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STUDIES OF THE MECHANISM OF ACTION OF THE TUMOUR-INHIBITORY NITROSOUREAS

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Abstract—BCNU [1,3 bis-(2-chloroethyl)-1-nitrosourea] and some related nitrosoureas have been shown to have a wide spectrum of action against a number of transplanted rodent tumours. No correlation was found between the chemical instability of a nitrosourea and its antitumour activity. Unlike difunctional alkylating agents, the nitrosoureas inhibit the incorporation of tritiated precursors in DNA, RNA and protein to equal extents, the inhibition of tritiated thymidine incorporation into DNA occurring within 5 min of incubating cells with BCNU. Although the biological half life of BCNU was found to be very short (15 min by bioassay) a single injection was as effective against the established and widely-disseminated TLX5 lymphoma as against the early transplant. BCNU interfered specifically with the incorporation of labelled thymidine triphosphate into DNA, but no inhibition of DNA polymerase could be demonstrated at physiological dose levels. In their mechanism of action and in their biological properties the tumour inhibitory nitrosoureas are quite distinct from the bifunctional alkylating agents.

BCNU [1,3 bis-(2-chloroethyl)-1-nitrosourea, NSC 409964] and related nitrosoureas are used in man in the treatment of various forms of cancer, especially melanoma, brain tumours and Hodgkin's disease[1–7]. Early studies suggested that they were similar to the bifunctional alkylating agents in their action, and cross-resistance studies with tumours made resistant to the latter, offered further evidence of a similarity between the two classes of agents[8]. It was also shown that the tumour inhibitory nitrosoureas could decompose in polar solvents to form alkylating entities[9]. However, there are differences between the two classes of compounds both in the spectrum of tumours they affect in man and animals[10, 11] and in their reaction with cellular macromolecules[12, 13]. This paper presents further evidence that the anti-tumour nitrosoureas are quite distinct from the biological alkylating agents in a number of aspects and that they probably exert their anti-cancer effect by a different mechanism.

MATERIALS AND METHODS

Anti-tumour tests. Tumours were obtained by subcutaneous implantation of tumour fragments (Walker, PC6) or cell suspensions (TLX5, R1) into female CBA/Lac mice (TLX5, R1), Balb/C mice (PC6) or male Wistar rats (Walker). Compounds under test were usually injected 3 days after tumour implantation, except for the Walker tumour (1 day after transplantation) and the PC6 tumour where treatment was delayed until the tumour was 3 weeks old and 2–3 g in weight. Injections were given intraperitoneally as a single dose, or as five consecutive daily doses. For the Walker and PC6 tumours, the drug effectiveness was assessed by tumour weight at the end of the experiment, and selectivity expressed as a therapeutic index, the ratio between the LD_{50} and the minimum dose to cause complete tumour regression (PC6) or complete inhibition of growth (Walker)[14]. For the R1 and TLX5 lymphomas, the effectiveness of an agent was assessed by its ability to extend the life-span of mice transplanted with a known number of tumour cells.

Tumours with acquired resistance to BCNU were developed by treating successive passages with gradually increasing doses of BCNU until there was no anti-tumour effect even after the administration of the maximum tolerated dose.

Growth rates of the ascites tumours were measured by total removal of the cells in the peritoneal cavity and counting, on a Coulter Counter Model FN (Coulter Electronics, Dunstable, Bedford.). Groups of three mice were used to determine the cell number on each day after transplantation and the results are the mean of two independent experiments.

To measure the 'biological' half-life of BCNU, the drug was injected intraperitoneally at a tumour-effective dose and TLX5 ascites tumour cells injected intraperitoneally at various time intervals afterwards. Comparison of the survival times with mice injected with only the tumour cells at the same time gave a measure of the period during which BCNU or its active metabolites were present in the animal at tumour-effective doses. In a similar experiment to measure the stability of BCNU and other related compounds, the drug was dissolved in 1 mM phosphate buffer, pH 7·4 at 37° at a cytotoxic concentration and was added at intervals to a suspension of TLX5 lymphoma cells ($1{\cdot}5 \times 10^6$ cells/ml) in TC 199/horse serum (60:40, v/v). The drug and cell-suspension were incubated together at 37° for 2 hr and then injected intraperitoneally into CBA/Lac female mice. The cytotoxic effect of the residual drug was assessed by comparing the life span of these mice with those having received only the untreated cell suspension.

