Center Probe Report

Project Title: High Throughput Fluorescence Polarization Screen for Bcl-B Phenotype Converters (Chemical modulators of anti-apoptotic protein Bcl-B)

Grant Number: 1 X01 MH077632-01
(This probe project was initiated by the Center to take advantage of secondary assays developed at the Center for the Bfl-1 probe project and a hypothesis that inhibitors to a non-canonical binding site of Bcl-B could be found).

Screening Center Name: San Diego Center for Chemical Genomics (SDCCG)

Principal Investigator of Screening Center: Dr. John C. Reed

Parental Assay Provider & Institution: John C. Reed, M.D., Ph.D. Burnham Institute for Medical Research

Assay or Pathway Target: Anti-apoptotic protein Bcl-B

Probe PubChem Compound Identifier (CID/SID): CID-5816499

Assay provider information: Dr. Kenneth Yip, Burnham Institute for Medical Research (PI: Dr. John C. Reed, Burnham Institute for Medical Research)

Specific Aim:
It has been recently reported in literature that several of anti-apoptotic Bcl-2 family proteins, such as Bcl-B and Bcl-2, could be converted to become pro-apoptotic upon binding with TR3, steroid/thyroid/nuclear receptor protein TR3/Nur77/NR4A. The aim of this project is to identify small molecule chemical probes that bind to Bcl-B within its TR3-binding site.

Significance:
Chemical probes with the above mechanism of action (MOA) would provide novel chemical probes to advance the functional elucidation of Bcl-B/TR3 interaction, and perhaps provide starting points for developing small molecules that could directly recapitulate the pro-apoptotic effect of TR3 on Bcl-B, in a novel, non-BH3-dependent manner. This would provide an alternative mechanism and binding site to the current pro-apoptotic compounds being currently developed.

Rationale:
Apoptosis represents the typical route of programmed cell death under non-pathological circumstances[1-4]. The inhibition of apoptosis is a causative or contributing factor in cancer and autoimmune disease[1,5]. Particularly common is the dysregulation of the Bcl-2 family, a group of proteins that function as critical regulators of programmed cell death[6]. The over-expression of anti-apoptotic Bcl-2 family proteins has been associated with

cancer chemoresistance, and thus restoring chemosensitivity by inhibiting these proteins has emerged as an attractive strategy for improving cancer therapy[4,9]. Currently, the most common strategy for inhibiting these proteins is based on mimicking the BH3 domains of pro-apoptotic Bcl-2 family members. In this regard, synthetic compounds ABT-737 (and ABT-263), GX15-070, and HA-14-1, as well as natural products such as (-)-Gossypol and (-)-epigallocatechin gallate (EGCG), bind to a hydrophobic cleft on the surface of anti-apoptotic Bcl-2 family proteins, thus inhibiting their cytoprotective activity[4,9-13].

TR3 (NR4A1; HMR; NP10; GFRP1; NAK1; NUR77; NGFIB) is an orphan member of the steroid/thyroid/retinoid nuclear receptor superfamily that translocates from cellular nuclei to mitochondria upon phosphorylation[14,15]. At mitochondria, TR3 binds and converts several antiapoptotic Bcl-2 family members (Bcl-B, Bcl-2, and Bfl-1) into pro-apoptotic proteins[16,17]. TR3 binds to Bcl-B with the highest affinity among the six human anti-apoptotic Bcl-2 family members. Bcl-B is a protein that is over-expressed in plasma cells and multiple myeloma and recently shown to be highly expressed in several types of cancer[17,18]. A 9-mer TR3 peptide derived from the full length TR3 protein with polyarginine tail (TR3-r8) has been found to recapitulate the binding selectivity of the full-length TR3 protein[17].

Screening center information

Assay Implementation and Screening

PubChem Bioassay Name: High Throughput Fluorescence Polarization Screen for Bcl-B Phenotype Converters

List of PubChem bioassay identifiers generated for this screening project (AIDS): AID 1240 (Bcl-B/TR3 Chembridge collection).

List of relevant AIDs that may be used as counterscreen information: 432 (Bfl-1/Bid), 621 (Bfl-1/Bid TR-FRET), 781 (14-3-3/Bad), 950 (Bcl-2/Bim), 951 (Bcl-B/Bim), 952 (Bcl-W/Bim), 1007 (Bcl-XL/Bim), 1008 (Bfl-1/Bim), 1009 (Mcl-1/Bim), 1021 (Mcl-1/Bid), 1022 (Mcl-1/Noxa), 1070 (Bfl-1/Bid SAR), 1231 (Bfl-1/Bid Summary).

