Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical Pharmacology 69 (2005) 1463–1472
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Differential inhibition of cellular glutathione reductase activity by isocyanates generated from the antitumor prodrugs Cloretazine™ and BCNU

Kevin P. Rice, Philip G. Penketh, Krishnamurthy Shyam, Alan C. Sartorelli *

Department of Pharmacology and Developmental Therapeutics Program, Cancer Center, Yale University School of Medicine, New Haven, CT 06520, USA

Received 23 December 2004; accepted 11 February 2005

Abstract

The antitumor, DNA-alkylating agent 1,3-bis[2-chloroethyl]-2-nitrosourea (BCNU; Carmustine), which generates 2-chloroethyl isocyanate upon decomposition in situ, inhibits cellular glutathione reductase (GR; EC 1.8.1.7) activity by up to 90% at pharmacological doses. GR is susceptible to attack from exogenous electrophiles, particularly carbamoylation from alkyl isocyanates, rendering the enzyme unable to catalyze the reduction of oxidized glutathione. Evidence implicates inhibition of GR as a cause of the pulmonary toxicity often seen in high-dose BCNU-treated animals and human cancer patients. Herein we demonstrate that the prodrug Cloretazine™ (1,2-bis[methylsulfonyl]-1-[2-chloroethyl]-2-[(methylamino)carbonyl]hydrazine; VNP40101M), which yields methyl isocyanate and chloroethylating species upon activation, did not produce similar inhibition of cellular GR activity, despite BCNU and Cloretazine™ being equally potent inhibitors of purified human GR (IC_{50} values of 55.5 μM and 54.6 μM, respectively). Human erythrocytes, following exposure to 50 μM BCNU for 1 h at 37 °C, had an 84% decrease in GR activity, whereas 50 μM Cloretazine™ caused less than 1% inhibition under the same conditions. Similar results were found using L1210 murine leukemia cells. The disparity between these compounds remained when cells were lysed prior to drug exposure and were partially recapitulated using purified enzyme when 1 mM reduced glutathione was included during the drug exposure. The superior antineoplastic potential of Cloretazine™ compared to BCNU in animal models could be attributed in part to the contribution of the methyl isocyanate, which is synergistic with the co-generated cytotoxic alkylating species, while at the same time unable to significantly inhibit cellular GR.

Keywords: Cloretazine™; BCNU; Glutathione reductase; DNA alkylating agents; Methyl isocyanate; 2-Chloroethyl isocyanate

1. Introduction

Cloretazine™ is a novel sulfonylhydrazine anti-cancer compound with broad spectrum activity in murine models and a drug candidate currently in Phase II clinical trials against acute myelocytic leukemia. The antineoplastic activity of Cloretazine™ is a function of two reactive electrophiles co-generated by base-catalyzed activation in situ, one with alkylating activity and the other with carbamoylating activity [1]. The alkylating species generated from Cloretazine™, 90CE (Fig. 1), chloroethylates DNA at the O^6 position of guanine then forms a highly cytotoxic crosslink with a complementary cytosine base [2]. This crosslink is believed to be the critical cytotoxic event largely responsible for the therapeutic action of Cloretazine™ [2]. The carbamoylating species generated from Cloretazine™ is MiC, a very thiophilic electrophile. In a cell, MiC will carbamoylate sulfhydryl groups, including cysteine residues in proteins.

The clinically useful nitrosourea BCNU also decomposes in aqueous solution, yielding electrophilic species with chloroethylating, carbamoylating, vinylating, hydroxyethylating, and aminoethylating activities [3–5]. BCNU, as is the case with Cloretazine™, is believed to owe its therapeutic efficacy to DNA-crosslinking [6]. Its carbamoylating activity is a function of CEiC, which has often been considered a deleterious consequence of the drug's activation [5,7]. Other structurally distinct nitrosoureas yield different isocyanates whose reactivity and pharma-

Abbreviations: BCNU, 1,3-bis[2-chloroethyl]-2-nitrosourea; GR, glutathione reductase; MiC, methyl isocyanate; CEiC, 2-chloroethyl isocyanate; AGT, O^6-alkylguanine-DNA alkyltranferase

* Corresponding author. Tel.: +1 203 785 4533; fax: +1 203 737 2045.
E-mail address: alan.sartorelli@yale.edu (A.C. Sartorelli).

0006-2952/$ – see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2005.02.016

cology differ greatly [8]. The activation of Cloretazine™ yields insignificant levels of non-therapeutic vinylating, aminoethylating, and hydroxyethylating activities [2], all of which only add to the toxicity of BCNU. While MiC generation is perhaps unnecessary for the chloroethylating activity of Cloretazine™, preclinical findings suggest that MiC is an active participant in therapeutic efficacy [1].

Therapeutically beneficial or not, in vivo targets of isocyanates are likely to be numerous due to the promiscuous nature of carbamoylation chemistry. We previously identified AGT (EC 2.1.1.63) as a target for carbamoylation by Cloretazine™-derived MiC in vitro [1,9]. AGT activity has the capacity to produce resistance to DNA alkylating agents by repairing the primary lesion, which for BCNU and Cloretazine™ is the chloroethylation of the O^6 position of guanine in DNA. Findings suggested that at least part of the contribution of MiC to cytotoxicity may be due to inactivation of AGT by carbamoylation; however, synergism existing between the two reactive electrophiles in cells lacking AGT indicated that other targets for MiC carbamoylation are involved [1]. Pre-clinical and clinical studies involving BCNU identified GR as a target for inhibition by reactive isocyanates [10]. GR is a critical enzyme in the homeostasis of intracellular concentrations of GSH [11]. GSH is the primary line of defense for many toxic insults, including reactive oxygen species and xenobiotics, and is also involved in other redox pathways [11,12]. It is possible that decreased GR activity in a cancer cell could facilitate the cytotoxicity of BCNU by inducing apoptosis, depressing the activity of multidrug resistance proteins, and/or increasing sensitivity to the toxic byproducts of BCNU decomposition [13–15]. However, there is no evidence that these consequences are specific to neoplastic tissue. In fact, there are well-documented harmful consequences of a systematic depression of GR activity, particularly in pulmonary tissue [13,16], where cells are steadily bathed in reactive oxygen species, as well as the intestine [11,17], liver [18], and kidney [19], where an environment of depleted GSH renders these tissues hypersensitive to toxic insults.

In its reduced form, GR is very susceptible to inactivation by the carbamoylating activity of BCNU, which has been documented both in vitro [20,21] and in vivo [22,23]. There is some evidence to suggest that the active site of GR was alkylated rather than carbamoylated [24,25], but most reports indicate that carbamoylation is primarily responsible for the inhibitory activity [21]. However, with intracellular concentrations of glutathione and cysteine in the millimolar range [17], isocyanates are likely to react nonspecifically with these low molecular-weight thiol compounds with greater frequency than with the active site of a particular enzyme.

Carbamoylated metabolites of CEiC are also active against GR in vitro [26,27], as are MiC and its metabolites [27–29], although the MiC metabolites are substantially less inhibitory against GR than CEiC metabolites. Little information exists on the effects of MiC-generating prodrugs on in vivo GR activity.