Cytotoxicity test in vitro. The concentration of drug required to kill 99·9% of the tumour cell population was assessed by incubating various

concentrations of each drug with freshly removed and washed TLX5 lymphoma cells in the medium and at the cell concentration described above. After 2 hr incubation at 37°, 0·1-ml aliquots of the suspension were injected into each of a group of five animals. From the survival time of the animals so treated, the concentration of drug to kill 99·9% of the tumour cells could be estimated, since in previous work it has been shown that a linear inverse correlation exists between the number of tumour cells implanted and survival time over the range 10^6–10^3 cells.

In experiments to measure the ability of certain compounds to prevent the cytotoxicity of BCNU, the cells in suspension were incubated with the 'protecting agent' for 30 min prior to the addition of the BCNU.

To assay the effect of various agents on the uptake of tritiated thymidine ([*methyl*-^{3}H]thymidine), uridine ([6-^{3}H]uridine) and leucine (D,L-[4,5-^{3}H]leucine) by tumour cells *in vitro*, cell suspensions at a concentration of $3·0 \times 10^6$ cells/ml were prepared as described above. The cell suspensions were incubated with the drug at 37° for up to 4 hr and then the tritiated precursors added to a final concentration of 3 μCi/ml. The incorporation of the label into the DNA, RNA or protein of the cells was then assayed at 20-min intervals for the next 80 min, 1-ml aliquots being filtered through Whatman GFC glass fibre filters and then washed with isotonic saline, 0·2 M perchloric acid and finally isotonic saline. After drying in air, the filter papers were placed in plastic vials containing 10 ml scintillation fluid (AR Toluene, 385 g; dioxan, 285 g; ethanol, 99·5%, v/v, 230 ml; naphthalene, 80 g/l. and butyl-PPO 7 g/l.) and counted in a scintillation counter (Packard Tricarb Model 3375).

Measurement of thymidine deoxyribotides. TLX5 tumours of the same age (6 days after transplantation of 10^6 cells) were always used. The tumour cells were washed free of erythrocytes with lysing medium[15] and resuspended at a cell concentration of $1·5 \times 10^7$ cells/ml in TC 199/horse serum (60:40, v/v). To these cells was added [^{3}H-*methyl*]thymidine to a final concentration of 3 μCi/ml and after 30 min at 37° BCNU was also added to a concentration of 20 or 8 μg/ml. The incubation at 37° was continued for a further hour and the cells then collected by centrifugation, after which they were drained free of excess medium and the labelled thymidine and thymidine deoxyribonucleotides were extracted by the method of Giraldi and Baldini[16] using two successive washes (250 μl) of 70% (v/v) ethanol at 70°. The labelled thymidine and its phosphates were separated by thin layer chromatography on precoated cellulose plates (Camlab., Cambridge) using isobutyric acid–ammonia–water (19:2:1, v/v) and were located by running the cell extract (10 μl) with the appropriate unlabelled marker compounds.

DNA polymerase assay. The TLX5 lymphoma was harvested 6 days after passaging and washed free of erythrocytes as described above. After the cell number had been determined, a crude cell extract of DNA polymerase was prepared and assayed according to the method of Roychoudhury and Bloch[17], the incorporation of 1 μCi of dTTP ([^{3}H-*methyl*]dTTP, sp. act. 21·6 Ci/m-mole) into DNA being determined by stopping the reaction with ice-cold 1 M perchloric acid (500 μl) and washing the PCA insoluble material onto a glass fibre filter and assaying for radioactivity as described earlier. The effect of BCNU was investigated by preincubating the crude polymerase preparation for 30 min at 4° with BCNU at a final concentration of 20 μg/ml, and assaying the activity of the residual enzyme.

RESULTS AND DISCUSSION

The effect of BCNU on a number of transplanted tumours is shown in Table 1 and is compared with a typical alkylating agent (cyclophosphamide), antimetabolite (methotrexate), triazene 5,(3,3 - dimethyl - 1 - triazeno) - 4 - carbethoxy - 2 - phenylimidazole and platinum complex (*cis*-dichlorobis(cyclopentylamine)platinum II). The Walker and PC6 tumours are known to be highly sensitive to alkylating agents and the therapeutic index of cyclophosphamide against these two tumours is high. The R1 and the TLX5 lymphomas, however, although growing rapidly, are insensitive to the alkylating agents and no increase in life-span was obtained, even at the maximum tolerated dose of cyclophosphamide. The platinum compounds are thought to act in a similar way to the alkylating agents and the similarity is apparent since the platinum complex has a similar spectrum of action as cyclophosphamide. Methotrexate and purine and pyrimidine antimetabolites, such as 6-mercaptopurine and cytosine arabinoside respec-

