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Role of Thymidine Kinase and Thymidylate Synthetase in the Cytostatic, Antimetabolic, and Antitumor Effects of the Carbocyclic Analogue of 5-Nitro-2′-deoxyuridine

A Comparison with 5-Nitro-2′-deoxyuridine

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SUMMARY

The carbocyclic analogue of 5-nitro-2′-deoxyuridine (NO_2dUrd), in which the sugar moiety is replaced by a cyclopentane ring and which was designated C-NO_2dUrd, has been evaluated for its cytostatic, antimetabolic, and antitumor properties. The following findings are noted. C-NO_2dUrd is about 500- to 2000-fold less inhibitory to tumor cell proliferation *in vitro* than NO_2dUrd. Phosphorolysis of C-NO_2dUrd by rabbit liver extracts was not observed under conditions where NO_2dUrd was readily converted to 5-nitrouracil (NO_2Ura). Also, C-NO_2dUrd is converted to its 5′-monophosphate (C-NO_2dUMP) by dThd kinase nearly as efficiently as the true nucleoside NO_2dUrd. This metabolic conversion is necessary for the inhibitory effect of C-NO_2dUrd on tumor cell proliferation in cell culture. The principal target enzyme for the cytostatic action of C-NO_2dUrd is 2′-deoxythymidylate (dTMP) synthetase. C-NO_2dUMP, the active metabolite of C-NO_2dUrd, has a much lower affinity for dTMP synthetase than does NO_2dUMP. This is the first demonstration of the interaction of a carbocyclic pyrimidine nucleotide analogue with dTMP synthetase. Neither NO_2dUrd nor C-NO_2dUrd exerts any significant antitumor activity in mice bearing L1210 or P388 leukemia; for NO_2dUrd, this failure may be related to a rapid degradation to its inactive metabolite, NO_2Ura; for C-NO_2dUrd, it is most likely due to a decreased affinity of C-NO_2dUMP for its target enzyme, dTMP synthetase.

INTRODUCTION

Carbocyclic nucleoside analogues, in which the normal sugar moiety is replaced by a cyclopentane ring, have recently received much attention as potentially useful antiviral and/or antitumor drugs. Such carbocyclic analogues have been synthesized for a number of antitumor drugs (araC,[1] FdUrd) and antiviral drugs (BVdUrd, IdUrd). The carbocyclic analogue (C-FdUrd) of FdUrd inhibited the proliferation of cultured L1210 cells at an ID_{50} of 2.5 μg/ml, but failed to increase the life span of mice bearing P388 or L1210 leukemia at doses at which FdUrd significantly increased the life span (1). Whereas C-FdUrd and FdUrd were phosphorylated at a similar rate, phosphorolytic cleavage of C-FdUrd to FUra was not observed under conditions that readily converted FdUrd to FUra.

The carbocyclic analogues of Cyd and araC (designated C-Cyd and C-araC, respectively) have also been synthesized (2), and these compounds were found to increase the life span in L1210 leukemia-bearing mice by 82 and 104% (3, 4), when administered for 9 days at a daily dose of 200 or 150 mg/kg/day, respectively. C-Cyd was markedly active against human influenza virus *in vitro* but did not offer much activity against influenza *in vivo* (5).

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[1] The abbreviations used are: araC, 1-β-D-arabinofuranosylcytosine; C-araC, carbocyclic analogue of araC; FdUrd, 5-fluoro-2′-deoxyuridine; C-FdUrd, carbocyclic analogue of FdUrd; BVdUrd, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine; C-BVdUrd, carbocyclic analogue of BVdUrd; IVdUrd, (*E*)-5-(2-iodovinyl)-2′-deoxyuridine; C-IVdUrd, carbocyclic analogue of IVdUrd; IdUrd, 5-iodo-2′-deoxyuridine; C-IdUrd, carbocyclic analogue of IdUrd; BVdCyd, (*E*)-5-(2-bromovinyl)-2′-deoxycytidine; C-BVdCyd, carbocyclic analogue of BVdCyd; NO_2dUrd, 5-nitro-2′-deoxyuridine; C-NO_2dUrd, carbocyclic analogue of NO_2dUrd; NO_2dUMP, 5-nitro-2′-deoxyuridine-5′-monophosphate; C-NO_2dUMP, carbocyclic analogue of NO_2dUMP; NO_2Ura, 5-nitrouracil; dTMP, 2′-deoxythymidylate; C-dUrd, carbocyclic dUrd; C-Cyd, carbocyclic analogue of Cyd; HSV-1, herpes simplex virus type 1; TK^-, thymidine kinase deficient; IP, intraperitoneal.