Primary Assay Description as defined in PubChem

Bcl-B is an anti-apoptotic member of the Bcl-2 family that is prominently expressed in normal plasma and several types of cancer including multiple myeloma, small cell lung cancer, prostate cancer, and breast cancer. TR3 (NR4A1; HMR; NP10; GFRP1; NAK1; NUR77; NGFIB) is an orphan member of the steroid/thyroid/retinoid nuclear receptor superfamily that translocates from cellular nuclei to mitochondria upon exposure to various pro-apoptotic stimuli. At mitochondria, TR3 binds to Bcl-B and converts it into a pro-apoptotic protein. A specific 9-amino acid sequence within the full length TR3 is able to form a complex with Bcl-B and induce its phenotypic conversion. This interaction forms the basis for a high throughput fluorescence polarization screen for compound mimetics. Such compounds may be useful as chemical probes for understanding the functional mechanism of Bcl-B, and may have therapeutic relevance in autoimmune disease and cancer.

This work's aim is to identify chemical probes capable of targeting Bcl-B/TR3

protein-protein interactions through a fluorescence polarization assay (FPA) using FITC-TR3 peptide. The screening was performed at the San Diego Center for Chemical Genomics (SDCCG) as part of the Molecular Libraries Screening Centers Network (MLSCN). This assay represents a selectivity screening for Bfl-1 anti-apoptotic protein, screened at SDCCG (AID 432). Bfl-1 assay XO1 submission, MH077632-01, Chemical Inhibitors of Anti-Apoptotic Protein Bfl-1, Assay Provider Dr. John C. Reed, Burnham Institute for Medical Research, La Jolla, CA.

PubChem Protocol

Bcl-B fluorescence polarization assay materials:

1) GST-Bcl-B protein and FITC-TR3-r8 peptide were obtained from Prof. John Reed's laboratory (Burnham Institute for Medical Research, San Diego, CA).
    a) Bcl-B is a GST-fusion protein lacking their C-terminal transmembrane domains (~20 amino acids)
    b) FITC-TR3-R8 is FITC-Ahx-FSRSLHSLL-GX-R8 peptide, where Ahx is an N-aminohexanoic acid linker; X is N-aminocaproic acid; and R8 is 8 residues of D-arginine
2) Assay Buffer: 25 mM HEPES-KOH,20 mM β-glycerophosphate, 0.005% Tween-20, pH 7.5, 1 mM TCEP.
3) Bcl-B working solution - 44.4 nM GST-Bcl-B were freshly prepared in the assay buffer and kept on ice prior to use.
4) FITC-TR3-R8 working solution – 44.4 nM peptide in the assay buffer

Bcl-B HTS protocol:

