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Authors
Guillory, Xavier
Wolter, Madita
Leysen, Seppe
et al.

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Fragment-based Differential Targeting of PPI Stabilizer Interfaces

Xavier Guillory, Madita Wolter, Seppe Leysen, João Filipe Neves, Ave Kuusk, Sylvia Genet, Bente Somsen, John Kenneth Morrow, Emma Rivers, Lotte van Beek, Joe Patel, Robert Goodnow, Heike Schoenherr, Nathan Fuller, Qing Cao, Richard G. Doveston, Luc Brunsveld, Michelle R. Arkin, Paola Castaldi, Helen Boyd, Isabelle Landrieu,* Hongming Chen,* and Christian Ottmann*

ABSTRACT: Stabilization of protein–protein interactions (PPIs) holds great potential for therapeutic agents, as illustrated by the successful drugs rapamycin and lenalidomide. However, how such interface-binding molecules can be created in a rational, bottom-up manner is a largely unanswered question. We report here how a fragment-based approach can be used to identify chemical starting points for the development of small-molecule stabilizers that differentiate between two different PPI interfaces of the adapter protein 14-3-3. The fragments discriminately bind to the interface of 14-3-3 with the recognition motif of either the tumor suppressor protein p53 or the oncogenic transcription factor TAZ. This X-ray crystallography driven study shows that the rim of the interface of individual 14-3-3 complexes can be targeted in a differential manner with fragments that represent promising starting points for the development of specific 14-3-3 PPI stabilizers.

INTRODUCTION

PPI modulation is one of the most promising and active fields in current drug discovery and chemical biology. However, PPI modulation is in many instances understood synonymously with PPI inhibition, disregarding the complementary strategy of stabilizing PPIs. This is despite the fact that a multitude of natural products and a growing number of synthetic compounds validate the feasibility and value of small-molecule PPI stabilization. Stabilizers of PPIs display a number of advantages like their uncompetitive nature and their potential for higher specificity. They bind to composite pockets at the interface of their target protein complexes that naturally can show a much higher structural variability compared for example to active sites of enzymes or ligand binding pockets of receptors. Consequently, the potential to develop selective PPI-stabilizing compounds might be higher than with ligands that bind to conventional drug targets. An especially interesting target for PPI modulation is the adapter protein 14-3-3. This “hub” protein, consisting of a family of seven isoforms, interacts with several hundred partner proteins and regulates the activity of many disease-related proteins like C-Raf (cancer), Tau (Alzheimer’s), and CFTR (cystic fibrosis).

Here, we focused on the interaction of 14-3-3 with the transcriptional coactivator TAZ and the tumor suppressor protein p53. TAZ interacts with 14-3-3 via a motif surrounding S89, which, as part of the Hippo signaling pathway, is phosphorylated by LATS1/2, resulting in cytoplasmic retention of TAZ and thus its functional deactivation. Loss of this regulation leads to abrogation of contact inhibition, which is one of the prerequisites of cancer formation and growth of solid tumors. Binding of 14-3-3 to p53, following phosphorylation of T387 of p53 in response to DNA damage, is protective against MDM2-mediated degradation. In addition, 14-3-3 binding to p53 facilitates p53 dimer–dimer interaction and stabilization of the functional p53 tetramer.
14-3-3 thus plays an important role in maintaining the tumor suppressor role of p53 by stimulating p53’s transcriptional activity. Enhancing the binding of 14-3-3 to these two cancer-related proteins with a small-molecule 14-3-3 PPI stabilizer could convey a positive therapeutic effect in many cancer types.

Importantly, the potential “druggability” of 14-3-3 proteins has been validated by nature in the form of the natural products class of the fusicoccanes. These diterpene glycosides have been shown to stabilize a number of 14-3-3 PPIs and display biological activities that might be of therapeutic benefit in cancer,\textsuperscript{5,16−20} cystic fibrosis,\textsuperscript{10,17,21} and axon regeneration.\textsuperscript{22} The eponymous molecule, the fungal toxin fusicoccin A (FC-A), was shown to stabilize the interaction of 14-3-3 proteins with the regulatory C-terminus of the plant plasma ATPase, thereby activating this important proton pump.\textsuperscript{23} Later it was shown that the related compound cotylenin A displays a remarkable anticancer activity while showing a very low general toxicity.\textsuperscript{24} One possible target for this activity was suggested by us to be the negative regulatory complex between the N-terminus of the oncogenic kinase C-Raf and 14-3-3 proteins.\textsuperscript{7} The crystal structure of cotylenin A in complex with 14-3-3\textsubscript{ζ} and the diphospho peptide C-Raf\textsuperscript{pS233pS259} showed that the natural product binds to a gap in the interface pocket simultaneously, establishing contacts to both 14-3-3 and the C-Raf-derived peptide. Recently, an optimized semisynthetic fusicoccane was shown to display enhanced antiproliferative activity against cancer cells.\textsuperscript{25} Furthermore, we reported that the natural product FC-A displayed a beneficial effect for a potential therapy of breast cancer and cystic fibrosis by stabilizing the inhibitory interaction of 14-3-3 with estrogen receptor α\textsuperscript{26} or the chloride channel CFTR.\textsuperscript{11} Although the fusicoccanes are very useful proof-of-principle molecules, their optimization is not without synthetic challenges. Therefore, additional approaches to identify starting points for the development of PPI stabilizers are needed. Because traditional high throughput-screening (HTS) were so far rewarded with only limited success,\textsuperscript{27} alternative methods like fragment-based drug discovery (FBDD) holds the most promise to achieve this goal.

In this study, we report the identification of fragments that bind to the distinct interfaces of 14-3-3 with the partner proteins p53 and TAZ, hinting at the possibility to introduce specificity at an early stage of fragment-based optimization of 14-3-3 PPI stabilizing ligands.

**RESULTS**

Screening for Fragments That Bind to the Interface of 14-3-3 with TAZ and p53. For the identification of interface-binding fragments that might be developed into PPI-stabilizing compounds, we employed a crystallography-based screen of fragment cocktails.\textsuperscript{28} Here, crystals of the binary 14-3-3 complexes were soaked in solutions of fragment mixtures (100 cocktails of five each, part of the X-ray crystallography subset of the AstraZeneca fragments collection) at a final concentration of 10 mM per fragment. This screen identified two scaffolds that bound to a shallow pocket that is located in the central binding channel of 14-3-3. Both fragment scaffolds

