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Letter

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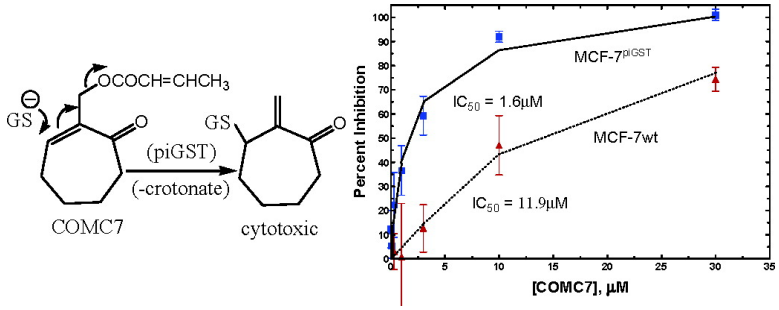

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Selective Inhibition of MCF-7^{piGST} Breast Tumors Using Glutathione Transferase-Derived 2-Methylene-cycloalkenones

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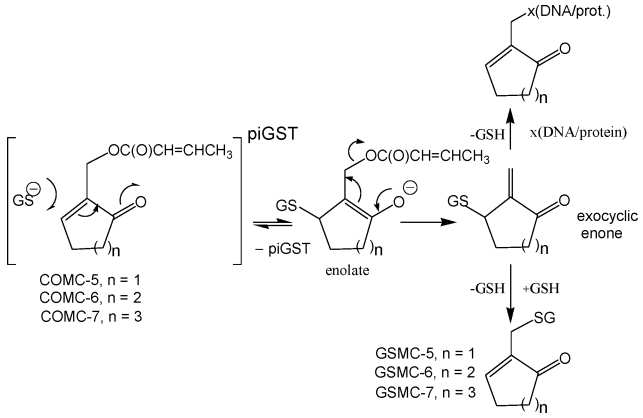
Abstract: Human glutathione (GSH) transferase (hGSTP1-1) catalyzes the conversion of antitumor 2-crotonyloxymethyl-2-cycloalkenones (COMCs) to highly reactive exocyclic enone alkylating agents. In vitro efficacy studies show that the cytotoxicities of the COMCs directly correlate with the level of expression of GSTP1-1 in MCF-7^{piGST} versus MCF-7wt breast tumors, indicating that the exocyclic enones are the actual cytotoxic species. The COMCs are a potentially important new class of prodrugs, which can specifically target multidrug-resistant tumors overexpressing hGSTP1-1.

Overcoming the multidrug resistance (MDR) phenotype is one of the most important challenges facing medicinal enzymology.[1−4] While some anticancer drugs give impressive short-term benefits to cancer patients, repeated exposure often leads to overexpression of MDR proteins that render the drugs less effective over time. The glutathione transferases (GSTs) are one class of MDR proteins that often play a role in detoxifying antitumor drugs by catalyzing their conjugation to the ubiquitous cofactor GSH. To complete detoxification, the conjugate is then pumped out of the cell via the resistance-associated-protein MRP-1, a member of the ATP binding cassette transporter superfamily.[5] The expression of either mu- or pi-GSTs in cultured MDR breast tumor cell lines often parallels the degree of drug resistance and can be as high as 10-fold greater than that found in wild-type tumors.[1,2] One long-standing strategy for overcoming MDR is to use isozyme-specific reversible[6] and/or irreversible[7] inhibitors of the GSTs in combination with antitumor agents.

An intriguing alternative strategy is to use isozyme specific substrates that will be catalytically converted to toxic products by the transferases, thus leading to enhanced antitumor activity in MDR tumors overexpressing transferase activity. Indeed, GSH derivatives have been described, which form nitrogen mustards in the presence of GST.[8,9] An aromatic *N*,*N*-dialkyl diazenium diolate salt has also been reported that releases apoptotic nitric oxide (NO) in the presence of GST.[10] Recently, we suggested that antitumor 2-crotonyloxymethyl-2-cycloalkenones (COMCs) might function similarly, Scheme 1.[11,12]

Scheme 1. Proposed Reaction Mechanism of GSTP1-1 with the COMCs



This hypothesis follows from the fact that GSTP1-1 catalyzes the conversion of the COMC derivatives to the enolate, which subsequently forms exocyclic enones capable of either reacting with another equivalent of GSH to give the GSMC derivative or alkylate biomacromolecules like DNA.[13] Here, we describe for the first time the results of in vitro efficacy studies with MCF-7^{piGST} breast tumor cells, which show that the transiently formed exocyclic enone does indeed account for most of the tumoricidal activity of the COMCs.