Table 1. Spectrum of action of various antitumour agents on various transplantable animal tumours

	Walker	PC6	R1	TLX5	TLX5/R‡
Cyclophosphamide	30	136	0	0	26·5
$(RNH_2)_2Pt(Cl)_2$*	7	200	0	0	0
Methotrexate	1·7	0	65	55	57
Triazene†	1·5	86	98	67	0
BCNU	20	96	'cures'	197	0

Results obtained with the Walker and PC6 tumours are expressed as therapeutic indices. Results obtained with the R1 and TLX5 lymphomas are expressed as percentage increase in survival times.

* *cis*-Dichlorodicyclopentylamine Platinum (II).

† 5-(3,3-dimethyl-1-triazeno)-4-carbethoxy-2-phenylimidazole (BRL51242).

‡ Tumour with acquired resistance to BCNU.

tively, have only a slight or insignificant effect on the Walker and PC6 tumours, but in contrast to the alkylating agents they are active against the R1 and TLX5 lymphomas. BCNU has a spectrum of action different from either of these two classes of compound inasmuch that it is very active against the lymphomas and the Walker and PC6 tumours. The dimethyl triazenes have a similar wide spectrum of action and further evidence of a similarity between the two classes is provided (Table 1) by the demonstration that the TLX5 lymphoma with acquired resistance to BCNU is also cross-resistant to the triazene. Methotrexate, however, can still inhibit the growth of the resistant tumour and cyclophosphamide, which had no effect on the parent lymphoma, has a slight but significant effect on the line with acquired resistance. Thus absence of cross-resistance between the alkylating agents and BCNU implies that the two classes of agents exert their anti-tumour effects by independent and dissimilar pathways.

BCNU is the only compound tested to date which can bring about long-term survival in mice implanted with 10^5 TLX5 lymphoma cells, although even with this drug, the effect is only obtained at a dose which causes some mortality. Nevertheless, the high selectivity of action of this agent can be seen from Table 2. Even when treatment of the lymphoma is delayed until day 7, at which time the tumour is widely disseminated throughout the body, it is still possible to obtain long extensions of survival time and, furthermore, the anti-tumour effect appears to be improved as the tumour becomes more widely dispersed.

Figures 1 and 2 show the effect of various doses of BCNU on the growth curves of the ascites TLX5 lymphoma and the line with acquired resistance to BCNU. The two tumours have identical growth rates and have a period of exponential growth until a cell number of approximately 10^9 cells is reached, a plateau and a degenerative phase follow and during the latter the animals die. The method used, direct counting of all the cells in the peritoneal cavity, obviously gives no indication of the rates of dissemination of the cells into the body organs and this may be continuing after the plateau phase has been reached in the peritoneal cavity. The insensitivity of the resistant line can also been in Fig. 2. Single intraperitoneal injections of up to 20 mg/kg of BCNU have no effect on the progression of cell growth in the resistant line, the growth curves of both the treated and untreated cells being identical. Similar treatment of the sensitive TLX5 lymphoma with 10 mg/kg of BCNU results in a diphasic response in the growth curve (Fig. 1a). After an initial reduction in cell number, there is a slow recovery followed by a log phase and subsequent plateau and degenerative phases. The plateau phase in treated animals (9 days) is longer than the corresponding phase in the untreated animals (6 days). This may reflect a reduction in the proliferative fraction in the treated cells as reported by Young and DeVita[18] for BCNU or an alteration in the cell cycle time, particularly the duration of the S phase (T_s)[19, 20]. Furthermore, the alteration in the T_s duration has been shown to persist for some considerable time[21].

