Cancer Chemother Pharmacol (1981) 6: 121–125

Cancer Chemotherapy and Pharmacology

Effect of Concurrent Calcium Leucovorin Infusion on 5-Fluorouracil Cytotoxicity Against Murine L1210 Leukemia

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Summary. *We examined the effect of concurrent SC infusion of calcium leucovorin (LV) on the action of 5-fluorouracil (FUra) against mouse L1210 leukemia implanted either SC or IP. Mice bearing the SC tumor treated with FUra (100 mg/kg, IP, day 1) plus infusion with either LV (11.5 mg · kg^{-1} · day^{-1}, days 1–4), or 0.9% NaCl (days 1–4) resulted in an identical increase in median lifespan (ILS) of 28%. Similar experiments with FUra (100 mg/kg) plus LV infusion (115 mg · kg^{-1} · day^{-1}) or FUra (200 mg/kg) plus LV infusion (115 mg · kg^{-1} · day^{-1}) resulted in 50% and 59% ILS, respectively, which were not different from that obtained with the same doses of FUra plus 0.9% NaCl infusion. Mice bearing the IP tumor treated with FUra (100 mg/kg, IP, day 1) plus infusion with either LV (11.5 mg · kg^{-1} · day^{-1}, days 1–4) or 0.9% NaCl (days 1–4) had an identical 56% ILS. Similar experiments with FUra (100 mg/kg) plus LV infusion (115 mg · kg^{-1} · day^{-1}) or FUra (200 mg/kg) plus LV infusion (115 mg · kg^{-1} · day^{-1}) resulted in 67% and 94% ILS, respectively, which were not different from those obtained with the same doses of FUra plus 0.9% NaCl infusion. Treatment of normal mice with FUra (200 mg/kg, IP, day 0) plus LV infusion (115 mg · kg^{-1} · day^{-1}, days 0–3) was no more toxic than FUra plus 0.9% NaCl infusion, judging by similar transient decreases in body weight and no mortality. The data indicate that concurrent infusion with the LV failed to enhance the action of FUra against the mouse L1210 leukemia.*

Introduction

The major growth-inhibitory effect of 5-fluorouracil (FUra) has been associated with its anabolite 5-fluorodeoxyuridine 5′-monophosphate (FdUMP), which with 5,10-methylenetetrahydrofolate (5,10-MeTHF) forms a ternary covalent complex with thymidylate synthetase (TS), inactivates the enzyme, and thereby blocks DNA synthesis [15, 20]. Despite its covalent bonds, the ternary complex slowly dissociates, an effect which is decreased by increasing levels of 5,10-MeTHF [14, 15, 17, 19, 20]. The formation and disappearance of FdUMP in tumor and sensitive host tissues is an important determinant of responsiveness to FUra [1, 3, 14, 18]. In addition, FUra affects ribosomal RNA maturation as a consequence of 5-fluorouridine 5′-triphosphate incorporation [12].

In mouse L1210 leukemia cells in culture, which are first depleted of reduced folates, both the cytotoxicity of 5-fluorodeoxyuridine (FdUrd) and the intracellular amounts of the FdUMP : 5,10-MeTHF : TS complex are greatly reduced [21]. Furthermore, the potency of FUra against cultured tumor cells is increased by the addition of leucovorin (calcium salt of dl,L-5-formyltetrahydrofolate, LV) [6, 22]. We hypothesized, therefore, that high intracellular levels of 5,10-MeTHF, which presumably could be attained by administration of LV, might increase the therapeutic efficacy of FUra by enhancing both the formation and the stability of the ternary complex in tumor as against sensitive host tissues. This report describes an attempt to enhance the efficacy of FUra against the mouse L1210 leukemia by concurrent SC infusion of LV.

Materials and Methods

Drugs. FUra, LV, and MTX were obtained from Dr V. L. Narayanan, Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. FUra or MTX solutions were prepared in 2% $NaHCO_3$ just prior to use, at final concentrations which permitted injections of 0.01 ml/g and 0.02 ml/g mouse body weight, respectively. LV was prepared in 0.9% NaCl just prior to use. Sodium pentobarbital was obtained from Veterinary Laboratories, Inc., Lenaxa, Kansas, USA.

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0344-5704/81/0006/0121/$ 01.00

Animals and Tumors. Male C57BL/6 × DBA/2 F_1 (hereafter called $B6D2F_1$) mice weighing 20–23 g, and male DBA/2 mice weighing 18–20 g were obtained from Harlan-Sprague-Dawley Laboratories (Madison, Wisconsin, USA). The mouse L1210 lymphocytic leukemia in the ascites form, obtained from Mason Research Institute, Worcester, Masachusetts, USA, was maintained as previously described [14].

