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Towards the next generation of dual Bcl-2/Bcl-x_L inhibitors

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ABSTRACT

Structural modifications of the left-hand side of compound **1** were identified which retained or improved potent binding to Bcl-2 and Bcl-x_L in in vitro biochemical assays and had strong activity in an RS4;11 apoptotic cellular assay. For example, sulfoxide diastereomer **13** maintained good binding affinity and comparable cellular potency to **1** while improving aqueous solubility. The corresponding diastereomer (**14**) was significantly less potent in the cell, and docking studies suggest that this is due to a stereochemical preference for the R_S versus S_S sulfoxide. Appending a dimethylaminoethoxy side chain (**27**) adjacent to the benzylic position of the biphenyl moiety of **1** improved cellular activity by approximately three-fold, and this activity was corroborated in cell lines overexpressing Bcl-2 and Bcl-x_L.

The B-cell lymphoma 2 (Bcl-2) family of proteins play a central role in regulating cell death through the outer mitochondrial pathway of apoptosis. Antiapoptotic members, such as Bcl-2, Bcl-x_L, and Mcl-1, are comprised of four Bcl homology (BH) domains. Three of these domains (BH1–BH3) are shared by the proapoptotic Bcl-2 effector proteins Bax and Bak, while the proapoptotic activator proteins (e.g., Bim, Bid) only contain BH3. In a normal healthy cell, antiapoptotic proteins sequester their apoptotic counterparts through BH3 domain binding. In response to cellular injury, Bax and Bak are activated, resulting in oligomerization. Oligomer insertion into the outer mitochondrial membrane forms an exit pore through which cytochrome *c* enters the cytosol. Subsequent initiation of the caspase cascade culminates in cell death.[1]

Pro-survival members of the Bcl-2 family of proteins are overexpressed in a variety of neoplastic malignancies, and this dysregulation has been identified as a critical component of tumor development and chemotherapy resistance.[2] As a result of overexpression, proapoptotic proteins are perpetually sequestered, and the intrinsic apoptotic pathway is blocked. A developing treatment to restore this process in cells that are 'primed for death' is the inhibition of antiapoptotic Bcl-2 proteins with BH3-mimetics.[3] Two such examples are ABT-737[4] and ABT-263[5] (Navitoclax; Fig. 1), which bind to Bcl-2, Bcl-x_L, and Bcl-w with subnanomolar affinity. Navitoclax, which is an orally bioavailable second generation Bcl-2 inhibitor, is in Phase I/II clinical trials for solid tumors and hematologic malignancies.[5,6]

Dose-limiting observations for Navitoclax include thrombocytopenia, which is a target-driven toxicity resulting from Bcl-x_L inhibition in platelets.[7a] Bcl-x_L inhibition also induces transient thrombocytopathy, leading to increased tail-bleeding time in mice.[7b] Thrombocytopenia has been managed in the clinic by starting with low lead-in dosing prior to dose escalation to avoid severe platelet nadirs,[6d] and blood platelet levels have been shown to revert to pre-dosing levels after cessation of Navitoclax administration.[6b] Therefore, a desirable outcome in developing second generation dual Bcl-2/Bcl-x_L inhibitors is an amelioration or at least attenuation of the severity and duration of thrombocytopenia in patients. An alternative approach is to develop a selective Bcl-2 inhibitor and thereby side-step thrombocytopenia as a dose-limiting toxicity.[8]

Herein we discuss structural modification of the left-hand side of known triflone **1**[9] (Fig. 1, green box). Modifications of the right-hand side of this compound will be discussed in a future publication. This tool compound was selected because its synthetic accessibility enabled the rapid profiling of scaffold changes. One goal of these studies was to improve aqueous solubility, with the aim of increasing the fraction absorbed and, ultimately, bioavailability. We were also keen to selectively improve Bcl-2 potency to determine if this might translate to an improved safety profile in vivo.

As a protein–protein interaction inhibitor that binds to a hydrophobic BH3 groove,[10] Navitoclax is not surprisingly very lipophilic ($c\log P = 12.4$). There are a number of functionalities, such as the

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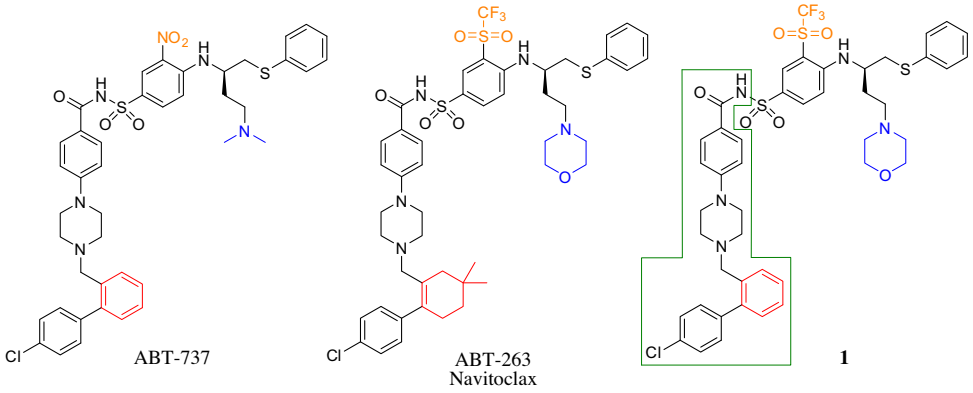


Figure 1. Structures of ABT-737, ABT-263, and **1**.

biphenyl and aryl acylsulfonamide, which likely contribute to its poor aqueous solubility. Additionally, the high acidity of the acylsulfonamide (pK_a 3.4) is suspected of reducing cellular permeability.[5] If this moiety could be replaced, a number of properties might therefore be improved.

