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COMPARATIVE STUDY ON THE INFLUENCE OF TWO 2-CHLOROETHYLNITROSOUREAS WITH DIFFERENT CARBAMOYLATING POTENTIAL TOWARDS GLUTATHIONE AND GLUTATHIONE-RELATED ENZYMES IN DIFFERENT ORGANS OF THE RAT

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The influence of two CNUs with similar alkylating but strongly different carbamoylating activity towards the glutathione system was investigated in different organs. Both CNUs influence the glutathione system of the bone marrow in a similar manner, irrespective of their carbamoylating potential. In contrast, glutathione reductase activity in the other organs was strongly decreased by the potent carbamoylator BCNU, whereas no or only minor effects were produced by its weakly carbamoylating counterpart HECNU.

The results confirm that bone marrow toxicity of CNUs primarily results from alkylation and not from carbamoylation. Other organ-related toxic effects, however, are probably a result of carbamoylating reactions exerted by BCNU. This applies especially to lung toxicity that has been observed frequently as a major side effect in clinical trials with BCNU.

KEY WORDS: Nitrosoureas, bone-marrow toxicity, glutathione, glutathione reductase, isocyanate.

ABBREVIATIONS: CNU: 2-chloroethylnitrosourea, BCNU: 1,3-bis-(2-chloroethyl)-1-nitrosourea, HECNU: 1-(2-chloroethyl)-3-(2-hydroxyethyl)-1-nitrosourea, GSH: glutathione reduced form, GSSG: glutathione oxidised form, GSSG-R: glutathione reductase, GS-Tr: glutathione transferase, CDNB: 1-chloro-2,4-dinitrobenzene

INTRODUCTION

2-Chloroethylnitrosoureas (CNUs) are effective anticancer drugs which belong to the group of alkylating and DNA crosslinking agents. Their value as anticancer agents, however, is limited by toxic side effects, in particular by their cumulative toxicity towards the bone marrow and other organ related adverse effects.[1,2] In the clinical application, bone marrow toxicity has been found to be dose-limiting. Another major problem associated with the clinical use especially of 1.3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) is the occurrence of pulmonary toxicity. Lung damage and pulmonary fibrosis have been found in about 30% of patients treated with BCNU.[3,4] Induction of lung damage has also been observed in mice and in rats after repeated application.[5–7]

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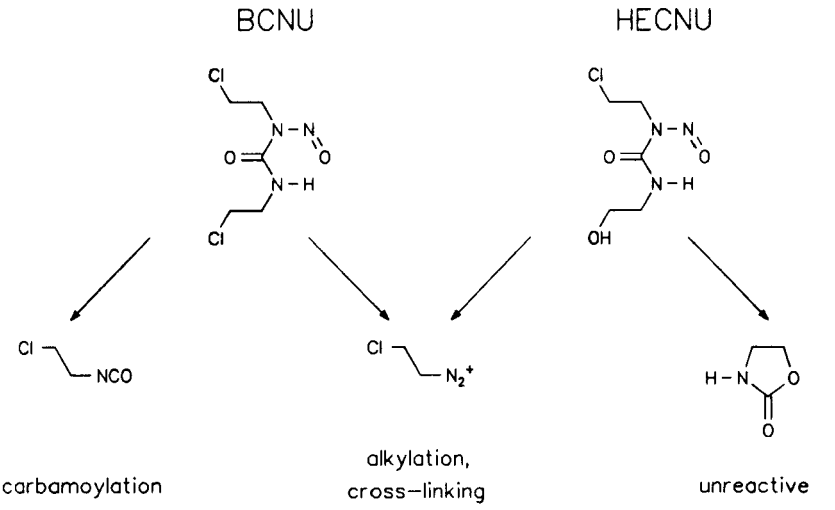

FIGURE 1 Decomposition of BCNU and HECNU.

