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Effects of the New Nitrosourea Derivative, Fotemustine, on the Glutathione Reductase Activity in Rat Tissues *In Vivo* and in Isolated Rat Hepatocytes

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Abstract—*Fotemustine, a new clinically active nitrosourea, is demonstrated herein to be a poor inhibitor of glutathione reductase activity from rat liver, lung and kidney cytosols. In order to show that an intracellular step of activation does not lead to a toxic intermediary metabolite, rat hepatocytes were incubated with fotemustine. Their glutathione-related pathways were checked and shown not to be altered, while under similar experimental conditions BCNU was shown to be dramatically harmful. Furthermore, association of fotemustine with a H_2O_2 production leading drug, diquat, was shown to be inefficient—while BCNU is efficient—in potentiating the diquat toxicity. Considering the role of glutathione level in the detoxification of mutagens and carcinogens, the advantage of foemustine over BCNU in therapeutic use seems substantiated.*

INTRODUCTION

AMONGST the cytotoxic drugs used as clinical anticancer agents, nitrosoureas form a major class of compounds. It has been established that their antitumor activity is mainly mediated through deoxyribonucleic acid alkylation [1–4]. The side-effects of these compounds (cumulative bone marrow, pulmonary and renal toxicities), limit their use in the clinic [5–7]. It has been hypothesized that some of these toxicities are correlated to a specific inhibition of glutathione reductase (GR; EC 1.6.4.2) [8, 9]. This enzyme plays a key role in the glutathione pathway and contributes to the maintenance of the intracellular pool of glutathione responsible for detoxification of xenobiotics and chemical alkylating species [10, 11].

Fotemustine [12] is a novel nitrosourea currently in phase III clinical trials which has already shown activity against malignant melanoma [13]. Both in preclinical (Cudennec, unpublished data) and therapeutic studies [14] this compound has demonstrated a far less general toxicity than classical nitrosoureas.

The question then arose of whether a low glutathione reductase inhibiting ability could account for the weak toxicity of fotemustine. The present study reports experimental evidence that fotemustine is devoid of specific inhibiting properties on several rat tissue-derived glutathione reductases both *in vivo* and *in vitro*.

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MATERIALS AND METHODS

Treatment, subcellular fractionation and isolated hepatocytes experiments

Fischer F344 male rats (Iffa-Credo), weighing 300–330 g were treated by i.p. injection (2 ml) of the compounds (either fotemustine or BCNU, 30 mg/kg) in suspension in hydroxypropylcellulose solution (0.2% in 0.15 M NaCl). A control group of animals received the vehicle only, under the same conditions. Twenty-four hours after treatment, the animals were sacrificed by decapitation. The organs (liver, kidneys and lungs) were rapidly removed and extensively washed in a TES buffer (Tris 50 mM; pH 8.6; EDTA 1 mM; sucrose 250 mM). The organs were homogenized in TES using an Ultraturrax, then submitted to a low-speed centrifugation (500 ***g***, 60 min at 4°C) and cytosols were obtained by $CaCl_2$ precipitation of the microsomal fraction by adding to the homogenates 20% (v/v) of a $CaCl_2$ solution (0.1 M) under constant stirring for 30 min and then centrifugated at 500 ***g*** for 60 min at 4°C.

A second set of experiments were done in which animals were sacrificed 1–7 days after a single treament with fotemustine or BCNU (20 mg/kg,

i.p.). A third group of animals was treated with 10, 20, 40, 60 or 80 mg/kg i.p. of fotemustine or BCNU and sacrificed after 24 h. Controls were treated by the vehicle only under identical schedules. In the last two experimental groups, cytosols were prepared from the lungs by the method described above.

A fourth set of experiments was conducted on isolated hepatocytes. Hepatocytes were prepared from male Sprague–Dawley rats by the collagenase technique described by Moldeus *et al.* [15]. One million cells per ml were incubated in rotating round-bottomed flasks in Krebs–Hepes buffer, pH 7.4 under a 95% O_2 and 5% CO_2 atmosphere. The viability of hepatocytes was assessed by the trypan blue exclusion test as described by Moldeus *et al.* [15].

Biochemical analysis

For the cytosolic GR, activities were measured by the method of Zanetti [16] by following spectrometrically the oxidation of NAD(P)H at 340 nm at 30°C. Protein concentration was evaluated by the method of Lowry *et al.* [17] using bovine serum albumin as standard.