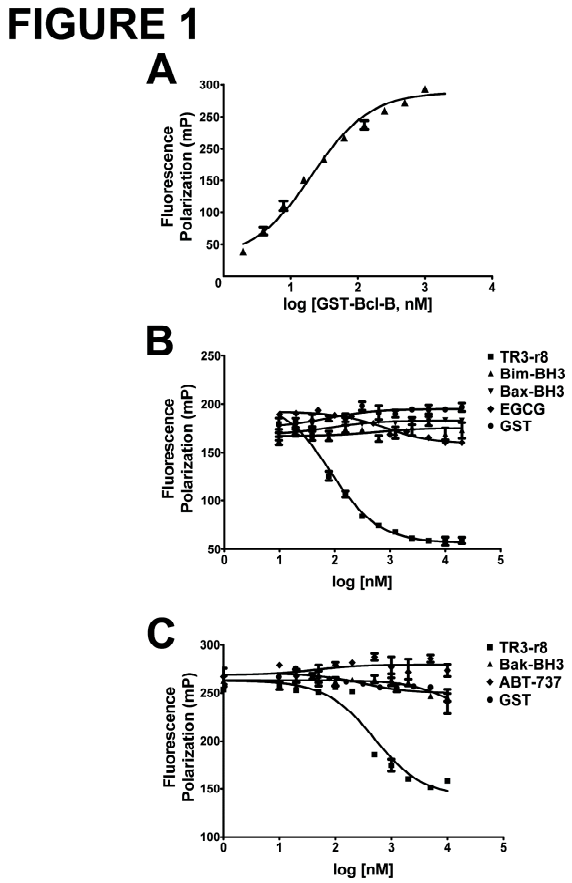
1) 2 uL of 37.5 mg/L compounds in 10% DMSO were dispensed in columns 3-24 of Greiner 384-well black small-volume plates (784076) using a BioMek FX liquid handler. Columns 1 (positive controls) and 2 (negative controls) were added with 2 uL of 10% DMSO.
2) 9 uL of assay buffer were added to column 1 using a WellMate bulk dispenser (Matrix).
3) 9 uL of Bcl-B working solution was added to columns 2-24 using a WellMate bulk dispenser.
4) 9 uL of freshly prepared FITC-TR3-r8 working solution was added to the whole plate using a WellMate bulk dispenser.
5) Final concentrations of the components in the assay were as follows:
    a. 22.5 mM HEPES-KOH, 18 mM β-glycerophosphate, 0.005% Tween-20, pH 7.5, 1 mM TCEP
    b. 20 nM FITC-TR3-r8 (columns 1-24)
    c. 20 nM Bcl-B(columns 2-24)
    d. 1% DMSO (columns 1-24)
    e. 3.75 mg/L compounds (columns 3-24)
6) Plates were incubated for 10 min at room temperature protected from direct light.
7) Fluorescence polarization was measured on an Analyst HT Multi-mode Plate reader (Molecular Devices, Sunnyvale, CA) using fluorescein

filters (ex/em – 485/530 nm, dichroic mirror 505 nm).

8) Data analysis was performed using CBIS software (ChemInnovations, Inc).
9) Fluorescence intensity of each sample was normalized to the average fluorescence intensity value of the plate negative control wells to calculate F_ratio parameter.

Center Summary of the Primary Screen and Confirmatory Screen:
Assay development and validation: The fluorescence polarization (FP) assay, which measures binding of a FITC-labeled to a TR3 derived short synthetic peptide, was developed by Dr. Dayong Zhai in Prof. John Reed's lab (BIMR) in collaboration with Dr. Eduard Sergienko of the SDCCG. As a first step, we determined the limit of detection for FITC-TR3-R8 to provide signal distinguishable from background. The concentration of FITC-TR3-R8 used in the subsequent experiments was equal 20 nM, 10-fold over the limit of detection Then we determined apparent Kd for the binding of GST-Bcl-B to FITC-TR3-R8. The apparent Kd for GST-Bcl-B was ~20 nM (Figure 1A). To determine if TR3-R8 could displace FITC-TR3-r8 from GST-Bcl-B, increasing concentrations of the unlabeled TR3-r8 were incubated with a FITC-TR3-r8/GST-Bcl-B solution (Figure 1B). Indeed, unlabeled TR3-r8 was able to displace FITC-TR3-r8 in a concentration-dependent fashion and the displacement was with EC50 = 82.8 nM.

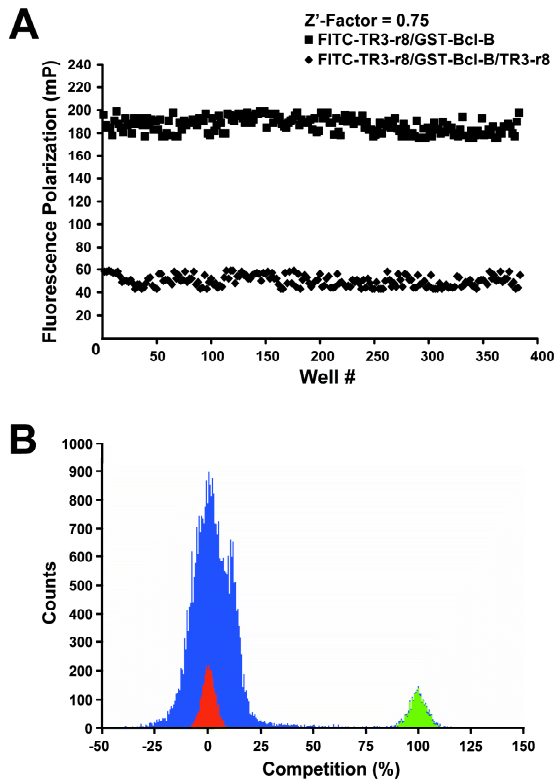
Figure 1: ***TR3-R8 Binds to Bcl-B.*** **(A)** Increasing concentrations of GST-Bcl-B were incubated with 20 nM FITC-TR3-R8 in the assay buffer (25 mM HEPES-KOH, 20 mM β-glycerophosphate, pH 7.5 buffer containing 0.005% Tween-20, 1 mM TCEP) for 10 min, after which fluorescence polarization was measured. **(B)** GST-Bcl-B (20 nM) was incubated with FITC-TR3-r8 (20 nM) and either TR3-R8, Bim-BH3, Bax-BH3, EGCG, or GST in the assay buffer for 10 min, after which fluorescence polarization was measured. **(C)** GST-Bcl-2 (200 nM) was incubated with FITC-TR3-R8 (20 nM) and either TR3-R8, Bak-BH3, ABT-737, or GST in a PBS, 0.005% Tween-20, pH 7.5 buffer for 10 min, after which fluorescence polarization was measured. All data shown represent mean ± standard deviation (n=3).



