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THERAPEUTIC RESPONSE OF LEUKEMIC MICE TREATED WITH FLUORINATED PYRIMIDINES AND INHIBITORS OF DEOXYURIDYLATE SYNTHESIS*

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Abstract—The therapeutic efficacy of combinations of fluorinated pyrimidines and inhibitors of either ribonucleotide reductase or deoxycytidylate deaminase was evaluated for the treatment of the L1210 mouse leukemia in DBA/2 mice. Therapeutic synergisms were observed with optimal combinations of 5-fluoro-2′-deoxyuridine and either hydroxyurea or guanazole. In addition, mice treated with guanazole combined with 5-fluorouracil survived longer than was observed with any dose of guanazole or with 5-fluorouracil alone. Tetrahydrodeoxyuridine, a potential prodrug of a transition-state analog of deoxycytidylate deaminase, did not have antitumor activity by itself nor did it improve the therapeutic response of leukemic mice to 5-fluoro-2′-deoxyuridine. These results are consistent with the hypothesis that deoxyuridylate accumulation was limited by inhibition of ribonucleotide reductase but not by administration of tetrahydrodeoxyuridine. It is suggested that combination chemotherapy with fluorinated pyrimidines and inhibitors of deoxyuridylate synthesis may improve the therapeutic response to these drugs.

The fluorinated pyrimidines, 5-fluorouracil (FUra‡) and its 2′-deoxyribonucleoside (FdUrd), are potent cytotoxic compounds that are clinically useful in the management of various human adult carcinomas [1–3]. Both fluorinated pyrimidines can be metabolized to 5-fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP) and to 5-fluorouridine-5′-triphosphate which can be incorporated into RNA [4]. Individual circumstances in which incorporation of the fluorouracil base into RNA is apparently the cause of cytotoxicity [5, 6] and other cases in which RNA incorporation is apparently unrelated to cytotoxicity [6, 7] have been defined. The extent to which incorporation of FUra into RNA is responsible for either the chemotherapeutic response to fluorinated pyrimidines under clinical conditions or for the dose-limiting toxicity of normal human tissues remains obscure. On the other hand, FdUMP is known to be a potent inhibitor of thymidylate synthase [8, 9] and forms a covalent complex with this enzyme and with its cofactor, 5,10-methylenetetrahydrofolate [10, 11]. The resultant inhibition of thymidylate synthesis *de novo* and, hence, of DNA synthesis is marked and is thought to be sufficient to explain the chemotherapeutic effects of the fluorinated pyrimidines in most circumstances [4].

Exposure of neoplastic cells in culture and some rodent tumors *in vivo* to FUra results in the accumulation of intracellular 2′-deoxyuridine-5′-monophosphate (dUMP) [12–17] to levels that have been shown to greatly reduce the inactivation of purified thymidylate synthase by FdUMP [12, 17, 18]. In addition, although FdUMP binding to thymidylate synthase is covalent [10, 11], the reactions leading to covalent bond formation are reversible, and free enzyme can be regenerated at a biologically significant rate [11, 17, 18]. Hence, the accumulation of dUMP might limit or terminate the inhibition of thymidylate synthase by fluorinated pyrimidines and, thus, might be a key limitation for their therapeutic efficacy against refractory tumors.

dUMP is formed in mammalian tissues primarily by two enzymatic pathways, namely, the reduction of UDP by ribonucleotide reductase (and the subsequent phosphorylation to UTP followed by cleavage to the monophosphate by a specific hydrolase) and the deamination of dCMP by deoxycytidylate deaminase. Several inhibitors of ribonucleotide reductase are known, including guanazole (GZ) [19] and hydroxyurea (HU) [20]. Both these compounds are reversible enzyme inhibitors and both readily pass through the plasma membrane. A specific transition state inhibitor of deoxycytidylate deaminase, 3,4,5,6-tetrahydrodeoxyuridylate (H_4dUMP), has been described [21]. Although H_4dUMP, as a

