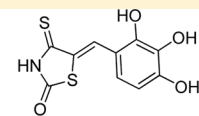
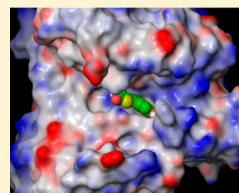


5-Arylidene thioxothiazolidinones as Inhibitors of Tyrosyl–DNA Phosphodiesterase I

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S Supporting Information

ABSTRACT: Tyrosyl–DNA phosphodiesterase I (Tdp1) is a cellular enzyme that repairs the irreversible topoisomerase I (Top1)–DNA complexes and confers chemotherapeutic resistance to Top1 inhibitors. Inhibiting Tdp1 provides an attractive approach to potentiating clinically used Top1 inhibitors. However, despite recent efforts in studying Tdp1 as a therapeutic target, its inhibition remains poorly understood and largely underexplored. We describe herein the discovery of arylidene thioxothiazolidinone as a scaffold for potent Tdp1 inhibitors based on an initial tyrphostin lead compound **8**. Through structure–activity relationship (SAR) studies we demonstrated that arylidene thioxothiazolidinones inhibit Tdp1 and identified compound **50** as a submicromolar inhibitor of Tdp1 ($IC_{50} = 0.87 \mu M$). Molecular modeling provided insight into key interactions essential for observed activities. Some derivatives were also active against endogenous Tdp1 in whole cell extracts. These findings contribute to advancing the understanding on Tdp1 inhibition.

**50**Tdp1 $IC_{50} = 0.87 \mu M$
Tdp1(BSA) $IC_{50} = 2.8 \mu M$

■ INTRODUCTION

Topoisomerase I (Top1) is an essential enzyme that catalyzes the relaxation of supercoiling and the release of torsional strains during DNA replication, transcription, recombination, and chromatin remodeling.^{1,2} This enzymatic function involves a dynamic equilibrium between the cleavage of the DNA substrate and the religation of the resulting transient Top1–DNA cleavage complex, which features a 3′-phosphotyrosyl bond (Figure 1). Inhibitors of Top1 stall this equilibrium by binding to and stabilizing an irreversible ternary Top1–DNA–inhibitor complex, preventing the religation of the 5′-hydroxyl group.^{3,4}

This inhibition creates a DNA lesion and underlies the use of camptothecin (CPT)-based Top1 inhibitors, such as topotecan and irinotecan, for treating ovarian, lung, and colon cancers^{5,6} and the development of the indenoisoquinolines as non-camptothecin Top1 inhibitors.⁷ Stalled Top1–DNA complexes are repaired by Tdp1, a cellular enzyme conserved from yeast to humans and which hydrolyzes the unique 3′-phosphotyrosyl linkage of the Top1–DNA cleavage complex.⁸ Other than Top1 inhibitors, cleavage complexes can also be generated by endogenous DNA lesions such as strand breaks, abasic sites, base mismatches, and specific oxidized or modified bases and carcinogenic DNA adducts.^{9–11} The repair of the Top1–DNA cleavage complex begins with a nucleophilic attack of the histidine 263 of Tdp1 onto the 3′-phosphotyrosyl bond to form

a Tdp1–DNA covalent intermediate (Figure 1 C) and release Top1.¹² Hydrolysis of this intermediate turns over Tdp1 and generates a DNA product with a 3′-phosphate (Figure 1 D and E). Further repair presumably involves the polynucleotide kinase phosphatase (PNKP), a bifunctional enzyme with 5′-kinase and 3′-phosphatase activities that catalyze both the hydrolysis of the 3′-phosphate and the phosphorylation of the 5′-DNA end to enable their rejoining.⁸ The critical role of Tdp1 in the cellular repair of Top1-mediated DNA damage has been established through three lines of evidence. First, knocking out Tdp1 in yeast¹³ and in vertebrate cells^{14,15} renders such cells hypersensitive to camptothecin. Second, Tdp1-defective SCAN1 (spinocerebellar ataxia with axonal neuropathy-1) cells, which have a Tdp1 mutation N493R and accumulate the normally transient Tdp1–DNA repair complex (Figure 1C), are highly sensitive to CPT and accumulate DNA strand breaks upon treatment with CPT.^{14,16} Third, overexpression of wild-type Tdp1 protects cells against CPT-induced cell death, whereas the H263A mutant does not.¹⁷ These observations provide the rationale for developing Tdp1 inhibitors for combination therapy with Top1 inhibitors.^{18,19}

Tdp1 belongs to the phospholipase D (PLD) superfamily,²⁰ which is defined by a conserved HKD catalytic motif²¹ and a

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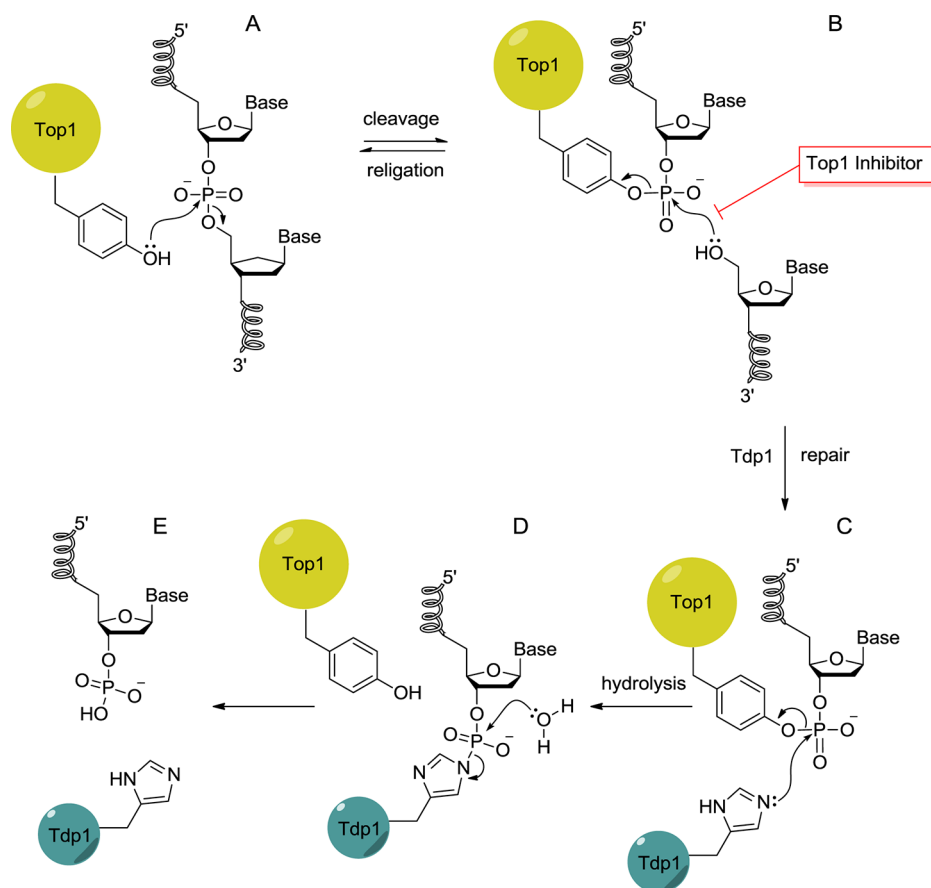


Figure 1. Tdp1 in the repair of the stalled Top1–DNA complex: (A) noncovalent Top1–DNA complex; (B) transient Top1–DNA complex, which is trapped in the presence of a Top1 inhibitor; (C) the stalled Top1–DNA complex (cleavage complex) is repaired by Tdp1 via a transient Tdp1–DNA covalent intermediate; (D) Top1 is released and the Tdp1–DNA covalent intermediate resolved by hydrolysis; (E) 3'-phospho–DNA–end product.

signature catalytic mechanism¹² featuring a covalent phosphohistidine intermediate (Figure 1C). Two such motifs are present in the N- and C-terminal domains of human Tdp1, both containing the highly conserved histidine and lysine residues (H263/K265 and H493/K495). The importance of these residues for Tdp1 catalytic activity is manifested through the observations that the mutation of H263 generates a catalytically inactive enzyme and mutating H493, K265, or K495 results in compromised enzymatic activity.^{20,22} Thus, Tdp1 represents a distinct member of the PLD superfamily with unique HKN motifs. Although the mechanism of Tdp1 catalysis is well established, its inhibition remains poorly understood and largely unexplored. Early studies on Tdp1 discovered molecular probes that inhibit Tdp1 at millimolar range, including aminoglycoside antibiotics and the ribosome inhibitors,²³ as well as tungstates and vanadates which served as transition state mimics for crystallographic studies.^{24,25} Later on, a few micromolar inhibitors (Figure 2) were identified through a high-throughput screening (HTS) assay,²⁶ among which 3 was confirmed to specifically inhibit Tdp1 through SAR.¹¹ Recently, Cushman and co-workers reported that a subset of indenoisoquinoline derivatives potently inhibit both Top1 and Tdp1, making them the first Top1/Tdp1 dual inhibitors.²⁷ Nevertheless, though inhibiting Tdp1 at micromolar concentrations, these compounds remain far from therapeutic development. Therefore, there is a need to continue to identify potent and specific small molecule inhibitors to

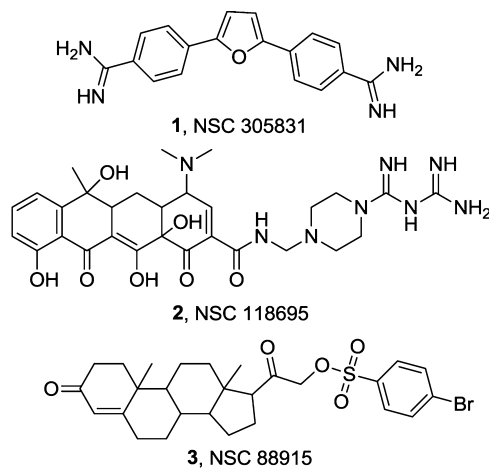


Figure 2. Known inhibitors of Tdp1.

further advance the understanding of Tdp1 functions and develop therapeutic candidates. Herein we report the design, synthesis, molecular modeling, and biological evaluations of thioxothiazolidinones as novel and potent Tdp1 inhibitors.

