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ANTITUMOUR IMIDAZOTETRAZINES—IV

AN INVESTIGATION INTO THE MECHANISM OF ANTITUMOUR ACTIVITY OF A NOVEL AND POTENT ANTITUMOUR AGENT, MITOZOLOMIDE (CCRG 81010, M & B 39565; NSC 353451)

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Abstract—8-Carbamoyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4-(3H)-one–mitozolomide (CCRG 81010, M & B 39565, NSC 353451) is a potent inhibitor of the growth of a number of experimental tumours and can potentially decompose to give either an isocyanate or the monochloroethyltriazene (MCTIC). *In vitro* CCRG 81010 is not cross-resistant with the bifunctional alkylating agents against the Walker carcinoma. To investigate the mechanism of the antitumour activity of CCRG 81010 a comparison has been made with BCNU and MCTIC on precursor incorporation into macromolecules in TLX5 mouse lymphoma cells. Whereas BCNU produces a rapid and extensive inhibition of both (methyl ^{3}H) thymidine and [5-^{3}H]uridine incorporation into acid-insoluble material, neither CCRG 81010 or MCTIC have an early effect on precursor incorporation. Inhibition of precursor uptake is also not produced by concentrations of 2-chloroethylisocyanate that inhibit intracellular glutathione reductase activity. The potential carbamoylating activity of CCRG 81010 has also been assessed by comparing its effect with that of BCNU and 2-chloroethyl isocyanate on enzymes known to be inhibited by carbamoylation. Such enzymes, glutathione reductase, chymotrypsin and γ-glutamyltranspeptidase are not inhibited by CCRG 81010 under conditions where BCNU and 2-chloroethyl isocyanate show complete inhibition of enzyme activity, suggesting an absence of carbamoylating species. The results suggest that the most likely antitumour metabonate produced from CCRG 81010 is the triazene MCTIC.

8-Carbamoyl-3(-2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one* (mitozolomide CCRG 81010, I) is a cycloaddition product of 5-diazoimidazole-4-carboxamide (II) and 2-chloroethyl isocyanate (III) [1]. This molecule might be expected to decompose to give reactive species with potential antitumour activity [1]. CCRG 81010 exhibits essentially curative activity against the experimental tumours L1210, P388, TLX5, Lewis lung, PC6A plasmacytoma and Colon 38 carcinoma [2] and is currently undergoing a phase 1 clinical trial.

The potential chemical decomposition pathways for CCRG 81010 are shown in Fig. 1. Although most of the species depicted are potentially toxic, attention has been focused primarily on those which have already been implicated in the mode of action of established antitumour agents. CCRG 81010 is theoretically capable of generating 2-chloroethylisocyanate (III), which has been suggested as constituting a major element of the cytotoxicity of the nitrosoureas to the TLX5 lymphoma [3], and the triazene (MCTIC), (IV) which is an incipient chloroethylcarbonium species [4] which can alkylate nucleophiles by an SN_2 type mechanism. In the alkylation process 5-aminoimidazole-4-carboxamide (V) is liberated [1]. MCTIC has been shown to have very effective antitumour activity on the L1210 system [5] and has been suggested as the agent responsible for the antitumour activity of BCTIC. Both the triazenes [6] and the nitrosoureas [7] cause an inhibition of precursor incorporation into macromolecules. Previous investigations with BCNU [8] have shown a marked depression of the incorporation of [^{3}H]thymidine into macromolecules. This effect has been attributed mainly to lesions in DNA produced by chloroethylation since it is the incipient chloroethylcarbonium ion that will react preferentially with nucleic acids and not the isocyanate (III) [3]. Isocyanates are characterized by their ability to carbamoylate proteins and this effect is probably responsible for the inhibition of glutathione reductase [8], chymotrypsin [9] and transglutaminase [10] by the nitrosoureas.

In an attempt to define the cytotoxic species responsible for the antitumour activity of CCRG 81010 (I) a comparison has been made with BCNU and MCTIC (IV) of precursor incorporation into macromolecules as well as the effect on enzymes known to be inhibited by nitrosoureas.

* Abbreviations—8-carbamoyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one, CCRG 81010, MB 39565, NSC 35341; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; BCTIC, 5-[3,3-bis(2-chloroethyl)-1-triazenyl] imidazole-4-carboxamide; MCTIC, 5-[3-(2-chloroethyl)-1-triazenyl]imidazole-4-carboxamide; CNU, 2-chloroethyl-nitrosourea.

