Molecular Basis of the Antitumor Activities of 2-Crotonyloxymethyl-2-cycloalkenones

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The antitumor activity of 2-crotonyloxymethyl-2-cyclohexenone (COMC-6) is not the result of the GSH conjugate (GSMC-6) formed inside tumor cells, as the diethyl ester prodrug form of GSMC-6 displays little antitumor activity with B16 melanotic melanoma in vitro (IC_{50} > 460 μM) versus COMC-6 (IC_{50} 0.041 μM) and its five- and seven-membered ring homologues. Antitumor activity probably results from a reactive intermediate that forms during conjugation of the COMCs with intracellular GSH.

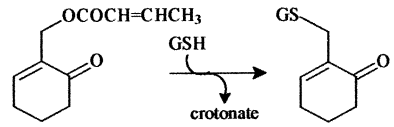
Introduction

The *Streptomyces* metabolite 2-crotonyloxymethyl-(4*R*,5*R*,6*R*)-4,5,6-trihydroxy-2-cyclohexenone (COTC) and its synthetic analogue 2-crotonyloxymethyl-2-cyclohexenone (COMC-6) are potent antitumor agents against both murine and human tumors in culture and in tumor-bearing mice.[1−3] As such, these compounds have attracted considerable interest as synthetic targets.[4] Early investigators proposed that antitumor activity might arise from competitive inhibition of the methylglyoxal-detoxifying enzyme glyoxalase I (GlxI) by the covalent adducts arising from the S_N2 displacement of crotonate by intracellular glutathione (GSH); e.g., COMC-6 gives 2-glutathionylmethyl-2-cyclohexenone (GSMC-6) in the presence of GSH, Scheme 1.[1,3]

GlxI plays an important role in detoxifying intracellular methylglyoxal, which is formed during normal carbohydrate metabolism.[5] Indeed, tight-binding enediol analogue inhibitors of GlxI retard the growth of both murine and human tumors in culture and in tumor-bearing mice by causing the accumulation of intracellular methylglyoxal.[6,7] However, the inhibitors showing antitumor activity have K_i values in the nanomolar concentration range with human erythrocyte GlxI. In contrast, the GSH adducts of COTC and COMC-6 were shown to be poor competitive inhibitors of human GlxI, with K_i values in the 100−200 μM range.[8,9] Therefore, antitumor activity is unlikely to arise from inhibition of GlxI. Nevertheless, this finding does *not* exclude the possibility that the GSH adducts are toxic to tumor cells by some other mechanism.

Here, we summarize the results of cell growth inhibition studies that exclude the possibility that the GSH adducts are responsible for cytotoxicity and support our previously proposed hypothesis that cytotoxicity instead arises from an intermediate exocyclic enone formed during the conjugation reaction between COMC-6 and GSH. Also reported is the synthesis and cytotoxicities of the five- and seven-membered ring homologues COMC-5 and COMC-7, respectively.

Scheme 1



COMC-6 GSMC-6

Synthesis. The synthesis of COMC-6 and GSMC-6 have previously been described.[9] The homologues COMC-5, and COMC-7 were synthesized from the corresponding, commercially available 2-cycloalkenones utilizing the Baylis−Hillman reaction to prepare the 2-hydroxymethyl-2-cycloalkenones, which were then crotonylated using crotonic anhydride.[4] The [*glycyl,glutamyl*] diethyl ester of the GSH−COMC-6 adduct (GSMC-6$(Et)_2$) was prepared by acid-catalyzed esterification of GSMC-6 in ethanolic HCl. The diethyl ester was then by purified to greater than 95% purity using reverse-phase HPLC.