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Carbocyclic analogues of 5-iodo-dUrd, 5-bromo-dUrd, dThd, 5-methylamino-dUrd, 5-butylamino-dUrd, and 5-dimethylamino-dUrd have also been synthesized (6). Of this series, the carbocyclic analogue of 5-iodo-dUrd (C-IdUrd) was the most potent inhibitor of HSV-1 (minimum inhibitory concentration, 0.1–0.4 μg/ml). All carbocyclic compounds were inactive against a TK^- variant of HSV-1 (6), indicating that to be effective as antiviral agents the carbocyclic analogues had to be activated by the virus-induced dThd kinase (7, 8). We have recently synthesized the carbocyclic analogues of BVdUrd: IVdUrd and BVdCyd (9). C-BVdUrd, C-IVdUrd, and C-BVdCyd proved at least as selective, but slightly less potent, in their antiherpetic activity than BVdUrd, IVdUrd, and BVdCyd. It was further ascertained that the selective antiherpetic activity of C-BVdUrd and C-IVdUrd depended on a specific phosphorylation by the HSV-1-encoded dThd kinase (10).

Based on these experimental data, it seemed particularly interesting to synthesize the carbocyclic analogue of 5-nitro-dUrd. Indeed, NO_2dUrd is a potent cytotoxic drug: it strongly inhibits the proliferation of tumor cells *in vitro* (11, 12) and can therefore be regarded as a potential antitumor agent *in vivo*. NO_2dUrd proceeds through the same anabolic and catabolic steps as does dThd. It is metabolized to NO_2dUMP by dThd kinase, and NO_2dUMP is a potent inhibitor of dTMP synthetase (12–15), a pivotal enzyme in DNA synthesis (16). However, the nucleotide(s) of NO_2dUrd may be converted back to the nucleoside by the action of phosphatases, and the nucleoside itself is subject to phosphorolytic cleavage by pyrimidine nucleoside phosphorylases (17), which release the free pyrimidine base NO_2Ura. The latter can then be further catabolized by dihydrouracil dehydrogenase (18). The rapid degradation of NO_2dUrd to its inactive metabolite NO_2Ura may obviously affect the therapeutic efficacy of NO_2dUrd. Thus, the effectiveness of NO_2dUrd as an antitumor agent may be muted by its premature conversion to NO_2Ura.

It was postulated, therefore, that the carbocyclic counterpart of NO_2-dUrd, C-NO_2dUrd, which lacks the glycosidic bond of true nucleosides and as a consequence is not expected to be a substrate of pyrimidine nucleoside phosphorylases, would not be degraded to NO_2Ura. It was further surmised that C-NO_2dUrd might be more selective as an inhibitor of dTMP synthetase and more effective than NO_2dUrd *in vivo*.

These considerations led us to synthesize the carbocyclic analogue of 5-nitro-2′-deoxyuridine, C-NO_2dUrd, and the corresponding carbocyclic 5-nitro-dUrd-5′-monophosphate, C-NO_2dUMP, and compare their cytostatic, antimetabolic, and antitumor properties with those of the parent compounds, NO_2dUrd and NO_2dUMP.

MATERIALS AND METHODS

Cells. Murine leukemia L1210/0 cells, L1210/BdUrd (TK^-) cells, human lymphoblast Raji/0, and Raji/TK^- cells were cultivated as previously described (19). Rat hepatoma cells were maintained in Eagle's minimum essential medium, supplemented with 10% (v/v) inactivated fetal calf serum (GIBCO Bio-Cult, Glasgow, Scotland) and 2 mM L-glutamine (Flow Laboratories, Irvine, Scotland).

