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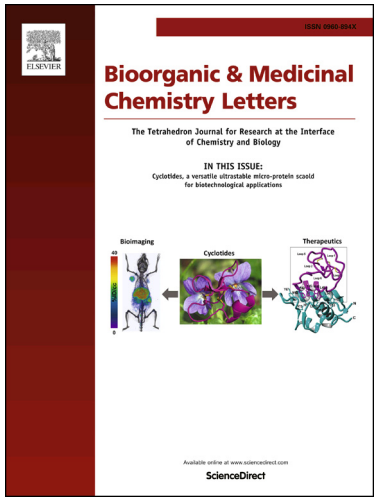

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Graphical Abstract

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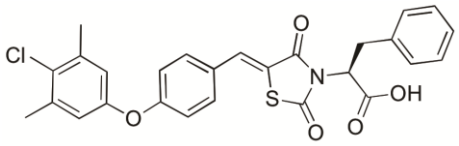

12b: K_i = 155 nM

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Discovery of Mcl-1 inhibitors based on a thiazolidine-2,4-dione scaffold

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ABSTRACT

Inspired by a rhodanine-based dual inhibitor of Bcl-x_L and Mcl-1, a focused library of analogues was prepared wherein the rhodanine core was replaced with a less promiscuous thiazolidine-2,4-dione scaffold. Compounds were initially evaluated for their abilities to inhibit Mcl-1. The most potent compound **12b** inhibited Mcl-1 with a K_i of 155 nM. Further investigation revealed comparable inhibition of Bcl-x_L (K_i = 90 nM), indicating that the dual inhibitory profile had been retained upon switching the heterocycle core.

The breakdown of apoptosis, a programmed cell-death mechanism, is one of the hallmarks of cancer and a crucial step in tumorigenesis.[1-3] Apoptosis is triggered by either cytotoxic stimuli, such as growth factor deprivation, UV and γ-irradiation, and stress (called the intrinsic or mitochondrial pathway) or by binding of an external ligand to a member of the tumor necrosis factor (TNF) receptor family (called the extrinsic or death receptor pathway). The intrinsic pathway is strictly regulated by the B-cell lymphoma 2 (Bcl-2) family of proteins,[4] which exerts its influence over cell fate primarily by interactions between three distinct subfamilies. These include the BH3-only family members such as Bad, Bid, Bim, Noxa, and Puma (death initiators), the pro-survival Bcl-2-like proteins such as Bcl-2 itself, Bcl-x_L, Bcl-w, and Mcl-1 (cell guardians), and the pro-apoptotic proteins such as Bax, Bak, and Bok (effector proteins). The Bcl-2 family of proteins is commonly described as a tripartite apoptotic switch.[5] External cytotoxic stress signals activate BH3-only members of the protein family and induce apoptosis by two distinct, but not mutually exclusive, mechanisms:[6-7] sequestration of the pro-survival Bcl-2 family proteins which liberates the pro-apoptotic proteins Bax and Bak[8] through their BH3 domains or by direct activation of Bax and Bak.[9] The pro-survival and pro-apoptotic Bcl-2 proteins neutralize each other's function to ensure apoptosis is tightly regulated. This is accomplished through the molecular recognition of the BH3 α-helix of pro-apoptotic proteins by hydrophobic grooves on the surfaces of the pro-survival proteins; specifically, conserved hydrophobic side chains along one face of the α-helix at positions *i*, *i*+3/4, *i*+7 and *i*+11 probe into sub-pockets p1 through p4. In addition, a conserved aspartate on the opposing face of the BH3 α-helix engages in a salt bridge interaction with a conserved arginine (Arg263 (Mcl-1), Arg139 (Bcl-x_L) and Arg143 (Bcl-2)).[10,11]

