

The Compound 13-D Selectively Induces Apoptosis in White Blood Cancers versus Other Cancer Cell Types

Karson S. Putt,^[b] Vitaliy Nesterenko,^[a] Robin S. Dothager,^[b] and Paul J. Hergenrother^{*[a, b]}

As general cytotoxins are still the backbone of anticancer chemotherapy, the identification of selective inducers of cell death in defined cancer types and subtypes is one of the major goals of modern oncology research. Thus, compounds identified with such selectivity have utility as probes of cancer-type-specific biological pathways, and optimized versions have potential in targeted anticancer therapy. Described herein is the discovery that compound 13-D selectively induces apoptotic cell death in white blood cancer cell lines but not in other cancer cell lines. Further

experiments indicate that this selectivity is not simply due to selective cell permeability. The compound localizes to both the nucleus and cytoplasm and arrests cells in the prophase/prometaphase of the cell cycle, and there is a very sharp dependence of activity on compound structure, with the trans- α,β -unsaturated amide of 13-D being critical for inducing cell death. The macromolecular target of 13-D could be involved in white blood cell-specific oncogenic pathways.

White blood cell cancers constitute a diverse array of neoplasms including leukemia, lymphoma, and myeloma. Leukemias are divided into four major types, acute myelogenous leukemia (AML), acute lymphocytic leukemia (ALL), chronic myelogenous leukemia (CML), and chronic lymphocytic leukemia (CLL). Lymphomas are divided into two major categories, Hodgkin's and non-Hodgkin's lymphoma, and multiple myeloma constitutes approximately 90% of all myeloma cases. The prognosis for patients receiving one of these diagnoses is highly variable. The drug Gleevec (imatinib) has been recently developed based on the known chromosomal translocation (the "Philadelphia chromosome") in patients with CML, a translocation that results in the production of the Bcr-Abl kinase fusion protein.^[1] Gleevec has had widely celebrated success as a selective inhibitor of this kinase, and the patient-response rate to this drug in some studies has been a remarkable 90%.^[2] Similarly, Hodgkin's lymphoma can be cured in >80% of cases.^[3] In contrast, the survival of patients with CLL (the most common type of leukemia in the western hemisphere) varies widely, with many dying soon after diagnosis.^[4] In addition some lymphomas, such as follicular lymphoma, are unresponsive to chemotherapeutic agents.^[5] Thus, while great strides have been made in the fight against certain white blood cell cancers, others remain untreatable or are only moderately responsive to even the most potent of chemotherapeutic agents.

The vast majority of anticancer agents can be classified as antimetabolites (methotrexate, fluorouracil), DNA-damaging agents (cisplatin, doxorubicin), or antimitotics (Taxol, vincristine).^[6] In general, antimetabolites and DNA-damaging agents arrest cellular growth in the S (DNA synthesis) phase of the cell cycle, while the antimitotics arrest cells in the M (mitosis) phase; after cell-cycle arrest, cells typically die through apopto-


sis. Such compounds have been the backbone of anticancer treatments for over five decades and have saved countless lives. The ultimate goal of personalized medicine, as it applies to anticancer therapy, however, is to move away from general cytotoxic agents and to develop discrete classes of drugs that are specific for distinct cancer types and subtypes.

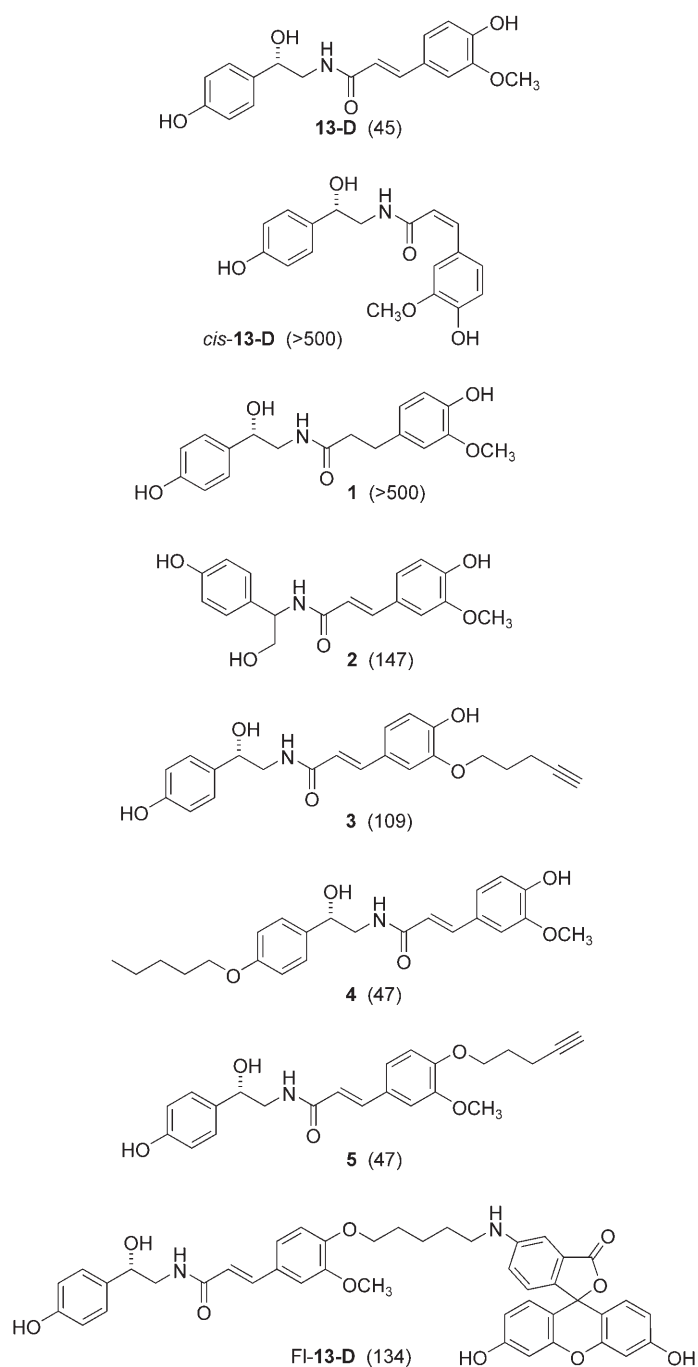
We previously constructed a library of 88 amides and evaluated their proapoptotic activity.^[7] After synthesis and purification, all compounds were evaluated for their ability to induce death in two white blood cancer cell lines, HL-60 (acute promyelocytic leukemia, a subtype of AML) and U-937 (histiocytic lymphoma, non-Hodgkin's type). Those compounds that were active death inducers were evaluated for their ability to induce death through apoptosis, and those proapoptotic agents thus identified were tested against noncancerous white blood cells isolated from the spleen of a mouse. Through this multitiered assay system, we discovered one particular amide (compound 13-D, Scheme 1) that selectively induced cell death in the two white blood cancer cell lines, but had no effect on the noncancerous mouse splenocytes.^[7]