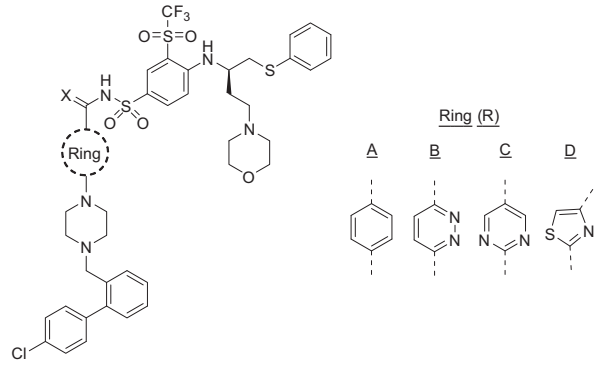
Conversion of the acylsulfonamide to the corresponding benzamidine analog **2**[11] (Table 1) resulted in a complete loss of cellular activity and poor solubility. Similarly, sulfonamide **3** was designed based on the ability of oxetane to act as a carbonyl isostere[15] but was inactive in both biochemical and cellular assays. A correlation between cellular activity and acyl sulfonamide acidity has been previously reported.[5b] Therefore, lack of cell potency for **2** (no calculated acidic centers; ACD pK_a v10, with correction library) and **3** (pK_a 9.0) was attributed to reduced acidity. However, other less quantifiable factors such as functional group binding incompatability (e.g., oxetane) were not ruled out.

Heterocyclic replacement of the phenyl ring of the aryl acylsulfonamide was also attempted as a means of reducing lipophilicity, improving solubility, and retaining or enhancing cellular activity. Pyridazine **4** (*c*log*P* = 8.7; pK_a ~3.6) was modestly active and offered an improvement in solubility, while pyrimidine **5** (*c*log*P* = 8.8; pK_a 3.5) demonstrated comparable binding to Bcl-2 and Bcl-x_L relative to **1**. Thiazole **6** (*c*log*P* = 9.8) was less active than analog **1**, despite an increase in acidity (pK_a 2.6).

As shown in Table 2 and similar to changes to the acylsulfonamide, heterocyclic replacements of the benzylpiperazine were well tolerated with respect to binding, but retaining cellular activity was a challenge (Table 2). For example, we designed piperazine analogs **8** and **9** with the goal of leveraging the polar character of phosphine oxides to enhance aqueous solubility. Analog **9** did indeed have improved solubility, but this was obviated by a lack of cell potency. Other point modifications such as thio ether **12** and sulfone **15** were equally tantalizing. For 4-substituted piperidines **16**–**19**, only the hydroxymethyl analog had appreciable activity in cells but was still 10-fold less active than **1**.[16]

Of the compounds in Table 2, only sulfoxide **13** met our criteria for in vitro potency and solubility, while the corresponding sulfoxide diastereomer (**14**) was inactive in the cell. The data suggest that the latter may be due to poor target inhibition (Bcl-2 FP IC_{50} 0.454 μM). Docking studies (Fig. 2) were used to evaluate potential differences stemming from changes in stereochemistry and build support for stereochemical assignment of the sulfoxide itself.

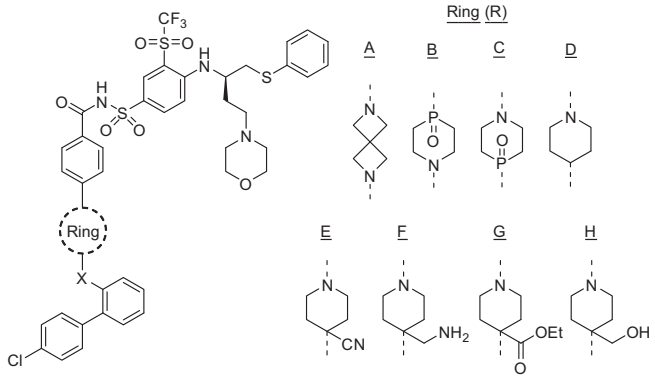
Table 1
Fluorescence polarization, cellular, and solubility data for **1**–**6**



Compd	R	X	Bcl-2 FP IC_{50}[a] (μM)	Bcl-x_L FP IC_{50}[a] (μM)	RS4;11 EC_{50}[b] (μM)	Sol[c] (μM)
1[9]	A	O	0.011	0.016	0.011	14
2[11]	A	NH	0.019	0.063	>3.19	<1
3	A	Oxetan-3,3-yl	>1	>1	>1.1	<1
4	B	O	0.270	0.378	0.563	38
5	C	O	0.018	0.039	0.177	3
6	D	O	0.119	0.098	0.385	2

[a] Fluorescence polarization assay.[12]
[b] Caspase-Glo® cellular assay.[13]
[c] Equilibrium solubility (pH 7.4).[14]

Table 2
Fluorescence polarization, cellular, and solubility data for **7**–**19**



Compd	R	X	Bcl-2 FP IC_{50}[a] (μM)	Bcl-x_L FP IC_{50}[a] (μM)	RS4;11 EC_{50}[b] (μM)	Sol[c] (μM)
7	A	CH_2	>0.468	0.077	0.610	<1
8	B	CH_2	>1.1	0.308	>1.1	4
9	C	CH_2	0.054	0.018	>1.1	21
10	D	O	0.250	0.038	>0.921	35
11	D	C=O	0.065	<0.010	>1.1	nt[d]
12	D	S	0.061	0.031	>0.516	27
13	D	S=O, isomer 1	0.019	0.011	0.090	26
14	D	S=O, isomer 2	0.545	0.054	>1.1	12
15	D	SO_2	0.087	0.021	>1.1	27
16	E	CH_2	<0.010	<0.010	0.504	2
17	F	CH_2	<0.010	<0.010	0.395	<1
18	G	CH_2	0.017	0.030	1.79	<1.5
19	H	CH_2	0.059	<0.010	0.112	6

[a] Fluorescence polarization assay.[12]
[b] Caspase-Glo® cellular assay.[13]
[c] Equilibrium solubility (pH 7.4).[14]
[d] Not tested.

