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Identification from a Combinatorial Library of a Small Molecule that Selectively Induces Apoptosis in Cancer Cells

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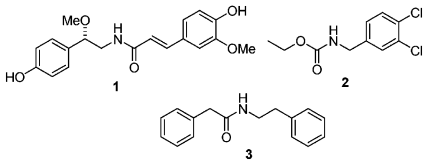
Apoptosis, or programmed cell death, is a highly conserved process used by multicellular organisms to rid themselves of unwanted or damaged cells.[1] Hallmarks of apoptosis include cellular membrane blebbing, cleavage of certain nucleases and polymerases, and activation of cysteine proteases known as caspases. From a medicinal perspective, small molecules that either inhibit or induce apoptosis have significant therapeutic potential.[2] For example, degenerative disorders such as Alzheimer's and Parkinson's diseases are thought to result from an aberrant increase in apoptosis;[3] in such cases apoptotic inhibitors hold considerable medicinal promise. In contrast, the onset of certain cancers has been traced to a missed apoptotic signal,[4] and in these cases compounds that induce apoptosis (such as etoposide, doxorubicin, and camptothecin) have proven to be powerful chemotherapeutic agents. However, for a compound to be medicinally useful it is critical that this apoptotic induction be *selective* for cancerous versus non-cancerous cells. Indeed, it is rare to find compounds that have the selectivity needed to merit serious consideration as chemotherapeutic agents.[5]

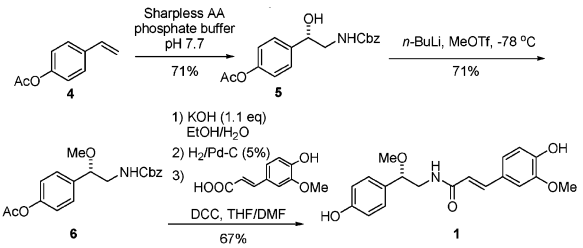
In searching for molecules that selectively induce apoptosis in cancer cells, we aimed to synthesize a combinatorial library of compounds whose members would likely be active in apoptotic assays. Among the classes of molecules that have displayed activity in either pro- or anti-apoptotic assays are *N*-acylated aromatic amines, as exemplified by a natural product from *Isodon excisus* (**1**),[6] carbamate **2**,[7] and *N*-phenethyl-2-phenylacetamide (NPPA) **3**.[8] Thus, due to its modular structure and biological activity, natural product **1** appeared as an attractive target for combinatorial derivatization and evaluation in apoptotic assays.



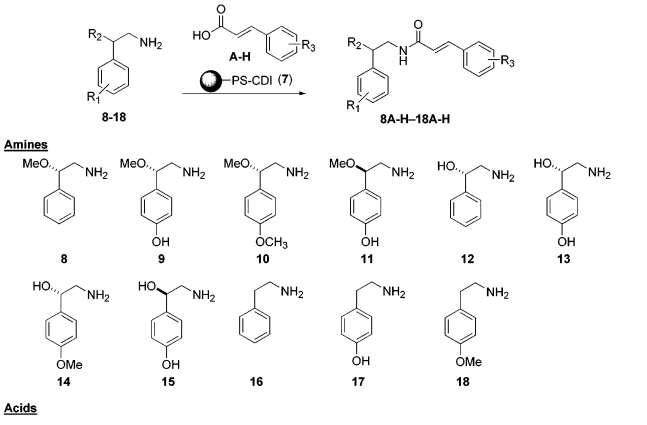
The synthesis of natural product **1** was performed as described in Scheme 1. Asymmetric aminohydroxylation under pH-controlled conditions[9] on styrene **4** gave the secondary alcohol **5** as the dominant regioisomer. The enantiomeric ratio of the product was 85:15, which was improved to >99:1 by crystallization. After *O*-methylation and deprotections, the resulting primary amine was *N*-acylated with ferulic acid activated by DCC to provide **1**. Compound **1** was modestly active (IC_{50} = 273 μM) in cytotoxicity assays with U-937 cells.

A library of derivatives of the natural product **1** was then created (Scheme 2), and its members were evaluated for their ability to induce cell death. The eight acid and eleven amine building blocks depicted in Scheme 2 were coupled in parallel, using the polymeric-supported carbodiimide **7**, to provide 88 amide products. After simple filtration to remove the resin, between 2 and 5 mg of amide was typically obtained. A determination was made of the precise milligram amount for every product, which allowed for all

Scheme 1. Synthesis of Natural Product **1**



Scheme 2. Synthesis of a Library of Compounds Based on **1**



molecules to be prepared as equimolar stock solutions for biological screening. Evaluation of every library member via LC−MS showed that all 88 of the amides were successfully synthesized and that the average purity of these products was 85% (see Supporting Information).

