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BIOCHEMICAL MODULATION OF 5-FLUOROURACIL WITH LEUCOVORIN OR DELAYED URIDINE RESCUE

CORRELATION OF ANTITUMOR ACTIVITY WITH DOSAGE AND FUra INCORPORATION INTO RNA

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Abstract—Two strategies for modulation of 5-fluorouracil (FUra) activity were compared *in vivo* in advanced murine $CD8F_1$ breast tumors with regard to three parameters: chemotherapeutic activity, inhibition of thymidylate synthase (TSase) activity, and incorporation of FUra into RNA, (FU)RNA. Inhibition of TSase by FUra was modulated by leucovorin (LV), and the incorporation of FUra into RNA was increased by the administration of otherwise lethal doses of FUra followed by uridine "rescue". Thymidylate synthase activity was inhibited substantially (49%) by low-dose FUra at 25 mg/kg, but was not further enhanced (48%) by repeated daily treatments at the same dose ($FUra_{25\times4}$). Inhibition of TSase was somewhat enhanced (55%) by the addition of LV to $FUra_{25\times4}$, and a greater therapeutic effect was obtained with $FUra_{25\times4}$ + LV over $FUra_{25\times4}$ alone. FUra as a single agent at the maximum tolerated weekly dose of 100 mg/kg inhibited TSase activity 66–73%. This inhibition was further enhanced slightly by the addition of LV (71–82%), and its therapeutic efficacy was greater than with $FUra_{25\times4}$ with or without LV. However, in contrast to low dose $FUra_{25\times4}$, the antitumor effect of $FUra_{100}$ was not enhanced by LV. (FU)RNA increased with FUra dose from 0.4 ($FUra_{25}$) to 2.2 nmol/mg DNA ($FUra_{100}$). At a very-high-dose of FUra (200–225 mg/kg) followed by uridine "rescue", TSase inhibition was not further enhanced, but both (FU)RNA (4.8 nmol/mg DNA) and the therapeutic efficacy were increased. Since TSase could not be further inhibited at doses above $FUra_{100}$, the increased chemotherapeutic efficacy correlated with increased (FU)RNA.

5-Fluorouracil (FUra§) causes cell death through at least two mechanisms, inhibition of thymidylate synthase (TSase) by the metabolite FdUMP, and incorporation of FUra (after conversion to FUrdTP) into RNA, (FU)RNA [1]. Although these mechanisms are not mutually exclusive, it is important to determine which pathways can be modulated to further improve the therapeutic activity of FUra.

In the present study, antitumor activity was measured in advanced murine $CD8F_1$ breast tumors after 3 weekly treatments of FUra using either leucovorin (LV) to enhance TSase inhibition by increased formation or stabilization of the ternary FdUMP–TSase–CH_2H_4PteGlu complex, or "very-high-dose" FUra in conjunction with uridine "rescue" [2] to enhance incorporation into RNA. Experiments were performed with six groups of tumor-bearing animals including a saline-treated control group, two groups on different schedules of FUra at equal weekly dose intensities ("low-dose" FUra, 25 mg/kg q.d. × 4/week; "high-dose" FUra, 100 mg/kg/week), both with and without the addition of LV, and the final group which received "very-high-dose" FUra at 200–225 mg/kg/week followed by delayed uridine "rescue". Biochemical experiments also were performed at the same dose levels to observe the effects of various doses and regimens on inhibition of TSase and on the incorporation of FUra into RNA.

MATERIALS AND METHODS

Materials. [6-^{3}H]Fluorouracil (20 Ci/mmol) and [5-^{3}H]deoxyuridine monophosphate (20 Ci/mmol) were purchased from Moravek. FUra, leucovorin and uridine were obtained from the Sigma Chemical Co. Leucovorin was also obtained from the Department of Health, Education and Welfare, USPHS, National Cancer Institute. These agents were dissolved in 0.85% NaCl immediately before use, and administered i.p. with the desired dose contained in 0.1 mL/10 g of mouse body weight.