Herein we report that compound 13-D shows an unusual cytotoxicity profile, in that it only induces cell death in white

[a] V. Nesterenko, Prof. Dr. P. J. Hergenrother
Department of Chemistry, University of Illinois, Roger Adams Laboratory
Urbana, IL 6180 (USA)
Fax: (+1) 217-244-8024
E-mail: hergenro@uiuc.edu

[b] K. S. Putt, R. S. Dothager, Prof. Dr. P. J. Hergenrother
Department of Biochemistry, University of Illinois, Roger Adams Laboratory
Urbana, IL 6180 (USA)

 Supporting information for this article is available on the WWW under <http://www.chembiochem.org> or from the author: full experimental protocols and NMR spectra of all new compounds.



Scheme 1. Various structural derivatives of **13-D** were incubated with HL-60 cells for 72 h. The IC_{50} values (given in parentheses [μM]) were determined by using the MTS bio-reduction assay.

blood cancer cell lines. We also show that this compound has no detectable effect on several noncancerous cell types, and we present further evidence showing that the death induction in white blood cancer cell lines is through apoptosis. In addition, our data indicate that, although **13-D** is toxic to white blood cell cancers only, the compound penetrates all cancer cell types; thus, its selective cytotoxicity is not merely a function of selective cell permeability. Compounds that selectively kill white blood cell cancers could be useful in dissecting the

underlying biology of leukemia, lymphoma, and myeloma, and the cellular target of **13-D** could be a useful macromolecule to target in the development of white blood cell-specific anti-cancer therapies.

Results

Evaluation of **13-D** in various cancer cell lines and normal cell types

As compound **13-D** had previously shown intriguing cancerous versus noncancerous selectivity in white blood cell cancers,^[7] it was evaluated against a variety of other cancer types by using the colorimetric bio-reduction of the MTS dye [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt] to determine cell death. This tetrazolium dye is quickly converted to the formazan product by metabolically active cells, and thus provides a sensitive readout of viable cell numbers that can be monitored spectroscopically. The cell line, cancer type, and IC_{50} value for death induction by **13-D** are displayed in Table 1. While **13-D** did kill the three

Table 1. Compound **13-D** is selective for white blood cell cancers.

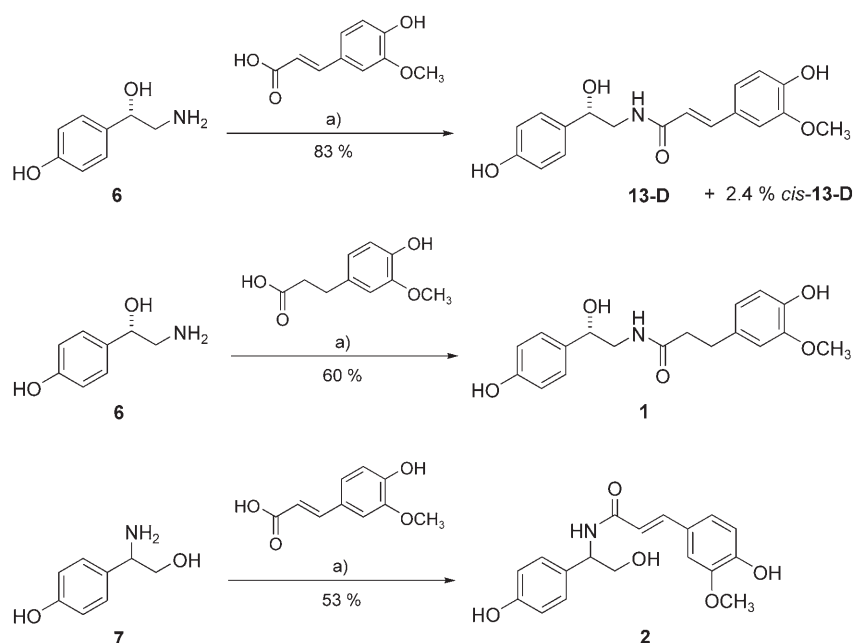
Cell line	Cancer ^[a] /cell ^[b] type	IC_{50} 13-D [μM]
U-937	lymphoma (human)	36
HL-60	leukemia (human)	45
K-562	leukemia (CML, human)	37
B16-F10	melanoma (mouse)	> 500
UACC-62	melanoma (human)	293
SK-MEL-5	melanoma (human)	> 500
CRL-1782	melanoma (human)	> 500
NCI-H226	mesothelioma (human)	> 500
Hs578t	breast cancer (human)	> 500
MDA-MB-231	breast cancer (human)	> 500
ACHN	renal cancer (human)	> 500
SK-N-SH	neuroblastoma (human)	> 500
MCF-10A	noncancerous breast epithelial (human)	> 500
Hs578Bst	noncancerous breast epithelial (human)	> 500
Hs888Lu	noncancerous lung fibroblast (human)	> 500
Bone marrow from healthy human donors		> 500