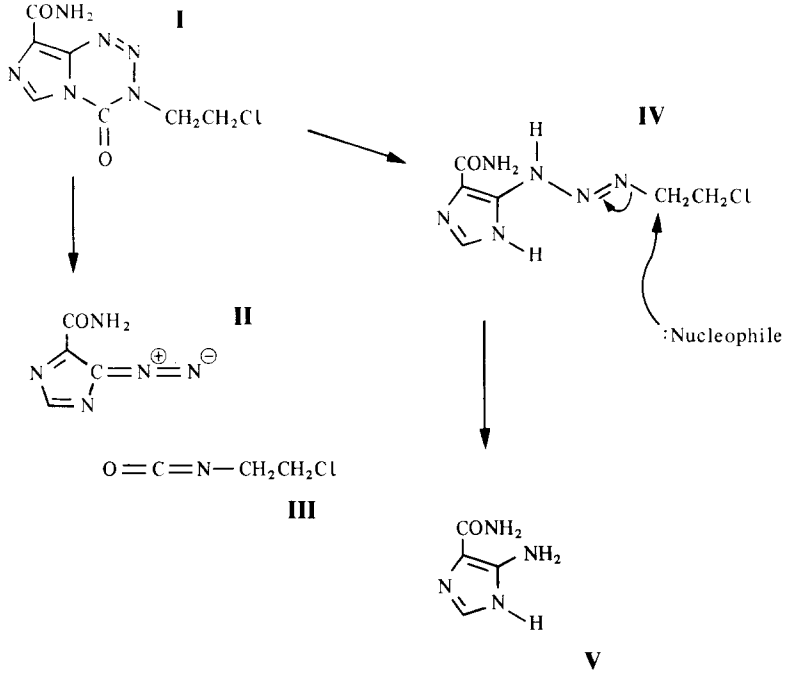

Fig. 1. Potential decomposition pathways of 8-carbamoyl-3-(2-chloroethyl)-imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (CCRG 81010: I).

MATERIALS AND METHODS

5-(Methyl-^{3}H)thymidine (sp.act. 5 Ci/mmol), [5-^{3}H]uridine (sp.act. 27 Ci/mmol), L[4,5-^{3}H] lysine (sp.act. 99 Ci/mmol) and [2,5,8-^{3}H] adenosine (sp. act. 40 Ci/mmol) were purchased from Amersham International, Bucks. Reagents for tissue culture were obtained from GIBCO Europe, Paisley, Scotland. Biochemical reagents were purchased from Sigma Chemical Co., Dorset, CCRG 81010 and MCTIC were synthesized by Professor M. F. G. Stevens in this department.

Cell culture. Cells were routinely grown in RPMI 1640 medium containing 10% foetal calf serum under an atmosphere of 10% CO_2 in air. Drug solutions were made up in 10% DMSO: saline, such that the final concentration of DMSO in the culture medium did not exceed 1%. Growth studies were performed as described [11] and growth inhibition was calculated from the linear part of the growth curves. For some experiments TLX5 ascites tumour cells were passaged in CBA/CA mice, removed in isotonic saline, and red cells were lysed, as necessary, by washing with 0.016 M Tris–HCl, pH 7.2, containing 7.5 g ammonium chloride/1. For precursor incorporation studies mouse ascites cells were used at a concentration of 1.0×10^6 cells/ml and tissue culture cells at 0.5×10^6 cells/ml. Cells were incubated with drugs for various periods of time as indicated in the figure legends.

Precursor incorporation. Pulse labelling was carried out for a 1 hr period and all precursors were used at a sp.act. of 2.5 μCi/ml. Incorporation into acid insoluble material was determined by filtering the cell suspension through a Whatman GF/C glass fibre disc with 0.9% NaCl, followed by 10 vol. of ice-cold 0.2 N perchloric acid and 5 vol of absolute ethanol. The discs were dried at 70° for 1 hr and the radioactivity was determined in a toluene, PPO/POPOP scintillation mixture. The size of the precursor pool was determined in parallel by lysing the cell pellets, obtained by centrifugation, with 0.5 M perchloric acid and the radioactivity in the supernatant, after neutralization with 5 M KOH, was determined in PCS (Hopkin and Williams, Romford) scintillation fluid.

The differential incorporation of adenosine into DNA and RNA was determined by first measuring total incorporation of radioactivity into acid-insoluble material as above. For incorporation of radioactivity into DNA parallel cell suspensions were mixed with an equal volume of 1 N NaOH and allowed to stand overnight. The alkali-insoluble material was washed onto GF/C discs, as above, with 20 vol. of 0.9% NaCl, followed by 5 vol. of absolute ethanol. The discs were then processed and counted as above. Incorporation of label into RNA was taken as the difference between total acid-insoluble material and alkali-insoluble material [12].

