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Discovery of Potent and Selective Benzothiazole Hydrazone Inhibitors of Bcl-X_L

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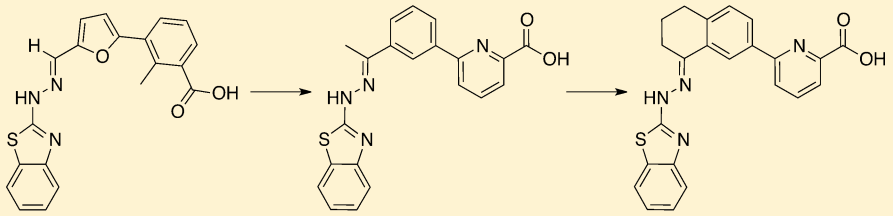
Supporting Information



ABSTRACT: Developing potent molecules that inhibit Bcl-2 family mediated apoptosis affords opportunities to treat cancers via reactivation of the cell death machinery. We describe the hit-to-lead development of selective Bcl-X_L inhibitors originating from a high-throughput screening campaign. Small structural changes to the hit compound increased binding affinity more than 300-fold (to IC_{50} < 20 nM). This molecular series exhibits drug-like characteristics, low molecular weights (M_w < 450), and unprecedented selectivity for Bcl-X_L. Surface plasmon resonance experiments afford strong evidence of binding affinity within the hydrophobic groove of Bcl-X_L. Biological experiments using engineered Mcl-1 deficient mouse embryonic fibroblasts (MEFs, reliant only on Bcl-X_L for survival) and Bax/Bak deficient MEFs (insensitive to selective activation of Bcl-2-driven apoptosis) support a mechanism-based induction of apoptosis. This manuscript describes the first series of selective small-molecule inhibitors of Bcl-X_L and provides promising leads for the development of efficacious therapeutics against solid tumors and chemoresistant cancer cell lines.

INTRODUCTION

The Bcl-2 family of proteins plays a central role in the regulation of apoptosis.[1] The interplay between its members commits cells toward survival or death. It participates in the removal of unwanted cells during embryogenesis and in maintenance of cell homeostasis in mature organisms. The link between the Bcl-2 family of proteins and cancer is now well established and amply documented.[2] Pro-survival proteins such as Bcl-2, Bcl-X_L, or Mcl-1, which promote cell survival, are overexpressed in a large number of tumor cell lines, thereby preventing these cells from undergoing natural apoptosis. Failure to remove damaged cells carrying potentially oncogenic DNA damage is a hallmark of cancer evolution.[3] Inability to respond to apoptotic stimuli has also been linked to metastasis and resistance to currently used cytotoxic agents and targeted therapeutics. Indeed, many such drugs rely on a functional Bcl-2 signaling pathway to exert their anticancer activity.[4] This body of evidence has made the pro-survival members of the Bcl-2 family of proteins a novel and promising target for treating cancers. The aim of this strategy is to target the core mechanism controlling cell death and thus re-establish the tumor cell's sensitivity to death signals.

The Bcl-2 family comprises three subfamilies, defined by their biological function (pro- or antiapoptotic) and their structure (presence of some or all the Bcl-2-Homology domains BH1 to BH4). The pro-survival subclass (Bcl-2 itself, Mcl-1, Bcl-X_L, and Bcl-w) maintains cell survival by keeping in check the pro-apoptotic activity of a second subclass, the pro-apoptotic Bax and Bak proteins. Their activity is focused on the mitochondrial membrane, where Bak is constitutively anchored and Bax converges upon apoptotic activation. A second class of pro-apoptotic proteins, the BH3-only proteins, responds to diverse cellular stress signals including DNA damage and acts as the upstream triggers of the "intrinsic" apoptotic pathway. The precise mechanism of apoptosis induction by the Bcl-2 family of proteins is still debated,[5] but all current models agree that upon pro-apoptotic signaling, BH3-only proteins are released and

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Chart 1. Structure of Previously Reported BH3-Mimetics Acylsulfonamide Derivatives 1, 2, and 3

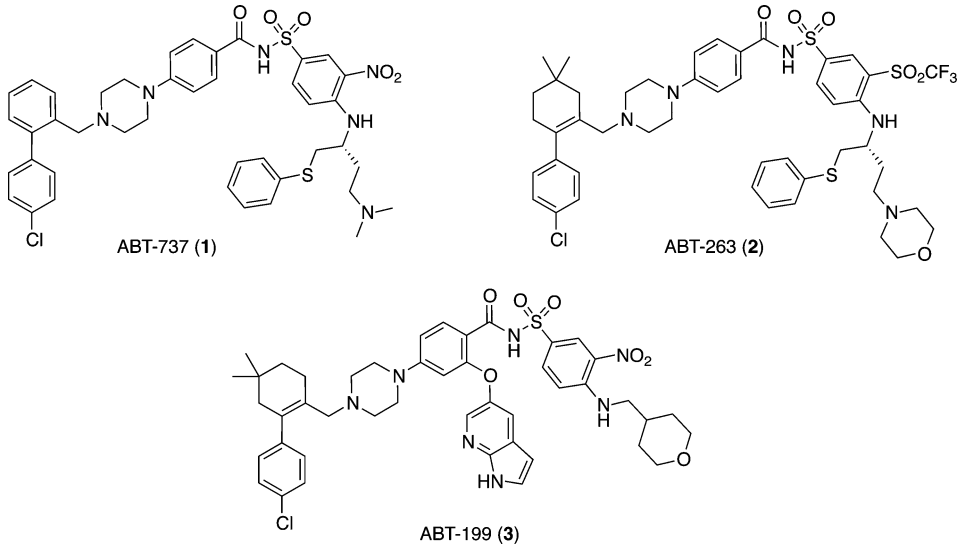
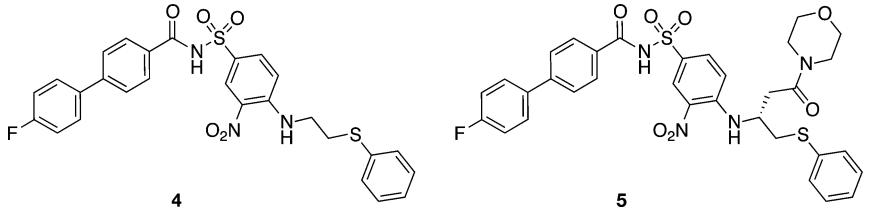


Table 1. Binding Affinities for Bcl-X_L: A Comparison between Published Data and LPA IC_{50} Values



4 5

assay	1	4	5
LPA (IC_{50}, μM)[a]	0.0005	2.2	0.15
FP (K_i, μM)	<0.0005[b]	0.036[c]	

[a]Average values from multiple separate repeats. [b]From ref 13. [c]From ref 27. LPA: luminescence proximity assay. FP: fluorescence polarization.

induce the downstream apoptotic response (cytochrome *c* release, caspase activation, cellular dismantlement) by indirectly or directly activating Bax/Bak.[6,7] Bax and Bak are therefore essential mediators of apoptosis induced by the BH3-only proteins.[8,9]

Replicating the pro-apoptotic activity of the BH3-only proteins using small molecules targeting the pro-survival subclass of proteins is currently an important goal of anticancer drug discovery. Despite major advances, only four small molecules modulating the activity of Bcl-2 proteins are currently in clinical trials.[10−12] One of these series of compounds has shown a mechanism-based mode of action, including Bax/Bak dependency: ABT-737, its related orally available analogue ABT-263, and ABT-199 (**1**, **2**, and **3**, respectively in Chart 1).[12−16] While **3** is a selective and potent inhibitor of Bcl-2, **1** and **2** are high-affinity ligands of Bcl-2, Bcl-w, and Bcl-X_L, replicating the binding profile of the BH3-only protein Bad and require the presence of Bax/Bak for cellular activity.[17] Because neither compound potently binds Mcl-1, their efficacy is limited to tumor cell lines with low levels of Mcl-1, although their spectrum of activity can be increased to a wider range of cancer cell lines when used in combination with drugs that neutralize Mcl-1.[18,19] Their on-target side effects include acute and reversible thrombocytopenia due to interference with Bcl-X_L's key role in regulating platelets' life-span.[20,21] Nevertheless, **1** has shown positive results in preclinical experiments in multiple blood-related malignancies and a subset of lung cancers.[13,22]

The paucity of successful examples of functional mimetics of the BH3-only proteins after two decades of interest in this target emphasizes the difficulty in developing Bcl-2 inhibitors and the need for new compounds, especially with different and/or selective binding profiles. Bcl-X_L is of particular interest. Although Bcl-2 was first recognized as an oncogene, more recent findings have shown the importance of Bcl-X_L in carcinogenesis, especially in solid tumors and in the specter of drug resistance.[23,24] It is not yet known how problematic thrombocytopenia due to Bcl-X_L abatement will be in a clinical setting, but the advantages of a potent inhibitor of Bcl-X_L might well outweigh the transient platelet reduction derived from targeting this protein.[21,22] In any case, the development of a compound with high affinity and high selectivity for Bcl-X_L would provide a valuable tool for investigating the role of this pro-survival protein in tumor development.

We have recently described small molecules that potently and selectively inhibit the pro-survival activity of Bcl-X_L.[25] Here we present a detailed account of this discovery and early hit-to-lead development of this series. We present an analysis of the mechanism of action of key compounds and show that they indeed act as functional mimetics of the BH3-only proteins. This work, based primarily on classical medicinal chemistry strategies, provides further evidence that the Bcl-2 pro-survival proteins are "druggable", but drug discovery in this field

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requires multiple assays and a deep understanding of the biology.

RESULTS

Hit Identification. Bcl-w-ΔC29 was chosen as the protein target for the initial primary assay due to its favorable solution properties and because its binding profile to BH3 ligands resembles that of Bcl-2 and Bcl-X_L. Our in-house library of approximately 100000 structurally diverse "hit-like" compounds was screened using an luminescent proximity assay (LPA).[26] This bead-based proximity competition assay was chosen for its robustness and its ability to detect weak binders with a manageable number of false positives. In parallel to the main assay involving Bcl-w, a counter-screen was also employed using beads carrying only GST-Ab to identify the compounds interfering with the assay technology (Supporting Information Figure S1). As references for the binding affinity, we prepared three compounds from a validated series of BH3-mimetics, published by AbbVie, for which binding affinities are known (compounds **1**, **4**, and **5**, Table 1).[14,27] Table 1 summarizes binding assay results for these control compounds against Bcl-X_L. The data show that the luminescence proximity assay (LPA) technology yields higher IC_{50} values compared to the calculated K_i values obtained by fluorescence polarization (FP) assays.[28]

The library of compounds was initially tested at 50 μM in a single-point assay, and all the selected hits were characterized with 5 or 11 point titration curves against Bcl-w and in the control counter-screen assay. At this stage, 60 compounds were selected for further analysis (see Figure S1 for full screening cascade, Supporting Information). Where available, new samples were sourced from the original vendors and thus only 46 compounds were obtained as fresh powder samples. These 46 reordered hits were characterized against Bcl-w, Bcl-X_L, Mcl-1, and in the counter-screen using 11-point titration curves and two distinct screening technologies: LPA and FP assays (Figure 1).[29] Only one compound (**6**) showed binding to Bcl-X_L in both the LPA and FP assays and lacked activity in the counter-screen assay (Table 2).[30] Other potentially interesting hits obtained during this process were characterized by one or more flaws (e.g., low selectivity compared to counter-screen) and were therefore abandoned. We also encountered a large number of "frequent-hitters", structures identified as pan-assay interference compounds or "PAINS",[31] such as rhodanine-like and fused tetrahydroquinoline derivatives.[32] Upon examination of the binding affinities across pro-survival proteins, **6** displayed some selectivity for Bcl-X_L with an IC_{50} below 10 μM, which was of a higher affinity than that measured for Bcl-w (Table 2). At this early stage, compound **6** also showed distinct selectivity over Mcl-1 and no activity in the counter-screen assay.

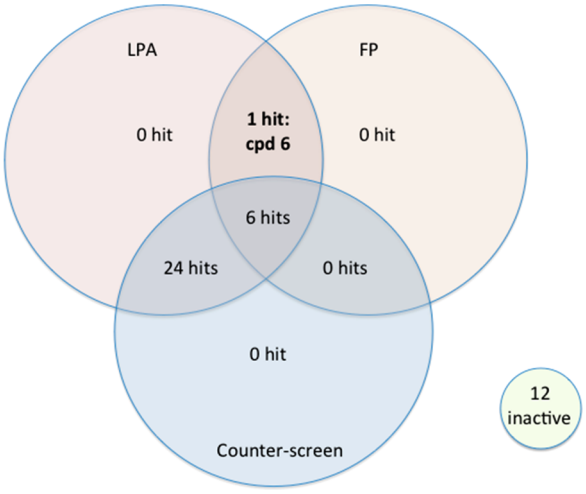
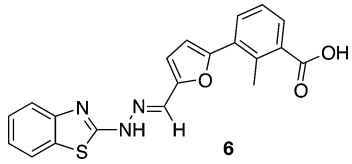


Figure 1. Identification of compound **6**. The final set of 46 reordered compounds was tested against Bcl-w, Bcl-X_L, and Mcl-1 using the luminescence proximity assay (LPA) and fluorescence polarization (FP) assays together with a counter-screen assay. Hits were compounds showing IC_{50} values below 60 μM against any of the three targets.

Table 2. Binding Data for the Sole Hit Compound 6[a]



6

	Bcl-w		Bcl-X_L			Mcl-1		counter-screen
compd no.	LPA	LPA	LPA	FP[b] (Bim)	FP[b] (Bak)	LPA	FP	LPA
6	33	>240	7.7	20	6.8	110	167	249

[a]IC_{50} values in μM. These values are from the original primary assay. In the remaining part of the text, IC_{50} will be reported as average of several experiments and therefore might differ slightly from the ones presented in this table. [b]Competition assay using the BH3 peptide indicated in bracket. LPA: Luminescence Proximity Assay. FP: Fluorescence Polarization.

Chemistry. The majority of the target compounds prepared for this study are based on a simple generic structure exemplified by compound **6**: a hydrazone moiety linking a biaryl group to a fused heterocyclic ring system (generally benzothiazole). A simple and obvious retrosynthetic analysis indicated that these compounds could be readily prepared from heteroaromatic hydrazines and biaryl formyl or acetyl precursors (Schemes1−3). Several methods could be envisaged to prepare the biaryl moiety. In Scheme 1, the direct precursor of compound **6** is 3-(5-formyl-2-furyl)-2-methylbenzoic acid **9a**, which is classically formed using a Meerwein arylation reaction between furaldehyde **7a** and 2-methyl-3-aminobenzoic acid **8a**.[33,34] Although this reaction afforded compounds in modest yields, it proved to be versatile and initially applicable to a wide range of substrates containing five-membered heterocyclic rings (Scheme 1). Thus, anilines **8a−i** were treated with sodium nitrite, followed by addition of formyl or acetyl furans, pyrroles, or thiophenes dissolved in acetone in the presence of copper(II) chloride (derivative **10j** was easily obtained from the reduction of **10i**). Compounds depicted in Schemes 1 and 3 were prepared using this method. Reverse biaryl structures, such as **34**, were also accessible using this protocol (Scheme 3). In the final step of the synthesis, a condensation of the aldehyde or ketone with hydrazine derivatives was performed in refluxing ethanol or acetic acid. The desired final products were obtained as precipitates from the reaction mixture and were easily isolated in high purity and reasonable yields.

SAR exploration of the benzothiazole section of the molecule required the preparation of derivatives with alternative groups at this position (Scheme 2). 2-Hydrazino-benzoxazole and 2-

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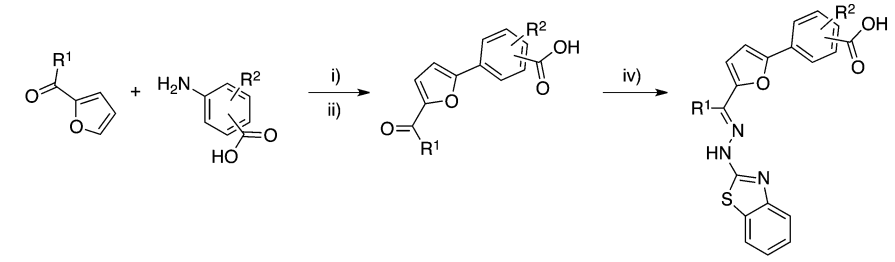
Scheme 1. Synthesis of Furyl-benzothiazole Hydrazone Derivatives[a]



7a: R^1 = H
7b: R^1 = CH_3

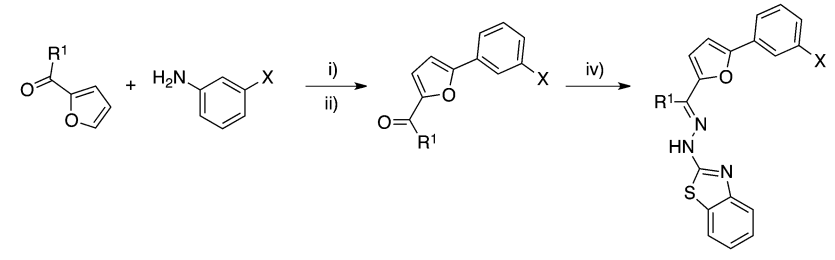
8a: R^2 = 2-Me, 3-COOH
8b: R^2 = H, 3-COOH
8c: R^2 = H, 4-COOH
8d: R^2 = H, 6-COOH
8e: R^2 = 6-Cl, 3-COOH
8f: R^2 = 4-Cl, 3-COOH
8g: R^2 = 2-Cl, 3-COOH
8h: R^2 = 4-NO_2, 3-COOH
8i: R^2 = 4-NH_2, 3-COOH
8j: R^2 = 5-COOH, 3-COOH

9: R^1 = H
9a: R^2 = 2-Me, 3-COOH
9b: R^2 =H, 3-COOH

10: R^1 = Me
10a: R^2 = H, 3-COOH
10b: R^2 = H, 4-COOH
10c: R^2 = H, 6-COOH
10d: R^2 = 6-Cl, 3-COOH
10e: R^2 = 4-Cl, 3-COOH
10f: R^2 = 2-Cl, 3-COOH
10g: R^2 = 5-COOH, 3-COOH
10h: R^2 = 2-Me, 3-COOH
10i: R^2 = 4-NO_2, 3-COOH
iii) → **10j**: R^2 = 4-NH_2, 3-COOH

6: R^1 = H, R^2 = 2-Me, 3-COOH
11: R^1 = H, R^2 =H, 3-COOH

12: R^1 = Me
12a: R^2 = H, 3-COOH
12b: R^2 = H, 4-COOH
12c: R^2 = H, 6-COOH
12d: R^2 = 6-Cl, 3-COOH
12e: R^2 = 4-Cl, 3-COOH
12f: R^2 = 2-Cl, 3-COOH
12g: R^2 = 5-COOH, 3-COOH
12h: R^2 = 2-Me, 3-COOH
12i: R^2 = 4-NO_2, 3-COOH
12j: R^2 = 4-NH_2, 3-COOH



7a: R^1 = H
7b: R^1 = CH_3

13b: X = $CONH_2$
13c: X = CH_2COOH

14: R^1 = H, X = NO_2

15: R^1 = Me
15a: X = $CONH_2$
15b: X = CH_2COOH

16: R^1 = H
16a: X = NO_2
11: X = COOH
v) → **16b**: X = COOMe

17: R^1 = Me
17a: X = $CONH_2$
17b: X = CH_2COOH

[a](i) $NaNO_2$, HCl, H_2O, 0 °C; (ii) $CuCl_2$, acetone; (iii) ammonium formate, Pd/C, MeOH; (iv) 2-hydrazino-benzothiazole, EtOH or AcOH, 60−80 °C; (v) H_2SO_4, MeOH.

hydrazino-1-methyl-benzimidazole (**19a−b**) were prepared simply by reacting the corresponding 2-chloro precursors (**18a−b**) with hydrazine. Substituted 2-hydrazino benzothiazoles **21a−f** were prepared from known substituted 2-amino-benzothiazole precursors **20a−f** using an acid-mediated displacement of the amino group in the presence of hydrazine hydrate and hydrazine hydrogen sulfate salt. The corresponding final compounds **22a−f**, **23**, and **24** were prepared according to the general synthesis using a hydrazone formation. Alternatively, commercially available aryl-hydrazine reagents were required to prepare compounds **25−27**.

As the project progressed, more diversity was required in the biaryl section of the target molecules. Thus, the Suzuki−Miyaura coupling reaction provided another route to these key intermediates with conditions tolerating a wide variety of aryl-, heteroaryl halides and various boronic acids or esters. Schemes 4−7 describe the synthesis of analogues using this method. Specifically, Scheme 4 describes analogues exploring 6/6, 6/5, and 5/6 biaryl systems prepared from commercially available boronic acids and esters **35** and **45**.

The synthesis of constrained compounds depicted in Schemes 5, 6, and 7 used essentially the same strategy based on a Suzuki−Miyaura coupling, however the cost of the commercially available borylated derivatives of picolinic acid and their extreme instability under classical coupling conditions led us to opt for the preparation of boronic ester derivatives of the arylketone precursors. Thus, the boronic esters **53** and **57a−b** were easily prepared from the corresponding commercially available bromoarylketones **52** and **56a−b**. Notably, the very efficient transformation described by Han et al. (Scheme 5) provided an excellent alternative to the oil bath heating method.[35] This procedure involved microwave irradiation of a mixture of the substrate and bispinacoldiboron in toluene at 150 °C for 5 min in the presence Pd(dppf)Cl_2, leading to the desired compounds **53** and **57a−b** in high yield and good purity. They were then engaged in the coupling/hydrazone formation sequence to furnish constrained derivatives **55** and **59a−b**.

A series of analogues exploring changes to the cyclic structure of the tetralone core (either by introducing

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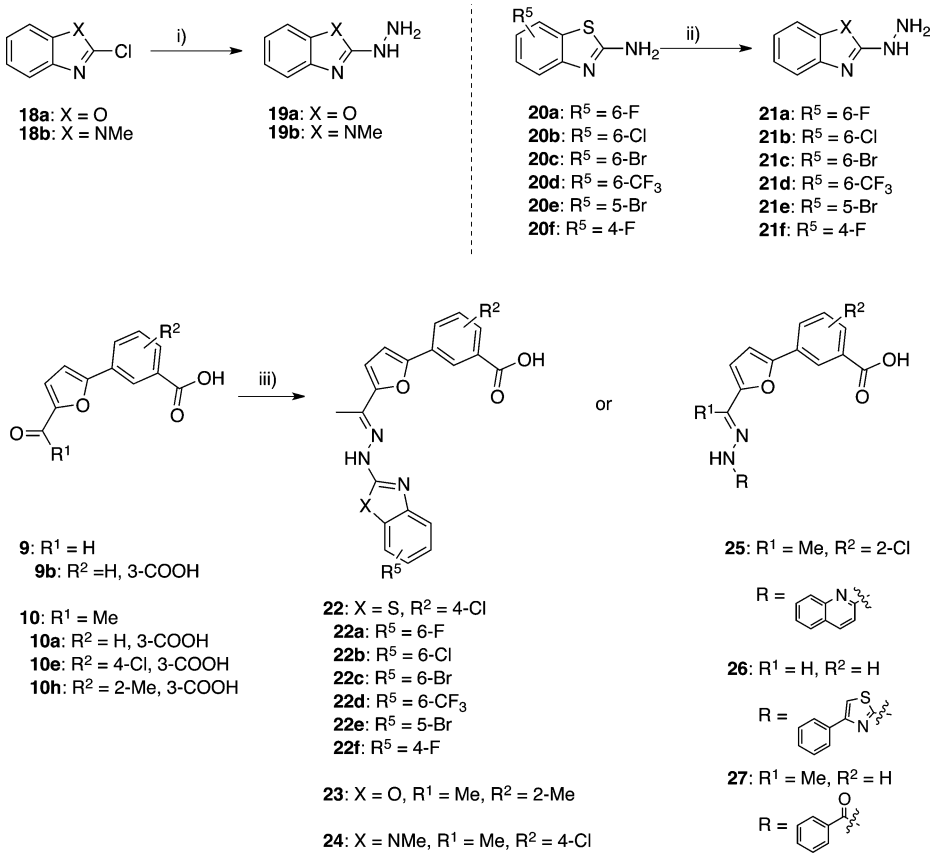
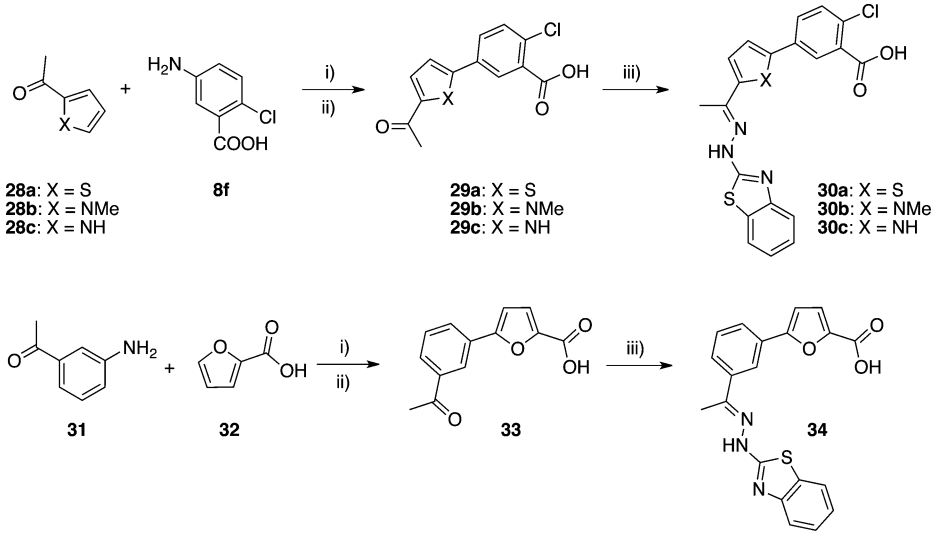
Scheme 2. Synthesis of Analogues with Various Replacements of the Benzothiazole Group[a]



[a](i) NH_2NH_2, EtOH, 70 °C; (ii) $NH_2NH_2.H_2O$, NH_2NH_2·HCl, ethylene glycol, 140 °C; (iii) Various R-NH-NH_2, EtOH or AcOH, 60−80 °C.