A three-tiered system was devised to identify compounds that selectively induce apoptosis in cancer cells. First, all compounds were screened at 100 μM in a high-throughput manner for their ability to induce death in two cancer cell lines, HL-60 (leukemia) and U-937 (lymphoma). Those molecules that showed cytotoxicity in both cell lines were then evaluated for their apoptotic versus necrotic properties. Those compounds that induced apoptosis then had their toxicity to non-cancerous white blood cells assessed.

Several of the 88 compounds were able to induce cell death at 100 μM in U-937 and HL-60 cells as measured by a dye bioreduction assay (Figure 1).[10] The three most potent of these compounds, **12-D**, **13-D**, and **15-D** were subsequently resynthesized, purified, and tested at multiple concentrations to determine IC_{50} values. Compound **13-D** was the most potent, with an IC_{50} value of 44 μM in U-937 cells, and compounds **12-D** and **15-D** had IC_{50} values of 61 and 109 μM, respectively.

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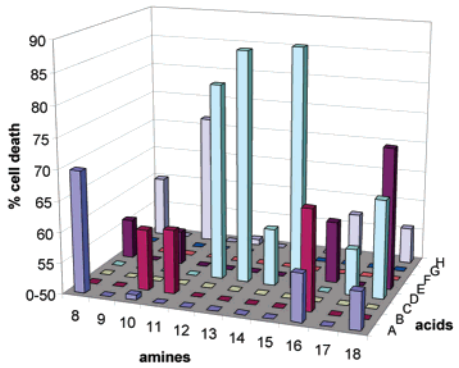

Figure 1. Induction of death in U-937 cells by library members at 100 μM.

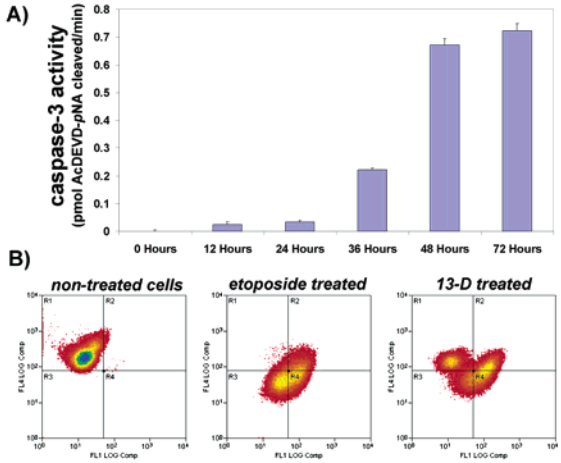

Figure 2. Induction of apoptosis by **13-D**. (A) Caspase-3 activity of U-937 cells treated with 100 μM **13-D**. (B) Flow cytometry of U-937 cells treated with 100 μM **13-D** and stained with JC-9 after 72 h; nontreated and etoposide-treated cells are shown as controls.

Further experiments assessed whether the observed death from **12-D**, **13-D**, and **15-D** was due to apoptosis or necrosis. In this regard, compound **13-D** appeared to be a fairly potent apoptotic inducer. Cells treated with this molecule showed several hallmarks of apoptotic cell death, including strong induction of caspase-3 activity (Figure 2A) and staining with the apoptotic-specific dye JC-9 (Figure 2B) as assessed by flow cytometry; JC-9 provides a sensitive readout on mitochondrial depolarization, and dyes of this class are commonly used to quantitate apoptosis.[11] In this case, 100 μM **13-D** had induced apoptosis in over 70% of the cell population after 72 h (as quantitated from Figure 2B). Additional evidence of apoptosis was provided by microscopy, which showed membrane blebbing and cell shrinkage in the **13-D** treated samples (see Supporting Information).

Finally, the selectivity of **13-D** for cancerous white blood cells over non-cancerous white blood cells was determined. For this experiment, the spleen from a euthanized mouse was harvested, and the splenocytes were isolated.[12] The T-cells were then stimulated to grow by the addition of concanavalin A. Remarkably, as the images in Figure 3A show, high concentrations (500 μM) of compound **13-D** showed virtually no toxicity toward the actively dividing splenocytes, while almost completely killing the cancerous U-937 cells. The IC_{50} of **13-D** was 44 μM for the cancerous U-937 cells, but greater than 1000 μM for the non-cancerous splenocytes. Additionally, in a separate experiment T-cells were purified from the heterogeneous splenocyte mixture, stimulated to grow with concanavalin A (in the presence or absence of 500 μM **13-D**), and

