

S_NAr Reactive Pyrazine Derivatives as p53-Y220C Cleft Binders with Diverse Binding Modes

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Purpose: The tumor suppressor p53 is most commonly mutated in human cancer. The structural mutant in the β -sandwich of the protein, p53-Y220C, is the ninth most common p53 mutant. The p53-Y220C mutant has a solvent-accessible hydrophobic pocket, leading to thermal destabilization of the protein. Screening of our covalent fragment library (CovLib) revealed the highly reactive pyrazine derivatives SN006 and SN007, which arylate among other cysteines in p53, the mutation-generated Cys220. Herein, comprehensive structure-activity relationship (SAR) studies of these intrinsically reactive CovLib hits were performed, aiming to identify improved stabilizers for p53-Y220C, with a more balanced reactivity profile, diverse binding modes and a better potential for chemical optimization.

Methods: The compounds were screened for enhanced stabilization of p53 wild type and its mutants using differential scanning fluorimetry (DSF). To confirm covalent modification, intact mass spectrometry was performed. Thiol reactivity profiles were determined using a standardized Glutathione-modifying (GSH) assay. The binding modes of the identified hits and covalent modification of Cys220 were elucidated by X-ray crystallography. Moreover, the influence of the hits on the DNA-binding affinity of full-length p53 was investigated employing a fluorescence polarization assay (FPA).

Results and Conclusion: The promising pyrazine derivatives SN006/7-3, SN006/7-8, and SN006/7-9 were identified, occupying different subsites of the Y220C binding pocket. The compound SN006/7-8 substantially stabilized the thermosensitive cancer mutant Y220C by up to 5.0 °C, representing a strong enhancement over SN006 (1.8 °C) and SN007 (2.0 °C).

Keywords: tumor suppressor p53 mutant stabilizers, covalent fragment-based drug discovery, structure-activity relationship, differential scanning fluorimetry, intact protein mass spectrometry, X-ray crystallography

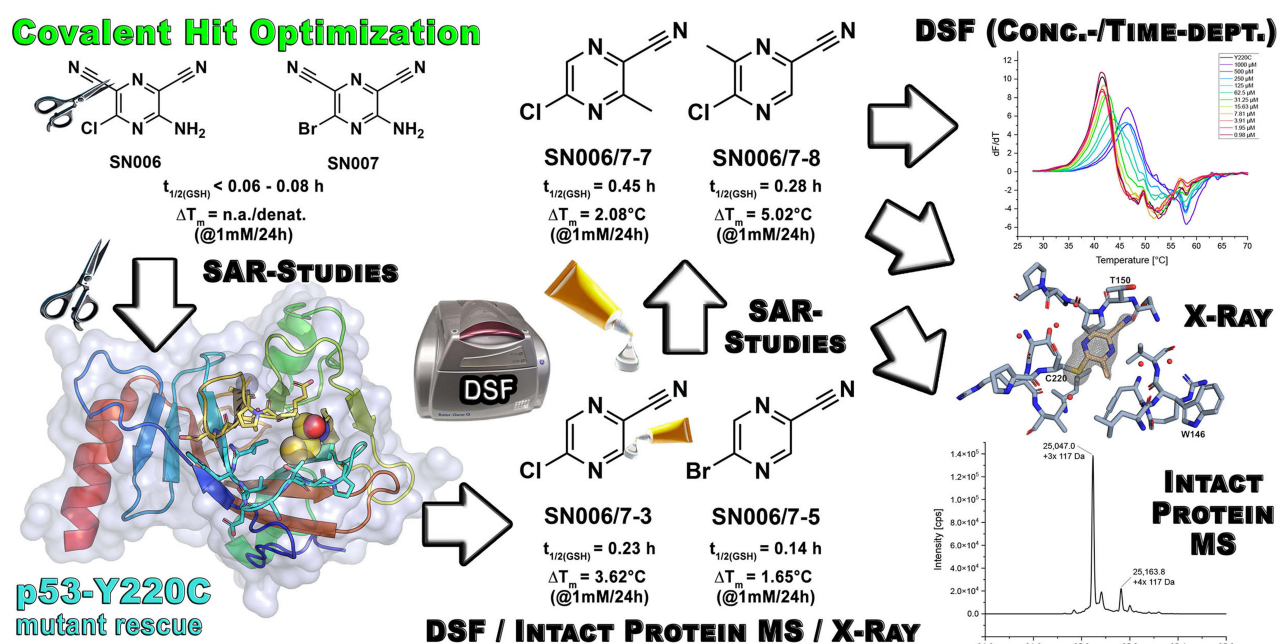
Introduction

The tumor suppressor protein p53, also known as the “guardian of the genome”, plays an essential role in the cell cycle.^{1–4} Activation of p53 in response to various stress signals leads to the regulation of the transcription of many genes, involved in cell cycle arrest, apoptosis, DNA-repair, and cellular senescence.^{5–7} In addition to the aforementioned canonical functions, it has been discovered that p53 is also involved in various processes, including autophagy, antiangiogenesis, protection against oxidative stress, metabolic homeostasis, ferroptosis, immune microenvironment, and regulation of stemness of cells.^{3,8–10}

In many tumors, a mutation of p53 is found, or p53 activity is reduced by disruption of associated signaling pathways such as the upregulation of the two negative regulators murine double minute 2 homolog (MDM2) and murine double minute 4 homolog (MDM4).^{10,11} The majority of the oncogenic p53 mutations are missense mutations located in the core domain of p53.^{7,12–15} In addition to the loss of function, many of these mutants have a dominant-negative effect on wild-type (WT) p53 by forming mixed tetramers.^{4,13,16} Moreover, some of the p53 mutants acquire other transcriptional

Graphical Abstract

Covalent Hit Optimization



regulatory functions, resulting in a gain-of-function phenotype.^{3,4,7} The missense core domain mutants can be categorized as DNA contact mutants (eg R273H) and structural mutants, which induce structural changes within the protein, folding variations or packing defects destabilizing the protein (eg R282W, Y220C).^{13,14,17} Mutations of p53 are related to rapid tumor progression, worse cancer prognosis, and chemoresistance.^{13,18,19} Therefore, p53 is an attractive yet challenging target for cancer therapy, and there is a great interest in identifying drugs that can rescue p53.

The structural Y220C β -sandwich mutant is the ninth most common p53 mutant and is estimated to account for 130 000 annual cases of human cancer.^{13,20–22} The mutation from tyrosine to cysteine creates a solvent-accessible hydrophobic crevice.^{13,14,23} The mutant p53-Y220C is strongly thermodynamically destabilized, and its melting temperature (T_m) is reduced by about 8 °C, resulting in rapid unfolding, denaturation, and aggregation at body temperature.^{13,24} However, the overall structure of the core domain remains intact.¹⁴ Furthermore, the mutation-induced Y220C cleft is far from the DNA-binding site, the dimerization interface, and not involved in essential protein contacts. Therefore, the crevice represents a possible binding pocket for stabilizing small molecules.^{13,14,23} A number of compounds were discovered that target the mutational cleft and enhance the thermal stability of the mutant Y220C protein.^{21,23–27} The *N*-ethylcarbazole based compound PK083 ($\Delta T_m = 0.8^\circ\text{C}$ at 125 μM PK083),^{23,25} the iodophenol derivative PK5196 ($\Delta T_m = 3.6^\circ\text{C}$ at 250 μM PK5196),²¹ the pyrazole derivative PK7088 ($\Delta T_m = 1.0^\circ\text{C}$ at 350 μM PK7088),²⁶ the aminobenzothiazole derivative MB710 ($\Delta T_m = 2.0^\circ\text{C}$ at 250 μM MB710),²⁷ and the nanomolar cleft binder JC744 ($\Delta T_m = 2.70^\circ\text{C}$ at 200 μM JC744)²⁴ are examples of stabilizers for the Y220C mutant. In addition, PMV Pharma has developed the small molecule PC14586 (Rezatapopt), which specifically targets and stabilizes the Y220C mutant and is currently evaluated in clinical trials for patients with advanced solid tumors harboring the p53-Y220C mutation (NCT04585750).^{28,29} Moreover, a covalent reactive compound, KG13, has been identified. KG13 contains an acrylamide warhead and targets Cys220 in the mutational cleft, thereby increasing the T_m of the Y220C mutant to levels comparable to those of the WT protein.³⁰

We recently employed a covalent fragment-based approach and screened a covalent fragment library (CovLib) with p53 and its mutants.^{31,32} The highly reactive pyrazines derivatives SN006 and SN007 were identified, reacting via nucleophilic aromatic substitution ($S_N\text{Ar}$) with cysteines in p53 and also with the Cys220 in the crevice. Thiol reactivity was determined with a standardized GSH assay, highlighting complete reaction with glutathione at the first

possible analysis time (20 min). Thus, their reaction half-time was initially reported by us as $t_{1/2} \ll 0.33\text{h}$.³² We estimate that at least 4–6 half-time durations need to pass, until none of the unreacted fragment is detectable by HPLC-UV. Consequently, a $t_{1/2}$ of 0.06 to 0.08 h (or even significantly below) is quite plausible based on the experimental method.

Covalent modification of Cys220 by SN006 was confirmed by X-ray crystallography. Nevertheless, SN006 and SN007 showed in line with the GSH assay a high reactivity and arylated cysteines in p53 non-specifically, resulting in a destabilization of the T-p53C protein at high compound concentrations.³¹ Therefore, we conducted structure-activity relationship (SAR) studies with the objective of identifying fragments with a more balanced reactivity profile, higher selectivity for Cys220, and different binding pose in the hydrophobic pocket.

Materials and Methods

Materials

The compounds SN006/7-1 to SN006/7-6 were purchased from Aldrich Market Select (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). The SAR compounds derived from SN006/7-3 and SN006/7-8 were purchased from the suppliers listed in [Table S1](#). Purity of the compounds was confirmed to be >90% by high-performance liquid chromatography (HPLC)-UV on an Ultimate 3000 HPLC-System (Thermo Fisher Scientific, Dreieich, Germany). Upon completion of the biophysical measurements, it was determined that the purity of fragments SN009 and SN001-15 was below the required threshold of 90%. The precise point at which this occurred could not be ascertained. Consequently, compounds SN009 and SN001-15 are not referenced in this paper. However, the corresponding results are included in the Supporting Information. The corresponding HPLC chromatograms are provided in [Figure S1](#) to [Figure S23](#). [Table S2](#) summarizes the analytical results.

Glutathione Assay

The glutathione (GSH) assay was performed as previously described³² using a method established by Keeley et al.³³ The following reaction conditions were applied: 250 μM fragment, 100 μM ketoprofen or ibuprofen as an internal standard, 5 mM GSH excess, 10% acetonitrile, and phosphate buffered saline (PBS) pH 7.4 at 37 °C. The samples were analyzed on an Ultimate 3000 HPLC-System (Thermo Fisher Scientific, Dreieich, Germany) with UV-detection after 0, 1, 2, 4, 8, 12, and 24 h. Highly reactive fragments (half-life ($t_{1/2}$) <1 h) were analyzed every 20 min. The reaction of the compounds with GSH was detected by measuring the decreasing area under the curve (AUC) of the compounds relative to the internal standard. OriginPro2020 (OriginLab, Northampton, MA, USA) was used to fit the relative AUC to the integrated rate equation of pseudo-first order kinetics:

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

The half-life $t_{1/2}$ was calculated from the pseudo-first order rate constant k according to the following equation:

$$t_{1/2} = \frac{\ln 2}{k}$$

The GSH measurements were performed in duplicates and multiple runs were averaged using error propagation. In addition, measurements were performed in PBS buffer without GSH to test the hydrolytic degradation. Contrary to the calculations of Keeley et al.,³³ the rate constants k_{GSH} and the corresponding $t_{1/2}$ were not corrected for the degradation reaction in pure buffer.