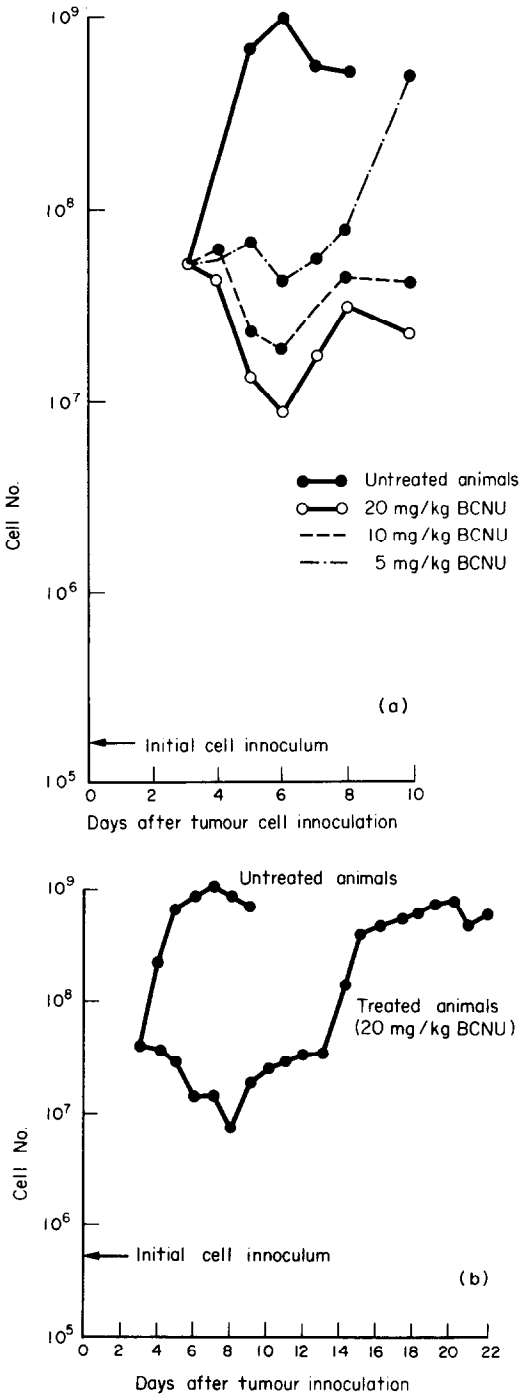


Fig. 1. (a) The growth curve of the TLX5 lymphoma with and without treatment with various doses of BCNU. Animals were treated three days after tumour inoculation. (b) The effect of BCNU (20 mg/kg) on the growth curve of the TLX5 lymphoma from the day of drug administration to the day of death in the treated animals. Animals were treated 3 days after tumour inoculation.

Despite the high selectivity of single doses of BCNU, the biological half-life is extremely short. Figure 3 is the result of an experiment where tumour cells were implanted intraperitoneally into groups of mice at different times after they had been injected with an effective dose of BCNU. After 12 min, 50% of the anti-tumour activity had

Table 2. Effect of BCNU (10 mg/kg) against the TLX5 lymphoma at various times after tumour inoculation

Dose of BCNU (mg/kg)	Day of treatment	Days of death	Mean	% Increase in survival time
10	3	14, 14, 15, 16, 16	15	50
10	4	14, 15, 15, 15, 15	14·8	48
10	5	16, 17, 18, 20, 24	19·2	92
10	6	15, 16, 17, 18, 21	17·2	72
10	7	17, 17, 21, 22, 22	19·8	98
10	8	17, 19, 21, 21, 23	20·2	102
10	9	10, 10, 10, 12, 17	11·8	18
Untreated controls		9, 9, 10, 10, 10, 10, 10, 10, 11, 11	10·0	—

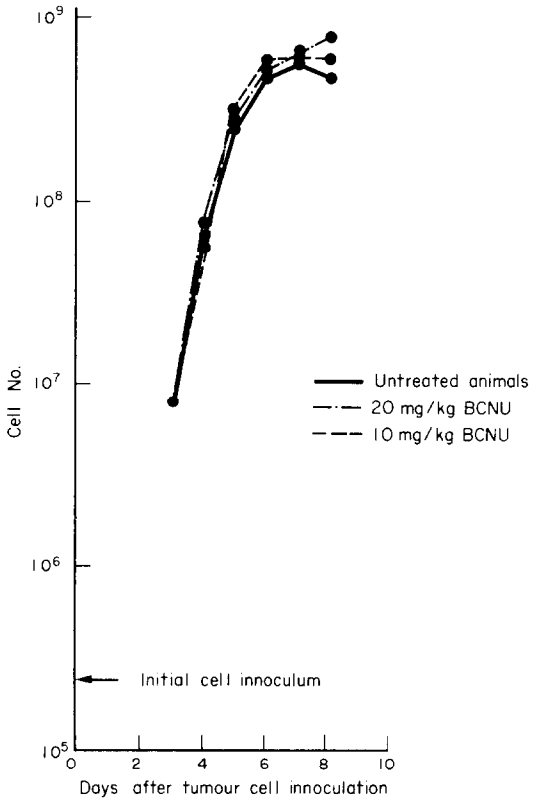


Fig. 2. The effect of BCNU at various doses on the growth curve of the TLX5 lymphoma with acquired resistance to BCNU. Animals were treated 3 days after inoculation.

