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EFFECT OF CARBAMATE THIOESTER DERIVATIVES OF METHYL- AND 2-CHLOROETHYL ISOCYANATE ON GLUTATHIONE LEVELS AND GLUTATHIONE REDUCTASE ACTIVITY IN ISOLATED RAT HEPATOCYTES*

KELEM KASSAHUN, CLAUDIA M. JOCHHEIM and THOMAS A. BAILLIE†

Department of Medicinal Chemistry, School of Pharmacy, University of Washington, Seattle, WA 98195, U.S.A.

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Abstract—The present study examined the effects of *S*-(*N*-methylcarbamoyl)glutathione (SMG), *S*-(*N*-methylcarbamoyl)-L-cysteine (L-SMC) and some analogs of these *S*-linked conjugates of methyl isocyanate (MIC) on the activity of glutathione reductase (GR) in freshly isolated rat hepatocytes and on the levels of reduced and oxidized glutathione (GSH and GSSG) in exposed cells. Both SMG and its monoethyl ester (0.5 mM) were found to inhibit GR weakly, although L-SMC proved to be an effective inhibitor of the enzyme (60 ± 4% activity remaining after a 4-hr incubation at 0.5 mM). The cysteine adduct (SCC) of 2-chloroethyl isocyanate (CEIC) was a strong inhibitor of GR (27 ± 1% activity remaining after a 1-hr incubation at 0.1 mM) and was essentially equipotent with the antitumor agent *N*,*N*′-bis(2-chloroethyl)-*N*-nitrosourea (BCNU). L-SMC depleted intracellular GSH in a time- and concentration-dependent manner up to 2 hr of incubation, beyond which time GSH levels began to recover. Exposure of cells to the enantiomeric conjugate, D-SMC, led to a similar concentration- and time-dependent inhibition of GR and fall in intracellular GSH, but in this case the depletion of GSH was extensive and was sustained throughout the 5-hr incubation period. Only a small amount (less than 10%) of the GSH that was lost from cells exposed to SMC was recovered in the medium, indicating that SMC did not cause efflux of GSH (most of the free cysteine released during breakdown of SMC was recovered in the medium). Experiments with hepatocytes exposed for 5 hr to SCC (0.1 mM) demonstrated that GSSG levels were elevated by 32 ± 5% relative to controls. Collectively, these results indicate that carbamate thioester conjugates of MIC and CEIC inhibit GR, probably via release of the free isocyanate at the cell surface, which then penetrates the hepatocyte. The inhibitory effects of the isocyanates on GR, coupled with their propensity to react spontaneously with GSH, combine to deplete significantly intracellular stores of GSH.

Key words: glutathione; glutathione reductase; carbamate thioesters; enzyme inhibition; isolated rat hepatocytes

Conjugation of reactive, electrophilic intermediates with GSH‡ normally represents a metabolic detoxification process, inasmuch as the resulting *S*-linked adducts are excreted into bile or, following biotransformation to the corresponding cysteine or *N*-acetylcysteine conjugates, are eliminated in urine [1]. In certain cases, however, the products that derive from this pathway are, or undergo transformation to, chemically reactive species, and therefore remain potentially toxic [2]. One example of this phenomenon occurs where the conjugation process is reversible under physiological conditions, such that the *S*-linked conjugate exists in equilibrium with the reactive intermediate from which it was derived. Organic isocyanates fall into this category, as illustrated by MIC, which reacts with GSH to form SMG and with cysteine to give SMC [3, 4]. These carbamate thioester conjugates, whose structures are shown in Fig. 1, have been shown to be reactive carbamoylating agents *in vitro* by virtue of their ability to transfer the elements of MIC to nucleophilic functional groups on peptides and proteins [5]. Effectively, therefore, SMG and SMC serve as latent forms of MIC and thus they may be considered as vehicles for the transport of this reactive, toxic isocyanate *in vivo* [6]. Other isocyanates behave in a similar fashion, such as *N*-(1-methyl-3,3-diphenylpropyl)isocyanate, which is formed as a metabolite of *N*-(1-methyl-3,3-diphenylpropyl)formamide in the rat [7], and CEIC, generated by chemical breakdown of the antitumor drug BCNU [8].

In light of the carbamoylating activity of *S*-linked

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† Corresponding author: Dr. Thomas A. Baillie, Department of Medicinal Chemistry, School of Pharmacy, BG-20, University of Washington, Seattle, WA 98195. Tel. (206) 685-2468; FAX (206) 685-3252.

