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Authors
Bracken, Jack D
Carlson, Andrew D
Frederich, James H
et al.

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Tailored fragments of roseophilin selectively antagonize Mcl-1 *in vitro*

Jack D. Bracken[‡], **Andrew D. Carlson**[‡], **James H. Frederich**[‡], **Mai Nguyen**[†], **Gordon C. Shore**[†], and **Patrick G. Harran**[‡]

[‡]Department of Chemistry and Biochemistry, University of California at Los Angeles, 607 Charles E. Young Drive East, Los Angeles, CA 90095, United States

[†]Department of Biochemistry and Goodman Cancer Research Center, McGill University, Montréal, Québec, Canada

Abstract

We have discovered a fragment of the natural product roseophilin, a member of the prodiginine family, that antagonizes Mcl-1 functions in a liposome-based assay for mitochondrial membrane permeabilization. By tailoring this substance such that it can participate in salt bridging with the protein surface, we have prepared the first prodiginine inspired structure that shows direct, saturable binding to a recombinant Bcl-2 family member *in vitro*.

Keywords

Roseophilin; Mcl-1; Bcl-2; Tetrazole; Obatoclax

Our laboratory is interested in small molecule mimics of regulatory proteins. Compounds that can restore apoptotic signaling in cancer cells are a particular focus. We described the first Smac mimetics in 2004.[1] Those molecules subsequently helped identify compounds that activate the TRAIL receptor.[2] The TRAIL / Smac mimetic combination selectively stimulates apoptosis by de-repressing caspase activities.[3] Attention has recently shifted to a second mechanism that results in apoptosis being suppressed in cancer; namely, the failed release of pro-apoptotic factors (including Smac) from the outer membrane space of mitochondria as gated by Bcl-2 proteins. Pro-survival Bcl-2 proteins prevent pore formations in the mitochondrial membrane needed to propagate apoptosis. Bcl-2 activities are countered by interactions with ‘BH3-only’ regulatory proteins – which involve key α-

Supplementary Data

Supplementary data (experimental procedures and copies of ^{1}H and ^{13}C NMR data for all new compounds) associated with this article can be found, in the online version, at http://

Dedicated to the memory of Professor Harry Wasserman and his seminal contributions to prodigiosin chemistry.

helix / domain interactions. These contacts have been studied intensely for two decades.[4] Early screens by one of us (GCS) identified the bacterial pigment streptorubin B (**1,** Figure 1) as an inhibitor of the Bcl-2 / Bax interaction.[5] This discovery led to the development of obatoclax (**2**), a simplified indolic variant of **1** that entered human clinical trials as a pan Bcl-2 antagonist.[6] In functional settings, obatoclax promotes apoptosis by countering activities of anti-apoptotic Bcl-2 family members, including myeloid cell leukemia protein 1 (Mcl-1). Mcl-1 based resistance has limited other Bcl-2 inhibitor programs.[7]

Obatoclax is a valuable structure, but it does have liabilities. Its pyrrolylpyrromethene core can generate reactive oxygen species when bound by copper[8] and, in protonated form, it can function as a chloride ion symporter.[9] In an attempt to identify variants of **2** lacking these activities, we looked to our recent synthesis of roseophilin (**3**)[10] for candidates. The heterocyclic domain of **3** harbors a furan in place of the central pyrrole present in **1** and **2**. We anticipated the coordination chemistries of roseophilin-type substances would differ markedly from obatoclax.

Synthetic **3**, along with numerous intermediates and their derivatives were assayed in a model system for mitochondrial outer membrane permeabilization (MOMP). This liposome based experiment recapitulates a signaling axis involving pro-apoptotic Bak, anti-apoptotic Mcl-1, and an active, truncated 15 kDa subunit of the pro-death protein Bid (tBid).[11] Bak and Mcl-1 are transmembrane proteins constitutively anchored in the mitochondrial outer membrane. tBid translocates to mitochondria upon cleavage of Bid by caspase-8 and stimulates MOMP by directly activating free Bak and by disengaging any activated Bak from Mcl-1. Our assay mimics these events using recombinant, lipidated Mcl-1 and Bak that are constitutively bound to unilamellar liposomes encapsulating calcein dye. tBid addition initiates Bak from Mcl-1. Our assay mimics these events using recombinant, lipidated Mcl-1 and Bak that are constitutively bound to unilamellar liposomes encapsulating calcein dye. tBid addition initiates Bak homo oligomerization and pore formations, which in turn results in calcein release and a fluorescence signal. Mcl-1 counters this tBid dependent activation of Bak, which is overcome by small molecule Mcl-1 antagonists (Figure 1).