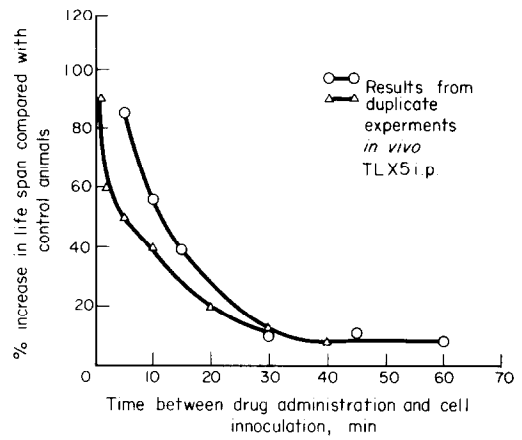


Fig. 3. The *in vivo* antitumour (biological) half-life of BCNU. The results are expressed as percentage increases in survival times relative to the time between administration of the drug (i.p.) and tumour inoculation (i.p.). The results are from two independent experiments.

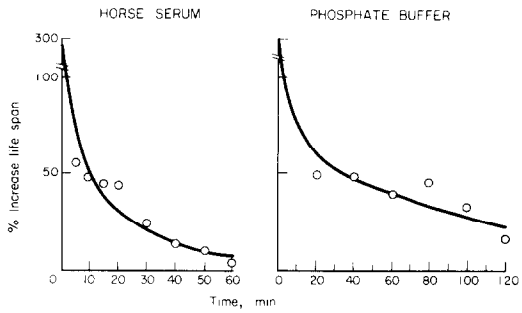
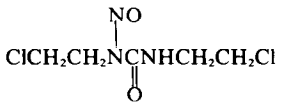
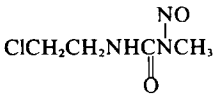
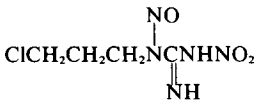
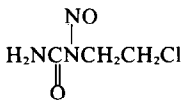
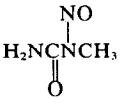
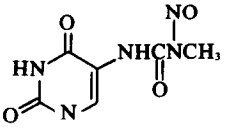
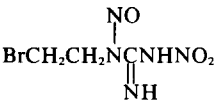
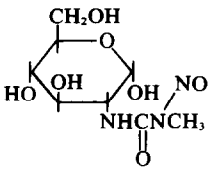


Fig. 4. The *in vitro* cytotoxic half-life of BCNU in horse serum and phosphate buffer. BCNU was incubated in either of the two solvents at 37° and aliquots taken at various time intervals and the residual cytotoxicity determined by bioassay as described in the text. Results are expressed as percentage increase in survival time relative to the time of incubation in the solvent.

been lost and no activity remained at all after 40 min. That this is probably due to chemical decomposition rather than deactivation by the host is indicated by measurements of the ability of BCNU to maintain its anti-tumour activity in various solvents. Figure 4 shows that BCNU in horse serum has lost half its anti-tumour activity after being in solution for 15 min, which compares favourably with the half-lives in plasma of 15 and 17 min reported by Loo and Dion[22] and Loo *et al.*[23] respectively.

Table 3 shows the effect of a series of compounds, structurally related to BCNU, on the growth of the TLX5 lymphoma. Although only a small number have been compared, the general formula $R\,CH_2N(NO)CO{\cdot}NH\,R^1$ (where R = H or CH_2Cl and R^1 = H or a variety of groups such as cyclohexyl or glucopyranosyl) appears to be essential for activity and is in agreement with studies on the L1210 leukaemia[24, 25] using a larger series of compounds. It has been proposed that BCNU acts after decomposition to reactive alkylating and carbamoylating species[12]. However, there was no apparent correlation between *in vitro* cytotoxicity and anti-tumour activity *in vivo*, the most cytotoxic compound (NSC 25959) of the series having no activity *in vivo* (Table 3). Moreover, some of the least toxic compounds *in vitro* had good activity in the whole animal. The possibility that the active compounds in the series had short half-lives in 1 mM phosphate buffer, such as had been seen with BCNU (Fig. 4), whereas the inactive compounds were more stable, was also investigated. A positive correlation would have suggested that chemical instability of the molecule was a prerequisite for anti-tumour action, and that it was the breakdown products of the nitrosoureas that were cytotoxic. No clear correlation was found, however, since a number of the inactive

