Prodrug Modification Increases Potassium Tricyclo[5.2.1.0^{2,6}]-decan-8-yl Dithiocarbonate (D609) Chemical Stability and Cytotoxicity against U937 Leukemia Cells

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ABSTRACT

Potassium tricyclo[5.2.1.0^{2,6}]-decan-8-yl dithiocarbonate (D609) is a selective antitumor agent, potent antioxidant, and cytoprotectant. It has the potential to be developed as a unique chemotherapeutic agent that may provide dual therapeutic benefits against cancer, e.g., enhancing tumor cell death while protecting normal tissues from damage. However, D609 contains a dithiocarbonate (xanthate) group [O–C(=S)S/O–C(=S)SH], which is chemically unstable, being readily oxidized to form a disulfide bond with subsequent loss of all biological activities. Therefore, we developed the synthesis of a series of S-(alkoxyacyl) D609 prodrugs by connecting the xanthate group of D609 to an ester via a self-immolative methyleneoxyl group. These S-(alkoxyacyl)-D609 prodrugs are designed to release D609 in two steps: esterase-catalyzed hydrolysis of the acyl ester bond followed by conversion of the resulting hydroxymethyl D609 to formaldehyde and D609. Three S-(alkoxyacyl) D609 prodrugs were synthesized by varying the steric bulkiness of the acyl group. These prodrugs are stable to ambient conditions, but readily hydrolyzed by esterases to liberate D609 in a controlled manner. More importantly, the lead prodrug methyleneoxybutyryl D609 is biologically more effective than D609 in inhibiting sphingomyelin synthase, thereby increasing the level of ceramide and inducing apoptosis in U937 leukemia cells. The prodrug has a significantly lower LD_{50} value than that of D609 (56.6 versus 117 μM) against U937 cells. These findings demonstrate that prodrug modification of the xanthate moiety with an alkoxyacyl group can improve D609 oxidative stability and enhance its antitumor activity.

Potassium tricyclo[5.2.1.0^{2,6}]-decan-8-yl dithiocarbonate (D609) was originally developed as a selective antitumor agent. Incubation of various transformed and malignant cells with low concentrations (<10 μg/ml) of D609 in an acidic condition (pH 6.8) reverted their morphology, growth pattern, and serum dependence to normal phenotypes (Amtmann et al., 1985; Amtmann and Sauer, 1987). When these cells were incubated with higher concentrations (>10 μg/ml) of D609 alone, or with low concentrations (5 or 10 μg/ml) of D609 plus C_{10-14} monocarboxylic acids (20–60 μg/ml) at physiological pH (7.4), significant cell death was induced (Amtmann and Sauer, 1987; Schick et al., 1989a; Porn-Ares et al., 1997). However, incubation of these cells with monocarboxylic acids alone had no significant effect on cell viability. The list of transformed and malignant cell types that are sensitive to D609 toxicity is expanding, including several drug-resistant tumor cell lines (Amtmann and Sauer, 1987; Schick et al., 1989a;Porn-Ares et al., 1997). In contrast, under the same in vitro cell culture conditions, D609 did not show any cytotoxicity against normal human fibroblasts or peripheral blood lymphocytes (Amtmann and Sauer, 1987). Moreover, D609 actually functions as a potent antioxidant and cytoprotectant and thus has the ability of protecting normal lymphocytes from ionizing radiation (IR)-induced oxidative damage and mice from IR-induced lethality (Zhou et al., 2001). These findings suggest that D609 is a unique chemotherapeutic agent. When it is used as an adjuvant with IR and/or chemotherapy, it may provide dual therapeutic benefits against can-

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ABBREVIATIONS: D609, potassium tricyclo[5.2.1.0^{2,6}]-decan-8-yl dithiocarbonate; IR, ionizing radiation; HPLC, high-performance liquid chromatography; PLE, porcine liver esterase; PC, phosphatidylcholine; DTNB, 5,5’-dithiobis(2-nitrobenzoic acid); PBS, phosphate-buffered saline; PBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; DMSO, dimethyl sulfoxide; SMS, sphingomyelin synthase; ESI/MS/MS, electrospray ionization/tandem mass spectrometry; AUC, area under the curve; NBD, 7-nitrobenz-2-oxa-1,3-diazol.
cer, e.g., protection of normal tissues and enhanced tumor cell death.