Test compounds. NO_2dUrd ws synthesized by nitration of 3′,5′-di-*O*-acetyl-2′-deoxyuridine with *N*-nitropyrazole (20); upon deblocking, NO_2dUrd was obtained in about 75% yield.[2] NO_2dUMP was provided by P. F. Torrence (National Institutes of Health, Bethesda, MD) (see also Refs. 21 and 22). It was obtained by nitration of dUMP with nitronium fluoroborate (21). C-NO_2dUrd was synthesized starting from the carbocyclic analogue of 2′-deoxyuridine (C-dUrd) (9). Reaction of the 3′,6′-diacetate of C-dUrd with 2 equivalents of nitronium tetrafluoroborate in sulfolane (21) gave C-NO_2-2′-dUrd-3′,6′-diacetate, which was deprotected with sodium methoxide in methanol. The reaction product C-NO_2dUrd was obtained with a yield of 77.5% (starting from C-dUrd-3′,6′-diacetate). C-NO_2dUMP was prepared by phosphorylation of C-NO_2dUrd following the $POCl_3$·trimethylphosphate method (23). The other nucleosides dUrd, dThd, dCyd, Urd, and Ura were purchased from Sigma Chemical Company (St Louis, MO), and 6-aminothymine was kindly provided by C. Desgranges (Unité 8 de Cardiologie de l'INSERM, Pessac, France).

Experimental analysis for C-NO_2dUrd [(+)-5-nitro-1-[(1α,3β,4α)-3-hydroxy-4-(hydroxymethyl)cyclopentyl]-2,4-(1H,3H)-pyrimidinedione]. R_f 0.47 (EtOAc/MeOH, 1:1), MS *m/z* 271 (M^+); UV λ_{max} at 244 and 312 nm (pH 1), 326 nm (pH 12); IR (KBr, 1%): 1710, 1620, 1510, 1465, 1390, 1355, 1345, 1330, 1285 cm^{-1}. ^1H NMR (CD_3OD, 90 MHz): δ 1.55–2.60 (m, 2 × CH_2 and C*H*CH_2OH), 3.70 (m, CH_2OH), 4.20 (m, CHOH), 5.05 (m, CHN), 9.10 (s, H-6) ppm (internal standard, tetramethylsilane). ^{13}C NMR (CD_3OD): 33.5 (C-5′), 40.4 (C-2′), 50.1 (C-4′), 59.0 (C-1′), 64.0 (C-6′), 73.7 (C-3′), 126.5 (C-5), 149.0 (C-6), 150.9 (C-2), 157.0 (C-4) ppm (internal standard, tetramethylsilane).

Experimental analysis of C-NO_2dUMP. R_f 0.30 (*n*-butyl-OH/H_2O/HOAc, 5:3:2); R_f 0.47 (MeOH/H_2O/NH_3 (33%), 85:10:5); UV λ_{max} at 243.5 and 311 nm (0.1 N HCl); 325 nm (0.2 N NaOH). ^{13}C NMR (D_2O): 32.9 (C-5′), 39.2 (C-2′), 47.6 (d, J = 7.3 Hz, C-4′), 58.7 (C-1′), 66.8 (d, J = 4.9 Hz, C-6′), 72.8 (C-3′), 125.9 (C-5), 150.0 (C-6), 151.2 (C-2), 157.8 (C-4) ppm (internal standard, CH_3OH: 49.9 ppm).

Radiochemicals. The radiolabeled nucleosides [*methyl*-^3H]dThd (specific radioactivity, 40Ci/mmol), [1′,2′-^3H]dUrd (specific radioactivity, 27 Ci/mmol), [5-^3H]dCyd (specific radioactivity, 20 Ci/mmol), [U-^{14}C]dCyd (specific radioactivity, 0.48 Ci/mmol), and [5-^3H]dUMP (specific radioactivity, 10 Ci/mmol) were obtained from the Radiochemical Centre (Amersham, England). [5-^3H]dUrd (specific radioactivity, 13 Ci/mmol) was obtained from Schwarz Mann Inc., New York.

Inhibition of tumor cell growth and metabolism. The methods for evaluating antitumor cell and antimetabolic activity using L1210 and Raji cells have been described previously (12). For the rat Novikoff hepatoma cell line, 10^4 cells were suspended in growth medium and added to microplate wells in the presence of varying concentrations of the test compounds. The cells were then allowed to proliferate for 72 hr at 37° in a humidified CO_2-controlled atmosphere. At the end of the incubation period, the cells were trypsinized and counted in a Coulter Counter (Coulter Electronics Ltd., Harpenden, England).

Inhibition of tritium release from [5-^3H]dUrd or [5-^3H]dCyd. Activity of dTMP synthetase in the intact L1210 cells was assessed by measuring the amount of tritium released from [5-^3H]deoxyuridylate formed in the cells from [5-^3H]dUrd or [5-^3H]dCyd in the presence or absence of the test compounds. The complete procedure has been described previously (24).

