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SPECIFIC PROTECTION AGAINST THE CYTOTOXICITY OF A NEW CLASS OF NITROSOUREAS BY PYRIMIDINE DEOXYRIBONUCLEOSIDES

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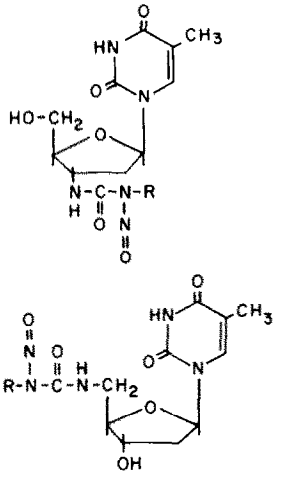
Abstract—Some of the pharmacological properties of a new class of compounds are described in which either a methyl- or a chloroethyl-nitrosourea functionality is attached to the 3′-position of 3′-deoxythymidine (3′-MTNU and 3′-CTNU) or to the 5′-position of 5′-deoxythymidine (5′-MTNU and 5′-CTNU). The pharmacological effects of these thymidine analogs vary considerably. The dose–response curves for the inhibition of leukemic L1210 cell growth by the 5′-derivatives were similar to that for 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), being characterized by a shoulder region and a linear component. However, the corresponding curves for the 3′-derivatives were biphasic, being initially steep and linear followed by a shallow portion. Pyrimidine 2′-deoxyribonucleosides (thymidine, deoxyuridine or deoxycytidine), but not pyrimidine ribonucleosides or purine nucleosides, can specifically prevent and reverse the inhibition of cell growth caused by the 3′-analogs. In contrast, the inhibition of cell replication induced by 5′-CTNU or BCNU was not decreased by the addition of thymidine, deoxyuridine or deoxycytidine. The protective effect was dependent on the relative concentrations of the protecting pyrimidine deoxyribonucleoside and the 3′-nitrosourea. Of particular importance is the finding that the addition of deoxycytidine to L1210 cells, treated previously for 24 hr with 3′CTNU, substantially reversed the growth inhibition, suggesting that competition for cellular transport is not the critical site of interaction and that alkylation may not be the primary mode of action. Soft agar cloning experiments revealed that deoxycytidine also protects L1210 cells from the lethal effects of 3′-CTNU. The protective interaction is not unique to L1210 cells since the growth inhibitory effects of 3′-CTNU on B16 melanoma cells could also be reduced by deoxycytidine. Under physiological conditions, 3′-CTNU decomposes to generate alkylating and carbamoylating species. In addition, however, a stable cytotoxic compound, 3′-amino-3′-deoxythymidine, is also formed. This aminonucleoside is generated in sufficient quantities to contribute to the pharmacological actions of 3′-CTNU and may account for the specific protective interaction exerted by pyrimidine 2′-deoxyribonucleosides.

The carrier portions of nitrosoureas affect their physical and chemical properties [1], and efforts to correlate some of these properties with biological activity have been made [2–5]. Of particular interest is the finding that bone marrow toxicity, a complication which limits the clinical usefulness of most nitrosoureas [6], is reduced in derivatives containing a glucose carrier [7, 8]. However, neither the alkylating nor the carbamoylating activity of the nitrosoureas correlated with myelosuppression [3, 4]. Thus, appropriate structural modifications can favorably alter the pharmacological properties of even highly reactive drugs.



R	NAME
$ClCH_2CH_2-$	3′-CTNU
CH_3-	3′-MTNU
$ClCH_2CH_2-$	5′-CTNU
CH_3-	5′-MTNU

Fig. 1. Chemical structures of the new nucleoside nitrosourea analogs: 3′-CTNU = 3′-[3-(2-chloroethyl)-3-nitrosoureido]-3′-deoxythymidine; 3′-MTNU = 3′-(3-methyl-3-nitrosoureido)-3′-deoxythymidine; 5′-CTNU = 5′-[3-(2-chloroethyl)nitrosoureido]-5′-deoxythymidine; and 5′-MTU = 5′-(3-methyl-3-nitrosoureido)-5′-deoxythymidine.