RESULTS AND DISCUSSION

Exploring Tdp1 as a therapeutic target was made possible by the development of a biochemical screening assay using recombinant human Tdp1.^{11,28} This gel-based assay is based

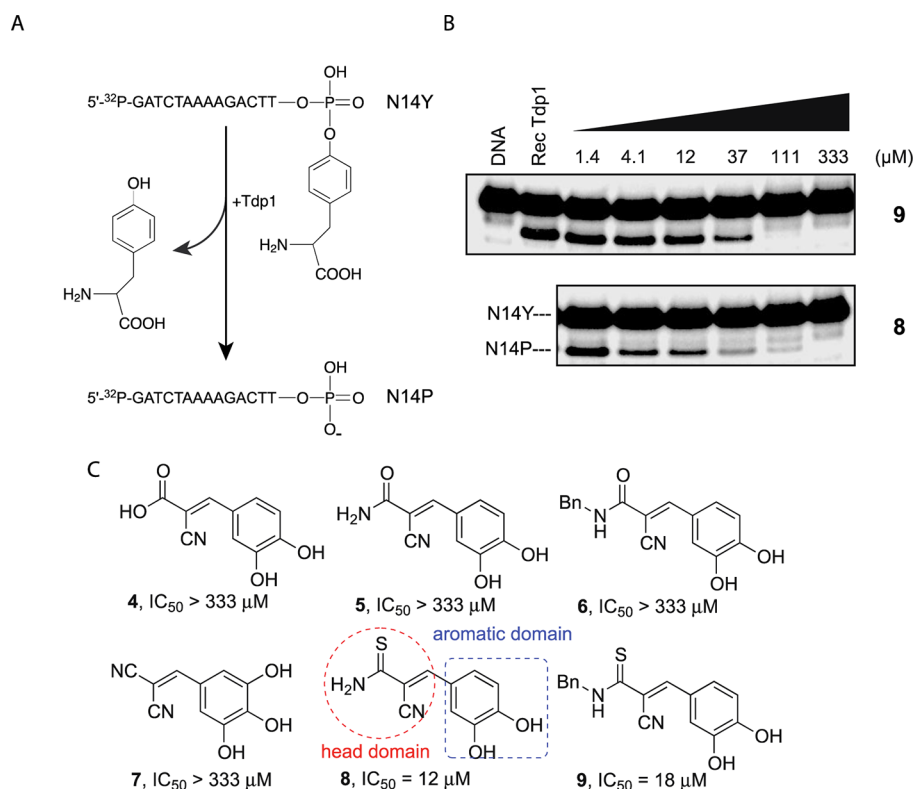


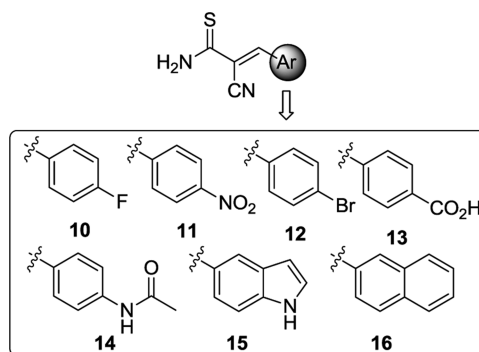
Figure 3. Inhibition of Tdp1 by tyrphostin derivatives. (A) Schematic representation of the Tdp1 biochemical gel-based assay. Tdp1 hydrolyzes the 3'-phosphotyrosine bond and converts the N14Y DNA substrate to a 3'-phosphate oligonucleotide product (N14P); (B) representative gel demonstrating concentration-dependent inhibition of Tdp1 by compounds 8 and 9; (C) representative compounds. The pharmacophore of lead compound 8 comprises two domains: the functional head domain and the aromatic moiety.

on the cleavage by Tdp1 of the 14-mer 5'-³²P-labeled 3'-phosphotyrosyl DNA substrate N14Y (5'-GATCTAAAA-GACTT-pY-3') to a 14-mer 5'-³²P-labeled 3'-phosphate DNA product N14P (Figure 3, A). This assay was applied to a small in-house chemical library constituted of benzylidenemalonitriles, a class of tyrphostins. This screening led to the identification of compounds 8 and 9 as Tdp1 inhibitors with IC₅₀ values of 12 and 18 μM, respectively (Figure 3, B and C). Notably, it was observed from this screening that only tyrphostins with a thioamide functionality (compounds 8 and 9) in the head domain inhibited Tdp1. The carboxylic acid (4), carboxamides (5 and 6), and cyano (7) analogues were completely inactive at the highest tested concentration (333 μM) (Figure 3, C).

On the basis of these initial screening results, derivatives 10–16 of lead compound 8 were prepared to explore the impact of the aromatic domain (Table 1). While carboxylic acid analogue 13 demonstrated potent inhibitory activity against Tdp1 (IC₅₀ = 12 μM, Table 1), all other analogues were inactive up to 333 μM. The phenolic hydroxyl group (compound 8) or the carboxylic acid functionality (compound 13) appears essential for the inhibition of Tdp1.

At this point, we shifted our focus onto constructing heterocyclic scaffolds mimicking the head domain of lead compound 8. Notably, rhodanine benzylidene derivatives such as compound 18 have been reported as inhibitors of phosphodiesterase,²⁹ PTP,³⁰ and recently as broad spectrum antiviral.³¹ To mimic the head domain of compound 8, we chose a few rhodanine-like five-membered heterocyclic scaffolds with wide applications in medicinal chemistry (Figure 4): thiohydantoin 19^{32–34} and 20,³⁵ thiazolidinedione 21,^{36–38}

Table 1. Aromatic Domain SAR of Lead Compound 8^a



compound	Tdp1 IC ₅₀ (μM)
10	>333
11	>333
12	>333
13	12
14	>333
15	>333
16	>333
1	31 ± 5.0

^aFuramide 1 was used as a positive control,²⁶ and its IC₅₀ value is expressed as mean ± SD from at least three independent experiments.

and thioxothiazolidinone 22.^{39,40} It must be pointed out that the medicinal chemistry value of these scaffolds is a subject of recent controversies. Many characterize these compounds as promiscuous frequent hitters that tend to produce pan-assay interference in a HTS setting.⁴¹ Others consider them

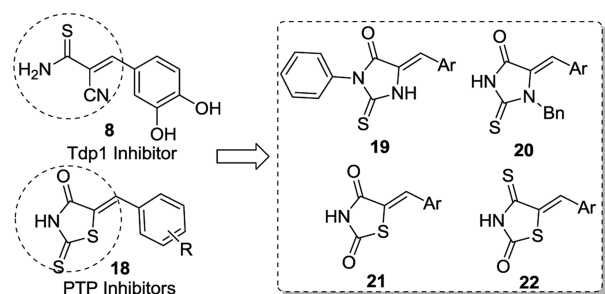
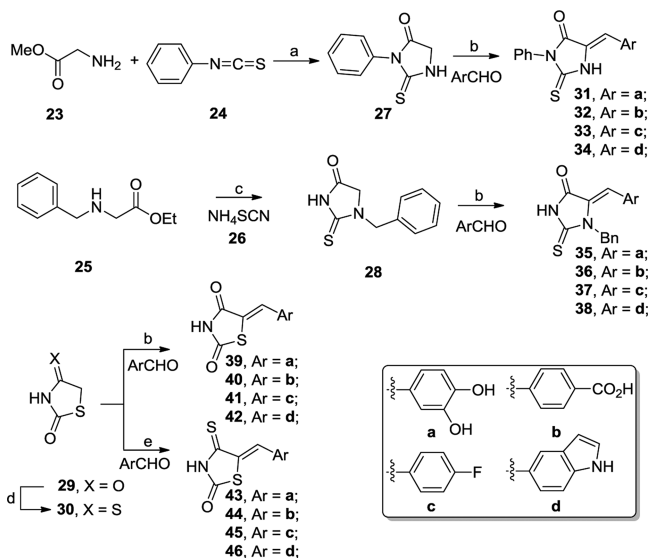


Figure 4. Design of heterocyclic arylidene scaffolds **19–22** based on lead compound **8** and PTP inhibitors **18**.

privileged scaffolds⁴² with an extraordinary ability to engage with important biological targets as evidenced by the clinical thiazolidinedione (**21**, aka glitazones) antidiabetic drugs.^{43–47} Recent systematic studies by Klein and co-workers found no aggregation-based promiscuity or unspecific reactivity toward protein,⁴⁸ arguing against the exclusion of these scaffolds in drug discovery efforts.

Synthetically, these scaffolds can be easily accessed through an aldol condensation between a heterocyclic ring (**27–30**) and an aromatic aldehyde (Scheme 1). The preparation of

Scheme 1. Synthesis of Scaffolds **19–22**^a



^aReagents and conditions: (a) EtOAc, reflux, 48 h, 92%; (b) EtOH, piperidine, reflux, 16 h, 41–78%; (c) 140 °C, 4 h, 72%; (d) Lawesson's reagent, toluene, reflux, 2 h, 98%; (e) NaOAc, AcOH, reflux, 2 h, 58–90%.

thiohydantoin intermediates **27** and **28** featured a reaction between a glycine derivative (**23** and **25**) and a thioisocyanate **24** or thiocyanate **26**. The thioxothiazolidinone intermediate **30** was synthesized from commercial thiazolidine-2,4-dione **29** using Lawesson's reagent. The key aldol condensation was performed under thermodynamic conditions to yield the more stable *Z*-isomers.⁴⁹ The assignment of this stereochemistry is consistent with literature reports.^{49–52}

Four analogues of each scaffold were prepared and tested against Tdp1. The assay results are summarized in Table 2. Notably, with the exception of **35**, which inhibited Tdp1 with an IC₅₀ value of 13 μM, analogues of the first three heterocyclic scaffolds were essentially inactive against Tdp1. By contrast, the

Table 2. Impact of the Head Domain on Tdp1 Inhibition^a

compound	Tdp1 IC ₅₀ (μM)
31	74
32	>333
33	>333
34	111–333
35	13 ± 3.0
36	>333
37	111–333
38	111–333
39	111–333
40	>111
41	>333
42	>111
43	2.6 ± 0.4
44	16 ± 8.8
45	27 ± 3.0
46	12 ± 2.2

^aIC₅₀ values are expressed as mean ± SD from at least three independent experiments.

thioxothiazolidinone scaffold (compounds **43–46**) consistently demonstrated significant potency. Of particular interest are the 3,4-dihydroxyl analogue **43** and the carboxylate analogue **44**, which inhibited Tdp1 at low micromolar concentrations (Table 2).

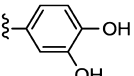
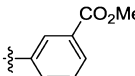
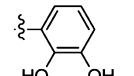
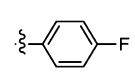
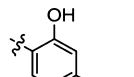
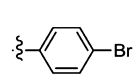
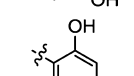
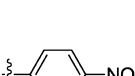
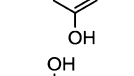
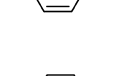
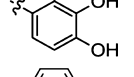
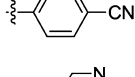
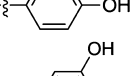
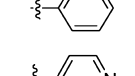
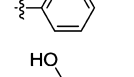
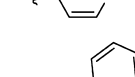
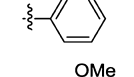
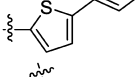
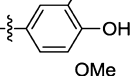
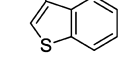
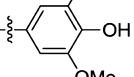
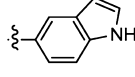
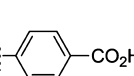
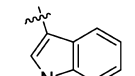
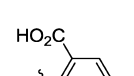
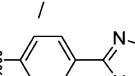
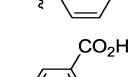
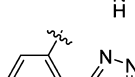
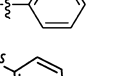
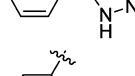
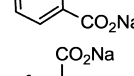
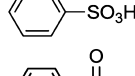
Having identified thioxothiazolidinone as the optimal substructure for the head domain, we then conducted a SAR on the aromatic domain. Toward this end, analogues with variable aromatic ring or substitution patterns were prepared based on the chemistry described in Scheme 1 (Table 3).

Analogues with two or three phenolic hydroxyl groups (**43**, **47–50**, Table 3) were by far the best inhibitors of the series, with **50** being a submicromolar Tdp1 inhibitor (Figure 5A and B). When only one free hydroxyl group was present (compounds **51–55**, Table 3, Figure 5A and B), a substantial drop in inhibitory activity was observed, suggesting that these free hydroxyl groups may provide key interactions with the enzyme possibly through hydrogen bonding.

The polyhydroxylated phenyl moiety, particularly the pyrogallol (1,2,3-trihydroxyphenyl) functionality in compound **50**, may potentially engage in excessive nonspecific protein binding. To assess the protein binding property of this functionality, **50** was tested against recombinant Tdp1 in WCE gel-based assay buffer containing bovine serum albumin (BSA, see Experimental Section) and exhibited an IC₅₀ of 2.8 μM. Knowing that BSA does not impact Tdp1 activity (Huang, S.H. and Pommier, Y., unpublished data), the 3-fold loss of potency may reflect nonspecific BSA binding, albeit to the extent typical of small molecule inhibitors. We have also tested **50** against recombinant Tdp2 in a gel-based assay and observed an IC₅₀ of 17 μM, a 20-fold increase when compared to that against Tdp1 (IC₅₀ = 0.87 μM). These studies strongly suggest that **50** is indeed a selective inhibitor of Tdp1. Efforts in bioisosteric replacement⁵¹ of catechol and pyrogallol moieties are currently underway, which could lead to more potent inhibitors with improved physicochemical properties.

