Center Probe Report

Project Title: Chemical inhibitors of anti-apoptotic protein Bfl-1
Grant Number: 1 X01 MH077632-01

Screening Center Name: San Diego Center for Chemical Genomics

Principal Investigator of Screening Center: Dr John C. Reed

Assay Provider & Institution: John C. Reed, Burnham Institute for Medical Research

Assay or Pathway Target: Apoptotic protein Bfl-1

Probe PubChem Compound Identifier (CID/SID): CID-2980973; SID-4265380 (sulfonylpyrimidine series).

Assay provider information

Specific Aim:

The aim of this project is to identify chemical probes of Bfl-1 through a fluorescence polarization assay (FPA) using FITC-Bid BH3 peptide.

Significance:

The Bcl-2 family proteins play a crucial role in tissue homeostasis and apoptosis (programmed cell death) and are therefore potential therapeutic targets for diseases, such as cancer.

Rationale:

Apoptosis is governed in part by B-cell lymphoma-2 (Bcl-2)-family proteins. The human genome contains six genes that encode anti-apoptotic members of the Bcl-2 family of which Bfl-1 is a member [1-4]. Other Bcl-2-family anti-apoptotic proteins include Bcl-2, Bcl-XL, Mcl-1, Bcl-W, and Bcl-B. The mouse ortholog of Bfl-1 has 4 gene copies (A1a-d). Bfl-1 interacts with other Bcl-2 family members, such as the pro-apoptotic BH3-interacting domain death agonist (BID). Bfl-1 plays a crucial role in tissue homeostasis and apoptosis (programmed cell death) *via* an intrinsic (mitochondrial) pathway and is therefore a potential therapeutic target for diseases, such as cancer, in which resistance to apoptosis is implicated.

Screening center information

<u>Assay Implementation and Screening</u>

PubChem Bioassay Name: HTS discovery of chemical inhibitors of anti-apoptotic protein Bfl-1

List of PubChem bioassay identifiers generated for this screening project (AIDS): 432, 621.

List of relevant AIDs that may be used as counterscreen information: 748.

Primary Assay Description as defined in PubChem:

<u>Protocol</u>

Bfl-1 assay materials:
1) Bfl-1 protein and FITC-Bid peptide (FITC-Ahx-EDIIRNIARHLAQVGDSMDR) were obtained from Prof. John Reed (Burnham Institute for Medical Research, San Diego, CA)

2) Assay Buffer: 25 mM Bis-Tris, pH 7.0, 1 mM TCEP, 0.005% Tween 20.
3) Bfl-1 working solution contained 7.4 nM Bfl-1 in assay buffer. Solution was prepared fresh and kept on ice prior to use.
4) FITC-Bid working solution contained 5.6 nM FITC-Bid in assay buffer.

Bfl-1 HTS protocol:
1) 4 uL of 100 uM compounds in 10% DMSO were dispensed in columns 3-24 of Greiner 384-well black small-volume plates (784076). Columns 1 and 2 were added with 4 uL of 10% DMSO.
2) Positive control wells, that contained no Bfl-1, were assigned to column 1 and were added 8 uL of assay buffer using WellMate bulk dispenser (Matrix).
3) 8 uL of Bfl-1 working solution was added to columns 2-24 using WellMate bulk dispenser (Matrix). Negative control wells that contained no compounds were assigned to column 2.
4) Plates were incubated for 1h at +4ºC.
5) 8 uL of freshly prepared FITC-Bid working solution was added to the whole plate using WellMate bulk dispenser (Matrix).
6) Final concentrations of the components in the assay were as follows:
a.20 mM Bis-Tris-HCl, pH 7.0, 0.8 mM TCEP, 0.004% Tween 20.
b.2.2 nM FITC-Bid (columns 1-24)
c.3.0 nM Bfl-1 (columns 2-24)
d.2% DMSO (columns 1-24)
e.20 uM compounds (columns 3-24)
7) Plates were incubated for 4h at room temperature protected from direct light.
8) Fluorescence polarization was measured on an Analyst HT plate reader (Molecular Devices, Inc) using fluorescein filters: excitation filter - 485 nM, emission filter 530 nM, dichroic mirror # 505 nM. The signal for each well was acquired for 100 ms.
9) Data analysis was performed using CBIS software (ChemInnovations, Inc).
10) Fluorescence intensity of each sample was normalized to the average fluorescence intensity value of the plate negative control wells to calculate F_ratio parameter.