Scheme 3. Synthesis of Analogues with Various Biarylic Moieties Using the Meerwein Reaction[a]



[a](i) $NaNO_2$, HCl, H_2O, 0 °C; (ii) $CuCl_2$, acetone; (iii) 2-hydrazino-benzothiazole, EtOH or AcOH, 60−80 °C.

heteroatoms or adding substitutions onto the ring) were prepared from the various precursors **60a−i** (Scheme 6, Supporting Information Scheme S1).[36] These precursors were then transformed into their respective boronic esters **61a−i** and reacted with 6-bromopicolinic acid to form biaryls **62a−i**. The final compounds **63a−i** were obtained by reacting the biaryl ketone with 2-hydrazinobenzothiazole in the usual manner. As oxidation of the advanced intermediate benzothiopyran-4-one with a free carboxylic **62f** failed to give the desired sulfone and sulfoxide products, a specific route had to be designed for these two derivatives. First, 6-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)thiochroman-4-one **61f** was coupled to *t*-butyl-6-bromopicolinate. This protected biaryl acid **64** was then easily converted into sulfoxide **65a** using *m*-CPBA or into sulfone **65b**

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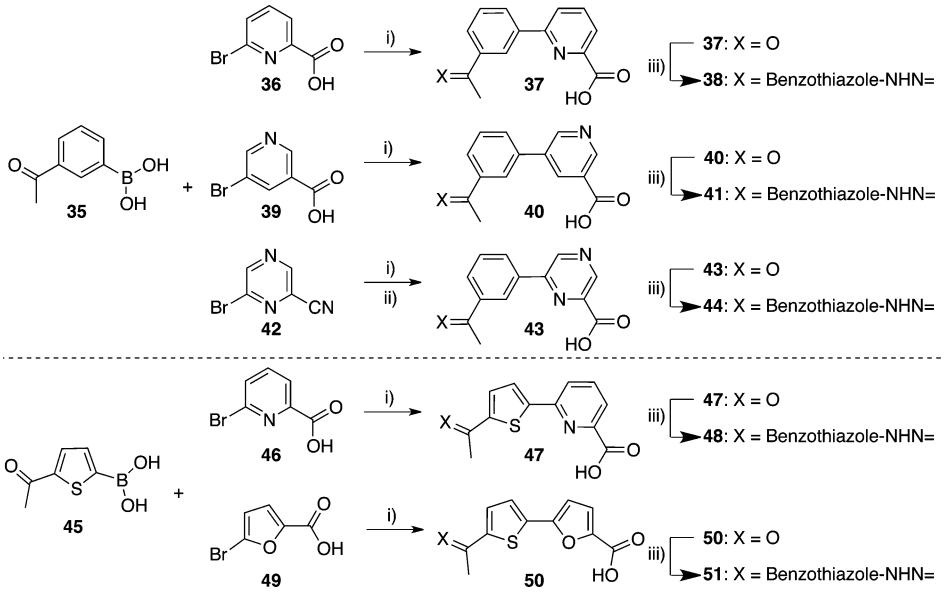
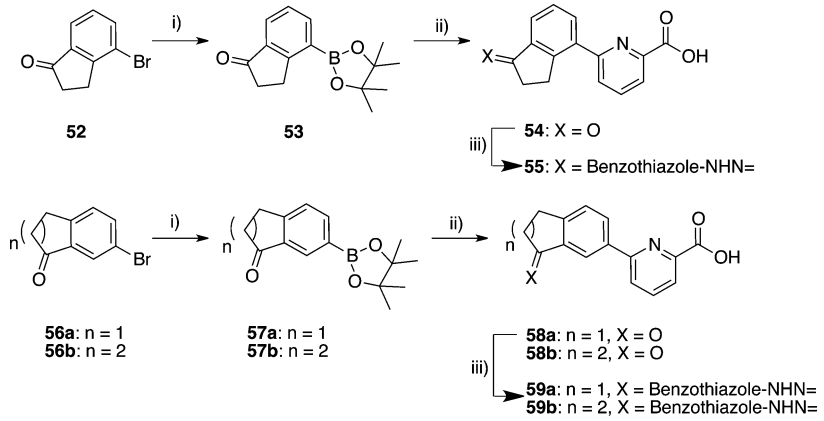
Scheme 4. Synthesis of Analogues with Various Biarylic Moieties Using a Suzuki–Miyaura Coupling Reaction[a]



[a](i) TBAB, K_2CO_3, $Pd(PPh_3)_2Cl_2$, dioxane/H_2O, 70 °C, μW; (ii) NaOH, MeOH/THF then HCl; (iii) 2-hydrazino-benzothiazole, EtOH or AcOH, 60–80 °C.

Scheme 5. Synthesis of Simple Conformationally Constrained Analogues[a]



[a](i) Bispinacoldiboron, $Pd(dppf)Cl_2$, KOAc, toluene, 150 °C, μW; (ii) 6-Bromopicolinic acid, TBAB, K_2CO_3, $Pd(PPh_3)_2Cl_2$, dioxane/H_2O, 70 °C, μW; (iii) 2-hydrazino-benzothiazole, EtOH or AcOH, 60–80 °C.

using hydrogen peroxide. Usual hydrazone preparation followed by *t*-butyl ester deprotection in formic acid resulted in the desired compounds **67a–b**.

The last set of compounds was prepared to explore the SAR of the terminal ring bearing the acid moiety (compounds **71a–b** and **74a–b**, Scheme 7). These compounds were prepared using the Suzuki–Miyaura route presented above using known five- and six-membered heterocyclic esters **68a–b** or acids **72a–b**. In the case where esters were used as starting material, an additional hydrolysis step was required prior to the preparation of the final compounds **71a–b**.

Hit-to-Lead Progression. After the identification of compound **6** as a promising hit with moderate activity against Bcl-X_L, an initial small set of close analogues was prepared to validate the screening results with indication of early SAR. The binding affinities measured with this set of compounds quickly confirmed the results obtained with **6** (Figure 2). Deletion of the methyl group on the terminal ring (compound **11**) did not affect the activity while the methylketohydrazone analogue **12a** and **12h** exhibited increased potency, bringing the IC_{50} values close to 1 μM. In addition to these positive assay results, this series also displayed a reasonable ligand binding efficiency (BE) value of 0.29 for compound **12a** compared to a BE of 0.21 for compound **4** at a similar level of potency.[37]

The next part of our study was aimed at increasing our understanding of the SAR and at improving the activity of the series. In the absence of structural information, these data would help formulate hypotheses about the binding mode of the molecules. We tried to elucidate the essential pharmacophore of the series by dissecting each section of the target compounds: the acidic moiety, the biaryl unit, the hydrazone linker, and the benzothiazole group. It quickly became evident that most of the elements constituting the core structure were essential for binding to Bcl-X_L. Table 3 presents results obtained for compounds where the nature and position of the terminal carboxylic acid are modified and where substituents have been added to the terminal ring.[38] While the derivative with the acid moiety in position 6 (**12b**, see Figure 2 for

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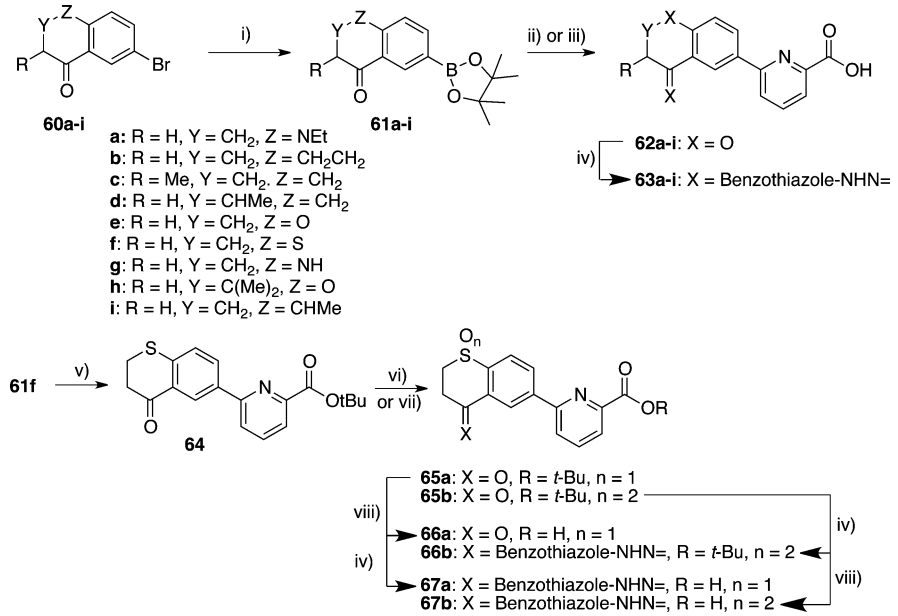
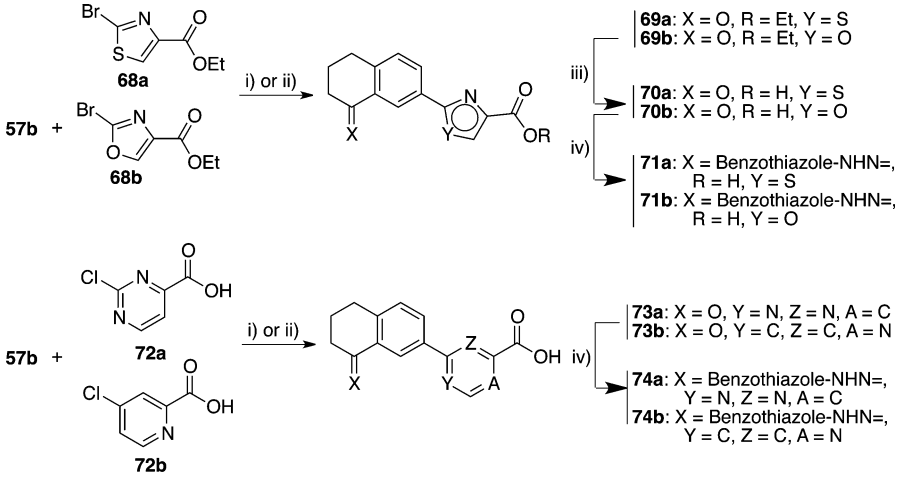
Scheme 6. Synthesis of Constrained Hydrazone Analogues[a]



[a](i) Bispinacoldiboron, Pd(dppf)Cl_2, KOAc, toluene, 150 °C, μM; (ii) 6-bromopicolinic acid, TBAB, K_2CO_3, Pd(PPh_3)$_2Cl_2$, dioxane/H_2O, 70 °C, μW; (iii) 6-bromopicolinic acid, Pd(PPh_3)$_2Cl_2$, Na_2CO_3, EtOH/DME/H_2O; (iv) 2-hydrazino-benzothiazole, EtOH or AcOH, 60−80 °C; (v) *t*-butyl 6-bromo-picolinate, TBAB, K_2CO_3, Pd(PPh_3)$_2Cl_2$, dioxane/H_2O, 70 °C, μW; (vi) n = 1: *m*-CPBA, $CHCl_3$; (vii) n = 2: *m*-CPBA, CH_2Cl_2; (viii) formic acid.

Scheme 7. Synthesis of Analogues of 59b with Alternative Terminal Aryl Rings[a]



[a](i) TBAB, K_2CO_3, Pd(PPh_3)$_2Cl_2$, dioxane/H_2O, 70 °C, μW; (ii) Pd(PPh_3)$_2Cl_2$, Na_2CO_3, EtOH/DME/H_2O; (iii) LiOH or NaOH, THF/MeOH; (iv) 2-hydrazino-benzothiazole, EtOH or AcOH, 60−80 °C.

terminal ring numbering) had no activity, the analogue **12c** (carboxylic acid in position 2) retained most of the original binding affinity although its binding affinity was difficult to assess accurately due to its low solubility. Exploration of ring substitutions indicated that position 6 tolerated a large range of substituents (from electron withdrawing to electron donating groups, compounds **12e** and **12i−j**, Table 3). Position 2 tolerates the presence of substituents such as a methyl (**6** and **12h**) and chloro (**12f**) group. Addition of a chlorine atom or a carboxylic acid on the opposite side of the ring (compounds **12d** and **12g**, at position 4 and 5, respectively, Table 3) did however reduce the affinity for Bcl-X_L. It can be deduced from these results that positions 2 and 4 of the terminal ring may point away from the target protein, whereas positions 5 and 6 may interact with the protein. The results observed with the orientation of the carboxylic acid on the ring suggest that an important electrostatic interaction is taking place with a residue located on the interacting pocket of Bcl-X_L. This conclusion was further supported by the lack of activity of compounds **16a−b** and **17a**, with nitro, ester, and amide replacements, respectively. Only the homologated compound **17b** retained activity similar to compound **12a**.

The benzothiazole moiety tolerated very little change without completely abrogating binding. Analogues with small substituents on positions 4 and 6 of the benzothiazole nucleus were the only compounds that retained some level of activity (Table 4, **22a−c**, **22e−f**). Conversely, derivative **22d** bearing a CF_3 group in position 6 of the benzothiazole was totally

dx.doi.org/10.1021/jm400556w | J. Med. Chem. XXXX, XXX, XXX−XXX

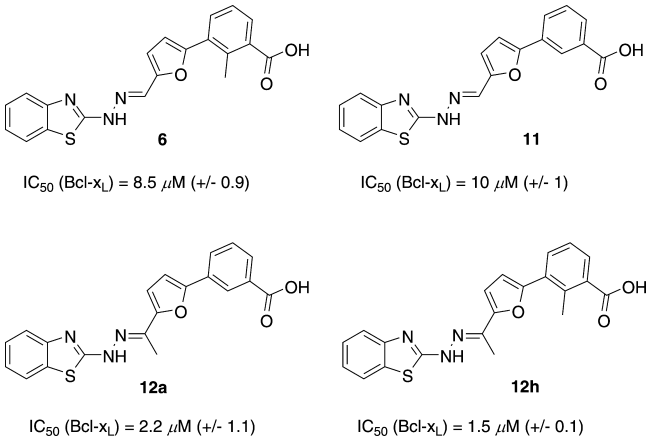
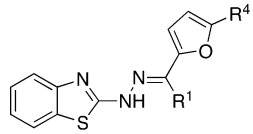


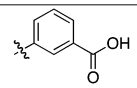
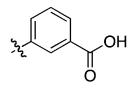
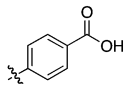
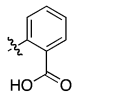
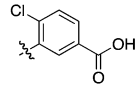
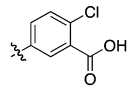
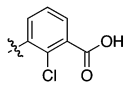
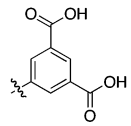
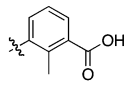
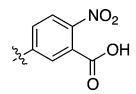
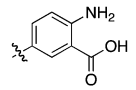
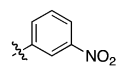
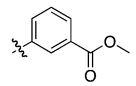
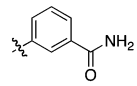
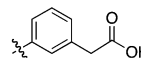
Figure 2. Binding affinities of compound **6** and its close analogues for Bcl-X_L. IC_{50} values shown with standard deviation if two separate experiments were performed, with standard error indicated with * if three or more.

inactive, suggesting that benzothiazole sits in a relatively narrow pocket. The exquisite match between the benzothiazole unit and the protein was also strikingly demonstrated by the lack of activity of the benzoxazole and methylbenzimidazole derivatives (**23** and **24**, respectively). These results indicate that hydrophobicity, as well as steric factors, contribute to the binding of the whole compound. Unsurprisingly, drastic changes to this section of the molecule (quinoline **25**, 5-phenylthiazole **26**, and benzoyl **27**) also led to loss in affinity for Bcl-X_L.

In contrast to other parts of the molecule, the biaryl unit was more tolerant to alteration; results presented in Table 5 indicate that 6/5, 5/6, and 5/5 biaryl sequences give very similar IC_{50} values between 1 and 5 μM (compare **12a**, **34**, and **51**, Table 5). The nature of the central five-membered heterocycle also seemed less important: thiophene, *N*-Me pyrrole, and pyrrole can replace the original furan motif without impairing the affinity for Bcl-X_L (compounds **30a**−**c**). Despite this tolerance to changes, none of these analogues imparted significantly better activity to the target molecules: compounds are either of similar or lower affinity. Satisfyingly, however, our exploration of the biaryl moiety led to picolinic acid derivative **38**, which bound more tightly to Bcl-X_L, with an IC_{50} of 0.12 μM (18 times more potent that compound **12a**). Importantly, the position of the picolinic acid nitrogen atom is crucial for affinity as a nicotinic acid derivative **41** reverts activity to micromolar levels. A pyrazinoic acid derivative **44** also exhibited sharply reduced affinity. Along with the lack of activity of the carboxylic acid replacement derivatives **16a**−**b** and **17a** (Table 3), these results confirm the importance of the acidic moiety for the overall binding affinity. The putative interaction between this group and a hydrogen bond donor from Bcl-X_L seems to be enhanced with a picolinic acid motif. Interestingly, with the picolinic acid, the 6/6 biaryl sequence seems to have reached an optimal geometry for efficient binding. While various 5/5, 5/6, and 6/5 biaryl sequences have little impact on the binding affinity (see compounds **12a**, **30a**, and **34**, Table 5), they are critical when the terminal ring is a picolinic acid: the thiophene derivative **48** binds to Bcl-X_L more weakly than related analogue **38**. Overall, we were encouraged that the increase in activity observed with compound **38** was due to a small change on the molecule, thereby indicating that optimization of other portions of the molecule might also lead to significant

Table 3. Influence of Changes the Benzothiazole Group Imparts on Binding Affinity[a]



Compounds	R^1	R^4	IC_{50} (Bcl-X_L, μM)
11	H		10.0 ± 1.1*
12a	Me		2.2 ± 1.1
12b	Me		>100
12c	Me		6.3 ± 4.6*
12d	Me		76
12e	Me		1.7 ± 0.4*
12f	Me		2.7
12g	Me		>100
12h	Me		1.5 ± 0.1*
12i	Me		1.8 ± 0.3*
12j	Me		6.7 ± 0.4
16a	H		>100
16b	H		>100
17a	Me		>100
17b	Me		5.4 ± 0.5*

[a] IC_{50} values shown with standard deviation if two separate experiments were performed, or with standard error indicated with * if three or more experiments were performed.

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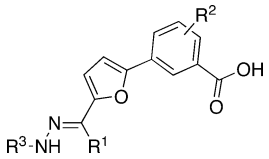
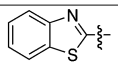
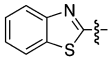
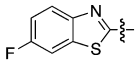
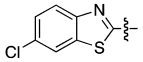
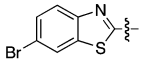
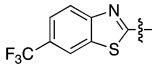
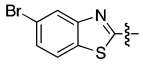
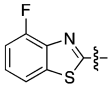
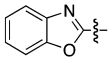
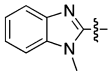
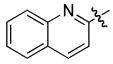
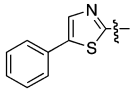
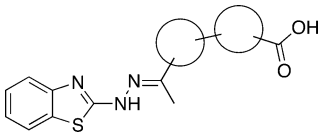
Table 4. Influence of Changes on the Benzothiazole Group on the Binding Affinity[a]

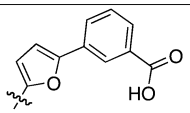
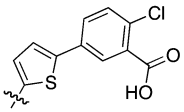
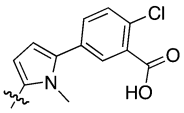
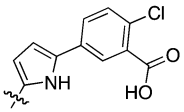
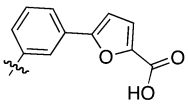
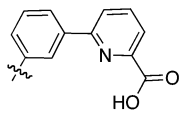
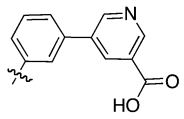
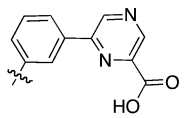
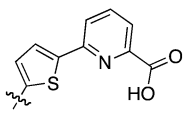
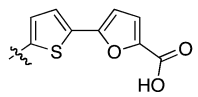


Compounds	R^1	R^2	R^3	IC_{50} (Bcl-X_L, μM)
11	H	-		10 ± 1.1*
12a	Me	-		2.2 ± 1.1
22a	Me	4-Cl		4.0 ± 1.1
22b	Me	4-Cl		17
22c	Me	4-Cl		9.3
22d	Me	4-Cl		>100
22e	Me	4-Cl		15
22f	Me	4-Cl		5.2 ± 1.1*
23	Me	2-Me		>100
24	Me	4-Cl		>100
25	Me	2-Cl		>100
26	H	-		>100
27	Me	-		>100

[a]IC_{50} values shown with standard deviation if two separate experiments were performed, or with standard error indicated with * if three or more experiments were performed.

Table 5. Biaryl Moiety[a]



Compounds	Biaryl	IC_{50} (Bcl-X_L, μM)
12a		2.2 ± 1.1
30a		1.6 ± 0.5*
30b		1.1
30c		1.5
34		1.3 ± 0.2*
38		0.12 ± 0.03*
41		8.1
44		3.8
48		1.2 ± 0.8*
51		1.7 ± 0.5*

[a]IC_{50} values shown with standard deviation if two separate experiments were performed, or with standard error indicated with * if three or more experiments were performed.

improvement in binding affinity without increasing the molecular weight.

The next increase in potency came from a consideration of the binding conformations available to our compounds. We reasoned that the free rotation around the aromatic-hydrazone bond would allow two major conformations maintaining coplanarity with the flanking aromatic rings (Figure 3a). One conformation would result in the molecule adopting an L-shape, whereas the other one would lead to a more linear pharmacophore. Constraining the molecule at this position could therefore potentially increase the binding affinity by locking the compound in its binding conformation. A first set of derivatives based on the indanone core confirmed our hypothesis. One of the conformations, represented by constrained compound **55**, showed decreased activity while the other constrained compound **59a** maintained an affinity close to compound **38** (0.13 vs 0.12 μM, respectively, Figure 3b). When the tetralone derivative **59b** was prepared, we observed a further increase in affinity for Bcl-X_L with an IC_{50} value of 23 nM (6-fold more potent than **38** and 100-fold more potent

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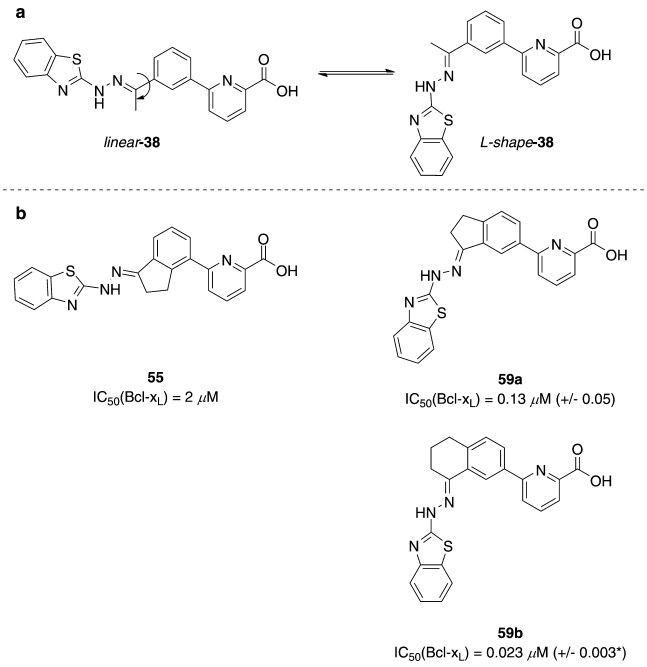

Figure 3. Introduction of constraints to the hydrazone core. IC_{50} values shown with standard deviation if two separate experiments were performed, with standard error indicated with * if three or more.

than **12a**, Figure 3b). Once again, a significant increase in binding efficiency was achieved with little increase in molecular weight.

In an effort to further enhance the binding affinity of the series, we explored derivatives of **59b** with variation of the saturated section of the tetralone moiety. These targets included constrained structures incorporating heteroatoms and substituted tetralones (Table 6). In general, this subset of compounds exhibited very good binding affinities for Bcl-X_L in a similar range compared to **59b** but with no significant increase in activity. The most potent analogues were the seven-membered ring analogue **63b** (IC_{50} = 10 nM), the β-methyl tetralone **63d** (IC_{50} = 17 nM), and the tetrahydroquinolone derivative **63g** (IC_{50} = 6 nM). Comparison of the five-, six-, and seven-membered ring analogues indicates that the angle induced on the structures by these rings does affect the binding affinity, with optimum binding observed for the seven-membered ring derivative **63b**. Similar levels of activity compared to **59b** were observed with chromanone derivative **63e** (IC_{50} = 55 nM) and the thiochromanone derivative **63f** (IC_{50} = 14 nM). Compounds with substitutents at the 4 position of the tetralone or related rings seemed to have decreased activity (see for example compound **63a** and **63i**, IC_{50} = 47 and 49 nM, respectively). The sulfoxide **67a** and sulfone **67b** derivatives of thiochromanone **63f** were also less potent. Although the β-methyltetralone derivative **63d** showed good potency, β-dimethylchromanone **63h** was less potent. Finally the α-methyltetralone **63c** suffered the largest loss in activity (IC_{50} = 260 nM), suggesting that either the methyl group in this position clashes with the protein or that it induces a steric clash with the hydrazone linker, forcing it into a nonbinding conformation.

