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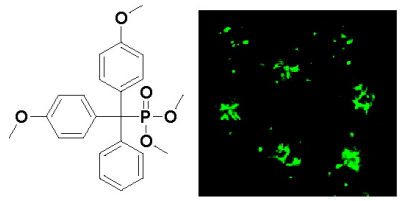
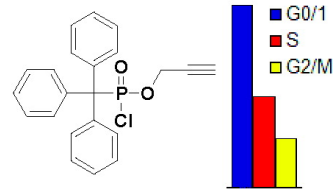


M-phase arrestor



G1-phase arrestor

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The Complex Role of the Triphenylmethyl Motif in Anticancer Compounds

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Abstract: Compounds incorporating the triphenylmethyl motif constitute an emerging family of potent anticancer agents. Although several small molecules containing this pharmacophore have now been identified, the mechanism of cell death induction for some of these compounds is unknown. In an effort to define their mechanism of action, and to distinguish subtypes within the group of compounds containing the triphenylmethyl moiety, we have created novel triphenylmethyl-containing small molecules and have evaluated them in a battery of biological assays. Here we show that several phosphonate and phosphonochloridates possessing the triphenylmethyl motif potently induce death of multiple cancer cell lines in culture. Further assays evaluating the ability to cause cell cycle arrest, inhibit tubulin polymerization, dissociate mitochondrial-bound hexokinase in cancer cells, and inhibit calcium-dependent potassium ion channels indicate that triphenylmethyl-containing compounds can be placed into at least four distinct categories, each with a different mechanism of action.

Introduction

Approximately one in four deaths in the U.S. are caused by cancer, and in 2005 cancer overtook heart disease as the top killer of Americans under age 85.[1] Despite dramatic advances in our understanding of the underlying biology of this disease, cancer death rates have remained constant (at roughly 200 deaths for every 100000 people) over the last 30 years,[1] highlighting the need for new therapeutic agents. We recently identified a family of compounds, the triphenylmethylamides (TPMAs), which induce apoptotic death in multiple melanoma cell lines.[2] Two representative TPMAs, **4BI** and **4A**, are shown in Figure 1. The TPMAs arrest growth of melanoma cells in the G1-phase of the cell cycle, substantially reduce cellular levels of active NFκB, and induce apoptosis. In addition, the TPMAs have reduced toxicity to normal cells derived from the bone marrow of healthy human donors, and high doses of these compounds are well tolerated by mice.[2]

Other compounds containing the triphenylmethyl motif also possess anticancer properties (Figure 1). Notably, the antifungal agent clotrimazole inhibits the growth of cancer cells in culture,[3] and has shown efficacy in various mouse models of cancer.[3,4] Further studies indicate that clotrimazole arrests cells in the G1-phase of the cell cycle.[5] The anticancer properties of clotrimazole have been linked to its ability to affect intracellular Ca^{2+} levels thereby inhibiting translation, in addition to inhibition of glycolysis by inducing the detachment of mitochondrial-bound hexokinase.[3,6] Other studies have shown that 3,3-diaryl-1,3-dihyroindoles also have antiproliferative activity against cancer cell lines in cell culture,[7–9] and this effect may also be a result of the inhibition of translation mediated by depletion of intracellular Ca^{2+} stores.[8,9] A clotrimazole derivative lacking imidazole functionality has also been shown to induce G1-phase cell cycle arrest and inhibit tumor growth in mouse xenograft models,[10] and even simple triphenylmethane derivatives potently induce death of cultured cancer cells.[5] Finally, a recent publication highlighted the activity of S-trityl-L-cysteine (STLC, Figure 1), a compound that showed potent antitumor activity in a screen against the NCI-60 cell line panel.[11] S-trityl-L-cysteine induces M-phase arrest by targeting the Eg5 kinesin spindle motor protein involved in bipolar spindle formation and maintenance.[11] As evaluated by the NCI, STLC has shown anticancer activity in over 20 mouse xenograft models, and is

(1) American Cancer Society. In *American Cancer Society Statistics for 2007*, see: http://www.cancer.org/docroot/PRO/content/PRO_1_1_Cancer_Statistics_2007_Presentation.asp.
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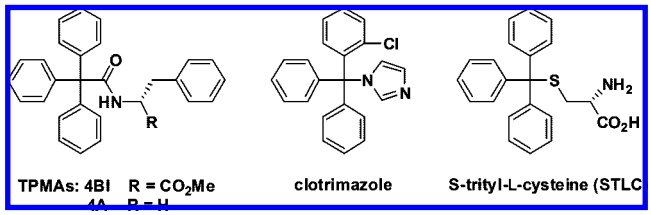


Figure 1. Triphenylmethyl-containing compounds with anticancer properties.

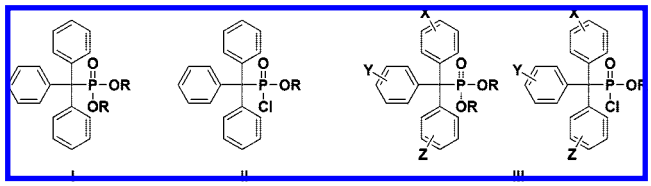


Figure 2. The three new classes of triphenylmethyl derivatives, called TPMPs, that are described in this manuscript: Class I compounds are phosphonates without ring substitution on the triphenylmethyl moiety. Class II compounds are phosphonochloridates without ring substitutions on the triphenylmethyl moiety. Class III compounds are phosphonates or phosphonochloridates with substituents on the triphenylmethyl moiety.

one of the 171 compounds in the NCI standard agent database that are of "special interest".[12]

The combined data indicates that triphenylmethyl-containing compounds have considerable potential as anticancer agents. However, the precise manner by which some compounds of this class exert their anticancer effect remains unknown. The goal of our current study was to build a unified picture of the anticancer mode of action of compounds containing this functional motif. Interestingly, although the triphenylmethyl moiety dominates the steric and conformational properties of the compounds described herein, our work shows that compounds containing this functional group can be placed into at least four distinct classes based on their mechanism of action and intracellular targets.