Irregularities in the mitochondrial apoptotic pathway are a root cause of cancer. The overexpression of Bcl-2-like proteins[12] or the loss of Bax or Bak function[13] contributes to tumorigenesis by boosting the survival of cells that would otherwise undergo apoptosis. In addition, defects in apoptosis contribute to the resistance of diverse tumors to cytotoxic drugs.[5] Consequently, small molecules disrupting the protein-protein interactions (PPIs) by binding to the hydrophobic cleft of Bcl-2-like survival proteins would be highly valuable as they are predicted to release pro-apoptotic proteins and restore normal levels of apoptosis.[10,11,14] Specifically, chemical agents that mimic the BH3 domains of the pro-apoptotic Bcl-2 family members might furnish novel antineoplastics, enhancing the chemical weaponry in the war on cancer.[15] In fact, this has been an intense area of research for over a decade.[11] Two of the best characterized BH3 mimetics are ABT-737 and its orally available derivative ABT-263 (navitoclax), reported by Abbott Laboratories through a combination of NMR-based screening and structure-based

design.[16,17] ABT-263 is a first-in-class Bcl-2 family inhibitor that restores the ability of cancer cells to undergo apoptosis.[17,18] It antagonizes the anti-apoptotic proteins Bcl-2, Bcl-x_L, and Bcl-w, thereby releasing Bax and Bak.[17,18] This selective inhibition of a subset of anti-apoptotic Bcl-2 protein family has produced encouraging clinical outcomes.[19] Indeed, the Bcl-2 selective inhibitor ABT-199[20] (venetoclax) recently received FDA approval for the treatment of patients with chronic lymphocytic leukemia who harbor a 17p chromosomal deletion.[21] Within the last few years, Mcl-1 selective inhibitors have begun to emerge,[22-28] and at least one clinical trial has been initiated.[29]

Many cancer cells are resistant to ABT-737 and ABT-263 due to existing high levels, or compensatory upregulation, of Mcl-1.[30,31] Downregulation of Mcl-1 by siRNA, miRNA, or Noxa upregulation overcomes this resistance and sensitizes cancer cells towards ABT-263.[32-33] This suggests that the dual inhibition of Mcl-1 and Bcl-2/Bcl-x_L/Bcl-w may be therapeutically more beneficial, either through engineering drugs with polypharmacological profiles, so-called "pan-Bcl-2" inhibitors,[34-40] or through adjuvant therapies. Thus, the expansion of small molecule chemical artillery to target single or multiple anti-apoptotic proteins involved in cancer progression continues to be an unmet medical need and is urgently required. Herein, we report the discovery of potent Mcl-1 inhibitors based on a thiazolidine-2,4-dione scaffold. Selected molecules were also evaluated for their abilities to inhibit Bcl-x_L.

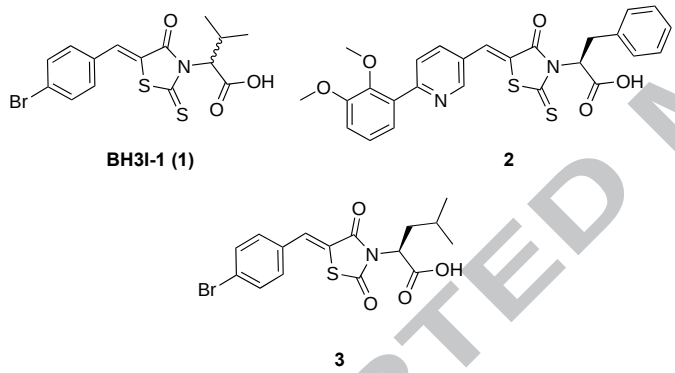


Figure 1: Lead rhodanine Mcl-1 inhibitors (**1** and **2**) and a novel thiazolidine-2,4-dione derivative (**3**).