We attempted to improve the pharmacological properties of the nitrosoureas by combining them with nucleosides [9]. It was hoped that such carriers would alter the metabolism or the uptake of the new agents, and that selectivity for cancer cells would be enhanced. Figure 1 shows the structures of the new compounds. These analogs possess methyl- or chloroethyl-nitrosourea groups at the 3′-position (3′-MTNU and 3′-CTNU) or the 5′-position (5′-MTNU and 5′-CTNU) of thymidine. These new nitrosoureas were found to be cytotoxic, and to display the alkylating and carbamoylating activities that are characteristic of other nitrosoureas [9]. We now report that the 3′-derivatives of this series are unusual in that their cytotoxicity can be prevented or reversed by pyrimidine 2′-deoxyribonucleosides (dThd, dCyd, dUrd).

MATERIALS AND METHODS

Chemicals. The syntheses of the nucleoside nitrosoureas were done as described previously [9]. Dr. Harry B. Wood, Jr., Chief, Drug Synthesis and Chemistry Branch, Division of Cancer Treatment of the National Cancer Institute, kindly provided the 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) used in these studies. The synthesis of 3′-aminothymidine has been reported previously [10]. The nucleosides were obtained from the Sigma Chemical Co., St. Louis, MO.

Cells. Murine leukemia L1210 cells were grown in Fischer's medium supplemented with 10% horse serum, 100 units/ml of penicillin, and 100μg/ml of streptomycin (Grand Island Biological Co., Grand Island, NY) at 37° in a humidified 5% carbon dioxide atmosphere as suspension cultures. Asynchronous exponentially growing cells were used in all growth experiments. Cells were suspended at a density of 1 to 1.2×10^4 cells/ml in 15 ml culture tubes and were counted at various times later with a model ZBI Coulter counter (Coulter Electronics, Hialeah, FL).

Within a 30-min period, the nitrosoureas were dissolved in 0.1 ml dimethylsulfoxide (DMSO), diluted with an appropriate volume of phosphate-buffered saline (PBS), pH 7.4, sterilized by membrane filtration (0.45 μM, Millipore Corp., Bedford, MA), and then added to the cultures. Data from most of the growth inhibition studies are expressed as the per cent of control (vehicle only) cell number, 72 hr after drug addition. The untreated cells grew exponentially throughout each experiment and had a population doubling time of about 13 hr. Each experimental point was done in duplicate or triplicate, and is the average value of two or three separate experiments.

B16 melanoma cells (obtained from Dr. John Lazo) were grown as monolayers in Dulbecco's modified minimal essential medium supplemented with 10% fetal calf serum (Grand Island Biological Co.). In growth experiments, approximately 5×10^4 cells per well were plated in six well tissue culture dishes (Costar, Cambridge, MA). The test compounds were added 24 hr later and the incubation was continued for 3 days. At daily intervals the cells were detached from the plates in PBS containing 2 mM EDTA and then counted. Control cells grew exponentially between 24 and 72 hr, with a population doubling time of 17 hr.

L1210 cell viability was assessed using the soft agar cloning procedure of Chu and Fischer [11]. In these experiments the test compounds were added to exponentially growing L1210 cells (4×10^4 cells/ml). After 48 hr of incubation the cells were centrifuged, washed in fresh medium, diluted appropriately, and then suspended in Fischer's medium containing 0.12% agar and 15% horse serum. The number of colonies formed was determined after 14 days of incubation at 37° in a humidified 5% CO_2 atmosphere.

High pressure liquid chromatography (h.p.l.c.). The formation of 3′-aminothymidine from 3′-CTNU was quantified by h.p.l.c. (column: ANC cation exchange; buffer: 0.3 M NH_4ClO_4, 0.1 M citric acid, pH 2.2; flow rate: 1 ml/min; temperature: 58°). Under these conditions, authentic 3′-aminothymidine [10] had a retention time of 48 min. 3′CTNU (2 mg/0.1 ml of DMSO) was diluted forty times with Fischer's medium, maintained at 37° and, at various time intervals, samples of the mixture were analyzed by h.p.l.c. The amount of 3′-aminothymidine formed was estimated by weighing the paper obtained from the spectral tracings generated by the u.v. (267 nm) detector.