A complete overview of all GSH assay data is provided in [Table S4](#) in the Supporting Information.

Molecular Biology

The expression and purification of T-p53C (94–312, M133L/V203A/N239Y/N268D), its mutants, and full-length (FL)-T-p53 (1–393, M133L/V203A/N239Y/N268D) were carried out as previously described.^{22,31,32} The lysate was loaded onto a Ni-NTA column (HisTrap FF, Cytiva) and eluted using a gradient of imidazole from 10–300 mM (Buffer: 50 mM KPi, pH = 8, 300 mM NaCl, 10/300 mM Imidazole, 2 mM TCEP). After cleaving the His-Tag using the tobacco etch virus

main protease, the sample was diluted 5–10 fold with heparin buffer A (50 mM KPi, pH = 7.5, 5 mM DTT) and loaded onto a heparin column (HiTrap Heparin HP, Cytiva). The sample was eluted setting a salt concentration of 800 mM (Buffer: 50 mM KPi, pH = 7.5, 5 mM DTT, 2 M NaCl). Final purification was performed via a size exclusion chromatography. For the core domain, a HiLoad 26/60 Superdex 75 pg (GE Healthcare) was used, while for the full length constructs a HiLoad 26/60 Superdex 200 pg was used (Buffer: 25 mM KPi, pH = 7.2, 5 mM DTT, 150 mM NaCl). This quadruple mutant is a frequently utilized p53 core domain construct, facilitating easier handling of p53 under lab conditions by enhancing its stability.^{34,35} The purity of the expressed proteins was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and protein mass was verified by ultra-high-performance liquid chromatography electrospray ionization mass spectrometry (UHPLC-ESI-MS). The protein sequences of all used constructs are listed in [Table S3](#). A construct with a deletion of the GGS overhang was utilized for X-ray crystallography.

Differential Scanning Fluorimetry (DSF)

DSF was used to investigate the impact of the compounds on the melting temperatures of T-p53C and its mutants. DSF measurements were performed as previously described^{22,31,32} using a Qiagen Rotor-Q Model-5-Plex HRM real-time PCR instrument (Qiagen, Hilden, Germany). SYPRO Orange (Life Technologies Corporation, Eugene, OR, USA) served as a fluorescent dye (5x final concentration). A constant heating rate of 270 °C/h²³ was used, while the temperature was raised from 28 °C to 60–70 °C. The excitation and emission filters were set at 470 nm and 610 nm, respectively. The DSF measurements were performed with 8 µM protein in phosphate buffer (25 mM KPi pH 7.2, 150 mM NaCl, 1 mM TCEP, 5% [v/v] DMSO). For the primary screening, a final fragment concentration of 1 mM was used, corresponding to a protein-to-compound ratio of 1:125.³⁶ For concentration-dependent DSF measurements, the compound concentration was varied by diluting the compounds in pure DMSO before adding to the protein solution. For time-dependent measurements, all samples were incubated at 20 °C while shaking. The T_m of T-p53C and its mutants was obtained by calculating the maxima of the first derivative of the melting curves using OriginPro2020 (OriginLab, Northampton, MA, USA). Finally, the T_m of the protein sample was subtracted from the T_m of the sample containing both protein and compound to calculate ΔT_m . All measurements were performed at least in triplicate and multiple runs were averaged according to the rules of error propagation. All DSF runs are summarized as [Table S5](#) – [Table S23](#) in the Supporting Information. In addition, the full set of DSF curves are provided as [Figure S26](#) – [Figure S39](#) and [Figure S41](#) – [Figure S49](#).

Intact Protein Mass Spectrometry

Sample preparation was performed as previously described.³¹ Therefore, the proteins in phosphate buffer (25 mM KPi pH 7.2, 150 mM NaCl, 5 mM DTT) were incubated with fragments, which were dissolved in DMSO yielding a protein-to-compound ratio of 1:100 and a final DMSO concentration of 5% [v/v]. The fragment concentration was varied by diluting the compound stock solution in DMSO before adding to the protein solution, resulting in a final DMSO concentration of 5% [v/v] and various molar ratios. The mixtures were incubated at 20 °C for 24 h while shaking at 150 rpm prior to intact protein mass analysis. The subsequent UHPLC-ESI-MS measurements, data acquisition, and data analysis were performed in accordance with the previously described procedure.^{22,31} Additional deconvoluted mass spectra are provided as [Figure S24](#) to [Figure S25](#), [Figure S40](#), and [Figure S50](#), complemented by [Table S3](#) in the Supporting Information.

Protein Crystallization

Crystallization of T-p53C-Y220C was carried out as previously described.³⁵ The T-p53C-Y220C protein (5 mg/mL) was mixed with reservoir solution (100 mM HEPES pH 7.2, 19% PEG4K, 10 mM DTT) and crystallized via the sitting drop vapor-diffusion technique and streak seeding. To generate complexes, compounds were dissolved to 5 mM and soaked for 1 day in cryo-protectant buffer (additional 20% glycerol).

Data Collection, Reduction, and Structure Refinement

Data sets were collected at the Swiss Light Source (Villingen, Switzerland) at beam lines X06SA (PXI). XDS was utilized for data processing and reduction.³⁷ Initial phases were obtained by molecular replacement using 4AGL²¹ or

8A92²² as a search model for PHASER³⁸ as part of the CCP4 suite.³⁹ Structure and phase improvements were performed by multiple cycles of manual model building in Coot⁴⁰ and structure refinement using PHENIX.⁴¹ Covalently bound fragment restraints were generated using JLigand⁴² in ACEdrg mode.⁴³ The unbiased omit-maps were generated by removing all covalently bound fragments and the entire Cys sidechain, followed by refinement with simulated annealing. All crystallographic and refinement statistics are provided as [Table S24](#) – [Table S26](#) in the Supporting Information.

Fluorescence Polarization Assay (FPA)

The FPA was performed in accordance with the previously described methodology.³¹ FL-T-p53 in phosphate buffer (25 mM KPi pH 7.2, 150 mM NaCl, 5 mM DTT, 5% [v/v] DMSO, 0.2 mg/mL BSA) was incubated with fragment (protein-to-compound ratio 1:200) for 24 h at 20 °C on a rotating shaker.³⁶ 5'-end fluorescein-labeled GADD45α DNA response element served as a probe (sequence of the forward strand: 5'-[6-FAM]-GTACAGAACATGTCTAAGCATGCTGGGGAC-3').^{36,44,45} Fluorescence polarization (FP) data were recorded on a CLARIOstar plate reader (BMG Labtech, Ortenberg, Germany) at 25 °C. Black non-binding polystyrene 96-well microplates (GBO, Frickenhausen, Germany) were utilized. Excitation and emission filters were set to 482 nm and 530 nm, respectively. The dichroic mirror was adjusted to 504 nm. During the one-hour measurement period, FP data were acquired at 10 min intervals and subsequently averaged. The normalized fluorescence polarization data were analyzed with OriginPro2020 (OriginLab, Northampton, MA, USA) according to the cooperative binding mechanism and fitted to the Hill equation with a linear drift term.^{36,46–48} Measurements were performed at least in quadruplicate and averaged according to the rules of error propagation.

Results

First Generation: Decomposition of the Initial CovLib Hits SN006 and SN007

The CovLib compounds SN006 and SN007, differing only in their leaving groups (LG), are highly reactive fragments with two electron-withdrawing nitrile groups, covalently modifying Cys220 in the hydrophobic pocket. However, due to their high reactivity, multiple cysteines in p53 were arylated. Therefore, we tried to decompose these initial hits, aiming for a simplified chemical lead structure for further development, showing attenuated reactivity and better selectivity, while maintaining or improving stabilization. Basically, we wanted to understand, which parts of SN006/7 are important for the stabilizing effect and the reactivity. [Figure 1](#) shows the tested SN006/7 SAR compounds and $t_{1/2}$ measured via GSH assay. 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 results of the DSF screen of T-p53C, the β-sandwich mutant Y220C, the DNA contact mutant R273H, the structural mutant R282W in the C-terminal helix (H2), and of the cysteine control mutant T-p53C-C124/182/277S with SN006/7 SAR compounds (1 mM) after 30 min, 4 h, and 24 h incubation time are depicted in [Tables S5–S7](#). The cysteine control mutant was designed to abolish three solvent-exposed, rather reactive cysteine residues, which are frequently found to be covalently modified.^{22,36,49,50} The DSF results of the identified hits with T-p53C and its mutants after 24 h of incubation are listed in [Table 1](#) and detailed results of the β-sandwich mutant Y220C incubated with the hits are listed [Table 2](#).

The tested fragments SN006/7-1 and SN006/7-6 had no considerable effect on the melting temperatures of T-p53C or T-p53C-Y220C 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 unreactive ($t_{1/2}$ GSH >100 h) and, therefore, did not stabilize T-p53C-Y220C.

All of the pyrazine derivatives SN006/7-2 to SN006/7-5 were identified as stabilizers by DSF. SN006/7-2 and SN006/7-4 have a halogen (chlorine or bromine, respectively) in the ortho position to the electron-withdrawing group (EWG), reducing their reactivity. No stabilization of T-p53C and its mutants was detected after only 30 min of incubation ([Table S5](#)). However, with increasing incubation time and target occupancy, the stabilizing effect increased. As shown in [Table 1](#), after 24 h of incubation, both compounds stabilized p53 and its mutants except for the triple cysteine control mutant. Nevertheless, it is unclear from the DSF results, whether covalent binding to Cys220 in the binding pocket of T-p53C-Y220C occurred, as all

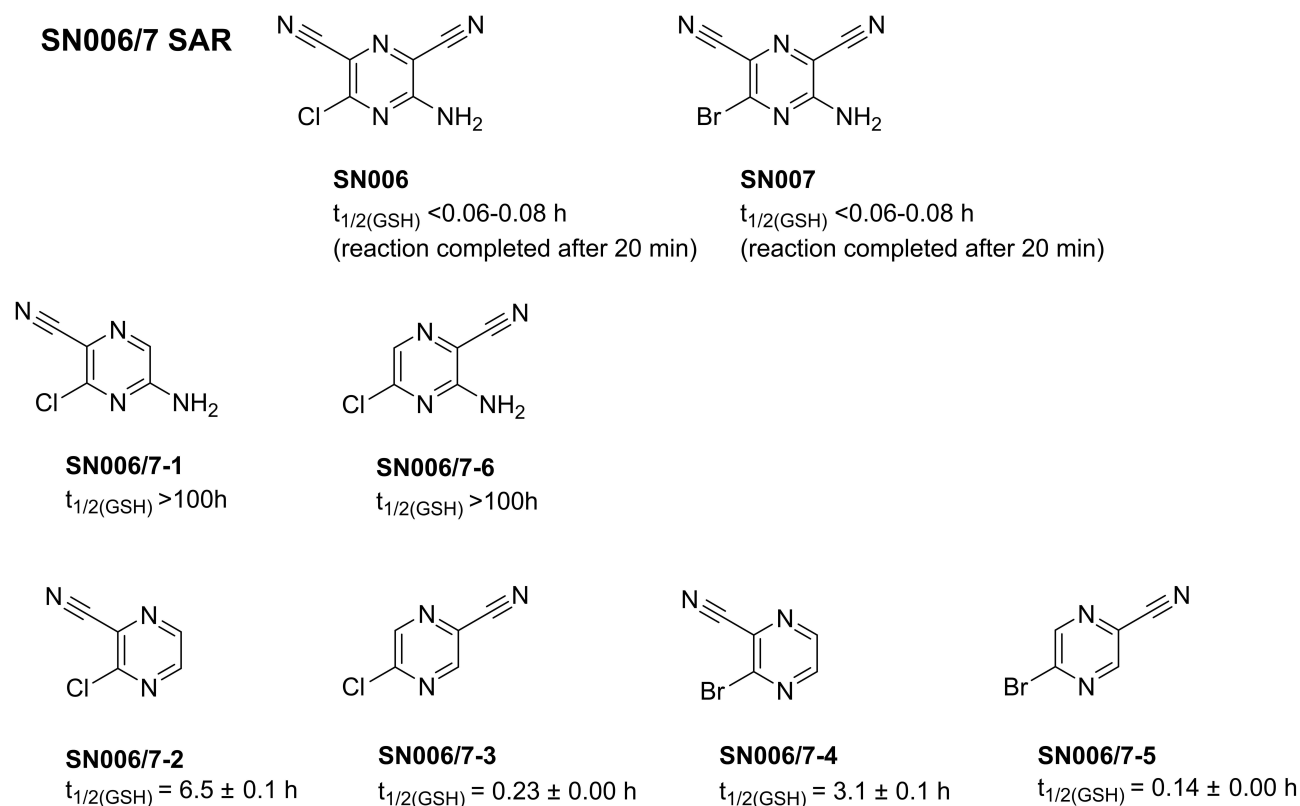


Figure 1 Overview of SN006 and SN007 SAR compounds and their $t_{1/2} \pm$ standard deviation (SD) measured via GSH assay.

mutants were stabilized. The melting curves of T-p53C supplemented with both compounds after 24 h were biphasic. The first derivatives showed two maxima (Figure S29A), corresponding to different arylated protein species with different melting points or non-covalently bound fragments with distinct melting points.^{51–53} A reversible covalent Pinner-type addition of proteinogenic nucleophiles to the nitrile group of the compounds^{33,54} is also conceivable, which could lead to differently modified protein species. Table S8 and Table 2 show that as the compound concentration decreased, the stabilization of T-p53C and T-p53C-Y220C also decreased.

