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Schedule-dependent antitumor and toxic effects of thymidine and 5-fluorouracil in AKR and L1210 leukemias*

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Summary. The effect of thymidine (dThd) on 5-fluorouracil (FUra) toxicity to both leukemia stem cells (LCFU) and hematopoietic stem cells (NCFU) was examined. A dose of 10 mg/mouse of dThd given within 1 h before FUra enhanced FUra cytotoxicity to NCFU by a factor of about 4. This effect was also reflected in the reduction of the LD_{10}. The dose-survival curve of FUra in AKR leukemia was modified by the prior administration of dThd 10 mg/mouse: the cytotoxic effect of FUra was enhanced by a factor of between 100 and 1000 throughout the dose range studied. These findings were reflected in the ILS studies. When given by 'high dose' infusion dThd had only a slight antitumor effect on AKR leukemia, and no effect on L1210. When large doses of dThd were infused concomitantly with FUra they ptoentiated its cytotoxicity against NCFU, AKR LCFU, and L1210 LCFU in a dose-dependent manner and by a maximum factor of about 70.

Introduction

Thymidine (dThd) has been utilized as a rescue agent to protect normal tissues from the toxicity induced by fluorodeoxyuridine [10, 14]. More recently it has been shown to potentiate the antitumor and toxic effects of FUra in some model murine tumors [17, 19, 22]. Previously, we examined the response of AKR and L1210 transplantable leukemias to this combination given by i.v. push, and we found that with proper scheduling, dThd, greatly increased the cytotoxicity of FUra in AKR leukemia, but only slightly in L1210 leukemia [17]. Because clinical trials of this combination have begun [13, 15, 20], we have carried out more extensive studies of the combination in experimental murine models. We have studied the toxicity of this combination to normal hematopoietic stem cells and to the whole animal and the modification of the dose-survival curve of FUra for AKR leukemina cells produced by the co-administration of dThd, and have determined the increase in lifespan of treated leukemic mice. Finally, because some studies report antitumor activity of dThd against human tumor xenografts [9, 12], experiments were performed to evaluate this agent at a 'high' dose and in a long-term infusion, both as a single agent and in combination, with FUra in both AKR and L1210 transplantable leukemias.

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Materials and methods

Drugs. 5-Fluorouracil (FUra) was obtained from Hoffmann-La Roche (Nutley, NJ) in 500 mg per 10-ml vials. dThd was purchased from Sigma Chemical Co. (St. Louis, Mo). A sterile 0.9% NaCl solution was used to dissolve and dilute the drugs to be injected to the mice. The prescribed doses were injected via the tail vein in 0.5 ml for the rapid i.v. push or in a total volume of 4.6 ml for the slow infusion experiments. A Harvard apparatus was used to deliver the drugs over a 22-h period [6]. The mice were kept in plastic restrainers for the duration of the infusion and were allowed food ad libitum.

Mice. AKR, DBA/2, and $CD2F_1$ (Balb/c × DBA/2) mice of both sexes bred in our own facility were used for the experiments; at the time of experiment were 7–8 weeks old and weighed 20–24 g.

Leukemia cells. The transplantable AKR leukemia is derived from a spontaneous lymphoma [1] and has been passaged in our laboratory as previously described [23]. Experimental mice received 10^6 cells via the tail vein and were treated 4 days later. The L1210 leukemia was obtained from the National Cancer Institute and is maintained and passaged as previously described [5]. The $CD2F_1$ mice used in the experiments received 2×10^6 leukemic cells 4 days before the drug treatment.

Assay for normal hematopoietic colony-forming units (NCFU). NCFU in the femoral marrow of mice were assayed by a method previously described [21]. Groups of five normal donors, AKR or $CD2F_1$, were sacrificed by cervical dislocation 24 h after the i.v. rapid injection or immediately after the end of the infusion. Their femurs were removed and a monodispersed cell suspension was obtained. These suspensions were diluted so that 0.5 ml of the final dilution produced a discrete number of macroscopic colonies in the spleen of recipient mice 9 days later. The recipient mice, 10–15 per group, were supralethally irradiated before receiving the bone marrow transplant.

The results are expressed in terms of surviving fraction of NCFU related to an untreated control group.

Assay for leukemia colony-forming units (LCFU). The assay for LCFU has been described previously [23]. Recipient mice were not irradiated and were killed 8 or 9 days after receiving the leukemia cells from the donors. The results are expressed in terms of surviving fraction of LCFU related to an untreated control group.