In this paper, the effects of Cloretazine™ on GR activity were investigated using purified enzyme, intact cells, and cell lysates. Findings are considered relative to BCNU. Clear differences in the inhibitory potency of BCNU and Cloretazine™ against cellular GR were observed and possible explanations for these findings are considered. The results offer evidence that the clinical efficacy and decreased toxicological profile of Cloretazine™ relative to that of BCNU can be explained in part by its production of MiC rather than CEiC.

2. Materials and methods

2.1. Enzymes and biochemicals

Cloretazine™, 101MDCE, and 90CE (Fig. 1) were synthesized, purified, and characterized in this laboratory

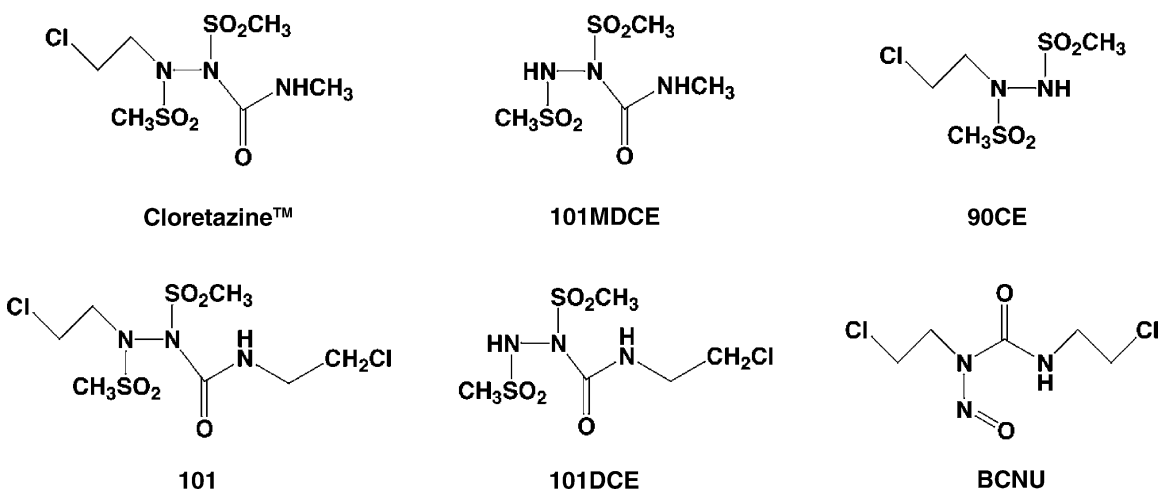

Fig. 1. Chemical structures of sulfonylhydrazine prodrugs and BCNU.

as described elsewhere [2,30]. BCNU was purchased from Sigma. Drugs were dissolved in dry DMSO to concentrations of 200 mM. Stock solutions were stored desiccated at −20 °C for up to 2 weeks; all dilutions were also prepared in dry DMSO. Glutaredoxin from *E. coli* was purchased from EMD Biosciences. Purified human glutathione reductase was purchased from Lab Frontier. All other enzymes, solvents, and biochemicals were obtained from Sigma.

2.2. Determination of the half-lives of sulfonylhydrazine prodrugs

The decomposition of sulfonylhydrazine prodrugs in 1 mM potassium phosphate buffer containing 20 μg/ml of phenol red was followed at 560 nm in a Beckman model-25 spectrophotometer as previously described [31]. In brief, this assay uses a pH indicator to detect the 2 molar equivalents of hydrogen ions released during the decomposition of the sulfonylhydrazine compounds.

2.3. Cell culture and treatment

The L1210 murine leukemia cell line was cultured in RPMI 1640 medium, supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, in an atmosphere containing 5% CO_2 at 37 °C. Cells were maintained at a density between 1×10^4 and 1×10^6 cells/mL. Cells were treated in fresh medium with drug or DMSO control for 1 h at 37 °C at a density of 2×10^6 cells/mL. Twenty million cells were harvested by centrifugation, washed in PBS, resuspended in 200 μL of 100 mM potassium phosphate buffer (pH 7.4), and lysed by three successive freeze–thaw cycles (−70 °C/37 °C). Lysates were clarified by centrifugation and normalized to the least concentrated sample using total protein concentration as determined by the Lowry assay kit from Bio-Rad.

2.4. Glutathione reductase activity from purified enzyme and L1210 cells

GR activity was determined by monitoring the oxidation of NADPH as measured by the decrease in absorbance at 340 nm. For reactions using purified GR, Cloretazine™, 101MDCE, 90CE, BCNU, or DMSO control was pre-incubated with GR (0.5 unit/mL from *S. cerevisiae*, 1 unit/mL from human) in 100 mM Tris–HCl (pH 8), 1 mM EDTA, and 100 μM NADPH for 3 h at room temperature unless stated otherwise. This incubation allows for at least three half-lives of drug activation (>87.5%) in the presence of the enzyme. For reactions using L1210 cells, treatment and lysis were carried out as described above. Standard assay conditions included 100 mM potassium phosphate buffer (pH 7.4), 1 mM EDTA, 300 μM NADPH, 500 μM GSSG, 0.1% DMSO, and purified, pre-incubated enzyme (10 μL added volume) or normalized cell lysate (15 μL added volume) in a total volume of 100 μL per reaction. This reaction was modified as necessary. Reactions were initiated with the pre-incubated enzyme or cell lysate and carried out in a UV-transparent 96-well microplate. Reaction progress was monitored by continuous spectrophotometric analysis using a SpectraMax250 plate reader (Molecular Dynamics). Absorbance readings were taken every 6 s and reaction velocity was calculated using the most linear region of the NADPH substrate depletion graph inside the first 3 min. All reactions were performed in triplicate. Data were normalized to the positive and negative controls and expressed as percent activity. IC_{50} values were calculated by fitting the data to the following hyperbolic equation: $1/(1 + (x/a)^b)$, where x refers to percent activity and a is solved as the IC_{50} value.

2.5. Glutathione reductase activity from erythrocytes

Plasma and erythrocytes were separated from whole human blood containing 1.5 mg/mL EDTA by centrifugation at 10,000 × *g* for 10 min. Erythrocyte pellets were washed four times with buffer (0.9% NaCl, 20 mM sodium phosphate, 10 mM glucose, pH 7.4). Drugs or the DMSO control were added to whole blood and incubated at 37 °C. At various times thereafter, 10 μl samples were removed and mixed with 90 μl of a lysis buffer composed of 100 mM EDTA (pH 8) and 0.5% SDS. This lysate was then stored on ice prior to assay for GR activity. Twenty microliters of lysate was added to a reaction mixture of 100 μM NADPH and 1 mM GSSG in 100 mM sodium phosphate buffer (pH 8.0) and the GR activity was measured by following the decrease in absorption at 340 nm at 37 °C in a Beckman model-25 spectrophotometer. Plasma contains very little GR activity, and the activity measured in whole blood essentially represents the erythrocytic content of this enzyme. Experiments using erythrocytes suspended in phosphate buffered saline plus glucose (cell density equivalent to whole blood) were performed in an identical manner. For experiments in which lysed blood was treated with drugs, 2 mM NADPH was first added to the whole blood. The blood was then lysed with three successive freeze–thaw cycles (−70 °C/37 °C) then treated immediately. The purpose of the NADPH was to maintain the GR in its reduced form, since it was found that upon cell lysis, the enzyme rapidly oxidized in the absence of NADPH and became resistant to inhibition. The drug induced loss of enzyme activity in lysed blood was measured in an identical manner to that used for whole blood, with aliquots being removed, mixed with lysis buffer at various time intervals, and assayed for GR activity.