RESULTS

Inhibition of cell growth. The effects of the various nucleoside nitrosoureas and BCNU on the replication of L1210 cells are shown in Fig. 2. The dose–response curves for 5′-CTNU and BCNU are typical for nitrosoureas [12] and are characterized by a shoulder region. 5′-MTNU was much less potent, inhibiting growth by 50 per cent at about 100 μM. In contrast, the

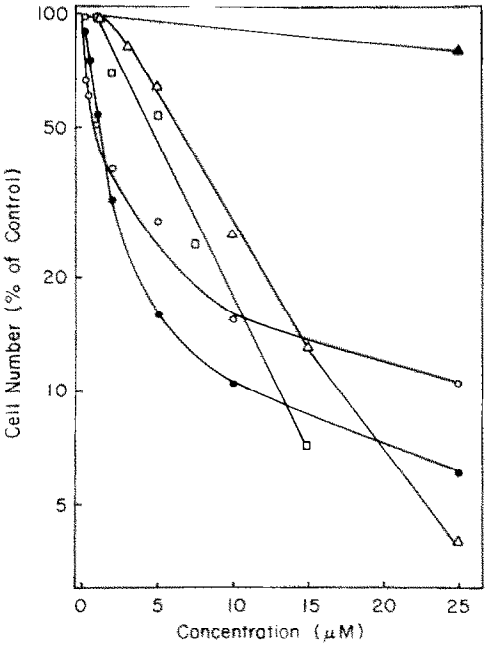


Fig. 2. Inhibition of L1210 cell growth by various nitrosoureas. L1210 cells were exposed to the indicated concentrations of either 3′-CTNU (●), 3′-MTNU (○), 5′-CTNU (△), 5′-MTNU (▲), or BCNU ( ). The cultures were incubated for 72 hr and the number of cells was determined. In a given experiment, each point was done in triplicate and each curve is compiled from at least three separate experiments. The data are expressed as the per cent of the cell number produced in the absence of drug.

dose–response curves of 3′-CTNU and 3′-MTNU are initially steep and linear, but become quite shallow at higher drug concentrations. These results suggest the presence of cell populations with different sensitivities to the 3′-derivatives and/or the possibility that the pharmacology of these compounds is different from that of other nitrosoureas.

Effects of nucleosides. The effects of various nucleosides on the cytotoxicity of the new nitrosoureas, which are analogs of thymidine, were investigated and a distinct pattern of interaction emerged. First, pyrimidine deoxyribonucleosides (dThd, dUrd, dCyd), but not pyrimidine ribonucleosides (Urd, Cyd) or purine nucleosides (Ado, dAdo, Guo, dGuo), were capable of protecting L1210 cells against the inhibition of growth caused by both of the 3′-derivatives (Fig. 3: 3′-MTNU, 5 μM; and Fig. 4: 3′-CTNU, 5 μM). Good protection

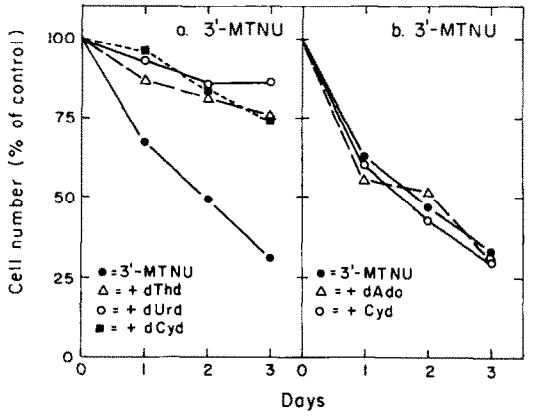


Fig. 3. Effects of nucleosides on the inhibition of L1210 cell growth caused by 3′-MTNU. L1210 cells were exposed to 5 μM 3′-MTNU (●) and 25 μM of either (a) dThd (△), dUrd (○), or dCyd (■), or (b) dAdo (△) or Cyd (○). The cultures were incubated for 72 hr and cell numbers were determined. The data, expressed as the per cent of control cell number, represent the average of at least two experiments, each done in duplicate.

