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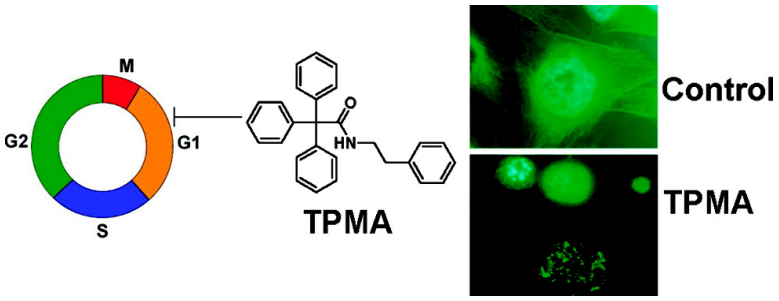

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Synthesis and Identification of Small Molecules that Potently Induce Apoptosis in Melanoma Cells through G1 Cell Cycle Arrest

Robin S. Dothager,[†] Karson S. Putt,[†] Brittany J. Allen,[‡] Benjamin J. Leslie,[‡] Vitaliy Nesterenko,[‡] and Paul J. Hergenrother*[,†,‡]

Contribution from the Department of Chemistry and Department of Biochemistry, Roger Adams Laboratory, University of Illinois, Urbana, Illinois 61801

Received November 23, 2004; E-mail: hergenro@uiuc.edu

Abstract: Late-stage malignant melanoma is a cancer that is refractory to current chemotherapeutic treatments. The average survival time for patients with such a diagnosis is 6 months. In general, the vast majority of anticancer drugs operate through induction of cell cycle arrest and cell death in either the DNA synthesis (S) or mitosis (M) phase of the cell cycle. Unfortunately, the same mechanisms that melanocytes possess to protect cells from DNA damage often confer resistance to drugs that derive their toxicity from S or M phase arrest. Described herein is the synthesis of a combinatorial library of potential proapoptotic agents and the subsequent identification of a class of small molecules (triphenylmethylamides, TPMAs) that arrest the growth of melanoma cells in the G1 phase of the cell cycle. Several of these TPMAs are quite potent inducers of apoptotic death in melanoma cell lines ($IC_{50} \sim 0.5\ \mu M$), and importantly, some TPMAs are comparatively nontoxic to normal cells isolated from the bone marrow of healthy donors. Furthermore, the TPMAs were found to dramatically reduce the level of active nuclear factor κ-B (NFκB) in the cell; NFκB is known to be constitutively active in melanoma, and this activity is critical for the proliferation of melanoma cells and their evasion of apoptosis. Compounds that reduce the level of NFκB and arrest cells in the G1 phase of the cell cycle can provide insights into the biology of melanoma and may be effective antimelanoma agents.

Introduction

A common trait of most cancers is the ability to evade the natural cell death process.[1] Although healthy cells from higher eukaryotes have tightly regulated mechanisms for apoptosis, or programmed cell death, cancerous cells often have multiple means by which they evade this pathway and achieve immortality. Virtually every point on the apoptotic cascade has been exploited by cancer, typically through aberrant expression levels or inactivating mutations in certain key proteins.[2−4] A goal of many anticancer treatments is to overcome this endogenous resistance to apoptosis, and indeed, the majority of anticancer drugs function by inducing apoptotic cell death.

The cell cycle is divided into four phases: G1, S (DNA synthesis), G2, and M (mitosis). Anticancer agents typically target the propensity of cancer cells to rapidly replicate their DNA and divide and thus arrest cell growth in the S or M phase of the cell cycle. For instance, cisplatin, doxorubicin, and cyclophosphamide cause DNA damage and S phase arrest,[5−7] while etoposide, taxol, and colchicine all target operations that affect mitosis and ultimately result in M phase arrest.[8,9]

Currently, 75% of cancer deaths are due to epithelial cancers;[10] some of these forms of cancer, such as advanced malignant melanoma, are largely incurable. The five-year survival rates for patients with disseminated melanoma is <5%[11] with an average survival time of 6−10 months.[12] The lack of sensitivity of melanoma to chemotherapy has been well-documented.[13,14] Common anticancer drugs, such as taxol, cisplatin, etoposide, doxorubicin, and several others, have shown no efficacy in large randomized trials,[15] and even popular combination therapies have provided no benefits to melanoma patients.[16] This lack of clinical efficacy is supported by in vitro studies testing drugs against melanoma cell lines.[17] In fact, only

[†] Department of Biochemistry.
[‡] Department of Chemistry.

(1) Hanahan, D.; Weinberg, R. A. *Cell* **2000**, *100*, 57−70.
(2) Makin, G.; Dive, C. *Trends Mol. Med.* **2003**, *9*, 251−255.
(3) Ehlert, J. E.; Kubbutat, M. H. *Onkologie* **2001**, *24*, 433−440.
(4) Zornig, M.; Hueber, A.; Baum, W.; Evan, G. *Biochim. Biophys. Acta* **2001**, *1551*, F1−F37.
(5) Jordan, P.; Carmo-Fonsecab, M. *Cell. Mol. Life Sci.* **2000**, *57*, 1229−1235.
(6) Minotti, G.; Menna, P.; Salvatorelli, E.; Cairo, G.; Gianni, L. *Pharmacol. Rev.* **2004**, *56*, 185−229.
(7) Schwartz, P. S.; Waxman, D. J. *Mol. Pharmacol.* **2001**, *60*, 1268−1279.
(8) Clifford, B.; Beljin, M.; Stark, G. R.; Taylor, W. R. *Cancer Res.* **2003**, *63*, 4074−4081.
(9) Jordan, M. A.; Wilson, L. *Nature Rev. Cancer* **2004**, *4*, 253.
(10) Adjei, A. A.; Rowinsky, E. K. *Cancer Biol. Ther.* **2003**, *S1*, S5−S15.
(11) McGovern, V. J.; Balch, C. M.; Milton, G. W. In *Cutaneous Melanoma: Clinical Management and Treatment Results Worldwide*; Lippincott, Williams & Wilkins: Philadelphia, 1985; pp 29−42.
(12) Jemal, A.; Thomas, A.; Murray, T.; Thun, M. *CA Cancer J. Clin.* **2002**, *52*, 23−47.
(13) Helmbach, H.; Rossmann, E.; Kern, M. A.; Schadendorf, D. *Int. J. Cancer* **2001**, *93*, 617−622.
(14) Serrone, L.; Zeuli, M.; Sega, F. M.; Cognetti, F. *J. Exp. Clin. Cancer Res.* **2000**, *19*, 21−34.
(15) Soengas, M. S.; Lowe, S. W. *Oncogene* **2003**, *22*, 3138−3151.

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one single-entity drug, dacarbazine (DTIC), has been approved by the FDA for treatment of late-stage melanoma, and this medicine provides complete remission in only 2% of patients.[14,18] Thus, as common anticancer drugs are profoundly ineffective in the treatment of disseminated melanoma, there is a clear need for compounds that are selectively cytotoxic to cancerous melanocytes and that act through nonstandard anticancer mechanisms. Some urgency is required in this regard, as the lifetime risk for melanoma is increasing and now estimated at 1 in 75, and it is a disease that is as likely to afflict the young as the old.[12]

The poor long-term prognosis for late-stage melanoma patients is due to the intrinsic resistance of malignant melanocytes to mechanisms of apoptotic death as induced by common anticancer drugs. In their natural role as a protectant from the harmful effects of the sun, melanocytes are bombarded with UV light, a potent DNA damaging agent. Therefore, it is not surprising that melanoma cells are exquisitely resistant to therapies that target DNA synthesis and replication through radiation and DNA alkylation; indeed, some lines of evidence indicate that such treatments cause melanoma cells to proliferate.[19] Recently, examination of apoptotic and cell checkpoint proteins has revealed clues to how melanoma cells evade cell death and continue to proliferate. It is now known that certain melanomas have a methylation-inactivated Apaf-1 signaling complex,[20] and/or exhibit upregulation of the caspase-inhibiting protein survivin.[21] In addition, a common chromosomal defect in melanoma cells is a deletion of the INK4a/ARF locus in the 9p21 region, resulting in the inactivation of the G1/S checkpoint through ablation of the p16INK4A protein.[22,23] A final trait of cancerous melanocytes is the constitutive upregulation of active nuclear factor κ-B (NFκB). This increased level of active NFκB is thought to contribute to the resistance of melanoma to apoptosis-inducing chemotherapeutic agents.[24,25]

Numerous trials have demonstrated that melanoma cells are resistant to standard anticancer drugs. The goal of this study was to identify compounds that are highly potent against cancer cells in culture and that act through a mechanism that arrests cell development in the G1 phase of the cell cycle. We hypothesized that such compounds might be effective against melanoma because, unlike S phase arresting compounds, melanoma cells should have no intrinsic resistance to G1 phase arrestors. In addition, a compound that arrests growth in the G1 phase would prevent the cell from passing through the critical G1/S checkpoint that is disrupted via mutation in melanoma cells and could have a novel macromolecular target.