Table 1 Identified Fragment Hits of the SN006/7 SAR DSF Screen and Their Impact on the T_m of T-p53C and Its Mutants After 24 h of Incubation. WT Refers to T-p53C; Mutant Labels (eg, Y220C) Indicate Mutants of T-p53C, Such as T-p53C-Y220C

Compound		WT $\Delta T_m \pm \text{SD } [^\circ\text{C}]^a$		Y220C $\Delta T_m \pm \text{SD } [^\circ\text{C}]^a$	R273H $\Delta T_m \pm \text{SD } [^\circ\text{C}]^a$	R282W $\Delta T_m \pm \text{SD } [^\circ\text{C}]^a$	C124/182/ 277S $\Delta T_m \pm \text{SD } [^\circ\text{C}]^a$
		1. Species	2. Species				
SN006/7-2		0.60 $\pm$ 0.12	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	2.55 $\pm$ 0.12	3.62 $\pm$ 0.11	0.23 $\pm$ 0.23	N/A ^b	0.55 $\pm$ 0.09

(Continued)

Table 1 (Continued).

Compound		WT $\Delta T_m \pm SD$ [°C] ^a		Y220C $\Delta T_m \pm SD$ [°C] ^a	R273H $\Delta T_m \pm SD$ [°C] ^a	R282W $\Delta T_m \pm SD$ [°C] ^a	C124/182/ 277S $\Delta T_m \pm SD$ [°C] ^a
SN006/7-4		0.65 ± 0.12	2.55 ± 0.12	2.00 ± 0.11	1.00 ± 0.09	1.85 ± 0.09	0.30 ± 0
SN006/7-5		-0.60 ± 0.12	0.30 ± 0.12	1.65 ± 0.30	-1.48 ± 0.28	N/A ^b	0.30 ± 0

Notes: ^a $\Delta T_m \pm SD$ after 24 h incubation time with a protein-to-compound ratio of 1:125 at 20 °C. Measurements were performed at least in triplicate, mean ΔT_m values $\pm$ SD are reported. ^bNot available (N/A). No evaluable melting curves and first derivatives with clearly defined melting points were obtained.

Table 2 Effect of SN006/7 SAR Hit Compounds on the T_m of T-p53C-Y220C

T-p53C-Y220C $\Delta T_m \pm SD$ [°C] ^a						
	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
SN006/7-2	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

Notes: ^a $\Delta T_m \pm SD$ of T-p53C-Y220C (8 μ M protein) with SN006/7 SAR hit compounds (1 mM, 250 μ M) after various incubation times at 20 °C. Measurements were performed at least in triplicate, mean ΔT_m values $\pm$ SD are reported.

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 (Table 2). 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 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.^{55,56} In addition, after 30 min of incubation, SN006/7-3 and SN006/7-5 exhibited a particularly pronounced stabilizing effect on the Y220C mutant (Table S5), indicating covalent modification of the mutation-induced cysteine in the crevice. Similar to the original compounds SN006 and SN007, the R282W mutant showed no suitable melting curves and first derivatives with clearly defined peaks after incubation with SN006/7-3 and SN006/7-5 (Figures S27B, S28E, and S30B). After 24 h of incubation, compound SN006/7-3, bearing a chlorine atom in para position to the EWG, strongly increased the melting temperature of T-p53C-Y220C by up to 3.62 °C (1 mM, 24 h). Moreover, 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 of 2.55 °C. Additionally, the compound slightly stabilized T-p53C-C124/182/277S by 0.55 °C (1 mM, 24 h), indicating 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 in the GSH assay compared to SN006/7-3 ($t_{1/2}$ GSH= 0.23 h), leading to hyperarylation and impairment of normal protein folding.¹⁰

Table 2 indicates that the overall stabilization of T-p53C-Y220C was reduced at 250 μ M SN006/7-3. 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 was already observed for the highly reactive CovLib compounds SN006

and SN007.³¹ The stabilization of T-p53C-Y220C by 2.65 °C after 24 h of incubation with 250 µM SN006/7-5 was comparable to the ΔT_m value of 2.87 °C measured with SN006/7-3 at the same concentration.

In summary, SN006/7-3 was identified as the best hit, primarily stabilizing the Y220C mutant. The best stabilization of T-p53C-Y220C by about 3.62 °C (1 mM, 24 h) was also more pronounced than the maximum stabilization by the original compounds SN006 (ΔT_m = 1.82 °C, 500 µM, 30 min) and SN007 (ΔT_m = 2.03 °C, 250 µM, 30 min).³¹

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 mass spectrometry (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 2](#).

The deconvoluted MS spectra of compounds SN006/7-2 ([Figure 2A](#)) and SN006/7-4 ([Figure 2C](#)) showed signals corresponding to the triple arylated protein. The highest signals 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 (ΔT_m = 1.38 °C, 1 mM, 24 h) compared to the bromine analog SN006/7-4 (ΔT_m = 2.00 °C, 1 mM, 24 h). Moreover, the 2-bromopyrazine derivative SN006/7-4 ($t_{1/2}$ GSH = 3.1 h) also had a shorter $t_{1/2}$ in GHS assay than the 2-chloropyrazine derivative SN006/7-2 ($t_{1/2}$ GSH = 6.5 h).

In the deconvoluted MS spectra of SN006/7-3 ([Figure 2B](#)) and SN006/7-5 ([Figure 2D](#)), 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 higher reactivity of SN006/7-3 and SN006/7-5, which have the π -accepting EWG in the para position.⁵⁶ Moreover, the spectrum of T-p53C-Y220C incubated with SN006/7-3 ([Figure 2B](#)) showed signals 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 2D](#)) only displayed signals 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.

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

The concentration-dependent DSF measurement with compound SN006/7-3 after 30 min of incubation ([Table 3](#) and [Figure 3A](#)) showed that the highest stabilization of T-p53C-Y220C was achieved with the highest tested compound concentration (1 mM, ΔT_m = 2.65 °C). In contrast to the original hits SN006 and SN007, a high compound concentration did not lead to a reduced stabilization of the protein. 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 (ΔT_m = 0.70 °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 1 mM compound concentration. In contrast, the SAR compound SN006/7-3 showed a characteristic concentration-dependent stabilization of T-p53C-Y220C ([Figure 3B](#)). 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 °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 °C.

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 S33](#) and [Table S10](#) show that a substantial increase in the melting temperature of T-p53C-Y220C was achieved after only 10 min of incubation (ΔT_m = 2.30 °C). This is consistent with the short

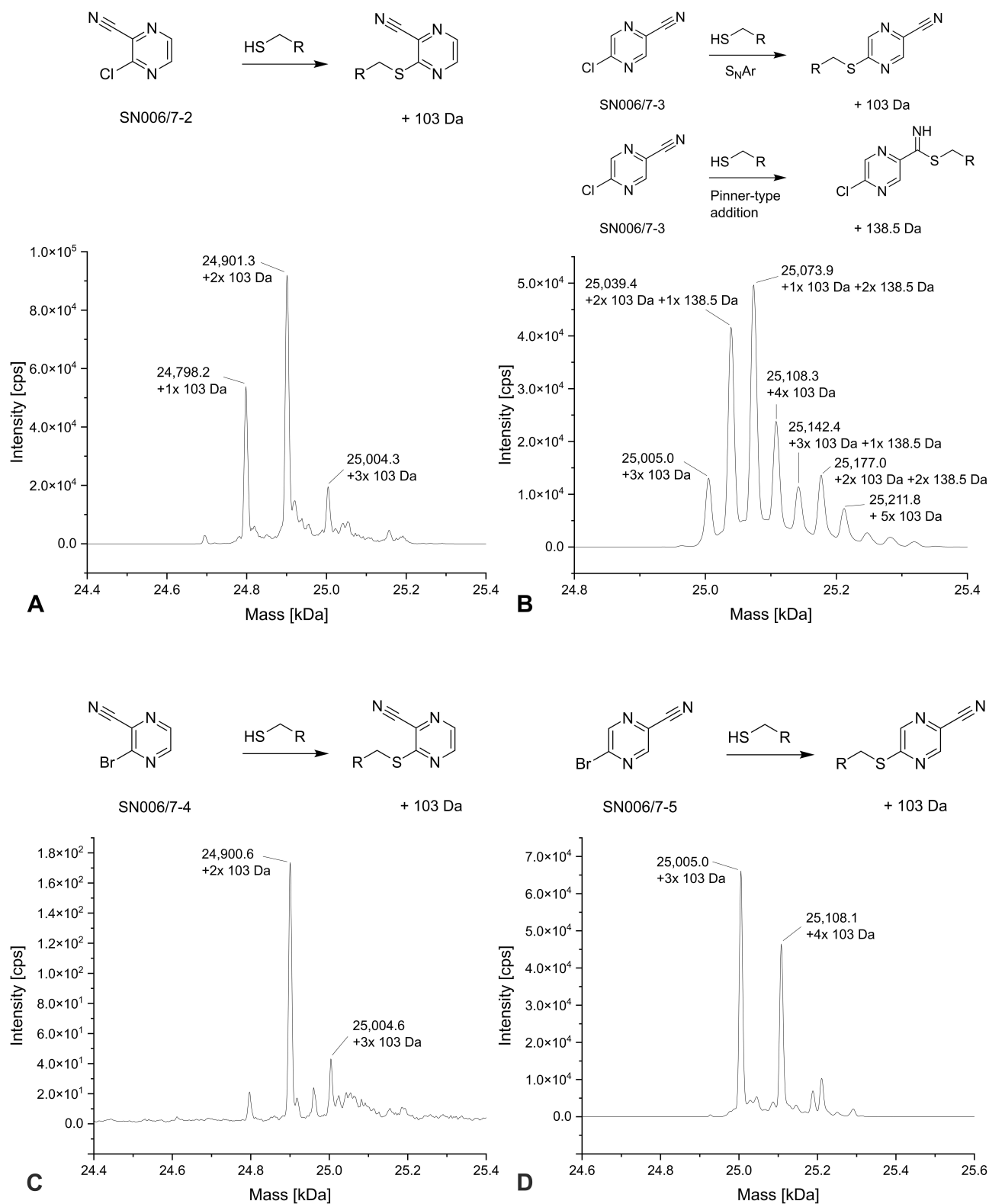


Figure 2 Deconvoluted MS spectra of T-p53C-Y220C with the identified SN006/7 SAR hits: SN006/7-2 (**A**), SN006/7-3 (**B**), SN006/7-4 (**C**), SN006/7-5 (**D**) at a protein-to-compound ratio of 1:100 after 24 h of incubation at 20 °C (theoretical mass of unmodified T-p53C-Y220C: 24,696.00 Da).