In a final exploration of the SAR of our series, we prepared a set of analogues of **59b** varying the terminal ring bearing a carboxylic acid (Table 7). We observed that a thiazole-4-carboxylic acid (compound **71a**) could efficiently replace the picolinic acid. However, replacement with an oxazole moiety was deleterious to the affinity. Previous results obtained in the nonconstrained series were also confirmed with compound **74a–b**, where changes from a picolinic acid to a pyrimidinic acid or to the other isomer of picolinic acid also severely decreased the activity. In particular, **74b** shows that the nitrogen atom on the ring not only modulates the acidity of the carboxylic acid but that its position in the ring is critical for obtaining maximum binding affinity.

Selectivity. Compound **6** was discovered from a screen using Bcl-w as the target protein. Our interest in Bcl-X_L, together with the preferential affinity of **6** for this pro-survival protein (Table 2), led us to explore the SAR against Bcl-X_L and develop more potent inhibitors of this pro-survival protein. With this new series of compounds in hand, we were interested in determining how the affinity profile across a panel of other pro-survival proteins had evolved during the hit-to-lead process. We assessed the binding affinity of key compounds of our series (Table 8) for Bcl-w, Bcl-2, and Mcl-1 using two different competition assay technologies. Using LPA assays and surface plasmon resonance (SPR), we evaluated the binding of the compounds for Mcl-1 and Bcl-w in addition to Bcl-X_L.[39] SPR also allowed us to assess binding to Bcl-2. We observed that the compounds derived from **6** rapidly developed a high selectivity for Bcl-X_L. Compound **12a**, which differs from **6** only by two minor changes (methyl groups on terminal aromatic ring and methylketohydazone instead of aldehydehydrazone), was found to have increased its affinity for Bcl-X_L without concomitantly gaining affinity for Bcl-w. Similarly, **12a** did not show any relevant activity for Mcl-1 and Bcl-2. The most potent compounds **59b** and **71a** exhibited some residual weak activity for Mcl-1, Bcl-2, and Bcl-w (at least 2 orders of magnitude less potent). We suspect that this activity is due to nonselective binding of these hydrophobic and acidic molecules rather than to selective interaction. Regardless of the nature of this binding, such low affinities are unlikely to interfere in the interpretation of biological data. We conclude from this analysis that the most potent compounds are indeed highly selective for Bcl-X_L.

Surface Plasmon Resonance: Binding Kinetics. To further analyze the binding characteristics of our compounds for Bcl-X_L, we developed an assay that directly measures equilibrium binding constants (K_D) using surface plasmon resonance technology (SPR, Supporting Information Figure S2). To measure only the specific binding of compounds in the hydrophobic groove without the nonspecific background binding (binding to other areas of the protein), the experimental design took advantage of the three detection spots available within each individual flow cell on the SPR measurement chip and of its capability to hydrodynamic addressing the individual spots (see Supporting Information).[40] Thus, one spot presented anti-GST antibody captured GST-Bcl-X_L prebound to a 26-mer Bim peptide (r1), the second spot presented anti-GST antibody captured GST-Bcl-X_L only (r2), and the third was left blank as a reference spot (r, see Supporting Information for experimental setup scheme). The high affinity of the Bim peptide to Bcl-X_L, due to extremely slow dissociation rate, makes it behave practically as an irreversible ligand over the time course of the experiment (~5 min).[41] Therefore, the observed difference of signals between r2 and r1 gives a measurement of the specific binding of any compound within the hydrophobic groove.[42] In addition to the direct equilibrium binding constants (K_D), the SPR experiments provided us with other valuable information such as the

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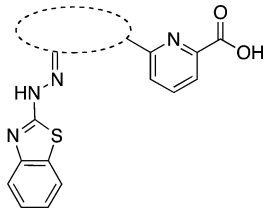
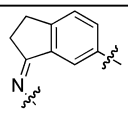
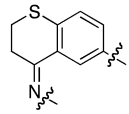
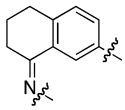
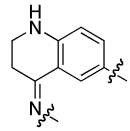
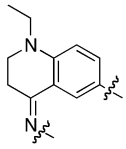
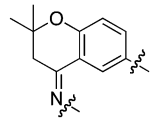
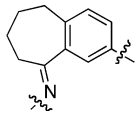
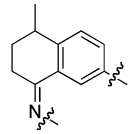
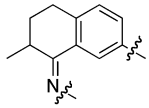
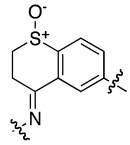
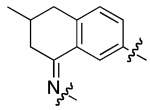
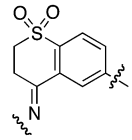
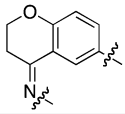
Table 6. Constrained Analogues[a]



Compounds	Core	IC_{50} (Bcl-X_L, μM)	Compounds	Core	IC_{50} (Bcl-X_L, μM)
59a		0.13 ± 0.05	**63f**		0.014 ± 0.003*
59b		0.023 ± 0.003*	**63g**		0.006 ± 0.001
63a		0.049 ± 0.008	**63h**		0.10 ± 0.02*
63b		0.010 ± 0.001	**63i**		0.047 ± 0.027*
63c		0.26 ± 0.06	**67a**		0.051 ± 0.020
63d		0.017 ± 0.006*	**67b**		0.078 ± 0.020
63e		0.055 ± 0.013*			

[a] IC_{50} values shown with standard deviation if two separate experiments were performed, or with standard error indicated with * if three or more experiments were performed.

stoichiometry and the kinetics of binding in the form of association (k_a) and dissociation rate (k_d) constants. We included compounds **12a**, **38**, and **59b** in this study as these represented milestones in terms of binding affinities during our hit-to-lead campaign. For reference, we also added two compounds from the acylsulfonamide series (**1** and **4**).

Results of these experiments are presented in Table 9 and Figure 4 (for detailed sensorgrams and summary of data, see Supporting Information Table S1). Pleasingly, the K_D values determined either from steady-state or kinetic analyses as measured by SPR are in relative agreement with the IC_{50} values obtained with the primary LPA binding assay, providing further evidence of binding by an independent assay technology. The steady-state curves also demonstrated that the compounds are binding in a 1:1 complex with Bcl-X_L (expected response for one small molecule binding onto the target protein, see Supporting Information).

As anticipated, the kinetic data provided more detailed insight into the structure–activity relationship of our series. With low micromolar affinity, compound **12a** classically displayed relatively fast association and dissociation rates. The kinetic data on picolinic acid derivative **38**, which gave us our first significant increase in activity, demonstrated that this improvement is due to both an increase in the association rate and a decrease in the dissociation rates (Figure 4C). Factors influencing the association rate are difficult to determine as multiple parameters can affect the dynamics of formation of the molecular complex. Although desolvation may contribute significantly to this parameter, we also propose that, compared to compound **12a**, compound **38** more readily recognizes the binding site of Bcl-X_L. We base this interpretation on the fact that the picolinic acid motif could provide an ideal interacting partner of guanidine groups. Arg 139 located within the hydrophobic groove may be a key recognition element attracting the picolinic acid derivatives toward its binding site.

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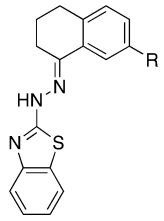
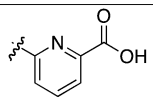
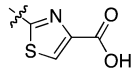
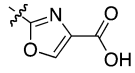
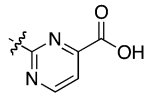
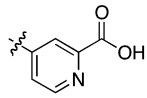
Table 7. Further Analogues of Compound 59b[a]



Compounds	R	IC_{50} (Bcl-X_L, μM)
59b		0.023 ± 0.003*
71a		0.024 ± 0.011*
71b		0.06 ± 0.01*
74a		0.34
74b		1.4

[a]IC_{50} values shown with standard deviation if two separate experiments were performed, or with standard error indicated with * if three or more experiments were performed.

This privileged interaction would decrease the number of possible nonproductive interactions between the molecule and the protein target, leading to an increase in the association rate.

With constrained compound **59b**, we anticipated that the association rate would also be affected. However, the results obtained with compound **38** suggest that this parameter is already nearly optimal (Figure 4C). Interestingly, we observed that the increase in binding affinity for **59b** is primarily due to a decreased dissociation rate (Figure 4C). This indicates that the complex of Bcl-X_L with **59b** is significantly more stable than with **12a** or **38**. Apart from an increased lipophilicity with the tetralone ring, it seems unlikely that **59b** creates any new interaction with the protein. Its conformational preference could, however, explain this result. The SAR leading to compound **38** indicated that this molecule comprises two essential pharmacophores linked by a flexible aryl-hydrazone bond: the benzothiazole/hydrazone moiety and the terminal aryl acid moiety. Because of the central rotatable bond, the two pharmacophores are partially independent. This conformational flexibility allows each pharmacophore to independently interact with the protein. Thus the three possible binding modes could be: benzothiazole only, biaryl acid only, or benzothiazole + biaryl acid. The constrained compound **59b** renders the two pharmacophores geometrically dependent and precludes them from adopting other nonbinding conformations. Thus: the half-life of the complex is extended.

Finally, comparison of the binding affinity and kinetic parameters of compound **1** put our results in perspective. Compound **59b** is a potent ligand of Bcl-X_L but is still at least 1 order of magnitude weaker than **1**. Visual comparison of the sensorgrams of **1** and **59b** clearly highlights the difference in residency time (i.e., dissociation rates) of the two compounds within the hydrophobic groove (Figure 4A,B).

Table 9. Surface Plasmon Resonance Results of Direct Binding of Various Ligands against Bcl-X_L (All Affinity Values in Micromolar)[a]

compd	IC_{50} (LPA, μM)	K_D (μM)[b]	k_a ($M \cdot s^{-1}$)	k_d (s^{-1})	K_d (μM)[c]
1	0.0005		7.91×10^5	2.93×10^{-4}	0.00013
4	2.2	0.9	4.75×10^5	0.76	1.9
12a	2.2	9	8.61×10^5	0.91	12
38	0.12	0.061	2.37×10^6	0.081	0.079
59b	0.023		2.91×10^6	9.92×10^{-3}	0.0041
Bim-26mer			3.75×10^6	5.36×10^{-4}	0.00014

[a]All values obtained from plasmon surface resonance are average of at least two separate experiments (see Supporting Information Table S1 for details). [b]Direct binding constant measured at steady state. [c]Direct binding constant calculated from kinetic parameters. k_a, association rate; k_d, dissociation rate.

Cytochrome *c* Release. We have described a series of compounds exhibiting potent and selective binding to Bcl-X_L. We next wanted to see if the affinity of the most potent analogues was sufficient to induce a mechanism-based apoptotic response in cells. At this stage, carefully assessing the mode of action of this series of compounds was of great importance as we intended to show that induction of apoptosis was indeed the result of selective and direct inhibition of Bcl-X_L. We first evaluated the compounds in a cytochrome *c* release assay in permeabilized cells. This assay circumvents both serum

Table 8. Binding Affinity of a Subset from the Benzothiazole Series against Members of the Bcl-2 Family

	LPA		SPR[a]			
compd	Bcl-X_L	Mcl-1	Bcl-X_L	Bcl-w	Mcl-1	Bcl-2
1	0.0005	>1	0.014 ± 0.001	0.066 ± 0.005	>10	0.007 ± 0.001
12a	2.2	>100	~15	>10	>10	>10
48	1.2	>100	0.75 ± 0.07	>10	>10	>10
74a	0.34	>100	0.077 ± 0.014	>10	>10	>10
74b	0.06	>100	0.041 ± 0.007	~5	~5	~5
59b	0.023	68 ± 4	0.02 ± 0.01	>10	>10	>10
71a	0.024	40 ± 10	0.013 ± 0.001	~5	~5	~5
38	0.12	>100	0.14 ± 0.02	>10	>10	>10
63a	0.049	42	0.088 ± 0.009	>10	>10	>10

[a]IC_{50} values in μM shown with standard deviation.

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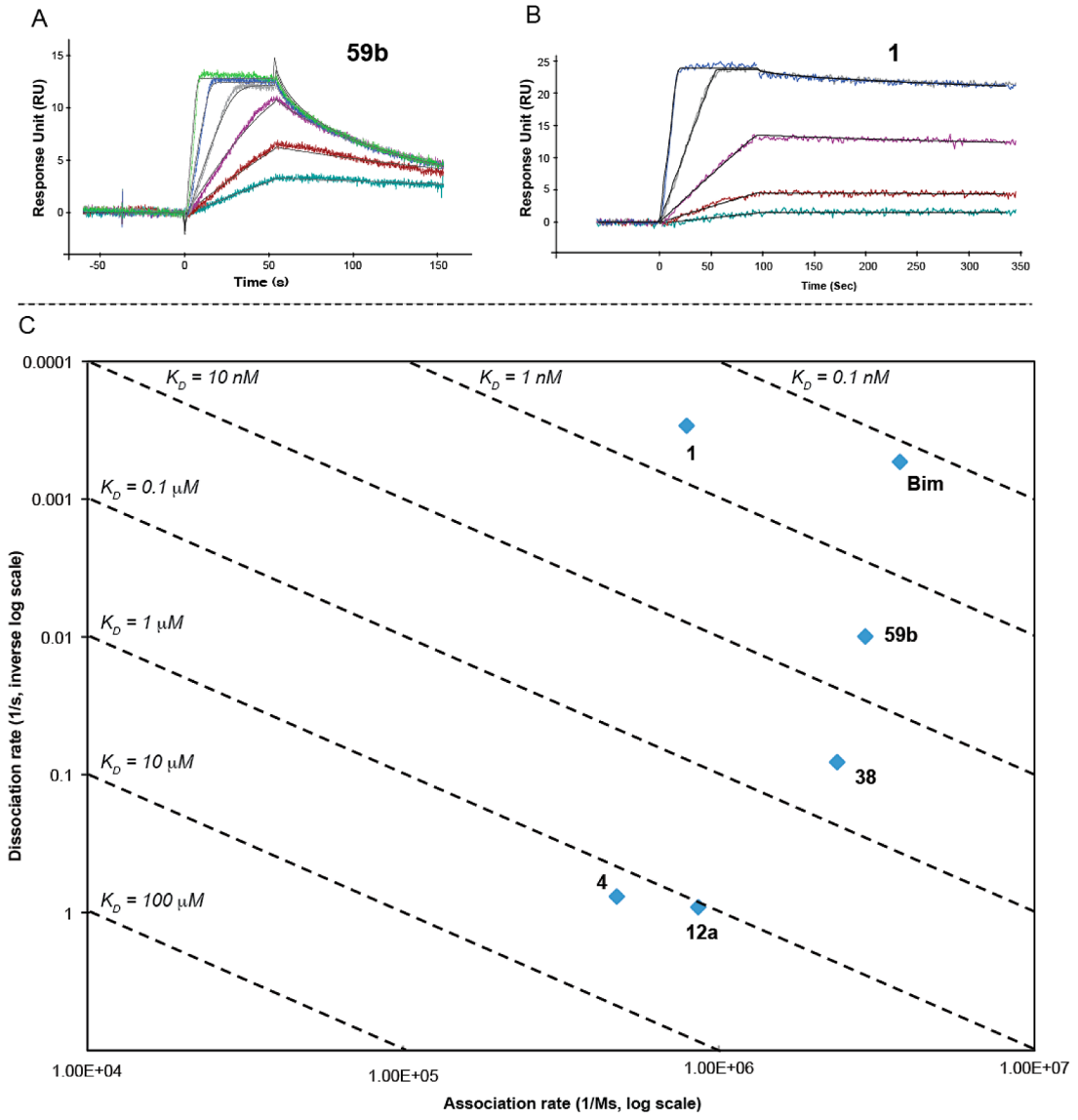

Figure 4. Kinetic analysis of three key benzothiazole analogues, two molecules from the acylsulfonamide series, and wt-Bim26mer peptide. (A) Sensorgrams for compound **59b** (concentrations: 100, 50, 25, 12.5, 6.25, 3.13 nM). (B) Sensorgrams for compound **1** (concentrations: 270, 90, 30, 10, 3.3 nM). (C) Kinetic plot for compounds **1**, **4**, **12a**, **38**, **59b**, and wt-Bim26mer.

protein binding and cell membrane permeability, either of which can compromise intact cell experiments. Mouse embryonic fibroblasts (MEF) were used in these experiments as a number of genetically modified lines are available for mechanism-of-action studies.[19] We utilized mitochondria from wild-type (*wt*) MEFs as well as genetically modified MEFs: *mcl-1*$^{-/-}$ or *bax*$^{-/-}$/*bak*$^{-/-}$ MEFs. Pro-apoptotic activity of BH3-only proteins such as Bim is abrogated if Bax and Bak are absent.[9] The Bax/Bak dependency is therefore necessary to demonstrate functional mimicry of the BH3-only protein activity. Bcl-X_L and Mcl-1 have also been shown to be the primary regulators of Bak's pro-apoptotic activity.[43] Mcl-1 knockout (KO) MEFs are therefore dependent on only Bcl-X_L for survival, hence compounds selectively inhibiting Bcl-X_L should induce cytochrome *c* release from mitochondria in these cells. Together, the experiments using *wt* MEFs, Mcl-1 KO, and Bax/Bak double knockout MEFs provide us with information on the ability of this chemical series to induce cytochrome *c* release by selectively inhibiting Bcl-X_L (in *mcl-1*$^{-/-}$ vs *wt* MEFs) and excluded nonspecific, off-target activities (in *bax*$^{-/-}$*bak*$^{-/-}$ MEFs).[44]

While no cytochrome *c* release was observed for selected compounds (**12a**, **48**, **74a**, and **38**) using mitochondria extracted from *wt* MEFs, the release was observed for compounds with activity around and below 0.5 μM using mitochondria derived from *mcl-1*$^{-/-}$ MEFs (Figure 5A). Interestingly, at the concentration tested (10 μM), compound **38** (IC_{50} = 0.12 μM) releases cytochrome *c* as efficiently as compound **59b** (IC_{50} = 0.023 μM) or **1** (IC_{50} = 0.0002 μM).[45] No cytochrome *c* release was observed with any compounds tested on the permeabilized Bax/Bak MEFs (Figure 5B). These initial results support a mechanism-based effect: the ability of the small molecules to provoke cytochrome *c* release, albeit related to reaching a certain threshold of binding affinity for Bcl-X_L, which is dependent on Bax/Bak.

Cell-Based Activity. The ability of the compounds to affect cell viability through a mechanism-based induction of apoptosis on whole cells was evaluated with the most potent analogues of

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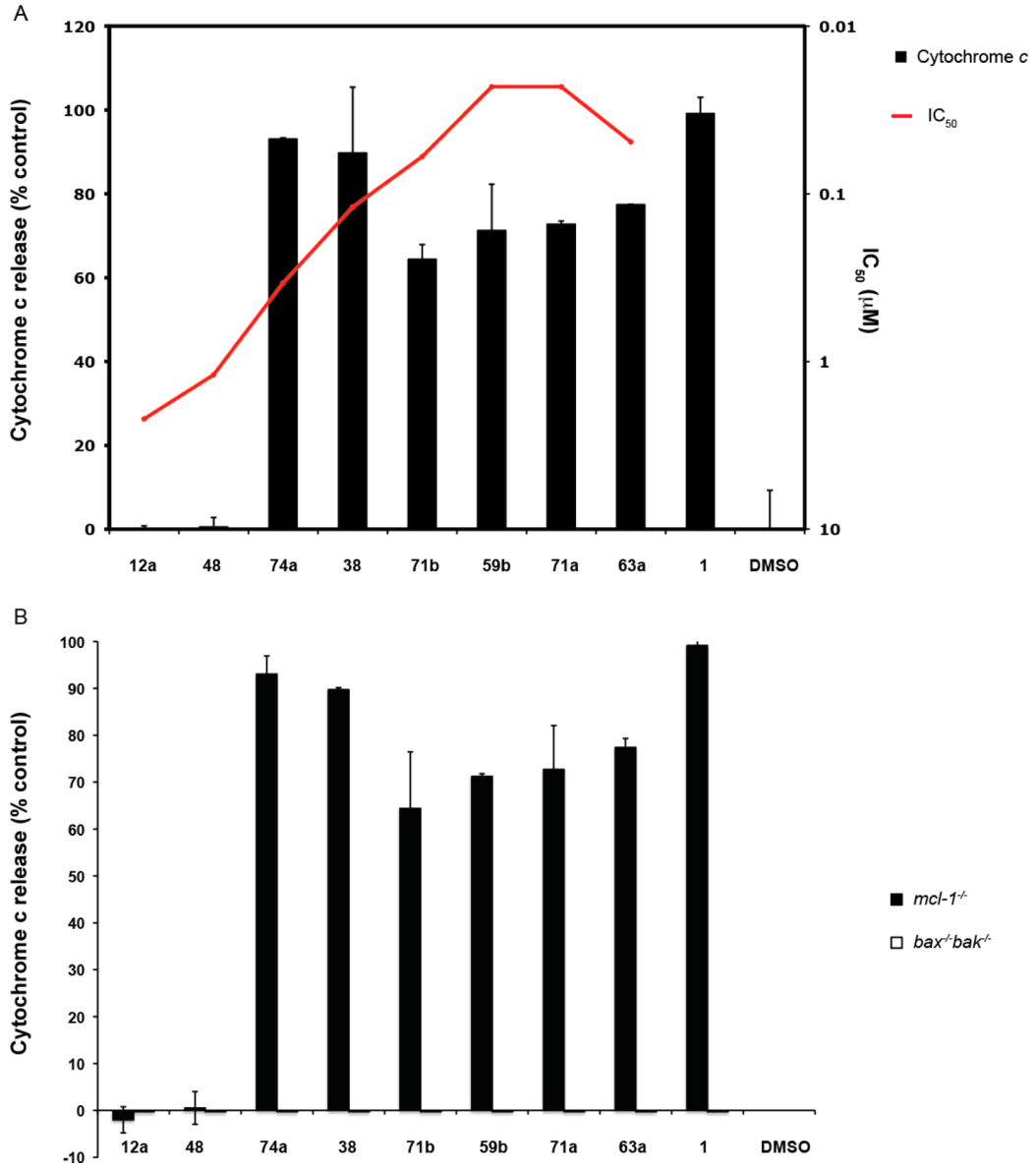

Figure 5. (A) Cytochrome *c* release experiments on *mcl-1*$^{-/-}$ MEF cells. In red: IC_{50} for each compound. Cells treated with compounds at a concentration of 10 μM. Abscissa: compounds. Left ordinate: cytochrome *c* release as % of that released by Bim 26-mer BH3 peptide. Right ordinate: binding IC_{50} in μM (LPA). Error bars: standard error. (B) Cytochrome *c* release experiments on *mcl-1*$^{-/-}$ MEF cells and bax$^{-/-}$/bak$^{-/-}$ MEF cells. Left ordinate: cytochrome *c* release as % of Bim 26-mer BH3 peptide. Abscissa: compounds. Error bars: Standard Error.

our series. In these experiments, the compounds were tested in *mcl-1*$^{-/-}$ and wild-type (*wt*) MEFs. In the wt cells, both Bcl-X_L and Mcl-1 are present and inhibit Bak's pro-apoptotic activity. Hence a compound selectively inhibiting the Bcl-X_L-dependent pathway should not significantly impair their viability because Mcl-1 can still prevent activation of Bak. Conversely, inhibiting the Bcl-X_L-dependent *mcl-1*$^{-/-}$ MEFs should result in apoptosis, while little or no difference between *mcl-1*$^{-/-}$ and wt MEF viability would suggest that the compounds are nonselective cytotoxics. As with the cytochrome *c* release assay, *bax*$^{-/-}$/*bak*$^{-/-}$ MEF cells were included as an additional control. Finally, the level of serum (fetal bovine serum, FBS) was varied between a low (1% v/v) and a higher concentration (10% v/v) to observe its effect on compound activity because previously developed molecules targeting the hydrophobic groove of pro-survival proteins have demonstrated affinity for the binding site of human serum albumin domain III (HSA).[46,47] Although binding kinetics may eventually favor binding to the target protein (through differences in dissociation rates),[48,49] strong binding to HSA would impact on the compounds' ability to efficiently reach their targets. As reported in other accounts in this field, great efforts have been devoted to separating the SAR for HSA and Bcl-X_L and Bcl-2.[13] Similarly, the series of compounds we have developed might bind strongly to serum proteins as they also are lipophilic acids.

The results of these experiments are presented in Table 10. In the presence of 1% serum, the most potent compounds from the series (as measured by LPA assays) impaired the viability of *mcl-1*$^{-/-}$ MEFs, with submicromolar EC_{50} values (**59b**, **63d**, and **70a**, EC_{50} values between 0.13 and 0.29 μM). Very weak or no activity was measured in the wild-type MEF and Bax/Bak DKO MEF cells, supporting a mechanism-based activity. However, as expected, the cell-based activity was strongly attenuated in the presence of 10% FBS. Only analogue **63d**

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Table 10. Cellular Activity for Selected Compounds[a]

compd	binding assay, IC_{50}[b]	EC_{50} (Mcl-$1^{-/-}$ MEF, 1% FBS)	EC_{50} (Mcl-$1^{-/-}$ MEF, 10% FBS)	EC_{50} (wt MEF, 10% FBS)	EC_{50} (Bax/Bak-DKO-MEF, 1% FBS)
1	0.0005	0.004	0.08	>5	>20
59b	0.023	0.29	1.9	>5	>20
63b	0.010	1.6	>20	>5	>20
63d	0.017	0.13	0.85	>5	6
63e	0.055	1.4	10	>5	>20
71a	0.024	0.26	1.7	>5	>13

[a]All values in μM as average of at least two replicates. [b]Measured by LPA. MEF: mouse embryonic fibroblasts. FBS: fetal bovine serum.

retained a submicromolar level of activity with an EC_{50} value of 0.85 μM. Although comparison with compound **1** shows that both potency and serum binding need to be improved to reach a therapeutically relevant level of cellular activity, the results obtained with our benzothiazole series of compounds illustrate moderate and selective cellular activity with low molecular weight molecules. This successful hit-to-lead campaign will allow further improvements toward more potent and drug-like compounds.