Herein we report the synthesis and screening of a library of 100 potential apoptotic inducers and the identification of compounds that arrest cell growth in the G1 phase of the cell cycle and induce apoptosis in melanoma cells. While the hits from this first library were quite toxic to melanoma cells, they were also found to be toxic to noncancerous cells from human bone marrow. Therefore, a second-generation library of 104 compounds was created and screened. Several of these compounds have powerful proapoptotic activity in melanoma cell lines and G1 phase arresting capacity. In addition, some of these compounds are markedly less toxic to noncancerous control cells. These compounds reduce the amount of active NFκB in the cell, which is known to be constitutively active in melanoma and directly linked to proliferation and apoptosis inhibition. Given the poor long-term survival prospects for patients with late-stage melanoma and the lack of any effective treatments, compounds discovered through this strategy can provide important insights into the biology of melanoma and have potential as chemotherapeutic agents.

Results and Discussion

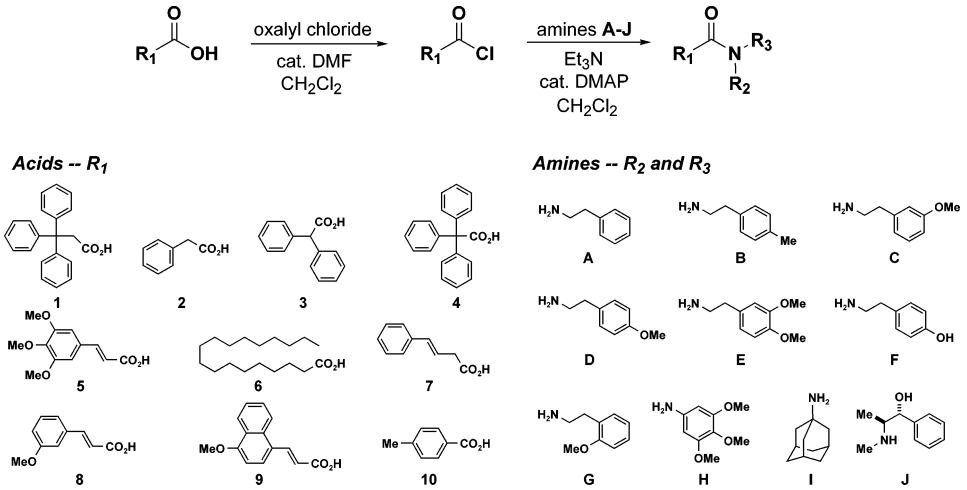
Synthesis and Screening of the First-Generation Library. To create compounds that might induce apoptosis, a small molecule library was designed based on the known apoptosis-inducing properties of certain amides.[26−28] The building blocks used to construct this library are depicted in Scheme 1. To form the amide bond, carboxylic acids **1**−**10** were first converted to the acid chlorides and then treated in parallel with amines **A**−**J** (1.05 equiv) and triethylamine (1.05 equiv). All reactions were performed on a 0.05 mmol scale such that approximately 20 mg of pure product would be obtained (assuming 100% yield for a compound of 400 MW). After the amidation reaction, all compounds were purified through small plugs of silica gel. This protocol provided a high yield (average yield = 94%) of highly pure compounds; all 100 compounds were assessed by HPLC−MS, and this library had an average purity of 89% (see Supporting Information for the exact purity of individual compounds). The precise milligram amount produced was determined for every compound, enabling each sample to be prepared at an exact and identical micromolar concentration for high-throughput biological testing.

The ability of the 100 amides thus produced to induce death in cancer cells was assessed by an assay based on the reduction of the dye MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt]. This tetrazolium dye is rapidly converted to the formazan product by living cells, and therefore provides a sensitive readout of cell life or death that can be monitored spectroscopically. As shown in Figure 1, several of the compounds rapidly (within 24 h) and potently induced death at 50 μM in the U-937 cell line, an easy-to-culture lymphoma cell line used during this initial screening stage. Data on the dose dependence of death-inducing activity was gathered for the most active compounds as identified through this high-throughput screen. As shown in Table 1, multiple compounds induced cell death with IC_{50} values in the low micromolar range.

Cell Cycle Analysis. The data in Figure 1 and Table 1 show obvious structure−activity trends. Certain amides derived from the triphenylpropionic acid **1** and triphenylacetic acid **4** building blocks induce a high degree of cell death in this assay, and

(16) Middleton, M. R.; Lorigan, P.; Owen, J.; Ashcroft, L.; Lee, S. M.; Harper, P.; Thatcher, N. *Br. J. Cancer* **2000**, *82*, 1158−1162.
(17) Schadendorf, D.; Worm, M.; Algermissen, B.; Kohlmus, C. M.; Czarnetzki, B. M. *Cancer* **1994**, *73*, 103−108.
(18) Anderson, C. M.; Buzaid, A. C.; Legha, S. S. *Oncology* **1995**, *9*, 1149−1154.
(19) Lev, D. C.; et al. *J. Clin. Oncol.* **2004**, *22*, 2092−2100.
(20) Soengas, M. S.; et al. *Nature* **2001**, *409*, 207−211.
(21) Grossman, D.; McNiff, J. M.; Li, F.; Altieri, D. C. *J. Invest. Dermatol.* **1999**, *113*, 1076−1081.
(22) Cannon-Albright, L. A.; et al. *Science* **1992**, *258*, 1148−1152.
(23) Fountain, J. W.; et al. *Proc. Natl. Acad. Sci.* **1992**, *89*, 10557−10561.
(24) Huang, S.; DeGuzman, A.; Bucana, C. D.; Fidler, I. J. *Clin. Cancer Res.* **2000**, *6*, 2573−2581.
(25) Makarov, S. S. *Mol. Med. Today* **2000**, *6*, 441−448.
(26) Nesterenko, V.; Putt, K. S.; Hergenrother, P. J. *J. Am. Chem. Soc.* **2003**, *125*, 14672−14673.
(27) Nguyen, J. T.; Wells, J. A. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 7533−7538.
(28) Hwang, S. Y.; et al. *Int. J. Oncol.* **2003**, *22*, 151−157.

Scheme 1. One Hundred Amides Were Synthesized in Parallel and Then Filtered through Small Plugs of Silica Gel[a]



[a] The compounds had an average purity of 89% as assessed by LC−MS.