Bfl-1 dose-response confirmation protocol:
1) Dose-response curves contained 10 concentrations of compounds obtained using 2-fold serial dilution. Compounds were serially diluted in 100% DMSO, and then diluted with water to 10% final DMSO concentration. 4 uL compounds in 10% DMSO were transferred into columns 3-22 of Greiner 384-well black small-volume plates (784076). Columns 1-2 and 23-24 contained 4 uL of 10% DMSO.
2) Columns 1-2 were reserved for positive controls and added with 8 uL of assay buffer using WellMate bulk dispenser (Matrix).
3) 8 uL of Bfl-1 working solution was added to columns 2-24 using WellMate bulk dispenser (Matrix). Columns 23-24 represent negative control wells.
4) Plates were incubated for 1h at +4ºC.
5) 8 uL of freshly prepared FITC-Bid working solution was added to the whole plate using WellMate bulk dispenser (Matrix).
6) Plates were incubated for 4h at room temperature protected from direct light.
7) Fluorescence polarization was measured on an Analyst HT plate reader (Molecular Devices, Inc) using fluorescein filters: excitation filter - 485 nm, emission filter - 530 nm, dichroic mirror - 505 nm. The signal for each well

was acquired for 100 ms.
8) Data analysis was performed using sigmoidal dose-response equation through non-linear regression.

Center Summary of the Primary Screen and Confirmatory Screen:

The fluorescence polarization (FP) assay, using a Bfl-1-binding synthetic peptide conjugated with FITC, was developed by Dr. Dayong Zhai in Prof. John Reed's lab (BIMR). The original assay was performed in 96-well plates, and demonstrated suboptimal behavior during its validation in 384-well plates at the San Diego Center for Chemical Genomics (SDCCG). The assay in its original configuration demonstrated a low signal window and was unstable over time (50% decrease of signal in less than 30 min). This constrained the assay provider to opt for the Bfl-1 screening to be performed at a 3-fold concentration above the dissociation constant of the Bfl-1/Bid-peptide complex. At this concentration one would expect a significant decrease in the assay sensitivity for compound inhibition.

The assay was optimized at the SDCCG to correct the above deficiencies. The FP assay conditions were modified to achieve a 30% increase in the assay window (3-fold relative to the K_d) which is a 33-fold total protein concentration reduction, and an improvement in the signal stability from 15 min to 24 h. The new concentration of Bfl-1 now equals the dissociation constant of its complex with the fluorescent probe. This resulted in a decrease in the assay protein requirements and therefore cost, and also an improved sensitivity which should lead to a higher number of compounds identified in the screen. Another issue observed during assay optimization was a time-dependent Bfl-1 binding to the fluorescent probe. To compensate for this phenomenon we reformatted the assay to include a 1h incubation of Bfl-1 in the presence of compounds and 4h incubation after the subsequent addition of the fluorescent probe. As expected, this was facilitated by the signal stabilization achieved at the assay development stage. The resulting assay was robust and characterized with a Z'-factor equal to 0.75. The assay was screened against the entire NIH small molecule library which at the time consisted of ~66,000 compounds.

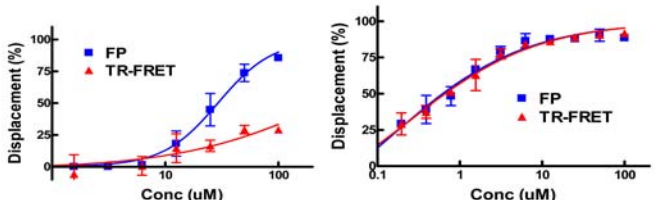


Figure 1. Exemplary Bfl-1 compounds in the primary and secondary assays.