DISCUSSION AND CONCLUSION

Targeting proteins of the Bcl-2 family remains a challenge. The signaling cascade involving this family relies on protein−protein interactions, still often considered as "undruggable".[50,51] Despite the considerable efforts devoted to the development of inhibitors of pro-survival proteins, only a handful of successful programs have been published.[10] By highlighting problems and their solutions, reports on new series of Bcl-2 inhibitors provide invaluable information to medicinal chemists who are interested in the design of new drugs against this class of protein. Here, we present novel selective inhibitors of Bcl-X_L derived from a high-throughput screening campaign. Other compounds partially selective for Bcl-X_L have been described, notably [(*R*)-4-(3-dimethylamino-1-phenylsulfanylmethyl-propylamino)-*N*-[4-(4,4-dimethyl-piperidin-1-yl)-benzoyl]-3-nitrobenzenesulfonamide (A-385358)[52] and (*R*)-4-(4-(biphenyl-3-ylmethyl)piperazin-1-yl)-*N*-(4-(4-(dimethylamino)-1-(phenylthio)butan-2-ylamino)-3-nitrophenylsulfonyl)-benzamide (W1191542)[53] have high affinities for Bcl-X_L (K_i = 0.17 nM and IC_{50} = 11 nM, respectively) but are still potent Bcl-2 inhibitors (K_i = 67 nM and IC_{50} = 130 nM, respectively). We have shown that structure-guided optimization of **55b** (IC_{50} (Bcl-X_L) = 23 nM) maintained this exquisite selectivity.[25]

Compounds directly inhibiting the pro-survival activity of Bcl-2 proteins should essentially replicate the functional activity of the pro-apoptotic BH3-only proteins. Along with potency, an important goal during the development of Bcl-2 inhibitors is the validation of their mechanism of action. Genuine inhibitors of the Bcl-2 family of proteins should exhibit some key characteristics, among others Bax/Bak-dependent cell killing and high affinity for the target protein[21,54] It is possible to develop assays that allow studies of the mechanism of action supporting these criteria. All the binding affinity data we have presented strongly confirms selective and direct binding to Bcl-X_L. We have observed a coherent SAR within the series with an increase in potency to low nanomolar levels. The binding affinity has been measured using three separate assay technologies: two competition-based assays (LPA and SPR) and one experiment measuring direct binding (SPR). The most potent compounds demonstrate low nanomolar affinities in all three assays (IC_{50} < 20 nM, K_D < 10 nM). These molecules also demonstrate selectivity for Bcl-X_L over other members of the family. This selectivity is a surprising result: most of the known small molecules reported to bind to the pro-survival family of proteins have more than one target, including **1**, which binds avidly to Bcl-2, Bcl-w, and Bcl-X_L.[14] The phenylpyrazole series of molecules developed by Celltech show preferential selectivity for Bcl-2 but retain some affinity for Bcl-X_L.[55] More recently, Abbvie disclosed a selective Bcl-2 inhibitor derived from the acylsufonamide **1** series.[12] Our results suggest a high level of structural complementarity between protein and compound. In particular, the SAR highlights that the benzothiazole and the hydrazone moieties are probably located in a narrow pocket able to accommodate a hydrophobic moiety and that key interactions occur between the protein and this portion of the molecule. The binding affinity results also suggest that a hydrogen bond-donating group located in the binding groove of Bcl-X_L interacts specifically with the terminal carboxylic acid and that this interaction is optimal with a picolinic acid motif. Subsequent structural biology studies have confirmed these binding hypotheses as well as shed some light as to why this series exhibits strong selectivity for Bcl-X_L.[25]

With biological tools, we have provided initial evidence that the benzothiazole series exert its cellular activity through a pathway involving Bcl-X_L. In both mitochondria cytochrome *c* and whole-cell assays, activity was only observed in the absence of Mcl-1 and is abrogated in the absence of Bax and Bak (Figure 5A,B). In the whole-cell assay, our results confirm that the compounds are not nonselective cytotoxic agents, as no activity was observed in wild-type MEF cells (Table 10). Whereas in mitochondria from permeabilized MEFs, cytochrome *c* release is observed with compounds possessing IC_{50} values of 0.5 μM or below, the cell-killing activity is only observed with more potent compounds (IC_{50} < 20 nM) and is strongly influenced by the level of serum protein in the media. Finally, our compounds are not yet sufficiently potent to show killing activity in Bcl-X_L-dependent cancer cell lines (data not shown).

One key question in the development of Bcl-2-family inhibitors is the level of potency required for cell-based activity and, ultimately, in vivo activity in animal models of cancer. Data available from the research program that led to **1** and from BH3-peptides indicates that, in biochemical binding assays, inhibition of the target protein(s) at subnanomolar concentrations is necessary. Compound **1** achieves this by covering a large area of interaction within the hydrophobic groove of Bcl-2 and Bcl-X_L. Its molecular weight is 812 Da, and its structure challenges most of the rules set out by Lipinski and others for good oral bioavailability.[56,57] Our work shows that it is possible to obtain potent ligands for Bcl-X_L while maintaining a reasonable molecular weight (414 Da for compound **59b**) and that, even for challenging targets such as the Bcl-2 proteins, hit-to-lead progression can be done through core optimization using classical medicinal chemistry.[58] However, further improvements are still required to reach therapeutically relevant levels of activity. Looking ahead, some important issues related to properties and structure will require attention in the next stage of our research. Once again, the sensitivity to serum protein levels will need to be addressed.

In conclusion, we have described a series of selective and potent ligands of Bcl-X_L. Initial evidence of mechanism-based

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activity has been provided supporting inhibition of a Bcl-X_L-driven pathway in a Bax/Bak dependent manner. Beyond its importance for the development of new drugs modulating the activity of Bcl-2 proteins, our work provides important tools that will help clarify the role of Bcl-X_L in the progression of various types of cancers and its involvement in other biological mechanisms such as normal tissue homeostasis.

EXPERIMENTAL SECTION

Chemistry. *General Chemistry Methods.* All nonaqueous reactions were performed in oven-dried glassware under an atmosphere of dry nitrogen unless otherwise specified. Tetrahydrofuran was freshly distilled from sodium/benzophenone under nitrogen. Dichloromethane was freshly distilled from CaH_2 under N_2. All other solvents were reagent grade. Petroleum ether describes a mixture of hexanes in the bp range 40−60 °C. Analytical thin-layer chromatography was performed on Merck silica gel $60F_{254}$ aluminum-backed plates and were visualized by fluorescence quenching under UV light. Flash chromatography was performed with silica gel 60 (particle size 0.040−0.063 mm). NMR spectra were recorded on a Bruker Avance DRX 300 with the solvents indicated (^{1}H NMR at 300 MHz). Chemical shifts are reported in ppm on the δ scale and referenced to the appropriate solvent peak. HRMS were recorded at the Australian National University Mass Spectrometry Facility using a Waters LCT Premier XE (ESI TOF mass spectrometer). Unless otherwise noted, all tested compounds were found to be >95% pure. Compounds **19a−b** and **20a−f** were obtained following known procedures. Compound **6** was originally purchased from Specs. Compound **14** is commercially available.

General Method A: Preparation of Meerwein Adducts. Preparation of 3-(5-formylfuran-2-yl)-2-methylbenzoic acid (**9a**). 3-Amino-2-methylbenzoic acid (1.5 g, 10 mmol) in 5 mL of water and 15 mL of concentrated HCl was treated over 5 min at 0−5 °C with $NaNO_2$ (0.8 g, 11.6 mmol) dissolved in 4 mL of water. Solutions of furaldehyde (1 g, 10.4 mmol) in 6 mL of acetone and $CuCl_2{\cdot}2H_2O$ (0.5 g, 3 mmol) in 3 mL of water were then added simultaneously. The reaction was stirred at room temperature for 10 h. After that time, a dark precipitate had formed. It was collected by filtration and dried in vacuo. The crude solid was purified by flash chromatography using SiO_2 (CH_2Cl_2/MeOH 95:5). A yellow solid was obtained (m = 230 mg, 10%). (^{1}H NMR (DMSO-d_6, ppm): δ 9.65 (s, 1H), 7.80 (d, J = 7.3 Hz, 1H), 7.77 (d, J = 7.0 Hz, 1H), 7.68 (d, J = 3.8 Hz, 1H), 7.43 (t, J = 7.9 Hz, 1H), 7.07 (d, J = 3.7 Hz, 1H), 2.57 (s, 3H). LCMS m/z 229.1 [M − 1].

*3-(5-Formylfuran-2-yl)-benzoic Acid (**9b**).* Prepared according to method A using 3-aminobenzoic acid (3.43 g, 25 mmol) and furaldehyde (2.4 g, 25 mmol). A brown solid was obtained (m = 2.59 g, 48%). ^{1}H NMR (DMSO-d_6, ppm): δ 9.61 (σ, 1H), 8.08 (d, J = 8.0 Hz, 1H), 7.96 (d, J = 7.8 Hz, 1H), 7.64 (d, J = 3.8 Hz, 1H), 7.61 (t, J = 7.8 Hz, 1H), 7.38 (d, J = 3.8 Hz, 1H). LCMS m/z 215.3 [M − 1].

*3-(5-Acetylfuran-2-yl)-benzoic Acid (**10a**).* Prepared according to method A using 3-aminobenzoic acid (2.75 g, 20 mmol) and 2-furyl methyl ketone (2.2 g, 20 mmol). A brown solid was obtained (m = 1.3 g, 28%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.37 (s, 1H), 8.11 (d, J = 7.8 Hz, 1H), 7.98 (d, J = 7.7 Hz, 1H), 7.65 (t, J = 7.8 Hz, 1H), 7.60 (d, J = 3.6 Hz, 1H), 7.35 (d, J = 3.7 Hz, 1H), 2.49 (s, 3H). LCMS m/z 229 [M − 1].

*4-(5-Acetylfuran-2-yl)-benzoic Acid (**10b**).* Prepared according to method A using 4-aminobenzoic acid (1.4 g, 10 mmol) and 2-furyl methyl ketone (1.1 g, 10 mmol). A light-brown solid was obtained (m = 0.96 g, 42%). ^{1}H NMR ($CDCl_3$, ppm): δ 8.85 (d, J = 8.3 Hz, 2H), 7.83 (d, J = 8.2 Hz, 2H), 7.26 (d, J = 4.7 Hz, 1H), 6.88 (d, J = 3.7 Hz, 1H), 2.52 (s, 3H). LCMS m/z 228.8 [M − 1].

*2-(5-Acetylfuran-2-yl)-benzoic Acid (**10c**).* Prepared according to method A using 2-aminobenzoic acid (3 g, 22 mmol) and 2-furyl methyl ketone (2.2 g, 20 mmol). A brown solid was obtained (m = 750 mg, 16%). ^{1}H NMR ($CDCl_3$, ppm): δ 9.85 (br s, 1H), 7.92 (d, J = 7.7 Hz, 1H), 7.71 (d, J = 5.8 Hz, 1H), 7.60 (t, J = 7.4 Hz, 1H), 7.49 (t, J = 7.6 Hz, 1H), 7.27 (d, J = 3.7 Hz, 1H), 6.75 (d, J = 3.6 Hz, 1H), 2.49 (s, 3H). LCMS m/z 228.8 [M − 1].

*3-(5-Acetylfuran-2-yl)-4-chlorobenzoic Acid (**10d**).* Prepared according to method A using 3-amino-4-chlorobenzoic acid (3.4 g, 20 mmol) and 2-furyl methyl ketone (2.0 g, 18 mmol). A brown solid was obtained (m = 4.2 g, 79%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.42 (d, J = 2 Hz, 1H), 7.94 (dd, J = 2.1 and 8.4 Hz, 1H), 7.75 (d, J = 8.3 Hz, 1H), 7.61 (d, J = 3.8 Hz, 1H), 7.39 (d, J = 3.8 Hz, 1H), 2.50 (s, 3H). LCMS m/z 263 [M − 1].

*5-(5-Acetylfuran-2-yl)-2-chlorobenzoic Acid (**10e**).* Prepared according to method A using 2-chloro-5-aminobenzoic acid (3.4 g, 20 mmol) and 2-furyl methyl ketone (2.0 g, 18 mmol). A brown solid was obtained (m = 1.1 g, 21%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.19 (δ, J = 2.3 Hz, 1H), 7.98 (dd, J = 2.3 and 8.5 Hz, 1H), 7.67 (d, J = 8.4 Hz, 1H), 7.58 (d, J = 3.8 Hz, 1H), 7.36 (d, J = 3.7 Hz, 1H), 2.47 (s, 3H). LCMS m/z 262.8 [M − 1].

*3-(5-Acetylfuran-2-yl)-2-chlorobenzoic Acid (**10f**).* Prepared according to method A using 2-chloro-3-aminobenzoic acid (1 g, 5.8 mmol) and 2-furyl methyl ketone (642 mg, 5.8 mmol). A brown solid was obtained (m = 137 mg, 9%). ^{1}H NMR (DMSO-d_6, ppm): δ 7.99 (δ J = 1.5 and 7.8 Hz, 1H), 7.73 (dd, J = 1.5 and 7.6 Hz, 1H), 7.57 (t, J = 8.3 Hz, 1H), 7.58 (d, J = 3.9 Hz, 1H), 7.32 (d, J = 3.8 Hz, 1H), 2.51 (s, 3H). LCMS m/z 263.2 [M − 1].

*5-(5-Acetylfuran-2-yl)isophthalic Acid (**10g**).* Prepared according to method A using 5-aminoisophthalic acid (718 mg, 3.96 mmol) and 2-furyl methyl ketone (436 mg, 3.96 mmol). A yellow solid was obtained (m = 515 mg, 47%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.54 (d, J = 1.5 Hz, 1H), 8.46 (t, J = 1.5 Hz, 1H), 7.59 (d, J = 3.8 Hz, 1H), 7.47 (d, J = 3.7 Hz, 1H), 2.49 (s, 3H). LCMS m/z 273.1 [M − 1].

*3-(5-Acetylfuran-2-yl)-2-methylbenzoic Acid (**10h**).* Prepared according to method A using 3-amino-2-methylbenzoic acid (1.5 g, 10 mmol) and 2-furyl methyl ketone (1.1 mg, 10 mmol). A light-brown solid was obtained (m = 235 mg, 10%). ^{1}H NMR (DMSO-d_6, ppm): δ 7.80 (dd, J = 1.1 and 7.9 Hz, 1H), 7.76 (d, J = 7.7 Hz, 1H), 7.57 (d, J = 3.7 Hz, 1H), 7.42 (t, J = 7.8 Hz, 1H), 6.99 (d, J = 3.7 Hz, 1H), 2.57 (s, 3H), 2.47 (s, 3H). LCMS m/z 169.2 [M − 1].

*5-(5-Acetylfuran-2-yl)-2-nitrobenzoic Acid (**10i**).* Prepared according to method A using 5-amino-2-nitrobenzoic acid (1 g, 5.5 mmol) and 2-furyl methyl ketone (605 mg, 5.5 mmol). A brown solid was obtained (m = 1.44 g, 48%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.23−8.13 (m, 3H), 7.62 (d, J = 3.8 Hz, 1H), 7.57 (d, J = 3.8 Hz, 1H), 2.51 (s, 3H). LCMS m/z 274.2 [M − 1].

*5-(5-Acetylfuran-2-yl)-2-aminobenzoic Acid (**10j**).* Ammonium formate (1.72 g, 27.3 mmol) was dissolved in 40 mL of dry methanol. Compound **10i** (500 mg, 1.82 mmol) was then added followed by Pd/C (10%, 220 mg). Once the reaction was complete as indicated by TLC (CH_2Cl_2/MeOH, 5:1), it was filtered through a plug of Celite. The solids were washed with MeOH. The filtrate was concentrated to an orange solid, which was suspended in water and filtered to give a brown solid dried in a vacuum oven (281 mg, 63%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.16 (d, J = 1.3 Hz, 1H), 7.71 (dd, J = 1.8 and 8.7 Hz, 1H), 7.51 (d, J = 3.5 Hz, 1H), 6.90 (d, J = 3.5 Hz, 1H), 6.85 (d, J = 8.7 Hz, 1H), 2.41 (s, 3H). LCMS m/z 246.1 [M + 1].

General Method B: Preparation of Hydrazones. Preparation of 3-(5-((2-(benzo[d]thiazol-2-yl)hydrazono)methyl)furan-2-yl)-2-methylbenzoic acid (**6**). Compound **9a** (100 mg, 0.44 mmol) was dissolved in 8 mL of ethanol with gentle warming. A solution of 2-hydrazinobenzothiazole (60 mg, 0.36 mmol) in 8 mL of ethanol was added to **9a**, and the reaction was heated to 80 °C and cooled down slowly. A brown precipitate appeared. It was collected by filtration, rinsed with cold ethanol, and dried in a vacuum oven (m = 54 mg, 40%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.06 (s, 1H), 7.80 (d, J = 8.4 Hz, 1H), 7.77 (d, J = 9.2 Hz, 1H), 7.70 (d, J = 7.7 Hz, 1H), 7.44−7.40 (m, 2H), 7.29 (t, J = 7.2 Hz, 1H), 7.10 (t, J = 7.8 Hz, 1H), 7.01 (d, J = 3.5 Hz, 1H), 6.92 (d, J = 3.5 Hz, 1H), 2.61 (s, 3H). LCMS m/z 378.6 [M + 1]. HRMS calculated mass, 378.0912 [M + 1]; measured, 378.0909 [M + 1]. Data identical to commercial sample from Specs.

*3-(5-((2-(Benzo[d]thiazol-2-yl)hydrazono)methyl)furan-2-yl)-benzoic Acid (**11**).* Procedure B was followed using compound **9b** (72 mg, 0.31 mmol) and 2-hydrazinobenzothiazole (60 mg, 0.36 mmol). A yellow solid was obtained (m = 72 mg, 64%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.31 (s, 1H), 8.06 (s, 1H), 8.01 (d, J = 7.7 Hz, 1H), 7.87 (d, J

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= 7.8 Hz, 1H), 7.77 (d, *J* = 7.6 Hz, 1H), 7.68−7.53 (m, 2H), 7.41 (d, *J* = 8.0 Hz, 1H), 7.30 (t, *J* = 7.9 Hz, 1H), 7.25 (d, *J* = 3.5 Hz, 1H), 7.10 (t, *J* = 7.4 Hz, 1H), 6.99 (d, *J* = 3.6 Hz, 1H). LCMS *m*/*z* 364.2 [M + 1].

*3-(5-(1-(2-(Benzo[d]thiazol-2-yl)hydrazono)ethyl)furan-2-yl)-benzoic Acid (**12a**).* Procedure B was followed using compound **10a** (50 mg, 0.22 mmol) and 2-hydrazinobenzothiazole (50 mg, 0.30 mmol). A light-brown solid was obtained (*m* = 55 mg, 66%). ^{1}H NMR (DMSO-d_6, ppm): δ 12.5 (br s, 2H), 8.32 (s, 1H), 8.02 (d, *J* = 8.0 Hz, 1H), 7.89 (d, *J* = 7.8 Hz, 1H), 7.71 (d, *J* = 7.8 Hz, 1H), 7.61 (t, *J* = 7.8 Hz, 1H), 7.31−7.25 (m, 2H), 7.22 (d, *J* = 3.5 Hz, 1H), 7.08 (t, *J* = 8.1 Hz, 1H), 7.01 (d, *J* = 3.5 Hz, 1H), 2.41 and 2.33 (s, 3H). LCMS *m*/*z* 378.4 [M + 1]. HRMS calculated mass, 378.0912 [M + 1]; measured, 378.0908 [M + 1].

*4-(5-(1-(2-(Benzo[d]thiazol-2-yl)hydrazono)ethyl)furan-2-yl)-benzoic Acid (**12b**).* Procedure B was followed using compound **10b** (140 mg, 0.65 mmol) and 2-hydrazinobenzothiazole (95 mg, 0.58 mmol). A cream solid was obtained (*m* = 128 mg, 58%). ^{1}H NMR (DMSO-d_6, ppm): Mixture of two isomers δ 8.02 (d, *J* = 8.2 Hz, 2H), 7.89 (d, *J* = 8.4 Hz, 2H), 7.72 (d, *J* = 7.6 Hz, 1H), 7.35−7.22 (m, 3H), 7.09 (t, *J* = 7.8 Hz, 1H), 7.03 (d, *J* = 3.5 Hz, 1H), 2.42 and 2.33 (2 s, 3H). LCMS *m*/*z* 378.7 [M + 1].

*2-(5-(1-(2-(Benzo[d]thiazol-2-yl)hydrazono)ethyl)furan-2-yl)-benzoic Acid (**12c**).* Procedure B was followed using compound **10c** (70 mg, 0.3 mmol) and 2-hydrazinobenzothiazole (50 mg, 0.3 mmol). A brown solid was obtained (*m* = 56 mg, 50%). ^{1}H NMR (DMSO-d_6, ppm): δ 7.74 (d, *J* = 7.8 Hz, 1H), 7.68 (d, *J* = 7.7 Hz, 1H), 7.63−7.57 (m, 2H), 7.45 (t, *J* = 7.5 Hz, 1H), 7.32−7.24 (m, 2H), 7.07 (t, *J* = 7.9 Hz, 1H), 6.96 (d, *J* = 3.5 Hz, 1H), 6.88 (d, *J* = 3.6 Hz, 1H), 2.30 (s, 3H). LCMS *m*/*z* 378.7 [M + 1]. HRMS calculated mass, 378.0912 [M + 1]; measured, 378.0911 [M + 1].

*3-(5-(1-(2-(Benzo[d]thiazol-2-yl)hydrazono)ethyl)furan-2-yl)-4-chlorobenzoic Acid (**12d**).* Procedure B was followed using compound **10d** (100 mg, 0.38 mmol) and 2-hydrazinobenzothiazole (50 mg, 0.3 mmol). A cream solid was obtained (*m* = 55 mg, 45%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.54 (d, *J* = 2.0 Hz, 1H), 7.87 (dd, *J* = 8.3 and 2.1 Hz, 1H), 7.72 (d, *J* = 8.3 Hz, 1H), 7.69 (d, *J* = 5.9 Hz, 1H), 7.34 (d, *J* = 3.6 Hz, 1H), 7.31−7.25 (m, 2H), 7.09 (t, *J* = 7.9 Hz, 1H), 7.05 (d, *J* = 3.7 Hz, 1H), 2.33 (s, 3H). LCMS *m*/*z* 413.0 [M + 1].

*5-(5-(1-(2-(Benzo[d]thiazol-2-yl)hydrazono)ethyl)furan-2-yl)-2-chlorobenzoic Acid (**12e**).* Procedure B was followed using compound **10e** (100 mg, 0.38 mmol) and 2-hydrazinobenzothiazole (50 mg, 0.30 mmol). An off-white solid was obtained (*m* = 80 mg, 65%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.15 (d, *J* = 2.1 Hz, 1H), 7.90 (dd, *J* = 8.4 and 2.2 Hz, 1H), 7.70 (d, *J* = 7.6 Hz, 1H), 7.65 (d, *J* = 8.5 Hz, 1H), 7.34−7.28 (m, 2H), 7.25 (d, *J* = 3.6 Hz, 1H), 7.09 (t, *J* = 8.0 Hz, 1H), 7.01 (d, *J* = 3.6 Hz, 1H), 2.32 (s, 3H). LCMS *m*/*z* 412.1 [M + 1]. HRMS calculated mass, 412.0517 [M + 1]; measured, 412.0518 [M + 1].

*3-(5-(1-(2-(Benzo[d]thiazol-2-yl)hydrazono)ethyl)furan-2-yl)-2-chlorobenzoic Acid (**12f**).* Procedure B was followed using compound **10f** (50 mg, 0.19 mmol) and 2-hydrazinobenzothiazole (31 mg, 0.19 mmol). An orange solid was obtained (*m* = 57 mg, 62%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.01 (d, *J* = 7.7 Hz, 1H), 7.71 (d, *J* = 7.5 Hz, 1H), 7.66−7.63 (m, 1H), 7.58 (t, *J* = 6.5 Hz, 1H), 7.37−7.22 (m, 3H), 7.09 (d, *J* = 7.8 Hz, 1H), 7.05 (d, *J* = 4.8 Hz, 1H), 2.33 (s, 3H). LCMS *m*/*z* 412.0 [M + 1].

*5-(5-(1-(2-(Benzo[d]thiazol-2-yl)hydrazono)ethyl)furan-2-yl)-isophthalic Acid (**12g**).* Procedure B was followed using compound **10g** (100 mg, 0.36 mmol) and 2-hydrazinobenzothiazole (60 mg, 0.36 mmol). An off-white solid was obtained (*m* = 90 mg, 59%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.51 (s, 2H), 8.39 (s, 1H), 7.69 (d, *J* = 7.4 Hz, 1H), 7.36 (d, *J* = 3.5 Hz, 1H), 7.30−7.26 (m, 2H), 7.09 (t, *J* = 8.1 Hz, 1H), 7.03 (d, *J* = 3.3 Hz, 1H), 2.36 (s, 3H). LCMS *m*/*z* 422.7 [M + 1].

