Brief Articles

Anticancer and Antimalarial Efficacy and Safety of Artemisinin-Derived Trioxane Dimers in Rodents

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In only four chemical steps from naturally occurring artemisinin (**1**), trioxane dimers **6** and **7** were prepared on a multigram scale in overall 32−44% yields. In mice, both isonicotinate *N*-oxide dimer **6** and isobutyric acid dimer **7** were considerably more antimalarially efficacious than clinically used sodium artesunate (**2**) via both oral and intravenous administration. In the transgenic adenocarcinoma of mouse prostate model, some of the trioxane dimers had potent anticancer activity.

Introduction

Strong interest has been directed at the dual medicinal value of 1,2,4-trioxanes and trioxane dimers as both antimalarial[1,2] and especially anticancer agents.[3−5] We reported this year on some orally active, antimalarial, anticancer, artemisinin-derived trioxane dimers with high stability and efficacy.[6] Two of these new chemical entities were shown in rodents to be more orally efficacious as antimalarials than either artelinic acid or clinically used sodium artesunate. Also, they are strongly inhibitory toward several human cancer cell lines.[6]

Prostate cancer is the most frequently diagnosed cancer in men in the U.S. and is the second highest cause of cancer deaths among men.[7] Recently transgenic mouse models have been developed that recapitulate the key features of the human disease. The *tr*ansgenic *a*denocarcinoma of *m*ouse *p*rostate (TRAMP) model utilizes the probasin promoter to express SV40 early genes (T and t) specifically in the epithelial cells of the prostate. With time, TRAMP male mice develop progressive prostatic disease culminating in poorly differentiated prostate cancer with frequent metastasis. Prostate cancer cell lines were established from a poorly differentiated prostatic tumor from a 32-week old TRAMP mouse.[8] Three rounds of dilutional cloning were performed to establish TRAMP clonal cell lines, C1A, C2D, C2G, and C2H. These cell lines have similar doubling times, with C1A growth the slowest (16.7 h) and C2G the fastest (10.2 h). All four cell lines are cytokeratin-positive, indicating they are epithelial in origin and have an intact androgen receptor signaling cascade, but none of the cell lines express the transgene in culture.[9] These cell lines were used to test the anticancer activity of the trioxane dimers.

Results and Discussion

Now we report on new trioxane dimers **6** and **7** prepared in only four chemical steps from natural artemisinin (**1**)[10] on a multigram scale in overall 32−44% yields (Scheme 1). Parent alcohol dimer **5**, reported by us this year,[6] was easily esterified in high yield to give isonicotinate *N*-oxide **6** and also easily oxidized in high yield to give carboxylic acid **7** (Scheme 1). Parent alcohol dimer **5**, isonicotinate *N*-oxide dimer **6**, and carboxylic acid dimer **7** are stable at 60 °C for at least 24 h; under these accelerating aging conditions, less than 5% decomposition was observed by ^{1}H NMR spectrometry.

By use of our standard in vitro assay,[11] the antimalarial potencies of dimers **6** and **7** against chloroquine-sensitive *Plasmodium falciparium* (NF54) parasites were determined (Table 1); for reference, the EC_{50} of artemisinin (**1**) was 8.8 ± 0.74 nM. The in vitro data in Table 1 show clearly that both *N*-oxide dimer **6** and carboxylic acid dimer **7** are very potent antimalarial compounds. The in vivo antimalarial efficacies of both of these two new dimers **6** and **7**, as measured in mice via either iv or oral administration according to a published protocol,[12] are considerably better than that of the clinically used sodium artesunate.[13] A small preliminary toxicity study of sodium artesunate and the two dimers **6** and **7** administered ip as a single dose to mice showed that carboxylic acid **7** is safer (fewer deaths and no effect on animal weight gain) than the other two trioxanes (Table 1).[14] An approximate therapeutic index

