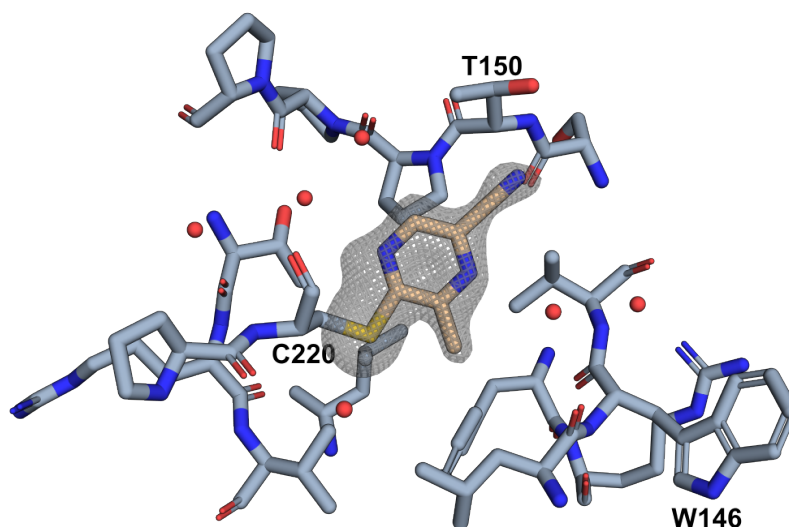


Figure 3.83: Overview of the binding mode of SN006/7-8 (soaked at 5 mM) into the Y220C induced crevice. The compound binds similarly to SN001, SN001-17, and SN006 in a Tyr-like conformation, with the nitrile group pointing towards subsite 1 and Thr150. The meta-methyl group points into the hydrophobic subsite 3. The unbiased omit map is shown at a contour level of 3 σ .

3.6.6 Effects of SN001-34, SN006/7-7, and SN006/7-8 on the DNA-Binding Capacity of FL-T-p53

The results of the FP measurements of FL-T-p53 incubated with SN001-34, SN006/7-7, or SN006/7-8 are depicted in Figure 3.84 and Table 3.49. As the original hits SN001-17 and SN006/7-3, these SAR compounds with additional electron-donating methyl groups at the heteroarene did not negatively affect the DNA-binding of FL-T-p53 to its response element GADD45 α after 24 h of incubation at a molar ratio of 1:200. The FP assay outcomes indicate that despite the modification of cysteine residues in p53 by the tested compounds, regular DNA-binding is maintained and regular transcription of p53 response elements is possible due to the thermal stabilization in cellular systems.

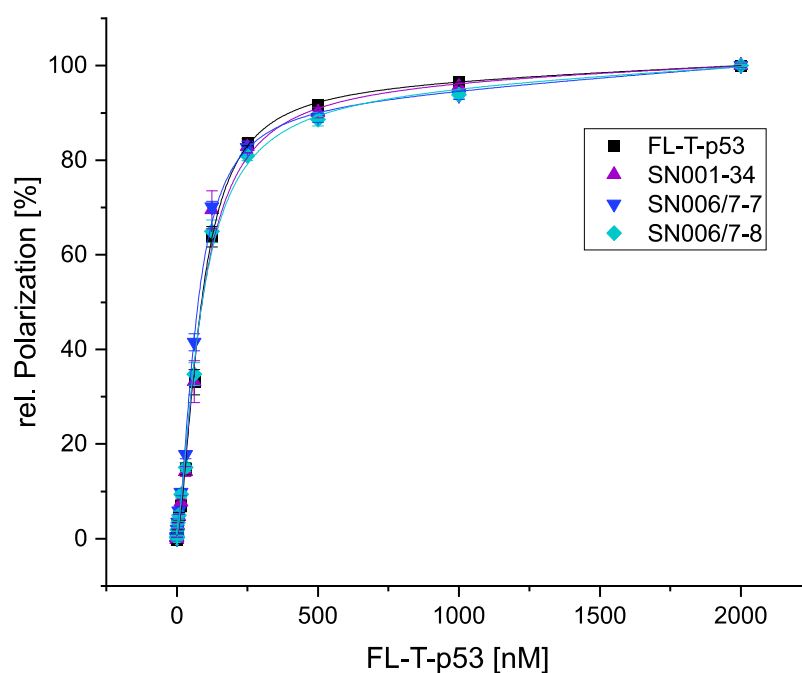


Figure 3.84: FP assay of FL-T-p53 incubated with SN001-34, SN006/7-7, and SN006/7-8 with GADD45 α . The relative fluorescence polarization data [%] of FL-T-p53 with 20 nM 5'-carboxyfluorescein-labeled GADD45 α DNA response element and 5% DMSO are shown. FL-T-p53 was incubated with SN001-34, SN006/7-7, and SN006/7-8 at a protein-to-compound ratio of 1:200 for 24 h at 20 °C [131]. FP data were recorded every 10 min for 1 h, and data were averaged. Measurements were performed at least one time in quadruplicate.

Table 3.49: Resulting K_d values $\pm$ SD of the direct titrations of FL-T-p53 with or without the addition of SN001-34, SN006/7-7, or SN006/7-8 (molar ratio 1:200) with GADD45 α DNA. Measurements were performed at least one time in quadruplicate.

	FL-T-p53	SN001-34	SN006/7-7	SN006/7-8
$K_d \pm SD$ [nM]	83 ± 4	87 ± 10	69 ± 6	84 ± 11

3.7 Structure-Activity Relationship Screen of the Identified Hit SN006/7-8

The SN001-17 and SN006/7-3 SAR compound screen revealed the promising fragment SN006/7-8 with improved stabilization of T-p53C-Y220C. The 5-chloro-6-methylpyrazine-2-carbonitrile SN006/7-8 covalently modifies Cys220, adopting a Tyr-like conformation in the mutational cleft, with the methyl group pointing into subsite 3 of the binding pocket. This prompted us to test additional SAR compounds with larger hydrophobic groups ortho to the LG to increase the selectivity for the pocket through improved hydrophobic interactions in subsite 3. However, no commercially available SAR compounds of SN006/7-8 with, for instance, ethyl or isopropyl groups at position 6 of the heteroarene were found. Hence, only 3,5-dichloropyrazine (SN006/7-9) or pyridine (SN006/7-10 to SN006/7-19) derivatives were purchased. The structures of the acquired SAR compounds are depicted in Figure 3.85.

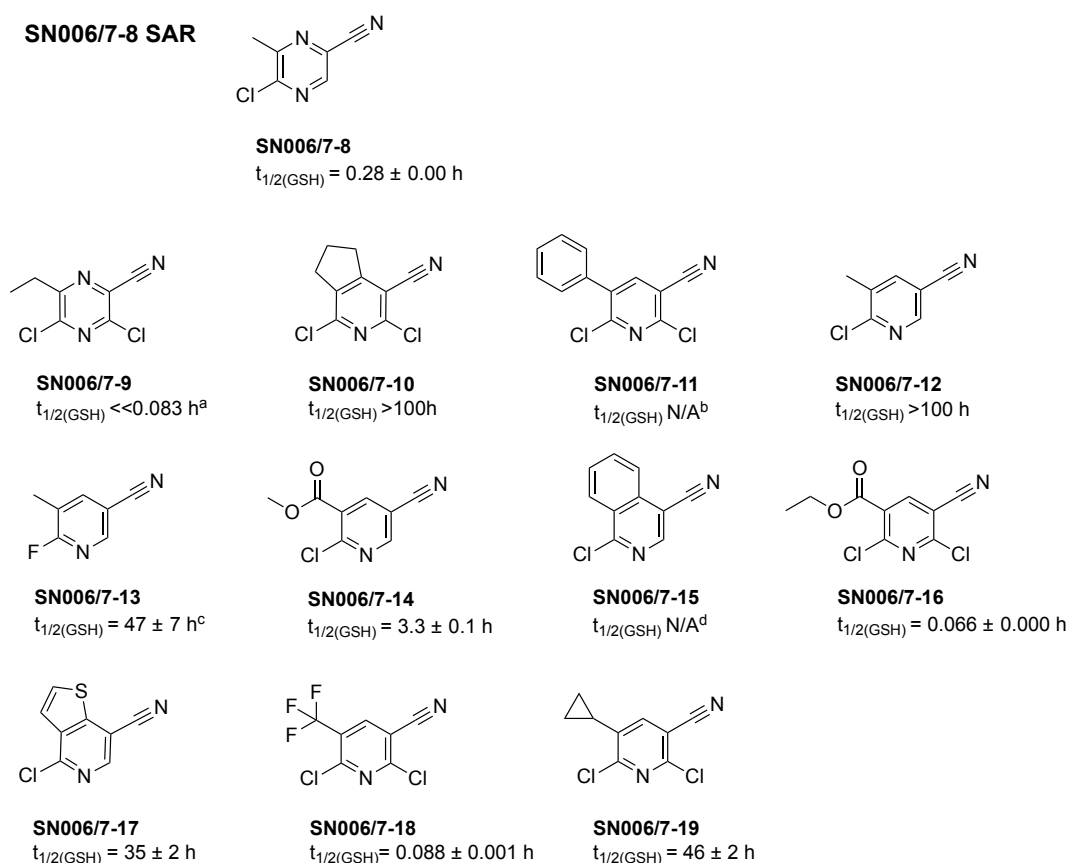


Figure 3.85: Overview of the tested SN006/7-8 SAR compounds and their $t_{1/2} \pm \text{SD}$ determined via GSH assay. The GSH assay was performed by M. Schwer. ^aThe fragment was already degraded at the initial measurement point (0 h). The value of 0.083 h corresponds to the approximate time between adding the fragment to the reaction mixture and starting the HPLC run. ^bThe compound was not soluble in ACN buffer mixture at the required concentration. ^cThe fragment appeared to precipitate during the measurement process. ^dThe purity of the compound could not be confirmed by HPLC-UV.

3.7.1 DSF Screen of the SN006/7-8 SAR Compounds with T-p53C and Its Mutants

The influence of the SN006/7-8 SAR compounds (1 mM) on the T_m of T-p53C and its mutants after 30 min, 4 h, and 24 h of incubation is shown in Table 3.50, Table 3.51, and Table 3.52.

The pyrazine derivative SN006/7-9 contains an ethyl group and an additional chlorine compared to the hit SN006/7-8. The introduction of a second electron-withdrawing chlorine in the ortho position to the EWG results in a highly reactive fragment with a $t_{1/2} < 0.083$ h in the GSH assay. Incubation with 1 mM SN006/7-9 for 30 min markedly lowered the T_m of T-p53C ($\Delta T_m = -4.85$ °C), T-p53C-Y220C ($\Delta T_m = -1.30$ °C), and T-p53C-C124/182/277S ($\Delta T_m = -2.45$ °C). However, the decline in the T_m of T-p53C-Y220C was the least pronounced, with a ΔT_m of -1.30 °C. After only 30 min incubation time, the first derivatives of the melting curves of the R273H and the R282W mutants did not display a peak (Figure 8.49). Furthermore, for all p53 mutants examined, no evaluable melting curves were retained after 4 h and 24 h of incubation, or a destabilization of the triple cysteine mutant occurred with a negative shift in the melting temperature. Fragment SN006/7-9 was also tested at a compound concentration of 250 μ M with T-p53C and T-p53C-Y220C, as shown in Table 3.53 and Table 3.54. Nevertheless, at this concentration, fragment SN006/7-9 only stabilized T-p53C-Y220C by up to 2.60 °C (1 mM, 24 h), indicating cleft binding. A higher stabilization of T-p53C-Y220C could be prevented by the high reactivity of the fragment, leading to unfavorable hyperarylation of the protein.

The 6-chloro and 6-fluoro-5-methylnicotinonitriles SN006/7-12 and SN006/7-13 did not affect the T_m of T-p53C and its mutant, even after 24 h of incubation. Consistent with the initial SAR compound screen, pyridine derivatives with an electron-withdrawing nitrile group and a halogen as the LG are less reactive or chemically stable and not suitable for the covalent modification and stabilization of p53 (Chapter 3.5.1). Similarly, SN006/7-17 also showed no raise in the melting temperatures of p53 and its mutants after various incubation times.

In addition, fragments SN006/7-10, SN006/7-11, and SN006/7-19 with two chlorine atoms, an electron-withdrawing CN group, and different electron-donating substituents at the pyridine scaffold, did not substantially influence the T_m of T-p53C, T-p53C-Y220C, or any of the tested mutants.

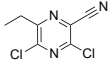
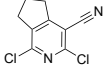
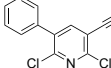
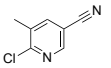
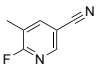
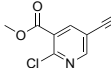
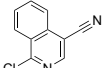
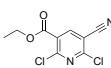
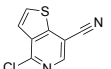
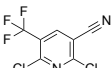
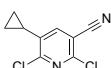
After 24 h of incubation at a compound concentration of 1 mM, the 1-chloroisoquinoline derivative SN006/7-15 slightly stabilized T-p53C-Y220C ($\Delta T_m = 0.58\text{ }^\circ\text{C}$), but destabilized T-p53C-R282W ($\Delta T_m = -0.90\text{ }^\circ\text{C}$) and T-p53C-R273H ($\Delta T_m = -1.40\text{ }^\circ\text{C}$). The maximum stabilization of T-p53C-Y220C by about $0.75\text{ }^\circ\text{C}$ was achieved at $250\text{ }\mu\text{M}$ SN006/7-15 after 24 h of incubation (Table 3.54). However, the increase in the melting temperature of T-p53C-Y220C was only minor compared to fragment SN006/7-8. Furthermore, the purity of SN006/7-15 was not confirmed by HPLC-UV, and the results should be interpreted with caution.

Compound SN006/7-14 with an additional electron-withdrawing methoxycarbonyl group at the pyridine ring increased the T_m of all p53 mutants tested in the DSF experiment after 24 h incubation time at a concentration of 1 mM. Nevertheless, as shown in Table 3.53 and Table 3.54, no notable stabilization of T-p53C and T-p53C-Y220C was observed at a concentration of $250\text{ }\mu\text{M}$. Compared to fragment SN006/7-12 ($t_{1/2}\text{ GSH} > 100\text{ h}$), the introduction of a second EWG in the ortho position to the LG in fragment SN006/7-14 ($t_{1/2}\text{ GSH} = 3.26\text{ h}$) increases the reactivity, leading to a stabilization of p53 and its mutants. Fragment SN006/7-14 stabilized all mutants comparably, and no pronounced stabilization of the Y220C mutant was observed. Therefore, binding into the Y220C pocket is not expected.

The pyridine derivatives SN006/7-16 and SN006/7-18 with electron-withdrawing ethoxycarbonyl group or CF_3 group and additional chlorine are highly reactive. Both compounds particularly stabilized T-p53C-Y220C, suggesting a covalent modification of Cys220. After 24 h of incubation, the T_m of T-p53C-Y220C was increased by up to $2.57\text{ }^\circ\text{C}$ with $250\text{ }\mu\text{M}$ SN006/7-16 and by up to $3.00\text{ }^\circ\text{C}$ with $250\text{ }\mu\text{M}$ SN006/7-18.

Overall, the SAR compound screen identified either less reactive or chemically stable compounds (SN006/7-10, SN006/7-11, SN006/7-12, SN006/7-13, SN006/7-17, SN006/7-19) or highly reactive compounds (SN006/7-9, SN006/7-16, SN006/7-18). In total, no better hit was discovered than fragment SN006/7-8, which was more selective for the Y220C binding pocket and achieved a higher stabilization of T-p53C-Y220C. Although fragments SN006/7-9, SN006/7-16, and SN006/7-18 represent hits that suggest cleft binding, they are also highly reactive and destabilized other p53 mutants tested. The screening indicates that for the development of SAR compounds with higher stabilization of T-p53C-Y220C and higher selectivity for the pocket, the scaffold of SN006/7-8 with EDGs other than the methyl group ortho to the LG is necessary.

Table 3.50: $\Delta T_m \pm SD$ of T-p53C and its mutants (8 μM) incubated with 1 mM SN006/7-8 SAR compounds for **30 min** at 20 °C. Measurements were performed at least in triplicate. Fragments with a ΔT_m of at least 0.50 °C are highlighted in bold [99, 130].

Compound	T-p53C	T-p53C- Y220C	T-p53C- R273H	T-p53C- R282W	T-p53C- C124/182/ 277S
	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]
SN006/7-9 	-4.85 $\pm$ 0.17	-1.30 $\pm$ 0.24	N/A ^a	N/A ^a	-2.45 $\pm$ 0.09
SN006/7-10 	0.20 $\pm$ 0.12	-0.15 $\pm$ 0.12	0 $\pm$ 0.12	-0.10 $\pm$ 0.09	0.15 $\pm$ 0
SN006/7-11^b 	0.25 $\pm$ 0.09	0.05 $\pm$ 0.12	0.15 $\pm$ 0.12	0.05 $\pm$ 0.27	0.60 $\pm$ 0
SN006/7-12 	-0.10 $\pm$ 0.12	-0.10 $\pm$ 0.12	-0.05 $\pm$ 0.09	-0.05 $\pm$ 0.12	-0.10 $\pm$ 0.09
SN006/7-13 	0 $\pm$ 0.12	-0.10 $\pm$ 0.12	-0.05 $\pm$ 0.09	-0.15 $\pm$ 0.12	0 $\pm$ 0
SN006/7-14 	0.30 $\pm$ 0.12	0.35 $\pm$ 0.12	0.10 $\pm$ 0.09	0.30 $\pm$ 0.24	0.15 $\pm$ 0
SN006/7-15^b 	0.05 $\pm$ 0.12	0.10 $\pm$ 0.17	-0.20 $\pm$ 0.09	-0.45 $\pm$ 0.32	0.55 $\pm$ 0.09
SN006/7-16 	-2.05 $\pm$ 0.17	-0.55 $\pm$ 0.12	N/A ^a	N/A ^a	-1.65 $\pm$ 0
SN006/7-17 	0.10 $\pm$ 0.09	-0.15 $\pm$ 0.12	-0.10 $\pm$ 0.12	-0.30 $\pm$ 0.24	-0.05 $\pm$ 0.09
SN006/7-18 	-2.00 $\pm$ 0.23	1.10 $\pm$ 0.12	-2.38 $\pm$ 0.30	N/A ^a	-1.75 $\pm$ 0.09
SN006/7-19 	0 $\pm$ 0.12	0 $\pm$ 0.12	-0.05 $\pm$ 0.09	-0.15 $\pm$ 0.12	-0.05 $\pm$ 0.09

^aNo evaluable melting curves and first derivatives with clearly defined melting points were obtained.

^bSolubility problems at a concentration of 1 mM.

Table 3.51: $\Delta T_m \pm SD$ of T-p53C and its mutants (8 μ M) incubated with 1 mM SN006/7-8 SAR compounds for 4 h at 20 °C. Measurements were performed at least in triplicate. Fragments with a ΔT_m of at least 0.50 °C are highlighted in bold [99, 130].

Compound	T-p53C	T-p53C- Y220C	T-p53C- R273H	T-p53C- R282W	T-p53C- C124/182/ 277S
	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]
SN006/7-9	N/A ^a	N/A ^a	N/A ^a	N/A ^a	-2.40 $\pm$ 0
SN006/7-10	0.20 $\pm$ 0.11	-0.03 $\pm$ 0.18	0 $\pm$ 0	-0.05 $\pm$ 0.09	0.40 $\pm$ 0.09
SN006/7-11 ^b	0.45 $\pm$ 0.14	0 $\pm$ 0.11	0.10 $\pm$ 0.09	-0.20 $\pm$ 0.17	0.40 $\pm$ 0.09
SN006/7-12	-0.08 $\pm$ 0.09	-0.03 $\pm$ 0.09	-0.20 $\pm$ 0.09	-0.15 $\pm$ 0.15	0 $\pm$ 0
SN006/7-13	0.03 $\pm$ 0.08	-0.03 $\pm$ 0.11	-0.15 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
SN006/7-14	0.80 $\pm$ 0.08	0.73 $\pm$ 0.11	0.15 $\pm$ 0	0.75 $\pm$ 0	0 $\pm$ 0
SN006/7-15 ^b	0.45 $\pm$ 0.36	0.45 $\pm$ 0.20	-0.40 $\pm$ 0.09	-0.45 $\pm$ 0	0.15 $\pm$ 0.15
SN006/7-16	-1.85 $\pm$ 0.32	1.70 $\pm$ 0.09	N/A ^a	N/A ^a	-0.80 $\pm$ 0.09
SN006/7-17	-0.08 $\pm$ 0.09	-0.15 $\pm$ 0	-0.20 $\pm$ 0.09	-0.30 $\pm$ 0.15	0 $\pm$ 0
SN006/7-18	-2.25 $\pm$ 0.12	1.40 $\pm$ 0.09	-2.60 $\pm$ 0.31	N/A ^a	-1.50 $\pm$ 0.15
SN006/7-19	0.10 $\pm$ 0.11	-0.15 $\pm$ 0	-0.05 $\pm$ 0.09	-0.25 $\pm$ 0.17	0 $\pm$ 0

^aNo evaluable melting curves and first derivatives with clearly defined melting points were obtained.

^bSolubility problems at a concentration of 1 mM.

Table 3.52: $\Delta T_m \pm SD$ of T-p53C and its mutants (8 μ M) incubated with 1 mM SN006/7-8 SAR compounds for **24 h** at 20 °C. Measurements were performed at least in triplicate. Fragments with a ΔT_m of at least 0.50 °C are highlighted in bold [99, 130].

Compound	T-p53C	T-p53C- Y220C	T-p53C- R273H	T-p53C- R282W	T-p53C- C124/182/ 277S
	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]	$\Delta T_m \pm SD$ [°C]
SN006/7-9	N/A ^a	N/A ^a	N/A ^a	N/A ^a	-2.55 $\pm$ 0
SN006/7-10	0.13 $\pm$ 0.10	0.10 $\pm$ 0.08	-0.05 $\pm$ 0.12	-0.25 $\pm$ 0.31	0.15 $\pm$ 0.15
SN006/7-11 ^b	0.32 $\pm$ 0.14	0.07 $\pm$ 0.09	0.20 $\pm$ 0.09	-0.10 $\pm$ 0.09	0.15 $\pm$ 0
SN006/7-12	-0.05 $\pm$ 0.12	0.05 $\pm$ 0.08	-0.15 $\pm$ 0.12	-0.15 $\pm$ 0	0 $\pm$ 0
SN006/7-13	0.05 $\pm$ 0.10	0.05 $\pm$ 0.08	0 $\pm$ 0.12	-0.10 $\pm$ 0.09	0 $\pm$ 0
SN006/7-14	0.97 $\pm$ 0.11 and 2.80 $\pm$ 0.14	1.18 $\pm$ 0.13	0.95 $\pm$ 0.09	1.10 $\pm$ 0.35	0.65 $\pm$ 0.09
SN006/7-15^b	0.05 $\pm$ 0.11	0.58 $\pm$ 0.23	-0.90 $\pm$ 0.12	-1.40 $\pm$ 0.09	-0.10 $\pm$ 0.17
SN006/7-16	0.20 $\pm$ 0.38	2.30 $\pm$ 0.25	N/A ^a	N/A ^a	0.95 $\pm$ 0.09
SN006/7-17	-0.08 $\pm$ 0.29	0.10 $\pm$ 0.09	-0.10 $\pm$ 0.17	-0.05 $\pm$ 0.09	-0.10 $\pm$ 0.17
SN006/7-18	-1.33 $\pm$ 0.16	2.25 $\pm$ 0.24	-1.55 $\pm$ 0.24	N/A ^a	-1.00 $\pm$ 0.09
SN006/7-19	-0.03 $\pm$ 0.14	0.10 $\pm$ 0.09	-0.05 $\pm$ 0.09	-0.10 $\pm$ 0.09	0 $\pm$ 0

^aNo evaluable melting curves and first derivatives with clearly defined melting points were obtained.

^bSolubility problems at a concentration of 1 mM.

Table 3.53: $\Delta T_m \pm SD$ of T-p53C (8 μ M protein) incubated with 1 mM or 250 μ M SN006/7-8 SAR hit compounds for 30 min, 4 h, and 24 h at 20 °C.

T-p53C						
$\Delta T_m \pm SD$ [°C]						
	1 mM 30 min	1 mM 4 h	1 mM 24 h	250 μM 30 min	250 μM 4 h	250 μM 24 h
SN006 /7-9	-4.85 ± 0.17	N/A ^a	N/A ^a	-2.85 ± 0.12	-2.30 ± 0.22	-0.13 ± 0.30
SN006 /7-14	0.30 ± 0.12	0.80 ± 0.08	0.97 ± 0.11 and 2.80 ± 0.14	0.10 ± 0.09	0.13 ± 0.08	0.47 ± 0.10
SN006 /7-15^b	0.05 ± 0.12	0.45 ± 0.36	0.05 ± 0.11	0.15 ± 0.12	0.18 ± 0.11	0.12 ± 0.14
SN006 /7-16	-2.05 ± 0.17	-1.85 ± 0.32	0.20 ± 0.38	-0.65 ± 0.09	1.13 ± 0.09	N/A ^a
SN006 /7-18	-2.00 ± 0.23	-2.25 ± 0.12	-1.33 ± 0.16	-0.70 ± 0.12	-0.73 ± 0.15	0.57 ± 0.35

^aNo evaluable melting curves and first derivatives with clearly defined melting points were obtained.^bSolubility problems at a concentration of 1 mM.**Table 3.54:** $\Delta T_m \pm SD$ of T-p53C-Y220C (8 μ M protein) incubated with 1 mM or 250 μ M SN006/7-8 SAR hit compounds for 30 min, 4 h, and 24 h at 20 °C.

T-p53C-Y220C						
$\Delta T_m \pm SD$ [°C]						
	1 mM 30 min	1 mM 4 h	1 mM 24 h	250 μM 30 min	250 μM 4 h	250 μM 24 h
SN006 /7-9	-1.30 ± 0.24	N/A ^a	N/A ^a	0.40 ± 0.17	0.93 ± 0.12	2.60 ± 0.19
SN006 /7-14	0.35 ± 0.12	0.73 ± 0.11	1.18 ± 0.13	0.20 ± 0.12	0.20 ± 0.11	0.38 ± 0.11
SN006 /7-15^b	0.10 ± 0.17	0.45 ± 0.20	0.58 ± 0.23	0.50 ± 0.12	0.56 ± 0.11	0.75 ± 0.16
SN006 /7-16	-0.55 ± 0.12	1.70 ± 0.09	2.30 ± 0.25	0.30 ± 0.12	1.95 ± 0.19	2.57 ± 0.08
SN006 /7-18	1.10 ± 0.12	1.40 ± 0.09	2.25 ± 0.24	2.15 ± 0.24	2.90 ± 0.09	3.00 ± 0.12

^aNo evaluable melting curves and first derivatives with clearly defined melting points were obtained.^bSolubility problems at a concentration of 1 mM.

3.7.2 Intact Protein Mass Spectrometry of T-p53C-Y220C with SN006/7-8 SAR Hits

Figure 3.85 shows the deconvoluted MS spectra of T-p53C-Y220C with the identified hits SN006/7-9, SN006/7-14, SN006/7-15, SN006/7-16, and SN006/7-18. All SN006/7-8 SAR DSF hits multiply modified the Y220C mutant after 24 h of incubation at a protein-to-compound ratio of 1:100.

The deconvoluted MS spectrum of fragment SN006/7-9 (Figure 3.86 A) showed up to triple arylated T-p53C-Y220C. Moreover, a peak for the native protein was evident. Similarly, in the spectrum of SN006/7-14 (Figure 3.86 B), up to three times modified protein was visible.

Figure 3.86 C shows that SN001-15 arylated the Y220C mutant up to two times, despite the low stabilization of the Y220C mutant in the DSF experiment by up to 0.75 °C (250 μ M, 24 h). The spectrum indicated that the single arylated protein species displayed the highest signal, and a peak for the native protein was also discernible. However, it should be noted that the results must be interpreted with caution as the purity of SN006/7-15 was not confirmed by HPLC-UV.

As expected, fragments SN006/7-16 and SN006/7-18, which contain an additional electron-withdrawing methoxycarbonyl or CF₃ group and an extra chlorine atom at the pyridine ring, exhibited multiple arylations and other covalent modifications of T-p53C-Y220C. In both deconvoluted MS spectra (Figure 3.86 D, E), there were additional peaks, indicating mass increases of the protein. However, these peaks could not be attributed to a specific compound reaction or protein modification.

In summary, the intact protein MS results emphasize that all tested SN006/7-8 SAR compounds generated multiple adducts of T-p53C-Y220C and did not exhibit a higher selectivity for the Y220C mutant than the previously discovered hit SN006/7-8.

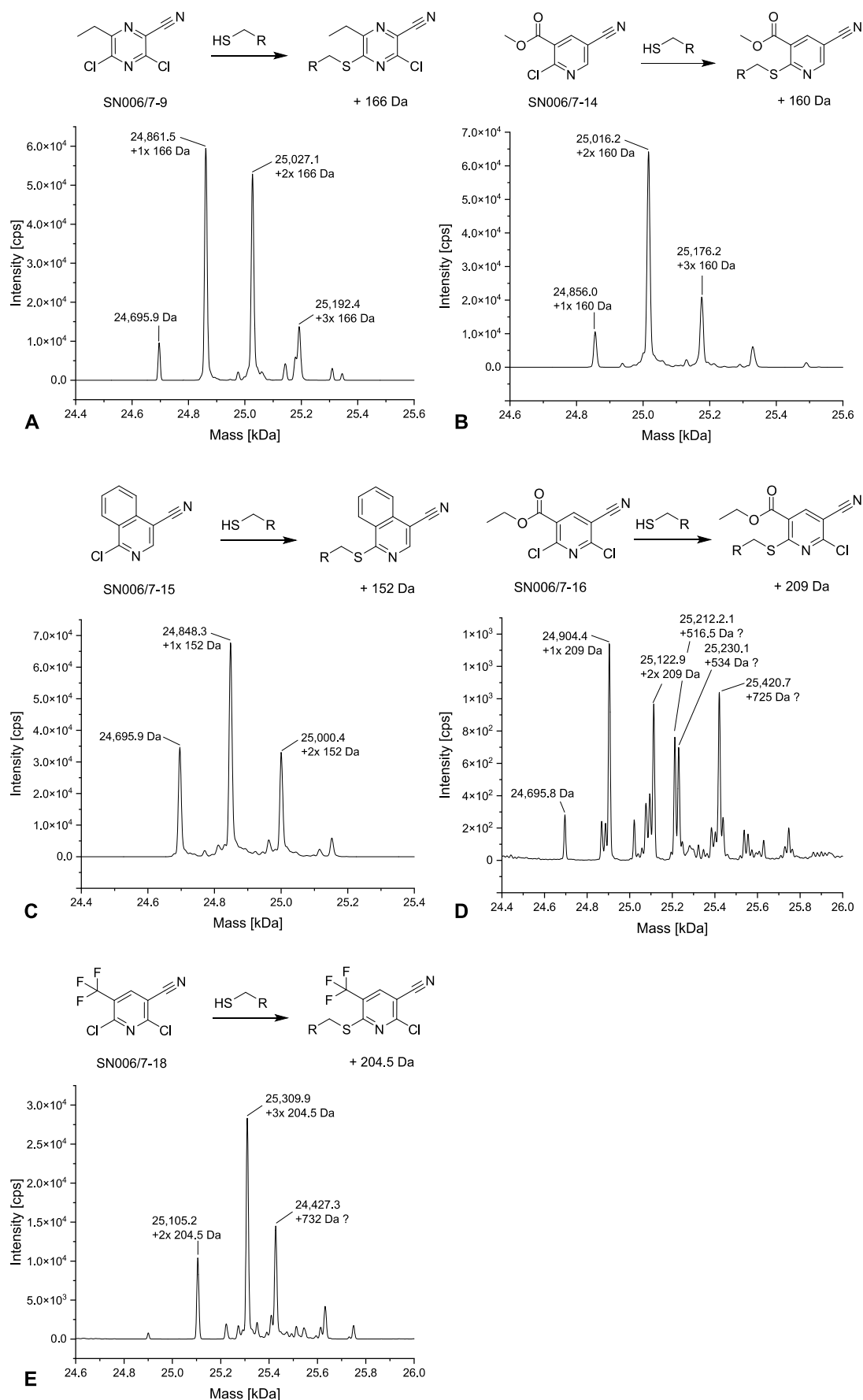


Figure 3.86: Deconvoluted MS spectra of T-p53C-Y220C with SN006/7-9 (A), SN006/7-14 (B), SN006/7-15 (C), SN006/7-16 (D), and SN006/7-18 (E) at protein-to-compound ratio of 1:100 after 24 h of incubation at 20 °C (theoretical mass of unmodified T-p53C-Y220C: 24,696.00 Da).

3.7.3 Determination of the Binding Mode of SN006/7-9 Using X-Ray Crystallography

The highly reactive fragment SN006/7-9 was identified as a cleft binder and Cys220 arylating compound using X-ray crystallography. As illustrated in Figure 3.87, compound SN006/7-9 reacted with Cys220 via an S_NAr reaction, with the displacement of the chlorine atom in the para position to the nitrile group. Similar to fragments SN001, SN006, SN001-17, and SN006/7-8, the compound SN006/7-9 also adopted a Tyr-like conformation, occupying the central cavity space. Its electron-withdrawing CN moiety pointed towards subsite 1 and Thr150. Moreover, the ortho-chlorine pointed towards subsite 2. As anticipated, the ethyl group at position 6 of the pyrazine ring pointed into the hydrophobic subsite 3. In contrast to the pyrazine derivative SN006/7-3, subsite 2 of the binding pocket was not occupied.

Additional modifications were found at Cys182 and Cys277 after soaking for one day with 5 mM SN006/7-9.

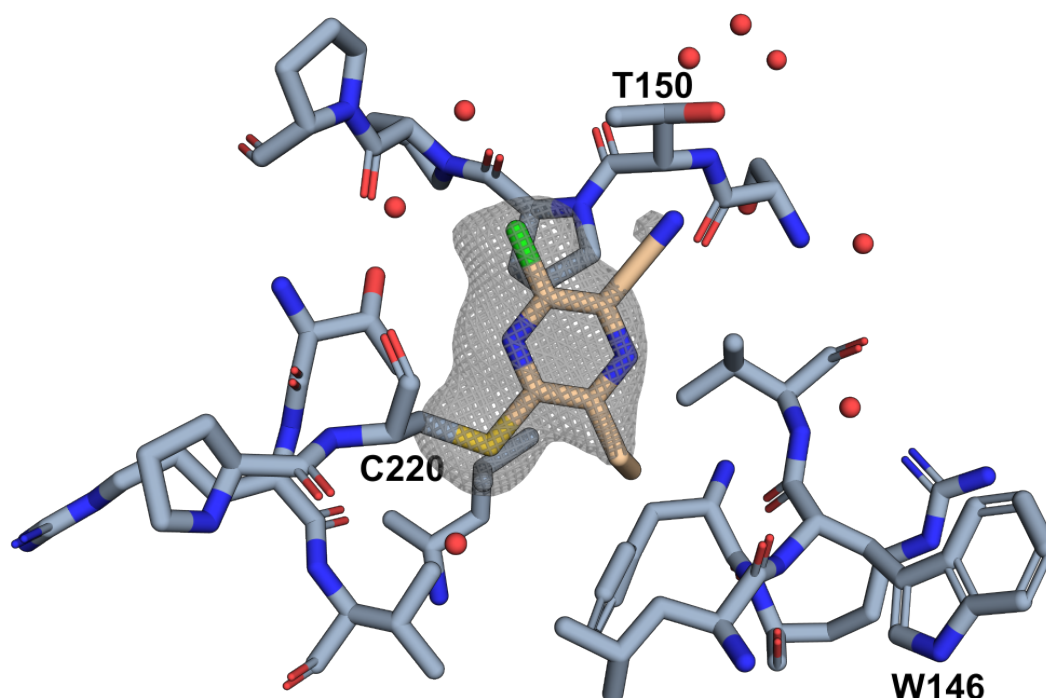


Figure 3.87: Overview of the binding mode of SN006/7-9 (soaked at 5 mM) into the Y220C induced crevice. The compound adopts a Tyr-like conformation, with the nitrile group pointing towards subsite 1 and Thr150. The ortho-chlorine points towards subsite 2, and the meta-ethyl group points into the hydrophobic subsite 3. The unbiased omit map is shown at a contour level of 3 σ .

4 Materials and Methods

4.1 Materials

Reagents, chemicals, and materials used in this work were obtained from standard vendors such as abcr GmbH (Karlsruhe, Germany), BLD Pharmatech Ltd. (Shanghai, China), Carl Roth (Karlsruhe, Germany), Jena Bioscience (Jena, Germany), Merck KGaA (Darmstadt, Germany), Sarstedt AG & Co. KG (Nümbrecht, Germany), Thermo Fisher Scientific (Waltham, MA, USA), VWR International (Bridgeport, NJ, USA). ^{15}N -labeled ammonium chloride was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA).

4.1.1 Screening Compounds

The screening compounds of the CovLib and the SN001, SN006/7, and VS004 SAR compounds were purchased from Aldrich Market Select (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) at purity levels of 90% or higher. The SN001-17, SN006/7-3, and SN006/7-8 SAR compounds were purchased from suppliers listed in Table 4.1. Purity was confirmed by HPLC on an Ultimate 3000 HPLC-System (Thermo Fisher Scientific, Dreieich, Germany) or ^1H nuclear magnetic resonance (NMR) spectroscopy (Bruker Avance III HD 400 MHz).

Table 4.1: Purity and suppliers of the SN001-17, SN006/7-3, and SN006/7-8 SAR compounds.

Compound	Name	Purity	Supplier
SN001-34	2,5,6-trichloro-4-methylnicotinonitrile	98%	BLD Pharmtech GmbH, Kaiserslautern, Germany
SN006/7-7	5-chloro-3-methylpyrazine-2-carbonitrile	95%	BLD Pharmtech Ltd., Shanghai, China
SN006/7-8	5-chloro-6-methylpyrazine-2-carbonitrile	95%	BLD Pharmtech Ltd., Shanghai, China
SN009	2-chloropyrimidine-5-carbonitrile	97%	BLD Pharmtech GmbH, Kaiserslautern, Germany
SN010	4-amino-2-chloropyrimidine-5-carbonitrile	97%	BLD Pharmtech GmbH, Kaiserslautern, Germany
SN011	6-chloropyridazine-3-carbonitrile	97%	BLD Pharmtech GmbH, Kaiserslautern, Germany
SN012	6-chloro-5-methylpyridazine-3-carbonitril	98%	BLD Pharmtech Ltd., Shanghai, China
SN006/7-9	3,5-dichloro-6-ethylpyrazine-2-carbonitrile	95%	AstaTech Inc., Bristol, PA, USA
SN006/7-10	1,3-dichloro-6,7-dihydro-5 <i>H</i> -cyclopenta[<i>c</i>]pyridine-4-carbonitrile	95%	Enamine Ltd., Kyiv, Ukraine
SN006/7-11	2,6-dichloro-5-phenylnicotinonitrile	95%	BLD Pharmtech Ltd., Shanghai, China
SN006/7-12	6-chloro-5-methylnicotinonitrile	98%	BLD Pharmtech Ltd., Shanghai, China
SN006/7-13	6-fluoro-5-methylnicotinonitrile	95%	BLD Pharmtech Ltd., Shanghai, China
SN006/7-14	methyl 2-chloro-5-cyanonicotinate	99.30%	ChemScene, Monmouth Junction, NJ, USA
SN006/7-15	1-chloroisoquinoline-4-carbonitrile	N/A	Angene International Limited, Nanjing, China
SN006/7-16	ethyl 2,6-dichloro-5-cyanonicotinate	97%	BLD Pharmtech Ltd., Shanghai, China
SN006/7-17	4-chlorothieno[3,2- <i>c</i>]pyridine-7-carbonitrile	N/A	Angene International Limited, Nanjing, China
SN006/7-18	2,6-dichloro-5-(trifluoromethyl)nicotinonitrile	99.05%	BLD Pharmtech Ltd., Shanghai, China
SN006/7-19	2,6-dichloro-5-cyclopropylnicotinonitrile	95%	BLD Pharmtech Ltd., Shanghai, China

4.2 Culture Media

The media were prepared with pure water from an ELGA PURELAB Ultra system (ELGA LabWater, Bucks, UK). The pH was adjusted to 7.0 using a strong base or acid, as required. Before usage, each medium was autoclaved (HICLAVE HV-85L, Manitowoc Foodservice, Manitowoc, USA) at 121 °C for 20 min.

For the expression of ^{15}N -labeled protein, M9 minimal medium was prepared [333]. Therefore, M9 salt solution (10x), 1 M MgSO_4 , and 1 mM CaCl_2 were autoclaved at 121 °C for 20 min. 20% glucose, biotin (1 mg/mL), thiamine (1 mg/mL), and 100x trace elements solution were sterilized using a 0.22 μm filter.

The compositions of all media used in the experiments are listed in Table 4.2.

Table 4.2: Compositions of the culture media.

Medium	Composition
LB (lysogeny broth)	15 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, optional 1.5-2% agar, pH 7.0
2xYT (2x yeast extract and tryptone)	16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl, pH 7.0
SOC (super optimal broth with catabolite repression)	20 g/L tryptone, 5 g/L yeast extract, 0.5 g/L NaCl, pH was adjusted to 7.4 and autoclaved for 20 min at 121 °C. 20 mL/L sterile filtered glucose (20%) and 10 mL/L sterilized MgCl_2 (1 M) were added.
M9 minimal medium (1 L)	100 mL M9 salt solution (10x) (75.2 g/L $\text{Na}_2\text{HPO}_4\cdot 2\text{H}_2\text{O}$, 30 g/L KH_2PO_4 , 5 g/L NaCl), 20 mL glucose (20% w/v), 1 mL MgSO_4 (1 M), 0.3 mL CaCl_2 (1 M), 1 mL biotin (1 mg/mL), 1 mL thiamine (1 mg/mL), 10 mL trace element solution (100x) (5 g/L EDTA, 0.83 g/L $\text{FeCl}_3\cdot 6\text{H}_2\text{O}$, 84 mg/L ZnCl_2 , 13 mg/L $\text{CuCl}_2\cdot 2\text{H}_2\text{O}$, 10 mg/L $\text{CoCl}_2\cdot 6\text{H}_2\text{O}$, 10 mg/L H_3BO_3 , 1.6 mg/L $\text{MnCl}_2\cdot 4\text{H}_2\text{O}$), 1 g $^{15}\text{NH}_4\text{Cl}$, 867 mL sterile water

4.3 Buffers for Protein Expression and Purification

The buffer recipes were created using a buffer calculator tool [334]. The recipes were prepared with consideration of the temperature at which the buffer will be used and the influence of ionic strength. All buffers were prepared with pure water from an ELGA PURELAB Ultra system (ELGA LabWater, Bucks, UK). Prior to utilization on the ÄKTA systems (Cytiva, Global Life Science Solution USA LLC, Marlborough, MA, USA), the buffers were filtered through a 0.22 µm filter. All buffers were stored at 4 °C.

4.3.1 TEV Protease

The recipes of the buffers for the expression and purification of TEV protease are displayed in Table 4.3.

Table 4.3: Buffers for the expression and purification of TEV protease.

Buffer	Composition
Lysis buffer	50 mM 2-amino-2-hydroxymethyl-propane-1,3-diol (TRIS), pH 7.5, 500 mM NaCl, 10 mM imidazole, 5 mM DTT
Elution buffer	50 mM TRIS, pH 7.5, 500 mM NaCl, 300 mM imidazole, 5 mM DTT

4.3.2 xT-/T-p53C, xT-/T-p53C-Y220C, T-p53C-R273H, xT-/T-p53C-R282W, T-p53C-C124/182/277S, T-p53C-Y220C-C124/182/229/275/277S, FL-T-p53

The recipes of the buffers for Ni²⁺-column, heparin column, and for gel filtration column for the expression of T-p53C and its mutants are listed in Table 4.4. For the expression of FL-T-p53, the lysis buffer was additionally supplemented with cOmplete™, EDTA-free Protease Inhibitor Cocktail (Roche Diagnostics GmbH, Mannheim, Germany).

Table 4.4: Buffers for the expression and purification of the xT-/T-p53C variants.

Buffer	Composition
Lysis buffer	50 mM KH ₂ PO ₄ /K ₂ HPO ₄ (KPi), pH 8.0, 300 mM NaCl, 10 mM imidazole, 2 mM TCEP, optional 1 tablet cOmplete™/ 50 mL lysis buffer
Elution buffer	50 mM KPi, pH 8.0, 300 mM NaCl, 250 mM imidazole, 2 mM TCEP
Heparin buffer A	25 mM KPi, pH 7.5, 5 mM DTT
Heparin buffer B	25 mM KPi, pH 7.5, 2 M NaCl, 5 mM DTT
SEC buffer	25 mM KPi pH 7.2, 150 mM NaCl, 5 mM DTT

4.3.3 JNK3

The recipes of the buffers for Ni²⁺-column, reverse Ni²⁺-column, and for gel filtration column for the expression and purification of JNK3 are depicted in Table 4.5.

Table 4.5: Buffers for the expression and purification of JNK3.

Buffer	Composition
Lysis buffer	50 mM TRIS, pH 7.4, 500 mM NaCl, 10 mM imidazole, 5 mM β -mercaptoethanol
Elution buffer	50 mM TRIS, pH 7.4, 500 mM NaCl, 300 mM imidazole, 5 mM β -mercaptoethanol
SEC buffer	50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.0, 100 mM NaCl, 2 mM MgCl, 5% [v/v] glycerol, 5 mM β -mercaptoethanol

4.4 Antibiotics

A stock solution of kanamycin and ampicillin was prepared in water at a concentration of 100 mg/mL. The stock solutions were sterile-filtered through a 0.22 μ m syringe filter. The ampicillin solution was freshly prepared. A 34 mg/mL stock solution of chloramphenicol in ethanol was utilized. The working concentrations of kanamycin and ampicillin were 50 μ g/mL and 34 μ g/mL of chloramphenicol, respectively.

4.5 Bacterial Strains

For the heat shock transformation, the following *E.coli* cell lines were utilized:

- *E.coli* DH5 α (Thermo Fisher Scientific, Waltham, MA, USA)
- *E.coli* BL21(DE3)pLysS (Novagen, Merck KGaA, Darmstadt, Germany)
- NEB 5-alpha Competent *E.coli* (High Efficiency) Cells (NEB #C2987, New England Biolabs, Ipswich, MA, USA)

E.coli DH5 α chemical competent cells were used for DNA replication, while *E.coli* BL21(DE3)pLysS cells were utilized for protein expression.