The majority of compounds we screened in this assay behaved like roseophilin itself. Namely, they showed little activity. However, an N-tosylated derivative of the pyrrolylfuran segment of the natural product did function in this format. At 2 μM concentrations (vs. 0.4 μM Mcl-1 in liposomes), compound **4** caused calcein release at a rate approaching that of obatoclax (data not shown). Additional experiments with **4** were complicated by its limited stability. However, acetylated variant **5** was a bench stable solid (m.p. = 162−165 °C) that was near equipotent to **4** in the liposome assay (Figure 1).[13]

An intriguing characteristic of obatoclax was that it blocked functions of Mcl-1 and other anti-apoptotic Bcl-2 proteins in membrane environments, yet showed limited/variable affinity for those proteins in isolation.[14] We asked whether this was also true of **5**. Recombinant Mcl-1 was expressed and purified from *E. coli*. We synthesized a 21-residue helical peptide from the BH3 domain of Mcl-1 and used that oligomer as a positive control for isothermal titration microcalorimetry (ITC) experiments.[12] Neither **2** nor **5** demonstrated measurable affinity for Mcl-1 by ITC (Figure 2). Compound **5** did not induce release of

calcein from liposomes lacking Mcl-1, suggesting its effects were not derived from interactions with Bak. Likewise, the compound showed no binding to truncated Bid (data not shown) – prepared by cleaving the full-length protein with TEV protease.[15] Like obatoclax, the functional attributes of **5** were not easily traceable to a binding event with isolated protein.

In 2013 Fesik reported a small molecule having quantifiable affinity for purified Mcl-1.[16] Benzothiophene carboxylate **7** (Figure 3A) was identified using Fesik's seminal fragment-based discovery methods.[17] X-ray co-crystallography showed the compound inserted into a binding pocket beneath the groove used by BH3 helical peptides to bind at the Mcl-1 surface (Figure 3C). Its carboxylic acid lay at the mouth of this pocket and formed a salt-bridge with R263 – an interaction normally made from the opposite direction by a conserved aspartic acid residue in BH3 peptides (see protein data bank entry 4HW4).

While benzothiophene **7** and acetyl furan **5** were discovered independently using completely different lines of inquiry, the resemblance of key elements in the structures was uncanny. In the Mcl-1 bound conformation of **7**, the distance between the carboxylate carbon and the center of the chlorodimethylphenoxy substituent was 8.4 Å. We could dock compound **5** into the same space occupied on Mcl-1 by **7** without unfavorable steric interactions (using AutoDock Vina).[18] In the docked conformation of **5**, wherein the carbonyl oxygen was in closest proximity to R263, the distance between the carbonyl carbon and the center of the tolyl group was 8.3 Å. We also observed that primary alcohol **8**, derived from reducing carboxylic acid **7** with $LiAlH_4$, no longer had affinity for Mcl-1 measurable by ITC (Figure 3B). This suggested that salt bridging was a critical aspect of the **7** / Mcl-1 interaction and that giving **5** the ability to interact with the protein similarly may be advantageous. Based on the docking experiments above, we chose to replace the methyl ketone in 5 with a carboxylic acid isostere – hoping to increase its affinity for Mcl-1 in the absence of membranes.

We metalated pyrrolofuran **9**[19] selectively at its 5' position with *n*-butyl lithium. Treatment of the lithiated species with ZnI_2 followed by tosylcyanide gave furanylnitrile **10** in 79% yield.[20] Desilylation of 10 and derivatization with *p*-TsCl afforded **11** in high yield. To complete the target isostere, azide was cycloadded to the nitrile in **11** using diethylaluminum azide.[21] This provided furanyl tetrazole **12** after careful workup with a Na_2HPO_4/H_3PO_4 buffer solution (pH = 2.1).[22] The overall yield of **12** from **9** was 33% (Scheme 1). A mixture of regioisomeric *N*-methyl tetrazoles **16**, which are unable to ionize via proton transfer, were prepared as controls.[23]

Tetrazoles **12** and **16** were tested for specific binding to Mcl-1 using ITC. To our delight, compound **12** showed strong, saturable binding to the protein, whereas its N-methylated congeners **16** did not (Figure 4A). Compound **12** bound Mcl-1 in an apparent 1:1 stoichiometry with K_D = 0.7 μM. There was no indication of solubility differences in ITC buffer that would account for its improved performance relative to **5** and **16**.