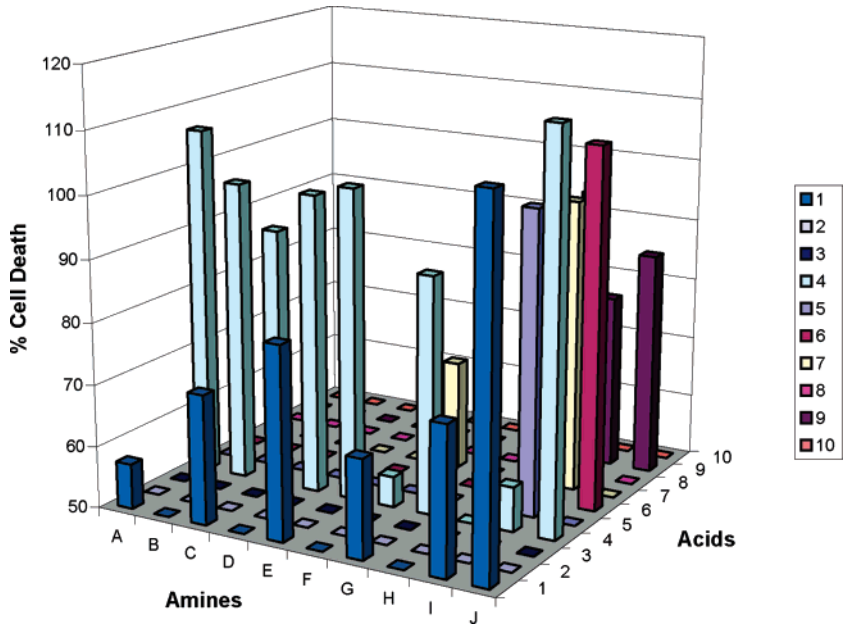


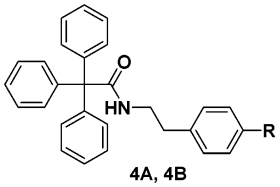
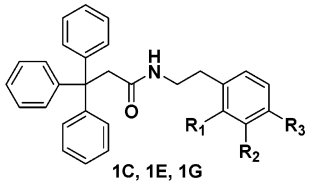
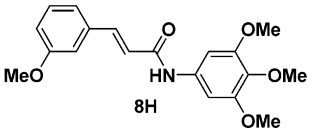
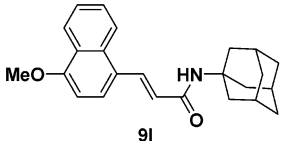
Figure 1. Percent of cell death induced in U-937 cells by the amide products depicted in Scheme 1. Cell death was evaluated after a 24-h incubation with compound at a concentration of 50 μM; death was quantitated by a MTS-bioreduction assay.

several other compounds have IC_{50} values in the low micromolar range. At this point, experiments were conducted to determine the phase of the cell cycle in which these compounds arrest cell growth. This is typically determined by assessing the DNA content of cells stained with propidium iodide as measured by flow cytometry. Flow cytometry data of U-937 cells treated with selected compounds are presented in Figure 2, along with control data. As expected, controls with compounds known to induce M phase arrest such as the microtubule stabilizer taxol (Figure 2A) show a pronounced shift to cell populations with 4n DNA, indicative of G2/M phase arrest. One of the active cell death inducers identified from this combinatorial library that was not derived from building blocks **1** or **4** also arrests cell growth in the G2/M phase (compound **8H**, Figure 2B). However, compounds **4A** and **1C**, derived from the triphenylacetic acid and triphenylpropionic acid building blocks, respectively, arrest cell growth in the G1 phase (Figure 2C,D; for percentages at various cell cycle phases see the Supporting Information).

Effect of TPMAs on Melanoma Cell Lines and NCI Screening Data. Given the interesting and somewhat unusual trait of G1 arrest, triphenylmethylamides (TPMAs) **4A** and **1C** were tested for their ability to induce death in three melanoma cell lines: UACC-62 (human), SK-MEL-5 (human), and B16-F10 (mouse). These melanomas have well-documented and distinct mutations in their apoptotic pathways, and the two human cell lines have been particularly well characterized. SK-MEL-5 expresses very little (less than 15% of melanocyte controls) Apaf-1,[20] a critical protein for apoptosome formation and caspase-9 activation. The UACC-62 cell line is negative for expression of p16INK4A and p14ARF;[20] these two proteins are expressed from the INK4A/ARF locus that is deleted in many melanomas. While the p14ARF gene is totally ablated from SK-MEL-5, this cell line still expresses the p16INK4A protein.[29] Both SK-MEL-5 and UACC-62 express wild-type p53

(29) Kumar, R.; Sauroja, I.; Punnonen, K.; Jansen, C.; Hemminki, K. *Genes Chromosomes Cancer* **1998**, *23*, 273−277.

Table 1. Potency of Death Induction for Compounds Identified as Hits in the High-Throughput Screen Depicted in Figure 1[a]

Compound		IC_{50} Value - U-937 cells	Position of cell cycle arrest
 4A, 4B	R = H	8.0 μM	G1
	R = Me	12.6 μM	G1
 1C, 1E, 1G	R_1 = H, R_2=OMe, R_3=H	14.5 μM	G1
	R_1=H, R_2=OMe, R_3=OMe	18.3 μM	G1
	R_1=OMe, R_2=H, R_3=H	15.7 μM	G1
 8H		8.0 μM	G2/M
 9I		7.1 μM	G2/M

[a] IC_{50} values were determined through analysis of the dose-dependent effect of each compound on U-937 cells in the MTS assay (see graphs in the Supporting Information). The effect of each compound on the cell cycle was determined by flow cytometric analysis of propidium iodide-stained cells.

protein at normal levels,[20] and both have upregulated expression of ABC multidrug efflux pumps.[30] This combination of mutations in critical proteins in the apoptotic cascade and aberrant expression levels of other proteins enable these melanoma cell lines to resist the effects of common anticancer agents.

Evaluation of TPMAs **4A** and **1C** showed that these compounds exhibit excellent death-inducing activity in the melanoma cell lines, with IC_{50} values ranging from 0.4 to 8.8 μM (Table 2). It was also found that these compounds induce cell cycle arrest in the G1 phase in these melanoma cell lines (see Supporting Information). Compounds **4A** and **1C** were submitted for testing in the National Cancer Institute's 60-cell line screen. The NCI data revealed that the compounds are quite active against a broad spectrum of cancers and confirmed the IC_{50} value of 600 nM against the UACC-62 melanoma cell line (for full NCI data on compounds **4A** and **1C**, see the Supporting Information). The triphenylmethyl group appears crucial to this activity, as diphenylmethyl derivatives such as compound **3A** show virtually no death induction (Table 2). In addition, the triphenylacetic and triphenylpropionic acid building blocks (compounds **1** and **4**) have minimal death-inducing activity. As extensively discussed later, it was determined that the TPMAs induce apoptosis in these melanoma cell lines. Very recently the proteasome inhibitor bortezomib (also called VELCADE or PS-341) was reported to be toxic to melanoma in cell culture.[31] Thus bortezomib was used as a control for the toxicity experiments, and its efficacy is reported in Table 2.

Toxicity of 4A and 1C to Human Bone Marrow. The vast majority of anticancer drugs are toxic to all rapidly dividing cell types, leading to deleterious side effects and a reduced therapeutic window. For instance, hematopoietic bone marrow is responsible for the production of red and white blood cells, and the anemia/neutropenia/leukopenia often observed in patients treated with chemotherapeutic agents is in a large part due to the toxicity of the anticancer drugs to cells in human bone marrow.[32] Cells obtained from bone marrow of a healthy human donor were used to determine the relative effect of the TPMAs on melanoma versus normal cells. Measurement of compound toxicity to healthy bone marrow cells is a common method for assessing the selectivity of putative anticancer agents.[33−36] These actively growing cells were treated with increasing concentrations of compounds **4A** and **1C**, and cell viability was evaluated after 72 h, with controls conducted in the absence of any compound. It was found that while compounds **4A** and **1C** are potent inducers of cell death in melanoma cells, they also have comparable toxicity to these

(30) Szakacs, G.; et al. *Cancer Cell* **2004**, *6*, 129−137.
(31) Amiri, K. I.; Horton, L. W.; LaFleur, B. J.; Sosman, J. A.; Richmond, A. *Cancer Res.* **2004**, *64*, 4912−4918.
(32) Groopman, J. E.; Itri, L. M. *J. Natl. Cancer Inst.* **1999**, *91*, 1616−1634.
(33) Konstantinov, S. M.; Topashka-Ancheva, M.; Benner, A.; Berger, M. R. *Int. J. Cancer* **1998**, *77*, 778−786.
(34) de Graaff, M.; Maliepaard, M.; Pluim, D.; Floot, B. J.; Slaper-Cortenbach, I. C.; Schellens, J. H. *Anticancer Drugs* **1999**, *10*, 213−218.
(35) LoRusso, P. M.; et al. *Invest. New Drugs* **1999**, *16*, 287−296.
(36) Oredipe, O. A.; Furbert-Harris, P. M.; Laniyan, I.; Griffin, W. M.; Sridhar, R. *Internation. Immunopharm.* **2003**, *3*, 1537−1547.