Two surrogate ligands (Fig. 2A) were generated for **13** and **14** wherein the triflone was replaced by hydrogen and the morpholine was changed to dimethyl amine to compensate for anticipated induced fit movements during binding. For convenience, we refer to these surrogate ligands as **13B** (*R* sulfoxide) and **14B** (*S* sulfoxide).[17]

As seen in Figure 2B and C the surrogate ligands were predicted to bind with very similar poses. The key difference between the two ligand poses (Fig. 2D and E) was the vector of the sulfoxide oxygen atom. For the *R* sulfoxide (**13B**) the oxygen atom points towards the solvent. The *S* sulfoxide oxygen (**14B**) points into the pocket in the direction of Ala104 and Phe97.[18a] Burial of the polar oxygen atom of **14B** into a hydrophobic region of the pocket is expected to result in unfavorable electrostatics and contribute to lower binding affinity.[18b] Based on this analysis we propose that **13** and **14** are the R_S and S_S sulfoxides, respectively.

Like changes made to the aryl ring of the acylsulfonamide, we postulated that incorporation of an aromatic nitrogen into the biphenyl moiety would decrease lipophilicity and potentially increase solubility (Table 3). This might also offer the opportunity to capitalize on the established plasticity[5b] of the P2 pocket of Bcl-2 and Bcl-x_L through perturbation of the orthogonality of the biaryl moiety itself.[10] Significant binding to both prosurvival proteins in fluorescence polarization assays was observed for many modifications. However, pyrimidine **20** was only marginally active and pyrazole **23** was completely inactive in the cellular assay, while a 20- to 40-fold decrease in activity was also observed for **21**, **22**, and **24** relative to **1**. No improvements in solubility were noted. In contrast, appending 2-oxo dimethylacetamide and dimethylaminoethoxy[20] side chains at the *ortho* and *meta* positions relative to the benzylic carbon maintained or improved binding and also gave good activity in the RS4;11 cellular assay. Dimethylamine **27** ($c\log P$ = 10.8) was particularly potent and compared favorably to **1**.

In terms of parameterization and as shown in Figure 3, the compounds described herein with $c\log P$'s greater than nine (right of red line) generally had improved cellular potency. Good RS4;11 activity was also secured when lowering lipophilicity compared to Navitoclax (black line). Lipophilicity may not be the only predictor of cellular activity, as compounds with PSA >170 (n = 2; Fig. 4) were generally observed to be less likely to induce apoptosis. A larger data set would help clarify this initial observation.

Select compounds were tested in cells overexpressing Bcl-2 and Bcl-x_L to validate the contributions of each prosurvival protein to activity (Table 4). All compounds demonstrated good agreement between RS4;11 cells and FDCP-1 cells overexpressing Bcl-2. This is consistent with the assertion that activity in RS4;11 cells is driven by Bcl-2 inhibition.[22] Four compounds (**5**, **13**, **16** and **26**) had only weak activity against Bcl-x_L and were largely selective for Bcl-2. Dimethylamine **27** was the most potent compound against all cell lines and compared favorably to both benchmark **1** and Navitoclax in terms of binding efficiency (BEI = 8.21).

In general, compounds were prepared using a late stage coupling (EDC, DMAP, DCM) of an acid and a known sulfonamide such as depicted for **1** in Scheme 1.[4,5a] While some compounds were derived from known acids, such as **2**,[11] **21**,[19] and **22**,[19] the synthesis of more esoteric moieties are described in Schemes 2–7. Both referenced methods and those detailed herein were used to prepare any compound not explicitly discussed.

Oxetane **3** (Scheme 2) was prepared from commercially available *tert*-butyl sulfinamide **29**. Lithium-halogen exchange of **30** with *tert*-butyl lithium, addition of the resulting anion to **29** and