![Figure 1. Identification of fragments binding to the interface of 14-3-3 with p53. (A) Fragment AZ-001. (B) Crystal structure of AZ-001 (yellow sticks) in complex with 14-3-3\textsubscript{σ} (white surface) and p53\textsuperscript{pT387} (orange sticks), PDB 6S40. The final 2F\textsubscript{o} − F\textsubscript{c} electron density maps are shown as blue mesh. (C) Detailed view of the binding pocket of AZ-001. The most prominent interaction is a salt-bridge (dotted black line) between the amidine of AZ-001 and carboxyl group of E14 of 14-3-3. (D) Fragment AZ-002. (E) Crystal structure of AZ-002 (yellow sticks) in complex with 14-3-3\textsubscript{σ} (white surface) and p53\textsuperscript{pT387} (orange sticks), PDB 6RWI. The final 2F\textsubscript{o} − F\textsubscript{c} electron density maps are shown as blue mesh. (F) Detailed view of the binding pocket of AZ-002. Also with AZ-002, the most prominent interaction is a salt-bridge (dotted black line) between the amidine of AZ-002 and carboxyl group of E14 of 14-3-3.](https://dx.doi.org/10.1021/acs.jmedchem.9b01942)
contain an amidine group (Figure 1A,D) that engages in a salt-bridge with the side chain carboxyl group of glutamic acid 14 (E14) of 14-3-3 (Figure 1C,F). The planar aromatic ring systems sit in a shallow pocket that is comprised of 14-3-3 residues E39, N42, and L43 (Figure 1C). The first fragment (AZ-001) can adopt two orientations with the chloride substituent either pointing toward or away from the peptide-binding channel of 14-3-3. The second fragment (AZ-002) shows a more defined orientation, with the benzyl substituent of the thiophene ring pointing away from the peptide, resting on an uncharged cushion comprised by the hydrocarbon part of C38 and E39 of helix 3 of 14-3-3. The second fragment (AZ-002) shows a more defined orientation, with the benzyl substituent of the thiophene ring pointing away from the peptide, resting on an uncharged cushion comprised by the hydrocarbon part of C38 and E39 of helix 3 of 14-3-3. Structural analogues of AZ-002, namely AZ-003 and AZ-004, were additionally selected from the screen and observed to bind to 14-3-3 in the presence of the p53pT387 but not contacting the peptide (Supporting Information (SI), Figures S1−S2).

**AZ-003 Interacts with Both 14-3-3 and TAZ and Induces a Conformational Change in the TAZpS89 Peptide.** In contrast to the 14-3-3/p53pT387 complex, the binding site of AZ-003 lies directly at the interface of 14-3-3 with the TAZpS89 peptide (Figure 2A,B). When this fragment was soaked into crystals of the binary complex, we observed a significant change in the conformation of the TAZ peptide (Figure 2C,D). Most obvious is the additional electron density at the C-terminus of the peptide, which allowed us to build three additional amino acids (L96-T98). Second, a peptide flip is observed between L94 and Q95 which allows the main chain carbonyl oxygen of Q95 to establish a polar contact with the amine group of AZ-003 (Figure 2D). Finally, the carbonyl oxygen of L96 is interacting in the same manner with this amine substituent. It is likely that these interactions are the reason for the observed changes in the conformation of the TAZ peptide because the similar structural analogue of AZ-004 does not invoke comparable structural differences (SI, Figure S3). Complementary protein-based NMR experiments allowed us to confirm the binding site of AZ-003 to 14-3-3σ and to the 14-3-3σ/TAZpS89 complex in solution (SI, Figures S4, S5). Further characterization of the effect of AZ-003 on the binary complex using fluorescence polarization (SI, Figure S6) revealed an increase in anisotropy in the control experiment (no protein), a sign of interferences which make this assay unsuitable for this particular fragment. Even though a stabilizing activity could not be confirmed by FP for AZ-003, based on the observed conformational change induced by the newly formed interactions with two residues of TAZpS89, we believe that fragment AZ-003 represents a promising chemical starting point that could be developed into a more potent...
orthosteric stabilizer, for example, by modulating the phenyl ring attached to the thiophene C4 position.

**Preliminary Fragment Extension Confers Selectivity toward 14-3-3σ in Complex with p53pT387 over TAZpS89.** On the basis of the structural data presented, fragments were designed and custom synthesized (SI, Scheme S1−S4), leading to the identification of fragments (Figure 3A) that were compatible with binding to the 14-3-3σ/p53pT387 complex, but not to the 14-3-3σ/TAZpS89 complex. Several structural features of the 14-3-3σ/p53pT387 complex have to be taken into consideration for the prospect of achieving specificity in stabilization. First, the sharp U-turn that the peptide is showing C-terminal of E388 is enabled by the consecutive presence of both a glycine (G389) and a proline (P390). This architecture is further supported by the unique feature that the entire protein ends three amino acids further, with the sequence DSD where both the side chain of the ultimate aspartate and the free C-terminus engage the guanidinium group of R60 in a salt bridge. Together with the rather unusual glutamate at position +1 (E388) after the phosphorylated threonine (pT387), this makes quite a unique interface (Figure 1B,E), which might allow for the development of a specific orthosteric 14-3-3σ/p53pT387 PPI stabilizer. With these requirements in mind, synthesized fragments AZ-005, AZ-006, and AZ-007 (Figure 3A) represent useful chemical starting points for structural optimizations toward 14-3-3σ/p53pT387 PPI stabilizers. The crystal structures of these fragments soaked into binary crystals of 14-3-3σ/p53pT387 showed that the nitrogen-bearing extensions of the core scaffold point toward the p53 peptide and reach into the water network between 14-3-3σ and p53 (Figure 3C). Because the TAZ peptide occupies the entire length of the 14-3-3 binding channel, these binding modes are expected to be incompatible with the presence of the TAZ peptide (SI, Figure S7). Consequently, we could not identify additional electron density after soaking these fragments into 14-3-3σ/TAZpS89 crystals, with the exception of AZ-006, which can form a disulfide bond with C38 and is therefore able to compete with TAZpS89 for binding to 14-3-3, resulting in a clear loss of electron density of the TAZ peptide, but of parts of the fragment, too (SI, Figure S8).

**AZ-008 Binds to and Shows Weak Stabilization of the 14-3-3σ/p53pT387 Complex.** For the goal of developing an orthosteric PPI stabilizer of the 14-3-3σ/p53pT387 complex, it seems necessary that an extended fragment based on AZ-005−AZ-007 has to reach the p53 peptide and preferentially engage the side chain of E388. These considerations led to the design of AZ-008 (Figure 4A), which in a docking calculation is predicted to contact the carboxyl group of E388 with its terminal amino group (P390). We tried to soak this compound into preformed 14-3-3σ/p53pT387 crystals and also undertook extensive cocrystallization trials, but unfortunately we were not able to detect any additional electron density by X-ray crystallography for compound AZ-008. We thus used WaterLOGSY NMR experiments to detect the potential interaction of AZ-008 to 14-3-3σ. Positive 1H signals of the ligand in the presence of the protein are indicative of binding. Positive NMR signals in the WaterLOGSY spectra of AZ-008, both in the presence or absence of the p53pT387 peptide, confirmed the interactions (SI, Figure S9). The two-dimensional spectrum (1H−15N TROSY-HSQC) of 14-3-3σ (15N2H labeled) was next used as reporter to further characterize the binding. Because the resonances in this spectrum are sensitive to the chemical environment of the
corresponding residues, a modification in the presence of a ligand of a resonance chemical shift value, and/or intensity, reports an interaction. In addition, chemical shift assignments of 14-3-3σ have been performed, allowing to link a resonance in the spectrum to a specific amino acid residue in the 14-3-3σ sequence. The addition of AZ-008 to 14-3-3σ or 14-3-3σ in the presence of the p53pT387 peptide resulted in detectable broadening (intensity decrease) and chemical shift value perturbation (CSP) of a few resonances in the 1H−15N 2D spectrum of the protein (SI, Figures S10−S11, Figures 4C,D, 5A). On the basis of the signal assignments, we concluded that addition of AZ-008 affected mainly the intensity of resonances of residues R41, N42, E115, and F119 of 14-3-3σ (Figure 4C,D, SI, Figure S11A,B). On the basis of the signal assignments, we concluded that addition of AZ-008 affected mainly the intensity of resonances of residues R41, N42, E115, and F119 of 14-3-3σ (Figure 4C,D, SI, Figure S11A,B), with no variation in the AZ-008 binding pocket mapping in the presence of the p53pT387 peptide (Figures 4D and 5A, and SI, Figure S10). These experiments indicated that binding of AZ-008 occurs in the same pocket in both cases, with or without the p53pT387 peptide. Visualizing the corresponding amino acid perturbation induced by AZ-008 on the 14-3-3σ structure clearly highlighted a binding site (Figure 4C), which can be matched to data from the co-crystal structure of 14-3-3σ and p53pT387 in the presence of its analogues AZ-001/002 (Figure 1). The binding pose resulting from docking AZ-008 to the 14-3-3σ/p53pT387 complex (Figure 4B) is in accordance with both the NMR data and the bound-conformation adopted by other AZ fragments based on this scaffold.

