Potentiation of 5-Fluorouracil-Leucovorin Activity by α2a-Interferon in Colon Adenocarcinoma Xenografts

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ABSTRACT

Previous studies using cultured colon adenocarcinoma cells demonstrated that a mixture of the diastereoisomers of the biologically active (6S) and inactive (6R) leucovorin or 5-formyl-H4PteGlu (LV) and recombinant human α2a-interferon (rIFN-α2a) in combination significantly increased the cytotoxicity of 5-fluorouracil (FUra) (by 10–14-fold) whereas FUra combined with single modulators was less potentiated (3-fold). Maximum cytotoxicity was achieved with 48-h drug exposures when drugs were applied continuously, and modulatory rIFN-α2a concentrations were obtained at ≥50 International units (IU)/ml. We therefore examined whether such interactions could occur in vivo using HC2/c1TK-3 colon adenocarcinoma xenografts, deficient in thymidine salvage. Potentiation of FUra activity was significantly greater when FUra was combined with both LV and rIFN-α2a in comparison to the use of single modulators using a 5-day schedule for 3 courses. In mice receiving LV, the maximum level of potentiation of FUra-induced growth inhibition was independent of the rIFN-α2a dose between 25,000 and 600,000 IU examined in contrast to rIFN-α2a used as a single modulator. After administration of 25,000 IU rIFN-α2a, plasma rIFN-α2a concentrations ≥50 IU/ml were maintained for 6–8 h, comparable to exposure times achievable clinically. Data indicate that intermittent rIFN-α2a exposure potentiated FUra-LV activity in vivo. The efficacy of FUra combined with dual versus single modulators will thus be of importance to evaluate in randomized phase III clinical trials in patients with colorectal cancer.

INTRODUCTION

We have previously shown significant potentiation (10–14-fold) of the cytotoxic activity of FUra1 against human colon adenocarcinoma cell lines using modulation with LV and rIFN-α2a (1). Cytotoxic effects were considerably greater than with the use of single modulators, where a 3-fold enhancement of FUra efficacy was obtained. Potentiation of FUra-LV cytotoxicity was influenced by the concentration of rIFN-α2a, and modulation was obtained at clinically achievable rIFN-α2a concentrations (≥50 IU/ml). Maximum interaction among FUra, LV, and rIFN-α2a required a minimum period of 48 h of continuous drug exposure (2).

The mechanism of FUra-LV interaction is well understood in the human colon adenocarcinoma cell lines and xenografts used in this laboratory (3–6). Under conditions of exposure to physiological folate, LV enhances FUra cytotoxicity by targeting FUra to the TS locus. Leucovorin has induced elevation of critical intracellular pools of 5,10-methylenetetrahydrofolate that enhance binding of the FUra metabolite FdUMP to TS within a covalent ternary complex. Enhanced complex stability mediated by elevated reduced folate pools further contribute to potentiation of the degree and duration of TS inhibition and to depleted dTTP pools, while drug resistance has been associated, in part, to inadequate pools of 5,10-methylenetetrahydrofolate (3).

Mechanistically, the locus of the interaction among FUra, LV, and rIFN-α2a has been identified in the GC/c1 colon adenocarcinoma cell line (2). FUra cytotoxicity in the presence of modulating agents was reversible by dThd. However, TS was determined not to be the primary drug interaction site since (a) direct assays of TS indicated no significant difference in the level, activity, or accumulation among FUra ± LV ± rIFN-α2a treatment groups; (b) rIFN-α2a did not potentiate the cytotoxic activity of 10-propargyl 5,8-dideazafolate, a TS inhibitory quinazoline; and (c) in TS-deficient cells rIFN-α2a cytotoxicity was not influenced by the concentration of dTdp. Data thus indicated the requirement for the nucleotide analogue (i.e., 5-fluoropyrimidine) in the interaction, and this suggested that potentiation of FUra by rIFN-α2a may be a consequence of increased incorporation of fraudulent nucleotide into DNA. The level of DNA single-strand and double-strand breaks obtained with FUra that were enhanced when combined with single modulators and further increased in the presence of dual modulators paralleled the cytotoxic activity of FUra-LV-rIFN-α2a combinations. Thus, rIFN-α2a may exert its effects by enhancement of FUra base excision or incorporation into DNA, events that subsequently become influenced by TS inhibition and dThd-less stress and are further potentiated by LV (2).