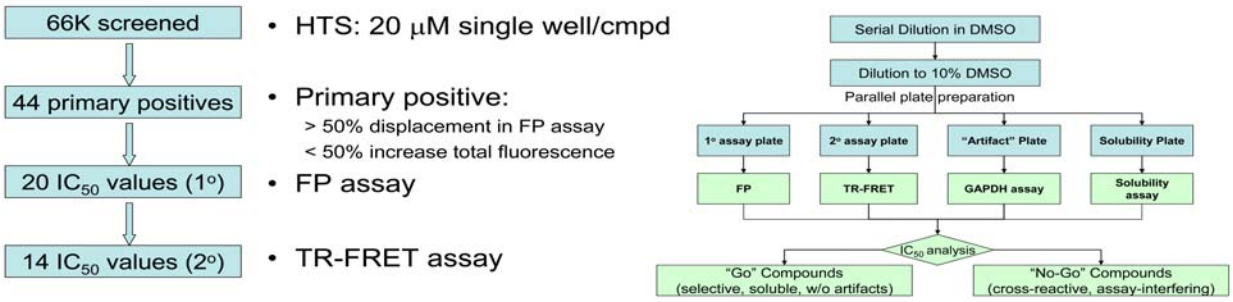
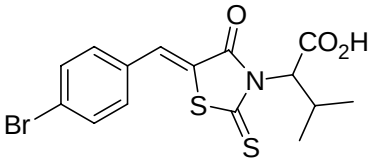


Figure 2. Flow chart for Bfl-1 screening and dose-response workflow.

Several new secondary assays were developed at the SDCCG to support hit confirmation and the hit-to-probe optimization process. A TR-FRET secondary assay utilized the same molecular interaction between the FITC-peptide and GST-tagged Bfl-1. Out of 20 compounds confirmed active in the primary screen, 14 demonstrated activity in the TR-FRET assay (see examples in figure 1). This assay was utilized as part of the routine dose-response panel for Bfl-1 hits (figure 2). Additional assays were developed for four other Bcl-2 family proteins and an additional FP assay using Bfl-1 with a different fluorescent peptide. A secondary HCS assay was also developed for chemical probe characterization based on the detection of intracellular caspase-9 activation characteristic of the mitochondrial (intrinsic) apoptotic pathway.

Comparative data on probe, similar compound structures and information on existing probes available to the public:

(Z)-2-(5-(4-Bromobenzylidene)-4-oxo-2-thioxothiazolidin-3-yl)-3-methylbutanoic acid (BH3I-1, see Figure 3) is a commercially available small molecule inhibitor of the Bcl-2 family proteins. As shown in figure 3, it possesses micromolar affinity for Bfl-1, Bcl-2, and Bcl-B in addition to others as previously reported (see reference [5] for details). In the assay utilized by the screening center which was performed in the presence of 0.005% Tween 20, the affinity of the compound in Bfl-1 assay was significantly lower (IC_{50} >> 20 uM). Thus, BH3I-1 is at best a modestly potent, non-selective tool.



BH3I-1

Bfl-1 (FP): IC_{50} = 4.65 μM
Bcl-2 (FP): IC_{50} = 1.14 μM
Bcl-B (FP): IC_{50} = 1.08 μM

Figure 3. Structure and *in vitro* assay data on BH3I-1, an inhibitor of Bcl-2 family

Probe Optimization

Chemical name of probe compound: ethyl 2-(4-(4-fluorophenyl)-6-(trifluoromethyl)pyrimidin-2-ylsulfonyl)acetate.

Draw probe chemical structure and show stereochemistry if known:

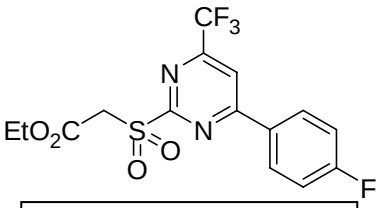


Figure 4. Structure of CID-2980973.

Describe mode of action for biological activity of probe: The probe binds reversibly and occupies the Bid BH3 binding site of the Bfl-1 protein. Thus it is expected to disrupt binding with tBid and potentially other BH3 containing proteins. Sulfonylpyrimidine hits appear as positives in multiple cytotoxicity assays deposited in *PubChem*. In our own HT1080 cytotoxicity assay (AID 620) performed as a secondary assay for the MT1-MMP project we also observed cytotoxicity of several sulfonylpyrimidine compounds (figure 5).