In contrast to other alkylating compounds CNUs decompose to yield two electrophilic reactants: a bifunctional alkylating 2-chloroethyldiazonium intermediate and a carbamoylating isocyanate (Figure 1). Structure and reactivity of the latter depend on the substituent at the N-3 position of the nitrosourea. In the presence of a nucleophilic hydroxy group in β-position of the N 3-substituent 2-oxazolidinone is formed by intramolecular carbamoylation. This results in quenching of carbamoylating activity.[8]

Current opinion favours alkylating- and subsequent crosslinking-reactions within DNA of tumor cells as molecular events most relevant to antitumor activity. Carbamoylation has not been found to contribute to any significant extent to anticancer activity of nitrosoureas.[9,10] The contribution of carbamoylating activity to CNU-related toxic effects, however, is still controversial.[11,12]

Investigations on nitrosoureas with different carbamoylating and alkylating potential have indicated that both, alkylation and carbamoylation, might be involved in bone marrow toxicity.[12] Experiences from *in vivo* studies and clinical trials, however, do not support carbamoylation to be of great relevance for bone marrow toxicity. For example, some weakly carbamoylating CNU's have been found to induce bone marrow toxicity to a similar extent as their strongly carbamoylating counterparts. With regard to longterm toxicity however, the weakly carbamoylating analogue 1-(2-chloroethyl)-3-(2-hydroxyethyl)-1-nitrosourea (HECNU) has been found to be significantly less toxic than BCNU.[2]

Nitrosoureas with strong carbamoylating potential have been shown to be potent inhibitors of glutathione reductase (GSSG-R: EC 1.6.4.2.)[13–16] Moreover, high doses of strongly carbamoylating CNUs also influence the glutathione status *in-vivo*. This might be due to a selective reaction of isocyanates with the thiol group of this tripeptide.[17]

GSH and GSH-related enzymes, including GSSG-R and GS-Tr (glutathione-S-transferase: EC 2.5.1.18), play an essential role in detoxifying reactions. The present

work describes effects of two clinically used nitrosoureas with strongly different carbamoylating activity, HECNU and BCNU, on GSH and GSH-related enzymes in target tissues of the rat.

MATERIALS AND METHODS

Drugs

Bis-1,3-(2-chloroethyl)-1-nitrosourea (BCNU) and 1-(2-chloroethyl)-3-(2-hydroxyethyl)-1-nitrosourea (HECNU) were synthesized according to published methods.[18] Their carbamoylating potential was determined by reaction with GSH as described earlier.[19]

GSH, GSSG o-phthaldialdehyde and NADPH were obtained from Serva GmbH (Heidelberg); 1-chloro-2.4-dinitrobenzene from Janssen (Berse-Belgium) was used after recrystallisation. All other chemicals were from E. Merck (Darmstadt).

Application and organ preparation

HECNU was dissolved in 0.9% saline solution, BCNU in a mixture of propanediol, cremophor and 0.9% saline solution 1/1/3 (v/w/v).

CNUs (0.15 mmol/kg body weight) were injected intraperitoneally to female Wistar rats (200–240 g); controls received an equivalent volume of solvent. At specified time points between 5 and 70 h after administration, blood samples were collected in heparinized tubes from the vena cava. After exsanguination, liver, lung und brain were removed, rinsed with 20 mM sodium phosphate buffer pH 7.7/0.9% NaCl-solution (standard buffer), weighed and immediately deep frozen in liquid nitrogen. The tissue samples were stored at −80°C until analysis. To obtain bone marrow samples, both femura were removed and flushed with 0.5 ml standard buffer per femur. These suspensions were also deep frozen until analysis. Cell numbers were counted in aliquots of blood samples and bone marrow suspensions. Glutathione content and enzymatic activities did not change to any significant extent during storage. The frozen organs were homogenized in a glass-glass hand potter in 4 ml standard buffer/g tissue (3 ml/g brain). Homogenates and cell suspensions of blood and bone marrow were centrifuged (40 min. at 0°C and 100 000 g). Enzyme activities and protein content were measured in the supernatant.

GSH-levels were determined in deproteinized samples. Protein was precipitated by addition of 0.5 ml 10% trichloroacetic acid (30 μM EDTA) to 5 ml homogenate or cell suspension. After centrifugation (2°C, 40 min., 100 000 g) the supernatant was used for thiol determination.

The content of GSH in the sample was measured using a method described by Hissin and Hilf[20] with o-phthaldialdehyde as derivatising agent. Fluorescence of the derivative was measured at 350 nm excitation and at 420 nm emission wavelength. Concentrations in the samples were calculated from standards.[20] Concentration of GSH in liver, lung and brain was normalized to wet tissue weight.