Inhibition of [methyl-^3H]dThd, [1′,2′-^3H]dUrd, [5-^3H]dCyd, and [U-^{14}C]dCyd incorporation into DNA. The procedures to measure the incorporation of [*methyl*-^3H]dThd, [1′,2′-^3H]dUrd, [5-^3H]dCyd, and [^{14}C]dCyd into cellular DNA have been described previously (12, 24). Briefly, to each microplate well were added 10^5 L1210 cells, a given amount of test compound, and 6.58 pmol (0.25 μCi) of [*methyl*-^3H]dThd, 9.3 pmol (0.25 μCi) of [1′,2′-^3H]dUrd, or 12.5 pmol (0.25 μCi) of [5-^3H]dCyd (for the experiments shown in Table 2), or 61 ng (0.28 μCi) of [5-^3H]dCyd or 61 ng (0.125 μCi) of [^{14}C]dCyd (for the experiments shown in Table 3). The specific activities of [5-^3H]dCyd and [^{14}C]dCyd in the latter experiments were chosen such that the count values

[2] M. J. Robins and J. Giziewicz, unpublished data.

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obtained with these radiolabeled nucleosides were similar. The cells were allowed to proliferate for 20 hr at 37°. At the end of this incubation period, trichloroacetic acid-insoluble material was assayed for radioactivity. It was ascertained by CsCl density gradient analysis that, under our test conditions, [*methyl*-^{3}H]dThd, [1′,2′-^{3}H]dUrd, [5-^{3}H]dCyd, and [^{14}C]dCyd were incorporated solely into DNA.

dThd kinase and dTMP synthetase assay. The procedures for determining the K_i and K_m values of murine leukemia L1210 dThd kinase and dTMP synthetase for 5-substituted pyrimidine nucleoside analogues have been described previously (15). The extracts from L1210 cells were assayed for dThd kinase activity in a standard reaction mixture containing 5 mM ATP, 5 mM $MgCl_2 \cdot 6H_2O$, 9 mM KF, 5 mM phosphoenolpyruvate, 5 μg of pyruvate kinase, 10 mM β-mercaptoethanol, 0.2 mM (0.1 μCi) [*methyl*-^{3}H]dThd, an appropriate amount of NO_2dUrd or C-NO_2dUrd, and 10 μl of cell extract in a total volume of 40 μl of 50 m M Tris-HCl, pH 8.0. The reaction mixture was incubated at 37° for 15 min and the reaction was terminated by addition of 75 μl of ice-cold 50 mM Tris-HCl buffer, pH 8.0. After boiling for 90 sec, the mixture was applied onto DE81 discs and washed with 1 mM NH_4OOCH, pH 8.2, ethanol, and ether. The filters were then assayed for radioactivity.

The L1210 extracts were assayed for dTMP synthetase activity in a standard reaction mixture containing 0.26 mM tetrahydrofolate, 5.0 mM formaldehyde, 15 mM β-mercaptoethanol, 0.1 M NaF, 45 μM (0.025 μCi) [5-^{3}H]dUMP, and an appropriate amount of NO_2dUMP or C-NO_2dUMP in a total volume of 30 μl of 50 mM potassium phosphate buffer, pH 7.5. The reaction was initiated by addition of 10 μl of the partially purified enzyme (15). The reaction mixture was incubated at 37° for 30 min and the reaction was terminated by addition of 160 μl of a charcoal suspension at 100 mg/ml in 2% trichloroacetic acid. After centrifugation for 10 min at 1000 × *g*, 100 μl of supernatant was assayed for radioactivity.

Nucleoside phosphorolysis. Enzymatic phosphorolysis of C-NO_2dUrd and NO_2dUrd was investigated with a dialyzed, partially purified, supernatant enzyme preparation from rabbit liver. The change in absorbance was continuously monitored at a wavelength of 346 nm, where the absorption difference between the deoxyribonucleoside and its free base was maximal. The reaction mixture (1 ml) contained 50 μl of cell extract and 0.1 mM nucleoside in potassium phosphate buffer, pH 7.4