Previously, Yuan's group screened a chemical library of 16,320 compounds and discovered the rhodanine derivative BH3I-1 (**1**, Figure 1) as an inhibitor of Bcl-x_L with a K_i of 2.4 μM.[41] In a subsequent report, Bernardo and colleagues described a library of analogues of BH3I-1 that are dual inhibitors of Bcl-x_L and Mcl-1, the most potent of which is **2** with K_i's of 3.7 μM (Bcl-x_L) and 7.6 μM (Mcl-1).[42] Rhodanines have gained notoriety as pan-assay interference compounds (PAINS), and are the amongst the worst offenders; to some degree, this has been attributed to their exocyclic sulfur atom that permits access to multiple hydrogen bonding interactions with limited restriction on geometries.[43] Towards the discovery of less promiscuous inhibitors of Mcl-1 (and Bcl-x_L), we considered adjusting the rhodanine core of BH3I-1 to a thiazolidine-2,4-dione (TZD), as exemplified by compound **3**. It should be noted that we are not necessarily condemning BH3I-1. However, devoid of an exocyclic sulfur atom, it was surmised that TZDs would begin with inherently improved selectivity profiles relative to the corresponding rhodanines, and thus represent improved leads for optimization, through access to fewer, and geometrically more restricted, hydrogen bonds. We enlisted the modeling package GOLD to determine a putative binding mode of **3** using Mcl-1 extracted from PDB ID: 4HW2.[24] One low-energy docked solution is presented in Figure 2. In the docking experiment, the only constraint was that the binding site was centered 10Å radius about the Nε of the guanidinium function of Arg263, which is near the middle of the BH3 binding crevice. As depicted, the carboxylic acid and one of the TZD ring carbonyls form electrostatic interactions with Arg263 (2.7 Å), recapitulating the salt bridge interaction between Arg263 and a conserved aspartic acid found on one face of the BH3 α-helices of pro-apoptotic Bcl-2 proteins. In addition, the 4-bromophenyl moiety points down into the hydrophobic p2 pocket, engaging in van der Waals interactions with various hydrophobic residues that include Val243, Leu246 and Met250. The *S*-isobutyl group of **3** appears to interact with the tetramethylene side chain of Arg263 and the methyl of Thr266. Interestingly, the docking solution suggests that groups larger than isobutyl of corresponding *R*-enantiomers may be able to engage in interactions with His224, Ala227 and Phe228. We hypothesized that expanding the bromophenyl moiety of **3** will afford more potent inhibitors owing to greater occupation of the p2 pockets of Mcl-1 and Bcl-x_L, as has been described previously.[11,17,24]

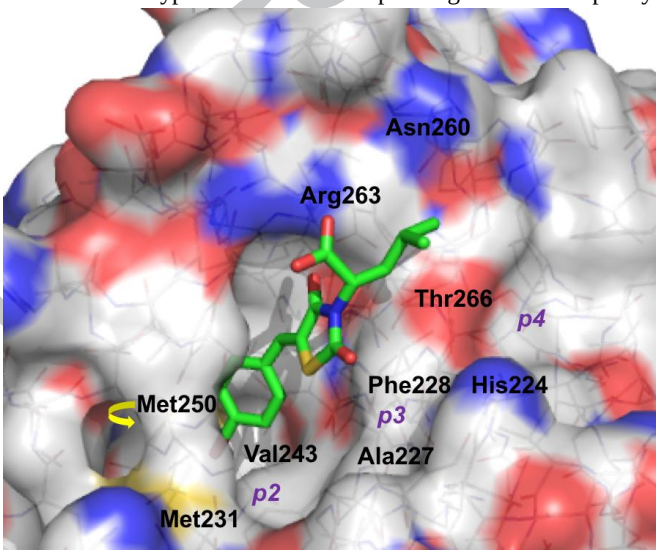
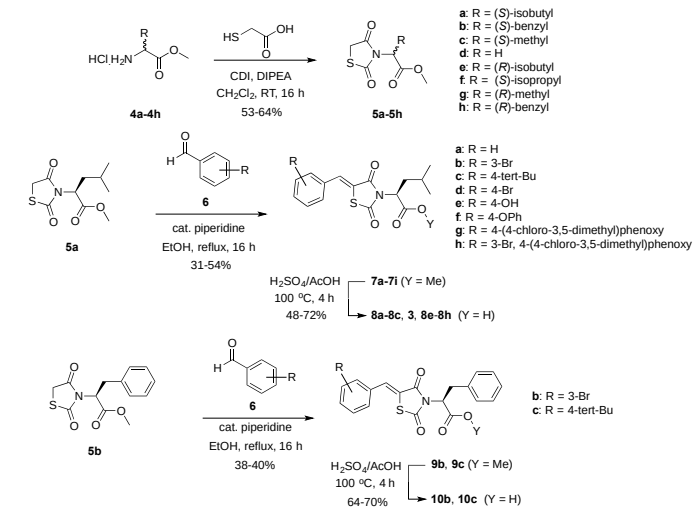
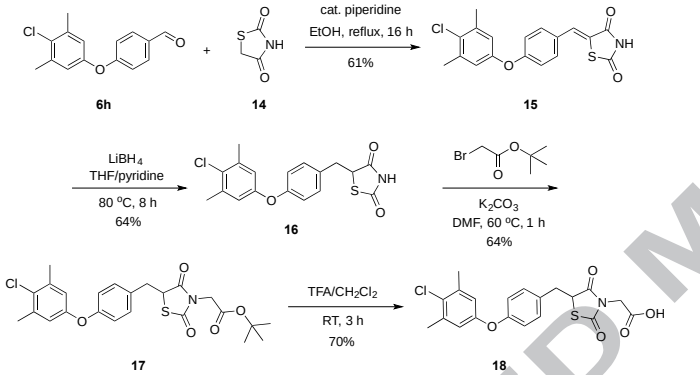


