Biological Mechanisms of Action of Novel C-10 Non-Acetal Trioxane Dimers in Prostate Cancer Cell Lines

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Received July 8, 2006

The mechanisms of action of three C-10 non-acetal trioxane dimers (TDs) were examined in human (LNCaP) and mouse (TRAMP-C1A and -C2H) prostate cancer cell lines. **1** (AJM3/23), **2** (GHP-TM-III-07w), and **3** (GHP-KB-06) inhibited cell growth with **3** being the most potent in C1A (GI_{50} = 18.0 nM), C2H (GI_{50} = 17.0 nM), and LNCaP (GI_{50} = 17.9 nM) cells. In comparison to a standard cytotoxic agent such as doxorubicin (GI_{50} = 45.3 nM), **3** (GI_{50} = 17.9 nM) inhibited LNCaP cell growth more potently. TDs induced G_0/G_1 cell cycle arrest in LNCaP cells and decreased cells in the S phase. These changes correlated with modulation of G_1 phase cell cycle proteins including decreased cyclin D1, cyclin E, and cdk2 and increased $p21^{waf1}$ and $p27^{Kip1}$. TDs also promoted apoptosis in LNCaP cells with increased expression of proapoptotic bax. These results demonstrate that TDs are potentially useful agents that warrant further preclinical development for treatment of prostate cancer.

Introduction

It is estimated that prostate cancer is the most prevalent cancer and the third leading cause of cancer death in American men.[1] Current treatment options for localized, early-stage prostate cancer include surgery, radiation therapy, and watchful waiting.[2] However, most patients relapse with advanced metastatic disease, that when treated with androgen deprivation therapy eventually becomes hormone refractory.[2] Clinical trials have been conducted with chemotherapeutic agents such as docetaxel, mitoxantrone, and doxorubicin in men with hormone refractory prostate cancer; however, these agents have limited efficacy and significant adverse side effects.[3] The poor clinical outcome of advanced metastatic prostate cancer highlights the urgent need to develop effective novel therapeutic agents for prevention and treatment of the disease.

Trioxane dimers (TDs[a]) are semisynthetic derivatives of the Chinese antimalarial compound 1,2,4-trioxane artemisinin (*Artemesia annua* L., qinghaosu).[4] The instability of first-generation TDs has prompted synthesis of more potent, orally active, and thermally and hydrolytically stable second-generation C-10 non-acetal TDs.[5−7] These compounds display potent antimalarial, antiproliferative, and anticancer activities.[5−7] They inhibit proliferation of chloroquine-sensitive *Plasmodium falciparum*,[5−7] murine keratinocytes,[5,6] and several malignant human cancer cell lines in the National Cancer Institute's (NCI) Developmental and Therapeutics Program.[5−7] Newly synthesized TDs are selectively cytotoxic in HeLa human cervical cancer cells compared to normal cervical cells.[8] In vivo, TDs decrease tumor mass in the NCI mouse hollow fiber assay.[5] Toxicity studies in antimalarial mouse models indicate that up to 125 mg/kg of TDs can be safely administered.[9] Although the antitumor modes of action of TDs have not been extensively characterized, the mechanisms of action of the parent compound (artemisinin) and its semisynthetic derivatives have been studied. Artemisinin derivatives inhibit proliferation, induce G_1 phase cell cycle arrest, and increase apoptosis in P388 leukemia cells.[10] mRNA expression profiling studies indicate that the antineoplastic activity of artemisinin derivatives in a diverse panel of tumor cell lines from the NCI correlates with changes in expression of genes involved in cell proliferation.[11] Further studies demonstrated that the cytotoxicity of an artemisinin derivative (artesunate) in a panel of 55 cancer cell lines results in induction of G_0/G_1 cell cycle arrest, inhibition of S phase progression, and changes in expression of cell cycle regulatory proteins.[12]

Cyclin/cyclin dependent kinase (cdk) complexes including cyclin D−cdk4/cdk6 and cyclin E−cdk2 complexes are important for progression of cells through the G_1 phase of the cell cycle and initiation of DNA replication.[13] Overexpression of cyclin D1[14] and cdk4[15,16] appears to be important for oncogenic transformation. The Cip/Kip family of cdk inhibitors, $p21^{Waf1}$ (p21) and $p27^{Kip1}$ (p27), impede cell cycle progression by inhibiting cyclin E−cdk2 complexes.[17] p21 induces G_1 or G_2 cell cycle arrest and inhibits DNA synthesis via inhibition of cdk activity and proliferating cell nuclear antigen (PCNA).[18]

We previously demonstrated that **1** (AJM3/23) inhibits the growth of prostate cancer clonal cell lines (C1A, C2D, C2G, and C2H).[9] These clonal cell lines were derived from a poorly differentiated tumor that developed in the transgenic adenocarcinoma of mouse prostate (TRAMP) model.[19] Probasin promoter-driven expression of SV40 early genes (T and t) in the prostatic epithelium leads to progressive development of prostate cancer in the TRAMP mouse model.[20] The mechanisms of action of three C-10 non-acetal TDs that were synthesized by chemical substitution at the C-10 position (Figure 1) were evaluated using human prostate cancer cells (LNCaP) and two clonal cell lines derived from the TRAMP mouse model (C1A and C2H). The molecular effects of **1**, **2** (GHP-TM-III-07w), and **3** (GHP-KB-06) in prostate cancer cells include inhibition of cell proliferation, regulation of cell cycle progression, and induction of apoptosis.

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[a] Abbreviations: TDs, trioxane dimers; GI_{50}, concentration that inhibits 50% cell growth; TRAMP, transgenic adenocarcinoma of mouse prostate; DOX, doxorubicin; PI, propidium iodide.