Continuous SC Infusion. The technique for continuous SC infusion of LV was modified from methods described previously [11, 16]. Mice were anesthetized with a single SC injection of sodium pentobarbital, 75 mg/kg. With the aid of an 18 gauge trocar needle, vinyl tubing (inner diameter 0.020 in., outer diameter 0.036 in., Becton-Dickinson, Ruthersford, NJ, USA), was inserted SC in the upper back of a mouse. The tubing was then taped to a small plastic splint, which was protected by a cylindrical plastic shield. The bottom of the shield was fixed to the back of the mouse with two ties of no. 00 silk surgical sutures (Ethicon, Somerville, NJ, USA). The shield was then anchored by wire to an overhead stand so that it was approximately perpendicular to the cage floor. The cannula was then connected to a 10-ml plastic syringe containing the infusion solution and the syringe was placed on a Harvard 940 infusion pump (Harvard Apparatus Corp., Dover, MA, USA). During the experiments, the animals were given food and water ad libitum. The cages were covered with a plastic cover with a slit cut in the center to allow the animals mobility. Syringes containing LV were covered with aluminum foil to protect against light exposure. Prior to administration, all solutions were sterilized by passage through 0.22 μ Millipore filters. In all experiments, animals not treated with drug(s) were given an equivalent volume of solvent.

Effect of LV Infusion on the Lethal Toxicity of Methotrexate (MTX) in Mice. $B6D2F_1$ mice were given a single IP injection of MTX (800 mg/kg). Twenty-four hours later the mice, which were randomly distributed into groups of eight, were each given a single IP injection of LV (1.35 mg/kg); SC infusion of LV (11.5 $mg \cdot kg^{-1} \cdot day^{-1}$) was then begun and continued for 5 days, after which the mice were removed from the infusion apparatus. Survivors were determined daily for 30 days.

The loading dose of LV (1.35 mg/kg) and the infusion rate (11.5 $mg \cdot kg^{-1} \cdot day^{-1}$; 2.97 ml/day) were calculated to produce a steady-state plasma concentration of LV of 2.5 μ*M*, based on the assumption of a plasma half-life for LV of 0.5 h in mice [2, 9].

Effect of Chemotherapy on Mice Bearing L1210 Leukemia Implanted SC. All chemotherapy experiments were routinely begun between 8 and 9 a.m. $B6D2F_1$ mice were given a single SC injection of 1×10^6 L1210 ascites cells. Twenty-four hours after tumor inoculation, the mice were randomly distributed into groups of eight each, placed on the infusion apparatus, and given a single IP injection of LV (1.35 or 13.5 mg/kg, as indicated), followed in 0.5 h by a single IP injection of FUra (100 or 200 mg/kg). LV infusion (11.5 or 115 $mg \cdot kg^{-1} \cdot day^{-1}$; 2.97 ml/day) was then begun and continued for 72 h. The doses of LV (13.5 mg/kg, IP, and 115 $mg \cdot kg^{-1} \cdot day^{-1}$) were calculated to produce a steady-state plasma concentration of LV of 25 μ*M* [2, 9]. Animals were observed for individual days of death and the median survival time (MST) and increase in median lifespan (%ILS) were determined as previously described [8].

Effect of Chemotherapy on Mice Bearing L1210 Leukemia Implanted IP. $B6D2F_1$ mice were each given a single IP injection of 1×10^6 L1210 ascites cells. Twenty-four hours after tumor inoculation the mice were randomly divided into groups of eight each and placed on the infusion apparatus. Mice were then treated with either FUra or FUra plus infusion with LV, as described above for mice inoculated SC with L1210 ascites cells. For mice bearing the L1210 tumor implanted either IP or SC, infusion with LV alone did not alter the MST.

Effect of Concurrent LV Infusion on FUra Toxicity. Normal $B6D2F_1$ mice in groups of six each were placed on the infusion apparatus. They were given a single IP injection of LV (13.5 mg/kg), followed in 0.5 h by a single IP injection of FUra (200 mg/kg) and a LV infusion (115 $mg \cdot kg^{-1} \cdot day^{-1}$; 2.97 ml/day) was begun and continued for 72 h. Mean body weights, as percent of pretreatment weights, and survivors were determined for 30 days after FUra.

Results

Effect of Delayed LV Infusion on the Lethal Toxicity of MTX in Mice

The lethal toxicity of MTX can be prevented by subsequent administration of LV, which restores MTX-depleted pools of reduced folates [10]. To test the ability of the LV infusion technique to supply essential intracellular reduced folates, we examined the effect of delayed LV infusion on the lethal toxicity of a high dose of MTX. A single injection of MTX (800 mg/kg) followed in 24 h by a 5-day infusion of either 0.9% NaCl or LV (11.5 $mg \cdot kg^{-1} \cdot day^{-1}$) resulted in three of eight and eight of eight survivors at day 30 respectively (data not shown).