*3-(5-(1-(2-(Benzo[d]thiazol-2-yl)hydrazono)ethyl)furan-2-yl)-2-methylbenzoic Acid (**12h**).* Procedure B was followed using compound **10h** (80 mg, 0.33 mmol) and 2-hydrazinobenzothiazole (40 mg, 0.24 mmol). A brown solid was obtained (*m* = 45 mg, 48%). ^{1}H NMR (DMSO-d_6, ppm): 1:2 mixture of two isomers δ 7.83 and 7.81 (2 d, *J* = 8.3 and 8.0 Hz, 1H), 7.70 (t, *J* = 6.8 Hz, 2H), 7.56 (d, *J* = 7.3 Hz, 0.3H), 7.41 (t, *J* = 7.7 Hz, 1H), 7.33−7.22 (m, 1.7H) 7.16 (d, *J* = 7.5 Hz, 0.3H), 7.07 (t, *J* = 8.2 Hz, 0.7H), 7.02 (d, *J* = 3.6 Hz, 1H), 6.99 (d, *J* = 3.6 Hz, 0.3H), 6.90 (d, *J* = 3.6 Hz, 0.7H), 2.38 and 2.31 (2 s, 3H). LCMS *m*/*z* 392.0 [M + 1].

*5-(5-(1-(2-(Benzo[d]thiazol-2-yl)hydrazono)ethyl)furan-2-yl)-2-nitrobenzoic Acid (**12i**).* Procedure B was followed using compound **10i** (250 mg, 0.91 mmol) and 2-hydrazinobenzothiazole (150 mg, 0.91 mmol). An off-white solid was obtained (*m* = 344 mg, 89%). ^{1}H NMR (DMSO-d_6, ppm): 14:86 mixture of two isomers δ 8.17−8.12 (m, 2H), 8.05 (dd, *J* = 1.7 and 8.5 Hz, 0.86H), 7.79 (d, *J* = 3.7 Hz, 0.14H), 7.70 (d, *J* = 7.7 Hz, 0.86H), 7.59−7.56 (m, 0.28H), 7.51 (d, *J* = 3.7 Hz, 0.86H), 7.34−7.23 (m, 2H), 7.12−7.01 (m, 2H), 2.42 (s, 0.42H), 2,34 (s, 2.58H). LCMS *m*/*z* 423.1 [M + 1]. HRMS calculated mass, 423.0758 [M + 1]; measured, 423.0754 [M + 1].

*5-(5-(1-(2-(Benzo[d]thiazol-2-yl)hydrazono)ethyl)furan-2-yl)-2-aminobenzoic Acid (**12j**).* Procedure B was followed using compound **10j** (67 mg, 0.28 mmol) and 2-hydrazinobenzothiazole (45 mg, 0.28 mmol). A white solid was obtained (*m* = 53 mg, 48%). ^{1}H NMR (DMSO-d_6, ppm): 29:71 mixture of two isomers δ 9.3 (br s, 3H), 8.15 (s, 1H), 7.71−7.64 (m, 2H), 7.46 (d, *J* = 3.7 Hz, 0.29H), 7.33−7.24 (m, 1.71), 7.07 (t, *J* = 7.3 Hz, 1H), 6.90−6.85 (m, 2H), 6.74 (d, *J* = 3.3 Hz, 1H), 2.41 (s, 0.87H), 2.30 (s, 2.13H). LCMS *m*/*z* 393.1 [M + 1]. HRMS calculated mass, 393.1016 [M + 1]; measured, 393.1018 [M + 1].

*3-(5-Formylfuran-2-yl)benzamide (**15a**).* Prepared according to method A using 3-aminobenzamide (1 g, 7.3 mmol) and 1-(furan-2-yl)ethanone (809 mg, 7.3 mmol). A white solid was obtained (*m* = 246 mg, 15%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.34 (s, 1H), 8.14 (s, 1H), 8.01 (d, *J* = 7.7 Hz, 1H), 7.92 (d, *J* = 6.9 Hz, 1H), 7.63−7.58 (m, 2H), 7.53 (s, 1H), 7.29 (d, *J* = 3.5 Hz, 1H), 2.5 (s, 3H). LCMS *m*/*z* 216.2 [M + 1].

*2-(3-(5-Acetylfuran-2-yl)phenyl)acetic Acid (**15b**).* Prepared according to method A using 2-(3-aminophenyl)acetic acid (0.9 g, 6.0 mmol) and 1-(furan-2-yl)ethanone (700 mg, 6.4 mmol). A yellow crystalline solid was obtained (*m* = 70 mg, 5%). ^{1}H NMR ($CDCl_3$, ppm): δ 7.72 (s, 1H), 7.70 (s, 1H), 7.41 (t, *J* = 7.9 Hz, 1H), 7.30 (d, *J* = 7.5 Hz, 1H), 7.27−7.26 (m, 2H), 6.79 (d, *J* = 3.7 Hz, 1H), 3.72 (s, 2H), 2.54 (s, 3H). LCMS *m*/*z* 245.4 [M + 1].

*2-(2-((5-(3-Nitrophenyl)furan-2-yl)methylene)hydrazinyl)-benzothiazole (**16a**).* Procedure B was followed using compound **14** (65 mg, 0.3 mmol) and 2-hydrazinobenzothiazole (50 mg, 0.3 mmol). An orange solid was obtained (*m* = 80 mg, 73%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.53 (t, *J* = 1.9 Hz, 1H), 8.23−8.16 (m, 2H), 8.08 (s, 1H), 7.80 (br s, 1H), 7.78 (t, *J* = 7.9 Hz, 1H), 7.43 (d, *J* = 3.6 Hz, 1H), 7.41 (br s, 1H), 7.30 (td, *J* = 8.5 and 1.2 Hz, 1H), 7.11 (td, *J* = 8.3 and 1.1 Hz, 1H), 7.03 (d, *J* = 3.7 Hz, 1H). LCMS *m*/*z* 365.3 [M + 1]. HRMS calculated mass, 365.0703 [M + 1]; measured, 365.0702 [M + 1].

*Methyl 3-(5-((2-(Benzothiazol-2-yl)hydrazono)methyl)furan-2-yl)benzoate (**16b**).* Compound **11** (50 mg, 0.14 mmol) was suspended in 20 mL of methanol and treated with a few drops of H_2SO_4. The reaction was then refluxed for 8 h. The cooled reaction mixture was concentrated and the residue taken up in CH_2Cl_2. The organic layer was washed with a cold dilute aqueous solution of $NaHCO_3$, dried over Na_2SO_4, and concentrated. An off-white solid was obtained (*m* = 40 mg, 76%). ^{1}H NMR ($CDCl_3$, ppm): δ 8.42 (s, 1H), 8.34 (s, 1H), 8.01 (dt, *J* = 6.2 and 1.7 Hz, 1H), 7.97 (dt, *J* = 7.9 and 1.3 Hz, 1H), 7.70 (d, *J* = 7.2 Hz, 1H), 7.65 (d, *J* = 7.9 Hz, 1H), 7.56−7.47 (m, 2H), 7.37 (t, *J* = 8.0 Hz, 1H), 7.00 (d, *J* = 3.7 Hz, 1H), 6.89 (d, *J* = 3.6 Hz, 1H), 3.98 (s, 3H). LCMS *m*/*z* 378.8 [M + 1]. HRMS calculated mass, 378.0907 [M + 1]; measured, 378.0907 [M + 1].

*3-(5-(1-(2-(Benzothiazol-2-yl)hydrazono)ethyl)furan-2-yl)-benzamide (**17a**).* Procedure B was followed using compound **12a** (100 mg, 0.44 mmol) and 2-hydrazinobenzothiazole (72 mg, 0.44 mmol). An off-white solid was obtained (*m* = 42 mg, 26%). ^{1}H NMR (DMSO-d_6, ppm): δ 11.71 (br s, 1H) 8.26 (s, 1H), 8.09 (s, 1H), 7.91 (d, *J* = 7.6 Hz, 1H), 7.82 (d, *J* = 7.6 Hz, 1H), 7.71 (br s, 1H), 7.57 (t, *J* = 6.8 Hz, 1H), 7.48 (s, 1H), 7.28 (s, 1H), 7.16 (s, 1H), 7.08 (s, 1H), 7.02 (s, 1H), 2.34 (s, 3H). LCMS *m*/*z* 377.1 [M + 1]. HRMS calculated mass, 377.1067 [M + 1]; measured, 377.1065 [M + 1].

*2-(3-(5-(1-(2-(Benzothiazol-2-yl)hydrazono)ethyl)furan-2-yl)-phenyl)acetic Acid (**17b**).* Procedure B was followed using compound

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12b (68 mg, 0.28 mmol) and 2-hydrazinobenzothiazole (54 mg, 0.33 mmol). A cream solid was obtained (m = 65 mg, 59%). ^{1}H NMR (DMSO-d_6, ppm): δ 7.72−7.66 (m, 3H), 7.42 (t, J = 7.6 Hz, 1H), 7.33−7.29 (m, 1H), 7.25 (t, J = 7.2 Hz, 2H), 7.11−7.05 (m, 1H), 7.08 (d, J = 3.5 Hz, 1H), 6.99 (d, J = 3.5 Hz, 1H), 3.65 (s, 2H), 2.32 (s, 3H). LCMS m/z 392.1 [M + 1]. HRMS calculated mass, 392.1063 [M + 1]; measured, 392.1062 [M + 1].

*2-Hydrazinylbenzoxazole (**19a**).* Hydrazine (1 mL, 32 mmol) was added to 2-chlorobenzo[d]oxazole (1 g, 6.5 mmol) dissolved in 1 mL of EtOH. The reaction temperature rose to 60−70 °C. It was stirred for 1 h and allowed to reach room temperature. A white precipitate appeared. It was collected by filtration and dried in vacuum (m = 735 mg, 76%). Analysis match previously reported data.[59]

*2-Hydrazino-1-methyl-1H-benzimidazole (**19b**).* The procedure used to prepare **19a** was followed for the preparation of **19b** from 2-chloro-1-methyl-1*H*-benzo[d]imidazole. Analysis matched previously reported data.[60]

General Method C: Preparation of Hydrazone Compounds from Substituted Benzothiazole. 2-Chloro-5-(5-(1-(2-(6-fluorobenzothiazol-2-yl)hydrazono)ethyl)furan-2-yl)-benzoic acid (**22a**): to a suspension of 6-fluorobenzothiazol-2-amine **21a** (84 mg, 0.5 mmol) in 2 mL of ethylene glycol were added successively hydrazine hydrate (234 μL, 4.1 mmol) and hydrazine dihydrochloride (210 mg, 2 mmol). The reaction was heated at 140 °C overnight, leading to the formation of 6-fluoro-2-hydrazinobenzothiazole. At room temperature, compound **10e** (132 mg, 0.5 mmol) dissolved in 4 mL of EtOH was added to the reaction mixture followed by 30 μL of acetic acid. This reaction was heated at 60 °C for 10 h. At room temperature, the precipitate was collected by filtration, washed with EtOH, and dried in a vacuum oven (m = 34 mg, 16% from **21a**). ^{1}H NMR (DMSO-d_6, ppm): δ 8.12 (d, J = 2.0 Hz, 1H), 7.85 (dd, J = 8.4 and 2.0 Hz, 1H), 7.66 (dd, J = 8.6 and 2.5 Hz, 1H), 7.61 (d, J = 8.5 Hz, 1H), 7.36−7.29 (m, 1H), 7.22 (d, J = 3.6 Hz, 1H), 7.10 (td, J = 9.1 and 2.6 Hz, 1H), 6.97 (d, J = 3.6 Hz, 1H), 2.28 (s, 3H). LCMS m/z 430.0 [M + 1].

*2-Chloro-5-(5-(1-(2-(6-chlorobenzothiazol-2-yl)hydrazono)ethyl)-furan-2-yl)-benzoic Acid (**22b**).* Procedure C was followed using 6-chlorobenzothiazol-2-amine (**21b**) on the same scale (m = 19 mg, 9% from **21b**). ^{1}H NMR (DMSO-d_6, ppm): δ 8.12 (d, J = 2.0 Hz, 1H), 7.85 (dd, J = 8.6 and 2.1 Hz, 1H), 7.84 (s, 1H), 7.61 (d, J = 8.4 Hz, 1H), 7.33−7.26 (m, 2H), 7.22 (d, J = 3.6 Hz, 1H), 6.99 (d, J = 3.5 Hz, 1H), 2.28 (s, 3H). LCMS m/z 446.1 [M + 1]. HRMS calculated mass, 446.0127 [M + 1]; measured, 446.0128 [M + 1].

*2-Chloro-5-(5-(1-(2-(6-bromobenzothiazol-2-yl)hydrazono)-ethyl)furan-2-yl)-benzoic Acid (**22c**).* Procedure C was followed using 6-bromobenzothiazol-2-amine (**21c**) on the same scale (m = 68 mg, 28% from **21c**). ^{1}H NMR (DMSO-d_6, ppm): δ 8.12 (d, J = 2.2 Hz, 1H), 7.95 (d, J = 1.8 Hz, 1H), 7.86 (dd, J = 8.4 and 2.2 Hz, 1H), 7.61 (d, J = 8.5 Hz, 1H), 7.39 (dd, J = 8.5 and 2.0 Hz, 1H), 7.25 (d, J = 8.6 Hz, 1H), 7.22 (d, J = 3.6 Hz, 1H), 6.99 (d, J = 3.6 Hz, 1H), 2.28 (s, 3H). LCMS m/z 491.8 [M + 1].

*2-Chloro-5-(5-(1-(2-(6-(trifluoromethyl)benzothiazol-2-yl)-hydrazono)ethyl)furan-2-yl)benzoic Acid (**22d**).* Procedure C was followed using 6-trifluoromethylbenzothiazol-2-amine (**21d**) on the same scale (m = 67 mg, 28% from **21d**). ^{1}H NMR (DMSO-d_6, ppm): d 8.18 (s, 1H), 8.02 (s, 1H), 7.79 (dd, J = 8.3 and 1.9 Hz, 1H), 7.59 (d, J = 8.3 Hz, 1H) 7.55 (d, J = 8.4 Hz, 1H), 7.48 (d, J = 8.5 Hz, 1H), 7.21 (d, J = 3.5 Hz, 1H), 7.04 (d, J = 3.5 Hz, 1H), 2.34 (s, 3H). LCMS m/z 480.1 [M + 1]. HRMS calculated mass, 480.0391 [M + 1]; measured, 480.0392 [M + 1].

*2-Chloro-5-(5-(1-(2-(5-bromobenzothiazol-2-yl)hydrazono)-ethyl)furan-2-yl)benzoic Acid (**22e**).* Procedure C was followed using 5-bromobenzothiazol-2-amine (**21e**) on the same scale (m = 30 mg, 12% from **21e**). ^{1}H NMR (DMSO-d_6, ppm): δ 8.11 (d, J = 2.2 Hz, 1H), 7.86 (dd, J = 8.4 and 2.3 Hz, 1H), 7.68 (d, J = 8.3 Hz, 1H), 7.61 (d, J = 8.5 Hz, 1H), 7.50 (s, 1H), 7.25−7.18 (m, 2H), 7.00 (d, J = 3.6 Hz, 1H), 2.29 (s, 3H). LCMS m/z 492.0 [M + 1]. HRMS calculated mass, 489.9622 [M + 1]; measured, 489.9625 [M + 1].

*2-Chloro-5-(5-(1-(2-(4-fluorobenzothiazol-2-yl)hydrazono)ethyl)-furan-2-yl)benzoic Acid (**22f**).* Procedure C was followed using 4-fluorobenzothiazol-2-amine (**21f**) on the same scale (m = 10 mg, 5% from **21f**). ^{1}H NMR (DMSO-d_6, ppm): δ 8.15 (s, 1H), 7.92 (d, J = 9.1 Hz, 1H), 7.89 (d, J = 10.2 Hz, 1H), 7.66−7.63 (m, 2H), 7.31−7.16 (m, 3H), 7.04 (d, J = 3.1 Hz, 1H), 2.32 (s, 3H). LCMS m/z 430.1 [M + 1]. HRMS calculated mass, 430.0423 [M + 1]; measured, 430.0424 [M + 1].

*3-(5-(1-(2-(Benzoxazol-2-yl)hydrazono)ethyl)furan-2-yl)-2-methylbenzoic Acid (**23**).* Procedure B was followed using compound **10h** (36 mg, 0.15 mmol) and **19a** (25 mg, 0.17 mmol). A white solid was obtained (m = 31 mg, 55%). ^{1}H NMR (DMSO-d_6, ppm): 1:2 mixture of two isomers δ 7.86 (d, J = 7.7 Hz, 0.3H), 7.78 (d, J = 8.1 Hz, 0.7H), 7.70 (t, J = 7.0 Hz, 1H), 7.51 (d, J = 7.7 Hz, 0.7H), 7.42 (d, J = 8.0 Hz, 1H), 7.39 (d, J = 8.7 Hz, 1H), 7.31 (d, J = 7.8 Hz, 0.3H), 7.21 (t, J = 7.1 Hz, 1H), 7.14−7.01 (m, 2H), 7.06 (d, J = 3.4 Hz, 1H), 6.89 (d, J = 3.4 Hz, 0.3H), 6.87 (d, J = 3.5 Hz, 1H), 2.59 (s, 0.9H), 2.58 (s, 2.1H), 2.32 (s, 2.1H), 2.31 (s, 0.9H). LCMS m/z 376.1 [M + 1]. HRMS calculated mass, 376.1292 [M + 1]; measured, 376.1292 [M + 1].

*2-Chloro-5-(5-(1-(2-(1-methyl-1H-benzimidazol-2-yl)hydrazono)-ethyl)furan-2-yl)benzoic Acid (**24**).* Procedure B was followed using compound **10e** (62 mg, 0.23 mmol) and **19b** (200 mg, 1.2 mmol). A dark solid was obtained (m = 33 mg, 35%). ^{1}H NMR (DMSO-d_6, ppm): δ 10.90 (br s, 1H), 8.11 (d, J = 2.2 Hz, 1H), 7.93 (dd, J = 8.5 and 2.3 Hz, 1H), 7.59 (d, J = 8.5 Hz, 1H), 7.22 (d, J = 3.5 Hz, 1H), 7.16−7.09 (m, 2H), 7.02−6.99 (m, 3H), 3.46 (s, 3H), 2.33 (s, 3H). LCMS m/z 409.1 [M + 1]. HRMS calculated mass, 409.1062 [M + 1]; measured, 409.1059 [M + 1].

*2-Chloro-5-(5-(1-(2-(quinolin-2-yl)hydrazono)ethyl)furan-2-yl)-benzoic Acid (**25**).* Procedure B was followed using compound **10e** (48 mg, 0.18 mmol) and 2-hydrazinylquinoline (29 mg, 0.18 mmol) and acetic acid as the solvent. A yellow solid was obtained (m = 41 mg, 56%). ^{1}H NMR (DMSO-d_6, ppm): δ 10.35 (br s, 1H), 8.19 (d, J = 7.1 Hz, 1H), 8.13−8.12 (m, 2H), 7.90 (d, J = 7.1 Hz, 1H), 7.79 (d, J = 6.6 Hz, 1H), 7.22−7.53 (m, 4H), 7.31−7.23 (m, 2H), 7.01 (br s, 1H), 2.34 (s, 3H). LCMS m/z 406.2 [M + 1].

*3-(5-((2-(4-Phenylthiazol-2-yl)hydrazono)methyl)furan-2-yl)-benzoic Acid (**26**).* Procedure B was followed using compound **9b** (72 mg, 0.33 mmol) and 2-hydrazinyl-4-phenylthiazole (95 mg, 0.5 mmol). A brown solid was obtained (m = 73 mg, 57%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.30 (s, 1H), 8.01 (d, J = 7.9 Hz, 1H), 7.97 (s, 1H), 7.90 (d, J = 7.9 Hz, 1H), 7.86 (d, J = 7.3 Hz, 2H), 7.60 (t, J = 7.8 Hz, 1H), 7.41 (t, J = 7.3 Hz, 2H), 7.34 (s, 1H), 7.30 (t, J = 7.3 Hz, 1H), 7.23 (d, J = 3.6 Hz, 1H), 6.95 (d, J = 3.6 Hz, 1H). LCMS m/z 390.1 [M + 1]. HRMS calculated mass, 390.0907 [M + 1]; measured, 390.0903 [M + 1].

*3-(5-(1-(2-Benzoylhydrazono)ethyl)furan-2-yl)benzoic Acid (**27**).* Procedure B was followed using compound **10a** (45 mg, 0.2 mmol) and benzohydrazide (30 mg, 0.22 mmol). A cream solid was obtained (m = 43 mg, 62%). ^{1}H NMR (DMSO-d_6, ppm): δ 10.78 (br s, 1H), 8.31 (s, 1H), 8.03 (d, J = 7.1 Hz, 1H), 7.90 (d, J = 7.5 Hz, 3H), 7.63−7.51 (m, 4H), 7.23 (s, 1H), 7.13 (s, 1H), 2.35 (s, 3H). LCMS m/z 349.1 [M + 1]. HRMS calculated mass, 349.1183 [M + 1]; measured, 349.1184 [M + 1].

*5-(5-(1-(2-(Benzo[d]thiazol-2-yl)hydrazono)ethyl)thiophen-2-yl)-2-chlorobenzoic Acid (**30a**).* The intermediate 5-(5-acetylthiophen-2-yl)-2-chlorobenzoic acid (**29a**) was prepared according to method A using 1-(thiophen-2-yl)ethanone (370 mg, 2.9 mmol) and **8f** (500 mg, 2.9 mmol). Then 50 mg (0.18 mmol) of this material was reacted directly with 2-hydrazinylbenzothiazole (32 mg, 0.2 mmol) using procedure B. A yellow solid was obtained (m = 35 mg, 41% from **29a**). ^{1}H NMR (DMSO-d_6, ppm): δ 8.02 (d, J = 1.9 Hz, 1H), 7.86 (dd, J = 8.4 and 2.0 Hz, 1H), 7.70 (d, J = 7.7 Hz, 1H), 7.62−7.59 (m, 2H), 7.44 (d, J = 3.8 Hz, 1H), 7.26−7.25 (m, 2H), 7.09−7.04 (m, 1H), 2.36 (s, 3H). LCMS m/z 428.6 [M + 1].

*5-(5-Acetyl-1-methyl-1H-pyrrol-2-yl)-2-chlorobenzoic Acid (**29b**).* Prepared according to method A using 1-(1-methyl-1*H*-pyrrol-2-yl)ethanone (500 mg, 4.1 mmol) and **8f** (697 mg, 4.1 mmol). A white solid was obtained (m = 299 mg, 26%). ^{1}H NMR (DMSO-d_6, ppm): δ 13.5 (br s, 1H), 7.82 (s, 1H), 7.64 (s, 2H), 7.19 (d, J = 4.1 Hz, 1H), 6.37 (d, J = 4.1 Hz, 1H), 3.80 (s, 3H), 2.42 (s, 3H). LCMS m/z 278.1 [M + 1].

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*5-(5-Acetyl-1H-pyrrol-2-yl)-2-chlorobenzoic Acid (**29c**).* Prepared according to method A using 1-(1*H*-pyrrol-2-yl)ethanone (500 mg, 4.6 mmol) and **8f** (787 mg, 4.6 mmol). An off-white solid was obtained (m = 328 mg, 27%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.30 (d, J = 2.3 Hz, 1H), 8.00 (dd, J = 8.5 and 2.3 Hz, 1H), 7.54 (d, J = 8.5 Hz, 1H), 7.08−7.06 (m, 1H), 6.79−6.77 (m, 1H), 2.39 (s, 3H). LCMS m/z 264.6 [M + 1].

*5-(5-(1-(2-(Benzothiazol-2-yl)hydrazono)ethyl)-1-methyl-1H-pyrrol-2-yl)-2-chlorobenzoic Acid (**30b**).* Procedure B was followed using compound **29b** (100 mg, 0.4 mmol) and 2-hydrazinylbenzothiazole (60 mg, 0.4 mmol). A light-brown solid was obtained (m = 44 mg, 29%). ^{1}H NMR (DMSO-d_6, ppm): δ 7.81 (d, J = 1.7 Hz, 1H), 7.67 (d, J = 7.9 Hz, 1H), 7.63−7.58 (m, 3H), 7.30−7.23 (m, 2H), 7.05 (td, J = 8.1 and 2.0 Hz, 1H), 6.67 (d, J = 3.9 Hz, 1H), 6.34 (d, J = 3.9 Hz, 1H), 3.91 (s, 3H), 2.34 (s, 3H). LCMS m/z 425.1 [M + 1].

*5-(5-(1-(2-(Benzothiazol-2-yl)hydrazono)ethyl)-1H-pyrrol-2-yl)-2-chlorobenzoic Acid (**30c**).* Procedure B was followed using compound **29c** (100 mg, 0.4 mmol) and 2-hydrazinylbenzothiazole (63 mg, 0.4 mmol). A brown solid was obtained (m = 124 mg, 79%). ^{1}H NMR (DMSO-d_6, ppm): 2:1 mixture of isomers δ 12.5 (br s, 1H), 12.20 (s, 0.3H), 11.23 (s, 0.7H), 8.30 (d, J = 2.1 Hz, 0.3H), 8.19 (d, J = 1.9 Hz, 0.7H), 8.00 (dd, J = 8.6 and 2.3 Hz, 0.3H), 7.91 (dd, J = 8.5 and 2.0 Hz, 0.7H), 7.69 (d, J = 7.7 Hz, 0.7H), 7.56−7.54 (m, 0.3H), 7.52 (d, J = 8.4 Hz, 0.7H), 7.30−7.22 (m, 1 + 2 × 0.33), 7.07−7.02 (m, 1H), 6.78 (t, J = 2.9 Hz, 0.3H), 6.71 (t, J = 3.2 Hz, 1H), 6.62 (t, J = 2.1 Hz, 0.7H), 2.39 (s, 0.9H), 2.30 (s, 2.1H). LCMS m/z 411.1 [M + 1]. HRMS calculated mass, 411.0677 [M + 1]; measured, 411.0676 [M + 1].