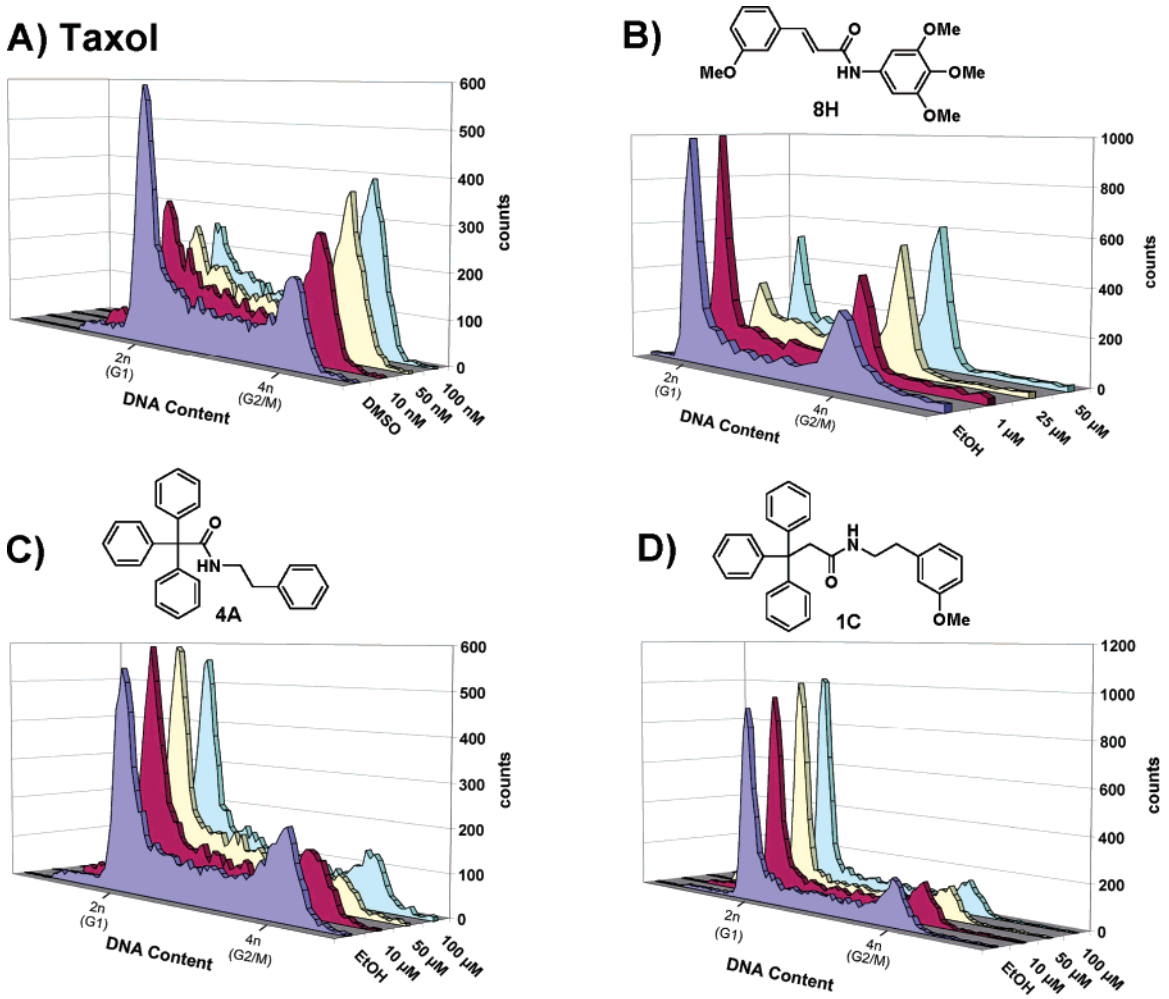


Figure 2. Analysis of cell populations (after 6 h of treatment) show that compounds such as taxol and **8H** arrest growth of U-937 cells in the G2/M phase of the cell cycle (A and B), whereas amides derived from triphenylacetic acid and triphenylpropionic acid arrest cell growth in the G1 phase of the cell cycle (C and D).

normal cells isolated from the bone marrow of healthy human donors (Table 2). It is also worth noting that common anticancer agents, such as cyclophosphamide[37] and etoposide, and the steroid prednisone (all of which are known S phase arrestors) have no effect on the melanoma cell lines but are quite toxic to the bone marrow-derived cells (Table 2). Bortezomib potently induces cell death in the melanoma cell lines, but is even more toxic to the bone marrow-derived cells (Table 2).

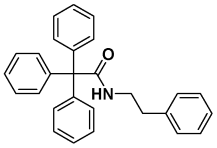
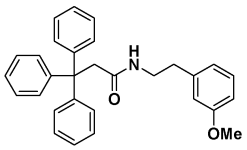
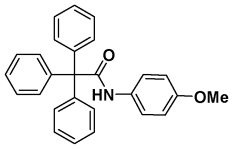
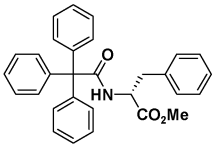
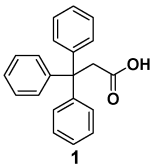
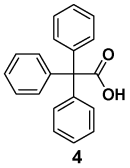
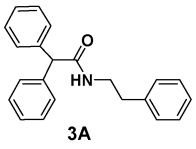
Second-Generation Library. To minimize general cytotoxicity and optimize potency for melanoma cells, a second-generation combinatorial library was constructed. Because multiple compounds containing amides derived from triphenylacetic and triphenylpropionic acid displayed powerful proapoptotic effects in melanoma cell lines and were shown to induce apoptosis and arrest the cell cycle in the G1 phase, a second library was synthesized on a 0.035-mmol scale using only the triphenylacetic acid (**4**) and triphenylpropionic acid (**1**) building blocks. The acid chlorides derived from the two acids were treated in parallel with 52 different amines to provide 104 amide products; this reaction scheme and the various building blocks are shown in Scheme 2. Again, all of the final products were purified via filtration through short plugs of silica gel. HPLC and mass spectrometry were used to characterize the entire library; in this case 94 of the 104 compounds were found to have been synthesized in good yields and were isolated in high purities. The average purity for these 94 compounds was 84% (see Supporting Information for exact purities of individual compounds). For IC_{50} value determinations of the most active compounds (below), samples utilized were all >97% pure as judged by HPLC analysis and ^{1}H NMR.

Potency of TPMAs in the Second-Generation Library. All amides thus produced were evaluated for induction of cell death in the melanoma cell lines, and the most active compounds then had their toxicity to the noncancerous bone marrow cells measured. In general, many of the TPMAs were potent death inducers in melanoma cell lines, with several displaying potencies in the 0.5−1.0 μM range. Of the hits from this library, two compounds in particular, **4AN** and **4BI**, showed markedly less (8−12-fold) toxicity to the noncancerous normal cells (Table 2).

Apoptotic Assays. To determine whether the observed death induced by the TPMAs was due to apoptosis or necrosis, common biochemical markers of apoptosis were monitored, including mitochondrial membrane depolarization, chromatin

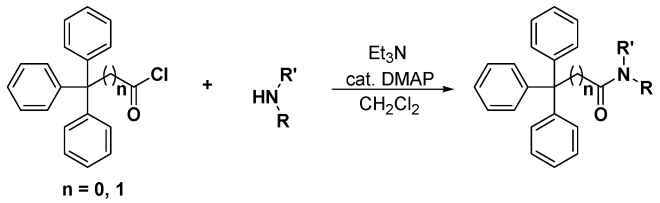
(37) Cyclophosphamide is activated in vivo to 4-hydroperoxycyclophosphamide (4-HC); however, both cyclophosphamide and 4-HC have been used in cell culture toxicity experiments. For an example of the recent uses of cyclophosphamide in cell culture experiments, please see: Rzeski, W.; et al. *Ann. Neurol.* **2004**, *56*, 351−360.