Animals. First generation syngeneic transplants of $CD8F_1$ breast tumors were obtained from a tumor cell brei made by pooling 3–4 spontaneous $CD8F_1$ murine mammary tumors [3, 4]. When the transplanted tumors were advanced in growth (3–4 weeks), the tumor-bearing mice were distributed among experimental groups so that mice carrying

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§ Abbreviations: FUra, 5-fluorouracil; Urd, uridine; LV, leucovorin (5-formyltetrahydrofolate); MTD, maximum tolerated dose (the dose that provides an LD_{10} over 4 weekly courses of treatment); TSase, thymidylate synthase (EC 2.1.1.45); (FU)RNA, FUra incorporated into RNA; and TKase, thymidine kinase.

tumors of approximately equal weight (averaging approximately 100 mg) were represented in each of four or six treatment groups (see Tables 1 and 2). Food and water were consumed *ad lib.* $FUra_{100}$ (100 mg/kg) is the weekly maximum tolerated dose (MTD) in the $CD8F_1$ murine tumor-host system. The dose (mg/kg) of FUra is indicated in subscript for each treatment group.

Tumor measurements. Two axes of the tumor (the longest axis, L, and the shortest axis, W) were measured with a Vernier caliper. Tumor weight was estimated according to the formula: tumor weight (mg) = L (mm) × W $(mm)^2/2$.

Biochemical studies. TSase and (FU)RNA were measured in tumors of separate groups of mice of the same age that received portions of the same tumor brei at the same time of transplant.

Measurement of "unbound" thymidylate synthase activity. Tumor tissues were excised, immediately frozen in liquid nitrogen and stored at −70° until the time of processing. Tumors (300–500 mg) were homogenized with a motor-driven teflon–glass homogenizer with 4 vol. of a solution of Tris–HCl (100 mM, pH 7.6), 2-mercaptoethanol (20 mM) and sodium fluoride (100 mM).

Other researchers have routinely added phosphatase inhibitors such as CMP, AMP [5, 6] or levamisole in the homogenization buffer. However, we obtained optimal activity with NaF alone (data not shown). In our hands, the addition of 1 mM levamisole provided no additional enhancement of TSase activity above that achieved with NaF alone, whereas the addition of 5 mM AMP or 15 mM CMP gave slight or substantial *apparent* inhibition of TSase activity, respectively, when included with NaF. Also, the addition of the protease inhibitor, phenylmethylsulfonyl fluoride (PMSF, 1 mM), to the homogenization buffer provided no enhancement of TSase activity.

Homogenates were centrifuged at 4° (100,000 *g*, 60 min or 12,000 *g*, 30 min) and the supernatant fractions were placed on ice. TSase activity was assayed by the release of tritium from [5-^{3}H]dUMP as described by Roberts [7]. The assay was conducted under conditions where activity was linear with time and added protein. The assay mixture (50 μL) contained [5-^{3}H]dUMP (10 μM, 1.0 Ci/mmol), $CH_2H_4PteGlu$ (100 μM) and 25 μL of protein. Protein was determined by the method of Lowry *et al.* [8].

Incorporation of precursors into RNA and DNA. [5-^{3}H]FUra (1.0 mCi/mmol) used to measure the time course of incorporation into nucleic acids was injected i.p. in saline. Tumor tissue was harvested at the indicated time after treatment and immediately frozen in liquid N_2. Tissues were homogenized in 10 mM Tris–HCl, 150 mM NaCl and 1 mM EDTA, pH 7.6. Sodium dodecyl sulfate was added to 0.5% and the homogenate was then sonicated for 30 sec, and digested with Pronase (20 μg/mL; predigested for 2 hr at 37°), for 60 min at 37°, and extracted with chloroform/isoamyl alcohol (24:1, v/v). Nucleic acids were precipitated with 10% trichloroacetic acid (TCA) to determine total incorporated radioactivity. Other samples were first treated with alkali (0.4 M NaOH, for 90 min at 37°) to determine alkali-stable acid-precipitable activity. Insoluble material was filtered onto GF-C filters, washed twice with ice-cold 5% TCA and then heated for 30 min at 50° in a scintillation vial with 1 mL of BCS scintillation fluid (Amersham)/1 M hyamine hydroxide in MeOH (ICN) (9:1, v/v). BCS fluid (10 mL) was added and the radioactivity was determined. The difference between the total and alkali-stable radioactivity was assumed to represent radioactivity in RNA. DNA was measured by the diphenylamine color reaction [9].

RESULTS

Therapeutic studies. In each of four separate experiments, $CD8F_1$ mice bearing advanced first passage $CD8F_1$ breast tumors were separated into six treatment groups of ten mice each. Each group received three courses of weekly treatment as follows: (1) saline-treated control; (2) "low-dose" FUra at 25 mg/kg q.d. × 4 ($FUra_{25\times4}$); (3) $FUra_{25\times4}$ + LV; (4) "high-dose" FUra at 100 mg/kg ($FUra_{100}$); (5) $FUra_{100}$ + LV; and (6) "very-high-dose" FUra at 200 or 225 mg/kg followed by uridine "rescue". Observations recorded 1 week after the last course of treatment in each experiment are presented in Table 1, and the statistical analysis of the pooled results of these four experiments is presented in Table 2.