*5-(3-(1-(2-(Benzothiazol-2-yl)hydrazono)ethyl)phenyl)furan-2-carboxylic (**34**) Acid.* 5-(3-Acetylphenyl)furan-2-carboxylic acid (**33**) was prepared according to method A using furan-2-carboxylic acid (1.1 g, 10 mmol) and 1-(3-aminophenyl)ethanone (1.4 g, 10.3 mmol). A brown solid was obtained (m = 70 mg, 3%) and treated directly with 2-hydrazinylbenzothiazole (40 mg, 0.24 mmol) using general procedure B. A pale-brown solid was obtained (35 mg, 31%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.17 (s, 1H), 7.87 (d, J = 7.8 Hz, 1H), 7.83 (d, J = 8.0 Hz, 1H), 7.71 (d, J = 7.3 Hz, 1H), 7.55 (t, J = 7.7 Hz, 1H), 7.36 (d, J = 3.6 Hz, 1H), 7.34 (d, J = 3.3 Hz, 1H), 7.29 (t, J = 7.7 Hz, 1H), 7.22 (d, J = 3.6 Hz, 1H), 7.09 (t, J = 7.7 Hz, 1H), 2.43 (s, 3H). LCMS m/z 378.1 [M + 1]. HRMS calculated mass, 378.0907 [M + 1]; measured, 378.0902 [M + 1].

General Method D: Formation of Biaryl Ketone Using the Suzuki Coupling Reaction. Preparation of 6-(3-acetylphenyl)picolinic acid (**37**): A mixture of 6-bromopicolinic acid (1.5 g, 7.4 mmol), 3-acetylphenylboronic acid (1.6 g, 9.6 mmol), tetrabutylammonium bromide (225 mg, 0.7 mmol), potassium carbonate (2.79 g, 20 mmol), and $Pd(PPh_3)_2Cl_2$ (270 mg, 0.4 mmol) in 30 mL of dioxane and 15 mL of water were heated at 100 °C in a microwave reactor (120W) for 60 min. After cooling, water and AcOEt were added and aqueous HCl was then added until the pH of aqueous phase reached 3. A precipitate appeared. It was collected by filtration and dried in a vacuum oven. As this solid contained traces of side products, it was redissolved in an aqueous solution of potassium carbonate (10%). The solution was then acidified to pH 4−5 with HCl 2N. The precipitate that formed was collected by filtration and dried in a vacuum oven. A white solid was obtained (m = 835 mg, 47%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.69 (t, J = 1.8 Hz, 1H), 8.43 (dd, J = 7.8 and 1.9 Hz, 1H), 8.30 (dd, J = 7.8 and 1.2 Hz, 1H), 8.11 (t, J = 7.7 Hz, 1H), 8.05 (td, J = 7.7 and 1.1 Hz, 2H), 7.69 (t, J = 7.8 Hz, 1H), 2.68 (s, 3H). LCMS m/z 242.2 [M + 1].

*6-(3-(1-(2-(Benzothiazol-2-yl)hydrazono)ethyl)phenyl)picolinic Acid (**38**).* Procedure B was followed using compound **37** (200 mg, 0.83 mmol) and 2-hydrazinylbenzothiazole (137 mg, 0.83 mmol). A white solid was obtained (m = 289 mg, 90%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.51 (t, J = 1.6 Hz, 1H), 8.21 (dd, J = 7.9 and 1.1 Hz, 1H), 8.15 (dt, J = 7.8 and 1.1 Hz, 1H), 8.08 (t, J = 7.7 Hz, 1H), 8.00 (dd, J = 7.6 and 1.1 Hz, 1H), 7.92 (dt, J = 7.8 and 1.1 Hz, 1H), 7.68 (d, J = 7.6 Hz, 1H), 7.56 (t, J = 7.8 Hz, 1H), 7.32 (d, J = 8.1 Hz, 1H), 7.25 (td, J = 7.2 and 1.1 Hz, 1H), 7.05 (td, J = 7.8 and 1.3 Hz, 1H), 2.34 (s, 3H). LCMS m/z 389.1 [M + 1]. HRMS calculated mass, 389.1067 [M + 1]; measured, 389.1063 [M + 1].

*5-(3-Acetylphenyl)nicotinic Acid (**40**).* Procedure D was followed using 5-bromonicotinic acid (700 mg, 1.5 mmol) and 3-acetylphenylboronic acid (341 mg, 2.1 mmol). A pale-yellow solid was obtained (m = 250 mg, 69%). ^{1}H NMR (DMSO-d_6, ppm): δ 9.18 (d, J = 2.3 Hz, 1H), 9.10 (d, J = 1.8 Hz, 1H), 8.53 (t, J = 2.1 Hz, 1H), 8.3 (t, J = 1.5 Hz, 1H), 8.04 (t, J = 7.8 Hz, 1H), 7.69 (t, J = 7.8 Hz, 1H), 2.69 (s, 3H). LCMS m/z 242.2 [M + 1].

*5-(3-(1-(2-(Benzothiazol-2-yl)hydrazono)ethyl)phenyl)nicotinic Acid (**41**).* Procedure B was followed using compound **40** (20 mg, 0.08 mmol) and 2-hydrazinylbenzothiazole (14 mg, 0.08 mmol). An off-white solid was obtained (m = 16 mg, 50%). ^{1}H NMR (DMSO-d_6, ppm): δ 9.16 (d, J = 1.9 Hz, 1H), 9.10 (d, J = 1.8 Hz, 1H), 8.52 (t, J = 2.1 Hz, 1H), 8.13 (s, 1H), 7.94 (d, J = 7.9 Hz, 1H), 7.80 (d, J = 7.8 Hz, 1H), 7.71 (d, J = 7.6 Hz, 1H), 7.60 (t, J = 7.7 Hz, 1H), 7.35 (d, J = 8.0 Hz, 1H), 7.28 (t, J = 7.2 Hz, 1H), 7.08 (t, J = 7.7 Hz, 1H), 2.45 (s, 3H). LCMS m/z 389.1 [M + 1]. HRMS calculated mass, 389.1067 [M + 1]; measured, 389.1067 [M + 1].

*6-(3-Acetylphenyl)pyrazine-2-carboxylic Acid (**43**).* Procedure D was followed using 6-chloropyrazine-2-carbonitrile (250 mg, 1.8 mm ol) and 3-acetylphenylboronic acid (354 mg, 2.2 mmol). A white solid was obtained (m = 100 mg, 25%). ^{1}H NMR ($CDCl_3$, ppm): δ 9.28 (s, 1H), 8.86 (s, 1H), 8.62 (t, J = 1.9 Hz, 1H), 8.27 (dd, J = 7.8 and 1.9 Hz, 1H), 8.11 (dd, J = 7.8 and 1.7 Hz, 1H), 7.66 (t, J = 7.8 Hz, 1H), 2.68 (s, 3H). Then 50 mg of the coupled product (0.22 mmol) were reacted with 3.3 mL of 6.25N aqueous NaOH in 5 mL of a 1:1 THF/MeOH mixture at 60 °C for 4 h. The reaction was cooled and concentrated to leave the basic aqueous phase. This solution was acidified with 6N HCl to pH 4, leading ro the formation of a precipitate. The aqueous phase was extracted twice with EtOAc, and the combined organic layers were dried over $MgSO_4$. Concentration afforded a pale-yellow solid (m = 20 mg, 38%). ^{1}H NMR (DMSO-d_6, ppm): δ 9.55 (s, 1H), 9.17 (s, 1H), 8.72 (t, J = 1.8 Hz, 1H), 8.47 (dd, J = 7.8 and 1.8 Hz, 1H), 8.12 (dd, J = 7.8 and 1.7 Hz, 1H), 7.74 (t, J = 7.8 Hz, 1H), 2.69 (s, 3H). LCMS m/z 241.3 [M + 1].

*6-(3-(1-(2-(Benzothiazol-2-yl)hydrazono)ethyl)phenyl)pyrazine-2-carboxylic Acid (**44**).* Procedure B was followed using compound **43** (35 mg, 0.15 mmol) and 2-hydrazinylbenzothiazole (26 mg, 0.16 mmol). A cream solid was obtained (m = 43 mg, 74%). ^{1}H NMR (DMSO-d_6, ppm): δ 9.48 (s, 1H), 9.14 (s, 1H), 8.55 (s, 1H), 8.19 J = 7.8 Hz, 1H), 7.98 (d, J = 8.4 Hz, 1H), 7.68 (d, J = 7.8 Hz, 1H), 7.61 (t, J = 7.8 Hz, 1H), 7.32 (d, J = 7.7 Hz, 1H), 7.27 (t, J = 8.0 Hz, 1H), 2.43 (s, 3H). LCMS m/z 390.1 [M + 1]. HRMS calculated mass, 390.1019 [M + 1]; measured, 390.1012 [M + 1].

*6-(5-Acetylthiophen-2-yl)picolinic Acid (**47**).* Procedure D was followed using 6-bromopicolinic acid (100 mg, 0.5 mmol) and 5-acetylthiophen-2-ylboronic acid **45** (109 mg, 0.65 mmol). A cream solid was obtained (m = 37 mg, 30%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.25 (dd, J = 7.8 and 1.1 Hz, 1H), 8.08 (t, J = 7.7 Hz, 1H), 8.01−7.97 (m, 3H), 2.57 (s, 3H). LCMS m/z 248.4 [M + 1].

*6-(5-(1-(2-(Benzothiazol-2-yl)hydrazono)ethyl)thiophen-2-yl)-picolinic Acid (**48**).* Procedure B was followed using compound **47** (25 mg, 0.10 mmol) and 2-hydrazinylbenzothiazole (17 mg, 0.10 mmol). A cream solid was obtained (m = 30 mg, 76%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.16 (dd, J = 7.9 and 1.1 Hz, 1H), 8.01 (t, J = 7.7 Hz, 1H), 7.92 (dd, J = 7.6 and 1.1 Hz, 1H), 7.86 (d, J = 4.0 Hz, 1H), 7.74 (d, J = 7.8 Hz, 1H), 7.47 (d, J = 4.0 Hz, 1H), 7.27 (d, J = 4.1 Hz, 2H), 7.10−7.04 (m, 1H), 2.39 (s, 3H). LCMS m/z 395.4 [M + 1].

*5-(5-Acetylthiophen-2-yl)furan-2-carboxylic Acid (**50**).* Procedure D was followed using 5-bromofuran-2-carboxylic acid **49** (200 mg, 1.04 mmol) and 5-acetylthiophen-2-ylboronic acid **45** (250 mg, 1.4 mmol). A yellow solid was obtained (m = 115 mg, 46%). ^{1}H NMR (DMSO-d_6, ppm): δ 7.96 (d, J = 4.2 Hz, 1H), 7.65 (d, J = 3.9 Hz, 1H), 7.35 (d, J = 3.6 Hz, 1H), 7.20 (d, J = 3.6 Hz, 1H), 2.56 (s, 3H). LCMS m/z 234.9 [M − 1].

*5-(5-(1-(2-(Benzothiazol-2-yl)hydrazono)ethyl)thiophen-2-yl)-furan-2-carboxylic Acid (**51**).* Procedure B was followed using compound **50** (20 mg, 0.08 mmol) and 2-hydrazinylbenzothiazole (15 mg, 0.9 mmol). A brown solid was obtained (m = 25 mg, 82%). ^{1}H NMR (DMSO-d_6, ppm): δ 7.72 (d, J = 7.8 Hz, 1H), 7.53 (d, J = 3.9 Hz, 1H), 7.44 (d, J = 3.9 Hz, 1H), 7.33 (d, J = 3.6 Hz, 1H), 7.27−

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7.25 (m, 2H), 7.10−7.07 (m, 1H), 7.04 (d, J = 3.6 Hz, 1H), 2.37 (s, 3H). LCMS m/z 384.1 [M + 1].

*4-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)-2,3-dihydro-1H-inden-1-one (**53**).* 4-Bromo-2,3-dihydro-1*H*-inden-1-one **52** (366 mg, 1.73 mmol), bis(pinacolato)diboron (572 mg, 2.24 mmol), potassium acetate (372 mg, 3.44 mmol), and $Pd(dppf)Cl_2.CH_2Cl_2$ (35 mg, 4 mol %) were stirred in 7 mL of dry methanol at 60 °C for 10 h. The reaction was cooled down to room temperature and filtered over Celite, and the solid residue was washed with AcOEt. Concentration of the filtrate afforded a brown solid, which was purified by flash chromatography on SiO_2 using AcOEt/petroleum ether (0:100 to 15:85). A white solid was obtained (m = 101 mg, 23%). ^{1}H NMR ($CDCl_3$, ppm): δ 8.04 (dd, J = 7.2 and 1.3 Hz, 1H), 7.84 (dd, J = 7.7 and 1.4 Hz, 1H), 7.37 (t, J = 7.2 Hz, 1H), 3.36−3.32 (m, 2H), 2.69−2.65 (m, 2H), 1.36 (s, 12H).

*6-(1-Oxo-2,3-dihydro-1H-inden-4-yl)picolinic Acid (**54**).* Procedure D was followed using 6-bromopicolinic acid **36** (56 mg, 0.28 mmol) and **53** (96 mg, 0.36 mol). A white solid was obtained (m = 56 mg, 62%). ^{1}H NMR ($CDCl_3$, ppm): δ 8.27 (dd, J = 7.7 and 1.1 Hz, 1H), 8.11 (t, J = 7.8 Hz, 1H), 7.95−7.90 (m, 3H), 7.58 (t, J = 7.6 Hz, 1H), 3.42−3.38 (m, 2H), 2.79−2.76 (m, 2H). LCMS m/z 254.0 [M + 1].

*6-(1-(2-(Benzothiazol-2-yl)hydrazono)-2,3-dihydro-1H-inden-4-yl)picolinic Acid (**55**).* Procedure B was followed using compound **53** (22 mg, 0.08 mmol) and 2-hydrazinylbenzothiazole (14 mg, 0.08 mmol). An off-white solid was obtained (m = 15 mg, 45%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.13−8.01 (m, 3H), 7.85 (dd, J = 7.6 and 1.1 Hz, 1H), 7.78 (dd, J = 7.7 and 1.1 Hz, 1H), 7.74 (br d, J = 6.6 Hz, 1H), 7.51 (t, J = 7.6 Hz, 1H), 7.38 (br d, J = 7.4 Hz, 1H), 7.28 (td, J = 7.3 and 1.3 Hz, 1H), 7.09 (td, J = 7.8 and 1.2 Hz, 1H), 3.47−3.43 (m, 2H), 2.97−2.93 (m, 2H). LCMS m/z 401.2 [M + 1].

*6-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)-2,3-dihydro-1H-inden-1-one (**57a**).* The procedure used for the preparation of compound **53** was used with 6-bromo-2,3-dihydro-1*H*-inden-1-one **56a** (183 mg, 0.86 mmol). A white solid was obtained (m = 50 mg, 23%). ^{1}H NMR ($CDCl_3$, ppm): δ 8.24 (d, J = 0.2 Hz, 1H), 8.00 (dd, J = 7.7 and 1.2 Hz, 1H), 7.47 (dd, J = 7.7 and 1.0 Hz, 1H), 3.18−3.14 (m, 2H), 2.71−2.67 (m, 2H), 1.35 (s, 12H).

*7-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)-3,4-dihydronaphthalen-1(2H)-one (**57b**).* The procedure used for the preparation of compound **53** was used with 7-bromo-3,4-dihydronaphthalen-1(2*H*)-one **56b** (200 mg, 0.89 mmol). A white solid was obtained (m = 160 mg, 66%). ^{1}H NMR ($CDCl_3$, ppm): δ 8.49 (d, J = 0.9 Hz, 1H), 7.87 (dd, J = 7.6 and 1.4 Hz, 1H), 7.24 (dd, J = 7.6 and 0.6 Hz, 1H), 2.97 (t, J = 6.1 Hz, 2H), 2.68−2.64 (m, 2H), 2.13 (q, J = 5.9 Hz, 2H), 1.33 (s, 12H).

*6-(3-Oxo-2,3-dihydro-1H-inden-5-yl)picolinic Acid (**58a**).* Procedure D was followed using 6-bromopicolinic acid **36** (34 mg, 0.17 mmol) and **57a** (50 mg, 0.2 mol). A brown solid was obtained (m = 25 mg, 60%). ^{1}H NMR ($CDCl_3$, ppm): δ 8.34 (d, J = 1.8 Hz, 1H), 8.29 (dd, J = 8.0 and 1.8 Hz, 1H), 8.19 (t, J = 4.3 Hz, 1H), 8.16 (dd, J = 7.3 and 1.3 Hz, 1H), 8.04 (dd, J = 4.4 and 0.7 Hz, 1H), 7.63 (dd, J = 8.0 and 0.8 Hz, 1H), 3.24−3.20 (m, 2H), 2.80−2.76 (m, 2H). LCMS m/z 254.2 [M + 1].

*6-(8-Oxo-5,6,7,8-tetrahydronaphthalen-2-yl)picolinic Acid (**58b**).* Procedure D was followed using 6-bromopicolinic acid **36** (139 mg, 0.424 mmol) and **57b** (150 mg, 0.55 mmol). A cream solid was obtained (m = 89 mg, 78%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.63 (d, J = 1.9 Hz, 1H), 8.35 (dd, J = 8.0 and 2.1 Hz, 1H), 8.22 (dd, J = 7.8 and 1.2 Hz, 1H), 8.07 (t, J = 7.7 Hz, 1H), 8.00 (dd, J = 7.7 and 1.2 Hz, 1H), 7.52 (d, J = 8.1 Hz, 1H), 3.02 (t, J = 6.0 Hz, 2H), 2.69−2.65 (m, 2H), 2.09 (q, J = 5.9 Hz, 1H). LCMS m/z 268.4 [M + 1].

*6-(3-(2-(Benzothiazol-2-yl)hydrazono)-2,3-dihydro-1H-inden-5-yl)picolinic Acid (**59a**).* Procedure B was followed using compound **58a** (25 mg, 0.1 mmol) and 2-hydrazinylbenzothiazole (25 mg, 0.15 mmol). A white solid was obtained (m = 16 mg, 40%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.36 (d, J = 1.4 Hz, 1H), 8.22 (dd, J = 7.8 and 1.1 Hz, 1H), 8.18 (dd, J = 8.0 and 1.8 Hz, 1H), 8.10 (t, J = 7.7 Hz, 1H), 8.02 (dd, J = 7.6 and 1.1 Hz, 1H), 7.75 (d, J = 7.6 Hz, 1H), 7.55 (d, J = 8.0 Hz, 1H), 7.38 (br d, J = 8.0 Hz, 1H), 7.29 (td, J = 7.4 and 1.2 Hz, 1H), 7.09 (td, J = 7.7 and 1.2 Hz, 1H), 3.19−3.15 (m, 2H), 3.02−2.98 (m, 2H). LCMS m/z 401.1 [M + 1]. HRMS calculated mass, 401.1067 [M + 1]; measured, 401.1066 [M + 1].

*6-(8-(2-(Benzothiazol-2-yl)hydrazono)-5,6,7,8-tetrahydronaphthalen-2-yl)picolinic Acid (**59b**).* Procedure B was followed using compound **58b** (34 mg, 0.13 mmol) and 2-hydrazinylbenzothiazole (32 mg, 0.19 mmol). A white solid was obtained (m = 25 mg, 47%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.81 (d, J = 1.9 Hz, 1H), 8.16−8.09 (m, 2H), 8.08 (dd, J = 8.0 and 2.0 Hz, 1H), 8.02 (dd, J = 6.7 and 2.0 Hz, 1H), 7.75 (d, J = 7.7 Hz, 1H), 7.36 (d, J = 8.01 Hz, 1H), 7.28 (td, J = 7.3 and 1.2 Hz, 1H), 7.10 (td, J = 7.8 and 1.2 Hz, 1H), 2.86−2.81 (m, 4H), 1.90 (q, J = 5.8 Hz, 1H). LCMS m/z 413.0 [M − 1]. HRMS calculated mass, 415.1223 [M + 1]; measured, 415.1219 [M + 1].

*1-Ethyl-6-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-2,3-dihydroquinolin-4(1H)-one (**61a**).* The procedure used for the preparation of compound **53** was used with 6-bromo-1-ethyl-2,3-dihydroquinolin-4(1*H*)-one **60a** (290 mg, 0.97 mmol). An off-white solid was obtained (m = 185 mg, 64%). ^{1}H NMR ($CDCl_3$, ppm): δ 8.38 (d, J = 1.7 Hz, 1H), 7.75 (dd, J = 8.6 and 1.7 Hz, 1H), 6.70 (d, J = 8.6 Hz, 1H), 3.54−3.45 (m, 4H), 2.72−2.67 (m, 2H), 1.31 (s, 12H).

*3-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)-6,7,8,9-tetrahydro-5H-benzo[7] Annulen-5-one (**61b**).* The procedure used for the preparation of compound **53** was used with **60b** (215 mg, 0.9 mmol). A light-brown solid was obtained (m = 145 mg, 56%). ^{1}H NMR ($CDCl_3$, ppm): δ 8.14 (d, J = 1.1 Hz, 1H), 7.83 (dd, J = 7.5 and 1.4 Hz, 1H), 7.19 (dd, J = 7.5 and 0.5 Hz, 1H), 2.95−2.91 (m, 2H), 2.73−2.69 (m, 2H), 1.92−1.75 (m, 4H), 1.33 (s, 12H).

*2-Methyl-7-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-3,4-dihydronaphthalen-1(2H)-one (**61c**).* A mixture of 7-bromo-2-methyl-3,4-dihydronaphthalen-1(2*H*)-one **60c** (152 mg, 0.64 mmol), bis(pinacolato)diboron (179 mg, 0.70 mmol), $Pd(dppf)Cl_2$.DCM (11 mg, 2 mol %), and potassium acetate (188 mg, 1.9 mmol) in 3 mL of toluene was heated at 150 °C for 6 min in a microwave oven (150 W). The crude mixture was passed through a pad of Celite eluting with AcOEt. The filtrate was concentrated, affording a brown solid, which did not require further purification and was used directly in the next step (273 mg, quantitative). ^{1}H NMR ($CDCl_3$, ppm): δ 8.48 (s, 1H), 7.86 (dd, J = 7.6 and 1.3 Hz, 1H), 7.23 (d, J = 7.6 Hz, 1H), 3.10−2.93 (m, 2H), 2.65−2.53 (m, 1H), 2.24−2.15 (m, 1H), 1.99−1.80 (m, 1H), 1.34 (s, 12H), 1.26 (d, J = 6.8 Hz, 3H).

*3-Methyl-7-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-3,4-dihydronaphthalen-1(2H)-one (**61d**).* The procedure used for the preparation of compound **61c** was used with **60d** (250 mg, 1.05 mmol). The crude residue was purified by flash chromatography on SiO_2 using AcOEt/petroleum ether (0:100 to 15:85). A white solid was obtained (m = 225 mg, 75%). ^{1}H NMR ($CDCl_3$, ppm): δ 8.48 (d, J = 0.9 Hz, 1H), 7.88 (dd, J = 7.6, 1.4 Hz, 1H), 7.24 (d, J = 7.7 Hz, 1H), 3.02−2.96 (m, 1H), 2.80−2.66 (m, 2H), 2.37−2.26 (m, 2H), 1.34 (s, 12H), 1.14 (d, J = 6.3 Hz, 3H).

*6-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)chroman-4-one (**61e**).* The procedure used for the preparation of compound **61c** was used with 6-bromochroman-4-one **60e** (1 g, 4.4 mmol). The crude residue was purified by flash chromatography on SiO_2 using AcOEt/petroleum ether (0:100 to 15:85). A white solid was obtained (m = 1.08 g, 89%). ^{1}H NMR ($CDCl_3$, ppm): δ 8.38 (d, J = 1.4 Hz, 1H), 7.87 (dd, J = 8.3 and 1.7 Hz, 1H), 6.94 (d, J = 8.3 Hz, 1H), 4.57−4.52 (m, 2H), 2.83−2.79 (m, 2H), 1.32 (s, 12H).

*6-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)thiochroman-4-one (**61f**).* The procedure used for the preparation of compound **61c** was used with 6-bromo-thiochroman-4-one **60f** (1 g, 4.1 mmol). The crude residue was purified by flash chromatography on SiO_2 using AcOEt/petroleum ether (0:100 to 15:85). An off-white solid was obtained (m = 840 mg, 78%). ^{1}H NMR ($CDCl_3$, ppm): δ 8.54 (d, J = 1.5 Hz, 1H), 7.75 (dd, J = 7.9 and 1.5 Hz, 1H), 7.26 (d, J = 8.0 Hz, 1H), 3.26−3.22 (m, 2H), 3.00−2.96 (m, 2H), 1.33 (s, 12H).

*6-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)-2,3-dihydroquinolin-4(1H)-one (**61g**).* The procedure used for the preparation of compound **53** was used with **60g** (115 mg, 0.51 mmol). A white solid was obtained (m = 60 mg, 35%). ^{1}H NMR ($CDCl_3$, ppm): δ 8.34 (d, J = 1.1 Hz, 1H), 7.69 (dd, J = 8.2 and 1.6 Hz, 1H), 6.65 (d, J = 8.1 Hz, 1H), 3.63−3.58 (m, 2H), 2.73−2.69 (m, 2H).