In order to assess fractional survival levels less than 10^{-7}, an end-point dilution assay was used, as initially proposed by Hewitt [8] and detailed previously [25]. The number of malignant cells was evaluated with the method of Fisher and Yates [7].

Host toxicity. Groups of five to ten AKR mice matched for age, sex, and weight were treated with dThd 10 mg/mouse, followed 15 min later by various doses of FUra; both drugs were administered by i.v. bolus. The survival at 30 days was recorded.

Increase in lifespan (ILS). Groups of five to ten AKR mice received 10^6 leukemic cells and 2 days later were treated with dThd 10 mg/mouse, followed 15 min later by various doses of FUra also given by rapid i.v. injection. The mice were checked daily for 30 days, and the median survival time was calculated to the nearest 0.5 day. The ILS of treated groups was expressed as the number of days beyond the median survival of an untreated control group.

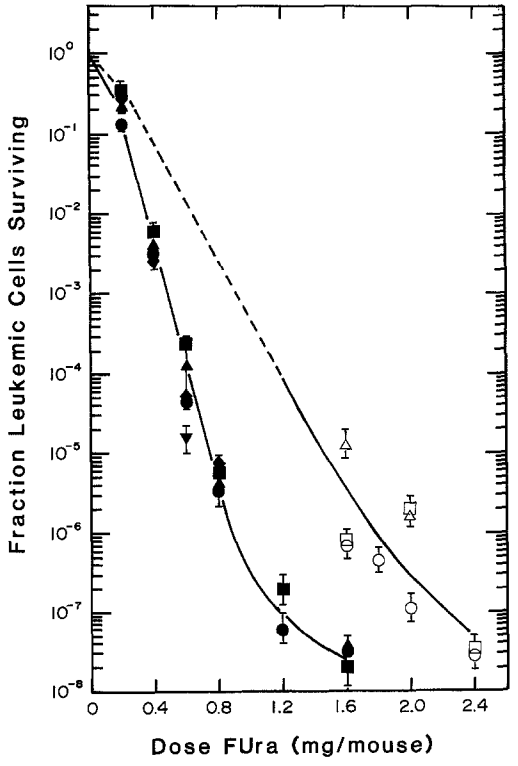

Fig. 1. Survival of AKR LCFU as a function of dose of FUra. *Open symbols,* FUra alone; *Closed symbols,* FUra administered 15 min after dThd 10 mg/mouse. *Dashed line* indicates previously published results of a single dose of FUra [24]. Different *symbols* show results recorded in different experiments. *Vertical bars* are ±1 SEM

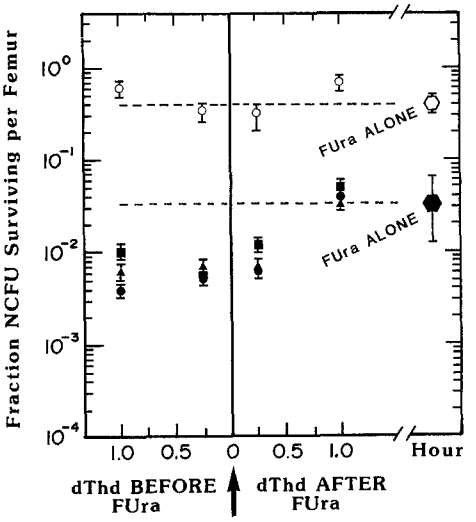

Fig. 2. Survival of NCFU as a function of sequence and interval between the administration of dThd (10 mg/mouse) and FUra (*open symbols* 0.6 mg/mouse, *closed symbols* 2.5 mg/mouse). *Dashed lines* indicate survival of NCFU after FUra alone. Symbols and errors as in Fig. 1

Results

LCFU dose-survival relationship

We first examined the effect of dThd pretreatment on the dose-response relationship of FUra for AKR leukemia. We choose a dThd dose of 10 mg/mouse, preceding the administration of FUra by 15 min. The results are shown in Fig. 1. The dose-survival for FUra alone up to 1.2 mg/mouse has been published [24], and is shown in Fig. 1 as a dashed line. The LCFU assay has a lower limit of about 10^{-6} for the surviving fraction, and therefore an end-point dilution assay with a limit of sensitivity of 10^{-8} was used to define the curve further. A straight line was drawn through these data and demonstrated a different slope from that observed at higher survival levels. A dose-survival curve was similarly constructed for the combination dThd-FUra and revealed greater cell killing throughout the range of FUra doses studied.