FUra treatment of these $CD8F_1$ breast tumors produced statistically significant tumor growth inhibition, relative to saline-treated controls, at both the "low-dose" FUra (25 mg/kg q.d. × 4/week) and the "high dose" FUra (100 mg/kg/week) regimens. However, despite the fact that these two regimens were designed to deliver the same dose intensity of FUra, it is apparent that the schedule of administration markedly affected the results of treatment. The "high-dose" regimen (group 4) resulted in a mean tumor size of 710 mg, which was found to be significantly smaller than the mean tumor weight of 1531 mg obtained with the "low-dose" FUra regimen (group 2; $P < 0.001$).

The magnitude of the modulatory, enhancing effect of LV on the activity of FUra also appeared to be somewhat dependent upon the schedule of administration of FUra. Where LV was added to "low-dose" FUra (group 3) the mean tumor weight was 963 mg, which was significantly smaller than the 1531 mg obtained in mice treated with the same regimen of FUra without LV (group 2), $P < 0.001$. However, when LV was added to high dose FUra (group 5), the mean tumor weight (600 mg) did not prove to be significantly different compared to mice treated with "high-dose" FUra alone (710 mg, group 4).

Very-high-dose FUra (200 or 225 mg/kg/week) with uridine rescue produced the greatest degree of tumor growth inhibition of all the treatment groups in each of the four experiments. The mean tumor growth inhibition produced by the very-high-dose FUra regimen was found to be significantly greater than that produced by $FUra_{100}$ either alone (group 4) or with modulatory LV (group 5), $P < 0.02$.

Biochemical studies. The biochemical effects of LV addition to FUra were investigated initially by

Table 1. Comparative effects on body weight change, host toxicity and tumor growth inhibition after $FUra_{25\times4}$ or $FUra_{100}$, each with or without LV, or with $FUra_{200\text{-}225}$ followed by uridine "rescue"

Treatment**	Exp. 2352			Exp. 2353			Exp. 2357			Exp. 2351		
	Body wt change	Dead/ Total	Tumor wt (mg)	Body wt change	Dead/ Total	Tumor wt (mg)	Body wt change	Dead/ Total	Tumor wt (mg)	Body wt change	Dead/ Total	Tumor (mg)
1. Saline	−8	2/10	4436	+5	0/10	4432	−3	4/10	6414	+7	1/10	2752
2. $FUra_{25\times4}$	−13	1/10	1827	3	0/10	1323	0	0/10	1623	−6	0/10	1377
3. $FUra_{25\times4}$ + LV	−21	3/10	974	−4	0/10	870	−3	0/10	1137	+1	0/10	873
4. $FUra_{100}$	−1	0/10	736	+7	0/10	431	0	0/10	789	+5	0/10	885
5. $FUra_{100}$ + LV	0	1/10	816	0	0/10	580	−5	0/10	555	−6	0/10	468
6. $FUra_{200\text{ or }225}$ + UR rescue	−6	0/10	533	−2	1/9	340	−10	1/10	243	−1	0/10	345

*Three weekly courses of the indicated treatment were administered with a 1-week interval between courses. Subscripts = mg/kg. Observations were recorded 1 week after the last weekly course of treatment. Tumor size at initiation of treatment: Exp. 2351-120 mg; Exp. 2352-143 mg; Exp. 253-182 mg; Exp. 2357-180 mg. First-generation $CD8F_1$ breast tumors were used. $FUra_{25\times4}$: each weekly course consisted of FUra at 25 mg/kg q. d. × 4. $FUra_{25\times4}$ + LV: each weekly course consisted of FUra as described above with LV at 100 mg/kg administered 1 hr before, and 2 hr after each dose of FUra. $FUra_{100}$: each weekly course consisted of a single dose of FUra at 100 mg/kg. $FUra_{100}$ + LV: each weekly course consisted of FUra as described above with LV at 100 mg/kg administered at 1 hr before and again at 2, 18, and 26 hr after FUra. $FUra_{200\text{ or }225}$ + UR rescue: each weekly course consisted of a single dose of FUra at 200 or 225 mg/kg followed 2 hr later by UR at 1500 mg/kg with UR at 3500 mg/kg given at intervals of 3, 8 and 5 hr.