‡ Abbreviations: GSH, glutathione; GSSG, glutathione disulfide; GR, glutathione reductase; SMG, *S*-(*N*-methylcarbamoyl)glutathione; SMC, *S*-(*N*-methylcarbamoyl)-cysteine; SCC, *S*-(*N*-[2-chloroethyl]carbamoyl)cysteine; MIC, methyl isocyanate; CEIC, 2-chloroethyl isocyanate; BCNU, *N*,*N*′-bis(2-chloroethyl)-*N*-nitrosourea; CCNU, *N*-(2-chloroethyl)-*N*′-cyclohexyl-*N*-nitrosourea; NMF, *N*-methylformamide; and MS/MS, tandem mass spectrometry.

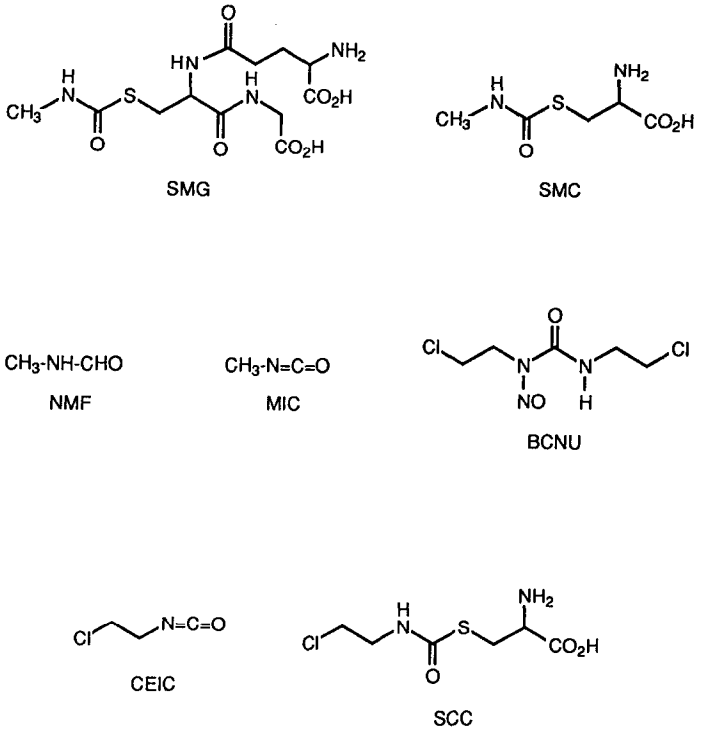

Fig. 1. Structures of compounds referred to in the text.

conjugates of isocyanates, it may be anticipated that this family of carbamate thioesters will exhibit a variety of biological effects. Indeed, it has been reported that SMG is cytotoxic toward isolated mouse hepatocytes [9], inhibits the growth of murine TLX5 lymphoma cells in culture [9], and is toxic to mouse embryos in culture [10]. Recently, we have shown that both the glutathione and *N*-acetylcysteine conjugates of CEIC act as potent inhibitors of rat liver GR *in vitro*, probably due to carbamoylation of the enzyme by CEIC [8]. Since GR plays a key role in maintaining GSH homeostasis through the reduction of GSSG to the corresponding thiol, it represents an important component of the antioxidant defense mechanism of the cell [11, 12] and has been considered as a potential target for novel chemotherapeutic agents. In this connection, we recently investigated the effects of SMG and SMC on purified yeast GR in a cell-free system and found that both conjugates inhibit the enzyme [13]. Moreover, mechanistic studies suggested that the inhibition is selective and irreversible and results from carbamoylation of a sulfhydryl group at the active site of the enzyme [13]. The present investigation, which represents an extension of these studies, was designed to evaluate the inhibitory properties of SMG and SMC toward GR in an intact mammalian cell system and to determine the associated effects on intracellular GSH levels. Freshly isolated rat hepatocytes were selected for this purpose, and the inhibitory properties of SMG and SMC were compared with those of SCC, the cysteine conjugate of CEIC.

