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Design, synthesis, and characterization of new embelin derivatives as potent inhibitors of X-linked inhibitor of apoptosis protein

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Abstract—X-Linked inhibitor of apoptosis protein (XIAP) is a promising molecular target for the design of new anticancer drugs aiming at promoting apoptosis in cancer cells. We have previously identified embelin as an inhibitor of XIAP through computational structure-based database screening. Herein, we report the design, synthesis, and evaluation of new embelin analogues as inhibitors of XIAP. Our efforts led to the identification of new and more potent inhibitors. For example, compound **6g** has a K_i value of 180 nM binding to XIAP BIR3, in a competitive binding assay and represents a promising lead compound for further optimization.
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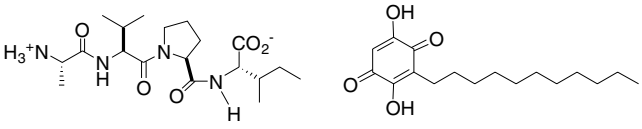
Apoptosis, or programmed cell death, is a genetically regulated cell death mechanism.[1] Dysfunction of the apoptosis machinery plays a major role in many human diseases, including cancer. X-Linked inhibitor of apoptosis protein (XIAP) is a potent and effective cellular inhibitor of apoptosis.[2–5] XIAP has been found to be overexpressed in many human cancer cell lines.[6] XIAP is considered as a promising cancer therapeutic target because inhibition of XIAP can promote apoptosis in cancer cells with overexpression of XIAP and sensitize cancer cells to apoptosis induction.[7] One of the major cellular functions of XIAP is the inhibition of the activity of caspase-9 by binding to caspase-9 through its BIR3 domain and trapping caspase-9 in its inactive form.[8] Smac/DIABLO protein (second mitochondria-derived activator of caspase or direct IAP-binding protein with low pI) has been discovered as an endogenous cellular inhibitor of XIAP,[9,10] and promotes apoptosis in cells at least in part by binding to the BIR3 domain of XIAP where caspase-9 binds and relieving the inhibition of XIAP to caspase-9.[11] There is a strong research interest in the design of peptidomimetics and non-peptidic small-molecule inhibitors to target the XIAP BIR3 domain where Smac and caspase-9 bind.[12–20] Such small-molecule inhibitors are predicted to promote apoptosis in cancer cells and may have great therapeutic potential to be developed as an entirely new class of anticancer drugs.



Through structure-based database searching, we have previously discovered embelin as a fairly potent, non-peptide, small-molecule inhibitor of XIAP.[20] Embelin was determined to bind to the XIAP BIR3 domain with a K_i value of 0.40 μM in our competitive fluorescence-polarization (FP)-based assay (Table 1). To the best of our knowledge, embelin is the only known class of non-peptide inhibitor that binds to the XIAP BIR3 domain, whose chemical structure is not related to the AVPI peptide in Smac. Hence, embelin represents a promising initial lead for optimization toward our ultimate goal of developing a new class of anticancer drugs to target XIAP. In this paper, we wish to report our design, synthesis, and biochemical evaluation of a series of new embelin analogues as inhibitors of XIAP.

Keywords: XIAP; Embelin; Small-molecule inhibitors.
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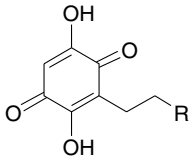
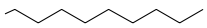
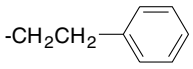
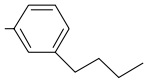
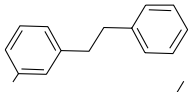
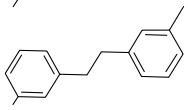
Table 1. Binding affinities to the XIAP BIR3 in an FP-based binding assay for **6a**–**g**



Compound	R	$K_i \pm$ SD (μM) FP-based binding assay
1		0.40 ± 0.13
6a	H	10.4 ± 1.3
6b	–$CH_2CH_2CH_2CH_2CH_2Me$	1.25 ± 0.9
6c		1.3 ± 0.3
6d		0.71 ± 0.17
6e		0.48 ± 0.3
6f		0.38 ± 0.09
6g		0.18 ± 0.09

Embelin consists of the dihydroxyquinone core and a long hydrophobic tail. Our modeling predicted that the hydrophilic dihydroxyquinone core forms a number of hydrogen bonds with XIAP and the hydrophobic tail interacts with the hydrophobic pocket where the isoleucine residues in the AVPI Smac peptide bind. In the present study, we have kept the dihydroxyquinone core intact and focused our modifications on the hydrophobic tail portion of the molecule.