observing the time course of TSase activity using pooled tumor samples. As shown in Fig. 1, TSase activity was inhibited by 49% after the first of four daily $FUra_{25}$ treatments. This inhibition did not appear to be cumulative since TSase was inhibited to the same degree (48%) after two further daily injections. TSase activity gradually returned to control levels after the final injection. Addition of LV to low-dose $FUra_{25\times4}$ showed a trend toward enhancing the inhibition of TSase. After a single injection of $FUra_{25}$ + LV, TSase was inhibited 55%. This level of inhibition was maintained until the last injection, and TSase activity then gradually returned to control levels. However, at high-dose $FUra_{100}$, a single bolus dose was sufficient to inhibit TSase 66% and this inhibition was maintained over at least 4 days when TSase was inhibited 73%. Addition of LV to high-dose FUra showed a similar trend to that at low-dose $FUra_{25\times4}$ in that the degree of inhibition appeared to be enhanced slightly by the addition of LV. However, TSase activities of separate tumors of the same transplant group can vary, with standard deviations of 15% being common. Therefore, while a trend toward enhancement of TSase inhibition by LV was observed, it appeared not to be statistically significant.

In another experiment (Fig. 2A), TSase inhibition was substantially inhibited (75%) by treatment with $FUra_{25}$, and was further inhibited (84%) after treatment with high-dose $FUra_{100}$, but was not further significantly enhanced by treatment at still higher FUra doses (i.e. $FUra_{115}$; $FUra_{225}$ + Urd). Although the very-high-dose of $FUra_{225}$ with uridine rescue showed the best chemotherapeutic activity, the inhibition of TSase by $FUra_{225}$ was not significantly greater than that produced by $FUra_{100}$ (Fig. 2A) and further increases in dose beyond $FUra_{100}$ did not provide further decreases in TSase activity.

The incorporation of FUra into RNA after treatment with increasing doses of FUra was also measured in animals transplanted with the same tumor brei in the same experiment (Fig. 2B). (FU)RNA increased in a linear fashion from 0.4 nmol/mg DNA after one treatment with $FUra_{25}$ to 2.2 nmol/mg DNA at $FUra_{100}$. The highest amount of (FU)RNA (4.8 nmol/mg DNA) was found at the very-high-dose of $FUra_{225}$.

In another experiment, the time courses of both TSase inhibition and (FU)RNA accumulation were investigated at either $FUra_{100}$, or $FUra_{225}$ followed by two of the scheduled Urd "rescue" doses (Urd_{1500} and Urd_{3500}). The results are shown in Fig. 3. TSase activity was inhibited (Fig. 3B) over the duration of the experiment, and there were no significant differences between the TSase activities of the $FUra_{100}$ group and the $FUra_{225}$ group. However, even after receiving two doses of Urd, the (FU)RNA content of the $FUra_{225}$ group was significantly higher than that of the $FUra_{100}$ group (Fig. 3A).

Since there were no significant differences between the TSase activities in tumors of $FUra_{100}$-treated and $Fura_{225}$-treated groups, and (FU)RNA was significantly higher in the tumors of $FUra_{225}$-treated animals, and the greatest antitumor activity was observed in the $FUra_{225}$-treated groups, the cor-

Table 2. Statistical analysis of pooled tumor growth inhibition values obtained after treatment with $FUra_{25\times4}$ or $FUra_{100}$, each with or without leucovorin, or with $FUra_{200\text{-}225}$ followed by uridine "rescue"*

Treatment	Body weight change (g)	Dead/ total (N)	Tumor weight (mg)
1. Saline	+1	7/40	4417 ± 328
2. $FUra_{25\times4}$	−4	1/40	1531 ± 124†
3. $FUra_{25\times4}$ + LV	−6	3/40	963 ± 85†
4. $FUra_{100}$	+3	0/40	710 ± 51†
5. $FUra_{100}$ + LV	−1	1/40	600 ± 82
6. $FUra_{200}$ or $_{225}$ + Urd "rescue"	−5	2/39	370 ± 46†

* Treatment groups are described in the legend to Table 1. The tumor weight values are means ± SEM. N = total number of tumor-bearing animals evaluated.
† Significantly different ($P < 0.05$) compared to the preceding group.

