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THE ROLE OF ISOCYANATES IN THE TOXICITY OF ANTITUMOUR HALOALKYLNITROSOUREAS

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Abstract—The cytotoxicity of the antitumour nitrosoureas BCNU and CCNU and the isocyanates which they liberate (chloroethylisocyanate and cyclohexylisocyanate respectively) has been measured utilising an *in vitro–in vivo* bioassay. Lines of the TLX5 lymphoma and L1210 leukaemia were used which were either sensitive or resistant to nitrosoureas *in vivo*. An estimated logarithmic cell kill produced by each compound *in vitro* (before injecting the cells into animals) was calculated by reference to assays of the survival time of animals given from 2×10^5 to 2×10^0 cells of each line. Resistance to both BCNU and CCNU was observed *in vitro* in the cell lines of the TLX5 lymphoma made resistant to either BCNU or a dimethyltriazene *in vivo*. The latter tumour was cross-resistant *in vivo* to nitrosoureas. Resistance *in vitro* to nitrosoureas was also observed in a line of L1210 leukaemia which had had resistance to BCNU induced *in vivo*. The nitrosourea resistant TLX5 lymphomas were cross-resistant *in vitro* to both cyclohexylisocyanate and chloroethylisocyanate whereas the nitrosourea resistant L1210 line showed no cross-resistance to cyclohexylisocyanate and marginal cross-resistance to chloroethylisocyanate. The results suggest that the TLX5 lymphoma, which is naturally resistant to alkylating agents of the 2-chloroethylamine type, may be sensitive *in vivo* to nitrosoureas as a consequence of the intracellular release of isocyanates. This hypothesis was supported by the finding that the resistant TLX5 lymphoma showed no cross-resistance to other electrophilic agents, for example formaldehyde, monomethyltriazene or HN2. The transport of nitrosoureas into the sensitive and resistant cell lines was similar in profile and there was no difference in the concentration of non-protein thiols.

The antitumour nitrosoureas, such as BCNU and CCNU, are generally considered to exert their cytotoxicity via the alkylation and cross-linking of DNA [1], a two-stage reaction which occurs when the nitrosoureas cleave spontaneously (Fig. 1) to liberate a 2-halogenoethyldiazohydroxide (II) and an isocyanate (III). The 2-halogenoethyldiazohydroxide reacts with nucleic acids whereas the isocyanate moiety does not, but instead reacts preferentially with cellular proteins by carbamoylating them [2]. The role of isocyanates in the cytotoxicity of nitrosoureas remains largely speculative [1] but is considered to be of minor importance since nitrosoureas which have been designed to have low carbamoylating activity still retain their antitumour effects [3].

In studies of the mechanism of action of other classes of antitumour agents which similarly cleave to release a number of reactive species, each being potentially capable of exerting cytotoxic effects, we have chosen to compare the *in vitro* cytotoxicity of each isolated species to cell lines which are either sensitive or resistant to the progenitor compound *in vivo*. It has previously been argued by us that if a cytotoxic species is observed to preferentially kill cells of sensitive lines *in vitro* in comparison to those which are resistant then this species is likely to be responsible for the elements of selective toxicity, or antitumour effect, observed *in vivo*. On the other hand, when a species has equivalent *in vitro* cytotoxicity to both the sensitive and resistant lines it was argued that this represents a non-selective cytotoxicity [4].

In this study we report the results of such experi-

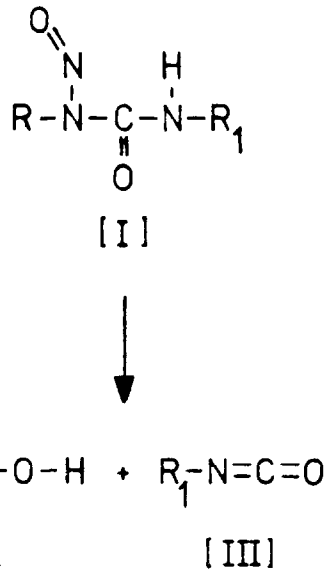

Fig. 1. The decomposition of alkylnitrosoureas (I) to yield an alkyl diazohydroxide (II) and isocyanate (III). BCNU: $R = R_1 = CH_2CH_2Cl$; CCNU: $R = CH_2CH_2Cl$, R = cyclohexyl (C_6H_{11}); CLZ: $R = CH_2CH_2Cl$, R_1 = 2-D-glucopyranosyl; ACNU: $R = CH_2CH_2Cl$, R_1 = 1-(4-amino-2-methylpyrimidin-5-yl)methyl.