[a] Cancerous cell lines were treated with **13-D** for 72 h, and the IC_{50} was determined by using the MTS bio-reduction assay. [b] Noncancerous cell types were treated with **13-D** for 72 h, and the IC_{50} was determined by using the MTS bio-reduction assay. No toxicity was observed in any non-cancerous cell types.

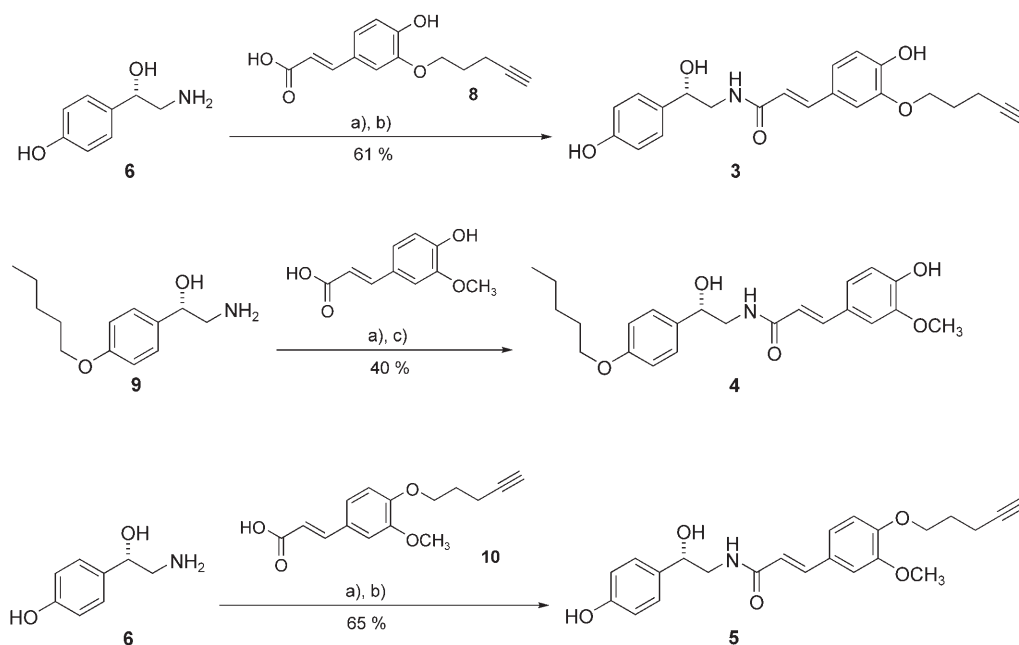
white blood cell cancer lines in the panel (HL-60, U-937, and K-562), it had little or no effect on a wide range of other cancer cell lines including melanoma, lung, breast, renal, and neuroblastoma. Consistent with the previous data obtained with mouse splenocytes, **13-D** showed no toxicity to a battery of normal human cells, including nontumorigenic breast and lung cells and white blood cells derived from the bone marrow of healthy human donors (Table 1).

Structure–activity relationship for 13-D

To further define the relationship between the structure of 13-D and its proapoptotic function, several derivatives were synthesized and evaluated. Analogues of 13-D were synthesized in which the double-bond geometry was varied, in which the double bond was eliminated, and in which the phenolic hydroxyls were alkylated. Compound 13-D, *cis*-13-D, a derivative in which the double bond was saturated (1), and a hydroxymethyl compound (2) were prepared. As shown in Scheme 2,



Scheme 2. Synthesis of derivatives of 13-D. a) 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI), 1-hydroxybenzotriazole (HOBT), THF/DMF 8:1.

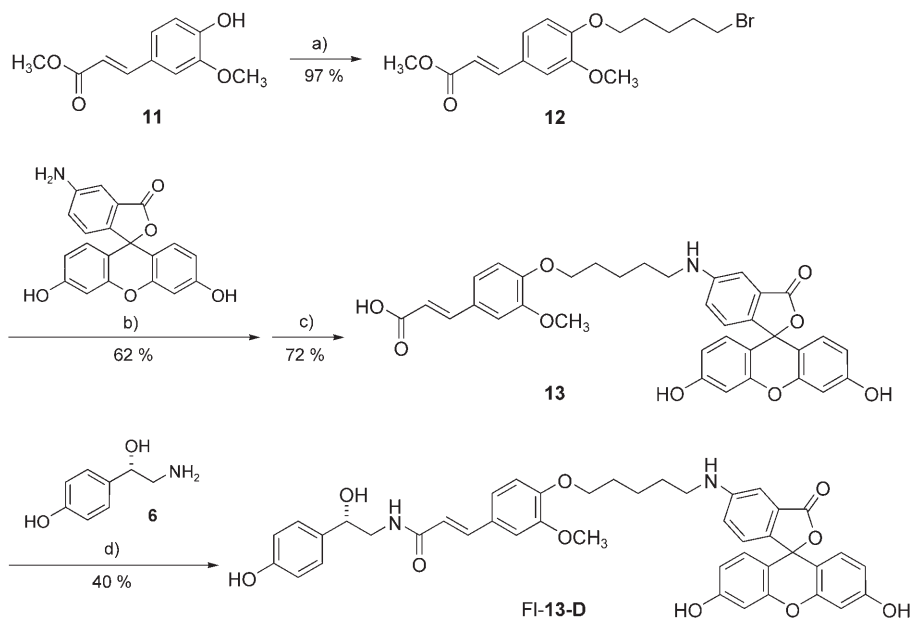


Scheme 3. Synthesis of derivatives of 13-D with modifications on the aromatic rings. a) EDCI, HOBT; b) THF/DMF 8:1; c) THF.