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Scheme 1

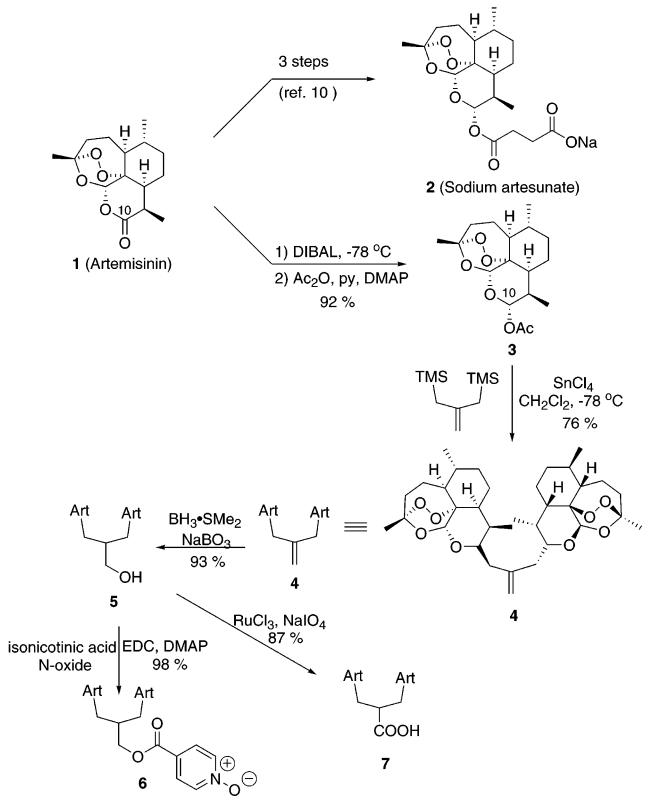
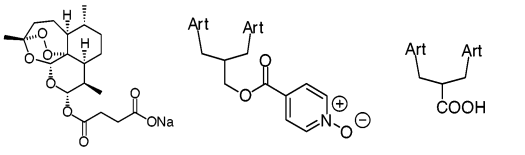


Table 1. Antimalarial Activities and Safety Data



property	2	6	7
Antimalarial Activity			
in vitro (EC_{50}, nM)[a]	1.5	0.53	2.4
in vivo (ED_{50}, (mg/kg)/day × 4)			
iv	5.5	0.8	1.5
po	8.0	2.0	4.5
Safety (mg/kg ip × 1)			
total deaths			
125	0/3	0/3	0/3
250	1/3	1/3	0/3
500	3/3	3/3	1/3
750		2/3	3/3
weight gain	markedly reduced	reduced	unaffected
Therapeutic Index			
max tolerated/ED_{50} iv	23	156	167

[a] The standard deviation for each set of quadruplicates was an average of 12% (≤36%) of the mean. R^2 values for the fitted curves were ≥0.993. Artemisinin activity is the mean ± standard deviation of concurrent control ($n = 3$).

calculation showed that both *N*-oxide **6** and carboxylic acid **7** are 6 times better than clincially used sodium artesunate (**2**).

Four TRAMP cell lines were used to test the anticancer activity of the trioxane dimers.[8] The C1A and C2D cell lines are nontumorigenic, and the C2G and C2H cell lines are tumorigenic and metastatic. Interestingly,

Table 2. Growth Inhibition of Prostate Cancer Cell Lines[a]

	IC_{50} (nM)			
	C1A	C2D	C2G	C2H
5	23.3	18.5	15.4	9.2
6	84.6	62.1	47.4	36.1
7	158.7	231.4	134.0	116.0
Gemzar	9.0	11.9	3.7	4.7
doxorubicin (Adriamycin)	75.9	46.5	28.7	30.3

[a] MTT assay was used to determine growth inhibition of C1A, C2D, C2G, and C2H. IC_{50} was determined using calcusyn.

the tumorigenic and metastastic cell lines C2G and C2H were the most responsive to the trioxane dimers. The order of anticancer activity of the trioxane dimers was consistent among all four cell lines, with alcohol dimer **5** being the most effective followed by *N*-oxide dimer **6**; carboxylic acid dimer **7** was the least effective. The most potent agent, alcohol dimer **5**, had an IC_{50} of 15.4 and 9.2 nM in the most aggressive cell lines C2G and C2H, respectively (Table 2). For comparison, gemcitabine is included in Table 2. Gemcitabine (Gemzar) is an FDA-approved chemotherapeutic agent for the treatment of human pancreatic cancer and non-small-cell lung cancer. It is an antimetabolite that has a wide range of anticancer activities. Gemcitabine inhibits the proliferation and formation of colonies in LNCaP, PC3, and DU145 human prostate cancer cell lines.[15] However, gemcitabine causes numerous side effects. Doxorubicin (Adriamycin) is an antiproliferative and cytotoxic anthracycline antibiotic that is used for the treatment of various solid tumors. However, this highly potent anticancer agent is associated with cardiac toxicity.

