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The Effect of 3-Deazauridine and Dipyridamole on Uridine Utilization by Mice

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Abstract—*The inhibition of uridine utilization by 3-deazauridine, an inhibitor of uridine kinase, and by dipyridamole, an inhibitor of the facilitated transport of nucleosides was examined. 3-Deazauridine* (500 mg/kg) *markedly inhibited* (>70%) *the formation of uracil nucleotides from uridine in liver, kidney, and* L1210 *tumor cells. The degree of inhibition is greatly reduced by* 6 hr *after administration of the drug. Dipyridamole* (100 mg/kg) *did not significantly reduce salvage of uridine by liver or kidney and produced only small, transient reductions in salvage by* L1210 *tumors. Dipyridamole pretreatment did not alter the rate of clearance of uridine from the plasma.*

INTRODUCTION

CELLS have distinct enzymatic pathways for synthesis of nucleotides *de novo* or by salvage of preformed nucleosides, purines, or pyrimidines. The relative contribution of each process to the nucleotide requirement of the cells under physiological conditions is not known. Although effective inhibitors of pyrimidine nucleotide synthesis *de novo* are available, no corresponding inhibitors of uridine salvage have been shown to be effective *in vivo*. Thus the continued operation of the salvage pathway may reduce the effectiveness of inhibitors of pyrimidine nucleotide synthesis as antitumor agents. Indeed, Karle *et al.* have shown that the cytostatic action of *N*-phosphonacetyl-L-aspartate (PALA, an inhibitor of aspartate transcarbamylase) is prevented by concentrations of uridine lower than those normally present in human plasma [1]. These results suggest that a concurrent inhibition of *de novo* and salvage routes to pyrimidine nucleotides may be more effective than inhibition of synthesis *de novo* alone.

The formation of uracil nucleotides by cells from exogeneous uridine requires entry into the cell followed by phosphorylation by uridine kinase (EC 2.7.1.48). Upon phosphorylation the uridine is trapped intracellularly and rapidly further phosphorylated to the di- and tri-phosphates. The process of accumulation of nucleosides as nucleotides is often referred to as nucleoside salvage. Thus inhibitors of the facilitated transport of nucleosides or of uridine kinase may reduce utilization of circulating uridine. We have examined the ability of 3-deazauridine and dipyridamole to inhibit uridine salvage *in vivo*. 3-Deazauridine is a substrate for mammalian uridine kinase [2] and effective in the treatment of murine L1210 leukemia [3]. Dipyridamole is a potent inhibitor of nucleoside transport by cells in culture, effective at micromolar concentrations [4, 5], but the effect of dipyridamole on salvage of circulating uridine by tissues of the mouse has not been directly assessed. Such studies of dipyridamole action in animals are crucial, because *in vivo* additional factors not present in cultures, such as rate of tissue perfusion, may determine the rate of nucleoside salvage.

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MATERIALS AND METHODS

Dipyridamole was a gift of Boehringer–Ingelheim Corp. Dipyridamole is poorly soluble in water and was prepared as a solution at 100 mg/ml by dropwise addition of HCl at 37°C, then diluted to 10 mg/ml with 0.9% sodium chloride just prior to use. In a few instances, as indicated, the dipyridamole was dissolved in dimethyl sulfoxide. All other biochemicals were purchased from Sigma Chemical Co., St. Louis, MO. [5^3H]Uridine (28 Ci/mmol) was purchased from Moravek Biochemicals, Brea, CA.

Male CDF_1 mice weighing 25–28 g were used for all experiments, and had free access to Lab-Chow and water.

Incorporation of radiolabeled uridine into total acid-soluble uracil nucleotides and RNA was measured as described previously [6]. In brief, the mouse was killed by cervical dislocation 20 min after administration of labeled nucleoside, and spleen, kidney, and liver rapidly removed and frozen in liquid nitrogen. A perchloric acid extract of each tissue homogenate was prepared, neutralized, and treated with phosphodiesterase to hydrolyze all nucleotides to the corresponding 5′-monophosphates. The radiolabeled uracil nucleotides were then estimated by separation of UMP by high performance anion exchange chromatography followed by measurement by liquid scintillation counting. Labeled nucleotides in RNA were measured following mild alkaline hydrolysis of the precipitated macromolecules.