High Throughput Screening (HTS) and confirmation:
To determine the quality and reproducibility of the FITC-TR3-R8/GST-Bcl-B fluorescence polarization assay for use in HTS, GST-Bcl-B was incubated with either FITC-TR3-R8 alone (negative control) or FITC-TR3-R8 and TR3-R8 (positive control) in a 384-well plate. The assay demonstrated robust performance, with a Z'-factor of 0.75 (Figure 2A. Having failed to obtain confirmable hits from the NIH library, we restored to screening a ~50,000 library (ChemBridge DIVERSet library) for compounds that displaced FITC-TR3-R8 from GST-Bcl-B (Figure 2B), as measured by ≥50% decrease in fluorescence polarization and that did not increase fluorescence intensity by ≥25% (thus eliminating compounds that interfered with the assay due to

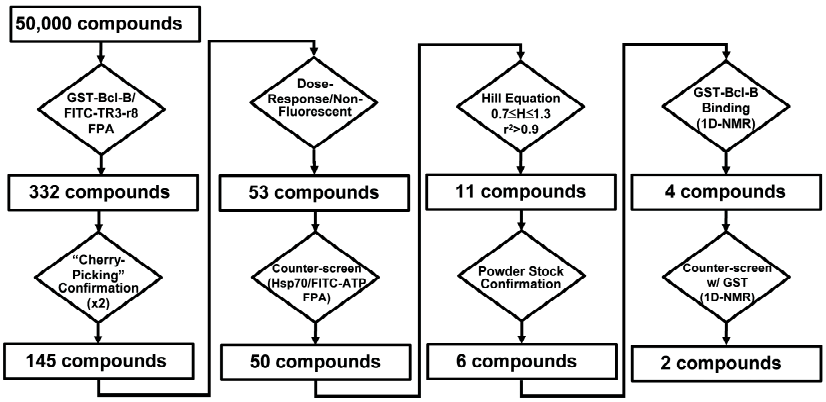
Figure 2: ***Bcl-B/TR3-r8 Fluorescence Polarization Compound Library Screen*. (A)** GST Bcl-B (20 nM) was incubated with either FITC-TR3-R8 (20 nM; *squares*) or with FITC-TR3-r8 (20 nM) and TR3-R8 (5 μM; *diamonds*) for 10 min, after which fluorescence polarization was measured. The data represent fluorescence polarization (mP) measured in individual wells, constituting 192 replicates for each condition. **(B)** GST-Bcl-B (20 nM) was incubated with either FITC-TR3-R8 (20 nM) alone (negative control; *red*), FITC-TR3-r8 (20 nM) and 5 μM TR3-R8 (positive control; *green*), or FITC-TR3-R8 (20 nM) and 3.75 mg/L ChemBridge DIVERSet compounds (*blue*). Fluorescence polarization was measured as previously described.