Early investigators hypothesized that the in vitro antitumor activity of COMC-6, a simple derivative of an antitumor metabolite from *Streptomyces*, was the result of competitive inhibition of the methylglyoxal-detoxifying enzyme glyoxalase I by GSMC-6.[14−16] The formation of GSMC-6 was proposed to result from a simple in-line displacement reaction between GSH and COMC-6.[17] However, subsequent studies excluded GSMC-6 from being the cytotoxic species, as the IC_{50} value of GSMC-6 (IC_{50} > 400 μM), indirectly delivered into murine melanotin melanoma B16 cells as the diethylester prodrug, is nearly 10^4-fold greater than that of COMC-6 alone ($IC_{50} = 0.05 \pm 0.03$ μM).[18]

The discovery that the formation of GSMC-6 does *not* involve an in-line displacement mechanism, but rather involves the formation of an intermediate exocyclic enone (Scheme 1),[11,12] suggested that tumoricidal activity might arise either directly from the Michael acceptor properties of COMC-6 and/or those of the exocyclic enone. Indeed, incubation of COMC-6 with model dinucleotides or single-stranded 16-mer oligo-nucleotides, either in the presence or in the absence of GSH/GSTP1-1, results in the formation of 2-methylene cyclohexenone adducts of the exocyclic amino group of the guanine residues.[13] Thus, tumoricidal activity could potentially reflect genotoxicity by either COMC-6 and/or the exocyclic enone derived from COMC-6. On purely chemical grounds, the exocyclic enone might be expected to account for most of the tumoricidal activity, as the

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exocyclic enone derived from COMC-6 is about 13-fold more reactive than COMC-6 with GSH (used as a model nucleophile).[11] However, within the confines of a highly compartmentalized tumor cell, reactivity alone might not be the only, or even the major, factor determining cytotoxicity.

To resolve this ambiguity, the relative toxicities of COMC-6 and COMC-7 were evaluated with MCF-7wt breast tumor cells versus MCF-7^{piGST} overexpressing GSTP1-1. We reasoned that if COMC is directly responsible for tumoricidal activity (assuming a steady-state kinetic model applies), overexpression of GST activity would protect tumor cells by lowering the steady-state concentration of intracellular COMC and, therefore, decrease the rate of covalent modification of biomacromolecules important to cell viability. Alternatively, if the COMC derivative *and* the exocyclic enone are equally toxic to tumor cells, overexpression of GST would have little effect on cytotoxicity. Finally, if the exocyclic enone is primarily responsible for antitumor activity, overexpression of GST activity would actually increase the cytotoxicity of the COMC derivative by increasing the steady-state concentration of the exocyclic enone ($[exo]_{piGST}$) in tumor cells overexpressing hGSTP1-1 versus wild-type tumor cells ($[exo]_{wt}$).

Since this general strategy is being explored by several different laboratories, it is instructive to note that if the last condition applies, the degree of tumor selective toxicity will equal the ratio of the net rates of formation of exocyclic enone in the two types of cells, wherein each net rate is equal to the sum of the nonenzymatic and enzymatic rates of formation of the exocyclic enone. The ratio of these two rates will give the ratio of the steady-state concentrations of the exocyclic enone in each tumor cell line

$$\frac{[exo]_{piGST}}{[exo]_{wt}} = \frac{k_1[GSH] + (k_{cat}/K_m^{comc})[piGST]_{piGST}}{k_1[GSH] + (k_{cat}/K_m^{comc})[piGST]_{wt}} \quad (1)$$

where $k_1[GSH]$ is the pseudo-first-order rate constant for the nonenzymatic formation of the exocyclic enone.

This equation follows from the minimum kinetic mechanisms that control the steady-state concentrations of exocyclic enone inside tumor cells. In the nonenzymatic case,

$$\text{COMC-X} \xrightarrow[\text{(-crotonate)}]{k_1[GSH]} [exo] \xrightarrow{k_2} \text{nucleophilic adducts} \quad (2)$$

where k_2 is the sum of the pseudo-first-order rate constants for reaction of the intermediate ([exo]) with all intracellular nucleophiles, including GSH. In the enzymatic case, a similar scheme applies,

$$\text{COMC-X} \xrightarrow[\text{(-crotonate)}]{k_{cat}/K_m^{comc}[piGST]} [exo] \xrightarrow{k_2} \text{nucleophilic adducts} \quad (3)$$

