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# HEPATOCYTE TOXICITY OF MECHLORETHAMINE AND OTHER ALKYLATING ANTICANCER DRUGS

## ROLE OF LIPID PEROXIDATION*

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**Abstract**—The alkylating anticancer drugs, mechlorethamine (HN2), chlorambucil, cyclophosphamide, carmustine and lomustine readily induced cytotoxicity in isolated rat hepatocytes. Hepatocyte glutathione (GSH) was depleted rapidly following addition of the drugs. Lipid peroxidation ensued following GSH depletion and before cytotoxicity occurred. Furthermore, cytotoxicity was delayed by the antioxidants butylated hydroxyanisole (BHA) and α-tocopherol, the ferric iron chelator desferoxamine or the radical trap 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO) even when added 10 min later. HN2 was much less toxic to hepatocytes under nitrogen and caused much less lipid peroxidation than under aerobic conditions. Cytotoxicity induced by HN2 was also prevented by choline, suggesting that a choline carrier is responsible for HN2 uptake in the hepatocytes. Various sulfur compounds acted as antidotes for HN2 cytotoxicity. Thiosulfate was still effective when added 30 min after HN2. Depletion of GSH in the hepatocytes markedly increased their susceptibility to HN2. However, BHA, desferoxamine or TEMPO protected these hepatocytes from HN2. This suggests that antioxidants could prove useful in preventing the increased risk of hepatotoxicity if GSH-depleting agents are used to overcome tumor resistance to nitrogen mustards.

The nitrogen mustards are an important class of antitumor agents and are components of many combination therapeutic regimens. However, the clinical usefulness of these anticancer drugs is severely limited by drug resistance. Studies carried out using cultured tumor cell models have attributed this drug resistance to be due to increased detoxification as a result of elevated glutathione (GSH‡) and GSH-*S*-transferase activity [1]. Recently it has been demonstrated that the cellular resistance can be overcome by depleting GSH by DL-buthionine sulfoximine (BSO), a potent inhibitor of γ-glutamyl cysteine synthase [2].

However, depletion of GSH in the host tissue potentiates toxicity to some host tissues by alkylating anticancer drugs [3]. In the following, the molecular mechanisms of cytotoxicity by alkylating anticancer drugs, in particular mechlorethamine [*N*-methylbis(2-chloroethyl)amine, HN2, Fig. 1], on isolated hepatocytes have been investigated. It was observed that these drugs rapidly deplete GSH, and cause a marked increase in lipid peroxidation before cytotoxicity ensues. Furthermore cytotoxicity was prevented with antioxidants such as butylated hydroxyanisole (BHA) or the iron chelator desferoxamine. This suggests that antioxidants could prove useful in preventing hepatotoxicity that may occur when BSO is used to overcome tumor resistance to nitrogen mustards.

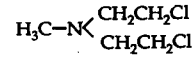

Fig. 1. Mechlorethamine [*N*-methylbis(2-chloroethyl)-amine, $HN_2$.

### MATERIALS AND METHODS

Male Sprague–Dawley rats (250–280 g) were obtained from Charles River (St. Constant, Quebec) and fed a standard chow diet (Rodent Laboratory No. 5001, Woodlyn Laboratories Ltd., Guelph, Ontario). HN2, trypan blue, dithiothreitol, BHA, and 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO) were purchased from the Aldrich Chemical Co. (Milwaukee, WS). α-Tocopherol was obtained from the Sigma Chemical Co. (St. Louis, MO). Collagenase (from *Clostridium histoliticum*), HEPES and bovine serum albumin were obtained from Boehringer-Mannheim (Montreal, Quebec). All other chemicals used were of analytical grade. HPLC grade solvents were purchased from Caledon (Georgetown, Ontario).

Hepatocytes were prepared by collagenase perfusion of the rat liver [4]. Isolated cells ($1 \times 10^6$ cells/mL) were suspended in Krebs–Henseleit buffer,

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‡ Abbreviations: GSH, glutathione; GSSG, oxidized glutathione; HN2, mechlorethamine; BHA, butylated hydroxyanisole; TEMPO, 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl; BCNU, bischloroethyl nitrosourea; CCNU, chloroethyl cyclohexyl nitrosourea; BSO, DL-buthionine sulfoximine; and MDA, malondialdehyde.