Table 2. IC_{50} Values for the Induction of Cell Death by Selected TPMAs in Melanoma Cell Lines and Normal Cells from Human Bone Marrow[a]

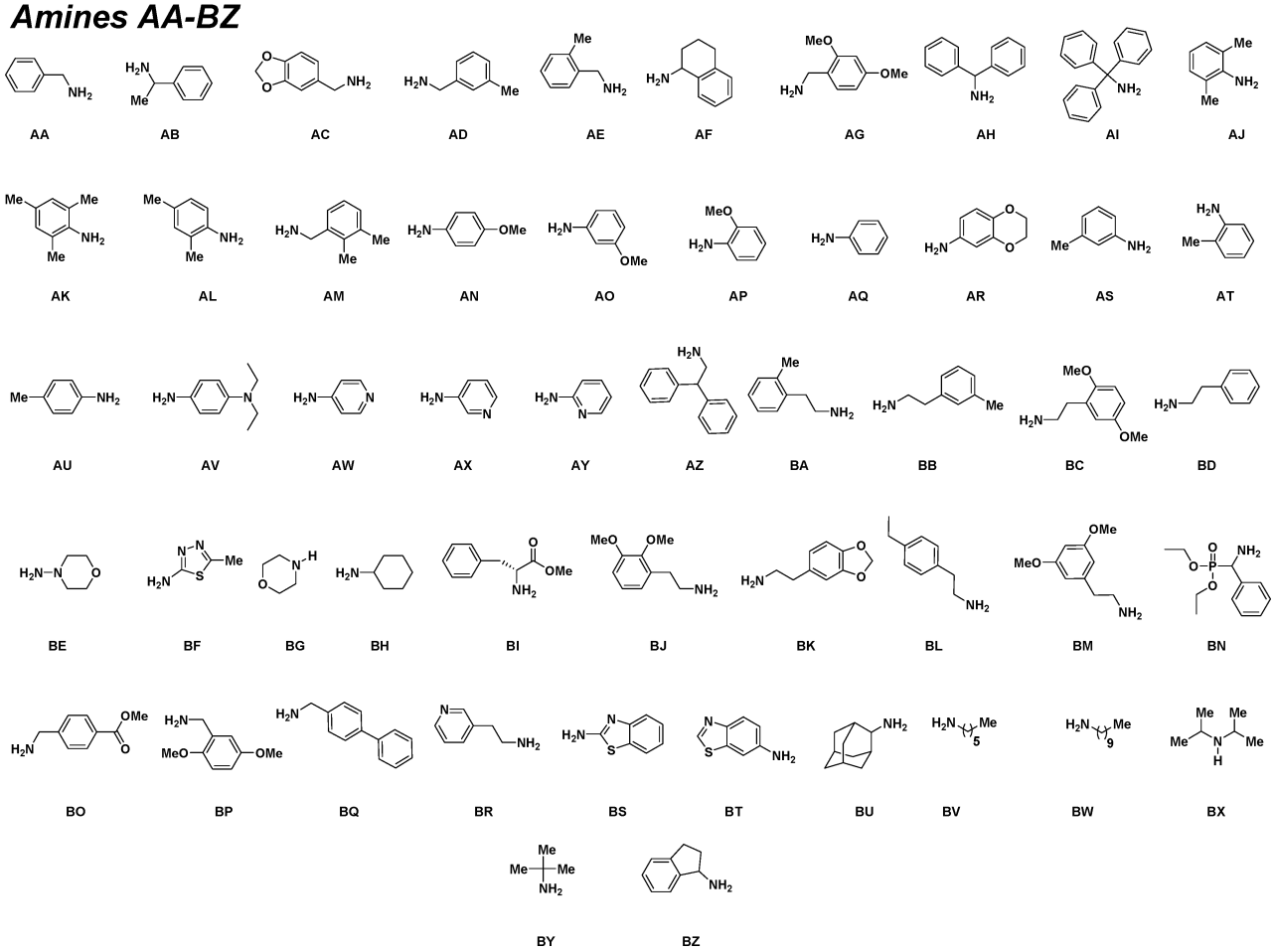
	IC_{50} values			
Compound	**UACC-62**	**B16-F10**	**SK-MEL-5**	**Bone marrow**
 4A	0.49 μM	0.42 μM	8.8 μM	0.50 μM
 1C	1.34 μM	0.85 μM	3.3 μM	0.99 μM
 4AN	0.69 μM	0.60 μM	0.83 μM	4.77 μM
 4BI	0.62 μM	0.80 μM	0.57 μM	7.00 μM
 1	25.4 μM	>100 μM	84.8 μM	3.49 μM
 4	11.8 μM	>100 μM	53.1 μM	2.16 μM
 3A	77.0 μM	>100 μM	49.5 μM	45.2 μM
Bortezomib	35.2 nM	10.6 nM	9.4 nM	6.0 pM
Cyclophosphamide	>100 μM	>100 μM	>100 μM	0.17 μM
Prednisone	>100 μM	>100 μM	>100 μM	0.15 μM
Etoposide	>100 μM	>100 μM	35.6 μM	8.3 pM

[a] All IC_{50} values were determined after a 72-h treatment with the compound. All compounds listed were evaluated at a purity of >97% as assessed by HPLC (See the Supporting Information).

Scheme 2. Second-Generation Library of Triphenylmethylamides[a]



Amines AA-BZ



[a] The compounds were synthesized in parallel, and after filtration through silica gel the amide products had an average purity of 84%.

condensation, and phosphatidylserine exposure. The depolarization of the mitochondrial membrane facilitates the release of proapoptotic proteins, such as cytochrome *c* and AIF (apoptosis inducing factor) from the mitochondria into the cytosol.[38] Several dyes, including JC-9, have been developed that provide a sensitive readout of this process.[39] As shown in Figure 3A, treatment of UACC-62 cells with 50 μM of compound **4BI** (the most selective antimelanoma agent of the TPMAs) shifts the fluorescence of this dye, consistent with mitochondrial membrane depolarization.

Another common biochemical hallmark of apoptosis is chromatin condensation.[38] Certain DNA intercalators exhibit a greater fluorescence when in the presence of condensed chromatin and are therefore used as an apoptotic indicator as measured by flow cytometry and/or microscopy. As seen in Figure 3B, the nuclei of UACC-62 melanoma cells treated with 50 μM of **4BI** contain highly condensed chromatin (as visualized with the Hoechst-33258 dye), indicative of apoptosis.

A final event in apoptosis is the exposure of phosphatidylserine on the outer leaflet of the cell membrane; this membrane perturbation allows for recognition of apoptotic cells by phagocytes, ultimately leading to the engulfment of the apoptotic cell and the recycling of the various cellular components. Phosphatidylserine exposure can be detected by the binding of fluorescently labeled Annexin V. As shown in Figure 3C, compound **4BI** (50 μM) causes apoptosis as measured by Annexin V staining and flow-cytometry. Compounds **4A** and **4AN** were also shown to induce apoptotic death in UACC-62, as evaluated by the three assays described above (see Supporting Information for images).

TPMA-Treated Cells Have Reduced Levels of Active NFκB. There is currently no effective therapeutic strategy for disseminated melanoma, as this cancer is resistant to both radiation treatments and chemotherapy. As a consequence, the five-year survival rates for patients with advanced melanoma

(38) Blatt, N. B.; Glick, G. D. *Bioorg. Med. Chem.* **2001**, *9*, 1371−1384.
(39) Cossarizza, A.; Baccarani-Contri, M.; Kalashnikova, G.; Franceschi, C. *Biochem. Biophys. Res. Commun.* **1993**, *197*, 40−45.

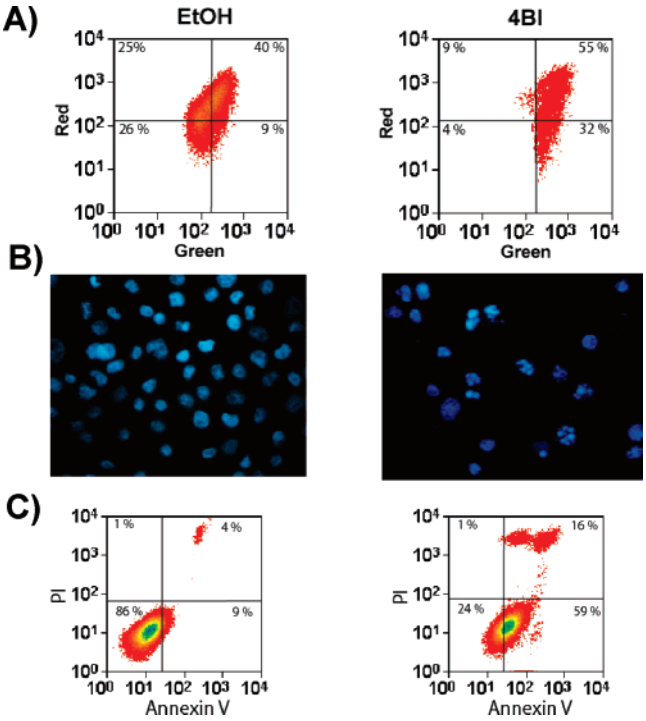


Figure 3. Induction of apoptosis in UACC-62 (human melanoma) cells by treatment with 50 μM of compound **4BI** (right) versus control (left). (A) Mitochondrial membrane depolarization (as measured by the dye JC-9), (B) chromatin condensation (as visualized with Hoechst-33258), and (C) phosphatidylserine externalization on the surface of UACC-62 cells (as observed with fluorescently labeled Annexin V) all indicate that **4BI** induces apoptotic cell death.