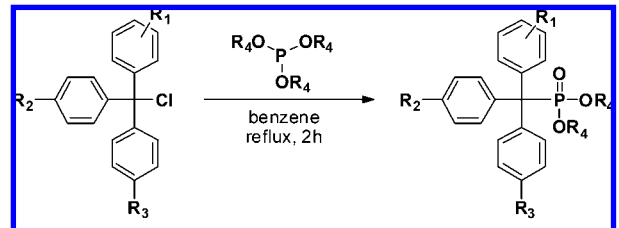
Results

Two of the most potent triphenylmethyl-containing anticancer compounds described in the literature are STLC and the TPMAs. Interestingly, these compounds appear to have very different modes of action. STLC induces M-phase cell cycle arrest, through the inhibition of the kinesin Eg5.[11] The TPMAs, conversely, induce G1-phase cell cycle arrest;[2] the biological target of the TPMAs is unknown. For this study we have designed several novel classes and subclasses of triphenylmethyl-containing compounds and have categorized them according to their cell cycle arresting properties. Compounds that arrested cellular growth in a discrete phase of the cell cycle and that were also potent death inducers were then evaluated in further biological assays.

Design and Synthesis of Novel Triphenylmethyl-Containing Compounds. Previous work had shown that the triphenylmethyl functional group was critical for activity of the TPMAs, as diphenylmethyl-containing derivatives are largely inactive against cultured cancer cells.[2] The diphenyl version of STLC is also inactive.[13] The substituent projecting off of the amide

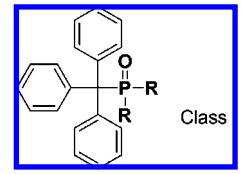
(12) For NCI standard agent database, see: http://dtp.nci.nih.gov/docs/cancer/searches/standard_agent_table.html.
(13) Ogo, N.; Oishi, S.; Matsuno, K.; Sawada, J.; Fujii, N.; Asai, A. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 3921–4.

Scheme 1. Synthesis of Triphenylmethyl Phosphonates of Class I and III by the Arbuzov Reaction



Triphenylmethyl chloride	Phosphite	TPMP	Yield
1. R_1, R_2, R_3 = H	R_4 = Me	TPMP-I-2	97%
2. R_1, R_2, R_3 = H	R_4 = Et	TPMP-I-3	95%
3. R_1, R_2, R_3 = H	R_4 = Bu	TPMP-I-6	92%
4. R_1 = 4-OMe; R_2, R_3 = H	R_4 = Me	TPMP-III-1	62%
5. R_1 = 4-OMe; R_2 = OMe, R_3 = H	R_4 = Me	TPMP-III-2	93%
6. R_1 = 4-OMe; R_2 = OMe, R_3 = H	R_4 = Et	TPMP-III-3	98%
7. R_1 = 4-OMe; R_2, R_3 = OMe	R_4 = Me	TPMP-III-4	67%
8. R_1 = 4-OMe; R_2, R_3 = OMe	R_4 = Et	TPMP-III-5	82%
9. R_1 = 2-Cl; R_2, R_3 = H	R_4 = Me	TPMP-III-6	25%

Table 1. The Evaluation of Class I TPMPs against the Human Melanoma Cell Lines SK-MEL-5 and UACC-62 in Cell Culture[a]



Compound	R	SK-MEL-5 (IC_{50} μM)	UACC-62 (IC_{50} μM)
TPMP-I-1	OH	>100	>100
TPMP-I-2	O—	12.3 ± 2.9	16.0 ± 3.3
TPMP-I-3	O	15.0 ± 5.6	13.5 ± 5.9
TPMP-I-4	O CF_3	15.1 ± 5.4	26.9 ± 8.6
TPMP-I-5	O	8.5 ± 3.0	15.3 ± 6.5
TPMP-I-6	O	6.6 ± 2.1	5.8 ± 1.6
TPMP-I-7	O	10.4 ± 2.8	12.1 ± 1.7
TPMP-I-8	O	9.9 ± 1.5	12.8 ± 2.4
TPMP-I-9	O	38 ± 16	48 ± 27
TPMP-I-10	O	5.4 ± 1.4	3.3 ± 0.4
TPMP-I-11	O OH HO	>100	>100
TPMP-I-12	O O	14.3 ± 4.8	13.8 ± 2.6

[a] Biomass was assessed by the sulforhodamine B assay after a 72 h incubation. Average IC_{50} values and standard deviations were determined from at least three independent experiments.

nitrogen of the TPMAs, however, appears to have little influence on the compound's anticancer properties. For example, many different amide derivatives, including amides of phenethylamine, 4-methyoxy-phenylamine, and 2-(3-methyoxy-phenyl)-ethylamine are all potent inducers of apoptosis in cancer cells in culture.[2] With the goal of obtaining a diverse array of triphenylmethyl-containing compounds for mechanistic studies, a series of derivatives were synthesized in which the amide in the TPMAs was replaced by a phosphonate or phosphonochloridate. This replacement allows the polarity of the linkage to the triphenylmethyl motif to be maintained, while permitting the projection of one additional substituent. These compounds

Scheme 2. Synthesis of *p*-Hydroxy-Substituted Triphenylmethyl Phosphonate **TPMP-III-8**

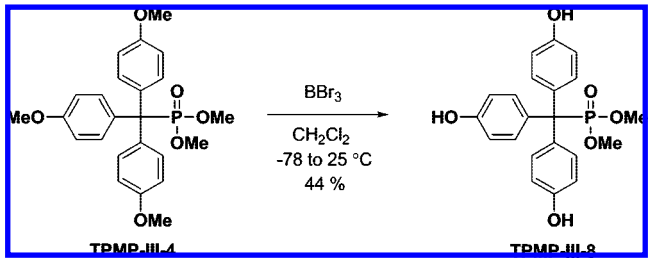
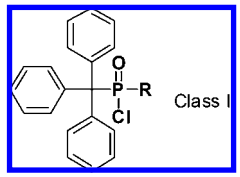


Table 2. The Evaluation of Class II TPMPs against the Human Melanoma Cell Lines SK-MEL-5 and UACC-62 in Cell Culture[a]



Compound	R	SK-MEL-5 (IC_{50} μM)	UACC-62 (IC_{50} μM)
TPMP-II-1	Cl	>100	>100
TPMP-II-2	O	3.3 ± 1.6	4.3 ± 1.3
TPMP-II-3	O	8.4 ± 3.9	7.5 ± 1.5

[a] Biomass was assessed by the sulforhodamine B assay after a 72 h incubation. Average IC_{50} values and standard deviations were determined from at least three independent experiments.