Clearance of [5^3H]uridine from the plasma was determined by sampling blood (50 μl) from the retro-orbital sinus with a heparinized micropippette at the indicated times. The final sample (20 min) was taken from an incision at the neck to avoid contamination with residual blood from earlier samples. The plasma was separated by 1 min of centrifugation in a table-top centrifuge at 8800 ***g*** and 25 μl of the plasma was mixed with 0.2 ml of ice-cold 0.5 M perchloric acid to precipitate the proteins. Radioactivity was determined by scintillation counting in ACS® (Amersham, Arlington Hts, IL) and corrected for efficiency by an internal standard. The radiolabel present in uridine was measured by separating the fraction containing uridine by chromatography followed by liquid scintillation counting. The chromatographic system was a 15 cm × 0.39 cm Resolve® C18 reversed phase column (Waters Corp., Milford, MA) eluted with 1.5 ml/min 0.2 M potassium phosphate pH 5.1. One-ml fractions were collected for counting. Uridine was well resolved from $^3H_20$, uracil and β-alanine by this system.

RESULTS AND DISCUSSION

Pretreatment of mice with 3-deazauridine (500 mg/kg) markedly decreased salvage of [5^3H]uridine by the liver, kidney, spleen (Fig. 1) and by both subcutaneous and ascites forms of the L1210 tumor (Fig. 2). Incorporation into both uracil nucleotides and RNA was reduced about equally, although some differences were seen. At 0.5 hr after injection of 3-deazauridine the inhibition of salvage was >80% in liver, kidney, and L1210 tumor, based on the decreased incorporation into uracil nucleotides. The inhibition of uracil nucleotide formation in the spleen was somewhat less (66%). The capacity to form uracil nucleotides by salvage or uridine recovered rapidly in all tissues, approaching control values by 6 hr after treatment. A statistically significant increase in the formation of labeled uracil nucleotides by L1210 ascites was observed 6 hr after treatment (Fig. 2).

3-Deazauridine is a substrate of uridine kinase and would therefore be expected to reduce the rate

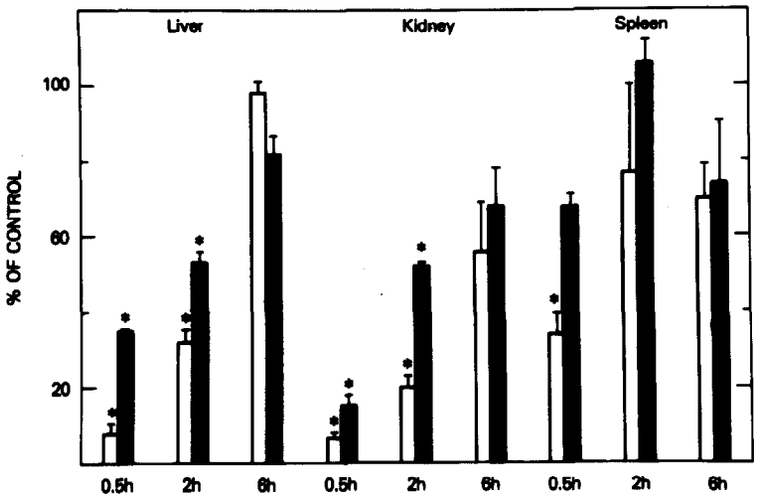

Fig. 1. The effect of 3-deazauridine on incorporation of [^{3}H]uridine into uracil nucleotides and RNA. Male CDF$_1$ *mice received* 500 mg/kg *3-deazauridine (or* 0.9% *sodium chloride for controls) by intraperitoneal injection. At the indicated time intervals following injection of 3-deazauridine each mouse received* 20 μCi *of [^{3}H]uridine* (0.2 ml, 100 μCi/ml *in* 0.9% *sodium chloride) by intravenous injection into the tail vein. Twenty minutes later mice were killed by cervical dislocation and incorporation of labeled uridine into uracil nucleotides (open bars) and RNA (closed bars) determined as described under Materials and Methods. The data given is the mean* ± S.E.M. *from determinations on three to five mice. Control values did not vary significantly with time of prior treatment with* 0.9% *sodium chloride and were therefore pooled for comparison. Control values for uracil nucleotides and RNA were* 0.87 ± .07, 0.17 ± .02; 1.1 ± .08, 0.31 ± .02; *and* 0.53 ± .06, 0.31 ± .04 μCi/g *for liver, kidney, and spleen respectively,* n = 11. *All values statistically different from controls at* $P < 0.05$ *by Student's t-test are indicated by an asterisk.*