MATERIALS AND METHODS

Chemicals. The following compounds were supplied by the Aldrich Chemical Co. (Milwaukee, WI): CEIC, MIC, and L- and D-cysteine. Baker's yeast GR (EC 1.6.4.2), NADPH, GSH and GSSG were purchased from the Sigma Chemical Co. (St. Louis, MO). Units of activity for the GR were reported by the manufacturer as follows: 1.0 U catalyses the reduction of 1.0 μmol of GS-SCoA/min at 37° and pH 7.4. The carbamate thioester conjugates SMG, SMC and SCC were obtained by synthesis, as outlined below. Monobromobimane was supplied by Calbiochem (La Jolla, CA), while BCNU was purchased from Bristol Laboratories (Evansville, IN). Collagenase (from *Clostridium histolyticum*) was obtained from the Worthington Biochemical Corp. (Freehold, NJ).

SMG and the L-enantiomer of SMC were prepared by adding MIC (dissolved in acetone) to a solution of the corresponding thiol in aqueous acetonitrile [9]. The D-enantiomer of SMC was obtained in a similar fashion, using D-cysteine in place of L-cysteine, and the product afforded NMR and mass spectra that were essentially identical to those of L-SMC [9]. The monoethyl ester of SMG was prepared by bubbling dry HCl gas (generated from H_2SO_4 and NaCl) through a solution of SMG in ethanol. Analysis of the resulting ester by ionspray MS gave an abundant ion at m/z 393 (MH^+), collision-induced dissociation of which in an MS/MS experiment afforded product ions at m/z 207 ([ethyl cysteinylglycine + H]$^+$) and 290 ([MH − ethyl

glycine]$^+$). On the basis of these product ions, it was concluded that the site of esterification was the glycine residue, the carboxyl group of which typically is the more reactive of the two carboxylic acid moieties in GSH conjugates [9].

SCC was synthesized as follows. L-Cysteine (121 mg, 1 mmol) was dissolved in a mixture of acetonitrile and 1% HCl (7:3, v/v) and treated dropwise with CEIC (102 μL, 1.2 mmol) in acetone (1 mL). The resulting mixture was stirred for 2.5 hr at room temperature, the solvent was removed *in vacuo*, and the product (85% crude yield) was purified by HPLC. The latter was performed on a semi-preparative C_{18} column (250 mm × 10 mm i.d.; 5 μm), eluted with aqueous acetonitrile (83:17, v/v; pH 3.3) at a flow rate of 3.0 mL/min. The identity of the product was confirmed by NMR and ionspray MS analysis. NMR: (δ) 3.38–3.46 (m, 2H, Cys-β), 3.57–3.70 (m, 4H, Cl-CH_2-CH_2-) and 4.26 (dd, 1H, J = 3.87 and 8.15 Hz, Cys-α). MS: m/z 227 (75%; MH^+, ^{35}Cl), 229 (29%; MH^+, ^{37}Cl) and 122 (72%; [Cys + H]$^+$).

Instrumentation. Proton NMR spectra were obtained (in D_2O) at 300 MHz on a VXR-300 (Varian Associates, Palo Alto, CA) spectrometer. Chemical shifts are reported in ppm (δ) relative to residual H_2O (δ 4.67). Mass spectrometry was performed on a Perkin Elmer-Sciex API III triple quadrupole mass spectrometer equipped with an atmospheric pressure ion source and an ionspray interface maintained at 5 kV. Samples were dissolved in 50% aqueous acetonitrile containing 0.06% trifluoroacetic acid and were introduced into the instrument by direct infusion. MS/MS experiments were performed as described previously [8]. HPLC analyses were carried out on a Shimadzu LC-600 liquid chromatograph equipped with an analytical C_{18} column (250 mm × 4.6 mm i.d.) and an HP 1046A fluorescence detector.