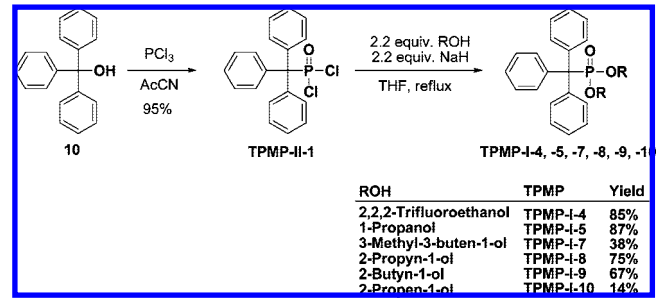
can be placed into three structural classes: phosphonates (Figure 2, class I), phosphonochloridates (Figure 2, class II), and ring-substituted derivatives of class I and II (Figure 2, class III). As a group these compounds are called triphenylmethyl phosophonates/triphenylmethyl phosphonochloridates (TPMPs, see Figure 2). The list of TPMPs synthesized and described herein is given in Tables 1–3.

The synthetic routes to these compounds are shown in Schemes 1-6. Phosphonates bearing dimethyl, diethyl, or dibutyl groups were synthesized in moderate to good yields (25−98%, average of 79%) via the Arbuzov reaction between the commercially available trimethyl-, triethyl-, or tributylphosphite and the triphenylmethyl chlorides (compounds **1**−**9**, Scheme 1). Reactions were complete after heating to reflux in benzene for 2 hours. Compound **TPMP-III-7** was synthesized as previously described,[14] and **TPMP-III-8** was created through the demethylation of the *para*-methoxy triphenylmethyl phosphonate **TPMP-III-4** (Scheme 2).

Owing to the limited availability of trialkylphosphites, displacement of the chlorines of triphenylmethyl phosphonyl dichloride **TPMP-II-1** with alkoxides was employed to gain access to a wider variety of phosphonates (Scheme 3). Treatment of dichloride **TPMP-II-1** with 2.2 equiv of alkoxide yielded primarily phosphonates of Class I. As shown in Scheme 3, six Class I TPMPs were synthesized through this route, with yields ranging from 14−87% (av 59%). The use of 1.3 equiv of alkoxide allowed access to phosphonochloridates of Class II and III (Scheme 4, av 51%). Two additional reactions were used to produce two other TPMPs. Reaction of **TPMP-II-1** with the

(14) Iliopulos, M. I.; Wieder, H. *Angew. Chem.* **1965**, *77*, 618–19.

Scheme 3. Synthesis of Phosphonates of Class I by Treatment of the Phosphonyldichloride **TPMP-II-1** with 2.2 equiv of Alkoxide



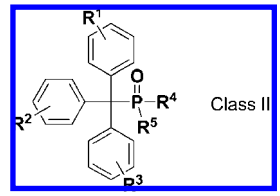
alkoxide 1,2-ethanediol yielded the ring closed phosphonate **TPMP-I-12** (Scheme 5), and dihydroxylation of **TPMP-I-10** afforded the phosphonate **TPMP-I-11** bearing alkyl groups with hydroxyl functionality (Scheme 6).

Determination of IC_{50} Values. All compounds were evaluated for their ability to induce death in the UACC-62 and SK-MEL-5 cell lines, both human malignant melanoma cell lines. The UACC-62 cell line has known mutations in the *braf*, *pten*, and *cdkn2a* genes,[15] and expresses Apaf-1 at intermediate levels.[16] The SK-MEL-5 cell line is a human melanoma derived from an auxiliary node of a Caucasian female.[17] It has known mutations in the *braf* and *cdkn2a* genes.[18] Additionally, there is little-to-no expression of Apaf-1 in this cell line, making it resistant to apoptotic stimuli that require apoptosome formation.[16]

The 25 TPMPs synthesized and the four compounds from Figure 1 were evaluated at a range of doses to obtain dose−response curves from which IC_{50} values could be calculated (see Supporting Information for dose response curves). The IC_{50} value determinations for active compounds (IC_{50} values < 100 μM) were made on at least three different occasions, in triplicate each time, according to the following protocol. UACC-62 or SK-MEL-5 cells (3000 cells/well in 96-well plates) were incubated in the presence of compounds for 72 h at 37 °C. The biomass was quantified by staining with sulforhodamine B; the results for each compound class are displayed in Tables 1, 2, and 3. In general, select compounds in each structural class had low micromolar potency against these melanoma cell lines; the most potent compounds were taken into the cell cycle evaluation, described below. The IC_{50} values for the compounds in Figure 1 were also determined alongside the TPMPs, and the values are as follows (against the UACC-62 and SK-MEL-5 cell lines, respectively): **4BI** = 11.1 ± 1.1 and 13.1 ± 0.9 μM, **4A** = 13.4 ± 0.7 and 13.8 ± 3.0 μM, clotrimazole = 9.0 ± 1.2 and 11.2 ± 0.6 μM, STLC = 0.8 ± 0.1 and 0.7 ± 0.3 μM.

Evaluation of Cell Cycle Arrest. As mentioned, TPMAs and clotrimazole are reported to arrest the growth of cancer cells in the G1-phase of the cell cycle, while STLC induces arrest in the M-phase. To determine the cell cycle arresting properties of the novel phosphorus-containing analogues of the TPMAs, HL-60 cells (human leukemia cell line) were treated with the most potent compounds at 20 μM for 12 h. HL-60 cells grow rapidly and in suspension (not adhered to the culture flask) in culture, and thus readily lend themselves to cell cycle analysis experiments. The population of cells in the different phases of the cell cycle was measured by propidium iodide staining and flow cytometry. Interestingly, the TPMPs displayed a range of cell cycle arresting properties. The data is displayed for the previously known compounds (Figure 3A), the new compounds

Table 3. The Evaluation of Class III TPMPs against the Human Melanoma Cell Lines SK-MEL-5 and UACC-62 in Cell Culture[a]