For the hepatocyte experiments, BCNU and fotemustine were dissolved in ethanol (<0.5% final concentration). GR activity was measured as described by Eklöw *et al.* [18]. Reduced glutathione (GSH) was determined by HPLC as monobromobimane adducts according to Cotgreave and Moldeus [19] while glutathione disulfide (GSSG) was determined by the method of Reed *et al.* [20]. Lipid peroxidation was estimated by the measurement of malondialdehyde formation through the thiobarbiturate procedure [21].

RESULTS

In vivo *treatment by nitrosoureas*

At a single dose under i.p. treatment, BCNU and fotemustine exhibit an optimal antitumor activity at 30 mg/kg body wt. Table 1 indicates the GR activity in the three organs removed from treated animals 24 h after administration of such an amount of drugs. While GR activity was drastically depressed after BCNU, it remains roughly unchanged after fotemustine treatment.

Figure 1 shows the effect on rat pulmonary GR activity of a single i.p. treatment by 20 mg/kg of BCNU or fotemustine during 7 days after the administration. At no time was the enzymatic activity inhibited by fotemustine pretreatment. *A contrario*, BCNU-treated samples demonstrated a noteworthy weak activity as soon as day 1 after treatment. Low values lasted up to 1 week after the BCNU injection.

Figure 2 depicts the results of a dose–effect study of BCNU or fotemustine on pulmonary GR, 24 h after i.p. administration. Toxicological data carried out prior to these experiments indicated that, under identical test conditions, LD_{50}s were about 40 mg/kg for BCNU and 50 mg/kg for fotemustine (data not shown). Maximal values of dosage in the present experiment (80 mg/kg) thus represent highly toxic amounts from a general toxicological point of view. Only a slight reduction of GR activity occurred after 60 or 80 mg/kg of fotemustine, while more than 60% GR activity were inhibited after treatment with as few as 10 mg/kg BCNU (a dosage with

Table 1. Effects of 30 mg/kg *BCNU or fotemustine on glutathione reductase activities from rat organs,* 24 h *after i.p. treatment*

Organs	Glutathione reductase activities (μmol/min/mg proteins)		
	Controls*	BCNU* treated	Fotemustine* treated
Liver	15.3 ± 0.8	7.6 ± 0.5	13.9 ± 1.7
Lungs	3.1 ± 0.4	0.8 ± 0.1	4.73 ± 1.1
Kidneys	9.6 ± 0.9	2.9 ± 0.3	8.3 ± 0.5

*Four animals per group. Activities are expressed as means ± S.E.M.

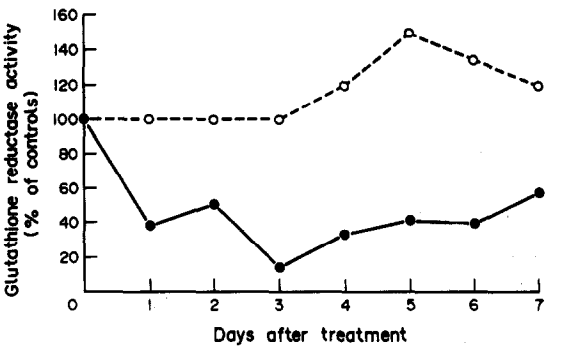

Fig. 1. Pulmonary glutathione reductase activity after a single i.p. treatment by 20 mg/kg *BCNU* (●—●) *or fotemustine* (O---O).

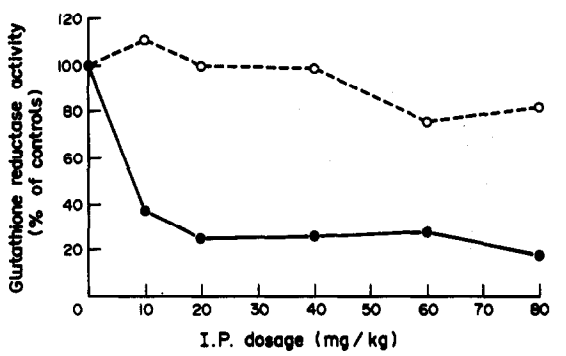

Fig. 2. Comparative dose–effect analysis of i.p. administered BCNU (●—●) *and fotemustine* (O---O) *on pulmonary glutathione reductase in rat* (24 h *after injection*).

borderline antitumor activity). No marked increased inhibition was seen for amounts greater than 20 mg/kg BCNU.