Interestingly, addition of AZ-008 also induced chemical shift perturbations of the 1H NMR signals of the bound peptide (Figure 5B, SI, Figure S12). In particular, the 1H signals (Figure 5B) of the Hδ methyl protons of L383 of the p53pT387 peptide are affected by AZ-008 binding despite the fact that they are not close to the fragment binding site. Perturbation of these signals thus did not correspond to a direct effect due to the local change of environment. This data rather suggests that the binding of the fragment impacted the global binding of the peptide to 14-3-3σ. Moreover, the extent of decrease of the intensity of specific resonances in the 2D spectrum of 14-3-3σ in the presence of AZ-008 reports on the amount of complex present in solution. The intensity of resonances corresponding to amino acid residues located in, or close to, the p53pT387 binding site specifically decreased with the addition of AZ-008 to a solution containing the 14-3-3σ/p53pT387 complex (Figure 5C,D) and, reversely, the extent of CSP increased (SI, Figure S11C). Combined with the results of other methods, this observation indicates that the amount of 14-3-3σ/p53pT387 complex present in solution was increased when both ligands were present, suggesting that the complex was therefore stabilized in the presence of AZ-008. Further experiments using fluorescence polarization (Figure 5E, SI, Figure S13) and surface plasmon resonance (Figure 5F, SI, Figure S14) also suggested that AZ-008 is able to stabilize the
interaction between 14-3-3σ and a p53pT387 peptide (32mer) containing the complete C-terminal domain. The enthalpically favorable contact between the amino substituent of AZ-008 and E388 of p53pT387 observed in the docking complex described above helps to explain the observed effect. Although this effect is objectively small, it appears to be tangible as it was confirmed quantitatively by two separate methods, but also qualitatively by NMR. Moreover, the 2-fold effect observed here is in accordance with the change in activity that small, noncovalent molecular fragments are expected to induce. Most importantly, these results hint at the possibility of transforming inactive fragments initially identified in X-ray crystallography and/or NMR studies into active molecules through rational design of structural extensions.

### DISCUSSION AND CONCLUSIONS

Targeted stabilization of PPIs is an underexplored concept in drug discovery despite numerous examples from natural products and synthetic molecules advocating for this principal strategy.2,3,30 Successful drugs that operate by stabilizing PPIs have almost exclusively only been recognized as PPI stabilizers after their therapeutic relevance was shown and in many cases even long after FDA approval. One famous example of such drugs is thalidomide and its derivatives, for example, lenalidomide,31 which are collectively called IMiDs (immuno-modulatory drugs). Lenalidomide acts as a "molecular glue" that hijacks a ubiquitin ligase for the targeted degradation of a neosubstrate protein.32 Another example is the history of the immunosuppressants rapamycin and FK506 which puzzled the...
community for a long time, how these natural products actually convey their therapeutic effect. Eventually it was shown that although they initially bind to the same primary receptor protein, FKBP12, their actual functional targets are the phosphatase calcineurin (FKS06) and the protein kinase mTOR (rapamycin) that were “glued” to FKBP12 by the natural products and thus inactivated.33,34

A third, recent success story of the PPI induction and stabilization concept is proteolysis targeting chimeras (PRO-TACs), a technology employing heterobifunctional small molecules that facilitate the interaction of a target protein with a ubiquitin E3 ligase.35 This approach has not only attracted a lot of attention in the chemical biology community for a long time, how these natural products actually convey their therapeutic effect. Eventually it was shown that although they initially bind to the same primary receptor protein, FKBP12, their actual functional targets are the phosphatase calcineurin (FKS06) and the protein kinase mTOR (rapamycin) that were “glued” to FKBP12 by the natural products and thus inactivated.33,34

As our example has shown, specific and fragment-driven success examples of IMiDs and “molecular glue” natural products, PRO-TACs may define a new conceptional era of pharmacologic intervention where an enhancement or induction of a native regulatory process (targeted protein degradation) instead of a direct protein interaction, is the region where orthosteric PPI stabilizers are currently explored by many pharma companies and at least half a dozen start-up companies. Together with the aforementioned, rather serendipity-driven success examples of IMiDs and once that the natural product class of the fusicocaines can be promoted. We and others have shown in the past that binding to 14-3-3 protein complexes can be identified.43,44 and 1 mer6700 crystals: 14-3-3 ΔC/p53pT387 crystals: 14-3-3 ΔC and acetylated p53T387 peptide, both in crystallization buffer (25 mM HEPES pH 7.5, 100 mM NaCl, 10 mM MgCl2 and 2 mM β-mercaptoethanol), were combined in a 1:2 14-3-3ΔC/TAZpS89 molar ratio with a final protein concentration of 12 μL/mL. After overnight complexation, hanging-drop crystallization wells were setup using a 1:1 ratio of precipitation buffer (95 mM HEPES pH 7.1, 0.19 mM CaCl2, 5% glycerol, and 27% PEG400) and complexation mixture for a total drop volume of 2 μL. Crystals were grown within 10 days at 4 °C.

14-3-3 ΔC/p53T387 crystals: 14-3-3 ΔC and acetylated p53T387 peptide, both in crystallization buffer (25 mM HEPES pH 7.5, 100 mM NaCl, 10 mM MgCl2, and 2 mM β-mercaptoethanol), were combined in a 1:2 14-3-3ΔC/TAZpS89 molar ratio with a final protein concentration of 12 μL/mL. After overnight complexation, hanging-drop crystallization wells were setup using a 1:1 ratio of precipitation buffer (95 mM HEPES pH 7.1, 0.19 mM CaCl2, 5% glycerol, and 27% PEG400) and complexation mixture for a total drop volume of 2 μL. Crystals were grown within 10 days at 4 °C.

The compounds screened were part of the X-ray crystallography subset of the AstraZeneca fragments collection. Fragment soaking was performed on crystals of 10 days and older by adding 0.2 μL of a 100 mM stock solution in dimethyl sulfoxide to the 2 μL drop for a final concentration of 20 μM. Crystals were flash-cooled in liquid nitrogen.

Data sets were collected either on an in-house Rigaku Micromax-003 (Rigaku Europe, Kemings Sevenoaks, UK) sealed tube X-ray source and a Dectris Pilatus 200 K detector (DECTRIS Ltd., Baden-Daettwil, Switzerland) or at the DESY PETRA III synchrotron beamline P11 (DESY, Hamburg, Germany). All data sets were indexed, integrated, and scaled using either iMosflm or DIALS.
followed by AIMLESS. Phasing was performed using molecular replacement in Molrep using PDBe PDB SN75 (14-3-3/TAZ) or SMC H (14-3-3/TAZ) as search model. Phaser, Coot, and phenix.refine were thereafter used in alternating cycles of model building and refinement.