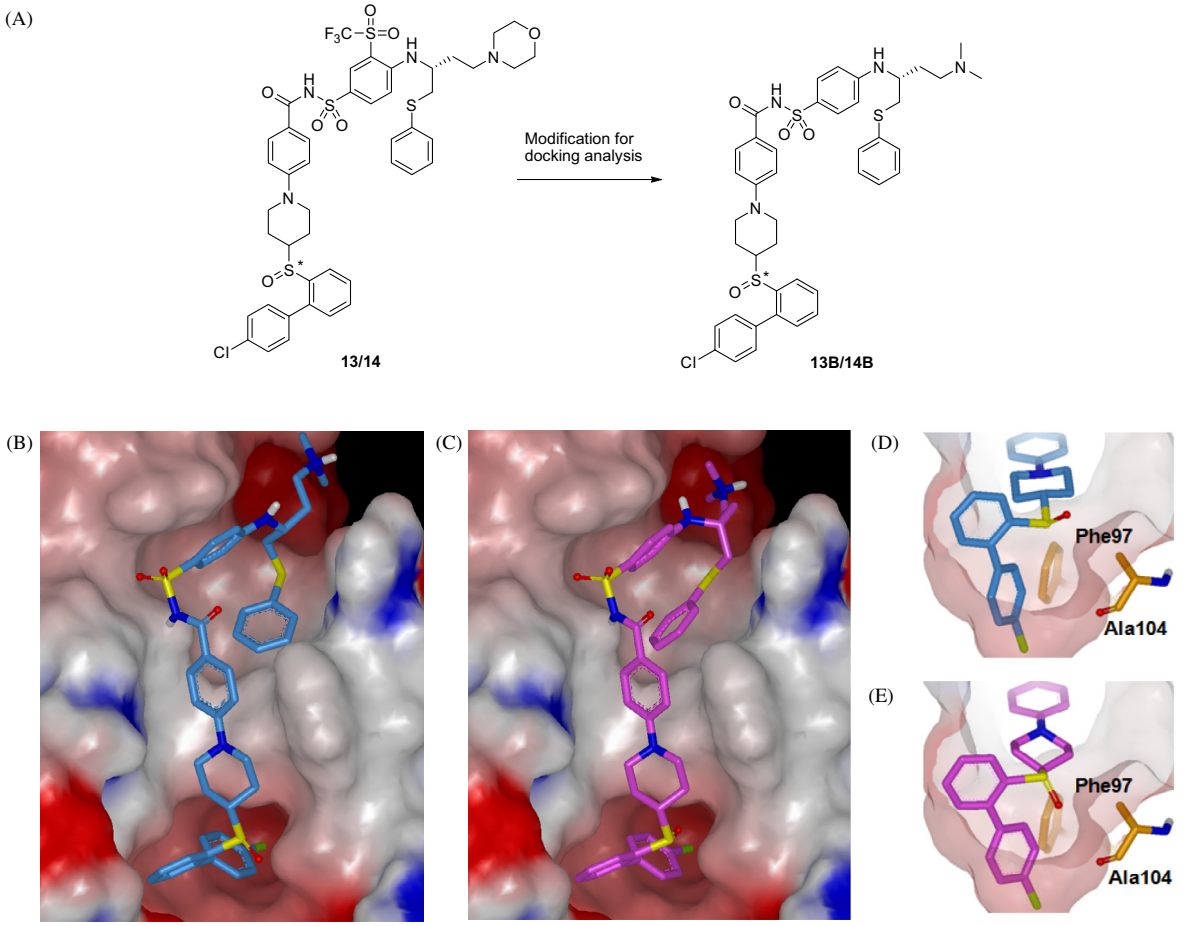
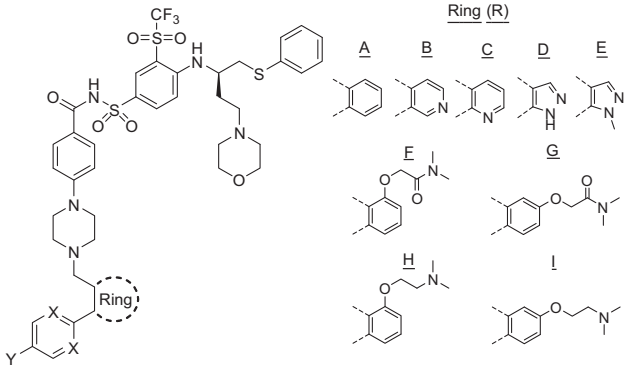


Figure 2. (A) Structural modifications used to create surrogate ligands **13B**/**14B** for rigid protein docking with Bcl-x_L. (B) Docking pose of **13B** (blue). (C) Docking pose of **14B** (magenta). (D) and (E) Close-up views **13B** and **14B**, respectively, which highlight the positioning of the sulfoxide oxygen atom relative to Phe97 and Ala104.[18a]

Table 3
Fluorescence polarization, cellular and solubility data for **20**–**28**



Compd	R	X	Y	Bcl-2 FP IC_{50}[a] (μM)	Bcl-x_L FP IC_{50}[a] (μM)	RS4;11 EC_{50}[b] (μM)	Sol[c] (μM)
20	A	N	Cl	0.021	0.021	3.98	<1
21[19]	B	CH	Cl	<0.010	0.015	0.201	nt[d]
22[19]	C	CH	Cl	0.026	0.025	0.374	nt[d]
23	D	CH	H	0.040	0.038	>10	<1
24	E	CH	H	<0.010	<0.010	0.420	<1
25	F	CH	Cl	<0.010	<0.010	0.018	<1
26	G	CH	Cl	<0.010	<0.010	0.051	2
27	H	CH	Cl	<0.010	nt[d]	0.004	<1
28	I	CH	Cl	<0.010	<0.010	0.023	<1

[a] Fluorescence polarization assay.[12]
[b] Caspase-Glo® cellular assay.[13]
[c] Equilibrium solubility (pH 7.4).[14]
[d] nt = not tested.

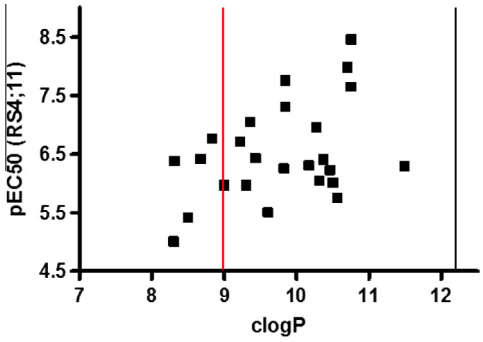


Figure 3. Cellular activity in RS4;11 as a function of lipophilicity (*c*log*P*). Compounds with Bcl-2 FP IC_{50} > 1.1 have been omitted. Compounds herein with a *c*log*P* > 9 (right of red line) generally have improved cellular activity. The *c*log*P* of Navitoclax (12.4) is shown as a black line.