2.6. Glutathione peroxidase activity

Glutathione peroxidase (EC 1.11.1.9) activity was measured by coupling the oxidation of GSH, using cumene hydroperoxide as the oxidant, to its reduction using GR, thereby allowing measurement by NADPH consumption as

described above. Cloretazine™, 101MDCE, 90CE, BCNU, or DMSO control was pre-incubated (200 μM final concentration) with 1.25 units/mL bovine liver glutathione peroxidase in 100 mM Tris–HCl (pH 8) and 1 mM EDTA for 3 h at room temperature. Standard assay conditions included 100 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 300 μM NADPH, 1 mM GSH, 200 μM cumene hydroperoxide, 0.1% DMSO, 1 unit/mL of yeast GR, and 0.125 unit/mL of glutathione peroxidase (10 μL added volume) in 100 μL total volume. Reactions were initiated with the pre-incubated peroxidase and carried out in a UV-transparent 96-well microplate. Reaction progress was monitored using a SpectraMax250 plate reader. Absorbance readings were taken every 6 s and reaction velocity was calculated using the most linear region of the NADPH substrate depletion graph inside the first 3 min. All reactions were performed in triplicate.

2.7. Glutathione S-transferase activity

Glutathione *S*-transferase (EC 2.5.1.18) activity was measured using 1-chloro-2,4-dinitrobenzene as the GSH acceptor and monitoring the accumulation of *S*-(2,4-dinitrobenzyl)glutathione, which absorbs 340 nm light. Cloretazine™, 101MDCE, 90CE, BCNU, or DMSO was pre-incubated (200 μM) with 3 units/mL of bovine liver glutathione *S*-tranferase in 100 mM Tris–HCl (pH 8) and 1 mM EDTA for 3 h at room temperature. Standard assay conditions included 100 mM potassium phosphate buffer (pH 6.6), 1 mM EDTA, 2.5 mM GSH, 2 mM 1-chloro-2,4-dinitrobenzene, 0.1% DMSO, and 0.3 unit/mL of glutathione *S*-transferase (10 μL added volume) in 100 μL total volume. Reactions were initiated with the pre-incubated enzyme and carried out in a UV-transparent 96-well microplate. Reaction progress was monitored using a SpectraMax250 plate reader. Absorbance readings were taken every 6 s and reaction velocity was calculated using the most linear region of the A_{340} versus time graph inside the first 2 min. All reactions were performed in triplicate.

2.8. Glutaredoxin activity

Glutaredoxin (EC 1.20.4.1) activity was measured using 2-hydroxyethyl disulfide to generate the requisite conjugate as described elsewhere [32]. Cloretazine™, 101MDCE, 90CE, BCNU, or DMSO control was pre-incubated (200 μM) with 5 units/mL of *E. coli* glutaredoxin in 100 mM Tris–HCl (pH 8) and 1 mM EDTA for 3 h at room temperature. Standard assay conditions included 100 mM Tris–HCl (pH 8.0), 1 mM EDTA, 300 μM NADPH, 1.4 mM GSH, 700 μM 2-hydroxyethyl disulfide, 1 unit/mL of GR, 0.1% DMSO, and 0.5 unit/mL of glutaredoxin (10 μL added volume) in 100 μL total volume. The reaction mixture was incubated for 15 min before enzymes were added, allowing the spontaneous formation of 2-hydroxyethyl glutathione disulfide. Reactions were initiated with the pre-incubated glutaredoxin and carried out in a UV-transparent 96-well microplate. Reaction progress was monitored using a SpectraMax250 plate reader. Absorbance readings were taken every 6 s and reaction velocity was calculated using the most linear region of the NADPH substrate depletion graph inside the first 1 min. All reactions were performed in triplicate.

3. Results

3.1. Cloretazine™, BCNU, and 101MDCE (but not 90CE) inhibited purified yeast and human glutathione reductase

The inhibition of GR by Cloretazine™, BCNU, 101MDCE, and 90CE was assessed by incubating the purified enzyme with drug for 3 h at room temperature in the presence of 100 μM NADPH. Pre-incubation allowed for the activation of the prodrugs. Inhibition of GR by carbamoylation is dependent upon NADPH as reported elsewhere [10]. Cloretazine™, 101MDCE, and BCNU inhibited the activity of purified human GR with IC_{50} values of 54.6 ± 4.3, 32.7 ± 7.7, and 55.5 ± 2.2 μM, respectively (Fig. 2A). In contrast, 90CE did not inhibit human GR until millimolar concentrations were achieved, which greatly exceeded any realistic pharmacological concentration. All compounds capable of generating a carbamoylating moiety in aqueous solution were inhibitory. With IC_{50} values of the same order of magnitude, there seemed to be no discernable difference between the inhibitory potency of the CEiC-generator BCNU and the MiC-generators, Cloretazine™ and 101MDCE. Similar results were obtained using purified GR from baker's yeast (Fig. 2B). The IC_{50} values for Cloretazine™, 101MDCE, and BCNU with this enzyme were 44.9 ± 7.3, 29.4 ± 3.3, and 37.9 ± 8.3 μM, respectively. Again, 90CE was a very poor inhibitor ($IC_{50} > 1$ mM).

3.2. Glutathione reductase inhibition by Cloretazine™, BCNU, and 101MDCE is a function of the half-life of the compound in aqueous solution

An extended pre-incubation afforded the prodrugs the opportunity for requisite activation via base-catalyzed decomposition in advance of substrate introduction. The half-lives of 1,2-bis(methylsulfonyl)hydrazines and BCNU decrease with increasing temperature and pH. In pH 8 buffer at 25 °C, the $T_{1/2}$ of Cloretazine™ is just under 1 h. The decomposition of BCNU has similar kinetics [21]. $T_{1/2}$ values for 90CE and 101MDCE are considerably less (0.6 and 3.6 min, respectively, at pH 7.4 and 37 °C) [2]. Since an assessment of the extent of the inhibitory potency of these compounds depends upon time, pre-incubation time-courses were carried out using 200 μM of each compound. Cloretazine™ and BCNU, with their similar $T_{1/2}$ values,