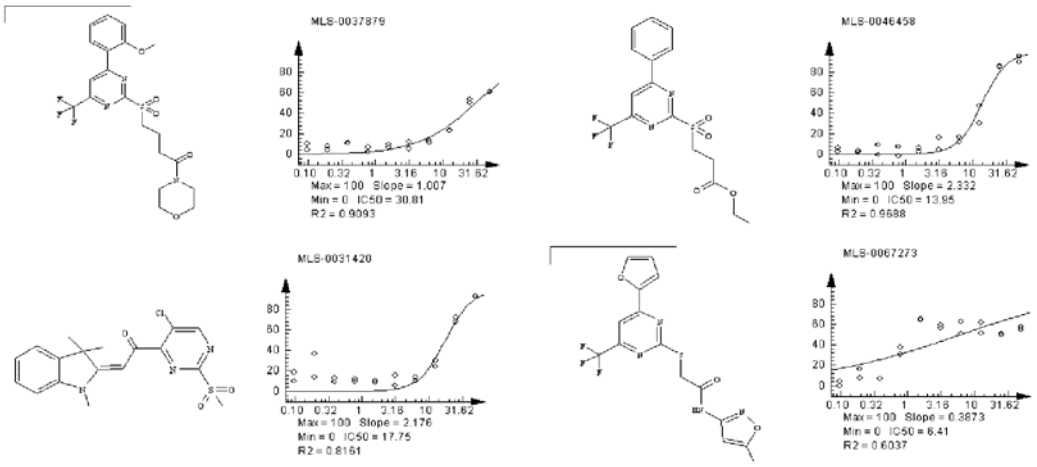


Figure 5 Cytotoxic effects of several sulfonylpyrimidines in the HT1080 assay. HT1080 cells were cultured in microtiter wells with various concentrations of compounds for 24 hrs and cell death was assessed by Alamar Blue staining, reporting data as % cytotoxicity.

To determine if the sulfonylpyrimidine compounds exert their cytotoxic effect via the intrinsic (mitochondrial) pathway for apoptosis, caspase-9 activity assays were developed and performed. Caspase-9 activation occurs in early stages of apoptosis following Cyt C release from mitochondria. We therefore developed a cell-based assay for caspase-9 activation. This assay is based on utilization of FITC-labeled caspase-9 specific irreversible inhibitor FITC-LEHD-FMK for intracellular detection of the activated enzyme. We have optimized the conditions for the detection of caspase-9 in HeLa cell line including the cell density and time of exposure to the compounds. In this assay we tested the sulfonylpyrimidine CID-2980973 compound and demonstrated its pro-apoptotic effect in the HeLa cells (figure 6, next page). As shown in figure 6 the cells that were treated with the Bfl-1 inhibitor exhibit intensely bright FITC staining (green channel) indicating the level of active caspase-9 in the cell. The cells also have brighter nuclear staining with DAPI (blue channel) consistent with chromatin condensation during apoptosis. The images were analyzed using cell count readout.

