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Pro-apoptotic meiogynin A derivatives that target Bcl-xL and Mcl-1

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ABSTRACT

The biological evaluation of a natural sesquiterpene dimer meiogynin A **1**, is described as well as that of five non-natural analogues. Although active on a micromolar range on the inhibition of Bcl-xL/Bak and Mcl-1/Bid interaction, meiogynin A **1** is not cytotoxic on three cell lines that overexpress Bcl-xL and Mcl-1. Contrarily, one of its analogues **6** with an inverted configuration on the side chain and an aromatic moiety replacing the cyclohexane ring was active on both target proteins, cytotoxic on a micromolar range and was found to induce apoptosis through a classical pathway.

Multicellular organisms use apoptosis to regulate tissue homeostasis through the elimination of useless or potentially harmful cells. Key players of apoptosis are caspases, a family of proteases whose activation is induced by two major signalling pathways among which the intrinsic pathway that is regulated by proteins of the Bcl-2 family. This family is divided into anti-apoptotic members such as Bcl-2, Bcl-xL or Mcl-1 and pro-apoptotic members such as Bax, Bak or Bid. The anti-apoptotic proteins bind and thus neutralize the pro-apoptotic ones *via* a hydrophobic cleft through protein-protein interactions. Over the past decade, it was shown that overexpression of the anti-apoptotic Bcl-2, Bcl-xL or Mcl-1 proteins is involved in the development of many kinds of cancers or confers resistance to apoptosis induced by standard anticancer therapies.^{1,2} For example, proteins of the Bcl-2 family play a central role in lymphocytes biology and are very often deregulated in lymphoid malignancies.³ Consequently, targeting this family of proteins is a highly promising strategy for tumor treatment. The feasibility of disrupting protein-protein interactions between anti- and pro-apoptotic members of the Bcl-2 family, using small-molecule inhibitors has been successfully established.⁴ Three small-molecule inhibitors have entered human clinical trials⁵ and at least three other compounds are in preclinical studies. Anti-apoptotic protein inhibitors can be classified in two distinct groups:

- Pan-inhibitors targeting various proteins of this family (Bcl-xL, Bcl-2 and Mcl-1), including *R*-(-)-gossypol (AT-101) and obatoclax⁶ that are currently in clinical trials. They are active at a micro-molar concentration on the target proteins and are more efficient in combination with other drugs.

- Selective inhibitors of a subset of proteins, targeting either Bcl-xL like navitoclax⁷ and WEHI-539⁸, or Bcl-2 like ABT 199⁸ or Mcl-1. In the latter case, the only reported compounds¹⁰ are not druggable but are rather regarded as potent leads. All these compounds are highly active with a *K_i* at a nano-molar level. Nevertheless, for those used in clinical trials, resistance due to overexpression of the non-targeted proteins (mainly Mcl-1) was identified suggesting that efficient treatment may require simultaneous inhibition of multiple anti-apoptotic Bcl-2 proteins.¹¹

Natural products or derivatives of natural products represent 75% of the 175 drugs used in cancer chemotherapy.¹² In 2008, we conducted a biological screening on Malaysian plant extracts, leading to the isolation of a new sesquiterpene dimer that we named meiogynin A **1**.¹³ This compound was found to be a natural antagonist of the Bcl-xL/Bak association. Its biomimetic asymmetric total synthesis was then disclosed¹⁴ and enabled the elaboration of three additional unnatural analogues **2-4**. Later on, two aromatic compounds **5** and **6** were also prepared (Figure 1).¹⁵ In this paper, the biological evaluation of meiogynin A **1**, as well as that of its non-natural analogs **2-6**, is disclosed.

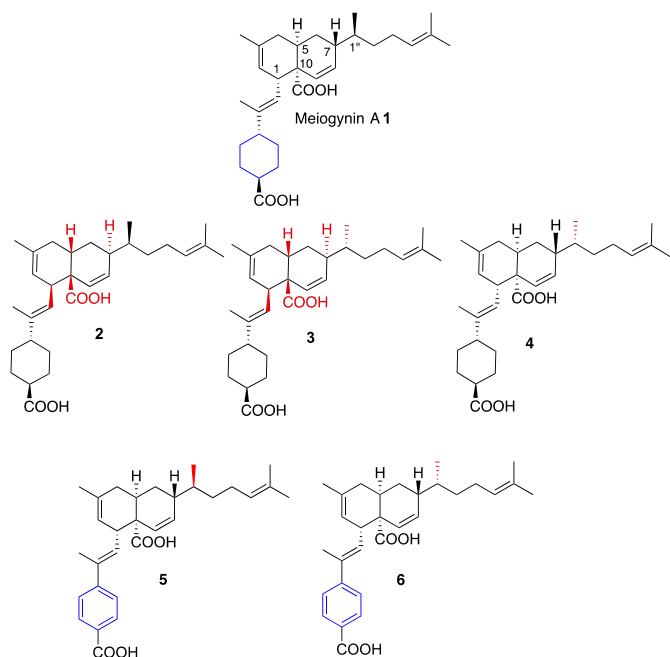


Figure 1. Structure of natural meioyginin A 1 and synthetic analogues 2-6.