Clinically, FUra-LV combinations have demonstrated significantly increased response rates (33–48%) in comparison to...

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1 The abbreviations used are: FUra, 5-fluorouracil; LV, a mixture of the diastereoisomers of the biologically active (6S) and inactive (6R) leucovorin or 5-formyl-H4PteGlu; rIFN-α2a, recombinant human α2a-interferon; IFN, interferon; FdUMP, 5-fluoro-2'-deoxyuridylic acid; TS, thymidylate synthase; TK, thymidine kinase; dTdp, deoxythymidine; AUC, area under curve; CR, complete response; PR, partial response of ≥50% reduction in tumor volume that also includes CRs; C, no regrowth during the observation period; CLs, clearance; IU, International units.
the use of FUra administered alone (7–15%) when evaluated in phase III randomized clinical trials conducted in patients presenting with colon cancer (7–10). In addition, significant increases in time to disease progression (8–10) and in patient survival (8, 9) have been indicated. Thus, by building on this combination with additional therapeutic approaches, response rates and surviviorship may be improved. rIFN-α2a has been evaluated for this purpose, commencing in single institutional trials. Initially, FUra and rIFN-α2a combinations were evaluated in previously untreated colon carcinoma patients and response rates (26–63%) greater than those anticipated for FUra treatment alone, and equal to those obtained with FUra-LV combinations, were reported (11–13). In a subsequent phase II multiinstitutional trial, Walder et al. (14) confirmed a high response rate of 42%. The potential for the interaction of FUra and rIFN-α2a at a locus distinct from that of FUra and LV, along with their nonoverlapping toxicities, suggested that rIFN-α2a would be of importance to evaluate in combination with FUra-LV. Subsequently, Gre pro et al. (15) evaluated the triple combination in previously untreated colon carcinoma patients and determined a response rate of 45%.

As demonstrated from both preclinical and clinical studies, the use of rIFN-α2a in combination with FUra-LV clearly has potential for improving therapeutic outcome in patients with colon carcinoma. However, several key issues remain to be addressed that will determine the utility of rIFN-α2a as a modulator of FUra-LV action. The minimum dose of rIFN-α2a that can be used to achieve modulatory plasma concentrations must be determined. In colorectal carcinoma patients, the rIFN-α2a dose may be limited by fatigue and neurological toxicities (16, 17) and also by elevation in the plasma concentrations of FUra (15). The duration of rIFN-α2a exposure must be sufficient to enhance FUra-LV action, and it is evident from preclinical studies that modulation has not been achieved with single, short (2-h) exposures (2). The interdependence of these two parameters on FUra-LV modulation at clinically achievable plasma concentrations of rIFN-α2a is of importance for clinical therapy. Finally, whether the effect of dual modulation versus the effect of single modulators significantly enhances the therapeutic activity of FUra will impact on the application of rIFN-α2a in clinical trials.

Previsly, we have extensively characterized the therapeutic activity of FUra-LV combinations in human colon adenocarcinoma xenografts maintained in immune-deprived mice (18). We have therefore used these models to address some of the key issues outlined above regarding the influence of rIFN-α2a action on the in vivo activity of FUra or FUra-LV combinations. In the studies presented, we have examined the therapeutic utility of FUra, FUra-LV, FUra-rIFN-α2a, and FUra-LV-rIFN-α2a combinations. The xenograft models used were the parental HxGC3/cl colon adenocarcinoma that salvages dThd and HxGC3/cTKc3, a subline selected for its inability to salvage dThd because of a deficiency in TK-7. HxGC3/cTKc3 tumors may be more representative of the situation in humans, where reduced dThd salvage capability due to considerably lower plasma dThd concentrations has been demonstrated (18, 19). Specifically, we have examined (a) the influence of dose of rIFN-α2a on FUra or FUra-LV-induced antitumor activity in mice; (b) pharmacokinetics of rIFN-α2a given to mice; and (c) duration of systemic exposure to rIFN-α2a at modulatory dose levels.