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ments which were aimed at assessing the contribution which chloroethylisocyanate and cyclohexylisocyanate, derived from the breakdown of BCNU and CCNU respectively, may make towards the selective toxicity which these nitrosoureas demonstrate *in vivo* to the TLX5 lymphoma and L1210 leukaemia.

MATERIALS AND METHODS

Materials. CCNU, [^{14}C]cyclohexyl-CCNU (11.7 mCi/mmole) and BCNU were obtained as generous gifts from the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD), CLZ was the gift of Dr. K. Tew, Georgetown University, Washington, DC, nitrogen mustard (HN2) from the Boots Company (Nottingham, U.K.), cyclohexylisocyanate and chloroethylisocyanate were obtained from Aldrich Chemicals Ltd. (Gillingham, U.K.) and Eastman–Kodak Ltd. (Kirkby, U.K.) respectively. All other chemicals were purchased from Sigma U.K. Ltd. (Poole, U.K.).

Tumours. The TLX5 lymphomas sensitive and resistant to either BCNU or ethyl-5-(3,3-dimethyl-1-triazeno)-2-phenylimidazole-4-carboxylate have been described previously [4, 5] and were obtained from the Chester Beatty Research Institute, London. The L1210 leukaemia sensitive to nitrosoureas was obtained from Dr. G. Atassi, Institute Jules Bordet, Brussels, and that resistant to BCNU from Dr. A. E. Bogden, Mason Research Institute, Worcester, Massachusetts. The TLX5 lymphomas were passaged every 7 days as ascites in male CBA mice and the L1210 leukaemias similarly in male BDF_1 mice.

Antitumour tests and in vitro–in vivo *bioassays.* The methods have been described fully elsewhere [4] for the TLX5 lymphomas, and exactly the same procedures were utilised here with the L1210 leukaemias using female BDF_1 mice as hosts. The measurement of survival time of animals which had received 2×10^5 to 2×10^0 cells was made after cells had been incubated under the conditions of the *in vitro–in vivo* bioassay. *In vivo* antitumour tests were performed in all cases with subcutaneous tumours and the drugs were administered i.p. as a single dose on the third day after transplantation.

Non-protein thiol assay. Tumour cells were harvested from a routine passage as ascites and washed with cell-lysis medium [6]. After centrifugation at 2000 *g* for 5 min the tumour cell pellet was homogenised in 5% trichloracetic acid (5 ml/g cells). The preparations were centrifuged at 300 *g* for 10 min and 0.5 ml of the supernatant added to 3.2 ml 0.4 M phosphate buffer (pH 8.0) before the addition of 0.3 ml of 0.01 M 5,5′-dithiobis-(2-nitrobenzoic acid) in phosphate buffer [7]. Duplicate samples were incubated at room temperature for 30 min and the absorbance measured at 412 nm using a Cecil CE 5095 spectrophotometer. Standards prepared with reduced glutathione were assayed concurrently and protein content was determined by the method of Lowry *et al.* [8] using bovine serum albumin as the standard.

Transport studies. 10^7 cells/ml of either the TLX5 lymphomas or L1210 leukaemias were incubated in RPMI 1640 medium for 0.25 hr before the addition of 0.88 μCi/ml of [^{14}C]cyclohexyl CCNU. 10^6 cells were removed at time periods between 0 and 120 min and were placed into tubes in which 100 μl of a mixture of Dow Corning 550 silicon oil and corn oil (10 parts to 3) was layered above 50 μl of 98% formic acid. The tubes were centrifuged at 9000 *g* for 2 min using a Beckman microfuge B, frozen in liquid nitrogen and then cut across the oil layer. Each part was placed into scintillation vials, thawed, 10 ml NEN 260 was added as scintillant, and samples were counted using a Packard Tricarb 2606 scintillation counter. Preliminary experiments had shown that there was less than 0.5% carryover of entrapped extracellular media and more than 95% carryover of either leukaemia or lymphoma cells through the oil layer into the formic acid.