FIGURE 2



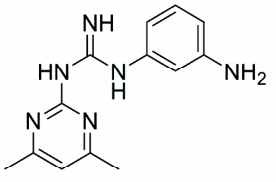
their fluorescence) were determined to be "hits". The primary screen yielded 332 hits. A number of assays were then performed to eliminate non-specific or irreproducible hits (Figure 3). First, the hit compounds were re-tested at the screening concentration; 145 reproducible hits were observed, representing a confirmed hit rate of 0.29%. Second, compound dose-response curves were generated using the fluorescence polarization assay. Compounds that did not display a sigmoidal dose-response curve, or that increased fluorescence intensity, were eliminated, leaving 53 hits.

FIGURE 3 **Workflow for Characterization of Bcl-B-Binding Compounds**



Probe Optimization:

Chemical name of probe compound(s): CID-5816499 (CB5804000) N–(3–aminophenyl)–N'–(4,6–dimethyl–2–pyrimidinyl)guanidine



5804000

Draw probe chemical structure(s) and show stereochemistry if known:

Describe mode of action of for biological activity of probe:
Probe compound interferes with the binding of TR3 to a non-BH3 portion of the Bcl-B anti-apoptotic protein. This is a very novel non-canonical mode of binding distinct from many inhibitors of the Bcl-2 family of proteins that disrupt binding via the BH3 domains.

Description of any secondary screens used to optimize probe structure:
The remaining compounds were then counter-screened using an unrelated, FITC-based fluorescence polarization assay (FITC-ATP/GST-Hsp70), leaving 50 compounds that appeared to be specific for inhibition of the FITC-TR3-R8/GST-Bcl-B assay. Fourth, further analyses of the previously generated dose-response curves identified 11 compounds with statistically "well-fitted" curves, using the Hill equation ($0.7 \leq H \leq 1.3$, $r2 > 0.9$)[20]. These stringent criteria eliminate compounds that may act in a cooperative or anti-cooperative manner[20]. Fifth, new powdered-stocks of the active compounds were then dissolved and tested, with 6 compounds confirming. Finally, 1D-NMR was used to test the compounds for binding to GST-Bcl-B versus GST (as a negative control) (Figure 4). Of the six compounds, two (Chembridge IDs 5804000 and 5954623) bound to GST-Bcl-B in a concentration dependent manner. These compounds did not bind to GST, thus indicating specificity. Several compounds did not bind to either GST-Bcl-B or GST,

serving as internal negative controls for this experiment (e.g. compound 2011727, Figure 4; data not shown).