Effect of Concurrent LV Infusion on the Antitumor Activity of FUra Against Mice Bearing L1210 Leukemia Implanted SC

Treatment of mice bearing the L1210 SC tumor with FUra (100 mg/kg) plus concurrent infusion with either LV (11.5 $mg \cdot kg^{-1} \cdot day^{-1}$) or 0.9% NaCl both resulted in a 28% ILS (Table 1, Expt 1). Similarly FUra (100 mg/kg) plus LV (115 $mg \cdot kg^{-1} \cdot day^{-1}$) (Expt 2) or FUra (200 mg/kg) plus LV (115 $mg \cdot kg^{-1} \cdot day^{-1}$) (Expt 3) also resulted in an ILS which was no different than that obtained with FUra (100 or 200 mg/kg, as indicated) plus 0.9% NaCl infusion.

Effect of Concurrent LV Infusion on the Antitumor Activity of FUra Against Mice Bearing L1210 Leukemia Implanted IP

To exclude the possibility that the failure of LV to enhance the action of FUra against the L1210 tumor implanted SC might be related to the impaired access of reduced folate to the tumor, experiments were also performed with L1210 ascites cells implanted IP.

Table 1. Effect of FUra plus concurrent LV infusion on the survival times of B6D2F$_1$ mice bearing L1210 leukemia implanted SC[a]

Experiment	Group	Treatment				
		Day 1 Drug		Days 1–4 Drug		
		LV Dose (mg/kg)	FUra Dose (mg/kg)	LV Dose (mg/kg/day)	MST (range)	ILS (%)
1	1	1.35	–	11.5	9.0 (8–11)	–
	2	–	100	–	11.5 (10–13)	28
	3	1.35	100	11.5	11.5 (11–13)	28
2	1	13.5	–	115	8.0 (8)	–
	2	–	100	–	12.0 (11–14)	50
	3	13.5	100	115	12.0 (11–12)	50
3	1	13.5	–	115	8.5 (8– 9)	–
	2	–	200	–	14.0 (12–14)	65
	3	13.5	200	115	13.5 (12–14)	59

[a] Groups of eight mice each were given SC inoculations of 1×10^6 L1210 ascites cells on day 0. Twenty-four hours later they were given a single IP injection of LV followed in 0.5 h by a single IP injection of FUra, and a LV infusion was begun and continued for 3 days. Animals were observed for individual day of death and MST determined as described in *Materials and Methods* section

Table 2. Effect of FUra plus concurrent LV infusion on the survival times of B6D2F$_1$ mice bearing L1210 leukemia implanted IP[a]

Experiment	Group	Treatment				
		Day 1 Drug		Days 1–4 Drug		
		LV Dose (mg/kg)	FUra Dose (mg/kg)	LV Dose (mg/kg/day)	MST (range)	ILS (%)
1	1	1.35	–	11.5	9.0 (8–10)	–
	2	–	100	–	14.0 (11–15)	56
	3	1.35	100	11.5	14.0 (11–15)	56
2	1	13.5	–	115	7.5 (7– 9)	–
	2	–	100	–	12.5 (7–16)	67
	3	13.5	100	115	12.5 (11–15)	67
3	1	13.5	–	115	8.0 (7– 9)	–
	2	–	200	–	15.5 (14–17)	94
	3	13.5	200	115	15.5 (14–17)	94

[a] Groups of eight mice each were given IP inoculations of 1×10^6 L1210 ascites cells on day 0. Twenty-four hours later they were given a single IP injection of either LV or 0.9% NaCl followed in 0.5 h by a single IP injection of FUra, and a LV infusion was begun and continued for 3 days. Animals were observed for individual day of death and MST determined as described in *Materials and Methods* section

Mice inoculated IP with L1210 ascites cells and treated with FUra (100 mg/kg) plus concurrent infusion with either LV (11.5 mg · kg^{-1} · day^{-1}) or 0.9% NaCl both resulted in a 56% ILS (Table 2, Expt 1). Similarly, FUra (100 mg/kg) plus LV (115 mg · kg^{-1} · day^{-1}) (Expt 2) or FUra (200 mg/kg) plus LV (115 mg · kg^{-1} · day^{-1}) (Expt 3) also resulted in an ILS which was no different from that obtained with FUra plus 0.9% NaCl infusion.