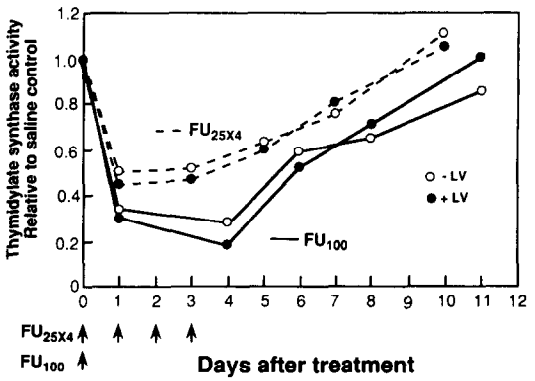

Fig. 1. Effect of leucovorin (LV) treatment on FUra inhibition of tumor thymidylate synthase *in vivo* over time. Mice were treated with $FU_{25\times4}$ ± LV or FU_{100} ± LV. Subscripts = mg/kg. Activity was measured prior to scheduled FUra treatment on the day indicated. Tumors from three mice were pooled, homogenates were prepared, and thymidylate synthase activity was determined. LV treatment: LV_{100}-2 hr→$FUra_x$-4.5 hr→LV_{100}-16.5 hr→LV_{100}-6.5 hr→LV_{100}.

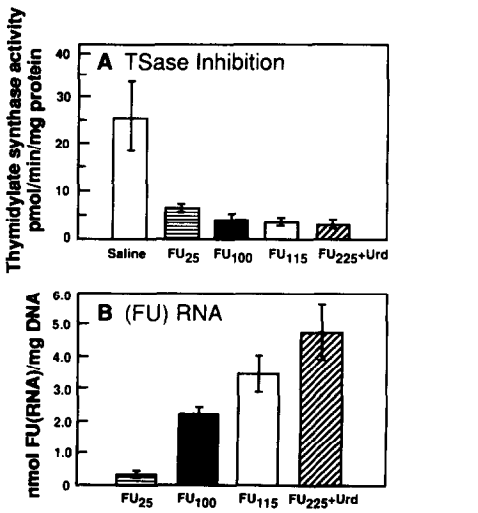

Fig. 2. Comparison of (A) the effect of TSase inhibition (N = 5) and (B) incorporation of FUra into RNA (N = 4) with increasing dose of FUra. The schedule for uridine "rescue" was FU_{225}-2 hr→Urd_{1500}. Tumors were excised and frozen 5 hr after FUra treatment. Values are means ± SD.

relation with enhanced antitumor activity was with the level of (FU)RNA which, in turn, correlated with FUra dosage.

DISCUSSION

The schedule and dose of leucovorin for *in vivo* studies have been reviewed recently by Mini and coworkers [10]. In prior studies by our group [11] leucovorin was administered i.p. at 500 mg/kg at −1, +4, +21 and +28 hr with FUra at 100 mg/mL. This dose and schedule of LV were found to be effective in enhancing the activity of FUra (i.e. occasionally synergistic but with increased host toxicity). Other researchers [12, 13] reported enhanced therapy in tumor-bearing mice with LV administered (50 or 100 mg/kg) at −1 and 0 hr in combination with FUra at 100 mg/kg. For this study, the 100 mg/kg dose of leucovorin was employed and was found to produce an enhanced antitumor effect in combination with FUra at 25 mg/kg. The schedule of LV administration was similar to that utilized previously [11].

In the $CD8F_1$ mammary tumor model, the dose of FUra appeared to be the key factor with regard to both of the two biochemical mechanisms of FUra action studied here and also the attendant therapeutic effects. Greater incorporation of FUra into RNA and greater antitumor activity were seen with greater doses of FUra. The duration of TSase inhibition was not increased by LV + FUra over that of high-dose FUra, and once TSase was inhibited to a nearly maximal extent (i.e. at the MTD of $FUra_{100}$), subsequent improvement in therapeutic activity was dependent upon other mechanisms of which the increased incorporation of FUra into RNA appeared to be of great importance. This increase of (FU)RNA can be accomplished by several strategies such

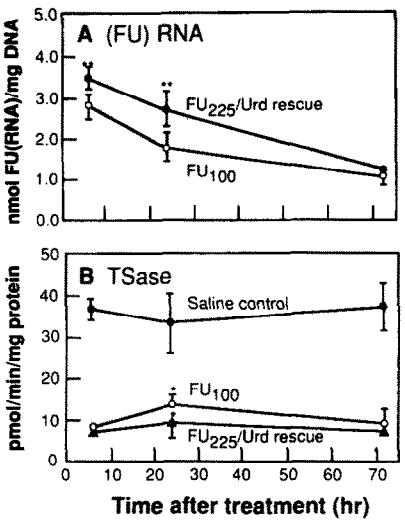

Fig. 3. Time course of (A) (FU)RNA and (B) thymidylate synthase inhibition after treatment with FU_{100} or with FU_{225} followed by uridine "rescue". Uridine schedule: FUra-2 hr → Urd_{1500}-2.5 hr → Urd_{3500}-17 hr → Urd_{3500}-8 hr → Urd_{3500}. Key: (*) not significant; and (**) significantly different from FU_{100} ($P < 0.05$).

as using biochemical modulators of FUra [*N*-(phosphonacetyl)-L-aspartate (PALA), methotrexate (MTX), 6-methylmercaptopurine riboside (MMPR)] or "rescue" of very-high-dose FUra with Urd [1].