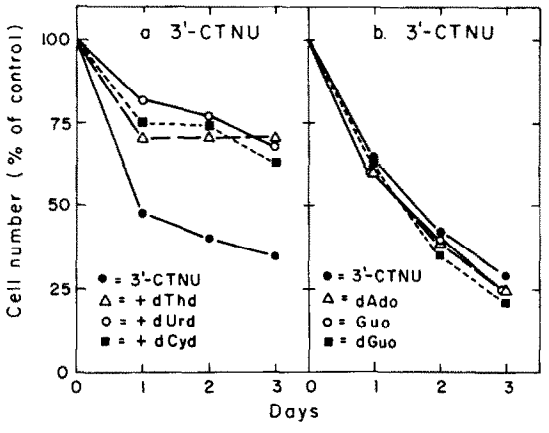


Fig. 4. Effects of nucleosides on the inhibition of L1210 cell growth caused by 3′-CTNU. L1210 cells were exposed to 5 μM 3′-CTNU (●) and 25 μM of either (a) dThd (△), dUrd (○), or dCyd (■), or (b) dAdo (△), Guo (○), or dGuo (■). The experimental conditions and the expression of data are the same as those described in the legend of Fig. 3.

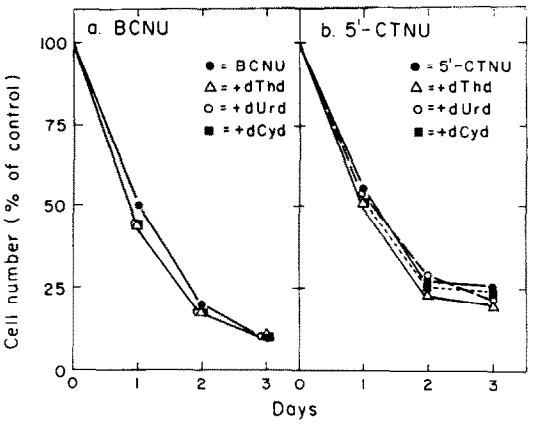


Fig. 5. Effects of nucleosides on the inhibition of L1210 cell growth caused by BCNU and 5′-CTNU. L1210 cells were exposed to (a) BCNU (8 μM) or (b) 5′-CTNU (10 μM) and either dThd (△), dUrd (○), or dCyd (■). The experimental conditions and the expression of data are the same as those described in the legend of Fig. 3.

was exerted by each of the three pyrimidine deoxyribonucleosides (25 μM). Representative examples of the ineffective nucleosides are presented in figs. 3 and 4; however, for each derivative all of the previously mentioned nucleosides were tested at a concentration of 25 μM. Thus, the protective effect is specific for pyrimidine deoxyribonucleosides. Second, the protective effect is also specific for the type of nucleoside nitrosourea. As shown in Fig. 5, the addition of pyrimidine deoxyribonucleosides did not reduce the cytotoxicity of either BCNU or 5′-CTNU. The importance of both the nature of the carrier and the position of attachment of the nitrosourea functionality is clearly illustrated.

The degree of protection conferred by the pyrimidine deoxyribonucleoside is dependent on the concentration of the added nucleoside. This relationship is shown in Fig. 6. Virtually complete protection was achieved

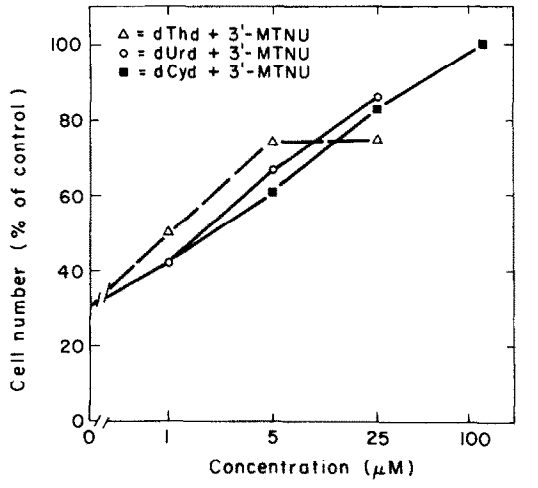


Fig. 6. Pyrimidine deoxyribonucleoside protection of 3′-MTNU-induced growth inhibition of L1210 cells. L1210 cells were treated with 5 μM 3′-MTNU and the indicated concentration of either dThd (△), dUrd (○), or dCyd (■). The experimental conditions and the expression of data are the same as those described in the legend of Fig. 3.