Compound	R^1	R^2	R^3	R^4	R^5	SK-MEL-5 (IC_{50} μM)	UACC-62 (IC_{50} μM
TPMP-III-1	4-OMe	H	H	OMe	OMe	12.4 ± 3.3	14.0 ± 7.1
TPMP-III-2	4-OMe	4-OMe	H	OMe	OMe	2.2 ± 0.6	2.5 ± 0.8
TPMP-III-3	4-OMe	4-OMe	H	OEt	OEt	10.2 ± 5.1	12.6 ± 6.0
TPMP-III-4	4-OMe	4-OMe	4-OMe	OMe	OMe	3.9 ± 0.8	5.0 ± 1.0
TPMP-III-5	4-OMe	4-OMe	4-OMe	OEt	OEt	10.3 ± 3.7	12.6 ± 3.2
TPMP-III-6	2-Cl	H	H	OMe	OMe	15.2 ± 0.8	8.2 ± 5.2
TPMP-III-7	4-OH	4-OH	H	OMe	OMe	>100	>100
TPMP-III-8	4-OH	4-OH	4-OH	OMe	OMe	>100	>100
TPMP-III-9	4-OMe	H	H	O	Cl	5.4 ± 1.4	3.3 ± 0.4
TPMP-III-10	4-Me	4-Me	4-Me	O	Cl	5.4 ± 2.9	5.8 ± 1.9

[a] Biomass was assessed by the sulforhodamine B assay after a 72 h incubation. Average IC_{50} values and standard deviations were determined from at least three independent experiments.

that induce G1-phase cell cycle arrest (Figure 3B), and the new compounds that induce G2/M-phase cell cycle arrest (Figure 3C). With this identification of several novel TPMPs that potently kill cancer cells (namely **TPMP-II-2**, **TPMP-III-2**, **TPMP-III-4**) and induce arrest in either the G1- or the G2/M-phase of the cell cycle, we set out to compare these novel compounds with the known compounds (from Figure 1) in assays examining the effect of compounds on mitosis, mitochondrial-bound hexokinase dissociation, and the effect of compounds on calcium-dependent potassium channels.

Effects on Mitosis and Tubulin Polymerization. STLC has previously been shown to induce the arrest of cellular growth in the M-phase of the cell cycle, and cells treated with STLC display a "monoaster" phenotype when examined by fluorescence microscopy staining for tubulin and DNA.[11] This monoaster phenotype is indicative of compounds that target the kinesin Eg5, and indeed STLC has been shown to be a potent and reversible Eg5 inhibitor in vitro (IC_{50} = 500 nM).[11] Figure 4 shows the effect of both STLC and **TPMP-III-2** on microtubules as examined by confocal microscopy. Consistent with data in the literature, cells treated with STLC display the "monoaster" phenotype. Interestingly, while **TPMP-III-2** also induces arrest in the M-phase of the cell cycle, cells treated with this compound do not show the monoaster phenotype but rather possess fragmented mitotic spindles characteristic of cells treated with antimicrotubule compounds (Figure 4).

Given this data, we assessed the effect of **TPMP-III-2** and several of the other triphenylmethyl-containing compounds on in vitro tubulin polymerization. Tubulin polymerization can be monitored spectrophotometrically by the increase in optical density at 340 nm. Compounds such as Taxol are known to stabilize microtublules (and give an increased rate of polymerization in this assay), whereas compounds such as nocodazole prevent tubulin polymerization. As shown by the data in Figure 5, **TPMP-III-2** is clearly a compound that prevents the polymerization of tubulin. The G1-phase arrestor **TPMP-I-2** had no effect in this tubulin polymerization assay (Figure 5). Interestingly, 4,4′-dimethoxytriphenylmethanol (structurally similar to **TPMP-III-2**) did not affect tubulin polymerization, demonstrating the necessity of both the phosphonate moiety and the *para*-methoxy functionality (Figure 5). STLC and clotrimazole also did not affect tubulin polymerization, while **TPMP-III-1** and **TPMP-III-4** inhibited tubulin polymerization to a modest degree at higher compound concentrations, consistent with their G2/M-phase arresting properties (See Supporting Information Figures S1 and S2).

Effect of Compounds on the Detachment of Mitochondrial-Bound Hexokinase. Clotrimazole and other antifungal azole-derivatives such as bifonazole, econazole, miconazole, and ketoconazole have been identified as calmodulin antagonsists because of their ability to inhibit calmodulin-dependent phosphodiesterase activity in vitro.[19] Clotrimazole exhibits a greater than 54-fold selective inhibition of calmodulin-dependent versus calmodulin-independent phosphodiesterase activity in vitro with an IC_{50} value of 18 μM.[19] As calmodulin activity plays a central role in the maintenance of cellular metabolism, calmodulin antagonists may exert an antiproliferative effect by disrupting metabolic events such as glycolysis.[20] In fact, treatment of melanoma B16−F10 cells with clotrimazole and bifonazole causes detachment of the key glycolytic enzymes phosphofruc-