4.6 Vectors

All p53 genes used in this work were subcloned into the pET24a(+)-HLT vector between the BamHI and EcoRI restriction sites. The HLT-domain consists of a 6x His-tag, followed by a lipoyl domain [335] (1-85) from *Bacillus stearothermophilus* and a TEV protease cleavage site, ENLYFQ'XYZ [336]. The TEV protease cuts proteins after these six amino acids (ENLYFQ'GGS), and the amino acids GGS are left at the N-terminus after cleavage. For crystallization experiments, a pET24a(+)-HLT vector with a deletion of the GGS overhang after the TEV cleavage site was applied. The constructs without GGS overhang are hereafter referred to as xT-p53C. For all experiments, the thermostable mutant of p53, containing the mutations M133L/V203A/N239Y/N268D, was utilized [163]. The genes for FL-T-p53 and T-p53C-Y220C-CL were synthesized by BioCat (BioCat GmbH, Heidelberg, Germany) and subcloned into the pET24a(+)-HLT vector between the BamHI and EcoRI restriction site. Table 4.6 represents all vectors used in this work.

Table 4.6: Vectors for protein expression.

Insert	Vector	Cleavage Site	Antibiotic resistance
T-p53C (94–312, M133L/V203A/N239Y/N268D)	pET24a(+)- HLT	ENLYFQ'G (TEV protease)	kanamycin
xT-p53C (94–312, M133L/V203A/N239Y/N268D)	pET24a(+)- HLT	ENLYFQ'S (TEV protease)	kanamycin
T-p53C-Y220C (94–312, M133L/V203A/N239Y/N268D/Y220C)	pET24a(+)- HLT	ENLYFQ'G (TEV protease)	kanamycin
xT-p53C-Y220C (94–312, M133L/V203A/N239Y/N268D/Y220C)	pET24a(+)- HLT	ENLYFQ'S (TEV protease)	kanamycin
T-p53C-R273H (94–312, M133L/V203A/N239Y/N268D/R273H)	pET24a(+)- HLT	ENLYFQ'G (TEV protease)	kanamycin
xT-p53C-R273H (94–312, M133L/V203A/N239Y/N268D/R273H)	pET24a(+)- HLT	ENLYFQ'S (TEV protease)	kanamycin
T-p53C-R282W (94–312, M133L/V203A/N239Y/N268D/R282W)	pET24a(+)- HLT	ENLYFQ'G (TEV protease)	kanamycin
xT-p53C-R282W (94–312, M133L/V203A/N239Y/N268D/R282W)	pET24a(+)- HLT	ENLYFQ'S (TEV protease)	kanamycin
T-p53C-C124/182/277S (94–312, M133L/V203A/N239Y/N268D/ C124S/C182S/C277S)	pET24a(+)- HLT	ENLYFQ'G (TEV protease)	kanamycin
T-p53C-Y220C-C124/182/229/275/277S (94–312, M133L/V203A/N239Y/N268D/ Y220C/C124S/C182S/C229S/C275S/C277S)	pET24a(+)- HLT	ENLYFQ'G (TEV protease)	kanamycin
FL-T-p53 (1–393, M133L/V203A/N239Y/N268D)	pET24a(+)- HLT	ENLYFQ'G (TEV protease)	kanamycin
JNK3 (39–402)	pET24a(+)- HLT	ENLYFQ'G (TEV protease)	kanamycin
TEV	pRK792		ampicillin

4.7 Methods in Molecular Biology

4.7.1 Preparation of CaCl₂ Competent Cells

First, a 100 mL 2xYT preculture of the desired cells was prepared. For *E.coli* DH5 α (Thermo Fisher Scientific, Waltham, MA, USA) cells, no antibiotic was added; for *E.coli* BL21(DE3)pLysS cells (Novagen, Merck KGaA, Darmstadt, Germany), 34 μ g/mL chloramphenicol was included. The preculture was incubated overnight at 37 °C and 180 rpm on a Multitron II (INFORS HT, Bottmingen-Basel, Switzerland). Next, 100 mL 2xYT medium with the appropriate antibiotic was inoculated with 1-3 mL preculture and cultured to an OD₆₀₀ of 0.4 to 0.6 at 37 °C and 180 rpm. The cells were harvested by centrifugation using a Heraeus Multifuge X3FR (Fisher Scientific GmbH, Schwerte, Germany) at 3,500 rpm, 4 °C for 10 min. The pellet was subsequently washed in 30 mL of ice-cold 0.1 M CaCl₂ solution and re-centrifuged at 3,000 rpm, 4 °C for 5 min. Cells were thereafter resuspended in 20 mL of ice-cold 0.1 M CaCl₂ solution and incubated on ice for 20 min. Another centrifugation step was then carried out at 3,000 rpm, 4 °C for 5 min. Finally, the pellet was resuspended in 1 mL of ice-cold 0.1 M CaCl₂ solution containing 15% glycerol, aliquoted to 100 μ L, and flash frozen at -80 °C.

4.7.2 Heat Shock Transformation

For the heat shock transformation, 100 μ L of CaCl₂ competent *E.coli* cells were slowly thawed on ice. Next, 2 μ L of plasmid DNA solution was added to the competent cells. Thereafter, the mixture was incubated on ice for 30 min. Subsequently, the heat shock was performed at 42 °C for exactly 2 min. After placing on ice for 5 min, 1 mL of SOC-medium without antibiotics was added for regeneration. The mixture was incubated for 1 h at 37 °C while shaking at 300 rpm. Next, 200 μ L of the cell suspension was plated on LB plates containing the appropriate antibiotic (low concentration). The remaining suspension was then centrifuged for 3-5 min at 3,000 rpm. After discarding the supernatant, except for 200 μ L, the pellet was resuspended, and the suspension was also plated onto a selection plate (high concentration). Finally, both plates were incubated overnight at 37 °C.

4.7.3 Primers for PCR

All primers for PCR mutagenesis were designed with the NEBaseChanger[®] tool [162]. Primers were ordered in a 0.025 μ M scale from Merck KGaA (Darmstadt, Germany), purified by desalting. Lyophilized primers were dissolved in an appropriate volume of autoclaved pure water to 100 μ M and subsequently diluted to 10 μ M for PCR mutagenesis. The sequences of all primers used in this work are listed in Table 4.7.

Table 4.7: Primers for PCR mutagenesis.

Primer	Sequence
T-p53C-R273H-for	5'-CTTTGAGGTGCATGTTTGTGCCTG-3'
T-p53C-R273H-rev	5'-CTGTCCCGTCCCAGTAGA-3'
T-p53C-R282W-for	5'-TGGGAGAGACTGGCGCACAGAGG-3'
T-p53C-R282W-rev	5'-GGACAGGCACAAACACGCAC-3'

4.7.4 PCR Mutagenesis

All mutations were introduced by PCR using the Q5[®] Site-Directed Mutagenesis Kit (New England Biolabs, Ipswich, MA, USA). First, the reagents depicted in Table 4.8 were mixed in a PCR tube.

Table 4.8: Reagents for PCR mutagenesis.

Component	Volume
Q5 Hot Start High-Fidelity 2x Master Mix	12.5 μ L
10 μ M Forward Primer	1.25 μ L
10 μ M Reverse Primer	1.25 μ L
Template DNA (1-25 ng/ μ L)	1 μ L
Nuclease-free water	9.0 μ L

Thereafter, the mixture was transferred to a TC-9639 Thermal Cycler (Benchmark Scientific, Sayreville, NJ, USA), and the PCR program listed in Table 4.9 was executed. The appropriate annealing temperatures for each primer were determined and selected using the NEBaseChanger[®] tool [162].

Table 4.9: PCR cycling conditions.

Step	xT-/T-p53C-R273H		xT-/T-p53C-R282W	
	Temp.	Time	Temp.	Time
Initial Denaturation	98 °C	30 sec	98 °C	30 sec
	98 °C	10 sec	98 °C	10 sec
25 Cycles	67 °C	20 sec	70 °C	20 sec
	72 °C	3 min	72 °C	3 min
Final Extension	72 °C	2 min	72 °C	2 min
Hold	4 °C		4 °C	

After each PCR, the resulting PCR product was purified with the QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany) to increase the effectiveness of the subsequent transformation. The purification of the PCR products was carried out according to the manual. Subsequently, the purified PCR product was subjected to qualitative analysis by agarose gel electrophoresis in accordance with the methodology outlined in Section 4.7.6.

For the R282W mutations, a preparative agarose gel had to be performed because additional impurities were visible on the gel. After excising the desired DNA signal from the agarose gel, extraction and purification were performed using the QIAquick® Gel Extraction Kit and QIAquick® PCR & Gel Cleanup Kit (Qiagen, Hilden, Germany).

The next experimental step involved the KLD reaction. Therefore, the reagents listed in Table 4.10 were assembled. The mixture was further incubated at room temperature for 30 min. The KLD treatment ligated the PCR product and removed the template DNA.

Table 4.10: KLD reaction.

Component	Volume
PCR product	2 µL
2x KLD Reaction Buffer	5 µL
10x KLD Enzyme Mix	1 µL
Nuclease-free water	2 µL

Next, a heat shock transformation into NEB 5-alpha Competent *E.coli* (High Efficiency) Cells (NEB #C2987, New England Biolabs, Ipswich, MA, USA) was performed. In

deviation from the protocol described in Chapter 4.7.2, the heat shock was carried out only for 30 sec at 42 °C according to the kit instructions.

4.7.5 Isolation and Purification of Plasmid DNA

After mutagenesis and transformation into NEB 5-alpha Competent *E.coli* (High Efficiency) Cells (NEB #C2987, New England Biolabs, Ipswich, MA, USA), single clones were picked under sterile conditions and used to inoculate 7 mL of LB or 2xYT media containing 50 µg/mL kanamycin. Cells were grown overnight at 37 °C while shaking at 180 rpm on a Multitron II (Infors HT, Bottmingen-Basel, Switzerland). The overnight bacterial cultures were harvested by centrifugation at 4,000 g for 15 min at 4 °C. Next, the QIAprep® Spin Miniprep Kit (Qiagen, Hilden, Germany) was used for DNA isolation. Therefore, the Quick-Start Protocol (Qiagen, Hilden, Germany) was followed for lysis and DNA purification. Alternatively, the Monarch Plasmid Miniprep Kit (New England Biolabs, Ipswich, MA, USA) was used for DNA preparation. The concentration of the isolated plasmid DNA was further measured at 260 nm using a BioPhotometer plus (Eppendorf AG, Hamburg, Germany) equipped with a LabelGuard™ Microliter Cell (Implen GmbH, Munich, Germany). For sequencing, the samples were sent to Eurofins Genomics Germany GmbH (Ebersberg, Germany). The analysis of the sequencing results and the verification of the point mutation were carried out with CLC Sequence Viewer (Qiagen Bioinformatics, Aarhus, Denmark).

Subsequently, a Maxiprep was performed for each plasmid. Therefore, the correctly mutated plasmid DNA was retransformed into *E.coli* DH5α cells (Thermo Fisher Scientific, Waltham, MA, USA). A single clone was picked under sterile conditions and used to inoculate 100 mL of LB or 2xYT media containing 50 µg/mL kanamycin. The overnight bacterial culture was harvested by centrifugation at 4,000 g for 20 min at 4 °C. The Qiagen® Plasmid Maxi Kit and the Plasmid Buffer Set (Qiagen, Hilden, Germany) were utilized for DNA preparation. Concentration measurements and sequence analysis were performed as previously described. Finally, the plasmid DNA was stored at -20 °C in TRIS buffer (10 mM TRIS, pH 8.5).

4.7.6 Agarose Gel Electrophoresis

For analysis of the PCR products, a 0.8% agarose gel in TRIS-acetate-EDTA (TAE) buffer (50 mL) was produced. The composition of the TAE buffer is shown in Table 4.11. A mixture of 3 μ L Gel Loading Dye, Blue (6x) (New England BioLabs, Ipswich, MA, USA) and 2 μ L PCR product was filled into the gel pockets. For a preparative gel, the PCR product was mixed with loading dye at a ratio of 5:1, and 40 μ L of the mixture was loaded into the pockets. The Gene Ruler™ 1 kb DNA Ladder (Merck KGaA, Darmstadt, Germany) served as a marker. The agarose gel electrophoresis was conducted for 1 h to 1.5 h at 100 V. After the run, the gel was stained with SYBR™ Gold nucleic acid gel stain (Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA). The gels were analyzed on a Gel Doc Xr+ gel documentation system (Bio-Rad Laboratories Inc., Hercules, CA, USA), and pictures of the gel were taken with the Image Lab™ Software (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Table 4.11: Recipe of the TAE buffer for agarose gel electrophoresis.

Buffer	Composition
TAE buffer 1x	40 mM TRIS, pH 8.0, 20 mM acetic acid, 1 mM EDTA

4.8 Protein Expression and Purification

4.8.1 Expression and Purification of TEV Protease

The plasmid of the TEV protease (prK792) with the S129P mutation from David Waugh [337] was purchased from Addgene (ID: #8830, Watertown, MA, USA). First, a heat shock transformation of the TEV plasmid into CaCl_2 competent *E.coli* BL21(DE3)pLysS cells (Novagen, Merck KGaA, Darmstadt, Germany) was performed. After overnight incubation at 37 °C on LB plates, containing ampicillin (50 μ g/mL) and chloramphenicol (37 μ g/mL), the bacterial colonies were washed from the plates using 10 mL of LB medium with both antibiotics. The solution was then transferred into 90 mL of the same media. The cells were grown in this pre-culture at 37 °C and 180 rpm on a Multitron II (Infors HT, Bottmingen-Basel, Switzerland) until reaching an OD_{600} of around 1. Each 10 mL of this culture was used to inoculate 6 L of 2xYT medium containing both

antibiotics. These main cultures were grown at 37 °C and 220 rpm. At an OD₆₀₀ of 0.4, the temperature was lowered to 20 °C, and the cells were induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). The TEV protease was expressed overnight at 20 °C and 220 rpm. The next morning, cells were harvested by centrifugation at 4 °C, 4,000 rpm for 30 min using a J6-MI centrifuge (Beckman-Coulter, Brea, CA, USA). The cell pellets were resuspended in ice-cold lysis buffer, and a spatula of deoxyribonuclease (DNase) and ribonuclease (RNase) was added to the cell suspension. Next, the cells were lysed by sonication using a Bandelin Sonopuls HD 3200 with KE76 sonotrode (Bandelin Electronic GmbH & Co. KG, Berlin, Germany). The sonication procedure comprises five cycles (3.6 kJ per cycle) of 4 min programs with alternating 15 s pulses (20% amplitude) and 30 s pauses. The cell solution was kept on ice during sonication and stirred between each cycle. After sonication, the lysate was centrifuged for 1 h at 18,500 rpm and 4 °C in an Avanti J-30 I with a pre-chilled JA-30.50 Ti rotor (Beckman-Coulter, Brea, CA, USA). The supernatant was sterile filtered on ice through a 0.22 μm filter (rapid-FILTERMAX, TTP®, Trasadingen, Switzerland) and subsequently purified on a Nickel-NTA column (HisTrap™ FF, Cytiva Sweden AB, Uppsala, Sweden) using an ÄKTA purification system (Cytiva, Global Life Science Solution USA LLC, Marlborough, MA, USA). The Nickel-NTA affinity column was pre-equilibrated with lysis buffer, and the sample (50 mL) was loaded onto the column. Thereafter, a wash step with five column volumes (CV) of lysis buffer was performed. The flow-through and wash step were collected for SDS-PAGE analysis. The protein was eluted from the column with a 300 mM concentration of imidazole using a linear gradient from 0% to 100% elution buffer in 15 min. Fractions of 3 mL were collected and analyzed by SDS-PAGE. The fractions containing the TEV protease were pooled and concentrated to around 1 mg/mL using a Vivaspin® Turbo 15 (molecular weight cut-off (MWCO)= 10 kD, Sartorius, Göttingen, Germany). Finally, the protein solution was flash frozen in liquid nitrogen and stored at -80 °C. The purity of the protein was verified by SDS-PAGE.

4.8.2 Expression and Purification of xT-/T-p53C, xT-/T-p53C-Y220C, T-p53C-R273H, xT-/T-p53C-R282W, T-p53C-C124/182/277S, T-p53C-Y220C-C124/182/229/275/277S

The expression and purification of xT-/T-p53C and all different point mutations were carried out according to the literature [99]. The plasmids were transformed into chemically competent *E.coli* BL21 (DE3) pLysS (Novagen, Merck KGaA, Darmstadt, Germany) cells. The cell suspension was either plated onto selection plates containing kanamycin (50 µg/mL) and chloramphenicol (37 µg/mL) and treated as described in Section 4.8.1, or directly pipetted into 100 mL 2xYT media supplemented with both antibiotics. Next, the 2xYT pre-culture was incubated overnight at 180 rpm on a Multitron II (Infors HT, Bottmingen-Basel, Switzerland) at 37 °C. The next morning, each 10 mL of this culture was used to inoculate 4 L of 2xYT medium containing kanamycin (50 µg/mL), chloramphenicol (37 µg/mL), and ZnSO₄ (1 mM). These main cultures were grown at 37 °C and 220 rpm. At an OD₆₀₀ of 0.6-0.8, the temperature was reduced to 24 °C, and the cells were induced with 1 mM IPTG. One drop of Antifoam (Merck KGaA, Darmstadt, Germany) was added, and cells were cultured at 24 °C, 220 rpm for 16-24 h. Cells were harvested by centrifugation at 4 °C and 4,000 rpm for 30 min using a J6-MI centrifuge (Beckman-Coulter, Brea, CA, USA). The cell pellets were resuspended in ice-cold lysis buffer (25 mL lysis buffer per 1 L 2xYT), and a spatula of DNase and RNase was added to the cell suspension. The cell solution was kept on ice during the following procedure. The cells were lysed by sonication (20% amplitude, five cycles (3.6 kJ per cycle) of 4 min programs with alternating 15 s pulses and 30 pauses) using a Bandelin Sonopuls HD 3200 with KE76 sonotrode (Bandelin electronic GmbH & Co. KG, Berlin, Germany). Afterwards, the lysate was centrifuged for 1 h at 18,500 rpm and 4 °C in an Avanti J-30 I with a pre-chilled JA-30.50 Ti rotor (Beckman-Coulter, Brea, CA, USA). The supernatant was sterile filtered through a 0.22 µm filter (rapid-FILTERMAX, TTP®, Trasadingen, Switzerland). Next, 50 mL of the filtered solution was loaded onto two connected 5 mL HisTrap™ FF columns (Cytiva Sweden AB, Uppsala, Sweden) equilibrated with lysis buffer using the ÄKTA purifier (Cytiva, Global Life Science Solution USA LLC, Marlborough, MA, USA). The column was washed with lysis buffer until a baseline UV signal was reached. The flow-through and the wash step were collected for an SDS-PAGE analysis. The protein was eluted from the column with elution buffer using a linear gradient from 0% to 100% over 15 min. Fractions of 3 mL were collected and analyzed by SDS-PAGE. Fractions

containing the p53 protein were pooled and supplemented with 1:5-1:10 [v/v] TEV protease (1 mg/mL). The mixture was cleaved overnight at 4 °C while stirring. The digested protein was mixed with heparin buffer A in a ratio of 1:5-8 [v/v]. Subsequently, the diluted protein (75 mL each) was loaded onto two connected 5 mL HiTrap™ Heparin HP columns (Cytiva Sweden AB, Uppsala, Sweden) equilibrated with heparin buffer A using the ÄKTA pure™ 25 M (Cytiva, Global Life Science Solution USA LLC, Marlborough, MA, USA). The purification program consisted of a wash step with 25 mL of heparin buffer A, followed by the elution with 20 mL of 40% heparin buffer B, and a wash step with 20 mL of 100% heparin buffer B. Fractions of 2 mL were collected during the elution, analyzed by SDS-PAGE, and those containing the digested protein were pooled. The protein was further purified on a HiLoad™ 26/60 Superdex 75 pg gel filtration column (Cytiva Sweden AB, Uppsala, Sweden) equilibrated with SEC buffer. Fractions of 4 mL were collected and analyzed by SDS-PAGE. Fractions containing the pure protein were pooled. Finally, the purified protein was concentrated to 50-100 µM using a Vivaspın® Turbo 15 (MWCO= 10 kD, Sartorius, Göttingen, Germany), flash frozen in liquid nitrogen, and stored at -80 °C. The purity of the protein was monitored by SDS-PAGE. The protein mass was confirmed by intact protein mass spectrometry. Protein concentrations were measured spectrophotometrically using the molar extinction coefficients (ϵ^{280}) of the respective proteins at 280 nm, calculated from the ProtParam tool on the Expasy server [338].

4.8.3 Expression and Purification of ¹⁵N-Labeled T-p53C-Y220C

For the expression of ¹⁵N-labeled T-p53C-Y220C, the transformed cells were plated onto 2xYT selection plates and incubated overnight at 37 °C. The next morning, a single clone was picked under sterile conditions and used to inoculate 100 mL of unlabeled minimal media. For this pre-culture, the ¹⁵NH₄Cl was exchanged with unlabeled NH₄Cl. Next, the pre-culture was incubated overnight at 37 °C and 180 rpm. Then, the expression of the labeled protein was performed in M9 minimal medium instead of 2xYT medium. The composition of the minimal medium is described in Table 4.2. All subsequent expression and purification steps were identical to those described in Chapter 4.8.2, except for the expression medium used.

4.8.4 Expression and Purification of FL-T-p53

The expression and purification of FL-T-p53 were mainly carried out as described in Chapter 4.8.2. Deviating from this protocol, cOmplete™, EDTA-free Protease Inhibitor Cocktail (Roche Diagnostics GmbH, Mannheim, Germany) was added to the lysis buffer (1 tablet per 50 mL lysis buffer). In addition, the final gel filtration step was performed on a HiLoad™ 26/60 Superdex 200 pg column (Cytiva Sweden AB, Uppsala, Sweden). Moreover, the purified FL p53 protein was concentrated using a Vivaspin® Turbo 15 (MWCO= 30 kD, Sartorius, Göttingen, Germany).

4.8.5 Expression and Purification of JNK3

The expression and purification of JNK3 (39-402), using a pET24a(+)_HLT_JNK3 construct, were carried out as previously described [192]. The plasmid was transformed into chemically competent *E.coli* BL21 (DE3) pLysS (Novagen, Merck KGaA, Darmstadt, Germany) cells. The cell suspension was plated onto selection plates containing kanamycin (50 µg/mL) and chloramphenicol (37 µg/mL). After overnight incubation at 37 °C, the bacterial colonies were washed from the plates using 10 mL of LB medium with both antibiotics. The solution was then transferred into 90 mL of the same media. The cells were grown in this pre-culture at 37 °C and 180 rpm on a Multitron II (Infors HT, Bottmingen-Basel, Switzerland) until reaching an OD₆₀₀ of around 1. Each 10 mL of this culture was used to inoculate 6 L of 2xYT medium containing both antibiotics. These main cultures were grown at 37 °C and 220 rpm. At an OD₆₀₀ of 0.5, the temperature was reduced to 20 °C, and the cells were induced with 0.8 mM IPTG. The cells were cultured at 20 °C, 220 rpm for 16-18 h. Cells were harvested by centrifugation at 4 °C, 4,000 rpm for 30 min using a J6-MI centrifuge (Beckman-Coulter, Brea, CA, USA). The cell pellets were resuspended in ice-cold lysis buffer. DNase and RNase were added to the cell suspension, and the cells were lysed by sonication. Afterwards, the lysate was centrifuged for 1 h at 18,500 rpm and 4 °C in an Avanti J-30 I with a pre-chilled JA-30.50 Ti rotor (Beckman-Coulter, Brea, CA, USA). The supernatant was sterile filtered through a 0.22 µm filter (rapid-FILTERMAX, TTP®, Trasadingen, Switzerland). Next, 50 mL of the filtered solution was loaded onto two connected 5 mL HisTrap™ FF columns (Cytiva Sweden AB, Uppsala, Sweden) equilibrated with lysis buffer using the ÄKTA purifier (Cytiva, Global Life Science Solution USA LLC, Marlborough, MA, USA). The column was washed with lysis buffer until a baseline UV signal was reached. The protein was eluted from the column with

30% elution buffer. Fractions containing the JNK3 protein were pooled and supplemented with 1:5-1:10 [v/v] TEV protease (1 mg/mL). The mixture was cleaved overnight at 4 °C on a stirrer while dialyzing with a regenerated cellulose sleeve (Zellutrans/Roth, Dialysiermembran T1, Sartorius, Göttingen, Germany) in 5 L lysis buffer without imidazole. Next, a reverse nickel chromatography step was performed to remove the His-tag and the His-tagged TEV protease. Before the SEC step, the protein was concentrated using a Vivaspin® Turbo 15 (MWCO = 30 kDa; Sartorius, Göttingen, Germany). The protein was then purified on a HiLoad™ 26/60 Superdex 75 pg gel filtration column (Cytiva Sweden AB, Uppsala, Sweden) equilibrated with SEC buffer. Fractions containing the pure protein were pooled. Finally, the purified protein was concentrated to 50 µM using a Vivaspin® Turbo 15 (MWCO= 30 kD, Sartorius, Göttingen, Germany), flash frozen in liquid nitrogen, and stored at -80 °C. The purity of the protein was monitored by SDS-PAGE. Protein concentrations were measured spectrophotometrically using the molar extinction coefficients (ϵ^{280}) of the respective protein at 280 nm, calculated from the ProtParam tool on the Expasy server [338].

4.8.6 SDS-PAGE

The progression of the protein purification was monitored using a 10% polyacrylamide BIS-TRIS gel. The recipes for the separating and collection gel for five gels are listed in Table 4.12. The 10% ammonium persulfate (APS) solution was freshly prepared before usage. Table 4.12 shows the composition of the loading dye as a 5x stock solution and the recipe for the 5x 3-(N-morpholino)propanesulfonic acid (MOPS) running buffer. For SDS-PAGE analysis, 20 µL of the samples were taken and mixed with 5 µL of loading buffer and heated to 95 °C for 2 min. Then, 5 µL of each sample was loaded into the gel pockets, along with the Blue Prestained Protein Standard, Broad Range (11-190 kDa) (New England BioLabs, Ipswich, MA, USA) as a marker. Electrophoresis was run at 200 V and 100 mA for 40 min. Finally, the gel was stained with ReadyBlue™ Protein Gel Stain (Merck KGaA, Darmstadt, Germany).

Table 4.12: Recipes for the gels, loading buffer, and running buffer.

Buffer	Composition
Collection gel	11.4 mL BIS-TRIS buffer 3.5x, 5.3 mL polyacrylamide 40 (29:1), 23.3 mL water, 30 μ L <i>N,N,N',N'</i> -tetramethylethylenediamine (TEMED), 150 μ L APS (10%), 2 mg bromophenol blue
Separating gel	50 mL BIS-TRIS buffer 3.5x, 16.7 mL polyacrylamide 40 (29:1), 19 mL water, 40 μ L TEMED, 200 μ L APS (10%)
BIS-TRIS buffer (3.5x)	1.25 M BIS-TRIS HCl, pH 6.6
Loading buffer (5x)	250 mM TRIS, pH 6.8, 30% glycerol, 5 mM DTT, 10% SDS, 0.02% bromophenol blue
MOPS buffer (5x)	250 mM MOPS, 250 mM TRIS, 4 mM EDTA, 0.25% SDS, 5 mM $\text{Na}_2\text{S}_2\text{O}_5$

4.9 Differential Scanning Fluorimetry

The effect of the fragments on the melting temperature of different p53 mutants was monitored by differential scanning fluorimetry. Before each DSF measurement, a buffer exchange to DSF buffer (Table 4.13) was performed using a HiPrep™ 26/10 desalting column (Cytiva Sweden AB, Uppsala, Sweden). The high-resolution melt analysis was conducted on a Qiagen Rotor-Q Model-5-Plex HRM real-time PCR instrument (Qiagen, Hilden, Germany) using a Rotor-Disc 72 (Qiagen, Hilden, Germany) filled with strip tubes (0.1 mL, Qiagen, Hilden Germany), each containing 20 μ L sample volume. Excitation and emission filters were set to 470 and 610 nm, respectively. During DSF experiments, the temperature was ramped from 28 °C to 60-70 °C with a constant heating rate of 270 °C/h [100]. SYPRO™ Orange protein gel stain (Life Technologies Corporation, Eugene, OR, USA) served as a fluorescent dye. The protein (8 μ M final concentration) was mixed with compounds dissolved in DMSO and SYPRO Orange (5x final concentration) dissolved in DSF buffer, yielding a final DMSO concentration of 5% [v/v] [131]. For the control sample, containing protein without compounds, pure DMSO was added. The primary compound screen was performed with a final compound concentration of 1 mM (protein-to-compound ratio 1:125) [131]. For subsequent concentration-dependent measurements, the compound concentration was varied by diluting the compound stock solution in pure DMSO before adding it to the protein solution. For time-dependent DSF experiments, samples containing protein and compound or protein and pure DMSO were incubated at 20 °C while shaking. SYPRO Orange was then added to the mixture immediately before measurement. The

melting temperature of the protein in the presence or absence of the compounds was determined from the maximum of the first derivative of the melting curve using OriginPro2020 (OriginLab, Northampton, MA, USA). ΔT_m was calculated by subtracting the T_m of the protein sample without compound from the T_m of the samples containing protein and compound. All samples were measured at least in triplicate. From Section 3.4 onwards, multiple runs were averaged using error propagation.

Table 4.13: Recipe for the DSF buffer.

Buffer	Composition
DSF buffer	25 mM KPi, pH 7.2, 150 mM NaCl, 1 mM TCEP

4.10 Intact Protein Mass Spectrometry

4.10.1 Sample Preparation

Protein in SEC buffer (25 mM KPi, pH 7.2, 150 mM NaCl, 5 mM DTT) was incubated with 100 equivalents of the compound dissolved in pure DMSO, resulting in a final DMSO concentration of 5% [v/v] [131]. For the HEFLib compound 4482, a molar ratio of 1:125 was applied. In certain cases (SN002, VS004, VS004-1, VS004-2) reactions with DTT occurred in the MS experiments and therefore DTT was excluded from measurements. A control sample, containing pure DMSO instead of the compound, was included in each series of measurements. For concentration-dependent MS measurements, the compound concentration was varied by diluting the compound stock solution in pure DMSO before adding it to the protein solution. All samples were incubated at 20 °C on a rotating shaker. The incubation time was 4 h for reactive compounds and 24 h for less reactive fragments. After incubation, all samples were filtered through 0.22 μ m sterile, polyvinylidene difluoride (PVDF) Rotilabo®-syringe filters (Carl Roth GmbH+Co.KG, Karlsruhe, Germany), pipetted into HPLC vials, and immediately subjected to UHPLC-ESI-MS analysis.

4.10.2 UHPLC-ESI-MS Measurements, Data Processing

The UHPLC-ESI-MS measurements were performed by Dr. Simon Jaag, Benedikt Masberg, and Cornelius Knappe.

Separation was performed on a UHPLC-system (Agilent, Waldbronn, Germany) with a 1290 Infinity binary pump (GA220A) and a thermostated column compartment (G1316C). For online desalting, a Valco EHMA diverter valve (2-pos/6-port, VICI, Schenkon, Switzerland) was incorporated between the column and the ion source. The temperature of the column was adjusted to 50 °C. H₂O + 0.1% [v/v] formic acid served as mobile phase A, and ACN + 0.1% [v/v] formic acid served as mobile phase B. The following gradient with a flow rate of 0.5 mL/min was applied: 5% B for 2 min (flow to waste, desalting of the sample), 5-80% B for 8 min, 80% B for 2 min, 5% B for 5 min. MS experiments were performed in positive ionization mode with a TripleTOF 5600+ mass spectrometer (Sciex, Darmstadt, Germany) with a Duospray ion source (ESI interface). The following conditions were applied: curtain gas 35 psi, nebulizing gas 50 psi, heater gas 40 psi, ion spray voltage floating 5100 V, source temperature 550 °C, collision energy: 30 V, and declustering potential 220 V. Mass spectra in TOF (time-of-flight) MS mode were acquired over the m/z range from 500-5000 with an accumulation time of 500 ms. To optimize advanced MS parameters for protein analysis, the IntactProteinMode script (Sciex) was utilized. Data acquisition was carried out using Analyst TF 1.8.1 software (Sciex). PeakView 2.2.0 software (Sciex) was used for data analysis, and the mass spectra were deconvoluted with the BioToolKit (2.2.0).

For relative quantification of the arylated protein species, XICs were generated using the +20 charge state of all species, which was the most abundant. All resulting peaks in the XICs were integrated to calculate the total peak area and, subsequently, the relative peak area [%] for each species.

4.11 $^1\text{H}/^{15}\text{N}$ -Heteronuclear Single Quantum Coherence NMR

This work was performed by Jason Stahlecker and Dr. Murray Coles.

For $^1\text{H}/^{15}\text{N}$ -HSQC-NMR experiments, 70 μM ^{15}N -labeled T-p53C-Y200C in SEC buffer, 1 mM compound, and a final concentration of 5% DMSO- d_6 [v/v] were applied [98]. Therefore, 523 μL of uniformly labeled T-p53C-Y220C in SEC buffer was mixed with 27.5 μL of compound dissolved in DMSO- d_6 . For compounds SN006 and SN007, a final concentration of 500 μM was chosen due to solubility issues. To test the solubility of the compounds, a control sample was pipetted, containing 523 μL of SEC buffer and 27 μL of compound dissolved in DMSO- d_6 . All $^1\text{H}/^{15}\text{N}$ -HSQC-NMR experiments were performed on an 800 MHz Bruker Avance-III at 293 K. Spectra were acquired with 1024 data points in the direct (^1H , f_2) and 128 data points in the indirect dimension (^{15}N , f_1), respectively. Data was processed and analyzed with Bruker Topspin 4.1 and NMRFAM-SPARKY 3.19 [339]. Figures were generated with nmrglue python (v.0.8) package [340]. Peak shifts were considered significant if

$$\Delta\delta(^1\text{H}/^{15}\text{N}) = \sqrt{(\Delta\delta(^1\text{H}))^2 + \left(\frac{\Delta\delta(^{15}\text{N})}{5}\right)^2} \quad \text{Equation 1}$$

was larger than 0.04 ppm [98, 341].

4.12 Fluorescence Polarization Assay

4.12.1 Preparation of the Probe

GADD45 α , a non-apoptotic DNA-recognition element of p53, served as a probe for the FP assay [130, 131]. The sequences of the forward and reverse strands are depicted in Table 4.14. The forward strand was labeled with 6-carboxyfluorescein (6-FAM) at the 5' end [81, 130, 292, 342, 343]. The oligonucleotides were synthesized by Merck KGaA (Darmstadt, Germany) on a 0.025 μM scale and purified using HPLC. The forward and reverse strands were diluted with autoclaved water to a concentration of 100 μM and mixed. The annealing was performed in a TC-9639 Thermal Cycler (Benchmark Scientific, Sayreville, NJ, USA) by heating to 95 $^\circ\text{C}$ for 5 min, followed by cooling at a rate 1 $^\circ\text{C}/\text{min}$ until reaching 22 $^\circ\text{C}$ [292]. The probe was then stored at -20 $^\circ\text{C}$.

Table 4.14: GADD45 α recognition element as a probe for the FP assay.

Oligonucleotides	Sequence
FAM-GADD45 α forward	5'-[6-FAM]-GTACAGAACATGTCTAAGCATGCTGGGGAC-3'
GADD45 α reverse	5'-GTCCCCAGCATGCTTAGACATGTTCTGTAC-3'

4.12.2 Direct Titration of FL-T-p53 with GADD45 α

The DNA-binding capacity of stabilized full-length p53 to its 6-FAM-labeled DNA-recognition element GADD45 α in the presence or absence of compounds was investigated by FP measurements. Titrations were performed on a CLARIOstar plate reader (BMG Labtech GmbH, Ortenberg, Germany) using black, non-binding polystyrene 96-well microplates (Greiner Bio One, Frickenhausen, Germany) at 25 °C. Excitation and emission filters were set to 482 nm and 530 nm, respectively, and the dichroic mirror to 504 nm. To ensure adequate sensitivity, the focal height and the detector gains were adjusted before each measurement [329, 344]. Buffer conditions for all screens are displayed in Table 4.15. 0.2 mg/mL bovine serum albumin (BSA) was added to prevent unspecific binding. By dissolving the compounds in pure DMSO, a final well concentration of 5% DMSO was achieved. For the direct titration of FL-T-p53 to GADD45 α without compounds, pure DMSO was added. FL-T-p53 was incubated with compounds at a protein-to-compound ratio of 1:200 for 2 h (reactive fragments) or 24 h (less reactive fragments) while shaking at 20 °C [62]. Next, the protein compound mixture with a protein start concentration of 2000 nM was serially diluted by a factor of two. Thereafter, 190 μ L of the diluted protein compound mixture and 10 μ L of the 6-FAM labeled GADD45 α recognition element (diluted in FP buffer) were mixed, leading to a final probe concentration of 20 nM. An additional measurement point, containing only GADD45 α and FP buffer without protein, was included as a blank polarization value to determine the signal baseline of the free, unbound probe. Measurements were performed at least in quadruplicate. The FP data were recorded every 10 min during the one-hour measurement, and the resulting FP values were averaged. Multiple measurements were averaged using error propagation. Absolute polarization values were normalized and plotted in OriginPro2020 (OriginLab, Northampton, MA, USA). According to the cooperative binding mechanism, curves were fitted to a modified Hill equation, including a linear drift term [83, 130, 131, 343, 345]:

$$r_{obs} = r_{START} + (r_{END} - r_{START}) \frac{[P]^n}{K_d^n + [P]^n} + r * [P] \quad \text{Equation 2}$$

r_{obs} represents the observed anisotropy, r_{START} and r_{END} are the initial anisotropy value of the unbound DNA and the anisotropy upon binding of p53 to DNA, $[P]$ is the p53 concentration, K_d represents the dissociation constant, n is the Hill coefficient, r is the constant of the linear drift term [83, 345].

Table 4.15: Composition of the FP assay buffer.

Buffer	Composition
FP assay buffer	25 mM KPi, pH 7.2, 150 mM NaCl, 5 mM DTT, 0.2 mg/mL BSA

4.13 Isothermal Titration Calorimetry

Before each ITC measurement, a buffer exchange into ITC buffer (Table 4.16) was performed using a HiPrep™ 26/10 desalting column (Cytiva Sweden AB, Uppsala, Sweden). Thereafter, the protein was concentrated to 80-120 μ M, and 5% DMSO [v/v] was added. A 100 mM stock solution of the fragments in pure DMSO was prepared. The compounds were then diluted with ITC buffer to 5 mM and a final DMSO concentration of 5% [v/v]. ITC experiments were performed on a MicroCal iTC200 system (Malvern Panalytical, Malvern, UK). The temperature of the measuring cell was set to 25 °C, and the temperature of the jacket was set to 15 °C. After an initial delay of 120 s, 0.5 μ L over 2 s were pre-injected. Next, 19 injections with 2 μ L over 4 s with 180 s spacing between each injection were performed. During the run, the solution was stirred at 1,000 rpm, and the reference heating rate was adjusted to 10 μ cal/s [196]. After the equilibration period, the heat rate of the cell was greater than 9 μ cal/s. Before the protein was filled into the cell, the cell was washed twice with ITC buffer. After each measurement, the cell and syringe were washed multiple times, and once a week, the cell was cleaned with 14% [v/v] Decon 90 (Decon, Hove, UK). The ITC runs were analyzed using MicroCal in Origin 7 (OriginLab, Northampton, MA, USA).

Table 4.16: Composition of the ITC buffer.

Buffer	Composition
ITC buffer	50 mM HEPES, pH 7.4, 100 mM NaCl, 2 mM MgCl ₂ , 1 mM TCEP

4.14 Saturation Transfer Difference NMR

Dr. Marcel Dammann and Dr. Markus Kramer performed the STD experiments, Jason Stahlecker and Dr. Marcel Dammann conducted the analysis.

The STD experiments were performed as previously described [196]. Before NMR measurements, a buffer exchange of the protein to 100 mM sodium phosphate, pH 7.0, 250 mM NaCl, and 2 mM MgCl₂ was performed. The spectra were recorded using a Bruker Avance III HDX 700 with a 5 mm Prodigy TCI cryo probehead. A standard ¹H NMR experiment with 1 k scans and water suppression through presaturation was conducted for the protein. The pulse sequence published by Mayer and Mayer (1999) [346], Mayer and Mayer (2001) [347] was adapted for the STD experiments. The on-resonance frequency was determined from the ¹H NMR spectra of the protein to be between 0.5 ppm and 0.6 ppm, while 40 ppm was used as off-resonance frequency. To achieve interleaved acquisition of the on- and off-resonance, a pseudo-2D scheme was applied. Saturation was achieved using Gaussian pulses with a length of 50 ms and 60 dB of attenuation, with an interpulse delay of 1 ms, resulting in an excitation bandwidth of about 42 Hz. The screening involved 16 scans of on- and off-resonance scans each, with a 3 s saturation time. The samples had final concentrations of 20 μM protein, 10% [v/v] DMSO-d₆, and 1 mM of each of the two fragments. A reference spectrum was obtained for each compound through a ¹H NMR experiment. All NMR experiments were conducted at 25 °C.

4.15 X-Ray Crystallography

4.15.1 Crystallization of xT-p53C-Y220C

The xT-p53C-Y220C construct with a deletion of the GGS overhang after the TEV cleavage site was used for crystallization experiments. Protein crystallization was performed as previously described [99, 146]. Crystals were obtained by sitting drop vapor diffusion technique using MRC Maxi 48-Well Crystallization Plates (Jena Bioscience, Jena, Germany). The reservoir was filled with 200 μL of crystallization buffer (Table 4.17). The protein in SEC buffer was concentrated to 5 mg/mL, and 2 μL of the protein solution was mixed with 2 μL of reservoir solution. Crystals were grown at 19 °C within a few days or were obtained by performing streak seeding.

The crystal structures of the compounds in complex with the protein were obtained by soaking. Therefore, the compounds were dissolved to the respective concentrations (5 mM, 15 mM, 40 mM, or saturated solution) in cryo-protectant buffer (crystallization buffer with the addition of 20% glycerol). A 300 mM compound stock solution in 100% DMSO was used for the preparation of the 5 mM and 15 mM compound solutions. The 40 mM compound solution was prepared by dissolving the compound directly in cryo-protectant buffer without the addition of DMSO. After adding the protein crystals into a 2 μ L drop of cryo-protectant buffer with compound, the crystals were soaked for 1-2 days.

Table 4.17: Composition of the crystallization buffer.

Buffer	Composition
Crystallization buffer	100 mM HEPES, pH 7.15, 19% polyethylene glycol (PEG) 4000, 10 mM DTT

4.15.2 Data collection and Structure Refinement

This work was done by Jason Stahlecker.

The protein crystals were measured at the Swiss Light Source (SLS) (Villingen, Switzerland) at the beamlines X06SA (PXI). The data were processed and reduced using XDS [348]. Molecular replacement was performed to obtain initial phases. Therefore, crystal structures with the PDB ID 4AGL [98] or 8A92 [99] served as a search model for PHASER [349] as part of the CCP4 suite [350]. Structure and phase improvements were done by multiple cycles of manual model building in Coot [351] and structure refinement using PHENIX [352]. Covalently bound compound restraints were generated using JLigand [353] in ACEdrp mode [354]. The unbiased omit-maps were obtained by removing all covalently bound fragments and the entire cysteine side chain, followed by refinement with simulated annealing.

4.16 Glutathione Assay

The GSH assay was performed by Martin Schwer.

The performed GSH stability studies were based on the established protocol by Keely et al. for heterocyclic electrophilic fragments [197]. The reaction conditions were 250 μ M of compound, 100 μ M ketoprofen or ibuprofen as an internal standard, 5 mM GSH excess, 10% ACN, and PBS buffer pH 7.4. The reaction temperature was set to 37 °C. The mixture was analyzed with an Ultimate 3000 HPLC-System (Thermo Fisher Scientific, Dreieich, Germany) with UV detection after 0, 1, 2, 4, 8, 12, and 24 h. For reactive fragments ($t_{1/2} < 1$ h), measurements were performed every 20 min. The reaction of the compounds with GSH was detected by measuring the decreasing area under the curve (AUC) relative to the internal standard. The relative AUC was fitted to the integrated rate equation of pseudo-first order kinetics using OriginPro2020 (OriginLab, Northampton, MA, USA):

$$\text{relative AUC} = e^{-kt} \quad \text{Equation 3}$$

Half-life $t_{1/2}$ was calculated from the pseudo-first order rate constant k using the following equation:

$$t_{1/2} = \frac{\ln 2}{k} \quad \text{Equation 4}$$

All GSH stability measurements were performed at least as duplicates. Multiple runs were averaged using error propagation. In contrast to the calculations by Keeley et al. [197], the rate constants k_{GSH} and the corresponding half-lives were not corrected for the degradation reaction in pure buffer. To test the hydrolytic degradation, measurements were performed in PBS buffer without GSH for each compound.

4.17 V_{max} and Adduct Formation Energy Calculations

This work was done by Dr. Markus Zimmermann.

4.17.1 MP2 Structure Optimizations and Single Point Calculations

Geometry optimizations and single point calculations were performed using TURBOMOLE 7.4.1. [355]. The study employed a triple- ζ basis set (def2-TZVPP) [356]. MP2 calculations were performed in combination with the resolution of identity (RI) technique and the frozen core approximation [356-359]. The default setting in

TURBOMOLE was used to attribute frozen core orbitals, considering all orbitals with energies below 3.0 au as core orbitals. The SCF convergence criterion was increased to 10^{-8} Hartree for all calculations. The heavy atoms of the model systems remained fixed during optimization.

4.17.2 Adduct Formation Calculations

The ligand and the halogen bond accepting moiety, represented as *N*-methylacetamide, were optimized using MP2/TZVPP. During optimization, heavy atoms were kept frozen. For the calculations of the putative adduct formations, the modified ligands were freely optimized using MP2/TZVPP-level of theory. Thereafter, the ligands were matched onto the geometry of the crystal structure using three atoms of the ligand. The complex formation energies were computed through single points as the difference between the complex and the sum of the two separate molecules.