FIGURE 4

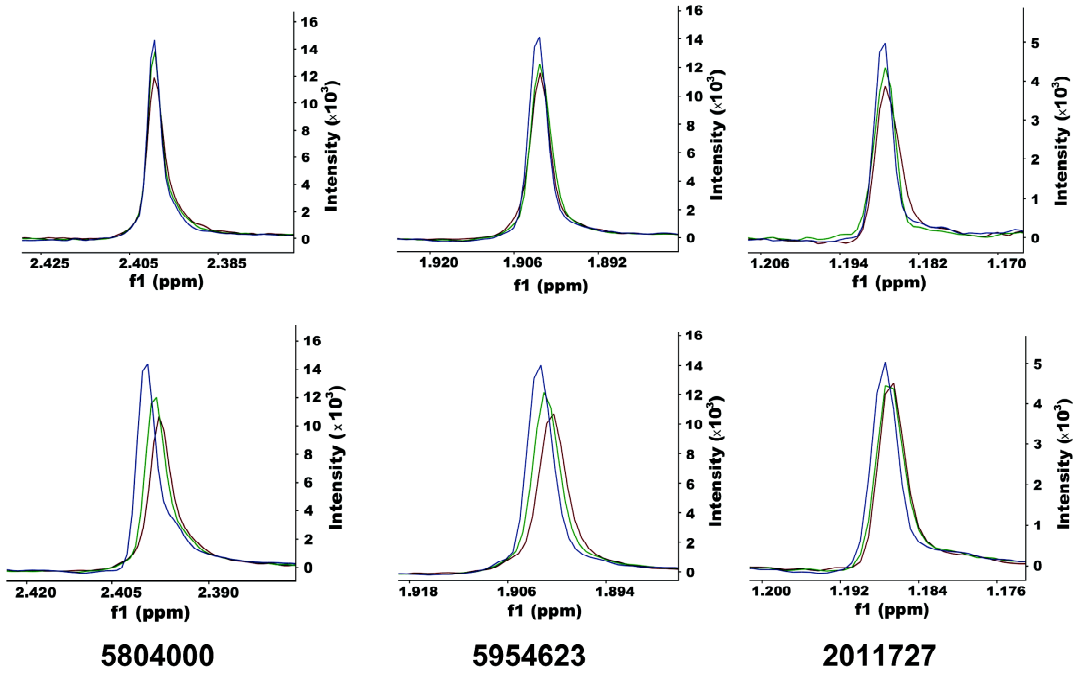


Figure 4*: 1H-NMR spectra confirmation of compound binding to Bcl-B.* Compounds CB-5804000, CB-5954623, and 2011727 (negative control) were diluted in PBS/D2O buffer to 1000 μM, then incubated with either GST (*upper row*) or GST-Bcl-B (*lower row*) at 0 (*blue*), 10 (*green*) or 20 μM (*red*) concentrations. The most prominent peak shift for each compound is shown.

Identification of a novel non-canonical binding site inhibitor:
The assay provider's laboratory previously showed that BH3 peptides derived from Bim (Bim-BH3) and Bax (Bax-BH3), as well as the compound EGCG (epigallecatechin), can displace FITC-BH3 peptides from GST-Bcl-B[19]. However, none of these reagents displaced FITC-TR3-R8 (Figure 1B), suggesting that FITC-TR3-r8 binds in a *non-canonical* manner. This experiment was repeated using a FITC-TR3-R8/GST-Bcl-2 fluorescence polarization assay. Bak-BH3 peptide and BH3 mimetic small molecule ABT-737 have been previously shown to displace FITC-BH3 peptides from Bcl-2[19]. However, Bak-BH3 and ABT-737 did not effectively displace FITC-TR3-r8 from Bcl-2, whereas TR3-R8 effectively competed for binding (Figure 1C).

Comparative data on probe, similar compound structures and information on existing probes available to the public:
Prior to this work no compounds were known to bind in the TR-3 binding site, therefore, it represents a unique and novel chemical probe.

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:
The two Bcl-B-specific compounds, CB-5804000 (Burnham Institute for Medical Research compound BIM-0034173) and CB-5954623 (BIM-0001329), were evaluated using the FITC-TR3-r8/GST-Bcl-B fluorescence polarization assay and yielded EC50 values of 5.6 μM and 2.1 μM, respectively (Figure 5A, 5B). Unlabeled TR3-r8 peptide (positive control) also displaced FITC TR3-r8 as expected, while 5729206 (negative control), a HTS-negative compound, did not (Figure 5B). The compounds were then tested for their ability to displace BH3 peptides from Bcl-B. FITC-labeled BH3 peptides have been previously shown to bind GST-Bcl-B in a fluorescence polarization assay[19]. When unlabeled BH3 peptides derived from Bim or Bax were added, they effectively displaced FITC-Puma-BH3 from GST-Bcl-B