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‡ Abbreviations: FUra, 5-fluorouracil; FdUrd, 5-fluoro-2′-deoxyuridine; FdUMP, 5-fluoro-2′-deoxyuridine-5′-monophosphate; dUMP, 2′-deoxyuridine-5′-monophosphate; dCMP, 2′-deoxycytidine-5′-monophosphate; H_4dUrd, 3,4,5,6-tetrahydrodeoxyuridine; H_4dUMP; 3,4,5,6-tetrahydrodeoxyuridine-5′-monophosphate; GZ, guanazole, 3,5-diamino-1,2,4-triazole; HU, hydroxyurea; and %ILS, increase in lifespan of leukemic mice, expressed as percent of control survival.

nucleotide, is probably incapable of entering cells, the corresponding nucleoside, 3,4,5,6-tetrahydrodeoxyuridine (H_4dUrd) might undergo lethal synthesis to H_4dUMP. In this study, we attempted to improve the therapeutic response to the fluorinated pyrimidines by coadministration of these inhibitors of the major pathways of dUMP synthesis. To evaluate this approach, we chose treatment of the ascites form of the L1210 mouse leukemia with FdUrd as a system in which we were reasonably assured that RNA-mediated toxic effects were minimal [7] and which demonstrated a measurable but limited therapeutic response.

MATERIALS AND METHODS

FUra and FdUrd were gifts of Hoffmann-LaRoche (Nutley, NJ); GZ, HU and human placental alkaline phosphatase (3 units/mg) were purchased from the Sigma Chemical Co. (St. Louis, MO). Other chemicals were reagent grade. DEAE cellulose was purchased from Eastman Chemicals (Rochester, NY). H_4dUrd was made from deoxycytidine by catalytic hydrogenation with 5% rhodium on alumina for 18 hr under 45 psi of H_2 as described by Hanze [22] and was purified by silica gel chromatography. Purified H_4dUrd gave a single spot on silica gel TLC in two solvent systems [chloroform–methanol (4:1), R_f = 0.75; and acetone–ethanol (19:1), R_f = 0.60]. NMR and mass spectrometry analysis of the product were consistent with the assigned structure. Tritiated H_4dUrd was similarly synthesized from [5-^{3}H]deoxycytidine (Schwartz-Mann, Orangeburg, NY) after dilution of its specific activity to 0.5 Ci/mmole. H_4dUMP was prepared from dCMP by the same technique and was purified on a 0.64 cm^2 × 35 cm column of DEAE cellulose that was eluted with a linear gradient of ammonium formate (pH 4.5) (0.003 to 0.3 M) with an initial mixer volume of 250 ml [23].

L1210/0 leukemia cells were originally obtained from Dr. T. A. Khwaja of the USC Cancer Center and were maintained by weekly serial passage of 10^5 cells in DBA/2 mice. DBA/2 female mice (Texas Inbred, Inc., Houston, TX), 18–21 g, were used for all experiments and were housed for at least 1 week prior to tumor inoculation. Mice were inoculated with 10^6 L1210 cells i.p. on day zero and drugs were injected i.p., usually starting 24 hr after tumor inoculation. Drugs were dissolved in sterile phosphate-buffered saline and were injected in a volume of 0.01 ml/g body weight. H_4dUrd was dissolved immediately prior to injection. Combinations of drugs were administered as a single solution. Leukemic mice treated with vehicle alone died in 6.9 ± 0.6 days (N = 50) after inoculation of L1210 cells. The therapeutic results shown represent a composite of two experiments, with five animals per point in each experiment (except where noted), and the data are expressed as means ± one standard deviation. The significance of differences between sample means was statistically tested using a one-sided *t*-test. The origins of the cultured cells and the methods of culture used in these experiments have been described previously [7].