Table 3 Concentration-Dependent Effects of Compound SN006/7-3 on the T_m of T-p53C-Y220C After 30 min and 24 h Incubation Time

SN006/7-3 [μ M]	30 min $\Delta T_m \pm SD$ [$^{\circ}$ C] ^a	24 h $\Delta T_m \pm SD$ [$^{\circ}$ C] ^a
1000	2.65 $\pm$ 0.17	3.55 $\pm$ 0.14
500	1.90 $\pm$ 0.09	3.43 $\pm$ 0.08
250	0.80 $\pm$ 0.23	2.75 $\pm$ 0.20
125	0.70 $\pm$ 0.23	1.95 $\pm$ 0.08
62.5	0.30 $\pm$ 0	1.45 $\pm$ 0.10
31.25	0.20 $\pm$ 0.09	0.98 $\pm$ 0.08
15.63	-0.05 $\pm$ 0.09	0.45 $\pm$ 0.06
7.81	0.10 $\pm$ 0.09	0.20 $\pm$ 0.08
3.91	0 $\pm$ 0.15	0.18 $\pm$ 0.08
1.95	0.05 $\pm$ 0.09	0 $\pm$ 0.06
0.98	-0.05 $\pm$ 0.09	0 $\pm$ 0.06

Notes: ^a $\Delta T_m \pm SD$ of T-p53C-Y220C (8 μ M protein) after 30 min and 24 h incubation time with different concentrations of compound SN006/7-3 at 20 $^{\circ}$ C. Measurements were performed at least in triplicate, mean ΔT_m values $\pm$ SD are reported.

$t_{1/2}$ of 0.23 h for fragment SN006/7-3 in the GSH assay. After about 2 h of incubation at 20 $^{\circ}$ C, the increase in the ΔT_m values plateaued, and temperature-sensitive cancer mutant T-p53C-Y220C was maximally stabilized.

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

A cysteine light (CL) construct of T-p53C-Y220C (T-p53C-Y220C-C124/182/229/275/277S, T-p53C-Y220C-CL) was employed as a control in the DSF and intact protein MS analysis of SN006/7-3. Given the limited number of competitively accessible cysteine residues in the cysteine light construct, we sought to conduct a more detailed investigation into the possible arylation of SN006/7-3 at Cys220 in the hydrophobic pocket.³⁰

The DSF results with the Y220C cysteine light mutant are depicted in [Figure S34](#) and [Table 4](#). SN006/7-3 stabilized T-p53C-Y220C-CL by up to 2.40 $^{\circ}$ C (1 mM, 24 h). At a compound concentration of 250 μ M, the stabilization decreased, as previously observed for T-p53C-Y220C. However, the stabilization of T-p53C-Y220C-CL was lower compared to T-p53C-Y220C (ΔT_m = 3.62 $^{\circ}$ C, 1 mM, 24 h), indicating that the labeling of cysteines other than Cys220 also has a stabilizing effect. Additionally, SN006/7-3 caused a smaller increase in the T_m of T-p53C-Y220C-CL than original compound SN006 (ΔT_m = 3.05 $^{\circ}$ C, 1 mM, 24 h).³¹ However, the deconvoluted MS spectrum of T-p53C-Y220C-CL with SN006 showed peaks with more than one fragment attached to the protein.³¹ As depicted in [Figure 4](#), in the deconvoluted MS spectrum of the Y220C cysteine light mutant with the SAR compound SN006/7-3, one signal corresponded to the protein species with one molecule attached via S_NAr , while the higher signal corresponded to protein with one molecule 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.

SN006/7-3 Adopts an Alternative Binding Pose in the Y220C Induced Pocket

The X-ray structure of T-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 5](#). 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 cysteines by SN006/7-3 via S_NAr was detected. The 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 the pyrazine derivative SN006/7-3 reacted with Cys220 via an S_NAr reaction with displacement of the chlorine atom.

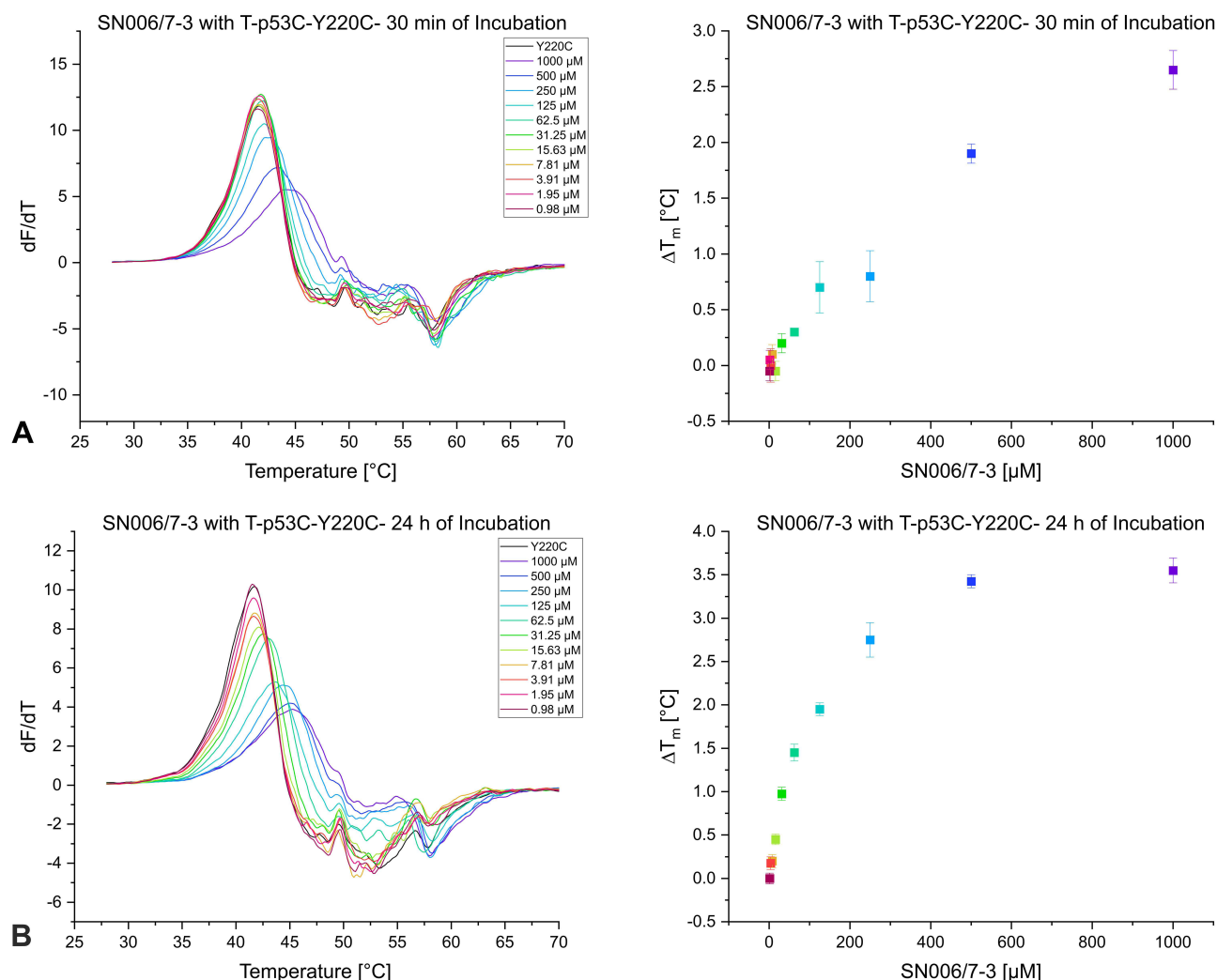


Figure 3 Concentration-dependent DSF measurements of T-p53C-Y220C with SN006/7-3. The first derivatives of the melting curves and resulting ΔT_m values of T-p53C-Y220C (8 μ M protein) with various concentrations of compound SN006/7-3 after 30 min (A) and 24 h (B) of incubation at 20 °C are shown.

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 and SN006. The CovLib hits SN001 and SN006 adopted a Tyr-like conformation, occupying the central cavity and mimicking the WT Tyr220. Their electron-withdrawing nitrile groups pointed towards Thr150 and subsite 1. The meta-fluorine (SN001) or meta-nitrile moiety (SN006) pointed towards subsite 3. However, subsite 2 of the Y220C binding pocket was not occupied by SN001 and SN006.³¹ In contrast, as shown in Figure 5, the pyrazine derivative SN006/7-3 was positioned deeply in subsite 2 of the Y220C

Table 4 Effect of Compound SN006/7-3 on the T_m of T-p53C-Y220C-CL

T-p53C-Y220C-CL $\Delta T_m \pm SD$ [°C] ^a						
	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 $\pm$ 0.10	1.80 $\pm$ 0	2.40 $\pm$ 0.12	0.20 $\pm$ 0.09	0.55 $\pm$ 0.17	1.25 $\pm$ 0

Notes: ^a $\Delta T_m \pm SD$ of T-p53C-Y220C-CL (8 μ M protein) with SN006/7-3 (1 mM, 250 μ M) after various incubation times at 20 °C. Measurements were performed at least in triplicate, mean ΔT_m values $\pm$ SD are reported.