(15) The Cancer Genome Project. http://www.sanger.ac.uk/perl/genetics/CGP/cosmic?action=sample&id=905976.
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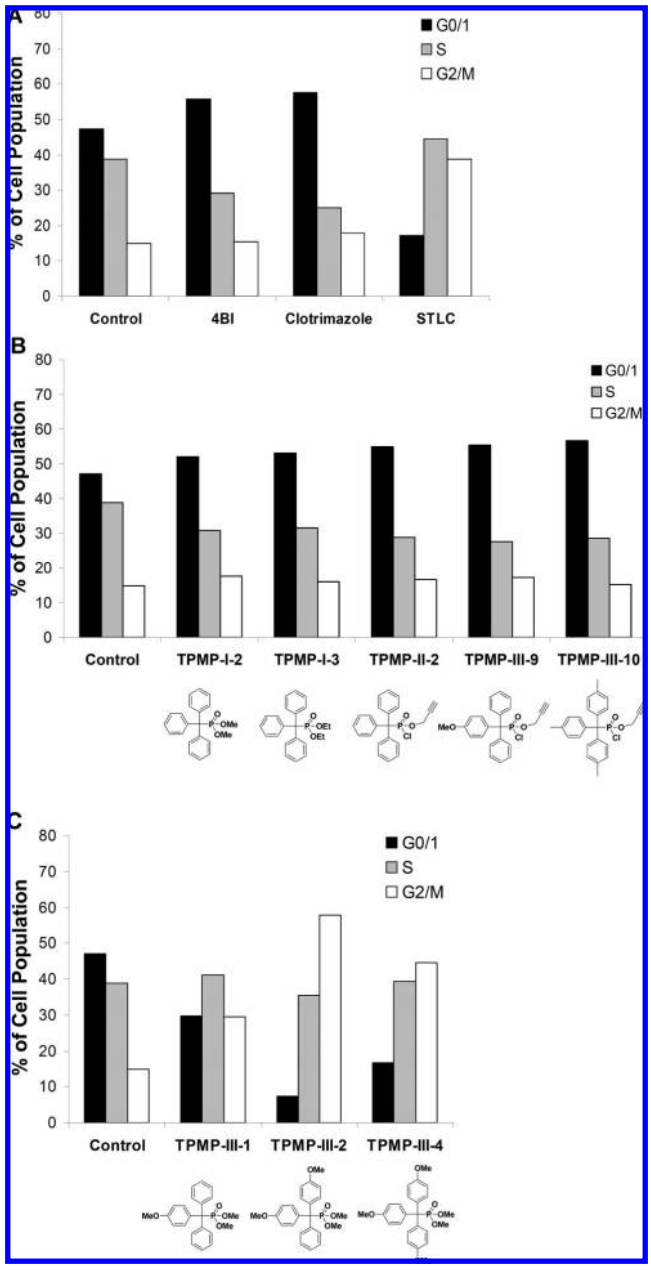

Figure 3. Cell cycle arresting properties of compounds containing the triphenylmethyl group. HL-60 cells (human leukemia) were treated with the indicated compound at 20 μM for 12 h, and the cell cycle distribution was examined by propidium iodide staining and flow cytometry. (A) The cell cycle arresting properties of known triphenylmethyl compounds that possess anticancer activity. Compound **4BI** and clotrimazole induce cell cycle arrest in the G1-phase whereas STLC induces M arrest. (B) Certain TPMPs induce G1-phase cell cycle arrest, (C) while others induce arrest in the G2/M-phase of the cell cycle.

tokinase and aldolase from the cytoskeleton and hexokinase from the outer mitochondrial membrane prior to affecting cell viability.[21–23] As elevated glycolytic activity is a major hallmark of cancer, compounds that inhibit glycolysis may prove to be effective anticancer agents.[24,25] It has been proposed that clotrimazole's ability to cause mitochondrial-bound hexokinase

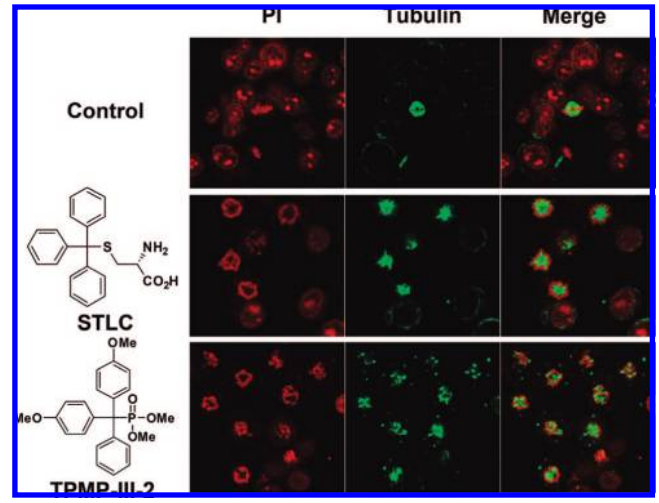

Figure 4. The effect of STLC and **TPMP-III-2** on microtubule assembly in HeLa cells after an 18 h incubation in the presence of 20 μM compound. Cells were fixed and the nuclei and microtubules were stained with propidium iodide (PI) and a FITC-conjugated antitubulin antibody, respectively. The "monoaster" phenotype is visible for STLC, whereas **TPMP-III-2** induces fragmented mitotic spindles.

detachment is the result of an allosteric interaction rather than a result of calmodulin antagonistic acitivity.[26] This is supported by the inability of other calmodulin antagonists to induce the dissociation of mitochondrial-bound hexokinase.[22]

Given the structural similarity of clotrimazole and the other triphenylmethyl-containing compounds, it was of interest to determine whether these compounds could induce detachment of hexokinase from the mitochondrial outer membrane and thereby exert their antiproliferative effect by inhibiting glycolysis and reducing cellular ATP levels. Melanoma B16−F10 cells were incubated in the presence of modest concentrations of compound (15 μM) for a short period of time (2 h) after which the cells were lysed and hexokinase activity of the mitochondrial-rich fraction was assessed and compared to vehicle-treated cells. As shown in Figure 6, treatment of cells with clotrimazole induced a 71% reduction in mitochondrial-bound hexokinase. This result is consistent with previous observations.[22] Other triphenylmethyl compounds which exhibit equal or significantly greater antiproliferative activity than clotrimazole in cell culture were not able to detach mitochondrial-bound hexokinase as effectively as clotrimazole under the same conditions (Figure 6), suggesting that these compounds induce death through an alternate mechanism.

Effect of Compounds on Gardos Channel Inhibition. In addition to its calmodulin antagonist activity and ability to cause the detachment of hexokinase from mitochondria, clotrimazole is also a potent inhibitor of the calcium-dependent potassium ion channel, also known as the Gardos channel.[27] A binding site model has been proposed for the inhibition of the Gardos channel by clotrimazole and another triphenylmethane derivative based on site directed mutagenesis.[28] Inhibitors of the Gardos channel show promise for the treatment of sickle cell disease

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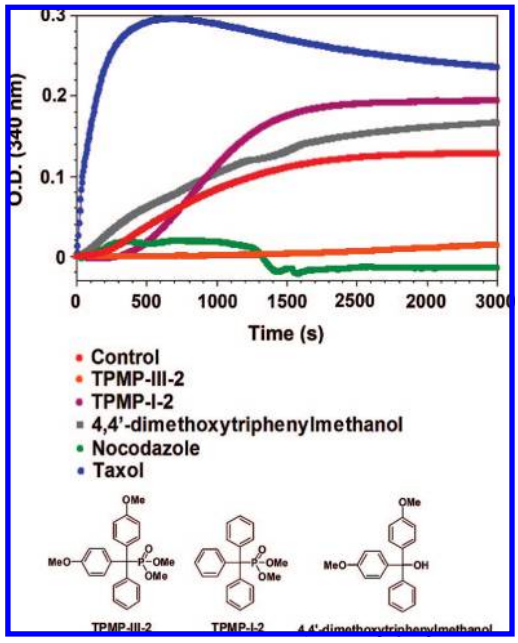

