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5-Hexyl-2′-deoxyuridine Blocks the Cytotoxic Effects of 5-Fluorodeoxyuridine or Deoxyadenosine in Leukemia L1210 Cells in Culture[1]

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ABSTRACT

Antitumor agents which block the *de novo* synthesis of nucleotides can be circumvented by the presence of salvage pathways for the reutilization of nucleobases and nucleosides. Studies have been carried out which show that 5-hexyl-2′-deoxyuridine (HdUrd) effectively blocks the cytotoxic effects of deoxyadenosine and fluorodeoxyuridine in L1210 cells. Although HdUrd (500 μM) had essentially no effect on the growth of L1210 cells in culture, the total uptake of [^{14}C]cytidine into these cells was inhibited 99% by this concentration of HdUrd. The inhibitory effects of fluorodeoxyuridine (FdUrd) and deoxyadenosine could be completely prevented by the presence of HdUrd (200 μM). The growth inhibitory effects of fluorouracil were not prevented by HdUrd. Dipyridamole prevented the inhibition of L1210 cell growth by FdUrd but not by deoxyadenosine or fluorouracil. 5-Isopropyl-, 5-pentyl-, and 5-octyldeoxyuridine were not effective in preventing the cytotoxic effects of deoxyadenosine. The data suggest that HdUrd might be useful in blocking the salvage of nucleosides, thereby potentiating the effects of inhibitors of *de novo* nucleotide synthesis.

INTRODUCTION

The cytotoxic effects of antitumor agents which are directed at key enzymes in *de novo* pathways of purine and pyrimidine nucleotide syntheses can be circumvented by the effective salvage of nucleobases and nucleosides (1, 2). For example, the cytotoxic effects of acivicin [which inhibits CTP synthetase (3) and GMP synthetase (4)] were prevented by including cytidine and guanosine in the culture medium (5). Studies have been carried out which show that compounds such as dipyridamole and NBMPR[3] are effective inhibitors of nucleoside transport (6–8). When dipyridamole or NBMPR was used in combination with antimetabolites, the salvage of nucleosides was blocked and the inhibition of cell growth was maintained (5, 9–14).

In the studies reported here, the effects of 5-hexyl-2′-deoxyuridine in blocking the cytotoxic effects of 5-fluorodeoxyuridine or deoxyadenosine are compared to the effects of dipyridamole.

MATERIALS AND METHODS

Materials. The 5-alkyldeoxyuridines were synthesized and obtained from Dr. Laszlo Otvos of the Central Research Institute for Chemistry, Hungarian Academy of Sciences, Budapest, Hungary. The nucleosides, dipyridamole, hydroxyurea, gentamicin sulfate and MTT were purchased from Sigma Chemical Co., St. Louis, MO. [^{14}C]Cytidine (400 mCi/mmol) was purchased from Research Products International Corp., Mount Prospect, IL. The RPMI 1640 culture medium, horse serum, and sodium bicarbonate were purchased from GIBCO, Grand Island, NY. Erythro-9-(2-hydroxyl-3-nonyl)adenine (EHNA) was purchased from Burroughs-Wellcome, Research Triangle, NC. The L1210 cells were obtained from the American Type Culture Collection, Rockville, MD.

Growth of L1210 Cells in Medium. L1210 cells were grown in suspension culture in RPMI 1640 culture medium supplemented with 10% horse serum, sodium bicarbonate (2 g/liter), and gentamicin sulfate (50 mg/liter). The cells were grown at 37°C in a humidified incubator with 5% CO_2/95% air. The cells were seeded at 150,000 cells/ml; 24 h later the drugs were added and the cells counted 72 h later. The cultures were set up in triplicate. Cell counts were made on a Coulter Counter, Model ZBI.