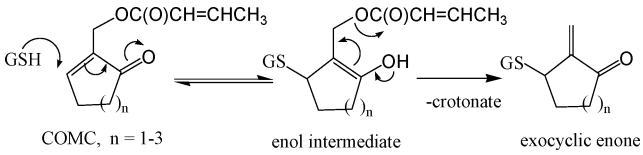
except that the kinetic properties of the enzyme control the rate of formation of [exo] in the forward direction. Under physiological conditions, the GSH binding site on GST is nearly saturated with GSH, as [GSH] equals about 2−8 mM in both normal cells and tumor cells[19,20]

Table 1. Observed Second-Order Rate Constants (k_1) for Reaction of GSH with COMC Derivatives and Their Kinetic Properties with GSTP1-1 (pH 6.5)[a]

derivative	k_1, mM^{-1} min^{-1}	$(k_{cat}/K_m^{comc}) \times 10^{-3}$, mM^{-1} min^{-1}
COMC-5	0.102 ± 0.006	1.0 ± 0.4
COMC-6	0.067 ± 0.003	1.4 ± 0.4
COMC-7	0.015 ± 0.002	1.0 ± 0.3

[a] Conditions: COMC-X (~0.05 mM), GSH (~1.0 mM), potassium phosphate buffer (0.1 M), pH 6.5, EDTA (0.05 mM), ethanol (0−5%), 25 °C.

Scheme 2



and, therefore, is $\gg K_m^{GSH}$ (~0.2 mM),[1] and [COMC] $\ll K_m^{comc}$. Under steady-state conditions,

$$\frac{d[exo]_{ss}}{dt} = \left\{k_1[GSH] + \frac{k_{cat}}{K_m}[GST]\right\}[COMC\text{-}X] - k_2[exo]_{ss} \approx 0 \quad (4)$$

Solving for $[exo]_{ss}$ for the two different cell types and dividing gives eq 1.

Thus, differential toxicity is greatest when the enzymatic rate of formation of the exocyclic enone greatly exceeds the nonenzymatic rate ($k_1[GSH] \ll (k_{cat}/K_m^{comc})[piGST]$. Under these conditions, the ratio $[exo]_{piGST}/[exo]_{wt}$ in eq 1 approaches the ratio of piGST activity in tumor cells overexpressing GSTP1-1 versus that in wild-type tumor cells. In contrast, if $k_1[GSH] \gg (k_{cat}/K_m^{comc})[piGST]$, $[exo]_{piGST}/[exo]_{wt}$ approaches unity and all discrimination is lost.

The COMC derivatives were selected for study, because there is a systematic change in the magnitudes of the observed second-order rate constants for reaction with GSH to give the exocyclic enones (k_1), but little change in the kinetic properties of the COMCs with GSTP1-1, Table 1.[11]

These trends can be rationalized in terms of a reaction mechanism involving an enol intermediate, Scheme 2.

In the nonenzymatic reaction, the decrease in k_1 with increasing ring size can be understood in terms of poorer orbital overlap in the enone-like transition states flanking the enol intermediate. Poorer orbital overlap could result from the increase in "ring-pucker" with increasing ring size. This explanation is consistent with the observation that the chemical shift of the ring vinyl proton of COMC-7 is least deshielded (δ 6.79 ppm), while that of the most reactive enone COMC-5 is most deshielded (δ 7.58 ppm).[11] The chemical shift of the ring vinyl proton of the enone having intermediate reactivity (COMC-6) is between these two chemical shifts (δ 6.98).

In the enzyme-catalyzed reaction, hGSTP1-1 has been proposed to catalyze the Michael addition reaction only, as the enol intermediate appears to dissociate from the active site prior to forming the exocyclic enone free in solution.[11] This tentative conclusion is based on the observation that crotonate undergoes stereorandom elimination from the enol intermediate during formation of the exocyclic enone. The fact that k_{cat}/K_m^{comc} is nearly

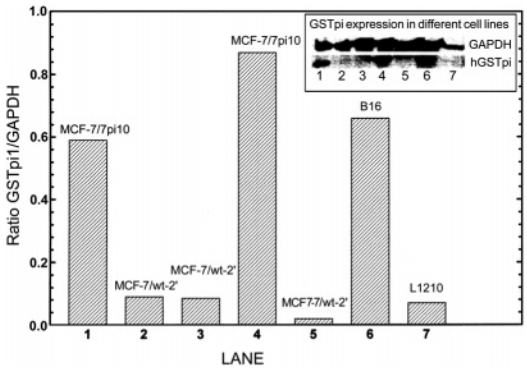


Figure 1. Relative concentrations of GST-pi in MCF-7/7pi vs MCF-7/wt.[22]

