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-779754; SID-24810089, SID: 3332609, SID: 26514158, SID: 1008291, SID: 8214517, SID: 8043994, SID: 6898290, SID: 45153601, SID: 3962106; MLS-0067130 (maleimide 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) and is therefore a potential therapeutic target for diseases, such as cancer, in which resistance to apoptosis is implicated.

Screening center information

Assay Implementation and Screening

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:

Protocol

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 that should lead to a higher number of compounds identified in the screen. Another issue that was observed during assay optimization was 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 a 4h incubation after the subsequent addition of the fluorescent probe. Needless to say, this was enabled 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.

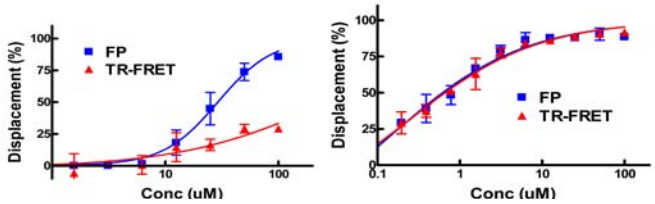


Figure 1. Exemplary Bfl-1 compounds in the primary and secondary assays.

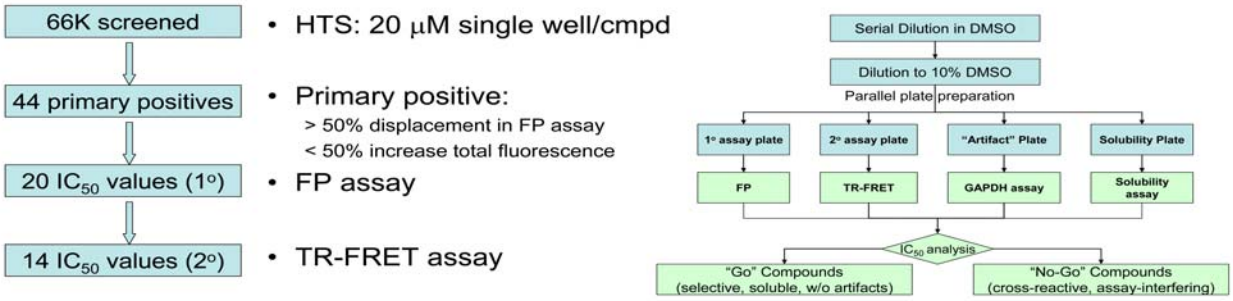
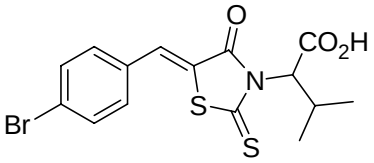


Figure 2. Flow chart for Bfl-1 screening and dose-response workflow.

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 as well as an 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 and 1 mM TCEP, 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 proteins.

Probe Optimization

Chemical name of probe compound: 3-chloro-1-(3,4-dichlorophenyl)-4-(dimethylamino)-1H-pyrrole-2,5-dione.

Draw probe chemical structure and show stereochemistry if known:

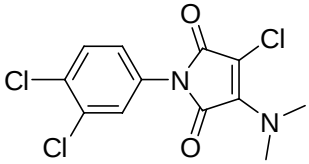


Figure 4. Structure of CID-779754.

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.

To validate the biological activity of the probe in a cellular readout that is Bfl-1 dependent, CID-779754 was tested in an assay measuring the effect of compounds on primary mast cell survival (see Appendix 2 for details). In these studies it was found that CID-779754 inhibited the activation-induced survival of human mast cells, but that the compound is not toxic to normal human cells.

Has this compound been provided to the MLSMR: The compound was recently included in the MLSMR collection as CID-779754.

Commercial vendor information if available for probe or analogs that have been purchased: The structures and compound identifiers for purchased chloromaleimide derivatives are shown in Table 1.

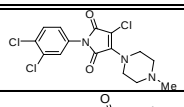
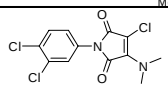
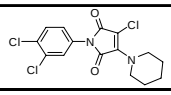
CID #	Structure	SID #	Vendor	Cat. No.	MLS #
1180676		26514112	Chembridge	6500959	0053105
779754		3962106	Chembridge	5354778	0067130
1180675		26514119	Chembridge	5344200	0066990

Table 1. Structures and compound identifiers for commercially available chloromaleimide derivatives.