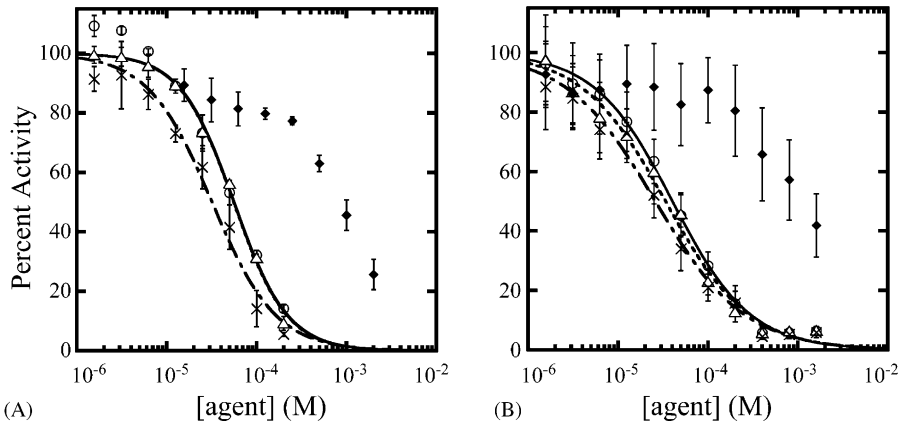

Fig. 2. Inhibition of purified GR from human (A) and yeast (B) sources. Cloretazine™ (○), BCNU (△), 101MDCE (×), and 90CE (◆) were serially diluted in DMSO such that equal volumes were added to pre-incubation mixtures with enzyme. 3 h pre-incubations were carried out in the presence of 100 μM NADPH at 25 °C in Tris–HCl, pH 8, prior to the addition of substrates at saturating concentrations. Reaction velocities were calculated as described in Section 2. All values were normalized to a DMSO control and reported as triplicate averages with standard deviations.

exhibited a direct relationship between pre-incubation time and GR inhibition that leveled off after 1 h (Fig. 3A). The other carbamoylating agent, 101MDCE with a $T_{1/2}$ value of 3.6 min, achieved its maximum inhibitory effectiveness much sooner at approximately 10 min. There was no discernable relationship between time and inhibition using 90CE. Conversely, when the compounds were exposed to buffer before a 3 h pre-incubation with GR, inhibitory compounds became ineffective (Fig. 3B).

3.3. Glutathione peroxidase, glutathione S-transferase, and glutaredoxin activities are unaffected by Cloretazine™, BCNU, 101MDCE, and 90CE

There are several other enzymes that utilize glutathione in some form as a substrate. We examined whether three of the most studied enzymes of glutathione metabolism were similarly affected by carbamoylation. Previous studies with BCNU revealed that glutathione peroxidase, which catalyzes the oxidation of GSH, and glutathione *S*-transferase, which delivers GSH to an available electrophile, were not significantly inhibited by BCNU or by the CEiC/GSH conjugate [23,28]. We confirmed these findings in this study and further showed that Cloretazine™ does not inhibit these enzymes significantly (Table 1). In addition, we assayed these agents against the activity of glutaredoxin, which generates GSSG from a mixed disulfide and GSH. Neither Cloretazine™ nor BCNU imposed any significant inhibition of glutaredoxin activity.

3.4. Glutathione reductase activity from cells treated with methyl isocyanate-generating compounds remains largely unchanged while 2-chloroethyl isocyanate-generating compounds dramatically diminish activity

Incubation of whole human blood with BCNU at a concentration of 50 μM caused an approximate 84% loss

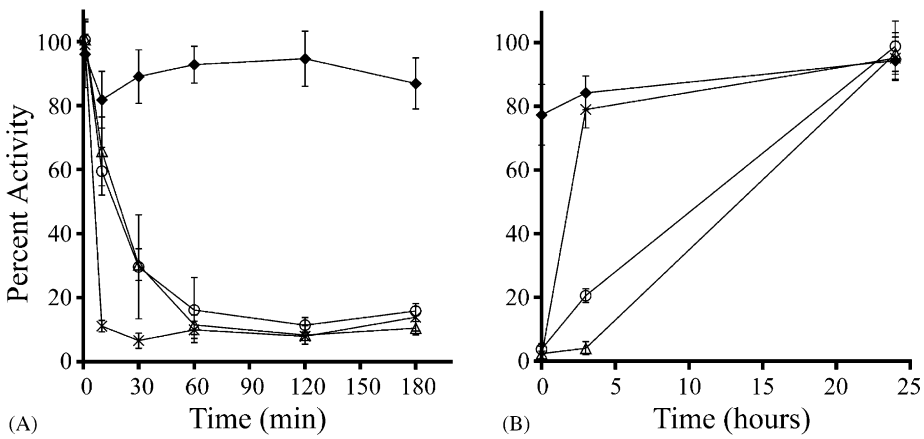

Fig. 3. Time course for the inhibition yeast GR by Cloretazine™ (○), BCNU (△), 101MDCE (×), and 90CE (◆); (A) 200 μM compound was pre-incubated with enzyme. Ten microliter aliquots of the pre-incubations were removed at indicated time points and assayed for activity; (B) 200 μM compound was pre-incubated in buffer alone. Aliquots were removed at the indicated times and incubated with GR for 3 h at 25 °C in Tris–HCl, pH 8, with 100 μM NADPH. After addition of saturating substrate concentrations, GR activity was assayed. All values were normalized to a DMSO control and reported as triplicate averages with standard deviations.

Table 1
Inhibition of the enzymes of glutathione metabolism by sulfonylhydrazines and BCNU

Agent	Percent original activity[a]			
	GR	Glutathione peroxidase	Glutathione *S*-transferase	Glutaredoxin
Cloretazine™	9.0 ± 1.3	108 ± 12	77.4 ± 1.1	87.8 ± 22.0
90CE	80.5 ± 6.4	95.9 ± 9.4	93.4 ± 3.9	87.3 ± 12.7
101MDCE	5.5 ± 0.7	94.8 ± 7.8	76.0 ± 1.6	85.4 ± 22.0
BCNU	9.8 ± 2.3	94.4 ± 9.4	84.8 ± 2.0	83.6 ± 15.2

[a] Agents (200 μM) were pre-incubated with enzymes for 3 h at 25 °C, pH 8 before addition of saturating substrate concentrations. Reaction progress for each enzyme reaction was monitored as described in Section 2. All reaction velocities were normalized to DMSO controls and reported as triplicate averages with standard deviations.

in GR activity over the course of 1 h (Fig. 4A). This degree of inhibition is similar to that observed in patients receiving therapeutic doses of BCNU [23]. In contrast, treatment with Cloretazine™ produced no significant inhibition of GR activity in whole blood, even at a 20-fold greater concentration over the same time course (Fig. 4B). At the same time, analogues of Cloretazine™ that lack either chloroethylating activity (101MDCE) or carbamoylating activity (90CE) had at best only nominal effects on GR. One millimolar 90CE caused only a 20% inhibition of GR (Fig. 4C) and 101MDCE caused no significant inhibition, even at a concentration of 1 mM (Fig. 4D). MiC itself was devoid of GR inhibitory activity in whole blood (Fig. 4E). However, all agents capable of producing CEiC (BCNU, 101, and 101DCE) were potent inhibitors of GR at 50 μM as was CEiC itself (Fig. 4A, F, G and H). These results also confirm that carbamoylation via CEiC, rather than 2-chloroethylation, is responsible for the inhibition of GR. This finding stands in contrast to other reports suggesting that the alkylating activities of some nitrosoureas can indeed inhibit GR under some circumstances in vitro [24,25]. Another possibility was that CEiC itself was alkylating rather than carbamoylating the active site of GR by first hydrolyzing into 2-chloroethylamine. This compound could cyclize to form an ethylenimine capable of alkylating thiol groups. However, 2-chloroethylamine did not inhibit whole blood GR activity at concentrations as high as 1 mM (Fig. 4I). It remains possible that the chloroethyl side chain of a GR-conjugated carbamoyl group could subsequently alkylate a secondary nucleophilic center in the GR active site. Such reactivity might explain the superiority of CEiC over MiC as an inhibitor of GR in cells as discussed elsewhere [27].