Results and Discussion

To test the possibility that GSMC-6 is responsible for tumoricidal activity, the antitumor activity of the diethyl ester GSMC-6$(Et)_2$ was compared with that of COMC-6. The diethyl ester should indirectly deliver GSMC-6 into cells by a process involving diffusion across the cell membrane, followed by esterase-catalyzed deethylation to give COMC-6. This prodrug strategy has previously been used to deliver enediol analogue inhibitors of GlxI into tumor cells.[6]

Accumulation studies confirmed this prediction. B16 melanotic melanoma in tissue culture was incubated with 50 μM GSMC-6$(Et)_2$ or COMC-6. As a function of time, cell pellets were lysed and fractionated by RPHPLC. The concentration of GSMC-6 was determined by comparison of the integrated intensity of the corresponding peak from the HPLC with the appropriate standard curve (see Supporting Information). Incubation of the

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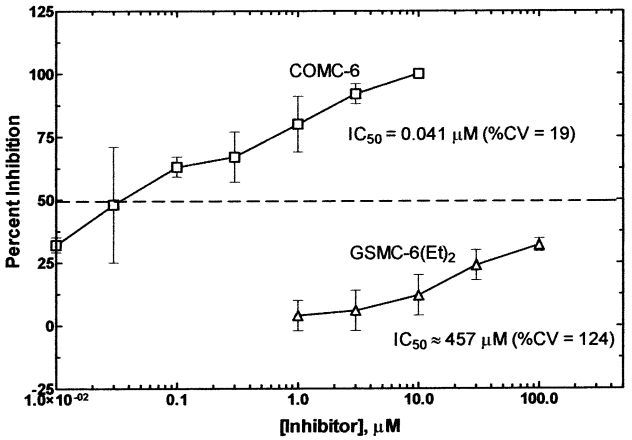
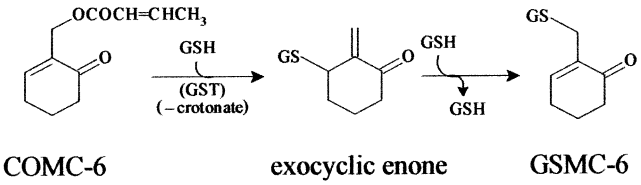


Figure 1. Growth inhibition of B16 cells in the presence of COMC-6 and GSMC-6(Et)$_2$. Conditions/methods given in text.

Scheme 2



cells with COMC-6 showed significant accumulation of GSMC-6 at 30 s (0.041 nmol/ 10^7 cells) and 10 min (0.074 nmol/ 10^7 cells). No GSMC-6 was detected at later times. Incubation with GSMC-6(Et)$_2$ showed significant GSMC-6 at 30 s (0.037 nmol/10^7 cells), but not at later times.[10]

While incubation of B16 cells with either of these species results in significant intracellular accumulation of GSMC-6, only COMC-6 shows dramatic antitumor activity, with an IC_{50} value 10^{-4}-fold that of GSMC-6(Et)$_2$, Figure 1. Therefore, the antitumor activity of COMC-6 cannot simply be due to the adduct GSMC-6, but must arise either directly from unconjugated COMC-6 or from an intermediate formed during the conjugation reaction between GSH and COMC-6.

Indeed, kinetic studies and intermediate trapping experiments show that conjugation involves an initial Michael addition of GSH to COMC-6 to give a highly reactive exocyclic enone intermediate, which subsequently reacts with GSH in bulk solvent to give GSMC-6, Scheme 2.[9]

This was first revealed by the observation that human placental glutathione transferase (GST) catalyzes the initial Michael addition reaction resulting in biphasic kinetics wherein the second step is rate determining overall. In the absence of enzyme, the rate constant for reaction of GSH with the exocyclic enone is about 12-fold larger than that for reaction of GSH with COMC-6. Therefore, the antitumor activity of COMC-6 could reasonably result from reaction of the exocyclic enone with proteins and/or nucleic acids critical to cell function.[11] Mass spectral studies show that COMC-6 alkylates model oligonucleotides in the presence of GSH via a mechanism in which the exocyclic enone is probably the alkylating species.[12]

The respective five- and seven-membered ring homologues of COMC-6 (Scheme 3) have also been shown to