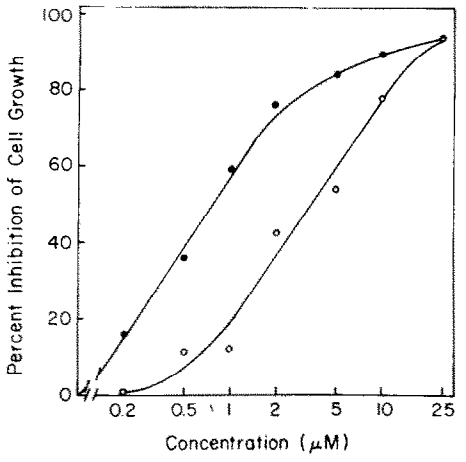


Fig. 7. Effect of dCyd (○) on the inhibition of cell growth caused by 3′-CTNU (●). In this experiment, 50 μM dCyd and the indicated concentration of 3′-CTNU were added to L1210 cells with each point done in triplicate. Cell numbers were determined 72 hr later and the results are expressed as the per cent inhibition of control cell growth.

when dCyd (100 μM) was added to cells treated with 3′-MTNU (5 μM). Protection by dThd and dUrd was dose-dependent at the lower concentrations; however, the growth inhibitory effects of these compounds limited their usefulness at the higher doses. The addition of dCyd (50 μM) to L1210 cells exposed to varying concentrations of 3′-CTNU resulted in a shift of the dose–response curve to the right (Fig. 7). The results demonstrate a consistent protective effect over a wide range of 3′-CTNU concentrations.

Reversal of growth inhibition. The cytotoxic effects of 3′-CTNU can be reversed as well as protected by dCyd. Figure 8 illustrates the effects caused by adding

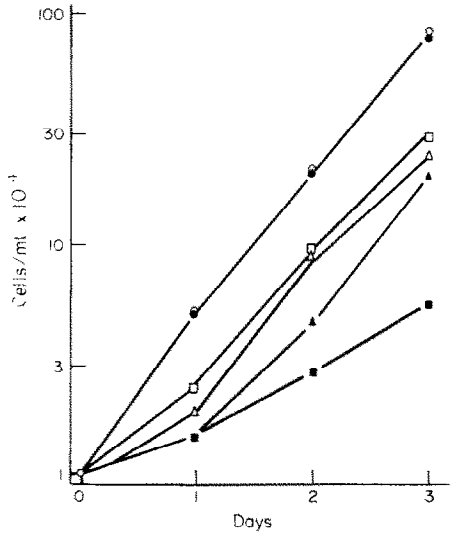


Fig. 8. Reversal of 3′-CTNU (10 μM) inhibition of L1210 cell growth by dCyd (50 μM). Exponentially growing cells were treated at 0 hr with PBS (●), 50 μM dCyd (○), 10 μM 3′-CTNU alone (■), or in combination with 50 μM dCyd added at 0 hr (□), 6 hr (△), or 24 hr (▲). Cells were counted after 24, 48 and 72 hr of incubation.

Table 1. Effects of deoxycytidine on the cytotoxicity of 3′-CTNU*

Condition	L1210 cell viability (cloning efficiency)	B16 cell growth (per cent of growth)
Control	80 ± 3	
Deoxycytidine (100 μM)	86 ± 11	98 ± 2
3′-CTNU (10 μM)	<1	53 ± 2.6
3′-CTNU + deoxycytidine	21 ± 9.6	76 ± 3.5

* L1210 cells were exposed to 3′-CTNU (10 μM) for 48 hr in the presence or absence of deoxycytidine (100 μM). The viability of the cells was then determined using soft agar cloning, as described in materials and Methods. The number of b16 cells present after exposure to 3′-CTNU (10 μM) in the presence or absence of deoxycytidine (100 μM) for 72 hr was determined, as described in Materials and Methods. The control cells grew exponentially during this time period, with a population doubling time of 17 hr. The data are expressed as the means ± S.D. of three separate experiments.

dCyd (50 μM) at different times following the treatment of L1210 cells with 3′-CTNU (10 μM). Substantial reversal of the growth inhibitory effects of 3′-CTNU was evident even when dCyd was added 24 hr after the nitrosourea.