In vitro *treatment of rat isolated hepatocytes by nitrosoureas*

Rat hepatocytes at a density of 10^6 cells per ml were exposed to 50 or 100 μM fotemustine or 50 μM BCNU for 2 h at 37°C. Reduced glutathione (GSH) content, GR activity and cell viability were followed during this experiment and reported in Fig. 3.

In vitro treatment by drugs appears to produce a similar effect as *in vivo* on GR activity (Fig. 3a). No gross variation of GR occurred during the treatment with fotemustine, but 30 min at 50 μM BCNU totally abolished this enzymatic activity.

Intracellular GSH measurements (Fig. 3b) indicate that 50 μM fotemustine was without a deleterious effect on the GSH pool. In the presence of 100 μM fotemustine, a minor decrease of GSH was recorded from 30 min onward, which never exceeded 30% of control values. BCNU (50 μM) was demonstrated in this experiment to drastically lower the GSH pool as soon as 15 min after the onset of the treatment.

As already shown, the nitrosourea-induced cytotoxicity developed during the course of this experiment much faster for BCNU than for fotemustine (Fig. 3c).

To obtain further insight into glutathione metabolism–nitrosourea interactions, we compared the effects of fotemustine (100 μM) and BCNU (50 μM) on diquat-induced (1 mM) (i) GSH oxidation assessed through GSH (Fig. 4a) and GSSG (Fig. 4b) content measurements; (ii) lipid peroxidation evaluated by the MDA method (Fig. 4c); and (iii) cytotoxicity (Fig. 4d). The potentiating effect of BCNU on diquat toxicity was confirmed in the present experiment whatever the parameter considered. The only promoting effect of fotemustine was an initial decrease in intracellular GSH (Fig. 4a), which was not enhanced by further incubation. Fotemustine co-treatment does not provoke a substantial GSSG increase, lipid peroxidation or cytotoxicity with diquat as compared to the control.

DISCUSSION

The present study deals with the comparative effect of BCNU and fotemustine, two nitrosoureas active as antitumor agents in the clinic, on glutathione metabolism. Both compounds were used *in vivo* in rats in order to measure potentially deleterious consequences of the treatments on lung, liver and kidney GR, and *in vitro* on isolated rat hepatocytes in which GSH/GSSG pools were measured and GR activity assessed.

The overall results clearly demonstrate that—in the range of dosage used for fotemustine—this new