Figure 2: A low-energy GOLD docking solution of **3** with Mcl-1 extracted from PDB ID: 4HW2. Ligand and protein coloured by atom type. Black labels indicate Mcl-1 residues, purple labels describe sub-pockets within the BH3-binding crevice.

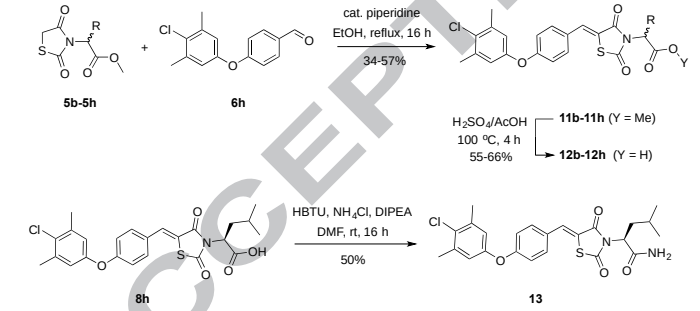
The TZD target molecules were synthesized according to a short and straightforward synthetic pathway (Schemes 1 and 2). Briefly, the methyl ester of the appropriate amino acid **4a**–**4h**, was condensed with thioglycolic acid in the presence of carbonyl-1,1-diimidazole (CDI) to construct the *N*-substituted TZD rings of compounds **5a**–**5h**. Knoevenagel condensations of compounds **5a**–**5h** with various aldehydes delivered compounds **7a**–**7h**, **9b**–**9c** and **11b**–**11i**. Finally, the methyl esters of **7a**–**7i**, **9b**–**9c** and **11b**–**11h** were hydrolyzed under acid catalyzed conditions to furnish the final molecules **8a**–**8c**, **3**, **8e**–**8h**, **10b**–**10c** and **12b**–**12h**, respectively. As depicted in Scheme 3, primary carboxamide **13** was generated through activation of carboxylic acid **8h** with HBTU followed by quenching with NH_4Cl. Deletion of the *N*-substitution of compounds **12b**–**12h** and reduction of the double bond of **12d** were accomplished through the *de novo* synthesis described in Scheme 3 to yield **15** and **18**, respectively.



Scheme 1



Scheme 2



Scheme 3

The abilities of our inhibitors to bind Mcl-1 were quantified by a fluorescence polarization competition assay (FPCA), as described by us previously.[28] Briefly, compounds were titrated into 96-well plates in order to compete with a fluorescently-labeled Bak-BH3 peptide for binding to Mcl-1. The IC_{50} values resulting from the changes in fluorescence polarization were then converted to K_i values using the Nikolovska-Coleska equation.[44] For further details, please see the Experimental Section.