Glutathione reductase activity was determined spectrophotometrically at 25° by following the oxidation of NADPH at 340 nm. The assay mixture contained 0.2 M KCl, 1 mM EDTA, cell extract and 1 mM oxidized glutathione in 0.1 M phosphate, pH 7.0. The reaction was initiated by the addition of NADPH to a final concentration of 0.1 mM. The protein content of the cell supernatant was determined by the method of Lowry *et al.* [13] using bovine serum albumin as a standard.

γ-Glutamyl transpeptidase was determined as

described [14]. This method is based on the production of *p*-nitroaniline which has a maximal absorbance at 405 nm.

Chymotrypsin activity was determined by measuring the rate of hydrolysis of N-benzoyl-L-tyrosine ethyl ester at 256 nm as described [15].

RESULTS

In vitro cytotoxicity. The *in vitro* LD_{50} of CCRG 81010, BCNU and MCTIC towards the TLX5 lymphoma and Walker carcinoma is shown in Table 1. The three agents show similar toxicity towards the TLX5 lymphoma sensitive to triazenes and nitrosoureas (TLX5). There is no difference in the LD_{50} of CCRG 81010 towards Walker carcinoma either sensitive (WS) or resistant (WR) to alkylating agents [16].

Enzyme studies. The effect of incubation with equitoxic concentrations of CCRG 81010 and BCNU on the activity of glutathione reductase in intact TLX5 cells is shown in Fig. 2. As reported [9] BCNU produces a rapid inhibition of enzyme activity, which is complete within 2 hr of treatment. In contrast CCRG 81010 had no significant effect on enzyme activity, even after 24 hr incubation, and no inhibition is observed with higher concentrations of the drug (Fig. 3). Inhibition of glutathione reductase is probably related to the carbamoylating activity of BCNU [9], since neither MCTIC or the alkylating agent chlorambucil have any effect on enzyme levels (results not shown), while 2-chloroethyl isocyanate produces complete inhibition at concentrations similar to BCNU. There is no alteration in the specific activity of glutathione reductase in tumours resistant to BCNU and CCRG 81010 (Table 2), suggesting

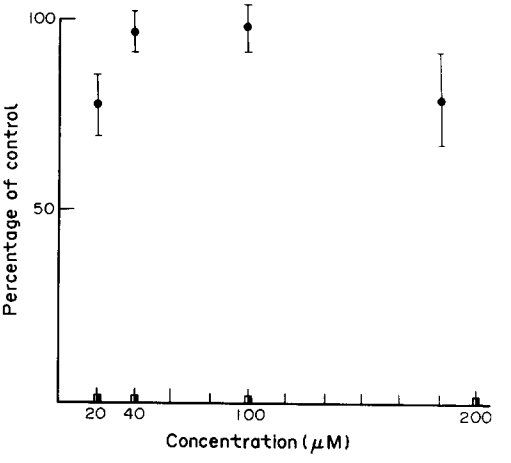

Fig. 3. Effect of different concentrations of CCRG 81010 (●——●), BCNU (□——□) and 2-chloroethyl isocyanate (■——■) on glutathione reductase activity of TLX5(S) cells 24 hr after drug treatment.

Table 1. Sensitivity of TXL5 lymphoma and Walker carcinoma to CCRG 81010, BCNU and MCTIC

Drug	Cell line	LD_{50} (μM)
CCRG 81010	TLX5	4.0 ± 1.0
	WS	17
	WR	21
BCNU	TLX5	1.0 ± 2.0
MCTIC	TLX5	4.0 ± 1.0

Table 2. Specific activity of glutathione reductase in TLX5 lymphoma a d L1210 leukaemia

Cell line	Specific activity (μmoles/sec/mg protein)
TLX5*	1.2×10^{-1}
TLX5T†	0.82×10^{-1}
L1210/S*	0.78×10^{-1}
L1210/R†	0.73×10^{-1}