Table 1. Decrease by antioxidants of hepatocyte cytotoxicity induced by alkylating anticancer drugs

| Additions | Trypan blue uptake (%) at time 0.5 hr | 1.0 hr | 2.0 hr | 3.0 hr |
|---|---|---|---|---|
| None | 21 ± 3 | 22 ± 2 | 21 ± 2 | 23 ± 4 |
| Cyclophosphamide (1.5 mM)*, † | 28 ± 3 | 36 ± 3 | 46 ± 4 | 95 ± 5 |
| + BHA (0.1 mM) | 21 ± 2 | 31 ± 3 | 31 ± 3 | 34 ± 3 |
| Chlorambucil (4 mM)‡, § | 41 ± 3 | 60 ± 5 | 90 ± 8 | 100 |
| + BHA (0.1 mM) | 34 ± 3 | 42 ± 4 | 51 ± 5 | 63 ± 7 |
| Carmustine (BCNU; 0.15 mM)†, ‡ | 32 ± 3 | 38 ± 3 | 64 ± 5 | 92 ± 7 |
| + BHA (0.1 mM) | 30 ± 3 | 32 ± 3 | 44 ± 5 | 69 ± 7 |
| Lomustine (CCNU; 0.25 mM)*, † | 32 ± 3 | 56 ± 5 | 69 ± 6 | 100 |
| + BHA (0.1 mM) | 29 ± 3 | 36 ± 4 | 41 ± 4 | 59 ± 6 |
| Mechlorethamine (HN2; 1 mM)†, ‡ | 29 ± 3 | 49 ± 5 | 54 ± 5 | 90 ± 8 |
| + BHA (0.1 mM) | 30 ± 3 | 36 ± 4 | 42 ± 4 | 45 ± 6 |

Values are means ± SD from three separate experiments.
* Hepatocytes isolated from phenobarbital-pretreated rats.
† Hepatocytes suspended in Krebs–Henseleit buffer, pH 7.4.
‡ Hepatocytes isolated from normal rats.
§ Hepatocytes suspended in Krebs–Henseleit buffer, pH 6.5.

pH 7.4, containing 12.5 mM HEPES and incubated in rotating round bottom flasks in a water bath (37°) under an atmosphere of 10% $O_2$/85% $N_2$/5% $CO_2$ or under hypoxic conditions—95% $N_2$/5% $CO_2$. Cell viability (normally 80–90%) was determined by trypan blue inclusion (final concentration 0.16%) as described [4]. Cells were preincubated for 30 min prior to the addition of other chemicals. Control hepatocytes were unaffected even under hypoxic conditions for at least 3 hr. Glutathione-depleted hepatocytes were isolated from rats injected i.p. with BSO as described [5].

GSH and oxidized glutathione (GSSG) levels in the hepatocytes and medium were determined by the HPLC method of Reed *et al.* [6] after derivatization with iodoacetic acid and fluoro-2,4-dinitrobenzene using a μBondapak $NH_2$ column (3.9 mm i.d. × 3.0 cm). Lipid peroxidation was measured by the thiobarbituric acid test and expressed as the amount of malondialdehyde (MDA) formed using an absorption coefficient of $1.56 \times 10^5$ mol·cm$^{-1}$ at 535 nm [7].

Statistical significance of differences between treatment groups in these studies was determined by Student's *t*-test. The minimal level of significance chosen was $P < 0.05$.

## RESULTS

$HN_2$, chlorambucil, cyclophosphamide, carmustine and lomustine caused cytotoxicity to isolated hepatocytes that was prevented/delayed by BHA (Table 1). As shown in Fig. 2, the cytotoxicity of HN2 was dependent on HN2 concentration. To determine the molecular mechanism for the toxicity, different compounds in conjunction with the HN2 were added to the incubation medium to determine their ability to modulate the toxic response. As shown in Table 2, choline prevented cytotoxicity, suggesting that HN2 cytotoxicity required the uptake of HN2 by the choline transporter. The thiol