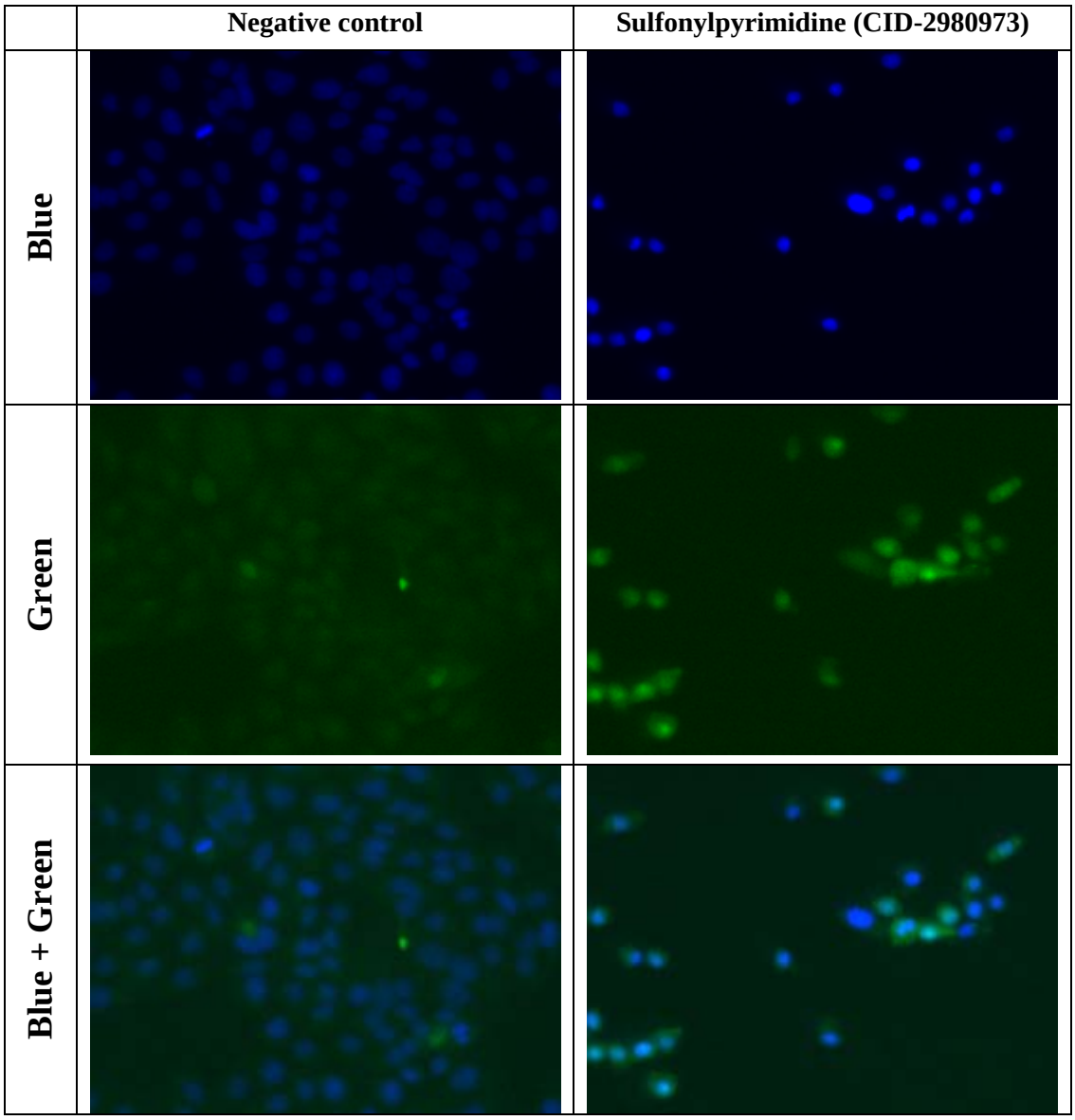
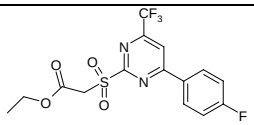
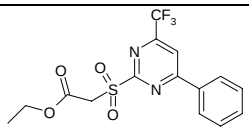
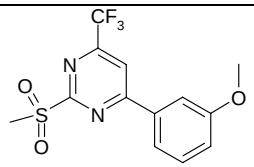
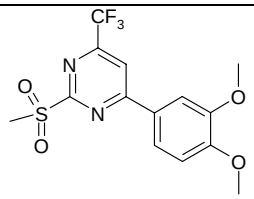
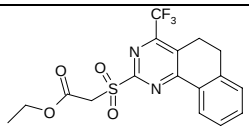


Figure 6 Caspase-9 activation assay test for probe CID-2980973. HeLa cells were treated with the compounds, then FITC-LEHD-fmk was added and left overnight, cells were then fixed, stained with DAPI, and imaged on IC100 microscope.

Has this compound been provided to the MLSMR: Yes.

Commercial vendor information if available for probe or analogs that have been purchased:

Structure	SDCCG ID	Vendor	Vendor ID	PubChem CID	PubChem SID
	MLS-0047123	ChemBridge	7979975	2980973	26514105
	MLS-0009480	ChemBridge	7663066	1151859	26514108
	MLS-0066988	ChemBridge	9004100	751044	26514110
	MLS-0051609	ChemDiv	Not available	3244140	26514115
	MLS-0066989	Asinex	BAS 03153297	1151895	26514118

Description of any secondary screens used to optimize probe structure:
In the course of this work numerous secondary assays were utilized to select the most promising chemical series and to perform SAR studies on the scaffold. The results from some of the assays performed on CID-2980973 are shown in figure 7. A TR-FRET assay was used to confirm the displacement of FITC-Bid BH3 peptide from its complex with Bfl-1. Since the TR-FRET assay is based on the same binding event between the Bid peptide and the Bfl-1 protein, the assay provided an alternative read-out and ensured that the observed compound effect does not result from the signal interference. This assay was an important part of the "go/no go" decision process for initiating the chemistry follow-up studies on the scaffold. It also provided additional confirmation throughout our routine SAR studies.