**Production of 15N-H Labeled 14-3-3ΔC for NMR Spectroscopy.** The 15N-H labeled 14-3-3ΔC (ΔC, cleaved after T23) for NMR studies was expressed in *Echerichia coli* BL21 (DE3) cells transformed with a pReOExHbt vector carrying the cDNA to express an N-terminally His6-tagged human 14-3-3ΔC. Briefly, the cells were grown in 1 L of deuterated M9 minimal medium supplemented with 2 g/L 13C6H5O, 1 g/L 15N ammonium chloride, 0.4 g/L Isogeo 15N12CO, 100 μg/mL ampicillin and the recombinant protein was then purified by affinity chromatography using a Ni-NTA column (GE Healthcare). The His6-tag was further cleaved by the TEV protease and the protein was further dialyzed overnight at 4 °C against NMR buffer (100 mM sodium phosphate, pH 6.8, 50 mM NaCl), concentrated, aliquoted, flash frozen, and stored at −80 °C. A detailed protocol can be found at 29

15N-H TROSY-HSQC NMR Spectroscopy. 1H-15N transverse relaxation optimized spectroscopy-heteronuclear single quantum coherence spectroscopy (TROSY-HSQC) spectra were acquired in 3 mm tubes (sample volume 200 μL) using a 900 MHz Bruker Avance Neo spectrometer, equipped with a cryoprobe. The spectra were recorded at 32 °C, in a buffer containing 100 mM sodium phosphate, 50 mM NaCl, pH 6.8, 4% (v/v) DMSO-d6, 1 mM DTT, EDTA-free protease inhibitor cocktail (Roche, Basel, Switzerland), and 10% (v/v) D2O. The experiments were recorded with 3072 complex data points in the direct dimension and 128 complex data points in the indirect dimension, with 184 scans per increment. For the evaluation of the binding of AZ-008 to the 14-3-3/TAZ complex, spectra of 15N2H labeled 14-3-3ΔC 100 μM were recorded in the presence and absence of 200 μM AZ-008, 500 μM p53ΔT387 peptide, and simultaneously 500 μM p53ΔT387 peptide and 2000 μM p53ΔT387 peptide, 500 μM AZ-008, and in the presence of 1 mM AZ-008 or 1% DMSO control. KD values were obtained by first fitting the non-normalized data using the “log(agonist) vs response (three parameters)” equation of GraphPad Prism, then taking the fitted upper plateau for data normalization, subsequently using the “Sigmoidal dose-response (variable slope)” equation with a constrain to 100% activity.

**Fluorescence Polarization (FP) Assay.** Fluorescence polarization measurements were conducted in FP-buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 0.01% TWEEEN-20, 10 μg/mL BSA). Protein titrations: dilution series of 14-3-3 were made in triplet in low binding Corning black round bottom 384-well plates in FP-buffer containing a fixed concentration of labeled 32mer peptide (10 nM TAMRA-p53) and 1% DMSO or 1 mM AZ-008. Plate was incubated at room temperature protected from light for 1 h and polarization was measured using PHERAstar (BMG Labtech) microplate reader (λex, 485 nm; λem, 535 nm) and TAMRA (λex, 535 nm; λem, 580 nm). KD values were obtained by first fitting the normalized data using the “log(agonist) vs response (three parameters)” equation of GraphPad Prism, then taking the fitted upper plateau for data normalization, subsequently using the “Sigmoidal dose-response (variable slope)” equation with a constrain to 100% activity. Compound titrations: dilution series of the fragments dissolved in DMSO were made with fixed concentration of labeled peptides (FITC-TAZ-p53:10 nM; TAMRA-p53T387:10 nM) and protein (40 nM and 10 μM 14-3-3 for TAZ and p53, respectively) (Control: DMSO concentration 1% v/v). The Corning 384-well plates (black, low volume, low binding, round-bottom) were incubated at 4°C protected from light overnight and polarization was measured using Tecan Infinite F500 plate reader (Thermo Fisher) using appropriate excitation and emission wavelength for FITC (λex, 485 nm; λem, 535 nm) and TAMRA (λex, 535 nm; λem, 580 nm).

**Synthesis and Characterization of Fragments.** General Chemistry Statement. All solvents and chemicals used were of reagent grade. Purity and characterization of compounds were established by a combination of LC−MS and NMR techniques. LC-MS spectra were obtained on a Shimadzu UFLC fitted with a Shimadzu LCMS-2020 mass spectrometer and a Waters BEH C18 (50 mm x 2.1 mm, 1.7 μm) or Shim-pack XR-ODS (50 mm x 3.0 mm, 2.2 μm) or Phenomenex Gemini-NX 3μ C18 110A (50 mm x 3.0 mm, 3 μm) column at a flow rate of 1.2 mL/min 95% A to 5% B over 2.0 min with a 0.6 min hold, where A = 0.1% formic acid or
0.05% TFA in water and B = acetonitrile. Purity was determined by UV absorption at a wavelength of 254 nm, and the mass ion was determined by electrospray ionization (ESI, Micromass Instrument). All final compounds were found to be of 95% purity or greater, as assessed by LC–MS and 1H NMR. 1H NMR spectra were recorded on a 300 or 400 MHz using Bruker spectrometers and using DMSO-d₆, CDCl₃, or CD₃OD as solvent depending on solubility. Data for 1H NMR were reported as chemical shifts (in parts per million from internal standard tetramethylsilane), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, h = heptet, m = multiplet, br = broad) and integration. Preparative high-performance liquid chromatography (HPLC) was performed on a Waters or Phenomenex column using decreasingly polar mixtures of water (containing 1% formic acid or 1% aqueous NH₄OH) and acetonitrile. Purification by flash column chromatography (FCC) was typically performed using silica gel (Merck 7734 grade), and solvent mixtures and gradients are recorded herein. All reactions were performed under nitrogen at room temperature (rt) unless otherwise stated.

### Synthesis Pathway to 4-[(2-Amino-1-methyl-ethyl)amino]-6-(sulfanylmethyl)benzothiophene-2-carboxamide (AZ-006) (Scheme 51)

**Step a:** Methyl 7-[(1-((tert-butoxycarbonyl)amino)propan-2-yl)amino]-benzothiophene-2-carboxylate (1). Xanthops (341 mg, 0.59 mmol) and Pd(dbta)₂ (270 mg, 0.30 mmol) were added to Cs₂CO₃ (2.884 g, 8.85 mmol), methyl 7-bromobenzothiophene-2-carboxylate (800 mg, 2.95 mmol), and tert-buty1(2-aminopyropyl)-carbamate (617 mg, 3.54 mmol) in toluene (20 mL) at 20 °C under nitrogen. The resulting solution was stirred at 110 °C for 4 h. The crude product was purified by flash silica chromatography, elution gradient 0–10% EtOAc in petroleum ether. Pure fractions were evaporated to dryness to afford the title compound (1000 mg, 93%) as a solid. MS (ESI) [M + H]⁺ = 404.