approaches zero. It is becoming increasingly clear that melanoma differs significantly at a molecular level from other forms of cancer. For instance, while a very high percentage (>50%) of cancers have a mutation in the p53 gene,[4] less than 5% of melanomas have this mutation.[40] Instead, recent evidence has indicated that melanoma cells have defects elsewhere in the apoptotic machinery.[15] For example, certain melanomas have a methylation-inactivated Apaf-1 signaling complex[20] and/or upregulation of the caspase-inhibiting protein survivin.[21] Due to these distinct differences in the apoptotic proteins, it is not surprising that drugs effective against other forms of cancer show no efficacy versus melanoma cells in vitro and in vivo.

Given the potency of the TPMAs against multiple melanoma cell lines and their unusual G1 arresting properties, their mechanism of action is potentially novel and therefore worthy of further exploration. Toward this end, the ability of the TPMAs to affect the cellular level of active NFκB was explored. The transcription factor NFκB is constitutively active in many melanoma cell lines, and this activated NFκB translocates to the nucleus and initiates the transcription of a variety of anti-apoptotic proteins;[41,42] the precise cause of this constitutive activity is not completely understood. High levels of active NFκB are known to promote transition from the G1 to the S phase of the cell cycle by inducing the transcription of cyclin D1.[43] Thus, UACC-62 cells were treated with **4A** and the cellular levels of active NFκB were assessed. As shown in Figure 4, compound **4A** causes a marked decrease in the amount of active NFκB, as assessed (with an antibody to active NFκB) by both flow cytometry and microscopy. This reduction of the levels of active NFκB by **4A** may block the progression from G1 to S phase in melanoma cells and lead to the G1 arrest observed. As NFκB is constitutively active in many different cancers,[41,42,44] this ability of the TPMAs to cause reduction of active NFκB is a possible explanation for the general potency of these compounds in multiple cancer types (as seen through the NCI screen).

The proteosome inhibitor bortezomib, which also reduces active levels of NFκB in cancer cells,[45] has been clinically approved for treatment of multiple myeloma.[46] Recently, bortezomib was shown to be effective against melanoma cell lines and in a mouse model of melanoma.[31] Indeed, control experiments we have performed indicate that bortezomib is quite toxic to melanoma cell lines (IC_{50} = 9−35 nM; Table 2). However, bortezomib is also extremely toxic to bone marrow from healthy human donors (IC_{50} = 6 pM; Table 2) and is toxic to mice at fairly low dosages (toxicity at 2 mg/kg with three times per week dosing[45]). In contrast, preliminary data indicate that mice can tolerate daily treatments with **4A** at concentrations as high as 100 mg/kg (unpublished data).

Described herein is the synthesis, identification, and optimization of several triphenylmethylamides that induce growth arrest in the G1 phase of the cell cycle and show excellent potency against three melanoma cell lines in cell culture. Further, multiple lines of evidence indicate that these compounds induce apoptosis in melanoma cells. Importantly, these melanoma cell lines have previously been shown to be resistant to common anticancer agents and have varied defects in their apoptotic pathways.[20] Additionally, certain TPMAs described herein have excellent potencies in melanoma cell lines relative to their toxicity to bone marrow cells derived from healthy human

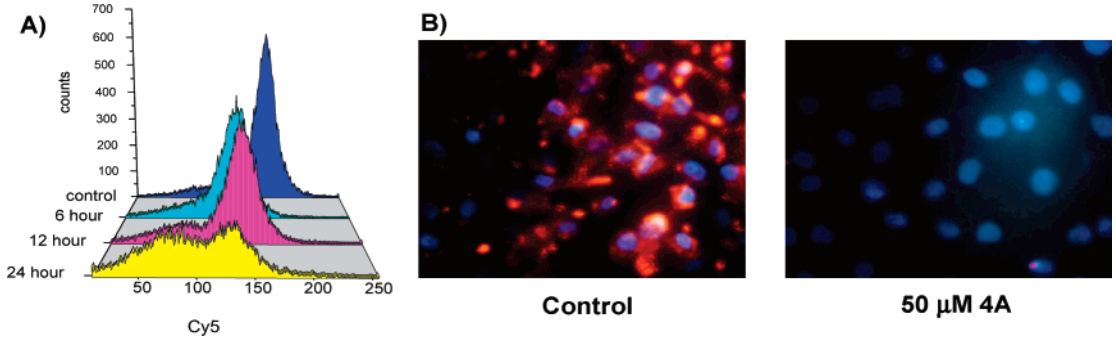


Figure 4. TPMAs reduce the levels of active NFκB in melanoma cells. (A) UACC-62 cells were treated with 50 μM of compound **4A** and then stained with an antibody to active NFκB and analyzed by flow cytometry over a 24-h period. (B) Untreated UACC-62 cells show marked levels of active NFκB when visualized by microscopy with an antibody to active NFκB. In contrast, UACC-62 cells treated with 50 μM of **4A** for 24 h (but still adherent) show very little active NFκB. Red = active NFκB, via Cy5-conjugated secondary antibody. Blue = Hoechst-stained nuclei.

donors. These compounds likely function at least in part by reducing the cellular levels of active NFκB. As the relationship between G1 cell cycle control and oncogenesis is still ill defined,[47] compounds that arrest cellular growth in this phase of the cell cycle may have novel macromolecular targets and can be used to help elucidate the underlying cancer biology. In addition, such compounds may be effective antimelanoma agents. Studies to identify the precise molecular target of the TPMAs and to determine the efficacy of these compounds in mouse models of melanoma are ongoing and will be reported in due course.

Experimental Section

Materials. MTS/PMS CellTiter 96 Cell Proliferation Assay reagent was purchased from Promega (Madison, WI). Cell culture media, Taxol, poly-L-lysine, and the Cy3 conjugated anti-mouse second antibody were purchased from Sigma (St. Louis, MO). Alexa Fluor 488 conjugate, JC-9, propidium iodide, and ProLong Antifade Kit were purchased from Molecular Probes (Eugene, OR). Microtiter plates and all other materials were purchased from Fisher (Chicago, IL). The antibody that recognizes the nuclear localization signal of the p65 subunit of active NFκB was purchased from Chemicon International (Temecula, CA).

Dry dichloromethane was distilled from P_2O_5 under dry air atmosphere. Triethylamine was stored over 3-Å molecular sieves. All reagents were obtained from common commercial sources and used without further purification.

Compound Analysis. All NMR experiments were recorded in $CDCl_3$, CD_2Cl_2, or CD_3OD on Varian Unity 400 & 500 MHz spectrometers with residual undeuterated solvent as an internal reference. Chemical shift, δ (ppm); coupling constants, J (Hz); multiplicity (s = singlet, d = doublet, t = triplet, q = quintet, m = multiplet); and integration are reported. High-resolution mass spectral data were recorded on a Micromass Q-Tof Ultima hybrid quadrupole/time-of-flight ESI mass spectrometer at the University of Illinois Mass Spectrometry Laboratory. Infrared spectra were recorded on a Perkin-Elmer Spectrum BX spectrophotometer, referenced to a polystyrene standard, and the peaks are reported in cm^{-1}. Solvents for chromatography were reagent grade and were used without further purification. Silica gel chromatography was performed on EMD Biosciences silica gel 60 (230−400 mesh). Thin-layer chromatography plates (Merck, 245 nm fluorescent indicator) were visualized by UV and stained with cerium ammonium molybdate (CAM), iodine (I_2), ninhydrin, or DPIP (0.5 mg 2,6-dichloroindophenolate hydrate per mL in EtOH). Purified products were analyzed on a Varian 2510 HPLC equipped with an Alltech Alltima C18 column (20 mm × 2.1 mm × 3 μm) with an acetonitrile/0.1% trifluoroacetic acid (in Millipore MilliQ-filtered water) solvent gradient. Eluted solute was detected with a Waters 486 UV multiple wavelength absorbance detector operated at 254 nm. All acid and amine building blocks were used as received from common commercial suppliers, except **8** and **9**, which were prepared by Wadsworth−Emmons olefination following a procedure described in *Organic Synthesis* (Collect. Vol. 5, p 547) from commercially obtained *m*-anisaldehyde and 4-methoxynaphthalene-1-carbaldehyde, respectively.