Description of any secondary screens used to optimize probe structure: The secondary assays were described above.

Known probe properties: The compound has a metabolic half life of >60 min in the presence of rat liver microsomes when incubated at 37.5^{o}C (Chart 1), with 64% of the compound remaining at 1 hour. The plasma stability of the compound is also good, with 79% remaining after incubating at 37.5^{o}C for 1 hour in fresh rat plasma (Chart 2). The compound shows high permeability in the PAMPA assay, with a log P_e of -4.2, which correlates to a predicted human GI absorbtion of >50%.

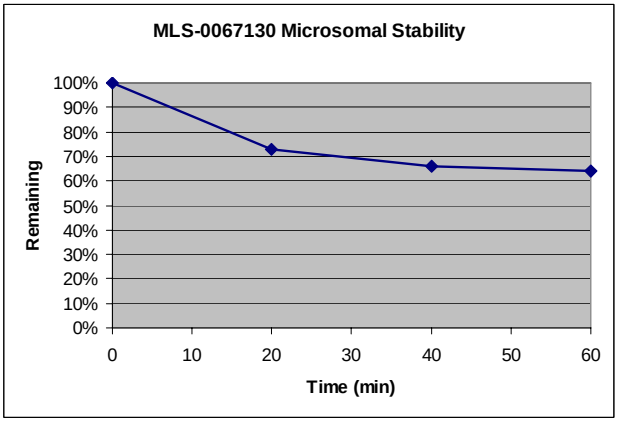


Chart 1. Probe Microsomal Stability, showing $t_{1/2}$ of >60 min.

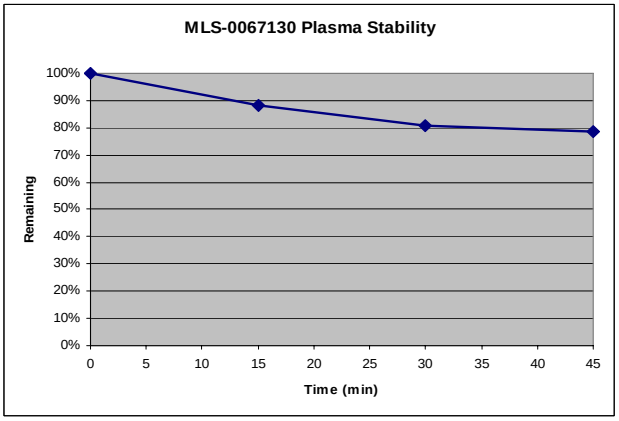


Chart 2. Probe Plasma Stability, 79% of compound remains after 1

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. It has some fluorescence in fluorescein channel at concentrations above 12.5 uM.

The compound is active in the biological studies described above and in the appendices, and is recommended as a valuable tool to study the biology of Bfl-1.

Center comments on chemistry strategy leading to probe identification:

Chloromaleimide Series. The original hit (CID-1180676) was confirmed to posess submicromolar potency in both the FP and TR-FRET assays (Table 3.1). It also appears to be highly selective, being inactive in 144 out of 151 assays tested as recorded in PubChem. Analogues of this compound were synthesized according to the synthetic pathway shown in Scheme 1 in the next section. Thus, substituted anilines **1** are reacted with dichloromaleic anhydride in acetic acid at reflux to furnish the corresponding dichloromaleimides **2**. Reaction of these products with various amines affords substitution of one of the chlorines to furnish the product aminomaleimides **3**. Using this methodology we prepared 280 analogues of CID-1180676 in order to optimize potency and selectivity for Bfl-1.

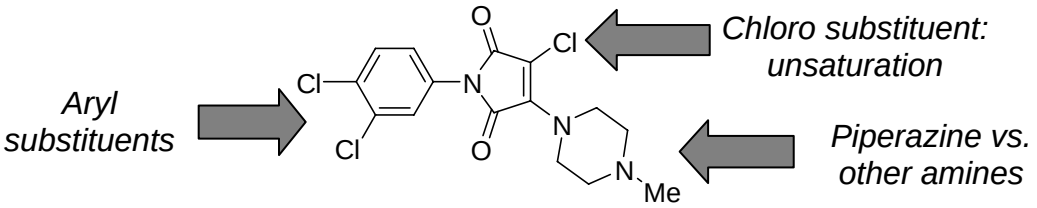


Figure 5. Summary of approach to chloromaleimide SAR.