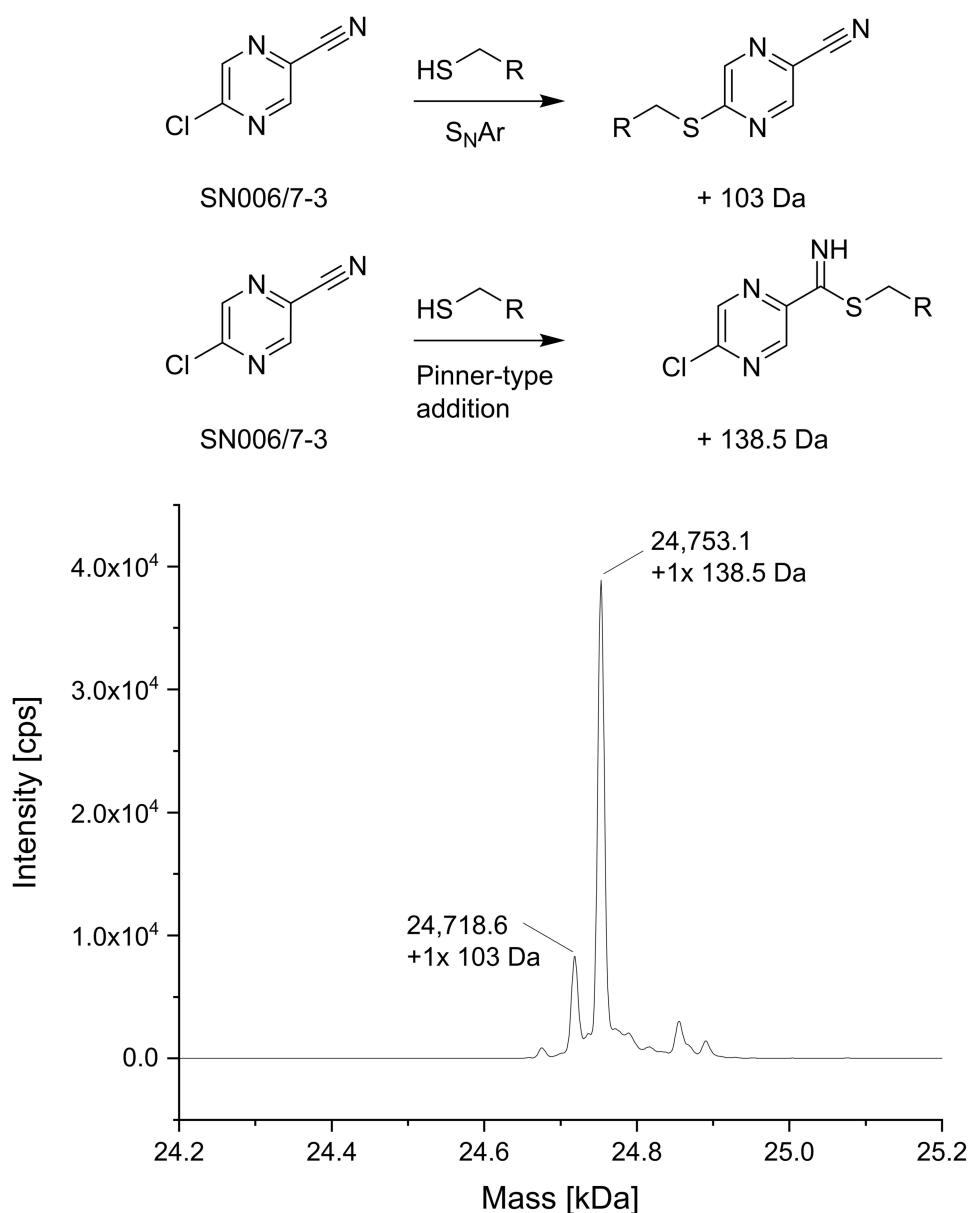


Figure 4 Deconvoluted MS spectrum of T-p53C-Y220C-CL with SN006/7-3 at a molar ratio of 1:100 after 24 h of incubation at 20 °C (theoretical mass of unmodified T-p53C-Y220C-CL: 24,615.68 Da).

binding pocket. Its electron-withdrawing nitrile group occupied subsite 2 of the mutational crevice. Nevertheless, subsite 1 and subsite 3 were not targeted by compound SN006/7-3.

Second Generation: Scaffold Hopping Based on SN006/7-3

The identified S_NAr reactive compound SN006/7-3, arylating Cys220 and occupying subsite 2 of the Y220C pocket, is still very reactive. Therefore, additional SAR fragments, which contain electron-donating methyl groups at the heteroarenes (Figure 6) were screened by DSF and intact protein MS. Since the previously identified cleft binders SN001, SN006, and SN006/7-3 are pyridine or pyrazine derivatives, additional six-membered heterocycles such as pyrimidines and pyridazines were tested.

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 SN006/7-3. In comparison to SN006/7-3 (*t*_{1/2} GSH= 0.23 h), the

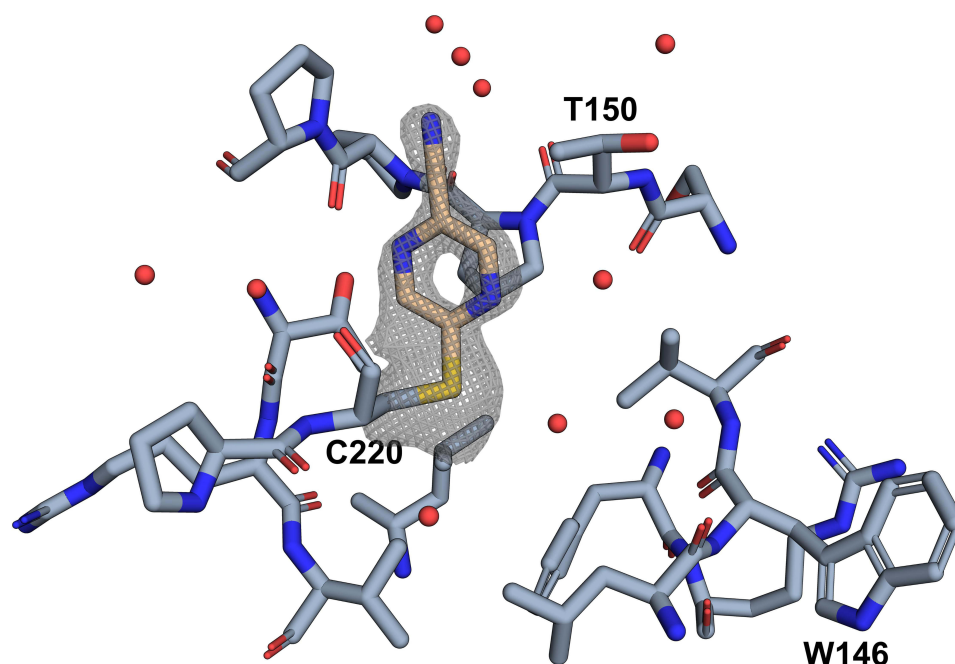


Figure 5 Overview of the binding mode of SN006/7-3 (soaked at 5 mM, PDB: 9G5H) into the Y220C induced crevice. The pyrazine derivative SN006/7-3 reacted with Cys220 via an S_NAr reaction. The compound adopts an alternative conformation, occupying subsite 2 of the binding pocket. The unbiased omit map is shown at a contour level of 3 σ .

SN006/7-3 SAR

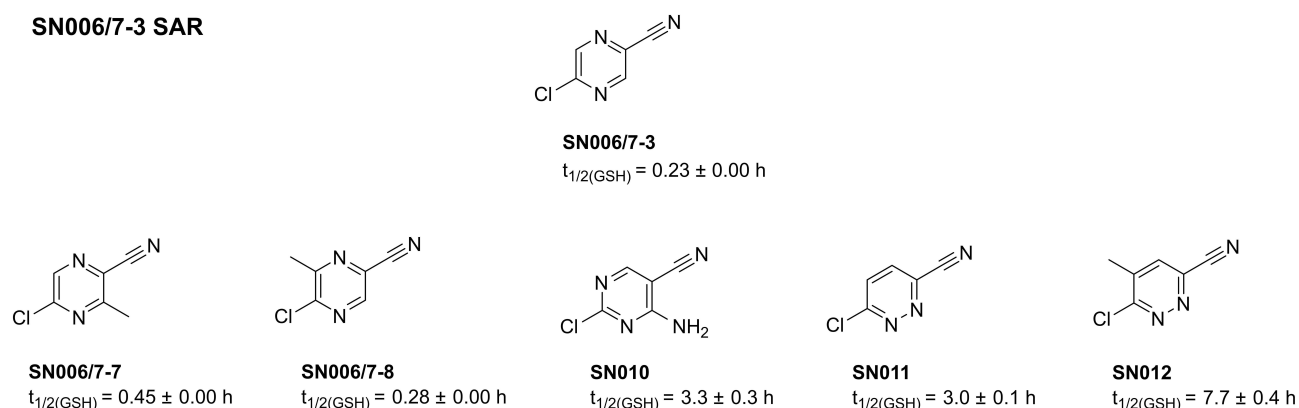


Figure 6 Overview of SN006/7-3 SAR compounds and their $t_{1/2} \pm \text{SD}$ measured via GSH assay.

GSH reactivity of SN006/7-7 ($t_{1/2} \text{ GSH} = 0.45 \text{ h}$) slightly diminished, whereas that of SN006/7-8 ($t_{1/2} \text{ GSH} = 0.28 \text{ h}$) was only minimally affected. Both compounds particularly stabilized T-p53C-Y220C, indicating cleft binding (Table 5). 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 S11). However, SN006/7-8 had a smaller stabilizing effect on T-p53C-Y220C, with a ΔT_m of 1.71 °C after 30 min (Table 6), 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 ($\Delta T_m = 1.28 \text{ °C}$, 1 mM) and SN006/7-8 ($\Delta T_m = 4.43 \text{ °C}$, 1 mM) (Table S12). 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 (Figure S36A, Figure S37A). 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 5 and Table 6). The introduction of a methyl group in the ortho

Table 5 Impact of the SN006/7-3 SAR Compounds on the T_m of T-p53C and Its Mutants After 24 h of Incubation. WT Refers to T-p53C; Mutant Labels (eg, Y220C) Indicate Mutants of T-p53C, Such as T-p53C-Y220C

Compound		WT $\Delta T_m \pm SD$ [°C] ^a		Y220C $\Delta T_m \pm SD$ [°C] ^a	R273H $\Delta T_m \pm SD$ [°C] ^a	R282W $\Delta T_m \pm SD$ [°C] ^a	C124/I82/ 277S $\Delta T_m \pm SD$ [°C] ^a
		1. Species	2. Species				
SN006/7-7		0.87 ± 0.09 ^b		2.08 ± 0.12	0.20 ± 0.17	N/A ^c	0.45 ± 0.12
SN006/7-8		1.03 ± 0.10	2.90 ± 0.17	5.02 ± 0.10	1.15 ± 0.12	N/A ^c	0.70 ± 0.09
SN010		0.82 ± 0.08 ^b		0.65 ± 0.23	1.20 ± 0.12	2.00 ± 0.09	0.85 ± 0.09
SN011		0.75 ± 0.08		1.10 ± 0.17	1.10 ± 0.09	1.70 ± 0.09	0.70 ± 0.09
SN012		0.55 ± 0.09		0.55 ± 0.19	0.40 ± 0.12	1.55 ± 0.09	0.55 ± 0.17

Notes: ^a $\Delta T_m \pm SD$ after 24 h incubation time with a protein-to-compound ratio of 1:125 at 20 °C. Measurements were performed at least in triplicate, mean ΔT_m values $\pm$ SD are reported. ^bMelting curves with two transitions, first derivatives of the melting curves showed an additional second shoulder. ^cNo evaluable melting curves and first derivatives with clearly defined melting points were obtained.

Table 6 Effect of SN006/7-3 SAR Compounds on the T_m of T-p53C-Y220C

T-p53C-Y220C $\Delta T_m \pm SD$ [°C] ^a						
	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
SN006/7-7	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
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

Notes: ^a $\Delta T_m \pm SD$ of T-p53C-Y220C (8 μ M protein) with SN006/7-3 SAR hit compounds (1 mM, 250 μ M) after various incubation times at 20 °C. Measurements were performed at least in triplicate, mean ΔT_m values $\pm$ SD are reported.

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 with the methyl group in the meta position to the LG resulted in a considerably lower stabilization of

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 S14 and Table 6). As the compound concentration decreased, the stabilization of T-p53C and T-p53C-Y220C also decreased. The highest increase in the melting temperatures was achieved with 1 mM SN006/7-7 and SN006/7-8 after a 24-hour incubation period. However, even at a compound concentration of 250 μ M, SN006/7-8 showed a higher stabilization of T-p53C-Y220C ($\Delta T_m = 4.45$ °C, 24 h) compared to SN006/7-7 ($\Delta T_m = 1.10$ °C, 24 h) and SN006/7-3 ($\Delta T_m = 2.87$ °C, 24 h).

The 2-chloropyrimidine SN010 caused a thermal stabilization of p53 and its mutants in the DSF experiment. 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. A pronounced stabilization of T-p53C-Y220C and binding in the Y220C pocket cannot be deduced from the DSF data for the 2-chloropyrimidine SN010. This discovery aligns with previous findings on pyrimidines substituted at position 2, such as the 2-sulfonylpyrimidine PK11000³⁶ or the 2-chloropyrimidine 4482.²² These compounds achieved general stabilization of p53, but did not bind into the Y220C pocket.

After 24 h of incubation, both pyridazine derivatives, SN011 and SN012, moderately stabilized all tested mutants within a similar range. Compared to the pyrazine analog SN006/7-3 ($\Delta T_m = 3.62$ °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 °C (1 mM, 24 h).

Overall, the results showed that the introduction of a methyl group, 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 a high stabilization of the cancer mutant Y220C and selective binding into the mutational cleft.