* Sensitive to nitrosoureas
† Resistant to nitrosoureas

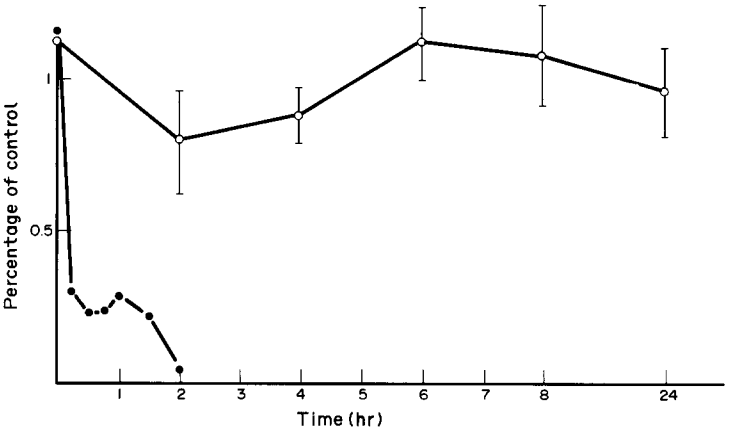

Fig. 2. Effect of time of incubation of TLX5(S) cells with CCRG 81010 (○——○) and BCNU (●——●) at equitoxic concentrations (21 and 23 μM) respectively on glutathione reductase activity. Incubations were carried out at a cell concentration of 10^6 cells/ml. At various time periods 20 ml of the cell suspension was removed and the cells were pelleted by centrifugation, washed in 0.9% NaCl and sonicated (MSE sonic oscillator, 20 Kc) in 400 μl of 0.1 M phosphate, pH 7.0 containing 0.2 M KCl and 1 mM EDTA. The supernatant obtained by centrifugation was used for the determination of glutathione reductase activity.

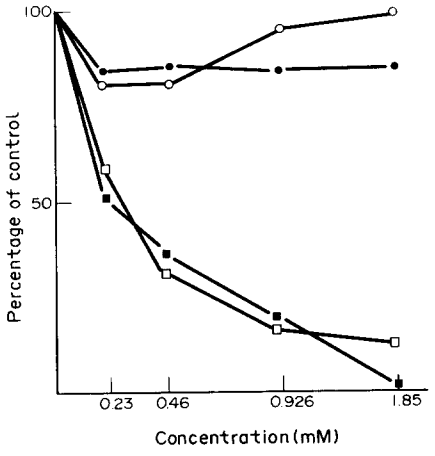

Fig. 4. Effect of incubation of CCRG 81010 (●, ○) and BCNU (■, □) with chymotrypsin for 2 hr (open symbols) and 4 hr (closed symbols). Both CCRG 81010 and BCNU were made up in 10% DMSO-saline solution and incubated with a solution of chymotrypsin (0.57 mg/ml) in water at 37°. At 2 and 4 hr aliquots were taken from the incubation mixtures and enzyme activity was determined as described [15].

that this enzyme may be unrelated to the cytotoxicity of these drugs.

The effect of BCNU and CCRG 81010 on chymotrypsin activity is shown in Fig. 4. As previously reported [10] BCNU produces a marked inhibition of enzyme activity with complete inhibition occurring within 2 hr at a concentration which causes 50% of inhibition of growth of the TLX5(S) lymphoma (Table 1). In contrast, under identical conditions, CCRG 81010 has no effect on chymotrypsin activity.

A similar situation occurs with γ-glutamyltranspeptidase (Fig. 5). As with chymotrypsin inhibition by BCNU is rapid and complete within 2 hr of enzyme incubation, while CCRG 81010 has no detectable effect on enzyme activity. These results suggest that decomposition of CCRG 81010 via the isocyanate (III) is small or non existent.

Effect on precursor incorporation. The concentration-dependent effect of CCRG 81010, BCNU, MCTIC and 2-chloroethyl isocyanate on the incorporation of (methyl ^{3}H)thymidine into acid-insoluble material is shown in Fig. 6(a–d). With BCNU there is a rapid, concentration-dependent decrease in (methyl ^{3}H)-thymidine incorporation, which is apparent within 1 hr of treatment, at which time the other agents have no effect on precursor incorporation. In contrast both MCTIC and CCRG 81010 produce a much slower inhibition of (methyl ^{3}H)-thymidine incorporation, which is maximal within 24 hr of treatment. The dose–response relationships for these two agents on (methyl ^{3}H) thymidine incorporation are similar. The effect of BCNU on (methyl ^{3}H)thymidine incorporation seems not to be due to isocyanate formation since 2-chloroethyl isocyanate has no effect on incorporation in intact cells at levels which cause complete inhibition of glutathione reductase (Fig. 3). The effect of BCNU and CCRG 81010 on (methyl ^{3}H)thymidine incorporation into acid-insoluble material does not result from perturbations of the size of the thymidine pool, since there is no effect on intracellular thymidine content until 24 hr after drug treatment (Fig. 7).