In conclusion, easily synthesized, thermally stable, trioxane dimers **5**−**7** have the following desirable characteristics:[16] (1) dimers **6** and **7** are considerably more antimalarially efficacious than sodium artesunate (**2**) via both oral and iv administration; (2) dimers **6** and **7** have a 6-fold better preliminary therapeutic index than antimalarial drug sodium artesunate; (3) both parent alcohol dimer **5** and *N*-oxide dimer **6**, but not carboxylic acid dimer **7**, very strongly inhibit the growth of prostate cancer cells.

Experimental Section[6]

Synthesis of Isonicotinate *N*-Oxide Dimer 6. To a stirring suspension of primary alcohol dimer **5** (0.14 g, 0.24 mmol) and commercially available (Aldrich) isonicotinic acid *N*-oxide (0.11 g, 0.76 mmol) in anhydrous methylene chloride (10 mL) was added 4-(dimethylamino)pyridine (0.11 g, 0.93 mmol) and 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (0.17 g, 0.90 mmol). A further 5 mL of anhydrous methylene chloride was added to wash down the flask walls, and the reaction mixture was stirred at room temperature for 4 h, at which time TLC analysis showed full consumption of starting material. Water (5 mL), 3 M HCl solution (10 mL), and methylene chloride (5 mL) were added, and organics were extracted with methylene chloride (3 × 30 mL), dried (Na_2SO_4), and concentrated in vacuo to give a colorless oil (0.23 g). Gradient column chromatography on silica (crude was dry-loaded) with elution first with 70% ethyl acetate/petroleum ether and then with 80% ethyl acetate/petroleum ether isolated *N*-oxide dimer **6** as a white solid (0.17 g, 0.23 mmol, 98%): mp 114−122 °C; $[\alpha]^{23.1}_D$ 51.7 ($CHCl_3$, *c* 0.67); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.22 (d, 2H, $J = 7.3$ Hz), 7.89 (d, 2H, $J = 7.3$ Hz), 5.32 (s, 1H), 5.29 (s, 1H), 4.52 (s, 1H), 4.51 (s, 1H), 4.51−4.54 (m, 1H), 4.39−4.32 (m, 1H), 2.67 (st, 1H, $J = 7.0$ Hz), 2.57 (st, 1H, $J = 7.0$ Hz), 2.42−2.26 (m, 3H), 2.07−1.97 (m, 2H), 1.97−1.85 (m, 2H), 1.84−1.74 (m, 4H), 1.70−1.47 (m, 6H), 1.41−

1.19 (m, 14H, including two singlets at 1.40 and 1.39), 1.00−0.91 (m, 2H), 0.96 (d, 3H, J = 5.6 Hz), 0.95 (d, 3H, J = 5.6 Hz), 0.88 (d, 3H, J = 6.9 Hz), 0.86 (d, 3H, J = 6.9 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 163.27, 139.35 (2), 127.26, 126.39 (2), 103.10, 102.80, 89.64, 89.01, 81.12, 81.09, 72.99, 70.52, 68.37, 52.22, 52.01, 44.21, 43.97, 37.56, 37.49, 36.63, 36.53, 34.40, 34.36, 33.98, 30.95, 30.58, 30.52, 29.68, 26.03, 25.98, 24.92, 24.88, 24.77, 24.75, 20.16, 20.07, 12.96, 12.62; IR (film) 2931, 2871, 1719, 1609, 1448, 1373, 1261, 1156, 1108, 1049, 1037, 1008, 925, 732 cm^{-1}; HRMS (ES) m/z calcd for $C_{40}H_{57}NO_{11}Na$ $[M + Na]^+$ 750.3824, found 750.3845. Anal. ($C_{40}H_{59}NO_{12}$) C, H, N.