Determination of glutathione reductase activity in isolated hepatocytes. Hepatocytes were isolated from male Sprague–Dawley rats (240–280 g) by collagenase perfusion, according to the procedure outlined by Moldéus *et al.* [14]. Initial cell viability, as measured by Trypan Blue exclusion, typically was >85%, and decreased by no more than 10% during a 5-hr incubation. Cells were incubated, at a concentration of 2×10^6 cells/mL, in Krebs–Henseleit buffer (pH 7.4) supplemented with HEPES (final concn 12.6 mM). Incubations were carried out at 37° in rotating 50-mL round-bottom flasks under an atmosphere of 95% O_2/5% CO_2. Stock solutions of test compounds in saline were prepared freshly before each experiment, and aliquots were added to the hepatocyte suspension to give the desired concentration in a final incubation volume of 10 mL. In the case of SMG and SMC, this concentration was 0.5 mM, whereas for SCC a value of 0.1 mM was employed since significant cell kill occurred at 0.5 mM. Aliquots (0.5 mL) of cell suspension were removed at intervals over 5 hr and disrupted by sonication with a micro-tip ultrasonic homogenizer (20 kHz, 3 sec). Following centrifugation, aliquots (0.1 mL) of the resulting supernatant were added to a solution of GSSG (0.50 mM) in 50 mM potassium phosphate buffer (pH 7.2) for assay of GR activity [13, 15]. The reaction was initiated by the addition of NADPH (0.20 mM) to give a final volume of 1.0 mL, and the rate of consumption of this cofactor was measured spectrophotometrically at 340 nm. Control GR activity did not decrease significantly up to 5 hr of incubation.

Measurement of GSH and cysteine in hepatocyte incubations. Aliquots (0.5 mL) of cell suspensions were removed at intervals from incubation medium and were pelleted by rapid centrifugation (5700 *g* for 5 sec on a bench-top centrifuge). In each case, the pellet was treated with water (0.5 mL), the resulting suspension was sonicated (as described above) to disrupt the cells, and the product was homogenized. Portions (0.1 mL) of these homogenates were treated with a stock solution of monobromobimane (10 mM, 0.1 mL), proteins were precipitated with 50% aqueous trichloroacetic acid (10 μL), and aliquots (20 μL) of the resulting solutions were subjected to HPLC analysis with fluorimetric detection for quantitative determination of intracellular GSH and cysteine [16]. A second sample of the cell suspension was centrifuged, as above, and an aliquot (0.1 mL) of the supernatant was derivatized directly with monobromobimane for assay of extracellular GSH and cysteine.

Measurement of GSSG in hepatocyte incubations. Aliquots (0.5 mL) of hepatocyte suspensions were sonicated as before to disrupt the cells. One portion (0.1 mL) was treated, in turn, with solutions of NADPH (50 mM, 0.1 mL) and GR (1.8 U, 10 μL), and the resulting mixture was incubated at 20° for 10 min to reduce the GSSG to GSH. After the addition of monobromobimane solution (2.7 mg/mL, 0.1 mL), the reaction mixture was incubated in the dark for an additional 10 min. Following precipitation of protein with 50% aqueous trichloroacetic acid (10 μL), aliquots (20 μL) of the supernatant were taken for analysis by HPLC with fluorimetric detection, as described by Cotgreave and Moldéus [16] to determine the "total" glutathione content. A second aliquot (0.1 mL) of the disrupted cells was treated with 50 mM potassium phosphate buffer (pH 7.2; 110 μL) in place of NADPH solution, and allowed to react directly with monobromobimane, as above, in order to determine the "free" GSH content. The difference between the values for "total" and "free" GSH were taken as a measure of the concentration of GSSG.

Statistical analysis. Statistical differences between treatment groups and controls (hepatocytes treated with saline) were evaluated using Student's t-test. The level of significance was set at $P < 0.05$. Results are expressed as means ± SEM for three separate hepatocyte preparations.

RESULTS

Incubation of hepatocytes for 5 hr with SMC, SMG and ethyl SMG at concentrations of up to 1.0 mM did not cause significant cytotoxicity, as measured by Trypan Blue exclusion. Similarly, SCC at a level of 0.1 mM did not result in significant cell kill over this time interval. As shown in Fig. 2, both SMG and its monoethyl ester (0.5 mM) inhibited rat liver GR in a time-dependent manner such that the

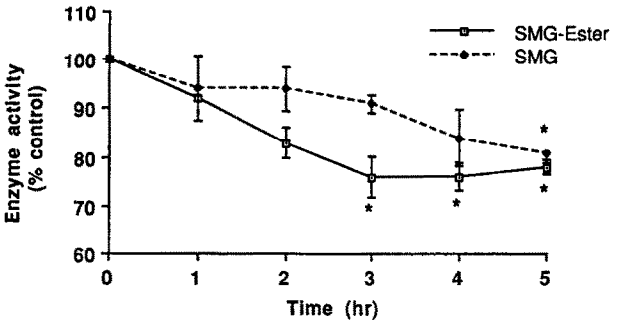

Fig. 2. Time-dependent inhibition of GR in isolated rat hepatocytes by carbamate thioesters. Hepatocytes were incubated with 0.5 mM each of SMG and its monoethyl ester, and enzyme activity was determined at the intervals shown. Results are the means ± SEM of three experiments. Key: (*) significantly different ($P < 0.05$) from the corresponding control value. GR activity in control hepatocytes was 788 ± 24 nmol GSH formed/min/10^6 cells (mean ± SEM, N = 4).