For the additional characterization of Bfl-1 compounds, we developed a

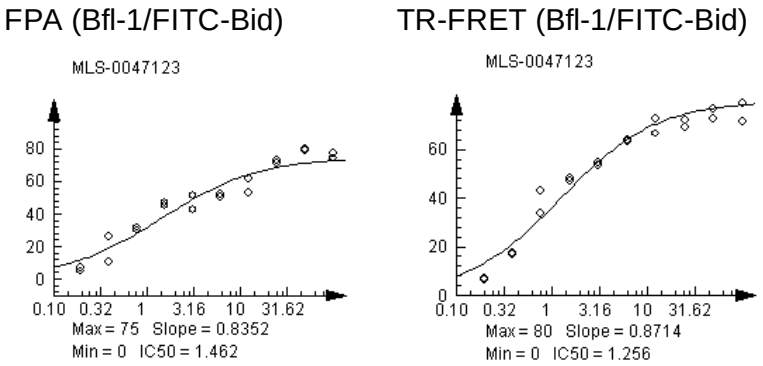


Figure 7. Effect of CID-2980973 in primary and secondary Bfl-1 assays. Units for Y-axes are % displacement.

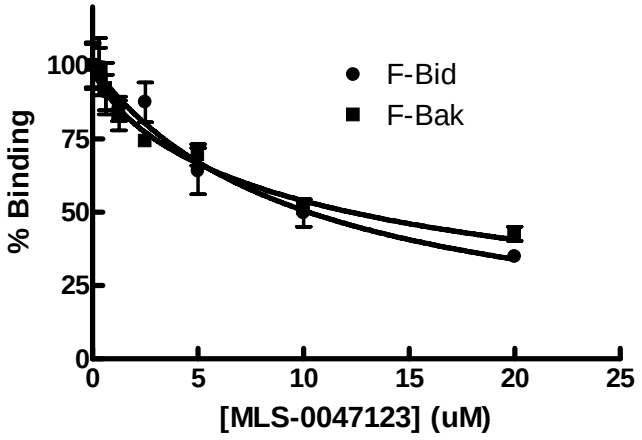


Figure 8. CID-2980973 in secondary fluorescence polarization assays using different binding partners for Bfl-1.

selectivity panel which included Bcl-2 and Bcl-B.

In addition, a fluorescence polarization assay using Bfl-1 and FITC-Bak BH3 peptide was developed. It was observed that CID-2980973 is equally potent in the assay employing both FITC-Bid and FITC-Bak BH3 peptide (figure 8), confirming that the compound is binding in the BH3 binding site.

Final confirmation of the pro-apoptotic effects of CID-2980973 was achieved in the HeLa cell caspase-9 activation assay described above.

Center comments on chemistry strategy leading to probe identification:

Bfl-1 Sulfonylpyrimidine Series. A sulfonylpyrimidine derivative (CID-1151859, Table 2) was identified as a hit in HTS as an inhibitor of Bfl-1. CID-1151859 was confirmed to posess micromolar potency in both the FP and TR-FRET assays. The synthetic chemistry employed to follow up on CID-1151859 is shown in Scheme 1 below. Thus, 1,3-diketones were reacted with S-substituted carbamimidothioates to afford the corresponding S-alkyl pyrimidines. Oxidation of these products at 25º C with mCPBA furnishes the desired sulfonylpyrimidine analogues. We synthesized and tested a series of compounds and *in vitro* data were generated to provide SAR data on the hit series. Initial studies focused on analogues in which the phenyl ring and/or the sulfone substituent were modified and some of these compounds are shown in Table 2. For example, a 3-methoxy substituent is tolerated (MLS-0066988) while a 3-, 4-dimethoxy derivative (MLS-0051609) is less potent than the parent molecule. It also appears that replacement of the phenyl ring with a heteroaryl moiety such as furan (MLS-0050257) or thiophene (MLS-0041503 and MLS-0051774) is viable. Truncating the sulfonyl side chain to a methylsulfone moiety as in MLS-0066988, MLS-0050257, MLS-0051609, and MLS-0051774 appears to lead to a modest reduction in potency compared with the lead compound. Homologation of the carbethoxymethylene side chain to the carbethoxyethylene variant (MLS-0046458) leads to a slight reduction in potency. More pronounced increases in side chain length, as in compounds MLS-0041503, MLS-0039077, MLS-0050749, and MLS-0008909 leads to further reduction in potency. Ultimately we selected the 4-fluorophenyl derivative CID-2980973, which is twice as potent as CID-1151859, as the Bfl-1 sulfonylpyrimidine series probe.