Glutathione-S-transferase activities were determined with 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate.[21] Glutathione reductase activity was measured by NADPH consumption using the method of Carlberg and Mannervik.[22] Protein concentration was determined with Biuret reagent.

TABLE I
Glutathione (GSH) levels and activities of glutathione transferase (GS-Tr) and glutathione reductase (GSSG-R) of untreated controls

organ	GSH	GS-Tr	GSSG-R
liver	5.2 ± 1.3	7860 ± 1080	73 ± 6.4
lung	0.8 ± 0.26	860 ± 161	51 ± 5.5
brain	1.0 ± 0.3	1800 ± 260	34 ± 6.6
blood	0.8 ± 0.2	6.2 ± 0.87	2.5 ± 0.8
bone marrow	1.1 ± 0.25	8.3 ± 2.2	6.6 ± 2.1

GSH contents in liver, lung and brain: μmol/g wet tissue; in blood and bone marrow: μmol/10^9 cells; GS-Tr activity in liver, lung and brain: nmol CDNB conjugated/min/mg protein; in blood and bone marrow: nmol CDNB conjugated/min/10^6 cells; GSSG-R activity in liver, lung and brain: nmol NADPH oxidized/min/mg protein; in blood and bone marrow: nmol NADPH oxidized/min/10^6 cells. Data are given as means ± SD (n = 10)

RESULTS

Mean levels of glutathione, glutathione transferase and glutathione reductase in untreated controls are given in Table I. The values are in good agreement with those reported in previous studies.[1,19,23-26]

In an earlier study we have developed an assay to determine the carbamoylating potential of CNUs by measuring their thiol group reactivity towards GSH.[19] BCNU was found to be highly reactive whereas HECNU exhibited negligible carbamoylating activity. This loss in thiol group reactivity results from intramolecular carbamoylation observed for HECNU, as shown in Figure 1.

GSH levels (percent of untreated controls) in different organs at various time points after application of BCNU and HECNU at therapeutically relevant dosage are given in Table II. In liver, lung, blood and brain, GSH levels are hardly influenced by CNU treatment. In contrast, a marked depletion of GSH to about 50% of control is induced in the bone marrow by BCNU and HECNU within 15 h.

TABLE II
GSH levels in percent of untreated control after treatment with BCNU and HECNU

		Time after application (h)					
		5	10	15	20	40	70
Liver	BCNU	95 ± 15	102 ± 15	91 ± 11	73 ± 17	86 ± 6	89 ± 14
	HECNU	100 ± 22	93 ± 16	108 ± 8	75 ± 7	79 ± 6	96 ± 11
Lung	BCNU	103 ± 12	102 ± 5	95 ± 9	88 ± 6	61 ± 23	104 ± 12
	HECNU	110 + 17	91 ± 5	83 ± 17	89 ± 12	85 ± 10	100 ± 2
Brain	BCNU	105 ± 4	105 ± 17	117 ± 12	100 ± 6	87 ± 7	92 ± 12
	HECNU	120 ± 13	108 ± 16	102 ± 14	102 ± 8	111 ± 11	96 ± 13
Blood	BCNU	98 ± 8	85 ± 12	90 ± 17	74 ± 16	79 ± 8	95 ± 13
	HECNU	105 ± 12	122 ± 6	93 ± 2	79 ± 5	85 ± 10	90 ± 9
Bone-marrow	BCNU	134 ± 12	90 ± 7	89 ± 13	69 ± 3	45 ± 9	61 ± 11
	HECNU	79 ± 25	80 ± 5	79 ± 15	48 ± 6	40 ± 12	39 ± 17

Mean values ± SD (n = 4)
100% values see Table I

TABLE III
GSH transferase levels in percent of untreated control after treatment with BCNU and HECNU