RESULTS

Serial logarithmic dilutions of the sensitive and resistant lines of both the TLX5 lymphoma and L1210 leukaemia and their injection into animals, after incubation *in vitro* under 'bioassay' conditions, showed that a linear inverse relationship exists between the survival time of the host and the number of cells injected (Fig. 2 shows an example of the TLX5 lymphoma and the line resistant to dimethyltriazenes). In the bioassays the control animals receive 2×10^5 untreated cells and the percentage increase in survival time for this number of cells is thus 0%. After calculation of the percentage increase in survival time for each log of cells injected, the relationship between log cell kill, percentage increase in survival time and drug concentration may be calculated. Figs. 3 A–F show such relationships with the data presented to show the concentration of drug required to give each log cell kill, by extrapolation. The results are the means of at least two experiments.

Each tumour line which was resistant *in vivo* to the nitrosoureas BCNU and CCNU (Table 1) was also resistant *in vitro* (Figs. 3 A–F). In the case of the two TLX5 lymphomas, made resistant *in vivo* to BCNU or to a dimethyltriazene [4, 5] there was

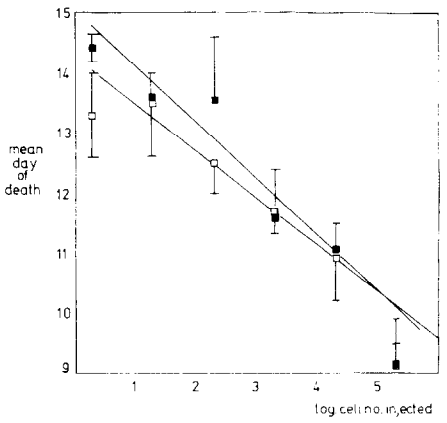

Fig. 2. An example of the relationship between the number of cells injected into host animals and their survival. Lines were fitted by the method of linear regression. TLX5S = ■, $r = -0.952$, TLX5RT = □, $r = -0.952$ ($n = 3$).

Table 1. Results of antitumour tests performed with BCNU, CCNU and CLZ using sensitive or resistant lines of the TLX5 lymphoma and L1210 leukaemia

Compound	Dose (mg/kg)	% Increase in survival time TLX5S	TLX5RT	TLX5RB	L1210S	L1210RB
BCNU	5	31		5	11	−2
	10	46	4	−3	24	2
	20	54	9	−1	54	15
	40	60	10	1	>200	9
	80	24	5	−20	−9	−22
CCNU	5	28		−1	22	0
	10	53	7	−1	46	9
	20	57	6	−1	102	9
	40	49	14	5	>200	30
	80	−2	−5	−13	−10	−9
CLZ	5	0	2			
	10	12	4		11	
	20	32	4		32	
	40	−8	−13		−32	
	80	−24	−27		−36	

S = sensitive line; RT = resistance induced to a dimethyltriazene; RB = resistance induced to BCNU.

cross-resistance to isocyanates (Figs. 3 A–D). This was not so with the L1210 resistant line in the case of cyclohexylisocyanate (Fig. 3 F) although some cross-resistance to chloroethylisocyanate was observed (Fig. 3 E). In an *in vivo* antitumour test of CLZ (Fig. 1) against the TLX5 lymphoma and L1210 leukaemia marginal but equivalent activity was observed (Table 1).