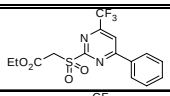
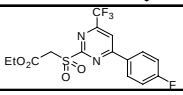
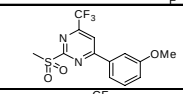
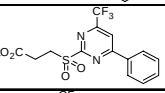
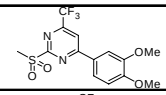
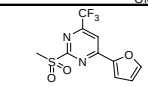
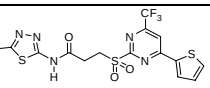
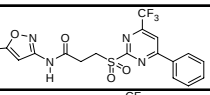
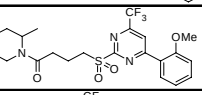
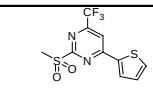
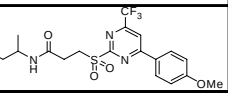
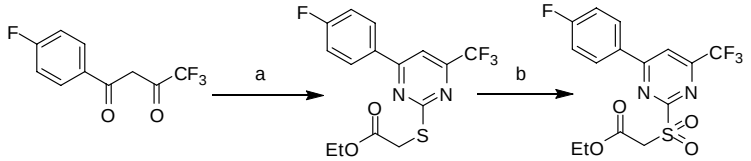
		Bfl-1 FP	Bfl-1 TR FRET
Compound #	**Structure**	**IC_{50} (uM)**	**IC_{50} (uM)**
CID-1151859 HTS Hit		4.6	2.9
CID-2980973 Probe		2.6	1.8
MLS-0066988		6.8	2.2
MLS-0046458		7.9	4.0
MLS-0051609		25.1	6.4
MLS-0050257		7.2	6.0
MLS-0041503		11.3	5.3
MLS-0039077		14.6	9.2
MLS-0050749		14.7	12.8
MLS-0051774		6.3	15.6
MLS-0008909		20.6	19.6

Table 2. Data on selected analogues of CID-1151859.

Reduced to practice detailed synthetic pathway for making probe



Scheme 1. Reagents and conditions: (a) ethyl 2-(carbamimidoylthio)acetate, EtOH, 100°C, 5h, 68%; (b) mCPBA, CH_2Cl_2, rt, 20h, 82%.

Known probe properties:
In vitro ADME/T profiling showed that CID-2980973 has a metabolic half life of 22 min in the presence of rat liver microsomes when incubated at 37.5^{o}C (Chart 1). The plasma stability of the compound is good, with 69% remaining after incubating at 37.5^{o}C for 1 hour in fresh rat plasma (Chart 2). The compound shows medium to low permeability in the PAMPA assay, with a log P_e of -5.1

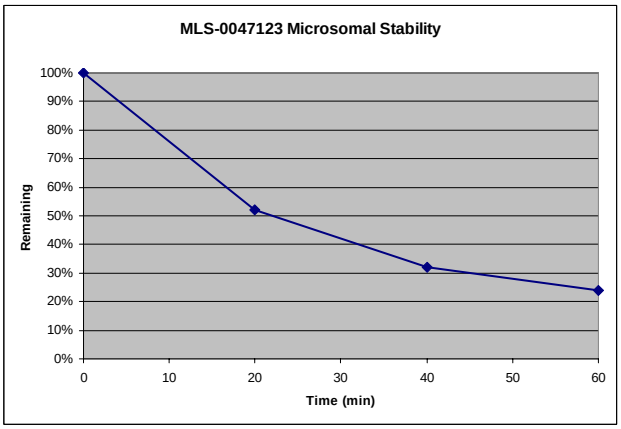


Chart 1. Probe Microsomal Stability, showing $t_{1/2}$ of 22 min.