Microculture Tetrazolium Assay. A modification of the MTT assay of Carmichael *et al.* (15), was used to determine cell growth. L1210 cells were seeded in 96-well plates at 2000 cells/well in 150 μl of culture medium. After 24 h of incubation, the drugs in 50 μl of culture medium were added to each well in triplicate. After exposure of the cells to drugs for 72 h, MTT (15 μl, 5 mg/ml phosphate-buffered saline) was added to the wells and the cells incubated for 4 h at 37°C. The plates were centrifuged for 10 min at 500 × *g* and the culture medium was aspirated carefully. Dimethyl sulfoxide (150 μl) was added to solubilize the formazan product. After thorough mixing the absorbance of each well was measured in a Dynatech MR700 microplate reader using the dual mode in which the absorbance at 630 nm was automatically substracted from the absorbance at 490 nm. In each experiment, five wells of control cells (no drug) were pooled and cell counts made on a Coulter counter, Model ZBI to relate the absorbance of the MTT assay to actual cell counts.

[^{14}C]Cytidine Metabolism. The L1210 cells were cultured in 7 ml of fresh medium at a final concentration of 4.24×10^6 cells/ml. The drug was added in a volume of 100 μl. The cells were incubated for 2 h in the presence and absence of 5-ethyl- or 5-hexyldeoxyuridine. [^{14}C]-Cytidine (400 mCi/mmol, 2 μCi/flask) was added and the incubation continued for 1 h. The cells were collected by centrifugation and subjected to the Schmidt-Thannhauser procedure (16) to separate the nucleotide pool, RNA and DNA fractions. Aliquots of each fraction were taken for radioactivity measurements. The flasks were set up in duplicate. The acid-soluble nucleotide pool fraction was neutralized with KOH, centrifuged to remove $KClO_4$, and the supernatant fluid lyophilized. The lyophylized material was dissolved in Tris-HCl buffer and treated with snake venom. Deoxycytidine was separated from cytidine on Dowex 1-borate columns (17).

Partition Coefficient Determination. The 5-alkyldeoxyuridines were dissolved in phosphate-buffered saline with a minimum of dimethyl sulfoxide. The concentrations of the drugs were approximately 100 μM. Aliquots (2 ml) of the 5-alkyldeoxyuridine aqueous solutions were transferred to conical glass centrifuge tubes and *n*-octanol (2 ml) was then added. The two-phase systems were vigorously mixed using a vortex mixer. The mixtures were centrifuged and the aqueous and *n*-octanol phases separated (18). The absorbance of each phase was measured at 260 nm. The partition coefficient was defined as the ratio of 5-alkyldeoxyuridine in the *n*-octanol layer to 5-alkyldeoxyuridine in the aqueous phase.

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[3] The abbreviations used are: NBMPR, nitrobenzylthioinosine; MTT, 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide; EHNA, erythro-9-(2-hydroxyl-3-nonyl)adenine; IC_{50}, concentration of drug required to cause 50% inhibition.

RESULTS

Effects of 5-Alkyldeoxyuridines on the Growth of L1210 Cells in Culture. The effects of a series of 5-alkyldeoxyuridines on the growth of L1210 cells in culture were determined. As seen in Table 1, 5-ethyldeoxyuridine inhibited L1210 cell growth with an IC_{50} of <20 μM. 5-Isopropyl- and 5-pentyldeoxyuridine, at a concentration of 500 μM, did not inhibit L1210 cell growth; 5-hexyldeoxyuridine, at 500 μM, inhibited L1210 cell growth by only 12%.

Effects of 5-Ethyldeoxyuridine and 5-Hexyldeoxyuridine on [^{14}C]Cytidine Metabolism in L1210 Cells in Culture. The effects of 5-ethyldeoxyuridine and 5-hexyldeoxyuridine on [^{14}C]cytidine metabolism in L1210 cells were studied. As seen in Table 2, 5-ethyldeoxyuridine, at concentrations which markedly inhibited L1210 cell growth, inhibited the uptake of [^{14}C]cytidine into the cells. However, the incorporation of [^{14}C]cytidine into DNA, via ribonucleotide reductase, was inhibited to a greater extent than the incorporation into RNA or total uptake. When the acid-soluble nucleotide pool was analyzed for the level of conversion of cytidine into deoxycytidine nucleotides it was found that 5-ethyldeoxyuridine had no effect at this step. Although 5-hexyldeoxyuridine did not inhibit L1210 cell growth (Table 1), the presence of 5-hexyldeoxyuridine markedly inhibited the overall uptake of [^{14}C]cytidine into the L1210 cells. The extent of inhibition of uptake of [^{14}C]cytidine into the L1210 cells was reflected in the decreased levels of [^{14}C]cytidine found incorporated into RNA or DNA.