The finding that the TLX5 lymphomas were cross-resistant *in vitro* to the isocyanates, particularly to the non-alkylating isocyanate cyclohexylisocyanate, suggested that this cross-resistance might be simply due to an increased concentration of cellular nucleophiles, as is the case on induction of resistance to alkylating agents when the level of non-protein thiols become elevated [9]. Measurement of the non-protein thiol content of the sensitive and resistant cell lines of the TLX5 lymphomas showed no significant difference between them (Table 2). In addition, no cross-resistance pattern between the nitrosoureas and other electrophilic species was observed, for example nitrogen mustard (Fig. 4) or formaldehyde (Fig. 5). In fact, it can be seen that the TLX5 resistant to the nitrosoureas *in vitro* is more sensitive to the effects of the alkylating agent HN2 (Fig. 4), a result similar to that reported *in vivo* by others [5]. We have previously reported that a methylating agent, a monomethyltriazene, has equal toxicity to the sensitive and resistant lines used here [4].

The transport of the [^{14}C]cyclohexyl CCNU was similar in both sensitive and resistant cell lines of the TLX5 lymphoma and L1210 leukaemia (Table 3). It was reported that the binding of both the alkylating and carbamoylating moieties of CCNU to cellular macromolecules was the same in the TLX5 lymphomas sensitive or resistant to nitrosoureas *in vivo* [10].

DISCUSSION

This study, in common with others, has demonstrated that the nitrosoureas are directly-acting cytotoxic drugs *in vitro* to cell lines which are sensitive to a single dose *in vivo* (Table 1, Figs. 3 A–F). In addition it has been shown that the induced *in vivo* resistance to nitrosoureas of certain of these lines can be estimated *in vitro*. The advantage of the *in vitro–in vivo* bioassay system used here is that tests were performed on cells which have not been selected for long-term growth *in vitro* but are instead the same as those used in the *in vivo* antitumour tests.

Using logarithmic dilutions of cells from each line (TLX5 lymphoma, L1210 leukaemia, sensitive or resistant to nitrosoureas *in vivo*) estimates were made of an apparent logarithmic cell kill achieved *in vitro*. This method assumes that increases in survival time of animals which have received treated cells are due to a reduction of viable cells rather than a slowing of cell cycle time or a change of immunogenicity, an assumption which is also applicable to the standard *in vivo* antitumour tests which measure survival time. Clonogenic assays performed *in vitro* (rather than *in vivo*, which is what this bioassay essentially does) may be able to more precisely quantitate cytotoxicity but suffer from the problem that cellular adaptation and selection may be necessary for growth *in vitro*. In the case of the L1210 leukaemia, for example, mercaptoethanol treatment of ascites cells was required in order to obtain growth *in vitro* [11] and we have anyway experienced difficulties in establishing a clonogenic assay for the TLX5 lymphoma.

The cross-resistance of the TLX5 lymphoma *in vitro* to the nitrosoureas BCNU and CCNU and their respective isocyanates was a surprising result (Figs. 3 A–D). This tumour is not sensitive to alkylating agents of the 2-chloroethylamine type *in vivo* [5], but is sensitive to the nitrosoureas (Table 1). It is widely accepted that although the nitrosoureas may alkylate cellular constituents, there are major differences between them and other alkylating agents with respect to their spectrum of activity and cross-resistance patterns [12]. Of additional interest