Unfortunately, D609 treatment exhibits only moderate antitumor activity in vivo. In combination with monocarboxylic acid, D609 induced extensive intratumoral cell death in athymic mice bearing a human nonsmall cell lung carcinoma (Sauer et al., 1990). Moreover, D609 plus monocarboxylic acid treatment also improved the effectiveness of tumor necrosis factor-α in this human xenograft tumor model (Amtmann and Sauer, 1990). However, administration of D609 alone produced no significant antitumor effect in vivo, and D609 plus monocarboxylic acid therapy also failed to show significant therapeutic benefit against murine 3-LL tumor and WEHI-3B myelomonocytic and L1210 lymphoid leukemia, even though these tumor cells are highly sensitive to amifostine, i.e., protection of normal tissues and enhanced tumor cell death.

1990; Sauer et al., 1990). The underlying mechanisms for the disparity between the in vitro and in vivo antitumor activities of D609 are not clear at present.

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D609 contains a xanthate group \([O\text{=}C\text{(}=\text{S})\text{]}\) and thus belongs to the family of chemicals called xanthates (Fig. 1) (Rao, 1971). Similar to the sulphydryl (–SH) moiety of other redox sensitive sulfur compounds, including glutathione and WR1065 (the active compound of amifostine), the xanthate group of D609 can readily oxidize to form a disulfide bond with subsequent loss of its biological activities (Rao, 1971; Zhou et al., 2001; Giron-Calle et al., 2002). This oxidative instability may contribute to the poor in vivo antitumor activity of D609 (Rao, 1971; Schick et al., 1989b; Amtmann and Sauer, 1990; Sauer et al., 1990). Therefore, we hypothesized that prodrug modification of the xanthate group of D609 through a metabolically labile linkage would protect D609 from rapid oxidation and lead to prodrugs with an increased stability and improved antitumor activity. To test this hypothesis, a series of S-(alkoxyacyl) D609 prodrugs was rationally designed and synthesized. These S-(alkoxyacyl) D609 prodrugs were designed and synthesized to improve the metabolic stability of D609.

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**Materials and Methods**

**Chemicals.** Anhydrous acetonitrile, paraformaldehyde, zinc chloride, acetyl chloride, chloromethyl butyrate, and chloromethyl pivalate were purchased from Fisher Scientific Co. (Pittsburgh, PA). High-performance liquid chromatography (HPLC) grade methanol was obtained from Curtin Matheson Science Inc. (Houston, TX). Porcine liver esterase (PLE; EC 3.1.1.1) was purchased from Sigma-Aldrich (St. Louis, MO). Porcine brain L-α-phosphatidylcholine (PC) and NBD C6-merocyanine were obtained from Avanti Polar Lipids (Alabaster, AL) and Molecular Probes (Eugene, OR), respectively.

**Synthesis and Characterization of S-(Alkoxycarbonyl) D609 Prodrugs.** D609 was synthesized and purified as described by Rao (1971), and its purity was determined to be >97%. Chloromethyl acetate was prepared as described by Bodor et al. (1983). The prodrugs of D609 were prepared and purified as illustrated in Fig. 2 and described below:

S-Methylexoxyacetyl D609 (1): Chloromethyl acetate (41 mg, 0.38 mmol) was added to a solution of D609 (100 mg, 0.38 mmol) in 15 ml of anhydrous acetone under nitrogen. The reaction mixture was stirred at room temperature for 8 h and then placed under reduced pressure to remove the solvent. The resulting suspension was extracted with dichloromethane (3 × 9 ml), the organic solutions combined, and the solvent evaporated. The resulting oil was separated by silica gel column chromatography (ethyl acetate/hexane, 1:10) to yield the target compound as a yellow oil (70 mg, 64%). 1H NMR and 13C NMR spectra were obtained using a Varian Inova 400-MHz NMR instrument (Palo Alto, CA) with tetramethylsilane as internal standard. 1H NMR (CDCl3, 400 MHz, δ ppm): 5.59 (s, 2 H, CH2), 5.44 (d, 1 H, CH, J = 10.0 Hz), 2.45–1.46 (m, 14 H), 2.06 (s, 3 H, CH3). 13C NMR (CDCl3, 100 MHz, δ ppm): 210.35, 170.70, 85.85, 66.74, 47.04, 44.43, 44.17, 41.08, 40.59, 34.12, 28.59, 27.43, 26.60, 21.14.