Analysis of tetrahydropyrimidines. Tetrahydropyrimidines were assayed following cleavage of the glycosidic bond by estimation of 2-deoxyribose with the highly sensitive thiobarbituric acid reaction [24, 25], using a standard curve of 2-deoxyribose (0–10 nmoles). For estimation of H_4dUrd, 2-deoxyribose was released by heating to 100° for 15 min in 0.01 N HCl. H_4dUrd was released from H_4dUMP by treatment of a sample containing up to 100 nmoles of H_4dUMP with 50 milliunits human placenta alkaline phosphatase for 2 hr in 10 mM bicarbonate buffer (pH 10.5).

Phosphorylation of [^{3}H]H_4dUrd or [^{3}H]thymidine by tissue homogenates. Six-day-old chick embryos or rapidly growing tumors were suspended in 2 vol. of 50 mM Tris (pH 7.5) containing 0.25 M sucrose, and the suspension was homogenized by ten strokes with a teflon pestle followed by two 10-sec bursts of sonic oscillation in a Bronson sonicator. The sonicates were centrifuged for 1 hr at 105,000 *g*. High-speed supernatant fractions (usually 10–15 mg of soluble protein) were incubated with [^{3}H]H_4dUrd (5.1 μM, 0.5 Ci/mmole) for 4 hr in a Tris–HCl buffer (pH 8.0) (120 mM) containing 12 mM ATP, 20 mM $MgCl_2$, 1% bovine serum albumin, 2 units/ml creatine phosphokinase, 4 mM creatinine phosphate and 10 mM NaF in a total volume of 1.5 ml. The reaction was stopped by chilling the incubation mix to 0°. The mixture was applied to a 0.64 cm^2 × 35 cm column of DEAE cellulose, and the column was eluted with a linear gradient of ammonium formate identical to that used for purification of H_4dUMP (see above). Salt concentrations were monitored by measurement of conductivity after dilution to the range 10–80 mM. In some experiments, the phosphorylation of thymidine or H_4dUrd was followed by an ion exchange disc method, as follows. The high-speed supernatant fractions were incubated with [^{3}H]thymidine (8 μM, 0.1 Ci/mmole) or [^{3}H]HdUrd (5.1 μM, 0.5 Ci/mmole) in the incubation mixture described above in a total volume of 125 μl. Incubation was terminated by spotting 50 μl onto a 25 mm disk of DE-81 paper (Whatman, Inc., Clifton, NJ) and dropping the disk into 95% ethanol (10 ml/disk). The disks were washed in batch, excess ethanol was blotted off, and the washing procedure was repeated two additional times. The dried disks were placed in scintillation vials, 1 ml of 0.1 N HCl/0.2 M KCl and 9 ml of scintillation fluid (RIA-Solve II, Research Products International, Inc., Mount Prospect, IL) were added, and radioactivity was determined by liquid scintillation spectrometry. The protein content of crude supernatant fractions was estimated by the biuret method [26].

RESULTS

Detection of tetrahydropyrimidines. The thiobarbituric acid assay [24, 25] proved to be a convenient method to quantitate H_4dUrd and its 5′-monophosphate. The glycosidic bond of H_4dUrd was readily cleaved in boiling 0.01 N HCl; 95% of the 2-deoxyribose that was released after 60 min was released by 10 min of acid hydrolysis (Table 1). However, substantial amounts of deoxycytidine were also cleaved by this treatment, as had been reported previously [25]. Hence, the assay for tetrahydropy-

Table 1. Release of 2-deoxyribose from tetrahydrodeoxyuridine and tetrahydrodeoxyuridylate*