Our approach to exploring the SAR around CID-1180676 is summarized in Figure 5. We defined three structural elements in the molecule to be investigated in the initial phase: (a) the maleimide chloro substituent and carbon-carbon double bond; (b) the substituents on the phenyl ring; and (c) the piperazine moiety. Therefore, we focused on these regions of the molecule and our efforts to date are described herein.

We identified two commercially available analogues, MLS-0066991 and MLS-0066987, (Figure 6) that test the importance of the maleimide chloro substituent and carbon-carbon double bond. Thus, MLS-0066991 lacks both the halogen and the unsaturation, while MLS-0066987 lacks the halogen but retains the double bond.

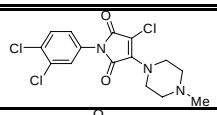
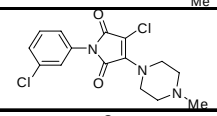
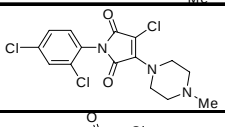
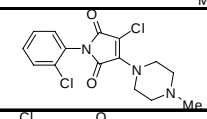
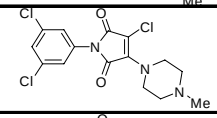
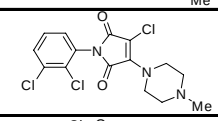
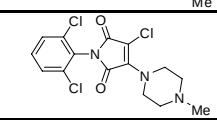
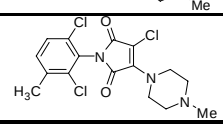
		Bfl-1 FP	Bfl-1 TR FRET
Compound #	**Structure**	**IC_{50} (uM)**	**IC_{50} (uM)**
CID-1180676		0.75	0.56
MLS-0090834		2.15	1.17
MLS-0090829		2.62	1.36
MLS-0090833		2.16	1.45
MLS-0090828		2.44	1.66
MLS-0090831		8.74	2.56
MLS-0090830		13.74	3.72
MLS-0090832		23.9	11.1

Table 2. SAR for substituents around phenyl ring.

Both compounds were inactive in the FP and TR-FRET assays, suggesting that these structural elements are important for Bfl-1 activity. It should be pointed out that we were concerned that the chloroenone functionality present in the structure may make the molecule susceptible to irreversible covalent protein labeling. As noted in a previous section, mechanistic studies indicate that this is not the case. We then focused an SAR study on the phenyl ring substituents. Using the initial lead (CID-1180676) as a template a series of analogues was synthesized in which the substituents on the phenyl ring were varied. The results of this study are summarized in Table 2. The results suggest that, with respect to the chloro substitution pattern, the 3,4-dichlorophenyl configuration present in the lead structure is preferred.

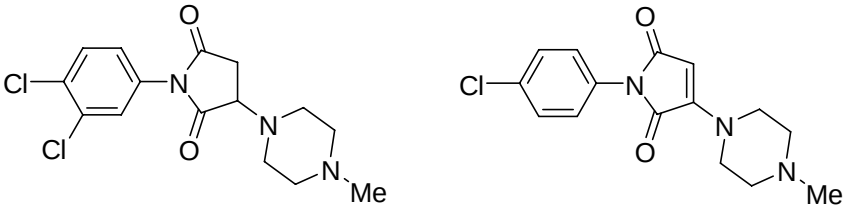


Figure 6. Structures of MLS-0066991 and MLS-0066987.

Based on these results we investigated the SAR around the maleimide amine substituent.