cis-13-D was isolated as a minor impurity in the 13-D synthesis, and compounds 1 and 2 were synthesized through amide couplings. Versions of 13-D with modifications on the aromatic rings (compounds 3, 4, and 5 in Scheme 1) were synthesized through amide couplings as shown in Scheme 3.

The death-inducing properties of these 13-D derivatives were then determined by using the HL-60 cell line (Scheme 1). Strikingly, any modification of the α,β -unsaturated olefin completely abolished activity; both *cis*-13-D and the fully saturated compound 1 had no effect on HL-60 cells. In contrast, it appeared that certain functionality

on the aromatic rings could be modified without drastically affecting activity. Compound 3 was approximately twofold less potent than 13-D, whereas compounds 4 and 5 had similar activity to the parent compound. Building on this information, a fluorescently labeled version of 13-D (compound FI-13-D), was constructed through the alkylation/amidation sequence shown in Scheme 4. This fluorescent conjugate was less water-soluble than 13-D and induced cell death in the HL-60 cell line with an IC_{50} of 134 μ M. Upon treatment of HL-60 cells with FI-13-D, membrane blebbing was observed by microscopy; this is consistent with FI-13-D inducing apoptotic cell death.



Scheme 4. Synthesis of fluorescently labeled **13-D** (FI-**13-D**). a) NaH, Br(CH₂)₃Br, THF; b) Cs₂CO₃, DMF; c) KOH, EtOH/H₂O 9:1; d) EDCl, HOBT, *i*PrOH.

Compound **13-D** induces death by apoptosis

Treatment of U-937 cells with **13-D** had been previously shown to induce depolarization of the mitochondrial membrane, cause an increase in caspase 3/7 activity, and induce cell shrinkage and membrane blebbing—all hallmarks of apoptotic cell death.^[7] Two other key hallmarks of apoptosis are the exposure of phosphatidylserine on the cell membrane and condensation of chromatin. Treatment of HL-60 cells with **13-D** did indeed lead to phosphatidylserine exposure on the cell surface (as measured by staining with fluorescently labeled annexin V) and chromatin condensation (as visualized with Hoechst-33258 dye). This data (displayed in Figure 1) is consistent with the previous data, which indicated that **13-D** induces death by apoptosis.^[7]

Compound **13-D** penetrates all cell types

A possible explanation for the selectivity of **13-D** for white blood cell cancers is that the compound is simply not able to permeate into the other cancer cell lines. To test this hypothesis, the various cancer cell lines were exposed to the fluorescent derivative of **13-D**, compound FI-**13-D**, and extensively washed, then the cellular fluorescence was examined by flow cytometry. As shown in Figure 2, the fluorescence sharply increases in all cell types upon exposure to FI-**13-D** (see the Supporting Information for the other cell lines), however the total increase in fluorescence for each particular cell line does not correlate with the toxicity of the molecule and appears to be dependent upon the volume of the cell. This result shows that FI-**13-D** penetrates all cell lines tested and suggests that **13-D** is similarly cell permeable, but once inside is only able to induce apoptotic death in white blood cell cancers.

Compound **13-D** induces cell cycle arrest in the G₂/M phase

To begin to explore the mechanism by which **13-D** induces apoptotic cell death, its cell cycle-arresting properties were examined. Treatment of HL-60 cells with increasing concentrations of **13-D** for 12 h leads to a clear arrest in the G₂/M phase of the cell cycle (Figure 3). Treatment with compound **1**, a non-toxic saturated version of **13-D**, did not affect cell-cycle progression (see the Supporting Information). To determine whether the arrest occurs in G₂ or a specific phase of mitosis, U-937 cells treated with **13-D** or the vehicle alone were fixed and stained with Hoechst dye. The number of cells in each phase of the cell cycle was counted by using a

fluorescent microscope; over 650 cells were counted in each group. As expected, the proportion of interphase cells showed a significant decrease ($p < 0.0005$) between the **13-D** treated cells and the vehicle control-treated cells; this is indicative of a mitotic arrest. A significant increase in the proportion of prophase cells ($p < 0.0005$) and a much smaller, but still significant,