S-Methylexoxybutyryl D609 (2): Prodrug 2 was prepared similarly to S-methylexoxyacetyl D609 (1), except that the commercially available chloromethyl butyrate was used. The yield was 73%. 1H NMR (CDCl3, 400 MHz, δ ppm): 5.58 (s, 2 H, CH2), 5.44 (d, 1 H, CH, J = 10.0 Hz), 2.45–1.45 (m, 14 H), 2.26 (t, 2 H, CH2), 1.67–1.59 (m, 4 H, 2 CH3), 0.92–0.88 (t, 3 H, CH3, J = 7.4 Hz). 13C NMR (CDCl3, 100 MHz, δ ppm): 210.23, 173.11, 85.72, 66.51, 47.03, 28.59, 27.43, 26.60, 21.14.

![Fig. 1. Structure and main metabolic pathway of D609.](image-url)
S-Methylenoxyxipivalyl D609 (3): It was prepared in a way similar to that of S-methylenoxyxacyetyl D609 (1), except that the commercially available chloromethyl pivalate was used. The yield was 86%. ^1H NMR (CDCl3, 400 MHz, δ ppm): 5.54 (s, 2 H, CH2), 5.44 (d, 1 H, CH, J = 10.0 Hz), 2.46–1.399 (m, 14 H), 1.15s (9 H, 3 CH3). ^13C NMR (CDCl3, 100 MHz, δ ppm): 210.30, 178.11, 87.87, 66.61, 47.50, 46.12, 42.75, 39.92, 39.22, 32.25, 31.93, 30.14, 28.04, 27.24.

HPLC Analysis. A reverse-phase HPLC assay was developed for the quantitative analysis of D609 and S-(alkoxyacyl) D609 prodrugs using a Gilson HPLC system (Middleton, WI). The system was equipped with a 306-pump and a GAT LCD 501-detector set at 290 nm for the analysis. A 3.9 × 150 mm Nova-Pack C18 column (5-μm particle size) was used with a mobile phase consisting of 100% methanol at a flow rate of 1.0 ml/min. The retention times were D609, 0.95 ± 0.01 min; prodrug 1, 1.77 ± 0.03 min; prodrug 2, 1.97 ± 0.01 min; and prodrug 3, 2.08 ± 0.01 min.

Detection of D609 with 5,5′-Dithiobis(2-nitrobenzoic Acid) (DTNB). DTNB is a commonly used reagent for the detection of free thiol compounds (Lauderback et al., 2003). DTNB stock solution was prepared in phosphate-buffered saline (PBS) and added in excess to a sample containing D609 as specified in figure legends. D609 rapidly reacts with DTNB to produce a mixed disulfide plus the stable thiolate anion, 5-thio-2-nitrobenzoate, which can be quantified by measuring the optical density value at 412 nm using a Vmax plate reader (Molecular Devices Corp., Sunnyvale, CA), as described previously (Lauderback, et al., 2003). The concentration of D609 (micro-
mol) was calculated based on a linear D609-DTNB standard curve.

Cell Viability Assay. The MTT assay was used to quantify viable U937 cells (Hansen et al., 1989). In brief, U937 cells were harvested, washed, and resuspended in complete medium at a concentration of 5 × 10^6 cells/ml. Aliquots (100 μl) of the cell suspension were added to wells of a 96-well microtiter plate with the addition of 100 μl of complete medium (control) or various concentrations of D609 or a D609 prodrug (diluted in complete medium). After 48-h incubation, the plates were centrifuged to remove the supernatants from the culture, and 50 μl of MTT at a concentration of 5 μg/ml in PBS was added to each well. The plates were incubated for 4 h at 37°C to allow for the formation of a colored formazan. The formazan was solubilized by lysing the cells with 100 μl of lysis buffer containing 20% (w/v) dodecylsulfate and 50% (v/v) N,N-dimethyl formamide, pH 4.7. Absorbance of the formazan was measured at 595 nm using a Vmax plate reader (Molecular Devices Corp.). The viability of the cells was expressed as a percentage of control calculated by the formula A_c/A_t × 100, where A_c and A_t represent the absorbance of drug-treated and untreated control cells, respectively, and expressed as a percentage of control.

Apoptosis Assays. U937 cells (5 × 10^5/ml) were cultured with vehicle (0.5% DMSO) or 177 μM D609 or prodrug 2. After 24-h incubation, the cells were harvested, washed, and then fixed in 70% ethanol at 4°C for 24 h. They were stained with a propidium iodide staining solution (PBS containing 50 μg/ml propidium iodide, 100 U/ml RNase A, and 0.1 mM EDTA) for 2 h at room temperature before flow cytometric analysis (10,000 events/sample). The percentage of apoptotic cells was determined by quantification of the sub-G0/G1 population using a FACSCaliber (BD Biosciences, San Jose, CA).