Synthesis of Carboxylic Acid Dimer 7. A 25 mL round-bottomed flask was charged with primary alcohol dimer **5** (132 mg, 0.218 mmol, 1.0 equiv), ethyl acetate (4 mL), acetonitrile (4.0 mL), and H_2O (1.3 mL). To this mixture was added ruthenium(III) chloride hydrate (4.5 mg, 0.022 mmol, 0.1 equiv) and sodium periodate (326 mg, 1.53 mmol, 7.0 equiv) (on addition of ruthenium chloride, the solution turned black). After being stirred for 30 min at room temperature (the solution turned pale-orange), the reaction mixture was poured into a mixture of 30 mL of ethyl acetate and 30 mL of saturated aqueous NH_4Cl solution. The aqueous layer was extracted with ethyl acetate (30 mL × 2). The organic layers were then combined and dried over $MgSO_4$, filtered through Celite, and concentrated under reduced pressure. The crude mixture was purified by flash column chromatography (1% acetic acid in 30% ethyl acetate/hexane) to afford the isobutyric acid **7** as a white solid (126 mg, 0.204 mmol, 94%): mp 105−110 °C. Further purification by medium-pressure liquid chromatography (MPLC, LiChroprep Si60 (40−63 μm), EM Science) with the same solvent system (1% acetic acid in 30% ethyl acetate/hexane) removed unknown impurities and gave a white solid (118 mg, 0.190 mmol, 87%): mp 110−113 °C; $[\alpha]^{23.4}_D$ 81.7 (CH_2Cl_2, c 0.14); ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.30 (s, 1H), 5.29 (s, 1H), 4.30−4.20 (m, 2H), 2.91−2.85 (m, 1H), 2.75−2.55 (m, 2H), 2.35−2.23 (m, 2H), 2.15−1.95 (m, 3H), 1.94−1.50 (m, 11H), 1.46−1.20 (m, 15H, including two singlets at 1.39 and 1.38), 0.98−0.86 (m, 2H), 0.94 (d, J = 6.0 Hz, 6H), 0.86 (d, J = 7.6 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 179.9, 103.3, 103.1, 89.0, 88.6, 81.1, 81.0, 74.3, 72.8, 52.3, 52.2, 44.4, 44.2, 41.8, 37.4, 37.3, 36.5, 36.5, 34.4, 31.5, 31.4, 30.3, 30.2, 25.9, 25.8, 24.7, 24.6, 20.2, 20.1, 13.1, 12.7; IR ($CHCl_3$) 3500 (br), 2940, 2875, 1705, 1450, 1377, 1279, 1187, 1094, 1053, 1011, 941, 877, 826, 734 cm^{-1} ; HRMS(ES) m/z calcd for $C_{34}H_{52}O_{10}Na$ $[M + Na]^+$ 643.3457, found 643.3470. Anal. ($C_{34}H_{52}O_{10}$) C, H.

MTT Assay for Cell Viability. The MTT assay is a colorimetric assay used to measure cell viability. This assay measures the reduction of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (Sigma-Aldrich, St. Louis, MO) to an insoluble, blue formazan product in the mitochondria of live cells.

Cell Culture. TRAMP-C1A, -C2D, -C2G, and -C2H were cultured as previously described.[8] C1A, C2D, C2G, and C2H were seeded in 100 μL of media at 2.5 × 10^4, 2.0 × 10^4, 2.0 × 10^4, and 1.5 × 10^4 cells/mL, respectively, in 96-well plates. TRAMP-Cs were cultured in DMEM high glucose (Invitrogen Corporation, Grand Island, NY) supplemented with 10% non-heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 5 mg/mL insulin (Sigma-Aldrich, St. Louis, MO), 25 U/mL penicillin−streptomycin (Invitrogen Corporation, Grand Island, NY), and 10^{-8} M dihydrotestosterone (Sigma-Aldrich, St. Louis, MO). Plates were incubated in 5% CO_2 at 37 °C. After 24 h of growth, the media was replaced with 100 mL of phenol-red-free DMEM supplemented as described above with the additon of trioxane dimers dissolved in 100% ethanol. **Assay for Cell Viability.** MTT assays were performed 0, 24, 48, and 72 h after cells were plated. A solution of 0.5% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO) in PBS was made. An amount of 20 mL of 0.5% MTT solution was added to each well, and the cells were incubated for an additional 4 h. Cells were solubilized with 100 mL of 20% SDS/0.02 M HCl and incubated overnight. The optical density was measured at 570 nm with a spectrophotometer. All data were analyzed using Microsoft Excel. IC_{50} values were determined with Calcusyn (Biosoft, Cambridge, U.K.).

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