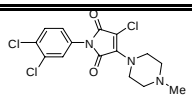
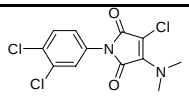
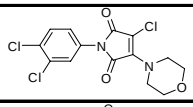
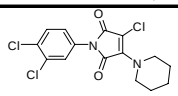
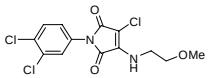
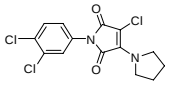
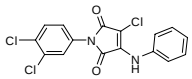
		Bfl-1 FP	Bfl-1 TR FRET
Compound #	**Structure**	**IC_{50} (uM)**	**IC_{50} (uM)**
CID-1180676		0.75	0.56
MLS-0067130		0.46	0.57
MLS-0067124		1.41	1.14
MLS-0066990		no fit	1.3
MLS-0067127		12.6	4.96
MLS-0067126		no fit	no fit
MLS-0067128		no fit	no fit

Table 3. SAR for amine substituents on the maleimide ring.

Thus, a series of analogues was prepared in which the piperazine moiety in the lead structure was

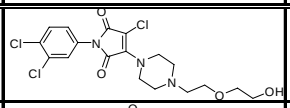
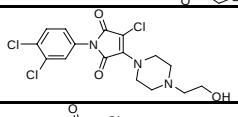
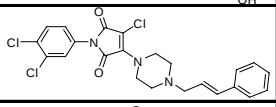
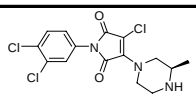
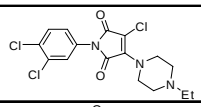
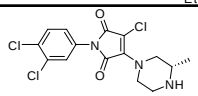
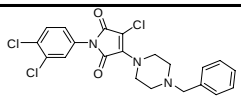
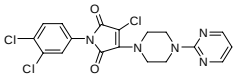
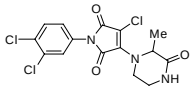
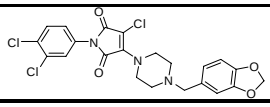
		Bfl-1 FP	Bfl-1 TR FRET
Compound #	**Structure**	**IC_{50} (uM)**	**IC_{50} (uM)**
MLS-0090861		0.47	0.42
MLS-0090874		0.56	0.59
MLS-0090859		0.59	0.73
MLS-0090857		1.16	1.03
MLS-0090860		1.67	0.97
MLS-0090858		1.32	1.25
MLS-0090866		1.62	1.64
MLS-0090868		1.9	2.46
MLS-0090884		5.63	2.03
MLS-0090865		4.07	2.14

Table 4. SAR for derivatives in which the piperazine has been modified.

replaced with different amines including dimethylamine, morpholine, piperidine, methoxyethylamine, pyrrolidine and aniline (see Table 3). In this series we found that the dimethylamine analogue (MLS-0067130) was equipotent with the lead compound while the morpholine derivative (MLS-0067124), with IC_{50} values of 1.4 μM and 1.14 μM in the FP and TR-FRET assays, respectively, was slightly less potent than the lead compound. The other analogues in this series were less potent still, with the pyrrolidine and aniline derivatives being inactive at the doses tested.

We then decided to synthesize a series of analogues in which the maleimide and phenyl portions of the molecule were retained intact while the piperazine moiety was modified with various substituents. The most active compounds generated in this phase of the investigation are shown in Table 4. The compounds synthesized include derivatives in which a variety of substituents are appended to the distal nitrogen of the piperazine moiety. In some examples the substituents are lipophilic (as in MLS-0090859, MLS-0090860, MLS-0090866 and MLS-0090865) while other examples are more hydrophilic in nature (e.g. MLS-0090861, MLS-0090874 and MLS-0090868).

Other analogues, exemplified by MLS-0090857, MLS-0090858, and MLS-0090884, are modified at the level of the piperazine carbon framework. Interestingly this study led to the identification of MLS-0090861, containing a (2-hydroxyethoxy)ethyl substituent as an analogue with slightly improved potency (IC_{50} = 0.47 uM) and enhanced aqueous solubility (data not shown) compared with the lead structure (see Figure 3.3). Based on these studies, taken together with the mechanistic and confirmatory studies, the dimethylamine derivative MLS-0067130 was selected as a research probe for Bfl-1.