Exp.	Compound	Quantity (nmoles)	Incubation time (min) With 0.01 N HCl (100°)	Incubation time (min) With alkaline phosphatase (37°)	2-Deoxyribose released (nmoles)
1	2-Deoxyribose	9.1	0		9.6
			3		9.4
			10		9.5
			60		8.4
	Deoxycytidine	10.0	0		0
			3		1.1
			10		2.8
	Tetrahydrodeoxyuridine	10.0	0		2.1
			3		7.6
			10		8.7
			60		9.2
2	2-Deoxyribose	9.1		0	9.3
				5	9.4
				10	9.4
				60	8.7
	Deoxycytidylate	10.1		0	0.4
				5	0.4
				10	0.4
				60	0.4
	Tetrahydrodeoxyuridylate	10.0†		0	25
				5	47
				10	60
				60	110

* Duplicate tubes were incubated either with 0.01 HCl or with 50 milliunits of placental alkaline phosphatase in 10 mM bicarbonate buffer (pH 10.5). Acid-treated samples were then assayed for 2-deoxyribose by the thiobarbaturic acid method. The reaction of alkaline phosphatase was terminated by boiling these samples for 5 min prior to assay for 2-deoxyribose; 10 nmoles of tetrahydrodeoxyuridine treated with alkaline phosphatase alone in this manner released 1.6 nmoles of 2-deoxyribose.

† The estimate of the amount of tetrahydrodeoxyuridylate used in this experiment was based on the hydrolysis of nucleotide at 55° in N HCl for 20 hr. Obviously, enzymatic hydrolysis gives much higher yields of sugar.

rimidines in the presence of deoxycytidine compounds was performed by the thiobarbituric reaction without prior acid treatment since substantial, but not quantitative, cleavage of the glycosidic bond of H_4dUrd occurred under the conditions of the assay (Table 1). Extended incubation with alkaline phos-

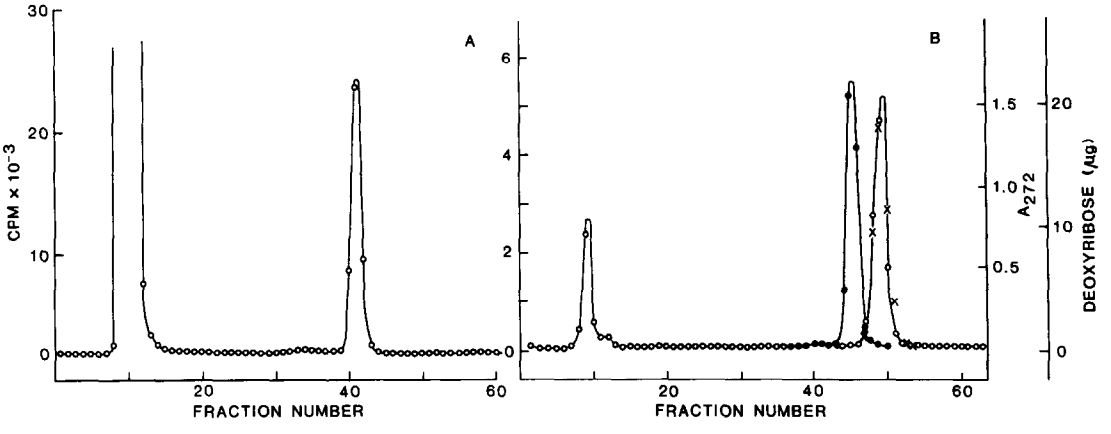

Fig. 1. Phosphorylation of H_4Urd by chick embryo extracts. The 105,000 *g* supernatant fraction of chick embryos (15.5 mg protein) was incubated with 5.1 μM [^{3}H]H_4Urd for 4 hr at 37° in the presence of ATP. The incubation mixture was then chromatographed on DEAE cellulose (0.64 cm² × 35 cm) and eluted with a linear gradient of ammonium formate (0.003 to 0.3 M), pH 4.5 (A). The peak eluting at tubes 39–44 was pooled and lyophilized. dCMP (1 μmole) and H_4dUMP (1 μmole) were added to a portion of this pool (39,000 cpm) and the mixture was chromatographed on a second column of DEAE cellulose (0.64 cm² × 35 cm) (B). Key: radioactivity (○), A_{272} (dCMP) (●), and deoxyribose released by alkaline phosphatase (×).