Both curves showed a biphasic relationship between survival and dose. The $D_{1/2}$ for FUra alone was 0.09 for the first phase and 0.15 for the second. The $D_{1/2}$ for the combination was 0.05 for the first and 0.18 for the second phase.

NCFU

We next examined the time-dependent effect of dThd 10 mg/mouse given by i.v. push on FUra toxicity on NCFU. Previously for LCFU [17], we had observed the greatest potentiation when dThd was administered from 1 h to immediately before FUra. Two doses of FUra were examined: 0.6 mg per mouse (the same dose as was used in the previous study) and 2.5 mg per mouse, a dose that reduces the NCFU survival to about 2×10^{-2}, the same level to which 0.6 mg per mouse reduces the number of LCFU. The results are shown in Fig. 2. The administration of dThd had no effect on the toxiticy of 0.6 mg per mouse, but did enhance the toxicity of 2.5 mg per mouse by a factor of about 4 when given either 1 h or 15 min before or

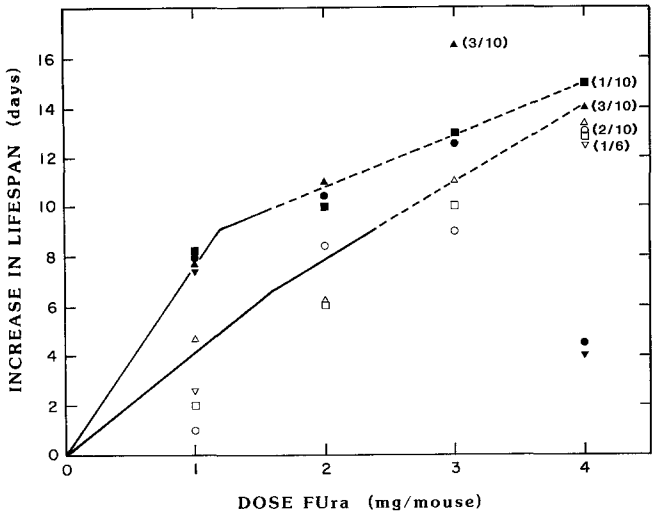

Fig. 3. Absolute ILS for leukemic mice following administration of FUra alone *(open symbols)* or in combination with dThd *(solid symbols)*. Fractions in *brackets* indicate: 30-day survivors/total treated. *Solid lines,* calculated ILS; *dashed lines,* extrapolation of the calculated ILS beyond the range examined in Fig. 1

15 min after the administration of FUra, and did not have any effect when given 1 h after.

Toxicity

Several experiments were carried out with doses of FUra ranging from 1 to 8 mg per mouse. The LD_{10} and LD_{50} (i.e., the doses of FUra or the combination dThd-FUra that resulted in the death of either 10% or 50%, respectively, of a group of mice within 30 days after treatment) were determined by Probit analysis. For FUra alone the values were 2.5 and 4.8, respectively. A dThd dose of 10 mg per mouse 15 min before FUra lowered the LD_{10} to 1.7 and the LD_{50} to 3.3 mg/mouse, which constituted a reduction to approximately 68% of the dose obtained for FUra alone.

Increase in lifespan (ILS)

Assuming day 0 as the day of cell inoculum, the majority of untreated mice (>65%) died between day 5 and day 6 (range 4.5–5.5). At least three separate experiments were carried out for each dose of FUra or the combination dThd-FUra, and the results are shown in Fig. 3. The predicted ILS was calculated from the dose-survival curve with the aid of the formula derived by Bruce et al. [3], assuming a doubling time for AKR leukemic cells of 0.38 days or approximately 9 h. For FUra doses of up to 3 mg/mouse the addition of dThd resulted in a consistently longer median survival time than that obtained with FUra alone. At the FUra dose level of 4 mg/mouse the results of the combination were widely scattered. The agreement between the experimental results and the calculated ILS is excellent in the dose range examined, except for the combination dThd at FUra 4 mg/mouse.