4.17.3 2D Electrostatic Potential Plots and $V_{\max}$ Calculations

The ligands were oriented using an in-house script after their geometry optimization. This was done by placing the halogen atom into the negative X-axis and the respective ring system into the XZ-plane. Electrostatic potentials were calculated on a regular 2D grid using TURBOMOLE 7.4.1 [355]. A custom Python script was used for visualization. The $V_{\max}$ values were extracted at 0.02 au electron isodensity.

4.18 Structural Filtering for Library Design

This work was done by Marc U. Engelhardt.

The Aldrich Market Select Data Bank, which contains approximately 18 million compounds as of April 2023, was filtered employing a customized Python script using the RDKit package. Compounds larger than 22 heavy atoms and smaller than six heavy atoms were removed, as well as those with a logP value >2. LogP values were calculated by RDKit according to Wildman et al. [360].

4.19 Figures, Tables, and General Tools

Chemical structures were generated using ChemDraw 21.0.0 (Perkin Elmer, Waltham MA, USA). Protein figures were prepared using PyMOL 2.3.3 (Schrödinger Inc., New York, NY, USA). Plots were generated using OriginPro2020 (OriginLab, Northampton, MA, USA). Figures were created with Inkscape 1.3.2 (Free Software Foundation, Inc. Boston, MA, USA).

4.20 Author Contributions

The research was envisioned by Prof. Dr. Frank M. Böckler. All experimental methods, data analysis, and data reprocessing, unless otherwise stated below, were planned and performed by Theresa Klett.

Mutagenesis experiments were performed by Theresa Klett. Theresa Klett prepared p53 and its mutants, and ^{15}N -labeled protein by heterologous expression. Theresa Klett performed and analyzed the DSF experiments. Theresa Klett prepared the MS samples; Dr. Simon Jaag, Benedikt Masberg, Cornelius Knappe, and Prof. Dr. Michael Lämmerhofer conducted the UHPLC-ESI-MS experiments; Theresa Klett, Dr. Simon Jaag, Benedikt Masberg, Cornelius Knappe, and Prof. Dr. Michael Lämmerhofer analyzed the MS experiments. Jason Stahlecker and Dr. Murray A. Coles carried out and analyzed $^1\text{H}/^{15}\text{N}$ -HSQC experiments. FP experiments were performed and analyzed by Theresa Klett. Dr. Marcel Dammann and Dr. Markus Kramer performed the STD experiments, Jason Stahlecker and Dr. Marcel Dammann did the analysis. Jason Stahlecker and Theresa Klett conducted p53 protein crystallization experiments. Prof. Dr. Thilo Stehle granted access to the SLS beamline. Jason Stahlecker performed protein data reduction and structure refinement. Martin Schwer performed and analyzed the GSH assay. Dr. Markus Zimmermann performed V_{max} and adduct formation energy calculations. Marc Engelhardt performed all computational investigation, including the customized fragment filtering process. Theresa Klett, Martin Schwer, Prof. Dr. Frank M. Böckler, and Prof. Dr. Matthias Gehring selected the final CovLib compounds. Theresa Klett, Larissa N. Ernst, Jason Stahlecker, and Prof. Dr. Frank M. Böckler selected the SAR compounds.

5 Summary and Outlook

The tumor suppressor protein p53, also known as the “guardian of the genome,” plays a pivotal role in the cell cycle [2-5]. The activation of p53 in response to various stress signals leads to the regulation of transcription of numerous genes involved in cell cycle arrest, apoptosis, DNA-repair, and cellular senescence [6, 7, 9]. In many tumors, a mutation of p53 is found, or p53 activity is reduced as a consequence of the disruption of associated signaling pathways [12]. The majority of oncogenic p53 mutations are missense mutations located in the core domain of p53 [6, 69, 73, 77, 92]. Mutations of p53 can eliminate an essential DNA contact (contact mutants) or induce structural changes that destabilize the protein (structural mutants) [55, 69, 73]. The R273H mutant represents a classical DNA contact mutant, removing an important DNA contact without disturbing the architecture of the core domain or the thermodynamic stability of the protein [69, 73]. The hot spot mutant R282W is a structural mutant located in the DNA-binding helix H2, perturbing the loop-sheet-helix motif. However, this alteration results in a significant reduction in thermodynamic stability, while DNA-binding is maintained at lower temperatures [73, 77]. The structural β -sandwich mutant Y220C is the ninth most common p53 mutant with estimated 130,000 annual cancer cases [73, 97-99]. The Y220C mutant is markedly thermodynamically destabilized. The melting temperature is reduced by approximately 8 °C, leading to rapid denaturation and aggregation at body temperature [73, 101]. The mutation from tyrosine to cysteine creates a solvent-accessible hydrophobic cleft in the periphery of the β -sandwich, situated at a distance from the DNA-binding region. This cleft represents a binding pocket for stabilizing molecules [69, 73, 100].

In almost 50% of all human cancer types, there is a mutation of p53 [12]. Furthermore, p53 mutations are often associated with poor cancer prognosis and rapid tumor progression [73, 155, 156]. Hence, p53 represents a primary target for cancer therapy, and there is significant interest in reactivating p53 mutants to restore p53 function [6, 8, 12, 97, 105, 361]. However, targeting p53 has proven to be a challenging task, and none of the p53 drug discovery efforts have yet received FDA approval [1, 12, 104]. Therefore, this thesis aimed to identify compounds that target and stabilize p53 and its mutants using fragment-based approaches.

Figure 5.1 provides an overview of the overall structure, the main parts, and the key results of the thesis.

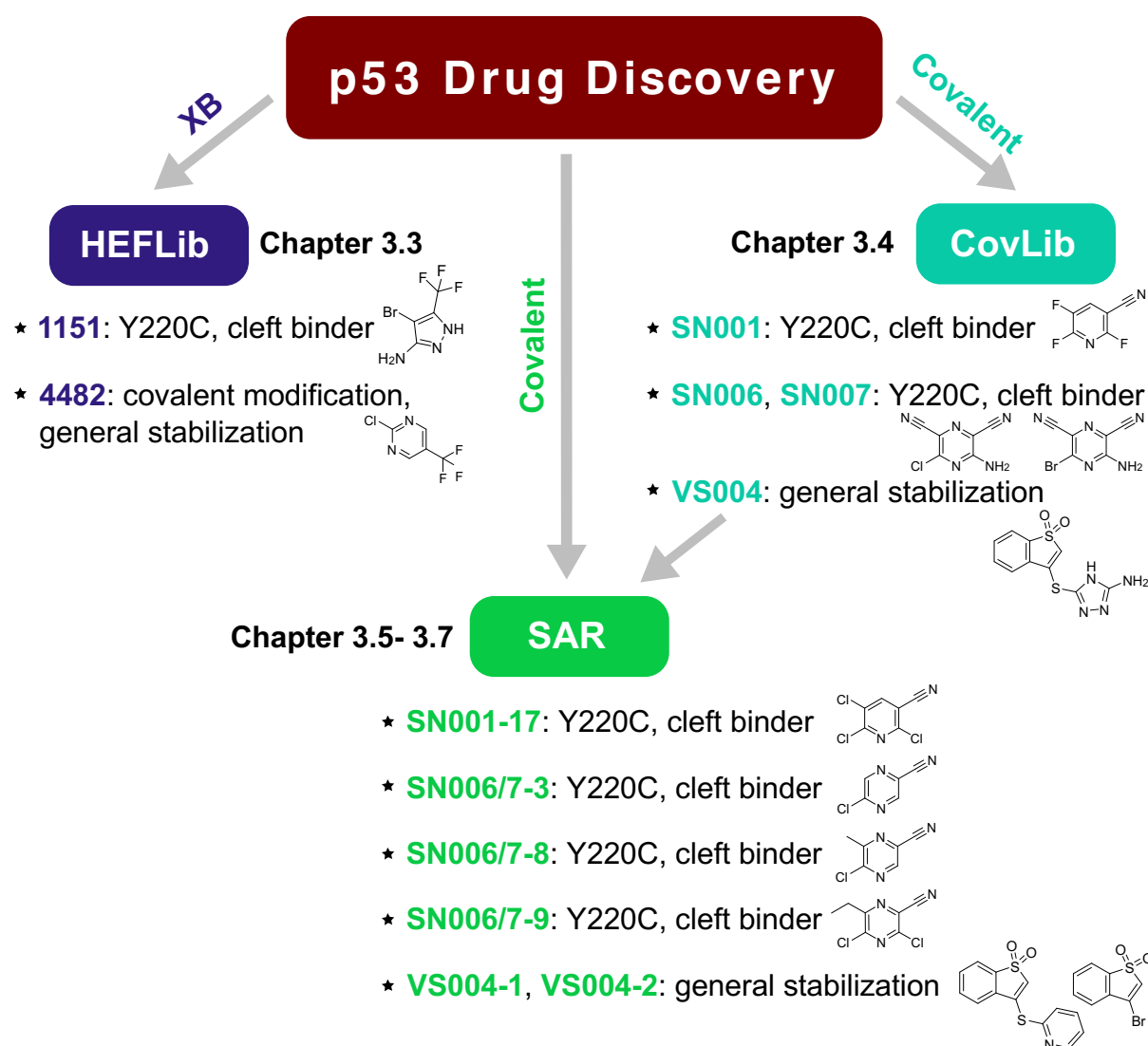


Figure 5.1: Overview of the overall structure, the major chapters, and the key findings of this thesis. The hits for p53 and its β -sandwich mutant Y220C identified by screening of the HEFLib, CovLib, and SAR compounds are presented.

In the first part of this thesis, the diversity-optimized Halogen-Enriched Fragment Library (HEFLib) was evaluated with T-p53C-Y220C and its challenging binding site. The goal of the screening was the discovery of new stabilizing hits with unconventional binding modes in the mutational cleft based on halogen bonding. The primary biophysical screening technique employed was DSF, with thermal stabilization and an increase in the melting temperature of T-p53C-Y220C serving as the primary readout for a fragment hit. The DSF screen identified three hits: 1151, 1223, and 4482. Fragment 1151 significantly increased the melting temperature of T-p53C-Y220C by

0.80 °C. Moreover, the fragment was identified as a cleft binder using $^1\text{H}/^{15}\text{N}$ -HSQC-NMR, and its binding into the pocket was confirmed by a protein crystal structure. In one binding pose, the bromine of 1151 was oriented towards the backbone oxygen of Leu145, forming a halogen bond with an angle of 153.4° and a distance of 3.0 Å. The second hit, HEFLib compound 4482, is a reactive fragment that exhibited a half-life of 2.80 h in the GSH stability assay. The 2-chloropyrimidine showed a time-dependent stabilizing effect on T-p53C-Y220C, increasing the T_m from 0.50 °C after 30 min, to 0.90 °C after 4 h, and up to 1.75 °C after 24 h of incubation at 20 °C. Fragment 4482 reacted with proteinogenic nucleophiles via an $\text{S}_{\text{N}}\text{Ar}$ reaction, and the covalent modification of cysteine residues in p53 was confirmed by intact protein mass spectrometry. In conclusion, the discovery of fragment 4482 and fragment 1223, which corresponds to the published fragment PK11000, demonstrated the potential of mutation-independent rescue of p53 by covalent modification of surface-exposed cysteines, resulting in a generic stabilization of p53.

In the second part of the study, a covalent fragment-based approach was applied to identify compounds that target surface-exposed cysteines in p53, thereby stabilizing the challenging target p53 and its mutants. Therefore, a covalent fragment library (CovLib) was devised to encompass a comprehensive and diverse range of covalent reactive groups, also referred to as “warheads,” and reactivities. Initially, 20 fragments with four different warhead classes were selected. The CovLib comprises five α -cyanoacrylamides/acrylates (CA), three epoxides (EO), four vinyl sulfones (VS), and eight electron-deficient (hetero)arenes with leaving group ($\text{S}_{\text{N}}\text{Ar}/\text{SN}$) warheads. The GSH reactivity assessment of the CovLib revealed strong differences in reactivity among the warhead classes. All tested α -cyanoacrylamides/acrylates and epoxides were considered to be less reactive compounds. The vinyl sulfone and $\text{S}_{\text{N}}\text{Ar}$ fragments exhibited either high reactivity (VS001, VS004, SN001, SN006, SN007) or chemical stability (VS002, VS003, SN003, SN004, SN005, SN008). The only exception was SN002, which demonstrated moderate reactivity.

The CovLib was screened with T-p53C using intact protein mass spectrometry (MS) and DSF as the primary biophysical screening techniques. The covalent modification of T-p53C was confirmed by intact protein MS for six fragments, including two vinyl sulfones (VS001, VS004) and four $\text{S}_{\text{N}}\text{Ar}$ reactive fragments (SN001, SN002, SN006, and SN007). All hits caused multiple and non-specific modifications of T-p53C. DSF was used to cross-validate the intact protein mass spectrometry results. Only the vinyl

sulfone VS004 with a β -leaving group resulted in a substantial stabilization of T-p53C by about 5.06 °C (1 mM, 30 min). Additionally, VS001 impacted the T_m of T-p53C, but it emerged as a potential Pan-Assay Interference Compound. The highly reactive fragment SN001 only slightly lowered the melting temperature of T-p53C by -0.57 °C (1 mM, 30 min). Moreover, the S_NAr reactive compounds SN006 and SN007 strongly destabilized T-p53C. Fragment SN002, the only CovLib fragment with intermediate reactivity, did not affect the T_m of T-p53C even after 24 h of incubation, despite the modification of T-p53C in the MS experiment. The hit rates of the intact protein MS and DSF experiments are in principle consistent with the determined reactivities of the compounds in the GSH assay. The information obtained from characterizing and evaluating the initial CovLib fragments could be used to select additional compounds for library expansion. Moreover, the integration of more fragments of intermediate reactivity into the library could be considered to identify more selective hits for p53 and new targets of interest.

In addition, the CovLib was also screened with various p53 mutants using DSF. Besides T-p53C, the DNA contact mutant R273H, the structural mutant R282W in the DNA-binding helix H2, and the β -sandwich mutant Y220C were also examined. The vinyl sulfone VS004 achieved a general stabilization of T-p53C and its mutants by covalently modifying surface-exposed cysteines. In particular, VS004 stabilized the structural mutant R282W, exhibiting a ΔT_m value of 5.96 °C (1 mM, 30 min). Furthermore, the S_NAr -type electrophiles SN001, SN006, and its bromine analog SN007 were identified, which stabilized especially the β -sandwich mutant T-p53C-Y220C, indicating a covalent modification of the mutation-induced cysteine. Intensive DSF experiments revealed a concentration-dependent reaction behavior. The stabilization of T-p53C-Y220C increased as the concentration of these highly reactive fragments decreased. At a compound concentration of 250 μ M (molar ratio 1:31.25) and 30 min incubation time, the CovLib fragment SN001 stabilized T-p53C-Y220C by up to 4.45 °C. The $^1H/^{15}N$ -HSQC-NMR experiment with SN001 revealed prominent peak shifts, indicating a covalent modification of cysteines in T-p53C-Y220C and binding into the pocket. Moreover, intact protein MS measurements of T-p53C-Y220C incubated with SN001 confirmed multiple arylations of the protein. Additionally, concentration-dependent MS experiments and relative quantification revealed a decrease in the number of arylations with decreasing SN001 concentration. DSF and MS studies were performed with a Y220C cysteine light

protein with mutations of the surface-exposed cysteines to serine residues. The results demonstrated that fragment SN001 stabilized the protein by covalently modifying the mutation-induced Cys220. The impact of the CovLib compounds on the DNA-binding affinity of FL-T-p53 to its non-apoptotic response element GADD45 α was examined using FP measurements. The results revealed that the CovLib hits SN001 and VS004 had no adverse effect on the DNA-binding, while SN006 and SN007 reduced the DNA-binding capability of FL-T-p53 to GADD45 α . Finally, X-ray crystallography confirmed the covalent modification of Cys220 by SN001 and SN006 in the hydrophobic cleft. The pyridine derivative SN001 occupied the central cavity of the Y220C pocket, adopting a Tyr-like conformation and mimicking the wt Tyr220 lost by the mutation. SN001 reacted with cysteine residues in p53 via an S_NAr reaction, with the fluorine atom in the para position to the nitrile group acting as the leaving group (LG). The CN moiety pointed towards subsite 1 and Thr150. The ortho-fluorine was directed towards subsite 2, and the meta-fluorine pointed into the hydrophobic subsite 3. In the 40 mM-soaked structure, multiple modifications were found, including the arylation of Cys229, which led to a side-chain flip. The pyrazine derivative SN006 bound similarly into the hydrophobic pocket, adopting the same Tyr-like conformation.

In the third part of this thesis, extensive structure-activity relationship (SAR) studies were conducted. A large number of SAR compounds with varying halogenation patterns, LG, electron-withdrawing groups (EWGs), electron-donating groups (EDGs), and (hetero)arenes were tested. The objective was to reduce reactivity and enhance selectivity for specific cysteines in p53, particularly for Cys220 in the mutational cleft, ultimately leading to increased stabilization of the Y220C mutant. The SAR screen revealed a set of S_NAr reactive fragments that target Cys220 in the hydrophobic pocket of T-p53C-Y220C, thereby substantially stabilizing the temperature-sensitive cancer mutant. All identified fragments are pyridine or pyrazine derivatives with an electron-withdrawing CN moiety and a halogen in the para position, serving as the LG. Variations in EWGs or (hetero)arenes did not result in a higher stabilization of the Y220C mutant or more selective hits for Cys220. Moreover, the reduction in the number of halogen atoms attached to the pyridine ring resulted in a notable decline in the reactivity of the compounds, accompanied by a substantial decrease in the stabilization of the cancer mutant.

The replacement of the three fluorine atoms in SN001 with weaker electron-withdrawing chlorines results in compound SN001-17. The pyridine derivative

SN001-17 stabilized the β -sandwich mutant depending on both concentration and time. The stabilization of T-p53C-Y220C increased with lengthened incubation time and diminishing concentration of SN001-17. SN001-17 extensively stabilized the Y220C mutant by up to 5.19 °C at 250 μ M after 24 h of incubation. Thus, SN001-17 exhibited a higher maximum stabilization compared to the CovLib hit SN001 (ΔT_m = 4.45 °C, 250 μ M, 30 min). Furthermore, SN001-17 markedly increased the T_m of the Y220C cysteine light mutant by 5.20 °C (250 μ M, 24 h), indicating that the covalent modification of cysteine 220 in the hydrophobic pocket is responsible for the stabilization of T-p53C-Y220C and T-p53C-Y220C-CL. The crystal structure confirmed the covalent modification of Cys220 in the hydrophobic cleft. The pyridine derivative SN001-17 showed a similar binding pose as the CovLib fragment SN001, adopting the same Tyr-like conformation and occupying the central cavity. The chlorine atom in the ortho position to the nitrile group pointed towards subsite 2 and the meta-chlorine into subsite 3.

The 5-chloro-pyrazine-2-carbonitrile SAR hit SN006/7-3 increased the T_m of T-p53C-Y220C by up to 3.62 °C (1 mM, 24 h). The stabilization increased with increasing incubation time and compound concentration. SN006/7-3 exhibited multiple modifications of the Y220C mutant in the intact protein MS experiment. However, the arylation of Cys220 in the binding pocket was confirmed by X-ray crystallography. The pyrazine derivative SN006/7-3 adopted a different conformation compared to the pyridine derivatives SN001, SN001-17, and the pyrazine CovLib hit SN006. The compound was positioned deeply in subsite 2 of the binding pocket. Nevertheless, subsite 1 and subsite 3 were not targeted by SN006/7-3.

It is noteworthy that compound SN006/7-8, which features an electron-donating methyl group in the ortho-position relative to the LG of fragment SN006/7-3, substantially increased the melting temperature of the Y220C mutant by up to 5.02 °C (1 mM) after 24 h of incubation. The stabilization of T-p53C-Y220C increased with prolonged incubation time and increased compound concentration. The introduction of a methyl group in the ortho position to the LG enhanced the stabilizing effect of this scaffold compared to SN006/7-3 (ΔT_m = 3.62 °C, 1 mM, 24 h) without a methyl group and SN006/7-7 (ΔT_m = 2.08 °C, 1 mM, 24 h) with a methyl group in the meta position. The covalent modification of Cys220 by compound SN006/7-8 was confirmed by X-ray crystallography. In comparison to fragment SN006/7-3, pointing into subsite 2 of the hydrophobic pocket, fragment SN006/7-8 with the additional methyl group adopted the

same Tyr-like conformation as the pyridine derivatives SN001, SN001-17, and the pyrazine derivative SN006. The methyl group at position 6 of the pyrazine ring of SN006/7-8 protruded into the hydrophobic subsite 3.

Subsequently, to enhance the selectivity for the Y220C pocket through improved hydrophobic interactions in subsite 3, additional SAR compounds of SN006/7-8 with larger hydrophobic groups ortho to the LG were tested. The highly reactive fragment SN006/7-9 was identified as a Cys220 arylating fragment that also adopted a Tyr-like conformation in the Y220C pocket using X-ray crystallography. Overall, either unreactive compounds or highly reactive compounds, such as SN006/7-9, were identified, and no better hit was discovered than fragment SN006/7-8.

In conclusion, the covalent fragment-based approach led to the discovery of numerous hits for p53 and its mutants. The vinyl sulfone warhead-containing fragments VS004, VS004-1, and VS004-2 were detected, achieving a general stabilization of the p53 protein by covalent modification of surface-exposed cysteines. Guiley and Shokat recently identified the first Cys220 covalently modifying drug through structure-based design with warhead attachment to a known non-covalent cleft binder [143]. Herein, Cys220 arylating fragments were identified through an “electrophile first approach” [204], analyzing a covalent fragment library. The CovLib screen and subsequent SAR compound screen revealed many S_NAr reactive hits for the destabilized cancer mutant T-p53C-Y220C. The identified pyridine or pyrazine derivatives induced an enormous stabilization of the thermosensitive Y220C cancer mutant by covalent modification. Moreover, the discovered fragments showed different binding poses and targeted various subsites of the Y220C binding pocket.

Nevertheless, all identified fragment hits remain highly reactive, leading to multiple modifications of p53 and its mutants. Therefore, it is essential to strike a balance between reactivity and selectivity [232]. However, the small size of the identified fragments provides ample opportunities for optimization in the hydrophobic binding pocket. For instance, the selectivity for the pocket could be enhanced with larger hydrophobic groups than the methyl group in fragment SN006/7-8, pointing into the hydrophobic subsite 3. The subsequent stage in a fragment-based drug discovery process involves the linking, merging, and growing of the identified fragment hits to enhance the non-covalent binding fraction and thus the affinity and selectivity for the Y220C binding pocket through reversible interactions [145, 231, 232].

The testing of the identified hits in cellular assays also represents a next step in the drug discovery process. However, it is important to note that the p53-Y220C mutant is significantly thermodynamically destabilized [73, 80]. This raises the question of whether there is sufficient folded p53-Y220C at 37 °C to be modified by the covalent reactive fragments. Nevertheless, some of the non-covalent binders have demonstrated cellular activity at 37 °C, suggesting that there is enough p53-Y220C in its native state [137]. Furthermore, p53 has a short $t_{1/2}$ of 9 min and is therefore also kinetically unstable. Consequently, drugs typically have to react with the protein immediately during or shortly after the biosynthesis [82]. Thus, the pivotal question is whether there is sufficient p53 protein in its native state to react with covalent fragments or target covalent inhibitors (TCIs) with intermediate or low reactivities. TCIs normally exhibit a balanced reactivity profile [144]. Covalent fragments and TCIs with an intermediate or low level of reactivity may have half-lives that are too long to react with p53 in its native conformation. Considering the short half-life of the target, it would be prudent to discover fragments that target p53 and the cancer mutant p53-Y220C with reversible covalent warheads. This strategy offers the advantages of a reversible covalent reaction mechanism, such as diminished permanent off-target effects and toxicological risks [144, 250].

6 Zusammenfassung

Das Tumorsuppressorprotein p53, das auch als „Wächter des Genoms“ bezeichnet wird, spielt eine zentrale Rolle im Zellzyklus [2-5]. Die Aktivierung von p53 als Reaktion auf verschiedene Stresssignale führt zur Regulierung der Transkription vieler Gene. Diese sind unter anderem am Zellzyklus-Arrest, der Apoptose, der DNA-Reparatur und der zellulären Seneszenz beteiligt [6, 7, 9]. Bei vielen Tumoren liegt eine Mutation von p53 vor oder die Aktivität von p53 ist durch die Störung der mit p53 assoziierten Signalwege reduziert [12]. Bei der Mehrzahl der onkogenen p53-Mutationen handelt es sich um Missense-Mutationen in der DNA-bindenden Domäne von p53 [6, 69, 73, 77, 92]. Mutationen von p53 können einen essenziellen DNA-Kontakt eliminieren (Kontaktmutanten) oder strukturelle Veränderungen hervorrufen, die das Protein destabilisieren (Strukturmutanten) [55, 69, 73]. Die R273H-Mutante ist eine klassische DNA-Kontaktmutante, wodurch ein wichtiger DNA-Kontakt entfernt wird, ohne die Architektur der DNA-bindenden Domäne oder die thermodynamische Stabilität des Proteins zu beeinträchtigen [69, 73]. Die Hotspot-Mutante R282W ist eine Strukturmutante in der DNA-bindenden Helix H2, die das Loop-Sheet-Helix-Motiv stört. Dies führt zu einem starken Verlust an thermodynamischer Stabilität, wobei die DNA-Bindung bei niedrigeren Temperaturen erhalten bleibt [73, 77]. Die Y220C-Mutante ist eine Strukturmutante im β -Sandwich des Proteins. Sie ist die neunthäufigste p53-Mutation, die schätzungsweise 130 000 jährliche Krebsfälle verursacht [73, 97-99]. Die Y220C-Mutante ist thermodynamisch weitgehend destabilisiert. Die Schmelztemperatur ist um etwa 8 °C erniedrigt, was zu einer schnellen Denaturierung und Aggregation bei Körpertemperatur führt [73, 101]. Durch die Mutation von Tyrosin zu Cystein entsteht ein lösungsmittelzugänglicher und hydrophober Spalt in der Peripherie des β -Sandwichs, weit entfernt von der DNA-Bindungsregion. Dieser Spalt stellt eine Bindungstasche für stabilisierende Moleküle dar [69, 73, 100].

Nahezu 50 % aller Krebserkrankungen des Menschen sind auf eine Mutation von p53 zurückzuführen [12]. Darüber hinaus sind p53-Mutationen häufig mit einer schlechten Prognose und einem schnellen Fortschreiten der Krebserkrankung assoziiert [73, 155, 156]. Daher ist p53 ein primäres Angriffsziel für die Krebstherapie und es besteht ein großes Interesse an der Reaktivierung von p53-Mutanten, um die p53-Funktion wiederherzustellen [6, 8, 12, 97, 105, 361]. Die gezielte Adressierung von p53 hat sich jedoch als eine schwierige Herausforderung erwiesen und keine der Bemühungen zur

Entwicklung von p53-Medikamenten hat bisher zur Zulassung geführt [1, 12, 104]. Das Ziel dieser Arbeit war es daher, unter Anwendung von Fragment basierten Ansätzen, Substanzen zu identifizieren, die p53 und seine Mutanten adressieren und diese stabilisieren.

Im ersten Teil der Arbeit wurde die auf Diversität optimierte Halogen-Enriched Fragment Library (HEFLib) mit T-p53C-Y220C und seiner herausfordernden Bindungsstelle evaluiert. Ziel des Screenings war es, neue stabilisierende Hits mit unkonventionellen Bindungsmodi in der Mutationsspalte auf der Basis von Halogenbindungen zu finden. Als primäres biophysikalisches Screening-Verfahren wurde DSF genutzt, wobei die thermische Stabilisierung von T-p53C-Y220C als Hauptindikator für einen Fragmenttreffer diente. Beim DSF-Screening wurden drei Treffer identifiziert: 1151, 1223 und 4482. Das Fragment 1151 bewirkte eine signifikante Erhöhung der Schmelztemperatur von T-p53C-Y220C um 0,80 °C. Das Fragment wurde anschließend mittels $^1\text{H}/^{15}\text{N}$ -HSQC-NMR-Analyse als Spaltbinder identifiziert und seine Bindung in der Tasche durch eine Kristallstruktur des p53-Y220C Proteins im Komplex mit Fragment 1151 bestätigt. In einer Bindungsposition zeigte das Brom von 1151 auf den Rückgrat-Sauerstoff von Leu145 und bildete eine Halogenbindung mit einem Winkel von 153,4° und einem Abstand von 3,0 Å. Der zweite Treffer, die HEFLib-Verbindung 4482, ist ein reaktives Fragment und wies eine Halbwertszeit von 2,80 h im GSH-Stabilitätsassay auf. Das 2-Chlorpyrimidin zeigte eine zeitabhängige Stabilisierung von T-p53C-Y220C, wobei die Schmelztemperatur von 0,50 °C nach 30 Minuten, über 0,90 °C nach 4 Stunden, auf 1,75 °C nach 24 Stunden Inkubation bei 20 °C anstieg. Fragment 4482 reagierte in einer $\text{S}_\text{N}\text{Ar}$ -Reaktion mit proteinogenen Nukleophilen und die kovalente Modifikation von Cysteinen in p53 wurde durch Intaktprotein-Massenspektrometrie (MS) bestätigt. Zusammenfassend lässt sich sagen, dass die Entdeckung des Fragments 4482 und des Fragments 1223, welches dem bereits veröffentlichten Fragment PK11000 entspricht, das Potenzial einer mutationsunabhängigen Rettung von p53 aufzeigt. Die kovalenten Modifikationen oberflächenexponierter Cysteine durch diese Fragmente führen zu einer allgemeinen Stabilisierung von p53.

Im zweiten Teil der Dissertation wurde ein auf kovalenten Fragmenten basierender Ansatz verwendet, um Verbindungen zu identifizieren, die auf oberflächenexponierte Cysteine in p53 abzielen und somit das anspruchsvolle Zielprotein p53 und seine Mutanten stabilisieren. Daher wurde eine kovalente Fragmentbibliothek (CovLib) mit

dem Ziel entwickelt, ein breites Spektrum an kovalent reaktiven Gruppen („Warheads“) mit unterschiedlichen Reaktivitäten zu inkludieren. Zunächst wurden 20 Fragmente mit vier verschiedenen kovalenten reaktiven Gruppen ausgewählt. Die CovLib umfasst fünf α -Cyanoacrylamide/Cyanoacrylate (CA), drei Epoxide (EO), vier Vinylsulfone (VS) und acht elektronenarme (Hetero)aromaten mit Abgangsgruppe (S_NAr/SN). Die Analyse der GSH-Reaktivität der CovLib-Fragmente ergab starke Reaktivitätsunterschiede zwischen den einzelnen „Warhead“-Klassen. Alle getesteten α -Cyanoacrylamide/Cyanoacrylate und Epoxide waren als wenig reaktive Verbindungen einzustufen. Die Fragmente mit einer Vinylsulfonstruktur und die über S_NAr reagierenden Fragmente zeigten entweder eine hohe Reaktivität (VS001, VS004, SN001, SN006, SN007) oder waren chemisch stabil (VS002, VS003, SN003, SN004, SN005, SN008). Die einzige Ausnahme war SN002 mit einer mäßigen Reaktivität.

Die CovLib wurde mit T-p53C unter Verwendung von Intaktprotein-Massenspektrometrie und DSF als primäre biophysikalische Screening-Techniken untersucht. Die kovalente Modifikation von T-p53C wurde durch Intaktprotein-MS für sechs Fragmente bestätigt, darunter zwei Vinylsulfone (VS001, VS004) und vier Fragmente mit S_NAr „Warhead“ (SN001, SN002, SN006 und SN007). Alle Treffer verursachten multiple und unspezifische Modifikationen von T-p53C. DSF wurde zur Validierung der MS-Ergebnisse verwendet. Nur das Vinylsulfon VS004 mit einer Abgangsgruppe an der β -Position führte zu einer beträchtlichen Stabilisierung von T-p53C und einer Schmelztemperaturerhöhung von 5,06 °C (1 mM, 30 min). Darüber hinaus beeinflusste VS001 die Schmelztemperatur von T-p53C, erwies sich aber als potenzieller Pan-Assay Interference Compound. Das reaktive Fragment SN001 senkte die Schmelztemperatur von T-p53C nur geringfügig um -0,57 °C (1 mM, 30 min). Daneben destabilisierten die über S_NAr reagierenden Fragmente SN006 und SN007 T-p53C stark. Fragment SN002 beeinflusste die Schmelztemperatur von T-p53C auch nach 24 Stunden Inkubation nicht maßgeblich, trotz der kovalenten Modifikation von T-p53C im MS-Experiment. Die Ergebnisse, die aus der Charakterisierung und Evaluierung der ersten CovLib-Fragmente gewonnen wurden, können für die Auswahl weiterer Verbindungen zur Erweiterung der Bibliothek genutzt werden. Außerdem können mehr Fragmente mit mittlerer Reaktivität in die Bibliothek integriert werden, um selektivere Treffer für p53 und neue interessante Targets zu identifizieren.

Zusätzlich wurde die CovLib mittels DSF mit verschiedenen p53-Mutanten getestet. Neben T-p53C wurden auch die DNA-Kontaktmutante R273H, die Strukturmutante

R282W in der DNA-bindenden Helix H2 und die β -Sandwich-Mutante Y220C untersucht. Das Vinylsulfon VS004 bewirkte eine allgemeine Stabilisierung von T-p53C und seinen Mutanten durch kovalente Modifizierung oberflächenexponierter Cysteine. VS004 stabilisierte vor allem die R282W-Mutante mit einer Erhöhung der Schmelztemperatur um bis zu 5,96 °C (1 mM, 30 min). Zudem wurden die Fragmente SN001, SN006 und dessen Bromanalogon SN007 identifiziert, die insbesondere die β -Sandwich-Mutante T-p53C-Y220C stabilisierten, was auf eine kovalente Modifikation des mutationsbedingten Cysteins hinwies. Intensive DSF-Experimente zeigten ein konzentrationsabhängiges Reaktionsverhalten. Die Stabilisierung von T-p53C-Y220C nahm mit abnehmender Konzentration dieser reaktiven Fragmente zu. Bei einer Konzentration von 250 μ M (molares Verhältnis 1:31,25) und 30 Minuten Inkubationszeit stabilisierte das CovLib-Fragment SN001 T-p53C-Y220C um bis zu 4,45 °C. Zusätzlich ergab das $^1\text{H}/^{15}\text{N}$ -HSQC-NMR-Experiment mit SN001 auffällige Peakverschiebungen, die auf eine kovalente Modifikation von Cysteinen in T-p53C-Y220C und eine Bindung in die Tasche hindeuteten. Daneben bestätigten MS-Messungen von T-p53C-Y220C, das mit SN001 inkubiert wurde, mehrfache Arylierungen des Proteins. Des Weiteren ergaben konzentrationsabhängige MS-Experimente und relative Quantifizierungen eine Abnahme der Anzahl der Arylierungen mit abnehmender Konzentration von SN001. Außerdem wurden DSF- und MS-Versuche mit einem Cystein-armen Y220C-Protein mit Mutationen der oberflächenexponierten Cysteinen zu Serinen durchgeführt. Die Ergebnisse zeigten, dass das Fragment SN001 das Protein durch kovalente Modifizierung des mutationsbedingten Cys220 stabilisierte. Die Auswirkungen der CovLib-Verbindungen auf die DNA-Bindungsaffinität von FL-T-p53 zu seinem nicht-apoptotischen Response-Element GADD45 α wurden anhand von FP-Messungen untersucht. Die CovLib-Treffer SN001 und VS004 hatten keine nachteiligen Auswirkungen auf die DNA-Bindung, während SN006 und SN007 die DNA-Bindungsfähigkeit von FL-T-p53 an GADD45 α verringerten. Abschließend wurde die kovalente Modifikation von Cys220 in der hydrophoben Bindungstasche durch SN001 und SN006 mittels Röntgenkristallographie bestätigt. Das Pyridinderivat SN001 besetzte den zentralen Hohlraum der Y220C-Tasche und nahm eine Tyr-ähnliche Konformation an, vergleichbar mit Tyr220 im Wildtyp-Protein. SN001 reagierte in einer $\text{S}_{\text{N}}\text{Ar}$ -Reaktion mit Cysteinen in p53 mit dem Fluoratom in der para-Position zur Nitrilgruppe als Abgangsgruppe. Die Nitrilgruppe zeigte in Richtung Bindungsbereich 1 und Thr150.

Das ortho-Fluor war auf den Bindungsbereich 2 gerichtet und das meta-Fluor zeigte in den hydrophoben Bindungsbereich 3. In der 40 mM-Struktur wurden mehrere Modifikationen gefunden, darunter die Arylierung von Cys229, die zu einem Flip der Seitenketten führte. Das Pyrazinderivat SN006 band in ähnlicher Weise in die hydrophobe Tasche wie SN001 und nahm die gleiche Tyr-ähnliche Konformation an.

Im dritten Teil der Arbeit wurden umfangreiche Studien zu Struktur-Wirkungs-Beziehungen (SAR) durchgeführt. Es wurde eine große Anzahl von SAR-Verbindungen mit unterschiedlichen Halogenierungsmustern, Abgangsgruppen, elektronenziehenden Gruppen, elektronenliefernden Gruppen und (Hetero)aromaten getestet. Ziel war es, die Reaktivität zu verringern und die Selektivität für bestimmte Cysteine in p53, insbesondere für Cys220 in der hydrophoben Bindungstasche, zu erhöhen und dadurch eine höhere Stabilisierung von T-p53C-Y220C zu erreichen. Im Rahmen des SAR-Screens wurden eine Reihe von Fragmenten mit S_NAr „Warhead“ entdeckt, die auf das Cys220 in der hydrophoben Tasche von T-p53C-Y220C abzielten und die temperaturempfindliche Krebsmutante deutlich stabilisierten. Bei allen identifizierten Fragmenten handelte es sich um Pyridin- oder Pyrazinderivate mit einem elektronenziehenden Nitril-Rest und einem Halogen als Abgangsgruppe in der para-Position. Variationen in den elektronenziehenden Gruppen oder (Hetero)aromaten führten nicht zu einer höheren Stabilisierung der Y220C-Mutante oder selektiveren Treffern für Cys220. Darüber hinaus führte die Verringerung der Anzahl der an den Pyridinring gebundenen Halogenatome zu einer erniedrigten Reaktivität der Verbindungen und zu einer erheblich verminderten Stabilisierung der Krebsmutante.

Das Pyridinderivat SN001-17 mit drei Chloratomen stabilisierte die β -Sandwich-Mutante konzentrations- und zeitabhängig. Mit zunehmender Inkubationszeit und sinkender Konzentration von SN001-17 nahm die Stabilisierung von T-p53C-Y220C zu. SN001-17 stabilisierte die Y220C-Mutante bei 250 μ M nach 24 Stunden Inkubation um bis zu 5,19 °C. Damit erzielte SN001-17 eine höhere maximale Stabilisierung als der CovLib-Hit SN001 ($\Delta T_m = 4,45$ °C, 250 μ M, 30 min). Zusätzlich erhöhte SN001-17 die Schmelztemperatur der Cystein-armen Y220C-Mutante um 5,20 °C (250 μ M, 24 h), was darauf hinwies, dass die kovalente Modifikation des Cys220 in der hydrophoben Tasche für die Stabilisierung von T-p53C-Y220C und T-p53C-Y220C-CL verantwortlich ist. Die Kristallstrukturanalyse bestätigte die kovalente Modifikation des Cys220 im hydrophoben Spalt. Das Pyridinderivat SN001-17 zeigte eine ähnliche Bindungsposition wie das CovLib-Fragment SN001, indem es die gleiche Tyr-ähnliche

Konformation annahm und den zentralen Hohlraum besetzte. Das Chlor in ortho-Position zur Nitrilgruppe zeigte in Richtung Bindungsbereich 2 und das meta-Chlor in Richtung Bindungsbereich 3.

Der SAR-Screening Treffer SN006/7-3 erhöhte die Schmelztemperatur von T-p53C-Y220C um bis zu 3,62 °C (1 mM, 24 h). Die Stabilisierung nahm mit zunehmender Inkubationszeit und Konzentration der Verbindung zu. Die Verbindung SN006/7-3 führte im MS-Experiment zu multiplen Modifikationen der Y220C-Mutante. Die Arylierung des Cys220 in der Bindungstasche wurde durch Röntgenkristallographie bestätigt. Das Pyrazinderivat SN006/7-3 nahm eine andere Konformation an als die Pyridinderivate SN001, SN001-17 und der CovLib-Treffer SN006. Die Verbindung war im Bindungsbereich 2 der Bindungstasche positioniert. Bindungsbereich 1 und Bindungsbereich 3 wurden von der Verbindung SN006/7-3 jedoch nicht besetzt.

Interessanterweise erhöhte die Verbindung SN006/7-8 mit einer elektronenliefernden Methylgruppe in der ortho-Position zur Abgangsgruppe des Fragments SN006/7-3 die Schmelztemperatur der Y220C-Mutante nach 24-stündiger Inkubation erheblich um bis zu 5,02 °C (1 mM). Die Stabilisierung von T-p53C-Y220C nahm mit zunehmender Inkubationszeit und erhöhter Konzentration der Verbindung zu. Die Einführung einer Methylgruppe in der ortho-Position zur Abgangsgruppe erhöhte den stabilisierenden Effekt im Vergleich zu SN006/7-3 ($\Delta T_m = 3,62$ °C, 1 mM, 24 h) ohne Methylgruppe und SN006/7-7 ($\Delta T_m = 2,08$ °C, 1 mM, 24 h) mit einer Methylgruppe in der meta-Position. Die kovalente Modifikation von Cys220 durch die Verbindung SN006/7-8 wurde durch Röntgenkristallographie bestätigt. Im Gegensatz zu Fragment SN006/7-3, das in den Bindungsbereich 2 der hydrophoben Tasche zeigte, nahm das Fragment SN006/7-8 mit der zusätzlichen Methylgruppe die gleiche Tyr-ähnliche Konformation an wie die Pyridinverbindungen SN001 und SN001-17 und das Pyrazinderivat SN006. Dabei ragte die Methylgruppe an Position 6 des Pyrazinrings von SN006/7-8 in den hydrophoben Bindungsbereich 3 hinein.

Zusätzlich wurden weitere SAR-Verbindungen mit größeren hydrophoben Gruppen in ortho-Position zur Abgangsgruppe getestet, um die Selektivität für die Tasche durch verbesserte hydrophobe Wechselwirkungen im Bindungsbereich 3 zu erhöhen. Dabei wurde mittels Röntgenkristallographie das sehr reaktive Fragment SN006/7-9 als Cys220-modifizierendes Fragment gefunden, das ebenfalls eine Tyr-ähnliche

Konformation in der Y220C-Bindetasche annahm. Insgesamt wurden bei diesem SAR-Screening entweder unreaktive Verbindungen oder sehr reaktive Verbindungen wie SN006/7-9 identifiziert, wobei kein besserer Treffer als das Fragment SN006/7-8 entdeckt wurde.

Zusammenfassend lässt sich sagen, dass mit dem auf kovalenten Fragmenten basierenden Ansatz viele Treffer für p53 und seine Mutanten entdeckt wurden. Mit VS004, VS004-1 und VS004-2 wurden Fragmente identifiziert, die Vinylsulfon „Warheads“ enthalten und eine allgemeine Stabilisierung des p53-Proteins durch kovalente Modifizierung oberflächenexponierter Cysteine bewirkten. Guiley und Shokat identifizierten kürzlich das erste Molekül, das Cys220 kovalent modifiziert, durch strukturbasiertes Design mit Anbringung des „Warheads“ an einen bekannten nicht-kovalenten Spaltbinder [143]. In dieser Arbeit wurden Cys220-arylierende Fragmente durch einen „electrophile first approach“ [204] entdeckt, indem eine Bibliothek kovalenter Fragmente untersucht wurde. Beim Screening der CovLib und des anschließenden Screenings der SAR-Fragmente wurden viele Treffer mit einem S_NAr „Warhead“ für die destabilisierte Krebsmutante T-p53C-Y220C gefunden. Die identifizierten Pyridin-oder Pyrazinderivate bewirkten eine enorme Stabilisierung der thermosensiblen Y220C-Krebsmutante durch kovalente Modifikation. Darüber hinaus zeigten die entdeckten Fragmente unterschiedliche Bindungsposen und zielten in verschiedene Bindungsbereiche der Y220C-Bindungstasche.

Dennoch sind alle identifizierten Fragmenttreffer sehr reaktiv und führten zu mehrfachen Modifizierungen von p53 und seinen Mutanten. Daher ist es notwendig, ein Gleichgewicht zwischen Reaktivität und Selektivität zu finden [232]. Die geringe Größe der identifizierten Fragmente bietet jedoch zahlreiche Möglichkeiten zur Optimierung in der hydrophoben Bindungstasche. Beispielsweise könnte die Selektivität für die Tasche mit größeren hydrophoben Gruppen als der Methylgruppe im Fragment SN006/7-8, die in den hydrophoben Bindungsbereich 3 zeigen, verbessert werden. Außerdem umfasst die nächste Phase der Fragment-basierten Wirkstoffforschung das Verbinden, Verschmelzen und Vergrößern der identifizierten Fragmenttreffer. Dadurch kann der nicht-kovalente Bindungsanteil und damit die Affinität und Selektivität für die Y220C-Bindungstasche durch reversible Wechselwirkungen erhöht werden [145, 231, 232]. Die Testung der identifizierten Treffer in zellulären Assays ist ebenfalls ein nächster Schritt im Prozess der Wirkstoffentwicklung.

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8 Appendix

8.1 Protein Sequences

Table 8.1: All used protein sequences. For crystallography, a construct with a deletion of the GGS overhang was used.