The effect of CCRG 81010, BCNU, MCTIC and 2-chloroethyl isocyanate on [5-^{3}H]uridine incorporation into acid-insoluble material is shown in Fig. 8(a–d). Again BCNU produces a marked depression of precursor incorporation, which is dose related and is evident within 1 hr of treatment, while both CCRG 81010 and MCTIC produce little change in uridine incorporation until 24 hr. There is no effect of 2-chloroethyl isocyanate on [5-^{3}H]uridine incorporation in intact cells. No agent has any effect on the size of the intracellular uridine pool until 24 hr after treatment.

The effect of BCNU and CCRG 81010 on [2,5,8-^{3}H] adenosine incorporation into DNA and RNA (Fig. 9) closely parallels their effect on (methyl ^{3}H) thymidine and [5-^{3}H] uridine incorporation into acid-insoluble material (Figs. 6 and 8). The dose–response curves for inhibition of both DNA and RNA synthesis by BCNU are very similar, while CCRG 81010 appears to produce a slow, but preferential inhibition of DNA synthesis.

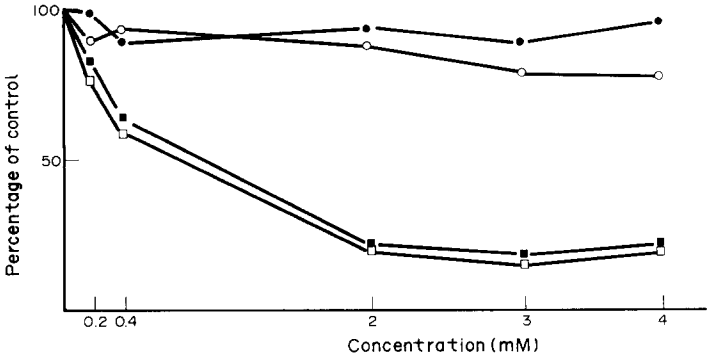

Fig. 5. Effect of incubation of CCRG 81010 (●, ○) and BCNU (■, □) with γ-glutamyltranspeptidase (10 mg/ml) for 2 hr (open symbols) and 4 hr (closed symbols). Enzyme activity was determined as described in Methods.