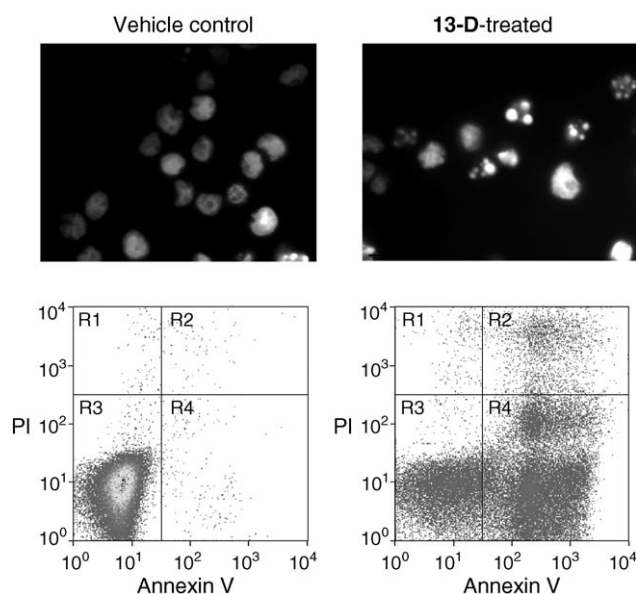


Figure 1. **13-D** induces apoptosis in HL-60 cells. Top: Chromatin condensation as visualized by Hoechst-33258 staining after 24 h of treatment with 100 μM **13-D**. Cells treated with compound **13-D** show the punctate nuclei that are indicative of apoptosis. Bottom: Exposure of phosphatidylserine as measured by Annexin V staining after 48 h of treatment with 100 μM **13-D**. Cells treated with compound **13-D** shift from the low Annexin V and low propidium iodide region to the high Annexin V and low propidium iodide region; this is indicative of apoptosis.

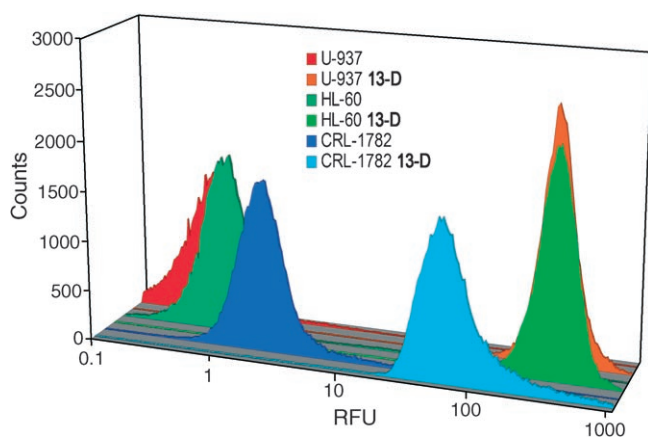


Figure 2. 13-D penetrates all cell types. Cells were treated with 50 μM FI-13-D for 24 h. After extensive washing, the fluorescent intensity of each cell was determined by flow cytometry. See the Supporting Information for other cell lines.

increase in the proportion of metaphase cells ($p=0.05$) were also observed between the 13-D- and vehicle-only-treated groups. Based on this data, it can be concluded that 13-D causes mitotic arrest in either the prophase or prometaphase of the cell cycle.

13-D localizes to both the nucleus and cytoplasm

Certain anticancer compounds have defined subcellular localization consistent with their mechanism of action. The fluorescent analogue FI-13-D was used to probe the subcellular localization of 13-D. Compound FI-13-D was incubated for 24 h with U-937 cells, and the cells were examined by fluorescence microscopy. The cellular nuclei were stained with the Hoechst dye, and fluorescently labeled phalloidin was used to stain actin. As shown by the images in Figure 4, FI-13-D does not appear to localize to any specific location, as fluorescence from this compound is observed in both the nucleus and the cytoplasm.

Discussion

The treatment of certain forms of CML has been revolutionized by the recent introduction of Gleevec into the pharmacopoeia. The Bcl-Abl kinase created by the "Philadelphia chromosome" translocation in patients with CML presents an unusual opportunity: a protein target that is present in cancer cells but not in noncancerous cells. Such unique proteins are not present in the majority of cancers, and one focus of anticancer research has therefore been on proteins whose levels are elevated (or reduced) in the cancer cell. Another way to discover novel anticancer targets and proteins that can be exploited in targeted cancer therapy is to first identify small molecules that selectively induce cell death in one type of cancer, then identify the precise mode of action and biological target of the small molecule. Compounds that selec-

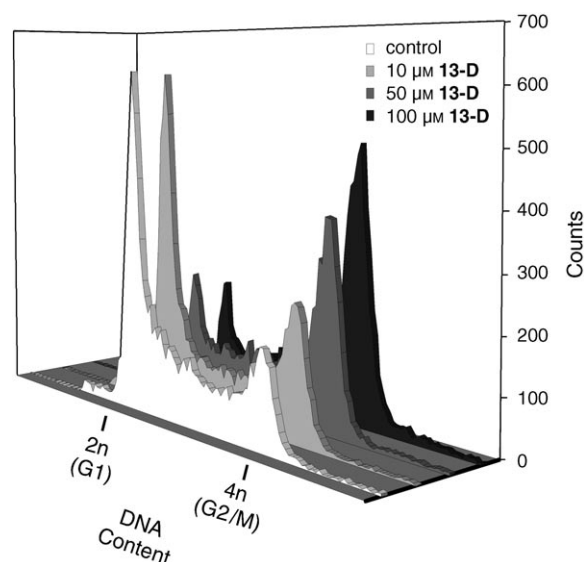


Figure 3. After 12 h of treatment with 13-D, cell-cycle analysis shows that HL-60 cells arrest in the G2M phase of the cell cycle.

tively induce death in one cell type over all others are thus valuable both as probes and as potential therapeutics.