These results are consistent with previous literature reports that stress the importance of (FU)RNA to the cytotoxicity of FUra. The recent review of FUra by Parker and Cheng [14] emphasized the therapeutic importance of (FU)RNA, and an earlier review by Valeriote and Santelli [15] stated that both DNA- and RNA-directed actions of FUra contribute to the overall cytotoxicity. Chabner [16] has stated that any prospective mechanism and therapy study of FUra should include measurements of both TSase activity and (FU)RNA. However, the relatively large number of reports attributing optimum therapeutic activity to the LV + FUra combination only report measurement of TSase activity; (FU)RNA has been neglected.

Our results reaffirm that the dose of FUra is an important determinant of the effect of FUra on its key biochemical targets, TSase activity and (FU)RNA; namely, the greater the dose, the greater the (FU)RNA effect, and the greater the antitumor activity. A review of pertinent literature over the past 15 years reveals identical findings. Thus, where both biochemical targets of FUra have been measured in the same therapeutic study, *low*-dose fluoropyrimidine resulted in cytotoxicity that was prevented by thymidine, whereas *high*-dose fluoropyrimidine produced growth-inhibition refractory to thymidine [17–22]. For example, Hakala and coworkers [19] reported that "cells were found to fall into two categories, those for which dTMP synthetase is growth-limiting at relatively *low* FUra concentration, and those for which it is not . . ." and "at '*high*' FUra concentrations the incorporation into RNA became growth-limiting for *all* cells".

Despite the fact that high-dose $FUra_{100}$ and low-dose $FUra_{25\times4}$ were administered in our studies at equal weekly-dose intensities, the difference in their schedule of administration also made a difference in the therapeutic result: $FUra_{100}$ produced superior therapeutic activity over $FUra_{25\times4}$.

In addition, other effects of high-dose FUra may overshadow any benefit of increased TSase inhibition by FUra, with or without LV. This is also consistent with reports in which a high-dose of either FdUrd alone [23] or FUra alone [24] produced the same degree of cell kill as a FUra–LV combination.

The lowest level of FUra used in the present study was 25 mg/kg. At this dose and also at $FUra_{100}$ there appeared to be a trend toward enhanced inhibition of TSase by leucovorin. However, even if statistical significance could be demonstrated, the magnitude of this enhancement is less than 5–10%.

Enhancement of the TSase inhibitory effect of FUra by LV has been demonstrated in *in vitro* [23, 24] and *in vivo* biochemical studies [25]. However, in these studies suboptimal doses of FUra were used in order to demonstrate the enhanced effect of LV treatment. Although similar low doses would not be used to treat cancer in the clinic, where drugs are typically escalated to limiting toxicity, these suboptimal doses of FUra have been referred to as "therapeutic" doses because cell growth inhibition does occur at these lower doses. It should be noted that higher doses of FUra alone produced equal or greater therapeutic effects than the combination of LV + FUra in several of these studies [19, 20, 24]. A suboptimal dose of FUra was employed in these studies only to magnify the biochemical interrelationships of LV + FUra in the formation of the ternary complex with TSase. These interrelationships are valid observations, but it may be misleading to consider exogenous LV necessary for the optimal therapeutic activity of FUra at clinical doses of FUra.

Although, all the data in our literature review and in the extensive review of Parker and Cheng [14] as well as our own results indicate that *high*-dose FUra can produce its effects by at least the two mechanisms of thymidylate synthase inhibition and incorporation of FUra into RNA, in a clinical regimen where FUra is being administered in a combination at relatively low dose it is likely that TSase inhibition is the main effect of FUra and it is possible that a subset of these patients could benefit from exogenous leucovorin. There is clinical evidence which suggests that leucovorin may be more effective against cancers with low levels of thymidylate synthase activity since an inverse relationship between TSase activity and drug (FUra + leucovorin) response was reported [26].