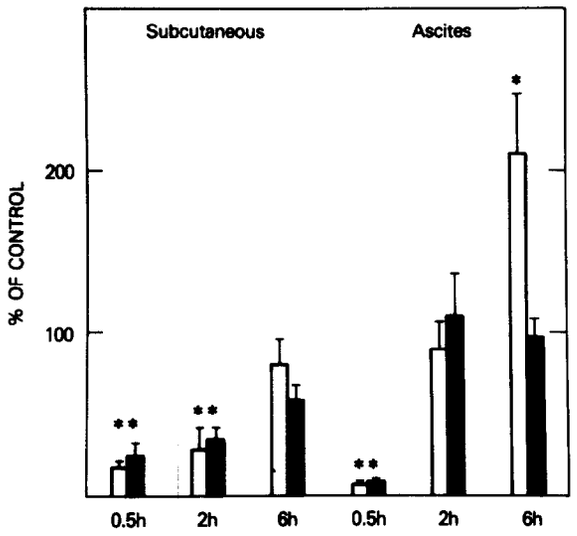

Fig. 2. The effect of 3-deazauridine pretreatment on salvage of uridine by L1210 *tumor cells. Male CDF$_1$ mice were inoculated with* 10^5 L1210 *cells by either intraperitoneal or subcutaneous injection and used for these experiments on day* 6 *or* 8 *respectively. Mice received* 500 mg/kg *3-deazauridine or saline (controls) by intraperitoneal injection, and then* 20 μCi [5^3H]*uridine (0.2 ml* × *100* μ *Ci/ml) by intravenous injection into the tail vein at the indicated time after 3-deazauridine. Twenty minutes after the* [5^3H]*uridine the mice were killed by cervical dislocation, tumors removed and incorporation of radiolabel into uracil nucleotides and RNA determined as under Materials and Methods. The data from three experiments is combined here. Control incorporation did not vary significantly with the time of saline injection, therefore all the controls were pooled. Control values were* 0.19 ± 0.02 *and* 0.23 ± 0.02 μCi/g *for uracil nucleotides and RNA respectively for ascites tumors* (n=20) *and* 0.19 ± .01 *and* 0.12 ± 0.01 μCi/g *for uracil nucleotides and RNA respectively in subcutaneous tumors* (n=16). *The bars indicate incorporation into uracil nucleotides (open bars) and RNA (closed bars) in six or seven treated tumors* ± S.E.M. *An asterisk indicates a value significantly different from the controls at* $P < 0.05$ *by Student's t-test.*

of phosphorylation of uridine by competition. Figures 1 and 2 indicate a marked reduction in formation of labeled uracil nucleotides from uridine in mice pretreated with 3-deazauridine. This reduction in uridine salvage may result from simple competitive inhibition, but alternatively could result from an allosteric inhibition of 3-deazauridine triphosphate, as recently demonstrated in cultured cells by Karle *et al.* [7]. Both mechanisms may be operating here and this experiment does not distinguish the two mechanisms. The inhibition of uridine salvage is short-lived even at the high dose employed here; for comparison, 100–300 mg/kg is effective in the treatment of L1210 leukemia [3]. This transient effect is consistent with the 30-min half-life previously reported for 3-deazauridine in mice [2]. Thus it seems unlikely that 3-deazauridine would be useful as an inhibitor of uridine kinase in combination with inhibitors of the *de novo* pathway, because the inhibition of uridine salvage by 3-deazauridine is rather transient. However, the data indicate that a nucleoside analog can reduce uridine salvage *in vivo*. A more potent or less toxic inhibitor capable of a prolonged effect is required for further development. A promising candidate, currently under investigation in this laboratory, is the carbocyclic analog recently described by Lim *et al.* [8].