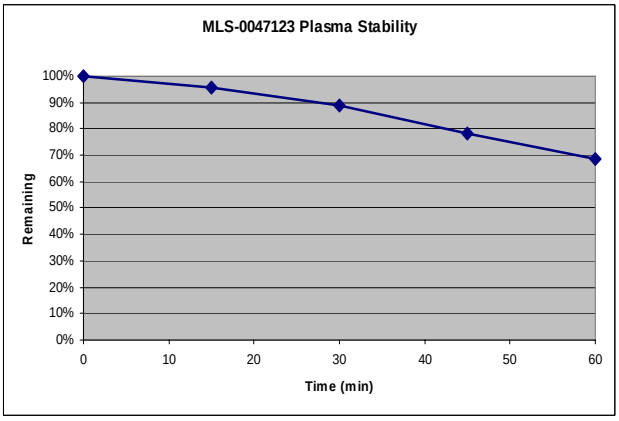


Chart 2. Probe Plasma Stability, 69% of compound remains at 1 h.

Center summary of probe properties (solubility, absorbance/fluorescence, reactivity, toxicity, etc.) and recommendations for the scientific use of probe as research tool: The compound demonstrated solubility at or below 100 uM in the biochemical assay. The compound is active in the biological studies described above, and could be recommended as a valuable tool to study the biology of Bfl-1.

Appendices

Comparative data showing probe specificity for target or for cell-based/phenotypic assays data should be shown in orthogonal cell-based assay systems that address the pathway of interest:

Bfl-1 and other Bcl-2 family proteins, participates in a very complex and intricate network of interactions with other representatives of the family. We developed two selectivity assays involving Bcl-2 and Bcl-B family members. CID-2980973 was inactive in the Bcl-B assay. This observation is expected, since Tr3 binds to Bcl-B at a site that is distinct from the BH3-binding peptide site. On the other hand, the compound was active in disrupting the Bcl-2/FITC-Bim BH3 peptide complex albeit with 5-fold lower potency than in the Bfl-1 assay (Figure 9), thus showing some selectivity.

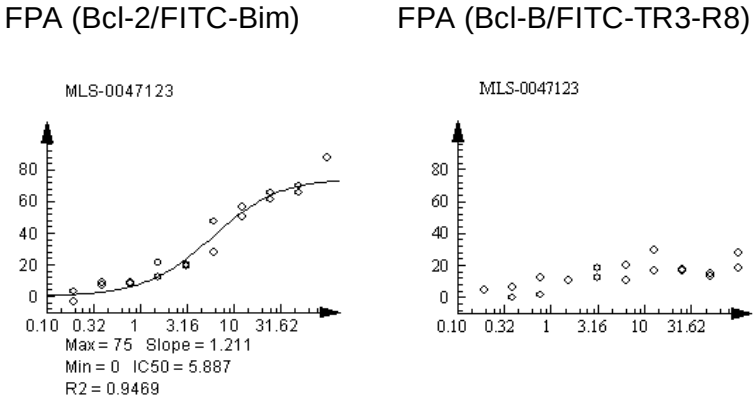


Figure 9. Effect of CID-2980973 in apoptotic protein selectivity assays. Units for Y-axes are % displacement.

The inhibition of Bcl-2 may have an important implication. Bfl-1 could be (at least, partially) substituted by other Bcl-2 family members in its biological functions, *i.e.* inhibiting just Bfl-1 may not be enough for a pro-apoptotic commitment of the cell with multiple members of anti-apoptotic Bcl-2 family members. Therefore, having probes with different selectivity profiles is important for characterization of different Bcl-2 proteins in biological systems. It is also known that Bcl-2-family members interact with multiple pro-apoptotic Bcl-2-family members potentially through the same BH3-domain binding site. This would mean that the same compound is expected to displace Bfl-1 from complex with diverse binding counterparts. To test this phenomenon, we developed a Bfl-1 assay using another BH3 peptide, FITC-Bak. As described above (see figure 8), a sulfonylpyrimidine compound displaced both FITC-Bid and FITC-Bak from their complex with Bfl-1.

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