Antitumor activity in vivo. Murine leukemia L1210 cells (provided by F. Schabel, Southern Research Institute, Kettering-Meyer Laboratory, Birmingham, AL) or P388/araC cells (provided by A. A. Avejera, National Cancer Institute, Bethesda, MD) were used for the *in vivo* studies on the antitumor activity of NO_2dUrd and C-NO_2dUrd. Both cell lines were maintained by weekly serial passage in DBA/2 mice. An equivalent number of five female mice was used in each set of experiments. DBA/2 mice (or BDF_1 mice) were inoculated IP with 10^6 P388 or L1210 cells on day 0, and the compounds were administered IP at a treatment regimen as indicated in Results under Antitumor Effects of 5-Nitro-dUrd and Carbocyclic 5-Nitro-dUrd. In the drug combination studies, 6-aminothymine, dThd, dUrd, Urd, and uracil were injected IP 10 to 15 min prior to the IP injection of NO_2dUrd.

RESULTS

Cytostatic effects of 5-nitro-dUrd and carbocyclic 5-nitro-dUrd in cell culture. NO_2dUrd and C-NO_2dUrd were compared for their inhibitory effects on the proliferation of murine leukemia (L1210/0 and L1210/BdUrd), rat (Novikoff) hepatoma, and human lymphoblast (Raji/0 and Raji/TK$^-$) cell lines. NO_2dUrd proved highly cytostatic for L1210/0, Novikoff hepatoma, and Raji/0 cells (Table 1). Its ID_{50} for these cell lines varied from 0.023 to 0.086 μg/ml. However, NO_2dUrd was about 2000-fold less cytostatic for L1210/BdUrd and Raji/TK$^-$ cells, two mutant cell lines which are deficient in dThd kinase activity (19).

As compared to NO_2dUrd, C-NO_2dUrd was about 1000-fold less effective in inhibiting the proliferation of L1210/0, Novikoff hepatoma, or Raji/0 cells (Table 1). Its ID_{50} for these cell lines ranged from 21.5 to 175 μg/ml, and its ID_{50} for both dThd kinase-deficient (L1210/BdUrd and Raji/TK$^-$) cell lines was further increased to >1000 and 873 μg/ml, respectively.

Antimetabolic effects of 5-nitro-dUrd and carbocyclic 5-nitro-dUrd. To elucidate the mechanism of action of NO_2dUrd and C-NO_2dUrd, and, more precisely, to pinpoint the molecular basis for the dramatic differences in cell growth-inhibiting effects, the following parameters were evaluated: (i) effect of the addition of dUrd, dThd, and dCyd on the inhibitory activity of NO_2dUrd and C-NO_2dUrd on L1210 cell proliferation; (ii) inhibitory effects of NO_2dUrd and C-NO_2dUrd on the incorporation of radiolabeled [1′,2′-^{3}H]dUrd, [*methyl*-^{3}H]dThd, and [5-^{3}H]dCyd into L1210 cell DNA; (iii) differential effects of NO_2dUrd and C-NO_2dUrd on the incorporation of dCyd into L1210 cell DNA via the dCyd → dCTP pathway or dCyd → dTTP pathway; (iv) inhibitory effects of NO_2dUrd and C-NO_2dUrd on the release of tritium from [5-^{3}H]dUrd and [5-^{3}H]dCyd; (v) enzymatic phosphorolysis of NO_2dUrd and C-NO_2dUrd; (vi) affinity of NO_2dUrd and C-NO_2dUrd for the cell-free L1210 dThd kinase; and (vii) affinity of NO_2dUMP and C-NO_2dUMP for the cell-free L1210 dTMP synthetase.

A 25- to 100-fold decrease in the cell growth-inhibiting effects of NO_2dUrd was observed upon addition of dUrd (125 μg/ml) or dCyd (500 μg/ml) (Table 2). For C-NO_2dUrd, this decrease was 10- to 20-fold. However, the addition of dThd (5 μg/ml) caused a much greater decrease in the cytostatic effects of the drugs: by at least 10,000-fold for NO_2dUrd and up to a total annihilation of the cytostatic activity for C-NO_2dUrd.

The inhibitory effects of NO_2dUrd and C-NO_2dUrd on the incorporation of [1′,2′-^{3}H]dUrd into L1210 cell DNA (ID_{50}, 0.010 and 3.95 μg/ml, respectively), closely matched their inhibitory effects on L1210 cell proliferation (Table 2). The ID_{50} of NO_2dUrd and C-NO_2dUrd for [5-^{3}H]dCyd incorporation were 5- to 25-fold higher than those for [1′,2′-^{3}H]dUrd. However, the most remarkable result was that neither compound inhibited the incorporation of [*methyl*-^{3}H]dThd into L1210 cell DNA even at a concentration of 1 mg/ml.