Figure 5. The effect of TPMPs (10 μM) on in vitro tubulin polymerization. As monitored by the optical density at 340 nm, **TPMP-III-2** inhibits the polymerization of tubulin, whereas **TPMP-I-2** and 4,4′-dimethoxytriphenylmethanol have no effect.

as they prevent red blood cell dehydration that may occur during oxygen−deoxygenation cycling.[29] In fact, several triphenylmethyl-containing compounds with potent Gardos channel inhibition properties have been previously described, and a triphenylmethylamide is currently in phase III clinical trials for sickle cell disease.[29] Interestingly, halide substitution on the triphenylmethyl group appears to be required for potent Gardos channel inhibition.[30]

Apart from the relevance of Gardos channel inhibition to sickle cell disease, the Gardos channel is also implicated in the proliferation of melanoma.[31,32] Blockers of the channels induce cell cycle arrest by causing membrane depolarization, which reduces Ca^{2+} influx that would otherwise promote growth and proliferation.[31,32] It was therefore of interest to assess the inhibition properties of triphenylmethyl-containing compounds on Gardos channel activity. As rubidium efflux is mediated by the Gardos channel,[33] human red blood cells were loaded with ^{86}Rb and incubated in the presence of various concentrations of compound or vehicle. Efflux was initiated by the addition of calcium and the calcium ionophore (A23187), and dose response curves were constructed upon analysis of rubidium content in the supernatant. As shown in Table 4, clotrimazole is a potent inhibitor of the Gardos channel with an average IC_{50} value of 0.92 μM, consistent with previously reported results.[34] The novel G1-phase arrestors **TPMP-I-2**, and **TPMP-II-2** exhibited good-to-modest inhibition with average IC_{50} values of 1.7 and 13.8 μM, respectively. The microtubule-inhibiting compound **TPMP-III-2**, the TPMAs, and STLC were poor inhibitors of the Gardos channel with IC_{50} values ranging from 35 to >100 μM. Given the potent death inducing abilities of these triphenylmethyl compounds relative to clotrimazole, their generally modest inhibition of the Gardos channel would suggest that the inhibition of calcium-dependent potassium channels is not the primary mechanism by which these compounds exert their anticancer activity.

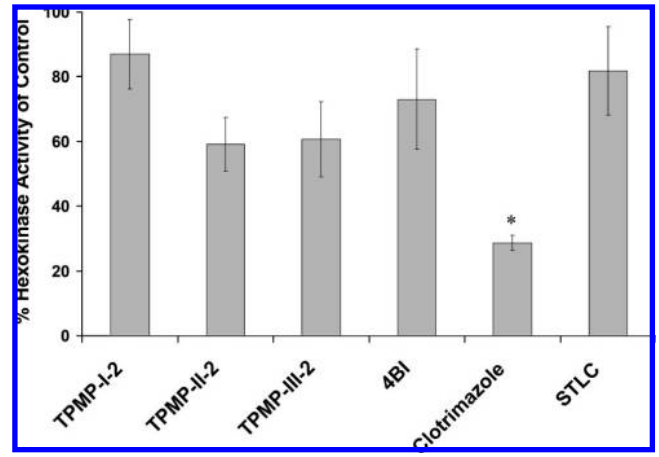

Figure 6. The assessment of mitochondrial-hexokinase activity in murine melanoma B16−F10 cells after 2 h treatment with compound (15 μM), as compared to vehicle-treated control cells. Clotrimazole causes a 71% reduction in mitochondrial-hexokinase activity whereas other triphenylmethyl containing compounds show limited effect (* = p values <0.012).

Effect of Compounds on Multiple Cell Lines. To investigate the general anticancer properties of the more potent TPMPs, compounds **TPMP-II-2**, **TPMP-III-2**, and **TPMP-III-4** were tested for their ability to induce death in a variety of cancer cell lines. As shown in Table 5, these compounds potently induced death in most of the cancer cell lines tested, with only the PC-12 rat adrenal cancer cell line being resistant. The microtubule-inhibiting compound **TPMP-III-2** was, in general, slightly more potent that the other two compounds in the majority of the cell lines.

Discussion

We recently reported a class of small molecules, the TPMAs, which induce arrest of cancer cell lines in culture in the G1-phase of the cell cycle.[2] From our initial analysis, the triphenylmethyl functional group was the critical pharmacophore, as diphenylmethyl derivatives had greatly reduced death-inducing properties. It was interesting to note other compounds in the literature that possess a triphenylmethyl motif, most notably STLC and clotrimazole also appear to be potent anticancer agents. We thus created a series of novel compounds containing this functional group, the TPMPs, and found that several of them induce death in cancer cell lines in culture. These compounds were then tested in a battery of biological assays, and the results were compared to the TPMAs, clotrimazole, and STLC. Interestingly, we have found that these structurally related compounds have very different mechanisms of action.