The carboxylic acid analogues also demonstrated good potency, which was not affected by the position of the substitution (**44**, **56–57**, Table 3). However, derivatization of the acid appeared to improve the activity, as the two sodium salts (**58** and **59**) and methyl ester **60** were all found to be

Table 3. Aromatic Domain SAR of Thioxothiazolidinone Scaffold 22^a

Cpd	Ar =	Tdp1 IC ₅₀ (μM)	Cpd	Ar =	Tdp1 IC ₅₀ (μM)
43		2.6 ± 0.4	61		51 ± 25
47		5.8 ± 1.1	45		27 ± 3.0
48		4.1 ± 1.0	62		30 ± 12
49		7.1 ± 0.50	63		19 ± 6.4
50		0.87 ± 0.07	64		28 ± 7.6
51		39 ± 9.4	65		44
52		26	66		75 ± 29
53		34 ± 13	67		46 ± 15
54		11 ± 4.0	68		34 ± 7.8
55		48 ± 16	46		12 ± 2.2
44		16 ± 8.8	69		40 ± 11
56		15 ± 1.2	70		32
57		15 ± 4.7	71		111
58		7.8	72		20 ± 3.5
59		8.5	73		29 ± 6.0
60		6.7 ± 0.40	74		2.2 ± 0.5

^aIC₅₀ values are expressed as mean ± SD from at least three independent experiments.

approximately twice as potent as the acid analogues. Other substitutions, including halogens (45 and 62), nitro (63), and cyano (64), all led to decreased inhibitory activity. In addition, replacing the benzene ring with other heterocycles, such as pyridines (65 and 66), thiophene (67), benzothiophene (68),

and indoles (46 and 69) also yielded less potent compounds. Finally, bioisosteric replacement⁵³ of the carboxylic acid was explored, and analogues with tetrazole (70 and 71), sulfonic acid (72), phosphonate (73), and phosphonic acid (74) were prepared. The biochemical assay identified compound 74 as a

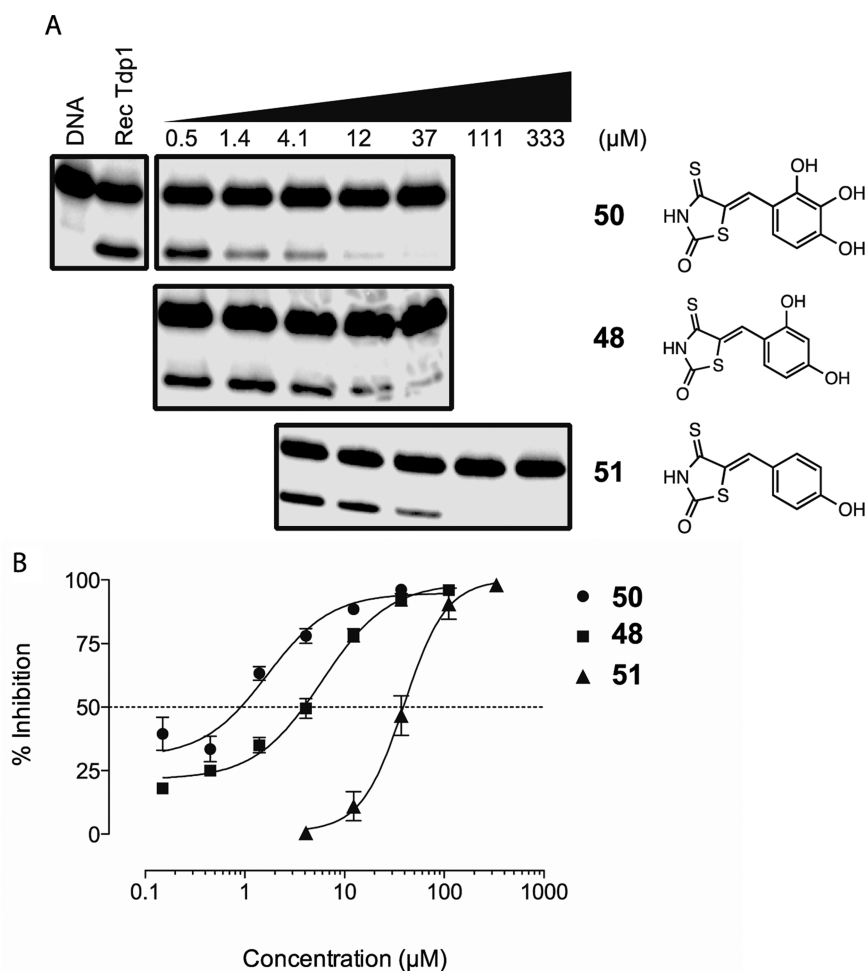


Figure 5. (A) Representative gel demonstrating concentration-dependent inhibition of Tdp1 by compounds **48**, **50**, and **51**. (C) Concentration–response curves. Each point represents the mean value \pm standard deviation of three independent experiments.

potent inhibitor ($IC_{50} = 2.2 \mu\text{M}$) with a 7.3-fold improvement in potency over **44**, demonstrating that the phosphonic acid functional group is a viable bioisostere of the carboxylic acid.

To further assess the ability of our compounds to inhibit human Tdp1 in a more complex pseudocellular environment, we performed secondary gel-based assays using the same N14Y DNA substrate while replacing recombinant Tdp1 by whole cell extracts (WCEs) as a source of endogenous Tdp1. For this purpose, the chicken Tdp1 gene in DT40 cells was knocked out and complemented with the human Tdp1 gene.¹⁵ Lysates were collected and used in place of recombinant Tdp1 in the gel assays.¹⁵ Compounds with a single digit micromolar IC_{50} from the recombinant Tdp1 assay were tested against WCE, and the results are shown in Table 4. Given the increased stringency of this assay, the observation that these compounds are generally active at micromolar concentrations in the WCE assay strongly corroborates the earlier biochemical results and confirms their inhibitory activities against endogenous Tdp1 in a pseudocellular environment. The confirmed inhibitory activity of this series of compounds, the SAR pattern and the concentration-dependent inhibition fashion, suggest that these controversial scaffolds can be valuable leads for inhibiting Tdp1.

Binding Analysis. The binding of our inhibitors to Tdp1 was directly measured using the Surface Plasmon Resonance (SPR) technology. In this testing, Tdp1 was amine coupled to a CMS sensor chip, and a dilution series of the representative

inhibitor **50** was run through the surface. A surface without coupled Tdp1 was used as a reference. As shown in Figure 6, a concentration-dependent binding of **50** on Tdp1 was observed (A). Similarly, a 14-base oligonucleotide modified with a biotin at the 5' end and a tyrosine at the 3' end (biotin-GATCTAAAAGACTT-Tyr) was immobilized onto the chip surface and tested against the same dilution series of **50** (Figure 6, B). No binding was observed between **50** and the N14Y oligomer, confirming the selective binding of our inhibitors to Tdp1 under assay conditions.

Modeling and Docking. Close examination of the active site identified three specific regions important for substrate binding (Figure 7A)—the hydrophobic (Y204, P461, W590), the catalytic (K265, H263, N283, H493, K495, N516), and an adjacent hydrophilic region (S400, S518, S536, E538). Both the catalytic and hydrophilic regions consist of positively charged and hydrophilic residues for the stabilization of the negatively charged phosphate backbone of the DNA (Figure 7A). The hydrophobic region is located at the top of the substrate channel that provides essential interactions to both the tyrosyl and the nucleoside group of the substrate. A docking study of the previously identified Tdp1 inhibitors (**1–3**) showed interactions to at least two of the three regions are required for binding (Figure 1S, Supporting Information). Docking of our first identified compounds **8** and **9** showed both inhibitors bind to the “floor” of the substrate channel at the catalytic and

Table 4. Assay Results with the DT40 WCE

Compound	Ar =	WCE Tdp1 IC ₅₀ (μM)
43		41
47		220
48		76
49		76
50		35
44		11
56		138
58		6.9
59		280
60		38
74		69

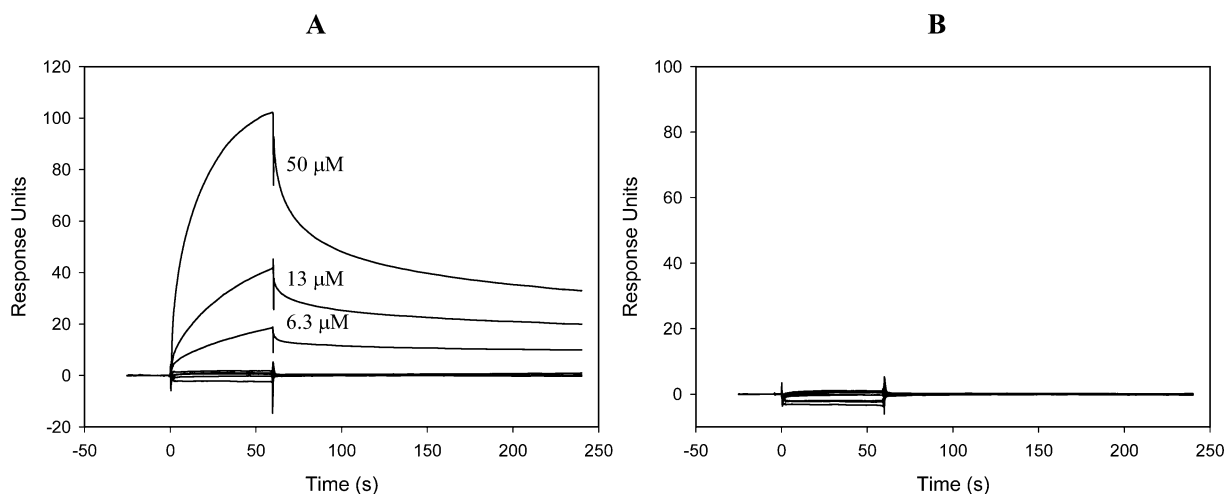


Figure 6. Binding of **50** to Tdp1 was examined using SPR spectroscopy. A concentration series of **50** (50, 25, 6.25, 3.12, 1.56, 0.78, and 0.39 μM) were injected over immobilized Tdp1 (A) or a tyrosyl-labeled oligonucleotide (GATCTAAAAGACTT-Tyr) (B). The theoretical maximal binding response to Tdp1 was 45 RU and to the oligonucleotide was 140 RU.

the hydrophilic regions (see **9** in Figure 7B). The essential interactions involved significant hydrogen bonding between the thiocarbonyl and the catalytic histidine and lysine residues as

well as hydrogen bonding between the catechol and the hydrophilic residues E538 and S536 (Figure 7B). The benzyl group of **9** was easily accommodated within the tyrosyl binding