**Step b:** tert-Butyl N-[2-[(2-(N-Hydroxycarbamimidoyl)-6-(hydroxymethyl)benzothiophen-6-yl)methyl acetate (4)]. Silver acetate (1.639 g, 9.82 mmol) was added to 4-bromo(6-bromomethyl)benzothiophene-2-carbonitrile (2.5 g, 7.55 mmol) in DMF (12 mL) at 20 °C under nitrogen. The resulting solution was stirred at 60 °C for 1 h. The reaction mixture was filtered through Celite. The crude product was purified by flash silica chromatography, elution gradient 2–8% EtOAc in petroleum ether. Pure fractions were evaporated to dryness to afford the title compound (2.70 g, 82%) as a white solid. 1H NMR (300 MHz, DMSO-d₆) δ 4.89 (s, 2H), 7.91 (s, 1H), 8.31 (s, 1H), 8.39 (s, 1H). 4 as solvent depending on solubility. Data for MS and 1H NMR. 1H NMR spectra were recorded on a 300 or 400 MHz using Bruker spectrometers and using DMSO-d₆ or CDCl₃ as solvent.

**Step f:** 4-(tert-Butyloxybenzamido)limino)-1-methyl-ethyl)amino)-2-cyano-benzothiophen-6-yl)methyl acetate (5). Pd(dbta)₂ (0.768 g, 0.84 mmol) was added to (4-bromo-2-cyanobenzoyl)[6-(hydroxymethyl)ethyl]methyl acetate (2.6 g, 8.38 mmol), tert-butyl(2-aminopyropyl)carbamate (1.75 g, 10.06 mmol), Cs₂CO₃ (4.10X), and Xantphos (0.970 g, 1.68 mmol) in toluene (25 mL) at 25 °C under nitrogen. The resulting solution was stirred at 130 °C for 3 h. The reaction mixture was filtered using a Buchner funnel, and the solvent was removed under reduced pressure. The crude product was purified by flash silica chromatography, elution gradient 1–10% EtOAc in petroleum ether. Pure fractions were evaporated to dryness to afford the title compound (1.700 g, 50.3%) as a solid. MS (ESI) [M + H]⁺ = 404.

**Step g:** tert-Butyl N-[2-[(N-Hydroxycarbamimidoyl)-6-(hydroxymethyl)benzothiophen-4-yl]amino]proplyl]carbamate. Hydroxylamine hydrochloride (0.861 g, 12.39 mmol) was added to (4-[(1-((tert-butoxybenzamido)limino)propan-2-yl)amino]-2-cyanobenzoyl)[6-(hydroxymethyl)ethyl]methyl acetate (1.0 g, 2.48 mmol), K₂CO₃ (2.055 g, 14.87 mmol) in MeOH (20 mL) at 25 °C under nitrogen. The resulting solution was stirred at 50 °C for 1 h. The reaction mixture was filtered through Celite. The crude product was purified by flash silica chromatography, elution gradient 0–20% MeCN in water. Pure fractions were evaporated to dryness to afford the title compound (1.68 g, 50.3%) as a solid. MS (ESI) [M + H]⁺ = 395.

**Step h:** tert-Butyl N-[2-[(2-(N-hydroxycarbamimidoyl)-6-(hydroxymethyl)benzothiophen-4-yl]amino]proplyl]carbamate. Nickel (0.2 g, 3.41 mmol) was added to tert-buty1(2-(2-[(N-hydroxycarbamimidoyl)-6-(hydroxymethyl)benzoyl]limino)proplyl]carbamate. The resulting solution was stirred at 100 °C for 2 h. The reaction mixture was diluted with EtOAc (250 mL) and washed sequentially with 0.1 M HCl (100 mL × 2). The organic layer was dried over Na₂SO₄, filtered, and evaporated to afford the title compound (0.5 g, 76%) as a solid. Taken through to the next step without further purification.

**Step d:** 4-Bromo-6-(bromomethyl)benzothiophene-2-carbonitrile (3). TFA (1.098 mL, 77.74 mmol) was added to a solution of 4-bromo-6-bromobenzoyl)[6-(hydroxymethyl)benzothiophene-2-carbonitrile (7 g, 25.91 mmol) and pyridine (10.48 mL, 129.56 mmol) in DCM (30 mL). The resulting solution was stirred at 25 °C for 0.5 h. The reaction mixture was diluted with DCM (200 mL) and washed sequentially with 0.1 M HCl (100 mL × 2). The organic layer was dried over Na₂SO₄, filtered, and evaporated to afford the title compound (6 g) as a solid. Taken through to the next step without further purification.

**Step c:** 4-Bromo-6-(bromomethyl)benzothiophene-2-carbonitrile. NBS (1.853 g, 10.41 mmol) was added in one portion to 4-bromo-6-bromobenzoyl)[6-(hydroxymethyl)benzothiophene-2-carbonitrile (2.5 g, 9.92 mmol) and BPO (0.240 g, 0.99 mmol) in CCl₄ (150 mL) at 90 °C. The resulting solution was stirred at 90 °C for 2 h. The reaction mixture was diluted with EtOAc (200 mL) and washed sequentially with saturated brine (150 mL × 2). The organic layer was dried over Na₂SO₄, filtered, and evaporated to afford crude product. The crude product was purified by flash silica chromatography, elution gradient 0–2% EtOAc in petroleum ether. Pure fractions were evaporated to dryness to afford the title compound (2.70 g, 82%) as a white solid. 1H NMR (300 MHz, DMSO-d₆) δ 4.89 (s, 2H), 7.91 (s, 1H), 8.31 (s, 1H), 8.39 (s, 1H). 4 as solvent depending on solubility. Data for MS and 1H NMR. 1H NMR spectra were recorded on a 300 or 400 MHz using Bruker spectrometers and using DMSO-d₆ or CDCl₃ as solvent.

**Step e:** 4-Bromo-2-cyano-benzothiophen-6-yl)methyl Acetate (4). Silver acetate (1.639 g, 9.82 mmol) was added to 4-bromo(6-bromomethyl)benzothiophene-2-carbonitrile (2.5 g, 7.55 mmol) in DMF (12 mL) at 20 °C under nitrogen. The resulting solution was stirred at 60 °C for 1 h. The reaction mixture was filtered through Celite. The crude product was purified by flash silica chromatography, elution gradient 40–50% MeCN in water. Pure fractions were evaporated to dryness to afford the title compound (2.100 g, 90%) as a solid. 1H NMR (300 MHz, DMSO-d₆) δ 2.13 (s, 3H), 5.23 (s, 2H), 7.82 (s, 1H), 8.22 (s, 1H), 8.38 (s, 1H). 4 as solvent depending on solubility. Data for MS for the 4-aminobenzothiophene-2-carboxamide. A solution of methyl 4-bromo-6-methylbenzoyl)[6-(hydroxymethyl)benzothiophene-2-carboxylate (6.5 g, 22.79 mmol) in NH₃ in MeOH (7 M) (75 mL) was stirred at 110 °C overnight. The mixture was concentrated to obtain the crude product, which was used in next step directly.

**Step b:** 4-Bromo-6-methylbenzothiophene-2-carboxamide. A solution of methyl 4-bromo-6-methylbenzoyl)[6-(hydroxymethyl)benzothiophene-2-carboxylate (6.5 g, 22.79 mmol) in NH₃ in MeOH (7 M) (75 mL) was stirred at 110 °C overnight. The mixture was concentrated to obtain the crude product, which was used in next step directly.
at 40 °C for 1 h under an atmosphere of hydrogen. The mixture was filtered through a Celite pad, and the solvent was removed under reduced pressure. The reaction mixture was adjusted to pH 8. The crude product was purified by flash silica chromatography, elution gradient 0–10% MeOH in DCM. Pure fractions were evaporated to dryness to afford the title compound (0.450 g, 78%) as a solid. MS (ESI) [M + H]+ = 579.