Analysis of Sphingomyelin Synthase (SMS) Activity. U937 cells (2 × 10^6/ml) were cultured with vehicle (0.5% DMSO) or 177 μM D609 or prodrug 2. After 0.5-, 1-, and 2-h incubation, the cells were harvested, washed, and then homogenized in ice-cold lysis buffer (250 mM sucrose, 5 mM HEPES, pH 7.4, 1 mM phenylmethylsulfonyl fluoride, and 20 μg/ml each of chymostatin, leupeptin, antipain, and pepstatin) by 15 passages through a 27-gauge × 0.5-inch needle. The cell lysates were first centrifuged at 1000g for 10 min at 4°C to remove all the unbroken cells and nuclei. The resultant supernatants were quantified for protein concentration using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA) and assayed for SMS activity as follows: aliquots of the cell lysates containing 50 μg of protein were preincubated for 10 min at 30°C in a total volume of 50 μl of incubation buffer (50 mM Tris-HCl, pH 7.4, 25 mM KCl, and 0.5 mM EDTA). The reaction was started by addition of 2 nmol of NBD-C(6)-ceramide and 12 nmol of PC to give a final volume of 50 μl and incubated for 30 min. The reaction was stopped by addition of 200 μl of chloroform/methanol [1:1 (v/v)]; the mixture was vortexed and kept on ice. The chloroform/methanol fraction was isolated, and the lipids were resolved by thin layer chromatography (silica gel) in chloroform/methanol/15 mM CaCl2 (90:52.5:12) (Luberto and Hanunn, 1998; Meng et al., 2004). The formation of NBD-C(6)-sphingomyelin was quantified by determination of the fluorescent intensity of NBD-C(6)-sphingomyelin using a PhosphorImager. Values for blanks were subtracted from total values of NBD-C(6)-sphingomyelin to yield the amount of NBD-C(6)-sphingomyelin produced in each sample.

Ceramide Analysis. The levels of various species of ceramide were measured using positive mode electrospray ionization (ESI)/MS/MS analysis at the Lipidomics Core facility (Department of Biochemistry and Molecular Biology, Medical University of South Carolina) as described previously (Pettus et al., 2003a,b). Briefly, ESI/MS/MS analysis of ceramide was performed on a Thermo Finnigan TSQ 7000 triple quadruple mass spectrometer, operating in a multiple reaction monitoring positive ionization mode. U937 cells (4 × 10^6/sample) were washed twice with PBS after they were harvested from cultures. The cell pellets were dissolved in methanol, and lipids were extracted as reported previously (Luberto and Hanunn, 1998; Meng et al., 2004). An aliquot of the lipid extracts was taken for inorganic phosphate determination and the remaining was evapo-
rated to dryness and reconstituted in 100 μl of methanol. The reconstituted samples were injected on the Surveyor/TSQ 7000 liquid chromatographic/mass spectrometric system, and gradient was eluted from the BDS Hypersil C8, 150 × 3.2 mm, 3-μm particle size column, with 1.0 mM methanolic ammonium formate/2 mM aqueous ammonium formate mobile phase system. Peaks for the target analytes and internal standards were collected and processed using the Xcalibur software system. Various species of ceramide were quantified using N-palmitoyl-D-erythro-sphingosine, C13 base (C13 ceramide) and N-heptadecanoyl-D-erythro-sphingosine, C18 base (C18 ceramide) as internal calibration standards. Calibration curves were constructed by plotting peak area ratios of synthetic standards corresponding to each target analyte with respect to the appropriate internal standard. The target analyte peak areas from the samples were similarly normalized to their respective internal standard and then compared with the calibration curves using a linear regression model. The results are expressed as picomoles of ceramide per nanomoles of lipid phosphate.

Statistical Analysis. The data were analyzed by analysis of variance. In the event that analysis of variance justified post hoc comparisons between group means, these were conducted using the Student-Newman-Keuls test for multiple comparisons. For experiments in which only single experimental and control groups were used, group differences were examined by unpaired Student’s t test. Differences were considered significant at p < 0.05.