L1210 cell viability. The effects of 3′-CTNU alone and in combination with dCyd on the viability of L1210 cells were assessed, utilizing a soft agar cloning technique [11]. As shown in Table 1, less than 1 per cent of the L1210 cells survived exposure to 10 μM 3′-CTNU for 48 hr. However, the additional presence of 100 μM dCyd conferred significant protection, as indicated by a 20-fold increase in the surviving fraction. Deoxycytidine alone did not alter the cloning efficiency of the cells.

Growth of B16 melanoma cells. 3′-CTNU inhibited the replication of B16 cells ($ED_{50} \sim 10$ μM), although to a lesser extent than L1210 cells ($ED_{50} \leqslant 0.5$ μM). Protection with dCyd was also demonstrated (Table 1). 3′-CTNU inhibited B16 cell growth by 47 per cent at 10 μM, but by 24 per cent if 100 μM dCyd was also present.

Formation of 3′-aminothymidine. We have shown that the new nucleoside nitrosoureas possess alkylating and carbamoylating activities [9]. It is possible that the

Table 2. Formation of 3′-aminothymidine from 3′-CTNU*

Time (hr)	Formation of 3′-aminothymidine (per cent of 3′-CTNU present at 0 hr)
0	1.2
1	10.5
2	13.7
3	18.8
5	21.2
23	22.9
96	20.4

* 3′-CTNU was incubated in Fischer's media at 37° and, at the indicated times, samples were analyzed for the presence of 3′-aminothymidine using h.p.l.c., as described in Materials and Methods.

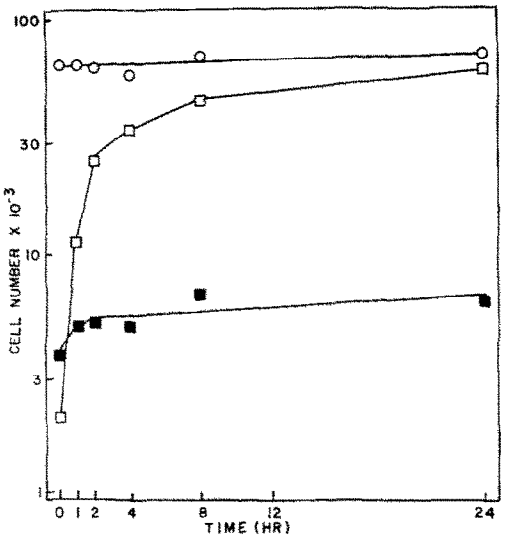


Fig. 9. Effect of incubating BCNU and 3′-CTNU at 37° on the inhibition of L1210 cell growth. Solutions of BCNU (0·75 mM) or 3′-CTNU (1.25 mM) in Fischer's medium were incubated at 37° in a 5% Co_2 atmosphere. At the indicated times, samples were removed and added to cultures of L1210 cells such that the final concentrations were 15 μM for BCNU (□) and 25 μM for 3′-CTNU (■). PBS (○) was added to the control cells. The cultures were incubated for 72 hr and then cell numbers were determined.

isocyanates thus formed could react with water to generate aminonucleosides. Since we have found that one such compound, 3′-aminothymidine, is a potent cytotoxic agent [10, 13], an effort was made to determine whether it is formed during the decomposition of 3′-CTNU. The nitrosourea was incubated in Fischer's medium at 37° and then analyzed by h.p.l.c. for the presence of 3′-aminothymidine. The data in Table 2 indicate that approximately 20 per cent of the 3′-CTNU was converted to 3′-aminothymidine and that the reaction was complete by 5 hr.

To determine if the formation of 3′-aminothymidine could account for some of the toxicity of 3′-CTNU, the nitrosourea was incubated in Fischer's medium at 37° for various lengths of time before assessing its effects on L1210 cell growth. BCNU was included as a positive control. The results in Fig. 9 clearly show that the cytotoxicity of BCNU rapidly decreased, whereas even after incubating 3′-CTNU for 24 hr marked inhibition of L1210 cell replication was evident.