From analysis of the data presented herein, the replacement of the TPMA amide with the phosphonate or phosphonochloridate motif provides many compounds possessing enhanced anticancer activity. Compounds **TPMP-I-2** through **-8**, **TPMP-I-10**, **TPMP-I-12**, **TPMP-II-2**, **TPMP-II-3**, and **TPMP-III-1**

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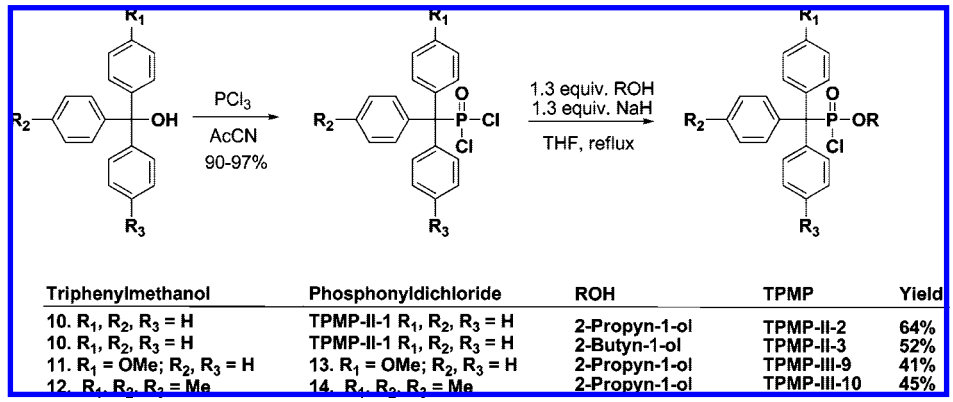
Scheme 4. Synthesis of Phosphonochloridates of Class II and III by Treatment of Phosphonyldichlorides with 1.3 equiv of Alkoxide

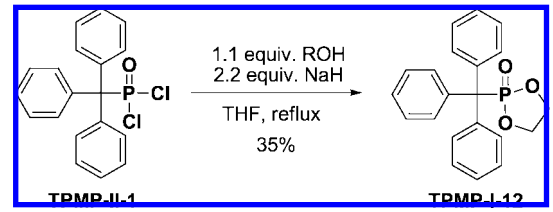


Triphenylmethanol	Phosphonyldichloride	ROH	TPMP	Yield
10. R_1, R_2, R_3 = H	TPMP-II-1 R_1, R_2, R_3 = H	2-Propyn-1-ol	TPMP-II-2	64%
10. R_1, R_2, R_3 = H	TPMP-II-1 R_1, R_2, R_3 = H	2-Butyn-1-ol	TPMP-II-3	52%
11. R_1 = OMe; R_2, R_3 = H	13. R_1 = OMe; R_2, R_3 = H	2-Propyn-1-ol	TPMP-III-9	41%
12. R_1, R_2, R_3 = Me	14. R_1, R_2, R_3 = Me	2-Propyn-1-ol	TPMP-III-10	45%

Table 4. Comparison of Various Triphenylmethyl-Containing Compounds and Their IC_{50} Values for ^{86}Rb Efflux Inhibition in Human Red Blood Cells

compound	av IC_{50} (μM)
clotrimazole	0.92
TPMP-I-2	1.7
TPMP-II-2	13.8
TPMP-III-2	56
4BI	35
4A	>100
STLC	>100

Scheme 5. Synthesis of the Ring-Closed Phosphonate **TPMP-I-12**



through **-6**, **TPMP-III-9** and **TPMP-III-10** all were reasonably potent versus both melanoma cell lines, with IC_{50} values ranging from 2.2 to 26.9 μM. An increase in activity was seen with compounds that had methyl ether substitutions on the aromatic rings; for example, **TPMP-III-2** and **TPMP-III-4** were quite potent against the melanoma cell lines, with IC_{50} values ranging from 2.2 to 5 μM. Compounds containing functional groups that increased aqueous solubility were not very potent, such as **TPMP-I-11**, **TPMP-III-7**, and **TPMP-III-8**.

The two phosphonochloridate derivatives created and evaluated were quite effective in killing melanoma cell lines (Table 2). One of these compounds in particular, **TPMP-II-2**, was quite potent in the induction of cell growth inhibition, with IC_{50} values of 3.3−4.3 μM. To our knowledge this is the first demonstration of biological activity of triphenylmethyl phosphonochloridates. Although certain compounds containing the phosphonochloridate moiety have been previously shown to rapidly hydrolyze in water,[35,36] our investigations show that compound **TPMP-II-2** is fully stable in aqueous solutions for multiple months (see Supporting Information Figure S3). This compound is also stable in both acidic and basic aqueous solutions; no evidence of degradation is observed when placed in aqueous solutions of pH 1.5 through pH 11.8 for 20 h (see Supporting Information Figure S3). Given their potencies, further study of this interesting class of compounds is warranted.

Additionally, while certain compounds containing the phosphonochloridate moiety have been demonstrated to irreversibly inhibit serine proteases such as trypsin, chymotrypsin, and acetylcholine esterase,[35–37] **TPMP-II-2** is only a modest chymotrypsin inhibitor in vitro with activities comparable to triphenylmethyl-containing compounds that do not possess functionalities that may irreversibly inhibit the serine protease (see Supporting Information). Clotrimazole is a potent chymotrypsin inhibitor, and it is in fact known to inhibit multiple enzymes nonspecifically.[38] Conversely, several of the TPMPs (such as **TPMP-I-2** and **TPMP-III-2**) do not show this promiscuous inhibition (see Supporting Information).

Because of the size and hydrophobic nature of the triphenylmethyl pharmacophore, it might be suspected that anticancer compounds possessing this functional group share a common mode of cell death induction. However, we were able to clearly separate the triphenylmethyl-containing compounds based on their cell cycle arresting properties. Further assays related to mitosis, mitochondrial-bound hexokinase detachment, and Gardos channel inhibition, were conducted on the most potent compounds. From the combined data a unified picture has emerged, revealing at least four distinct mechanisms of cell death induced by compounds containing the triphenylmethyl pharmacophore (Table 6): (1) STLC induces cell cycle arrest in the M-phase, consistent with its known inhibition of the kinesin Eg5. STLC has no effect on tubulin polymerization, nor does it inhibit calcium-dependent potassium ion channels. (2) Clotrimazole induces cell death through a much different process. It arrests cells in the G1-phase of the cell cycle, and it has been shown to cause the translocation of hexokinase from the mitochondrial outer membrane thus inhibiting glycolysis resulting in the depletion of ATP. Clotrimazole is also a potent inhibitor of calcium-dependent potassium ion channels, which can affect the transition from G1- to S-phase by modulating intracellular calcium levels.[31]

On the basis of the data reported herein neither the TPMAs nor the class I, II, or III TPMPs behave in a manner similar to STLC or clotrimazole, and we believe these compounds can be placed into two further mechanistic classes. (3) The third class of compounds are triphenylmethyl-containing derivatives that inhibit tubulin polymerization. **TPMP-III-2** and **TPMP-**