Effect of 5-Alkyldeoxyuridines on the Inhibition of L1210 Cell Growth by Deoxyadenosine. As seen in Fig. 1, deoxyadenosine in the presence of EHNA, 5 μM, inhibited cell growth with an IC_{50} value <25 μM. The addition of 5-hexyldeoxyuridine to the culture medium caused a marked increase in the IC_{50} for deoxyadenosine/EHNA. The inhibitory effects of deoxyadenosine were reduced by the presence of 5-hexyldeoxyuridine (200 or 400 μM). Very similar data were obtained when cell counts rather than the MTT assay were used.

Other 5-alkyldeoxyuridines were studied for their ability to prevent the inhibition of L1210 cell growth by deoxyadenosine/EHNA. As seen in Table 3, at the lowest concentrations of deoxyadenosine (25 μM), pentyldeoxyuridine gave only a partial protective response. 5-Octyldeoxyuridine was somewhat more protective than 5-pentyldeoxyuridine but much less effective than 5-hexyldeoxyuridine in protecting the L1210 cells from the inhibitory effects of deoxyadenosine. 5-Isopropyldeoxyuridine had essentially no effect on the inhibition of L1210 cell growth by deoxyadenosine.

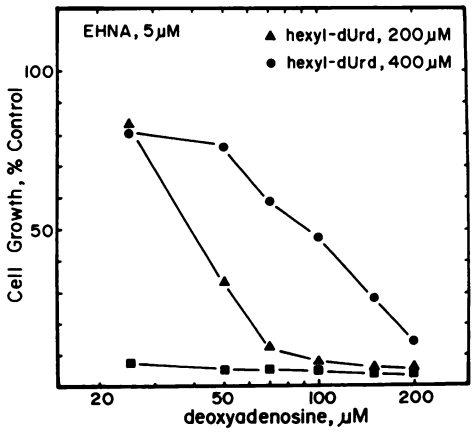

Fig. 1. Effect of 5-hexyl-2′-deoxyuridine on the inhibition of L1210 cell growth by deoxyadenosine. L1210 cells (2000 cells/well) were put into 96-well plates. After 24 h the drugs were added at the various concentrations and the cells incubated at 37°C. Three days later MTT was added to each well, incubated 4 h, and the plates treated as described in "Materials and Methods." Each drug concentration or drug combination was set up in triplicate. EHNA (5 μM) was added to protect deoxyadenosine from deamination and was added at the same time as the deoxyadenosine. The non-drug treated control cells grew out to a cell density of 49,800 cells/well. Deoxyadenosine (■) was added at the concentrations indicated; 5-hexyl-2′-deoxyuridine (*hexyl-dUrd*) was added at 200 μM (▲) or 400 μM (●), respectively, at the time of addition of deoxyadenosine.

Table 1 *Effects of 5-alkyldeoxyuridines on L1210 cell growth in culture*

Drug concentration (μM)	L1210 cell growth (% control[a]), 5-alkyldeoxyuridines			
	Ethyl-	Isopropyl-	Pentyl-	Hexyl-
500	7.3	100	100	88
375	7.3	100	100	100
250	9.4	100	100	100
125	10.2	100	100	100
62.5	16.3	100	100	100
31.25	21.8	100	100	100

[a] The control cells grew to a cell density of 141,000 cells/well.