**Step i:** tert-Butyl N-[4-[[tert-Butyloxy carbonyl]amino]-1-methyl-ethyl]amino]-6-(hydroxymethyl)benzothiophene-2-carboximido]carbamate (6). Boc-anhydride (0.920 mL, 3.96 mmol) was added slowly to tert-butyl (2-((2-carbamidomethyl-6-(hydroxymethyl)benzo)[b]thiophen-4-yl)amino)propyl)carbamate (0.4 g, 1.06 mmol), DMAP (0.129 g, 1.06 mmol) and triethylamine (1.841 ml, 13.21 mmol) in DMF (8 mL) at 0 °C under nitrogen. The resulting solution was stirred at 20 °C for 4 h. The reaction mixture was diluted with EtOAc (100 mL) and washed sequentially with saturated brine (20 mL x 3). The organic layer was dried over NaSO4, filtered, and evaporated to afford crude product. The crude product was purified by flash silica chromatography, elution gradient 0–10% MeOH in DCM. Pure fractions were evaporated to dryness to afford the title compound (0.450 g, 78%) as a solid. MS (ESI) [M + H]+ = 579.

**Step j:** tert-Butyl N-[4-[[tert-Butyloxy carbonyl]amino]-1-methyl-ethyl]amino]-6-(hydroxymethyl)benzothiophene-2-carboximido]carbamate (6). Boc-anhydride (0.920 mL, 3.96 mmol) was added slowly to tert-butyl (2-((2-carbamidomethyl-6-(hydroxymethyl)benzo)[b]thiophen-4-yl)amino)propyl)carbamate (0.4 g, 1.06 mmol) and triethylamine (0.291 mL, 2.37 mmol) in MeOH (5 mL) at 20 °C under nitrogen. The resulting solution was stirred at 20 °C for 4 h. The reaction mixture was diluted with EtOAc (100 mL) and washed sequentially with saturated brine (20 mL x 3). The organic layer was dried over NaSO4, filtered, and evaporated to afford crude product. The crude product was used in the next step directly without further purification.

**Step k:** S-[4-[[tert-Butyloxy carbonyl]amino]-1-methyl-ethyl]amino]-2-(N-tert-butoxy carbonyl)aminomethyl)benzothiophene-6-yl)methylmethanesulfonate. Potassium thioacetate (0.164 g, 1.44 mmol) was added dropwise to tert-butyl (2-((2-(tert-butoxy carbonyloxy)carbamidomethyl)6-(hydroxymethyl)benzo[b]thiophen-4-yl)amino)propyl)carbamate (0.4 g, 0.84 mmol) and triethylamine (0.291 mL, 2.09 mmol) in DCM (25 mL) at 0 °C under nitrogen. The resulting solution was stirred at 20 °C for 30 min. The reaction mixture was diluted with DCM (100 mL) and washed sequentially with saturated brine (25 mL x 3). The organic layer was dried over NaSO4, filtered, and evaporated to afford crude product. This was used in the next step directly without further purification.

**Step l:** S-[4-[[tert-Butyloxy carbonyl]amino]-1-methyl-ethyl]amino]-2-((4-((1-aminopropan-2-yl)amino)imidazol-4-yl)carboximidoyl)benzo[b]thiophene-2-carboxamidine (AZ-006). Trimethylphosphane (0.1% formic acid) was added slowly to methyl 5-bromo-4-phenylthiophene-2-carboxylate (1.600 g, 7.33 mmol) in DMF (5 mL) at 25 °C. The resulting solution was stirred at 20 °C for 4 h. The crude product was purified by flash chromatography, elution gradient 0–50% MeCN in water. Pure fractions were evaporated to dryness to afford the title compound (15.00 mg, 15.76%) as a solid. 1H NMR (300 MHz, DMSO-d6) δ 2.37 (m, 3 H), 4.19 (2 H, s), 6.54 (1 H, s), 7.08 (1 H, s), 8.17 (d, 1 H, J = 7.7 Hz). MS (ESI) [M + H]+ = 587.5.

**Synthesis Pathway to 5-(1H-Imidazol-4-yl)-4-phenyl-thiophene-2-carboxamidine (AZ-007) (SI, Scheme S3). Step a:** Methyl 5-Bromo-4-phenyl-thiophene-2-carboxylate (8). NBS (1.305 g, 7.33 mmol) was added to methyl 4-phenylthiophene-2-carboxylate (1.600 g, 7.33 mmol) in DMF (5 mL) at 25 °C. The resulting solution was stirred at 20 °C for 4 h. The crude product was purified by flash chromatography, elution gradient 0–50% MeCN in water. Pure fractions were evaporated to dryness to afford the title compound (1.600 g, 73.5%) as a solid. MS (ESI) [M + H]+ = 297/299.

**Step b:** Methyl 4-Phenyl-5-[1-((2-trimethylsilyl)ethoxy)methyl]-imidazol-4-yl)thiophene-2-carboxylate (9). A solution of Na2CO3 (490 mg, 4.63 mmol) in water (1.000 mL) was added to a stirred suspension of methyl 5-bromo-4-phenylthiophene-2-carboxylate (500 mg, 1.68 mmol), 4-(4,4,5,5-tetramethyl-1,3-dioxaborol-2-yl)-1-(1-((2-(trimethylsilyl)ethoxy)methyl)1H-1-imidazole (16) (500 mg, 1.54 mmol) and Pd(Ph3P)4 (178 mg, 0.15 mmol) in 1,4-dioxane (5 mL) at 25 °C under nitrogen. The resulting suspension was stirred at 90 °C overnight. The reaction mixture was diluted with EtOAc (200 mL), and washed sequentially with saturated brine (100 mL x 3). The organic layer was dried over NaSO4, filtered, and evaporated to afford crude product. The crude product was purified by flash C18-flash chromatography, elution gradient 0–60% MeCN in water. Pure fractions were evaporated to dryness to afford the title compound (300 mg, 46.9%) as an oil. MS (ESI) [M + H]+ = 415.

**Step c:** Methyl 5-(1H-Imidazol-4-yl)-4-phenyl-thiophene-2-carboxylate (10). HCl (1 mL, 32.91 mmol) was added to methyl 5-(1H-Imidazol-4-yl)thiophene-2-carboxylate (200 mg, 0.48 mmol) in MeOH (5 mL) at 25 °C under nitrogen. The resulting solution was stirred at rt overnight. The solvent was removed under reduced pressure. The crude product was purified by flash C18-flash chromatography, elution gradient 0–50% MeCN in water. Pure fractions were evaporated to dryness to afford the title compound (80 mg, 58.3%) as an oil. MS (ESI) [M + H]+ = 285.