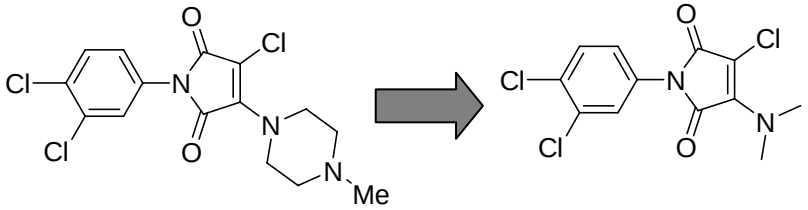
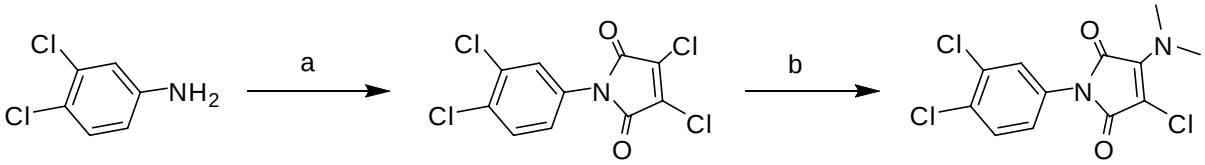


Figure 7. Summary of chloromaleimide Bfl-1 probe development.

Reduced to practice detailed synthetic pathway for making probe:



Scheme 1. Reagents and conditions: (a) Dichloromaleic anhydride, AcOH, 110°C, 3h; (b) $(CH_3)_2NH$, dioxane, 80°C, 20h; 57% overall.

Bfl-1 maleimide series Appendix 1

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, as with 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. A representative of the maleimide series was tested in these assays (Figure 8). As can be seen from Figure 8, a chloromaleimide derivative (CID-1180676) was selective for Bfl-1 and had no effect on either Bcl-2 or Bcl-B.

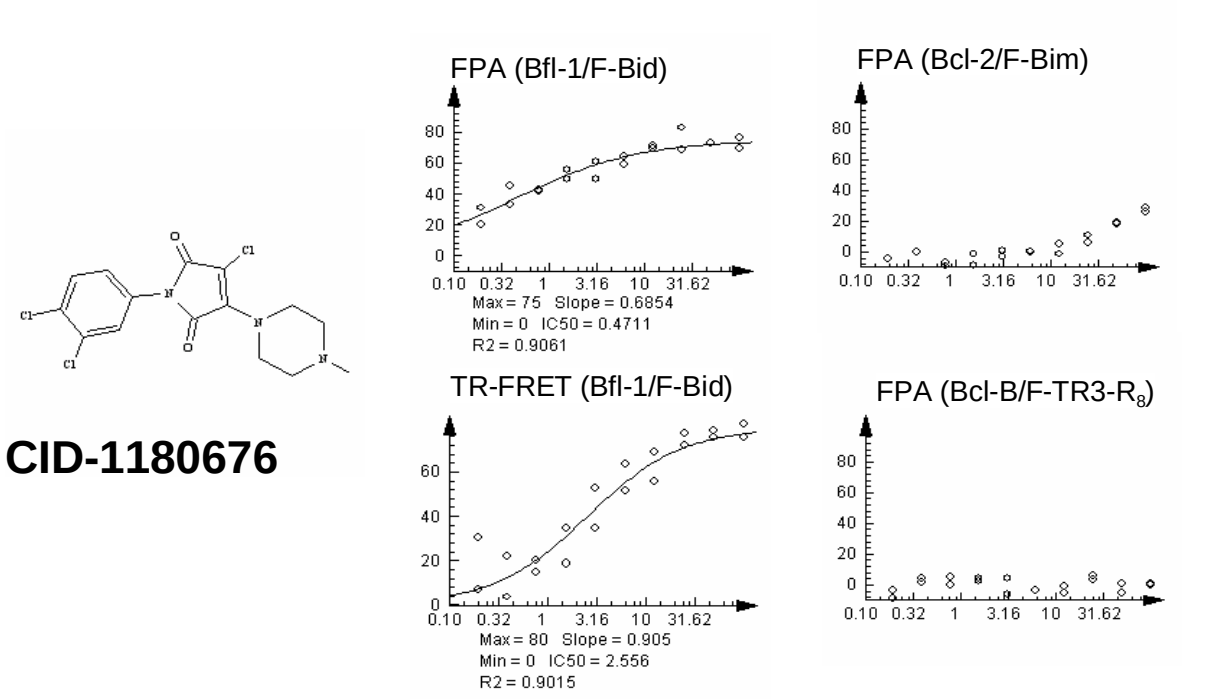


Figure 8. ***Selectivity of Bfl-1 antagonists.*** Representative of chloromaleimide scaffold screened against the Bcl-2 family panel. Compounds were tested in Bfl-1/FITC-Bid FPA and TR-FRET assays, Bcl-2/FITC-Bim and Bcl-B/FITC-TR3.