Table 2 *Effects of 5-alkyldeoxyuridines on [^{14}C]cytidine uptake and incorporation into RNA and DNA of L1210 cells in culture*

Compound μM	% Control[a]			
	Acid-soluble	RNA	DNA	Total
Ethyldeoxyuridine, 500	61	55	24	53
Ethyldeoxyuridine, 250	76	70	30	67
Ethyldeoxyuridine, 125	83	84	37	78
Hexyldeoxyuridine, 500	3	1	1	1
Hexyldeoxyuridine, 250	4	2	2	3
Hexyldeoxyuridine, 125	6	4	4	4

[a] The control values for the total [^{14}C]cytidine taken up by the cells, the acid-soluble fraction, and the RNA and DNA fractions were 42,700, 16,200, 21,100, and 5,400 cpm/10^6 cells, respectively.

Table 3 *Comparison of the effects of 5-alkyldeoxyuridines on the inhibition of L1210 cell growth by deoxyadenosine/EHNA*

Deoxyadenosine/EHNA[a] (μM)	L1210 cell growth (% control[b]), 5-alkyldeoxyuridines (200 μM)				
	None	Hexyl-	Octyl-	Pentyl-	Isopropyl-
25	3	92	35	26	6
50	2	61	26	3	3
75	2	30	14	3	3
100	2	19	10	2	3

[a] The concentration of EHNA was held constant at 5 μM. EHNA was added at the same time as the deoxyadenosine.
[b] The control cells grew to a cell density of 105,000 cells/ml.

Effects of 5-Hexyldeoxyuridine on the Inhibition of L1210 Cell Growth by Fluorodeoxyuridine or Fluorouracil. 5-Hexyldeoxyuridine reversed the inhibition of L1210 cell growth by fluorodeoxyuridine (Fig. 2). The complete inhibition of L1210 cell growth by fluorodeoxyuridine (10 nM) was totally prevented by the presence of 5-hexyldeoxyuridine (200 or 400 μM).

On the other hand, 5-hexyldeoxyuridine did not prevent the inhibition of L1210 cell growth by fluorouracil (Fig. 3). If anything, there was a slight increase in the inhibition of L1210 cell growth by the combination of fluorouracil plus 5-hexyldeoxyuridine.

Effect of 5-Hexyldeoxyuridine on the Inhibition of L1210 Cell Growth by Hydroxyurea. Hydroxyurea inhibited L1210 cell growth with an IC_{50} of 100 μM. 5-Hexyldeoxyuridine had no effect on the inhibition of L1210 cell growth by hydroxyurea (data not shown).

Effects of Dipyridamole on the Inhibition of L1210 Cell Growth by Fluorodeoxyuridine, Fluourouracil, or Deoxyadenosine. Dipyridamole is a known inhibitor of nucleoside transport (6–8). The effects of dipyridamole on the inhibition of L1210 cell growth by fluorodeoxyuridine, fluorouracil, or deoxyadenosine were studied to compare with the effects observed with 5-

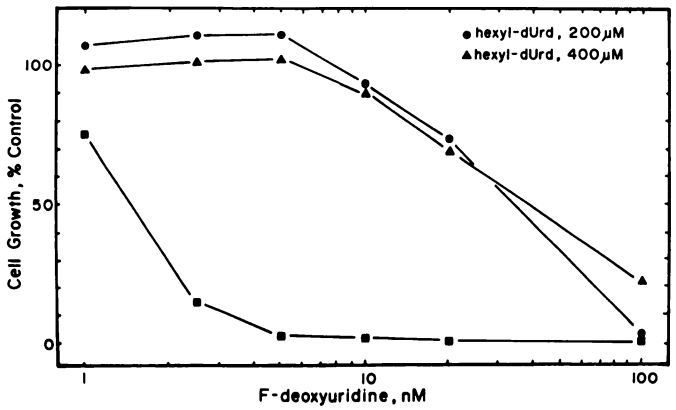

Fig. 2. Effect of 5-hexyl-2′-deoxyuridine on the inhibition of L1210 cell growth by 5-fluorodeoxyuridine. The experiments were set up as described in Fig. 1. The non-drug treated control cells grew out to a cell density of 50,000 cells/well. Fluorodeoxyuridine (■) was added at the concentrations indicated; 5-hexyl-2′-deoxyuridine (*hexyl-dUrd*) was added at 200 μM (●) or 400 μM (▲), respectively.