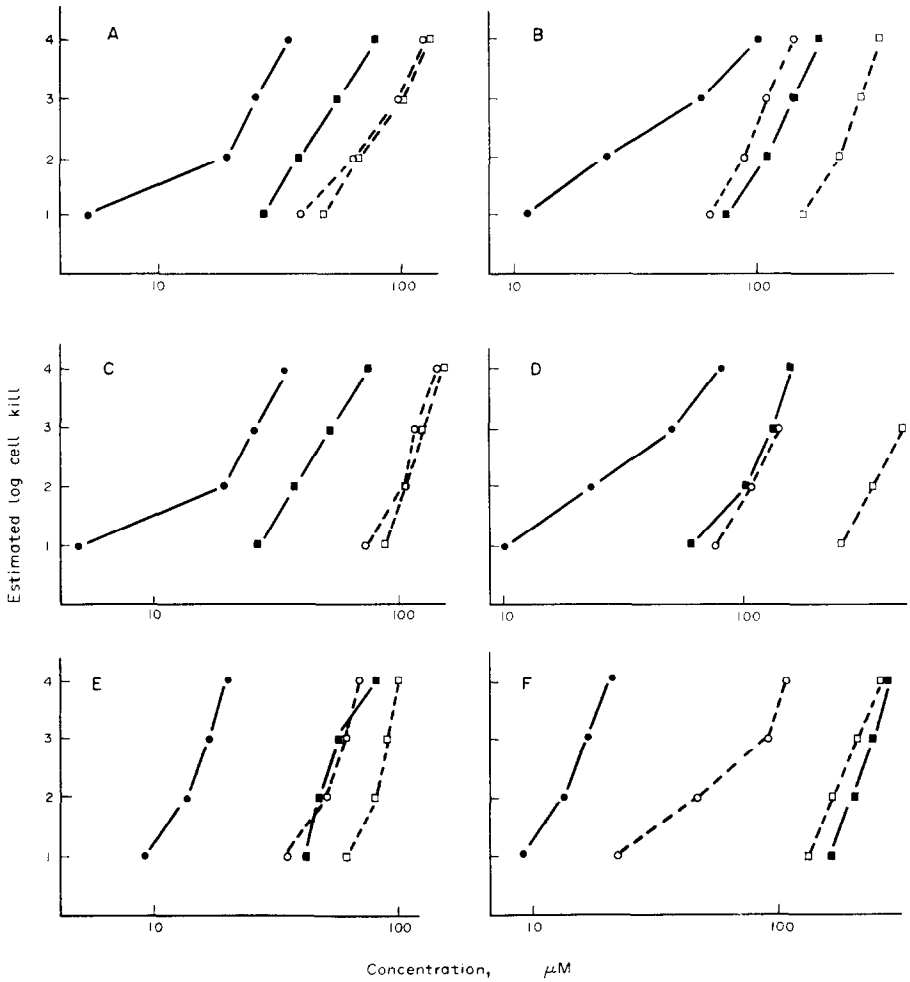

Fig. 3. Results of the *in vitro–in vivo* bioassays of BCNU, CCNU and the isocyanates produced upon their decomposition (chloroethylisocyanate and cyclohexylisocyanate) expressed as concentrations required to produce a number of log cell kills (see Materials and Methods). Solid lines closed symbols represent the nitrosourea sensitive cell lines (TLX5S or L1210S), broken lines open symbols represent nitrosourea resistant cell lines (RT, with resistance induced to a dimethyltriazene; RB, with resistance induced to BCNU). (A) ● = BCNU, TLX5S; ○ = BCNU, TLX5RT; ■ = chloroethylisocyanate, TLX5S; □ = chloroethylisocyanate, TLX5RT. (B) ● = CCNU, TLX5S; ○ = CCNU, TLX5RT; ■ = cyclohexylisocyanate, TLX5S; □ = cyclohexylisocyanate, TLX5RT. (C) ● = BCNU, TLX5S; ○ = BCNU, TLX5RB; ■ = chloroethylisocyanate, TLX5S; □ = chloroethylisocyanate, TLX5RB. (D) ● = CCNU, TLX5S; ○ = CCNU, TLX5RB; ■ = cyclohexylisocyanate, TLX5S; □ = cyclohexylisocyanate, TLX5RB. (E) ● = BCNU, L1210S; ○ = BCNU, L1210RB; ■ = chloroethylisocyanate, L1210S; □ = chloroethylisocyanate, L1210RB. (F) ● = CCNU, L1210S; ○ = CCNU, L1210RB; ■ = cyclohexylisocyanate, L1210S; □ = cyclohexylisocyanate, L1210RB.

Table 2. Non-protein thiol content of lines of the TLX5 lymphoma and L1210 leukaemia which are either nitrosourea sensitive (S) or resistant

Tumour line	Non-protein thiols (nmoles/g protein)
TLX5S	1.43 ± 0.8
TLX5RT	1.37 ± 0.63
L1210S	1.30 ± 0.46
L1210RB	1.38 ± 0.29

RT = resistance induced to a dimethyltriazene; RB = resistance induced to BCNU.

Figures show standard errors (n = 6).