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*2,2-Dimethyl-6-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-chroman-4-one (**61h**).* The procedure used for the preparation of compound **61c** was used with **60h** (200 mg, 0.76 mmol). The crude residue was purified by flash chromatography on SiO_2 using AcOEt/petroleum ether (5:95 to 30:70). A yellow solid was obtained (m = 293 mg, quantitative). ^{1}H NMR ($CDCl_3$, ppm): δ 8.34 (d, J = 1.7 Hz, 1H), 7.86 (dd, J = 8.3 and 1.7 Hz, 1H), 6.89 (d, J = 8.3 Hz, 1H), 1.44 (s, 6H), 1.31 (s, 12H).

*4-Methyl-7-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-3,4-dihydronaphthalen-1(2H)-one (**61i**).* The procedure used for the preparation of compound **61c** was used with **60i** (170 mg, 0.71 mmol). The crude residue was purified by flash chromatography on SiO_2 using AcOEt/petroleum ether (0:100 to 10:90). An off-white solid was obtained (m = 130 mg, 64%). ^{1}H NMR ($CDCl_3$, ppm): δ 8.46 (d, J = 1.4 Hz, 1H), 7.89 (dd, J = 7.7 and 1.4 Hz, 1H), 7.29 (d, J = 7.7 Hz, 1H), 3.12−3.01 (m, 1H), 2.82−2.71 (m, 1H), 2.62−2.52 (m, 1H), 2.27−2.16 (m, 1H), 1.92−1.81 (m, 1H), 1.36 (d, J = 7.1 Hz, 1H), 1.31 (s, 12H).

*6-(1-Ethyl-4-oxo-1,2,3,4-tetrahydroquinolin-6-yl)picolinic Acid (**62a**).* Procedure D was followed using 6-bromopicolinic acid (48 mg, 0.44 mmol) and **61a** (88 mg, 0.29 mmol). A white solid was obtained (m = 49 mg, 57%). ^{1}H NMR ($CDCl_3$, ppm): δ 8.50 (d, J = 2.2 Hz, 1H), 8.13.(dd, J = 9.0 and 2.5 Hz, 1H), 8.08 (dd, J = 6.7 and 1.8 Hz, 1H), 8.00−7.93 (m, 2H), 6.89 (d, J = 9.2 Hz, 1H), 3.61−3.58 (m, 2H), 3.56 (q, J = 7.1 Hz, 2H), 2.80−2.76 (m, 2H), 1.26 (t, J = 7.1 Hz, 3H). LCMS m/z 297.2 [M + 1].

*6-(9-Oxo-6,7,8,9-tetrahydro-5H-benzo[7]annulen-2-yl)picolinic Acid (**62b**).* Procedure D was followed using 6-bromopicolinic acid (83 mg, 0.41 mmol) and **61b** (137 mg, 0.48 mmol). A light-brown solid was obtained (m = 50 mg, 46%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.33 (d, J = 2.0 Hz, 1H), 8.23 (dd, J = 7.9 and 2.1 Hz, 1H), 8.17 (dd, J = 7.8 and 1.1 Hz, 1H), 8.03 (t, J = 7.7 Hz, 1H), 7.96 (dd, J = 7.6 and 1.1 Hz, 1H), 7.42 (d, J = 8.0 Hz, 1H), 2.99−2.95 (m, 2H), 2.73−2.69 (m, 2H), 1.82−1.67 (m, 4H). LCMS m/z 282.1 [M + 1].

*General Procedure E: 6-(7-Methyl-8-oxo-5,6,7,8-tetrahydronaphthalen-2-yl)picolinic Acid (**62c**).* 6-Bromopicolinic acid (146 mg, 0.72 mmol), **61c** (268 mg, 0.94 mmol), $Pd(PPh_3)_2Cl_2$ (22 mg, 4 mol %), and 348 μL of a 2 M sodium carbonate aqueous in 1.3 mL of a 1:1:1 mixture of water/DME/EtOH solution were stirred at 90 °C for 6 h. After this time, the reaction was concentrated. The residue was partitioned in a mixture of water/AcOEt. The aqueous phase was extracted three times with AcOEt. The combined organic layers were washed with water and brine and dried over Na_2SO_4 and concentrated. The residues was purified by flash chromatography using SiO_2 (petroleum ehter/AcOEt 5:95 to 50:50). An off-white solid was obtained (m = 100 mg, 49%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.62 (d, J = 2.0 Hz, 1H), 8.34 (dd, J = 8.0 and 2.0 Hz, 1H), 8.22 (dd, J = 7.7 and 1.1 Hz, 1H), 8.07 (t, J = 7.7 Hz, 1H), 8.00 (dd, J = 7.6 and 1.1 Hz, 1H), 7.50 (d, J = 8.1 Hz, 1H), 3.18−2.97 (m, 2H), 2.77−2.65 (m, 1H), 2.23−2.15 (m, 1H), 1.91−1.77 (m, 1H), 1.19 (d, J = 6.8 Hz, 1H).

*6-(6-Methyl-8-oxo-5,6,7,8-tetrahydronaphthalen-2-yl)picolinic Acid (**62d**).* Procedure D was followed using 6-bromopicolinic acid (120 mg, 0.6 mmol) and **61d** (222 mg, 0.77 mmol). A cream solid was obtained (m = 84 mg, 39%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.59 (d, J = 1.9 Hz, 1H), 8.19 (td, J = 6.8 and 1.8 Hz, 2H), 8.10−8.02 (m, 2H), 7.44 (d, J = 8.0 Hz, 1H), 3.12−3.06 (m, 1H), 2.87−2.73 (m, 2H), 2.44−2.34 (m, 2H), 1.19 (d, J = 6.2 Hz, 3H).

*6-(4-Oxochroman-6-yl)picolinic Acid (**62e**).* Procedure D was followed using 6-bromopicolinic acid (395 mg, 1.98 mmol) and **61e** (700 mg, 2.55 mmol). A cream solid was obtained (m = 434 mg, 81%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.55 (d, J = 2.1 Hz, 1H), 8.38 (dd, J = 8.8 and 2.4 Hz, 1H), 8.18 (dd, J = 7.9 and 1.2 Hz, 1H), 8.04 (t, J = 7.7 Hz, 1H), 7.96 (dd, J = 7.6 and 1.1 Hz, 1H), 7.19 (d, J = 8.7 Hz, 1H), 4.62 (m, 2H), 2.87 (m, 2H).

*6-(4-Oxothiochroman-6-yl)picolinic Acid (**62f**).* Procedure D was followed using 6-bromopicolinic acid (67 mg, 0.33 mmol) and **61f** (125 mg, 0.43 mmol). A light-brown solid was obtained (m = 40 mg, 33%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.73 (d, J = 2.0 Hz, 1H), 8.28 (dd, J = 8.4 and 2.2 Hz, 1H), 8.06 (t, J = 7.4 Hz, 1H), 7.99 (dd, J = 7.7 and 1.1 Hz, 1H), 7.53 (d, J = 8.4 Hz, 1H), 3.41−3.37 (m, 2H), 2.99−2.95 (m, 2H).

*6-(4-Oxo-1,2,3,4-tetrahydroquinolin-6-yl)picolinic Acid (**62g**).* Procedure D was followed using 6-bromopicolinic acid (24 mg, 0.12 mmol) and **61g** (40 mg, 0.15 mmol). A brown solid was obtained (m = 23 mg, 57%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.40 (d, J = 2.3 Hz, 1H), 8.15 (dd, J = 8.8 and 2.3 Hz, 1H), 8.04 (dd, J = 8.0 and 1.2 Hz, 1H), 7.96 (t, J = 7.5 Hz, 1H), 7.19 (br s, 1H), 6.89 (d, J = 8.8 Hz, 1H), 3.53−3.48 (m, 2H), 2.62−2.57 (m, 2H).

*6-(2,2-Dimethyl-4-oxochroman-6-yl)picolinic Acid (**62h**).* Procedure E was followed using 6-bromopicolinic acid (148 mg, 0.78 mmol) and **61h** (266 mg, 0.88 mmol). A white solid was obtained (m = 131 mg, 60%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.52 (d, J = 2.2 Hz, 1H), 8.38 (dd, J = 8.7 and 2.5 Hz, 1H), 8.18 (dd, J = 7.9 and 1.1 Hz, 1H), 8.04 (t, J = 7.7 Hz, 1H), 7.96 (dd, J = 7.6 and 1.1 Hz, 1H), 7.14 (d, J = 8.7 Hz, 1H), 2.78 (s, 2H), 1.44 (s, 6H).

*6-(5-Methyl-8-oxo-5,6,7,8-tetrahydronaphthalen-2-yl)picolinic Acid (**62i**).* Procedure D was followed using 6-bromopicolinic acid (70 mg, 0.35 mmol) and **61i** (130 mg, 0.45 mmol). A light-brown solid was obtained (m = 28 mg, 28%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.62 (d, J = 2.1 Hz, 1H), 8.37 (dd, J = 8.1 and 2.1 Hz, 1H), 8.22 (dd, J = 7.8 and 1.2 Hz, 1H), 8.07 (t, J = 7.7 Hz, 1H), 8.00 (dd, J = 7.7 and 1.2 Hz, 1H), 7.60 (d, J = 8.4 Hz, 1H), 3.22−3.13 (m, 1H), 2.83−2.73 (m, 1H), 2.69−2.58 (m, 1H), 2.28−2.17 (m, 1H), 1.93−1.81 (m, 1H), 1.40 (d, J = 7.0 Hz, 1H).

*6-(4-(2-(Benzothiazol-2-yl)hydrazono)-1-ethyl-1,2,3,4-tetrahydroquinolin-6-yl) picolinic Acid (**63a**).* Procedure B was followed using compound **62a** (30 mg, 0.1 mmol) and 2-hydrazinylbenzothiazole (17 mg, 0.1 mmol). A light-brown solid was obtained (m = 26 mg, 59%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.73 (d, J = 2.3 Hz, 1H), 8.06 (dd, J = 8.8 and 2.3 Hz, 1H), 8.03−7.99 (m, 2H) 7.89 (dd, J = 6.1 and 2.4 Hz, 1H), 7.70 (br d, J = 7.8 Hz, 1H), 7.29−7.24 (m, 2H), 7.09−7.04 (m, 1H), 6.93 (d, J = 8.9 Hz, 1H), 3.50 (q, J = 7.1 Hz, 2H), 3.36 (t, J = 6.8 Hz, 2H), 2.93 (t, J = 6.3 Hz, 2H), 1.13 (t, J = 7.0 Hz, 1H). LCMS m/z 444.2 [M + 1]. HRMS calculated mass, 444.1489 [M + 1]; measured, 444.1487 [M + 1].

*6-(9-(2-(Benzothiazol-2-yl)hydrazono)-6,7,8,9-tetrahydro-5H-benzo[7]annulen-2-yl)picolinic Acid (**63b**).* Procedure B was followed using compound **62b** (27 mg, 0.1 mmol) and 2-hydrazinylbenzothiazole (15 mg, 0.1 mmol). A light-brown solid was obtained (m = 15 mg, 35%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.19 (d, J = 1.9 Hz, 1H), 8.13 (dd, J = 7.9 and 1.1 Hz, 1H), 8.06 (d, J = 7.9 Hz, 1H), 8.04 (t, J = 7.6 Hz, 1H), 7.96 (dd, J = 7.5 and 1.1 Hz, 1H), 7.65 (d, J = 7.7 Hz, 1H), 7.35−7.30 (m, 2H), 7.23 (td, J = 6.2 and 1.2 Hz, 1H), 7.03 (td, J = 7.8 and 1.2 Hz, 1H), 2.80−2.74 (m, 4H), 1.77−1.73 (m, 2H), 1.64−1.60 (m, 2H). LCMS m/z 427.0 [M − 1]. HRMS calculated mass, 429.1380 [M + 1]; measured, 429.1383 [M + 1].

*6-(8-(2-(Benzothiazol-2-yl)hydrazono)-7-methyl-5,6,7,8-tetrahydronaphthalen-2-yl)picolinic Acid (**63c**).* Procedure B was followed using compound **62c** (50 mg, 0.18 mmol) and 2-hydrazinylbenzothiazole (29 mg, 0.18 mmol). A white solid was obtained (m = 38 mg, 49%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.87 (d, J = 1.9 Hz, 1H), 8.15−8.08 (m, 2H), 8.08 (dd, J = 8.0 and 2.0 Hz, 1H), 8.01 (dd, J = 6.5 and 2.2 Hz, 1H), 7.71 (d, J = 7.9 Hz, 1H), 7.36 (d, J = 8.0 Hz, 1H), 7.29−7.24 (m, 2H), 7.10−7.05 (m, 1H), 3.65−3.63 (m, 1H), 3.10−2.98 (m, 1H), 2.82−2.76 (m, 1H), 2.02−1.82 (m, 2H), 1.15 (d, J = 7.1 Hz, 1H). LCMS m/z 429.3 [M + 1]. HRMS calculated mass, 429.1380 [M + 1]; measured, 429.1383 [M + 1].

*6-(8-(2-(Benzothiazol-2-yl)hydrazono)-6-methyl-5,6,7,8-tetrahydronaphthalen-2-yl)picolinic Acid (**63d**).* Procedure B was followed using compound **62d** (50 mg, 0.18 mmol) and 2-hydrazinylbenzothiazole (29 mg, 0.18 mmol). A white solid was obtained (m = 53 mg, 69%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.81 (d, J = 1.7 Hz, 1H), 8.16−8.09 (m, 2H), 8.07 (dd, J = 8.0 and 1.9 Hz, 1H), 8.02 (dd, J = 6.8 and 1.9 Hz, 1H), 7.73 (d, J = 7.7 Hz, 1H), 7.35 (d, J = 8.0 Hz, 2H), 7.28 (t, J = 7.1 Hz, 1H), 7.09 (t, J = 8.0 Hz, 1H), 3.16 (dd, J = 17.6 and 3.7 Hz, 1H), 2.89 (dd, J = 15.4 and 2.4 Hz, 1H), 2.61−2.51 (m, 1H), 2.31−2.22 (m, 1H), 2.08−1.94 (m, 1H), 1.10 (d, J = 6.5 Hz, 1H). LCMS m/z 427.0 [M − 1]. HRMS calculated mass, 429.1380 [M + 1]; measured, 429.1378 [M + 1].

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*6-(4-(2-(Benzothiazol-2-yl)hydrazono)chroman-6-yl)picolinic Acid (**63e**).* Procedure B was followed using compound **62e** (200 mg, 0.74 mmol) and 2-hydrazinylbenzothiazole (122 mg, 0.74 mmol). A cream solid was obtained (m = 292 mg, 95%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.70 (d, J = 2.2 Hz, 1H), 8.10−8.02 (m, 3H), 7.98−7.93 (m, 1H), 7.67 (d, J = 7.7 Hz, 1H), 7.28−7.21 (m, 2H), 7.08−7.02 (m, 2H), 4.34−4.30 (m, 2H), 3.02−2.98 (m, 2H). LCMS m/z 415.0 [M − 1]. HRMS calculated mass, 417.1016 [M + 1]; measured, 417.1013 [M + 1].

*6-(4-(2-(Benzothiazol-2-yl)hydrazono)thiochroman-6-yl)picolinic Acid (**63f**).* Procedure B was followed using compound **62f** (20 mg, 0.07 mmol) and 2-hydrazinylbenzothiazole (12 mg, 0.07 mmol). A white solid was obtained (m = 10 mg, 33%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.88 (s, 1H), 8.13−8.05 (m, 2H), 8.02 (dd, J = 8.3 and 2.1 Hz, 1H), 7.97 (dd, J = 7.0 and 1.7 Hz, 1H), 7.68 (d, J = 7.7 Hz, 1H), 7.37 (d, J = 8.3 Hz, 1H), 7.28−7.22 (m, 2H), 7.08−7.03 (m, 1H), 3.15−3.10 (m, 4H). LCMS m/z 431.0 [M − 1]. HRMS calculated mass, 433.0787 [M + 1]; measured, 433.0784 [M + 1].

*6-(4-(2-(Benzothiazol-2-yl)hydrazono)-1,2,3,4-tetrahydroquinolin-6-yl)picolinic Acid (**63g**).* Procedure B was followed using compound **62g** (23 mg, 0.09 mmol) and 2-hydrazinylbenzothiazole (14 mg, 0.09 mmol). A light-yellow solid was obtained (m = 25 mg, 70%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.63 (d, J = 2.2 Hz, 1H), 8.04−7.95 (m, 3H), 7.88 (dd, J = 6.8 and 1.7 Hz, 1H), 7.70 (d, J = 7.7 Hz, 1H), 7.32−7.23 (m, 2H), 7.09−7.04 (m, 1H), 6.79 (d, J = 8.6 Hz, 1H), 6.58 (s, 1H), 3.38−3.26 (m, 2H), 2.88−2.84 (m, 2H). LCMS m/z 416.1 [M + 1].

*6-(4-(2-(Benzothiazol-2-yl)hydrazono)-2,2-dimethylchroman-6-yl)picolinic Acid (**63h**).* Procedure B was followed using compound **62g** (70 mg, 0.24 mmol) and 2-hydrazinylbenzothiazole (39 mg, 0.24 mmol). A white solid was obtained (m = 75 mg, 70%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.70 (d, J = 2.3 Hz, 1H), 8.13−8.08 (m, 3H), 7.97 (dd, J = 5.5 and 3.2 Hz, 1H), 7.69 (d, J = 7.9 Hz, 1H), 7.28−7.27 (m, 2H), 7.11−7.05 (m, 1H), 7.01 (d, J = 8.6 Hz, 1H), 3.01 (s, 2H), 1.39 (s, 6H). LCMS m/z 443.0 [M − 1]. HRMS calculated mass, 445.1329 [M + 1]; measured, 445.1332 [M + 1].

*6-(8-(2-(Benzothiazol-2-yl)hydrazono)-5-methyl-5,6,7,8-tetrahydronaphthalen-2-yl)picolinic Acid (**63i**).* Procedure B was followed using compound **62i** (22 mg, 0.08 mmol) and 2-hydrazinylbenzothiazole (13 mg, 0.08 mmol). A light-brown solid was obtained (m = 12 mg, 36%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.80 (d, J = 2.0 Hz, 1H), 8.15−8.08 (m, 3H), 8.01 (dd, J = 6.4 and 2.4 Hz, 1H), 7.75 (d, J = 7.5 Hz, 1H), 7.44 (d, J = 8.2 Hz, 1H), 7.39−7.37 (m, 1H), 7.29 (t, J = 7.0 Hz, 1H), 7.10 (t, J = 7.7 Hz, 1H), 3.04 (m, 1H), 2.88−2.85 (m, 1H), 2.04−1.97 (m, 1H), 1.78−1.69 (m, 1H), 1.31 (d, J = 7.0 Hz, 1H). LCMS m/z 429.2 [M + 1]. HRMS calculated mass, 429.1380 [M + 1]; measured, 429.1381 [M + 1].

*tert-Butyl 6-(4-Oxothiochroman-6-yl)picolinate (**64**).* Procedure D was followed using t-butyl 6-bromopicolinate (144 mg, 0.56 mmol) and **61f** (290 mg, 0.73 mmol). The product was purified by flash chromatography using SiO_2 (AcOEt/petroleum ether, 5:95 to 50:50). An off-white solid was obtained. It was triturated a diethyl ether/petroleum ether mixture. A white solid was obtained (m = 205 mg, 69%). ^{1}H NMR ($CDCl_3$, ppm): δ 8.68 (d, J = 2.0 Hz, 1H), 3.36 (dd, J = 8.4 and 2.2 Hz, 1H), 7.98−7.94 (m, 2H), 7.86 (t, J = 7.6 Hz, 1H), 7.41 (d, J = 8.3 Hz, 1H), 3.31−3.27 (m, 2H), 3.05−3.00 (m, 2H), 1.66 (s, 9H).

*tert-Butyl 6-(2,3-Dihydro-1-oxide-4H-1-benzothiopyran-4-yl)-picolinate (**65a**).* Compound **64** (50 mg, 0.15 mmol) dissolved in 0.3 mL of $CHCl_3$ was treated at room temperature every 5 min with 25 μL aliquots of a solution of m-CPBA (33 mg of 77% mixture, 0.15 mmol) in 0.3 mL of $CHCl_3$. The reaction was followed by TLC (AcOEt/Pet. Et. 30:70) and stopped after 1 h 30 min. A saturated aqueous solution of $NaHCO_3$ was added and the mixture vigorously stirred. The aqueous layer was decanted, and the organic layer was treated in the same manner two more times. It was then washed three times with water. The organic layer was applied directly onto a SiO_2 column and the mixture eluted with CH_2Cl_2/MeOH (100:0 to 98.5:1.5). A white solid was obtained (m = 48 mg, 89%). ^{1}H NMR ($CDCl_3$, ppm): δ 8.75 (d, J = 1.9 Hz, 1H), 8.67 (dd, J = 8.1 and 2.0 Hz, 1H), 8.05 (dd, J = 7.4 and 1.3 Hz, 1H), 8.02 (dd, J = 8.1 and 0.4 Hz, 1H), 8.01 (dd, J = 7.9 and 1.3 Hz, 1H), 7.93 (t, J = 7.4 Hz, 1H), 3.59−3.48 (m, 3H), 3.02−2.88 (m, 1H), 1.66 (s, 9H). LCMS m/z 358.1 [M + 1].

*tert-Butyl 6-(2,3-Dihydro-1,1-dioxide-4H-1-benzothiopyran-4-yl)-picolinate (**65b**).* Compound **64** (86 mg, 0.25 mmol) dissolved in 0.5 mL of CH_2Cl_2 was treated at 0 °C with a solution of m-CPBA (112 mg of 77% mixture, 0.5 mmol) in 0.5 mL of CH_2Cl_2. The reaction was warmed to room temperature and stirred for 3 h. A saturated aqueous solution of $NaHCO_3$ was added and the mixture vigorously stirred. The aqueous layer was extracted twice with CH_2Cl_2. The combined organic layers were washed with saturated aqueous $NaHCO_3$, dried over Na_2SO_4, and concentrated. The residue was purified by column chromatography using SiO_2 and AcOEt/petroleum ether (50:50 to 100:0). A yellow solid was obtained (m = 60 mg, 64%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.65 (s, 1H), 8.64 (dd, J = 8.3 and 1.9 Hz, 1H), 8.09−8.03 (m, 2H), 7.99−7.95 (m, 1H), 7.93 (t, J = 7.1 Hz, 1H), 3.75−3.71 (m, 2H), 3.46−3.41 (m, 2H), 1.64 (s, 9H).

*6-(2,3-Dihydro-1-oxide-4H-1-benzothiopyran-4-yl)picolinic Acid (**66a**).* Compound **65a** (25 mg, 0.07 mmol) was treated with 0.5 mL of formic acid. The reaction was stirred at room temperature for 20 h. The mixture was then concentrated, and diethyl ether was added to the residue. The solids were triturated. Once the solids had settled down, the ethereal layer was removed. The solids were triturated with diethyl ether two more times, and the resulting solids were dried in vacuum (m = 17 mg, 81%). ^{1}H NMR ($CDCl_3$, ppm): δ 8.79 (d, J = 1.7 Hz, 1H), 8.64 (dd, J = 8.1 and 2.0 Hz, 1H), 8.35 (dd, J = 7.7 and 1.2 Hz, 1H), 8.14 (t, J = 7.7 Hz, 1H), 8.07 (dd, J = 7.7 and 1.2 Hz, 1H), 8.05 (d, J = 8.0 Hz, 1H), 3.78−3.69 (m, 1H), 3.61−3.52 (m, 1H), 3.33−3.23 (m, 1H), 3.04−2.94 (m, 1H).

*tert-Butyl 6-(4-(2-(Benzo[d]thiazol-2-yl)hydrazono)-2,3-dihydro-1,1-dioxide-4H-1-benzothiopyran-4-yl)picolinate (**66b**).* Procedure B was followed using compound **65b** (28 mg, 0.08 mmol) and 2-hydrazinylbenzothiazole (12 mg, 0.08 mmol). A yellow solid was obtained (m = 32 mg, 82%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.99 (d, J = 1.4 Hz, 1H), 8.31 (dd, J = 8.3 and 1.8 Hz, 1H), 8.27 (dd, J = 7.9 and 1.0 Hz, 1H), 8.18 (t, J = 7.7 Hz, 1H), 8.05 (dd, J = 7.6 and 1.0 Hz, 1H), 8.01 (d, J = 8.2 Hz, 1H), 7.63 (br s, 1H), 7.33−7.28 (m, 2H), 7.15−7.10 (m, 1H), 3.78−3.74 (m, 2H), 3.46−3.42 (m, 2H), 1.63 (s, 9H). LCMS m/z 521.2 [M + 1].

*6-(4-(2-(Benzothiazol-2-yl)hydrazono)-2,3-dihydro-1-oxide-4H-1-benzothiopyran-4-yl)picolinic Acid (**67a**).* Procedure B was followed using compound **66a** (9 mg, 0.03 mmol) and 2-hydrazinylbenzothiazole (5 mg, 0.03 mmol). A white solid was obtained (m = 11 mg, 59%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.97 (d, J = 1.8 Hz, 1H), 8.34 (dd, J = 8.1, 1.9 Hz, 1H), 8.26 (dd, J = 7.8 and 1.1 Hz, 1H), 8.17 (t, J = 7.7 Hz, 1H), 8.09 (dd, J = 7.6 and 1.1 Hz, 1H), 7.90 (d, J = 8.4 Hz, 1H), 7.73 (d, J = 7.6 Hz, 1H), 7.32−7.27 (m, 2H), 7.14−7.09 (m, 1H), 3.45−3.15 (m, 4H). LCMS m/z 449.1 [M + 1].