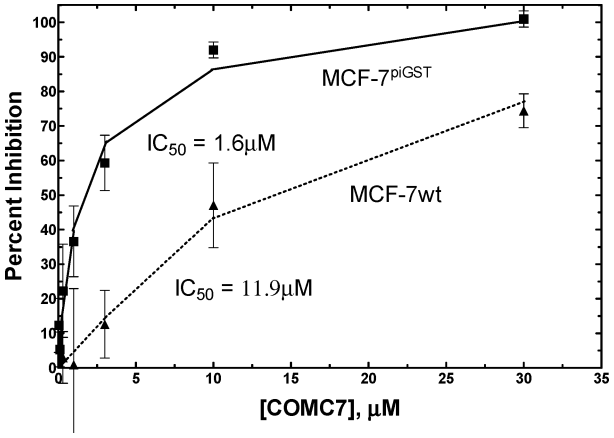


Figure 2. Dose−response curve for COMC7 with MCF-7^{piGST} versus MCF7wt.

invariant with ring size suggests that some step other than the Michael addition reaction is rate determining, possibly dissociation of the enol intermediate from the surface of the active site. Whatever the explanation, the substrate that should give the highest tumor selectivity should be COMC-7, given its relatively low reactivity with free GSH versus the kinetic efficiency with which it is converted to the exocyclic enone by the transferase (eq 1).

The influence of hGSTP1-1 concentration on the tumoricidal activities of COMC-6 and COMC-7 was evaluated using an MCF-7^{piGST} breast tumor cell line obtained from Charles Morrow, Wake Forest University.[21] These cells are stably transfected with piGST and, in our hands, were found to contain about 11-fold greater hGSTP1-1 protein than MCF-7wt, Figure 1.[22]

Indeed, COMC-6 and COMC-7 consistently show greater potency toward MCF-7^{piGST} than toward MCF-7wt (e.g., Figure 2). Therefore, these results are in accordance with the hypothesis that cytotoxicity is primarily derived from the exocyclic enone product of the piGST reaction and *not* the parent endocyclic enone.

In principle, the degree of discrimination between wild type and MCF-7^{piGST} tumors should be greatest for COMC-7 versus COMC-6, given the relative magnitudes of the kinetic constants for the enzymatic and nonenzymatic reactions (Table 2).

This assertion is based on eq 5,

$$\frac{[\text{exo}]_{\text{piGST}}}{[\text{exo}]_{\text{wt}}} = \frac{1 + R_{\text{piGST}}}{1 + R_{\text{wt}}} \qquad (5)$$

Table 2. Comparative IC_{50} Values for COMC-6 and COMC-7 with MCF-7wt versus MCF-7^{piGST} Overexpressing piGST Protein by a Factor of about 11[a]

cell line	COMC-6 (μM)	COMC-7 (μM)
MCF7wt	29.6 ± 7.0	10.8 ± 1.1
MCF7^{piGST}	13.2 ± 1.3	2.6 ± 1.0
ratio: (MCF7wt/MCF7^{piGST})	2.2 ± 0.6	4.2 ± 1.6

[a] Average (±SD) of three independent experiments, each carried out in triplicate.

which is a rearranged form of eq 1, where

$$R_{\text{piGST}} = (k_{\text{cat}}/K_{\text{m}}^{\text{comc}})[\text{piGST}]_{\text{piGST}}/k_1[\text{GSH}]$$

$$R_{\text{wt}} = (k_{\text{cat}}/K_{\text{m}}^{\text{comc}})[\text{piGST}]_{\text{wt}}/k_1[\text{GSH}].$$