**MATERIALS AND METHODS**

**Immune Deprivation of Mice.** Female 4-week-old CBA/CaJ mice (The Jackson Laboratory, Bar Harbor, ME) were immune deprived by thymectomy and subjected 3 weeks later to whole-body irradiation (950 cGy) using a 137Cs source. Mice received 3 × 106 nucleated bone marrow cells within 6–8 h of irradiation (18).

**Tumor Lines.** HxGC3/cl was originally derived from a poorly differentiated adenocarcinoma of the colon that was established as a xenograft in immune-deprived mice (HxGC3). Subsequently, the line was cultured and cloned (GC3/cl; Refs. 20–22) and reestablished as a xenograft in mice (HxGC3/cI). HxGC3/cITK-c3, a TK-deficient variant of HxGC3/cl was initially selected from GC3/cl in tissue culture (21, 22), and inoculated s.c. into immune-deprived mice to produce the xenografted line. All tumors were maintained as xenografts by passage in the s.c. space of female CBA/CaJ immune-deprived mice.

**Growth Inhibition Studies.** Mice bearing bilateral s.c. tumors were randomized and received drugs when the tumors had reached a diameter of ~0.5 cm. Tumor response was determined at 7-day intervals using digital calipers (Maxcal) interfaced with an IBM/PS2 microcomputer. Two perpendicular diameters were used to compute volumes (23). Growth inhibition was calculated from the difference in days required for treated tumors to grow to four times their volume at the start of treatment as compared with untreated controls. Each treatment group comprised six tumor-bearing mice. Relative tumor volumes were calculated using the formula $RTV = V_t / V_e$, where $V_t$ represents the tumor volume on day $t$ and $V_e$ indicates the tumor volume at the initiation of treatment. Volume regressions were also determined and expressed as CR, PR or C, and maximum percentage of treated/control ratio.

**Formulation and Administration of Drugs.** FUra (50 mg/ml), obtained from Solo-Pak Laboratories (Franklin Park, IL) as a pharmaceutical preparation, was diluted to a concentration of 15 mg/ml in sterile 0.9% saline. It was given to mice by i.v. bolus injection weekly via the tail vein for three courses (days 1, 8, and 15) after randomization of the animals. The dose used was 75 mg/kg (~225 mg/m2) given in a volume of 0.1 ml/20 g body weight and constituted the maximum tolerated dose on that schedule as described previously (18).

Leucovorin, purchased from Sigma Chemical Co. (St. Louis, MO), was given to mice by i.v. bolus injection daily at a dose of 500 mg/m2 on days 1–5, 8–12, and 15–19 immediately before FUra commencing on the first day of FUra injection as published previously (18). Leucovorin was dissolved in 0.9% sterile saline at a concentration of 23.6 mg/ml, and after additional filtration was injected at volumes of 0.17–0.27 ml/20 g body w as described previously (18). The dose relationship between mg/kg and mg/m2 body-surface area was determined for individual mice as reported previously (5, 6, 24).

rIFN-α2a was obtained, in part, as a generous gift from Hoffmann La Roche Inc., Nutley, NJ. The lyophilized powder representing 18 × 106 IU rIFN-α2a was initially dissolved in 1
were made in sterile 0.9% saline, and the agent was given i.p. to mice on days 1–5, 8–12, and 15–19 in volumes of 0.1 ml/10 g body weight at dose levels ranging from 25,000 to 600,000 IU/mouse. A 25,000-IU dose of rIFN-α2a in mice was equivalent to 3.3 \times 10^6 IU/m^2 or 5.7 \times 10^6 IU in humans. α2a-Interferon was given immediately before leucovorin. Because of the lack of interaction between high concentrations of rIFN-α2a and LV in cultured cells in the absence of FUra (1, 2) and the lack of antitumor activity or host toxicity of the combination in initial studies in mice, the agents were examined in combination in vivo as a control group.