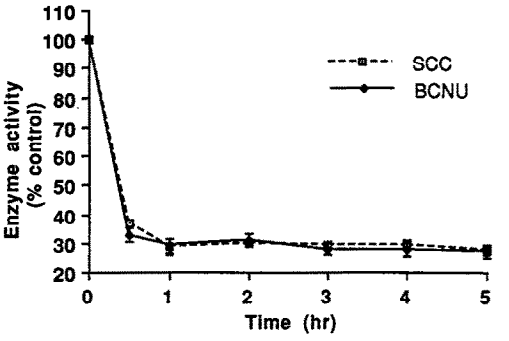

Fig. 4. Time-dependent inhibition of GR in isolated rat hepatocytes by BCNU and SCC. Cells were incubated with 0.1 mM each of BCNU and SCC, and GR activity was determined as described in the text. Results are the means ± SEM of three experiments. All time points from 30 min onwards were significantly different from control values ($P < 0.05$), although the corresponding values for BCNU and SCC did not differ from one another. GR activity in control hepatocytes was 788 ± 24 nmol GSH formed/min/10^6 cells (mean ± SEM, N = 4).

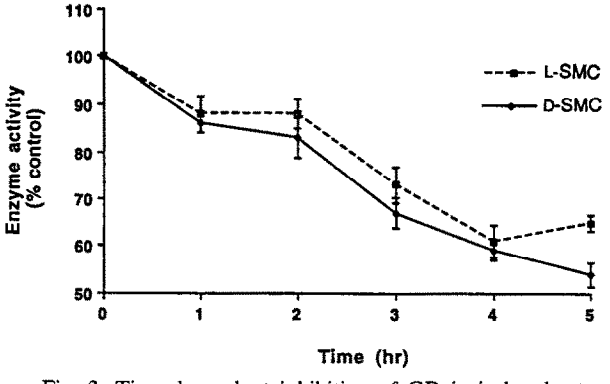

Fig. 3. Time-dependent inhibition of GR in isolated rat hepatocytes by the cysteine conjugates, L-SMC and D-SMC (0.5 mM). Results are the means ± SEM of three experiments. With the exception of the 1-hr time point for L-SMC, all values were statistically different from their respective controls ($P < 0.05$). However, the enantiomers did not differ from one another in potency ($P > 0.05$). GR activity in control hepatocytes was 788 ± 24 nmol GSH formed/min/10^6 cells (mean ± SEM, N = 4).

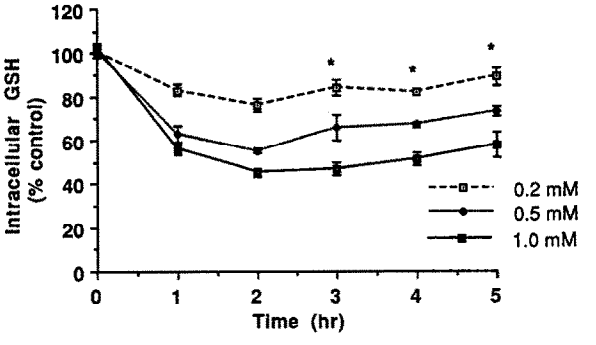

Fig. 5. Time-dependent depletion of GSH in isolated hepatocytes exposed to L-SMC. Hepatocytes were incubated with the indicated concentrations of L-SMC, and the intracellular concentration of GSH was determined by HPLC with fluorescence detection. Results are the means ± SEM of three experiments. All values were significantly different from the corresponding controls ($P < 0.05$) with the exception of those indicated with an asterisk. Glutathione levels in control hepatocytes at time zero were 42.4 ± 8.4 nmol/10^6 cells (mean ± SEM, N = 5).