10.1021/jm060803i CCC: $33.50 © 2006 American Chemical Society
Published on Web 11/29/2006

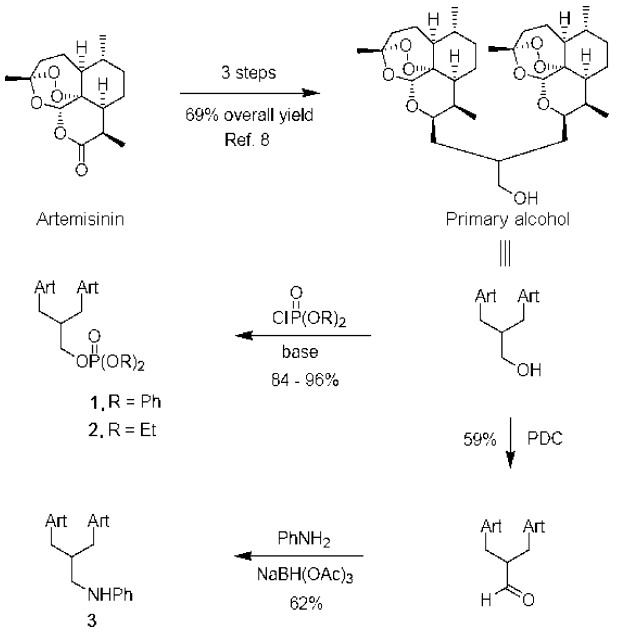

Figure 1. Chemical synthesis of C-10 non-acetal trioxane dimers. **1**−**3** were synthesized from trioxane dimer primary alcohol.

Table 1. GI_{50} of Trioxane Dimers in Prostate Cancer Cell Lines[a]

	GI_{50} (nM)		
compd	C1A	C2H	LNCaP
2	42.2	39.4	36.3
3	18.0	17.0	17.9
1	31.9	44.8	34.4
DOX	20.4	15.8	45.3

[a] Summary of GI_{50} values of trioxane dimers in prostrate cancer cells. Cells were treated with **1**, **2**, **3**, or DOX for 72 h. Cell viability was determined by MTT assay. GI_{50} is the concentration that inhibits 50% cell growth. These results are representative of at least three independent experiments.

Results

TDs Inhibit Proliferation of Prostate Cancer Cells. To assess the effect of TDs on cell growth, C1A, C2H, and LNCaP cells were treated with TDs or DOX for 72 h. Relative to other TDs, **3** was the most potent at inhibiting cell growth in C1A (GI_{50} = 18.0 nM), C2H (GI_{50} = 17.0 nM), and LNCaP (GI_{50} = 17.9 nM) cells (Figure 2 and Table 1). The efficacy of TDs was compared to the efficacy of doxorubicin (DOX), an anthracycline and standard chemotherapeutic agent that is used clinically to treat advanced prostate cancer.[21,22] DOX was used in these studies because antimalarial artemisinin derivatives share similar properties with DOX such as generation of reactive oxygen species.[23] **3** (GI_{50} = 17.9 nM) inhibited the growth of LNCaP cells more potently than DOX (GI_{50} = 45.3 nM, Table 1). At the highest dose tested, DOX (100 nM) inhibited LNCaP cell growth by 69%; however, **3** inhibited cell growth by 97% (Figure 2C). Interestingly, **3** (GI_{50} =17.0 nM, Table 1) was also as effective as DOX (GI_{50} = 15.8 nM, Table 1) in C2H cells that are aggressive and metastatic.

In subsequent studies, cells were treated with 50 nM TDs or DOX for 72 h because maximal inhibition was observed with all compounds at this dose (Figure 2). The effect of TDs on DNA synthesis was examined by BrdU incorporation assay. TDs (50 nM) inhibited BrdU incorporation in C1A, C2H, and LNCaP cells ($P < 0.0001$, Figure 3) after 72 h of treatment. Compared to other TDs, **3** strongly inhibited DNA synthesis (>90%) in C2H and LNCaP cells. Although cell growth inhibition studies indicated that **3** was more potent than DOX in LNCaP cells,

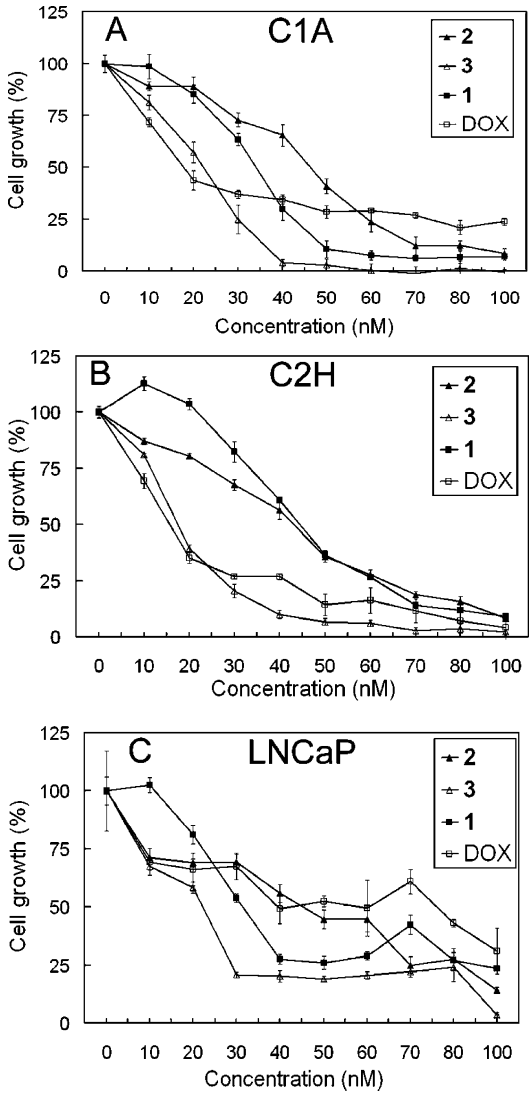

Figure 2. Inhibition of prostate cancer cell growth by trioxane dimers. (A) C1A, (B) C2H, and (C) LNCaP cells were treated with different concentrations of **1**−**3** or DOX for 72 h. Cell viability was determined by MTT assay, and results are reported as mean cell growth (%) of at least three replicate wells ± SD. Results are normalized to cell growth at time-point zero.

both compounds appear to be equally effective at inhibiting DNA synthesis. These differences may be attributable to the variability in the sensitivity of the assays used. LNCaP cells were used to further investigate the mechanisms of action of TDs.