Table 3. The *in vivo* antitumour activity and *in vitro* cytotoxicity of various structural analogues of BCNU

NSC number	Structure	Optimum single dose (mg/kg)	% Increase in survival time (% ILS)	Cytotoxic dose to give 99·9% cell kill (μg/ml)
409964		40	'cures'	4
25959		12	5·8*	3·125
35906		Inactive at all doses		9·325
47547		4·8	54·3	2·5
23909		160	50·4	64
406024		80	37·9	40
36885		200	4·9*	80
85998		160	11·3*	200

* Not significant activity.
Drugs were administered intraperitoneally.

nitrosoureas also had short half-lives in phosphate buffer.

The effects of lethal concentrations of BCNU on the incorporation of radioactive precursors into the macromolecules of the TLX5 lymphoma are shown in Fig. 5. The rate of incorporation of thymidine into DNA, uridine into RNA and leucine into protein is reduced within 2 hr incubation of the cells in the presence of BCNU. This is in clear contrast to the effects of an equitoxic dose of melphalan where the incorporation of thymidine into DNA is the only pathway inhibited. Furthermore, this effect of melphalan is only seen after the TLX5 lymphoma cells have been preincubated for a minimum of 4 hr with this agent. The rapid effect of BCNU is detailed in Fig. 6 in which the cells have been preincubated with the drug for 1 hr and there is a concomitant depression of thymidine incorporation. There is no such similar effect on the melphalan treated cells and indeed, no effect is seen until the cells have been preincubated for 4 hr (Fig. 6) when the degrees of inhibition of thymidine incorporation are similar in both BCNU and melphalan treated cells. The BCNU effect is almost immediate and this is shown in Fig. 7. The nitrosoureas is added to the cells, which are already incorporating thymidine into their DNA in a linear fashion, and 10 min after this addition to the cell suspension there is a reduction in incorporation.

A further difference between the action of the

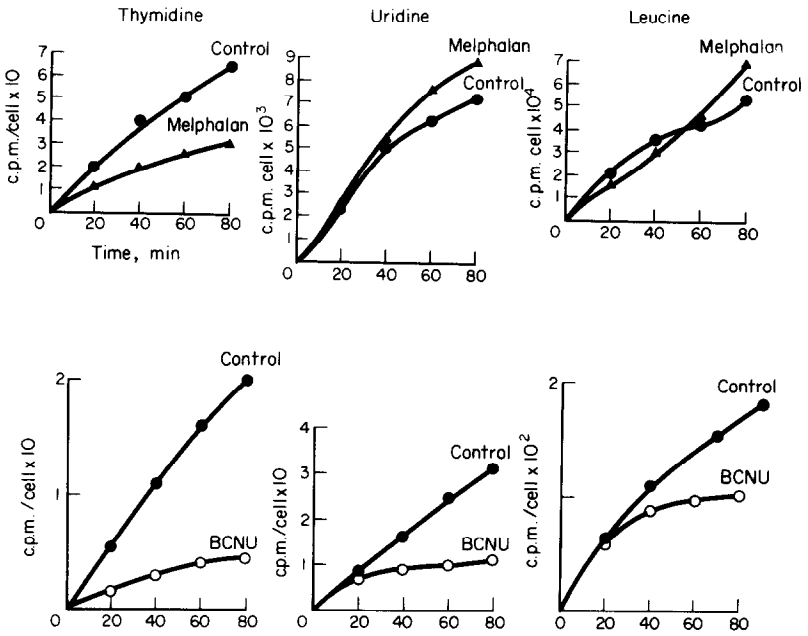


Fig. 5. A comparison of the effects of BCNU and Melphalan® on the incorporation of tritiated precursors into the DNA, RNA and protein of the TLX5 lymphoma. The cells and either of the drugs were incubated for 2 hr together and the precursor uptake performed as detailed in the text.