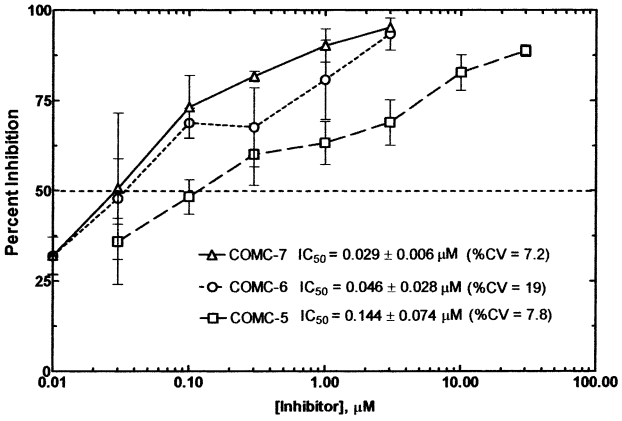
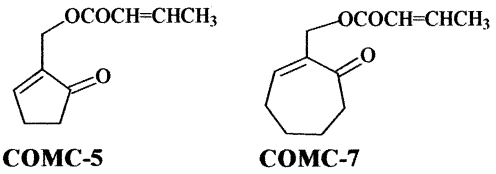


Figure 2. Growth inhibition of B16 cells in the presence of COMC-5, COMC-6 and COMC-7. Using Fisher's pairwise comparisons, the means of the IC_{50} values of COMC-6 and COMC-7 are not different from one another, but they are different from the mean IC_{50} for COMC-5. Conditions/methods given in text.

Scheme 3



undergo conjugate additions with GSH that involve intermediate exocyclic enones.[13]

Therefore, these species should also exhibit antitumor activity. Indeed, all three COMCs are toxic to B16 murine melanoma in vitro, Figure 2. The similar shapes and parallel shift of the dose−response curves suggest a similar mechanism of cytotoxicity for each compound.

The cytotoxicity of COMC-6 was also examined with HT29(wt) human colon adenocarcinoma versus HT29 (MDR) cells that overexpress p-glycoprotein. This experiment was prompted by a previous observation that COTC displays enhanced toxicity against certain types of drug-resistant neoplastic cells versus wild-type cells.[14] In the present study, the IC_{50} value for HT29 (wt) cells was 0.80 μM (%CV = 13) while that for HT29 (MDR) was 1.8 μM (%CV = 37), a change of only 2-fold (see Supporting Information). By comparison, a 17-fold difference in cytotoxicity of vincristine against these cell lines was noted in this laboratory: HT29 (wt), IC_{50} = 0.001 μM (%CV = 0.01); HT29 (MDR), IC_{50} = 0.017 μM (%CV = 17).

Conclusions

The central outcome of this work is that the GSH conjugate of COMC-6 (GSMC-6) cannot be responsible for the antitumor activity of COMC-6, contrary to previous suggestions. The mechanism of cytotoxicity is not clear, but probably involves alkylation of critically important protein(s) and/or nucleic acid(s) by the exocyclic enone intermediate that is formed during the conjugation of GSH with the COMCs. The cytotoxic species also appears to be a poor substrate for the MDR-associated p-glycoprotein, given the similar IC_{50} values of COMC-6 with HT29 (wt) versus HT29 (MDR). This finding might best be rationalized by the fact that the glutathionylated exocyclic enone is multiply charged,

and that p-glycoprotein most readily transports uncharged, hydrophobic antitumor agents.[15] This hypothesis might help to explain a previous observation that adriamycin-, aclarubicin-, and bleomycin-resistant sublines of murine lymphoblastoma L5178Y cells are no less sensitive to COTC than the parental cell line.[14]

Experimental Section

Proton and carbon-13 NMR spectra were recorded on a Bruker AF-300, a Varian VXR-400 or a Varian Unity-500 spectrometer. Chemical shifts were reported as δ scale in parts per million (Supporting Information). Spectra obtained were referenced to residual deuterated solvent peaks. Electrospray mass spectra were acquired in the positive ion mode using a Micromass Quattro 1 Triple Quadrupole Tandem Mass Spectrometer at the Cornell University Mass Spectrometry Facility. (Supporting Information).