Compound **12** also successfully drove pore formations in the artificial membranes of our liposomal assay (Figure 4B). Its activity exceeded that of **5** and obatoclax and was Mcl-1

dependent. Bak loaded proteoliposomes lacking Mcl-1 did not release calcein dye when treated with **12**. Moreover, when Mcl-1 was replaced with Bcl-xL in the assay, **12** also had no effect (Figure 5). This suggested a selectively for Mcl-1 over Bcl-xL which could be of value going forward. Toxic thrombocytopenia caused by ABT-737 *in vivo* has been correlated with Bcl-xL inhibition.[25]

The discovery of Mcl-1 antagonist **5** and a rational means to convert that molecule into a prodginine-like structure (i.e. **12**) having specific affinity for purified Mcl-1 holds considerable promise. Inhibition of anti-apoptotic Bcl-2 proteins by obatoclax is thought dependent on a supporting membrane environment. This could be true for **5** as well wherein its activity in liposomes (Figure 1) may be driven by hydrophobic effects. In contrast, data for the **12** / Mcl-1 interaction suggests a significant electrostatic component. Consistent with this idea, comparably performing Mcl-1 ligands could be generated using ionizable groups other than a tetrazole. We synthesized N-acyl sulfonamides **14** and **15** via primary carboxamide **13** as outlined in Scheme 1.[26,27] Both **14** and **15** functioned in the liposomal assay and bound to recombinant Mcl-1 in vitro (Figure 4C). While their affinity for the purified protein (K_D = 3.4 and 2.3 μM respectively) was less than that for **12**, these derivatives are amenable to further medicinal chemistry. We are currently attempting to confirm hypothetical binding modes for **12** (Figure 3D), **14** and/or **15** using X-ray co-crystallography. We hope to understand performance of this compound series in detail and, ultimately, to identify refined structures that potently inhibit Mcl-1 dependent cancer cell growth in culture and block Mcl-1 dependent tumor progression *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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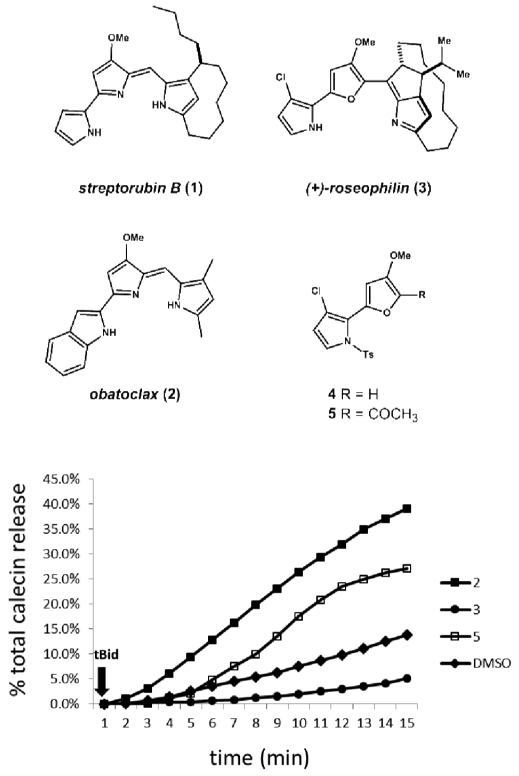


Figure 1.

Obatoclax (**2**) and roseophilin segment **5**, in contrast to roseophilin (**3**), counter Mcl-1 activity and promote Bak-mediated permeablization of liposomal membranes. Data normalized to DMSO control. See text and SI for assay details.