In 2001 a natural product from *Isodon excisus* was isolated and reported to possess antiapoptotic properties.^[8] Through independent synthesis and subsequent biological assays, however, we^[7] and others^[9] found this compound to be devoid of antiapoptotic activity. A combinatorial library based on the structure of this natural product was synthesized, and, through a multitiered assay protocol, compound 13-D was identified as a small molecule that induces apoptotic death in cancerous white blood cells but not in noncancerous control cells isolated from the spleen of a mouse. Described herein is the discov-

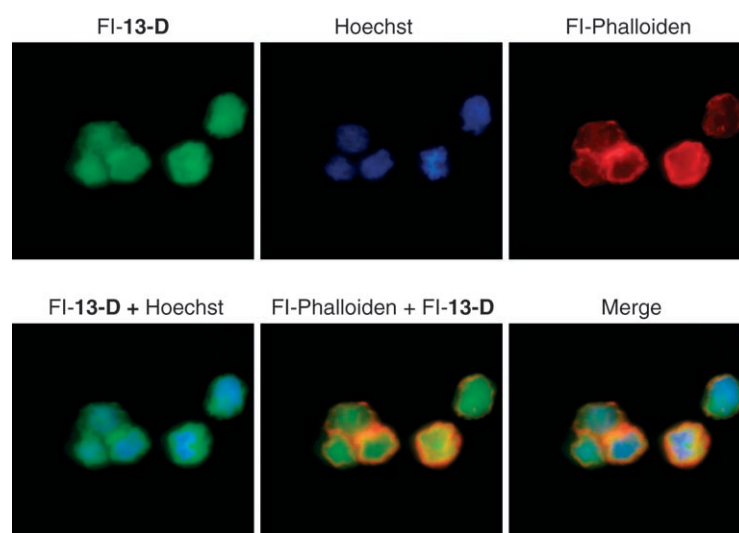


Figure 4. U-937 cells were treated with 100 μM FI-13-D for 24 h. The cells were then fixed, stained with FI-phalloidin and Hoechst dye and visualized by fluorescent microscopy. As is apparent in the images, FI-13-D (green) is present in the cytosol and the nucleus and shows no defined subcellular localization. Hoechst dye (blue) is used as a nuclear stain, while FI-phalloidin (red) binds to actin and thus defines the cytoplasmic space.

ery that compound **13-D** is not only selective for white blood cell cancers over noncancerous cell types, but is also selectively toxic to white blood cancer cell lines over all other cancer cell lines. The lack of toxicity of **13-D** to nontumorigenic cell types is also of note (Table 1). Bone marrow cells are a rapidly dividing normal cell type that is often killed during chemotherapeutic regimens, thus limiting dosing and treatment length. In cell culture, the bone marrow obtained from healthy human donors is considered a good predictor of the toxicity of small molecules,^[10–13] and indeed many clinically used anticancer drugs are toxic to such bone marrow cells with IC_{50} values in the nanomolar to picomolar range.^[14] Compound **13-D** had no observed toxicity to bone marrow cells, with an IC_{50} value of $> 500 \mu\text{M}$ after 72 h of incubation. Such selective cytotoxicity is fairly unusual, as the majority of anticancer compounds are cell-cycle-arresting agents that induce death in all rapidly dividing cell types.

From the structure–activity relationship data (Scheme 1), the *trans* α,β -unsaturated amide functional group of **13-D** appears to be critical for activity, as neither the reduced compound (**1**) nor the version with the *cis* olefin geometry (*cis*-**13-D**) showed any death-inducing activity. Certain phenolic hydroxyls could also be derivatized without loss of activity, an observation that allowed for the synthesis of a fluorescently labeled version of **13-D**, compound FI-**13-D**. Compound FI-**13-D** was then used in a variety of experiments to probe the cell permeability and subcellular localization of **13-D**. The selective induction of cell death by **13-D** in white blood cancer cell lines does not seem to be a function of its ability to penetrate cells. As shown in Figure 2, compound FI-**13-D** penetrates all cell types, as indicated by the increase in fluorescence observed in the presence of the compound relative to the background fluorescence of the cell. However, as indicated by the data in Figure 4, compound FI-**13-D** does not appear to localize specifically to the cytoplasm or the nucleus, but is instead observed in both locations. This is in contrast to certain anticancer drugs that have a defined subcellular localization. The fact that **13-D** apparently penetrates all cell types but induces apoptosis only in white blood cancers suggests that the level of its macromolecular target might be elevated in white blood cancers relative to other cancerous and noncancerous cell types.

Compounds that selectively induce death in one cancer cell type can be useful both as therapeutics and in the illumination of cell-type-specific oncogenic pathways. Herein is described the discovery that compound **13-D** is selectively proapoptotic in white blood cancer cell lines. Elucidation of the exact macromolecular target of **13-D** will ultimately be required for a full explanation of its interesting anticancer profile.

Experimental Section

Materials: RPMI-1640, Iscore's modified double minimal essential medium (IDMEM), Eagle's minimal essential medium, and FITC-conjugated tubulin antibody were purchased from Sigma (St. Louis, MO). Annexin V Alexa Fluor 488 conjugate, propidium iodide, Hoechst dye, Alexa Fluor 568 phalloidin conjugate, and ProLong Gold Antifade reagent were purchased from Molecular Probes

(Eugene, OR). MTS/PMS CellTiter 96 cell proliferation assay reagent was purchased from Promega (Madison, WI). Fetal bovine serum was purchased from Biomed (Foster City, CA). 96-well microtiter plates, microscope slides, microscope coverslips, horse serum, eppendorf tubes, and all other reagents were purchased from Fisher (Chicago, IL).