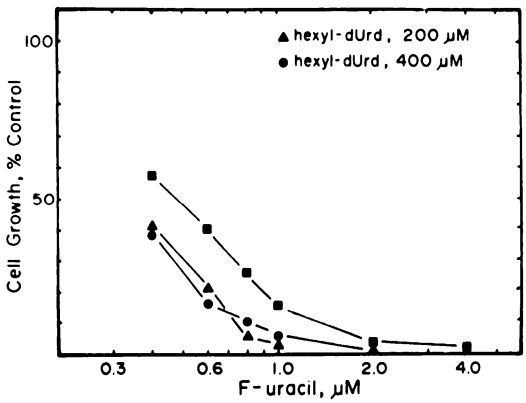

Fig. 3. Effect of 5-hexyl-2′-deoxyuridine on the growth of L1210 cells in the presence of 5-fluorouracil. The experiments were set up as described in the legend to Fig. 1. The non-drug treated control cells grew out to a cell density of 101,000 cells/well. Fluorouracil (■) was added at the concentrations indicated; 5-hexyl-2′-deoxyuridine was added at 200 μM (▲) or 400 μM (●), respectively.

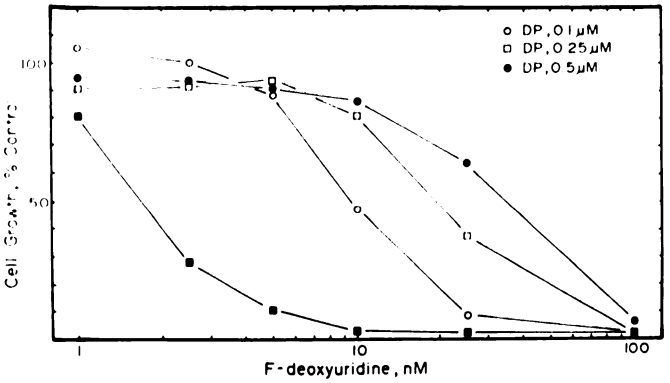

Fig. 4. Effect of dipyridamole on the inhibition of L1210 cell growth by 5-fluorodeoxyuridine. The experiments were set up as described in the legend to Fig. 1. The non-drug treated control cells grew out to a cell density of 140,000 cells/well. Fluorodeoxyuridine (■) was added at the concentrations indicated; dipyridamole (*DP*) was added at 0.1 μM (○), 0.25 μM (□), and 0.5 μM (●), respectively.

hexyldeoxyuridine. As seen in Fig. 4, dipyridamole, in a concentration-dependent manner prevented the inhibition of L1210 cell growth by fluorodeoxyuridine. These concentrations of dipyridamole had no effect on L1210 cell growth. The data in Fig. 5 show that the inhibitory effects of fluorouracil (over a 10-fold range of concentrations) on L1210 cell growth were not

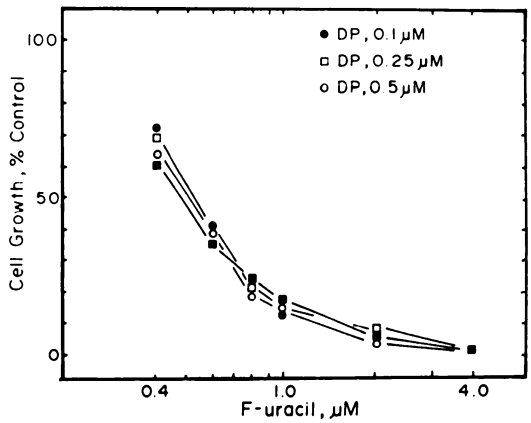

Fig. 5. Effect of dipyridamole on the inhibition of L1210 cell growth by 5-fluorouracil. The experiments were set up as described in the legend to Fig. 1. The non-drug treated control cells grew out to a cell density of 91,600/well. Fluorouracil (■) was added at the concentrations indicated; dipripidamole (*DP*) was added at 0.1 μM (●), 0.25 μM (□), and 0.5 μM (○), respectively.