When evaluated for their differential effects on the incorporation of dCyd into L1210 cell DNA via the dCyd → dCTP or dCyd → dTTP pathway, NO_2dUrd and C-NO_2dUrd inhibited both pathways at comparable concentrations, albeit with NO_2dUrd at a 1000-fold lower concentration than C-NO_2dUrd (Table 3). These concentrations were of the same order of magnitude as those required for inhibition of L1210 cell proliferation.

As to the inhibitory effects of NO_2dUrd and C-NO_2dUrd on tritium release from [5-^{3}H]dUrd and [5-^{3}H]dCyd in intact cells, again NO_2dUrd inhibited tritium release from these radiolabeled precursors at equivalent concentrations; C-NO_2dUrd did also, but only at a con-

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TABLE 1

Inhibitory effects of NO_2dUrd and C-NO_2dUrd on the growth of several tumor cell lines

Compound	ID_{50}[a]				
	Murine leukemia L1210/0	Murine leukemia L1210/BdUrd[b]	Rat Novikoff hepatoma	Human lymphoblast Raji/0	Human lymphoblast Raji/TK$^-$[c]
			μg/ml		
NO_2dUrd	0.023	46	0.043	0.086	163
C-NO_2dUrd	23	>1000	21.5	175	873

[a] The data represent the mean values for at least two to three separate experiments. The range of the individual values was between 5 and 30% of the indicated values.

[b] L1210/BdUrd corresponds to a TK$^-$ mutant of the parental (L1210/0) cell line.

[c] Raji/TK$^-$ corresponds to a TK$^-$ mutant of the parental (Raji/0) cell line.

TABLE 2

Inhibitory effects of NO_2dUrd and C-NO_2dUrd on L1210 cell growth (in the presence of dUrd, dThd, and dCyd) and DNA synthesis (as monitored by the incorporation of several radiolabeled precursors)

Compound	ID_{50}[a]						
	For cell growth				For DNA synthesis as monitored by the incorporation of		
	No additions	Upon addition of dUrd (125 μg/ml)[b]	Upon addition of dThd (5 μg/ml)[b]	Upon addition of dCyd (500 μg/ml)[b]	[*methyl*-^{3}H]dThd	[1',2'-^{3}H]dUrd	[5-^{3}H]dCyd
				μg/ml			
NO_2dUrd	0.023	1.81	218	0.533	>1000	0.010	0.247
C-NO_2dUrd	23.5	433	>1000	240	>1000	3.95	18.1

[a] The data represent the mean values for at least two separate experiments. The range of the individual values was between 5 and 30% of the indicated values.

[b] Maximum concentrations of dUrd, dThd, and dCyd that were not by themselves inhibitory to L1210 cell growth.

TABLE 3

Inhibitory effects of NO_2dUrd and C-NO_2dUrd on the incorporation of dCyd into L1210 cell DNA via the dCyd → dCTP pathway or dCyd → dTTP pathway (A) and tritium release from [5-^{3}H]dCyd and [5-^{3}H]dUrd in intact L1210 cells (B)

Compound	ID_{50}[a]	
	dCyd → dCTP pathway[b]	dCyd → dTTP pathway[c]
	μg/ml	
A. NO_2dUrd	0.095	0.043
C-NO_2dUrd	77.3	49.7
	[5-^{3}H]dCyd	[5-^{3}H]dUrd
B. NO_2dUrd	0.056	0.033
C-NO_2dUrd	4.47	3.82

[a] The data represent the mean values for at least three separate experiments. The range of the individual values was between 10 and 45% of the indicated values.

[b] As monitored by the incorporation of radiolabeled material into DNA with [5-^{3}H]dCyd as precursor.

[c] As monitored by subtracting the amount of radiolabeled material incorporated into DNA with [5-^{3}H]dCyd as precursor from the amount of radiolabeled material incorporated into DNA with [^{14}C]dCyd as precursor.

centration 100-fold higher than that required for NO_2dUrd (Table 3).

The presumed resistance of C-NO_2dUrd to phosphorolytic cleavage was confirmed by incubating this compound with a rabbit liver enzyme extract. Under conditions where NO_2dUrd was readily phosphorolyzed to its base NO_2Ura, no conversion of C-NO_2dUrd to NO_2Ura could be detected (data not shown).