First, as a point of reference the K_i for the binding of BH3I-1 to Mcl-1 was determined to be 44 μM using the previously reported IC_{50} data[42] and an online calculator[45] associated with reference 44. As indicated in Table 1, compound **8a** exhibited no inhibition of Mcl-1. However, the introduction of hydrophobic groups into the unsubstituted phenyl ring of compound **8a** resulted in improved inhibitory activity of Mcl-1, both in the *meta* and *para* positions, although a polar hydroxyl in the *para* position was not tolerated. Increasing the size of the peripheral aryl group of compounds **8f** to **8g** resulted in improved inhibition but the addition of a bromine atom into the internal phenyl ring (**8g**) resulted in a slight drop in activity. Substitution of the (*S*)-isobutyl groups of compounds **8b** and **8c** with (*S*)-benzyl groups generated inhibitors with greater activities. For example, homologue **10b** (K_i = 290 nM) was almost ten-fold more potent than **8b** (K_i = 2.13 μM). In the series of 4-(4-chloro-3,5-dimethylphenyl) derivatives **12b**–**12h**, the larger the *N*-substituent, the more active the compound, with **12b** the most potent of the series (K_i = 155 nM). Another observation is that in every case, *S*-enantiomers exhibited greater affinities than their *R*-counterparts, in contrast to our speculation with the docking result for **3**. For example, compound **12b** bound Mcl-1 five times more strongly than enantiomer **12h**, and **8g** was more than twice as active as enantiomer **12e**. Given that the chiral centre appeared to impact binding affinities, it was important to determine their optical integrities. To this end, we prepared diastereomeric amides of enantiomers **12b** and **12h** with enantiomerically-pure (*S*)-phenethylamine (1.1 eq of each of HBTU, HOAt, DIPEA in DMF, rt, 1 h). Analysis of the ^{1}H NMR spectra of the products indicated an approximate 2:1 inseparable mixture of two amides, suggesting 33% racemization; efforts will be made to obtain enantiomerically-pure compounds in a follow-up full paper so that the effect of the chiral centre can be more carefully scrutinized. Methyl ester **7g** and carboxamide **13** exhibited no binding affinities to Mcl-1, consistent with the requirement for a salt bridge interaction with Arg263. In line with this finding, complete removal of the *N*-substituent, as in **15**, also resulted in an inactive inhibitor. Finally, reduction of the double bond of **12d** yielded inhibitor **18** with a four-fold lower binding affinity, which may be ascribed to the generation of a more flexible inhibitor that suffered a greater entropic penalty upon binding. This finding is especially significant, since two features that may render molecules such as BH3I-1 promiscuous[43] have been modified or removed: the rhodanine core has been replaced, and the heterocycle is no longer aromatic.

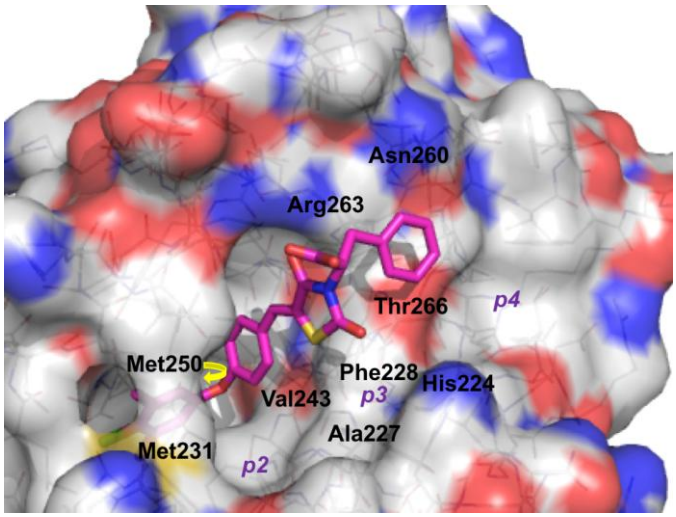


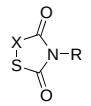
Figure 3: A low-energy GOLD docking solution of **12b** with Mcl-1 extracted from PDB ID: 4HW2. Ligand and protein coloured by atom type. Black labels indicate Mcl-1 residues, purple labels describe sub-pockets within the BH3-binding crevice.