A series of embelin analogues **6a**–**g** with different hydrophobic tails were designed and synthesized. The synthesis of compounds **6a**–**g** is shown in Scheme 1. Briefly, commercially available or easily prepared corresponding triphenylphosphonium bromide **2a**–**g** were treated with 1:1 equivalent of *n*-butyllithium, followed by reaction with aldehyde **3**, which was prepared according to a published method[21] and hydrogenation afforded the key intermediates **4a**–**g**. Oxidation of **4a**–**g** with ceric ammonium nitrate gave [1,2]benzoquinones **5a**–**g**.[22] The final target compounds **6a**–**g** were obtained by the treatment of **5a**–**g** with 70% perchloric acid and concentrated hydrochloric acid at room temperature for 48 h.[23]

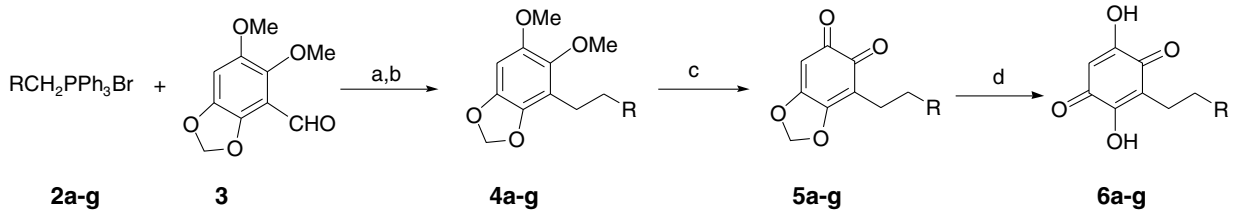
Compounds **6a**–**g** were tested for their binding affinities to recombinant XIAP BIR3 protein using our established quantitative fluorescence-polarization (FP)-based competitive binding assay[24] and compared directly to embelin (**1**) and the AVPI Smac peptide. The results are summarized in Table 1. In our FP-based binding assay, embelin (**1**) and the Smac AVPI peptide were determined to have K_i values of 0.40 and 0.58 μM, respectively.

Compound **6a** was designed to test the importance of the $C_{11}H_{23}$ long hydrophobic tail, in which the $C_{11}H_{23}$ tail was replaced by a much shorter ethyl group. Our FP-based binding assay showed that compound **6a** has a K_i value of 10.4 μM to XIAP BIR3, thus 25 times less potent than embelin. This suggests that the $C_{11}H_{23}$ long hydrophobic tail is critical for the binding of embelin to XIAP BIR3. Based upon this result, we have designed and synthesized compound **6b** with an *n*-octyl side chain. Compound **6b** has a K_i value of 1.25 μM. Hence, although compound **6b** is 3 times less potent than embelin, it is 8 times more potent than compound **6a**, further confirming the importance of the long hydrophobic tail in the binding of embelin to XIAP BIR3.

The crystal and NMR structures of XIAP BIR3 in complex with Smac protein or peptide showed that the binding of Smac to XIAP BIR3 is mediated primarily by the AVPI four amino acid residues in Smac and a well-defined surface binding groove in XIAP BIR3.[25,26] While the alanine residue in the Smac AVPI-binding motif forms an extensive hydrogen bonding network with XIAP BIR3, the proline residue has hydrophobic contacts with Trp323 in XIAP. Our modeling suggested that the hydrophilic dihydroxyquinone core in embelin mimics the alanine residue in the Smac AVPI peptide to form a hydrogen bonding network. We have designed two new analogues, **6c** and **6d**, to explore whether a phenyl ring would be able to mimic the interaction between the proline ring in the Smac AVPI peptide and Trp323 in XIAP. As can be seen, while compound **6c** with a $(CH_2)_4$ linker between the dihydroxyquinone group and the phenyl ring has a K_i value of 1.3 μM, compound **6d** with a $(CH_2)_2$ linker has a K_i value of 0.71 μM. Hence, compound **6d** is 2 and 15 times more potent than **6c** and **6a**, respectively. Thus, we have made further modifications based upon compound **6d**.