FIGURE 5

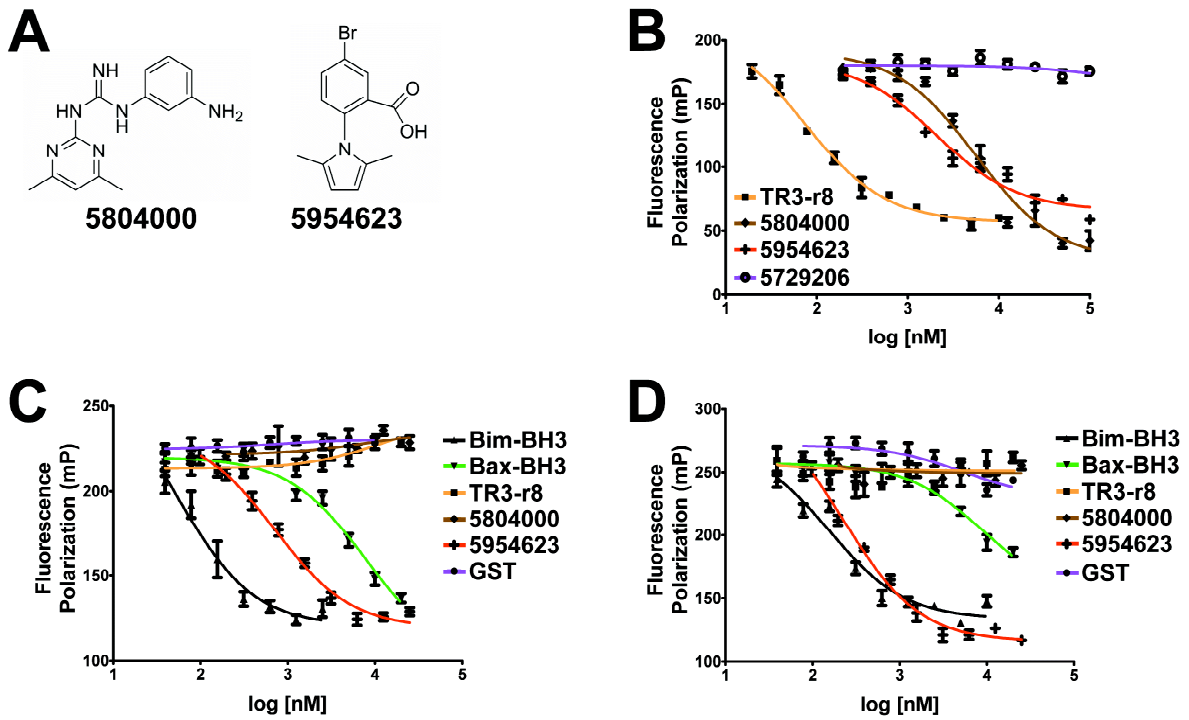


Figure 5: ***Characterization of Compound Competition with FITC-TR3-r8 for Bcl-B Binding by FPA.*** **(A)** Structures of confirmed-compound hits. **(B)** GST-Bcl-B (20 nM) was incubated with FITC-TR3-r8 (20 nM) and either unlabeled TR3-r8 or the indicated compounds in a 25 mM HEPES-KOH, 20 mM β-glycerophosphate, 0.005% Tween-20, pH 7.5 buffer for 10 min, after which fluorescence polarization was measured. 5729206 is a negative control compound. **(C)** GST-Bcl-B (16.4 nM) was incubated with FITC-Puma-BH3 (5 nM) and various concentrations of peptides, compounds, or GST in PBS pH 7.5, 0.005% Tween-20 for 10 min, after which fluorescence polarization was measured. **(D)** GST-Bcl-B (8.8 nM) was incubated with FITC-Bim-BH3 (5 nM) and different concentrations of peptides, compounds, or GST in PBS pH 7.5, 0.005% Tween-20 for 10 min, after which fluorescence polarization was measured. All data shown represent mean ± standard deviation (n=3).

(Figure 5C). Although compound CB-5954623 displaced FITC-Puma-BH3, CB-5804000 did not (Figure 5C). Similarly, in a fluorescence polarization assay using a FITC-labeled peptide derived from the BH3 region of Bim (FITCBim- BH3) and GST-Bcl-B, adding unlabeled BH3 peptides or compound CB-5954623 displaced FITC-Bim-BH3, while compound CB-5804000 did not (Figure 5D).

These results suggest that CB-5804000 binds to a region on Bcl-B that differs from the binding site of pro-apoptotic BH3 domains, and thus this compound meets our primary screening objective.

Describe mode of action for biological activity of probe: The probe binds reversibly and occupies the TR3 BH3 binding site of the Bcl-B protein. Thus it is expected to disrupt binding with TR-3 and potentially promote a pro-