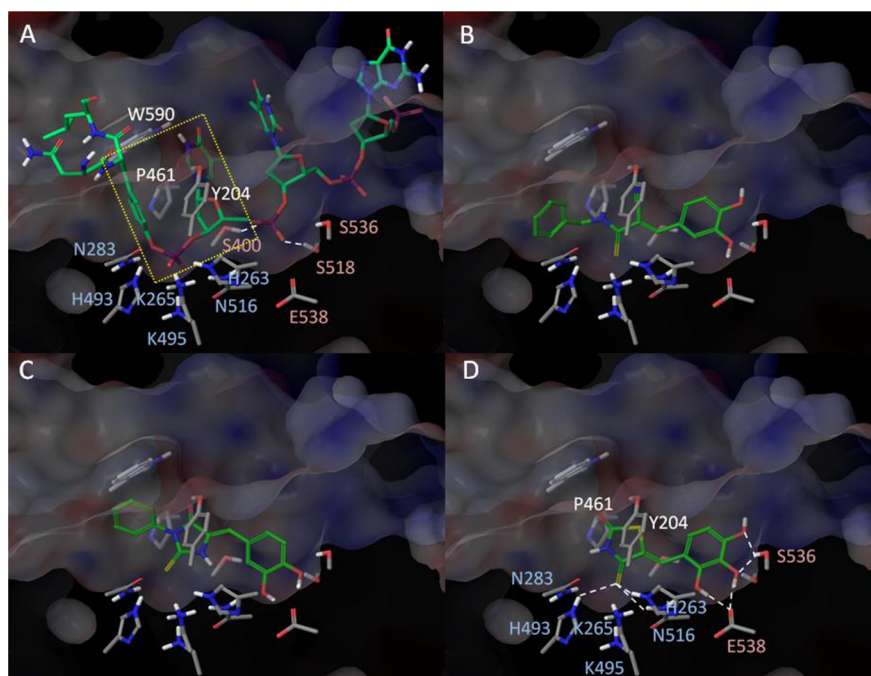


Figure 7. Modeled structure of Human Tdp1 (PDB: 1NOP)²⁵ with (A) Top1–DNA substrate, (B) **9**, (C) **31**, and (D) **50** docked within the active site. The model shows three specific regions important for substrate binding—the hydrophobic (Y204, P461, W590, labeled in white), the catalytic (K265, H263, N283, H493, K495, N516, labeled in blue), and an adjacent hydrophilic region (S400, S518, S536, E538, labeled in red). Effective binding of the hydrolytic tyrosyl–nucleosidic group of the Top1–DNA substrate involves the hydrophobic and the catalytic regions (boxed in yellow).

site, which supports the observed similar activities between **8** and **9**. Replacement of the catechol group with halobenzene or a large aromatic group significantly disrupts this observed mode of binding as the hydrophilic region energetically disfavors such substitution. For the heterocyclic head domain, placement of the catechol group directly adjacent to the thiocarbonyl is essential for maintaining the observed mode of binding. Placement of the thiocarbonyl at the 1,3 position to the catechol results in the thiocarbonyl group pointing slightly away from the catalytic lysine and histidine residues toward N283, which results in a weaker activity observed with **31** (Figure 7C). Addition of a benzyl group at the NH position of the heterocyclic ring (**35**–**38**) is prohibited as it leads to steric clashes with the interfacial region between the catalytic and hydrophilic sites. One advantage observed for the heterocyclic head is the ability to interact with P461 and Y204 within the hydrophobic region as observed in the increase in activity from 12 μM of **8** to 2.6 μM of **43**. The ability to interact with all three regions enhances affinity to retain the observed mode of binding and enables SAR modification of the catechol group which would otherwise lead to significant loss of activities (**10**–**12**, **14**–**16**). As observed in Table 3, replacement of the catechol by heteroaromatic groups and halobenzene after heterocyclization is tolerable within the hydrophilic region with only a 10-fold loss of activity. The replacement of the hydroxyl groups of catechol by negatively charged groups such as carboxylate, tetrazole, sulfonate, and phosphonate within the hydrophilic site leads to a slight shift in mode of binding, similar to that observed in **31** (Figure 7C). The observed effect depends significantly on the size, the electronegativity of the group, and the position of the aromatic substitution. Finally, addition of a third hydroxyl group to catechol (**50**) enables the formation of an additional hydrogen bond with E538 within the

hydrophilic site that correlates directly with the improved activity of 0.87 μM (Figure 7D).

CONCLUSIONS

On the basis of the initial lead compound **8** identified from screening a small focused library against Tdp1, we have discovered 5-arylidene thioxothiazolidinone as a novel scaffold for low micromolar Tdp1 inhibitors. SAR studies revealed that analogues with multiple hydroxyl groups or a carboxylic acid derivative on the terminal phenyl ring consistently inhibited Tdp1 in the biochemical assay. Among these, compound **50** was identified as a submicromolar inhibitor. A modeling study identified the most likely mode of inhibition that corroborates the observed SAR. In addition, bioisosteric replacement of the carboxylic acid also led to the discovery of phosphonic acid **74** as a potent Tdp1 inhibitor. Results from the more stringent secondary WCE gel-based assay confirm the observed inhibitory activities of these compounds.

EXPERIMENTAL SECTION

Chemistry. General Procedures. All commercial chemicals were used as supplied unless otherwise indicated. Dry solvents (THF, Et₂O, CH₂Cl₂, and DMF) were dispensed under argon from an anhydrous solvent system with two packed columns of neutral alumina or molecular sieves. Flash chromatography was performed on a Teledyne Combiflash RF-200 with RediSep columns (silica) and indicated mobile phase. All reactions were performed under an inert atmosphere of ultrapure argon with oven-dried glassware. ¹H and ¹³C NMR spectra were recorded on a Varian 600 MHz or a Varian 400 MHz spectrometer. Mass data were acquired on an Agilent TOF II TOS/MS spectrometer capable of ESI and APCI ion sources. Analysis of sample purity was performed on a Varian Prepstar SD-1 HPLC system with a Varian Microsorb-MW 100-5 C18 column (250 mm × 4.6 mm). HPLC conditions: solvent A = H₂O, solvent B = MeCN; flow rate = 1.0 mL/min; compounds were eluted with a gradient of 5%

MeCN/H₂O to 80% MeCN for 40 min. Purity was determined by total absorbance at 254 nm. All tested compounds have a purity \geq 98%. Compounds 4–9 were purchased from Axxora along with other tyrphostins as a small focused library.

General Procedure a for the Aldol Condensation. To a mixture of an aromatic aldehyde (2.0/mmol, 1 equiv) and cyanothioacetamide (2.0 mmol, 1 equiv) in ethanol (5 mL) was added one drop of *N*-methylmorpholine, and the resulting mixture was stirred at 35 °C for 30–60 min. The reaction mixture was evaporated to give the crude product. The obtained solid was further purified by column chromatography (SiO₂, EtOAc/hexane).

(E)-2-Cyano-3-(4-fluorophenyl)prop-2-enethioamide (10). Prepared from 4-fluorobenzaldehyde following the general procedure A. Yield 62%; mp 155–158 °C; ¹H NMR (600 MHz, DMSO-*d*₆) δ 10.10 (s, 1H), 9.61 (s, 1H), 8.06 (s, 1H), 8.06–7.98 (m, 2H), 7.43–7.41 (m, 2H); ¹³C NMR (150 MHz, DMSO-*d*₆) δ 192.9, 165.4, 146.2, 133.5, 129.2, 117.3, 112.8, 101.2; HRMS-ESI(–) *m/z* calcd for C₁₀H₆FN₂S 205.0236 [M – H][–], found 205.0214.

(E)-2-Cyano-3-(4-nitrophenyl)prop-2-enethioamide (11). Prepared from 4-nitrobenzaldehyde following the general procedure A. Yield 58%; mp 171–173 °C; ¹H NMR (600 MHz, DMSO-*d*₆) δ 8.24–8.26 (m, 3H), 7.81 (d, *J* = 7.2 Hz, 2H), 7.63 (br, 2H); ¹³C NMR (150 MHz, DMSO-*d*₆) δ 194.6, 148.4, 148.0, 132.3, 131.9, 124.3, 123.7, 118.9; HRMS-ESI(–) *m/z* calcd for C₁₀H₆N₃O₂S 232.0259 [M – H][–], found 232.0181.

(E)-3-(4-Bromophenyl)-2-cyanoprop-2-enethioamide (12). Prepared from 4-bromobenzaldehyde following the general procedure A. Yield 59%; mp 176–179 °C; ¹H NMR (600 MHz, DMSO-*d*₆) δ 10.13 (s, 1H), 9.64 (s, 1H), 8.01 (s, 1H), 7.84 (d, *J* = 8.4 Hz, 2H), 7.77 (d, *J* = 8.4 Hz, 2H); ¹³C NMR (150 MHz, DMSO-*d*₆) δ 192.7, 146.0, 133.0, 132.5, 131.8, 126.4, 116.7, 113.8; HRMS-ESI(–) *m/z* calcd for C₁₀H₆BrN₂S 264.9435 [M – H][–], found 264.9444.

(E)-4-(3-Amino-2-cyano-3-thioxoprop-1-en-1-yl)benzoic Acid (13). Prepared from 4-formylbenzoic acid following the general procedure A. Yield 63%; mp >300 °C; ¹H NMR (600 MHz, DMSO-*d*₆) δ 13.12 (s, 1H), 10.06 (s, 1H), 9.51 (s, 1H), 8.18 (s, 1H), 7.98 (d, *J* = 8.4 Hz, 2H), 7.87 (d, *J* = 8.4 Hz, 2H); ¹³C NMR (150 MHz, DMSO-*d*₆) δ 192.2, 167.3, 161.9, 151.8, 140.1, 138.0, 132.6, 130.2, 128.5, 115.3; HRMS-ESI(–) *m/z* calcd for C₁₁H₇N₂O₃S 231.0228 [M – H][–], found 231.0257.

(E)-N-(4-(3-Amino-2-cyano-3-thioxoprop-1-en-1-yl)phenyl)acetamide (14). Prepared from *N*-(4-formylphenyl)acetamide following the general procedure A. Yield 74%; mp 231–236 °C; ¹H NMR (600 MHz, DMSO-*d*₆) δ 10.36 (s, 1H), 9.98 (s, 1H), 9.48 (s, 1H), 8.01 (s, 1H), 7.92 (d, *J* = 8.9 Hz, 2H), 7.73 (d, *J* = 8.9 Hz, 2H), 2.07 (s, 3H); ¹³C NMR (150 MHz, DMSO-*d*₆) δ 193.2, 169.7, 147.4, 143.7, 132.4, 126.8, 119.5, 117.4, 110.4, 24.8; HRMS-ESI(+) *m/z* calcd for C₁₂H₁₂N₃SO 246.0701 [M + H]⁺, found 246.0705.

(E)-2-Cyano-3-(1H-indol-5-yl)prop-2-enethioamide (15). Prepared from 1H-indole-5-carbaldehyde following the general procedure A. Yield 70%; mp 189–191 °C; ¹H NMR (600 MHz, DMSO-*d*₆) δ 11.58 (s, 1H), 9.90 (s, 1H), 9.41 (s, 1H), 8.24 (d, *J* = 10.2 Hz, 2H), 7.81 (d, *J* = 8.4 Hz, 1H), 7.53 (d, *J* = 7.8 Hz, 1H), 7.48–7.47 (m, 1H), 6.59 (s, 1H); ¹³C NMR (150 MHz, DMSO-*d*₆) δ 193.7, 150.6, 138.8, 128.6, 128.3, 125.8, 123.9, 123.4, 118.1, 113.1, 108.3, 103.5; HRMS-ESI(+) *m/z* calcd for C₁₂H₁₀N₃S 228.0595 [M + H]⁺, found 228.0581.

(E)-2-Cyano-3-(naphthalen-2-yl)prop-2-enethioamide (16). Prepared from 2-naphthaldehyde following the general procedure A. Yield 67%; mp 141–144 °C; ¹H NMR (600 MHz, DMSO-*d*₆) δ 10.13 (s, 1H), 9.66 (s, 1H), 8.44 (s, 1H), 8.23 (s, 1H), 8.09–8.05 (m, 2H), 8.02 (d, *J* = 7.8 Hz, 1H), 7.99 (d, *J* = 8.4 Hz, 1H), 7.66 (t, *J* = 7.2 Hz, 1H), 7.61 (t, *J* = 7.2 Hz, 1H); ¹³C NMR (150 MHz, DMSO-*d*₆) δ 193.1, 147.6, 134.9, 133.1, 130.2, 129.7, 129.5, 129.3, 128.5, 127.9, 125.5, 117.1, 113.1; HRMS-ESI(+) *m/z* calcd for C₁₄H₁₁N₂S 239.0643 [M + H]⁺, found 239.0650.