activity remaining after 5 hr was *ca.* 80% of control values. The effects of ethyl SMG were more marked than those of SMG at earlier time points, although the difference between the two compounds was not significant after 5 hr of incubation. L-SMC (0.5 mM) proved to be a more effective inhibitor of the enzyme than these GSH conjugates, causing a decrease in GR activity to *ca.* 60% of control values after 4 hr of incubation (Fig. 3). Similar results were obtained with the D-isomer of this cysteine conjugate (Fig. 3). At a concentration of 0.1 mM, SCC and BCNU proved to be potent inhibitors of rat liver GR in that their maximum effect (GR activity decreased to 27% of control values) was evident as early as the 1-hr time point and was maintained throughout the 5-hr study (Fig. 4). SCC and BCNU (0.1 mM) were essentially equipotent as inhibitors of GR, consistent with their proposed function as latent forms of the active inhibitor, CEIC.

In light of the ability of the above conjugates to inhibit GR in isolated rat hepatocytes, it became of interest to determine their effects on free GSH levels in exposed cells. In the case of SMG and ethyl SMG, both compounds were found to decompose slowly

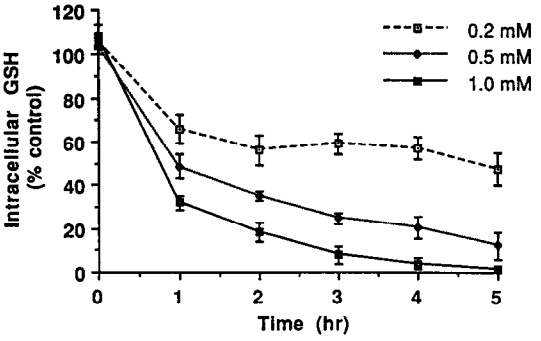

Fig. 6. Time-dependent depletion of GSH in isolated hepatocytes exposed to D-SMC. Hepatocytes were incubated with the indicated concentrations of D-SMC, and the intracellular concentration of GSH was determined by HPLC with fluorescence detection. Results are the means ± SEM of three experiments. All values were significantly different from the corresponding controls ($P < 0.05$). Glutathione levels in control hepatocytes at time zero were 42.4 ± 8.4 nmol/10^6 cells (mean ± SEM, N = 5).

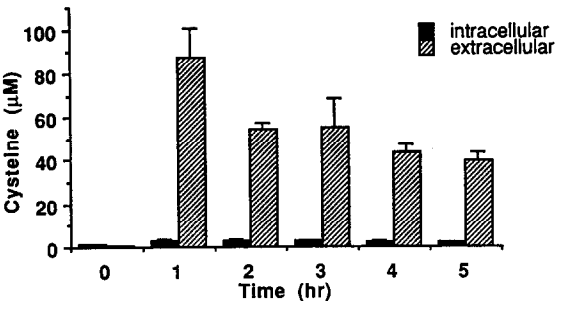

Fig. 8. Release of cysteine in incubations of isolated hepatocytes with D-SMC (0.5 mM). The cysteine was quantified by HPLC in both the pellet ("intracellular" fraction) and supernatant ("extracellular" fraction) obtained by rapid centrifugation of hepatocyte suspensions. The values at time zero correspond to the normal concentrations of L-cysteine in hepatocyte preparations, while the increased levels at all subsequent sampling times are attributed to D-cysteine liberated from D-SMC. Results are the means ± SEM of three experiments.

to free GSH in aqueous medium at pH 7.4, and therefore it was not possible to discern the effects of the inhibitors on *endogenous* GSH levels. However, when cells were exposed to L-SMC (which also decomposes in aqueous solution, although to L-cysteine), concentrations of intracellular GSH fell in a time- and concentration-dependent fashion during the first 2 hr of incubation, beyond which point GSH levels increased (Fig. 5). The possibility that the observed recovery of intracellular GSH was due to its *de novo* biosynthesis from the L-cysteine liberated during decomposition of L-SMC was tested by studying the effects of the enantiomeric conjugate, D-SMC. Exposure of cells to the latter isomer led, once again, to a time- and concentration-dependent decrease in intracellular GSH (Fig. 6). However, GSH levels did not recover in these experiments, but continued to fall throughout the incubation period. Indeed, at the highest concentration examined (1.0 mM), D-SMC depleted intracellular GSH to barely detectable levels at the 5-hr time point (Fig. 6). SCC (0.1 mM) also depleted intracellular GSH stores (Fig. 7) but did not affect extracellular GSH levels (data not shown). This indicated that SCC did not cause significant efflux of GSH from cells.