Table 4
RS4;11 and FDCP-1 cellular activity

Compd	RS4;11 EC_{50}[a] (μM)	Bcl-2 FDCP-1 IC_{50}[b] (μM)	Bcl-x_L FDCP-1 IC_{50}[c] (μM)	BEI[d]
Navitoclax	0.014	<0.015	0.060	8.06
1	0.011	0.014	0.064	8.46
5	0.177	0.340	2.09	7.15
13	0.090	0.434	1.72	7.22
16	0.504	0.545	2.82	6.51
25	0.018	0.045	0.226	7.43
26	0.051	0.187	2.09	6.99
27	0.004	0.004	0.033	8.21
28	0.023	0.022	0.138	7.42

[a] Caspase-Glo® cellular assay.[13]
[b] Cellular assay with over-expression of Bcl-2.[21]
[c] Cellular assay with over-expression of Bcl-x_L.[21]
[d] BEI = Binding Efficiency Index = (RS4;11 pEC_{50} × 1000)/MW.

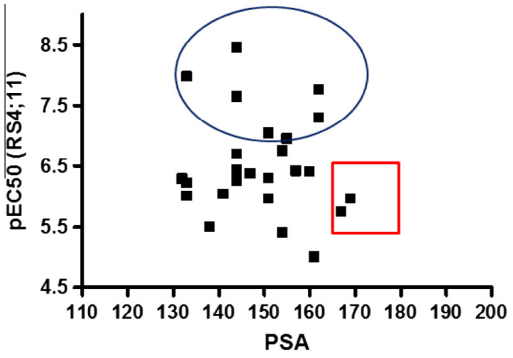


Figure 4. Observed cellular (RS4;11) activity versus polar surface area (PSA). Marked regions suggest possible preferred (blue circle) and potentially less preferred (red box) PSA values for BH3 mimetics described herein with good cell potency. PSA for Navitoclax = 133.4.

deprotection with dilute hydrochloric acid in methanol afforded **31**. Addition to known sulfonyl chloride **32**[5a] followed by S_NAr displacement with chiral amine **34**[23] afforded the corresponding thiophenyl Boc carbamate. Boccleavage was accomplished by adding a minimal amount of concentrated aqueous hydrochloric acid. Reductive amination with commercially available aldehyde **36** then provided **3**.

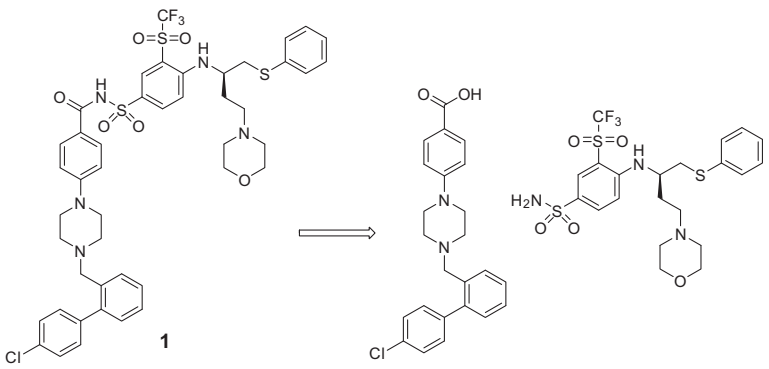
Phosphine oxide **8** was prepared starting from iodide **37**. Palladium-catalyzed coupling with diethyl phosphite in the presence of potassium acetate[24] and a subsequent three step conversion[25] of the resulting aryl phosphonate to its divinyl analog provided **38** (Scheme 3). Cyclization through the addition of benzyl amine, deprotection with α-chloroethyl chloroformate, and reprotection with di-*tert*-butyl dicarbonate afforded bromide **39**. Suzuki coupling to install a vinyl moiety was followed by ozonolysis and oxidation with potassium permanganate to provide the corresponding benzoic acid. Simultaneous esterification and Boc-cleavage in the presence of refluxing hydrochloric acid in methanol provided **40**. Reductive amination and hydrolysis gave acid **41**.

Phosphine oxide **9** was prepared from dibromide **42** by first displacing the benzylic bromide with diethylphosphite[26] and then using a Suzuki coupling to provide **43** (Scheme 4). Conversion of the phosphonate to benzyl piperidine **44** in a manner analogous to that described in Scheme 3 was followed by deprotection, addition to ethyl 4-fluorobenzoate, and hydrolysis to yield **45**.

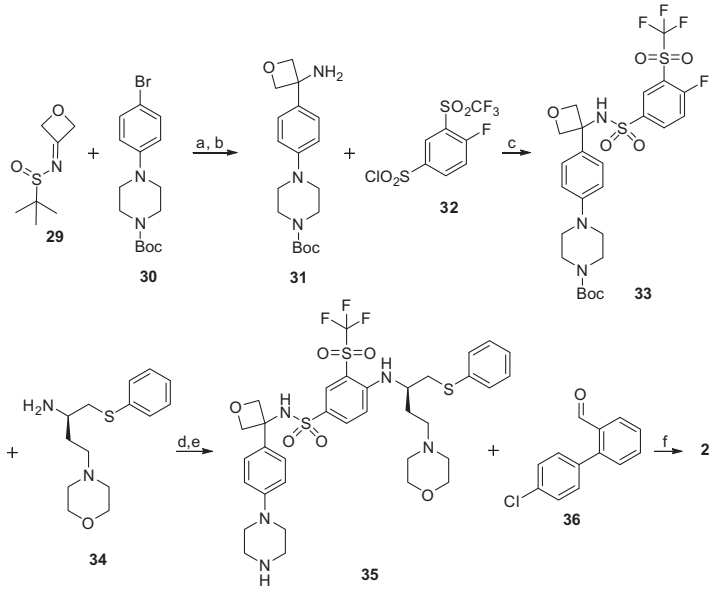
Sulfoxides **13** and **14** were generated starting with alkylation of thiol **47** with bromide **46** (Scheme 5). Oxidation of **48** to the corresponding sulfoxide and deprotection to give **49** was followed by additon to ethyl *p*-fluorobenzoate. However, the only isolated product was ethyl ester **50**. The same chemistry using the sulfone analog of **49** was successful.