Results

Rational Design and Synthesis of Prodrug Forms of D609. A series of S-(alkoxylacyl)-D609 prodrugs was rationally designed and synthesized as illustrated in Fig. 2. The syntheses of the alkoxycarbonyl prodrugs 1, 2, and 3 were developed based on the work of Nudelman et al. (2001). Briefly, chloromethyl acetate was synthesized according to the procedure of Bodor et al. (1983). The reaction of this alkylating agent with the potassium salt of D609 generated the desired S-methylalkoxycarbonyl D609 (1), which was isolated by column chromatography in 64% yield. The synthesis of the desired S-methylalkoxybutyryl D609 (2) and S-methylalkoxyxypivalyl D609 (3) was accomplished by the same procedure, beginning with the commercially available chloromethyl butyrate and chloromethyl pivalate, respectively. The yield of 2 and 3 after column chromatography was 73 and 86%, respectively. The purity of these prodrugs was >97% by HPLC analysis and the identity of these compounds was confirmed by 1H NMR and 13C NMR spectroscopy.

Comparison of the Stability of D609 and Its Prodrugs. An HPLC assay was initially developed to examine the stability of D609 and D609 prodrugs. The assay showed that D609 rapidly disappeared in saline solution at room temperature (24°C) with a t1/2 of about 19.5 min (Figs. 1 and 3A). The disappearance of D609 in saline is likely due to its oxidation, because the rate of the disappearance was accelerated even further by the addition of low concentrations of mild oxidants, such as H2O2 (data not shown). Because of its rapid disappearance after being dissolved in saline, a linear standard curve of D609 could not be constructed using the HPLC analysis, and thus the results were expressed as net area under curves (AUC). Compared with D609, D609 prodrugs 1, 2, and 3 are highly stable and their concentrations barely changed during a 3-h incubation in saline, suggesting that no significant spontaneous oxidation and hydrolysis of these compounds occurred (Fig. 3B). Actually, even after 48-h incubation in saline the concentrations of these D609 prodrugs remained steady (data not shown).

Esterase-Catalyzed Hydrolysis of D609 Prodrugs. The three S-(alkoxylacyl)-D609 prodrugs are designed to release D609 in two steps: 1) esterase-catalyzed hydrolysis of the acyl ester bond (k1), followed by 2) conversion of the resulting hydroxymethyl D609 to formaldehyde and D609 (k2) (Fig. 4). To determine the hydrolytic property of these D609 prodrugs, 1, 2, and 3 (300 μM in 15% DMSO/PBS, pH 7.4) were incubated with 0.1 unit/ml PLE at 37°C. After various times during incubation, the rate of hydrolysis of these D609 prodrugs was monitored by HPLC analysis. The release of D609 was determined by measuring the colorimetric assay of D609 with DTNB, because the concentrations of D609 can be measured more accurately by DTNB than by
added to rat plasma. The complete hydrolysis of prodrug reached a plateau in less than 100 s after the prodrug was dissolved in DMSO and then diluted into rat plasma (300 μM in 15% DMSO/plasma). After incubation at 37°C, aliquots (100 μl) of the plasma were removed at various times and immediately mixed with an equal volume of DTNB in acetonitrile (3 mM DTNB). Acetonitrile was used to quickly inactivate plasma esterases and to precipitate plasma proteins. After removal of the precipitated plasma proteins by centrifugation, the concentrations of the D609 prodrug and D609 in the clear plasma supernatants were determined by HPLC and DTNB assays, respectively, as described above. As shown in Fig. 6, prodrug 2 underwent rapid hydrolysis in plasma. The complete hydrolysis of prodrug 2 in plasma was achieved within 60 s. The $K_{obs}$ and $t_{1/2}$ for prodrug 2 are 9.168 × 10⁻² s⁻¹ and 7.559 s, respectively. Correspondingly, the concentrations of D609 in plasma went up rapidly and reached a plateau in less than 100 s after the prodrug was added to rat plasma. The complete hydrolysis of prodrug 2 resulted in the release of 88% of D609 based on the initial molar quantity of the prodrug.