		Time after application (h)					
		5	10	15	20	40	70
Liver	BCNU	88 ± 8	131 ± 19	127 ± 14	104 ± 11	113 ± 18	98 ± 12
	HECNU	110 ± 10	103 ± 5	123 ± 9	112 ± 15	104 ± 10	80 ± 9
Lung	BCNU	98 ± 2	139 ± 10	139 ± 12	95 ± 22	104 ± 10	103 ± 10
	HECNU	95 ± 3	117 ± 9	141 ± 15	122 ± 14	106 ± 2	75 ± 11
Brain	BCNU	100 ± 8	110 ± 11	105 ± 3	95 ± 6	115 ± 8	94 ± 16
	HECNU	108 ± 10	104 ± 10	101 ± 17	103 ± 3	106 ± 0	107 ± 11
Blood	BNCU	100 ± 18	95 ± 13	75 ± 5	97 ± 9	103 ± 27	99 ± 4
	HECNU	104 ± 3	97 ± 2	90 ± 0	88 ± 3	99 ± 4	93 ± 8
Bone-marrow	BCNU	109 ± 28	106 ± 23	56 ± 4	54 ± 8	48 ± 10	53 ± 7
	HECNU	119 ± 25	113 ± 6	50 ± 14	48 ± 13	46 ± 6	38 ± 7

Mean values + SD ($n = 4$)
100% values see Table I

Effects of nitrosoureas on glutathione transferase activities in different organs are shown in Table III. BCNU and HECNU exhibit nearly identical time courses of effects in all organs examined. Only a weak and transient decrease is observed in liver, lung, brain and blood, recovering at about 70 h. In the bone marrow, however, both compounds induce a decrease in GS-Tr activity to less than 50% of control, without apparent recovery within 70 h.

Effects on glutathione reductase are shown in Figure 2. In all organs strong differences between the effects of BCNU and HECNU become apparent. In blood, liver, lung and brain BCNU causes a strong and long-lasting decrease whereas GSSG-R-activity in these organs is hardly affected by HECNU. In the bone marrow, however, both compounds induce a marked decrease in GSSG-R, with apparently stronger effects being exerted by BCNU. After BCNU, a nadir of 25% is observed at 15 h, GSSG-R activity remaining below 40% of untreated control even at 70 h. HECNU induces a less steep decrease in GSSG-R activity.[8]

DISCUSSION

Effects of therapy with BCNU on the status of glutathione and glutathione-reductase in various organs have been described in several studies.[13,15,17] Inhibitory effects on GSSG-R were found in blood, liver, lung and kidney.[15] To our knowledge the bone marrow has not been studied yet. The influence of CNU-therapy on GS-Tr, another important detoxifying GSH-related enzyme, was also measured in the present investigation. The data show a dramatic difference between BCNU and HECNU with respect to their inhibitory potential towards GSSG-R in blood, liver, brain and lung. Effects in blood are mainly reflecting those to GSSG-R of erythrocytes, as previously shown.[13,14]

GSSG-R is a major defense system against oxidative stress. Since pulmonary tissue is exposed to a continuous oxidative challenge, its integrity is depending on highly effective antioxidative defense systems. One of the major late effects of clinical therapy