Table 4 *Partition coefficients of 5-alkyldeoxyuridines*

5-Alkyldeoxyuridine	Partition coefficient[a]
Ethyl	0.25
Isopropyl	0.74
Pentyl	8.37
Hexyl	22.17
Octyl	50.43

[a] Ratio of amount of 5-alkyldeoxyuridine in octanol phase to amount of 5-alkyldeoxyuridine in aqueous phase.

reversed by the presence of dipyridamole. The inhibitory effects of deoxyadenosine were neither increased nor decreased by the concentrations of dipyridamole (0.1, 0.25, and 0.5 μM) which prevented the cytotoxic effects of 5-fluorodeoxyuridine (data not shown).

Partition Coefficients of 5-Alkyldeoxyuridines. The distribution of the 5-alkyldeoxyuridines between the *n*-octanol and aqueous layers was determined for the series of 5-alkyldeoxyuridines. As seen in Table 4, 5-ethyl- and 5-isopropyldeoxyuridine partitioned primarily in the aqueous phase; 5-pentyl-, 5-hexyl-, and 5-octyldeoxyuridine partitioned in the octanol phase. As expected, 5-octyldeoxyuridine had the largest partition coefficient.

DISCUSSION

Inhibitors of the *de novo* pathways for nucleotide synthesis have been used in the treatment of human tumors. One of the limitations of the use of these inhibitors is that the inhibition of *de novo* pathways can be circumvented by the presence of very effective salvage pathways for nucleobases and nucleosides. It has been shown that the levels of the salvage enzymes (phosphoribosyltransferases and kinases) are much in excess over the levels of the rate-limiting enzymes in the *de novo* pathways (*e.g.*, IMP dehydrogenase, ribonucleotide reductase) (2).

Studies have been carried out which show that compounds such as nitrobenzylthioinosine and dipyridamole (6–8) are inhibitors of nucleoside transport. Dipyridamole and NBMPR potentiated the effects of inhibitors of the *de novo* synthesis pathways by decreasing the uptake of nucleosides which could be used in the salvage pathways (5, 9–14).

In the studies reported here, it was shown that 5-hexyl-2′-deoxyuridine inhibited the total uptake of [^{14}C]cytidine into L1210 cells without having a significant effect on the growth

of L1210 cells in culture at concentrations as high as 500 μM. In contrast, 5-ethyldeoxyuridine inhibited [^{14}C]cytidine incorporation into DNA to a greater extent than the incorporation into RNA or total uptake. 5-Ethyldeoxyuridine was an inhibitor of L1210 cell growth with an IC_{50} of less than 20 μM. Previous studies had shown that 5-ethyldeoxyuridine was inhibitory to the growth of a series of tumor cells, phosphorylated and incorporated into DNA (19, 20).

5-Hexyldeoxyuridine inhibited the cellular uptake of [^{14}C]-cytidine and prevented the growth inhibition of L1210 cells by 5-fluorodeoxyuridine and deoxyadenosine. In each case, the effects of fluorodeoxyuridine and deoxyadenosine at concentrations of drug which completely inhibited L1210 cell growth could be overcome by the presence of 5-hexyldeoxyuridine. The data suggest that 5-hexyldeoxyuridine blocked the uptake of these cytotoxic nucleosides. On the other hand, 5-hexyldeoxyuridine did not prevent the inhibition of L1210 cell growth by 5-fluorouracil indicating a specificity towards nucleosides rather than nucleobases. The presence of 5-hexyldeoxyuridine caused a slight increase in the inhibition of L1210 cell growth by 5-fluorouracil (Fig. 3). This may be due to the inhibition of the uptake of the small concentrations of uridine or thymidine in the culture medium which would serve to reverse the inhibitory effects of 5-fluorouracil (21). 5-Hexyldeoxyuridine did not alter the growth inhibitory effects of hydroxyurea, a nonnucleoside inhibitor of ribonucleotide reductase.