The reporting of only one parameter [e.g. TSase activity and not (FU)RNA] also can be misleading. For example, Fig. 1 indicates that $FUra_{100}$ with or without LV produces much stronger inhibition of TSase than low-dose $FUra_{25}$. Since the therapeutic results clearly demonstrated that $FUra_{100}$ gives antitumor activity greater than $FUra_{25\times4}$, in the absence of (FU)RNA data one interpretation would be that the biochemical basis for this greater therapeutic activity is that TSase is more strongly

inhibited at the higher dose. Yet an examination of both the TSase and (FU)RNA data (Fig. 2) provides the alternative and appropriate explanation that (FU)RNA adds a complementary effect to the already pronounced TSase inhibition.

Over recent years the importance of (FU)RNA has been recognized, and the potential effects of (FU)RNA are beginning to be investigated [27–31].

One contributory effect due to (FU)RNA appears to be the loss of thymidine kinase (TKase) [32]. Other short half-life, proliferation-related enzymes such as ornithine decarboxylase [32] and/or certain regulatory proteins (transcription factors) which are coded for by short half-life mRNAs may also be preferentially affected by (FU)RNA. These additional effects may also contribute to the cellular injury produced by the TSase block by FdUMP; however, the (FU)RNA-related loss of TKase means that both the salvage (i.e. TKase) and *de novo* (i.e. TSase) pathways producing thymidylate for DNA synthesis are blocked simultaneously. On this basis alone, it is not surprising that the greater doses of FUra, which produce a greater amount of (FU)RNA, lead to greater antitumor activity. In addition, the dose of FUra need not necessarily be raised beyond the MTD of FUra alone, as there are a number of biochemical modulators (PALA, MTX, MMPR, Urd rescue) that can produce a therapeutically important increase in (FU)RNA [1].