Protein	Sequence (N'-C')
T-p53C	GGSSSSVPSQKTYQGSYGFR LGFLHSGTAKSVTCTYSPALNKLFC QLAKTCPVQLWVDSTPPPGTRVRAMAIYKQSQHMTEVVRRCPPHH ERCSDSDGLAPPQH LIRVEGNLRAEYLDDRNTFRHSVVVPYEPPE VGSDCTTIHYNMCMYSSCMGGMNRRPILTIITLEDSSGNLLGRDSF EVRVCACPGRDRRTEENLRKKGEPHHELPPGSTKRALPNNT
xT-p53C (crystallography)	SSSVPSQKTYQGSYGFR LGFLHSGTAKSVTCTYSPALNKLFCQLA KTCVPVQLWVDSTPPPGTRVRAMAIYKQSQHMTEVVRRCPPHHERC SDSDGLAPPQH LIRVEGNLRAEYLDDRNTFRHSVVVPYEPPEVGS DCTTIHYNMCMYSSCMGGMNRRPILTIITLEDSSGNLLGRDSFEVR VCACPGRDRRTEENLRKKGEPHHELPPGSTKRALPNNT
T-p53C-Y220C	GGSSSSVPSQKTYQGSYGFR LGFLHSGTAKSVTCTYSPALNKLFC QLAKTCPVQLWVDSTPPPGTRVRAMAIYKQSQHMTEVVRRCPPHH ERCSDSDGLAPPQH LIRVEGNLRAEYLDDRNTFRHSVVVPCEPPE VGSDCTTIHYNMCMYSSCMGGMNRRPILTIITLEDSSGNLLGRDSF EVRVCACPGRDRRTEENLRKKGEPHHELPPGSTKRALPNNT
xT-p53C-Y220C (crystallography)	SSSVPSQKTYQGSYGFR LGFLHSGTAKSVTCTYSPALNKLFCQLA KTCVPVQLWVDSTPPPGTRVRAMAIYKQSQHMTEVVRRCPPHHERC SDSDGLAPPQH LIRVEGNLRAEYLDDRNTFRHSVVVPCEPPEVGS DCTTIHYNMCMYSSCMGGMNRRPILTIITLEDSSGNLLGRDSFEVR VCACPGRDRRTEENLRKKGEPHHELPPGSTKRALPNNT
T-p53C-R273H	GGSSSSVPSQKTYQGSYGFR LGFLHSGTAKSVTCTYSPALNKLFC QLAKTCPVQLWVDSTPPPGTRVRAMAIYKQSQHMTEVVRRCPPHH ERCSDSDGLAPPQH LIRVEGNLRAEYLDDRNTFRHSVVVPYEPPE VGSDCTTIHYNMCMYSSCMGGMNRRPILTIITLEDSSGNLLGRDSF EVHVCACPGRDRRTEENLRKKGEPHHELPPGSTKRALPNNT
xT-p53C-R273H (crystallography)	SSSVPSQKTYQGSYGFR LGFLHSGTAKSVTCTYSPALNKLFCQLA KTCVPVQLWVDSTPPPGTRVRAMAIYKQSQHMTEVVRRCPPHHERC SDSDGLAPPQH LIRVEGNLRAEYLDDRNTFRHSVVVPYEPPEVGS DCTTIHYNMCMYSSCMGGMNRRPILTIITLEDSSGNLLGRDSFEVH VCACPGRDRRTEENLRKKGEPHHELPPGSTKRALPNNT
T-p53C-R282W	GGSSSSVPSQKTYQGSYGFR LGFLHSGTAKSVTCTYSPALNKLFC QLAKTCPVQLWVDSTPPPGTRVRAMAIYKQSQHMTEVVRRCPPHH ERCSDSDGLAPPQH LIRVEGNLRAEYLDDRNTFRHSVVVPYEPPE VGSDCTTIHYNMCMYSSCMGGMNRRPILTIITLEDSSGNLLGRDSF EVRVCACPGRDWRTEENLRKKGEPHHELPPGSTKRALPNNT
xT-p53C-R282W (crystallography)	SSSVPSQKTYQGSYGFR LGFLHSGTAKSVTCTYSPALNKLFCQLA KTCVPVQLWVDSTPPPGTRVRAMAIYKQSQHMTEVVRRCPPHHERC SDSDGLAPPQH LIRVEGNLRAEYLDDRNTFRHSVVVPYEPPEVGS DCTTIHYNMCMYSSCMGGMNRRPILTIITLEDSSGNLLGRDSFEVR VCACPGRDWRTEENLRKKGEPHHELPPGSTKRALPNNT

T-p53C- C124/182/277S	GGSSSSVPSQKTYQGSYGFR LGFLHSGTAKSVTSTYSPALNKLFC QLAKTCPVQLWVDSTPPPGTRVRAMAIYKQSQHMTEVVRRCPPH ERSSDSGLAPPQH LIRVEGNLRAEYLDDRNTFRHSVVVPYEPPE VGSDCTTIHYNMCMYSSCMGGMNRRPILTIITLEDSSGNLLGRDSF EVRVCASPGRDRRTEEEENLRKKGEPHHELPPGSTKRALPNNT
T-p53C-Y220C- C124/182/229/275/ 277S (T-p53C-Y220C- CL)	GGSSSSVPSQKTYQGSYGFR LGFLHSGTAKSVTSTYSPALNKLFC QLAKTCPVQLWVDSTPPPGTRVRAMAIYKQSQHMTEVVRRCPPH ERSSDSGLAPPQH LIRVEGNLRAEYLDDRNTFRHSVVVPCEPPE VGSDSTTIHYNMCMYSSCMGGMNRRPILTIITLEDSSGNLLGRDSF EVRVSASPGRDRRTEEEENLRKKGEPHHELPPGSTKRALPNNT
FL-T-p53	GGSMEEPQSDPSVEPPLSQETFS DLWKLLPENNVLSPLPSQAMDD LMLSPDDIEQWFTEDPGPDEAPRMPEAAPPVAPAPAAPTPAAPAP APSWPLSSSVPSQKTYQGSYGFR LGFLHSGTAKSVTCTYSPALNK LFCQLAKTCPVQLWVDSTPPPGTRVRAMAIYKQSQHMTEVVRRCPPH HERCSDSDGLAPPQH LIRVEGNLRAEYLDDRNTFRHSVVVPYEP PEVGSDCTTIHYNMCMYSSCMGGMNRRPILTIITLEDSSGNLLGRD SFEVRVCACPGDRDRRTEEEENLRKKGEPHHELPPGSTKRALPNNTS SSPQPKKKPLDGEYFTLQIRGRERFEMFRELNEALELKDAQAGKEP GGSRAHSSHLKSKKGQSTSRHKKLMFKTEGPDSD
JNK3	GGSMSKSKVDNQFYSVEVGDSTFTVLKRYQNLKPIGSGAQGIVCA AYDAVLDRNVAIKKLSRPFQNQTHAKRAYREL VLMKCVNHKNIIISLL NVFTPQKTLEEFQDVYLVME LMDANLCQVIQMELDHERMSYLLYQ MLCGIKHLHSAGIIHRDLKPSNIVVKSDCTLKILDFGLARTAGTSFMM TPYVVTRYRAPEVILGMGYKENVDIWSVGCIMGEMVRHKILFPGR DYIDQWNKVIEQLGTPCPEFMKKLQPTVRNYVENRPKYAGLTFPKL FPDSLFPADSEHNKLKASQARDLLSKMLVIDPAKRISVDDALQHPYI NVWYDPAEVEAPPPQIYDKQLDEREHTIEEWKELIYKEVMNSE
TEV	GHHHHHHHGESLFGPRDYNPISSTICHLTNESDGHTTSLYIGIGFG PFIITNKH LFR RNNGTLLVQSLHGVFKVKN TTTTLQQHLIDGRDMIIR MPKDFPPFPQKLKFREPQREERICLVTTNFQTKSMSSMVSDTSCT FPSSDGIFWKHWIQTKDGQCGSPLVSTRDGFIVGIHSASNFTNTNN YFTSVPKNFMELLTNQEAQQWVSGWRLNADSVLWGGHKVFMPK PEEPFQPVKEATQLMNRRRRR

8.2 Protein Expression and Purification

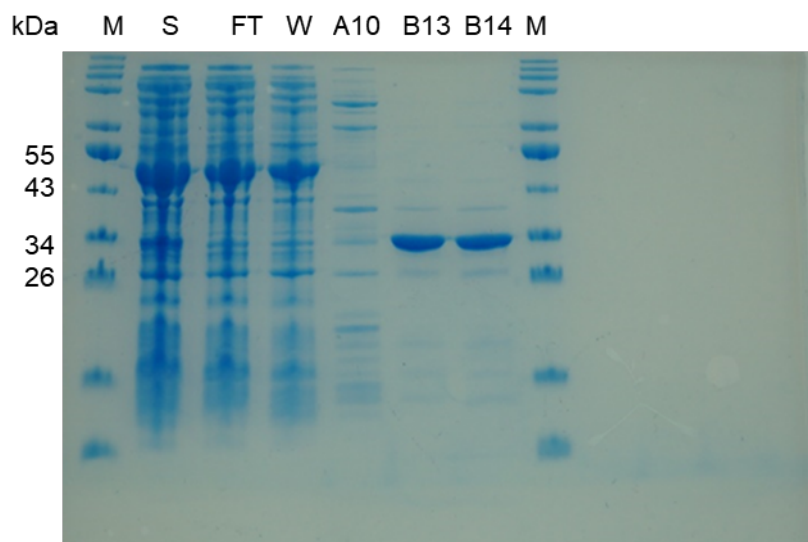


Figure 8.1: SDS-PAGE after the nickel affinity chromatography step of the TEV protease. M= marker, S= supernatant, FT= flow-through, W= wash step, A10= discarded fraction containing impurities, B13-B14= pooled fractions containing the TEV protease with a size of 28.615 kDa.

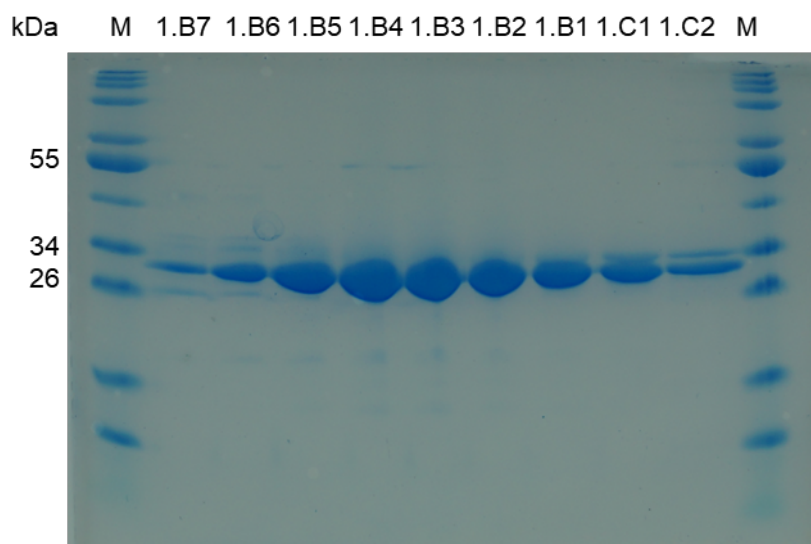


Figure 8.2: SDS-PAGE after the SEC run of xT-p53C-Y220C. M= marker, 1.B7-1.B1= pooled fractions containing the pure xT-p53C-Y220C protein with a size of 24.495 kDa, 1.C1-1.C2= discarded fractions with an additional signal over the protein signal.

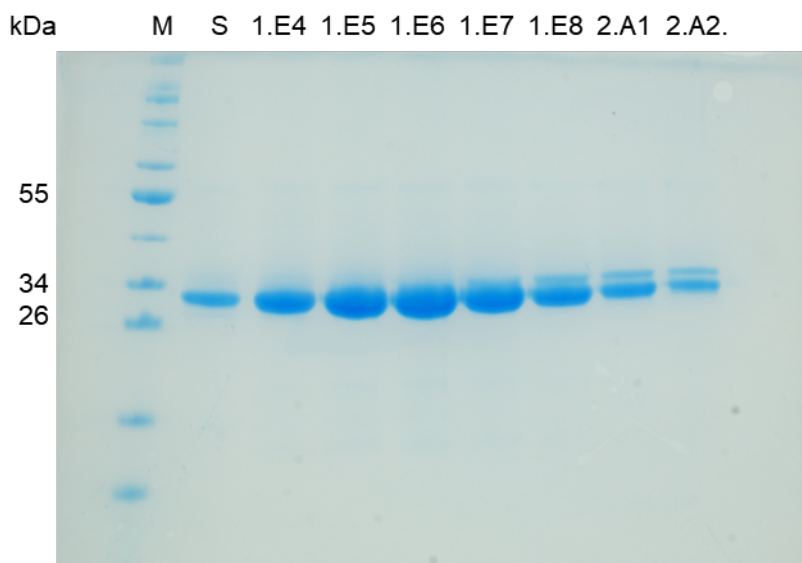


Figure 8.3: SDS-PAGE after the SEC run of xT-p53C. M= marker, S= concentrated xT-p53C after the heparin column, 1.E4-1.E8= pooled fractions containing the pure xT-p53C protein with a size of 24.555 kDa, 2.A1-2.A2= discarded fractions with an additional signal over the protein signal.

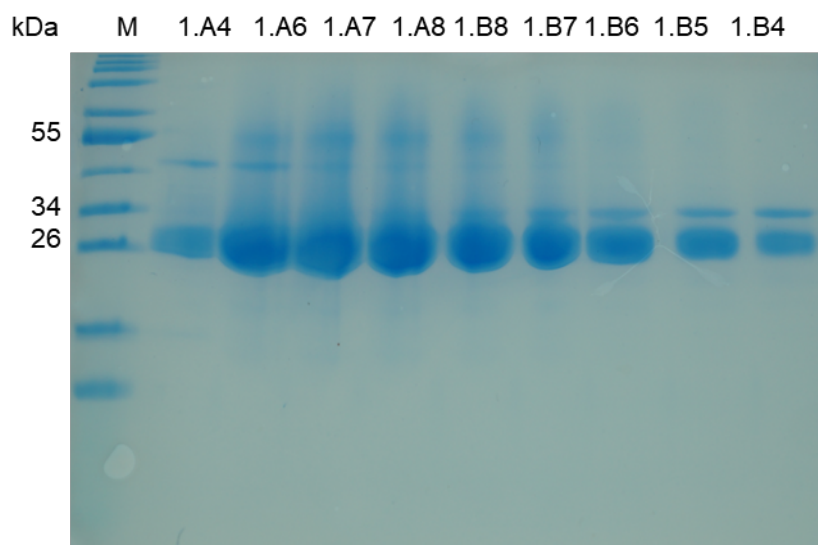


Figure 8.4: SDS-PAGE after the SEC run of xT-p53C-R282W. M= marker, S= concentrated xT-p53C-R282W after the heparin column, 1.A4-1.B7= pooled fractions containing the pure xT-p53C-R282W protein with a size of 24.585 kDa, 1.B6-1.B4= discarded fractions with an additional signal over the protein signal.

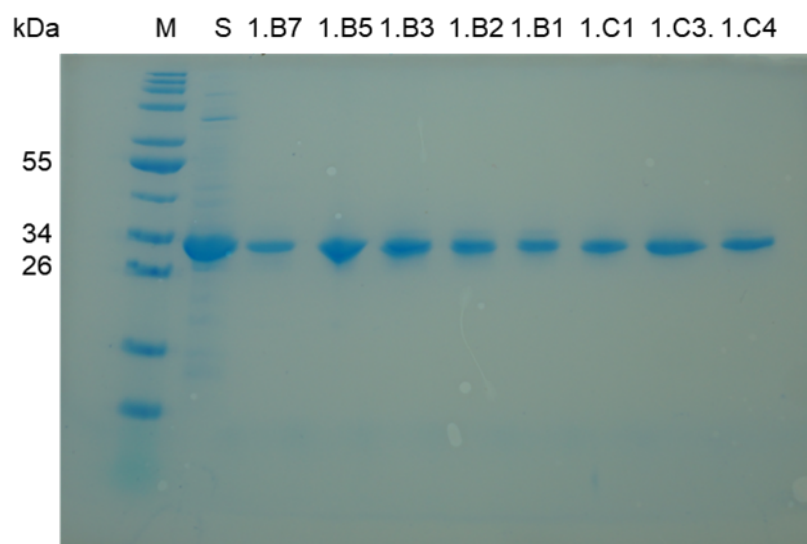


Figure 8.5: SDS-PAGE after the SEC run of ^{15}N -labeled T-p53C-Y220C. M= marker, S= concentrated ^{15}N -labeled T-p53C-Y220C protein after the heparin column, 1.B7-1.C4= pooled fractions containing the pure ^{15}N -labeled T-p53C-Y220C protein with a size of 24.696 kDa.

8.3 Crystallographic Data Collection and Refinement Statistics of the xT-p53C-Y220C Crystal Soaked with 1151

Table 8.2: Crystallographic data collection and refinement statistics of the dataset of xT-p53C-Y220C with 1151 corresponding to the PDB code 8A92.

	Compound 1151 (PDB: 8A92)
Wavelength [Å]	0.92
Space group	P2 ₁ 2 ₁ 2 ₁
Cell dimension	
a, b, c [Å]	65.1, 74.1, 105.2
α , β , γ [°]	90, 90, 90
Resolution range [Å]	50-1.37 (1.45-1.37)
Redundancy	13.2 (13.3)
Completeness [%]	100 (100)
Mean I/ σ (I)	14.4 (1.0)
R-meas [%]	11.7 (256.8)
CC1/2 [%]	99.9 (49.7)
Wilson B [Å ²]	22.8
Resolution included [Å]	43.68-1.37
R _{work} /R _{free} [%]	13.32/18.32
Bond RMSD [Å]	0.008
Angle RMSD [°]	0.99
Ramachandran [%] (favored/allowed/outliers)	98.98/1.02/0
Rotamer outliers [%]	1.43
All-atom Clashscore	1.44
Average B factor [Å ²]	
Overall	22.34
Protein	20.03
Ligand	41.4
Water	35.58

8.4 Primary Screening of the CovLib with T-p53C Using DSF

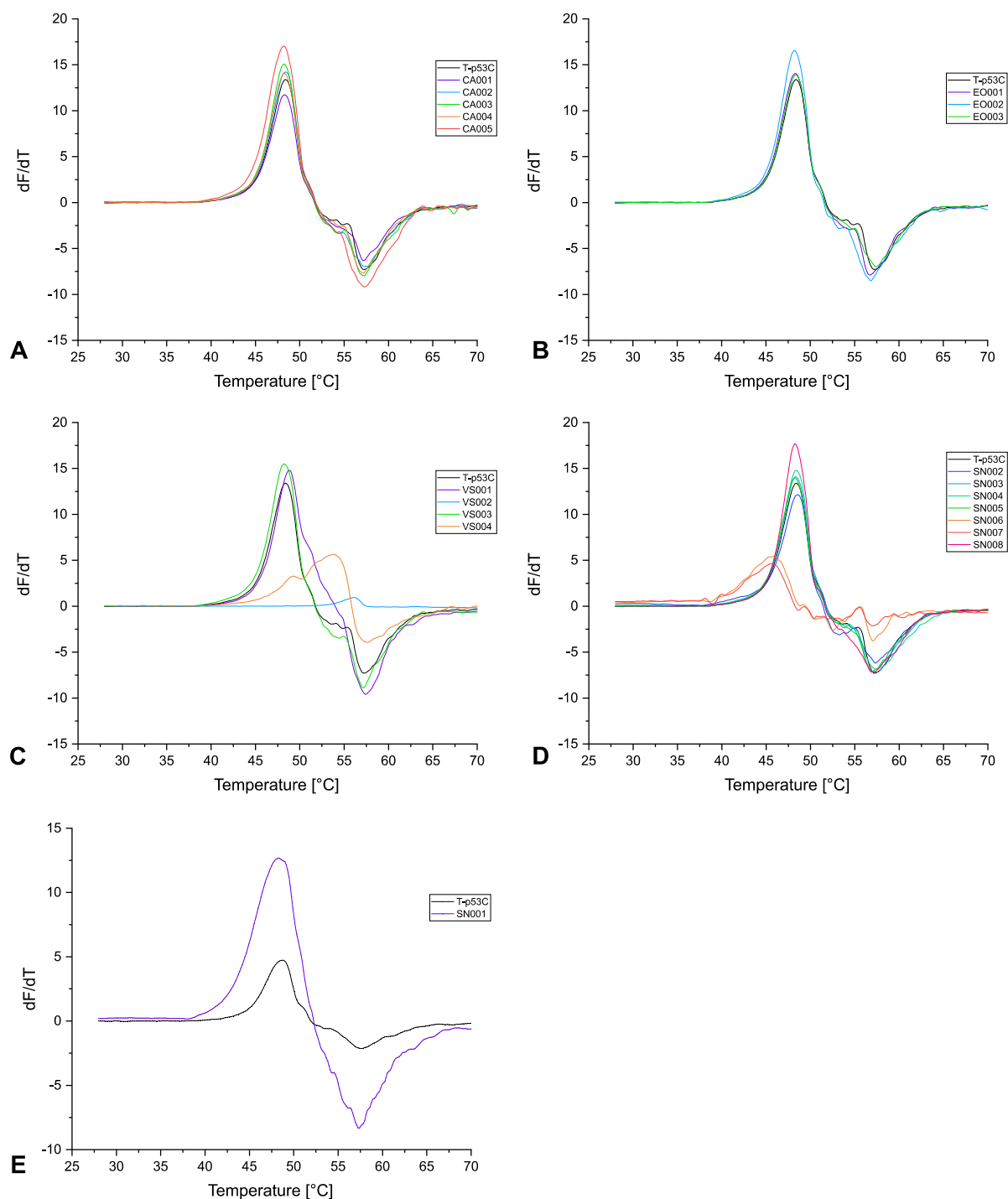


Figure 8.6: DSF screen of the CovLib with T-p53C after 4 h of incubation. The first derivatives of the melting curves of α -cyanoacrylamides/acrylates (A), epoxides (B), vinyl sulfones (C), S_NAr (D), and SN001 (E) fragments (1 mM) with T-p53C (8 μ M protein) after 4 h of incubation at 20 °C are shown.

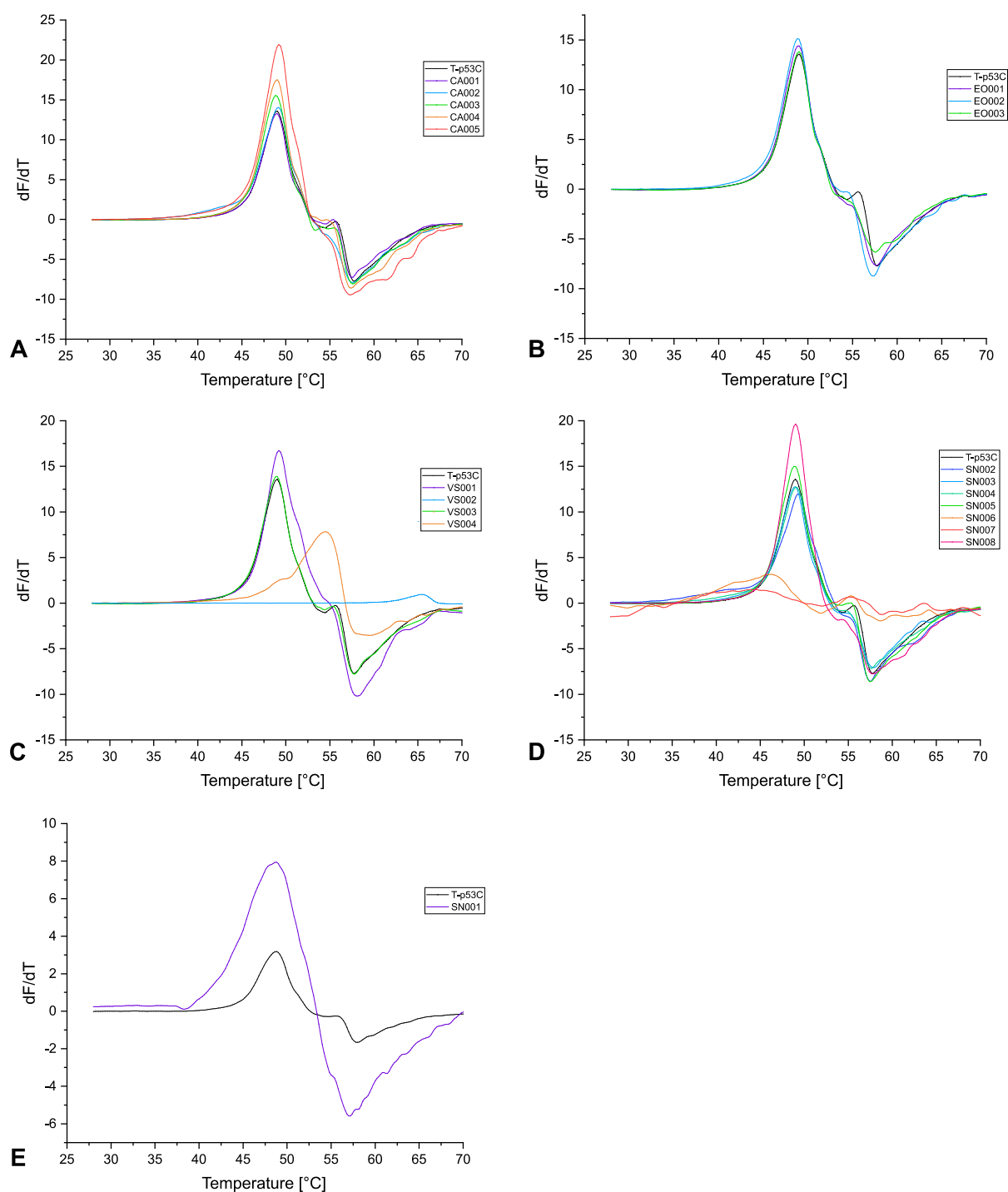


Figure 8.7: DSF screen of the CovLib with T-p53C after 24 h of incubation. The first derivatives of the melting curves of α -cyanoacrylamides/acrylates (A), epoxides (B), vinyl sulfones (C), S_NAr (D), and SN001 (E) fragments (1 mM) with T-p53C (8 μ M protein) after 24 h of incubation at 20 °C are shown.

8.5 DSF Screen of the CovLib with T-p53C and Its Mutants

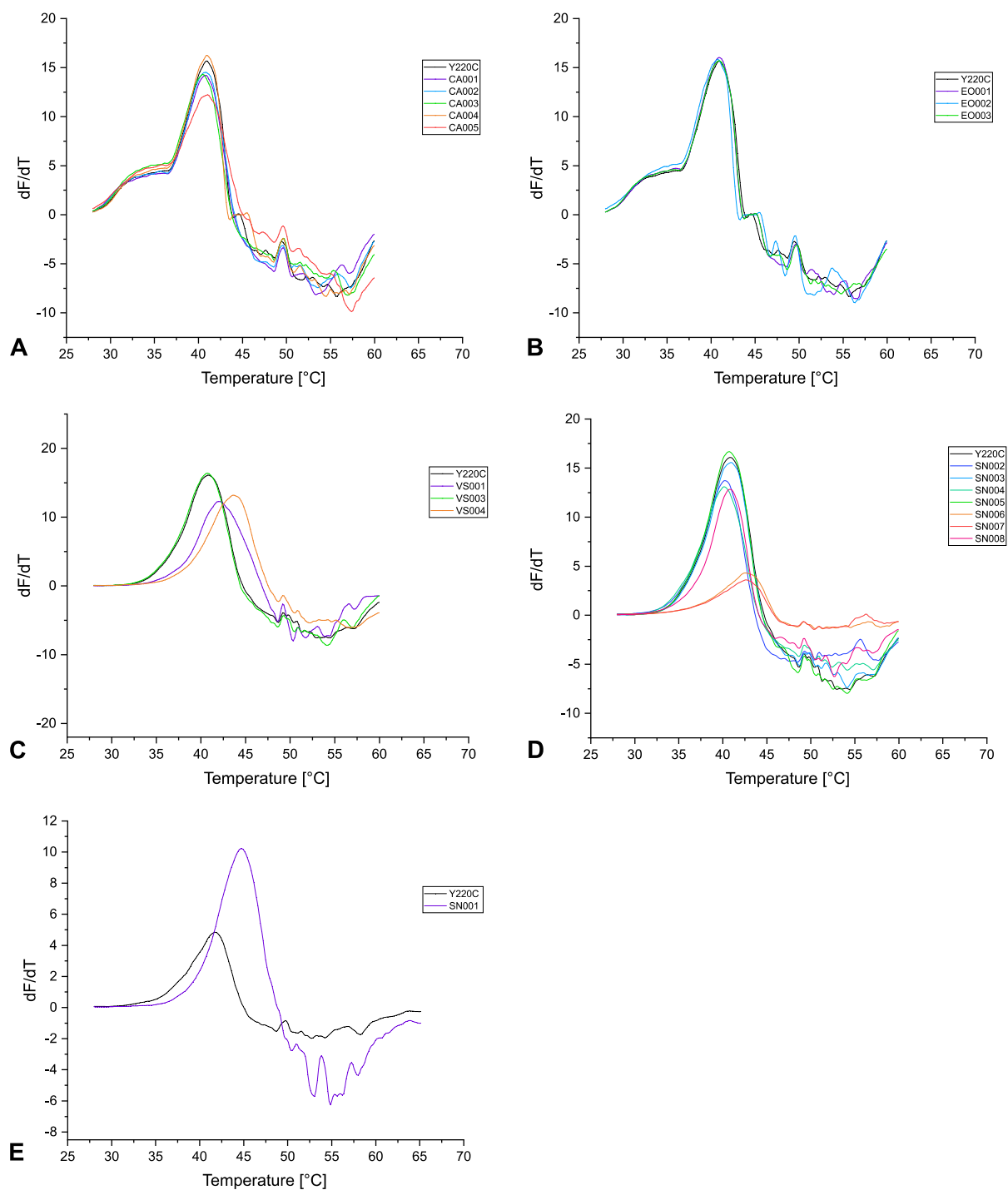


Figure 8.8: DSF screen of the CovLib with T-p53C-Y220C. The first derivatives of the melting curves of α -cyanoacrylamides/acylates (A), epoxides (B), vinyl sulfones (C), S_NAr (D), and SN001 (E) fragments (1 mM) with T-p53C-Y220C (8 μ M protein) after 30 min of incubation at 20 °C are shown.

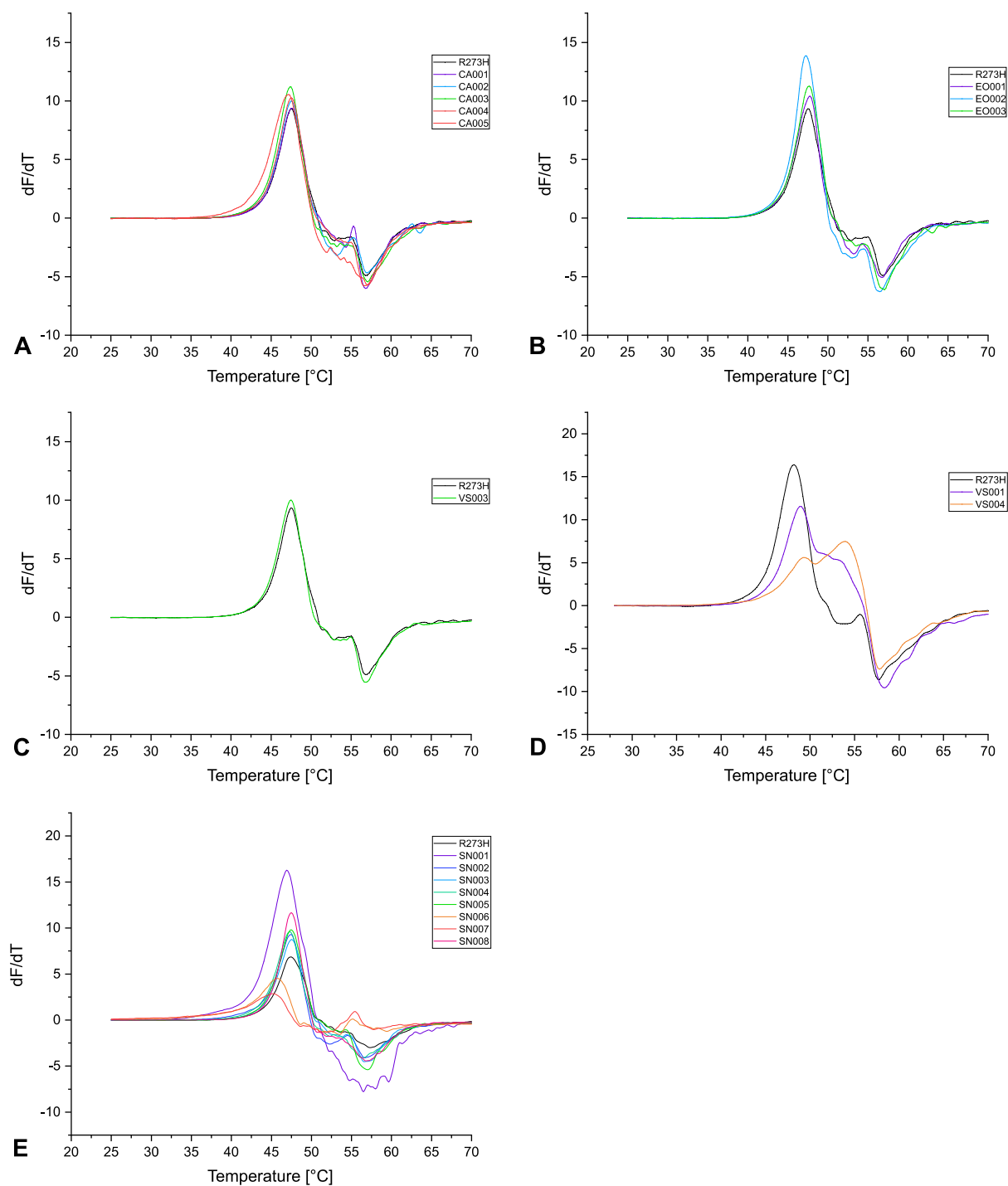


Figure 8.9: DSF screen of the CovLib with T-p53C-R273H. The first derivatives of the melting curves of α -cyanoacrylamides/acrylates (A), epoxides (B), VS003 (C), VS001, VS004 (D), and SAr (E) fragments (1 mM) with T-p53C-R273H (8 μ M protein) after 30 min of incubation at 20 °C are shown.

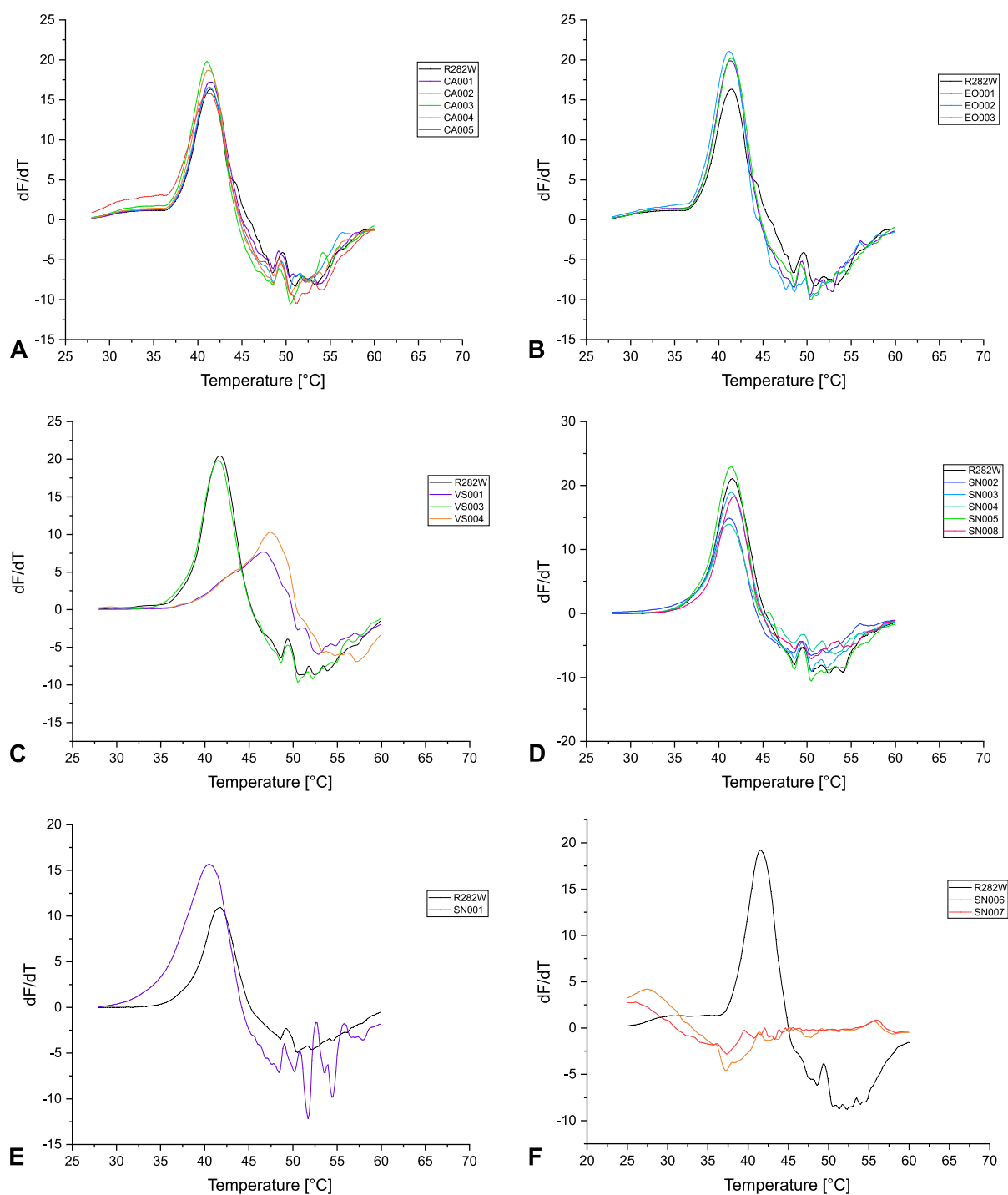


Figure 8.10: DSF screen of the CovLib with T-p53C-R282W. The first derivatives of the melting curves of α -cyanoacrylamides/acrylates (A), epoxides (B), vinyl sulfones (C), S_NAr (D), SN001 (E), and SN006, SN007 (F) fragments (1 mM) with T-p53C-R282W (8 μ M protein) after 30 min of incubation at 20 °C are shown.

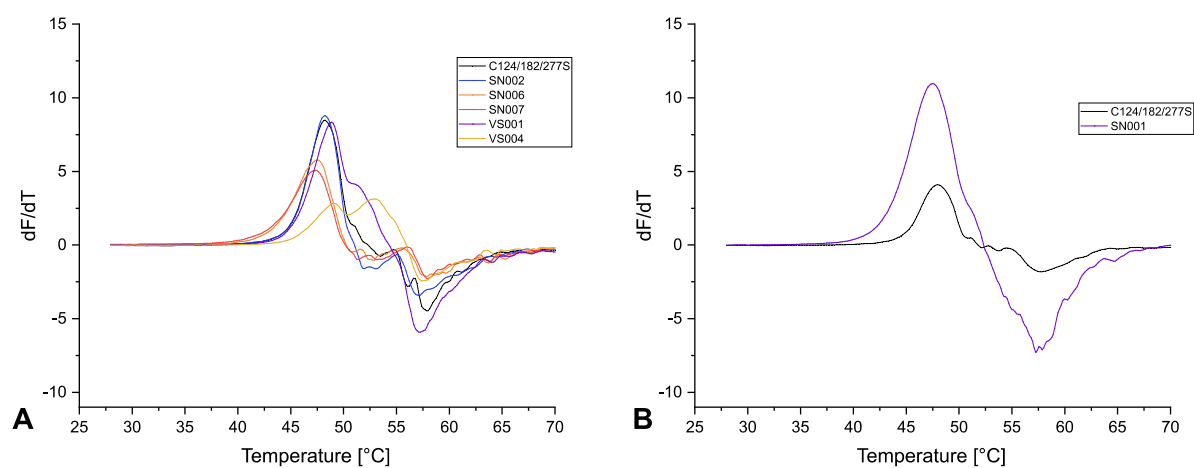


Figure 8.11: DSF screen of the CovLib hits with T-p53C-C124/182/277S. The first derivatives of the melting curves of the CovLib hits SN002, SN006, SN007, VS001, VS004 (**A**), and SN001 (**B**) (1 mM) with T-p53C-C124/182/277S (8 μ M protein) after 30 min of incubation at 20 °C are shown.

8.6 Crystallographic Data Collection and Refinement Statistics of the xT-p53C-Y220C Crystals Soaked with SN001 or SN006

Table 8.3: Crystallographic data collection and refinement statistics of the dataset of xT-p53C-Y220C soaked with 5 mM SN001 corresponding to the PDB code 8RBA. Parenthesized values show the respective value of the highest resolution bin.

	Compound SN001 5 mM (PDB: 8RBA)
Wavelength [Å]	1
Space group	P2 ₁ 2 ₁ 2 ₁
Cell dimension	
a, b, c [Å]	64.91, 70.71, 105.33
α , β , γ [°]	90, 90, 90
Resolution range [Å]	50-1.57 (1.66-1.57)
Redundancy	9.28 (8.87)
Completeness [%]	97.1 (90.5)
Mean I/ σ (I)	9.47 (1.04)
R-meas [%]	14.7 (212.3)
CC1/2 [%]	99.8 (54.7)
Wilson B [Å ²]	30
Resolution included [Å]	47.82-1.57
R _{work} /R _{free} [%]	15.01/18.26
Bond RMSD [Å]	0.009
Angle RMSD [°]	0.986
Ramachandran [%] (favored/allowed/outliers)	99.74/0.26/0
Rotamer outliers [%]	3.20
All-atom Clashscore	2.54
Average B factor [Å ²]	
Overall	28.76
Protein	26.14
Ligand	39.78
Water	41.02

Table 8.4: Crystallographic data collection and refinement statistics of the dataset of xT-p53C-Y220C soaked with 40 mM SN001 corresponding to the PDB code 8RBB. Parenthesized values show the respective value of the highest resolution bin.

	Compound SN001 40 mM (PDB: 8RBB)
Wavelength [Å]	1
Space group	P2 ₁ 2 ₁ 2 ₁
Cell dimension	
a, b, c [Å]	65.08, 70.99, 105.94
α , β , γ [°]	90, 90, 90
Resolution range [Å]	50-1.69 (1.79-1.69)
Redundancy	12.54 (8.56)
Completeness [%]	99.5 (96.9)
Mean I/ σ (I)	19.43 (1.03)
R-meas [%]	8.4 (201.2)
CC1/2 [%]	99.9 (56.9)
Wilson B [Å ²]	38.74
Resolution included [Å]	47.97-1.69
R _{work} /R _{free} [%]	16.38/20.73
Bond RMSD [Å]	0.01
Angle RMSD [°]	1.018
Ramachandran [%] (favored/allowed/outliers)	99.74/0.26/0
Rotamer outliers [%]	1.82
All-atom Clashscore	2.46
Average B factor [Å ²]	
Overall	41.5
Protein	39.27
Ligand	63.90
Water	50.27

Table 8.5: Crystallographic data collection and refinement statistics of the dataset of xT-p53C-Y220C soaked with 5 mM SN006 corresponding to the PDB code 8RBC. Parenthesized values show the respective value of the highest resolution bin.

	Compound SN006 5 mM (PDB: 8RBC)
Wavelength [Å]	1
Space group	P2 ₁ 2 ₁ 2 ₁
Cell dimension	
a, b, c [Å]	65.91, 71.09, 107.06
α , β , γ [°]	90, 90, 90
Resolution range [Å]	50-2.06 (2.18-2.06)
Redundancy	13.37 (13.5)
Completeness [%]	100 (100)
Mean I/ σ (I)	10.48 (1.01)
R-meas [%]	18.8 (342)
CC1/2 [%]	99.9 (73.7)
Wilson B [Å ²]	48.19
Resolution included [Å]	48.33-2.06
R _{work} /R _{free} [%]	22.14/26.02
Bond RMSD [Å]	0.007
Angle RMSD [°]	0.889
Ramachandran [%] (favored/allowed/outliers)	99.23/0.77/0
Rotamer outliers [%]	2.19
All-atom Clashscore	2.20
Average B factor [Å ²]	
Overall	49
Protein	48.5
Ligand	59.6
Water	48.87

8.7 DSF Screen of SN001 SAR Compounds with T-p53C and Its Mutants

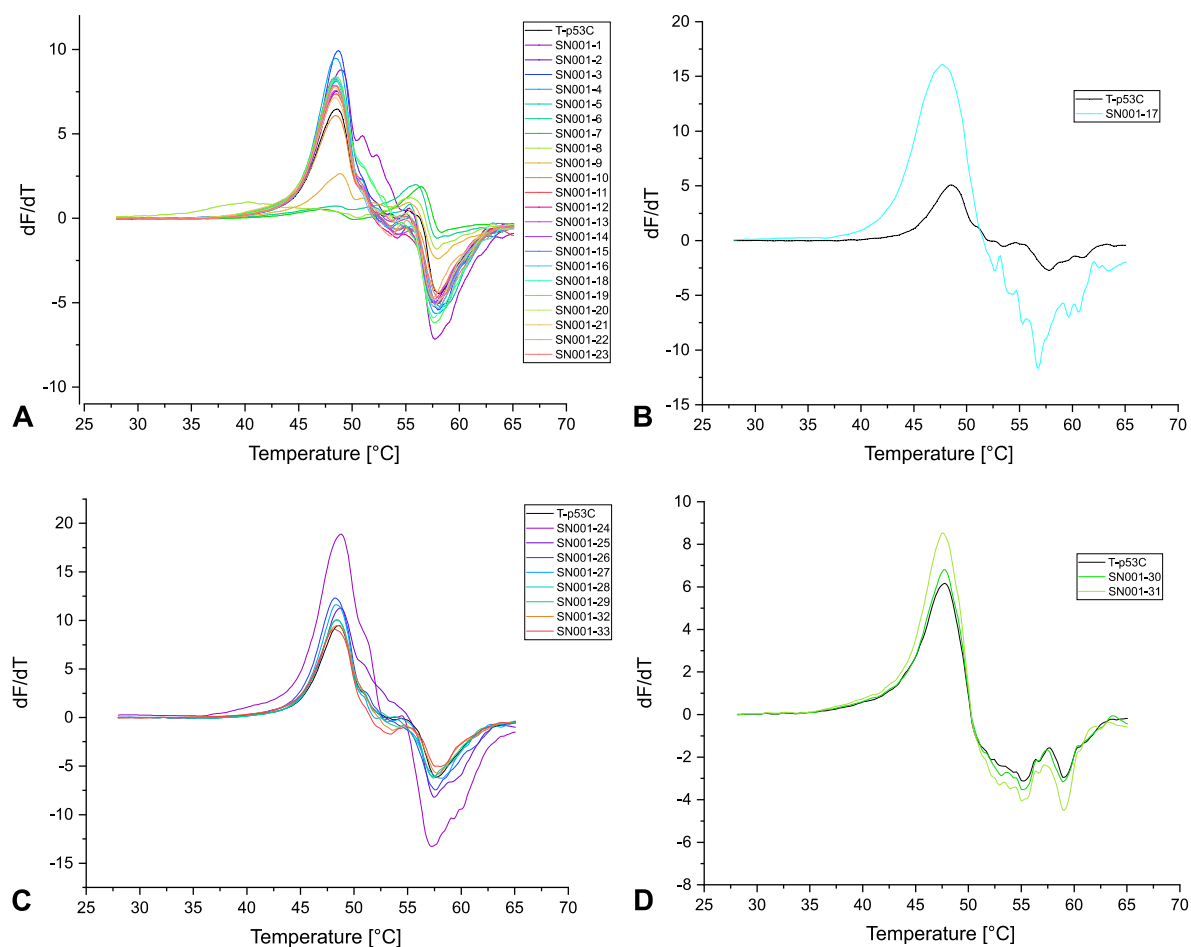


Figure 8.12: DSF screen of SN001 SAR compounds with T-p53C after 30 min of incubation. The first derivatives of the melting curves of SN001 SAR compounds (**1 mM**): SN001-1 to SN001-23 (**A**), SN001-17 (**B**), SN001-24 to SN001-29, SN001-32, SN001-33 (**C**), SN001-30, and SN001-31 (**D**) with **T-p53C** (8 μ M protein) after **30 min** of incubation at 20 °C are shown.