Cell culture conditions: U-937, HL-60, CRL-1872, ACHN, NCI-H226, MCF-10A, Hs888Lu, Hs578Bst, SK-MEL-5, MDA-MB-231, and UACC-62 cells were grown in RPMI 1640 medium supplemented with 10% FBS. SK-N-SH, B16-F10, and Hs578t cells were grown in Eagle's minimal essential medium with Earle's BSS, sodium bicarbonate (1.5 g L^{-1}), and 10% FBS. PC-12 cells were grown in RPMI 1640 medium supplemented with 5% FBS and 10% horse serum. Human bone marrow was grown in IDMEM supplemented with 40% FBS. All cell lines were incubated at 37°C in CO_2/air (5:95). U-937, HL-60, and K562 cells were split every two to three days, as needed. Human bone marrow was thawed from frozen stock and immediately diluted and used for experiments. All other cells were split when they reached approximately 90% confluency.

Determination of IC_{50} values in various cell lines: Medium containing various concentrations of **13-D** or **13-D** derivatives ($50 \mu\text{L}$) was added to each well of a 96-well plate except control wells, which contained only DMSO. U-937, HL-60, K562, and human bone marrow cells were harvested by centrifugation. All other cell lines were first trypsinized before centrifugation. Cells were then resuspended in medium and diluted to either 0.5×10^6 cells per mL for U-937, HL-60, K562, and human bone marrow cells or 50000 cells per mL for all other cell lines. Aliquots ($50 \mu\text{L}$) of the cell solutions were then added to each well, and the plates were incubated for 72 h. Cell death was quantitated by the addition of the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS)/PMS (phenazine methosulfate) CellTiter 96 cell proliferation assay reagent ($20 \mu\text{L}$) to each well. Viable cells are able to enzymatically modify the MTS dye to the formazan product. The plates were then incubated at 37°C for approximately 1 h until the colored formazan product formed. The absorbance was measured at 490 nm in a Spectra Max Plus 384 plate reader (Molecular Devices, Sunnyvale, CA).

Annexin V staining: Medium ($500 \mu\text{L}$) containing **13-D** ($200 \mu\text{M}$) or only DMSO as a control was added to a 24-well plate. HL-60 ($500 \mu\text{L}$) cells at a concentration of 2×10^6 cells per mL were then added to the wells. The cells were incubated for 48 h at 37°C . Cells were harvested by centrifugation and washed twice in PBS. The cells were then washed in annexin V binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl_2 , pH 7.4) and resuspended in annexin V binding buffer ($100 \mu\text{L}$). Annexin V, Alexa Fluor 488 conjugate ($5 \mu\text{L}$) was added, and the tubes were incubated at room temperature for 15 min protected from light. Annexin V binding buffer ($400 \mu\text{L}$) was then added, followed by the addition of an aqueous solution of propidium iodide ($1 \mu\text{L}$, 1 mg mL^{-1}). The fluorescent intensity of each cell was determined by flow cytometry at 525 nm (green channel) and 675 nm (red channel). At least 50000 cells were analyzed in each experiment.

Condensed chromatin staining: Medium ($500 \mu\text{L}$) containing **13-D** ($100 \mu\text{M}$) or only DMSO as a control was added to a 24-well plate. HL-60 cells ($500 \mu\text{L}$, 2×10^6 cells per mL) were then added to the wells. The cells were incubated for 48 h and harvested by centrifugation. The cells were then washed in PBS buffer ($1 \times$) followed by the addition of ice-cold 100% ethanol. The cells were fixed overnight at 4°C . Fixed cells were incubated with Hoechst-33258 ($2 \mu\text{g mL}^{-1}$) for 30 min at room temperature. A drop of cells was

then placed on a microscope slide and covered with a coverslip (No. 1 thickness). Condensed chromatin was observed at 400× magnification on a Zeiss Axiovert 100 microscope.

Permeability of 13-D fluorescein conjugate: A solution of each cell line (1 mL, 1×10^6 cells per mL) was added to a 24-well plate. A solution of FI-13-D in DMSO (5 μ L, 10 mM) or only DMSO was added to the cells. The cells were incubated for 24 h protected from light. Adherent cell lines were trypsinized, and suspension cells were directly transferred to 1.7 mL eppendorf tubes. Cells were washed in PBS (5×) and resuspended in PBS (500 μ L). The fluorescent intensity of each cell was determined by flow cytometry by using the 525 nm green channel. At least 25000 cells were analyzed in each experiment.

Cell-cycle analysis by flow cytometry: RPMI-1640 plus 10% FBS medium (500 μ L) was added to each well of a 24-well plate. Varying concentrations of 13-D or vector only were added to the plate. HL-60 cells were harvested by centrifugation at 250g for 5 min. Cells were then resuspended in RPMI-1640 plus 10% FBS, counted by using a hemocytometer, and diluted to 2×10^6 cells per mL. The cell solution (500 μ L) was then added to each well to bring the final concentration to 1×10^6 cells per well. The cells were then incubated with the compounds for 12 h and harvested by centrifugation. The cells were then washed with PBS (1×) and resuspended in ice-cold 100% ethanol. After being harvested, the cells were stored at 4 °C overnight. The cells were pelleted out of the ethanol by centrifugation, washed in PBS (1×), and resuspended in PBS (50 μ L) containing RNase A (100 μ g mL⁻¹). The cells were incubated at 4 °C for 4 h. An aliquot (400 μ L) of an aqueous solution of propidium iodide (50 μ g mL⁻¹) was then added, and the cells were analyzed by flow cytometry by using the 675 nm red channel.