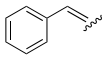
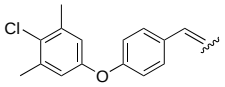
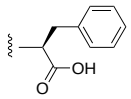
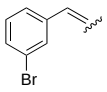
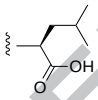
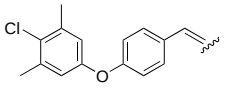
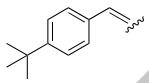
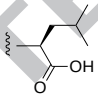
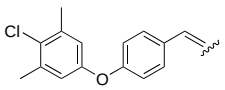
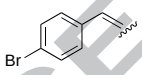
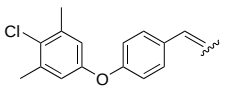
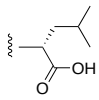
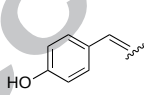
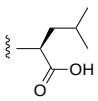
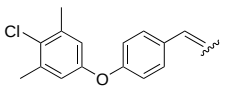
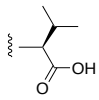
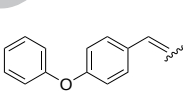
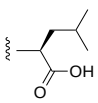
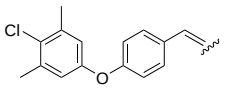
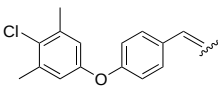
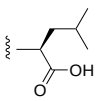
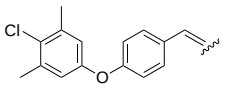
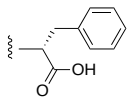
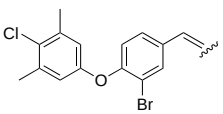
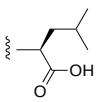
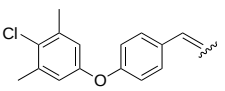
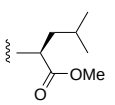
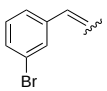
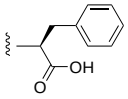
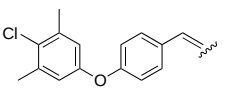
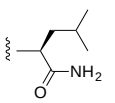
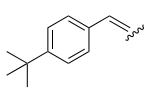
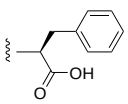
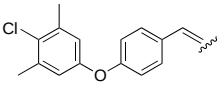
We then evaluated the binding affinities of two of the most potent compounds to Bcl-x_L; **8g** and **12b** bound with K_i values of 60 nM and 90 nM, respectively. These improved binding affinities to Bcl-x_L over Mcl-1 is mirrored by similar findings

with related rhodanine-based inhibitors, and suggests the core scaffold modulation has not adversely affected the dual inhibitory profiles of the original rhodanine lead.[42] GOLD docking studies were performed with **12b**, as shown in Figure 3, again with PDB ID: 4HW2. A semi-transparent surfacing has been utilized to indicate that the sizeable p2 pocket (deepest point Met250) is capable of accommodating the large 4-chloro-3,5-dimethylphenyl moiety). Although the salt bridge between the ligand's carboxylic acid and Arg263 is now much weaker (4.3 Å), GOLD is not capable of taking into account the flexibility of the protein. Finally, direct binding of our inhibitors to Mcl-1 was confirmed by an orthogonal technique. 2D ^{1}H-^{15}N heteronuclear single quantum coherence (HSQC) spectra of ^{15}N-Mcl-1 were collected in the absence (black) and presence (red) of **12b**, and overlaid in Figure 4. As illustrated, there were multiple chemical shift perturbations indicating that **12b** bound directly to Mcl-1. However, due to broad loss of peaks from intermediate chemical exchange, we cannot determine specifically where the compound is interacting.