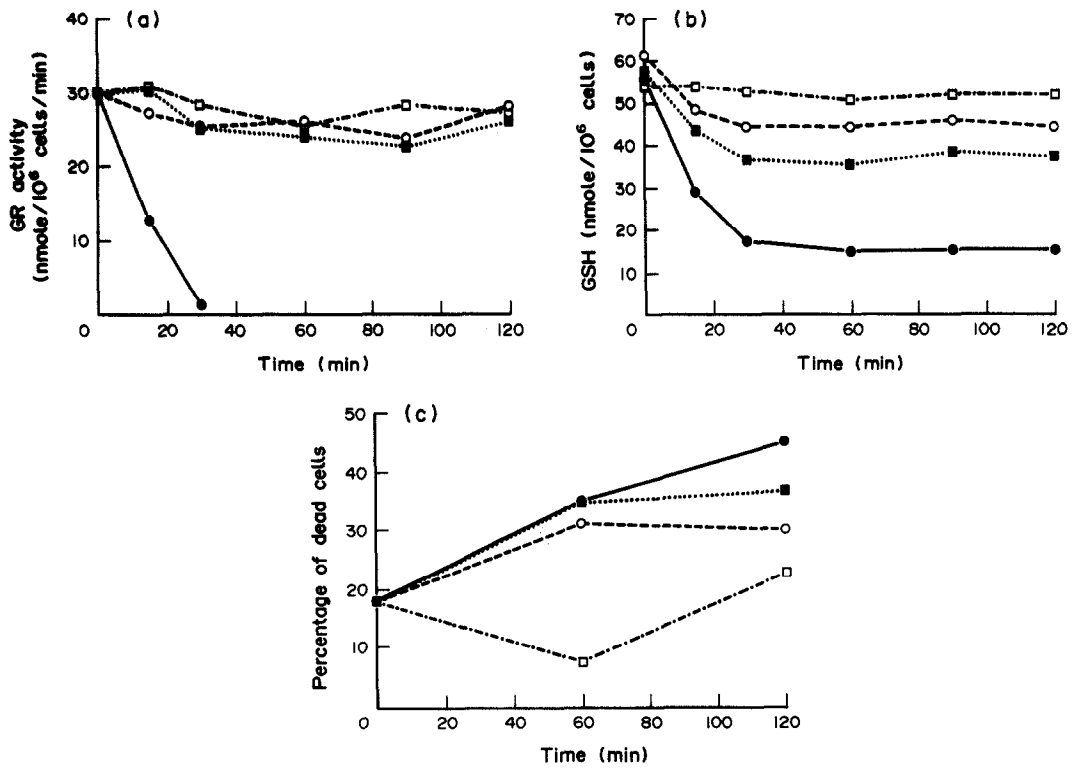

Fig. 3. In vitro *effects of fotemustine and BCNU on glutathione reductase activity (a), GSH pool (b) and viability of isolated rat hepatocytes (c). Isolated cells were exposed either to* 50 μM *BCNU* (●—●), 50 μM *fotemustine* (○---○) 100 μM *fotemustine* (■···■), *untreated cells* (□·····□) *were processed in parallel.*

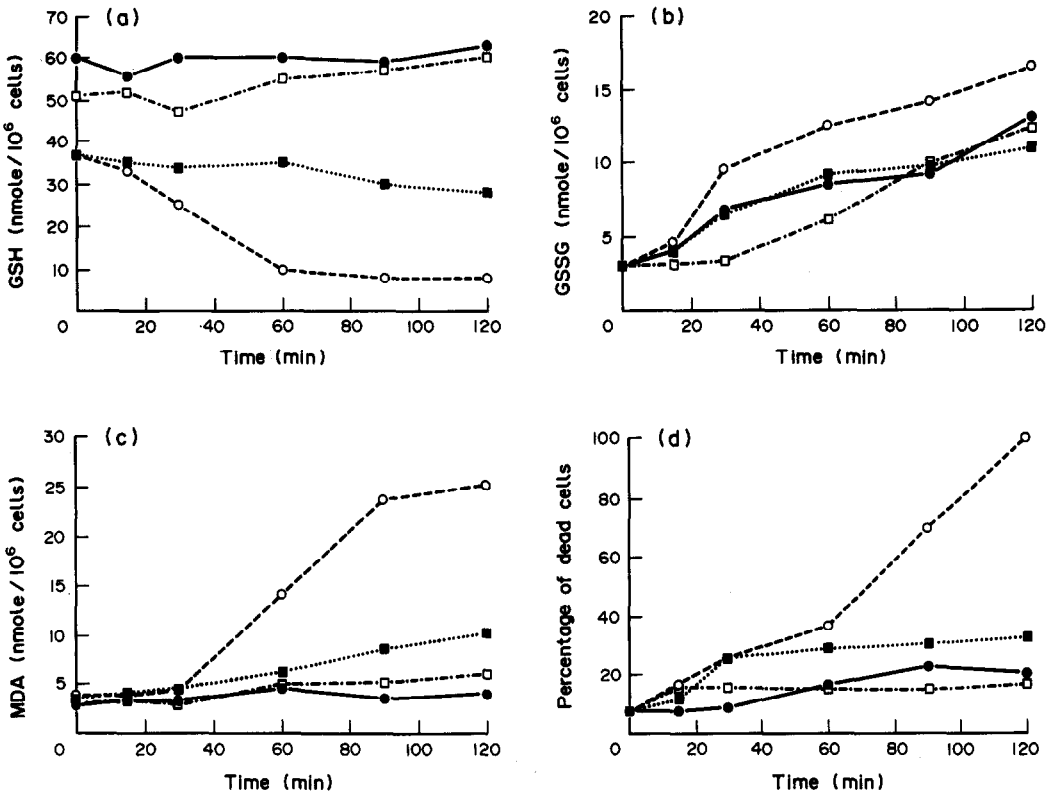

Fig. 4. In vitro *effects of co-treated of rat hepatocytes by diquat* (1 mM *and fotemustine* (100 μM) *or BCNU* (50 μM). *Cellular GSH content* (*a*), *GSSG content* (*b*), *lipid peroxidation* (*c*) *and toxicity* (*d*) *were measured* (*control* □—·—·—□; *diquat* ●—●; *BCNU + diquat* ○— — —○; *fotemustine + diquat* ■···■).