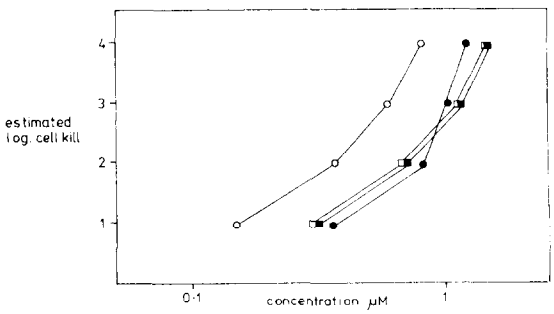

Fig. 4. Results of the *in vitro–in vivo* bioassays of nitrogen mustard. ● = TLX5S; ○ = TLX5RT; ■ = L1210S; □ = L1210RB (see caption of Fig. 3 for explanation of tumour types).

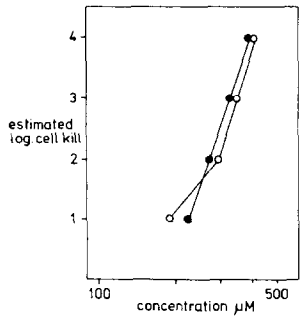

Fig. 5. Results of the *in vitro–in vivo* bioassays of formaldehyde. ● = TLX5S; ○ = TLX5RT (see caption of Fig. 3 for explanation of tumour types).

to us was the finding that the TLX5 lymphoma with induced resistance to a dimethyltriazine *in vivo* was cross-resistant to the nitrosoureas *in vivo* [5]. We thus considered that an *in vitro* analysis of the cytotoxic effects of the isolated cytotoxic fragments generated from a nitrosourea, particularly the isocyanates, to this triazene resistant tumour would be interesting since it had not been exposed to either the alkylating or carbamoylating fragments of nitrosoureas *in vivo* as resistance was induced.

The cross-resistance to the isocyanates observed in this study (Figs. 3 A–D) appeared to be specific for this chemical species (cf. Figs. 4 and 5) and also largely specific to the TLX5 lymphoma (cf. Figs. 3 E and F). In the case of cyclohexylisocyanate the degree of resistance observed when a greater than 99.9% cell kill was obtained was similar to that observed with the progenitor nitrosourea (CCNU) (Fig. 3B). Generally the concentrations of isocyanates required to give equivalent cytotoxicity as the nitrosoureas (using the TLX5 lymphoma) was 2–3 fold that of the progenitor nitrosourea. It has been argued that isocyanates play little role in the cytotoxicity of the nitrosoureas since when they were added to L1210 cells over a time period calculated to give a (concentration × time) profile similar to that of their release from their progenitors little toxicity was observed [13]. However the nitrosoureas are latentiated forms of isocyanate which are transported intact into tumour cells [14] and the addition of extracellular isocyanates at these concentrations may not be equivalent to the addition of an intact nitrosourea.

Table 3. The ratio of cellular uptake, with time, of [^{14}C]cyclohexyl CCNU into resistant and sensitive cell lines of the TLX5 lymphoma and L1210 leukaemia

Time (min)	cpm TLX5RT / cpm TLX5S	cpm L1210RB / cpm L1210S
0	0.994	1.41
10	0.971	1.54
20	0.968	1.32
40	0.978	1.31
80	0.987	1.34
120	0.987	1.24

S = sensitive line; RT = resistance induced to a dimethyltriazene; RB = resistance induced to BCNU.

Cross-resistance between the isocyanates and nitrosoureas could not be explained by difference in cellular transport of the nitrosourea in sensitive and resistant lines (Table 3) nor to the elevation of non-protein thiols, which may inactivate both the alkylating and carbamoylating activity of the nitrosoureas (Table 2).

These results suggest that the carbamoylating activity of CCNU and BCNU may constitute a major element of the cytotoxicity of these nitrosoureas to the TLX5 lymphoma. In contrast, the failure to find cross-resistance between the nitrosoureas and isocyanates using the L1210 leukaemia, suggests that, as is generally accepted, alkylation may be the major cause of nitrosourea cytotoxicity to this tumour. The L1210 leukaemia resistant to BCNU showed some degree of cross-resistance to chloroethylisocyanate (Fig. 3E) and it may be that the ability of this molecule to cross-link cellular macromolecules is of relevance.