**Statistical Analyses of Antitumor Activity.** The results of studies were evaluated and compared by one-way analysis of variance using the number of days required to reach 4 times the original tumor volume as the dependent variable followed by the Newman-Keuls range test for differences between means (18, 25). Only tumors from mice that survived the entire study were included in the analyses, and any tumor that failed to reach 4 times its original volume was assigned a default value of 140 days for the maximum duration of the study. The percentage of tumors showing partial and/or complete regressions, along with the extent of regression, were also analyzed. Data are presented as the mean and SE.

**Pharmacokinetic Analyses.** Plasma concentrations of rIFN-α2a were determined following i.p. injection of non-tumor-bearing immune-deprived mice at dose levels of 25,000 and 200,000 IU/dose. Time points, using 4 mice at each point, ranged from 0.25 to 6 h following rIFN-α2a administration. Additionally, groups of mice receiving 25,000 IU rIFN-α2a on day 1 were injected with the same dose level of rIFN-α2a at 24-h intervals over a period of 5 days. On day 5, samples were obtained between time 0 (just before injection) and 6 h (4 mice/point) after rIFN-α2a administration. Blood was drawn by cardiac puncture into heparinized syringes and plasma prepared (18).

Plasma concentrations of rIFN-α2a were measured by an adaptation of an enzyme immunoradiometric assay (Celltech Limited, Berkshire, United Kingdom; Ref. 26). Calibrators were prepared using pure rIFN-α2a (Hoffman La Roche Inc.) in pooled mouse plasma over a concentration range of 25–2500 IU/ml. The lower limit of sensitivity of the assay was 15 IU/ml.

A one-compartment model was fit to the rIFN-α2a plasma concentration-time data by the use of maximum likelihood estimation (27). Parameters estimated included volume of the central compartment (Vd,), and elimination rate constant (Ke). Calculated parameters included t_{1/2}, AUC versus time curve, maximum drug concentration, systemic CLs, and length of time plasma concentrations of rIFN-α2a exceeded 50 IU/ml.

For purposes of estimating systemic exposure to rIFN-α2a in humans from existing clinical dosing schedules, a simulation analysis was performed. A one-compartment model was fit to concentration versus time data from a previously published study in humans (28). The parameters of Vd, and Ke were used to simulate cumulative rIFN-α2a systemic exposure for two clinically relevant dosage schedules [i.e., 5 \times 10^6 IU/m^2 daily for 5 consecutive days (15) and 9 \times 10^6 IU on alternate days for three doses; (Ref 17)].

RESULTS

**Potentiation of FUra-LV Activity by rIFN-α2a in Vivo.** Previous reports in the literature indicated that a standard dose of 200,000 IU IFN-α in mice induced growth inhibition of xenografted tumors (29, 30) or was modulatory to the action of other agents (30, 31). Consequently, this dose level of rIFN-α2a was initially evaluated for its modulatory effects on FUra or FUra-LV activity in mice bearing HxGC3/c1TK–c3 xenografts, deficient in dThd salvage (Fig. 1, left). In mice receiving no treatment or LV-rIFN-α2a only, tumors grew at similar rates and reached four times their pretreatment size at similar times (35 and 38 days, respectively), indicating no direct effect of LV-rIFN-α2a on tumor growth. When FUra was given once weekly for 3 weeks, volume regressions were obtained and growth was delayed by 33.5 days relative to controls. When FUra was combined with rIFN-α2a or LV as single modulators, volume regressions increased and further increases in growth delay were obtained. However, when FUra was combined with both modulators, additional and significant decreases in tumor volume and further enhancement of growth inhibition (P < 0.001) was evident, effects considerably greater than those achieved with single modulators (Fig. 1).

Since data obtained with cultured GC3/c1 cells had indicated that FUra-LV-rIFN-α2a cytotoxicity was reversible by dThd (1, 2), modulation of FUra activity was subsequently determined in HxGC3/c1 xenografts capable of dThd salvage (Fig. 1, right). In this experiment, the dose level of rIFN-α2a was increased by 3-fold to 600,000 IU, although drug administration schedules remained the same. In contrast to the HxGC3/c1TK–c3 xenografts, no volume regressions or growth inhibition were induced in HxGC3/c1 tumors when FUra was combined with dual modulators, confirming results obtained with cultured cells.