We finally compared NO_2dUrd and C-NO_2dUrd for their affinities for L1210 dThd kinase (with [*methyl*-^{3}H]dThd as substrate) and NO_2dUMP and C-NO_2dUMP for their affinities for L1210 dTMP synthetase (with [5-^{3}H]dUMP as substrate). The K_i/K_m value of dThd kinase for C-NO_2dUrd was only 2-fold higher than the K_i/K_m value for NO_2dUrd (Table 4). However, the K_i/K_m value of dTMP synthetase for C-NO_2dUMP was about 80-fold higher than the K_i/K_m value of dTMP synthetase for NO_2dUMP. For both NO_2dUrd and C-NO_2dUrd, as well

TABLE 4

Kinetics for the inhibition of L1210 dThd kinase and L1210 dTMP synthetase by NO_2dUrd, C-NO_2dUrd, NO_2dUMP, and C-NO_2dUMP, respectively

K_m values for the individual experiments ranged from 11.7 to 23.5 μM for dThd kinase with [*methyl*-^{3}H]dThd as the radiolabeled substrate and from 1.27 to 3.12 μM for dTMP synthetase with [5-^{3}H]dUMP as the radiolabeled substrate. The average K_m values of dThd kinase and dTMP synthetase were 17.3 and 1.34 μM, respectively. The data represent the mean values for at least two to five separate experiments. The range of the individual values was between 10 and 30% of the indicated values.

Compound	dThd kinase		dTMP synthetase	
	K_i/K_m	Type of inhibition	K_i/K_m	Type of inhibition
NO_2dUrd	65	Competitive		
C-NO_2dUrd	133	Competitive		
NO_2dUMP			0.005	Competitive
C-NO_2dUMP			0.398	Competitive

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as NO_2dUMP and C-NO_2dUMP, the inhibition of dThd kinase and dTMP synthetase was competitive with respect to the natural substrate (Table 4).

Antitumor effects of 5-nitro-dUrd and carbocyclic 5-nitro-dUrd. NO_2dUrd and C-NO_2dUrd were further evaluated for their antitumor activities *in vivo*, i.e., in two murine leukemia (L1210 and P388/araC) models.

NO_2dUrd caused a slight increase in life span of mice (8 to 13%), when injected IP 3 times a day for 5 days into L1210 cell-bearing mice at a total dose of 150, 300, or 600 mg/kg. When injected IP on days 1, 3, and 5 after tumor cell inoculation (total dose, 300 mg/kg), the increase in life span was only 6% (data not shown). C-NO_2dUrd administered to L1210 cell-bearing mice on days 1, 3, and 5 after tumor cell inoculation at a total dose of 450 mg/kg was devoid of any antitumor activity. When injected IP into P388/araC cell-bearing mice on days 1, 3, 5, 7, and 9 after tumor cell inoculation at a total dose of 600 mg/kg, neither NO_2dUrd nor C-NO_2dUrd demonstrated any significant increase in the life span of mice (data not shown).

In an attempt to increase the antitumor effects of NO_2dUrd, it was combined with 6-aminothymine [an inhibitor of pyrimidine nucleoside (dThd) phosphorylase (25)] or Ura, Urd, dUrd, or dThd (the latter four being either substrate or product of dThd phosphorylase or Urd phosphorylase). NO_2dUrd was injected IP at 120 mg/kg on days 1, 3, 5, 7, and 9 after IP inoculation of the P388/araC cells to DBA/2 mice, and 6-aminothymine was administered IP at 50 mg/kg 10 to 15 min prior to each injection of NO_2dUrd. In another set of experiments, NO_2dUrd was injected IP at 100 mg/kg on days 1, 3, and 5 after IP inoculation of the L1210 cells into DBA/2 mice, and Ura, Urd, dUrd, or dThd was administered IP at 200 mg/kg 10 to 15 min prior to each injection of NO_2dUrd. None of these combinations resulted in an enhancement of the antitumor effect of NO_2dUrd (data not shown).