*6-(4-(2-(Benzothiazol-2-yl)hydrazono)-2,3-dihydro-1,1-dioxide-4H-1-benzothiopyran-4-yl)picolinic Acid (**67b**).* Compound **66b** (15 mg, 0.03 mmol) was treated with 0.2 mL of formic acid. The reaction was stirred at room temperature for 16 h. The reaction mixture was concentrated. The residue was triturated in methanol. The solids were collected by filtration, rinsed with methanol, and dried under vacuum. A yellow solid was obtained (m = 11 mg, 82%). ^{1}H NMR (DMSO-d_6, ppm): δ 9.03 (d, J = 1.6 Hz, 1H), 8.38 (dd, J = 8.3 and 1.8 Hz, 1H), 8.28 (dd, J = 7.8 and 1.2 Hz, 1H), 8.19 (t, J = 7.7 Hz, 1H), 8.11 (dd, J = 7.6 and 1.2 Hz, 1H), 8.01 (d, J = 8.3 Hz, 1H), 7.73 (d, J = 7.8 Hz, 1H), 7.32−7.30 (m, 2H), 7.15−7.10 (m, 1H), 3.78−3.74 (m, 2H), 3.46−3.41 (m, 2H). LCMS m/z 465.1 [M + 1]. HRMS calculated mass, 465.0686 [M + 1]; measured, 465.0687 [M + 1].

*Ethyl 2-(8-Oxo-5,6,7,8-tetrahydronaphthalen-2-yl)thiazole-4-carboxylate (**69a**).* Procedure D was followed using ethyl 2-bromothiazole-4-carboxylate **68a** (153 mg, 0.65 mmol) and **57b** (250 mg, 0.92 mmol). The product was purified by flash chromatography using SiO_2 (AcOEt/petroleum ether, 0:100 to 15:85). A white solid was obtained (m = 62 mg, 22%). ^{1}H NMR ($CDCl_3$, ppm): δ 8.50 (d, J = 1.9 Hz, 1H), 8.27 (dd, J = 8.0 and 2.1 Hz, 1H), 8.17 (s, 1H), 7.37 (d, J = 7.6 Hz, 1H), 4.45 (q, J = 7.1 Hz,

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1H), 3.02 (t, J = 6.2 Hz, 1H), 2.73−2.69 (m, 2H), 2.18 (quint., J = 6.0 Hz, 1H), 1.44 (t, J = 6.0 Hz, 1H).

*Ethyl 2-(8-Oxo-5,6,7,8-tetrahydronaphthalen-2-yl)oxazole-4-carboxylate (**69b**).* Procedure D was followed using ethyl 2-bromooxazole-4-carboxylate **68b** (50 mg, 0.28 mmol) and **57b** (94 mg, 0.36 mmol). The product was purified by flash chromatography using SiO_2 (AcOEt/petroleum ether, 5:95 to 40:60). A white solid was obtained (m = 43 mg, 54%). ^{1}H NMR ($CDCl_3$, ppm): δ 8.69 (d, J = 2.0 Hz, 1H), 8.27 (s, 1H), 8.25 (dd, J = 8.0 and 2.0 Hz, 1H), 7.37 (d, J = 8.0 Hz, 1H), 4.42 (q, J = 7.1 Hz, 1H), 3.02 (t, J = 6.0 Hz, 2H), 2.71−2.67 (m, 2H), 2.18 (quint., J = 5.9 Hz, 2H), 1.40 (t, J = 7.1 Hz, 3H).

*2-(8-Oxo-5,6,7,8-tetrahydronaphthalen-2-yl)thiazole-4-carboxylic Acid (**70a**).* Compound **69a** (56 mg, 0.19 mmol) was dissolved in 2 mL of MeOH and treated with 2 mL of a 1 M NaOH aqueous solution. The reaction was stirred at 90 °C for 2.5 h. After this time, the reaction was concentrated. Then 10% aqueous citric acid solution was added to the residue followed by AcOEt. The aqueous layer was extracted twice with AcOEt. The combined organic layers were washed with brine, dried over Na_2SO_4, and concentrated. A light-yellow solid was obtained (m = 46 mg, 89%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.51 (s, 1H), 8.40 (s, 1H), 8.12 (d, J = 8.1 Hz, 1H), 7.53 (d, J = 7.5 Hz, 1H), 3.02−3.04 (m, 2H), 2.69−2.68 (m, 2H), 2.10−2.07 (m, 2H). LCMS m/z 274.1 [M + 1].

*2-(8-Oxo-5,6,7,8-tetrahydronaphthalen-2-yl)oxazole-4-carboxylic Acid (**70b**).* Compound **69b** (43 mg, 0.15 mmol) was dissolved in 0.2 mL of MeOH and treated with 0.2 mL of a 1 M NaOH aqueous solution. The reaction was stirred at 90 °C for 5 min until all solids disappeared and at room temperature for 1 h. After this time, the reaction was concentrated. Water and diethyl ether were added. The aqueous phase was extracted two times with diethyl ether. It was then acidified to pH 1 with 2 M HCl. A white solid precipitated. It was collected by filtration, rinsed with water, and dried in a vacuum oven. A white solid was obtained (m = 23 mg, 61%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.84 (s, 1H), 8.44 (d, J = 1.9 Hz, 1H), 8.13 (dd, J = 8.0 and 2.0 Hz, 1H), 7.57 (d, J = 8.0 Hz, 1H), 3.02 (t, J = 5.7 Hz, 2H), 2.69−2.65 (m, 2H), 2.08 (quint, J = 5.7 Hz, 1H).

*2-(8-(2-(Benzothiazol-2-yl)hydrazono)-5,6,7,8-tetrahydronaphthalen-2-yl)thiazole-4-carboxylic Acid (**71a**).* Procedure B was followed using compound **70a** (40 mg, 0.15 mmol) and 2-hydrazinylbenzothiazole (24 mg, 0.15 mmol). The solids obtained were stirred in hot methanol. After cooling, the solids were isolated by filtration and dried in vacuum. A white solid was obtained (m = 24 mg, 39%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.55 (d, J = 1.9 Hz, 1H), 8.48 (s, 1H), 7.85 (dd, J = 8.0 and 2.0 Hz, 1H), 7.72 (d, J = 7.7 Hz, 1H), 7.34 (d, J = 8.0 Hz, 1H), 7.32 (d, J = 7.1 Hz, 1H), 7.25 (td, J = 7.2 and 1.1 Hz, 1H), 7.06 (t, J = 7.8 Hz, 1H), 2.80−2.77 (m, 4H), 1.87−1.84 (m, 2H). LCMS m/z 421.1 [M + 1]. HRMS calculated mass, 421.0787 [M + 1]; measured, 421.0787 [M + 1].

*2-(8-(2-(Benzothiazol-2-yl)hydrazono)-5,6,7,8-tetrahydronaphthalen-2-yl)oxazole-4-carboxylic Acid (**71b**).* Procedure B was followed using compound **70b** (23 mg, 0.09 mmol) and 2-hydrazinylbenzothiazole (15 mg, 0.09 mmol). A brown solid was obtained (m = 22 mg, 61%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.84 (s, 1H), 8.66 (d, J = 1.9 Hz, 1H), 7.90 (dd, J = 7.9 and 1.9 Hz, 1H), 7.77 (d, J = 8.1 Hz, 1H), 7.41 (d, J = 8.0 Hz, 1H), 7.36 (d, J = 8.5 Hz, 1H), 7.29 (t, J = 7.2 Hz, 1H), 7.09 (t, J = 7.9 Hz, 1H), 2.85−2.82 (m, 4H), 1.91−1.88 (m, 2H). LCMS m/z 405.1 [M + 1]. HRMS calculated mass, 405.1016 [M + 1]; measured, 405.1017 [M + 1].

*2-(8-Oxo-5,6,7,8-tetrahydronaphthalen-2-yl)pyrimidine-4-carboxylic Acid (**73a**).* Procedure E was followed using 2-chloropyrimidine-4-carboxylic acid **72a** (63 mg, 0.28 mmol) and **57b** (100 mg, 0.37 mmol). A light-brown solid was obtained (m = 57 mg, 76%). ^{1}H (DMSO-d_6, ppm): δ 9.16 (d, J = 5.0 Hz, 1H), 8.96 (d, J = 1.9 Hz, 1H), 8.61 (dd, J = 8.0 and 2.0 Hz, 1H), 7.92 (d, J = 5.0 Hz, 1H), 7.56 (d, J = 8.4 Hz, 1H), 3.04, (t, J = 6.0 Hz, 2H), 2.70−2.66 (m, 2H), 2.14−2.06 (m, 2H). LCMS m/z 269.1 [M + 1].

*4-(8-Oxo-5,6,7,8-tetrahydronaphthalen-2-yl)picolinic Acid (**73b**).* Procedure D was followed using 4-chloropicolinic acid **72b** (100 mg, 0.63 mmol) and **57b** (206 mg, 0.76 mmol). A pale-yellow solid was obtained (m = 78 mg, 46%). ^{1}H NMR ($CDCl_3$, ppm): δ 8.77 (dd, J = 5.1 and 0.7 Hz, 1H), 8.29 (d, J = 1.9 Hz, 1H), 8.24 (d, J = 2.1 Hz, 1H), 8.05 (dd, J = 8.0 and 2.2 Hz, 1H), 7.98 (dd, J = 5.1 and 1.9 Hz, 1H), 7.55 (d, J = 8.2 Hz, 1H), 3.03 (t, J = 5.7 Hz, 2H), 2.69−2.65 (m, 2H), 2.09 (quint., J = 6.1 Hz, 2H).

*2-(8-(2-(Benzothiazol-2-yl)hydrazono)-5,6,7,8-tetrahydronaphthalen-2-yl)pyrimidine-4-carboxylic Acid (**74a**).* Procedure B was followed using compound **73a** (50 mg, 0.19 mmol) and 2-hydrazinylbenzothiazole (31 mg, 0.19 mmol). A light-brown solid was obtained (m = 61 mg, 77%). ^{1}H NMR (DMSO-d_6, ppm): δ 9.21 (d, J = 1.7 Hz, 1H), 9.19 (d, J = 5.0 Hz, 1H), 8.35 (dd, J = 8.0 and 1.8 Hz, 1H), 7.91 (d, J = 5.0 Hz, 1H), 7.75 (d, J = 7.7 Hz, 1H), 7.39 (d, J = 8.0 Hz, 1H), 7.36 (br s, 1H), 7.29 (t, J = 7.2 Hz, 1H), 7.10 (t, J = 7.5 Hz, 1H), 2.86−2.81 (m, 4H), 1.92−1.89 (m, 2H). LCMS m/z 416.1 [M + 1]. HRMS calculated mass, 416.1176 [M + 1]; measured, 416.1178 [M + 1].

*4-(8-(2-(Benzothiazol-2-yl)hydrazono)-5,6,7,8-tetrahydronaphthalen-2-yl)picolinic Acid (**74b**).* Procedure B was followed using compound **73b** (18 mg, 0.07 mmol) and 2-hydrazinylbenzothiazole (11 mg, 0.07 mmol). A pale-yellow solid was obtained (m = 26 mg, 90%). ^{1}H NMR (DMSO-d_6, ppm): δ 8.80 (d, J = 5.1 Hz, 1H), 8.43 (s, 1H), 8.32 (s, 1H), 7.93 (dd, J = 5.1 and 1.6 Hz, 1H), 7.74 (dd, J = 7.8 and 1.7 Hz, 1H), 7.71 (d, J = 7.8 Hz, 1H), 7.41−7.35 (m, 2H), 7.28 (t, J = 7.1 Hz, 1H), 7.08 (t, J = 7.4 Hz, 1H), 2.84−2.79 (m, 4H), 1.90−1.87 (m, 2H). LCMS m/z 415.2 [M + 1]. HRMS calculated mass, 415.1223 [M + 1]; measured, 415.1223 [M + 1].

AlphaScreen Assay. The aim of the BH3 proteins AlphaScreen assay is to identify active small molecules inhibiting the protein−protein interaction between Bcl-w or Bcl-X_L and BH3-only proteins such as Bim or BH3-peptides derived from Bak and Bax.

Reagents and Materials. GST-Bcl-w (ΔC29), GST- Bcl-X_L, GST-Mcl-1, and biotinylated-GST (for the counter-screen) proteins were prepared and provided by Peter Czabotar.[17,61] These were stored as stock solutions at −80 °C. The biotinylated-Bak and biotinylated-Bim 26mer peptides were obtained from Auspep and were stored as 500 μM stock solutions in 100% DMSO at −20 °C. The AlphaScreen GST (glutathione *S*-yransferase) detection kits were obtained from Perkin-Elmer Lifesciences. White, 384, low-volume assay plates and polypropylene, 384, compound storage plates were obtained from Greiner Bio One and Matrical, respectively. Optical-grade, adhesive plate seals for assay plates were obtained from Applied Biosystems, while adhesive aluminum foil seals used for compound plate storage were obtained from Beckman-Coulter. DMSO was purchased from AnalaR. Assay buffer (25 mM HEPES/25 mM Tris-HCl/50 mM NaCl, pH 7.5) stock was warmed to room temperature from 4 °C, followed by the addition of 5 mM DTT (aliquots stored at −20 °C), 0.1 mg/mL casein (sodium salt; aliquots stored at −20 °C), and 0.03% v/v Tween 20 (all final concentrations) fresh on a daily basis.

Methods. AlphaScreen Assay. AlphaScreen is a bead-based technology that facilitates the measurement of the interaction between molecules. The assay consisted of two hydrogel coated beads which, when brought into close proximity by a binding interaction, allow the transfer of singlet oxygen from a donor bead to an acceptor bead. Upon binding and excitation with laser light at 680 nm, a photosensitizer in the donor bead converts ambient oxygen to a more excited singlet state. This singlet oxygen then diffuses across to react with a chemiluminescer in the acceptor bead. Fluorophores within the same bead are activated, resulting in the emission of light at 580−620 nm. Screening of the test compounds was performed using the AlphaScreen GST (glutathione *S*-transferase) detection kit system (Perkin-Elmer Lifesciences). Briefly, test compounds were titrated into the assay, which consisted of GST tagged Bcl-X_L ΔC25 protein (0.6 nM final concentration) and biotinylated Bim BH3-26mer peptide, biotin-DLRPEIRIAQELRRIGDEFNETYTRR (5.0 nM final concentration), anti-GST coated acceptor beads, and streptavidin coated donor beads (both bead types at a final concentration of 15 μg/mL) and a room temperature incubation time of 4 h before reading. More specifically: (i) a 384-well plate was prepared with 4.75 μL of buffer and 0.25 μL of compound stock (20 mM in DMSO) per well; (ii) binding partners were mixed, in one tube Bcl-X_L was added with the

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acceptor beads, while in the second tube the biotinylated BH3 peptide was added with the donor beads; (iii) these two pairs of binding partners were preincubated for 30 min; (iv) 10 μL of the acceptor beads/Bcl-X_L protein complex was then added to each of the 384 wells; (v) plates were sealed and incubated at room temperature for a further 30 min; (vi) 10 μL of the donor bead/BH3 peptide complex was then added to each of the 384 wells; (vii) plates were sealed, covered with foil, and incubated for a further 4 h and then read. Assay buffer contained 50 mM Hepes pH 7.4, 10 mM DTT, 100 mM NaCl, 0.05% Tween20, and 0.1 mg/mL casein. Bead dilution buffer contained 50 mM Tris-HCl, pH 7.5, 0.01% Tween20, and 0.1 mg/mL casein. The final DMSO concentration in the assay was 1.0% (v/v). Assays were performed in 384-well white Optiplates (Perkin-Elmer Lifesciences) and analyzed on the Perkin-Elmer Fusion Alpha plate reader (Ex680, Em520−620 nM).

The conditions for the assay with Mcl-1 and Bcl-w were essentially the same using the following final concentrations: GST-Mcl-1 (0.8 nM), Biot-Bak (4 nM), GST-Bcl-w (0.9 nM), and Biot-Bim (3 nM). The counter-screen assay was performed using the donor and acceptor beads in presence of Biotinylated-GST (final concentration 2 nM).

Determination of Selectivity Profile by Biacore S3000 SPR Analysis. Recombinant Proteins. Expression and purification of the loop-deleted form of human Bcl-x_L (Δ27−82, ΔC24) and Bcl-2 ΔC22, Bcl-x_L ΔC24, Bcl-w C29S/A128E ΔC29, and human/mouse chimeric Mcl-1 (ΔN170,ΔC23) for Biacore studies was performed as described previously.[17,61,62]

Binding Affinity Measurements: Solution Competition Assays. Solution competition assays were performed using a Biacore 3000 instrument as described previously.[63] Briefly, pro-survival proteins (10 nM) were incubated with varying concentrations of compounds for at least 2 h in running buffer (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.005% (v/v) Tween 20, pH 7.4) prior to injection onto a CM5 sensor chip on which either a wild-type BimBH3 peptide or an inert BimBH3 mutant peptide was immobilized. Specific binding of the pro-survival protein to the surface in the presence and absence of compounds was quantified by subtracting the signal from the Bim mutant channel from that obtained on the wild-type Bim channel. The IC_{50} was calculated by nonlinear curve-fitting of the data with Kaleidagraph (Synergy Software).

Surface Plasmon Resonance Assay (Biacore S51). A. Methods for Potent Binders (K_d < 5 nM). Immobilization of Anti-GST antibody: Anti-GST antibody surfaces were prepared by using standard amine-coupling procedures as instructed by the GST Capture Kit from Biacore. The running buffer was 10 mM $NaH_2PO_4{\cdot}H_2O$, 40 mM $Na_2HPO_4{\cdot}2H_2O$, 150 mM NaCl, 1.0 mM EDTA, 0.03% Tween 20, 5% DMSO, pH 7.4. Flow cells (both spot 1 and spot 2) were activated by injecting 200 μL of NHS (11.5 mg/mL) and EDC (75 mg/mL). Then 50 μL of anti-GST antibody (30 μg/mL) in 10 mM sodium acetate pH 5.0 was injected for 10 min at 5 μL/min, followed by the injection of 175 μL of ethanolamine (1 M) and injection of Biadesorb1 solution (0.5% SDS, 36 s) and of Biadesorb2 solution (50 mM glycine pH 9.5, 40 s) at 30 μL/min at the end of the run. These resulted in about 10000 RU anti-GST antibody immobilized.

Capture of GST-Bcl-X_L: The running buffer utilized was the same as for immobilization of anti-GST antibody. GST-Bcl-X_L (0.1 mg/mL to 0.2 mg/mL) in the running buffer was injected at 10 μL/min for 3 min across spot 2 only, resulting in the capture of about 1200 RU protein.

Kinetic analysis of potent small molecules and GST-Bcl-X_L interactions: A concentration series of each compound was injected at a flow rate of 90 μL/min over three spots (spot 1, immobilized with anti-GST antibody only; spot 2, coated with GST-Bcl-X_L captured by the anti-GST antibody; spot r, free) at 25 °C. The association time and dissociation time were 90 and 240 s, respectively. The buffer blank was also injected periodically for double referencing. The buffer samples containing 4−6% DMSO were injected for solvent correction. The antibody surface was regenerated between binding cycles with 40 s injection of 10 mM glycine-HCl, pH 2.2, and 40 s injection of 0.05% SDS. The protein was injected at 10 μL/min for 3 min across spot 2 only at the beginning of each cycle.

Data Analysis: All sensorgrams were processed by using double referencing. (1) The response from the reference spot (spot 1) was subtracted from the binding response (spot 2), followed by solvent correction. (2) The response from an average of buffer injection was subtracted. To obtain kinetic rate constants (k_a and k_d), corrected response data were then fitted to a one to one binding site model, which includes correction for mass transport limitations. The equilibrium dissociation constant (K_d) was determined by k_d/k_a.

B. Methods for Weaker Binders (K_d > 5 nM). Immobilization of anti-GST antibody: The anti-GST antibody was immobilized on both spot 1 and spot 2 of the same flow cell. The method used was similar to the one described for tight binders.

Capture of GST-Bcl-X_L: The GST-Bcl-X_L (about 1300 RU) was captured on both spot 1 and spot 2 of the same flow cell using a method similar to the one described for tight binders.

Kinetic analysis of nonpotent small molecules and GST-Bcl-X_L interactions: First, 30 nM of 26-mer-wt-Bim (10 μL/min × 3 min) was loaded into spot 1 only at the beginning of each cycle using hydronamic addressing resulting in Bcl-X_L BH3 binding groove "pre-blocked" with wt-Bim 26mer. A concentration series of each compound was then injected at a flow rate of 90 μL/min over three spots (spot 1, coated with Bcl-X_L and Bim peptide; spot 2, coated with GST-Bcl-X_L; spot r, free) at 25 °C. The association and dissociation measurement times were 60 and 120 s, respectively. The buffer blanks were also injected periodically for double referencing. The buffer samples containing 4−6% DMSO were injected for solvent correction. The sensorgrams of (spot 1−spot r) reflect the nonspecific binding to the GST-Bcl-X_L protein (prior to DMSO solvent correction). The sensorgrams of (spot 2−spot 1) reflect the specific binding to the GST-Bcl-X_L protein's binding groove (prior to DMSO solvent correction).

Data analysis: The same method described for tight binders was used.

Cytochrome *c* Assay Release Assay. Mouse embryonic fibroblasts (*mcl-1*$^{-/-}$ and *bax*$^{-/-}$/*bak*$^{-/-}$) (~2 × 10^6) were permeabilized in 20 mM HEPES pH 7.2, 100 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose, and 0.05% (w/v) digitonin (Calbiochem) supplemented with protease inhibitors (Roche) for 10 min on ice. These mitochondria-containing crude lysates were then incubated with 10 μM compound or peptide at 30 °C for 1 h before pelleting and removal of the supernatant (soluble fraction). The relative amount of cytochrome *c* in 3 μL (5% of the total) of this fraction was determined by ELISA (Rat/Mouse Total Cytochrome *c* DuoSet IC; R&D Systems), performed according to the manufacturer's instructions.

Cell-Based Assays. Mouse embryonic fibroblasts (wild-type, *mcl-1*$^{-/-}$ and *bax*$^{-/-}$/*bak*$^{-/-}$) were routinely cultured in Iwaki 75-cm^2 tissue-culture flasks (cat. no. 3123-075) and were grown in DMEM/Kelso consisting of 10% heat-inactivated fetal calf serum (FCS; Hyclone cat. no. SH30396.03), 0.1 mM asparagine (Fluka cat. no. 11149), and 3.5 μM of 2-mercaptoethanol (Sigma cat. no. M7522).

For assays, cells were seeded in the above media except containing only 1% FCS. Cells were seeded in 384-well tissue-culture-grade black plates with flat, clear bottoms (Corning cat. no. 3712) at a calculated dilution necessary to achieve a density of 1 × 10^4 cells ml^{-1} (500 cells per well in 50 μL media) and incubated overnight at 37 °C with 5% CO_2 in air. Compounds were made up in 384-well 50 μL V-bottomed plates (Matrical cat. no. MP101-2-PP), from which 100 nL was added to the cells, resulting in a highest final concentration of compound of 20 μM serially titrated. The plates were then returned to 37 °C overnight. On the final day, 10 μL of prewarmed CellTiter-Blue (Promega, cat. no. G8081) was added to all wells of the assay plates and incubated at 37 °C for a further period of 4−8 h before plates were read using an EnVision 2103 (Perkin-Elmer) plate reader used to measure the viability via the detection of the fluorescent dye, resorufin, at λ_{ex} 535 nm/λ_{em} 590 nm. Data were saved in ".csv" format and imported into an ActivityBase database (IDBS Solutions). The percent inhibition was calculated using the following equation:

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$$\%\text{inhibition} = 100 \times \left(1 - \left[\frac{(x - \mu^-)}{\mu^+ - \mu^-}\right]\right)$$

x = CPS obtained after sample compound treatment, μ^- = CPS obtained for the negative controls (columns 24), and μ^+ = CPS obtained for the positive controls (columns 23).

IC_{50} values were obtained by nonlinear least-squares fitting of the data using the four-parameter logistic fit (XLFit 4 eq 205)

$$y = A + ((B - A)/(1 + ((C/x)^D)))$$

The quality of the assay results was monitored by determination of the Z' factor for each assay plate, where $Z' \geq 0.5$ for the results was considered as robust.[64]

■ ASSOCIATED CONTENT

Supporting Information

Screening cascade, the synthesis of **60a−d**, all steady state curves, sensorgrams and parameters from SPR experiments for compounds **1**, **4**, **12a**, **38**, **59b**, and wtBim-26mer, Biacore 3000 competition assay details. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS USED

AS, AlphaScreen (amplified luminescent proximity homogeneous assay); Bak, Bcl-2 homologous antagonist/killer; Bax, Bcl-2 associated X protein; Bcl-2, B-cell lymphoma 2; Bcl-X_L, B-cell lymphoma extra large; Bcl-w, Bcl-2-like protein 2; Bim, Bcl-2 interacting mediator of cell death; BE, binding efficiency; BH, Bcl-2 homology domain; DKO, double knockout; FBS, fetal bovine serum; HSA, human serum albumin domain III; KO, knock out; Mcl-1, myeloid cell leukemia sequence 1; LPA, luminescence proximity assay; MEF, mouse embryonic fibroblasts; RU, resonance units; SPR, surface plasmon resonance; TMS, trimethylsilyl; wt, wild-type

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