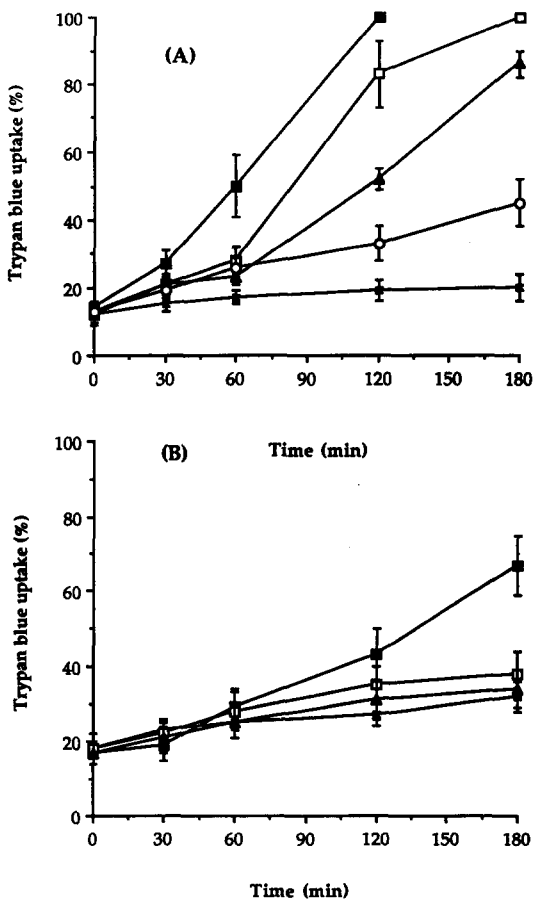


Fig. 2. Mechlorethamine (HN2) induced cytotoxicity towards isolated rat hepatocytes ($10^6$ cells/mL) under (A) aerobic conditions and (B) hypoxic conditions. Cell viability was determined by trypan blue inclusion at various time points. Key: control cells (×); HN2: 0.4 mM (○), 0.7 mM (▲), 1.0 mM (□) and 1.5 mM (■). Values are means ± SD of three separate experiments.

Table 2. Effects of modulators on the cytotoxicity of mechlorethamine (HN2) towards isolated hepatocytes under aerobic conditions

| Conditions | Trypan blue uptake (%) at time 0.5 hr | 1.0 hr | 2.0 hr | 3.0 hr |
|---|---|---|---|---|
| (a) Normal hepatocytes | | | | |
| Control | 22 ± 2 | 21 ± 3 | 23 ± 3 | 22 ± 2 |
| HN2 treated* | 22 ± 2 | 29 ± 4 | 56 ± 6 | 93 ± 6 |
| + Dithiothreitol (10 mM) at 10 min | 23 ± 3 | 23 ± 2 | 25 ± 3 | 28 ± 3 |
| + Thiosulfate (5 mM) at 10 min | 25 ± 2 | 23 ± 3 | 30 ± 3 | 35 ± 4 |
| + Thiosulfate (5 mM) at 30 min | 25 ± 2 | 30 ± 3 | 42 ± 3 | 51 ± 4 |
| + *N*-Acetylcysteine (10 mM) at 10 min | 26 ± 2 | 30 ± 4 | 40 ± 3 | 38 ± 3 |
| + *N*-Acetylcycteine (10 mM) at 30 min | 27 ± 3 | 31 ± 3 | 48 ± 4 | 85 ± 6 |
| + Choline (1 mM) at 0 min | 22 ± 2 | 29 ± 3 | 35 ± 3 | 52 ± 5 |
| + BHA (0.1 mM) at 10 min | 26 ± 3 | 26 ± 4 | 43 ± 4 | 45 ± 5 |
| + α-Tocopherol (0.1 mM) at 10 min | 24 ± 3 | 27 ± 3 | 38 ± 4 | 42 ± 3 |
| + Desferoxamine (0.2 mM) at 10 min | 25 ± 3 | 26 ± 3 | 39 ± 3 | 46 ± 5 |
| + TEMPO (1 mM) at 10 min | 22 ± 2 | 29 ± 3 | 39 ± 3 | 42 ± 4 |
| (b) GSH-depleted hepatocytes† | | | | |
| Control | 22 ± 2 | 23 ± 2 | 22 ± 3 | 24 ± 2 |
| HN2 treated* | 20 ± 2 | 44 ± 2 | 84 ± 8 | 100 |
| + BHA (0.1 mM) at 10 min | 24 ± 3 | 23 ± 4 | 34 ± 4 | 43 ± 3 |
| + Desferoxamine (0.2 mM) at 10 min | 23 ± 3 | 25 ± 3 | 31 ± 3 | 39 ± 4 |
| + TEMPO (1 mM) at 10 min | 22 ± 4 | 27 ± 2 | 34 ± 3 | 37 ± 3 |

Values are means ± SD of three separate experiments.
* Isolated hepatocytes ($10^6$ cells/mL) were incubated with 1 mM HN2.
† Hepatocyte GSH was depleted 70% beforehand by isolating hepatocytes from rats treated *in vivo* with BSO [5].

compound dithiothreitol (10 mM) added 10 min before or after HN2 also prevented cytotoxicity. Other thiol compounds such as thiosulfate and *N*-acetylcysteine when added to the hepatocytes 10 min after the HN2 also reduced cytotoxicity. Furthermore, thiosulfate but not *N*-acetylcysteine were still effective as an HN2 antidote when added 30 min after HN2.