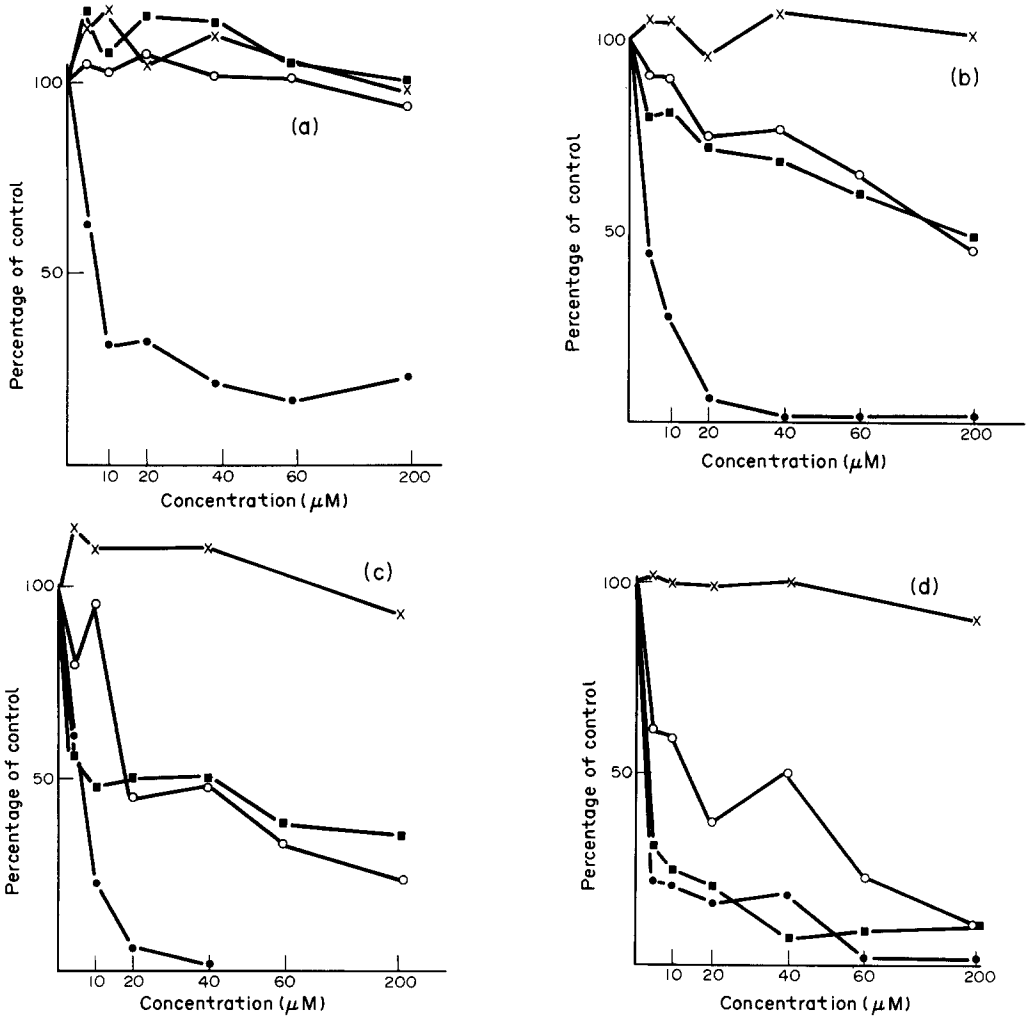

Fig. 6. Effect of CCRG 81010 (○——○), MCTIC (■——■), BCNU (●) and 2-chloroethylisocyanate (×——×) on the incorporation of (methyl-^{3}H)thymidine into acid-insoluble material of TLX5(S) cells (a) 1 hr (b) 3 hr (c) 8 hr (d) 24 hr after drug treatment. Cultures were pulse labelled for 1 hr and the incorporation of radioactivity into acid-insoluble material was determined as described in methods.

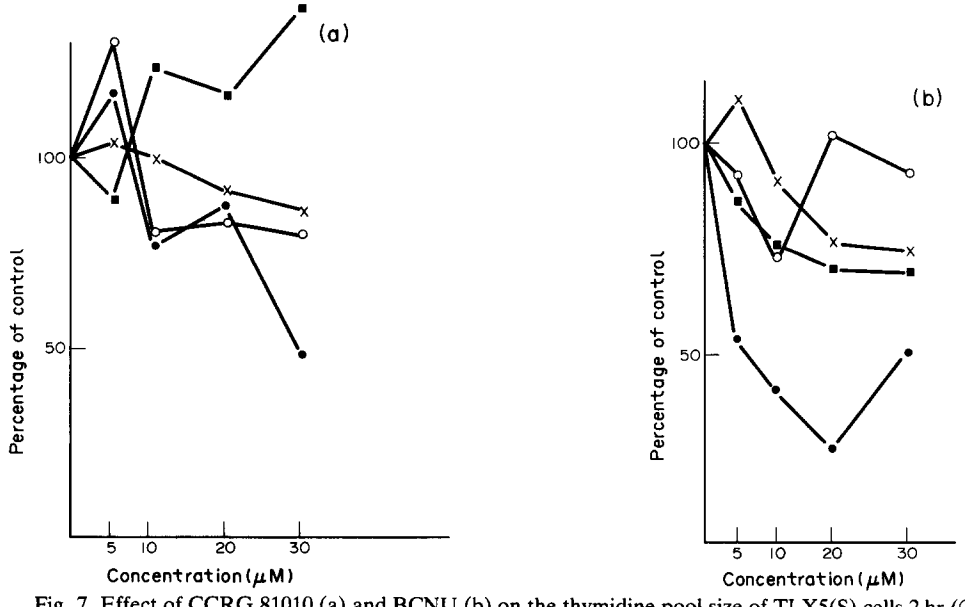

Fig. 7. Effect of CCRG 81010 (a) and BCNU (b) on the thymidine pool size of TLX5(S) cells 2 hr (○), 4 hr (×), 6 hr (■) and 24 hr (●) after drug treatment.