Intact protein MS confirmed covalent modification of T-p53C-Y220C by all tested SN006/7-3 SAR compounds (Figure S40). The deconvoluted MS spectra of T-p53C-Y220C with the pyrazine derivatives SN006/7-7 and SN006/7-8 are depicted in Figure 7. 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. Moreover, in the deconvoluted MS spectrum of SN006/7-7 (Figure 7A) with T-p53C-Y220C, an additional small signal corresponding to the two times arylated protein was evident. The number of modifications was reduced compared to the original fragment SN006/7-3 (Figure 2B). Furthermore, the deconvoluted

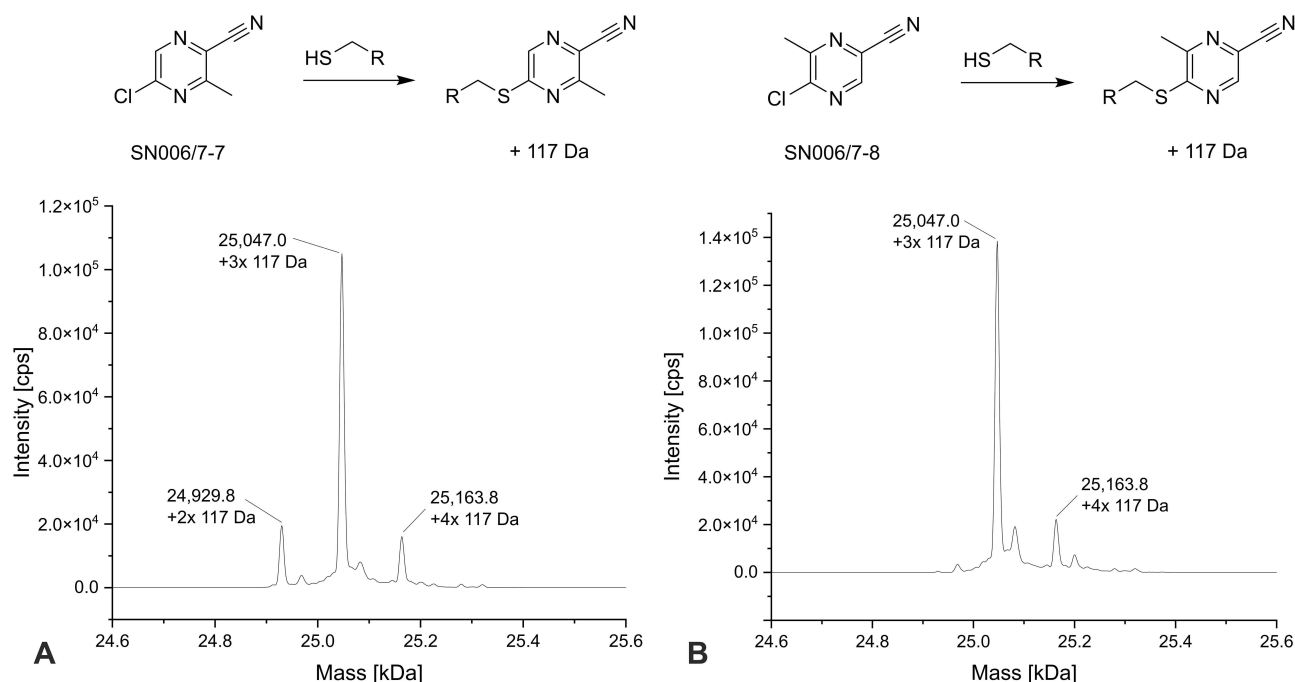


Figure 7 Deconvoluted MS spectra of T-p53C-Y220C with SN006/7-7 (A) and SN006/7-8 (B) at a protein-to-compound ratio of 1:100 after 24 h of incubation at 20 °C (theoretical mass of unmodified T-p53C-Y220C: 24,696.00 Da).

MS spectrum of SN006/7-3 showed numerous signals where the protein was modified by reversible covalent addition to the nitrile group. In contrast, only signals for the protein arylated via S_NAr were detectable in the spectra with the methylated SAR compounds SN006/7-7 and SN006/7-8.

SN006/7-8 Induced a Time and Concentration-Dependent Pronounced Stabilization of the Cancer Mutant T-p53C-Y220C

SN006/7-8 showed a distinct stabilization of the Y220C mutant up to a lowest effective compound concentration of 250 μM after only 30 min of incubation (Figure 8 and Table 7), in contrast to its analog SN006/7-7 (Figure S41 and Table S16). Nevertheless, the maximum stabilization achieved by fragment SN006/7-8 ($\Delta T_m = 1.83^\circ\text{C}$, 1 mM) after 30 min was lower than that of the original fragment SN006/7-3 ($\Delta T_m = 2.65^\circ\text{C}$, 1 mM, Table 3). Additionally, SN006/7-3 stabilized up to a lowest effective compound concentration of 125 μM after 30 min of incubation. After 24 h incubation time, the stabilization of T-p53C-Y220C by compound SN006/7-8 with 4.98°C (1mM) was much more pronounced than by fragments SN006/7-3 ($\Delta T_m = 3.55^\circ\text{C}$, 1 mM, Table 3) and SN006/7-7 ($\Delta T_m = 2.15^\circ\text{C}$, 1 mM, Table S16). Even at concentrations of 500 μM and 250 μM , compound SN006/7-8 achieved substantially higher ΔT_m values than SN006/7-3. 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

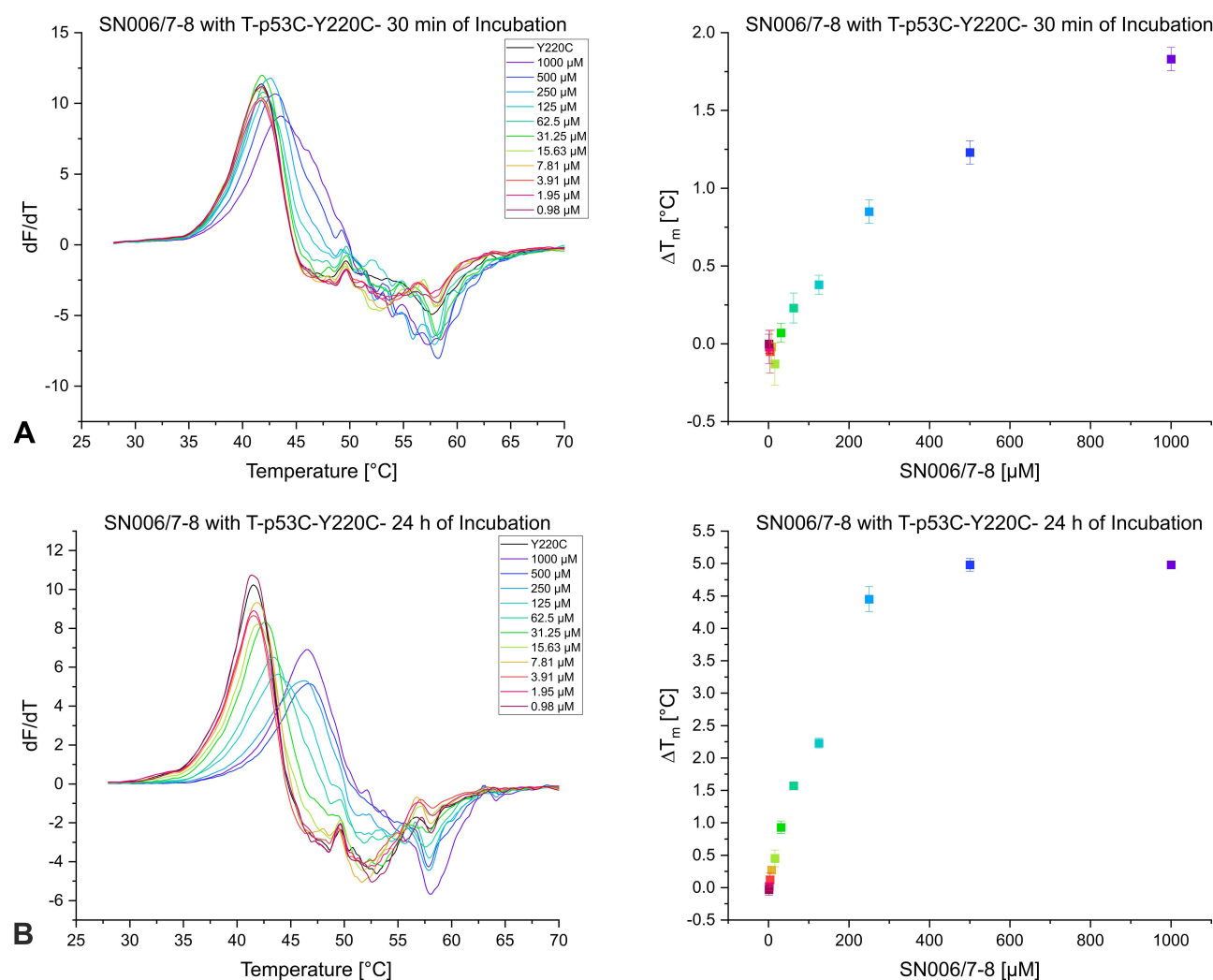


Figure 8 Concentration-dependent DSF measurements of T-p53C-Y220C with SN006/7-8. The first derivatives of the melting curves and resulting ΔT_m values of T-p53C-Y220C (8 μM protein) with various concentrations of compound SN006/7-8 after 30 min (A) and 24 h (B) of incubation at 20°C are shown.

Table 7 Concentration-Dependent Effects of Compound SN006/7-8 on the T_m of T-p53C-Y220C After 30 min and 24 h Incubation Time

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

Notes: ^a $\Delta T_m \pm SD$ of T-p53C-Y220C (8 μ M protein) after 30 min and 24 h incubation time with different concentrations of compound SN006/7-8 at 20 $^{\circ}$ C. Measurements were performed at least in triplicate, mean ΔT_m values $\pm$ SD are reported.

markedly 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 S43](#) and [Table S18](#), SN006/7-8 stabilized T-p53C-Y220C by up to 1.43 $^{\circ}$ 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 S42](#) and [Table S17](#)), whereas with SN006/7-3 ([Figure S33](#) and [Table S10](#)) a higher stabilization of T-p53C-Y220C and thus target occupancy was already achieved at shorter incubation times.

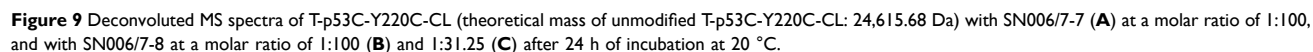
Evaluation of SN006/7-7 and SN006/7-8 with T-p53C-Y220C-CL

The identified T-p53C-Y220C stabilizing hits SN006/7-7 and SN006/7-8 were further investigated with the Y220C cysteine light mutant ([Figure S44](#), [Table 8](#) and [Figure 9](#)). SN006/7-8 increased the T_m of the Y220C cysteine light mutant by up to 3.68 $^{\circ}$ C (1 mM, 24 h). As previously noted for T-p53C-Y220C, the fragment SN006/7-8, which has a methyl group in the ortho position to the LG, resulted in a higher stabilization of T-p53C-Y220C-CL compared to its constitutional isomer SN006/7-7 (ΔT_m = 1.50 $^{\circ}$ C, 1 mM, 24 h) and SN006/7-3 (ΔT_m = 2.40 $^{\circ}$ C, 1 mM, 24h). In addition, a comparably high stabilization of T-p53C-Y220C-CL was measured after 24 h of incubation with 1 mM (ΔT_m = 3.68 $^{\circ}$ C) and 250 μ M (ΔT_m = 3.58 $^{\circ}$ 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 (ΔT_m = 5.02 $^{\circ}$ C, 1 mM, 24 h), indicating that the modification of cysteines besides Cys220 also has a stabilizing effect.