**Prodrug Modification Increases D609 Tumor Cytotoxicity.** D609 is a selective tumor cytotoxic agent that has the ability to induce tumor cell death by apoptosis (Amtmann and Sauer, 1987; Porn-Ares et al., 1997; Bettaieb et al., 1999; Meng et al., 2004). To determine whether prodrug modification increases the biological activity of D609 against tumor, we compared the tumor cell cytotoxicity of prodrug 2 with that of D609 in U937 leukemia cells. As shown in Fig. 7A, incubation of U937 cells with prodrug 2 and D609 resulted in a dose-dependent reduction in cell viability. The decrease in cell viability was associated with an increase in the number of the sub-G₀/G₁ cells (Fig. 7B), indicating that both prodrug 2 and D609 are capable of inducing apoptosis in U937 leukemia cells. However, the cells treated with prodrug 2 showed a significantly greater reduction in cell viability and increase in the sub-G₀/G₁ cells than D609-treated cells, suggesting that prodrug 2 is more cytotoxic to U937 cells than D609 (Fig. 7). This suggestion is confirmed by the fact that prodrug 2 has a significantly lower LD₅₀ value than that of D609 (56.6 versus 117 μM) against U937 cells. Similarly, prodrug 2 also exerted a greater cytotoxicity than D609 against Jurkat T-cell leukemia cells (LD₅₀ of prodrug 2, 44.26 μM versus D609, 63.97 μM) and STM91-01 malignant rhabdoid tumor cells (LD₅₀ of prodrug 2, 87.10 μM versus D609, 545.75 μM). In contrast, prodrug 2 was less toxic to the normal human diploid fibroblasts-W138 cells than D609 (LD₅₀ of prodrug 2, 333.43 μM versus D609, 267.51 μM). This result indicates that prodrug modification not only increases the cytotoxicity of D609 against tumor cells, but more importantly, it also reduces its toxicity to normal cells.

**Prodrug Modification Increases the Inhibitory Effect of D609 on SMS.** We have recently identified that SMS is a potential molecular target of D609 (Luberto and Hannun, 1998; Meng et al., 2004). Inhibition of SMS activity increases the intracellular level of ceramide and decreases that of diacylglycerol in favor of induction of tumor cell apoptosis (Luberto and Hannun, 1998; Meng et al., 2004). Thus, we compared the effect of prodrug 2 with that of D609 on SMS in U937 cells. A preliminary study showed that the enzymatic activity of SMS in U937 cell lysates was linear with the amount of protein and time of the reaction (data not shown). Based on this preliminary assay, the optimal conditions of the assay were selected. Under these conditions, incubation of U937 cells with D609 or prodrug 2 (177 μM) resulted in a time-dependent inhibition of SMS activity (Fig. 8). However, the inhibition was significantly greater in prodrug 2-treated cells than that of D609-treated cells ($p < 0.05$), demonstrating that prodrug modification significantly increased the inhibitory effect of D609 on SMS.

**Prodrug Modification Augments D609-Induced Increase in Ceramide.** The effects of D609 and prodrug 2 on
the level of ceramide in U937 cells were also examined because D609 can increase the level of ceramide via inhibition of SMS and stimulation of the de novo synthesis of ceramide (Luberto and Hannun, 1998; Meng et al., 2004; Perry and Ridgway, 2004). An ESI/MS/MS analysis was used to profile the changes in the levels of various species of ceramide in U937 cells after they were incubated with 177\textsuperscript{H}D609 or prodrug 2. The cells treated with D609 or prodrug 2 showed a significant increase in almost all species of ceramide, except that the level of C\textsubscript{24}-ceramide was not changed in the cells treated with D609 and that of C\textsubscript{18:1}-ceramide was below detection limits for all cells examined (Table 2; data not shown). In general, the cells treated with prodrug 2 exhibited a greater increase in the levels of various species of ceramide than those treated with D609. Specifically, U937 cells treated with D609 exhibited an about 1.61-fold increase in the level of total ceramide compared with that of vehicle-treated cells ($p < 0.001$). The increase (1.84-fold) was significantly greater in the cells treated with prodrug 2 than that of D609-treated cells ($p < 0.05$).

Discussion

A “prodrug” is a pharmacologically inactive compound that can be converted into an active drug by metabolizing enzymes in the body, by nonmetabolic reactions, or by using both strategies (Sinkula and Yalkowsky, 1975; Smith and Clark, 1998). Prodrug modification of an active drug can be achieved by attaching a metabolically labile group that blocks the active or reactive site of the drug, which leads to decreased metabolic inactivation and increased chemical stability of the compound. Ultimately, this can result in the improvement of the pharmacokinetics, safety, and therapeutic efficacy of the active compound (Capizzi, 1999; Culy and Spencer, 2001). This strategy is extremely useful for developing prodrugs of redox-sensitive sulfur compounds because these compounds contain a sulphydryl (–SH) moiety that can be readily oxidized to a disulfide with subsequent loss of biological activities. For example, WR1065, a potent nucleo-