Tr3 binds to Bcl-B at a site that is distinct from the BH3-binding peptide site so the selectivity in this assay is expected. On the other hand, this observation confirmed that CID-1180676 does not interfere with the fluorescence polarization read-out of the FITC-based assay, *i.e.* it is not an artifact of the Bfl-1/FITC-Bid assay. Another significant observation is that a chloromaleimide failed to interfere with the Bcl-2/FITC-Bim complex, demonstrating a much higher selectivity profile than BH3I-1. The latter observation 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 be a great asset for uncovering Bfl-1-specific biology, but this effect is anticipated to be cell-type specific (see for example Appendix 2 and Bfl-1 sulfonylpyrimidine series probe report).

Bfl-1 maleimide series Appendix 2. Regulation of Activation-induced Mast Cell Survival in Allergy.

Hypothesis: Apoptosis can be induced in mast cells during allergic reaction by inhibiting the prosurvival protein Bfl-1/A1.

Objectives: We aim to investigate the sensitivity of primary mast cells to a small molecule inhibitor of Bfl-1. Human mast cells are activated by cross-linkage of the high affinity IgE-receptor or Ionomycin, and the effect of CID-779754 on mast cell survival is determined.

Background: Mast cells are inflammatory cells of hematopoietic origin, distributed in almost all tissues in the body. Upon activation, mast cells release their granule content (e.g., histamine and proteases like tryptase, chymase and metalloproteinases), generate lipid mediators and secrete cytokines and growth factors. Although best known for their role in allergic reaction, mast cells are today considered to be versatile effector and regulatory cells, involved in many disorders such as autoimmunity (1). More recent findings suggest also that mast cells are more central to both innate and acquired immune responses than previously believed (2). Mast cells are also believed to be a part of tumorigenesis in the development of several types of cancer. Thus, there are several acute and chronic inflammations where mast cells are important both for the initiation as well as the perpetuation of the inflammation. Today several drugs are used that targets mast cell secreted inflammatory mediators, like histamine, leukotrienes and TNF-α. Using mast cells as a direct pharmaceutical target is a plausible new way to treat these disorders.

In several previous studies we investigated the role of different Bcl-2 family members in the regulation of mast cell survival (mice and human). During allergic activation of mast cells (mice and human) through aggregation of the high-affinity IgE-receptor, the prosurvival gene A1/Bfl-1 is upregulated (3, 4). A1/Bfl-1 deficient mast cells do not exhibit such activation-induced mast cells (3). Furthermore, sensitized A1 deficient mice show reduced active cutaneous anaphylaxis and reduced number of mast cells after provocation (Xiang et al. submitted). Thus, these data suggest that A1/Bfl-1 is crucial for the survival of mast cells during an allergic reaction.

Bcl-2 family members regulate survival and apoptosis by interacting with each other, froming heterodimers. Studies were previously reported the binding of A1/Bfl-1 to other. However, no such studies have been performed in mast cells. The first study showed that overexpressed FLAG-tagged Bfl-1 heterodimerized with Bak and Bik (5). More recent studies suggest that A1/Bfl-1 forms heterodimers primarily with BH3-only proteins. A study by Chen et al. showed that Noxa is very strict in its partnership, only binding to Mcl-1 and Bfl-1; whereas Bim and Puma are more promiscuous, binding to several prosurvival proteins including Bfl-1 (6). In another study it was described that the interaction between Bfl-1 and Bim stabilizes the Bfl-1-protein, thereby reducing the turnover of Bfl-1 and amplifying the capacity of Bfl-1 to protect cells against apoptosis (7). This is of particular interest since we have shown that IgE-receptor activation not only induces Bfl-1-expression but also Bim (8, 9). Finally, Werner et al described that Bfl-1 binds to Bid, both full-length and truncated forms, thereby blocking the formation of heterodimers between tbid and Bax/Bak in the mitochondrial membrane (10).