Subcellular localization of 13-D: U-937 cells (1 mL, 1×10^6 cells per mL) were added to the wells of a 24-well plate in RPMI-1640 plus 10% FBS medium. A solution of FI-13-D in DMSO (10 μ L, 10 mM) or DMSO only was added to the wells. The cells were incubated for 24 h protected from light, and then harvested by centrifugation at 250g for 5 min and washed with PBS (3×). The cells were fixed in 4% formaldehyde for 20 min at 37 °C and then washed with PBS (1×) and plated onto polylysine-coated coverslips for 2 h. The cells were then permeabilized with 0.5% TX-100 in PBS for 10 min. This was followed by washing the cells with 0.1% TX-100 in PBS and staining with Alexa Fluor 568-conjugated phalloidin (20 μ g mL⁻¹) for 20 min. The cells were washed with 0.1% TX-100 in PBS (1×) and then stained with Hoechst dye (15 μ g mL⁻¹) for 20 min. The cells were washed with 0.1% TX-100 in PBS (2×) and with PBS only (1×). Cells on coverslips were mounted onto glass slides with ProLong Gold Antifade reagent. Localization of the 13-D fluorescein conjugate was viewed at 400× magnification on a Zeiss Axiovert 100 microscope.

Mitotic index of 13-D: U-937 cells (1 mL, 1×10^6 cells per mL) were added to the wells of a 24-well plate in RPMI-1640 plus 10% FBS medium. A solution of 13-D in DMSO (10 μ L, 10 mM) or DMSO only was added to the wells. The cells were incubated for 24 h and then harvested by centrifugation at 250g for 5 min. The cells were washed with PBS (2×) and then fixed in 4% formaldehyde for 20 min at 37 °C. The cells were washed with PBS (1×) and plated onto polylysine-coated coverslips for 2 h. The cells were then permeabilized with 0.5% TX-100 in PBS for 10 min. This was followed by washing with 0.1% TX-100 in PBS and blocking with 3% BSA in

PBS for 30 min. The cells were stained with a FITC-conjugated antitubulin antibody in PBS with 3% BSA for 1 h. The cells were washed once with 0.1% TX-100 in PBS and then stained with Hoechst dye (15 μ g mL⁻¹) for 20 min. This was followed by washing with 0.1% TX-100 in PBS (2×) and with PBS only (1×). Cells on coverslips were mounted onto glass slides with ProLong Gold Antifade reagent. The cells were visualized and counted with a Zeiss Axiovert 100 microscope at 400× magnification. In a given area on the coverslip, the number of interphase cells and cells of the various mitotic phases were counted. The area on the coverslip was marked by using OpenLab imaging software so that double counting could be avoided. Statistical analysis was performed to determine the difference between the population proportions by using the standard t-test.

Acknowledgements

We thank Prof. Ronald Hoffman (University of Illinois–Chicago Cancer Center) for the generous gift of human bone marrow. We acknowledge the assistance of the Flow Cytometry Facility of the Biotechnology Center and the imaging technology group of the Beckman Institute of Advanced Science and Technology, University of Illinois. We are grateful to the National Science Foundation (NSF-CAREER Award to P.J.H.) for funding this research. K.S.P. is supported by a fellowship from the American Chemical Society Division of Medicinal Chemistry. P.J.H. is a fellow of the Alfred P. Sloan Foundation.

Keywords: apoptosis • bioorganic chemistry • cancer • cell cycle • white blood cells

- [1] R. Ren, *Nat. Rev. Cancer* **2005**, 5(3), 172–183.
- [2] J. Cortes, H. Kantarjian, *J. Clin. Oncol.* **2005**, 23, 6316–6324.
- [3] J. M. Connors, *J. Clin. Oncol.* **2005**, 23, 6400–6408.
- [4] P. Kokhaei, M. Palma, H. Mellstedt, A. Choudhury, *Ann. Oncol.* **2005**, 16, ii113–ii123.
- [5] W. Hiddemann, C. Buske, M. Dreyling, O. Weigert, G. Lenz, R. Forstpointner, C. Nickenig, M. Unterhalt, *J. Clin. Oncol.* **2005**, 23(26), 6394–6399.
- [6] V. Malhotra, M. C. Perry, *Cancer Biol. Ther.* **2003**, 2(4 Suppl 1), S2–4.
- [7] V. Nesterenko, K. S. Putt, P. J. Hergenrother, *J. Am. Chem. Soc.* **2003**, 125, 14672–14673.
- [8] C. Lee, J. Kim, H. Lee, S. Lee, Y. Kho, *J. Nat. Prod.* **2001**, 64, 659–660.
- [9] X. Xing, P. Ho, G. Bourquin, L.-A. Yeh, G. D. Cuny, *Tetrahedron* **2003**, 59, 9961–9969.
- [10] S. M. Konstantinov, M. Topashka-Ancheva, A. Benner, M. R. Berger, *Int. J. Cancer* **1998**, 77, 778–786.
- [11] M. de Graaff, M. Maliepaard, D. Pluim, B. J. Froot, I. C. Slaper-Cortenbach, J. H. Schellens, *Anticancer Drugs* **1999**, 10, 213–218.
- [12] P. M. LoRusso, R. Parchment, L. Demchik, J. Knight, L. Polin, J. Dzubow, C. Behrens, B. Harrison, G. Trainor, T. H. Corbett, *Invest. New Drugs* **1999**, 16, 287–296.
- [13] O. A. Oredipe, P. M. Furbert-Harris, I. Laniyan, W. M. Griffin, R. Sridhar, *Int. Immunopharmacol.* **2003**, 3, 1537–1547.
- [14] R. S. Dohager, K. S. Putt, B. J. Allen, B. J. Leslie, V. Nesterenko, P. J. Hergenrother, *J. Am. Chem. Soc.* **2005**, 127, 8686–8696.

Received: May 29, 2006

Published online on October 19, 2006