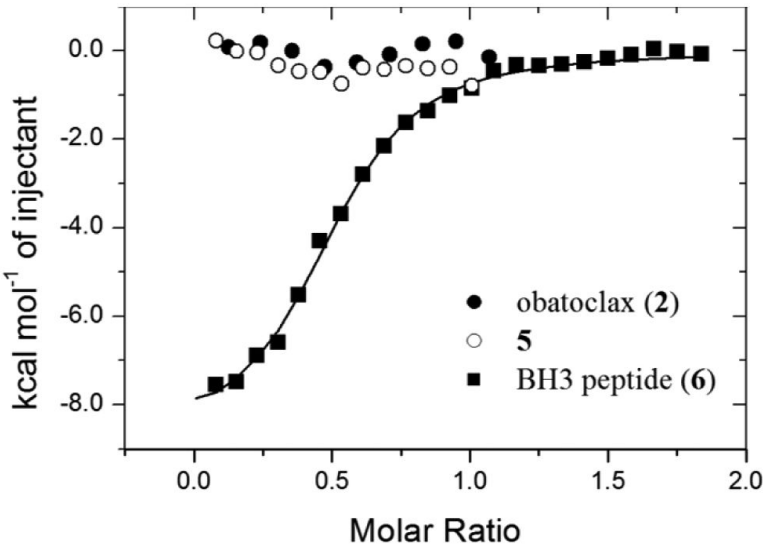


Figure 2.

A peptide corresponding to the Mcl-1 BH3 domain (**6**, Ac-KALETLRRVGDGVQRNHETAF-$CONH_2$) binds Mcl-1 *in vitro* (K_D = 1.1 ± 0.06 μM).[12] Neither compound **5** nor obatoclax (**2**) exhibit similar behavior. Titration microcalorimetry data shown are averaged values from triplicate analyses. See SI for details.

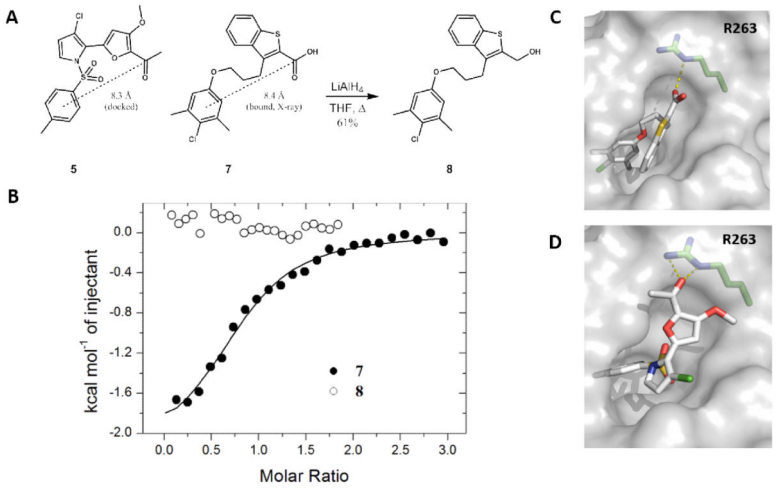
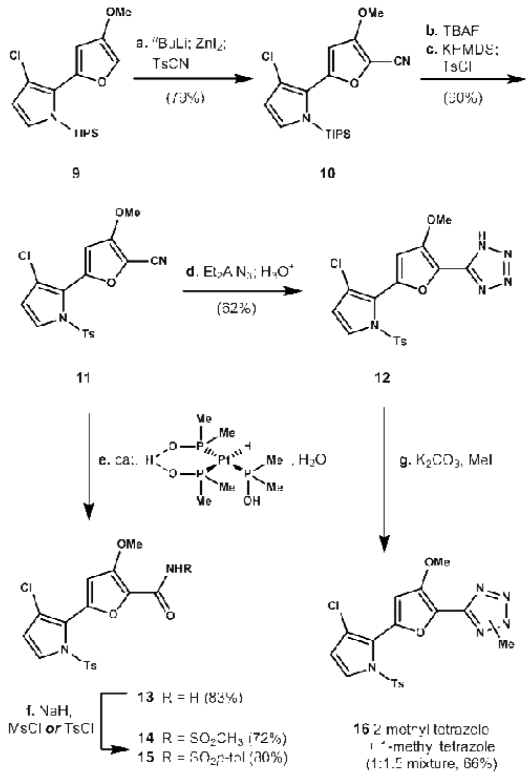


Figure 3.