In light of the studies described we were eager to test small molecule inhibitors of Bfl-1 identified by the Burnham (San Diego) Center for Chemical Genomics to inhibit the interaction between Bfl-1 and Bid. The experiments were performed on human mast cells as described below.

Results:

CID-779754 is not cytotoxic to in vitro developed human mast cells

To investigate whether CID-779754 is cytotoxic to cord blood derived human mast cells (CBMCs), a colorimetric assay based on the measurement of lactate dehydrogenase (LDH) activity was used (Figure A1). In this assay no cytoxicity was observed for CID-779754 at 0.1 – 100 μM. The positive control in Figure A1 is a control included in the LDH kit.

CID-779754 prevents activation-induced mast cell survival

The effect of CID-779754 at 1 μM was tested on CBMCs derived from 12 different individuals. CBMCs were activated by aggregation of the high-affinity IgE-receptor (FcεRI) ± CID-779754. The percentage cell survival upon IgE-receptor activation was compared to cells not activated and the delta survival was calculated. CID-779754 totally inhibited the activation-induced survival in 6 out of 11 CBMCs (Figure A2). In 5 out of 11 the effect was partially reduced. One donor did not exhibit activation-induced survival.

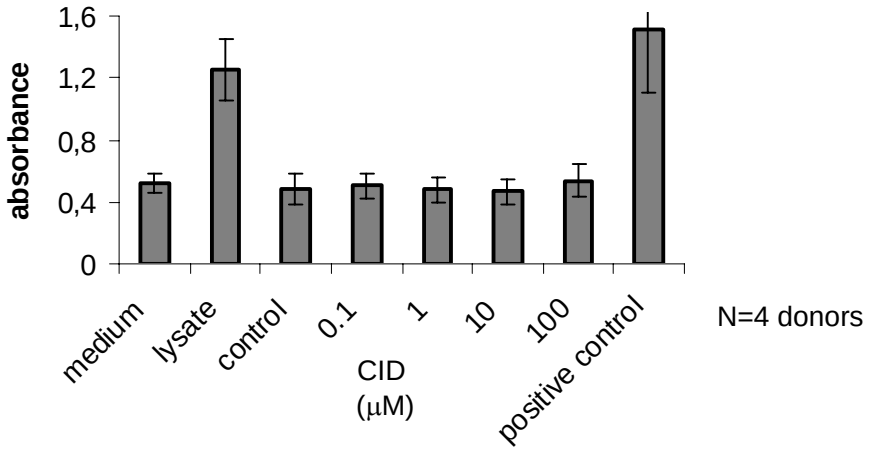


Figure A1. CID-779754 is not toxic to human cord blood-derived mast cells (CBMC) up to 100 μM

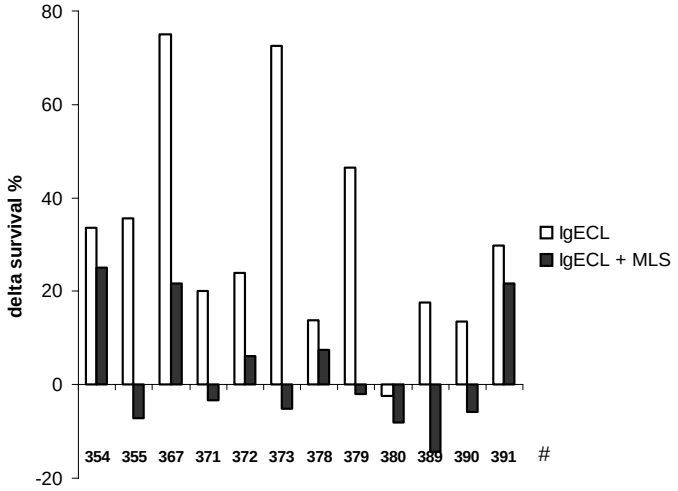


Figure A2. CID-779754 treatment reduces IgECL-induced survival of CBMCs.