Despite the wide range of $T_{1/2}$ values for agents that were inhibitory to GR in whole blood (CEiC, BCNU, 101, 101DCE) from 17 s for CEiC [33] up to 40 min for BCNU [21], the time courses of inhibition of GR in whole blood were very similar, with half maximal inhibition occurring after 15–20 min, even with the very short-lived CEiC. This finding indicated that either CEiC is latentiated by reacting reversibly with a blood component(s) or that more stable adducts of CEiC were responsible for the direct inhibition of the GR enzyme. Both explanations involve chemical reactions preceding the carbamoylation of the enzyme. This conclusion was confirmed in experiments in which 100 μM CEiC was incubated with plasma at 37 °C for 100 min. These mixtures were then added to an equal volume of washed blood cells to give an equivalent concentration of 50 μM. This mixture was further incubated for 100 min at 37 °C and then assayed for GR activity. Aging of CEiC in phosphate buffer caused it to rapidly lose its inhibitory potency and only a few minutes of aging was required for loss of activity (data not shown). However, inhibitory activity persisted at high levels (75 ± 4%) when the CEiC was aged in plasma, even after 100 min of incubation at 37 °C.

Studies using L1210 leukemia cells gave similar results to those seen with human erythrocytes in that CEiC, but not MiC, precursors were strongly inhibitory. Cells were treated with 50 μM drug in fresh medium, then harvested, lysed, and normalized to total protein concentration. Lysates from cells exposed to 50 μM BCNU showed a 94.2 ± 3.2% inhibition of GR activity. However, like-concentrations of Cloretazine™ only modestly inhibited GR activity upon cell lysis (28.2 ± 2.9% of control activity). This finding indicates that the resistance of GR to inhibition within cells by MiC was not restricted to human erythrocytes. L1210 cells were selected due to their high sensitivity to Cloretazine™ in pre-clinical studies.

3.5. The effects of drug treatment on GR activity in cell lysates is equivalent to treatment of whole cells

One possible explanation for the difference between the inhibitory potencies of precursors of MiC and those of CEiC in whole cells could be a difference in the permeability of erythrocytes to their respective parent compounds, or if inhibitory adducts formed by the reaction of CEiC and MiC with plasma components were different. To test this possibility, erythrocytes in samples of whole blood were lysed in the presence of NADPH (to maintain GR in the reduced state upon lysis) and these lysates were treated with either Cloretazine™ or BCNU for various periods of time. GR activity was then measured as a function of time. The results were essentially identical to those obtained in intact cells; approximately 80% inhibition of GR activity was observed in BCNU treated lysates, while Cloretazine™ treatment produced no inhibition of GR, even at 1 mM. These results indicate that selective permeability was not a factor in the

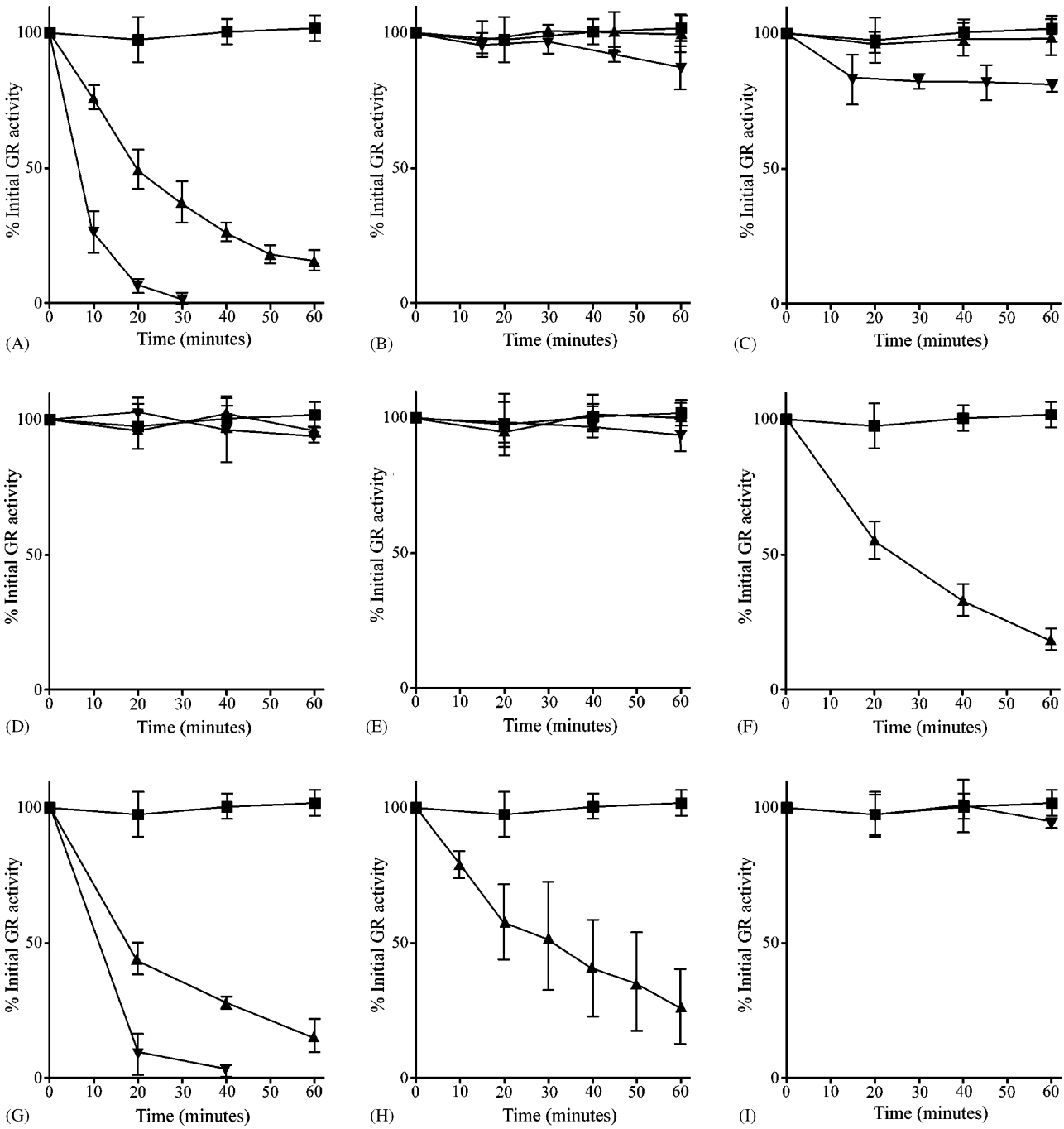

Fig. 4. Inhibition of erythrocytic GR activity by agents at 50 μM (▲) and/or 1 mM (▼) concentrations as compared to 1% DMSO (■). BCNU (A), Cloretazine™ (B), 90CE (C), 101MDCE (D), MiC (E), 101 (F), 101DCE (G), CEiC (H), and 2-chloroethylamine (I) were added to whole blood and incubated at 37 °C. At the indicated times, 10 μL aliquots were removed and lysed with 0.5% SDS. Saturating substrates were then added to the lysates and GR activity was assayed by monitoring the oxidation of NADPH at 340 nm. Data are reported as triplicate averages of the initial reaction velocity.

inability of MiC precursor agents to inhibit GR in whole cells.