**Influence of rIFN-α2a Dose on FUra ± LV Modulation.** Since significant potentiation of the in vivo activity of FUra had been obtained in HxGC3/c1TK–c3 xenografts by dual modulation with rIFN-α2a administered at a dose of 200,000 IU, the influence of rIFN-α2a dose (systemic exposure) on therapeutic activity was examined. In these experiments, the rIFN-α2a dose was increased to 600,000 IU to examine whether FUra or FUra-LV activity could be potentiated further. In addition, the dose level of rIFN-α2a was reduced to 25,000 IU (considered to be a low dose level in mice; Ref. 29) to determine the minimum dose exhibiting a modulatory effect. Data for rIFN-α2a combined with FUra alone as a single modulator, or combined with LV in dual modulation studies, are shown in Tables 1 and 2 and Fig. 2.

**Tumor Growth Inhibition.** The time required in days for tumors to reach four times the volume at the initiation of treatment has been compared for control and all drug-treated groups (Table 1). The differences between untreated (42.1 days) and FUra-treated (69.6 days) tumors, or between FUra-treated and FUra-LV-treated (88.6 days) groups were significantly different (P < 0.05). When the efficacy of FUra treatment was compared with or without rIFN-α2a, the time required to reach the value at four times the volume ranged from 77.1 to 118.8 days, significantly different at rIFN-α2a doses of 25,000 and 600,000 IU (P < 0.001). Modulation of FUra activity by rIFN-
Fig. 1 Modulation by rIFN-α2a of the activity of FUra or FUra-LV against HxGC_/cITK c3 (left) or HxGC_/c1 (right) xenografts maintained in immune-deprived mice. ○, control; ●, LV-rIFN-α2a; □, FUra; ■, FUra-LV; ▽, FUra-LV-rIFN-α2a. Six mice bearing bilateral tumors were randomized to each group, and results represent the mean ± SD for tumor volumes determined at each time point. Drug treatments were conducted as follows: FUra, 75 mg/kg i.v. days 1, 8, and 15; LV, 500 mg/m² i.v. daily on days 1-5, 8-12, and 15-19; rIFN-α2a, 25,000 IU (HxGC_/TK c3) or 600,000 IU (HxGC_/c1) i.p. daily on days 1-5, 8-12, and 15-19. Tumor volumes were determined at weekly intervals.

Table 1 Delay in tumor growth induced by FUra-LV-rIFN-α2a combinations in HxGC_/cITK c3 xenografts

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose of rIFN-α2a (IU)</th>
<th>Days to 4X ± SE</th>
<th>n</th>
<th>Days to 4X ± SE</th>
<th>n</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>42.1 ± 3.5</td>
<td>50</td>
<td>69.6 ± 3.2</td>
<td>29</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FUra</td>
<td></td>
<td>69.6 ± 3.2</td>
<td>29</td>
<td>88.6 ± 4.1</td>
<td>52</td>
<td>&lt;0.05</td>
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<td>FUra-LV-rIFN-α2a</td>
<td>25,000</td>
<td>96.5 ± 5.4</td>
<td>35</td>
<td>115.0 ± 4.7</td>
<td>35</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>200,000</td>
<td>77.1 ± 5.4</td>
<td>10</td>
<td>111.2 ± 8.3</td>
<td>8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>600,000</td>
<td>118.8 ± 11.5</td>
<td>12</td>
<td>124.2 ± 7.9</td>
<td>12</td>
<td>&gt;0.05</td>
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</tbody>
</table>

α2a alone appeared to be dependent on dose and was greatest at the highest dose level of rIFN-α2a used. Data for FUra-LV activity compared with FUra-rIFN-α2a responses were similar (P > 0.05) at the 25,000- and 200,000-IU dose levels of rIFN-α2a (P > 0.05), but were also greater at the high rIFN-α2a dose level (P < 0.05). When FUra was combined with both modulators however, days required to reach four times the volume increased (range, 111.2-124.2 days) and was independent of the rIFN-α2a dose. Comparison of FUra-LV-rIFN-α2a responses with the response to FUra-LV indicated significant differences over the entire dose range of rIFN-α2a (P < 0.05). Similarly, a comparison of FUra-LV-rIFN-α2a responses with the responses to FUra-rIFN-α2a at equivalent rIFN-α2a dose levels indicated increased FUra activity in the presence of dual modulators at doses of rIFN-α2a ≤ 200,000 IU (P < 0.05), but not at the 600,000-IU rIFN-α2a dose.