Bfl-1 maleimide series Appendix 2 References.

1. Benoist, C., and Mathis, D. 2002. Mast cells in autoimmune disease. *Nature* 420:875-878.
2. Galli, S.J., Nakae, S., and Tsai, M. 2005. Mast cells in the development of adaptive immune responses. *Nat Immunol* 6:135-142.
3. Xiang, Z., Ahmed, A.A., Möller, C., Nakayama, K.-i., Hatakeyama, S., and Nilsson, G. 2001. Essential role of the pro-survival bc-2 homolog A1 in mast cell survival following allergic activation. *J. Exp. Med.* 194:1561-1569.
4. Xiang, Z., Möller, C., and Nilsson, G. 2006. IgE-receptor activation induces survival and bfl-1 expression in human mast cells but not basophils. *Allergy* 61:1040-1046.
5. Holmgreen, S.P., Huang, D.C., Adams, J.M., and Cory, S. 1999. Survival activity of Bcl-2 homologs Bcl-w and A1 only partially correlates with their ability to bind pro-apoptotic family members. *Cell death Differ.* 6:525-532.
6. Chen, L., Willis, S.N., Wei, A., Smith, B.J., Fletcher, J.I., Hinds, M.G., Colman, P.M., Day, C.L., Adams, J.M., and Huang, D.C. 2005. Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. *Mol Cell* 17:393-403.
7. Herold, M.J., Zeitz, J., Pelzer, C., Kraus, C., Peters, A., Wohlleben, G., and Berberich, I. 2006. The stability and anti-apoptotic function of A1 are controlled by its C-terminus. *J. Biol. Chem.* 281:13663-13671.
8. Alfredsson, J., Puthalakath, H., Martin, H., Strasser, A., and Nilsson, G. 2005. Proapoptotic Bcl-2 family member Bim is involved in the control of mast cell survival and is induced together with Bcl-XL upon IgE-receptor activation. *Cell Death Differ* 12:136-144.
9. Alfredsson, J., Möller, C., and Nilsson, G. 2006. IgE-receptor activation of mast cells regulates phosphorylation and expression of forkhead and Bcl-2 family members. *Scand. J. Immunol.* 63:1-6.
10. Werner, A.B., de Vries, E., Tait, S.W., Bontjer, I., and Borst, J. 2002. Bcl-2 family member Bfl-1/A1 sequesters truncated bid to inhibit is collaboration with pro-apoptotic Bak or Bax. *J Biol Chem* 277:22781-22788.
11. Möller, C., Alfredsson, J., Engstrom, M., Wootz, H., Xiang, Z., Lennartsson, J., Jönsson, J., and Nilsson, G. 2005. Stem cell factor promotes mast cell survival via inactivation of FOXO3a mediated transcriptional induction and MEK regulated phosphorylation of the pro-apoptotic protein Bim. *Blood* 106:1330-1336.

Bfl-1 maleimide series Bibliography for Main Text

1. Reed, J. C., Doctor, K. S., and Godzik, A. (2004). The domains of apoptosis: a genomics perspective. *Sci STKE 2004*, re9.
2. Reed, J. C., and Pellecchia, M. (2005). Apoptosis-based therapies for hematologic malignancies. *Blood* ***106***, 408-418.
3. Oltersdorf, T., Elmore, S. W., Shoemaker, A. R., Armstrong, R. C., Augeri, D. J., Belli, B. A., Bruncko, M., Deckwerth, T. L., Dinges, J., Hajduk, P. J., *et al.* (2005). An inhibitor of Bcl-2 family proteins induces regression of solid tumours. *Nature* ***435***, 677-681.
4. Palmer, A. E., Jin, C., Reed, J. C., and Tsien, R. Y. (2004). Bcl-2-mediated alterations in endoplasmic reticulum Ca2+ analyzed with an improved genetically encoded fluorescent sensor. *Proc Natl Acad Sci U S A* ***101***, 17404-17409.
5. Zhai, D., Jin, C., Satterthwait, A. C. and Reed, J. C. (2006) Comparison of chemical inhibitors of antiapoptotic Bcl-2-family proteins. *Cell Death and Differentiation* ***13***, 1419–1421.