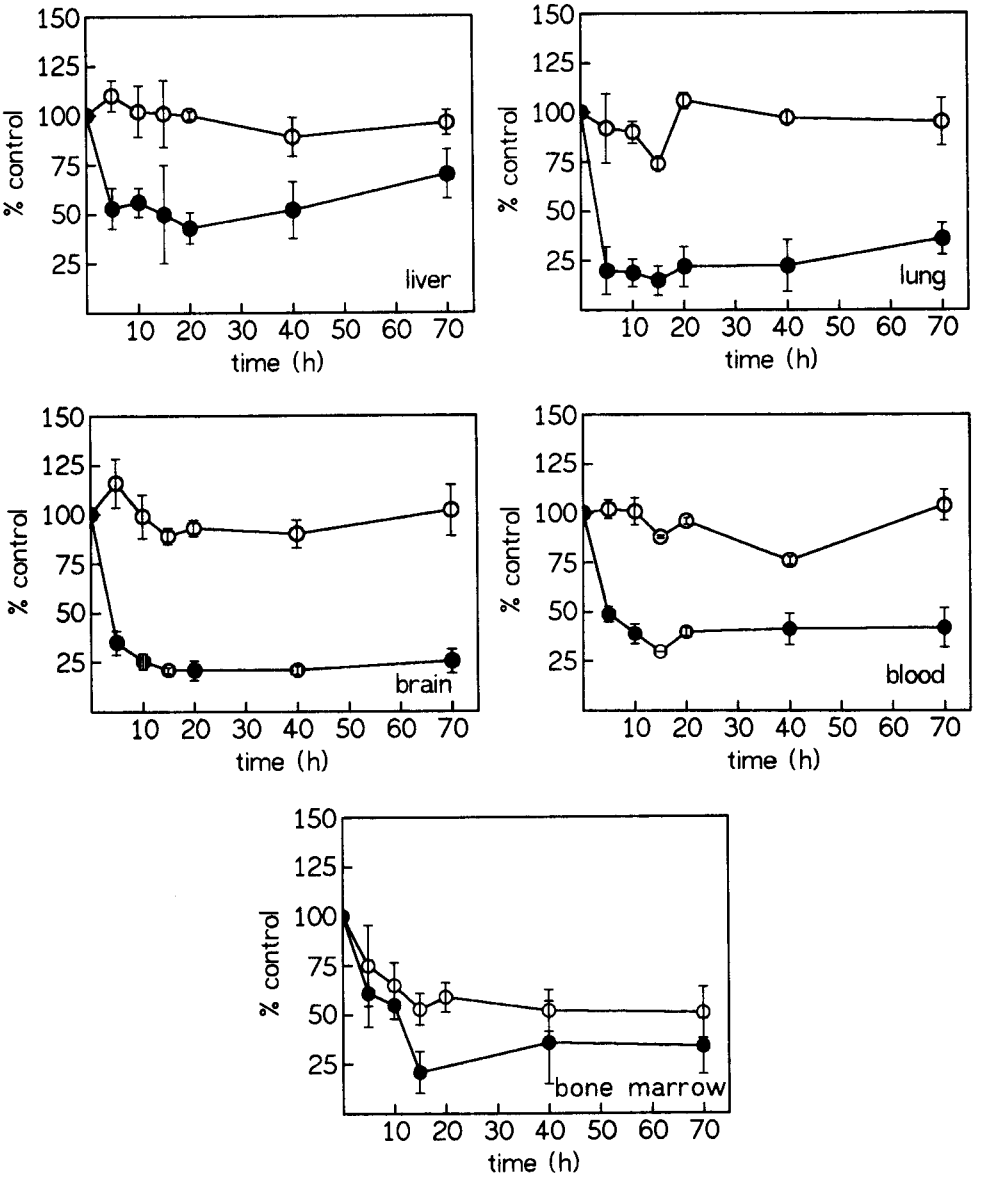

FIGURE 2 Glutathione reductase activity in liver, lung, brain, blood and bone-marrow at different time points after administration of BCNU and HECNU.

with BCNU is pulmonary fibrosis, observed in about 30% of all cases treated.[4,7,27] Lung toxicity of BCNU is suspected to be caused by its detrimental effects on the glutathione system,[7,28] and hence on antioxidative defense.

HECNU is not damaging glutathione reductase *in vivo* to any significant extent. This correlates with a significantly diminished long-term toxicity in rats, as compared

to BCNU.[2] Accordingly, lung toxicity has not been observed in clinical studies with HECNU.[29]

In contrast to the differential effectiveness of BCNU and HECNU towards GSSG-R in lung, liver, blood and brain, GSSG-R of the bone marrow is affected by both compounds to a similar extent. The specific sensitivity of the bone marrow towards CNUs is also reflected by a similar response of GSH-levels and GS-Tr activities. These parallel effects most probably arise as a result of generalized cytotoxicity to bone marrow cells by these myelosuppressive agents. It can be concluded that CNU-induced myelotoxicity is not depending on carbamoylation since the very weakly carbamoylating HECNU induces myelotoxicity similar to that of the potent carbamoylating agent BCNU.[30]

In summary, although carbamoylation does not appear to be important for toxicity to the bone marrow, the data suggest an important role for overall long term toxicity. Especially pulmonary toxicity induced by BCNU treatment, in many cases life-threatening or fatal, appears to correlate with carbamoylation. In clinical phase I and phase II studies no toxicity to the lung has been observed after iv. application of HECNU.

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