Because thiol compounds prevented cytotoxicity, the effects of HN2 on hepatocyte GSH levels were measured. As shown in Fig. 3, hepatocyte GSH was depleted 50% in 10 min without any increase in GSSG levels by a cytotoxic concentration of HN2. Hepatocyte GSH levels were depleted 85% by 1 hr but cytotoxicity did not ensue until 2 hr. *In vitro* studies also revealed that HN2 reacted with GSH to form GSH conjugates (data not shown). Because of the involvement of GSH in HN2 metabolism, the effect of depleting GSH on hepatocyte susceptibility to HN2 was investigated. As shown in Table 2, depleting hepatocyte GSH with buthionine sulfoximine beforehand significantly increased the HN2 susceptibility towards isolated hepatocytes. GSH levels were also depleted before cytotoxicity ensued with chlorambucil, cyclophosphamide, carmustine and lomustine (results not shown).

In HN2-treated hepatocyes, lipid peroxidation (as determined by MDA formation) increased in a time-dependent manner (Fig. 4). MDA formation was much more rapid with GSH-depleted hepatocytes. The antioxidant BHA or the iron chelator desferoxamine prevented lipid peroxidation (Fig. 4). Furthermore, the antioxidants BHA and α-tocopherol or the iron chelator desferoxamine or the radical trap TEMPO also prevented or delayed

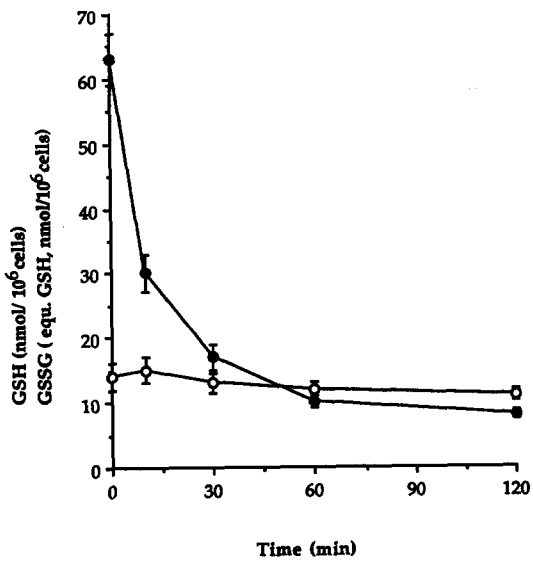


Fig. 3. GSH depletion induced in isolated rat hepatocytes by HN2 (1.0 mM). Key: GSH (●) and GSSG (○). GSH and GSSG were determined by HPLC as described in Materials and Methods. Values are means ± SD of three separate experiments.

HN2 cytotoxicity in both normal and GSH-depleted hepatocytes over a 3-hr period (Table 2). Also, as shown in Fig. 2B, hepatocytes were much more resistant to HN2 under hypoxic conditions. Lipid

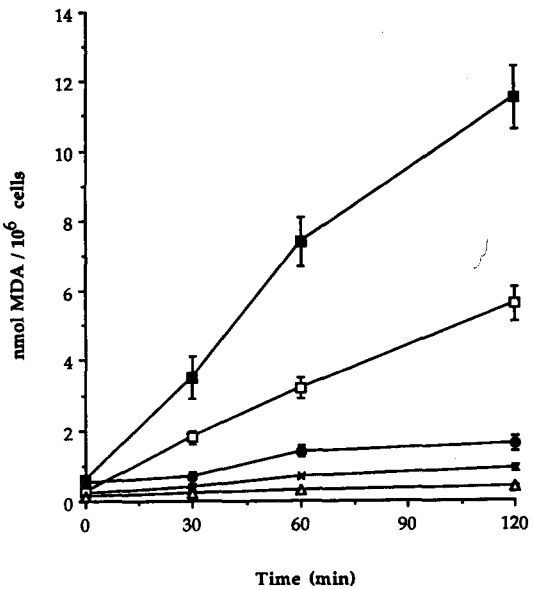


Fig. 4. Lipid peroxidation (MDA formation) induced in isolated rat hepatocytes by HN2 (1.0 mM). Key: control cells (×), HN2 (□), HN2 + BHA (△); and GSH-depleted cells (●), + HN2 (■). Values are means ± SD of three separate experiments.

peroxidation (MDA formation) was also decreased markedly under hypoxic conditions (results not shown).