A/B. Binding of Fesik's benzothiophene **7** to purified Mcl-1 was detectable by ITC (K_D = 1.0 ± 0.41 μM). This was not the case for derived primary alcohol **8**. **C**. PyMOL rendering of compound **7** bound to Mcl-1 in the solid state (PDB:4HW3). **D**. Pyrrolofuran **5** could be docked (Autodock Vina)[18] into the same space on Mcl-1 occupied by **7**, wherein its carbonyl group oriented proximal to R263, analogous to the carboxylate group in **7**.



Scheme 1.

Reagents and conditions: (a) ^{n}BuLi, THF, −78 °C, 15 min; ZnI_2, 0 °C, 3 min; TsCN, −78 °C to rt, 3 h, 79%; (b) TBAF, THF, rt, 10 min; (c) KHMDS, THF, 0 °C, 15 min; TsCl, 0 °C to rt, 1 h, 90% (two steps); (d) Et_2AlN_3, PhMe, 85 °C, 24 h; H_3O^+, rt, 30 min, 62%; (e) 20 mol % Parkin's catalyst, H_2O, 100 °C, 8 h, 83%; (f) NaH, MsCl, THF, 0 °C to rt, 3 h, 72%; or NaH, TsCl, THF, 0 °C to rt, 3 h, 80%; (g) K_2CO_3, MeI, MeCN/THF (1:1), 0 °C to rt, 5 h, 66%

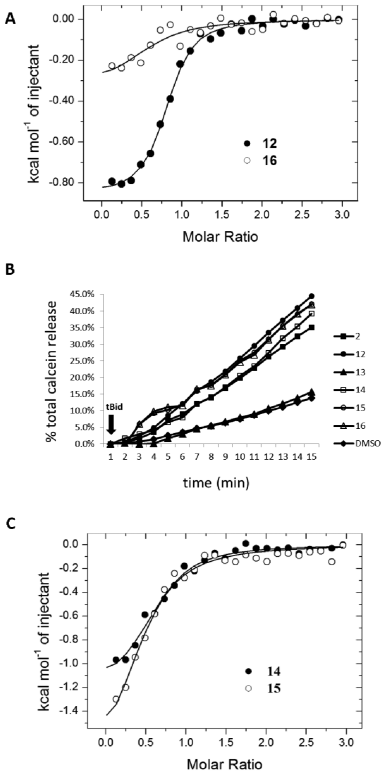


Figure 4.

Compounds **12**, **14** and **15** bind directly to recombinant Mcl-1 in vitro (**A** & **C**) and stimulate Bak-mediated permeablization of liposomal membranes (**B**). [K_D (ITC) for **12**, **14**, and **15** = 0.7 ± 0.12 μM, 3.4 ± 0.49 μM, and 2.3 μM ± 0.16 μM, respectively].

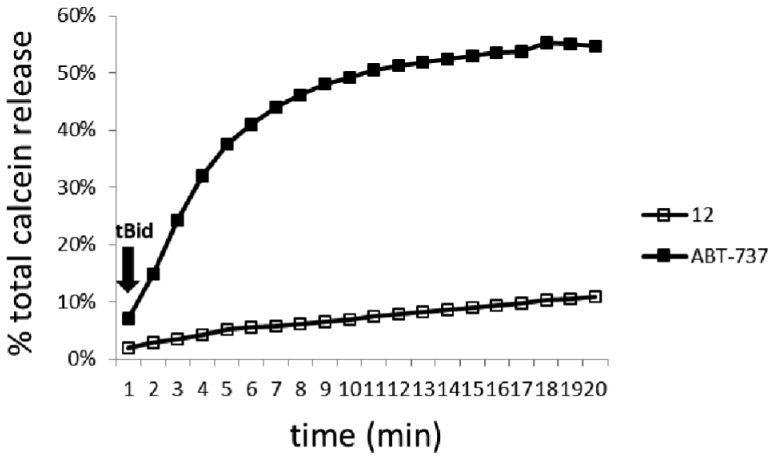


Figure 5.

Tetrazole **12** did not stimulate MOMP-like pore formations in liposomes that contain Bcl-xL in place of Mcl-1. ABT-737, a known antagonist of Bcl-xL,[24] was used as a positive control.