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Table 5. The Effect of **TPMP-II-2**, **TPMP-III-2**, and **TPMP-III-4** on Multiple Cancer Cell Lines in Cell Culture[a]

cell line	cancer type	TPMP-II-2 (IC_{50} μM)	TPMP-III-2 (IC_{50} μM)	TPMP-III-4 (IC_{50} μM)
SK-MEL-5	melanoma (human)	3.3 ± 1.6	2.2 ± 0.6	3.9 ± 0.8
UACC-62	melanoma (human)	4.3 ± 1.3	2.5 ± 0.8	5.0 ± 1.0
PC-12	adrenal (rat)	77 ± 25	10.6 ± 0.6	23 ± 6
HL-60	leukemia (human)	2.3 ± 0.2	1.4 ± 0.4	2.2 ± 0.3
U-937	lymphoma (human)	4.5 ± 0.2	1.7 ± 0.7	2.3 ± 0.1
IGROV-1	ovarian (human)	1.2 ± 0.1	2.9 ± 1.3	4.3 ± 3.1
MCF-7	breast (human)	9.2 ± 0.8	5.1 ± 0.2	6.8 ± 1.9
SK-N-SH	neuroblastoma (human)	4.0 ± 2.6	4.2 ± 0.4	7.1 ± 4.4

[a] Cells were treated with the compounds for 72 h and death was quantified via the methods described in the Materials and Methods section. Average IC_{50} values and standard deviations were determined from at least three independent experiments.

Scheme 6. Synthesis of Triphenylmethyl Phosphonate **TPMP-I-11** via the Dihydroxylation of **TPMP-I-10**

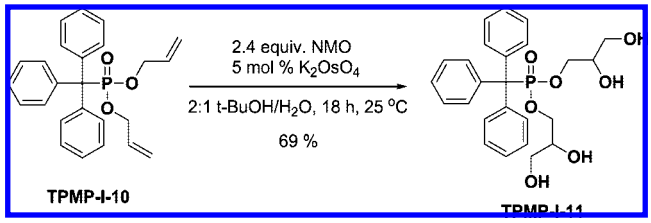

Table 6. Comparison of Various Anticancer Triphenylmethyl-Containing Compounds Based on Their Activity in a Variety of Assays

compound	cell cycle arrest	affects microtubules?	mitchondrial-hexokinase dissociation ability	Ca^{2+}-dependent K^+ channels	mechanism of action
STLC	M	no	weak	no effect	Eg5 kinesin inhibitor
clotrimazole	G1	no	strong	inhibitor	intracellular Ca^{2+} depletion, translation inhibition
TPMP-III-2	M	inhibitor	weak	no effect	antitubulin
4BI/4A	G1	no	weak	no effect	unknown
TPMP-I-2	G1	no	weak	inhibitor	unknown
TPMP-II-2	G1	no	weak	inhibitor	unknown

III-4 inhibit tubulin polymerization in vitro and induce M-phase arrest of cells in culture. There does appear to be a relationship between the antimitotic effect and the degree of methoxy substitution in these compounds. These are the first examples of triphenylmethyl-containing compounds that are able to inhibit tubulin polymerization. (4) TPMAs and TPMPs that are G1-phase arrestors (such as **TPMP-I-2**, **TPMP-III-2**) are in a final category. In fact, TPMAs and TPMPs with no functionality on the triphenylmethyl moiety all appear to induce cell death in a similar fashion (causing G1-phase arrest), regardless of whether they contain an amide, phosphonate, or phosphonochloridate functional group. Although **TPMP-I-2** and **TPMP-III-2** and TPMAs are modest inhibitors of calcium-dependent potassium ion channels in comparison to clotrimazole, the potency of Gardos channel inhibition does not correlate with the antiproliferative efficacy observed in cell culture. The precise biological target of these compounds remains unknown at this point.

Several important pieces of information can be gleaned from this data. First, assessment of these derivatives shows that subtle changes in chemical composition can dramatically alter the mechanism of action of a small molecule. In this case, one level of mechanistic discrimination is made through the connection of the triphenylmethyl motif with either cysteine (STLC), imidazole (chotrimazole), or amides, phosphonates, phosphonochloridates (TPMAs/TPMPs). Mechanistically distinct classes of compounds further arise when substitutions are made on the aryl rings of TPMAs and TPMPs. Second, a major drawback of the antitubulin drugs (such as Taxol and the Vinca alkaloids) is the inability to penetrate the blood-brain-barrier (BBB) due to active efflux by P-glycoproteins, thus rendering these compounds less useful for CNS tumors.[39] In fact, few compounds that have tubulin as their biological target penetrate the BBB.[39] The lipophilic nature of the triphenylmethyl functional group and the known BBB penetrance of clotrimazole[40] suggest that derivatives based on **TPMP-III-2** may provide the rare antimitotic that penetrates the BBB. Third, triphenylmethyl phosphonates and triphenylmethyl phosphonochloridates are novel and potent anticancer compounds, and the G1-phase arresting subclass of these agents appears to operate by a unique mode of action, distinct from clotrimazole. As shown in Table 5, several of these compounds are potent death inducers to a variety of cancer cell lines in culture.

In summary, we have discovered that phosphonate and phosphonochloridate analogues of triphenylmethylamides induce apoptosis in melanoma and other cancer cell lines and arrest cellular growth in the G1- or M-phases of the cell cycle. Preclinical studies with the most potent compounds and experiments directed toward the identification of the precise biological targets of the G1-phase arrestors are underway and will be reported in due course.

Acknowledgment. We thank Professor Timothy Mitchison (Harvard Medical School) for the gift of tubulin and Professor John A. Katzenellenbogen and Dr. Kathryn E. Carlson for their guidance regarding the Gardos channel inhibition assay. We also acknowledge the assistance of the cell flow cytometry and mass spectrometry facilities at the University of Illinois, Urbana−Champaign. We are grateful to the American Cancer Society (Grant RSG-07-026-01) for funding this work.

Supporting Information Available: Complete reference 34; materials and methods; NMR spectral characterization; IC_{50} curves for cell culture experiments; Gardos channel inhibition and chymotrypsin inhibition; supporting figures. This material is available free of charge via the Internet at http://pubs.acs.org.

JA8020999

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