TABLE 1
Hydrolysis of D609 prodrugs by esterase

<table>
<thead>
<tr>
<th>Prodrug</th>
<th>$k_{obs}$ ($\min^{-1}$)</th>
<th>$t_{1/2}$ ($\min$)</th>
<th>D609 (%)</th>
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<tr>
<td>1</td>
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<td>71</td>
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<td>3</td>
<td>2.984 x 10^{-2}</td>
<td>23.22</td>
<td>60</td>
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philic sulfur antioxidant, exhibits poor pharmacokinetics and inferior therapeutic efficacy, which precludes its use as a therapeutic agent in clinic. The phosphorothioate modification of WR1065 produces a prodrug, e.g., amifostine, in which the \(-\text{SH}\) group is protected as a \(\text{S}-\text{phosphate ester}\). The phosphate group can be removed by phosphatase on the cell membrane with the release of the active compound WR1065 near its target. Therefore, amifostine exhibits higher stability, reduced toxicity, superior pharmacokinetics, and improved therapeutic efficacy compared with that of WR1065 (Spencer and Goa, 1995; Capizzi, 1999; Culy and Spencer, 2001; Poggi et al., 2001).

D609 is a member of a new class of nucleophilic sulfur pharmaceutical agents that contains a xanthate group \([-\text{C}(=\text{S})\text{S}^-/\text{C}(=\text{S})\text{SH}]\). Similar to the \(-\text{SH}\) group of WR1065, the xanthate group of D609 can be easily oxidized to form a disulfide bond (Rao, 1971; Zhou et al., 2001; Giron-Calle et al., 2002). This oxidative instability may contribute to its poor antitumor activity in vivo (Schick et al., 1989b; Amtmann and Sauer, 1990; Sauer et al., 1990). Therefore, the development of prodrug modifications of the xanthate group of D609 would be anticipated to lead to agents with increased oxidative stability, and thus improved pharmacokinetic and therapeutic efficacy for cancer therapy. Although the phosphorothioate modification has been commonly used to generate prodrugs for various redox-sensitive sulfur compounds, including WR1065 (Spencer and Goa, 1995; Capizzi, 1999; Culy and Spencer, 2001; Poggi et al., 2001), our initial attempts to synthesize the phosphorothioate analog of D609 were unsuccessful owing to the instability of the resulting mixed anhydride (data not shown). Therefore, a series of \(\text{S}-(\text{alkoxyacyl})\) D609 prodrugs was rationally designed and synthesized by connecting D609 to an acyl moiety at the xanthate group via a self-immolative methylenoxyl group. These \(\text{S}-(\text{alkoxyacyl})\)-D609 prodrugs are designed to release D609 in two steps: 1) esterase-catalyzed hydrolysis of the

Fig. 7. Prodrug modification increases D609 tumor cell cytotoxicity. A, effects of prodrug 2 and D609 on U937 cell viability. U937 cells \((5 \times 10^5/\text{ml})\) were incubated in 96-well plates for 48 h with several concentrations of prodrug 2 or D609. No exogenous esterase was added as FBS contains sufficient esterases (about 1 unit/ml) to hydrolyze the prodrug. Cell viability was analyzed by MTT assay. The results are expressed as a percentage relative to control untreated cells and presented as means \(\pm\) S.E.M. of triplicates. A representative assay of three independent assays is shown. B, prodrug 2 and D609 induce apoptosis in U937 cells. U937 cells \((5 \times 10^5/\text{ml})\) were incubated with vehicle (0.5% DMSO) or 177 \(\mu\text{M}\) D609 or prodrug 2 in vehicle. Apoptotic cell death was analyzed by determination of the sub-\(G_{\text{sub}}\) cells using a flow cytometer. Representative flow cytometric analyses are shown.
In addition, the rates and pharmacokinetic parameters of the D609 prodrugs. This observation suggests that further modification of the acyl ester bond. This is followed by prodrug 2. Among these prodrugs, prodrug 1 had the shortest $t_{1/2}$, which was followed by prodrug 2 and then by prodrug 3, indicating that an increase in the steric bulkiness of the acyl group hinders the esterase-catalyzed hydrolysis of the acyl ester bond. This observation suggests that further modification of the S-(alkoxyacyl) group may allow tailoring of the hydrolysis rates and pharmacokinetic parameters of the D609 prodrugs. In addition, the S-(alkoxyacyl) modification strategy may be applicable to the development of other redox active sulfur compounds to produce ester prodrugs. These ester prodrugs should have better drug absorption and distribution properties than phosphorothioate-modified sulfur prodrugs, because phosphorothioate-modified prodrugs, such as amifostine, exist as an ionized phosphorothioic acid molecule at a physiological pH (7.4), which contributes to their poor distribution and rapid clearance in urine (Spencer and Goa, 1995; Capizzi, 1999; Culy and Spencer, 2001; Poggi et al., 2001).