Synthesis of the First-Generation Library of 100 Amides. Amines **A**−**J** were coupled with the 10 acid chlorides derived from acids **1**−**10** in parallel on a 0.05 mmol scale. Acid chlorides of **1**−**10** were synthesized in parallel through addition of 3 equiv of oxalyl chloride and a catalytic (1 mol %) amount of *N*,*N*-dimethylformamide to a stirred solution of the corresponding carboxylic acid in dry dichloromethane. After 12 h, solvent and excess oxalyl chloride were removed under reduced pressure and the resulting acid chlorides were used immediately. Stock solutions of each amine (0.102 M), each acid chloride (0.17 M), and triethylamine (0.21 M), to which 5 mol % DMAP was added, were prepared in dry CH_2Cl_2. To 100 glass vials was added 0.25 mL of a stock solution of dry triethylamine (5.3 mg, 0.0525 mmol, 1.05 equiv), followed by addition of a 0.5-mL aliquot of each amine **A**−**J** (0.051 mmol, 1.02 equiv). The vials were capped and cooled in an ice bath to 0−5 °C. At this point, 0.3-mL aliquots of the corresponding acid chlorides (0.05 mmol) were added, and the vials were capped and shaken on an orbital shaker at 300 rpm for 48h, during which time the reactions were allowed to warm to ambient temperature. All reaction mixtures were diluted with 1 mL of CH_2Cl_2 and passed through 1.5-in. silica gel columns packed in Pasteur pipets with hexanes. The columns were eluted with 2 mL of 1:1/hexanes:ethyl acetate mixture into tared vials. Solvents were removed under reduced pressure, samples were dried under vacuum (0.01 mmHg) in a desiccator, and weights were recorded for all samples. The average isolated yield of the 100 compounds was 94%. Each sample was analyzed by high-throughput LC/MS (ESI), which allowed for determination of the purity of each compound and confirmation of each molecular ion peak. The presence of the molecular ion peak was confirmed for 89 compounds. Compounds **6A**−**6J** failed to be identified by LC/MS and were analyzed by ESI-MS, ^{1}H NMR, and ^{13}C NMR, all of which confirmed the formation of the desired product. Compounds **5A**−**5J** were additionally analyzed by HPLC. Formation of compound **3F** could not be confirmed by any analytical method. The purity of each sample was determined from the corresponding LC trace (average purity was 89%.)

Synthesis of Second-Generation Library of Triphenylmethyl Amides. A library of 104 triphenylmethyl amides was generated by coupling 52 amines with the acid chlorides derived from **1** and **4** in parallel on a 0.035-mmol scale. The two acid chlorides were synthesized by known protocols,[48,49] recrystallized from hexane, and stored in a desiccator until use. To each of 104 glass vials was added 0.5-mL aliquots of a stock solution of dry triethylamine (0.077 M, 0.037 mmol) and 4-*N*,*N*-dimethylaminopyridine (0.004 M, 5 mol %) in dry CH_2Cl_2. Aliquots of amine (0.5 mL of a 0.074 M solution, 0.037 mmol) and acid chloride (0.25 mL of a 0.14 M solution, 0.035 mmol) stock solutions in dry CH_2Cl_2 were added to the triethylamine/DMAP solution, and vials were capped. An additional equivalent of triethylamine was added to those amines that were purchased as the HBr or HCl salt. Vials were mixed on an orbital shaker at 225 rpm for 48 h. Crude reaction mixtures were passed through 1.5-in. silica gel columns packed in Pasteur pipets. Solvent was removed, and weights were recorded for all isolated products. High-throughput HR-ESI mass spectrometry was performed on all 104 compounds. Ten compounds failed to show positive identification (M + 1) as the dominant peak. These compounds (**1AI**, **4AI**, **1AW**, **1AX**, **4AX**, **1BE**, **4BE**, **1BR**, **1BX**, and **4BX**) were not included in yield and purity calculations. The average isolated yield of the 94 compounds was 77%. Purity (average purity 84%) was quantified by HPLC. Generally, the 52 amides obtained from building block **4** were synthesized in higher yields (85% vs 69%) and purity (90% vs 78%) than those from **1**.

Cell Culture Conditions. U-937, HL-60, SK-MEL-5, and UACC-62 cells were grown in RPMI 1640 media supplemented with 10% FBS, B16-F10 cells were grown in Eagle's minimal essential medium with Earle's BSS supplemented with 10% FBS, and human bone

(40) Satyamoorthy, K.; Bogenrieder, T.; Herlyn, M. *Trends Mol. Med.* **2001**, *7*, 191−194.
(41) Monks, N. R.; Biswas, D. K.; Pardee, A. B. *J. Cell Biochem.* **2004**, *92*, 646−650.
(42) Aggarwal, B. B. *Cancer Cell* **2004**, *6*, 203−208.
(43) Joyce, D.; Albanese, C.; Steer, J.; Fu, M.; Bouzahzah, B.; Pestell, R. G. *Cytokine Growth Factor Rev.* **2001**, *12*, 73−90.
(44) Bours, V.; Dejardin, E.; Goujon-Letawe, F.; Merville, M. P.; Castronovo, V. *Biochem. Pharmacol.* **1994**, *47*, 145−149.
(45) Sunwoo, J. B.; Chen, Z.; Dong, G.; Yeh, N.; Crowl Bancroft, C.; Sausville, E.; Adams, J.; Elliott, P.; Van Waes, C. *Clin. Cancer Res.* **2001**, *7*, 1419−1428.
(46) Rajkumar, S. V.; Richardson, P. G.; Hideshima, T.; Anderson, K. C. *J. Clin. Oncol.* **2005**, *23*, 630−639.
(47) Massague, J. *Nature* **2004**, *432*, 298−306.
(48) Seto, C. T.; Whitesides, G. M. *J. Am. Chem. Soc.* **1993**, *115*, 905−916.
(49) Bollyky, L. J.; Whitman, R. H.; Clarke, R. A.; Rauhut, M. M. *J. Org. Chem.* **1967**, *32*, 1663−1667.

marrow cells were grown in Iscove's modified Dulbecco's medium supplemented with 40% FBS. All cell lines were incubated at 37 °C in a 5% CO_2, 95% air atmosphere. U-937 and HL-60 cells were split every 2−3 days as needed and UACC-62 and B16-F10 cells were split when they reached approximately 80% confluency at a 1:6 ratio.

Cell Death Assay and IC_{50} Value Determination in White Blood Cells. To each well of a 96-well flat-bottom microtiter plate was added 50 μL RPMI-1640 + 10% FBS media. During the initial screen, library compounds dissolved in DMSO were added to the plates to give a 50 μM solution. To determine the IC_{50} values, active compounds were added to a 96-well plate in varying concentrations. Controls were performed in which only DMSO (containing no compound) was transferred into wells. U-937 or HL-60 cells were harvested by centrifugation at 250*g* for 5 min. Cells were then resuspended in RPMI-1640 + 10% FBS, counted using a hemocytometer and diluted to 8×10^5 cells/mL. A 50-μL aliquot of the cell solution was then added to each well. The cells were then incubated with the compounds for 24 or 72 h. Cell death was quantitated by adding 20 μL of the MTS/PMS CellTiter 96 Cell Proliferation Assay reagent to each well. The plates were incubated at 37 °C for approximately 1 h until the colored product formed. The absorbance was then measured at 490 nm in a Spectra Max Plus 384 plate reader (Molecular Devices, Sunnyvale CA).