Table 1: Mcl-1 inhibitory structure–activity relationships of substituted thiazolidine-2,4-diones.[a,b] [a]IC_{50} data from a fluorescence polarization competition assay with Mcl-$1^{172-371}$ and fluorescein isocyanate (FITC)-labeled Bak-BH3 peptide were converted to K_i values using the Nikolovska-Coleska equation.[44]

In summary, starting from the rhodanine-derived lead BH3I-1, we have developed a focused library of thiazolidine-2,4-dione-based dual inhibitors of Mcl-1 and Bcl-x_L. Compounds were readily accessed in three steps, and their inhibitory activities were initially evaluated against Mcl-1. In general, greater inhibition of Mcl-1 was realized when larger hydrophobic groups were substituted off the benzylidene aryl moiety. Likewise, large hydrophobic groups at the chiral carbon elicited more potent inhibition. The most potent compound, **12b**, inhibited Mcl-1 with a K_i of 155 nM. Further investigation revealed the dual inhibitory profile of the parent rhodanines had been retained as **12b** inhibited Bcl-x_L with a K_i of 90 nM. Rhodanine derivatives have become infamous as PAINS compounds. Herein, we have replaced the promiscuous scaffold with a reputably more selective thiazolidine-2,4-dione core,[43] and discovered compounds with affinities of more than an order of magnitude greater than the lead BH3I-1. Armed in the knowledge that switching the heterocyclic core is tolerated, further work is now



Compound	X	R	K_i (μM)	Compound	X	R	K_i (μM)
8a			>100	**12b**			0.155 ± 0.018
8b			2.13 ± 0.97	**12c**			1.94 ± 0.19
8c			2.22 ± 1.22	**12d**			2.34 ± 0.25
3			8.78 ± 3.27	**12e**			1.00 ± 0.11
8e			>100	**12f**			1.19 ± 0.08
8f			12.7 ± 2.9	**12g**			2.78 ± 0.30
8g			0.419 ± 0.053	**12h**			0.878 ± 0.088
8h			0.791 ± 0.125	**7g**			>100
10b			0.290 ± 0.054	**13**			>100
10c			0.845 ± 0.193	**15**			>100
				18			10.2 ± 3.6

underway to focus on a more elaborate structure–activity study, particularly with expansion of the *N*-substituent in order to make greater contacts with both proteins.

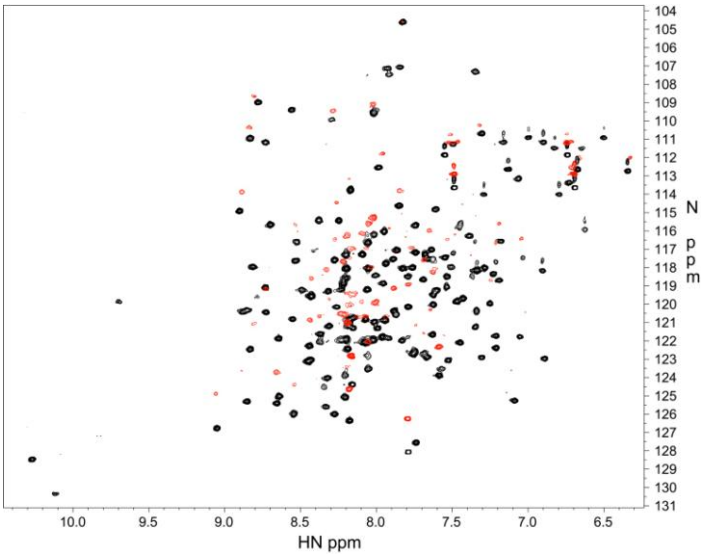


Figure 4: 2D ^{1}H-^{15}N HSQC NMR spectra overlay of apo-Mcl-1 (black) and **12b**-bound Mcl-1 (red). The NMR samples contained 131 mM ^{15}N-labeled Mcl-1, (182 mM **12b**), 20 mM HEPES, pH 6.8, 50 mM NaCl, 3 mM dithiothreitol, 20% D_2O, and 5% D_6-DMSO.

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Supplementary Material

Complete experimental procedures, and ^{1}H and ^{13}C NMR spectra of all final molecules.