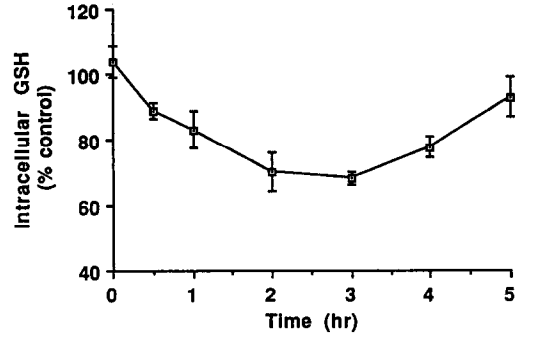

Fig. 7. Time-dependent depletion of intracellular GSH in hepatocytes exposed to SCC (0.1 mM). Results are the means ± SEM of three experiments. Glutathione levels in control hepatocytes at time zero were 42.4 ± 8.4 nmol/10^6 cells (mean ± SEM, N = 5).

As noted above, incubation of either enantiomer of SMC with hepatocytes was accompanied by the release of free cysteine, and this is shown for the case of D-SMC in Fig. 8. Greater than 95% of the liberated cysteine was found to be present in the extracellular medium, where highest concentrations were reached after 1 hr. Interestingly, less than 10% of the GSH that was lost from hepatocytes incubated with the enantiomers of SMC was recovered in the extracellular medium (even at the 1 mM concentration), indicating that SMC did not cause extensive efflux of GSH. Therefore, the observed loss of intracellular GSH in hepatocytes exposed to SMC was viewed as being due to a combination of two factors, namely (i) oxidation of GSH to GSSG, which could not be recycled effectively to GSH by the impaired GR, and (ii) conjugation of GSH with MIC to generate SMG. In a preliminary experiment, GSSG levels were determined in cells exposed to SCC (0.1 mM) for 5 hr, when it was found that the combined intracellular and extracellular concentrations of the disulfide were 32 ± 5% higher (mean ± SEM, N = 3) than those in corresponding control incubations where the inhibitor was absent. Since this increase in GSSG is much less than stoichiometric with the corresponding fall in GSH concentrations, it seems likely that consumption of GSH through conjugation with CEIC (and possibly also protein *S*-thiolation) must account for the bulk of the observed GSH loss.

DISCUSSION

The present *in vitro* study has demonstrated that two carbamate thioester conjugates of MIC, viz. SMG and SMC, inhibit rat liver GR in a time-dependent manner. SMG was a relatively weak inhibitor of the enzyme, as was its lipophilic ethyl ester derivative [17, 18], although SMC proved to be considerably more potent than either of the GSH conjugates. The corresponding cysteine adduct of CEIC, viz. SCC, was the most effective inhibitor of the series, being equipotent with BCNU, the classical inhibitor of this enzyme [19]. A similar rank order was observed in our recent studies on the inhibition of purified yeast GR by SMG, SMC and BCNU [13], and thus the results from the present study with mammalian tissue are corroborated by those from the cell-free system.

Studies on the mechanism of action of GR traditionally have made extensive use of the nitrosourea antitumor agent BCNU, which has found widespread use as a potent and selective inhibitor of the enzyme and which has been employed as a tool to investigate the effects of GR on cellular function [15, 20]. A number of lines of evidence indicate that the active inhibitory species derived from BCNU is the isocyanate CEIC [8, 19] which carbamoylates a key sulfhydryl group at the active site of the enzyme [21]. Both SMG and SMC have been shown to act as carbamoylating agents *in vitro*, inasmuch as the elements of MIC are transferred selectively from these conjugates to sulfhydryl groups on peptides and proteins [5], and therefore it is likely that SMG and SMC inhibit GR by a directly analogous mechanism [13]. Transfer of the elements of MIC from SMG and SMC would be expected to result in the liberation of free GSH and cysteine, respectively, and studies on the chemical stability of these conjugates in aqueous medium have verified that this is indeed the case [13]. In the present study, incubation of SMC with isolated rat hepatocytes again led to the release of free cysteine, which was found almost exclusively in the medium, as opposed to the intracellular compartment. This finding suggests that SMG and SMC act via the extracellular release of MIC, which penetrates the cell either as the free isocyanate (whose half-life in neutral aqueous solution has been estimated at 2–5 min [22]), or via labile conjugates with sulfhydryl groups on membrane proteins. Once inside the cell, MIC carbamoylates, and thereby inhibits, GR and other susceptible enzymes. According to this view, the enhanced inhibitory potency of SCC relative to that of SMG or SMC could be due to the higher lipophilicity of the isocyanate (CEIC) released from this conjugate, resulting in more effective interaction with the active site thiol of GR. In this connection, it should also be noted that the GSH conjugate of CEIC has been reported to exhibit *alkylating*, as well as carbamoylating activity [23]. It is possible, therefore, that after GR becomes carbamoylated by CEIC, intramolecular cross-linking occurs to afford a more extensively modified (and thus more severely inhibited) enzyme. Interestingly, this hypothesis would be consistent with X-ray crystallographic data on the BCNU-inhibited human erythrocyte GR, which failed to detect the presence of a chlorine atom in the putative *S*-(2-chloroethyl)carbamoyl cysteine residue at the active site [21].