Table 2. Phosphorylation of [^{3}H]H_4Urd by tumor cytosols

Tissue	Nucleoside monophosphate formed [nmoles·hr^{-1}·(mg protein)$^{-1}$]	
	H_4Urd	TdR
Chick embryo	5.8×10^{-3}	0.84
L1210*	0.72×10^{-3}	23.2
Lewis lung	0.79×10^{-3}	2.2
P388*	0.80×10^{-3}	0.96
B16 melanoma	2.7×10^{-3}	5.3
13762 adenocarcinoma	3.5×10^{-3}	10.6
CCRF-CEM*	18.0×10^{-3}	33.0

* Exponentially growing tissues culture cells were used for these determinations.

phatase followed by boiling under alkaline conditions caused release of 2'-deoxyribose from H_4dUMP without significant cleavage of the glycosidic bond of dCMP (Table 1).

Phosphorylation of [^{3}H]H_4dUrd by chick embryo extracts. When [^{3}H]H_4dUrd was incubated with high-speed supernatant fractions of 6-day-old chick embryos in the presence of ATP, an anionic product was detected (Fig. 1) that eluted from DEAE cellulose at a position characteristic of a nucleoside monophosphate [23]. Since the [^{3}H]H_4dUrd used in these experiments was synthesized from [^{3}H]deoxycytidine, the small amount of nucleotide detected could represent dCMP formed from trace contamination with unreacted [^{3}H]deoxycytidine. To investigate this possibility, the labeled anion (Fig. 1A) was lyophilized, authentic samples of H_4dUMP and dCMP were added, and the mixture was again chromatographed on a column of DEAE cellulose. The elution profile of this column (Fig. 1B) demonstrates that the labeled species cochromatographed with H_4dUMP and was separated from dCMP.

Treatment of mice bearing the L1210 leukemia with FdUrd and H_4dUrd. FdUrd was found to have minimal but measurable activity against the L1210 leukemia. The dose–response curve on a daily times five schedule was quite broad, with an optimal increase in lifespan (ILS) of 25% centering at 75 mg·kg^{-1}·day^{-1} (data not shown); little weight loss was noted on this schedule at doses below 100 mg·kg^{-1}·day^{-1}.

When H_4dUrd was administered daily for 9 days starting on the day of tumor inoculation, no detectable change in the survival of tumor bearing mice was observed at any dose from 65 to 1000 mg·kg^{-1}·day^{-1}. In addition, H_4dUrd on this schedule at doses from 65 to 1000 mg·kg^{-1}·day^{-1} had no effect on the therapeutic activity of 75 mg·kg^{-1}·day^{-1} of FdUrd administered daily times five starting on day 1. Thus, DBA/2 mice treated with vehicle control or H_4dUrd at doses from 65 to 1000 mg·kg^{-1}·day^{-1} died 7.6 ± 0.5 days (N = 5) and 7.3 ± 0.6 days (N = 29), respectively, after the inoculation of 10^6 leukemic cells. Leukemic mice treated with H_4dUrd at these doses in combination with FdUrd survived 9.0 ± 0.5 days (N = 30) while mice treated with FdUrd alone survived 9.0 ± 0.7 days (N = 5).

Phosphorylation of [^{3}H]H_4dUrd by cytosol preparations of a spectrum of tumors. The ability of