Thioether **51** formed smoothly when **48** was deprotected and added to ethyl *p*-fluorobenzoate. This ester was hydrolyzed and carried on to provide **12**. Oxidation of **51** with sodium periodate under reflux conditions afforded the corresponding sulfoxide, which was resolved using normal phase chiral HPLC. The faster eluting sulfoxide ethyl ester provided **52a**, which, upon coupling with the triflone sulfonamide of Scheme 1, yielded **13**.

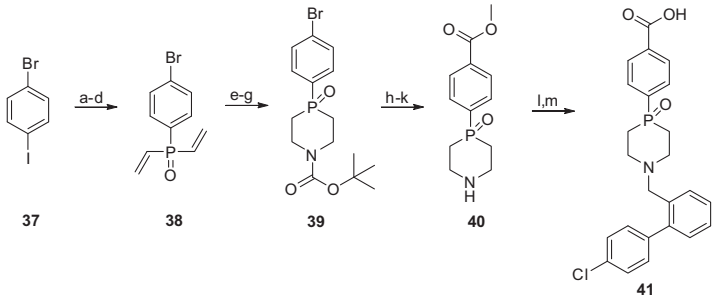
Substituted piperidines **16**–**19** were prepared using a synthetic strategy such as that depicted in Scheme 6. Palladium-catalyzed coupling of **53** and **54** afforded **55** in low yield. In the case of **18** and **19**, ethyl piperidine-4-carboxylate was added directly to *tert*-butyl 4-fluorobenzoate in a method analogous to that described



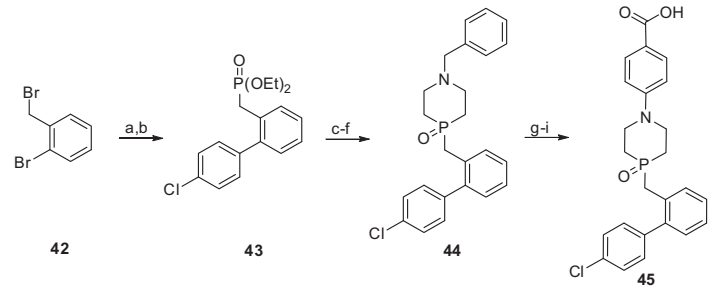
Scheme 1. Retrosynthesis of **1**.



Scheme 2. Reagents and conditions: (a) *t*-butyllithium, THF, −78 °C, 52%; (b) 3 M HCl in MeOH, MeOH, 76%; (c) DIPEA, DCM/EtOAc, 49%; (d) DIPEA, DMF, 50 °C, 61%; (e) cHCl, 96%; (f) $NaBH(OAc)_3$, DCE, 68%.



Scheme 3. Reagent and conditions: (a) $(EtO)_2HPO$, Et_3N, $Pd(dppf)Cl_2$, KOAc, THF, 68 °C, 22 h, 76%; (b) TMSBr; (c) $(COCl)_2$, DMF, CH_2Cl_2; (d) vinyl-MgBr, THF, −78 °C, 53% (three steps); (e) $BnNH_2$, H_2O, 90 °C, 60%; (f) (i) α-chloroethylchloroformate, toluene, reflux, (ii) MeOH, reflux; (g) Boc_2O, Et_3N, DCM, 35% (two steps); (h) vinyl boroxane–pyridine complex, K_2CO_3, $Pd(PPh_3)_4$, 9:1 DME/H_2O, 80 °C; (i) O_3, MeOH, −78 °C, 97% (two steps); (j) $KMnO_4$, acetone, 80%; (k) 3 M HCl in MeOH, reflux; (l) **36**, $NaBH(OAc)_3$, DCE, 50% (two steps); (m) LiOH, THF/MeOH/H_2O, 100 °C, 93%.

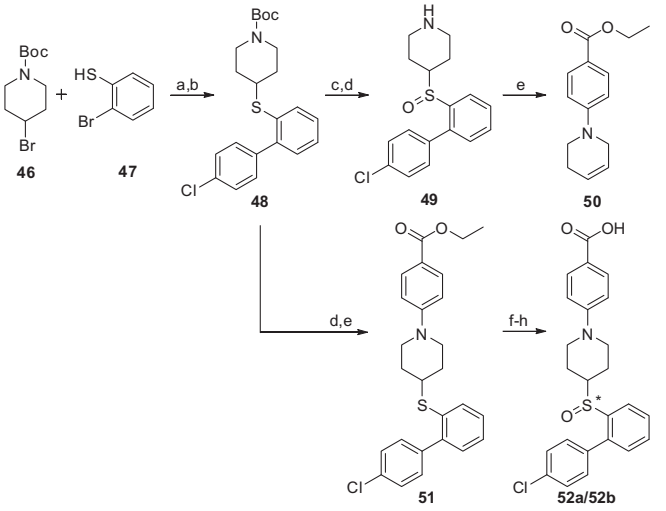


Scheme 4. Reagents and conditions: (a) $KN(TMS)_2$, $(EtO)_2HPO$, THF, −78 °C, 96%; (b) *p*-Cl–$C_6H_5B(OH)_2$, $Pd(PPh_3)_4$, K_2CO_3, 9:1 DME/H_2O, 90 °C, 93%; (c) TMSBr; (d) $(COCl)_2$, DMF, CH_2Cl_2; (e) vinyl-MgBr, THF/Et_2O, −78 °C, 45% (three steps); (f) $BnNH_2$, H_2O, 90 °C, 60%; (g) (i) α-chloroethylchloroformate, toluene, reflux, (ii) MeOH, reflux, 72%; (h) 4-F–$C_6H_4CO_2Et$, DIPEA, DMSO, 120 °C, 41%; (i) LiOH, THF/MeOH/H_2O, 100 °C, 91%.