TDs Regulate Cell Cycle Progression in LNCaP Cells. The effect of TDs on the cell cycle profile of LNCaP cells was examined by PI staining and flow cytometry. Compared to control, TDs induced G_0/G_1 cell cycle arrest in LNCaP cells (Figure 4). Thus, modulation of G_1 phase cell cycle regulators may be a key mechanism of action of TDs in LNCaP cells. Consistent with earlier findings that TDs inhibited DNA synthesis in LNCaP cells (Figure 3), a dramatic reduction in the S phase population was observed (Figure 4), indicating a block in G_1−S transition. DOX dramatically promoted accumulation of LNCaP cells in the G_2/M phase and decreased the G_1 and S phase populations (Figure 4), consistent with previous findings by Martinez et al.[24] The effects of DOX in the cell cycle include induction of G_2/M phase cell cycle arrest.

Deregulation of G_1 phase cell cycle regulators is critical for tumor progression, and genetic alterations of cell cycle proteins

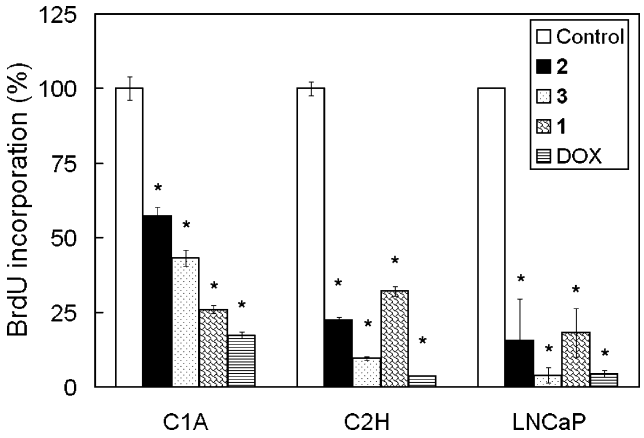

Figure 3. Inhibition of DNA synthesis in C1A, C2H, and LNCaP cells. Cells were treated with vehicle (control) or 50 nM **1**, **2**, **3**, or DOX for 72 h. DNA synthesis was measured by BrdU incorporation assay. Results are reported as mean BrdU incorporation (%) ± SEM normalized to control group: (∗) $P < 0.0001$ by ANOVA.

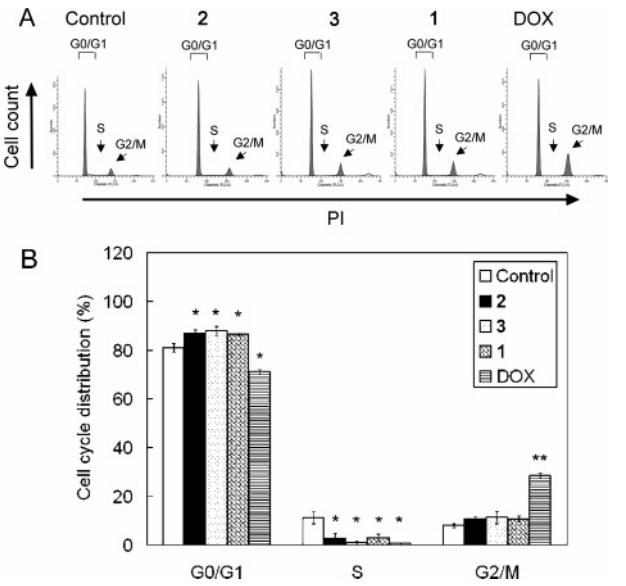

Figure 4. Induction of G_0/G_1 cell cycle arrest in LNCaP cells. Cells were treated with vehicle (control) or 50 nM **1**, **2**, **3**, or DOX for 72 h. Cell cycle distribution was determined by PI staining and flow cytometry. Shown are (A) histograms of cell cycle distribution and (B) mean cell cycle distribution (%) in the G_0/G_1, S, and G_2/M phases of three independent experiments ± SEM: (∗) $P < 0.05$, (∗∗) $P < 0.0001$ by ANOVA.

occur frequently in human cancers.[25] The effect of TDs on protein expression of G_1 phase cell cycle regulatory proteins was examined in LNCaP cells by Western blot analysis following treatment for 72 h. Cyclin D1−cdk4/6 and cyclin E−cdk2 complexes promote G_1 phase cell cycle progression.[13] TDs had minimal effects on cdk4 expression, as evidenced by a 10−30% decrease (Figure 5A). However, **1** and **3** increased cdk6 expression (Figure 5A). This coincided with a dramatic inhibition of cyclin D1 expression by TDs (Figure 5A). The effect of TDs on the cyclin E−cdk2 complex was also examined. TDs markedly inhibited cdk2 protein expression and kinase activity, as demonstrated by decreased phosphorylation of histone H1, a cdk2 substrate (Figure 5B). These changes correlated with decreased expression of cyclin E (Figure 5B) that forms a complex with cdk2.