The isoleucine residue in the Smac AVPI peptide binds to a hydrophobic pocket in XIAP BIR3 and plays an important role for their binding. Our modeling suggested that the long hydrophobic tail in embelin interacts with this hydrophobic pocket in XIAP. We have thus designed and synthesized a series of new analogues based upon compound **6d** in an attempt to capture the hydrophobic interaction between the isoleucine residue in the Smac AVPI peptide and the hydrophobic pocket in XIAP BIR3.

Compound **6e** with an *n*-butyl group attached to the *meta*-position on the phenyl ring in compound **6d** has a K_i value of 0.48 μM, which is slightly more potent than **6d**. Encouraged by this result, we replaced the *n*-butyl group with a phenylethyl group since it was previously shown that replacement of the isoleucine in the Smac



Scheme 1. Synthesis of compounds **6a**–**g**. Reagents and conditions: (a) *n*-BuLi, THF, 0 °C, 10 min; (b) H_2, 10% Pd–C, EtOAc, room temperature; (c) CAN, CH_3CN–H_2O, 0 °C, 1 h; (d) $HClO_4$, HCl, dioxane, room temperature, 48 h.

AVPI peptide by a phenylalanine residue increased the binding affinity of the resulting peptide.[27] This resulted in compound **6f**, which has a K_i value of 0.38 μM binding to XIAP and is as potent as embelin.

Compound **6g** was designed and synthesized to further explore the interaction between the terminal phenyl ring and XIAP by installation of a methyl group on the *meta*-position the binding affinity of the resulting peptide.[28] Compound **6g** has a K_i value of 0.18 μM and is thus 2 times more potent than embelin for binding to XIAP BIR3.

We have evaluated compound **6g** for its activity in inhibition of cell growth in the MDA-MB-231 (2LMP) human breast cancer line and the PC-3 human prostate cancer cell line. Both of these two cancer cell lines have high levels of XIAP (data not shown). The results are shown in Figure 1. As can be seen, compound **6g** is effective in inhibition of cell growth with IC_{50} values of 5.0 and 5.5 μM in the MDA-MB-231 and PC-3 cell lines, respectively.

In summary, embelin represents a novel class of non-peptide small-molecule inhibitor of XIAP. Our present study focused on the modifications of the hydrophobic tail in embelin. Our study yielded new inhibitors with binding affinities better than embelin and provided preliminary structure–activity relationship for this class of inhibitors. The most potent inhibitor **6g** binds to XIAP BIR3 with a K_i value of 180 nM. Importantly, **6g** is effective in inhibition of cell growth in human breast and prostate cancer cell lines with high levels of XIAP. Hence, compound **6g** represents a promising new lead compound for further optimization toward our ultimate goal of developing a new class of anticancer drugs by targeting XIAP and promoting apoptosis in cancer cells.

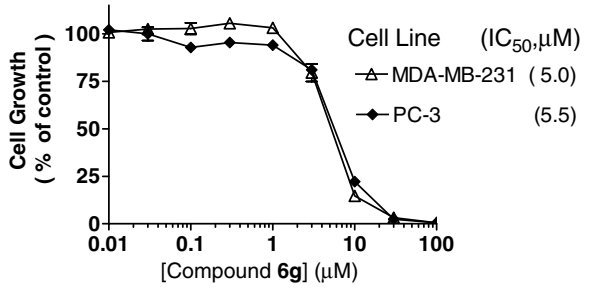


Figure 1. Inhibition of cell growth by compound **6g** in the MDA-MB-231 (2LMP) human breast cancer cell line and the PC-3 human prostate cancer cell line. Cells were treated by compound **6g** for 4 days and cell growth was determined using the WST assay.

Acknowledgments

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28. NMR and elemental analysis data for compound **6g** (2,5-dihydroxy-3-{2-[3-(2-*m*-tolyl-ethyl)-phenyl]-ethyl}-[1,4]benzoquinone). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.67 (br s, 2H), 7.27–7.14 (m, 2H), 7.12–6.97 (m, 6H), 6.05 (s, 1H), 2.88 (s, 4H), 2.78 (s, 4H), 2.35 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 142.1, 141.8, 141.1, 137.9, 129.2, 128.6, 128.4, 128.2, 126.6, 126.3, 126.0, 125.4, 115.9, 102.3, 38.0, 33.8, 24.6, 21.4; Anal. Calcd for $C_{23}H_{22}O_4$: C, 76.22; H, 6.12. Found: C, 75.88; H, 5.95.