Volume Regressions. The maximum percentage of T/C for all treatment groups is shown in Fig. 2, and the percentage of PR and CR responses for tumors that did not regrow during C are presented in Table 2. FUra treatment alone induced 62% PR that was increased significantly (P < 0.01) to 83% PR, 12% CR, and 10% C in the presence of LV. When FUra was combined with rIFN-α2a, volume regressions were dependent on the rIFN-α2a dose, being significantly greater at the 600,000-IU dose of rIFN-α2a only (P < 0.001) in comparison to FUra or FUra-LV treatment groups. Additionally, FUra-LV-rIFN-α2a-treated tumors were smaller and demonstrated less rIFN-α2a dose dependency, but were significantly smaller than in the FUra-LV-treated group, only at the highest dose level of rIFN-α2a (P < 0.01). However, when FUra-rIFN-α2a activity was compared either in the absence or in the presence of LV at equivalent rIFN-α2a dose levels, tumor volume regressions...
were significantly greater in groups receiving LV at doses of rIFN-α2a ≤ 200,000 IU (P < 0.05), but not at the 600,000-IU rIFN-α2a dose.

Taken together, these data suggested that (a) rIFN-α2a significantly modulated the activity of FUra-LV; (b) modulation of FUra-LV-induced growth inhibition was independent of the dose of rIFN-α2a over the dose range examined; and (c) the use of dual modulation had greater effects on FUra-induced tumor growth inhibition and volume regression than the use of single modulators. Hence maximum potentiation of FUra by rIFN-α2a dose level of 600,000 IU has previously been shown that FUra (75 mg/kg) given i.v. to mice once weekly for 3 weeks was the maximum tolerated dose. Toxicity, as determined from deaths occurring within the first 3 weeks after completion of therapy, was not increased in the presence of LV (500 mg/m²; Ref. 18). From the data presented in Table 3, addition of rIFN-α2a did not enhance the toxicity of FUra-LV in tumor-bearing mice.

**rIFN-α2a Pharmacokinetics.** Concentrations of rIFN-α2a achieved in the plasma of mice following i.p. administration of the agent at dose levels of 25,000 IU on days 1 and 5, and at 200,000 IU on day 1, are shown in Fig. 3. These are dosages equivalent to 3.3 x 10⁵ and 26.4 x 10⁶ IU/m², respectively, in humans. Peak plasma levels occurred by 1 h after rIFN-α2a administration. Model estimated peak plasma levels of rIFN-α2a achieved were 3400 and 4500 IU/ml (25,000-IU dose) on days 1 and 5, respectively.

Pharmacokinetic analyses of these data are shown in Table 4. The AUC and systemic CLs on days 1 and 5 were similar for the 25,000-IU rIFN-α2a dose. The elimination half-life was lower (0.87–1.1 h) for the 25,000-IU dose level in comparison to the 200,000-IU dose (t1/2 = 16.4 h). The elimination half-life for the 25,000-IU dose was 26% greater on day 5 compared with day 1. The AUC for 200,000 IU rIFN-α2a was considerably greater and CLs lower than predicted from data obtained at the lower rIFN-α2a dose, suggesting saturation of elimination processes.

The duration of maintenance of rIFN-α2a concentrations above 50 IU/ml, a minimal modulatory concentration of FUra-LV cytotoxicity determined for 72-h continuous exposures in cultured cells (1) is shown in Table 4. For the 25,000-IU dose level of rIFN-α2a, plasma concentrations of 50 IU/ml were maintained for 6 h on day 1 and 8 h on day 5, and for the 200,000-IU dose for >24 h.