The effect of TDs on expression of the Cip/Kip family of cdk inhibitors (p21 and p27) was evaluated. Compared to control, TDs increased expression of p21 (9- to 11-fold) and

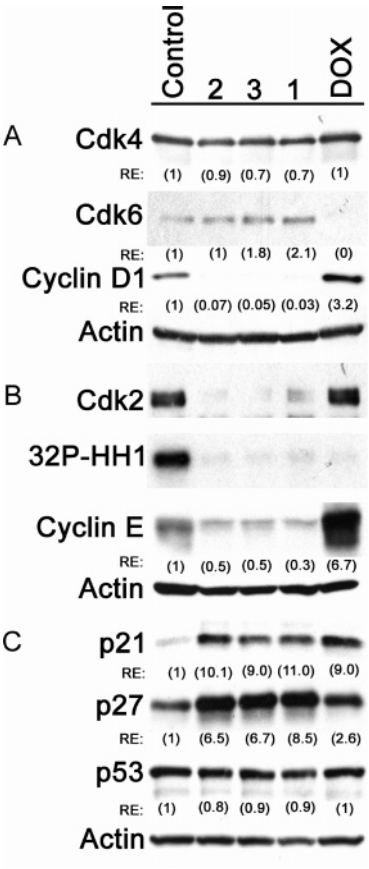

Figure 5. Regulation of G_1 phase cell cycle regulators in LNCaP cells. Cell lysates were collected following treatment with vehicle (control) or 50 nM **1**, **2**, **3**, or DOX for 72 h. Shown are (A) Western blot analysis with anti-cdk4, -cdk6, and -cyclin D1, (B) Western blot analysis with anti-cdk2 and -cyclin E and cdk2 kinase assay. ^{32}P-HH1, γ-^{32}P-histone H1, and (C) Western blot analysis with anti-p21, -p27, and -p53. Actin was used as loading control. RE, relative expression of proteins, was determined by densitometry and normalized to actin.

p27 (6.5- to 8.5-fold) (Figure 5C). Induction of p21 expression may be mediated by p53-dependent or -independent pathways.[26] TDs did not increase p53 expression relative to control (Figure 5C). This suggests that at the doses tested, induction of p21 expression by TDs in LNCaP cells does not correlate with increased p53 levels.

TDs Increase Apoptosis in LNCaP Cells. To further examine the mechanisms of action of TDs in LNCaP cells, apoptosis was measured by annexin V staining. Cells in early stages of apoptosis stain positive for annexin V only, while late-stage apoptotic and necrotic cells stain positive for annexin V and 7-AAD.[27] TDs increased early apoptosis by ∼4- to 6-fold compared to control (Figure 6A), with **3** having the greatest effect. In contrast, DOX induced late apoptosis and necrosis in LNCaP cells (Figure 6A). The apoptotic effect of TDs in LNCaP cells was accompanied by increased expression of proapoptotic bax (Figure 6B) and a decrease in antiapoptotic Bcl-2 by **1** and **3** (Figure 6B).

Discussion

The antiproliferative effects of artemisinin-derived C-10 non-acetal TDs in prostate cancer cells include inhibition of cell proliferation, induction of G_0/G_1 cell cycle arrest, and promotion of apoptosis. These effects were accompanied by changes in expression of key proteins in the G_1 phase of the cell cycle and increased expression of proapoptotic bax. **3** was the most potent

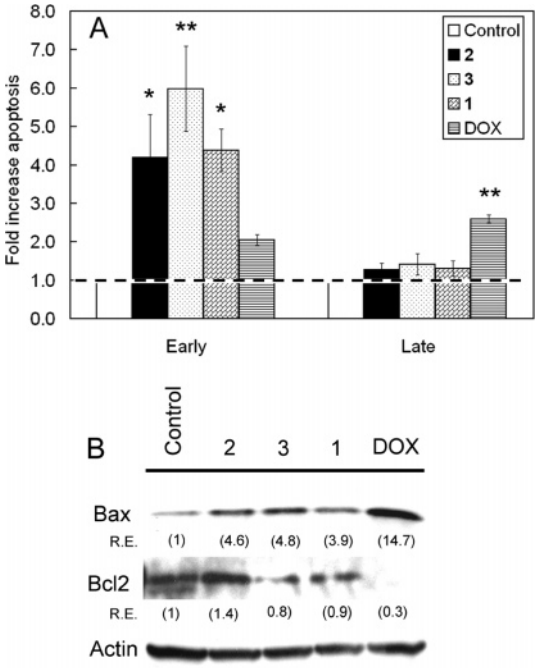

Figure 6. Induction of apoptosis in LNCaP cells. Cells were treated with vehicle (control) or 50 nM **1**, **2**, **3**, or DOX for 72 h. Apoptosis was measured by annexin V-PE/7-AAD staining. Shown are (A) mean fold increase in early-stage (annexin V positive cells) and late-stage (annexin V and 7-AAD positive cells) apoptosis ± SEM normalized to control group (fold increase = 1.0): (∗) $P < 0.05$, (∗∗) $P < 0.001$ by ANOVA. (B) Western blot analysis was performed with anti-bax and -Bcl-2. Actin was used as loading control. RE, relative expression of proteins was determined by densitometry and normalized to actin.

TD tested, as it elicited the most profound growth inhibition in C1A and LNCaP cells. Interestingly, compared to the other agents, **3** has different chemical substituents in the linker position (Figure 1). The linkers of **1** and **2** contain phosphate esters;[28] however, in **3**, the linker has a basic aniline functionality that can hydrogen-bond with various biological hydrogen-bond donors. We speculate that this chemical difference may account for the increased efficacy of **3** in prostate cancer cell lines.