**Step d:** 5-(1H-Imidazol-4-yl)-4-phenyl-thiophene-2-carboxamidine (AZ-007). trimethylaluminum (81 mg, 1.13 mmol) was added to ammonium chloride (60.2 mg, 1.13 mmol) in toluene (3 mL) at 0 °C. The resulting suspension was stirred at rt for 1 h. A solution of methyl 5-(1H-Imidazol-4-yl)thiophene-2-carboxylate (80 mg, 0.28 mmol) in toluene (3.00 mL) was added dropwise to the reaction at 25 °C under nitrogen. The resulting solution was stirred at 110 °C for 2 h. The reaction mixture was filtered through silica and washed with DCM:MeOH (5:1). The solvent was removed under reduced pressure. The crude product was purified by preparative HPLC (XBridge Prep C18 OBD column, 5 μ silica, 19 mm diameter, 150 mm length), using decreasingly polar mixtures of water (containing 0.1% formic acid) and MeCN as eluents. Fractions containing the desired compound were evaporated to dryness to afford the title compound (15.00 mg, 15.76%) as a solid. 1H NMR (400 MHz, methanol-d4) δ 6.65–6.70 (1 H, m), 7.40–7.55 (5 H, m), 7.75 (1 H, s), 7.88 (1 H, s), 8.49 (1 H, br s). MS (ESI) [M + H]+ = 269.
Step a: NaH (150 mg, 3.74 mmol) was added slowly to 5-bromo-1H-imidazole (500 mg, 3.40 mmol) in THF (10 mL) at 0 °C under nitrogen. The resulting suspension was stirred at rt for 1 h. The mixture was concentrated and diluted with EtOAc (250 mL) and washed sequentially with saturated brine (150 mL x 3). The organic layer was dried over Na2SO4, filtered, and evaporated to afford crude product. The crude product was purified by flash silica chromatography, elution gradient 0–50% MeCN in water. Pure fractions were evaporated to dryness to afford 5-bromo-1H-imidazole (900 mg, 95%). As a colorless oil. MS (ESI) [M + H]+ = 127. m/z (ES+). Step b: 4-Bromo-3-(2-(trimethylsilyl)ethoxy)methyl-1H-imidazole (900 mg, 95%) was colorless oil. The crude product was purified by flash silica chromatography, elution gradient 0–10% MeOH in DCM. Pure fractions were evaporated to dryness to afford the title compound (0.550 g, 78%) as a gum. *H NMR (300 MHz, DMSO-d6) δ 1.33–1.45 (m, 6H), 4.95–5.09 (m, 1H), 7.21–7.38 (m, 1H), 8.11–8.24 (m, 1H), 8.85 (s, 1H). MS (ESI) [M – H]- = 260.

Step e: 2-Cyano-7-isopropoxy-benzothiophene-4-carboxylic Acid (13). BuLi in THF (2.0, 15.61 mL, 31.22 mmol) was added dropwise to 4-bromo-7-isopropoxybenzo[b]thiophene-2-carbonitrile (0.8 g, 2.70 mmol) in THF (50 mL) and dry ice (2.97 mmol, 1.1 equiv) at −78 °C under nitrogen. The resulting solution was stirred at −78 °C for 10 min. The reaction was incomplete, and further dry ice (2.97 mmol) was added in one portion and the solution was stirred at −78 °C for a further 30 min. The reaction mixture was quenched with saturated NH4Cl (25 mL), extracted with EtOAc (3 × 100 mL), and the organic layer was dried over Na2SO4, filtered, and evaporated to afford colorless oil. The crude product was purified by flash silica chromatography, elution gradient 0–10% MeOH in DCM. Pure fractions were evaporated to dryness to afford the title compound (0.350 g, 41.2%) as a solid. *H NMR (300 MHz, DMSO-d6) δ 7.75 (d, J = 8.6, 1H), 7.68–7.77 (d, J = 8.4, 1H), 8.32–8.39 (s, 1H).

Step f: tert-Butyl N-[2-(2-Cyano-7-isopropoxy-benzothiophene-4-carboxylic acid)-methyl]carbamate (14). HATU (1.040 g, 2.74 mmol) was added to 2-cyano-7-isopropoxybenzo[b]thiophene-4-carboxylic acid (0.55 g, 2.10 mmol), tert-butyl (2-aminomethyl)carbamate (0.405 g, 2.33 mmol), and triethylamine (1.467 mL, 10.52 mmol) in DMF (10 mL) at 20 °C under nitrogen. The resulting solution was stirred at 20 °C for 1 h. The reaction mixture was diluted with EtOAc (100 mL) and washed sequentially with saturated brine (25 mL x 3). The organic layer was dried over Na2SO4, filtered, and evaporated to afford crude product. The crude product was purified by flash silica chromatography, elution gradient 0–10% EtOAc in petroleum ether. Pure fractions were evaporated to dryness to afford the title compound (0.350 g, 41.2%) as a solid. *H NMR (300 MHz, DMSO-d6) δ 1.33–1.43 (m, 6H), 7.23–7.37 (m, 1H), 8.12–8.26 (m, 1H), 8.90 (s, 1H). MS (ESI) [M + H]+ = 404.

Step g: N-(2-Aminomethyl)-2-(2-cyano-7-isopropoxy-benzothiophene-4-carboxamide). TFA (3 mL, 38.94 mmol) was added to tert-butyl (2-(2-cyano-7-isopropoxybenzo[b]thiophene-4-carboxamido)ethyl)carbamate (0.35 g, 0.87 mmol) in DCM (12 mL) at 20 °C under nitrogen. The resulting solution was stirred at rt for 1 h. The solvent was removed under reduced pressure. The reaction mixture was adjusted to pH 7. The crude product was purified by flash silica chromatography, elution gradient 0–20% MeOH in DCM. Pure fractions were evaporated to dryness to afford the title compound (0.250 g, 95%) as a solid. *H NMR (300 MHz, DMSO-d6) δ 1.33–1.44 (m, 15H), 3.56–3.67 (m, 2H), 6.96 (s, 1H), 7.26–7.35 (d, J = 8.4, 1H), 7.83–7.93 (d, J = 8.3, 1H), 8.71 (s, 1H). MS (ESI) [M + H]+ = 304.

Step h: N-(2-Aminomethyl)-2-(N-hydroxy carbamimidoyl)-7-isopropoxy-benzothiophene-4-carboxamide (AZ-008). Nickel (0.2 g, 3.41 mmol) was added to N-(2-aminomethyl)-2-(N-hydroxy carbamimidoyl)-7-isopropoxy-benzothiophene-4-carboxamide (0.15 g, 0.45 mmol) and acetic acid (0.5 mL, 8.73 mmol) in MeOH (5 mL) at 25 °C under nitrogen. The resulting solution was stirred at 40 °C for 1 h under an atmosphere of hydrogen. The mixture was filtered through a Celite pad. The solvent was removed under reduced pressure. The
crude product was purified by preparative HPLC column: Xselect CSH OBD column 30 mm × 150 mm, 5 μm; mobile phase A, water (0.05%TFA), mobile phase B, MeCN; flow rate, 60 mL/min. Fractions containing the desired compound were evaporated to dryness to afford the title compound (0.025 g, 8.67%) as a white solid. 1H NMR (300 MHz, methanol-d6) δ 1.43–1.52 (d, J = 6.0, 6H), 3.18–3.29 (t, J = 5.9, 2H), 3.65–3.79 (t, J = 6.0, 2H), 4.94–5.04 (m, 1H), 7.14–7.24 (d, J = 8.5, 1H), 7.90–7.99 (d, J = 8.3, 1H), 8.87 (s, 1H). 13C NMR (126 MHz, DMSO-d6) δ 126.25, 159.88, 158.88, 158.60, 154.39, 138.36, 132.01, 131.91, 130.72, 128.51, 124.14, 108.77, 71.88, 39.05, 22.16. MS (ESI) [M + H]+ = 321. HRMS (ES+): for C13H15N2O5S [M + H]+: calc 321.1385, found 321.1392.