3.6. Reduced glutathione completely protects purified GR from inhibition by Cloretazine™ but only partially protects purified GR from BCNU inhibition

In an effort to explain the different inhibitory potencies of carbamoylating species against purified GR and GR present in cells or cell lysates, the in vitro assay was modified slightly. The exposure of purified human GR to these agents was carried out in the presence of 1 mM GSH at 37 °C for 1 h. The cytoplasm of cells is rich in available nucleophiles, including up to 3 mM GSH, with which isocyanates could react. When GR was exposed to 200 μM Cloretazine™ in the presence of 1 mM GSH, the enzyme activity was completely protected from the strong inhibition observed in the absence GSH. However, 1 mM

Table 2
GSH protection from GR inhibition by Cloretazine™ and BCNU at 37 °C

Agent	Percent original activity[a]	
	Without GSH	1 mM GSH
Cloretazine™	3.1 ± 0.9	102 ± 1.9
BCNU	3.8 ± 1.5	70.5 ± 1.0

[a] Agents (200 μM) were pre-incubated with GR with or without 1 mM GSH for 1 h at 37 °C, pH 8. Reaction velocities were measured by monitoring the depletion of NADPH at 340 nm, normalized to the DMSO control, and reported as triplicate averages with standard deviations.

glutathione was less effective at protecting GR from inhibition by BCNU, with only 70.5 ± 1.0% of GR activity remaining (Table 2).

4. Discussion

The clinical significance of isocyanates generated from the in situ decomposition of antitumor nitrosourea and sulfonylhydrazine alkylating agents remains largely enigmatic. Arguments have been made for advantageous [34], deleterious [35], and insignificant [33] consequences of isocyanates formed from nitrosoureas as cancer chemotherapeutic agents. It seems likely that isocyanates are indeed therapeutically significant, but in both positive and negative ways. That MiC is important to the cytotoxicity of Cloretazine™ is quite evident, with synergism existing between the chloroethylating and carbamoylating activities of Cloretazine™, as measured by clonogenic cytotoxicity assays using Chinese hamster ovary cells [1]. The precise mechanism by which MiC participates in such a synergistic interaction is not known.

There is further, more anecdotal evidence that supports a contribution of carbamoylation towards the therapeutic efficacy of DNA-chloroethylating antitumor compounds. There are at least three reported classes of highly active antitumor agents that can be engineered to co-generate chloroethylating and carbamoylating activities: nitrosoureas, sulfonylhydrazines, and acyltriazenes [36,37]. All nitrosoureas possess carbamoylating activity, while sulfonylhydrazines and acyltriazenes can exhibit cytotoxicity by alkylation in the absence of carbamoylating activity. The clinical pursuance of Cloretazine™ was due in part to the characterization of a series of sulfonylhydrazines, including several alkyl isocyanate-generating analogues, which revealed that the MiC-generating agent Cloretazine™ was the most efficacious as an antineoplastic agent [30,38]. Similarly, the acyltriazene with the greatest antitumor activity possessed methylcarbamoylating activity [36]. No clinically used nitrosourea generates a MiC carbamoylating agent. The experimental antineoplastic agents caracemide [39] and *N*-methylformamide [40] generate MiC metabolites in situ without chloroethylating activity.

Despite potential benefits of isocyanates in cancer therapy, there are examples of isocyanate toxicity. Perhaps the most pronounced of which was a 1984 chemical plant explosion in Bhopal, India, which released massive amounts of MiC into the atmosphere, resulting in the death of more than 3000 people within the first 3 days alone [41]. Both acute and delayed toxicity was observed and attributed to MiC and more stable adducts of MiC formed in the body [29]. High concentrations of isocyanates are toxic to the lungs, kidneys, and liver [29,41]. However, even at its highest doses, Cloretazine™ never yields isocyanate concentrations that approach the bolus exposure absorbed by many Bhopal residents. At lower doses, direct actions of isocyanates should be influenced in cells by an overwhelming concentration (mM) of low molecular weight thiol compounds, such as GSH, relative to the concentrations (nM–μM) of active-site thiols in critical enzymes. However, inhibition of GR by BCNU observed in our study was more potent in cells and cell lysates than with purified enzyme. It is possible, perhaps likely, that the isocyanate itself is not the reactive inhibitory species in cells, but rather a transiently carbamoylated metabolite that transfers its carbamoyl group to a receptive nucleophile in the enzyme. An isocyanate–glutathione conjugate might not only be a simple carbamoyl donor for GR, but one that has specific affinity for the GR active site. The potent effects of serum in extending the duration of the inhibitory action of short-lived CEiC lends support to the idea that such conjugates are important in the inhibition of GR in biological systems.

In a simple enzymatic system, devoid of competing thiol nucleophiles, Cloretazine™ (MiC generator) and BCNU (CEiC generator) inhibit GR, a thiol-dependent enzyme, equivalently. However, this behavior was not reproduced in cellular studies, where Cloretazine™ was nearly ineffective as an inhibitor of erythrocyte GR activity, even at concentrations as high as 1 mM, and only marginally effective against GR activity in L1210 leukemia cells. BCNU and other CEiC precursors were, however, very potent inhibitors of GR activity in both cell types in the 50 μM concentration range. A major contributing factor to the poor inhibition observed in whole cells by precursors of MiC compared to those of CEiC possibly relates to the properties of their respective GSH adducts. Both isocyanates readily form carbamate thioesters with GSH, but the CEiC/GSH adduct is much more strongly inhibitory towards GR than its MiC/GSH counterpart [26,27]. In support of this possibility, the inclusion of GSH in our experiments with purified GR produced results that were more similar to those found with intact cells. It is likely that in the complex cellular environment multiple factors play a role in the disparate inhibition of cellular GR by Cloretazine™ and BCNU.