The binding affinities of natural and unnatural sesquiterpene dimer type-compounds **1-6** for Bcl-xL and Mcl-1 were evaluated by competition against fluorescently labeled peptides Bak and Bid respectively, using an assay adapted from Qian and co-workers.¹⁶

The results of the biological assays are summarized in Table 1.

The binding affinities for Bcl-xL are closely related to the absolute configuration of the five stereocenters. Thus, the enantiomer **3** of meioyginin A **1** is inactive, whereas the diastereomer **4** that only differs from **1** by the configuration at C1'' is three times more potent. Its enantiomer **2** is moderately active. The aromatic analogues behave the same way: compound **6** (analogue of **4**) is slightly more active than **5** (analogue of natural **1**). In contrast, binding affinities for Mcl-1 are all on the same level regardless of their absolute configuration. The potency of the aromatic analogues **5** and **6** are a little weaker than **1** and **4**. Nevertheless, taken together, these results emphasize the fact that changing the cyclohexane moiety by an aromatic group has no real influence on the inhibition of Bcl-xL/Bak and Mcl-1/Bid interaction.

Table 1. Inhibition of Bcl-xL/Bak and Mcl-1/Bid interaction

Compound	K _i Bcl-xL/Bak (μM) ^{a,b}	K _i Mcl-1/Bid (μM) ^{a,b}
1	9.0 ± 0.4	3.8 ± 0.2
2	100 ± 13	14.4 ± 0.9
3	40 ± 9	12.9 ± 0.4
4	2.3 ± 0.7	4.2 ± 0.9
5	7.5 ± 0.5	15.9 ± 0.6
6	4.6 ± 1.5	9.1 ± 0.5

^a K_i is the concentration corresponding to 50% of inhibition of the binding of the labeled reference compound Bak to Bcl-xL and Bid to Mcl-1, and corrected for experimental conditions according to Cheng and Prussoff.¹⁷ ^b Values are reported as the means of three independent determinations.

The cytotoxicities of all the compounds were evaluated on Burkitt lymphoma (BL) cell lines (BL2, BL2/B95) and lymphoblastoid (Remb1) cell lines (LCL), that were previously shown to express both Bcl-xL and Mcl-1.¹⁸ Cells were treated, or not, with various concentrations of compounds **1-6** and cytotoxicities were evaluated by MTT assays. As shown in

Figure 2, compound **6**, contrarily to meioyginin A **1**, was cytotoxic for the three cell lines since more than 50% of the cells were killed after 24 h of treatment with 10 μM of this compound. IC₅₀ of compound **6** were determined to be of 8.5 μM for BL2 and 8.6 μM for BL2/B95 and Remb1. In contrast, the other compounds **2-5** were not cytotoxic for the cells (IC₅₀ > 20 μM, data not shown).

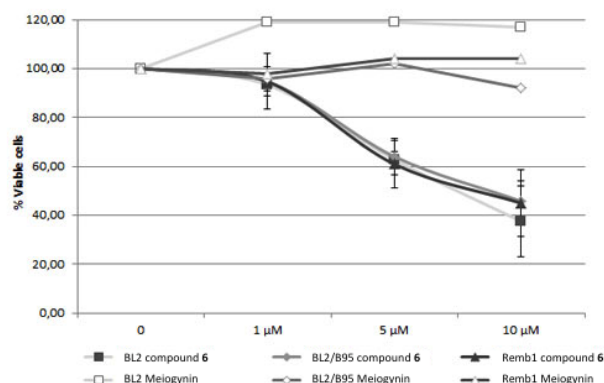


Figure 2. Cytotoxicity evaluation of compound **6** in BL and LCL. Cells were treated, or not, with various concentration of compound **6** for 24 h. After cell lysis, an MTT assay was performed and cell viability was determined by reading the absorbance at 550 nm. Results are expressed in percentage as compared to untreated cells. The values presented (mean ± s.d.) are from three independent experiments.

We then confirmed that the cytotoxic effect of compound **6** was due to its ability to induce apoptosis. Apoptosis was first assessed by flow cytometry, after labeling the cells with annexin-V-FITC and PI. As shown in Figure 3, treatment with 7.5 μM of compound **6** for 24 h strongly induced apoptosis in all cell lines (71 ± 3.8%, 52 ± 14% and 54 ± 14% of apoptotic cells for BL2, BL2/B95 and Remb1 respectively).