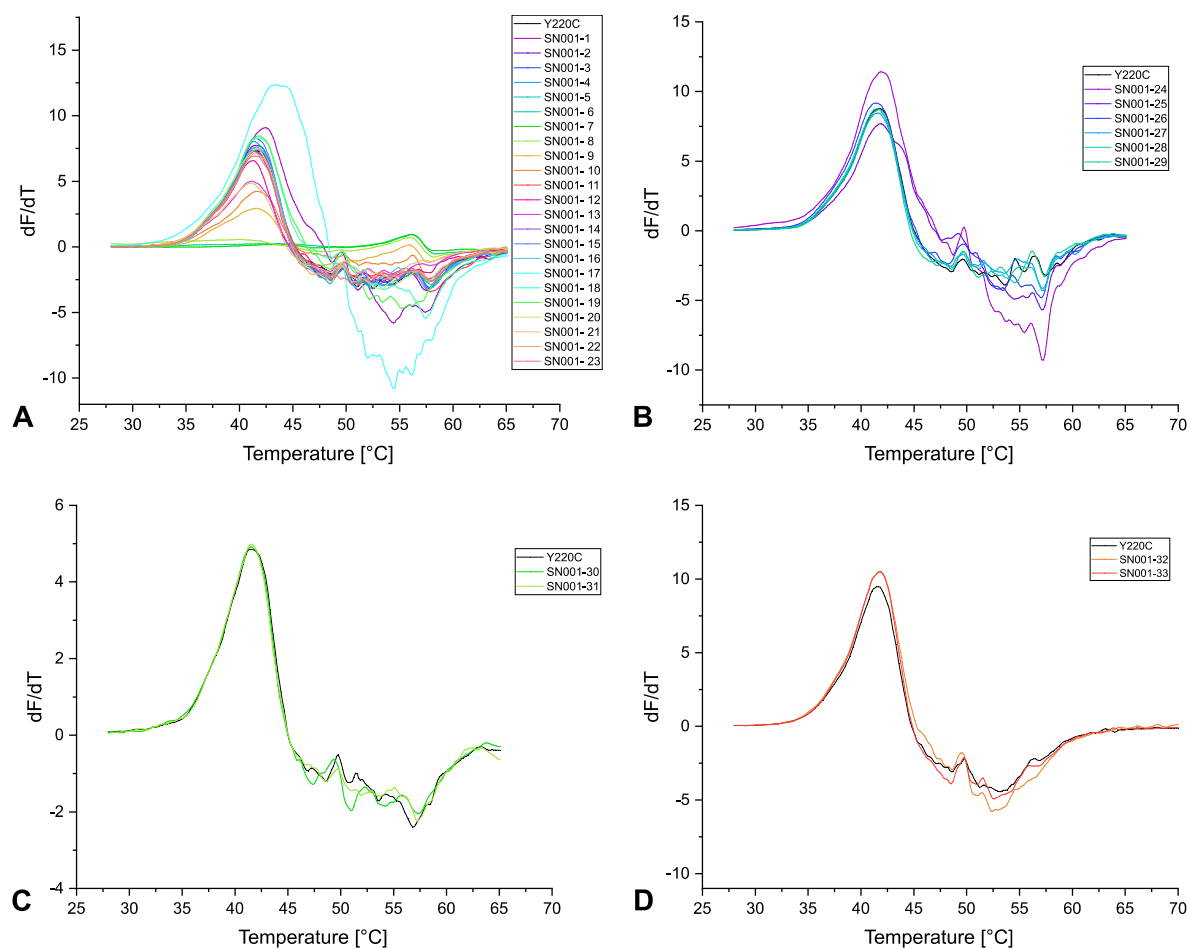


Figure 8.13: DSF screen of SN001 SAR compounds with T-p53C-Y220C after 30 min of incubation. The first derivatives of the melting curves of SN001 SAR compounds (**1 mM**): SN001-1 to SN001-23 (**A**), SN001-24 to SN001-29 (**B**), SN001-30, SN001-31 (**C**), SN001-32, and SN001-33 (**D**) with **T-p53C-Y220C** (8 μ M protein) after **30 min** of incubation at 20 °C are shown.

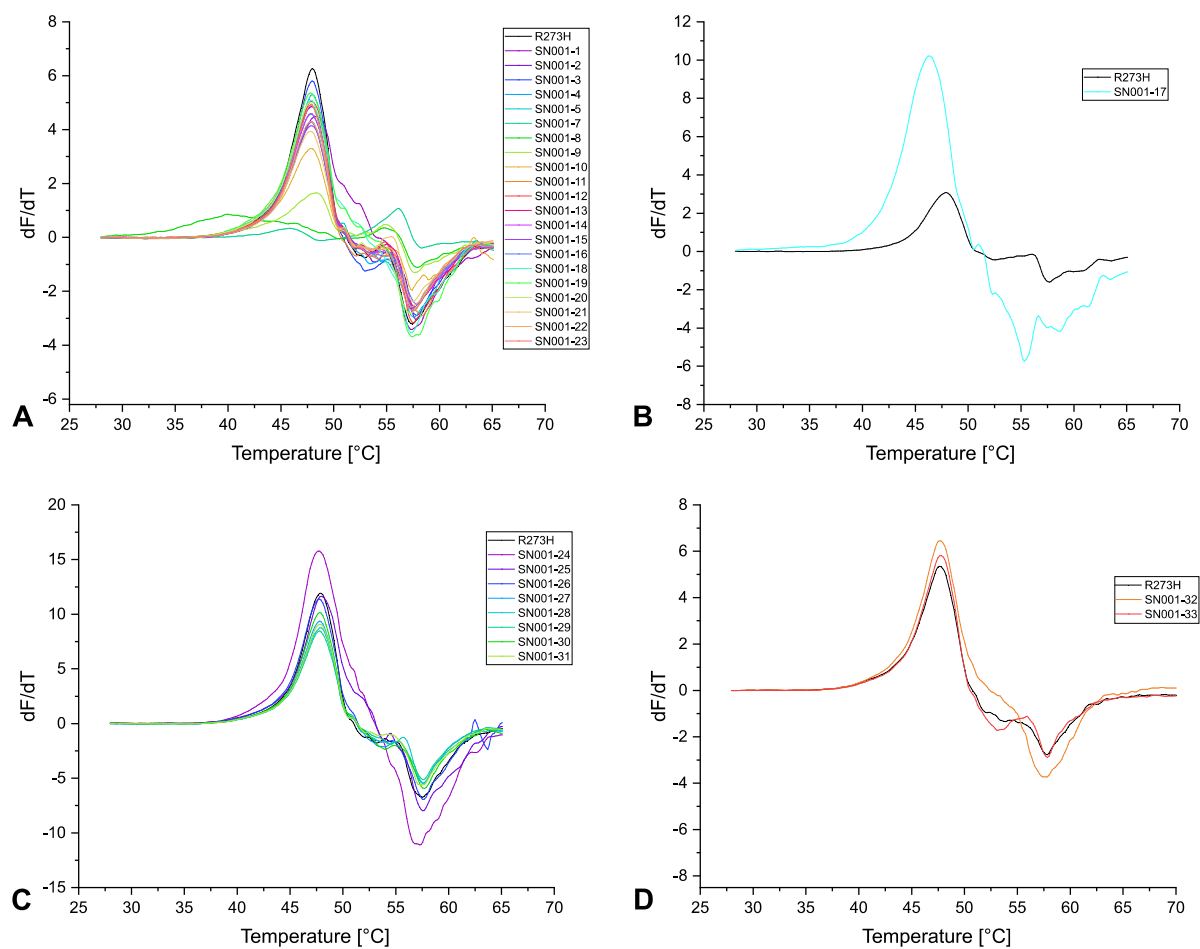


Figure 8.14: DSF screen of SN001 SAR compounds with T-p53C-R273H after 30 min of incubation. The first derivatives of the melting curves of SN001 SAR compounds (1 mM): SN001-1 to SN001-23 (A), SN001-17 (B), SN001-24 to SN001-31 (C), SN001-32, and SN001-33 (D) with T-p53C-R273H (8 μM protein) after 30 min of incubation at 20 $^{\circ}\text{C}$ are shown.

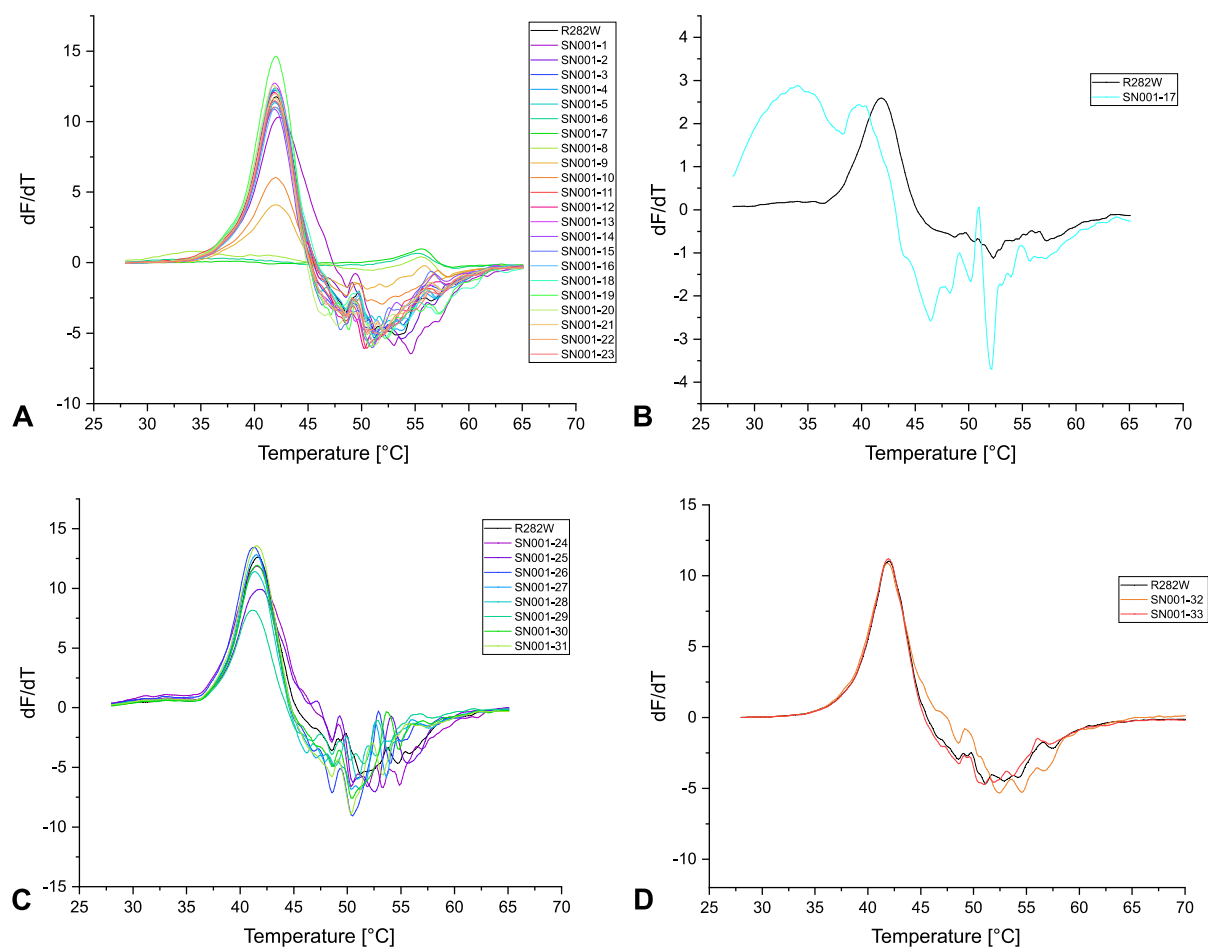


Figure 8.15: DSF screen of SN001 SAR compounds with T-p53C-R282W after 30 min of incubation. The first derivatives of the melting curves of SN001 SAR compounds (1 mM): SN001-1 to SN001-23 (A), SN001-17 (B), SN001-24 to SN001-31 (C), SN001-32, and SN001-33 (D) with T-p53C-R282W (8 μ M protein) after 30 min of incubation at 20 °C are shown.

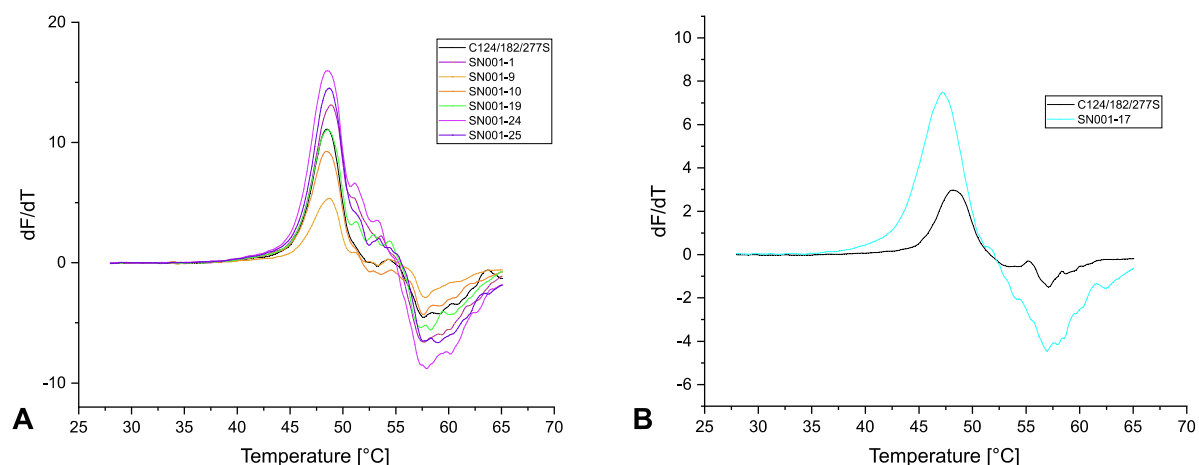


Figure 8.16: DSF screen of SN001 SAR hits with T-p53C-C124/182/277S after 30 min of incubation. The first derivatives of the melting curves of SN001 SAR compounds (1 mM): SN001-1, SN001-9, SN001-10, SN001-19, SN001-24, SN001-25 (A), and SN001-17 (B) with T-p53C-C124/182/277S (8 μ M protein) after 30 min of incubation at 20 °C are shown.

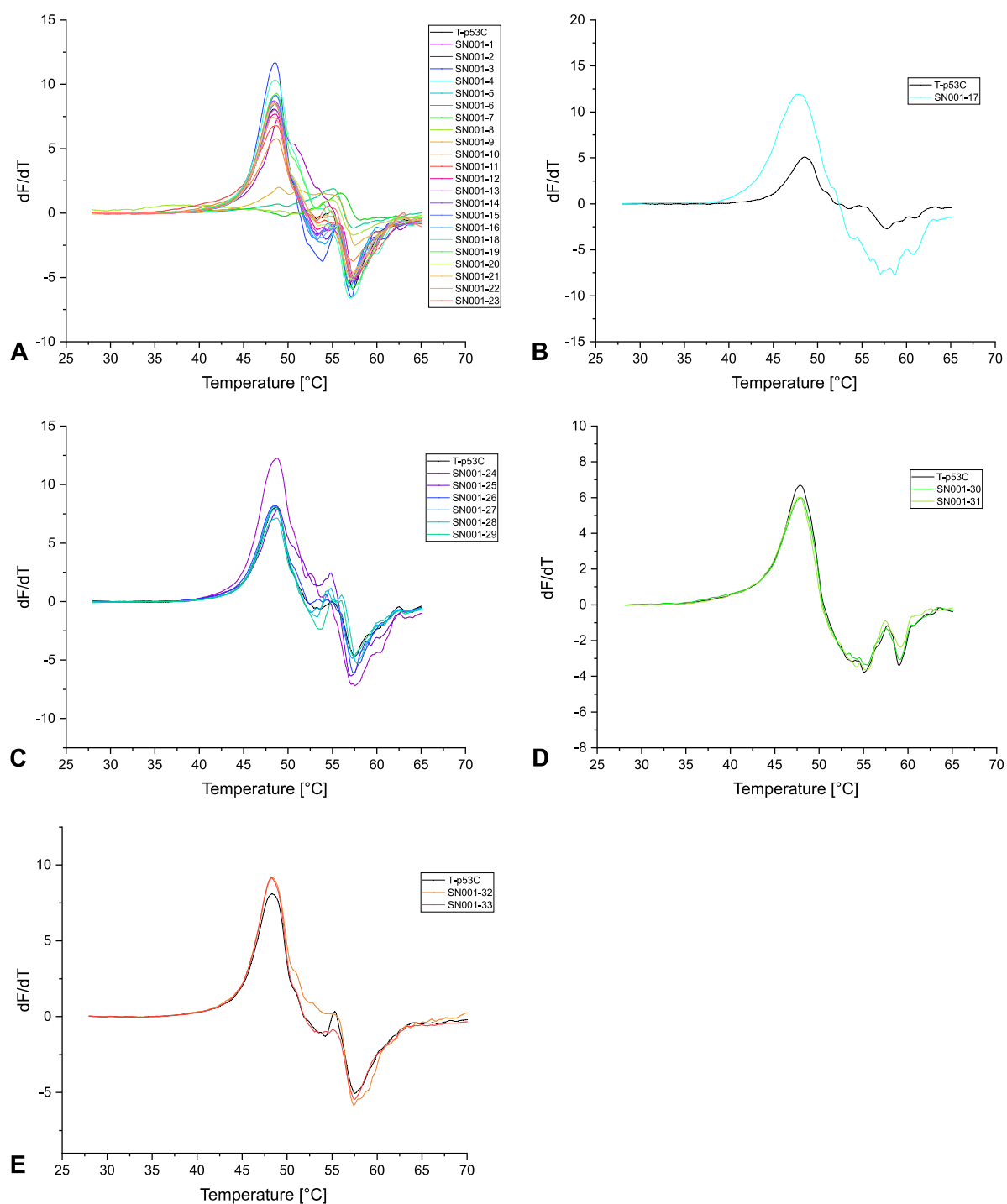


Figure 8.17: DSF screen of SN001 SAR compounds with T-p53C after 4 h of incubation. The first derivatives of the melting curves of SN001 SAR compounds (1 mM): SN001-1 to SN001-23 (A), SN001-17 (B), SN001-24 to SN001-29 (C), SN001-30, SN001-31 (D), SN001-32, and SN001-33 (E) with T-p53C (8 μM protein) after 4 h of incubation at 20 °C are shown.

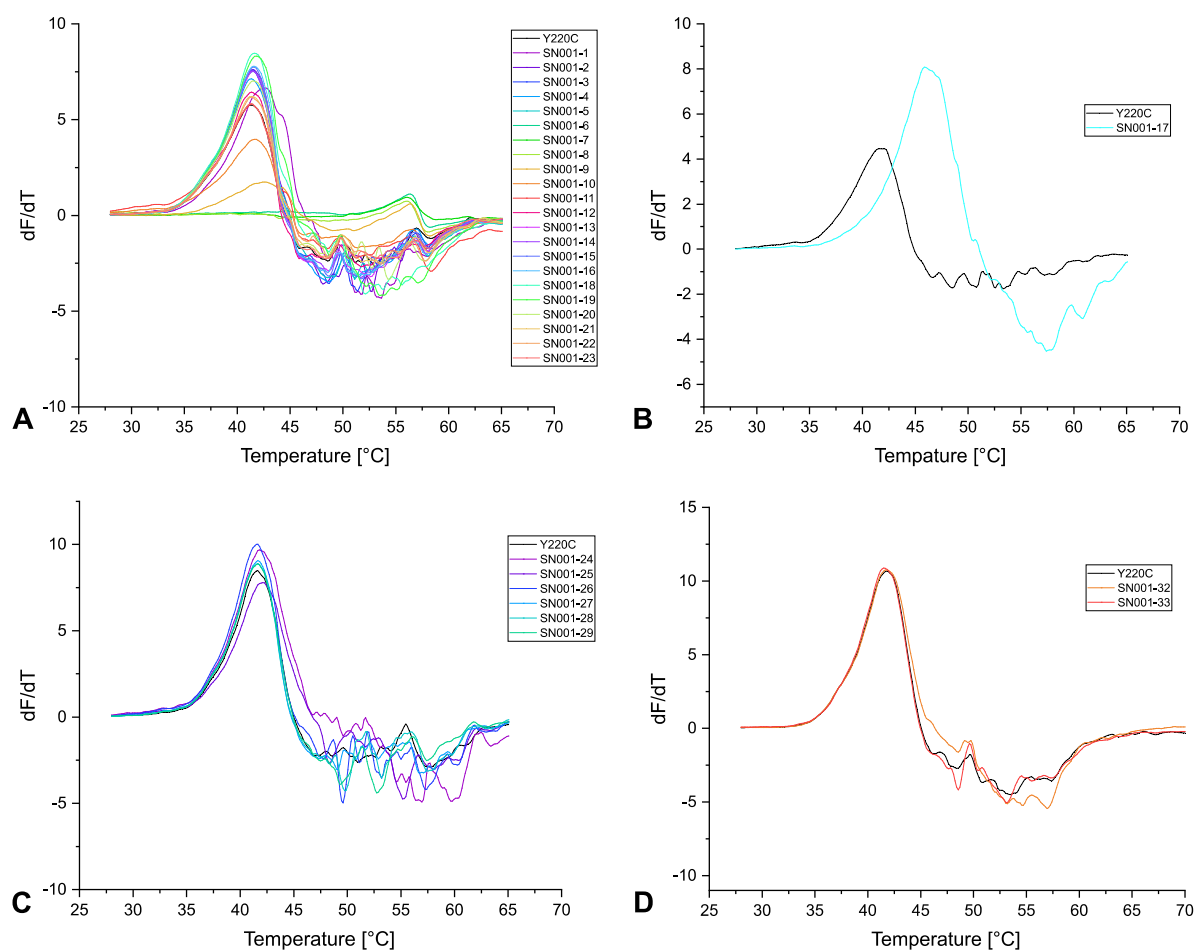


Figure 8.18: DSF screen of SN001 SAR compounds with T-p53C-Y220C after 4 h of incubation. The first derivatives of the melting curves of SN001 SAR compounds (**1 mM**): SN001-1 to SN001-23 (**A**), SN001-17 (**B**), SN001-24 to SN001-29 (**C**), SN001-32, and SN001-33 (**D**) with **T-p53C-Y220C** (**8 μ M** protein) after **4 h** of incubation at **20 °C** are shown.

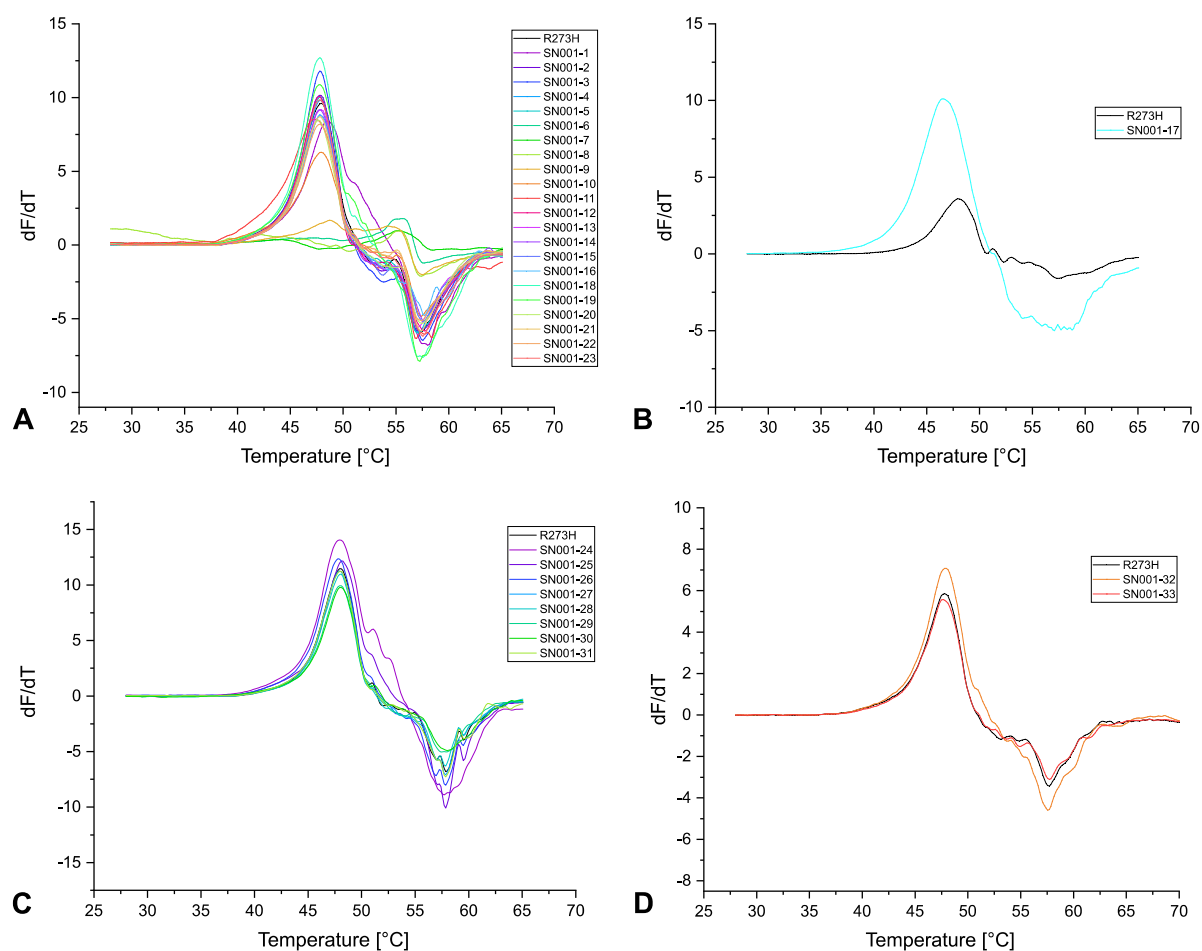


Figure 8.19: DSF screen of SN001 SAR compounds with T-p53C-R273H after 4 h of incubation. The first derivatives of the melting curves of SN001 SAR compounds (**1 mM**): SN001-1 to SN001-23 (**A**), SN001-17 (**B**), SN001-24 to SN001-31 (**C**), SN001-32, and SN001-33 (**D**) with **T-p53C-R273H** (**8 μ M** protein) after **4 h** of incubation at **20 °C** are shown.

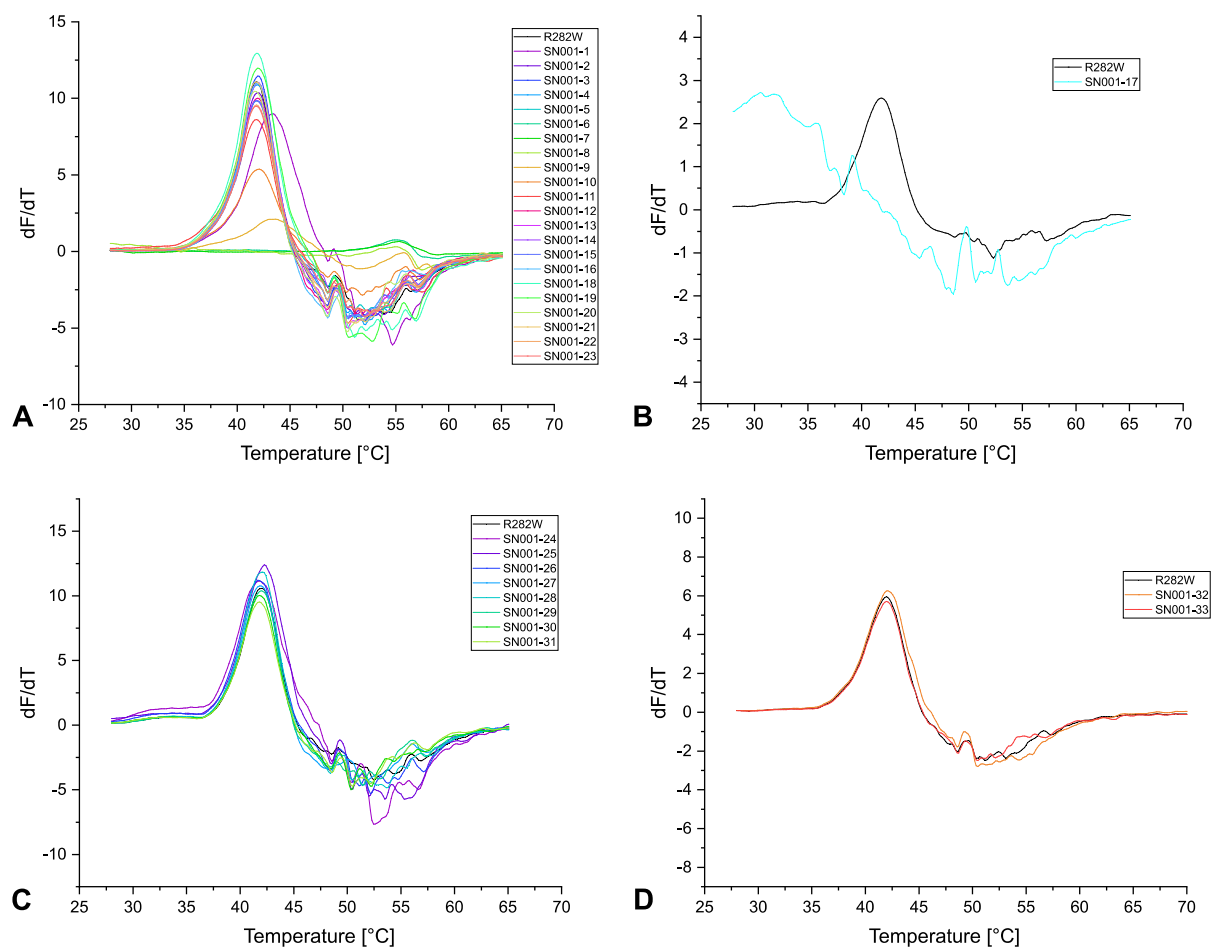


Figure 8.20: DSF screen of SN001 SAR compounds with T-p53C-R282W after 4 h of incubation. The first derivatives of the melting curves of SN001 SAR compounds (1 mM): SN001-1 to SN001-23 (A), SN001-17 (B), SN001-24 to SN001-31 (C), SN001-32, and SN001-33 (D) with T-p53C-R282W (8 μM protein) after 4 h of incubation at 20 $^{\circ}\text{C}$ are shown.

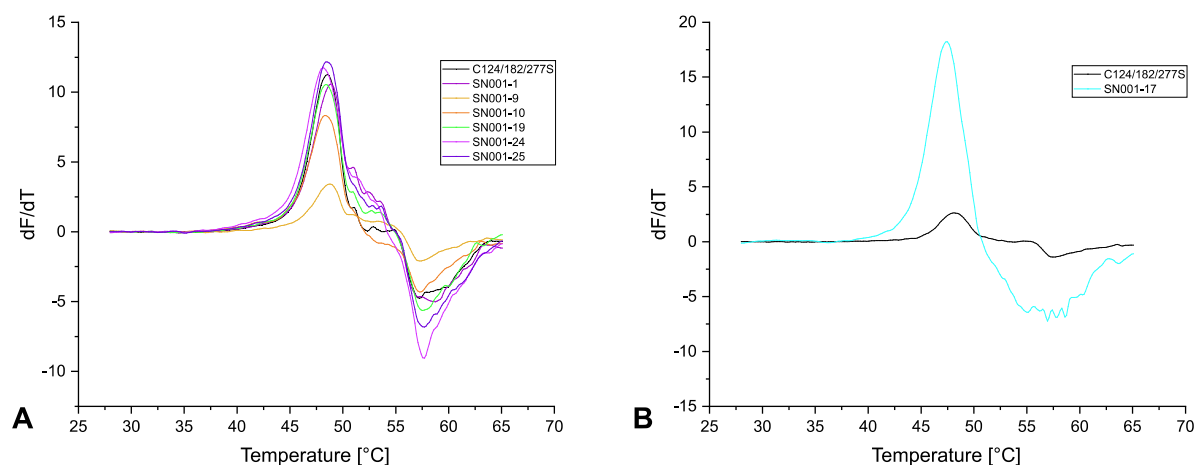


Figure 8.21: DSF screen of SN001 SAR hits with T-p53C-C124/182/277S after 4 h of incubation. The first derivatives of the melting curves of SN001 SAR compounds (1 mM): SN001-1, SN001-9, SN001-10, SN001-19, SN001-24, SN001-25 (A), and SN001-17 (B) with T-p53C-C124/182/277S (8 μM protein) after 4 h of incubation at 20 $^{\circ}\text{C}$ are shown.

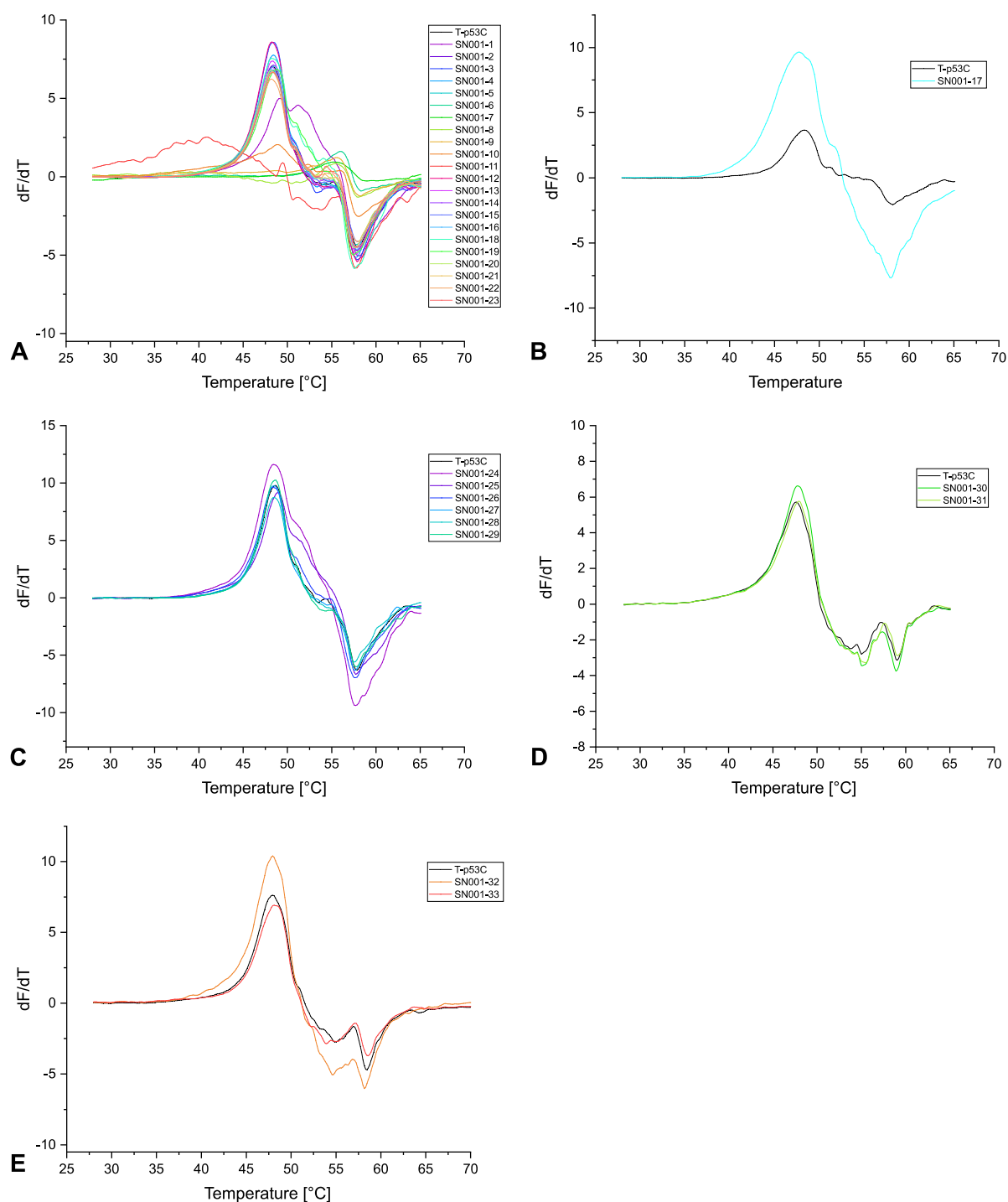


Figure 8.22: DSF screen of SN001 SAR compounds with T-p53C after 24 h of incubation. The first derivatives of the melting curves of SN001 SAR compounds (1 mM): SN001-1 to SN001-23 (A), SN001-17 (B), SN001-24 to SN001-29 (C), SN001-30, SN001-31 (D), SN001-32, and SN001-33 (E) with T-p53C (8 μ M protein) after 24 h of incubation at 20 °C are shown.

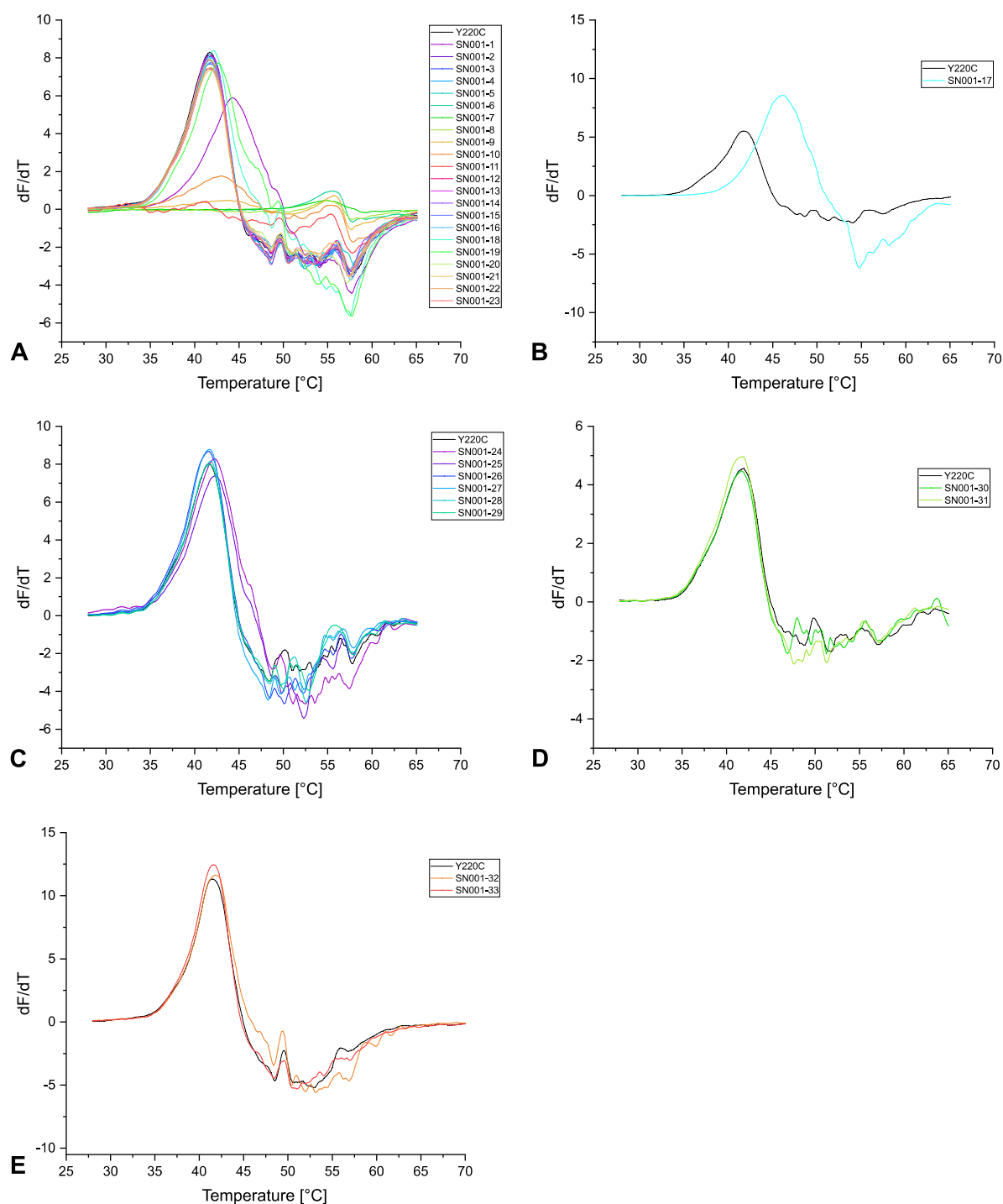


Figure 8.23: DSF screen of SN001 SAR compounds with T-p53C-Y220C after 24 h of incubation. The first derivatives of the melting curves of SN001 SAR compounds (**1 mM**): SN001-1 to SN001-23 (**A**), SN001-17 (**B**), SN001-24 to SN001-29 (**C**), SN001-30, SN001-31 (**D**), SN001-32, and SN001-33 (**E**) with **T-p53C-Y220C** (8 μ M protein) after **24 h** of incubation at 20 °C are shown.

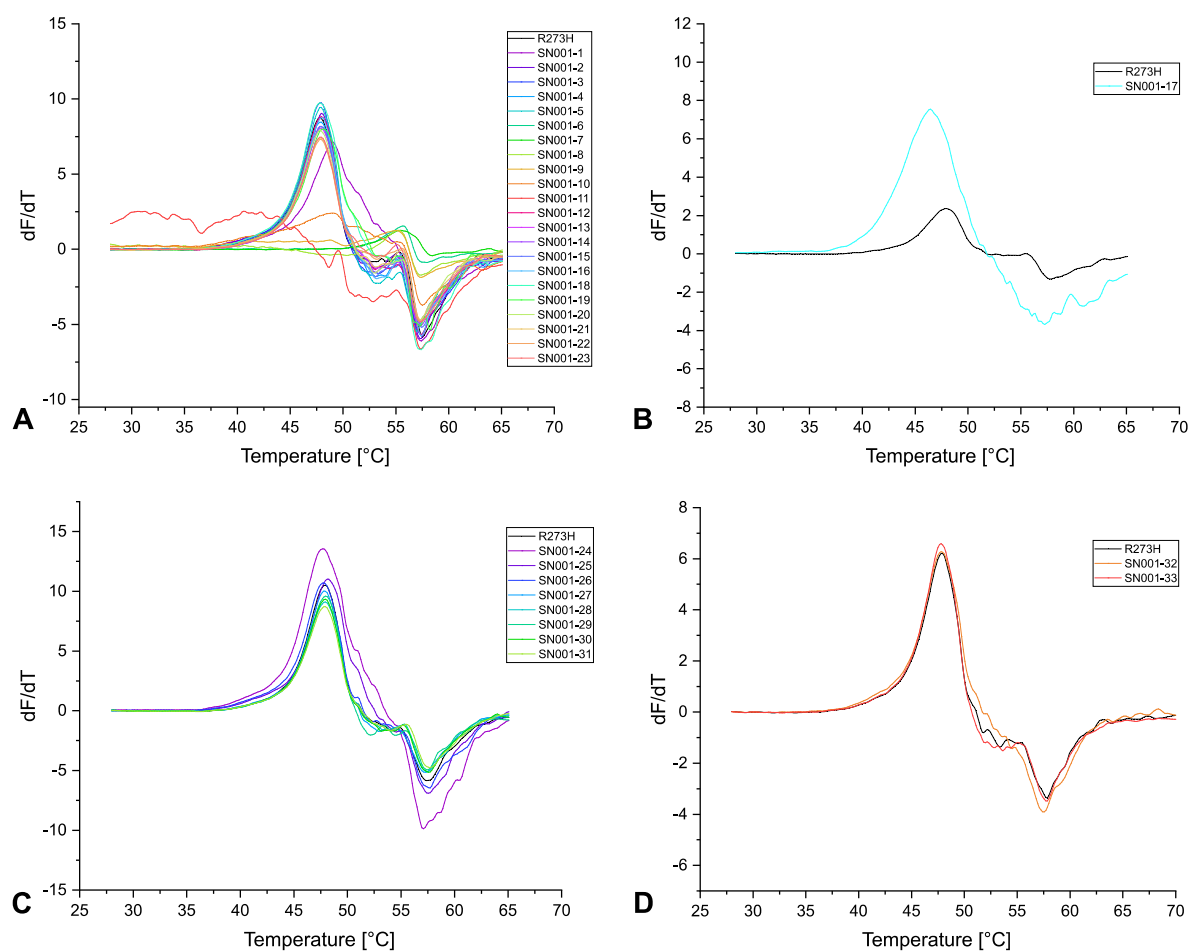


Figure 8.24: DSF screen of SN001 SAR compounds with T-p53C-R273H after 24 h of incubation. The first derivatives of the melting curves of SN001 SAR compounds (**1 mM**): SN001-1 to SN001-23 (**A**), SN001-17 (**B**), SN001-24 to SN001-31 (**C**), SN001-32, and SN001-33 (**D**) with **T-p53C-R273H** (8 μ M protein) after **24 h** of incubation at 20 °C are shown.