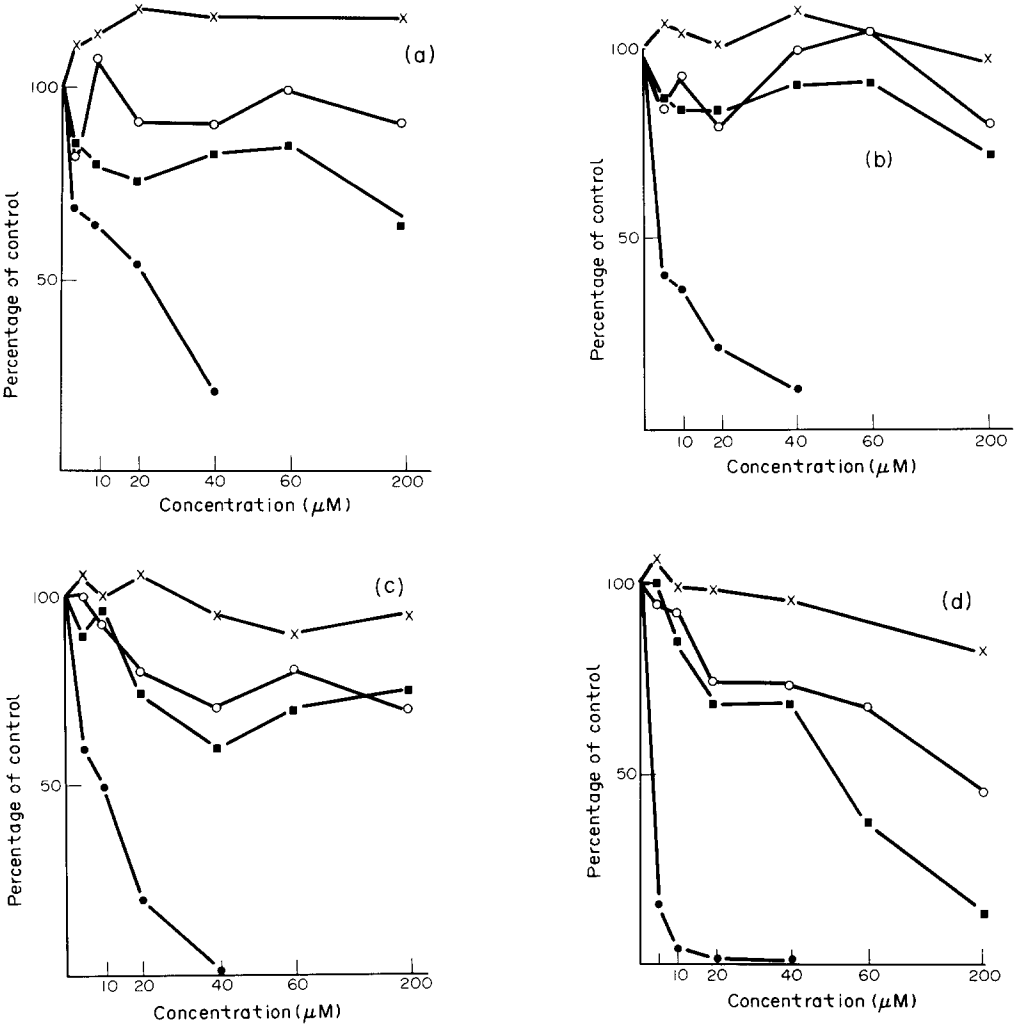

Fig. 8. Effect of CCRG 81010 (○——○), MCTIC (■——■), BCNU (●——●) and 2-chloroethyl isocyanate (×——×) on the incorporation of [5-^{3}H]uridine into acid-insoluble material of TLX5(S) cells. (a) 1 hr (b) 3 hr (c) 8 hr (d) 24 hr after drug treatment.

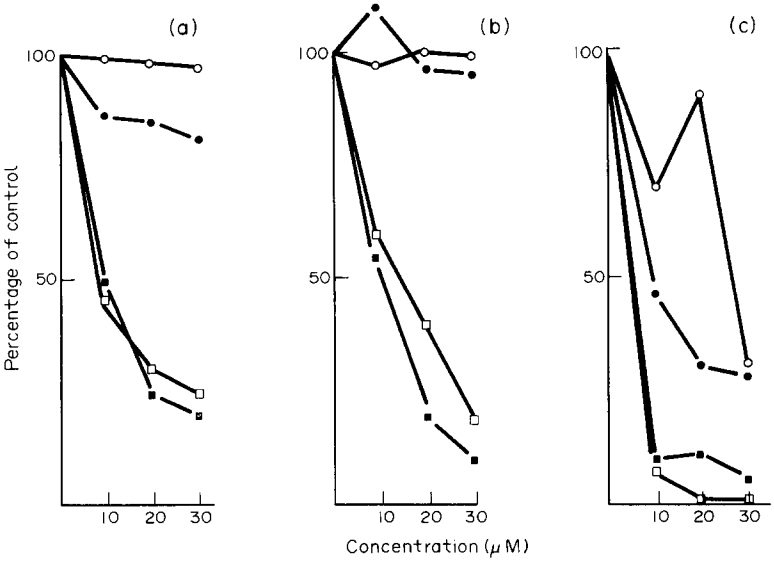

Fig. 9. Effect of CCRG 81010 (○, ●) and BCNU (□, ■) on adenosine incorporation into DNA (closed symbols) and RNA (open symbols) of TLX5(S) cells (a) 4 hr (b) 8 hr (c) 16 hr after drug treatment.