Uncontrolled cell cycle progression and evasion of apoptosis are hallmarks of cancer.[29] Therefore, therapeutic agents such as TDs that can simultaneously impede cell cycle progression and promote apoptosis in cancer cells are highly desirable. TDs inhibited DNA synthesis, induced G_0/G1 cell cycle arrest, and regulated expression of key G_1 phase cell cycle proteins in LNCaP cells. Aberrant proliferation of cancer cells involves deregulation of key G_1 phase cell cycle regulators,[25] and overexpression of cyclins and cdks provides a selective growth advantage to tumor cells.[25,30] Therefore, targeting cyclin−cdk complexes that promote tumor progression is therapeutically relevant for treatment of cancer. D-type cyclins are considered to be required for cell cycle entry and early G_1 phase cell cycle progression,[13] as inhibition of cyclin D1 specifically triggers G_1 phase cell cycle arrest.[31] Cyclin D1 is overexpressed in many human cancers,[32,33] including prostate cancer.[34] Cyclin D1 forms a complex with either of its catalytic partners, cdk4 or cdk6, to promote G_1 phase cell cycle progression.[30] Induction of G_0/G_1 cell cycle arrest by TDs in LNCaP cells coincided with inhibition of cyclin D1, thus inactivating the cyclin D1−cdk4/6 complex. This suggests that inhibition of progrowth signals such as cyclin D1 is critical for the anticancer effects of TDs in prostate cancer.

p21 and p27 impede cell cycle progression by inhibiting cyclin E−cdk2 complexes that promote G_1−S phase cell cycle progression.[17] The role of p21 expression in prostate cancer remains controversial. Overexpression of p21 in the nucleus has been associated with poor prognosis in prostate cancer;[35] however, other studies have indicated otherwise.[36] Decreased p27 expression is associated with poor prognosis in prostate cancer.[36] Interestingly, TDs strongly up-regulated p21 and p27 expression in LNCaP cells. These effects coincided with inhibition of cyclin E−cdk2, induction of G_0/G_1 cell cycle arrest, and inhibition of DNA synthesis in the S phase. p53 is a tumor suppressor gene that regulates expression of p21 following DNA damage.[37] However, p53-independent up-regulation of p21 expression has been reported.[38] Induction of p21 expression by TDs in LNCaP cells was not accompanied by increased p53 expression. Past studies by Efferth et al. indicate that artesunate (an artemisinin derivative) increases p21 expression in a p53-dependent and -independent manner in HCT-116 colon cancer cells.[12]

TDs promoted early apoptosis and increased expression of bax, a proapoptotic protein.[39] Wang et al. previously demonstrated that artesunate induces apoptosis in hepatic carcinoma in vivo by up-regulating bax.[40] Although TDs increased apoptosis in LNCaP cells, it appears that their major mechanism of action involves modulation of cell cycle progression. The cytostatic and cytotoxic properties of TDs in prostate cancer indicate that the biological mechanisms of action of these agents are diverse, thus rendering them more attractive for preclinical and clinical development for prostate cancer.

The efficacy of TDs was compared to DOX, a standard chemotherapeutic agent that is used clinically to treat several cancers, including prostate cancer.[22] DOX inhibited the growth of prostate cancer cells, decreased DNA synthesis, induced cell cycle arrest, modulated cell cycle regulatory proteins, and promoted apoptosis. These findings are consistent with previous findings that the antitumor effects of DOX are mediated by inhibition of DNA synthesis,[41] induction of cell cycle arrest,[24] and promotion of apoptosis.[42] Interestingly, **3** was more potent than DOX in human LNCaP cells. However, while cardiotoxicity is a major side effect of DOX,[43] no serious adverse side effects have been observed clinically with artemisinin derivatives.[44] In vivo studies with TDs[9] and other artemisinin derivatives[45] indicate that these compounds can be safely administered. The effect of TDs on cell cycle progression appears to differ from DOX. TDs induced G_1 phase cell cycle arrest, while DOX largely promoted accumulation of cells in the G_2/M phase. These findings are consistent with previous reports that DOX induces G_2/M arrest in LNCaP[24] and DU145[46] prostate cancer cell lines. DOX increased cyclin D1 expression, while cdk4 levels were unchanged. Although DOX reduced cdk6 levels, cyclin D1 can complex with cdk4 to facilitate G_1 phase cell cycle progression. The strong induction of G_2/M arrest by DOX may be an early event. It appears that DOX later induces G_1 cell cycle arrest, as evidenced by increased expression of G_1 phase cell cycle inhibitors p21 and p27, decreased cdk2 kinase activity, and inhibition of DNA synthesis in LNCaP cells. At the dose of DOX utilized in these studies, increased p21 expression did not correlate with up-regulation of p53 levels in LNCaP cells. These results appear to be contrary to studies by Martinez et al. that demonstrated that high doses of DOX (0.2 μg/mL, 345 nM) increase p21 and p53 expression in LNCaP cells.[24] In the current studies, LNCaP cells were treated with much lower doses of DOX (50 nM) that increased p21 levels with no change in p53 expression.

These are the first studies to specifically explore the anticancer effects and biological mechanisms of action of TDs in prostate cancer. The novel TDs tested in these studies inhibit the growth

of prostate cancer cells by inducing G_0/G_1 cell cycle arrest, inhibiting DNA synthesis, modulating expression of G_1 phase cell cycle regulators, and promoting apoptosis. We have identified a highly potent dimer (**3**) that was more effective than DOX in human LNCaP cells. In contrast to DOX, which causes cardiotoxicity, there are no serious adverse side effects associated with clinically used artemisinin derivatives.[47] On the basis of these results, further preclinical studies are warranted to develop TDs such as **3** for prostate cancer treatment.

Experimental Section

Semisynthetic Trioxane Dimers. The trioxane dimer primary alcohol[9] in Figure 1 was phosphorylated to give diphenyl phosphate ester **1** (96%) and diethyl phosphate ester **2** (84%). Separately, this dimer primary alcohol was oxidized into the corresponding aldehyde (59%) that was reductively aminated to produce aniline **3** (62%). Each of these new trioxane dimers is stable in the absence of solvent at 60 °C for at least 24 h and is hydrolytically stable in a solution of pH 7.4 H_2O/DMSO (1/4) at 25 °C for at least 12 h.