2-Crotonyloxymethyl-2-cyclopentenone (COMC-5). To a stirred solution of 2-hydroxymethyl-2-cyclopentenone[16] (0.54 g, 4.82 mmol) in CH_2Cl_2 (50 mL) under Ar was added crotonic anhydride (1.56 g, 9.79 mmol) and DMAP (50 mg, 0.41 mmol). Then pyridine (3.5 mL) was added. The mixture was stirred at room temperature for 2 h and then diluted with another 50 mL CH_2Cl_2. Saturated NH_4Cl aqueous solution (100 mL) was added and stirred overnight to quench the reaction. The aqueous and organic layers were separated and the aqueous layer was washed with CH_2Cl_2 (3 × 100 mL). The combined organic layers were dried over $MgSO_4$, filtered, and concentrated. Silica gel chromatography afforded COMC-5 as a colorless oil (0.61 g, 3.4 mmol, yield 71%): R_f 0.28 (1:1 petroleum ether:Et_2O).

2-Crotonyloxymethyl-2-cycloheptenone (COMC-7). This compound was prepared from 2-hydroxymethyl-2-cycloheptenone[16] following the general procedure used to prepare COMC-5. Silica gel chromatography afforded COMC-7 as a light yellow oil (yield 72%): R_f 0.58 (1:1 hexanes:EtOAc).

2-Glutathionylmethyl-2-cyclohexenone [*glycyl, glutamyl*] Diethyl Ester (GSMC-6(Et)$_2$). GSMC-6 (67.3 mg, 0.14 mmol) was dissolved in 30 mL of ethanolic HCl (8 N) and stirred at 50 °C for 3h (yield ~ 75%). The solvent was removed in vacuo and the crude product purified by reverse-phase C18 HPLC, using methanol/water (1:3) containing 0.25% acetic acid as an eluting solvent. The solvent was removed from the peak fractions to give the acetate salt of the diethyl ester with greater than 95% purity by HPLC. The NMR spectrum (D_2O/DSS) of GSMC(Et)$_2$ featured the expected ethyl and glutathionyl resonances and the downfield resonance (δ 7.13) characteristic of H3 in β,γ-unsaturated 2-cyclohexenones.

In Vitro Cytotoxicity Studies. B16 (2×10^4 cells) were plated in 24-well plates containing RPMI 1640/10% bovine calf serum, 10 μg/mL gentamicin and incubated at 37 °C under an atmosphere of 5% CO_2 and 95% humidified air. Drug was added at the indicated concentrations. After 72 h, cells were trypsinized, concentrated, and counted by trypan blue exclusion using a hemocytometer. IC_{50} values are the mean ± standard deviation of triplicate determinations carried out in three separate assays on different days. IC_{50} values were calculated using the Hill equation and the program Adapt.[17]

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Supporting Information Available: Analytical data, ^1H and ^{13}C NMR data, methods used in the accumulation studies, dose−response curves for inhibition of HT29(wt) and HT29-(MDR) by COMC-6. This material is available free of charge via the Internet at http://pubs.acs.org.

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(10) The short lifetime of GSMC-6 inside B-16 cells is rather surprising, given that GSMC-6 is stable in aqueous solution for hours. One reasonable possibility is that GSMC-6 is reacting with nucleophilic species in the cytosol of the cell that are not critical for cell survival, as GSMC-6 is itself a Michael acceptor.

(11) A direct test of this hypothesis would be to deliver the glutathionylated exocyclic enone (Scheme 2) into B16 cells as the diethyl ester and test whether the diethyl ester is indeed more toxic to tumor cells than GSMC-6(Et)$_2$. However this experiment could not be done, because of the instability of the exocyclic enone in buffered solution at pH 7.

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