For COMC-7, $[\text{exo}]_{\text{piGST}}/[\text{exo}]_{\text{wt}} \approx 4.2$ (Table 2) and $R_{\text{piGST}} \approx 11\, R_{\text{wt}}$, given that $[\text{piGST}]_{\text{piGST}}/[\text{piGST}]_{\text{wt}} \approx 11$ (Figure 1). Combining these relationships with eq 5 shows that for the cells transfected with piGST the enzymatic rate exceeds the nonenzymatic rate by a factor of about five: $R_{\text{piGST}} \approx 5.2$. For wild type cells, the nonenzymatic rate slightly exceeds the enzymatic rate: $R_{\text{wt}} \approx 0.47$. The predicted discrimination ($[\text{exo}]_{\text{piGST}}/[\text{exo}]_{\text{wt}}$) for COMC-6 can be calculated with eq 5, after multiplying R_{piGST} and R_{wt} for COMC-7 by the ratio of the second-order rate constants for the nonenzymatic reactions of COMC-6 and COMC-7 with GSH; i.e., $k_1^{\text{comc}-6}/k_1^{\text{comc}-7} = 0.224$. This gives $[\text{exo}]_{\text{piGST}}/[\text{exo}]_{\text{wt}} \approx 2.0$, which closely agrees with the experimental value of 2.2 ± 0.6 (Table 2). While the theoretical and experimental values agree within experimental error, it must be emphasized that a comparison of the ratios for COMC-6 and COMC-7 do not provide a rigorous test of the underlying theory, as the errors associated with the ratios are overlaping. Nevertheless, the above analysis provides a general framework for evaluating prodrugs subject to enzyme-catalyzed modifications.

The explanation for why COMC-7 is more potent than COMC-6 to MCF-7 cells is not clear, but could be related to the greater hydrophobicity of COMC-7 versus COMC-6. A similar trend was noted for murine melanotic melanoma B16 cells in vitro in which potency increases with ring size.[18] Alternatively, increasing toxicity with increasing ring size might also reflect the increase in the chemical stabilities of the COMC derivative and the corresponding exocyclic enone, which would allow more time for the exocyclic enones to reach the nucleus of the cell (covalently modifying DNA) before reacting with cytosolic GSH.

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(22) Approximately 40 μg of protein was loaded into each lane containing cell lines. Lane 1 contains the earliest passage in culture of the MCF-7/7pi stably transfected cell line (culture passage 2). Since the parental cell line, MCF-7/wt, grows more rapidly in culture, more cells were available from culture passage 2 for this cell line and the lysate from this cell line is presented in Lanes 2 and 3. Lane 4 is a later culture passage, passage 9, for the MCF-7/7pi to ensure that GST-pi is still expressed. Lane 5 is passage 11 for the parental line, MCF-7/wt, harvested on the same day as MCF-7/7pi passage 9. Lane 6 is the murine cell line, B16 melanoma, which has high concentrations of murine GST-pi. Lane 7 contains L1210 murine leukemia cells, which have low concentrations of murine GST-pi. Lane 8 contains the purified human GST-pi, which is the positive control. The later culture passages of MCF-7/7pi and MCF-7/wt were included to confirm that the expression of the stably transfected GST-pi does not change with passage. The murine cell lines were included to check for cross-reactivity of the antibodies and to confirm the concentrations of murine GST-pi in these cell lines. GAPDH, glyceraldehyde-3-phosphate dehydrogenase, served as the loading control for all cell lines. GAPDH is constitutively expressed in almost all tissues at high levels. Therefore, it is a useful marker when a loading/positive control is required in western blotting. The activity of GST-pi in each cell line was expressed as the ratio of GST-pi to GAPDH. Anti-human glutathione S-transferease P1-1 was obtained from Oxford Biomedical Research (antibody GS 72, Oxford, MI) and used at a dilution of 1/1000. Anti-GAPDH was obtained from Chemicom International (MAB374, Temecula, CA). Western blot method: Cell pellets were lysed in 10 vol of lysis buffer (50 mM Tris-HCl (pH 7.9) 100 mM NaCl, 1% NP-40m 2mM EDTA, 100mM NaF) containing fresh protein inhibitors (1 μg/mL pepstatin, 10 μg/mL aprotin, 5 μg/mL leupeptin, 5 mM PMSF, 0.1 μM microcystin, and 5 mM Na pyrophosphate). The tissue lysate was centrifuged at 13000*g* for 10 min. The supernatant was collected and protein concentration determined using a Bio-Rad protein assay system (Bio-Rad, Hercules, CA). Concentrations of bovine serum albumin between 0.05 and 0.5 mg/mL were used to obtain the calibration standard curve. Equal amounts of protein (40 μg) were denatured in 3X modified Laemmli sample buffer (Bio-Rad) and loaded on 4−15% gradient gels (Bio-Rad). The separated proteins were transferred to PVDF membranes and blotted with 5% nonfat milk in TBS for 1 h. The membranes were incubated with antibodies against GST-pi or GAPDH overnight. The immunoreactive signals were detected by ELC detection reagents (PerkinElmer Life Sciences, Boston, MA) following the manufacturer's instructions. The densities of the signals were quantified by densitometry with UN-SCAN-IT (Silk Scientific, Orem, UT).

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