1. To a solution of the dimer primary alcohol[9] (60 mg, 0.099 mmol) in CH_2Cl_2 (1 mL) at 0 °C was added pyridine (0.5 mL), 4-(dimethylamino)pyridine (1.2 mg, 9.8 μmol), and diphenyl phosphoryl chloride (61 μL, 0.29 mmol). The solution was warmed to room temperature and stirred for 16 h. The mixture was diluted with EtOAc (5 mL), and the reaction was quenched by addition of aqueous citric acid (1 N, 10 mL). The layers were separated, and the aqueous layer was extracted with EtOAc (3 × 3 mL). The combined organic solution was washed with aqueous citric acid (0.1 N, 2 mL), dried ($MgSO_4$), filtered, and concentrated. The residue was purified by flash column chromatography (elution with EtOAc/hexanes = 1:3) to provide **1** (80 mg, 96%) as a colorless oil: $[\alpha]^{24}_D$ + 68 (*c* 0.26, $CHCl_3$); IR (neat) 2919, 2861, 1589, 1485, 1455, 1376, 1290,1220, 1190, 1108, 1008, 944, 767, 686 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.33 (t, 4H, J = 7.7 Hz), 7.28−7.22 (m, 4H), 7.17 (t, 2H, J = 7.7 Hz), 5.30 (s, 1H), 5.29 (s, 1H), 4.58−4.51 (m, 1H), 4.50−4.37 (m, 2H), 4.25 (dd, 1H, J = 10.1, 5.9 Hz), 2.68 (q, 1H, J = 6.6 Hz), 2.52 (q, 1H, J = 6.6 Hz), 2.37−2.24 (m, 3H), 2.05−1.97 (m, 2H), 1.95−1.79 (m, 4H), 1.79−1.33 (m, 16H, including s at 1.40 and 1.37), 1.32−1.17 (m, 6H), 0.98−0.78 (m, 14H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 150.7 (d, 2C, J = 7.2 Hz), 129.7 (4C), 125.1 (2C), 120.2 (d, 2C, J = 3.9 Hz), 120.1 (d, 2C, J = 3.9 Hz), 103.2, 102.8, 89.5, 88.7, 81.14, 81.10, 73.8, 71.9 (d, J = 7.0 Hz), 70.8, 52.4, 52.1, 44.5, 44.1, 37.4, 37.3, 36.64, 36.58, 35.3 (d, J = 7.7 Hz), 34.5, 34.4, 30.5, 30.4, 30.2, 29.4, 26.11, 26.05, 24.9, 24.8, 24.7, 24.6, 20.2, 20.1, 13.2, 12.6; ^{31}P NMR (162 MHz, $CDCl_3$) δ −11.8; HRMS (ESI) calculated for $C_{46}H_{63}O_{12}PNa$ [$(M + Na)^+$] 861.3955, found 861.3879.

2. To a solution of the dimer primary alcohol[9] (103 mg, 0.170 mmol) in CH_2Cl_2 (1 mL) at 0 °C was added pyridine (42 μL, 0.52 mmol) and diethyl phosphoryl chloride (74 μL, 0.51 mmol). The solution was stirred for 4 h at room temperature, diluted with EtOAc (3 mL), and quenched with aqueous citric acid (0.1 N, 5 mL). The layers were separated, and the aqueous layer was extracted with EtOAc (3 × 3 mL). The combined organic solution was dried ($MgSO_4$) and concentrated. The resulting oil was purified by flash column chromatography (elution with EtOAc/hexanes = 1:1) to yield **2** (106 mg, 84%) as an oily solid: $[\alpha]^{24}_D$ +13 (*c* 0.30, $CHCl_3$); IR (thin film) 2941, 2880, 1454, 1373, 1271, 1103, 1037, 1011, 878, 843, 665 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 5.31 (s, 1H), 5.28 (s, 1H), 4.38 (m, 1H), 4.23 (q, J = 5.2 Hz, 2H), 4.18−4.05 (m, 5H), 2.68 (sextet, J = 6.8 Hz, 1H), 2.56 (sextet, J = 6.8 Hz, 1H), 2.30 (m, 2H), 2.1 (m, 1H), 2.0 (dm, J = 14.4 Hz, 2H), 1.94−1.18 (m, 32H including s at 1.38 and 1.37 and dt at 1.32 with J = 0.8, 7.0 Hz), 0.95−0.85 (m, 14H including d at 0.94 with J = 6.0 Hz and dd at 0.85 with J = 4.2, 7.4 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 103.1, 102.8, 89.4, 88.7, 81.2, 81.1, 74.0, 71.1, 69.8, 63.6, 63.5, 52.4, 52.2, 44.5, 44.2, 37.4, 37.4, 36.7, 36.6, 35.5, 35.5, 34.5, 34.4, 30.6, 30.5, 30.1, 29.5, 26.1, 26.0, 24.8, 24.8, 24.7, 24.7, 20.2, 20.1, 16.2, 16.1, 13.2, 12.7; HRMS (FAB) calculated for $C_{38}H_{64}O_{12}P$ [$(M + H)^+$] 743.4135, found 743.4144.