In contrast to 3-deazauridine, dipyridamole, a potent inhibitor of nucleoside transport, had only modest effects on the salvage of intravenously administered [5^3H]uridine. No decrease in uridine salvage was seen in liver or kidney, but a small effect was observed for the spleen at 2 hr (Fig. 3). Both subcutaneous and ascites forms of L1210

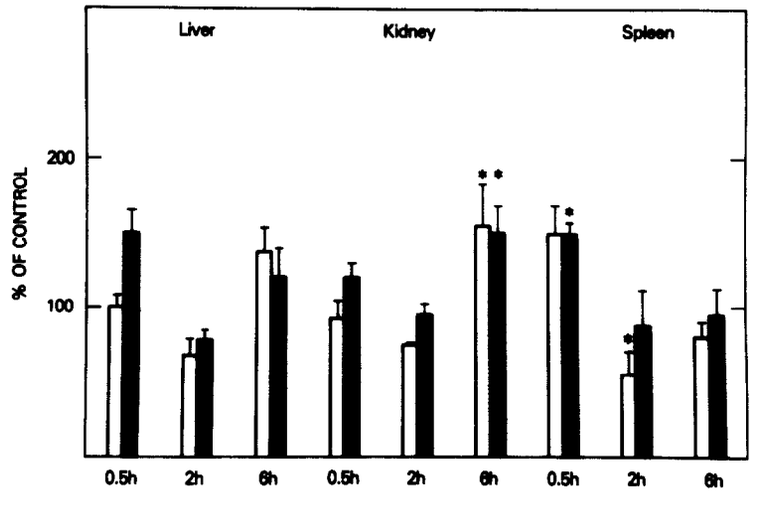

Fig. 3. The effect of dipyridamole on incorporation of [^{3}H]*uridine into uracil nucleotides and RNA. Male CDF$_1$ mice received* 100 mg/kg *of dipyridamole by intraperitoneal injection. At the indicated time after dipyridamole administration each mouse received* 10 μCi *of* [5^3H]*uridine* (0.2 ml, 100 μCi/ml *in* 0.9% *sodium chloride) by intravenous injection into the tail vein. Twenty minutes later the mice were killed by cervical dislocation and incorporation of* [5^3H]*uridine into uracil nucleotides (open bars) and RNA (closed bars) determined as described under Materials and Methods. The data given is the mean* ± S.E.M. *from determinations on three to five mice. Control values are given in the legend of Fig.* 1. *Statistical significance at* $P < 0.05$ *is indicated by an asterisk.*

experienced only a very small and short-lived decrease in uridine salvage after administration of dipyridamole (Fig. 4). These results indicate that dipyridamole does not prevent salvage of uridine from the circulation even at a dose of 100 mg/kg. This dose is higher than that employed previously by Nelson and Drake [9] for studies in mice. Their report indicated that dipyridamole at 10 mg/kg significantly increased the toxicity of methotrexate and maximally reduced uptake of [^{3}H]thymidine by mouse whole blood cells for 4 hr. In a single experiment dipyridamole dissolved in dimethyl sulfoxide was administered by intraperitoneal injection at 400 mg/kg and uridine salvage assessed by the protocol in the legend of Fig. 3. This treatment produced only a small decrease (<30%) in uridine salvage by liver and spleen measured 2 hr after dipyridamole injection. Thus even at a dose of 400 mg/kg the utilization of uridine by these tissues is not greatly inhibited.

Dipyridamole could effectively inhibit entry of uridine into cells without altering the balance of phosphorylation and catabolism intracellularly. Thus the result of inhibition of nucleoside transport may be a slower entry into cells, but ultimately the percent conversion to nucleotide may be unchanged. To examine this possibility, we measured the effect of pretreatment with dipyridamole on the clearance of intravenously injected [^{3}H]uridine. Dipyridamole (100 mg/kg) did not significantly change the rate of clearance of total radioactivity or of unmetabolized [^{3}H]uridine (Fig. 5).