DISCUSSION

The imidazotetrazinones represent a new group of antitumour agents with high cytotoxic potency against a wide range of experimental tumours. This study has aimed at determining the chemical species responsible for cytotoxicity. The lack of cross-resistance of CCRG 81010 towards a bifunctional alkylating agent resistant cell line is an observation also noted with the haloethylnitrosoureas [17] despite the postulates that both types of agent exert antitumour activity as a result of cross-linking of DNA [18, 19].

To distinguish between the potential decomposition pathways of CCRG 81010, either through an isocyanate, or an incipient chloroethyl carbonium ion, the effect of CCRG 81010 on enzymes known to be inhibited by isocyanates (glutathione reductase, chymotrypsin and γ-glutamyl transpeptidase) has been studied. In all cases under conditions where BCNU and 2-chloroethyl isocyanate show inhibition of enzyme activity CCRG 81010 has no inhibitory effect. These results suggest that isocyanate generation does not occur during the decomposition of CCRG 81010, either in the intact cell, or in aqueous solution.

The effect of CCRG 81010 on precursor incorporation into nucleic acids is very similar to that of the triazene, MCTIC, providing some support for the alternative decomposition pathway. Both agents have little immediate effect on either (methyl ^{3}H) thymidine or [5-^{3}H]uridine incorporation into acid-insoluble material, in contrast with BCNU, which produces a rapid and marked inhibition of precursor incorporation. The effect of BCNU on macromolecular synthesis is probably not mediated by carbamoylation as previously suggested [20] since 2-chloroethyl isocyanate has no effect on precursor incorporation into macromolecules at concentrations which produce complete inhibition of glutathione reductase activity in intact cells. Although nitrosoureas and isocyanates have been shown to inhibit DNA polymerase II activity, possibly through carbamoylation of the active site of the enzyme [21], the effect was only observed at high concentrations of the agents (1 mM), which are not likely to be achieved at normal pharmacological concentrations. The effect of BCNU on (methyl ^{3}H)thymidine incorporation is also mirrored by its effect on [2,5,8-^{3}H] adenosine incorporation into DNA suggesting rapid inhibition of new DNA synthesis.

Neither CCRG 81010 nor BCNU produce changes in the precursor pool size until 24 hr after treatment, suggesting that these agents do not interact with the cell membrane. This is confirmed by the lack of effect of CCRG 81010 on ^{86}Rb uptake, in contrast with the bifunctional alkylating agents [22].

CCRG 81010 has recently been shown to produce DNA interstrand crosslinking in L1210 murine lymphocytic leukaemia cells at similar levels to that produced by MCTIC and CNU [1-(2-chloroethyl)-1-nitrosourea] at equitoxic concentrations [23]. Similarly DNA interstrand cross-link formation also occurred in O^6-methylguanine repair-deficient SV-40 transformed (VA-13) hyman embryo cells treated with CCRG 81010, but only at low levels in a repair proficient cell line (IMR-90) [24]. In each case the peak of crosslinking occurred 9–12 hr after drug removal, at which time CCRG 81010 exerted an effect on (methyl ^{3}H)thymidine incorporation into acid-insoluble material. Since CCRG 81010, MCTIC and BCNU can potentially decompose to produce similar alkylating fragments, a similarity of their effects on DNA might be expected. However, differences in the interaction of chloroethyltriazenes and haloethylnitrosoureas with DNA have previously been shown by ethidium fluorescence assay [25, 26].

Both CCRG 81010 and MCTIC have similar effects on cell cycle progression, as determined by flow cytometry [27]. In each case there is a loss of the G_1 tumour population and an irreversible block of the cells in late S-G_2-M.

The above results would support chemical studies [1] that formation of MCTIC by CCRG 81010 is the primary decomposition pathway to produce cytotoxic species. Preliminary studies show no significant change in antitumour activity when CCRG 81010 is preincubated with mouse liver microsomes suggesting that metabolic activation is not required for activity. Stability studies show a rapid breakdown of CCRG 81010 in aqueous solution at pH 7.4 ($t_{\frac{1}{2}}$ = 98 min) [1] leading to the conclusion that cytoxicity may be exerted by a metabonate. Thus CCRG 81010 may act as a transport form of a highly reactive cytotoxic species.

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