3. A 100 mL round-bottom flask was charged with the dimer primary alcohol[9] (230 mg, 0.38 mmol), CH_2Cl_2 (40 mL), and pyridinium dichromate (PDC) (260 mg, 0.68 mmol). The mixture was stirred at room temperature overnight and filtered through a pad of Celite, and the filtrate was concentrated under reduced pressure. The crude mixture was purified by flash column chromatography (elution with EtOAc) to give the dimer aldehyde (130 mg, 59%) as a sticky white solid: ^1H NMR (400 MHz, $CDCl_3$) δ 9.86 (d, 1H, J = 1.2 Hz), 5.26 (s, 1H), 5.23 (s, 1H), 4.38−4.26 (m, 2H), 2.82 (m, 1H), 2.67 (m, 1H), 2.56 (m, 1H), 2.36−2.24 (m, 2H), 2.18−2.06 (m, 2H), 2.04−1.96 (m, 2H), 1.92−1.84 (m, 4H), 1.82−1.74 (m, 2H), 1.70−1.58 (m, 4H), 1.46−1.20 (m, 14H including s at 1.39 and 1.38), 0.96−0.86 (m, 14H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 205.6, 103.8, 103.1, 89.7, 89.2, 81.5, 81.4, 73.4, 70.9, 52.5, 52.3, 47.8, 44.5, 44.2, 37.72, 37.65, 36.8, 34.70, 34.65, 30.7, 30.5, 30.0, 28.9, 26.3, 26.2, 25.13, 25.11, 25.03, 24.95, 20.43, 20.37, 13.3, 12.7; HRMS (ESI) calculated for $C_{34}H_{52}O_9Na$ [$(M + Na)^+$] 627.3504, found 627.3509. A 25 mL round-bottom flask was charged with the dimer aldehyde (93 mg, 0.15 mmol) and CH_2Cl_2 (10 mL) at room temperature. To this was added aniline (70 μL, 0.78 mmol) and sodium triacetoxy borohydride (69 mg, 0.32 mmol). The mixture was stirred at room temperature for 5 h until complete consumption of starting material was observed by TLC analysis. The reaction was quenched with saturated aqueous $NaHCO_3$. The aqueous layer was extracted with CH_2Cl_2 (3 × 30 mL). The combined organic layers were dried ($MgSO_4$) and concentrated to give a pale-yellow oil. The crude product was purified by silica gel flash column chromatography (10−20% EtOAc in PE) to yield **3** (64 mg, 62%) as a white fluffy solid: mp 84−87 °C; IR (thin film) 3584, 3378, 3051, 2925, 2874, 2359, 1716, 1602, 1508, 1453, 1376, 1322, 1253, 1222, 1186, 1103, 1053, 1008, 940, 909, 878, 846, 827 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.16−7.11 (m, 2H), 6.70−6.60 (m, 3H), 5.34 (s, 1H), 5.31 (s, 1H), 4.46−4.26 (m, 2H), 3.29−3.15 (m, 2H), 2.72−2.56 (m, 2H), 2.36−2.27 (m, 2H), 2.22−2.16 (m, 1H), 2.10−1.99 (m, 2H), 1.93−1.86 (m, 2H), 1.79−1.71 (m, 5H), 1.65−1.55 (m, 5H), 1.50−1.21 (m, 14 H including s at 1.41 and 1.38), 0.96−0.84 (m, 14H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 149.1, 129.1, 116.3, 112.7, 103.2, 103.0, 89.4, 88.9, 81.2, 81.2, 73.9, 71.5, 52.3, 52.2, 47.3, 44.4, 44.2, 37.4, 37.3, 36.6, 36.6, 34.8, 34.4, 31.5, 31.1, 30.7, 30.5, 26.1, 26.0, 24.8, 24.8, 24.7, 24.7, 20.2, 20.1, 13.1, 12.8; HRMS (ESI) calculated for $C_{40}H_{59}NO_8Na$ [$(M + Na)^+$] 704.4133, found 704.4136.

Chemicals. Trioxane dimers (**1**−**3**) were dissolved in pure ethanol and stored at −80 °C. Doxorubicin (DOX, a gift from Dr. Enrico Mihich's laboratory) was dissolved in sterile water, protected from light exposure, and stored at −20 °C.

Cell Culture Conditions. TRAMP cells (C1A and C2H) were cultured in DMEM media (Invitrogen, Frederick, MD) supplemented with 10% FBS (Hyclone, Logan, UT), 10^{-8} M dihydrotestosterone (Sigma-Aldrich, St. Louis, MO), 5 μg/mL insulin (Sigma), and 25 U/mL penicillin/streptomycin (Invitrogen) as previously described.[9,19] LNCaP cells were cultured in RPMI media (Invitrogen) supplemented with 10% FBS, 1% L-glutamine (Invitrogen), 10 mM HEPES buffer (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 100 U/mL penicillin/streptomycin, and 2.4 mg/mL glucose (Sigma). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Ethanol (≤0.002%) was used as vehicle control for all experiments

MTT Assay. Cell viability was measured as previously described.[9] Briefly, C1A (2500 cells/well), C2H (1500 cells/well), and LNCaP (10 000 cells/well) were seeded in 96-well plates overnight. Cells were treated with media containing vehicle or different concentrations of TDs or DOX for an additional 72 h. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT, Sigma) solution was added to each well for 4 h. Cells were solubilized with 100 μL of 20% SDS/0.02 N HCl, and absorbance was measured at 570 nm using an ELISA plate reader (Molecular Devices, Sunnyvale, CA). The concentration of test compounds required to inhibit 50% of cell growth (GI_{50}) was determined using CalcuSyn (Biosoft, Ferguson, MO).

Propidium Iodide (PI) Staining. Subconfluent LNCaP cells were treated with vehicle or 50 nM of TDs and DOX for 72 h. Cells were harvested, washed with ice-cold PBS, and fixed with ice-cold 70% ethanol for at least 30 min at 4 °C. About 10^6 cells were resuspended in PBS containing PI (10 μg/mL, Sigma) and RNase A (100 μg/mL, Sigma) for 30 min in the dark. Cells were sorted by florescence activated cell sorting (FACS) analysis at the RPCI Laboratory of Flow Cytometry.

Bromodeoxyuridine (BrdU) Incorporation Assay. DNA synthesis was measured in CIA and C2H cells using a cell proliferation ELISA BrdU colorimetric kit (Roche, Indianapolis, IN) according to manufacturer's instructions. BrdU is a brominated analogue of thymidine that is incorporated into actively replicating DNA of cells in the S phase of the cell cycle.[48] Briefly, subconfluent cells were treated with vehicle or either 50 nM TDs or 50 nM DOX for 72 h. Cells were labeled with BrdU for 4 h, and BrdU incorporation was assessed by measuring absorbances at 370 nm using an ELISA plate reader.