Cell Cycle Analysis. For suspension cells, 500 μL of RPMI-1640 + 10% FBS media was added to each well of a 24-well plate. Test compounds and Taxol were added to the plate in varying concentrations. Controls were performed in which only the vector (DMSO or ethanol) was transferred into wells. U-937 cells were harvested by centrifugation at 250*g* for 5 min. Cells were then resuspended in RPMI-1640 + 10% FBS, counted using a hemocytometer, and diluted to 2×10^6 cells/mL. Then 500 μL of the cell solution was added to each well, bringing the final concentration of cells to 1×10^6 cells per well. The cells were then incubated with the compounds for 6 h and harvested by centrifugation.

For adherent cells, 1 mL of cells was seeded into the wells of a 24-well plate and grown to confluency. The media was replaced with media containing the test compounds or vector only. The cells were incubated with the compounds for various times, trypsinized, and harvested by centrifugation. The cells were then washed with PBS and resuspended in ice-cold 100% ethanol. After being harvested the cells were then stored at 4 °C overnight. The cells were pelleted out of the ethanol by centrifugation, washed in PBS, and resuspended in 50 μL PBS containing 100 μg/mL RNase A. The cells were incubated at 4 °C for 4 h. A 400-μL aliquot of a 50 μg/mL solution of propidium iodide was then added, and the cells were analyzed by flow cytometry.

Cell Death Assay and IC_{50} Value Determination in Melanoma Cells. To each well of a 96-well flat-bottom microtiter plate was added 50 μL of RPMI-1640 + 10% FBS or 50 μL of MEM + 10% FBS media. During the initial screen, library compounds dissolved in DMSO were added to the plates to give a 1 μM solution. To determine the IC_{50} values, active compounds were added to a 96-well plate in varying concentrations. Controls were performed in which only DMSO (containing no compound) was transferred into wells. UACC-62, SK-MEL-5, or B16-F10 cells were trypsinized and harvested by centrifugation at 250*g* for 5 min. Cells were then resuspended in the appropriate media, counted using a hemocytometer and diluted to 1×10^5 cells/mL. Then 50 μL of the cell solution was added to each well. The cells were then incubated with the compounds for 72 h. Cell death was quantitated by using sulforhodamine B. To accomplish this, the media was replaced with 200 μL of 10% trichloroacetic acid. The plate was incubated for 1 h at 4 °C. The cells were then washed five times with distilled water, and 200 μL of a 0.4% sulforhodamine B in a 1% acetic acid solution was added to the plate. The plate was then incubated for 20 min at room temperature. The plate was then washed five times with 1% acetic acid, and 200 μL of a 10 mM solution of unbuffered Tris was added. The plates were shaken to release the dye from the cells and the absorbance was measured at 520 nm in a Spectra Max Plus 384 plate reader (Molecular Devices, Sunnyvale CA).

Annexin V staining. UACC-62 cells (10 mL) were seeded into a 12.5 cm^2 flask and grown to confluency. The media was replaced with media containing the test compounds or vector only. The cells were incubated for various times, trypsinized, and harvested by centrifugation. The cells were then washed in PBS buffer before staining. After PBS washes, the cells were washed in 1 mL of Annexin V binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) and resuspended in 100 μL of Annexin V binding buffer. Then 5 μL of Annexin V, Alexa Fluor 488 conjugate was added, and the tubes were incubated at room temperature for 15 min protected from light. Annexin V binding buffer (400 μL) was then added, followed by the addition of 1 μL of a 1 mg/mL solution of propidium iodide. The fluorescent intensity of each cell was determined by flow cytometry at 525 nm (green channel) and 675 nm (red channel). At least 10 000 cells were analyzed in each experiment.

Mitchondrial Membrane Potential. UACC-62 cells (10 mL) were seeded into a 12.5 cm^2 flask and grown to confluency. The media was replaced with media containing the test compounds or vector only. The cells were incubated for various times, trypsinized, and harvested by centrifugation. The cells were then washed in PBS buffer before staining. After PBS washes, the cells were resuspended in 1 mL of PBS. JC-9 dye (10 μg) was added, and the cells were incubated at room temperature for 10 min protected from light. The cells were then washed two times with PBS and brought up in 500 μL of PBS. The fluorescent intensity of each cell was determined by flow cytometry at 525 nm (green channel) and 675 nm (red channel). At least 10 000 cells were analyzed in each experiment.

NFκB Staining. UACC-62 (10 mL) cells were seeded into a 12.5 cm^2 flask and grown to confluency. The media was replaced with media containing the test compounds or vector only. The cells were incubated for various times, scraped with a rubber policeman, and harvested by centrifugation. The cells were then washed twice in ice-cold PBS buffer before fixing at −20 °C in 100% ethanol. The fixed cells were incubated overnight at 4 °C, followed again by washing twice in PBS buffer. The cells were then resuspended in 100 μL of PBS buffer containing 10 μg/mL of an antibody that recognizes active NFκB. After incubation at room temperature for 2 h followed by two washes in PBS buffer, the cells were then resuspended in 1.0 mL of a 1:5000 dilution of an anti-mouse second antibody conjugated to Cy5 and were incubated at room temperature in the dark for 2 h. The cells were then washed twice in PBS buffer and resuspended in 400 μL of PBS buffer. The fluorescent intensity of each cell was determined by flow cytometry at 675 nm (red channel). At least 50 000 cells were analyzed in each experiment.

Microscopy of Condensed Chromatin and NFκB. To observe chromatin condensation, 1 mL of UACC-62 cells was seeded into a 24-well plate and grown to confluency. The media was replaced with media containing the test compounds or vector only. The cells were incubated for 24 h followed by resuspension by pipeting vigorously up and down. The cells were removed from the media by centrifugation. The cells were then washed in PBS buffer before being plated onto polylysine-coated no. 1 thickness coverslips. The coverslips were then incubated at 37 °C in a humidified incubator for 1 h. The coverslips with adhered cells were washed twice with PBS, fixed at 37 °C in 4% formaldehyde in BRB80 buffer (80 mM Pipes, pH 6.9; 1 mM EGTA; 1 mM $MgCl_2$) for 30 min and then rinsed again twice with PBS. Cells were permeabilized in PBS containing 0.5% Triton X-100 for 10 min followed by rinsing three times with PBS containing 0.1% Triton X-100. Coverslips were incubated with 2 μg/mL Hoechst-33258 in PBS for 30 min at room temperature. Coverslips were rinsed twice with PBS and mounted on glass slides using a ProLong Antifade Kit. Images of chromatin were obtained at 400× magnification on a Zeiss Axiovert 100 microscope. For immunofluorescence of NFκB, UACC-62 cells were grown on glass coverslips by adding 2 mL of cells to a 35×10 mm style tissue culture dish containing a single coverslip. The media

was replaced with media containing the test compounds or vector only and incubated for 24 h. The coverslips with adhered cells were removed from the media and washed twice with PBS, fixed at 37 °C in 4% formaldehyde in BRB80 buffer (80 mM Pipes, pH 6.9; 1 mM EGTA; 1 mM $MgCl_2$) for 30 min, and then rinsed again twice with PBS. Cells were permeabilized in PBS containing 0.5% Triton X-100 for 10 min followed by rinsing three times with PBS containing 0.1% Triton X-100. The cells on coverslips were then incubated for 10 min in PBS containing 2% BSA followed by incubation for 2 h in 10 μg/mL of anti-active NFκB antibody. The cells were then washed three times in PBS containing 0.1% Triton X-100 followed by a 2 h incubation in a 1:5000 dilution of anti-mouse second-antibody conjugated with Cy5. The cells were then washed three times with PBS containing 0.1% Triton X-100 and then incubated with 2 μg/mL Hoechst-33258 in PBS for 30 min at room temperature. Coverslips were rinsed twice with PBS and mounted on glass slides using a ProLong Antifade Kit. Fluorescent images of NFκB and Hoechst stained cells were obtained at 400× magnification on a Zeiss Axiovert 100 microscope.

Data Analysis. The data from all flow cytometry experiments was analyzed using Summit Software (Cytomation, Fort Collins, CO) and ModFit software (Verity Software House, Inc., Topsham, ME). IC_{50} values were determined using Table Curve (Systat, Richmond, CA) using a logistic dose−response model.

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Supporting Information Available: Tables listing purity of library members, characterization data for individual compounds, dose−response graphs used in IC_{50} determinations, NCI data, apoptotic data not shown in the text, and complete author lists for references with 10 or more authors. This material is available free of charge via the Internet at http://pubs.acs.org.

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