In comparative studies, the effects of dipyridamole on the inhibition of L1210 cell growth by 5-fluorodeoxyuridine, deoxyadenosine, and 5-fluorouracil were determined. Dipyridamole (0.1, 0.25, and 0.5 μM) prevented the inhibition of L1210 cell growth by 5-fluorodeoxyuridine and had no effect on the inhibition caused by 5-fluorouracil. However, these low concentrations of dipyridamole, (0.1, 0.25, and 0.5 μM) did not protect the L1210 cells from the cytotoxic effects of deoxyadenosine. (Higher concentrations of dipyridamole were not used in these studies as our L1210 cell line appeared to be more sensitive to dipyridamole than the other L1210 cell lines; at 1.0 μM dipyridamole, the growth of L1210 cells were inhibited approximately 25%.) Kang and Kimball (22) reported that dipyridamole enhanced the toxicity of deoxyadenosine/deoxycoformycin to L1210 cells in culture. The increase in toxicity correlated with the increase in the intracellular levels of dATP. Plagemann and Wohlhueter (23) concluded that this increased toxicity was not mediated at the level of nucleoside transport but was due to enhanced accumulation of dATP in the cells treated with dipyridamole.

Of the series of 5-alkyldeoxyuridines studied, only 5-hexyldeoxyuridine was effective in blocking the cytotoxic effects of deoxyadenosine. 5-Isopropyl- and 5-pentyldeoxyuridine essentially had no effect; 5-octyldeoxyuridine was only slightly effective. Partition coefficient studies indicated that there were marked differences in the distribution of these drugs between the octanol and aqueous phases; the order being: octyl->hexyl->pentyl->isopropyl->ethyldeoxyuridine. The apparent effect of these alkyldeoxyuridines on preventing the cytotoxic effects of deoxyadenosine did not correlate directly with the partition coefficient. These data may suggest that there is an optimal value for the distribution of the compounds between the lipid phase and aqueous phase of the membrane with 5-hexyldeoxyuridine approaching that value.

5-Hexyldeoxyuridine is not phosphorylated and is therefore not incorporated into DNA (24). Studies with [2-^{14}C]hexyldeoxyuridine showed that the majority of the radioactivity was associated with the Golgi apparatus (24). 5-Hexyldeoxyuridine is an inhibitor of the incorporation of glucosamine into various glycoconjugate fractions (24). The toxicity of 5-hexyldeoxyuridine is relatively low since the dosage level resulting in 50% lethality is 350 and 450 mg/kg in normal Swiss mice and Ehrlich tumor-bearing Swiss mice, respectively (24).

5-Ethyldeoxyuridine was not studied further for its ability to block the cytotoxic effects of deoxyadenosine or fluorodeoxyuridine as it clearly has other modes of action (19, 20).

In summary, these studies indicate that 5-hexyldeoxyuridine may be an inhibitor of nucleoside transport. L1210 cells have at least three nucleoside transporters (25, 26). Two of these involve mechanisms of facilitated diffusion but differ in sensitivity to the nucleoside transport inhibitor, nitrobenzylthioinosine (27), and a third which involves sodium-dependent nucleoside transport (25, 26, 28). Studies are underway to determine if 5-hexyldeoxyuridine is an inhibitor of nucleoside transport (23) and if so, which of the three nucleoside transporters in L1210 cells is involved (26–28). These results indicate that further studies on the utility of using 5-hexyldeoxyuridine in combination with inhibitors of *de novo* pathways for nucleotide synthesis are warranted.

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