There are other lines of evidence to suggest that Cloretazine™ is superior to BCNU as an antineoplastic agent. The nitrosoureas appear to generate a lower yield of the therapeutically important G–C cross-link compared to the sulfonylhydrazines [2] and generate reactive electrophiles

of questionable therapeutic relevance. Moreover, while the CEiC generated by BCNU possesses many of the properties of MiC that cause synergism with chloroethylating activity, it also produces deleterious effects not found with MiC. For example, 2-chloroethylamine, a hydrolysis product of CEiC, can form a mono-functional nitrogen mustard that can aminoethylate the N^7 position of guanine in DNA [5]. Mono-functional nitrogen mustards have low anticancer activity in relation to their mutagenicity and are therefore not considered desirable as therapeutic agents [42]. The CEiC/GSH conjugate has been reported to be damaging to DNA and strongly mutagenic in human and bacterial cells [43].

The strong inhibition of GR in intact cells by CEiC probably represents an additional non-therapeutic action, which could result in the selective sensitization of tissues such as the lung under high oxidant stress. This sensitivity could intensify when other toxic electrophiles, such as those generated by many chemotherapeutic agents, are co-administered with BCNU. For example, such interactions could contribute to the severe delayed lung toxicity observed in most patients receiving the high dose chemotherapy regimen aimed at breast cancer that utilizes cyclophosphamide, cisplatin, and BCNU [7,44]. The intestine is also exposed to high levels of extracellular GSH via a combination of endogenous synthesis and millimolar concentrations of hepatic GSH through biliary supply [11]. This reliance on GSH for homeostasis may make this tissue particularly sensitive to the severe and generalized GR inhibition caused by BCNU. This action may in part explain the lower incidence of gastrointestinal toxicity observed with Cloretazine™ [45] compared to BCNU [46].

The role of the carbamoylating activity of sulfonylhydrazine prodrugs and nitrosoureas requires further investigation in terms of both therapeutic benefits and detrimental effects. Such information may lead to improvements in the therapeutic indices of both classes of agents. Early clinical results suggest that Cloretazine™ is a relatively well-tolerated [45] and effective agent against acute myelocytic leukemia. Phase II clinical trials using combination therapy of Cloretazine™ with cytosine arabinoside demonstrated 20% complete remissions for patients with refractory acute myelocytic leukemia [47]. Substitution of BCNU with Cloretazine™ may prove to be equal or superior in chemotherapeutic regimens, while perhaps reducing the often-serious complications associated with BCNU toxicity.

References

[1] Baumann RP, Shyam K, Penketh PG, Remack JS, Brent TP, Sartorelli AC. 1,2-Bis(methylsulfonyl)-1-(2-chloroethyl)-2-[(methylamino)carbonyl]hydrazine (VNP40101M). II. Role of O^6-alkylguanine-DNA alkyltransferase in cytotoxicity. Cancer Chemother Pharmacol 2004; 53:288–95.

[2] Penketh PG, Shyam K, Sartorelli AC. Comparison of DNA lesions produced by tumor-inhibitory 1,2-bis(sulfonyl)hydrazines and chloroethylnitrosoureas. Biochem Pharmacol 2000;59:283–91.

[3] Naghipur A, Ikonomou MG, Kebarle P, Lown JW. Mechanism of action of (2-haloethyl)nitrosoureas on DNA: discrimination between alternative pathways of DNA base modification by 1,3-bis(2-fluoroethyl)-1-nitrosourea by using specific deuterium labeling and identification of reaction products by HPLC tandem mass spectrometry. J Am Chem Soc 1990;112:3178–87.

[4] Eisenbrand G, Muller N, Denkel E, Sterzel W. DNA adducts and DNA damage by antineoplastic and carcinogenic *N*-nitroso compounds. J Cancer Res Clin Oncol 1986;112:196–204.

[5] Gombar CT, Tong WP, Ludlum DB. Mechanism of action of the nitrosoureas. IV. Reactions of bis-chloroethyl nitrosourea and chloroethyl cyclohexyl nitrosourea with deoxyribonucleic acid. Biochem Pharmacol 1980;29:2639–43.

[6] Bodell WJ, Tokuda K, Ludlum DB. Differences in DNA alkylation products formed in sensitive and resistant human glioma cells treated with *N*-(2-chloroethyl)-*N*-nitrosourea. Cancer Res 1988;48:4489–92.

[7] Abushamaa AM, Sporn TA, Folz RJ. Oxidative stress and inflammation contribute to lung toxicity after a common breast cancer chemotherapy regimen. Am J Physiol Lung Cell Mol Physiol 2002;283: L336–45.

[8] Johnston TP, Montgomery JA. Relationship of structure to anticancer activity and toxicity of the nitrosoureas in animal systems. Cancer Treat Rep 1986;70:13–30.

[9] Penketh PG, Shyam K, Baumann RP, Remack JS, Brent TP, Sartorelli AC. 1,2-Bis(methylsulfonyl)-1-(2-chloroethyl)-2-[(methylamino)carbonyl]hydrazine (VNP40101M). I. Direct inhibition of O6-alkylguanine-DNA alkyltransferase (AGT) by electrophilic species generated by decomposition. Cancer Chemother Pharmacol 2004;53:279–87.

[10] Babson JR, Reed DJ. Inactivation of glutathione reductase by 2-chloroethyl nitrosourea-derived isocyanates. Biochem Biophys Res Commun 1978;83:754–62.

[11] Moriarty-Craige SE, Jones DP. Extracellular thiols and thiol/disulfide redox in metabolism. Ann Rev Nutr 2004;24:481–509.

[12] Meister A, Anderson ME. Glutathione. Ann Rev Biochem 1983; 52:711–60.

[13] Rahman Q, Abidi P, Afaq F, Schiffmann D, Mossman BT, Kamp DW, et al. Glutathione redox system in oxidative lung injury. Crit Rev Toxicol 1999;29:543–68.

[14] Vanhoefer U, Yin MB, Harstrick A, Seeber S, Rustum YM. Carbamoylation of glutathione reductase by *N*,*N*-bis(2-chloroethyl)-*N*-nitrosourea associated with inhibition of multidrug resistance protein (MRP) function. Biochem Pharmacol 1997;53:801–9.

[15] Zhang K, Mack P, Wong KP. Glutathione-related mechanisms in cellular resistance to anticancer drugs. Int J Oncol 1998;12: 871–82.

[16] Smith AC, Boyd MR. Preferential effects of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) on pulmonary glutathione reductase and glutathione/glutathione disulfide ratios: possible implications for lung toxicity. J Pharmacol Exp Ther 1984;229:658–63.

[17] Smith CV, Jones DP, Guenthner TM, Lash LH, Lauterburg BH. Compartmentation of glutathione: implications for the study of toxicity and disease. Toxicol Appl Pharmacol 1996;140:1–12.

[18] Pappas P, Vasiliou V, Nebert DW, Marselos M. Lack of response of the rat liver class 3 cytosolic aldehyde dehydrogenase to toxic chemicals, glutathione depletion, and other forms of stress. Biochem Pharmacol 1994;48:841–5.

[19] Satoh M, Shimada A, Zhang B, Tohyama C. Renal toxicity caused by cisplatinum in glutathione-depleted metallothionein-null mice. Biochem Pharmacol 2000;60:1729–34.