3-Phenyl-2-thioxoimidazolidin-4-one (27).⁵⁴ A suspension of methylglycinate hydrochloride (2.5 g, 20 mmol), phenylisothiocyanate (2.7 g, 20 mmol), and triethylamine (2.02 g, 2.8 mL, 20 mmol) in dry ethylacetate was refluxed (80 °C) for 2 days. Solvent was evaporated under reduced pressure. The residue was dissolved in EtOH, and

crystallization afforded 27 (3.5 g, 18 mmol, 92%) as a brown solid. ¹H NMR (600 MHz, CDCl₃) δ 7.53–7.46 (m, 3H), 7.33–7.32 (m, 2H), 4.23 (s, 2H).

Synthesis of 1-Benzyl-2-thiohydantoin (28). A suspension of *N*-benzyl glycine ethylester (3.86 g, 20 mmol) and ammonium thiocyanate (4.56 g, 60 mmol) was heated to 40 °C. A clear orange solution was observed which turned into dark red. After refluxing the reaction mixture for 4 h, the solution was immediately poured into 40 mL of (EtOH:H₂O). The crystalline white solid was collected by suction filtration, washed with 100 mL of (EtOH:H₂O), followed by diethyl ether (20 mL), and dried under vacuum to afford 28 (2.8 g, 13.7 mmol, 70%) as a pale yellow solid. ¹H NMR (600 MHz, DMSO-*d*₆) δ 11.84 (s, 1H), 7.27–7.36 (m, 5H), 4.85 (s, 2H), 4.07 (s, 2H).

4-Thioxo-thiazolidin-2-one (30).⁵⁵ To a solution of compound 29 (0.61 g, 5.21 mmol) in dry toluene (12 mL) was added Lawesson's reagent (2.2 g, 5.2 mmol). The reaction mixture was refluxed for 2 h and cooled to room temperature. The solid formed was filtered off and crystallized from acetone to afford 0.64 g (98%) of 30 as yellow crystals. ¹H (600 MHz, DMSO-*d*₆) δ 13.58 (s, 1H), 4.67 (s, 2H).

General Procedure B for Aldol Condensation.⁵⁶ An aromatic aldehyde (0.55 mmol, 1 equiv) and appropriate heterocyclic ring (27–29) (0.73 mmol, 1.3 equiv) were dissolved in ethanol (14 mL) which was followed by the addition of piperidine (90 μ L). The reaction mixture was refluxed for 6–14 h (monitored by TLC). The ethanol was evaporated, and the residue was dissolved in ethylacetate (100 mL) and washed with water (50 mL) and brine (50 mL). The organic layer was dried over Na₂SO₄, filtered, and concentrated to give the crude product. The obtained solid was further purified by crystallization from ethanol or by column chromatography to yield the desired compound.

(Z)-5-(3,4-Dihydroxybenzylidene)-3-phenyl-2-thioxoimidazolidin-4-one (31). Prepared from 3,4-dihydroxybenzaldehyde and compound 27 (0.14 g, 0.73 mmol, 1.3 equiv) following the general procedure B. Yield 51%; mp 214–220 °C; ¹H NMR (600 MHz, DMSO-*d*₆) δ 7.48–7.45 (m, 2H), 7.41–7.37 (m, 2H), 7.32 (d, *J* = 7.8 Hz, 2H), 7.14–7.13 (m, 1H), 6.76 (d, *J* = 8.4 Hz, 1H), 6.54 (s, 1H); ¹³C NMR (150 MHz, DMSO-*d*₆) δ 178.7, 169.8, 146.0, 145.4, 139.9, 137.1, 129.0, 128.2, 126.7, 123.3, 117.4, 111.6; HRMS-ESI(–) *m/z* calcd for C₁₆H₁₁N₂O₃S 311.0490 [M – H][–], found 311.0505.

(Z)-4-(5-Oxo-1-phenyl-2-thioxoimidazolidin-4-ylidene)methylbenzoic Acid (32). Prepared from 4-formylbenzoic acid and compound 27 (0.14 g, 0.73 mmol, 1.3 equiv) following the general procedure B. Yield 78%; mp > 300 °C; ¹H NMR (600 MHz, DMSO-*d*₆) δ 13.07 (s, 1H), 12.70 (s, 1H), 7.96 (d, *J* = 8.4 Hz, 2H), 7.89 (d, *J* = 8.4 Hz, 2H), 7.52–7.46 (m, 3H), 7.38 (d, *J* = 8.4 Hz, 2H), 6.70 (s, 1H); ¹³C NMR (150 MHz, DMSO-*d*₆) δ 179.6, 167.3, 164.4, 137.2, 133.7, 131.1, 130.6, 129.9, 129.3, 129.2, 111.3; HRMS-ESI(–) *m/z* calcd for C₁₆H₁₁N₂O₃S 323.0490 [M – H][–], found 323.0490.

(Z)-5-(4-Fluorobenzylidene)-3-phenyl-2-thioxoimidazolidin-4-one (33). Prepared from 4-fluorobenzaldehyde and compound 27 (0.14 g, 0.73 mmol, 1.3 equiv) following the general procedure B. Yield: 74%. ¹H NMR (600 MHz, DMSO-*d*₆) δ 12.69 (s, 1H), 7.98 (m, 2H), 7.61 (m, 2H), 7.37–7.56 (m, 5H), 6.78 (s, 1H).

(Z)-5-((1H-Indol-5-yl)methylene)-3-phenyl-2-thioxoimidazolidin-4-one (34). Prepared from 1H-indole-5-carbaldehyde and compound 27 (0.14 g, 0.73 mmol, 1.3 equiv) following the general procedure B. Yield 58%; mp 254–259 °C; ¹H NMR (600 MHz, DMSO-*d*₆) δ 12.25 (s, 1H), 11.33 (s, 1H), 8.14 (s, 1H), 7.54–7.36 (m, 8 H), 6.80 (s, 1H), 6.50 (s, 1H); ¹³C NMR (150 MHz, DMSO-*d*₆) δ 178.1, 164.4, 137.0, 133.9, 129.3, 129.2, 129.0, 128.6, 127.3, 124.7, 124.1, 123.9, 123.7, 116.8, 112.3, 102.5; HRMS-ESI(–) *m/z* calcd for C₁₈H₁₂N₃OS 318.0701 [M – H][–], found 318.0708.

(Z)-1-Benzyl-5-(3,4-dihydroxybenzylidene)-2-thioxoimidazolidin-4-one (35). Prepared from 3,4-dihydroxybenzaldehyde and compound 28 (0.15 g, 0.73 mmol, 1.3 equiv) following the general procedure B. Yield 63%; mp 252–255 °C; ¹H NMR (600 MHz, CD₃OD) δ 7.64 (s, 1H), 7.26–7.22 (m, 4H), 7.18–7.13 (m, 2H), 6.60 (d, *J* = 8.4 Hz, 1H), 6.27 (s, 1H), 5.28 (s, 2H); ¹³C NMR (150 MHz, CD₃OD) δ 175.9, 162.8, 147.9, 144.4, 135.6, 128.4, 127.2, 126.7, 125.0, 124.3,

123.3, 117.4, 114.5, 45.7; HRMS-ESI(-) m/z calcd for $C_{17}H_{13}N_2O_3S$ 325.0647 $[M - H]^-$, found 325.0637.

(Z)-4-((3-Benzyl-5-oxo-2-thioxoimidazolidin-4-ylidene)methyl)benzoic acid (36). Prepared from 4-formylbenzoic acid and compound **28** (0.15 g, 0.73 mmol, 1.3 equiv) following the general procedure B. Yield 64%; mp 209–211 °C; 1H NMR (600 MHz, DMSO- d_6) δ 7.91 (d, $J = 8.4$ Hz, 2H), 7.79 (d, $J = 8.4$ Hz, 2H), 7.33–7.32 (m, 5H), 6.49 (s, 1H), 5.35 (s, 2H); ^{13}C NMR (150 MHz, DMSO- d_6) δ 169.6, 165.9, 137.7, 136.4, 134.7, 130.4, 130.2, 129.0, 127.7, 127.4, 118.6, 43.7; HRMS-ESI(-) m/z calcd for $C_{18}H_{13}N_2O_3S$ 337.0647 $[M - H]^-$, found 337.0624.

(Z)-1-Benzyl-5-(4-fluorobenzylidene)-2-thioxoimidazolidin-4-one (37). Prepared from 4-fluorobenzaldehyde and compound **28** (0.15 g, 0.73 mmol, 1.3 equiv) following the general procedure B. Yield 47%; mp 135–138 °C; 1H NMR (600 MHz, DMSO- d_6) δ 12.62 (s, 1H), 8.02 (m, 2H), 7.24–7.34 (m, 5H), 7.20 (m, 2H), 6.64 (s, 1H), 5.32 (s, 2H); ^{13}C NMR (150 MHz, DMSO- d_6) δ 177.1, 163.2, 162.1, 135.8, 133.6, 133.5, 129.1, 128.8, 128.5, 127.9, 127.4, 119.8, 115.6, 45.7; HRMS-ESI(-) m/z calcd for $C_{16}H_{11}N_2O_3S$ 311.0654 $[M - H]^-$, found 311.0664.

(Z)-5-((1H-Indol-5-yl)methylene)-1-benzyl-2-thioxoimidazolidin-4-one (38). Prepared from 1H-indole-5-carbaldehyde and compound **28** (0.15 g, 0.73 mmol, 1.3 equiv) following the general procedure B. Yield 41%; 1H NMR (600 MHz, DMSO- d_6) δ 12.49 (s, 1H), 12.29 (s, 1H), 8.32 (s, 1H), 7.83 (d, 1H), 7.23–7.36 (m, 7H), 6.75 (s, 1H), 6.44 (s, 1H), 5.40 (s, 2H).

(Z)-5-(3,4-Dihydroxybenzylidene)thiazolidine-2,4-dione (39). Prepared from 3,4-dihydroxybenzaldehyde and commercially available 2,4-thiazolidinedione **29** (0.15 g, 1.3 mmol, 1.3 equiv) following the general procedure B. Yield 65%; mp > 300 °C; 1H NMR (600 MHz, DMSO- d_6) δ 9.47 (br, 2H), 7.37 (s, 1H), 6.97 (s, 1H), 6.87 (d, $J = 7.8$ Hz, 1H), 6.81 (d, $J = 7.8$ Hz, 1H); ^{13}C NMR (150 MHz, DMSO- d_6) δ 174.6, 171.9, 148.0, 146.1, 129.3, 125.8, 124.5, 123.4, 116.7, 116.5; HRMS-ESI(-) m/z calcd for $C_{10}H_6NO_4S$ 236.0018 $[M - H]^-$, found 236.0016.

(Z)-4-((2,4-Dioxothiazolidin-5-ylidene)methyl)benzoic Acid (40). Prepared from 4-formylbenzoic acid and commercially available 2,4-thiazolidinedione **29** (0.15 g, 1.3 mmol, 1.3 equiv) following the general procedure B. Yield 63%; mp > 300 °C; 1H NMR (600 MHz, DMSO- d_6) δ 13.17 (s, 1H), 12.68 (s, 1H), 8.02 (d, $J = 8.4$ Hz, 2H), 7.80 (s, 1H), 7.67 (d, $J = 8.4$ Hz, 2H); ^{13}C NMR (150 MHz, DMSO- d_6) δ 168.1, 167.6, 167.0, 137.4, 132.1, 130.9, 130.8, 130.7, 130.3, 126.4; HRMS-ESI(-) m/z calcd for $C_{11}H_6NO_4S$ 248.0018 $[M - H]^-$, found 247.9998.

(Z)-5-(4-Fluorobenzylidene)thiazolidine-2,4-dione (41). Prepared from 4-fluorobenzaldehyde and commercially available 2,4-thiazolidinedione **29** (0.15 g, 1.3 mmol, 1.3 equiv) following the general procedure B. Yield 78%; mp > 300 °C; 1H (600 MHz, DMSO- d_6) δ 7.55–7.54 (m, 2H), 7.28 (s, 1H), 7.23–7.26 (m, 2H); ^{13}C NMR (150 MHz, DMSO- d_6) δ 181.8, 175.2, 162.7, 161.1, 135.2, 132.6, 131.5, 122.4, 116.3, 116.1; HRMS-ESI(-) m/z calcd for $C_{10}H_5NO_2SF$ 222.0025 $[M - H]^-$, found 222.0026.