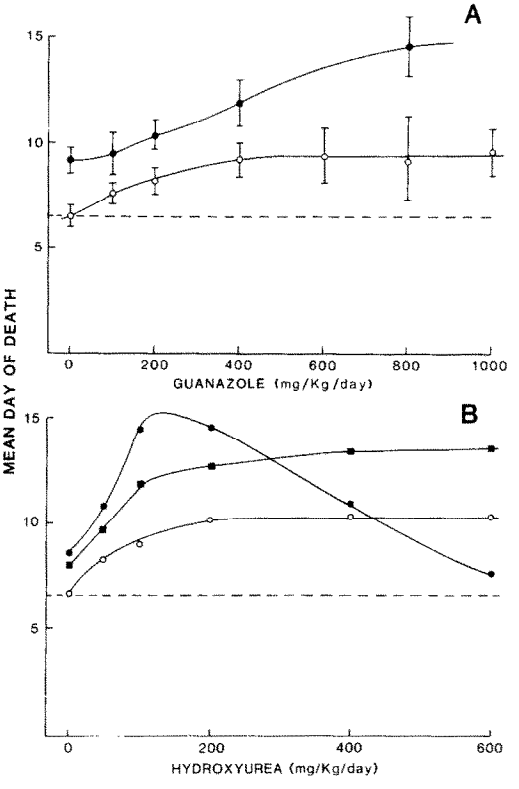

Fig. 2. (A) Survival of L1210-bearing mice treated with various doses of GZ with (●) or without (○) FdUrd. GZ was injected i.p. daily until the death of each animal; FdUrd (75 mg·kg^{-1}·day^{-1}) was given on days 1–5, 8, 11 and 14. The survival of mice treated with optimal FdUrd and GZ (800 mg·kg^{-1}·day^{-1}) in combination [14.4 ± 1.35 days (N = 10)] was significantly longer than that of mice treated with FdUrd alone [9.2 ± 0.6 days (N = 10)] or GZ alone [9.2 ± 1.0 days (N = 10)] ($P < 0.001$). The points represent means ± S.D. (B) Therapeutic response of the L1210 leukemia to HU alone (○) or in combination with FdUrd at 75 mg·kg^{-1}·day^{-1} (●) or 40 mg·kg^{-1}·day^{-1} (■). Hydroxyurea was injected i.p. daily until the death of each animal; FdUrd was given on days 1–5, 8, 11 and 14. The survival of mice treated with 100 mg·kg^{-1}·day^{-1} HU plus 75 mg·kg^{-1}·day^{-1} FdUrd [14.5 ± 2.0 (N = 10)] was significantly longer than that of mice treated with HU [10.3 ± 1.1 (N = 9)] or FdUrd [8.8 ± 0.8 (N = 10)] at optimal doses ($P < 0.001$). The dashed line in this and the subsequent figure shows the survival of vehicle-treated controls.

high-speed supernatant fractions of L1210 cells to phosphorylate [^{3}H]H$_4$dUrd was examined by chromatography on DEAE cellulose. As an internal control against artifactual loss of nucleotide by endogenous phosphatase action, the phosphorylation of [^{3}H]thymidine was monitored in parallel incubation mixtures of identical composition. The level of phosphorylation of [^{3}H]H$_4$dUrd carried out by L1210 supernatant fractions found in these experiments was three orders of magnitude lower than that seen with [^{3}H]thymidine and was substantially lower than that seen with chick embryo extracts (Table 2). In control experiments, it was found that the rate of phosphorylation of [^{3}H]H$_4$dUrd determined by DEAE cellulose column chromatography was identical with that found using a DE-81 disc technique such as that used in Table 2 for detection of [^{3}H]thymidine nucleotides (data not shown).

When the ability of cytosol fractions of various mouse, rat and human tumor cells to anabolize [^{3}H]H$_4$dUrd was examined, the level of phosphorylation of H$_4$dUrd was found to be detectable in all cases but was quite low relative to that observed in control incubations containing [^{3}H]thymidine as substrate (Table 2). In other experiments, it was found that [^{3}H]thymidylate was not detectably degraded to nucleoside during a 4-hr incubation with chick embryo extract in this sytem, suggesting that phosphatase activity was minimal during these incubations. It is of interest that the rate of phosphorylation of H$_4$dUrd in cytosols of CCRF-CEM human lymphoblastic leukemia cells was significant and exceeded that in chick embryo extracts. In all *in vitro* phosphorylation experiments performed, a single anionic metabolite was detected that eluted from DEAE cellulose with 54 ± 3 mM ammonium formate.