The finding that the nitrosourea resistant TLX5 lymphomas were cross-resistant to the isocyanates studied here suggests the hypothesis that carbamoylation is thus responsible for nitrosourea cytotoxicity to the TLX5 lymphoma and further, that nitrosoureas which have been found to have low carbamoylating activity, (as measured by *in vitro* reaction with radiolabelled lysine [15]) may be ineffective against this tumour *in vivo*. Such an agent is chlorozotocin (CLZ, Fig. 1). However, in a comparison of the *in vivo* antitumour activity of CLZ using the TLX5 lymphoma and the L1210 leukaemia, comparable, although marginal, antitumour effects were observed (Table 1). This result raises some doubts about our hypothesis, although the result is somewhat equivocal given that doubts exist concerning the correlation between the failure of CLZ to carbamoylate lysine *in vitro* and certain of its biological effects which suggest that it may be capable of carbamoylating cellular constituents [16]. Similar doubts were raised by Reed and Babson concerning the inactivation of glutathione reductase by the so-called 'non-carbamoylator' ACNU (Fig. 1), an inactivation presumably caused by the formation of an isocyanate [17]. Further experiments are in progress which aim to compare a range of nitrosoureas, with varying carbamoylating potentials, both for their *in vivo* antitumour effects against the TLX5 lymphoma and their *in vitro* cross-resistance patterns.

The logical consequence of our present findings with the TLX5 lymphoma is the synthesis and screening of novel molecules which release isocyanate but not halogenoalkyldiazohydroxides (Fig. 1). Preferably these should demonstrate kinetics of isocyanate release similar to that of the nitrosoureas. Reports of the synthesis of molecules which potentially fulfil these criteria and of their excellent *in vivo* antitumour activity will be presented at a later date.

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REFERENCES

1. K. W. Kohn, in *Recent Results in Cancer Research* (Eds. S. K. Carter, Y. Sakurai and H. Umerzawa), Vol. 76, p. 141. Springer, Berlin (1981).
2. C. J. Cheng, S. Fujimura, D. Grunberger and I. B. Weinstein, *Cancer Res.* **32**, 22 (1972).
3. L. C. Panasci, P. A. Fox and P. S. Schein, *Cancer Res.* **37**, 3321 (1977).
4. A. Gescher, J. A. Hickman, R. J. Simmonds, M. F. G. Stevens and K. Vaughan, *Biochem. Pharmac.* **30**, 89 (1981).
5. T. A. Connors and J. R. Hare, *Biochem. Pharmac.* **24**, 2133 (1975).
6. W. Boyle, *Transplantation* **6**, 761 (1968).
7. G. L. Ellman, *Archs Biochem. Biophys.* **82**, 70 (1955).
8. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. chem.* **193**, 265 (1951).
9. T. A. Connors, *Eur. J. Cancer* **2**, 293 (1966).
10. T. A. Connors and J. A. Hare, *Br. J. Cancer* **30**, 477 (1974).
11. T. Ishi, I. Hishinuma and S. Bannai, *J. cell. comp. Physiol.* **107**, 283 (1981).
12. G. P. Wheeler, J. A. Alexander and D. J. Adamson, *Cancer Res.* **40**, 3723 (1980).
13. J. Hilton, F. Maldarelli and S. Sargent, *Biochem. Pharmac.* **27**, 1359 (1979).
14. A. Begleiter, H. Y. P. Lam and G. J. Goldenberg, *Cancer Res.* **37**, 1022 (1977).
15. G. P. Wheeler, B. J. Bowdon and R. F. Struck, *Cancer Res.* **35**, 2974 (1975).
16. K. D. Tew and A. L. Wang, *Molec. Pharmac.*, in press.
17. J. R. Babson and D. J. Reed, *Biochem. Biophys. Res. Commun.* **83**, 754 (1978).