Table 8 Effect of SN006/7-7 and SN006/7-8 on the T_m of T-p53C-Y220C-CL

T-p53C-Y220C-CL $\Delta T_m \pm SD$ [$^{\circ}$ C] ^a						
	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
SN006/7-7	0.40 $\pm$ 0.32	0.75 $\pm$ 0	1.50 $\pm$ 0.10	0 $\pm$ 0	0.20 $\pm$ 0.09	0.63 $\pm$ 0.08
SN006/7-8	0.40 $\pm$ 0.09 ^b	4.10 $\pm$ 0.09	3.68 $\pm$ 0.21	0.10 $\pm$ 0.09	0.50 $\pm$ 0.09 ^c	3.58 $\pm$ 0.10

Notes: ^a $\Delta T_m \pm SD$ of T-p53C-Y220C-CL (8 μ M protein) with SN006/7-7 and SN006/7-8 (1 mM, 250 μ M) after various incubation times at 20 $^{\circ}$ C. Measurements were performed at least in triplicate, mean ΔT_m values $\pm$ SD are reported. ^bMelting curves with two transitions, first derivatives of the melting curves showed a second shoulder. ^cFirst derivatives of the melting curves displayed a broad curve.

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arylated protein. At a ratio of 1:31.25, an additional small signal was present for the native Y220C cysteine light protein (Figure 9C).

In summary, the DSF and intact protein MS results with the Y220C cysteine light mutant indicated that SN006/7-8, with a methyl group ortho 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.

SN006/7-8 Adopts a Tyr-Like Conformation in the Y220C Induced Crevice

The covalent modification of Cys220 by compound SN006/7-8 was confirmed using X-ray crystallography, as depicted in Figure 10. 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 derivative SN001 and the pyrazine derivative SN006.³¹ Fragment SN006/7-8 reacted with Cys220 via an S_NAr reaction with the chlorine atom as the LG. It occupied most of the space of the central cavity, mimicking the WT Tyr220. 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 PK7088²⁶ and MB710²⁷ 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.

Third Generation: Structure-Activity Relationship Screening of the Identified Hit SN006/7-8

The promising fragment SN006/7-8 with improved stabilization of T-p53C-Y220C 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 eg, 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 11.

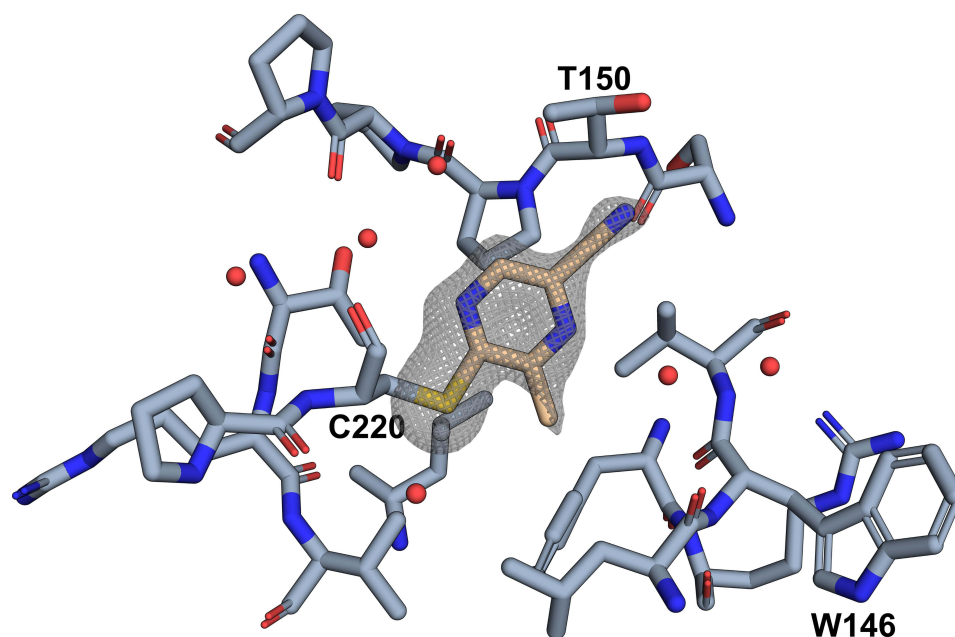
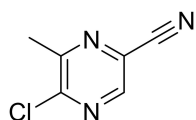


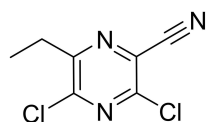
Figure 10 Overview of the binding mode of SN006/7-8 (soaked at 5 mM, PDB 9G6T) into the Y220C induced crevice. The compound binds similarly to SN001 and SN006 in a Tyr-like conformation, with the nitrile pointing towards subsite 1 and Thr150.³¹ The meta-methyl group points into the hydrophobic subsite 3. The unbiased omit map is shown at a contour level of 3 σ .

SN006/7-8 SAR



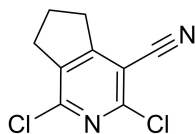
SN006/7-8

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



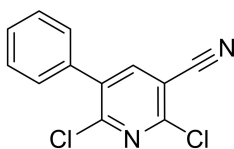
SN006/7-9

$$t_{1/2}(\text{GSH}) < 0.083 \text{ h}^a$$



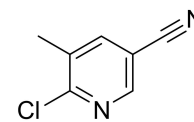
SN006/7-10

$$t_{1/2}(\text{GSH}) > 100 \text{ h}$$



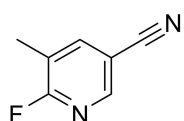
SN006/7-11

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



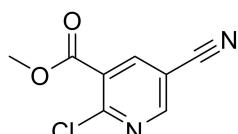
SN006/7-12

$$t_{1/2}(\text{GSH}) > 100 \text{ h}$$



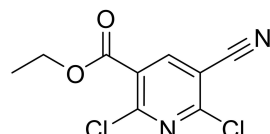
SN006/7-13

$$t_{1/2}(\text{GSH}) = 47 \pm 7 \text{ h}^c$$



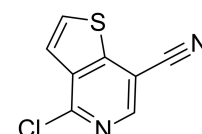
SN006/7-14

$$t_{1/2}(\text{GSH}) = 3.3 \pm 0.1 \text{ h}$$



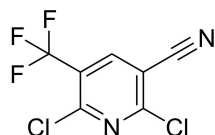
SN006/7-16

$$t_{1/2}(\text{GSH}) = 0.066 \pm 0.000 \text{ h}$$



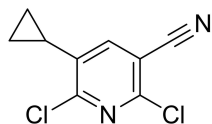
SN006/7-17

$$t_{1/2}(\text{GSH}) = 35 \pm 2 \text{ h}$$



SN006/7-18

$$t_{1/2}(\text{GSH}) = 0.088 \pm 0.001 \text{ h}$$



SN006/7-19

$$t_{1/2}(\text{GSH}) = 46 \pm 2 \text{ h}$$

Figure 11 Overview of the tested SN006/7-8 SAR compounds and their $t_{1/2} \pm \text{SD}$ determined via GSH assay. ^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.

The pyrazine fragment 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 $t_{1/2} \ll 0.083 \text{ h}$ in the GSH assay. Incubation with 1 mM SN006/7-9 for 30 min markedly lowered the T_m of T-p53C ($\Delta T_m = -4.85^\circ \text{C}$), T-p53C-Y220C ($\Delta T_m = -1.30^\circ \text{C}$), and T-p53-C124/182/277S ($\Delta T_m = -2.45^\circ \text{C}$) (Table S19). However, the decrease 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 S45). Furthermore, for all tested p53 mutants, no suitable melting curves were retained after 4 h and 24 h of incubation, or a destabilization of the triple cysteine control mutant occurred (Table 9). Fragment SN006/7-9 was also tested at a compound concentration of 250 μM with T-p53C and T-p53C-Y220C, as shown in Table S22 and Table 10. Nevertheless, at this concentration, fragment SN006/7-9 only stabilized the 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.

Table 9 Impact of the Identified SN006/7-8 SAR Hits on the T_m of T-p53C and Its Mutants After 24 h of Incubation. WT Refers to T-p53C; Mutant Labels (eg, Y220C) Indicate Mutants of T-p53C, Such as T-p53C-Y220C

Compound		WT $\Delta T_m \pm SD$ [°C] ^a		Y220C $\Delta T_m \pm SD$ [°C] ^a	R273H $\Delta T_m \pm SD$ [°C] ^a	R282W $\Delta T_m \pm SD$ [°C] ^a	C124/I82/ 277S $\Delta T_m \pm SD$ [°C] ^a
		1. Species	2. Species				
SN006/7-9		N/A ^b		N/A ^b	N/A ^b	N/A ^b	-2.55 ± 0
SN006/7-14		0.97 ± 0.11	2.80 ± 0.14	1.18 ± 0.13	0.95 ± 0.09	1.10 ± 0.35	0.65 ± 0.09
SN006/7-16		0.20 ± 0.38		2.30 ± 0.25	N/A ^b	N/A ^b	0.95 ± 0.09
SN006/7-18		-1.33 ± 0.16		2.25 ± 0.24	-1.55 ± 0.24	N/A ^b	-1.00 ± 0.09

Notes: ^a $\Delta T_m \pm SD$ after 24 h incubation time with a protein-to-compound ratio of 1:125 at 20 °C. Measurements were performed at least in triplicate, mean ΔT_m values $\pm$ SD are reported. ^bNo evaluable melting curves and first derivatives with clearly defined melting point were obtained.

Table 10 Effect of SN006/7-8 SAR Hits on the T_m of T-p53C-Y220C

T-p53C-Y220C $\Delta T_m \pm SD$ [°C] ^a						
	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
SN006/7-9	-1.30 ± 0.24	N/A ^b	N/A ^b	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
SN006/7-16	-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

Notes: ^a $\Delta T_m \pm SD$ of T-p53C-Y220C (8 μ M protein) with SN006/7-8 SAR hit compounds (1 mM, 250 μ M) after various incubation times at 20 °C. Measurements were performed at least in triplicate, mean ΔT_m values $\pm$ SD are reported. ^bNo evaluable melting curves and first derivatives with clearly defined melting points were obtained.

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. The pyridines with an electron-withdrawing nitrile group and a halogen as the LG are less reactive or chemically stable and not suitable for covalent modification and stabilization of p53. 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 ring, did not substantially influence the T_m of T-p53C, T-p53C-Y220C, and all tested mutants (Tables S19–S21).

Compound SN006/7-14 with an additional electron-withdrawing methoxycarbonyl group at the pyridine increased the T_m of all tested p53 mutants after 24 h incubation time at a concentration of 1 mM (Table 9). Nevertheless, as shown in

Table 10, no substantial stabilization of T-p53C-Y220C was observed at a concentration of 250 μ M. Compared to fragment SN006/7-12 ($t_{1/2}$ GSH >100 h), the introduction of a second EWG in the ortho position to the LG in fragment SN006/7-14 ($t_{1/2}$ GSH = 3.3 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 an additional chlorine atom are highly reactive. Both compounds particularly stabilized T-p53C-Y220C, suggesting a covalent modification of Cys220 (**Table 9**). After 24 h of incubation, the T_m of T-p53C-Y220C was increased by up to 2.57 $^{\circ}\text{C}$ with 250 μ M SN006/7-16 and by up to 3.00 $^{\circ}\text{C}$ with 250 μ M SN006/7-18.

The intact protein MS results emphasize that the hits SN006/7-9, SN006/7-14, SN006/7-16, and SN006/7-18 multiply modified T-p53C-Y220C after 24 h of incubation at a protein-to-compound ratio of 1:100 (**Figure S50**).

Overall, the SAR compound screen identified either less reactive or unreactive 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 screen indicates that for the development of more T-p53C-Y220C selective SAR compounds, the scaffold of SN006/7-8 is necessary with electron-donating groups (EDGs) other than the methyl group ortho to the LG.