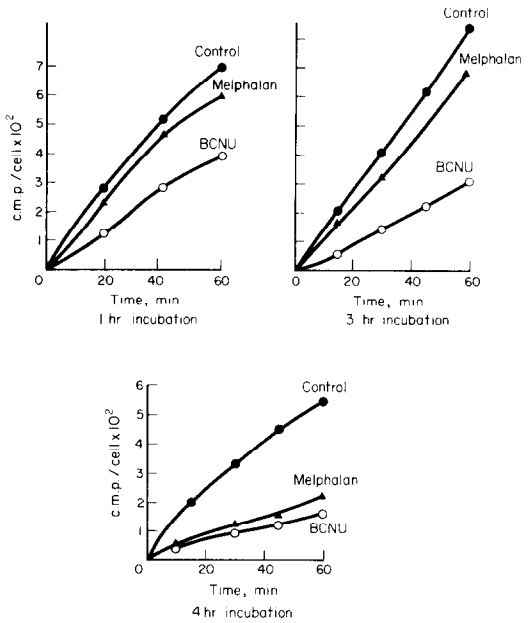


Fig. 6. The progressive effects of BCNU and Melphalan® on the incorporation of [^{3}H-*methyl*]thymidine into the TLX5 lymphoma. TLX5 cells were exposed to either of the drugs for various periods of time and the incorporation of the tritiated precursor determined thereafter as described in the text.

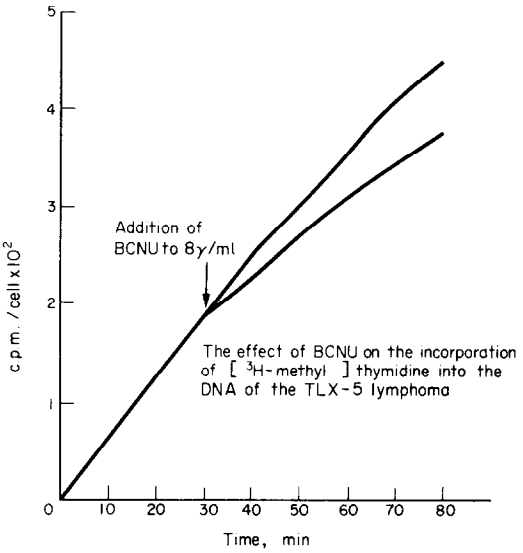


Fig. 7. The rate of BCNU induced inhibition of labelled thymidine incorporation into the TLX5 lymphoma. The cells were incubated with the tritiated thymidine and the incorporation into the cells followed. After 30 min a cytotoxic dose (8 μg/ml) of BCNU was added and the incorporation of thymidine in the TLX5 cells was followed for a further hour. The incorporation was compared with that in untreated cells.

alkylating agent melphalan and BCNU is evident from Fig. 8, which shows the formation of the mono-, di- and triphosphates from the added radioactive thymidine in the presence of either of the two drugs. Expressed as a percentage of the controls, melphalan at 20 μg/ml has little effect on the levels of the labelled thymidine nucleotides after 1½ hr incubation. BCNU, however, at an equitoxic dose (20 μg/ml) caused a build up of thymidine triphosphate and a reduction of thymidine incorporation of label into DNA, which suggested some interference with the utilisation or availability of thymidine triphosphate for DNA synthesis. Some inhibition of labelled thymidine incorporation into DNA after melphalan treatment was also evident but the features of the BCNU inhibition were absent. Although there was a rise in