DISCUSSION

The carbocyclic derivative C-NO_2dUrd proved considerably less inhibitory to the proliferation of murine, rat, and human tumor cell lines than its nucleoside counterpart NO_2dUrd (Table 1). All the antimetabolic data (Tables 2–4) point to dTMP synthetase as the principal target for the cytostatic action of NO_2dUrd and C-NO_2dUrd. It has been postulated previously that those dUrd analogues that are far more inhibitory to dUrd than dThd incorporation into cell DNA, and whose tumor cell-inhibiting effects are more readily reversed by dThd than dUrd, owe their antitumor activity to a selective inhibition of dTMP synthetase (12). According to the results presented in Table 2, this premise holds for both NO_2dUrd and C-NO_2dUrd. Inhibition of the release of tritium from [5-^{3}H]dUrd or [5-^{3}H]dCyd and the incorporation of dCyd as dTMP into DNA offer two additional criteria for any action targeted at dTMP synthetase (24). Both approaches again pointed to dTMP synthetase as the principal target for the cytostatic activity of NO_2dUrd and C-NO_2dUrd (Table 3).

The marked decrease in the cytostatic effects of NO_2dUrd and C-NO_2dUrd towards dThd kinase-deficient L1210 and Raji cells (Table 1) suggested that the cytostatic action of both compounds depended to a large extent on the dThd kinase activity of the host cell. The inferior cytostatic activity of C-NO_2dUrd, as compared to NO_2dUrd, could be due to either a lower affinity of C-NO_2dUrd for dThd kinase or a lower affinity of its 5′-monophosphate for dTMP synthetase. Indeed, a lower affinity of C-NO_2dUrd for dThd kinase should interfere with the activating role of this enzyme and thereby diminish the conversion of C-NO_2dUrd to its 5′-monophosphate, whereas a lower affinity for C-NO_2dUMP for dTMP synthetase would weaken an inhibitory action on this enzyme and thus reduce the suppressive effect on cell proliferation. To discriminate between these two possibilities, we determined the affinities of C-NO_2dUrd (C-NO_2dUMP) and NO_2dUrd (NO_2dUMP) for these two key enzymes and found a 2-fold decrease in the affinity of the carbocyclic nucleoside for dThd kinase, but a 80-fold decrease in affinity of the carbocyclic nucleotide for dTMP synthetase. Similar results were obtained with partially purified dTMP synthetase from human lymphoblast (MOLT/4F) cells (data not shown).

Thus, C-NO_2dUrd inhibited dTMP synthetase in intact cells to a much lesser extent than did NO_2dUrd. This implies that substitution of the 2′-deoxyribose moiety by a cyclopentane ring has an important negative influence on the affinity for dTMP synthetase. The decreased affinity for C-NO_2dUMP for dTMP synthetase may in turn account for the lack of antitumor activity of C-NO_2dUrd *in vivo*: in neither of the two test systems (mice inoculated with P388 or L1210 tumor cells) did C-NO_2dUrd treatment result in any significant increase in life span of the mice.

NO_2dUrd is an effective substrate for dThd phosphorylase (17). This degradation prevents the conversion of NO_2dUrd to its active metabolite, NO_2dUMP, and may obviously explain the lack of antitumor activity of NO_2dUrd in our two *in vivo* systems that we studied (murine L1210 and P388 leukemia). In an attempt to facilitate the conversion of NO_2dUrd to its 5′-monophosphate, we used NO_2dUrd in combination with either 6-aminothymine, Ura, Urd, dUrd, or dThd. With the addition of Ura, which is a substrate for the anabolic phosphorylase reaction, we wanted to direct this reaction towards nucleoside synthesis, and thus prevent the phosphorolytic cleavage of NO_2dUrd. With the use of 6-aminothymine, which is a well known potent inhibitor of dThd phosphorylase (25), we wanted to directly block phosphorolysis of NO_2dUrd. Finally, by adding any of the nucleosides Urd, dUrd, or dThd, we aimed at decreasing the rate of breakdown of NO_2dUrd by pyrimidine nucleoside phosphorylases by increasing the competition of the other nucleosides as a substrate for the catabolic phosphorylase reaction. However, despite all these attempts, no increase in the life span of the leukemic mice was observed. Neither 6-aminothymine, Ura, Urd, dUrd, nor dThd potentiated the antitumor activity of NO_2dUrd.

In conclusion, we have demonstrated that a carbocyclic pyrimidine derivative, whose corresponding nucleoside exerts its inhibitory effect on cell proliferation by inhi-

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bition of dTMP synthetase, has a markedly decreased affinity for this target enzyme. This finding should be taken into consideration when developing antitumor agents that are targeted at dTMP synthetase.

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