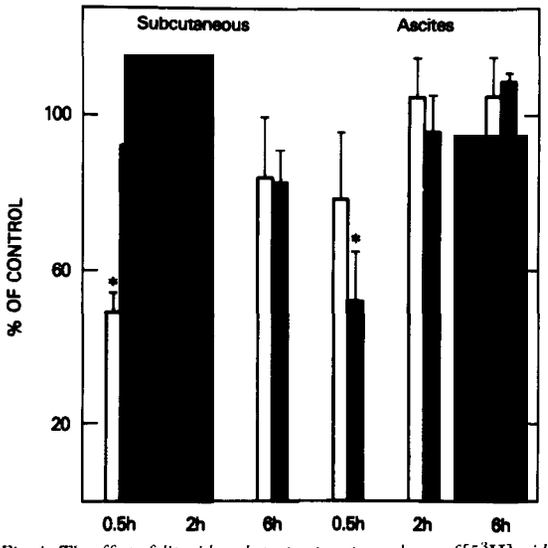

Fig. 4. The effect of dipyridamole pretreatment on salvage of [^{3}H]*uridine by* L1210 *tumor cells. Mice bearing* L1210 *tumors, as described in the legend to Fig. 2, received* 100 mg/kg *dipyridamole or saline (controls) by intraperitoneal injection, and then* 20 μCi [^{3}H]*uridine by intravenous injection into the tail vein at the indicated time after dipyridamole. Twenty minutes after the* [^{3}H]*uridine the mice were killed, tumors removed, and incorporation of label into uracil nucleotides (open bars) and RNA (closed bars) determined is described under Materials and Methods. The data from three experiments is combined here and control values were given in the legend of Fig. 3. The bars indicate the mean ± S.E.M. of determinations on four to nine tumors. An asterisk indicates a value significantly different from controls at* $P < 0.05$ *by Student's t-test.*

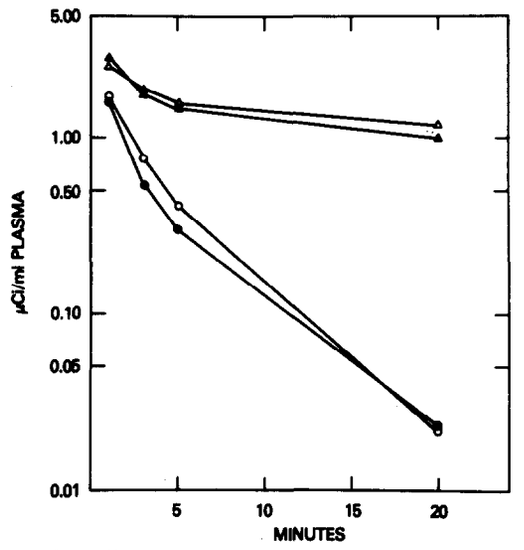

Fig. 5. The effect of dipyridamole pretreatment on the clearance of [^{3}H]*uridine from the plasma. Mice* (CDF_1) *received* 0.9% *sodium chloride (controls) or dipyridamole* (100 mg/kg) *by intraperitoneal injection into the tail vein. Two hours after this injection* 40 μCi *of* [^{3}H]*uridine* (0.2 ml × 200 μCi/ml) *was injected intravenously. The concentrations of total radioactivity* (Δ, Δ) and [^{3}H]*uridine.* (●, ○) *in plasma were determined as described under Materials and Methods. The mean values for controls* (Δ, ○) *and treated* (▲, ●) *mice are given for six mice from two independent experiments which are combined.*

Combinations of dipyridamole and acivicin [10–12], methotrexate [9, 13, 14], or *N*-phosphonacetyl-L-aspartate [15, 16] have been proposed and evaluated in cell culture systems and to a more limited extent *in vivo*. To our knowledge no studies reported have established that dipyridamole effectively blocks nucleoside salvage in treated animals. An earlier study indicated that an alternative inhibitor of nucleoside transport, *p*-nitrobenzylmercaptopurine riboside monophosphate, inhibited salvage of uridine by liver, but only to a limited extent, and salvage of uridine by the kidney increased in mice after treatment [6]. This report indicates that salvage of uridine from the blood is not greatly reduced in dipyridamole treated mice. It is possible that the salvage of hypoxanthine or of nucleosides other than uridine are inhibited by dipyridamole treatment. Uptake of cytidine by human colon carcinoma cells is reduced at much lower concentrations of dipyridamole than required to inhibit uptake of uridine [12], whereas cytidine and uridine uptake by hepatoma 3924A cells are about equally sensitive to inhibition by dipyridamole [10]. The assumption that nucleoside salvage is prevented by administration of dipyridamole is unwarranted and studies of combinations of dipyridamole and antimetabolites should consider the effectiveness of the inhibition of salvage achieved.

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