Therapeutic response of L1210 to combinations of fluorinated pyrimidines and inhibitors of ribonucleotide reductase. The effectiveness of FdUrd against the L1210 leukemia was improved significantly by coadministration of GZ (Fig. 2A), an inhibitor of the synthesis of deoxyribonucleotides (including dUMP) from ribonucleotides [19]. The therapeutic activity of GZ by itself on this schedule was limited (the maximum ILS observed was 45%); combined treatment with FdUrd and GZ gave a synergistic* effect on survival (an ILS of 125% with 800 mg·kg^{-1}·day^{-1} GZ). As shown in Fig. 2B, a similar synergism was observed from the combination of HU with 75 mg/kg FdUrd (116% ILS at 100 mg·kg^{-1}·day^{-1} HU). However, the combination of HU and FdUrd was apparently also synergistically toxic to the host, as evidence by the sharp decline in ILS seen at HU doses above 200 mg·kg^{-1}·day^{-1}. When the FdUrd used in these experiments was reduced to 40 mg·kg^{-1}·day^{-1}, a dose–response curve for the combination of HU plus FdUrd was similar to that seen with GZ and, again, a therapeutic synergism was observed (100% ILS at 600 mg·kg^{-1}·day^{-1} HU) ($P < 0.05$)† (Fig. 2B).

FUra is much more effective against the L1210 leukemia than is FdUrd; mice treated with 25 mg/kg FUra survive 90% longer than control animals (Fig. 3). When leukemic mice were treated with FUra and concurrent GZ, a striking synergism was observed (Fig. 3). At optimal doses of FUra and GZ, 25 mg/kg and 500 mg/kg, respectively, an ILS of 215% was observed. At doses of GZ of 600–1000 mg/kg in combination with FUra, some animals died before the control group (on days 5–8), while those that survived the marked weight loss and toxicity survived for extended periods free of disease but finally succumbed on days 17–28.

DISCUSSION

The inhibition of an enzyme on a metabolic pathway will usually cause accumulation of the substrate of the inhibited enzyme [28, 29]. Unless the concentration of substrate is subject to feedback control, the pool of substrate will expand until either the inhibition is overcome, the substrate becomes toxic to some other possibly unrelated enzyme, or the rate of loss of substrate from the cell equals its rate of accumulation [30, 31]. Potter [28], in defining the concept of "sequential inhibition", proposed the use of a second inhibitor to avert such metabolite accumulation as a general approach to combination chemotherapy. However, the generalization [28, 32] that two inhibitors of the same pathway must necessarily lead to a synergistic effect has been questioned on theoretical grounds [31, 33]. Inhibition of thymidylate synthase, either directly by fluorinated pyrimidines [14, 34] or indirectly by methotrexate [34], has been shown to expand the cellular content