■ ASSOCIATED CONTENT

* Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jmedchem.9b01942.

Additional results and X-ray crystallography tables (PDF)

Molecular formula strings (CSV)

PDB docking AZ-008 to p53 (PDB)

Accession Codes

Coordinates and structure factors have been deposited in the Protein Data Bank under the following accession codes. 14-3-3σ/TAZpS89/fragment complexes: 6RHC (AZ-003); 6SLW (AZ-004); 6RJE (AZ-005); 6SLX (AZ-010); 6RJL (AZ-018); 6RP6 (AZ-019). 14-3-3σ/p53pT387/Fragment complexes: 6S40 (AZ-001); 6RWI (AZ-002); 6RL3 (AZ-003); 6SQQ (AZ-004); 6RX2 (AZ-005); 6RSL (AZ-006); 6RWH (AZ-007); 6RWS (AZ-009); 6RWU (AZ-010); 6SIP (AZ-011); 6SIQ (AZ-012); 6SLV (AZ-013); 6SKS (AZ-014); 6RJZ (AZ-015); 6RMS (AZ-016); 6SIO (AZ-017); 6S39 (AZ-018); 6SC3 (AZ-019); 6SIN (AZ-020); 6RRK (AZ-21); 6RRM (AZ-022); 6RLI (AZ-023); 6RL6 (AZ-024); 6RL4 (AZ-025); 6RM7 (AZ-026). Authors will release the atomic coordinates and experimental data upon article publication.

■ AUTHOR INFORMATION

Corresponding Authors

Christian Ottmann — Laboratory of Chemical Biology, Department of Biomedical Engineering and Institute for Complex Molecular Systems, Eindhoven University of Technology, 5600MB Eindhoven, The Netherlands; Department of Organic Chemistry, University of Duisburg-Essen, 47057 Duisburg, Germany; orcid.org/0000-0001-7315-0315; Phone: +31 40 247-2835; Email: c.ottmann@tue.nl

Isabelle Landrieu — CNRS ERL9002 Integrative Structural Biology, F-59000 Lille, France; Univ. Lille, Inserm, CHU Lille, Institut Pasteur de Lille, U1167-RID-AGE, Risk Factors and Molecular Determinants of Aging-Related Diseases, F-59000 Lille, France; orcid.org/0000-0002-4883-2637; Email: isabelle.landrieu@univ-lille.fr

Hongming Chen — Hit Discovery, Discovery Sciences, Biopharmaceutical R&D, AstraZeneca, Gothenburg, 431 50 Mölndal, Sweden; Email: hongming.chen71@hotmail.com

Authors

Xavier Guillory — Laboratory of Chemical Biology, Department of Biomedical Engineering and Institute for Complex Molecular Systems, Eindhoven University of Technology, 5600MB Eindhoven, The Netherlands

Madita Wolter — Laboratory of Chemical Biology, Department of Biomedical Engineering and Institute for Complex Molecular Systems, Eindhoven University of Technology, 5600MB Eindhoven, The Netherlands

Seppe Lysis — Laboratory of Chemical Biology, Department of Biomedical Engineering and Institute for Complex Molecular Systems, Eindhoven University of Technology, 5600MB Eindhoven, The Netherlands

João Filipe Neves — CNRS ERL9002 Integrative Structural Biology, F-59000 Lille, France; Univ. Lille, Inserm, CHU Lille, Institut Pasteur de Lille, U1167-RID-AGE, Risk Factors and Molecular Determinants of Aging-Related Diseases, F-59000 Lille, France

Ave Kuusk — Laboratory of Chemical Biology, Department of Biomedical Engineering and Institute for Complex Molecular Systems, Eindhoven University of Technology, 5600MB Eindhoven, The Netherlands; Hit Discovery, Discovery Sciences, Biopharmaceutical R&D, AstraZeneca, Gothenburg, 431 50 Mölndal, Sweden

Sylvia Genet — Laboratory of Chemical Biology, Department of Biomedical Engineering and Institute for Complex Molecular Systems, Eindhoven University of Technology, 5600MB Eindhoven, The Netherlands

Bente Somsen — Laboratory of Chemical Biology, Department of Biomedical Engineering and Institute for Complex Molecular Systems, Eindhoven University of Technology, 5600MB Eindhoven, The Netherlands

John Kenneth Morrow — Department of Pharmaceutical Chemistry and Small Molecule Discovery Center (SMDC), University of California, San Francisco, California 94143, United States

Emma Rivers — Hit Discovery, Discovery Sciences, Biopharmaceutical R&D, AstraZeneca, Gothenburg, 431 50 Mölndal, Sweden

Lotte van Beek — Laboratory of Chemical Biology, Department of Biomedical Engineering and Institute for Complex Molecular Systems, Eindhoven University of Technology, 5600MB Eindhoven, The Netherlands

Joe Patel — Oncology and Discovery Sciences, IMED Biotech Unit, AstraZeneca, Walhalla, Massachusetts 02451, United States

Robert Goodnow — Oncology and Discovery Sciences, IMED Biotech Unit, AstraZeneca, Walhalla, Massachusetts 02451, United States

Heike Schoenherr — Oncology and Discovery Sciences, IMED Biotech Unit, AstraZeneca, Walhalla, Massachusetts 02451, United States

Nathan Fuller — Oncology and Discovery Sciences, IMED Biotech Unit, AstraZeneca, Walhalla, Massachusetts 02451, United States

Qing Cao — Oncology and Discovery Sciences, IMED Biotech Unit, AstraZeneca, Walhalla, Massachusetts 02451, United States

Richard G. Dovey — Laboratory of Chemical Biology, Department of Biomedical Engineering and Institute for Complex Molecular Systems, Eindhoven University of Technology, 5600MB Eindhoven, The Netherlands

Luc Brunsveld — Laboratory of Chemical Biology, Department of Biomedical Engineering and Institute for Complex Molecular Systems, Eindhoven University of Technology, 5600MB Eindhoven, The Netherlands; orcid.org/0000-0001-5675-511X
Michelle R. Arkin — Department of Pharmaceutical Chemistry and Small Molecule Discovery Center (SMDC), University of California, San Francisco, California 94143, United States; orcid.org/0000-0002-9366-6770

Paola Castaldi — Oncology and Discovery Sciences, IMED Biotech Unit, AstraZeneca, Waltham, Massachusetts 02451, United States

Helen Boyd — Hit Discovery, Discovery Sciences, Biopharmaceutical R&D, AstraZeneca, Gothenburg, 431 50 Möndal, Sweden

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jmedchem.9b01942

Author Contributions

X.G., M.W., and S.L. contributed equally. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

PPI, protein—protein interaction; FBDD, fragment-based drug discovery; FP, fluorescence polarization; SPR, surface plasmon resonance; FDA, Food and Drug Administration; PROTACs, proteolysis targeting chimeras; EtoAc, ethyl acetate; DMSO, dimethyl sulfoxide; DCM, dichloromethane; MeCN, acetonitrile; FA, formic acid; DMF, dimethylformamide; DMAP, 4-dimethylaminopyridine; THF, tetrahydrofuran; TFA, trifluoroacetic acid; NBS, N-bromosuccinimide; MS-Cl, methanesulfonyl chloride; CSP, chemical shift perturbation

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