DNA synthesis was measured in LNCaP cells by flow cytometry. Subconfluent cells were treated with vehicle or either 50 nM TDs or 50 nM DOX for 72 h. Cells were labeled for 30 min with 100 μg/mL BrdU (Sigma) and 1 μg/mL 5-fluoro-2′-deoxyuridine (FdU, Sigma). FdU enhances BrdU incorporation by inhibiting thymidylate synthetase.[49] Cells were harvested and fixed for flow cytometry as described previously. Approximately 10^6 cells were denatured with 2 N HCl, neutralized with 0.1 M sodium borate (pH 8.5), and incubated with mouse anti-BrdU antibody (BD PharMingen, San Diego, CA) in 0.5% BSA/PBS overnight at 4 °C. Cells were labeled with Alexa Fluor 488 goat antimouse secondary antibody (Molecular Probes, Eugene, OR) for 1 h at 4 °C. Samples were stained with PI and sorted by FACS analysis as previously described.

Annexin V Staining. Apoptotic cells were detected using the annexin V-PE apoptosis detection kit (BD PharMingen) according to the manufacturer's instructions. Annexin V detects cells in the earliest stages of apoptosis,[27] and 7-AAD is a vital dye that is excluded by viable cells. Briefly, subconfluent LNCaP cells were treated with vehicle or either 50 nM TDs or 50 nM DOX for 72 h. Cells were trypsinized, washed twice with ice-cold PBS, and stained with annexin V-PE and 7-AAD for 15 min in the dark. Apoptotic cells were detected by FACS analysis as previously described.

Western Blot Analysis. Subconfluent LNCaP cells were treated with vehicle or either 50 nM TDs or 50 nM DOX for 72 h. Cells were lysed with Triton X-100/SDS lysis buffer (10% Triton X-100, 10% SDS, 1.0 M Tris-Cl, pH 8.0, and 5.0 M NaCl) containing 1× protease inhibitor cocktail (BD PharMingen), 2 mM sodium orthovanadate, 2 mM EDTA, 12 mM β-glycerol phosphate, and 10 mM sodium fluoride. Proteins were quantified using the Dc protein assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. Proteins were resolved by SDS−PAGE and transferred to immobilon-P polyvinylidene fluoride (PVDF) membranes (Millipore Corp., Bedford, MA) overnight at 4 °C by electrophoresis. Membranes were blocked at room temperature for a minimum of 1 h using 5% (w/v) blotto (Santa Cruz Biotechnology, Santa Cruz, CA) in TBST (25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 2.7 mM KCl, and 0.05% Tween-20). Western blots were incubated overnight at 4 °C with these primary antibodies: cdk4 (1:1000, sc-260, Santa Cruz), cdk6 (1:1000, sc-177, Santa Cruz), cyclin D1 (1:1000, sc-753, Santa Cruz), cyclin E (1:1000, sc-247, Santa Cruz), p21 (1:1000, sc-397, Santa Cruz), p27 (1:1000, sc-528, Santa Cruz), p53 (1:1000, sc-126, Santa Cruz), bax (1:1000, sc-493, Santa Cruz), Bcl-2 (1:200, sc-492, Santa Cruz), and actin (1:6000, CP01, Calbiochem, San Diego, CA). Membranes were washed at least three times with TBST followed by incubation with HRP-conjugated secondary antibodies for a minimum of 1 h. Three additional washes were performed, and proteins were detected using Western Lightning chemiluminescence reagent (Perkin-Elmer Life Sciences, Boston, MA). Protein expression was quantified using the GS-800 calibrated densitometer and Quantity One software (Bio-Rad).

Kinase Assay. LNCaP cells were treated with vehicle, TDs, or DOX as described above. Cells were lysed with NP40 lysis buffer (10 mM HEPES, pH 7.9, 1 mM EDTA, 150 mM NaCl, 1% NP40) containing 1× protease inhibitor cocktail and phosphatase inhibitors (2 mM sodium orthovanadate, 2 mM EGTA, 10 mM sodium fluoride). Protein concentration was determined as described above. Lysates (100 μg) were immunoprecipitated with 2 μg of cdk2 antibody (sc-163-G, Santa Cruz) and protein A/G agarose (Santa Cruz) overnight at 4 °C with rocking. Immunoprecipitated proteins were washed twice with NP40 lysis buffer and once with kinase buffer (20 mM HEPES, pH 7.8, 10 mM $MgCl_2$, 20 mM β-glycerol phosphate, 1 mM DTT, 50 μM active sodium orthovanadate). Kinase activity was assayed by resuspending beads in kinase buffer containing 2 μg of histone H1 (cdk2 substrate), 50 μM active sodium orthovanadate, 20 μM ATP, and 5 μCi (γ-^{32}P) ATP, for 30 min at 30 °C. Kinase reactions were terminated with equal volume 2× laemmli buffer (Bio-Rad), and the mixture was boiled for 5 min. Samples were resolved on a SDS−PAGE gel and transferred to a PVDF membrane as described above. (γ-^{32}P)-Histone H1 was detected by autoradigraphy. The cdk2 protein expression was confirmed by immunoblot analysis with anti-cdk2 antibody (1:500).

Acknowledgment. This work was supported by NIH/NCI Grant 5R01CA95367-4 (to B.A.F), DOD Predoctoral Fellowship Grant 57-8046-01 (to A.A.A.), and NIH AI Grant 34885 (to G.H.P.). We thank Drs. Jennifer Black and Adrian Black of the Department of Pharmacology and Therapeutics at Roswell Park Cancer Institute for providing antibodies for cell cycle studies.

Supporting Information Available: HPLC traces and NMR spectra for trioxane dimers **1**−**3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM060803I