(Z)-5-((1H-Indol-5-yl)methylene)thiazolidine-2,4-dione (42). Prepared from 1H-indole-5-carbaldehyde and commercially available 2,4-thiazolidinedione **29** (0.15 g, 1.3 mmol, 1.3 equiv) following the general procedure B. Yield 67%; mp > 300 °C; 1H (600 MHz, DMSO- d_6) δ 12.42 (s, 1H), 11.41 (s, 1H), 7.87 (s, 1H), 7.82 (s, 1H), 7.51 (d, $J = 8.4$ Hz, 1H), 7.42 (s, 1H), 7.31 (d, $J = 8.4$ Hz, 1H); ^{13}C NMR (150 MHz, DMSO- d_6) δ 168.7, 167.9, 137.3, 134.8, 134.7, 128.6, 127.9, 127.7, 124.4, 124.2, 123.4, 119.2; HRMS-ESI(-) m/z calcd for $C_{12}H_7N_2O_2S$ 243.0228 $[M - H]^-$, found 243.0232.

General Procedure C for Aldol Condensation.⁵⁵ To a mixture of aromatic aldehyde (1 equiv), **30** (1 equiv), and sodium acetate (1 equiv) was added glacial acetic acid (3 mL). The mixture was heated under reflux for 2 h and then cooled to room temperature and poured into cold water. The yellow solid precipitate was collected by filtration. The obtained solid was further purified by crystallization from ethanol or by column chromatography to yield the desired compound.

(Z)-5-(3,4-Dihydroxybenzylidene)-4-thioxothiazolidin-2-one (43). Prepared from 3,4-dihydroxybenzaldehyde following the general

procedure C. Yield 63%; mp 210–214 °C; 1H NMR (600 MHz, DMSO- d_6) δ 13.62 (s, 1H), 7.92 (s, 1H), 7.05–7.03 (m, 2H), 6.86 (d, $J = 8.4$ Hz, 1H); ^{13}C NMR (150 MHz, DMSO- d_6) δ 195.2, 171.1, 150.1, 146.5, 137.9, 125.9, 125.3, 117.0, 116.9; HRMS-ESI(-) m/z calcd for $C_{10}H_6NO_3S_2$ 251.9789 $[M - H]^-$, found 251.9803.

(Z)-4-((2-Oxo-4-thioxothiazolidin-5-ylidene)methyl)benzoic Acid (44). Prepared from 4-formylbenzoic acid following the general procedure C. Yield 67%; mp > 300 °C; 1H NMR (600 MHz, DMSO- d_6) δ 13.22 (s, 1H), 8.08 (s, 1H), 8.02 (d, $J = 8.4$ Hz, 2H), 7.75 (d, $J = 8.4$ Hz, 2H); ^{13}C NMR (150 MHz, DMSO- d_6) δ 195.8, 170.8, 166.9, 137.6, 134.6, 132.4, 132.1, 130.8, 130.5; HRMS-ESI(-) m/z calcd for $C_{11}H_6NO_3S_2$ 263.9789 $[M - H]^-$, found 263.9808.

(Z)-5-(4-Fluorobenzylidene)-4-thioxothiazolidin-2-one (45). Prepared from 4-fluorobenzaldehyde following the general procedure C. Yield 53%; mp 192–194 °C; 1H NMR (600 MHz, CD₃OD) δ 8.01 (s, 1H), 7.57–7.55 (m, 2H), 7.16–7.13 (m, 2H); ^{13}C NMR (150 MHz, CD₃OD) δ 195.5, 170.2, 164.5, 162.8, 134.3, 132.5, 132.4, 130.4, 116.1, 115.9; HRMS-ESI(-) m/z calcd for $C_{12}H_7N_2OS_2$ 237.9797 $[M - H]^-$, found 237.9827.

(Z)-5-((1H-Indol-5-yl)methylene)-4-thioxothiazolidin-2-one (46). Prepared from 1H-indole-5-carbaldehyde following the general procedure C. Yield 49%; mp 192–194 °C; 1H NMR (600 MHz, CD₃OD) δ 8.24 (s, 1H), 7.81 (s, 1H), 7.41 (d, $J = 8.4$ Hz, 1H), 7.41 (d, $J = 8.4$ Hz, 1H), 7.24 (d, $J = 3.1$ Hz, 1H), 6.49 (d, $J = 3.1$ Hz, 1H); ^{13}C NMR (150 MHz, CD₃OD) δ 196.2, 172.8, 139.2, 137.6, 128.7, 126.5, 125.8, 125.2, 124.5, 123.7, 111.8, 102.4; HRMS-ESI(-) m/z calcd for $C_{12}H_7N_2OS_2$ 259.0000 $[M - H]^-$, found 259.0007.

(Z)-5-(2,3-Dihydroxybenzylidene)-4-thioxothiazolidin-2-one (47). Prepared from 2,3-dihydroxybenzaldehyde following the general procedure C. Yield 69%; mp 188–192 °C; 1H NMR (600 MHz, DMSO- d_6) δ 12.89 (s, 1H), 9.99 (s, 1H), 9.76 (s, 1H), 8.49 (s, 1H), 6.92 (d, $J = 6.6$ Hz, 1H), 6.88 (d, $J = 6.6$ Hz, 1H), 6.76 (t, $J = 8.4$ Hz, 1H); ^{13}C NMR (150 MHz, DMSO- d_6) δ 195.8, 171.7, 161.6, 147.6, 146.3, 132.7, 128.5, 121.7, 120.1, 118.6; HRMS-ESI(-) m/z calcd for $C_{10}H_6NO_3S_2$ 251.9789 $[M - H]^-$, found 251.9763.

(Z)-5-(2,4-Dihydroxybenzylidene)-4-thioxothiazolidin-2-one (48). Prepared from 2,4-dihydroxybenzaldehyde following the general procedure C. Yield 62%; mp 238–241 °C; 1H NMR (600 MHz, CD₃OD) δ 8.53 (s, 1H), 7.24 (d, $J = 8.4$ Hz, 1H), 6.31 (d, $J = 8.4$ Hz, 1H), 6.26 (s, 1H); ^{13}C NMR (150 MHz, CD₃OD) δ 194.3, 170.4, 161.7, 159.9, 132.1, 129.1, 112.8, 107.1, 101.2; HRMS-ESI(-) m/z calcd for $C_{10}H_6NO_3S_2$ 251.9789 $[M - H]^-$, found 251.9776.

(Z)-5-(2,5-Dihydroxybenzylidene)-4-thioxothiazolidin-2-one (49). Prepared from 2,5-dihydroxybenzaldehyde following the general procedure C. Yield 62%; mp 226–229 °C; 1H NMR (600 MHz, DMSO- d_6) δ 13.7 (s, 1H), 9.49 (s, 1H), 9.18 (s, 1H), 8.41 (s, 1H), 6.82–6.80 (m, 3H); ^{13}C NMR (150 MHz, DMSO- d_6) δ 195.7, 175.1, 152.2, 150.6, 132.3, 128.0, 121.4, 121.0, 117.6; HRMS-ESI(-) m/z calcd for $C_{10}H_6NO_3S_2$ 251.9789 $[M - H]^-$, found 251.9781.

(Z)-4-Thioxo-5-(2,3,4-trihydroxybenzylidene)thiazolidin-2-one (50). Prepared from 2,3,4-trihydroxybenzaldehyde following the general procedure C. Yield 60%; mp 227–231 °C; 1H NMR (600 MHz, CD₃OD) δ 8.54 (s, 1H), 6.81 (d, $J = 8.4$ Hz, 1H), 6.36 (d, $J = 8.4$ Hz, 1H); ^{13}C NMR (150 MHz, CD₃OD) δ 195.1, 171.4, 149.8, 148.6, 133.2, 132.9, 124.9, 119.9, 114.3, 107.5; HRMS-ESI(-) m/z calcd for $C_{10}H_6NO_4S_2$ 267.9738 $[M - H]^-$, found 267.9759.

(Z)-5-(4-Hydroxybenzylidene)-4-thioxothiazolidin-2-one (51). Prepared from 4-hydroxybenzaldehyde following the general procedure C. Yield 69%; mp 241–244 °C; 1H NMR (600 MHz, DMSO- d_6) δ 13.66 (s, 1H), 10.50 (s, 1H), 8.03 (s, 1H), 7.54 (d, $J = 8.9$ Hz, 2H), 6.92 (d, $J = 8.9$ Hz, 2H); ^{13}C NMR (150 MHz, DMSO- d_6) δ 195.3, 171.1, 161.2, 137.5, 137.4, 133.8, 133.7, 126.2, 124.9, 117.2; HRMS-ESI(-) m/z calcd for $C_{10}H_6NO_3S_2$ 235.9840 $[M - H]^-$, found 235.9858.

(Z)-5-(3-Hydroxybenzylidene)-4-thioxothiazolidin-2-one (52). Prepared from 3-hydroxybenzaldehyde following the general procedure C. Yield 67%; mp 222–225 °C; 1H NMR (600 MHz, DMSO- d_6) δ 13.86 (s, 1H), 9.86 (s, 1H), 7.97 (s, 1H), 7.32–7.31 (m, 1H), 7.10 (d, $J = 6.0$ Hz, 1H), 7.02 (s, 1H), 6.91 (d, $J = 7.8$ Hz, 1H); ^{13}C NMR (150 MHz, DMSO- d_6) δ 195.8, 175.0, 171.0, 158.4, 136.5, 134.9,

130.9, 122.4, 118.8, 116.5; HRMS-ESI(−) m/z calcd for $C_{10}H_6NO_2S_2$ 235.9840 $[M - H]^-$, found 235.9860.

(Z)-5-(2-Hydroxybenzylidene)-4-thioxothiazolidin-2-one (53). Prepared from 2-hydroxybenzaldehyde following the general procedure C. Yield 63%; mp 210–212 °C; 1H NMR (600 MHz, DMSO- d_6) δ 13.73 (s, 1H), 10.62 (s, 1H), 8.45 (s, 1H), 7.40 (d, $J = 7.2$ Hz, 1H), 7.35–7.32 (m, 1H), 6.97–6.92 (m, 2H); ^{13}C NMR (150 MHz, DMSO- d_6) δ 195.8, 186.0, 175.0, 171.2, 158.7, 132.2, 128.6, 126.0, 120.4; HRMS-ESI(−) m/z calcd for $C_{10}H_6NO_2S_2$ 235.9840 $[M - H]^-$, found 235.9855.

(Z)-5-(4-Hydroxy-3-methoxybenzylidene)-4-thioxothiazolidin-2-one (54). Prepared from 4-hydroxy-3-methoxybenzaldehyde following the general procedure C. Yield 83%; mp 195–198 °C; 1H NMR (600 MHz, DMSO- d_6) δ 13.66 (s, 1H), 10.1 (s, 1H), 8.04 (s, 1H), 7.22 (s, 1H), 7.16 (d, $J = 8.4$ Hz, 1H), 6.93 (d, $J = 8.4$ Hz, 1H), 2.47 (s, 3H); ^{13}C NMR (150 MHz, DMSO- d_6) δ 195.2, 171.0, 150.8, 148.6, 137.8, 126.4, 125.6, 123.3, 116.8, 115.2, 56.5; HRMS-ESI(−) m/z calcd for $C_{11}H_8NO_3S_2$ 265.9946 $[M - H]^-$, found 265.9937.