[20] Cohen MB, Duvel DL. Characterization of the inhibition of glutathione reductase and the recovery of enzyme activity in exponentially growing murine leukemia (L1210) cells treated with 1,3-bis(2-chloroethyl)-1-nitrosourea. Biochem Pharmacol 1988;37:3317–20.

[21] Becker K, Schirmer RH. 1,3-Bis(2-chloroethyl)-1-nitrosourea as thiol-carbamoylating agent in biological systems. Methods Enzymol 1995;251:173–88.
[22] Jopperi-Davis KS, Park MS, Rogers LK, Backes Jr CH, Lim IK, Smith CV. Compartmental inhibition of hepatic glutathione reductase activities by 1,3-bis(2-chloroethyl)-*N*-nitrosourea (BCNU) in Sprague–Dawley and Fischer-344 rats. Toxicol Lett 2004;147:219–28.
[23] Frischer H, Ahmad T. Severe generalized glutathione reductase deficiency after antitumor chemotherapy with BCNU [1,3-bis(chloroethyl)-1-nitrosourea]. J Lab Clin Med 1977;89:1080–91.
[24] Karplus PA, Krauth-Siegel RL, Schirmer RH, Schulz GE. Inhibition of human glutathione reductase by the nitrosourea drugs 1,3-bis(2-chloroethyl)-1-nitrosourea and 1-(2-chloroethyl)-3-(2-hydroxyethyl)-1-nitrosourea. A crystallographic analysis. Eur J Biochem 1988; 171:193–8.
[25] Schallreuter KU, Gleason FK, Wood JM. The mechanism of action of the nitrosourea anti-tumor drugs on thioredoxin reductase, glutathione reductase and ribonucleotide reductase. Biochim Biophys Acta 1990;1054:14–20.
[26] Davis MR, Kassahun K, Jochheim CM, Brandt KM, Baillie TA. Glutathione and *N*-acetylcysteine conjugates of 2-chloroethyl isocyanate. Identification as metabolites of *N*,*N*′-bis(2-chloroethyl)-*N*-nitrosourea in the rat and inhibitory properties toward glutathione reductase in vitro. Chem Res Toxicol 1993;6:376–83.
[27] Kassahun K, Jochheim CM, Baillie TA. Effect of carbamate thioester derivatives of methyl- and 2-chloroethyl isocyanate on glutathione levels and glutathione reductase activity in isolated rat hepatocytes. Biochem Pharmacol 1994;48:587–94.
[28] Jochheim CM, Baillie TA. Selective and irreversible inhibition of glutathione reductase in vitro by carbamate thioester conjugates of methyl isocyanate. Biochem Pharmacol 1994;47:1197–206.
[29] Slatter JG, Rashed MS, Pearson PG, Han DH, Baillie TA. Biotransformation of methyl isocyanate in the rat. Evidence for glutathione conjugation as a major pathway of metabolism and implications for isocyanate-mediated toxicities. Chem Res Toxicol 1991;4:157–61.
[30] Shyam K, Penketh PG, Loomis RH, Rose WC. Sartorelli AC. Antitumor 2-(aminocarbonyl)-1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-hydrazines. J Med Chem 1996;39:796–801.
[31] Penketh PG, Shyam K, Patton CL, Sartorelli AC. Spectrophotometric assay for processes involving changes in hydrogen ion concentration in aqueous solution. Anal Biochem 1996;238:46–9.
[32] Holmgren A, Aslund F. Glutaredoxin. Methods Enzymol 1995; 252:283–92.
[33] Hilton J, Maldarelli F, Sargent S. Evaluation of the role of isocyanates in the action of therapeutic nitrosoureas. Biochem Pharmacol 1978; 27:1359–63.
[34] Kann Jr HE, Kohn KW, Lyles JM. Inhibition of DNA repair by the 1,3-bis(2-chloroethyl)-1-nitrosourea breakdown product, 2-chloroethyl isocyanate. Cancer Res 1974;34:398–402.
[35] Gibson NW, Hickman JA. The role of isocyanates in the toxicity of antitumour haloalkylnitrosoureas. Biochem Pharmacol 1982;31: 2795–800.
[36] Smith RH, Scudiero DA, Michejda CJ. 1,3-Dialkyl-3-acyltriazenes, a novel class of antineoplastic alkylating-agents. J Med Chem 1990; 33:2579–83.
[37] Rouzer CA, Sabourin M, Skinner TL, Thompson EJ, Wood TO, Chmurny GN, et al. Oxidative metabolism of 1-(2-chloroethyl)-3-alkyl-3-(methylcarbamoyl)triazenes: formation of chloroacetaldehyde and relevance to biological activity. Chem Res Toxicol 1996;9:172–8.
[38] Finch RA, Shyam K, Penketh PG, Sartorelli AC. 1,2-Bis(methylsulfonyl)-1-(2-chloroethyl)-2-(methylamino)carbonylhydrazine (101M): a novel sulfonylhydrazine prodrug with broad-spectrum antineoplastic activity. Cancer Res 2001;61:3033–8.
[39] Slatter JG, Davis MR, Han DH, Pearson PG, Baillie TA. Studies on the metabolic fate of caracemide, an experimental antitumor agent, in the rat. Evidence for the release of methyl isocyanate in vivo. Chem Res Toxicol 1993;6:335–40.
[40] Kestell P, Gledhill AP, Threadgill MD, Gescher A. S-(*N*-Methylcarbamoyl)-*N*-acetylcysteine: a urinary metabolite of the hepatotoxic experimental antitumour agent *N*-methylformamide (NSC 3051) in mouse, rat and man. Biochem Pharmacol 1986;35:2283–6.
[41] Varma DR, Guest I. The Bhopal accident and methyl isocyanate toxicity. J Toxicol Environ Health 1993;40:513–29.
[42] Brookes P, Lawley PD. Reaction of mono- and di-functional alkylating agents with nucleic acids. Biochem J 1961;80:496–503.
[43] Stahl W, Lenhardt S, Przybylski M, Eisenbrand G. Mechanism of glutathione-mediated DNA damage by the antineoplastic agent 1,3-bis(2-chloroethyl)-*N*-nitrosourea. Chem Res Toxicol 1992;5:106–9.
[44] Jones RB, Matthes S, Shpall EJ, Fisher JH, Stemmer SM, Dufton C, et al. Acute lung injury following treatment with high-dose cyclophosphamide, cisplatin, and carmustine: pharmacodynamic evaluation of carmustine. J Natl Cancer Inst 1993;85:640–7.
[45] Giles F, Thomas D, Garcia-Manero G, Faderl S, Cortes J, Verstovsek S, et al. A Phase I and pharmacokinetic study of VNP40101M, a novel sulfonylhydrazine alkylating agent, in patients with refractory leukemia. Clin Cancer Res 2004;10:2908–17.
[46] Bashir R, Hochberg FH, Linggood RM, Hottleman K. Pre-irradiation internal carotid artery BCNU in treatment of glioblastoma multiforme. J Neurosurg 1988;68:917–9.
[47] Kornblau SM, Qui YH, Cade JS, Lemker EM, Giles F. Survivor cells predict which patients will respond to therapy with VNP40101M and Ara-C. Blood 2004;104:559a.