Infusion studies

The results of the infusion studies are summarized in Table 1. The infusion of dThd alone at a high dose had only a small antitumor effect in AKR leukemia and none in L1210. We chose a dose of 0.4 mg per mouse of FUra for the AKR leukemia and a dose of 3 mg/mouse for the L1210 leukemia, because these doses were similar in terms of reducing the surviving fraction of LCFU to about 10^{-2} [18]. The co-administration of dThd potentiated the cytotoxicity of FUra alone, as can be seen in the last column. For AKR leukemia the synergistic effect was more prominent with dThd doses of 100 and 250 mg/mouse, and for the L1210 leukemia, with 50 mg/mouse. However, approximately the same degree of potentiation was observed when NCFU were assayed (results not shown).

Discussion

The co-administration of dThd increased the toxicity of FUra in AKR mice. This effect has also been observed in humans [13] and in rats [4], and could be ascribed to the common degradative pathway involving the enzyme dihydrouracil dehydrogenase. It is possible, however, to achieve a therapeutic synergism if the enhancement in the antitumor effect is much higher than the enhancement in toxicity. This is the case in AKR leukemia, where we observed an excellent correspondence between the dose-survival curve and the ILS data up to FUra 2 mg/mouse; but unfortunately it was not possible to investigate the behav-

Table 1. Infusion of FUra and high-dose dThd in AKR and L1210 leukemias

AKR Dose of dThd (mg/mouse)	Surviving fraction[a] with			Potentiation index[b]
	dThd alone	FUra alone 0.4 mg/mouse	dThd plus FUra	
50	3.7×10^{-1}	1.2×10^{-2}	9.0×10^{-3}	0.5
100	3.9×10^{-1}	1.2×10^{-2}	1.9×10^{-4}	24.7
250	4.0×10^{-1}	1.2×10^{-2}	1.4×10^{-4}	34.2
L1210 Dose of dThd (mg/mouse)	Surviving fraction[a] with			Potentiation index[b]
	dThd alone	FUra alone 3 mg/mouse	dThd plus FUra	
50	1.3	8.9×10^{-2}	2.2×10^{-3}	72.7
100	1.1	8.9×10^{-2}	3.9×10^{-3}	25.1
250	9.7×10^{-1}	8.9×10^{-2}	5.7×10^{-3}	15.1

[a] Surviving fraction of LCFUs: Average of several separate experiments

[b] $\frac{\text{Theoretical additive level of survival}}{\text{Observed level of survival}}$

ior of the dose-survival curve beyond this point because of the limit of sensitivity of the assay. However, when the slopes were extrapolated to cover the dose range examined the agreement was still good between the predicted and observed ILS. When the mice were treated for the survival studies they harbored (estimating a doubling time of 9 h for these cells) about 4×10^7 leukemia cells; we should therefore have cured some mice in the groups treated with FUra 2 mg/mouse or more plus dThd. But in fact, we observed cures only in the groups treated with FUra 3 mg/mouse or more plus dThd. One possible explanation for the discrepancy between the two assays is that the leukemia cells can disseminate to the central nervous system and be protected from the cytotoxic action of anticancer agents. Another factor that makes the interpretation of these results difficult at higher doses is the interplay between toxicity and antitumor effects, which can influence the survival time in a manner that cannot be accurately predicted. It is thought that FUra acts biologically through two mechanisms: One is the inhibition of thymidylate synthetase by its metabolite 5-fluoro-2'-deoxyuridine, with consequent inhibition of DNA synthesis; the other is the incorporation of its metabolite 5-fluorouridine triphosphate into RNA, with still uncertain effects. The recent discovery of the incorporation of fluoropyrimidines into DNA of mammalian cells [11] has added another factor to the already complex picture, making it even more difficult to interpret cellular toxicity data based on the biochemical pharmacology of this agent. No rescue of normal hematopoietic stem cells from the toxicity induced by FUra was ever observed with dThd administered either as an i..v. bolus or as a prolonged infusion. This was also true for leukemic AKR and L1210 stem cells. We have tried different schedules and various doses of dThd, and although some caution is needed in the interpretation of these results, we conclude that, at least for the three cell lines examined, the inhibition of thymidylate synthetase is not the main mechanism of action of FUra.

When given alone as a prolonged infusion dThd was only moderately cytotoxic against AKR leukemia, and not at all against L1210 leukemia. This difference between these two cell lines, classified at T and B cells respectively, confirms the higher sensitivity of thymus-derived lymphocytes towards the growth inhibition of dThd [16].

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