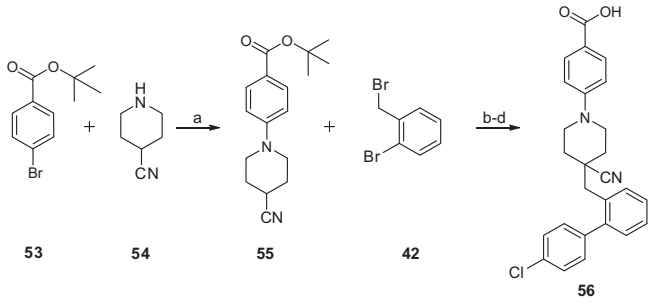
in Schemes 4 and 5. Alkylation of **55** with bromide **42** was followed by installation of the 4-chloro-phenyl moiety, and subsequent acidic deprotection of the *tert*-butyl ester furnished **56**.

Phenyl ethers **25**–**28** were prepared according to Scheme 7. Thus reductive amination of commercially available aldehyde **57** and piperazine **58** followed by Suzuki coupling and subsequent alkylation with 2-chloro dimethylacetamide afforded amide **59**. Acid precursors of **25** and **26** were subsequently prepared via hydrolysis of the appropriate analog using either potassium trimethylsilanoate (**25**) or lithium hydroxide (**26**). Conversion of **59** to the corresponding dimethylamine was smoothly effected using a Rh-catalyzed methodology.[27] Hydrolysis then afforded **60**.

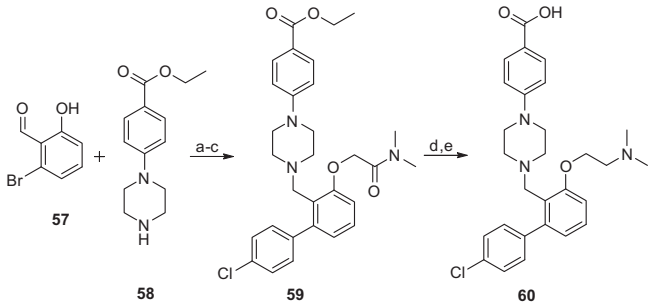
In summary, we have identified several compounds with potent activity in vitro Bcl-2/Bcl-x_L binding and cellular assays. Achieving cellular activity was challenging, and, given the high protein binding exhibited by ABT-737 and Navitoclax,[5b] we suspect that this



Scheme 5. Reagents and conditions: (a) K_2CO_3, DMF, 85 °C, 100%; (b) *p*-Cl–$C_6H_5B(OH)_2$, $Pd(PPh_3)_4$, K_2CO_3, 9:1 DME/H_2O, 90 °C, 93%; (c) *m*-CPBA, CH_2Cl_2, 91%; (d) cHCl, 83–90%; (e) DIPEA, DMSO, 120 °C, **50**: 41%, **51**: 81%; (f) $NaIO_4$, 3:1 MeOH/H_2O, reflux, 83%; (g) Chiral separation: AD column, 50% hexanes in (1:1 methanol/isopropanol) containing 0.1% diethylamine; (h) LiOH, THF/MeOH/H_2O, 100 °C, 74–77%.



Scheme 6. Reagents and conditions: (a) Pd_2dba_3, Cs_2CO_3, *tert*-butyl-XPhos, DMF, 90 °C, 22%; (b) LiHMDS, TMEDA, THF, −78 °C, 45%; (c) *p*-Cl–$C_6H_4B(OH)_2$, Na_2CO_3, toluene, reflux, 60%; (d) TFA, 99%.



Scheme 7. Reagents and conditions: (a) $NaBH(OAc)_3$, DCE, 98%; (b) 4-Cl–C_6H_4-$B(OH)_2$, $Pd(PPh_3)_4$, K_2CO_3, 9:1 DME/H_2O, 80 °C, 54%; (c) $ClCH_2C(O)NMe_2$, NaH, DMF, 92%; (d) $(PPh_3)_2(CO)RhH$, Ph_2SiH_2, THF, 54%; (e) LiOH, THF/MeOH/H_2O, 55 °C, 48%.

was at least partly due to non-specific binding and poor membrane permeability. Based on an analysis of the compounds of this Letter, parameter-based design of Bcl-2/Bcl-x_L inhibitors appears well served when physical properties such as PSA and *c*log*P* are considered. Significant increases in solubility through modification of acyl sulfonamide, biphenyl, and piperidine moieties were elusive because of both the lipophilic nature of this class of BH3 mimetics and the debatable impact of small changes on the physical properties of molecules of this size. Sulfoxide **13** was intriguing because, in addition to an improvement in solubility, docking studies suggest that potency resides with the *R* sulfoxide. Lastly, of all compounds tested, dimethylamine **27** was the most potent Bcl-2/Bcl-x_L inhibitor in RS4;11 and FDCP-1 cell assays, and both potency and binding efficiency compared favorably to **1** and Navitoclax.