(Z)-5-(4-Hydroxy-3,5-dimethoxybenzylidene)-4-thioxothiazolidin-2-one (55). Prepared from 4-hydroxy-3,5-dimethoxybenzaldehyde following the general procedure C. Yield 72%; mp 222–226 °C; 1H NMR (600 MHz, DMSO- d_6) δ 13.63 (s, 1H), 8.01 (s, 1H), 6.93 (s, 1H), 3.81 (s, 6H); ^{13}C NMR (150 MHz, DMSO- d_6) δ 195.0, 175.0, 171.0, 148.7, 140.1, 138.1, 126.6, 124.2, 109.3, 108.9, 56.6, 56.3; HRMS-ESI(−) m/z calcd for $C_{12}H_{10}NO_4S_2$ 296.0051 $[M - H]^-$, found 296.0069.

(Z)-2-((2-Oxo-4-thioxothiazolidin-5-ylidene)methyl)benzoic Acid (56). Prepared from 2-formylbenzoic acid following the general procedure C. Yield 58%; mp 206–209 °C; 1H NMR (600 MHz, CD $_3$ OD) δ 8.71 (s, 1H), 8.09 (d, $J = 7.8$ Hz, 1H), 7.67–7.65 (m, 1H), 7.62 (d, $J = 7.2$ Hz, 1H), 7.55–7.53 (m, 1H); ^{13}C NMR (150 MHz, CD $_3$ OD) δ 195.6, 171.2, 167.9, 137.0, 135.3, 133.1, 132.0, 131.9, 131.5, 130.7, 128.6; HRMS-ESI(−) m/z calcd for $C_{11}H_6NO_3S_2$ 263.9789 $[M - H]^-$, found 263.9808.

(Z)-3-((2-Oxo-4-thioxothiazolidin-5-ylidene)methyl)benzoic Acid (57). Prepared from 3-formylbenzoic acid following the general procedure C. Yield 58%; mp 242–244 °C; 1H NMR (600 MHz, DMSO- d_6) δ 8.15 (s, 1H), 8.07 (s, 1H), 8.00 (d, $J = 7.2$ Hz, 1H), 7.74 (d, $J = 7.2$ Hz, 1H), 7.52 (t, $J = 8.4$ Hz, 1H); ^{13}C NMR (150 MHz, DMSO- d_6) δ 195.7, 170.7, 166.9, 135.0, 134.2, 132.3, 131.5, 131.2, 130.3, 126.7; HRMS-ESI(−) m/z calcd for $C_{11}H_6NO_3S_2$ 263.9789 $[M - H]^-$, found 263.9807.

Sodium (Z)-4-((2-Oxo-4-thioxothiazolidin-5-ylidene)methyl)benzoate (58). Compound **44** (43 mg, 0.162 mmol) and NaOH (6.5 mg, 0.162 mmol) were dissolved in 0.7 mL (H $_2$ O:MeOH/3:2) and stirred at room temperature for 2 h. The solution was evaporated and dried to afford **58** (43.8 mg, 94%) as a yellow colored compound; mp > 300 °C; 1H NMR (600 MHz, DMSO- d_6) δ 7.99 (d, 2H), 7.97 (s, 1H), 7.55 (d, $J = 7.8$ Hz, 2H); ^{13}C NMR (150 MHz, DMSO- d_6) δ 210.5, 180.2, 170.1, 140.6, 140.5, 136.2, 131.4, 129.9, 128.8, 128.6, 117.1; HRMS-ESI(−) m/z calcd for $C_{11}H_6NO_3S_2$ 263.9789 $[M - Na]^-$, found 263.9838.

Sodium (Z)-2-((2-Oxo-4-thioxothiazolidin-5-ylidene)methyl)benzoate (59). Prepared from **56** following the procedure preparation of **58**. Yield 92%; mp 252–256 °C; 1H NMR (600 MHz, DMSO- d_6) δ 8.68 (s, 1H), 7.62 (d, $J = 7.8$ Hz, 1H), 7.54 (d, $J = 7.8$ Hz, 1H), 7.32 (t, 1H), 7.26 (t, 1H); ^{13}C NMR (150 MHz, DMSO- d_6) δ 210.5, 181.1, 172.5, 144.6, 140.4, 134.7, 131.4, 128.9, 127.3, 117.2; HRMS-ESI(−) m/z calcd for $C_{11}H_6NO_3S_2$ 263.9789 $[M - Na]^-$, found 263.9829.

(Z)-Methyl 4-((2-oxo-4-thioxothiazolidin-5-ylidene)methyl)benzoate (60). Prepared from methyl 4-formylbenzoate following the general procedure C. Yield 90%; mp 187–191 °C; 1H NMR (600 MHz, DMSO- d_6) δ 8.08 (s, 1H), 8.05 (d, $J = 8.4$ Hz, 2H), 7.79 (d, $J = 8.4$ Hz, 2H), 3.86 (s, 3H); ^{13}C NMR (150 MHz, DMSO- d_6) δ 195.8, 170.8, 165.9, 138.1, 134.3, 130.9, 130.4, 52.8; HRMS-ESI(−) m/z calcd for $C_{12}H_8NO_3S_2$ 277.9946 $[M - H]^-$, found 277.9965.

(Z)-Methyl 3-((2-oxo-4-thioxothiazolidin-5-ylidene)methyl)benzoate (61). Prepared from methyl 3-formylbenzoate following the general procedure C. Yield 79%; mp 200–203 °C; 1H NMR (600 MHz, DMSO- d_6) δ 8.19 (s, 1H), 8.12 (s, 1H), 8.04 (d, $J = 7.2$ Hz, 1H), 7.93 (d, $J = 7.2$ Hz, 1H), 7.68 (t, $J = 7.8$ Hz, 1H), 3.88 (s, 3H);

^{13}C NMR (150 MHz, DMSO- d_6) δ 195.7, 170.6, 165.8, 135.2, 134.7, 134.3, 131.3, 131.0, 130.7, 130.4, 52.5; HRMS-ESI(−) m/z calcd for $C_{12}H_8NO_3S_2$ 277.9946 $[M - H]^-$, found 277.9951.

(Z)-5-(2-Bromobenzylidene)-4-thioxothiazolidin-2-one (62). Prepared from 4-bromobenzaldehyde following the general procedure C. Yield 40%; mp 165–168 °C; 1H NMR (600 MHz, CD $_3$ OD) δ 8.28 (s, 1H), 7.64 (d, $J = 8.4$ Hz, 1H), 7.52 (d, $J = 8.4$ Hz, 1H), 7.38 (d, $J = 7.8$ Hz, 1H), 7.25 (d, $J = 7.8$ Hz, 1H); ^{13}C NMR (150 MHz, CD $_3$ OD) δ 195.2, 179.7, 141.0, 134.2, 133.8, 133.4, 131.4, 128.7, 127.8, 125.9; HRMS-ESI(−) m/z calcd for $C_{10}H_5NBrOS_2$ 297.8996 $[M - H]^-$, found 297.8992.

(Z)-5-(4-Nitrobenzylidene)-4-thioxothiazolidin-2-one (63). Prepared from 4-nitrobenzaldehyde following the general procedure C. Yield 58%; mp 192–194 °C; 1H NMR (600 MHz, DMSO- d_6) δ 8.32 (d, $J = 8.4$ Hz, 2H), 8.11 (s, 1H), 7.91 (d, $J = 8.7$ Hz, 2H); ^{13}C NMR (150 MHz, DMSO- d_6) δ 195.3, 172.9, 147.9, 139.9, 131.9, 130.6, 123.8; HRMS-ESI(−) m/z calcd for $C_{10}H_5N_2O_3S_2$ 264.9742 $[M - H]^-$, found 264.9757.

(Z)-4-((2-Oxo-4-thioxothiazolidin-5-ylidene)methyl)benzonitrile (64). Prepared from 4-formylbenzonitrile following the general procedure C. Yield 61%; mp 178–181 °C; 1H NMR (600 MHz, DMSO- d_6) δ 8.03 (s, 1H), 7.75 (d, $J = 8.4$ Hz, 2H), 7.68 (d, 1H, $J = 8.4$ Hz, 2H); ^{13}C NMR (150 MHz, DMSO- d_6) δ 195.7, 170.6, 138.1, 133.5, 133.2, 131.2, 118.8, 112.7; HRMS-ESI(−) m/z calcd for $C_{11}H_5N_2OS_2$ 244.9843 $[M - H]^-$, found 244.9861.

(Z)-5-(Pyridin-3-ylmethylene)-4-thioxothiazolidin-2-one (65). Prepared from nicotinaldehyde following the general procedure C. Yield 84%; mp 255–258 °C; 1H NMR (600 MHz, DMSO- d_6) δ 8.80 (s, 1H), 8.64–8.63 (m, 1H), 8.07 (s, 1H), 8.03 (d, $J = 8.4$ Hz, 1H), 7.56 (dd, $J = 6.0$ Hz, $J = 7.8$ Hz, 1H); ^{13}C NMR (150 MHz, DMSO- d_6) δ 195.7, 170.7, 152.1, 151.1, 136.7, 132.6, 132.2, 130.1, 124.7; HRMS-ESI(−) m/z calcd for $C_9H_5N_2OS_2$ 220.9843 $[M - H]^-$, found 220.9863.

(Z)-5-(Pyridin-4-ylmethylene)-4-thioxothiazolidin-2-one (66). Prepared from isonicotinaldehyde following the general procedure C. Yield 67%; mp 252–254 °C; 1H NMR (600 MHz, DMSO- d_6) δ 8.71 (d, $J = 5.1$ Hz, 2H), 7.97 (s, 1H), 7.59 (d, $J = 5.4$ Hz, 2H); ^{13}C NMR (150 MHz, DMSO- d_6) δ 198.2, 172.4, 153.8, 153.1, 126.1, 124.4, 111.8; HRMS-ESI(−) m/z calcd for $C_9H_5N_2OS_2$ 220.9843 $[M - H]^-$, found 220.9860.

(Z)-5-((5-Phenylthiophen-2-yl)methylene)-4-thioxothiazolidin-2-one (67). Prepared from 5-phenylthiophene-2-carbaldehyde following the general procedure C. Yield 65%; mp 250–255 °C; 1H NMR (600 MHz, DMSO- d_6) δ 13.76 (s, 1H), 8.27 (s, 1H), 7.86 (d, $J = 3.6$ Hz, 1H), 7.78 (d, $J = 7.8$ Hz, 2H), 7.75 (d, $J = 4.2$ Hz, 1H), 7.47 (t, $J = 7.8$ Hz, 2H), 7.41 (d, $J = 7.2$ Hz, 1H); ^{13}C NMR (150 MHz, DMSO- d_6) δ 194.3, 170.0, 151.9, 138.9, 137.6, 132.9, 129.7, 129.2, 127.5, 126.4, 126.5; HRMS-ESI(−) m/z calcd for $C_{14}H_8NOS_2$ 301.9768 $[M - H]^-$, found 301.9779.

(Z)-5-(Benzo[b]thiophen-3-ylmethylene)-4-thioxothiazolidin-2-one (68). Prepared from benzo[b]thiophene-3-carbaldehyde following the general procedure C. Yield 73%; mp 218–221 °C; 1H NMR (600 MHz, DMSO- d_6) δ 13.83 (s, 1H), 8.35 (s, 1H), 8.24 (s, 1H), 8.10 (d, $J = 7.8$ Hz, 1H), 8.01 (d, $J = 7.8$ Hz, 1H), 7.55 (t, $J = 7.2$ Hz, 1H), 7.50 (t, $J = 7.8$ Hz, 1H); ^{13}C NMR (150 MHz, DMSO- d_6) δ 195.2, 170.7, 139.5, 138.4, 132.8, 132.4, 131.1, 130.0, 125.4, 125.6, 123.8, 121.8; HRMS-ESI(−) m/z calcd for $C_{12}H_6NOS_2$ 275.9612 $[M - H]^-$, found 275.9628.