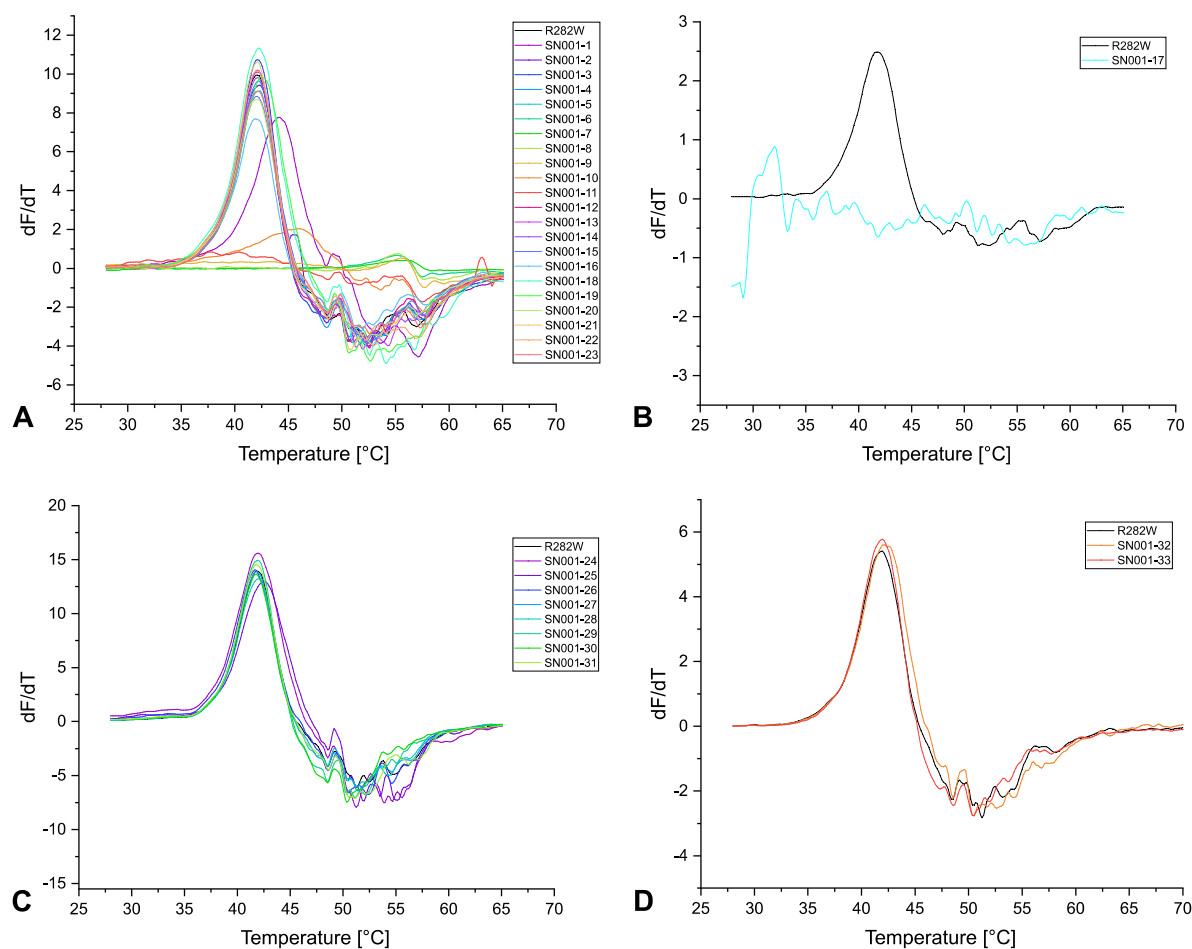


Figure 8.25: DSF screen of SN001 SAR compounds with T-p53C-R282W after 24 h of incubation. The first derivatives of the melting curves of SN001 SAR compounds (**1 mM**): SN001-1 to SN001-23 (**A**), SN001-17 (**B**), SN001-24 to SN001-31 (**C**), SN001-32, and SN001-33 (**D**) with **T-p53C-R282W** (**8 μ M** protein) after **24 h** of incubation at **20 °C** are shown.

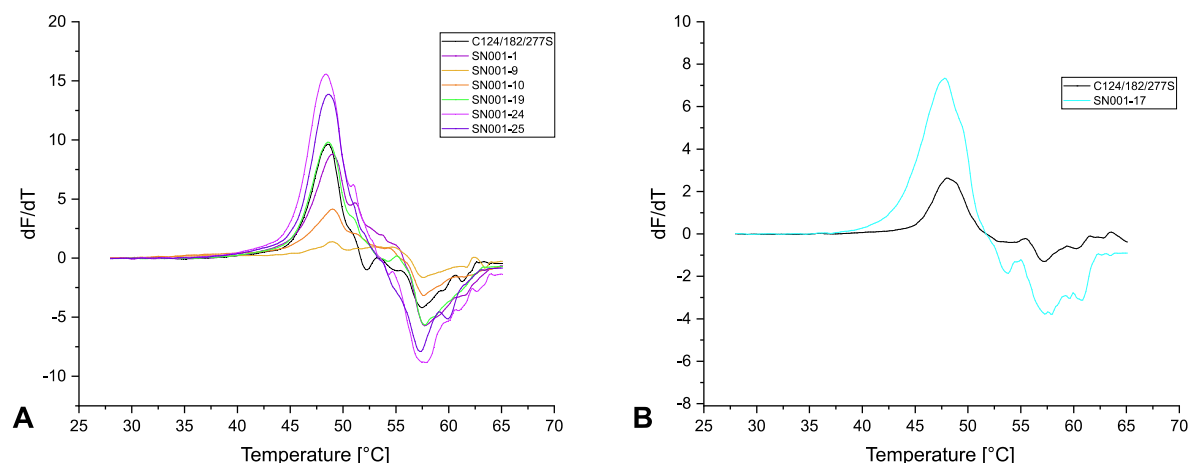


Figure 8.26: DSF screen of SN001 SAR hits with T-p53C-C124/182/277S after 24 h of incubation. The first derivatives of the melting curves of SN001 SAR compounds (**1 mM**): SN001-1, SN001-9, SN001-10, SN001-19, SN001-24, SN001-25 (**A**), and SN001-17 (**B**) with **T-p53C-C124/182/277S** (**8 μ M** protein) after **24 h** of incubation at **20 °C** are shown.

Table 8.6: $\Delta T_m \pm SD$ of T-p53C (8 μM) incubated with 250 μM SN001 SAR compounds for 30 min, 4 h, and 24 h at 20 °C. Measurements were performed at least in triplicate. Fragments with a ΔT_m of at least 0.50 °C are highlighted in bold [99, 130].

T-p53C			
$\Delta T_m \pm SD$ [°C]			
Compound	250 μM 30 min	250 μM 4 h	250 μM 24 h
SN001-1	0.15 $\pm$ 0	0.35 $\pm$ 0.17	0.40 $\pm$ 0.09
SN001-2	0 $\pm$ 0	0.15 $\pm$ 0.19	-0.05 $\pm$ 0.09
SN001-3	0 $\pm$ 0	0.05 $\pm$ 0.17	0 $\pm$ 0
SN001-4	0 $\pm$ 0	0.05 $\pm$ 0.17	0 $\pm$ 0
SN001-5	0 $\pm$ 0	0.05 $\pm$ 0.17	0.05 $\pm$ 0.09
SN001-6	N/A ^a	N/A ^a	N/A ^a
SN001-7	N/A ^a	N/A ^a	N/A ^a
SN001-8	N/A ^a	N/A ^a	N/A ^a
SN001-9	0 $\pm$ 0	0.35 $\pm$ 0.17	N/A ^a
SN001-10	0 $\pm$ 0	0.10 $\pm$ 0.19	0.10 $\pm$ 0.09
SN001-11	-0.05 $\pm$ 0.09	0.15 $\pm$ 0.19	0.05 $\pm$ 0.09
SN001-12	0 $\pm$ 0	0 $\pm$ 0.19	-0.05 $\pm$ 0.09
SN001-13	0 $\pm$ 0	0.10 $\pm$ 0.19	0 $\pm$ 0
SN001-14	0.05 $\pm$ 0.09	0.05 $\pm$ 0.17	0 $\pm$ 0
SN001-15	0 $\pm$ 0	0.05 $\pm$ 0.17	-0.05 $\pm$ 0.09
SN001-16	0 $\pm$ 0	0.10 $\pm$ 0.19	0 $\pm$ 0
SN001-17	0.10 $\pm$ 0.17	0.50 $\pm$ 0.09	0.35 $\pm$ 0.09
SN001-18	0 $\pm$ 0	0.05 $\pm$ 0.17	0 $\pm$ 0
SN001-19	0 $\pm$ 0	0.15 $\pm$ 0.19	0.05 $\pm$ 0.09
SN001-20	0 $\pm$ 0	0.05 $\pm$ 0.17	0.05 $\pm$ 0.09
SN001-21	0 $\pm$ 0	0.05 $\pm$ 0.17	0 $\pm$ 0
SN001-22	0 $\pm$ 0	0.05 $\pm$ 0.17	0 $\pm$ 0
SN001-23	0 $\pm$ 0	0 $\pm$ 0	-0.05 $\pm$ 0.09
SN001-24	0.05 $\pm$ 0.09	0 $\pm$ 0	0.20 $\pm$ 0.17
SN001-25	0.20 $\pm$ 0.09	0.10 $\pm$ 0.09	0.40 $\pm$ 0.12
SN001-26	0.10 $\pm$ 0.12	-0.15 $\pm$ 0	-0.10 $\pm$ 0.17
SN001-27	0 $\pm$ 0.12	-0.10 $\pm$ 0.09	-0.10 $\pm$ 0.09
SN001-28	0 $\pm$ 0.12	-0.05 $\pm$ 0.09	-0.05 $\pm$ 0.12
SN001-29	0 $\pm$ 0.12	0 $\pm$ 0	0.05 $\pm$ 0.17
SN001-30	0.10 $\pm$ 0.12	0.15 $\pm$ 0.12	0.10 $\pm$ 0.17
SN001-31	0.05 $\pm$ 0.27	0.05 $\pm$ 0.12	0.05 $\pm$ 0.12
SN001-32	0.15 $\pm$ 0.15	0.10 $\pm$ 0.12	0.20 $\pm$ 0.09
SN001-33	0 $\pm$ 0.15	0 $\pm$ 0.12	0 $\pm$ 0

^aNo evaluable melting curves and first derivatives with clearly defined melting points were obtained.

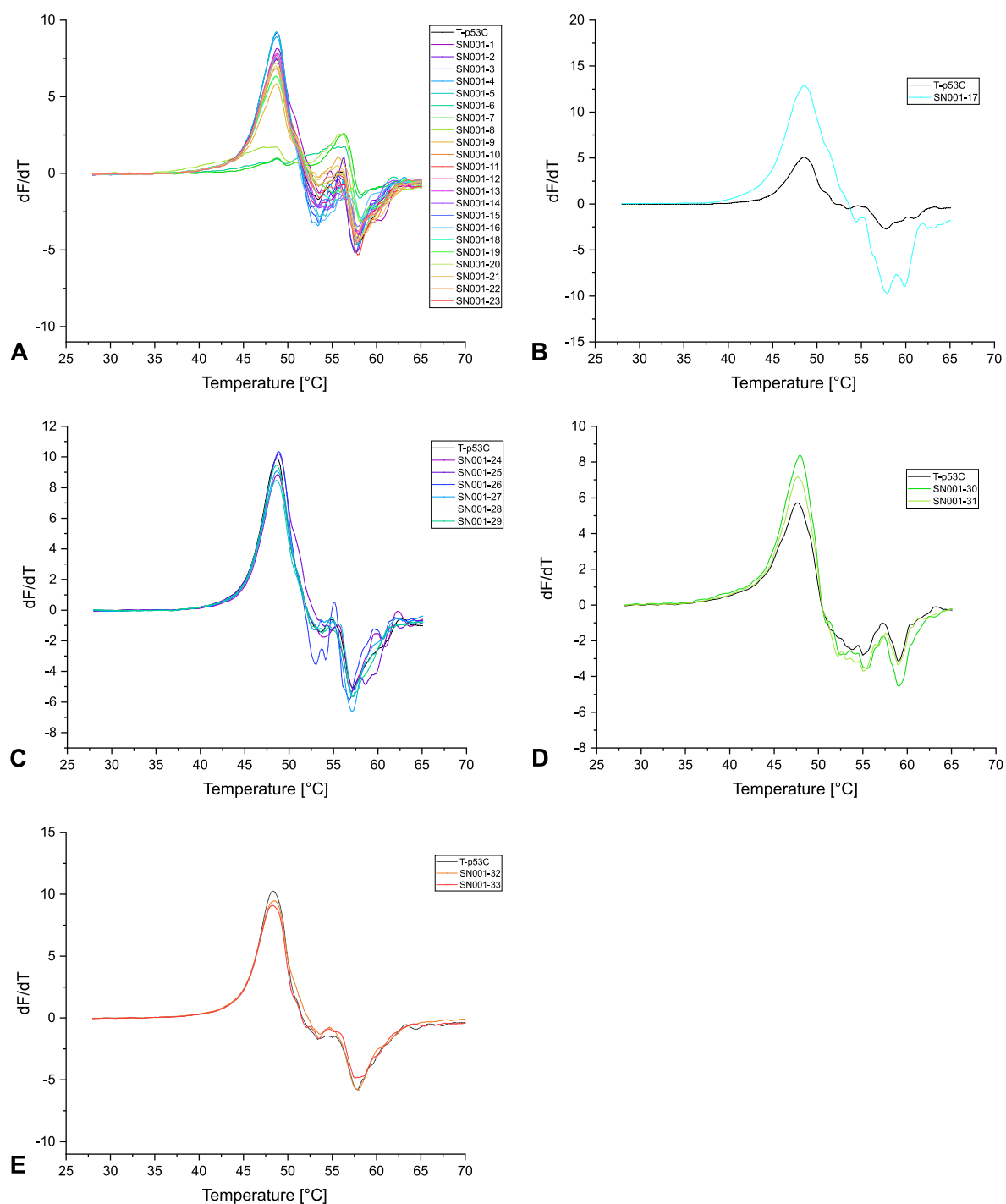


Figure 8.27: DSF screen of SN001 SAR compounds (250 μM) with T-p53C after 30 min of incubation. The first derivatives of the melting curves of SN001 SAR compounds (250 μM): SN001-1 to SN001-23 (A), SN001-17 (B), SN001-24 to SN001-29 (C), SN001-30, SN001-31 (D), SN001-32, and SN001-33 (E) with T-p53C (8 μM protein) after 30 min of incubation at 20 $^{\circ}\text{C}$ are shown.

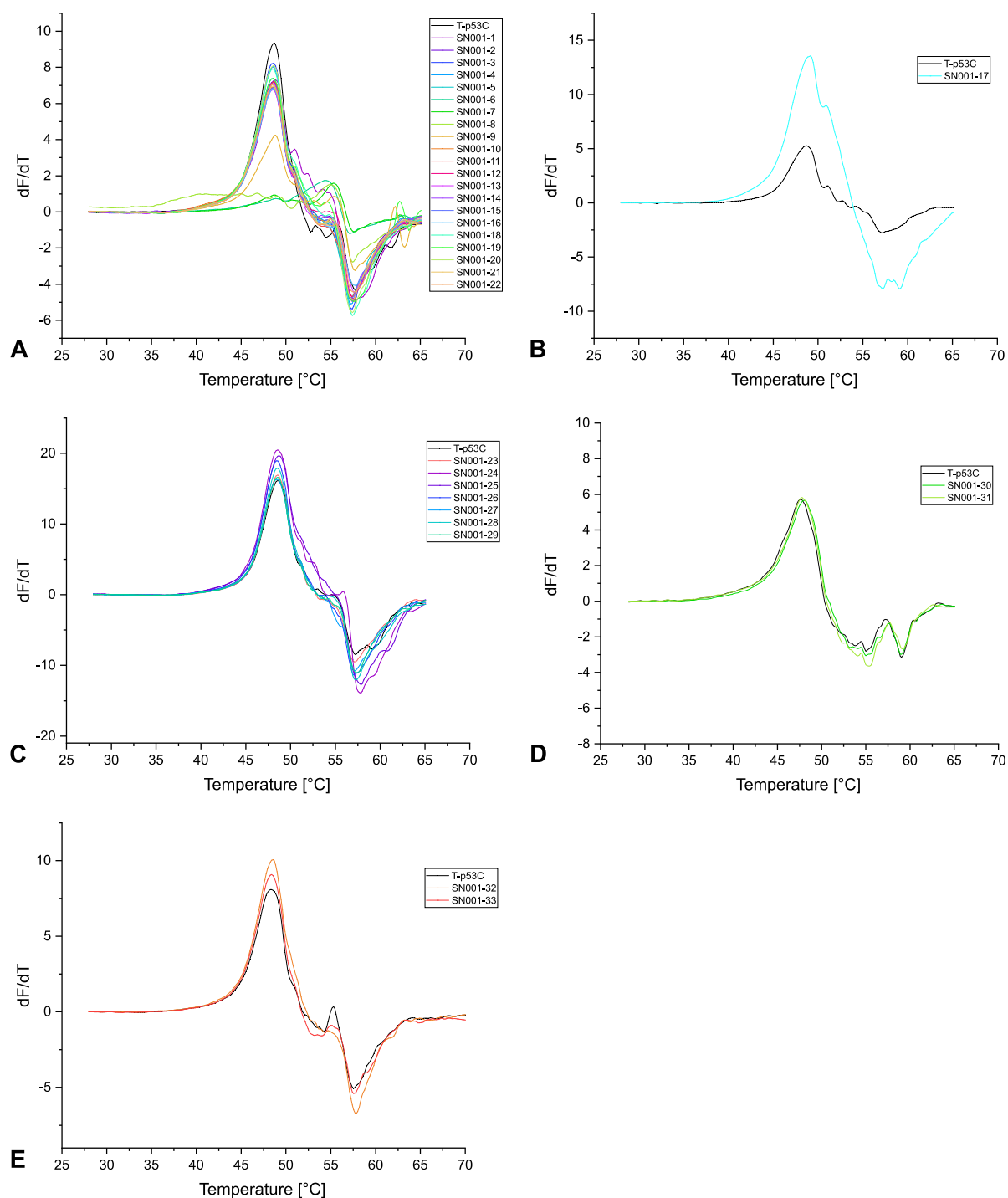


Figure 8.28: DSF screen of SN001 SAR compounds (250 μM) with T-p53C after 4 h of incubation. The first derivatives of the melting curves of SN001 SAR compounds (250 μM): SN001-1 to SN001-22 (A), SN001-17 (B), SN001-23 to SN001-29 (C), SN001-30, SN001-31 (D), SN001-32, and SN001-33 (E) with T-p53C (8 μM protein) after 4 h of incubation at 20 °C are shown.

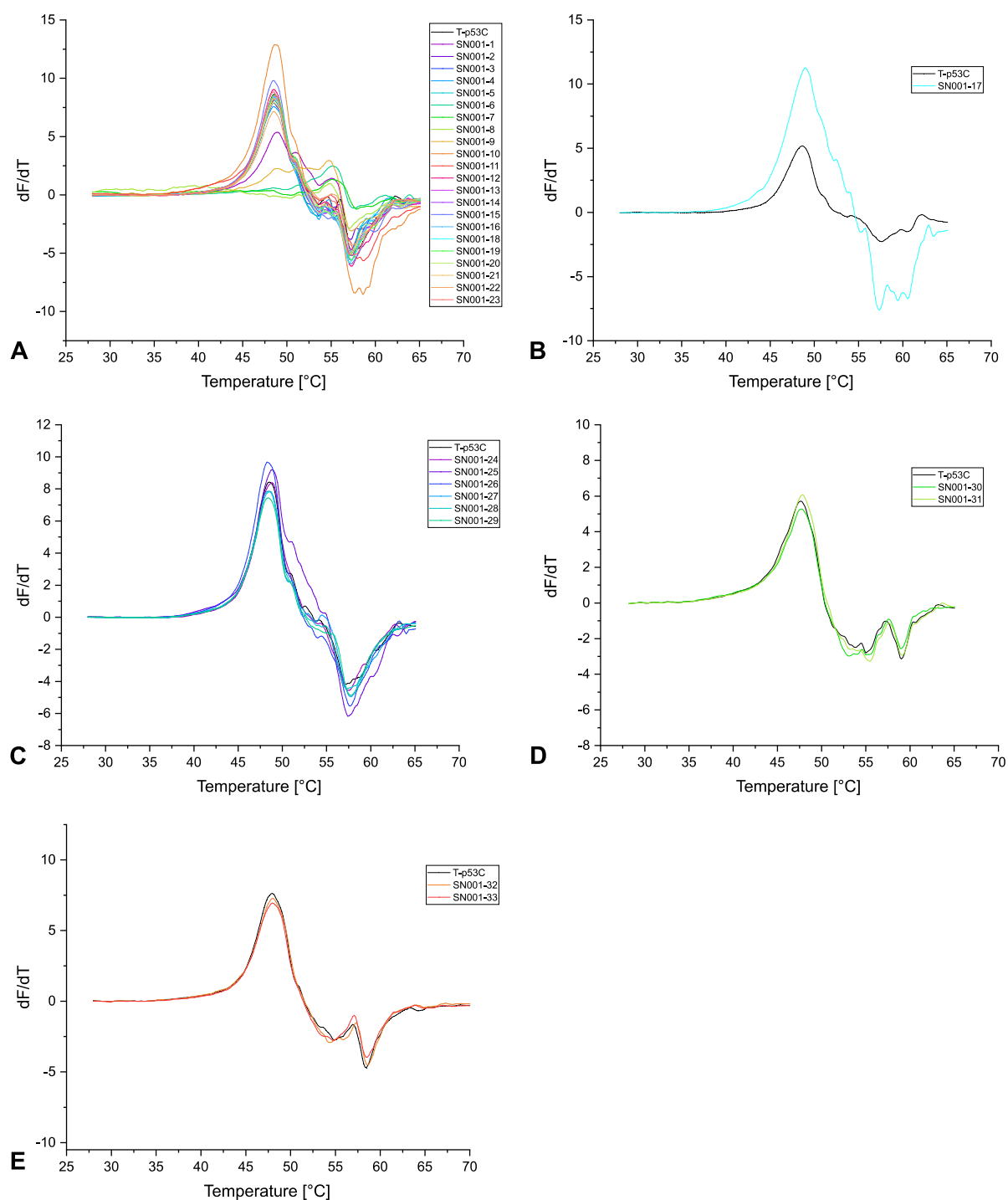


Figure 8.29: DSF screen of SN001 SAR compounds (250 μM) with T-p53C after 24 h of incubation. The first derivatives of the melting curves of SN001 SAR compounds (250 μM): SN001-1 to SN001-23 (A), SN001-17 (B), SN001-24 to SN001-29 (C), SN001-30, SN001-31 (D), SN001-32, and SN001-33 (E) with T-p53C (8 μM protein) after 24 h of incubation at 20 $^{\circ}\text{C}$ are shown.

Table 8.7: $\Delta T_m \pm$ SD of T-p53C-Y220C (8 μ M) incubated with 250 μ M SN001 SAR compounds for 30 min, 4 h, and 24 h at 20 °C. Measurements were performed at least in triplicate. Fragments with a ΔT_m of at least 0.50 °C are highlighted in bold [99, 130].

T-p53C-Y220C			
$\Delta T_m \pm$ SD [°C]			
Compound	250 μM 30 min	250 μM 4 h	250 μM 24 h
SN001-1	0.30 $\pm$ 0.19	0.25 $\pm$ 0.17	1.35 $\pm$ 0.32
SN001-2	0.20 $\pm$ 0.12	0.05 $\pm$ 0.09	0 $\pm$ 0.12
SN001-3	0.15 $\pm$ 0.12	0 $\pm$ 0	0.05 $\pm$ 0.09
SN001-4	0.05 $\pm$ 0.12	0.05 $\pm$ 0.09	0 $\pm$ 0.12
SN001-5	0.10 $\pm$ 0.09	0 $\pm$ 0	0.10 $\pm$ 0.12
SN001-6	N/A ^a	N/A ^a	N/A ^a
SN001-7	N/A ^a	N/A ^a	N/A ^a
SN001-8	-0.85 $\pm$ 0.12 ^b	N/A ^a	N/A ^a
SN001-9	0.30 $\pm$ 0.12	0.20 $\pm$ 0.09	1.90 $\pm$ 0.12
SN001-10	0.30 $\pm$ 0.12	0.10 $\pm$ 0.09	0.15 $\pm$ 0.12
SN001-11	0.25 $\pm$ 0.09	0.05 $\pm$ 0.09	0.15 $\pm$ 0.12
SN001-12	0.25 $\pm$ 0.09	0 $\pm$ 0	0.25 $\pm$ 0.19
SN001-13	0.20 $\pm$ 0.12	0 $\pm$ 0	0.15 $\pm$ 0.12
SN001-14	0 $\pm$ 0.19	0.05 $\pm$ 0.09	0 $\pm$ 0.12
SN001-15	0.20 $\pm$ 0.12	0.05 $\pm$ 0.09	0.05 $\pm$ 0.09
SN001-16	0.05 $\pm$ 0.12	0 $\pm$ 0	0.05 $\pm$ 0.09
SN001-17	0.55 $\pm$ 0.09	4.25 $\pm$ 0.09	5.19 $\pm$ 0.06
SN001-18	0.20 $\pm$ 0.12	0.05 $\pm$ 0.09	0.05 $\pm$ 0.09
SN001-19	0.25 $\pm$ 0.17	0.10 $\pm$ 0.09	0.30 $\pm$ 0.12
SN001-20	0.25 $\pm$ 0.09	0.05 $\pm$ 0.09	0.15 $\pm$ 0.12
SN001-21	0.20 $\pm$ 0.12	0.15 $\pm$ 0	0.15 $\pm$ 0.12
SN001-22	0.30 $\pm$ 0.15	0.10 $\pm$ 0.09	0.10 $\pm$ 0.19
SN001-23	0.25 $\pm$ 0.09	0.10 $\pm$ 0.09	0.15 $\pm$ 0.12
SN001-24	0.35 $\pm$ 0.17	0.40 $\pm$ 0.24	0.45 $\pm$ 0.24
SN001-25	0.50 $\pm$ 0.17	0.45 $\pm$ 0.12	0.95 $\pm$ 0.12
SN001-26	0.15 $\pm$ 0.12	-0.05 $\pm$ 0.12	0 $\pm$ 0.12
SN001-27	0.05 $\pm$ 0.09	0.05 $\pm$ 0.09	-0.10 $\pm$ 0.12
SN001-28	0 $\pm$ 0.12	0.05 $\pm$ 0.09	-0.05 $\pm$ 0.09
SN001-29	0.10 $\pm$ 0.12	0.05 $\pm$ 0.17	-0.05 $\pm$ 0.09
SN001-30	N/D	N/D	N/D
SN001-31	N/D	N/D	N/D
SN001-32	-0.10 $\pm$ 0.21	0.30 $\pm$ 0.12	0.25 $\pm$ 0.19
SN001-33	-0.10 $\pm$ 0.17	-0.15 $\pm$ 0.19	0 $\pm$ 0.24

^aNo evaluable melting curves and first derivatives with clearly defined melting points were obtained.

^bFirst derivatives of the melting curves displayed broad peaks with low fluorescence intensities.

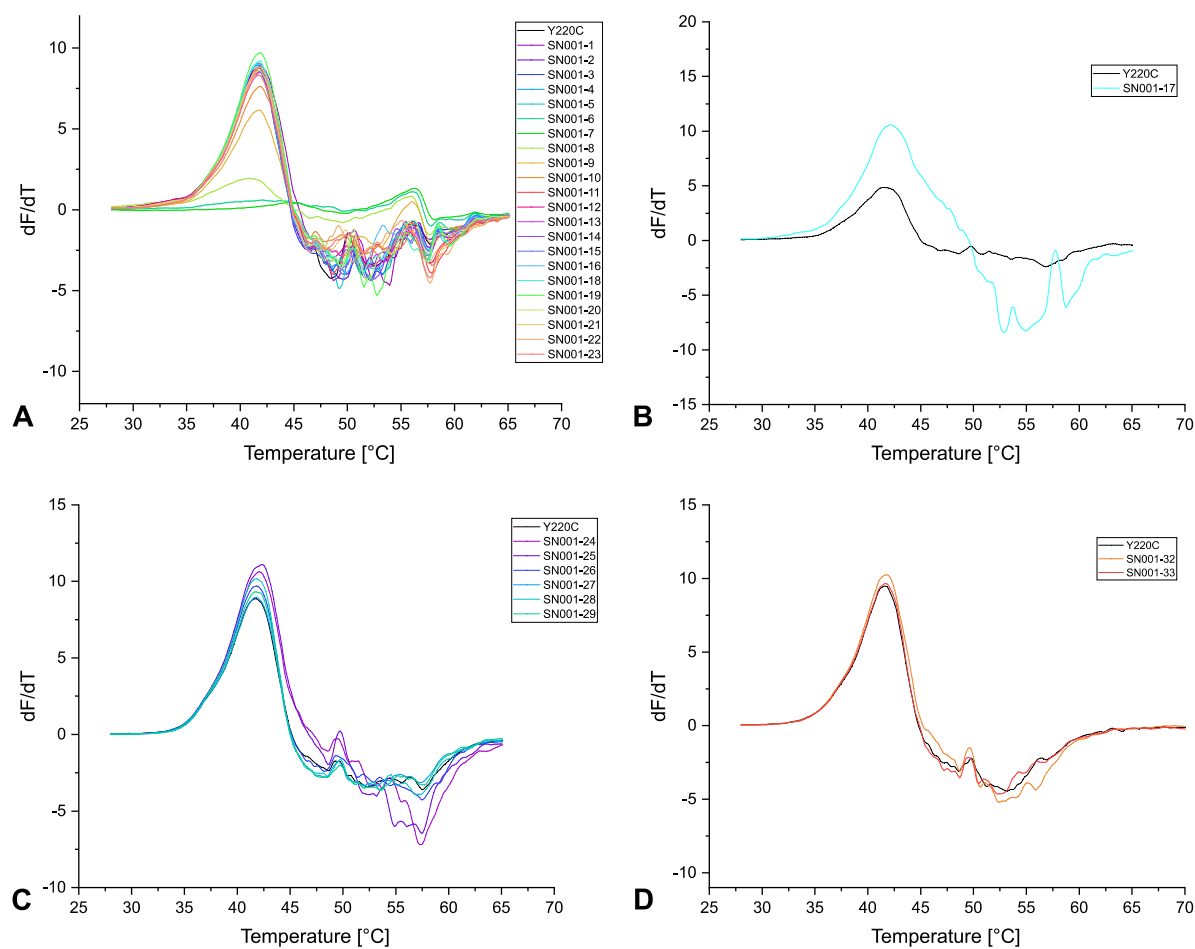


Figure 8.30: DSF screen of SN001 SAR compounds (250 μM) with T-p53C-Y220C after 30 min of incubation. The first derivatives of the melting curves of SN001 SAR compounds (250 μM): SN001-1 to SN001-23 (A), SN001-17 (B), SN001-24 to SN001-29 (C), SN001-32, and SN001-33 (D) with T-p53C-Y220C (8 μM protein) after 30 min of incubation at 20 °C are shown.

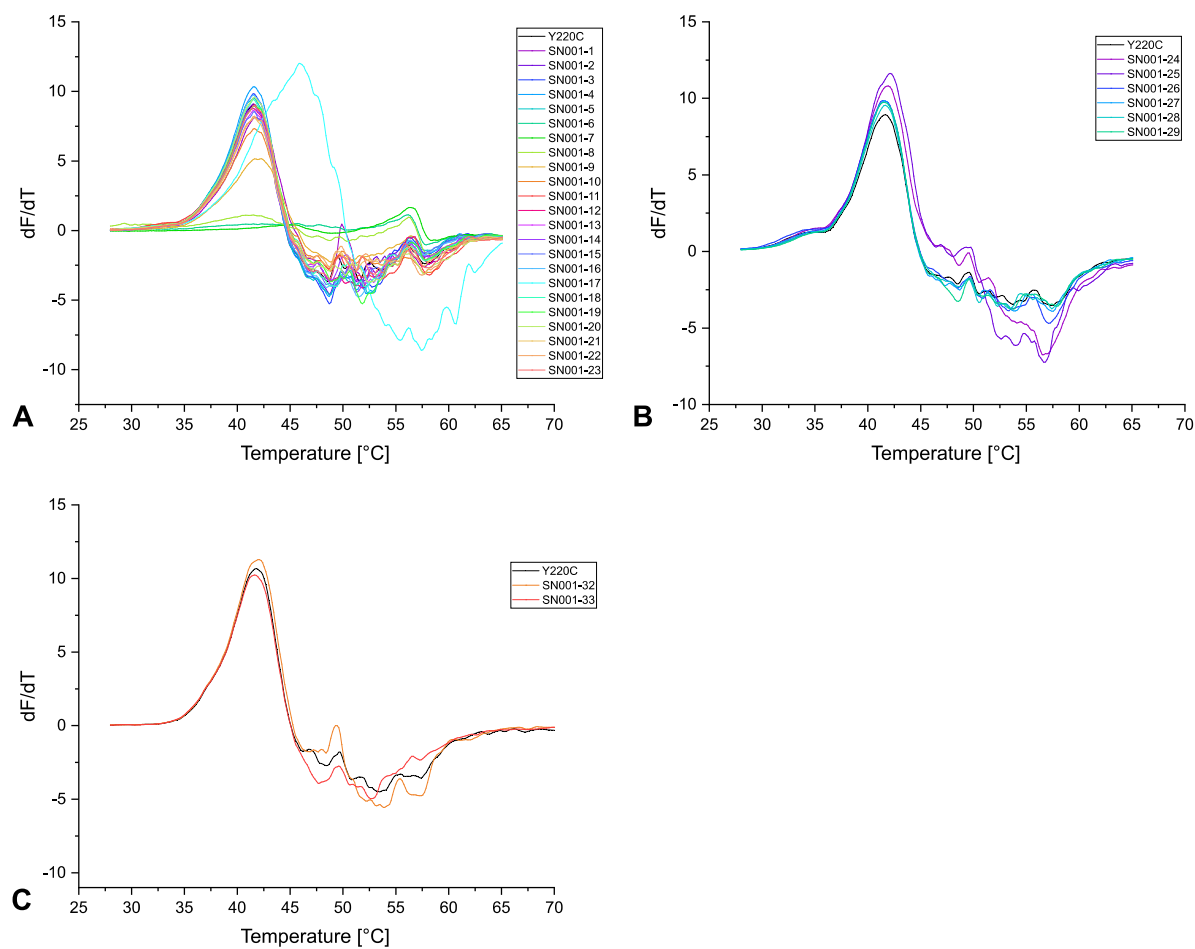


Figure 8.31: DSF screen of SN001 SAR compounds (250 μM) with T-p53C-Y220C after 4 h of incubation. The first derivatives of the melting curves of SN001 SAR compounds (250 μM): SN001-1 to SN001-23 (A), SN001-24 to SN001-29 (B), SN001-32, and SN001-33 (C) with T-p53C-Y220C (8 μM protein) after 4 h of incubation at 20 $^{\circ}\text{C}$ are shown.

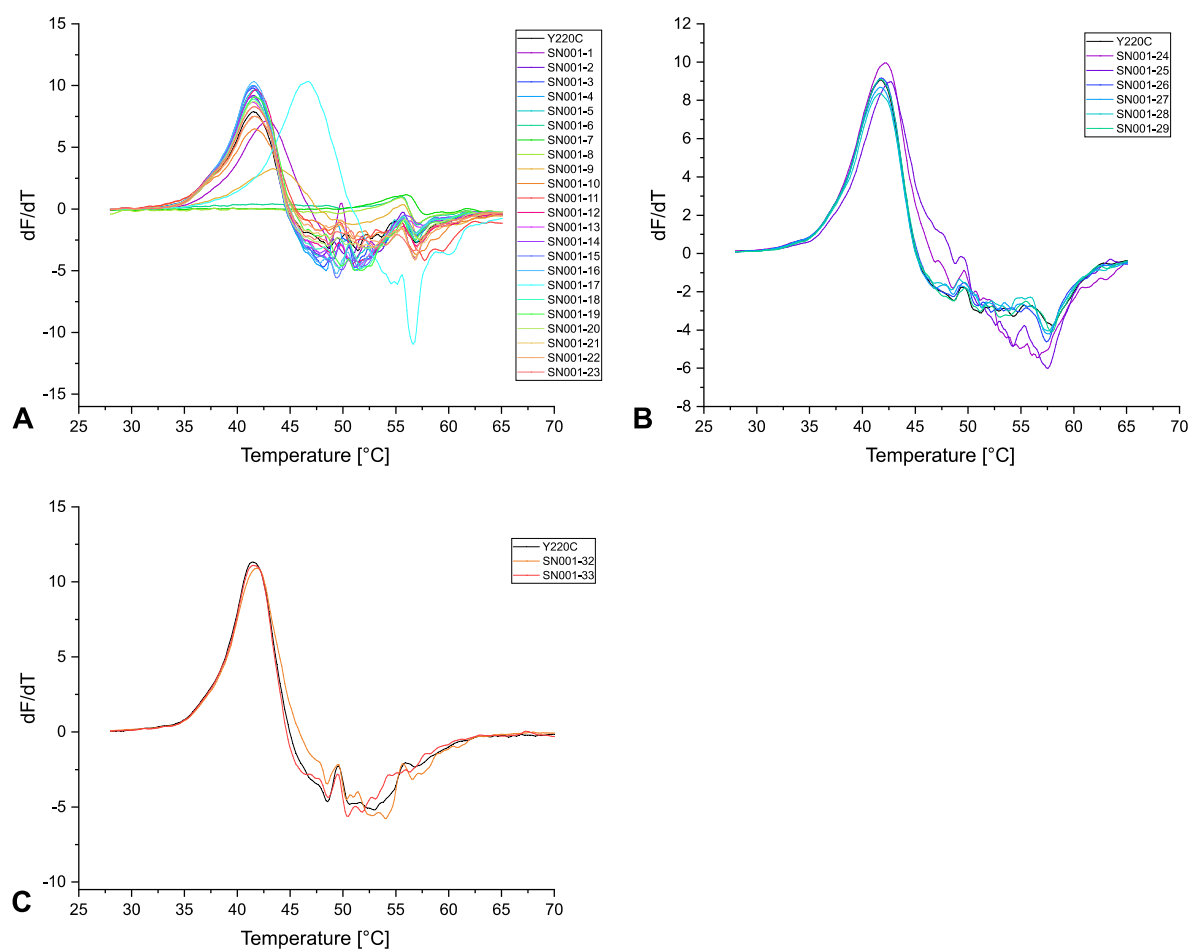


Figure 8.32: DSF screen of SN001 SAR compounds (250 μM) with T-p53C-Y220C after 24 h of incubation. The first derivatives of the melting curves of SN001 SAR compounds (250 μM): SN001-1 to SN001-23 (A), SN001-24 to SN001-29 (B), SN001-32, and SN001-33 (C) with T-p53C-Y220C (8 μM protein) after 24 h of incubation at 20 $^{\circ}\text{C}$ are shown.

8.8 Crystallographic Data Collection and Refinement Statistics of the xT-p53C-Y220C Crystal Soaked with SN001-17

Table 8.8: Crystallographic data collection and refinement statistics of the dataset of xT-p53C-Y220C soaked with 5 mM SN001-17 for two days. Parenthesized values show the respective value of the highest resolution bin.

	Compound SN001-17 5 mM
Wavelength [Å]	1
Space group	P2 ₁ 2 ₁ 2 ₁
Cell dimension	
a, b, c [Å]	65.47, 71.38, 106.44
α , β , γ [°]	90, 90, 90
Resolution range [Å]	50-1.72 (1.82-1.72)
Redundancy	8.33 (8.30)
Completeness [%]	98.4 (99.0)
Mean I/ σ (I)	11.05 (1.09)
R-meas [%]	11.3 (192.4)
CC1/2 [%]	99.9 (76.6)
Wilson B [Å ²]	35.75
Resolution included [Å]	48.26-1.72
R _{work} /R _{free} [%]	20.11/23.30
Bond RMSD [Å]	0.008
Angle RMSD [°]	0.890
Ramachandran [%] (favored/allowed/outliers)	99.21/0.79/0
Rotamer outliers [%]	1.83
All-atom Clashscore	2.15
Average B factor [Å ²]	
Overall	37.01
Protein	35.66
Ligand	50.38
Water	41.65

8.9 DSF Screen of SN006/7 SAR Compounds with T-p53C and Its Mutants

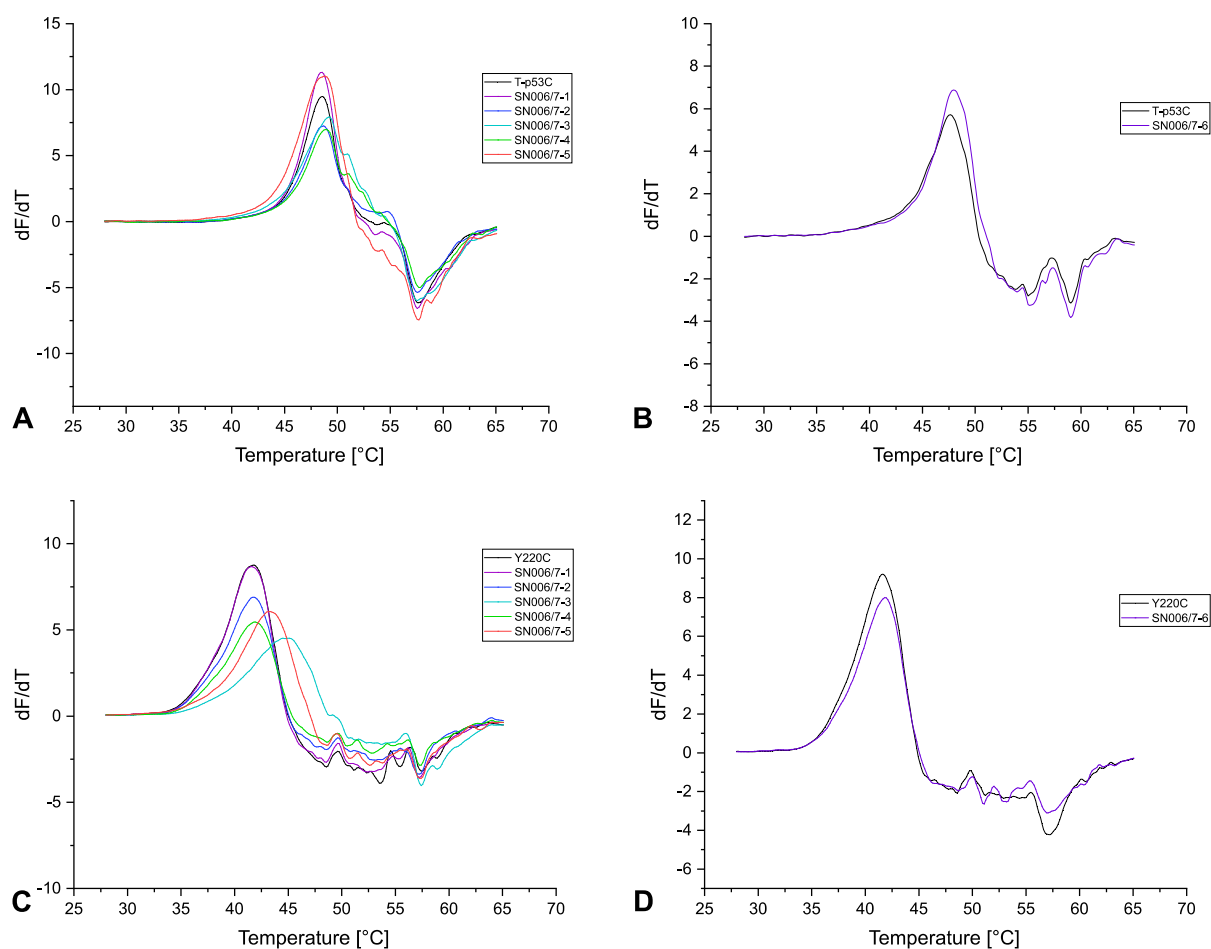


Figure 8.33: DSF screen of SN006/7 SAR compounds with T-p53C and T-p53C-Y220C after 30 min of incubation. The first derivatives of the melting curves of SN006/7-1 to SN006/7-5 (1 mM) (A), SN006/7-6 (1 mM) (B) with T-p53C (8 μ M protein), and of SN006/7-1 to SN006/7-5 (1 mM) (C), SN006/7-6 (1 mM) (D) with T-p53C-Y220C (8 μ M protein) after 30 min of incubation at 20 °C are shown.