antitumor agent is devoid of a specific inhibiting property on GSH pools and GR activity. Experiments carried out in parallel confirm the high toxicity of BCNU on these parameters as previously described by Maker *et al.* [22] and Cohen and Duvel [9]. It is noteworthy to consider that fotemustine did not demonstrate a toxic effect even at a dosage much higher than the lethal dose, the death of animals occuring in this case on days 6–9 after the treatment. On the contrary, BCNU displays its irreversible noxiousness on glutathione metabolism at dosages where the general toxicity is weak and antitumor potency borderline.

The harmfulness of BCNU-induced perturbations on glutathione metabolism appears clearly in experiments involving diquat. Diquat undergoes rapid redox cycling with concomitant production of superoxide and hydrogen peroxide [23]. Diquat is a model compound, since other xenobiotics were shown to produce similar effects [24]. In hepatocytes, the glutathione peroxidase/reductase system is able to handle the H_2O_2 formed and reduces it to water. Inhibition of the glutathione reductase and thus the decrease in GSSG reduction results in rapid depletion of GSH and increase in H_2O_2. Hydroxyl radicals are formed through heterocyclic cleavage at H_2O_2 and these radicals are presumably responsible for the toxicity of diquat in the presence of BCNU [23]. This potentiating effect of BCNU on diquat toxicity was coonfirmed in the present investigation. In the presence of BCNU, there was an increased loss of GSH, partly detectable as GSSG accumulation and massive lipid peroxidation within the hepatocytes.

On the other hand, in the presence of fotemustine, there was a minor increase in diquat-induced toxicity at the onset of the incubation. No further increase in toxicity was however observed after 30 min of incubation. The minor initial increase in toxicity is obviously not due to GR inhibition since there was no loss of GSH or increased lipid peroxidation.

The BCNU-induced inhibition of GR [25] as well as other enzymatic species [26, 27] was thought to be mediated through carbamoylation [4]. Therefore, it was hoped that the synthesis of less carbamoylating nitrosoureas will lead to less toxic compounds such as 3-hydroxy ethyl chloroethyl nitrosourea (HECNU) [28, 29]. On the other hand, the inhibition of GR appears not to be mandatory for antitumor potency. Fotemustine which was demonstrated in this study unable to modify glutathione metabolism and which was already shown in experimental models as well as in clinic to be a potent novel anticancer agent, is evidence that glutathione suppression is not a prerequisite or, at least, a

beneficial property in the basic mode of action of efficient antitumor nitrosoureas.

Considering that co-treatments with various other drugs may occur, as supporting care, during a nitrosourea-containing chemotherapy regimen, it would be of interest to maintain operative superoxide and hydrogen peroxide detoxifying mechanisms. This is valid even if Adriamycin® (ADR)—an active oxygen species producing compound—is associated with nitrosoureas in a polychemotherapy regimen, since it was previously established by Romine and Kessel [30] that ADR antitumor activity is not dependent on intracellular GSH concentration.

In addition, as it was demonstrated [31] that intracellular GSH may protect against carcinogenic events, it appears safer to use antitumor compounds devoid of deleterious properties against glutathione metabolism. This advantage of fotemustine may partly explain its low genetic toxicity, as reported elsewhere [32].

Fotemustine was previously shown (Cudennec, unpublished data) to be able to carbamoylate lysine in the standard Wheeler assay [33]. Prior to the present study, it could thus be thought to be a candidate for GR inhibition. It is not. As established recently by Karplus *et al.* [34], the cysteine in position 58 of the human GR enzyme appears to be the target for GR-inhibiting nitrosoureas such as BCNU. It will be of interest to know whether the accessibility of such a site to nitrosoureas (or its equivalent in rat GR) might govern the GR-inhibiting capability of these antitumor agents. A comparative analysis of the structural interactions of BCNU or fotemustine with GR could then be carried out in order to determine the validity of this hypothesis.

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