(Z)-5-((1-Methyl-1H-indol-3-yl)methylene)-4-thioxothiazolidin-2-one (69). Prepared from 1-methyl-1H-indole-3-carbaldehyde following the general procedure C. Yield 59%; mp 224–228 °C; 1H NMR (600 MHz, DMSO- d_6) δ 13.38 (s, 1H), 8.39 (s, 1H), 7.97 (s, 1H), 7.84 (d, $J = 8.4$ Hz, 1H), 7.58 (d, $J = 7.8$ Hz, 1H), 7.33 (t, $J = 7.2$ Hz, 1H), 7.28 (t, $J = 7.8$ Hz, 1H), 3.93 (s, 3H); ^{13}C NMR (150 MHz, DMSO- d_6) δ 193.3, 175.0, 170.5, 137.5, 134.2, 128.9, 128.1, 124.1, 123.5, 122.5, 118.7, 111.6, 34.0; HRMS-ESI(−) m/z calcd for $C_{13}H_9N_2OS_2$ 273.0156 $[M - H]^-$, found 273.0171.

(Z)-5-(4-(1H-Tetrazol-5-yl)benzylidene)-4-thioxothiazolidin-2-one (70). Prepared from 4-(1H-tetrazol-5-yl)benzaldehyde³¹ following the general procedure C. Yield 84%; 1H NMR (600 MHz, DMSO- d_6) δ

13.9 (s, 1H), 8.16 (d, $J = 8.4$ Hz, 2H), 8.10 (s, 1H), 7.88 (d, $J = 8.4$ Hz, 2H); ^{13}C NMR (150 MHz, $\text{DMSO}-d_6$) δ 195.7, 170.8, 134.1, 131.7, 128.2, 117.4.

(*Z*)-5-(2-(1*H*-Tetrazol-5-yl)benzylidene)-4-thioxothiazolidin-2-one (**71**). Prepared from 2-(1*H*-tetrazol-5-yl)benzaldehyde⁵⁷ following the general procedure C. Yield 41%; mp 206–210 °C; ^1H NMR (600 MHz, CD_3OD) δ 8.26 (s, 1H), 7.79 (d, $J = 7.8$ Hz, 1H), 7.70 (d, $J = 7.8$ Hz, 1H), 7.62 (t, $J = 7.2$ Hz, 1H), 7.56 (t, $J = 7.2$ Hz, 1H); ^{13}C NMR (150 MHz, CD_3OD) δ 195.1, 170.9, 133.7, 133.2, 131.0, 130.1, 130.0, 128.2, 112.2, 109.9; HRMS-ESI(–) m/z calcd for $\text{C}_{11}\text{H}_6\text{N}_5\text{O}_2\text{S}_2$ 288.0014 [$\text{M} - \text{H}$][–], found 288.0026.

(*Z*)-2-((2-Oxo-4-thioxothiazolidin-5-ylidene)methyl)benzenesulfonic Acid (**72**). Prepared from 2-formylbenzenesulfonic acid sodium salt following the general procedure C. Yield 27%; mp 218–221 °C; ^1H NMR (600 MHz, CD_3OD) δ 9.02 (s, 1H), 8.04 (d, $J = 7.8$ Hz, 1H), 7.63 (d, $J = 7.8$ Hz, 1H), 7.38 (t, $J = 7.8$ Hz, 1H), 7.25 (t, $J = 7.8$ Hz, 1H); ^{13}C NMR (150 MHz, CD_3OD) δ 192.5, 176.7, 144.8, 134.6, 132.8, 130.2, 129.0, 128.1, 127.5, 127.0; HRMS-ESI(–) m/z calcd for $\text{C}_{10}\text{H}_6\text{NO}_4\text{S}_3$ 299.9459 [$\text{M} - \text{H}$][–], found 299.9445.

(*Z*)-Diethyl (4-((2-Oxo-4-thioxothiazolidin-5-ylidene)methyl)phenyl)phosphonate (**73**). Prepared from diethyl (4-formylphenyl)phosphonate⁵⁸ following the general procedure C. Yield 51%; mp 151–154 °C; ^1H NMR (600 MHz, CD_3OD) δ 8.05 (s, 1H), 7.81–7.77 (m, 2H), 7.68–7.66 (m, 2H), 4.04 (q, $J = 6.6$ Hz, $J = 14.4$ Hz, 4H), 1.24 (t, $J = 7.2$ Hz, 1H); ^{13}C NMR (150 MHz, CD_3OD) δ 195.3, 138.1, 133.3, 132.3, 132.0, 131.9, 129.9, 129.8, 62.6, 15.2; HRMS-ESI(–) m/z calcd for $\text{C}_{14}\text{H}_{15}\text{NO}_4\text{P}_2$ 356.0180 [$\text{M} - \text{H}$][–], found 356.0213.

(*Z*)-4-((2-Oxo-4-thioxothiazolidin-5-ylidene)methyl)phenylphosphonic Acid (**74**).⁵⁹ To a solution of compound **73** (0.05 g, 0.14 mmol) in dry DCM (3 mL) was added TMSBr (0.3 g, 1.9 mmol) and stirred at room temperature for 16 h. The solvent was evaporated under vacuum and coevaporated with methanol. The reaction mixture was poured into water, and the solid separated was filtered off and washed with water to afford **74** (0.035 g, 84%). Mp 225–228 °C; ^1H NMR (600 MHz, CD_3OD) δ 8.14 (s, 1H), 7.91–7.88 (m, 2H), 7.71–7.61 (m, 2H); ^{13}C NMR (150 MHz, CD_3OD) δ 195.4, 170.2, 136.8, 133.9, 133.9, 131.6, 131.3, 131.2, 129.6, 129.5; HRMS-ESI(–) m/z calcd for $\text{C}_{10}\text{H}_7\text{NO}_4\text{P}_2$ 299.9554 [$\text{M} - \text{H}$][–], found 299.9580.

Biology. Tdp1 Gel-Based Assay.¹¹ $5'$ - ^{32}P -labeled DNA substrate (1 nM; N14Y; 5'-GATCTAAAAGACTT-pY-3') was incubated with 0.02 nM Tdp1 in the absence or presence of inhibitor for 15 min at room temperature in an assay buffer containing PBS IX, pH 7.4, 80 mM KCl, and 0.01% Tween-20. Reactions were terminated by the addition of 1 volume of gel loading buffer [99.5% (v/v) formamide, 5 mM EDTA, 0.01% (w/v) xylene cyanol, and 0.01% (w/v) bromophenol blue]. Samples were subjected to a 16% denaturing PAGE, and gels were exposed after drying to a PhosphorImager screen (GE Healthcare). Gel images were scanned using a Typhoon 8600 (GE Healthcare), and densitometric analyses were performed using the ImageQuant software (GE Healthcare).

WCE Tdp1 Gel-Based Assay. An amount of 1×10^7 DT40 knockout cells for chicken Tdp1 and complemented with human Tdp1 were collected and washed once with PBS. The supernatant was removed, and the pellet was resuspended with 50 μL of CellLytic M Cell Lysis Reagent (SIGMA C2978), pipetting several times over ice. After 15 min, the lysate was centrifuged at 12 000g for 10 min, and the protein concentration of the supernatant was determined using the Protein Assay Dye Reagent Concentrate (Bio-Rad #500–0006). $5'$ - ^{32}P -labeled DNA substrate (1 nM; N14Y; 5'-GATCTAAAAGACTT-pY-3') was incubated with 1 $\mu\text{g}/\text{mL}$ of whole cell extract in the absence or presence of inhibitor for 15 min at room temperature in an assay buffer containing 25 mM Tris HCl, pH 7.5, 80 mM KCl, 2 mM EDTA, 1 mM DTT, 40 $\mu\text{g}/\text{mL}$ of BSA, and 0.01% Tween-20. Reactions were terminated by the addition of 1 volume of gel loading buffer [99.5% (v/v) formamide, 5 mM EDTA, 0.01% (w/v) xylene cyanol, and 0.01% (w/v) bromophenol blue]. Samples were subjected to a 16% denaturing PAGE, and gels were exposed after drying to a PhosphorImager screen (GE Healthcare). Gel images were scanned

using a Typhoon 8600 (GE Healthcare), and densitometric analyses were performed using the ImageQuant software (GE Healthcare).

Surface Plasmon Resonance Analysis. Materials. Coupling reagents *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), and ethanolamine were purchased from GE Healthcare (Piscataway, NJ). Neutravidin was obtained from Pierce.

Binding experiments were performed on a Biacore T200 instrument (GE, Piscataway, NJ). Tdp1 was amine coupled to a CM5 sensor chip (GE Healthcare, Piscataway, NJ). The amine groups in the active site of Tdp1 were protected from modification by binding a 14 base oligonucleotide to Tdp1 during the coupling step. Specifically 1 μM Tdp1 was incubated with 2 μM of a 14 base oligonucleotide containing a phosphate group at the 3' end (GATCTAAAAGACTT) in 10 mM sodium acetate pH 4.5 for 20 min. The CM5 chip surface was activated for 7 min with 0.1 M NHS and 0.4 M EDC at a flow rate of 20 $\mu\text{L}/\text{min}$, and the Tdp1–oligonucleotide mixture was injected until approximately 6000 RUs were attached. Activated amine groups were quenched with an injection of 1 M solution of ethanolamine pH 8.0 for 7 min. Any bound oligonucleotide was removed by washing the surface with 1 M NaCl. A reference surface was prepared in the same manner without coupling of Tdp1. Using the same approach, 8000 RUs of Neutravidin were amine coupled to flow cells 3 and 4. A 14 base oligonucleotide modified with a biotin at the 5' end and a tyrosine at the 3' end (biotin-GATCTAAAAGACTT-Tyr) was captured in the flow cell 4. A dilution series of **50** (50, 25, 6.25, 3.12, 1.56, 0.78, and 0.39 μM) was prepared in running buffer [50 mM Tris/HCl, 80 mM KCl, 0.01% tween 20 (v/v), 5% DMSO (v/v), 2 mM EDTA, 1 mM DTT, 40 $\mu\text{g}/\text{mL}$ of BSA pH 7.5] and injected over all flow cells at 30 $\mu\text{L}/\text{min}$ at 25 °C. Following compound injections, the surface was regenerated with a 30 s injection of 1 M NaCl, a 30 s injection of 50% DMSO (v/v), and a 30 s running buffer injection. Each cycle of compound injection was followed by a buffer cycle for referencing purposes. A DMSO calibration curve was included to correct for refractive index mismatches between the running buffer and compound dilution series.

Modeling and Docking. The study was carried out using the Schrodinger modeling suite.⁶⁰ The X-ray crystallographic structure of human Tdp1 in complex with vanadate, DNA, and human Top1 derived peptide (PDB: 1NOP)²⁵ was used as the starting point of the study. The crystallographic vanadate ion was replaced with a pyrophosphate linker to generate the complete Top1–DNA covalent intermediate. All missing hydrogen atoms were added based on ionization states determined by PROPKA.⁶¹ Restraint energy was minimized with the implicit solvent model using the OPLS 2005 force field.⁶² All Tdp1 inhibitors were built and prepared with Ligprep to enumerate all possible ionization states, tautomers, and low ring energy conformers. The docking studies were carried out using Glide at standard precision without any constraint within the catalytic binding site of Tdp1. The van der Waals radii of nonpolar atoms for each of the ligands were scaled by a factor of 0.8 to account for structure variability to specific ligand binding. Visual inspection was employed to identify the potential mode of binding that best corroborates with our observed SAR study.

■ ASSOCIATED CONTENT

📄 Supporting Information

Docking of previously reported inhibitors (**1–3**). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

Tdp1, tyrosyl-DNA phosphodiesterase; Top1, topoisomerase I; SAR, structure-activity relationship; CPT, camptothecin; PLD, phospholipase D; HTS, high-throughput screening; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; WCE, whole cell extracts; SPR, Surface Plasmon Resonance

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