FIGURE 6

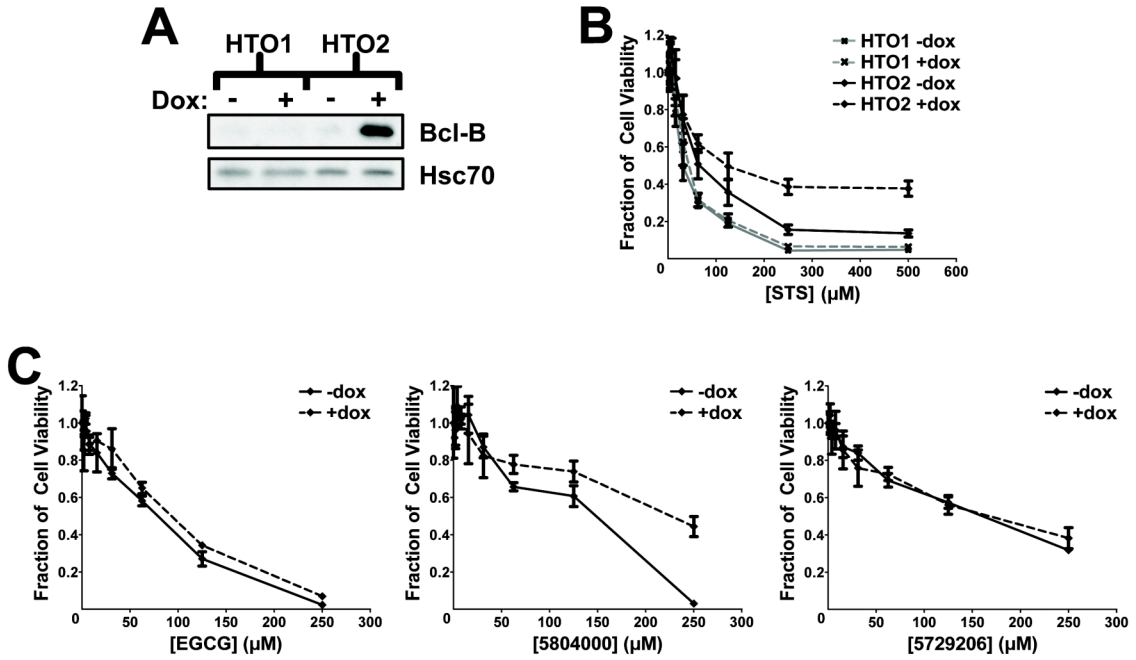


Figure 6: *CB-5804000 Exhibits Bcl-B-Dependent Biological Activity.* (A) HeLa Tet-On (HTO1) and HeLa Tet-On-Bcl-B (HTO2) cells were treated with or without doxycycline (dox) for 24 h to induce their tetracycline-responsive promoters. Protein lysates were collected, and immunoblotting was used to analyze Bcl-B and Hsc70 (for loading control) expression. (B) The cells were treated with or without doxycycline for 24 h, and then with staurosporine at the indicated concentrations for 24 h. Cell viability was measured using ATPlite. (C) HeLa Tet-On-Bcl-B (HTO2) cells were treated with or without doxycycline as above. Compounds were then added at the indicated concentrations, and cell viability was measured 24 h later using ATPlite. The data shown represent mean ± standard deviation (n=3)

apoptotic conformation observed in the presence of TR3. To test if CB-5804000 displays Bcl-B-dependent cellular activity, the assay provider's laboratory generated HeLa cells that expressed Bcl-B under the control of a doxycyline-activated promoter (HeLa Tet-On-Bcl-B; HTO2 cells) (Figure 6A). As expected, HeLa Tet-On-Bcl-B cells were more resistant to staurosporine-induced cell death in the presence of doxycycline-induced Bcl-B (Figure 6B). Control HeLa Tet-On cells that did not express Bcl-B in the presence of doxycycline (HTO1 cells) displayed the same level of staurosporine-induced cell death whether or not doxycycline was added, indicating that doxycycline was not confounding the HeLa Tet-On-Bcl-B results (Figure 6B). In the presence of doxycycline, HeLa Tet-On-Bcl-B cells were more resistant to EGCG (positive control) and CB-5804000, but not to 5729206 (negative control) (Figure 6C). For EGCG and CB-5804000, the concentrations required to reduce HeLa Tet-On-Bcl-B cell viability by 50% increased from ~79 μM and ~148 μM, respectively, in the absence of Bcl-B, to ~93 μM and ~226 μM, respectively, in the presence of Bcl-B. In contrast, the expression of Bcl-B did not affect 5729206-induced cell death. Thus, CB-5804000 acted in a Bcl-B-dependent manner in these genetically engineered cells.

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

Commercial vendor information if available for probe or analogs that have been purchased: Probe compound is available commercially from Chembridge Cat. No. 580400.

Center comments on chemistry strategy leading to probe identification: As these are the first reported compounds that inhibit via the TR3 binding site one in a BH3-dependent and the other in a novel non-canonical fashion, with low micromolar potency and cell activity, it meets the requirements of an initial probe for the Bcl-B/TR-3 interaction.

Center summary of probe properties (solubility, absorbance/fluorescence, reactivity, toxicity, etc.) and recommendations for the scientific use of probe as research tool: The study described here provides a novel TR3-derived chemical probe to study Bcl-B role in biological processes.

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