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12. Bcl-x_L and Bcl-2 FP binding affinity data were determined according to the procedures of Wang, J.-L.; Zhang, Z.-J.; Choksi, S.; Sjam, S.; Lu, Z.; Croce, C. M.; Alnemri, E. S.; Komgold, R.; Huang, Z. *Cancer Res.* **2000**, *60*, 1498. *Procedure:* Fluorescence polarization ('FP') assays were developed using c-terminal 6XHIS tagged Bcl-2 (aa 1–204) and a C-terminal 6XHIS tagged Bcl-x_L (aa 1–209). The tracer was a synthetic BH-3 peptide (Bim) conjugated to Fluorescein isothiocyanate (FITC-DLRPEIRIAQELRRIGDEFNETYTRR). Dilutions of either Bcl-2 (1.3 nM) or Bcl-x_L (0.8 nM) were added to serial dilutions of antagonist and incubated for one hour prior to the addition of 2 nM of fluorescent peptide tracer (Anaspec, Fremont, CA) in assay buffer. Final assay buffer conditions were 20 mM HEPES, pH 7.5, 1 mM DTT, 0.005% Tween-20 and 50 mM NaCl. Samples were read after incubation for 20 min. Fluorescence polarization values were plotted as a function of antagonist concentration, and IC_{50}'s are the average of at least two experiments. Values of 10 nM or less are reported as <0.010 μM to account for assay limitations when using the above concentrations for very potent compounds.

13. Apoptosis was assessed using Promega's Caspase-Glo® 3/7 Assay Kit (Catalog#G8092, Madison, WI) following the manufacturer's directions. *Protocol:* RS4;11 cells were plated in NUNC white opaque 96-well plates (Catalog#353296, Rochester, NY) at 10,000 cells per well in Sigma's RPMI Media (Catalog #R7509, St. Louis, Mo) containing 10% FBS (Thermofisher Catalog#SH30396 Waltham, MA). After 16 h, cells were treated in duplicate with compound and incubated at 37 °C for 6 h prior to caspase detection. DMSO was used as minimum caspase activity control, while the addition of ABT-263 was used as a measure of maximum caspase activity. An equal volume of Caspase-Glo 3/7 reagent was added to each well and incubated for 1 h prior to reading on a Tecan Ultra luminometer (Tecan, Durham, NC) to quantitate luminescence. EC_{50}'s are the average of at least two experiments.
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17. These modifications preserved the similarities and differences of these two ligands, allowing rigid protein docking on a publicly available protein structure. *Method:* The crystal structure of a quinazoline ligand bound to Bcl-x_L (PDB code-3QKD) was chosen for docking analysis due to the similarity of the cognate ligand with **13** and **14**. The protein complex was downloaded from the protein data bank (www.rcsb.org) and prepared for docking using the protein preparation workflow in Maestro (Schrodinger LLC, Portland, OR). Ligands for docking were also prepared in Maestro using the OPLS2005/GBSA setting for ligand minimization. Pose validation studies with the cognate ligand were carried out in both Glide (Schrodinger LLC, Portland, OR) and Gold (CCDC, Cambridge, UK). Default settings failed to produce desirable results and multiple constraints were evaluated. Satisfactory results were obtained in Gold using four hydrophobic constraints defined based on the pose of the cognate ligand. The hydrophobic constraints were 2 A spheres defined around the centroids of the (a) phenyl ring connected to the thioether linkage, (b) phenyl ring with the nitro substitution, (c) piperidine ring and (d) the terminal phenyl ring of the biphenyl moiety. The automatic GA settings were used with 200% search efficiency and the chemscore scoring function was utilized for docking. Energetic analysis was performed using the Embrace module of Macromodel (Schrodinger LLC, Portland, OR) in the interaction energy mode using the OPLS2005 potential.
18. (a) The Bcl-2 residues corresponding to Bcl-x_L residues Phe97 and Ala104 are Phe101 and Asp108, respectively. Based on the observation of available Bcl-2 structures, the Asp108 residue lies in between two helices, a region which is highly plastic, reorganizing itself in response to ligand binding. In the *apo* form (PDB code-1GJH) the residue points into the pocket but shows side chain (PDB code-1YSW) and backbone movements (PDB code-2O2F)] away from the ligand in response to ligand binding. The acid residue in Bcl-2 may be more rigid and therefore causes greater energetic destabilization due to lone pair clashes between the acid and sulfoxide with the *S*-sulfoxide than the *R*-sulfoxide, leading to the >10 fold difference in binding affinity between Bcl-2 and Bcl-x_L.
(b) Energetic analysis of the protein–ligand complex showed that the ligand–protein interaction energy for **13B** is energetically more favorable (−1053.92 kJ/mol) compared to **14B** (−1002.79 kJ/mol). A similar trend was observed for the electrostatic component of the interaction energy with **13B** being more stable (−759.05 kJ/mol) than **14B** (−707.05 kJ/mol). The van der Waals interaction energy was more comparable for **13B** (−294.87 kJ/mol) and **14B** (−295.74 kJ/mol).
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21. A cell viability assay was used to determine the relative inhibition of BCL-2 and BCL-x_L test compounds. FDCP-1 cells (parental line from DSMZ) were engineered by retroviral infection to over-express either human BCL-2 or human BCL-x_L. Both these cell lines, as well as the parental, were maintained at 37 °C in RPMI/10% FBS supplemented with 5 ng/mL IL-3. Prior to compound treatment, cells were first washed in No IL-3 media (otherwise identical to media above), then incubated for 48 h w/o IL3, during which time the parental cells died out. The surviving FDCP-1 BCL-2 and FDCP-1 BCL-x_L cells were then counted, resuspended in fresh No IL-3 media, and plated at approximately 5–7 K cells/well in 384 w microplates containing the test compounds, including the DMSO control (untreated wells), in triplicate. The cells were then incubated for an additional 24 h at 37 °C. After this incubation, a matching volume of Cell Titer Glo reagent was added to each well, the plate shaken at RT for at least 15 min, and the luminescence read using a Tecan Ultra 384 (Magellan). IC_{50}'s were determined and reported as a percentage of surviving cells in the untreated control wells. Matching +IL3 controls were treated in the same fashion, but in IL3-replete media, to verify that the compounds had minimal to no effect under those conditions.
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