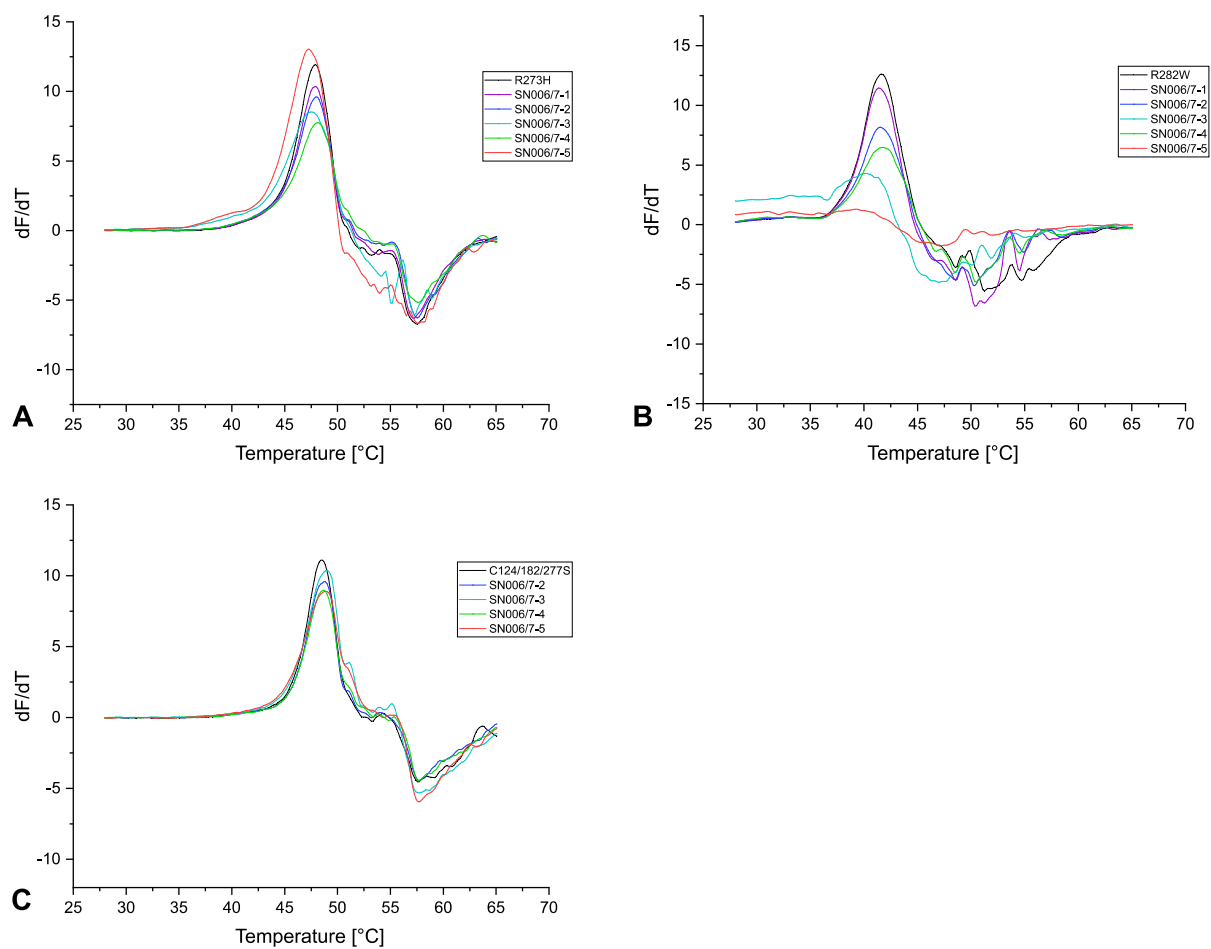


Figure 8.34: DSF screen of SN006/7 SAR compounds with T-p53C-R273H, T-p53C-R282W, and T-p53C-C124/182/277S after 30 min of incubation. The first derivatives of the melting curves of SN006/7 SAR compounds (1 mM) with T-p53C-R273H (A), T-p53C-R282W (B), and T-p53C-C124/182/277S (C) (8 μ M protein) after 30 min of incubation at 20 $^{\circ}$ C are shown.

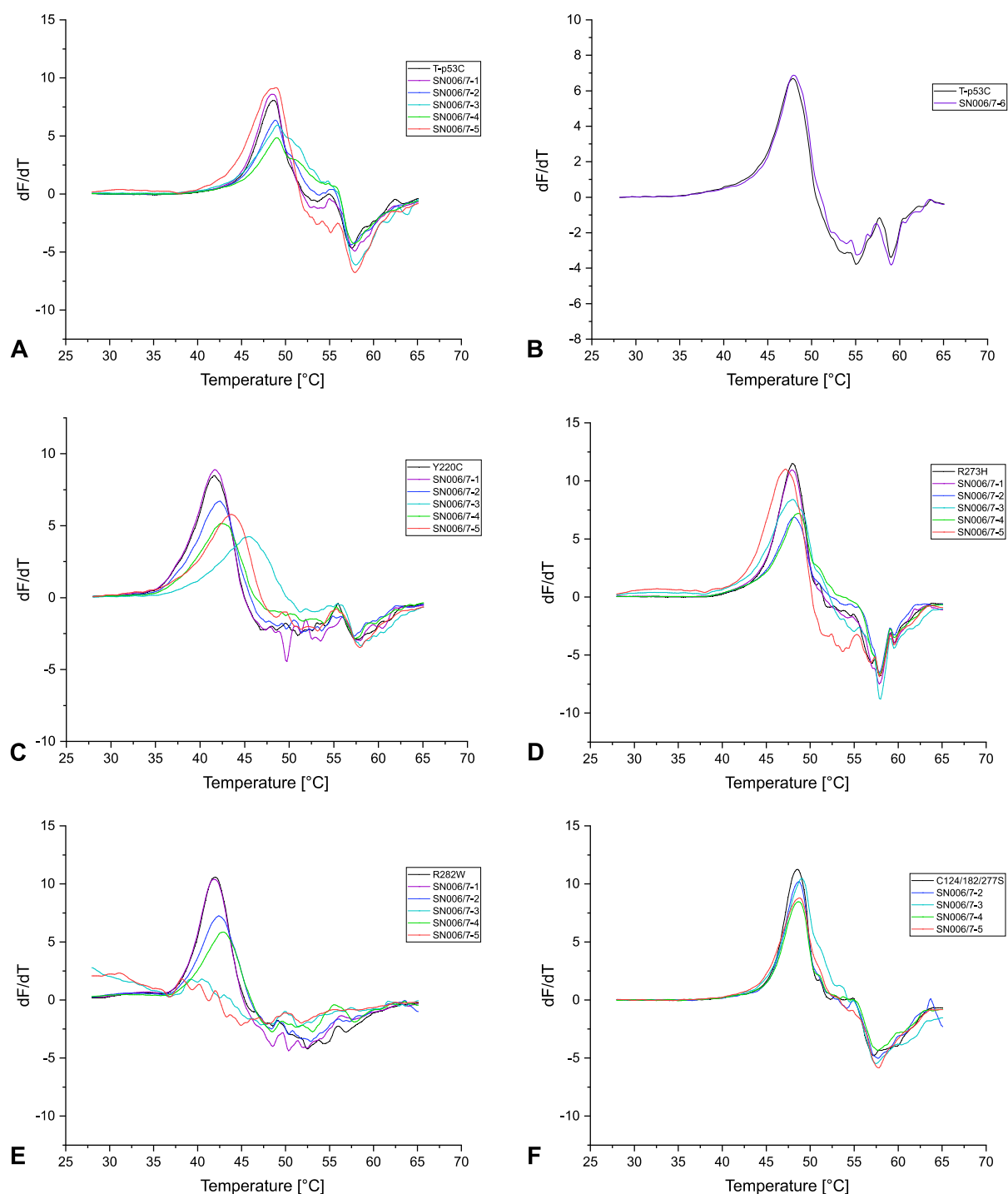


Figure 8.35: DSF screen of SN006/7 SAR compounds with T-p53C, T-p53C-Y220C, T-p53C-R273H, T-p53C-R282W, and T-p53C-C124/182/277S after 4 h of incubation. The first derivatives of the melting curves of SN006/7 SAR compounds (1 mM) with T-p53C (A, B), T-p53C-Y220C (C), T-p53C-R273H (D), T-p53C-R282W (E), and T-p53C-C124/182/277S (F) (8 μ M protein) after 4 h of incubation at 20 °C are shown.

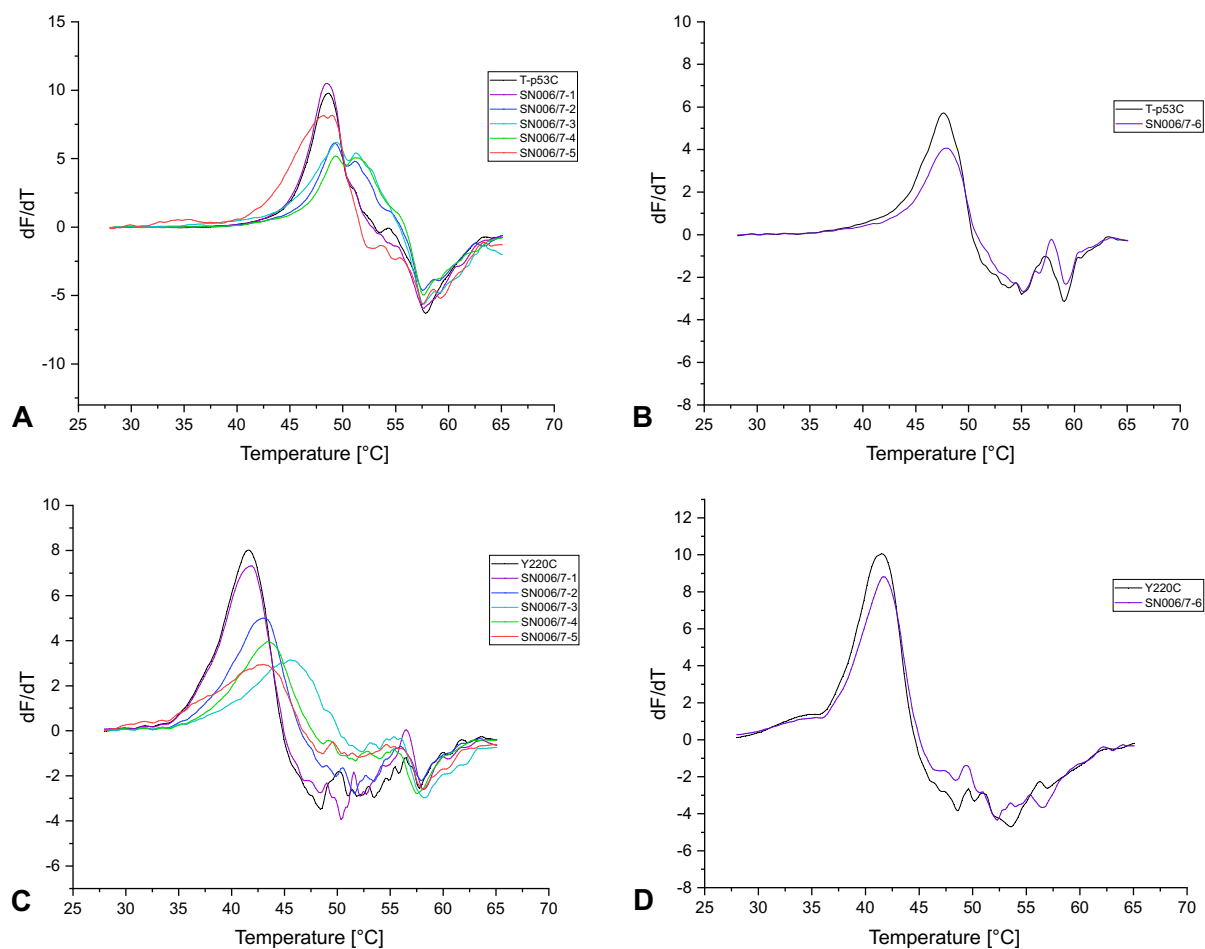


Figure 8.36: DSF screen of SN006/7 SAR compounds with T-p53C and T-p53C-Y220C after 24 h of incubation. The first derivatives of the melting curves of SN006/7-1 to SN006/7-5 (1 mM) (A), SN006/7-6 (1 mM) (B) with T-p53C (8 μ M protein), and of SN006/7-1 to SN006/7-5 (1 mM) (C), SN006/7-6 (1 mM) (D) with T-p53C-Y220C (8 μ M protein) after 24 h of incubation at 20 °C are shown.

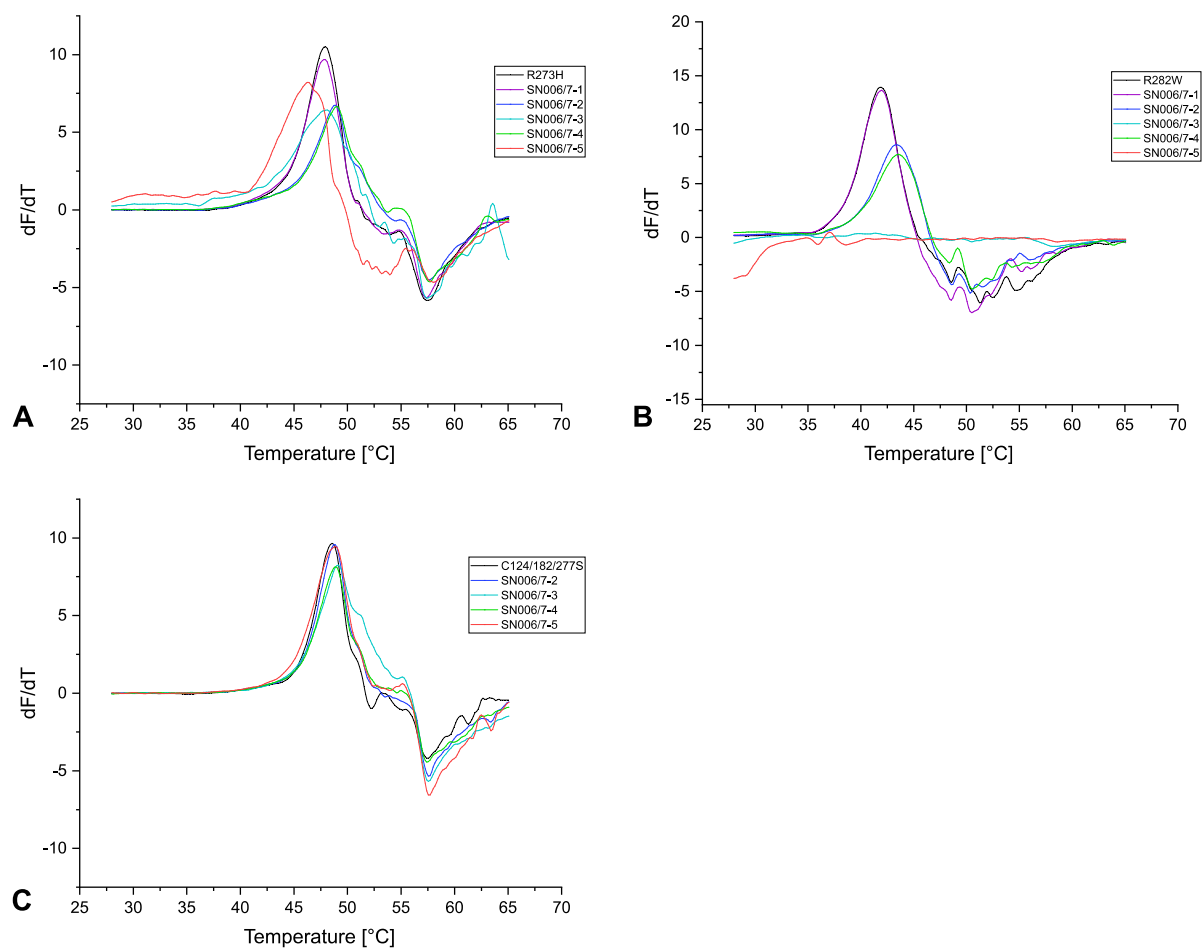


Figure 8.37: DSF screen of SN006/7 SAR compounds with T-p53C-R273H, T-p53C-R282W, and T-p53C-C124/182/277S after 24 h of incubation. The first derivatives of the melting curves of SN006/7 SAR compounds (1 mM) with **T-p53C-R273H** (A), **T-p53C-R282W** (B), and **T-p53C-C124/182/277S** (C) (8 μ M protein) after 24 h of incubation at 20 °C are shown.

Table 8.9: $\Delta T_m \pm SD$ of T-p53C (8 μM) incubated with 250 μM SN006/7 SAR compounds for 30 min, 4 h, and 24 h at 20 °C. Measurements were performed at least in triplicate. Fragments with a ΔT_m of at least 0.50 °C are highlighted in bold [99, 130].

T-p53C			
$\Delta T_m \pm SD$ [°C]			
Compound	250 μM 30 min	250 μM 4 h	250 μM 24 h
SN006/7-1	0 ± 0.12	-0.05 ± 0.09	0.05 ± 0.09
SN006/7-6	0.15 ± 0.12	-0.05 ± 0.09	0.05 ± 0.12
SN006/7-2	0.05 ± 0.09	0.05 ± 0.09	0.25 ± 0.12
SN006/7-3	0.50 ± 0.17^a	0.90 ± 0 and 2.60 ± 0.09	0.90 ± 0.12 and 2.85 ± 0.19
SN006/7-4	0.15 ± 0.17	0.20 ± 0.09	0.50 ± 0.09
SN006/7-5	0.50 ± 0.09^a	0.80 ± 0.09 and 2.50 ± 0.09	0.80 ± 0.09 and 2.65 ± 0.12

^aFirst derivatives of the melting curves displayed a broad peak.

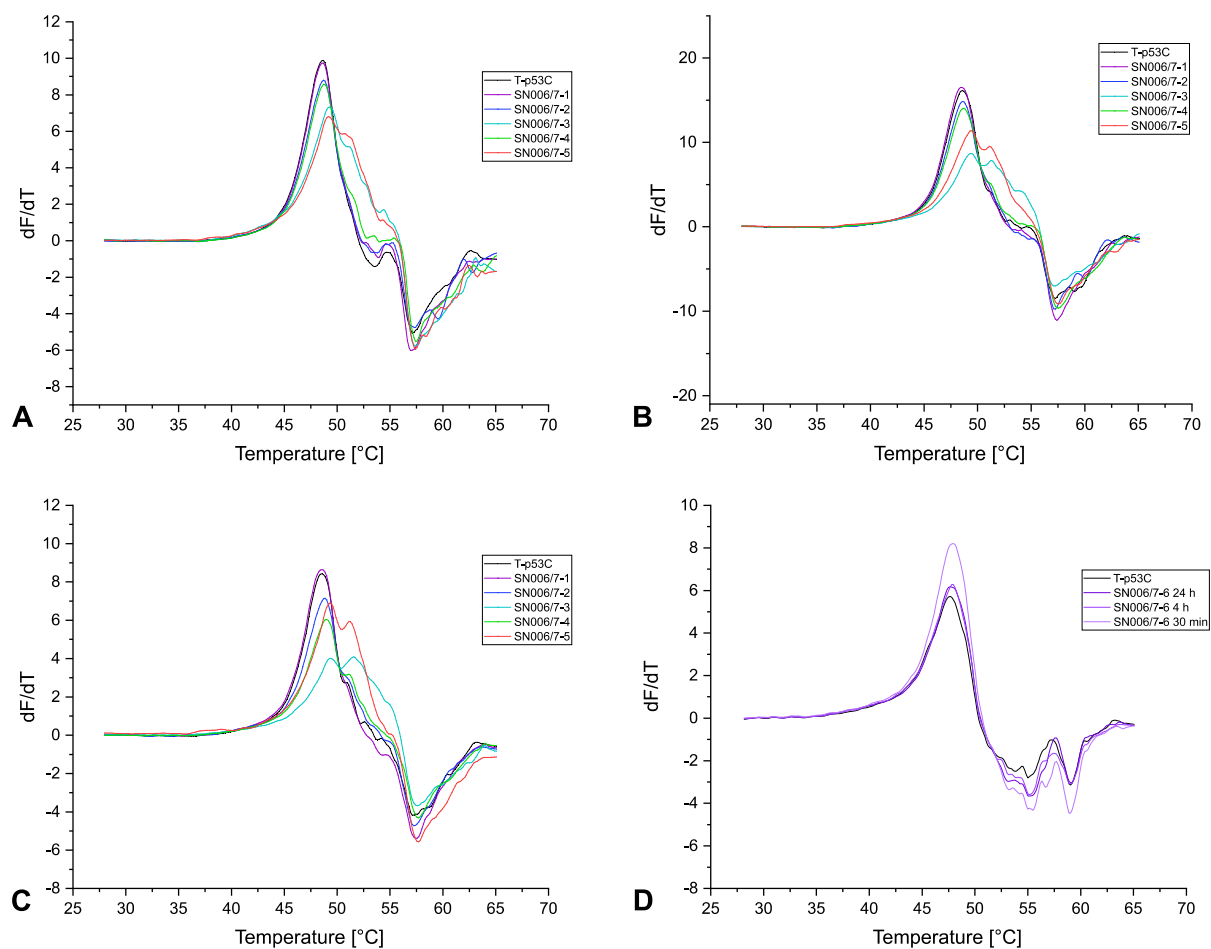


Figure 8.38: DSF screen of SN006/7 SAR compounds (250 μM) with T-p53C after 30 min, 4 h, and 24 h of incubation. The first derivatives of the melting curves of SN006/7-1 to SN006/7-5 (250 μM) with T-p53C (8 μM protein) after 30 min (A), 4 h (B), and 24 h (C) of incubation at 20 °C are shown. (D) The first derivatives of the melting curves of SN006/7-6 (250 μM) with T-p53C (8 μM protein) after 30 min, 4 h, and 24 h of incubation at 20 °C are presented.

Table 8.10: $\Delta T_m \pm SD$ of T-p53C-Y220C (8 μ M) incubated with 250 μ M SN006/7 SAR compounds for 30 min, 4 h, and 24 h at 20 °C. Measurements were performed at least in triplicate. Fragments with a ΔT_m of at least 0.50 °C are highlighted in bold [99, 130].

T-p53C-Y220C			
$\Delta T_m \pm SD$ [°C]			
Compound	250 μM 30 min	250 μM 4 h	250 μM 24 h
SN006/7-1	0.10 $\pm$ 0.12	0.05 $\pm$ 0.09	-0.10 $\pm$ 0.12
SN006/7-6	N/D	N/D	N/D
SN006/7-2	0.10 $\pm$ 0.12	0.10 $\pm$ 0.12	0.70 $\pm$ 0.09
SN006/7-3	0.88 $\pm$ 0.19	2.10 $\pm$ 0.12	2.87 $\pm$ 0.14
SN006/7-4	0.05 $\pm$ 0.31	0.25 $\pm$ 0.12	1.00 $\pm$ 0.09
SN006/7-5	1.30 $\pm$ 0.12	2.20 $\pm$ 0.12	2.65 $\pm$ 0.17

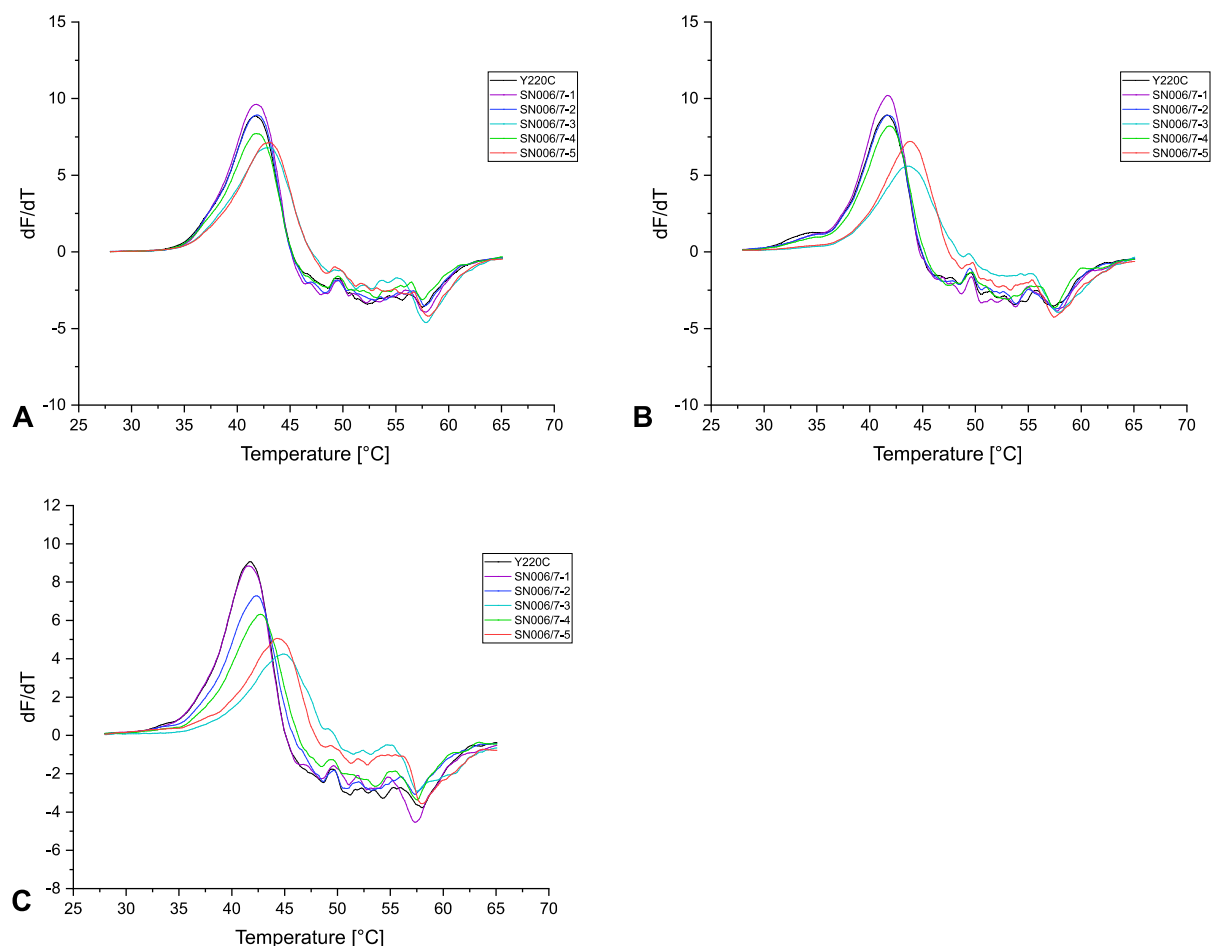


Figure 8.39: DSF screen of SN006/7 SAR compounds (250 μ M) with T-p53C-Y220C after 30 min, 4 h, and 24 h of incubation. The first derivatives of the melting curves of SN006/7 SAR compounds (250 μ M) with T-p53C-Y220C (8 μ M protein) after **30 min (A)**, **4 h (B)**, and **24 h (C)** of incubation at 20 °C are shown.

8.10 Crystallographic Data Collection and Refinement Statistics of the xT-p53C-Y220C Crystal Soaked with SN006/7-3

Table 8.11: Crystallographic data collection and refinement statistics of the dataset of xT-p53C-Y220C soaked with 5 mM SN006/7-3 for one day. Parenthesized values show the respective value of the highest resolution bin.

	Compound SN006/7-3 5 mM
Wavelength [Å]	1
Space group	P2 ₁ 2 ₁ 2 ₁
Cell dimension	
a, b, c [Å]	64.97, 70.90, 105.29
α , β , γ [°]	90, 90, 90
Resolution range [Å]	50-1.65 (1.75-1.65)
Redundancy	12.28 (7.46)
Completeness [%]	97.3 (85.0)
Mean I/ σ (I)	16.08 (1.08)
R-meas [%]	10.7 (153.7)
CC1/2 [%]	99.9 (62.2)
Wilson B [Å ²]	30.9
Resolution included [Å]	47.90-1.65
R _{work} /R _{free} [%]	15.44/20.37
Bond RMSD [Å]	0.006
Angle RMSD [°]	0.756
Ramachandran [%] (favored/allowed/outliers)	100/0/0
Rotamer outliers [%]	2.08
All-atom Clashscore	2.25
Average B factor [Å ²]	
Overall	28.70
Protein	26.15
Ligand	47.47
Water	41.37

8.11 DSF Screen of VS004 SAR Compounds with T-p53C and Its Mutants

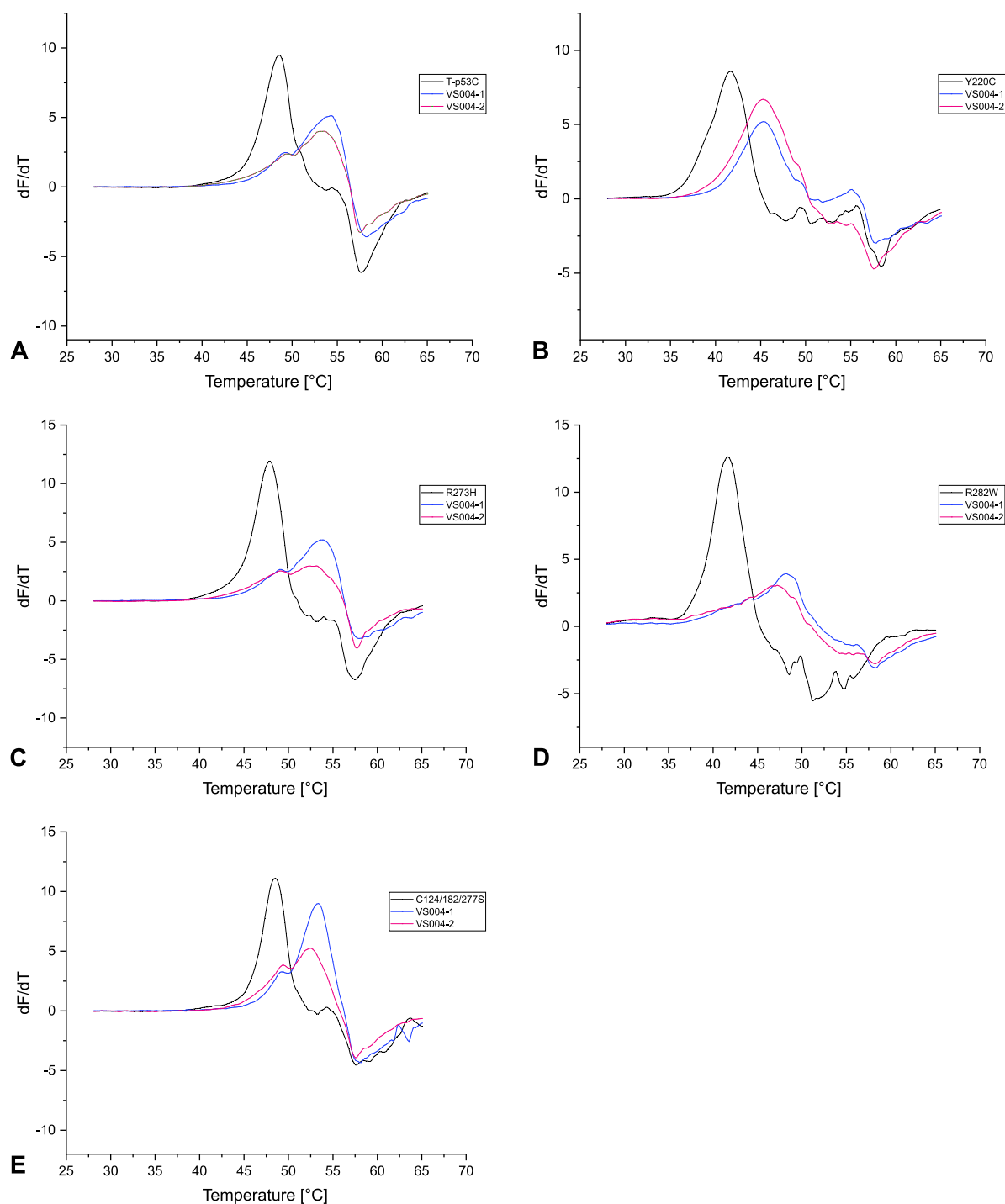


Figure 8.40: DSF screen of VS004 SAR compounds with T-p53C, T-p53C-Y220C, T-p53C-R273H, T-p53C-R282W, and T-p53C-C124/182/277S. The first derivatives of the melting curves of VS004 SAR compounds (1 mM) with **T-p53C** (A), **T-p53C-Y220C** (B), **T-p53C-R273H** (C), **T-p53C-R282W** (D), and **T-p53C-C124/182/277S** (E) (8 μ M protein) after 30 min of incubation at 20 °C are shown.

8.12 DSF Screen of SN001-17 and SN006/7-3 SAR Compounds with T-p53C and Its Mutants

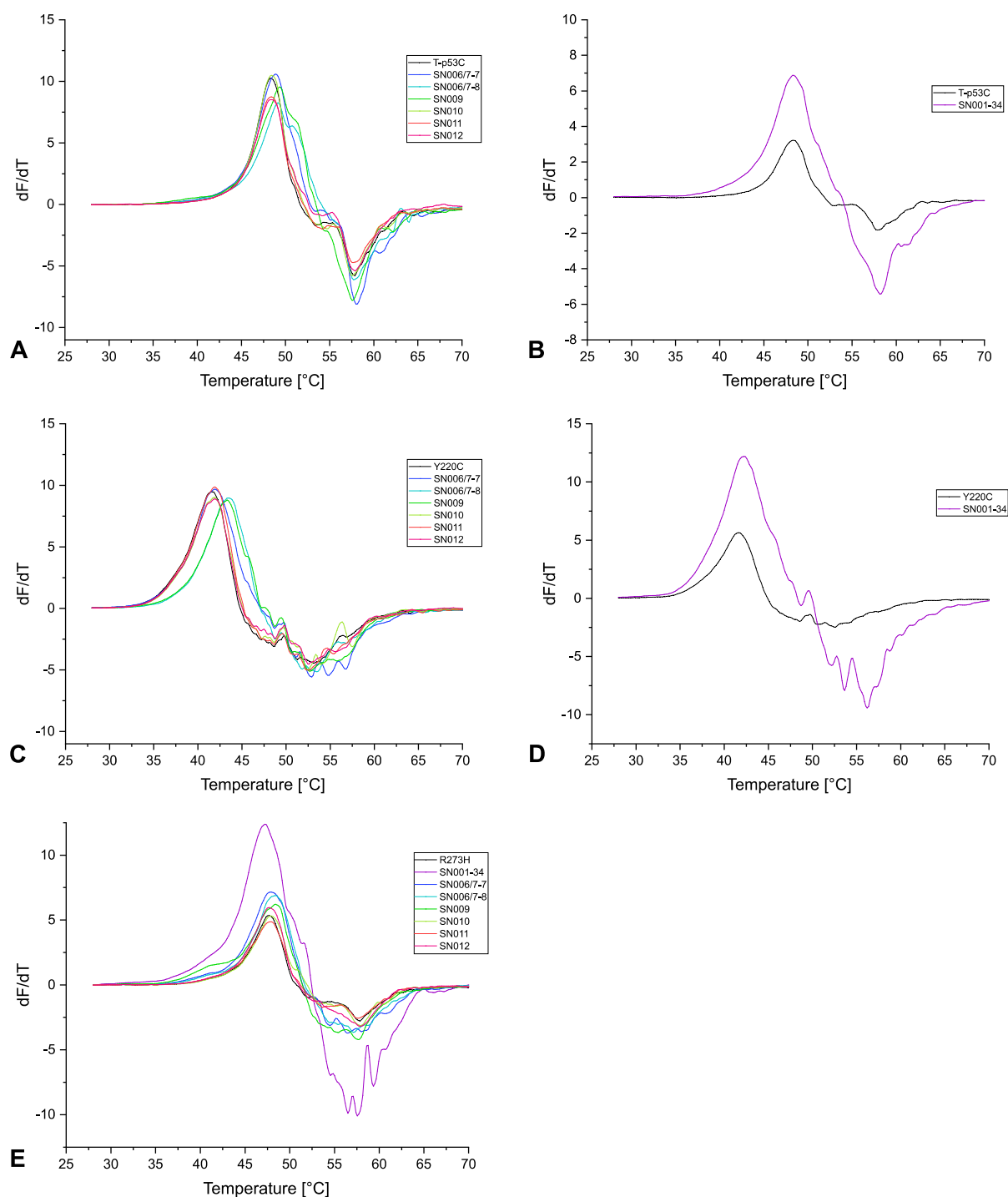


Figure 8.41: DSF screen of SN001-17 and SN006/7-3 SAR compounds with T-p53C, T-p53C-Y220C, and T-p53C-R273H after 30 min of incubation. The first derivatives of the melting curves of compounds (1 mM) SN006/7-7, SN006/7-8, SN009 to SN012 with **T-p53C** (A), SN001-34 with **T-p53C** (B), SN006/7-7, SN006/7-8, SN009 to SN012 with **T-p53C-Y220C** (C), SN001-34 with **T-p53C-Y220C** (D), and with **T-p53C-R273H** (E) (8 μ M protein) after 30 min of incubation at 20 °C are shown.

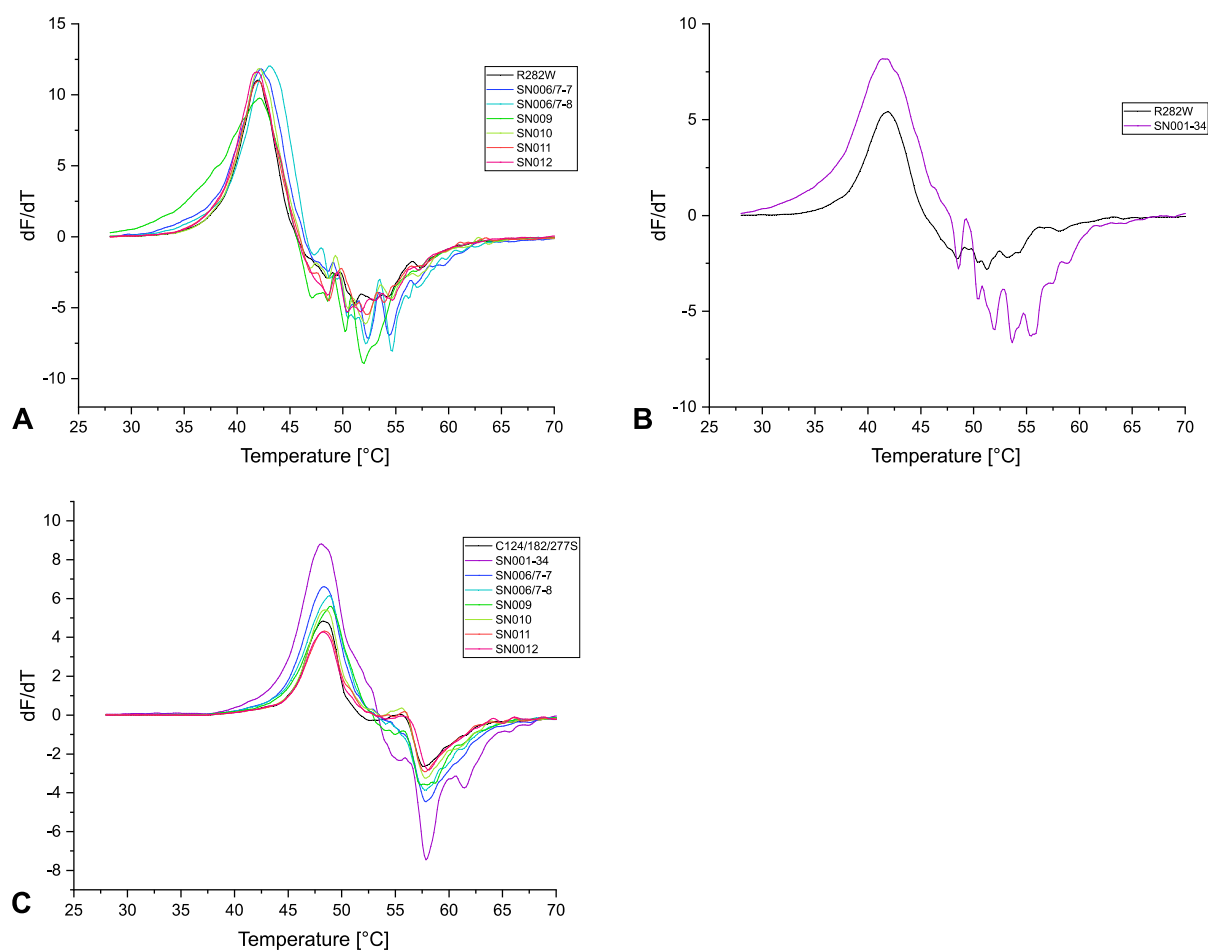


Figure 8.42: DSF screen of SN001-17 and SN006/7-3 SAR compounds with T-p53C-R282W and T-p53C-C124/182/277S after 30 min of incubation. The first derivatives of the melting curves of compounds (1 mM) SN006/7-7, SN006/7-8, SN009 to SN012 with **T-p53C-R282W** (A), SN001-34 with **T-p53C-R282W** (B), and with **T-p53C-C124/182/277S** (C) (8 μ M protein) after 30 min of incubation at 20 °C are shown.

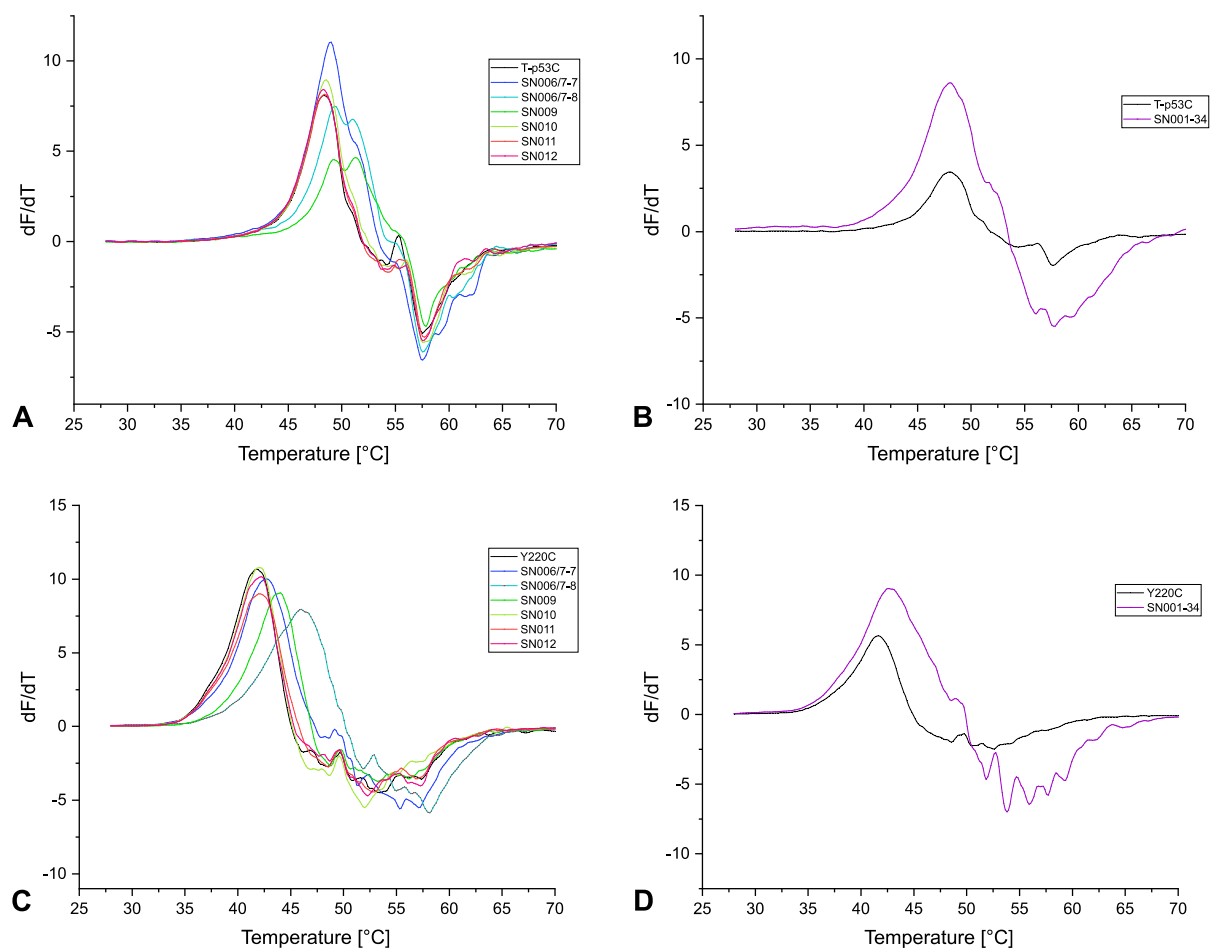


Figure 8.43: DSF screen of SN001-17 and SN006/7-3 SAR compounds with T-p53C and T-p53C-Y220C after 4 h of incubation. The first derivatives of the melting curves of compounds (**1 mM**) SN006/7-7, SN006/7-8, SN009 to SN012 with **T-p53C** (A), SN001-34 with **T-p53C** (B), SN006/7-7, SN006/7-8, SN009 to SN012 with **T-p53C-Y220C** (C), and SN001-34 with **T-p53C-Y220C** (D) (**8 μ M** protein) after **4 h** of incubation at **20 °C** are shown.