Effect of Concurrent LV Infusion on FUra Toxicity

LV infusion did not appear to increase the lethal toxicity of FUra (which might have resulted in early deaths), since in each experiment tumor-bearing mice treated with either FUra + LV or FUra + 0.9% NaCl died over approximately the same range of days (Tables 1 and 2). Nevertheless, it was important to

Table 3. Effect of concurrent LV infusion on FUra toxicity[a]

Group	Treatment			Mean body weight (%)		Day 30 survivors/ total treated
	Day 0 Drug		Days 0–3 Drug	Day 4	Day 7	
	LV Dose (mg/kg)	FUra Dose (mg/kg)	LV Dose (mg/kg/day)			
1	–	–	–	99	106	6/6
2	13.5	–	115	95	101	6/6
3	–	200	–	88	100	6/6
4	13.5	200	115	85	95	6/6

[a] Groups of six mice each were given a single IP injection of LV followed in 0.5 h by a single IP injection of FUra, and a LV infusion was begun and continued for 3 days. Mean body weight, as a percentage of day 0 total body weight, was determined on days 4 and 7 after FUra, and survivors were monitored daily for 30 days

exclude the possibility that the failure of LV to increase the efficacy of FUra may have been related to LV increasing the toxicity of FUra. When non-tumor mice were given a single IP injection of FUra (200 mg/kg), which is the optimal single IP dose of FUra [7], then concurrent infusion with LV (115 $mg \cdot kg^{-1} \cdot day^{-1}$) did not significantly increase FUra toxicity as measured by either changes in body weight at days 4 and 7, or the number of survivors at day 30, after FUra (Table 3).

Discussion

Using a newly modified technique for long-term continuous SC infusion in unrestrained mice, we have shown that concurrent infusion with LV does not enhance the action of a single IP injection of FUra against the mouse L1210 leukemia implanted either SC or IP. We have previously shown the persistence of free intracellular FdUMP after a single IP injection to mice bearing the L1210 solid or ascitic tumors. Furthermore, despite the presence of high levels of free FdUMP the incorporation of ^{3}H-deoxyuridine into tumor DNA, which is mediated via TS, was never completely inhibited [1, 14]. Therefore, in the experiments reported in this paper, the failure of LV infusion to increase the antitumor action of FUra, for example by increasing the formation of the FdUMP : 5,10-MeTHF : TS complex, was not due to a lack of either FdUMP or TS. Similarly, there is no reason to infer that LV infusion enhanced the stability of the ternary complex [4, 17] which was formed initially, since stabilizing the complex would presumably also have increased the action of FUra. Furthermore, the possibility that LV simultaneously increased the antitumor action of FUra and its toxicity to the host so that there was no net therapeutic gain is ruled out, since LV infusion did not increase the toxicity of FUra to normal non-tumor-bearing mice, as indicated by body weight changes and 30 day survivors.

The doses of LV which were infused in our experiments, i.e., 11.5 and 115 $mg \cdot kg^{-1} \cdot day^{-1}$, were calculated to produce plasma concentrations of LV of 2.5 and 25 μM, respectively. LV at 0.1 μM was optimal for growth of L1210 cells in culture [21]. Presumably, plasma LV concentrations of 25–250 times that required for optimal growth of L1210 cells would be expected to increase tumor intracellular levels of 5.10-MeTHF. Studies with cell cultures have indicated that the action of either FUra or FdUrd could be enhanced by concentrations of LV which were within the range of plasma levels of LV which we attempted to attain [5, 6, 21, 22].

Our study of FUra plus LV against the L1210 tumor does not exclude the possibility that other tumors might show an enhanced responsiveness to FUra plus LV. In a series of human colorectal tumors maintained in nude mice, measurement of TS activity after incubation of tumor cytosols with FdUMP, with or without added 5,10-MeTHF, showed that in nonresponsive tumors maximum TS inhibition occurred only with added 5,10-MeTHF. This suggests that 5,10-MeTHF availability may prove important in the formation of the ternary complex when high concentrations of FdUMP are present only for a short time [13]. It should be noted that concurrent SC infusion of LV (11.5 $mg \cdot kg^{-1} \cdot day^{-1}$ for 3 days) failed to increase the action of a single SC injection of FUra (100 mg/kg, IP) against rats inoculated IP with Walker 256 ascites cells (P. Klubes et al., unpublished work). The Walker 256 tumor is unresponsive to FUra despite initial high levels of FdUMP, which disappear within 24 h after FUra [14].

Acknowledgements. This work was supported by grant CH-160 from the American Cancer Society and grant CA 17601 from the National Cancer Institute, DHHS. We thank Mr Willie T. Wynn for his expert technical assistance. The helpful discussion with Dr Gerald B. Grindey for the infusion technique is gratefully acknowledged.

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Received March 28/Accepted July 10, 1981