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REFERENCES

1. Martin DS, Purine and pyrimidine biochemistry, and some relevant clinical and preclinical cancer chemotherapy research. In: *Metabolism and Action of Anti-Cancer Drugs* (Eds. Powis G and Prough RA), pp. 91–140. Taylor & Francis, London 1987.
2. Martin DS, Stolfi RL, Sawyer RC, Spiegelman S and Young CW, High-dose 5-fluorouracil with delayed uridine "rescue" in mice. *Cancer Res* **42**: 3964–3970, 1982.
3. Stolfi RL, Martin DS and Fugmann RA, Spontaneous murine mammary adenocarcinoma: Model system for evaluation of combined methods of therapy. *Cancer Chemother Rep, Part 1* **55**: 239–251, 1971.
4. Martin DS, Fugmann RA, Stolfi RL and Hayworth PE, Solid tumor animal model therapeutically predictive for human breast cancer. *Cancer Chemother Rep, Part 2* **5**: 89–109, 1975.
5. Houghton JA, Radparvar S, Torrance PM, Williams LG and Houghton PJ, Determination of thymidylate synthase activity in colon tumor tissues after treatment with 5-fluorouracil. *Biochem Pharmacol* **36**: 1285–1289, 1987.
6. Spears CP, Shahinian AH, Moran RG, Heidelberger C and Corbett TH, *In vivo* kinetics of thymidylate synthase inhibition in 5-fluorouracil-sensitive and -resistant murine colon adenocarcinomas. *Cancer Res* **42**: 450–456, 1982.
7. Roberts D, An isotopic assay for thymidylate synthase. *Biochemistry* **5**: 3547–3548, 1966.
8. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
9. Burton K, A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem J* **62**: 315–323, 1956.
10. Mini E, Trave F, Rustum YM and Bertino JR, Enhancement of the antitumor effects of 5-fluorouracil by folinic acid. *Pharmacol Ther* **47**: 1–19, 1990.
11. Martin DS, Stolfi RL and Colofiore JR, Failure of high-dose leucovorin to improve therapy with the maximally tolerated dose of 5-fluorouracil: A murine study with clinical relevance? *J Natl Cancer Inst* **80**: 496–501, 1988.
12. Nadel JC, van Groeningen CJ, Pinedo HM and Peters GJ, Schedule-dependency of *in vivo* modulation of 5-fluorouracil by leucovorin and uridine in murine colon carcinoma. *Invest New Drugs* **7**: 163–172, 1989.
13. Nadel JC, van Groeningen CJ, Pinedo HM and Peters GJ, *In vivo* potentiation of 5-fluorouracil by leucovorin in murine colon carcinoma. *Biomed Pharmacother* **42**: 387–393, 1988.
14. Parker WB and Cheng YC, Metabolism and mechanism of action of 5-fluorouracil. *Pharmacol Ther* **48**: 381–395, 1990.
15. Valeriote F and Santelli G, 5-Fluorouracil (FUra). *Pharmacol Ther* **24**: 107–132, 1984.
16. Chabner BA, Pyrimidine antagonists. In: *Pharmacological Principles of Cancer Treatment* (Ed. Chabner BA), pp. 183–212. WB Saunders, Philadelphia, 1982.
17. Wilkinson D and Crumly J, The mechanism of 5-fluorouridine toxicity in Novikoff hepatoma cells. *Cancer Res* **36**: 4032–4038, 1976.
18. Maybaum J, Ullman B, Mandel HG, Day JL and Sadee W, Regulation of RNA- and DNA-directed actions of 5-fluoropyrimidines in mouse T-lymphoma (S-49) cells. *Cancer Res* **40**: 4209–4215, 1980.
19. Evans RM, Laskin JD and Hakala MT, Assessment of growth-limiting events caused by 5-fluorouracil in mouse cells and in human cells. *Cancer Res* **40**: 4113–4122, 1980.
20. Radparvar S, Houghton PJ, Germain G, Pennington J, Rahman A and Houghton JA, Cellular pharmacology of 5-fluorouracil in a human colon adenocarcinoma cell line selected for thymidine kinase deficiency. *Biochem Pharmacol* **39**: 1759–1765, 1990.
21. Piper AA and Fox RM, Biochemical basis for the differential sensitivity of human T- and B-lymphocyte lines to 5-fluorouracil. *Cancer Res* **42**: 3753–3760, 1982.
22. Washtien WL, Comparison of 5-fluorouracil metabolism in two human gastrointestinal tumor cell lines. *Cancer Res* **44**: 909–914, 1984.
23. Ullman B, Lee M, Martin DW and Santi DV, Cytotoxicity of 5-fluoro-2′-deoxyuridine: Requirement for reduced folate cofactors and antagonism by methotrexate. *Proc Natl Acad Sci USA* **75**: 980–983, 1978.
24. Keyomarsi K and Moran RG, Folinic acid augmentation of the effects of fluoropyrimidines on murine and human leukemic cells. *Cancer Res* **46**: 5229–5235, 1986.
25. Houghton JA, Williams LG, de Graaf SSN Cheshire PJ, Rodman JH, Maneval DC, Wainer IW, Jadaud P and Houghton PJ, Relationship between dose rate of [6*RS*]leucovorin administration, plasma concentrations of reduced folates, and pools of 5,10-methylene-tetrahydrofolates and tetrahydrofolates in human colon adenocarcinoma xenografts. *Cancer Res* **50**: 3493–3502, 1990.
26. Horikoshi T, Danenberg KD, Stadlbauer THW, Volkenandt M, Shea LCC, Aigner K, Gustavsson B, Leichman L, Frösing R, Ray M, Gibson NW, Spears

CP and Danenberg PV, Quantitation of thymidylate synthase, dihydrofolate reductase, and DT-diaphorase gene expression in human tumors using the polymerase chain reaction. *Cancer Res* **52**: 108–116, 1992.
27. Doong S-L and Dolnick BJ, 5-Fluorouracil substitution alters pre-mRNA splicing *in vitro*. *J Biol Chem* **263**: 4467–4473, 1988.
28. Greenhalgh DA and Parish JH, Effects of 5-fluorouracil on cytotoxicity and RNA metabolism in human colonic carcinoma cells. *Cancer Chemother Pharmacol* **25**: 37–44, 1989.
29. Greenhalgh DA and Parish JH, Effect of 5-fluorouracil combination therapy on RNA processing in human colonic carcinoma cells. *Br J Cancer* **61**: 415–419, 1990.
30. Heimer R and Sartorelli AC, RNA polymerase II transcripts as targets for 5-fluorouridine cytotoxicity: Antagonism of 5-fluorouridine actions by α-amanitin. *Cancer Chemother Pharmacol* **24**: 80–86, 1989.
31. Armstrong RD, Lewis M, Stern SG and Cadman EC, Acute effect of 5-fluorouracil on cytoplasmic and nuclear dihydrofolate reductase messenger RNA metabolism. *J Biol Chem* **261**: 7366–7371, 1986.
32. Nord LD and Martin DS, Loss of murine tumor thymidine kinase activity *in vivo* following 5-fluorouracil (FUra) treatment by incorporation of FUra into RNA. *Biochem Pharmacol* **42**: 2369–2375, 1991.