The hydrolysis of S-(alkoxyacyl) D609 prodrugs by esterase produces a hydroxymethyl D609 intermediate that spontaneously breaks down to release the parent drug (D609) and formaldehyde. As such, the complete hydrolysis of prodrugs 1, 2, and 3 resulted in approximately 71, 93, and 60% molar recovery of D609, respectively. It has yet to be determined why these prodrugs release different amount of D609 after complete hydrolysis: all of the prodrugs give the same intermediate and final product; thus, this difference cannot be due to an alternative hydrolysis pathway of the hydroxymethyl-D609 nor can it be due to oxidation of D609. It is also interesting to note that the hydrolysis of prodrug 2, and the subsequent release of D609, was much faster with rat plasma than with PLE (0.1 unit/ml). The shorter half-life of prodrug 2 in rat plasma may be due to higher levels of esterases and/or to a greater susceptibility of this prodrug to the esterases present in rat plasma. At any rate, the hydrolysis of prodrug 2 in rat plasma resulted in the almost complete (88%) release of D609.

More importantly, it was found that in in vitro assays prodrug 2 was biologically more active against various human tumor cell lines but less toxic to normal human diploid fibroblasts than D609. This result suggests that the S-(alkoxyacyl) prodrug modification significantly improves the antitumor activity of D609, probably in part by increasing the chemical stability of D609. For example, it was shown that prodrug 2 has a greater apparent potency than D609 in induction of apoptosis in U937 cells and had a significantly lower LD$_{50}$ value than that of D609 (56.6 versus 117 μM). In addition, the increased tumor cell cytotoxicity of prodrug 2 was associated with an augmented inhibition of SMS, a potential molecular target of D609, resulting in a greater elevation in the ceramide levels in U937 cells. It has been suggested that D609 may selectively kill tumor cells by elevating the level of ceramide via direct inhibition of SMS. Therefore, these observations suggest that the tumor cell cytotoxicity of the S-(alkoxyacyl) prodrug is likely mediated by D609 released from the prodrug after its hydrolysis, because the prodrug induces tumor cell death by affecting the same molecular target and pathway.

In summary, the results presented in this report provide the proof-of-principal that prodrug modification by protecting the xanthate moiety of D609 with an alkoxyacyl group can improve D609 oxidative stability and antitumor activity. This prodrug modification strategy represents a novel approach for the development of redox-sensitive sulfur prodrugs. In addition, we predict that the S-(alkoxyacyl) modification of D609 can be further optimized by varying the alkyl groups to tailor the analogs for hydrolysis by esterases with

**TABLE 2**

D609- and prodrug 2-induced changes in ceramide profiles in U937 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>C16-Cer</th>
<th>dhC14-Cer</th>
<th>C16-Cer</th>
<th>C14-Cer</th>
<th>C16-Cer</th>
<th>C14-Cer</th>
<th>C14-Cer</th>
<th>Total Cer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0.019 (0.004)</td>
<td>0.405 (0.009)</td>
<td>1.027 (0.032)</td>
<td>0.057 (0.006)</td>
<td>0.007 (0.001)</td>
<td>0.494 (0.040)</td>
<td>0.357 (0.012)</td>
<td>2.363 (0.012)</td>
</tr>
<tr>
<td>D609</td>
<td>0.038 (0.004)</td>
<td>0.822 (0.035)</td>
<td>1.612 (0.064)</td>
<td>0.213 (0.023)</td>
<td>0.036 (0.005)</td>
<td>0.466 (0.077)</td>
<td>0.416 (0.060)</td>
<td>3.816 (0.197)</td>
</tr>
<tr>
<td>Prodrug 2</td>
<td>0.054 (0.008)</td>
<td>0.851 (0.042)</td>
<td>1.713 (0.134)</td>
<td>0.262 (0.044)</td>
<td>0.031 (0.002)</td>
<td>0.863 (0.057)</td>
<td>0.482 (0.037)</td>
<td>4.358 (0.222)</td>
</tr>
</tbody>
</table>

*Cer, ceramide; dh-Cer, dihydroceramide.

*<i>p < 0.05 to 0.001 versus vehicle.</i>

*<i>p < 0.05 to 0.001 versus D609.</i>
different rates and pharmacokinetics. This may eventually lead to a clinically useful drug that can be used as an adjuvant for cancer therapy by enhancing the antitumor activity of radiotherapy and chemotherapy, while reducing their side effects in normal cells.

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References


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