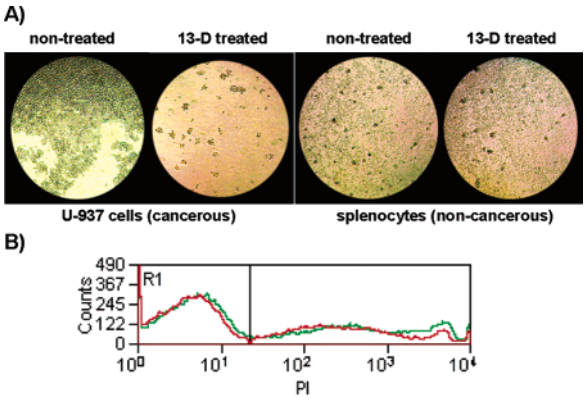

Figure 3. Cancerous U-937 cells are almost completely killed by **13-D**, whereas non-cancerous white blood cells show virtually no death. (A) Cells were treated with 500 μM **13-D** for 72 h. The IC_{50} of **13-D** for U-937 cells is 44 μM, whereas it is greater than 1000 μM for the splenocytes. (B) The T-cell population from the mouse splenocytes was purified and stimulated to grow, treated with 500 μM **13-D** for 72 h, and stained with propidium iodide. Flow cytometry analysis of the nontreated (green) and treated (red) samples indicates a viable cell population (R1) of 86 and 87%, respectively.

death was assessed after 72 h. Again, **13-D** had virtually no effect on these purified non-cancerous cells (Figure 3B).

In summary, we have identified a small molecule that selectively induces apoptosis in cancerous white blood cells but is nontoxic toward non-cancerous white blood cells. In the process, a facile synthetic route for the synthesis of natural product **1** and a library of its derivatives has been developed. Further studies on the biological basis of this selectivity will be reported in due course.

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Supporting Information Available: Full experimental protocols and characterization data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) For reviews on apoptosis see: (a) Blatt, N. B.; Glick, G. D. *Bioorg. Med. Chem. Lett.* **2001**, *9*, 1371−1384. (b) Newmeyer, D. D.; Ferguson-Miller, S. *Cell* **2003**, *112*, 481−490. (c) Huang, Z. *Chem. Biol.* **2002**, *9*, 1059−1072.
(2) (a) Reed, J. C. *Nat. Rev. Drug Dis.* **2002**, *1*, 111−121. (b) Makin, G.; Dive, C. *Trends Mol. Med.* **2003**, *9*, 251−255.
(3) (a) Hartmann, A. et al. *Proc. Natl. Acad. Sci.* **2000**, *97*, 2875−2880. (b) Mattson, M. P. *Nat. Rev. Mol. Cell Biol.* **2000**, *1*, 120−129. (c) Marx, J. *Science* **2001**, *293*, 2192−2194.
(4) Johnstone, R. W.; Ruefli, A. A.; Lowe, S. W. *Cell* **2002**, *108*, 153−164.
(5) Haskell, C. M., Ed. *Cancer Treatment*, 5th ed.; W. B. Saunders Company: Philadephia, 2001; pp 62−87.
(6) Lee, C.; Kim, J.; Lee, H.; Lee, S.; Kho, Y. *J. Nat. Prod.* **2001**, *64*, 659−660.
(7) Nguyen, J. T.; Wells, J. A. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 7533−7538.
(8) Hwang, S.-Y.; Paik, S.; Park, S.-H.; Kim, H.-S.; Lee, I.-S.; Kim, S.-P.; Baek, W. K.; Suh, M.-H.; Kwon, T. K.; Park, J.-W.; Park, J.-B.; Lee, J.-J.; Suh, S. I. *Int. J. Oncol.* **2003**, 151−157.
(9) Nesterenko, V.; Byers, J. T.; Hergenrother, P. J. *Org. Lett.* **2003**, *5*, 281−284.
(10) Neither compound **1** nor any library members showed activity in assays exploring inhibition of etoposide-induced apoptosis.
(11) Cossarizza, A.; Salvioli, S. *Methods Cell Biol.* **2001**, *63*, 467−486.
(12) Mouse splenocytes are commonly used to assess the toxicity of small molecules. For examples see: (a) Prater, M. R.; Gogal, R. M.; Blaylock, B. L.; Longstreth, J.; Holladay, S. D. *Food Chem. Toxicol.* **2002**, *40*, 1863−1873. (b) Blake, C. A.; Nair-Menon, J. U.; Campbell, G. T. *Endocrine* **1997**, *6*, 243−249. (c) Yamaura, K.; Ogawa, K.; Yonekawa, T.; Nakamura, T.; Yano, S.; Ueno, K. *Biol. Pharm. Bull.* **2002**, *25*, 201−205. (d) Li, Q.; Hirata, Y.; Piao, S.; Minami, M. *Toxicology* **2000**, *150*, 179−189.

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