DISCUSSION

A highly selective interaction between a new class of nitrosoureas and certain nucleosides is documented by the data presented. Pyrimidine 2′-deoxyribonucleosides, but not other nucleosides, can protect or reverse the inhibition of L1210 cell growth caused by the 3′-nitrosourea derivatives of thymidine, but not that caused by the 5′-analogs. These findings emphasize the importance of the group to which the nitrosourea functionality is attached. The pharmacology of these nitrosoureas is changed by the nature of the carrier, a nucleoside, and its position of attachment, the 3′- versus the 5′-position. The influence of a glucose carrier on the properties of nitrosoureas has been well documented by Schein *et al.* [3, 4, 8]. This structural modification results in reduced bone marrow toxicity in mice and humans [14], and this advantageous property is present whether the cytotoxic group is attached to the C-1 or the C-2 of glucose [15]. However, this change may introduce a new toxicity, diabetes, as in the case of streptozotocin [16]. The diabetogenic effect can be specifically modified by the administration of nicotinamide [17], and an enhancement of the antitumor activity of streptozotocin has been achieved by pretreatment with 3-*O*-methyl-D-glucose and nicotinamide [18]. These results indicate that interactions between nitrosoureas and certain tissues can be dependent on the nature of the carrier moiety.

The mechanism by which pyrimidine 2′-deoxyribonucleosides exert the protective effect shown in this study appears to be related to the formation of 3′-aminothymidine. Several lines of evidence suggest this. The data presented in Fig. 9 indicate that either 3′-CTNU is a stable, cytotoxic compound or that it decomposes to form such a species. An important characteristic of nitrosoureas is their chemical instability. They decompose under physiological conditions to yield carbonium ions and isocyanates [19], two reactive species which possess alkylating and carbamoylating activities [20] respectively. The fact that 3′-CTNU shares these properties [9] suggests the generation of a new compound. In addition, by using h.p.l.c. we have found that 3′-aminothymidine is formed during the incubation of 3′-CTNU at 37° (Table 2). In this regard, the hydrolysis of organic isocyanates to yield the corresponding carbamic acid and the amino derivative is well known [21]. We have reported that 3′-aminothymidine is a potent inhibitor of L1210 cell replication and that pyrimidine 2′-deoxyribonucleosides can prevent its cytotoxicity [10, 13]. Importantly, the specificity for that interaction is the same as that described for the protection of the cytotoxicity induced by 3′-CTNU (Fig. 4). The amount of 3′-aminothymidine formed during the decomposition of 3′-CTNU (about 20 per cent conversion, Table 2) is sufficient to contribute significantly to the pharmacology of the parent nitrosourea. For example, the replication of L1210 cells is inhibited by 93 per cent in the presence of 25 μM 3′-CTNU and, under these conditions, approximately 5 μM 3′-aminothymidine would be expected to be formed, a concentration which reduces L1210 cell growth by 85 per cent [13].

In contrast, 5′-aminothymidine does not inhibit L1210 cell replication [10] and, therefore, its formation would not be expected to contribute to the toxicity of the 5′-nucleoside nitrosoureas. Thus, it is not surprising that pyrimidine 2′-deoxyribonucleosides do not prevent the toxicity of 5′-CTNU (Fig. 5) and that the dose–response curve for cell growth inhibition is similar to that of BCNU and not that of 3′-CTNU (Fig. 1).

Interference by pyrimidine deoxyribonucleosides with the cellular transport of the nucleoside nitrosoureas or their breakdown products does not seem to be of major importance since reversal of cytotoxicity can be achieved as much as 24 hr after 3′-CTNU addition (Fig. 9). Nucleoside transport appears to be mediated by a common carrier [22] and thus it would not account for the selectivity of the protective effect. The reversibility also suggests that alkylation may not be the primary mode of action of the 3′-nucleoside nitrosoureas. How-

ever, 3′-CTNU does have twice the alkylating activity of BCNU [9], and the contribution of this effect to the pharmacology of 3′-CTNU, particularly at higher doses, needs to be assessed.

The results of the cloning experiments and growth inhibition studies are in agreement and indicate that dCyd can protect L1210 cells from the lethal toxicity of 3′-CTNU. More than twenty times as many cells survived exposure to 3′-CTNU if dCyd was also present. It will be of considerable interest to determine the response, *in vivo*, of both normal and cancerous mouse tissues to the effects of 3′-CTNU alone and in combination with pyrimidine 2′-deoxyribonucleosides.

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