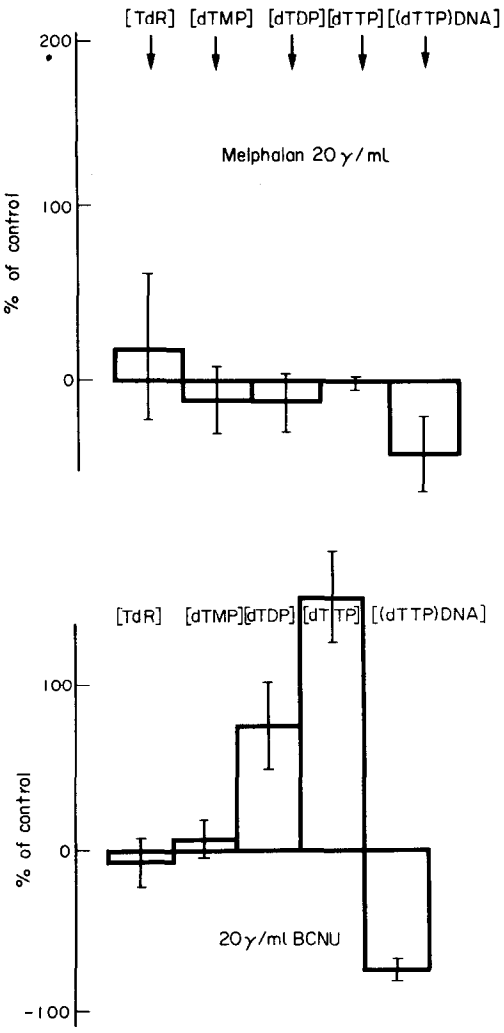


Fig. 8. The influence of BCNU and Melphalan® on the intracellular thymidine deoxyribonucleotide pools. Results are expressed as percentage deviation from the pool sizes in untreated cells. dTTP-DNA refers to the incorporation of [^{3}H]thymidine into DNA.

thymidine triphosphate due to the BCNU, no similar alteration in the levels of the deoxyadenosine, deoxyguanosine or deoxycytidine phosphates was observed. No explanation for this seemingly specific effect on thymidine triphosphate utilisation can be proposed, especially as it has been shown that BCNU has no effect on the DNA polymerase enzyme. In this context it is of interest that a number of anti-tumour agents cause a build-up of TTP in sensitive cells [26, 27], but unlike the effects observed with BCNU there is a concomitant rise of TDP and TMP. The absence of any effect on the DNA polymerase enzyme would appear to conflict with the results of Wheeler and Bowdon [28]: however, the doses used to elicit an effect on their enzyme preparations were somewhat higher (5×10^{-4}–1×10^{-3} M; 107–214 μg/ml). In our studies, doses above 40 μg/ml were not investigated since they represent excessive supralethal doses and thus may not reflect a specific physiological effect on thymidine metabolism.

A specific effect of BCNU on thymidine metabolism was further indicated in a series of cytotoxicity tests where the ability of a number of compounds to prevent the toxicity of either BCNU or melphalan was investigated (Table 4). If the TLX5 lymphoma cells were pretreated with thymidine, the cytotoxicity of 8 μg/ml of BCNU could in some experiments be significantly reversed: no other deoxyribonucleoside was capable of this effect. It was found that the best protection was obtained if the thymidine was added 30 min before the addition of BCNU to the tumour cells rather than the simultaneous addition of the two. However, the results were variable. The mechanism by which this reversal of BCNU cytotoxicity occurs is presently under investigation.

Considerable differences between BCNU and the alkylating agent melphalan have therefore been demonstrated in their spectrum of action, in their effects upon precursor incorporation into macromolecules and in the speed with which this latter effect occurs. It thus seems unlikely that the nitrosoureas and the alkylating agents have a

Table 4. The reversal of BCNU cytotoxicity by preincubation with thymidine

Treatment / Experiment	% Increase in survival time 1	2	3	4	5
BCNU 8 μg/ml	'cures'	'cures'	'cures'	'cures'	'cures'
BCNU 8 μg/ml + thymidine 1000 μg/ml	'cures'	—	—	—	—
BCNU 8 μg/ml + thymidine 500 μg/ml	'cures'	'cures'	—	80% 'cures'	—
BCNU 8 μg/ml + thymidine 250 μg/ml	15·4	29·5	—	80% 'cures'	20% 'cures'
BCNU 8 μg/ml + thymidine 125 μg/ml	—	25·0	11·1	40% 'cures'	80% 'cures'
BCNU 8 μg/ml + thymidine 62·5 μg/ml	—	—	33	42	80% 'cures'
BCNU 8 μg/ml + thymidine 31·25 μg/ml	—	—	'cures'	40% 'cures'	—

Varying doses of thymidine were incubated at 37° with TLX5 cells for 30 min after which the cytotoxicity of BCNU (8 μg/ml) was determined. Results are expressed as percentage increase in survival times and 'cures' refer to animals surviving longer than 30 days after tumour cell inoculation.

common mechanism of action. A specific effect on the utilisation of thymidine is implicated as an important feature of the mechanism of action of BCNU since its incorporation into DNA (as the labelled nucleotide) is rapidly and specifically inhibited and high concentrations of the nucleoside can significantly reverse the cytotoxicity of BCNU.

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