## DISCUSSION

Nitrogen mustards used as antineoplastic agents are believed to act by alkylating DNA which causes DNA strand cross-linking, thereby interfering with normal mitosis and cell division in rapidly proliferating tumor cells. Although little work has been carried out on the cellular metabolism of these drugs, recent studies have suggested that glutathione levels and enzymes related to glutathione metabolism may be important variables affecting drug cytotoxicity.

Although GSH conjugate formation with bifunctional nitrogen mustards has been reported previously [8–10], it is not clear to what extent GSH plays a role in modulating toxicity induced by these drugs. Cancer cells displaying resistance to these alkylating agents often have an increased intracellular concentration of GSH and a higher activity of glutathione-*S*-transferase(s) [11]. Increased glutathione-*S*-transferase(s) levels also paralleled amplification of certain transferase genes [12]. Therefore, the effect of depleting GSH on hepatocyte susceptibility to HN2 is of importance because of the interest in using GSH-depleting agents to overcome resistance to alkylating anticancer agents [2]. The above results with isolated hepatocytes show that HN2 caused GSH depletion well before cytotoxicity ensued. Depleting hepatocyte GSH before HN2 addition markedly enhanced cytotoxicity, showing that GSH plays a major role in HN2 detoxification.

To further determine the molecular cytotoxic mechanisms involved, various compounds were tested for antidotal activity by adding them 10–30 min after HN2 treatment. The thiol compounds dithiothreitol and *N*-acetylcysteine were highly effective at preventing cytotoxicity. Presumably they act as nucleophiles which conjugate to the cytotoxic aziridinium intermediate [13] formed when HN2 hydrolyses in aqueous solution to 2-hydroxyethyl-2-chloroethylmethylamine and bis-2-hydroxyethylmethylamine [14]. Thiosulfate was also very effective at preventing cytotoxicity even when added 30 min after HN2 treatment. Previously thiosulfate was shown to be very reactive towards HN2 and protected very effectively against HN2 toxicity in rats [15]. HN2 is transported into tumor cells by the choline transport carrier for choline, presumably because choline is a close structural analog of HN2 [16]. That choline at equimolar concentrations to HN2 also protected hepatocytes against HN2 indicates that the cytotoxic mechanism of HN2 requires HN2 uptake.

Lipid peroxidation as measured by MDA formation occurred in the hepatocytes 30–60 min after HN2 treatment and before cytotoxicity was apparent. Lipid peroxidation did not occur in the presence of BHA, α-tocopherol or desferoxamine and cytotoxicity was prevented even when added some time after HN2 treatment. However, lipid peroxidation occurred earlier and before 30 min when GSH-depleted hepatocytes were treated with HN2, suggesting that GSH plays an antioxidant role in hepatocytes. Furthermore, the protection of hepatocytes from HN2 by the nitroxide radical TEMPO [17] suggests that the lipid peroxidation is initiated by radicals formed during HN2 metabolism. The lack of cyanide-resistant respiration induced by HN2 (results not shown) suggests that activated oxygen radicals are not responsible for the TEMPO effect. Initiating radicals could therefore result from the one electron oxidative N-demethylation of HN2 [18] or reductive dechlorination of the mustard moiety.

Depletion of GSH levels in tumor cells with acquired drug resistance by BSO, an inhibitor of γ-glutamyl cysteine synthase, results in reversal of the resistance to nitrogen mustards [19–21]. The results presented here suggest that the current use of BSO in clinical trials to deplete tumor GSH levels so as to increase the efficacy of nitrogen mustard drugs against tumor cells exhibiting acquired drug resistance could increase the risk of tissue damage, e.g. hepatotoxicity. Depletion of GSH levels by BSO *in vivo* was shown previously to markedly increase hepatotoxicity induced by the anticancer drugs melphalan, BCNU or cyclophosphamide in mice [3] but the toxicity mechanism was not investigated. In the studies presented here, hepatocytes whose GSH had been depleted by BSO were also found to be more susceptible to HN2 probably as a result of increased lipid peroxidation. By contrast the susceptibility of tumor cells such as murine L5178Y lymphoma cells to HN2 was not affected by antioxidants and lipid peroxidation was

not induced (results not shown). Tumor cells in general are not susceptible to lipid peroxidation [22] and DNA cross-linking is believed to be responsible for the inhibition of tumor cell division by alkylating drugs [23]. Thus, protection of normal cells against lipid peroxidation induced cytotoxicity by antioxidants or chelators may prevent tissue toxicity without affecting the cytotoxicity of alkylating drugs towards tumor cells.

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