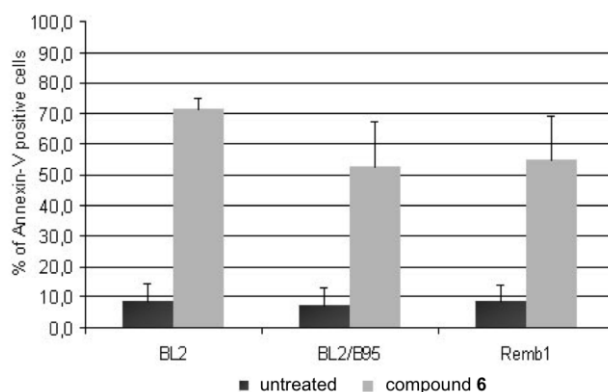


Figure 3. Effects of compound **6** treatment on the induction of apoptosis in BL and LCL. Cells were treated or not with 7.5 μM of compound **6** for 24 h. Cells were labeled with annexin-V-FITC and PI and analyzed with a FACSCalibur flow cytometer to determine the percentage of apoptotic cells. The values presented (mean ± s.d.) are from three independent experiments.

Next, apoptosis was confirmed by measuring the cleavage of polyadenosine diphosphate ribose polymerase (PARP), a direct target of caspase-3, by western blots. Thus, treatment with compound **6** induced a strong cleavage of PARP (Figure 4).

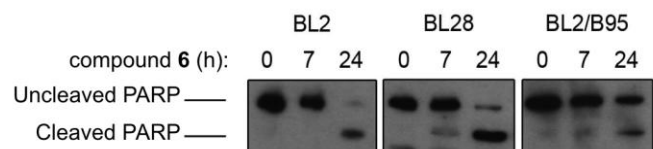


Figure 4. Effects of compound **6** treatment on PARP cleavage. Cells were treated, or not, with 7.5 μ M of compound **6** for 24 h. Apoptosis levels were determined by assessing polyadenosine diphosphate-ribose polymerase (PARP) cleavage on western blots. The data are representative of at least three independent experiments.

Finally, in order to identify the nature of the interactions between compound **6** and Bcl-xL, at the molecular level, docking calculations were carried out using a conformer of Bcl-xL generated previously using a ligand-driven conformer selection from molecular dynamics simulations^{19,20} that was found to be the most relevant for the interaction with compounds of the meioygnin A family.^{19,20} Compound **6** is accommodated in the binding site in a similar way to meioygnin A **1**, with interactions that are predominantly hydrophobic (Figure 5). The only notable exception is the ionic interaction between the carboxylate substituent of the bicyclic system and the side chain of Arg139, which most likely provides a major contribution to the overall binding affinity.

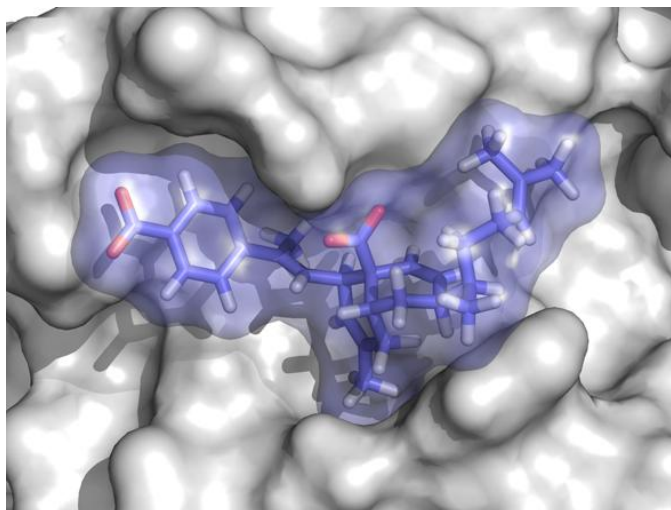


Figure 5. Docking complex of compound **6**, colored in blue, with a selected conformation of Bcl-xL.

In conclusion, the potential of synthetic analogues of a natural sesquiterpene dimer as dual Mcl-1 and Bcl-xL inhibitors was disclosed. Indeed, compound **6** was found to be active on these two proteins, cytotoxic at the micromolar range on three lymphoid cell lines and was shown to induce apoptotic cell death through a classical pathway involving caspase proteases. These compounds form a new family of dual Bcl-xL and Mcl-1 inhibitors. Work is currently underway in our laboratory²⁰ in order to elaborate new analogs of meioygnin A **1** with improved cytotoxicity and anti-apoptotic properties.

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