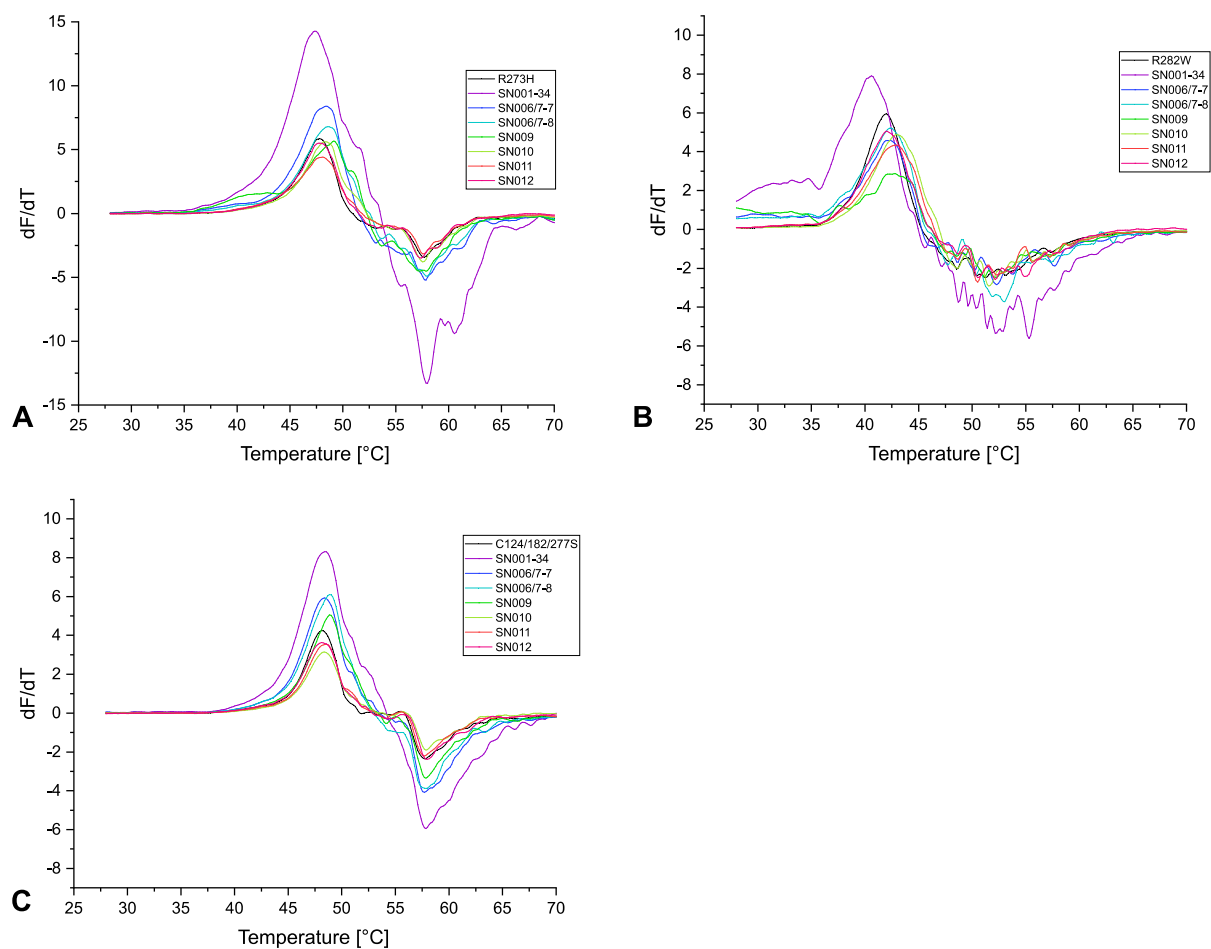


Figure 8.44: DSF screen of SN001-17 and SN006/7-3 SAR compounds with T-p53C-R273H, T-p53C-R282W, and T-p53C-C124/182/277S after 4 h of incubation. The first derivatives of the melting curves of SN001-17 and SN006/7-3 SAR compounds (1 mM) with **T-p53C-R273H** (A), **T-p53C-R282W** (B), and **T-p53C-C124/182/277S** (C) (8 μ M protein) after 4 h of incubation at 20 °C are shown.

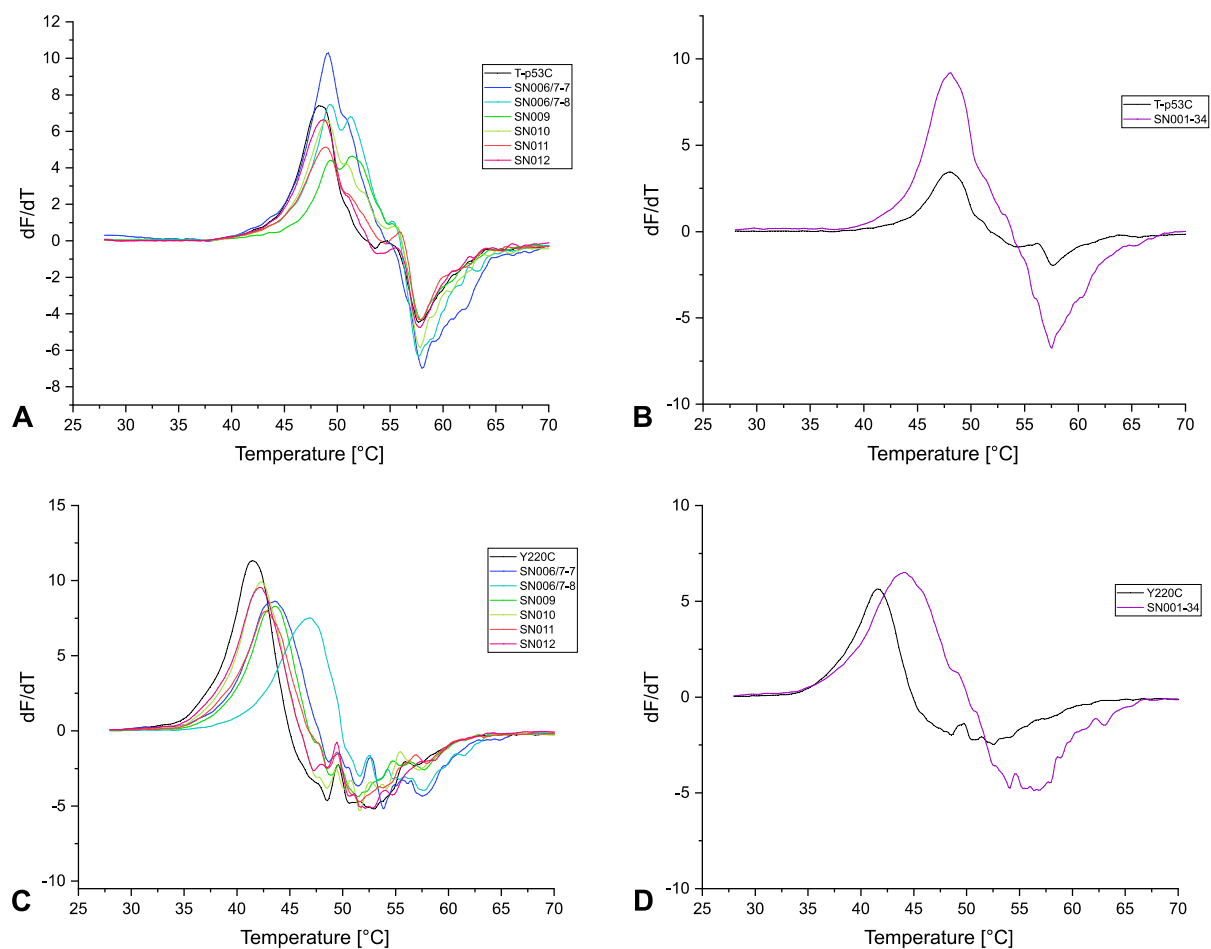


Figure 8.45: DSF screen of SN001-17 and SN006/7-3 SAR compounds with T-p53C and T-p53C-Y220C after 24 h of incubation. The first derivatives of the melting curves of compounds (**1 mM**) SN006/7-7, SN006/7-8, SN009 to SN012 with **T-p53C** (A), SN001-34 with **T-p53C** (B), SN006/7-7, SN006/7-8, SN009 to SN012 with **T-p53C-Y220C** (C), and SN001-34 with **T-p53C-Y220C** (D) (**8 μ M** protein) after **24 h** of incubation at **20 °C** are shown.

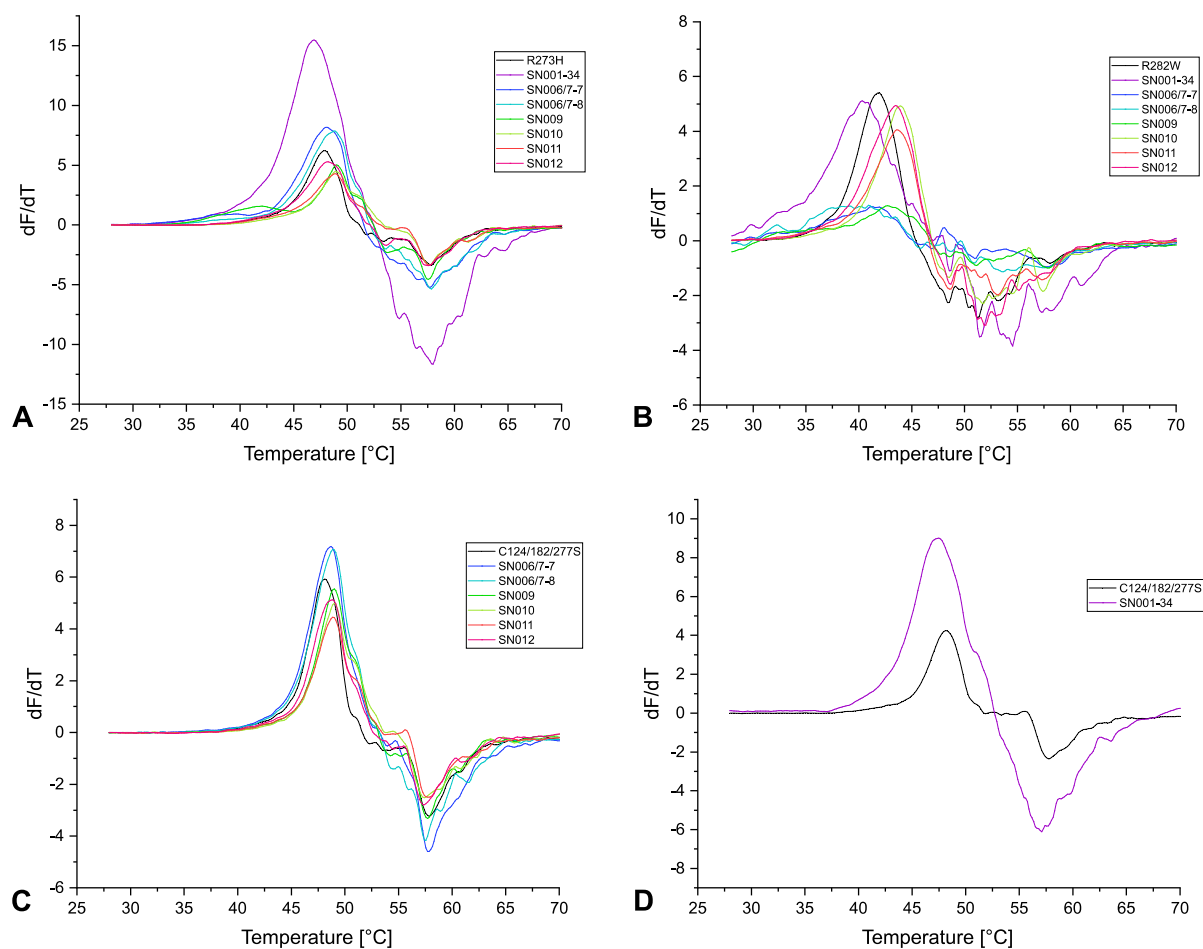


Figure 8.46: DSF screen of SN001-17 and SN006/7-3 SAR compounds with T-p53C-R273H, T-p53C-R282W, and T-p53C-C124/182/277S after 24 h of incubation. The first derivatives of the melting curves of SN001-17 and SN006/7-3 SAR compounds (1 mM) with **T-p53C-R273H** (A), **T-p53C-R282W** (B), SN006/7-7, SN006/7-8, SN009 to SN012 with **T-p53C-C124/182/277S** (C), and SN001-34 with **T-p53C-C124/182/277S** (D) (8 μM protein) after 24 h of incubation at 20 $^{\circ}\text{C}$ are shown.

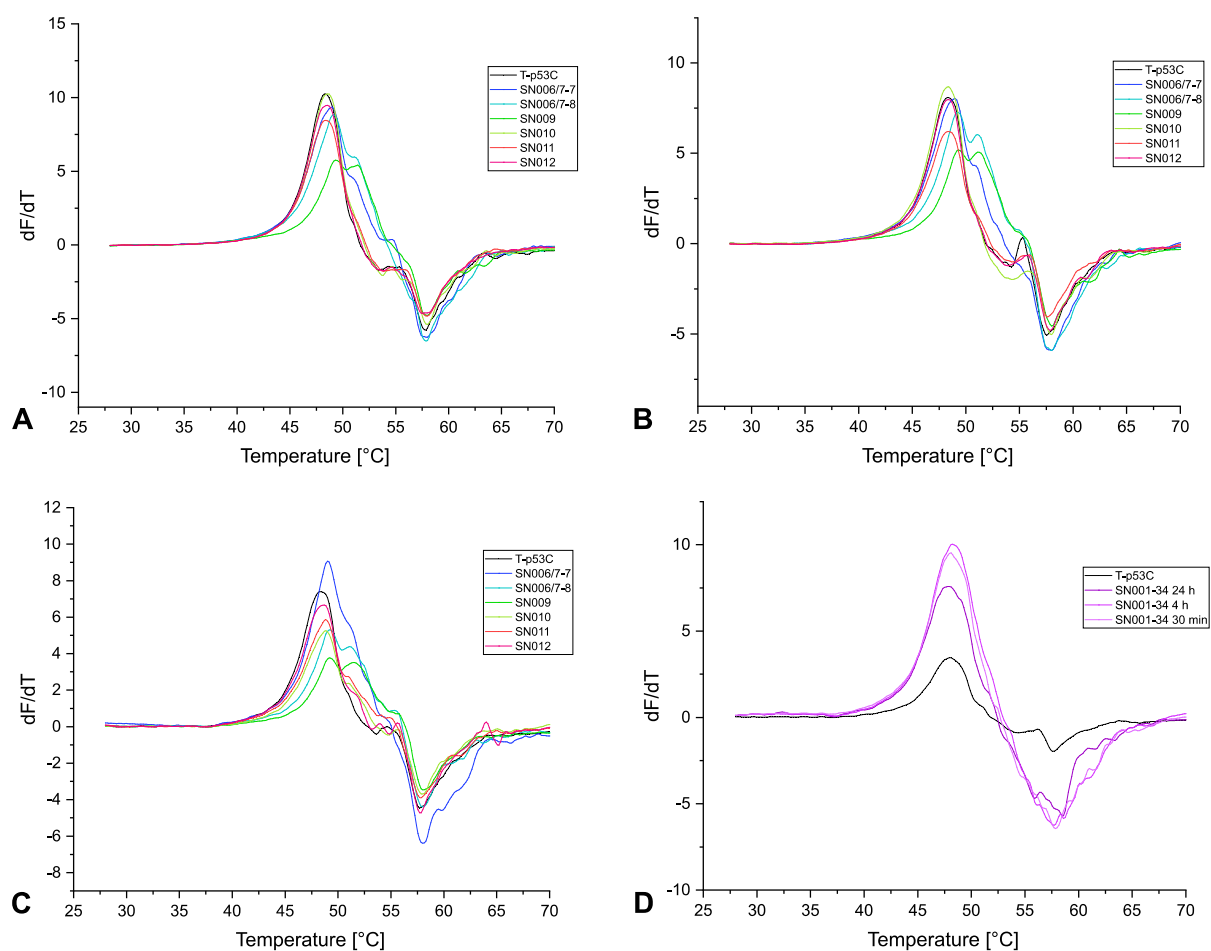


Figure 8.47: DSF screen of SN001-17 and SN006/7-3 SAR compounds (250 μM) with T-p53C after 30 min, 4 h, and 24 h of incubation. The first derivatives of the melting curves of SN006/7-3 SAR compounds (250 μM) with T-p53C (8 μM protein) after 30 min (A), 4 h (B), and 24 h (C) of incubation at 20 $^{\circ}\text{C}$ are shown. Moreover, the first derivatives of the melting curves of SN001-34 (250 μM) with T-p53C (8 μM protein) after 30 min, 4 h, and 24 h of incubation at 20 $^{\circ}\text{C}$ (D) are depicted.

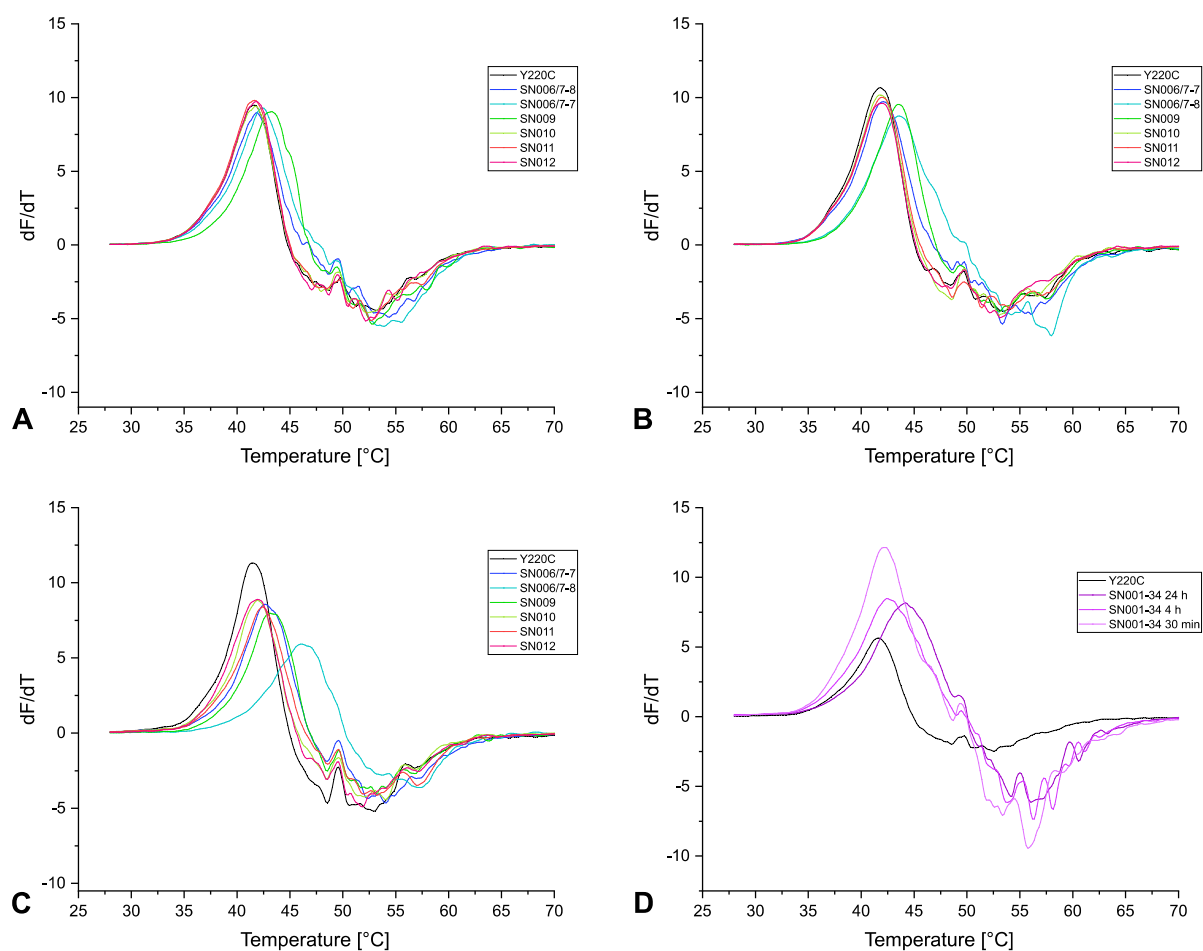


Figure 8.48: DSF screen of SN001-17 and SN006/7-3 SAR compounds (250 μM) with T-p53C-Y220C after 30 min, 4 h, and 24 h of incubation. The first derivatives of the melting curves of SN006/7-3 SAR compounds (250 μM) with T-p53C-Y220C (8 μM protein) after 30 min (A), 4 h (B), and 24 h (C) of incubation at 20°C are shown. Moreover, the first derivatives of the melting curves of SN001-34 (250 μM) with T-p53C-Y220C (8 μM protein) after 30 min, 4 h, and 24 h of incubation at 20 °C (D) are depicted.

8.13 Crystallographic Data Collection and Refinement Statistics of the xT-p53C-Y220C Crystal Soaked with SN006/7-8

Table 8.12: Crystallographic data collection and refinement statistics of the dataset of xT-p53C-Y220C soaked with 5 mM SN006/7-8 for one day. Parenthesized values show the respective value of the highest resolution bin.

	Compound SN006/7-8 5 mM
Wavelength [Å]	0.92
Space group	P2 ₁ 2 ₁ 2 ₁
Cell dimension	
a, b, c [Å]	65.19, 71.15, 105.73
α , β , γ [°]	90, 90, 90
Resolution range [Å]	50-1.56 (1.66-1.56)
Redundancy	12.05 (12.38)
Completeness [%]	100 (99.9)
Mean I/ σ (I)	13.02 (1.03)
R-meas [%]	11.7 (239.1)
CC1/2 [%]	99.9 (57.6)
Wilson B [Å ²]	30.68
Resolution included [Å]	48.06-1.56
R _{work} /R _{free} [%]	14.97/19.92
Bond RMSD [Å]	0.007
Angle RMSD [°]	0.812
Ramachandran [%] (favored/allowed/outliers)	99.74/0/0.26
Rotamer outliers [%]	1.49
All-atom Clashscore	2.58
Average B factor [Å ²]	
Overall	30.41
Protein	27.71
Ligand	45.96
Water	42.61

8.14 DSF Screen of SN006/7-8 SAR Compounds with T-p53C and Its Mutants

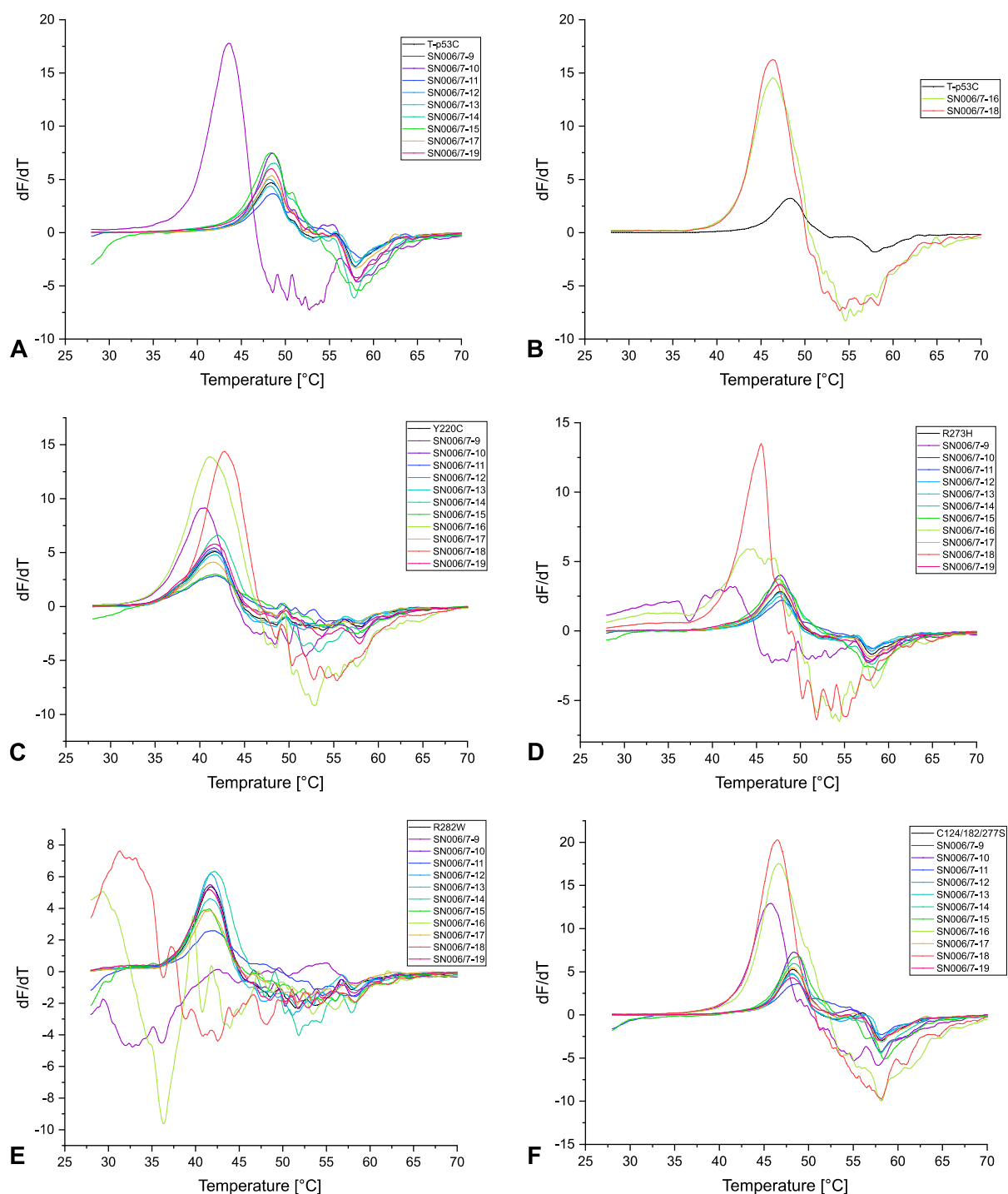


Figure 8.49: DSF screen of SN006/7-8 SAR compounds with T-p53C, T-p53C-Y220C, T-p53C-R273H, T-p53C-R282W, and T-p53C-C124/182/277S after 30 min of incubation. The first derivatives of the melting curves of SN006/7-8 SAR compounds (1 mM) with T-p53C (A), SN006/7-16 and SN006/7-18 with T-p53C (B), with T-p53C-Y220C (C), T-p53C-R273H (D), T-p53C-R282W (E), and T-p53C-C124/182/277S (F) (8 μ M protein) after 30 min of incubation at 20 °C are shown.

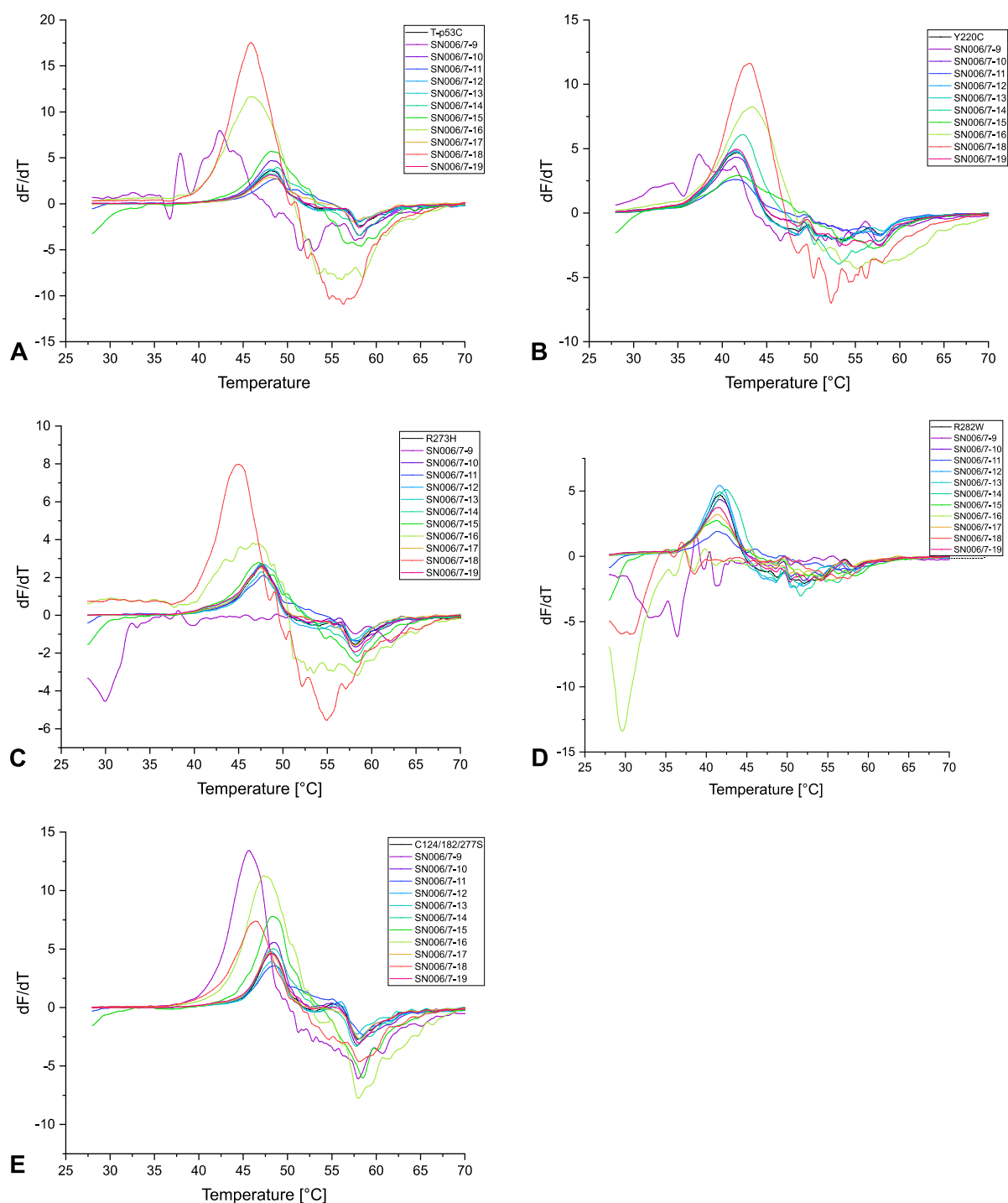


Figure 8.50: DSF screen of SN006/7-8 SAR compounds with T-p53C, T-p53C-Y220C, T-p53C-R273H, T-p53C-R282W, and T-p53C-C124/182/277S after 4 h of incubation. The first derivatives of the melting curves of SN006/7-8 SAR compounds (1 mM) with **T-p53C** (A), **T-p53C-Y220C** (B), **T-p53C-R273H** (C), **T-p53C-R282W** (D), and **T-p53C-C124/182/277S** (E) (8 μ M protein) after 4 h of incubation at 20 °C are shown.

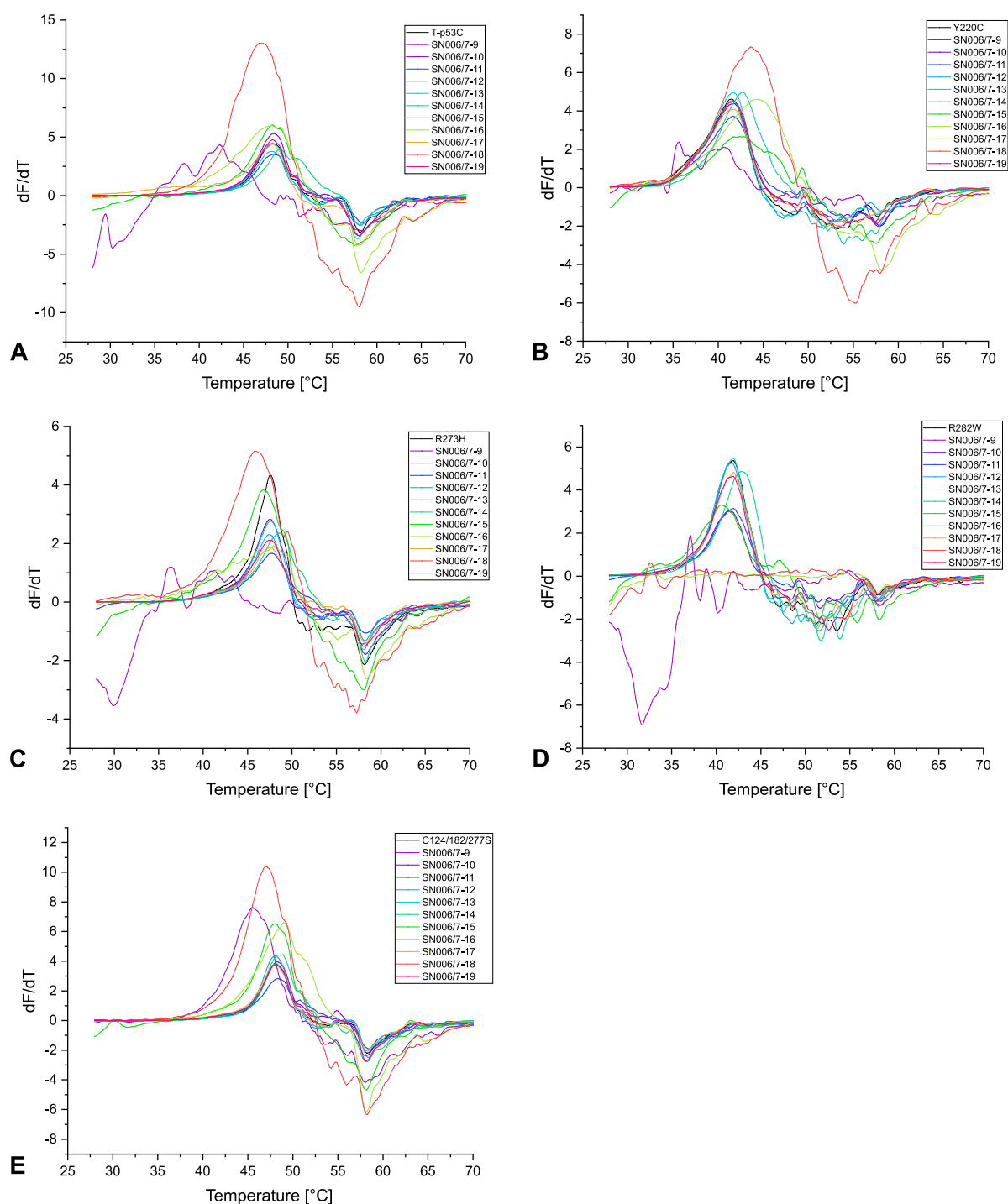


Figure 8.51: DSF screen of SN006/7-8 SAR compounds with T-p53C, T-p53C-Y220C, T-p53C-R273H, T-p53C-R282W, and T-p53C-C124/182/277S after 24 h of incubation. The first derivatives of the melting curves of SN006/7-8 SAR compounds (1 mM) with T-p53C (A), T-p53C-Y220C (B), T-p53C-R273H (C), T-p53C-R282W (D), and T-p53C-C124/182/277S (E) (8 μ M protein) after 24 h of incubation at 20 °C are shown.

Table 8.13: $\Delta T_m \pm$ SD of T-p53C (8 μ M) incubated with 250 μ M SN006/7-8 SAR compounds for 30 min, 4 h, and 24 h at 20 °C. Measurements were performed at least in triplicate. Fragments with a ΔT_m of at least 0.50 °C are highlighted in bold [99, 130].

T-p53C			
$\Delta T_m \pm$ SD [°C]			
Compound	250 μ M 30 min	250 μ M 4 h	250 μ M 24 h
SN006/7-9	-2.85 $\pm$ 0.12	-2.30 $\pm$ 0.22	-0.13 $\pm$ 0.30
SN006/7-10	-0.05 $\pm$ 0.09	-0.05 $\pm$ 0.08	-0.05 $\pm$ 0.10
SN006/7-11	0.10 $\pm$ 0.09	0.05 $\pm$ 0.09	-0.07 $\pm$ 0.14
SN006/7-12	-0.05 $\pm$ 0.09	0 $\pm$ 0.09	0 $\pm$ 0.11
SN006/7-13	0 $\pm$ 0.12	0.03 $\pm$ 0.08	0.07 $\pm$ 0.08
SN006/7-14	0.10 $\pm$ 0.09	0.13 $\pm$ 0.08	0.47 $\pm$ 0.10
SN006/7-15	0.15 $\pm$ 0.12	0.18 $\pm$ 0.11	0.12 $\pm$ 0.14
SN006/7-16	-0.65 $\pm$ 0.09	1.13 $\pm$ 0.09	N/A ^a
SN006/7-17	0.05 $\pm$ 0.12	0 $\pm$ 0.09	-0.08 $\pm$ 0.10
SN006/7-18	-0.70 $\pm$ 0.12	-0.73 $\pm$ 0.15	0.57 $\pm$ 0.35
SN006/7-19	-0.05 $\pm$ 0.09	-0.03 $\pm$ 0.08	0 $\pm$ 0.10

^aNo evaluable melting curves and first derivatives with clearly defined melting points were obtained.

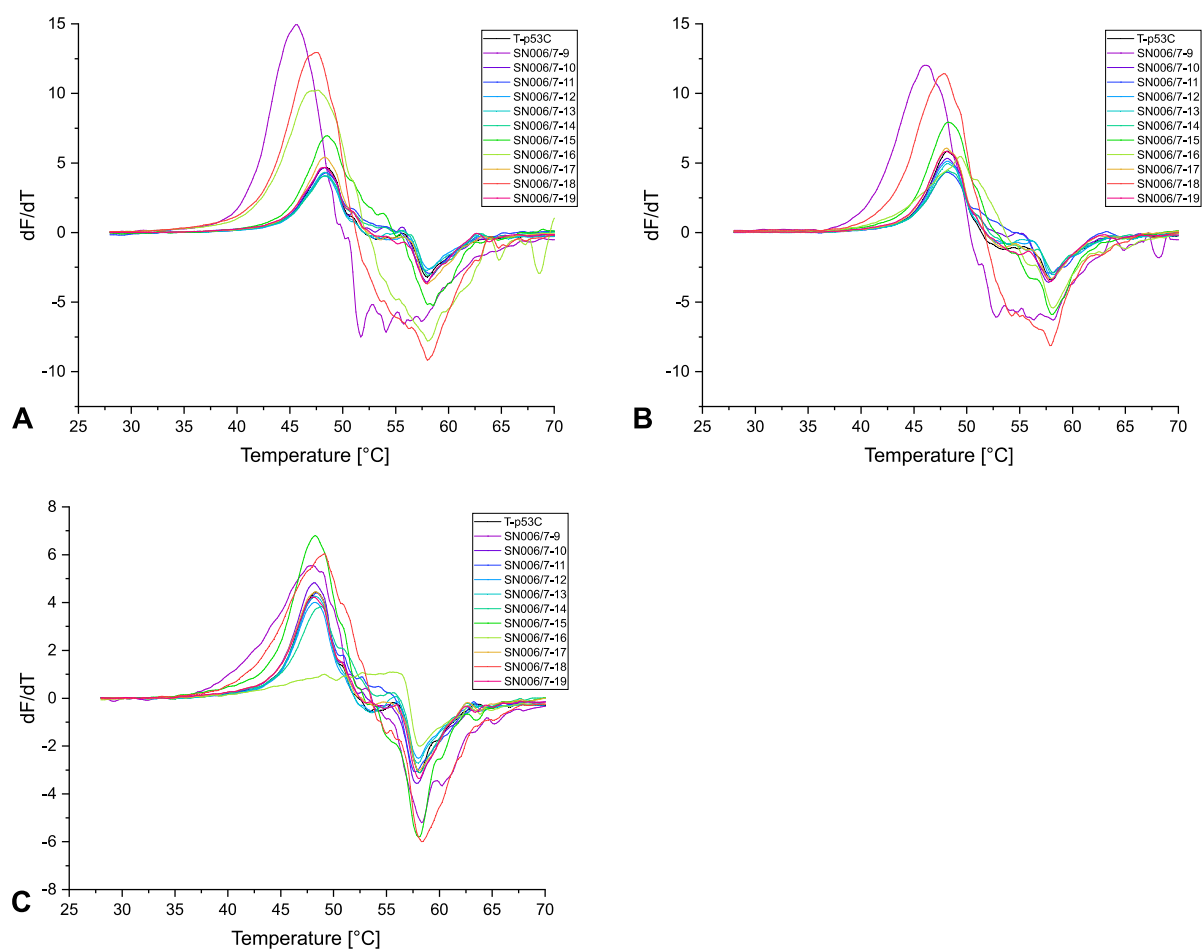


Figure 8.52: DSF screen of SN006/7-8 SAR compounds (250 μM) with T-p53C after 30 min, 4 h, and 24 h of incubation. The first derivatives of the melting curves of the SN006/7-8 SAR compounds (250 μM) with T-p53C (8 μM protein) after 30 min (A), 4 h (B), and 24 h (C) of incubation at 20 $^{\circ}\text{C}$ are shown.

Table 8.14: $\Delta T_m \pm SD$ of T-p53C-Y220C (8 μM) incubated with 250 μM SN006/7-8 SAR compounds for 30 min, 4 h, and 24 h at 20 °C. Measurements were performed at least in triplicate. Fragments with a ΔT_m of at least 0.50 °C are highlighted in bold [99, 130].

T-p53C-Y220C			
$\Delta T_m \pm SD$ [°C]			
Compound	250 μM 30 min	250 μM 4 h	250 μM 24 h
SN006/7-9	0.40 $\pm$ 0.17	0.93 $\pm$ 0.12	2.60 $\pm$ 0.19
SN006/7-10	-0.10 $\pm$ 0.12	-0.08 $\pm$ 0.11	0 $\pm$ 0.09
SN006/7-11	0.05 $\pm$ 0.12	0.23 $\pm$ 0.15	0.15 $\pm$ 0.09
SN006/7-12	0 $\pm$ 0.12	-0.03 $\pm$ 0.09	0 $\pm$ 0.09
SN006/7-13	0 $\pm$ 0.12	0.02 $\pm$ 0.10	0.02 $\pm$ 0.08
SN006/7-14	0.20 $\pm$ 0.12	0.20 $\pm$ 0.11	0.38 $\pm$ 0.11
SN006/7-15	0.50 $\pm$ 0.12	0.56 $\pm$ 0.11	0.75 $\pm$ 0.16
SN006/7-16	0.30 $\pm$ 0.12	1.95 $\pm$ 0.19	2.57 $\pm$ 0.08
SN006/7-17	0 $\pm$ 0.12	0.05 $\pm$ 0.09	0.10 $\pm$ 0.09
SN006/7-18	2.15 $\pm$ 0.24	2.90 $\pm$ 0.09	3.00 $\pm$ 0.12
SN006/7-19	0 $\pm$ 0.12	-0.10 $\pm$ 0.09	0.10 $\pm$ 0

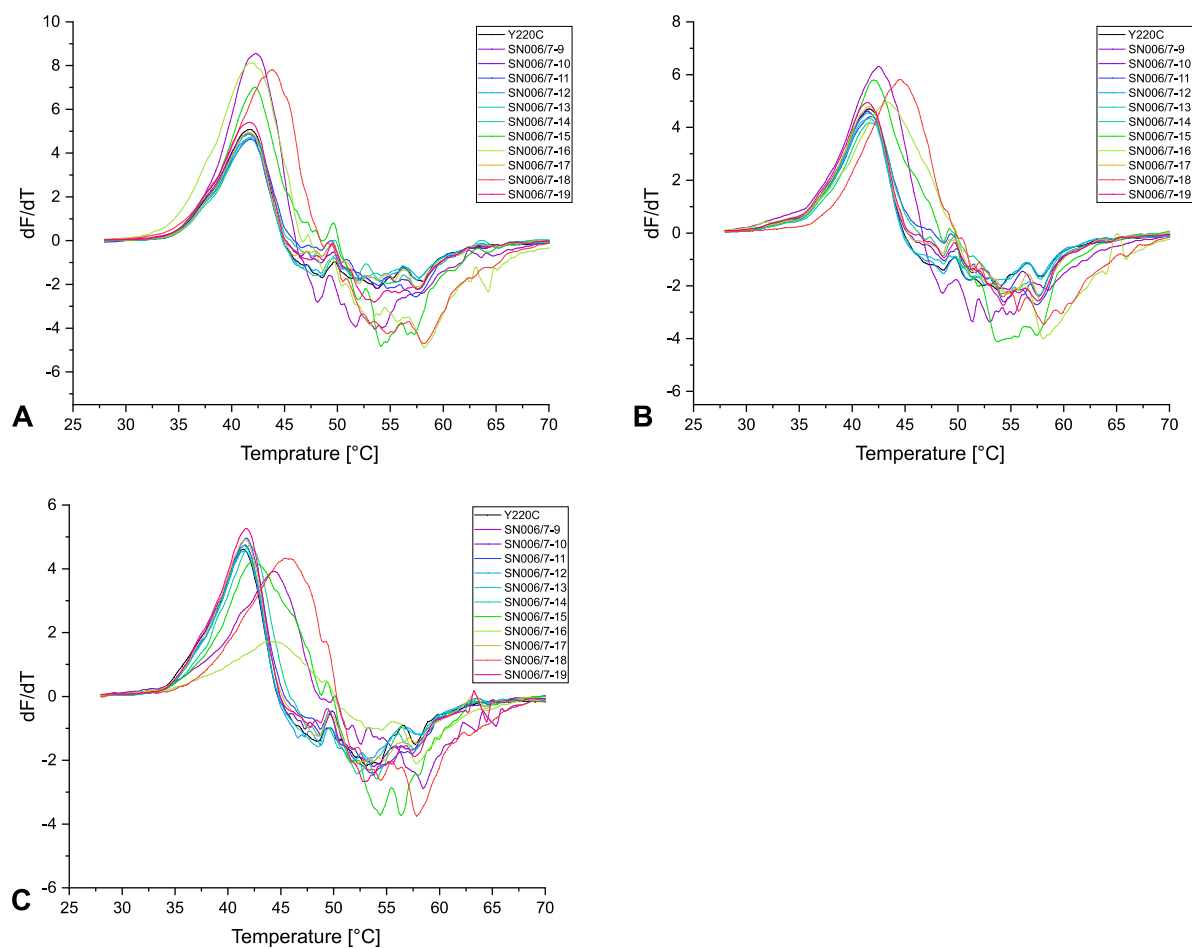


Figure 8.53: DSF screen of SN006/7-8 SAR compounds (250 μM) with T-p53C-Y220C after 30 min, 4 h, and 24 h of incubation. The first derivatives of the melting curves of the SN006/7-8 SAR compounds (250 μM) with T-p53C-Y220C (8 μM protein) after 30 min (A), 4 h (B), and 24 h (C) of incubation at 20 $^{\circ}\text{C}$ are shown.

8.15 Crystallographic Data Collection and Refinement Statistics of the xT-p53C-Y220C Crystal Soaked with SN006/7-9

Table 8.15: Crystallographic data collection and refinement statistics of the dataset of xT-p53C-Y220C soaked with 5 mM SN006/7-9 for one day. Parenthesized values show the respective value of the highest resolution bin.

	Compound SN006/7-9 5 mM
Wavelength [Å]	1
Space group	P2 ₁ 2 ₁ 2 ₁
Cell dimension	
a, b, c [Å]	64.82, 71.16, 105.60
α , β , γ [°]	90, 90, 90
Resolution range [Å]	50-1.64 (1.74-1.64)
Redundancy	9.13 (7.98)
Completeness [%]	96.1 (93.3)
Mean I/ σ (I)	10.71 (1.04)
R-meas [%]	12.4 (212.4)
CC1/2 [%]	99.7 (54.7)
Wilson B [Å ²]	34.64
Resolution included [Å]	47.92-1.64
R _{work} /R _{free} [%]	20.31/23.23
Bond RMSD [Å]	0.009
Angle RMSD [°]	0.987
Ramachandran [%] (favored/allowed/outliers)	99.74/0.26/0
Rotamer outliers [%]	3.22
All-atom Clashscore	5.23
Average B factor [Å ²]	
Overall	34.83
Protein	33.25
Ligand	51.96
Water	40.92

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Figure 1.5 and parts of Chapter 3.3:

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