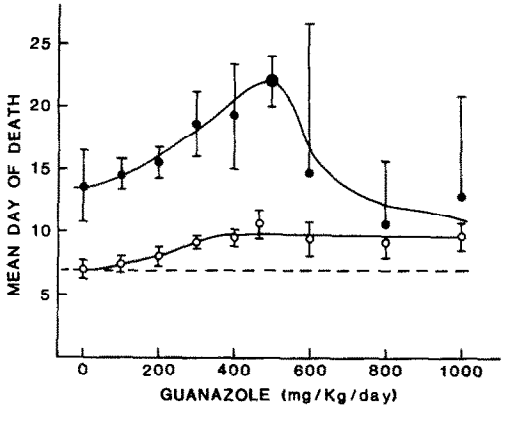

Fig. 3. Survival of leukemic mice treated with GZ alone (○) or in combination with FUra (●). GZ was injected i.p. daily until the death of each animal; FUra (25 mg·kg^{-1}·day^{-1}) was given on days 1–5, 8, 11, 14, 17 and 20. The survival of mice treated with FUra in combination with either 400 mg·kg^{-1}·day^{-1} GZ [19.2 ± 4.2 days (N = 10)] or 500 mg·kg^{-1}·day^{-1} GZ [22.0 ± 2.0 days (N = 5)] was significantly greater than that of mice treated with FUra alone [13.6 ± 2.8 days (N = 10)] or GZ alone at either 400 mg·kg^{-1}·day^{-1} [9.5 ± 0.7 days (N = 10)] or 500 mg·kg^{-1}·day^{-1} [10.6 ± 1.1 days (N = 5)].

* In this context, we use the definition of a synergism between two drugs as an antitumor effect of the two drugs in combination that is significantly greater than that obtained with either drug alone under identical conditions of treatment [27].

† One experiment, N = 5 in each group.

of dUMP dramatically in many cases and to cause deoxyuridine to spill out of the cell [34]. Such levels of dUMP prevent the binding of FdUMP to thymidylate synthase *in vitro* [12, 17, 18].

In these experiments, we attempted to expose tumor thymidylate synthase to FdUMP and to induce intermittent periods of depletion of the dUMP pool with inhibitors of either ribonucleotide reductase or deoxycytidylate deaminase. We have shown a distinct synergism against one murine tumor when fluorinated pyrimidines were combined with inhibitors of ribonucleotide reductase (Figs. 2 and 3), which would specifically depress deoxyribonucleotide synthesis with little or no effect on ribonucleotides [19]. Whether the therapeutic synergisms reported in this study are actually due to a diminished pool of dUMP in treated cells is not known at present.

H_4dUMP has been shown to be a unique inhibitor of chick embryo deoxycytidylate deaminase [21]. The potency of this interaction is thought to be the result of structural similarity of H_4dUMP to a transition state of the reaction in which the 4-carbon of the substrate temporarily assumes a tetrahedral configuration [35]. Since the anionic nature of H_4dUMP would prohibit entry into the cell, we evaluated H_4dUrd as a membrane-permeable precursor of the active inhibitor. It has been reported that incubation of H_4dUrd with minces of chick embryos leads to a significant inhibition of deoxycytidylate deaminase in crude supernatant fractions prepared from these minces [21]. Presumably, this reflected phosphorylation of H_4dUrd to the 5′-monophosphate, which is a potent inhibitor of deoxycytidylate deaminase, since H_4dUrd itself did not inhibit this enzyme at the concentrations used [21]. We have directly confirmed the phosphorylation of H_4dUrd by crude supernatant fractions of chick embryos as well as by supernatant fractions of various mouse, rat and human tumors (Fig. 1 and Table 2). Yet, treatment of mice bearing the L1210 leukemia with H_4dUrd alone or in combination with FdUrd did not result in inhibition of the growth of leukemic cells attributable to H_4dUrd, as measured by survival of mice bearing L1210 (see Results). A similar discrepancy was reported previously in experiments which showed that, although inhibition of deamination of dCMP could be demonstrated in chick embryo minces, the rate of DNA synthesis was not altered in these same minces [21]. These results may indicate either that inhibition of deoxycytidylate synthesis via deoxycytidylate deaminase is compensated for by a stimulation of ribonucleotide reductase, that blockade of deoxycytidylate deaminase by H_4dUMP is overcome by accumulation of dCMP, or that the rate of clearance of H_4dUrd *in vivo* prohibits the accumulation of cytotoxic levels of H_4dUMP. Finally, it should be noted that, whereas several potent inhibitors of ribonucleotide reductase are known and are chemotherapeutically active [19, 20, 36], there is a marked scarcity of specific inhibitors of deoxycytidylate deaminase. Our results are consistent with the hypothesis that inhibition of the synthesis of dUMP would improve the therapeutic activity of the fluoropyrimidines but that both major pathways of dUMP synthesis should be inhibited.

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