Based on the relatively potent inhibitory effects of SMC on rat liver GR, it was anticipated that intracellular GSH levels would fall upon exposure of hepatocytes to this carbamate thioester conjugate. This was indeed found to be the case, although the depletion of GSH proved to be only transient in nature. On the premise that the observed rebound in intracellular GSH concentrations following treatment of hepatocytes with L-SMC reflected *de novo* biosynthesis of GSH from the liberated L-cysteine (a portion of which will enter the cell [24]), the corresponding adduct with D-cysteine was investigated since this enantiomer of the amino acid is also taken up into hepatocytes [24] but is not incorporated into GSH and does not influence GSH levels [25]. Consistent with the above hypothesis, D-SMC inhibited GR and caused extensive and sustained depletion of GSH in exposed hepatocytes. Moreover, this conjugate-induced loss of intracellular GSH proved not to be the result of simple export of GSH from cells, and therefore the mechanism by which SMC and related *S*-linked isocyanate adducts deplete GSH pools differs from that of compounds such as α-naphthylisothiocyanate [26].

Several compounds with anticancer activity appear to yield isocyanates *in vivo*, either due to chemical decomposition, metabolic transformation, or to some combination of these processes. For example, caracemide, an experimental antitumor agent that contains *N*-methylcarbamate and *N*-methylurea functionalities, has been shown to produce the *N*-acetylcysteine conjugate of MIC in rats [27]. BCNU, the prototype chloroethylnitrosourea, affords the GSH and *N*-acetylcysteine adducts of CEIC in bile and urine, respectively [8], while CCNU [28] and fotemustine [29] yield similar conjugates of cyclohexyl- and 1-(diethylphosphono)ethyl isocyanate, respectively. NMF, which displays antitumor activity in animal models, undergoes cytochrome P450-mediated metabolic oxidation to MIC [30] which, in turn, is excreted in bile in the form of SMG [31] and in urine as the corresponding *N*-acetylcysteine conjugate [32]. In the case of both BCNU [33] and NMF [34], administration to rodents has been reported to cause significant decreases in hepatic GSH levels, and this effect has been attributed to the consumption of GSH through conjugation with reactive metabolites of these drugs (most likely the isocyanates CEIC and MIC, respectively). The results of the present study, however, raise the possibility that this depletion may be exacerbated by the inhibitory effects of *S*-linked metabolites of the isocyanates on GR.

Finally, it should be noted that BCNU and other carbamoylating nitrosoureas with GR inhibitory properties have been proposed as potential radiosensitizers for use in the treatment of radiation-resistant tumors [35, 36]. In view of the present finding that SCC, the cysteine conjugate of CEIC, is equipotent to BCNU as an inhibitor of GR *in vitro*, it would be interesting to assess the utility of SCC as a radiosynergist, particularly since SCC is stable as a solid, is quite water-soluble, and should

be less difficult to formulate than BCNU. It is also noteworthy that two other carbamate thioester conjugates with cysteine, namely ethyl *S*-(*N*-methylcarbamoyl)cysteine [37] and *S*-(*N*-ethylcarbamoyl)cysteine [38], have been reported to exhibit antitumor activity *in vivo*, and it is tempting to speculate that spontaneous release of MIC and ethyl isocyanate, respectively, may contribute to the oncolytic effects of these experimental agents. However, regardless of the mechanism of their antitumor activity, the results of the present study suggest that these compounds will serve as effective inhibitors of GR *in vivo*.

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