The Highly Reactive Fragment SN006/7-9 Also Adopts a Tyr-Like Conformation in the Mutational Cleft

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 12**, compound SN006/7-9 reacted with Cys220 via an $\text{S}_{\text{N}}\text{Ar}$ reaction with the displacement of the para-chlorine. In chain A (**Figure 12A**), similar to fragments SN001, SN006, and SN006/7-8, the compound SN006/7-9 also adopted a Tyr-like conformation, occupying the central cavity space.³¹ Its electron-withdrawing nitrile moiety pointed towards subsite 1 and Thr150, and the chlorine atom in ortho position 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 targeted.

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

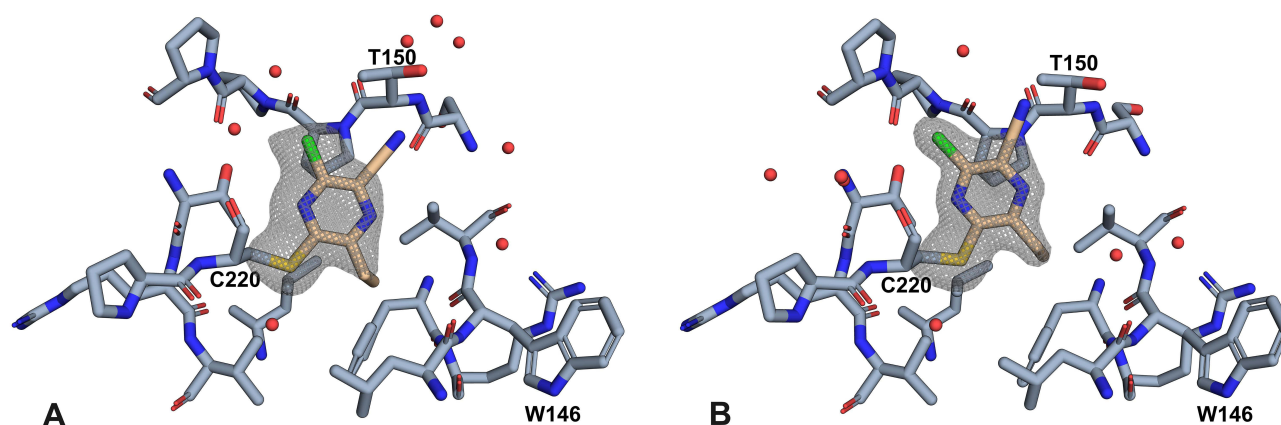


Figure 12 Overview of the binding mode of SN006/7-9 (soaked at 5 mM, PDB 9G6U) into the Y220C induced crevice. In chain A, the compound adopts a Tyr-like conformation, with the nitrile pointing towards subsite 1 and Thr150. The ortho-chlorine points towards subsite 2, and the meta-ethyl group points into the hydrophobic subsite 3 (**A**). In chain B, the binding pose of SN006/7-9 in the Y220C pocket is slightly altered, with SN006/7-9 oriented more towards subsite 2 (**B**). The unbiased omit maps are shown at a contour level of 3 σ .

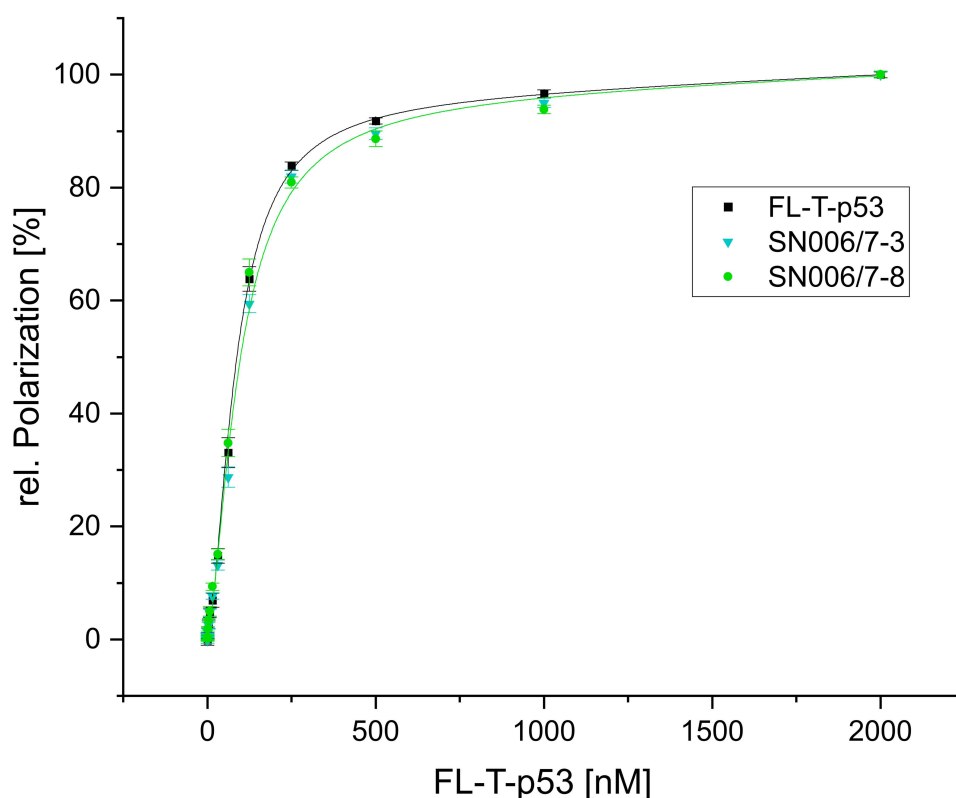


Figure 13 Relative fluorescence polarization data [%] of FL-T-p53 with 20 nM 5'-carboxyfluorescein-labeled GADD45 α response element and 5% DMSO.³⁶ FL-T-p53 was incubated with compounds SN006/7-3 and SN006/7-8 at a protein-to-compound ratio of 1:200 for 24 h at 20 °C.

The Identified Fragment Hits SN006/7-3 and SN006/7-8 Did Not Alter the DNA Binding Capacity of Full-Length p53

The effect of the SN006/7-3 and its methylated analog SN006/7-8 on the DNA binding affinity of full-length (FL)-T-p53 to its non-apoptotic response element GADD45 α was tested by FPA. Incubation of 2 h with the two original CovLib fragments SN006 and SN007 negatively affected the DNA binding of FL-T-p53 to GADD45 α .³¹ However, after 24 h of incubation with SN006/7-3 and SN006/7-8 at a high molar ratio of 1:200, the DNA-binding of FL-T-p53 to GADD45 α was maintained. The dissociation constant (K_d) for FL-T-p53 binding to GADD45 α was only minimally increased from 83 nM without compound to 95 nM with SN006/7-3 and was perfectly conserved with a K_d of 84 nM after incubation with SN006/7-8 (Figure 13 and Table 11). The FPA results indicate that despite the binding of SN006/7-3 and SN006/7-8 to cysteines in p53 and especially to Cys277 in the DNA binding region of p53,⁴ regular DNA binding of FL-T-p53 is maintained. Moreover, the FPA outcomes suggest that normal transcription of p53 response elements is possible due to the thermal stabilization in cellular systems.

Table 11 Resulting K_d Values $\pm$ SD of the Direct Titrations of FL-T-p53 With or Without the Addition of SN006/7-3 or SN006/7-8 With GADD45 α . The DMSO Column Indicates the Reference Measurement Without Adding Compound

	DMSO	SN006/7-3	SN006/7-8
$K_d \pm SD$ [nM] ^a	83 $\pm$ 4	95 $\pm$ 9	84 $\pm$ 11

Notes: ^aMeasurements were performed at least one time in quadruplicate.

Conclusion

In summary, we herein employed an “electrophile first approach”,⁵⁷ screening SAR fragments of the previously identified highly reactive CovLib hits SN006 and SN007 with the challenging target T-p53C-Y220C. We found a set of S_NAr reactive fragments, targeting Cys220 in the hydrophobic pocket of T-p53C-Y220C and stabilizing the thermosensitive cancer mutant. Moreover, the discovered fragments showed different binding poses and occupied various subsites of the Y220C binding pocket. All identified fragments, covalently addressing Cys220 in the binding pocket of T-p53C-Y220C, are pyrazines with an electron-withdrawing CN group and a halogen as the LG in para position to the EWG. Variations in the heteroarene did not lead to more Cys220 selective hits. 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).

The 5-chloro-pyrazine-2-carbonitrile (SN006/7-3) increased the T_m of T-p53C-Y220C by up to 3.62 °C. SN006/7-3 exhibited multiple modifications of the Y220C mutant in the intact protein MS experiment. However, arylation of the 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 and the pyrazine CovLib hit SN006.³¹ The compound occupied subsite 2 of the binding pocket.

Interestingly, compound SN006/7-8, with an electron-donating methyl group in the ortho position 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 introduction of a methyl group in the ortho position to the LG increased the stabilizing effect of this scaffold compared to SN006/7-3 ($\Delta T_m = 3.62$ °C, 1 mM, 24 h) without a methyl group and SN006/7-7 ($\Delta T_m = 2.08$ °C, 1 mM, 24 h) with a methyl group in the meta position. Covalent modification of Cys220 by compound SN006/7-8 was confirmed by X-ray crystallography. 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 derivative SN001 and the pyrazine derivative SN006.³¹ The methyl group at position 6 of the pyrazine ring of SN006/7-8 protruded into the hydrophobic subsite 3.

To increase 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 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.

We have demonstrated that after incubation with SN006/7-3 and SN006/7-8 FL-T-p53 retains its potential to bind the GADD45a response element. However, in vivo experiments are essential to fully investigate the complexity of signaling and downstream effect activation.

In conclusion, all identified fragments are still highly reactive but represent a promising basis for optimization in a subsequent drug discovery process. To reduce the reactivity and increase the selectivity for the mutation-induced Y220C pocket with non-covalent interactions, the identified fragments can be merged, linked, and grown.^{58–60}

Abbreviations

AUC, area under the curve; CL, cysteine light (C124/182/229/275/277S); CovLib, covalent fragment library; DSF, differential scanning fluorimetry; EDG, electron-donating group; EWG, electron-withdrawing group; FBDD, fragment-based drug discovery; FL, full-length; FP, fluorescence polarization; FPA, fluorescence polarization assay; GSH, glutathione; HPLC, high-performance liquid chromatography; K_d , dissociation constant; LG, leaving group; MDM2, mouse double minute 2 homolog; MDM4, mouse double minute 4 homolog; MS, mass spectrometry; N/A, not available; PBS, phosphate buffered saline; SAR, structure-activity relationship; SD, standard deviation; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; S_NAr , nucleophilic aromatic substitution; $t_{1/2}$, half-life; T_m , melting temperature; T-p53C, quadrupole thermostable mutant of the p53 core-domain, 94–312, M133L/V203A/N239Y/N268D; UHPLC-ESI-MS, ultra-high-performance liquid chromatography electrospray ionization mass spectrometry; WT, wild-type.

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Author Contributions

All authors made a significant contribution to the work reported: F.M.B. envisioned the research. T.K. and F.M.B. conceptualized the experiments and designed the study. T.K. prepared the proteins by heterologous expression and performed the DSF studies. TK prepared the MS samples. S.J.J., B.M., C.K., and M.L. performed the UHPLC-ESI-MS experiments. S.J.J., B.M., C.K., T.K., and M.L. analyzed the UHPLC-ESI-MS experiments. Crystallographic data reduction and refinement was performed by J.S. and T.S. FPA experiments were performed by T.K. M.S. performed the Glutathione Assay. T.K. conducted data analysis and reprocessing of the experimental data. T.K., J.S., and F.M.B. wrote the manuscript. S.J.J., B.M., C.K., M.L., M.S., and T.S. critically reviewed the manuscript. All authors have agreed on the journal to which the article has been submitted. All authors agreed on all versions of the article at any stage of the submission, revision, publication and proofing process, particularly including the final version accepted for publication. All authors agree to take responsibility and be accountable for the contents of the article.

Disclosure

The authors report no conflicts of interest in this work.

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