Pharmacokinetic modeling of two clinical dosing schedules used in humans is shown in Fig. 4. Cumulative systemic exposure (AUC) following five daily doses of rIFN-α2a (5 x 10⁶ IU/m²) was 5270 IU/h/ml, and following three doses administered on alternate days, 3310 IU/h/ml. Although in mice at the 200,000-IU rIFN-α2a dose level accumulation was suggested, simulations in humans were more consistent with the lower dosage studied in the mice where no accumulation was apparent between days 1 and 5 as evidenced by no changes in CLs or AUC. The simulation study also indicated that plasma concentrations of rIFN-α2a ≥50 IU/ml would be maintained for periods of 8 h following rIFN-α2a administration, consistent with the systemic exposure observed for the lowest rIFN-α2a dose in the xenograft study.

**DISCUSSION**

In this study we extend the relationship between rIFN-α2a concentrations that modulate FUra-LV cytotoxicity determined in vitro to the level of rIFN-α2a systemic exposure associated with dosages modulating the cytotoxicity of FUra and FUra-LV in mice bearing human colon adenocarcinoma xenografts. Further we show that the length of time above an in vitro modulatory concentration is similar between the mouse xenograft model and pharmacokinetic simulations of dosing schedules used clinically.

rIFN-α2a has been shown to modulate the in vitro cytotoxicity of FUra and FUra-LV against two human colon adenocarcinoma cell lines, GC3/c1 and VRC3/c1 (1). Previously we had demonstrated, using cultured colon adenocarcinoma cells, that 500 IU/ml and 5,000 IU/ml rIFN-α2a produced similar and significant modulation of FUra-LV cytotoxicity (10–14-fold; >95% reduction in clonogenic survival) when cells were exposed continuously for 72 h (1). Reduction in clonogenic survival by 60% was achievable when FUra-LV were combined with as low as 50 IU/ml rIFN-α2a for 72 h (1). In addition, in the presence of LV lower modulatory concentrations of rIFN-α2a could be combined with a noncytotoxic FUra concentration to achieve a cytotoxic response. In this in vitro system maximal cytotoxicity required a minimum of 48 h of continuous drug exposure (2).

With the knowledge that rIFN-α2a modulated the activity of FUra in vitro, we evaluated three rIFN-α2a doses (25,000 IU, 200,000 IU, and 600,000 IU) against human colon adenocarcinoma xenografts in combination with FUra and FUra-LV to investigate the potential for enhancing in vivo therapeutic activity. Using HxGC3/c1TK c3 xenografts deficient in dThd salvage, significant potentiation of FUra-LV activity was obtained in the presence of rIFN-α2a, determined by enhanced volume...
### Table 2 Volume regressions induced by FUra-LV-rIFN-α2a combinations in HxGC/c1TK^c3 xenografts

<table>
<thead>
<tr>
<th>Group</th>
<th>% PR</th>
<th>% CR</th>
<th>% C</th>
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<td>FUra-LV</td>
<td>83</td>
<td>12</td>
<td>10</td>
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<tr>
<th>Dose of rIFN-α2a (IU)</th>
<th>% PR</th>
<th>% CR</th>
<th>% C</th>
<th>n</th>
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<tr>
<td>25,000</td>
<td>31</td>
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<td>0</td>
<td>10</td>
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<tr>
<td>600,000</td>
<td>100</td>
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<td>10</td>
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**FUra-LV > FUra, P < 0.01; FUra-rIFN-α2a > FUra, P < 0.001 at 600,000 IU only; FUra-rIFN-α2a > FUra-LV, P < 0.05 at 600,000 IU only; FUra-LV-rIFN-α2a > FUra-LV, P < 0.01 at 600,000 IU only. The PR group contains all tumors that achieved at least 50% reduction in tumor volume and hence also includes CRs.**

### Table 3 Toxicity of FUra-LV-rIFN-α2a combinations in tumor-bearing mice

<table>
<thead>
<tr>
<th>Group</th>
<th>% Survival</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>30</td>
</tr>
<tr>
<td>FUra</td>
<td>97</td>
<td>30</td>
</tr>
<tr>
<td>FUra-LV</td>
<td>100</td>
<td>30</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dose of rIFN-α2a (IU)</th>
<th>% Survival</th>
<th>% Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>25,000</td>
<td>100</td>
<td>18</td>
</tr>
<tr>
<td>200,000</td>
<td>83</td>
<td>100</td>
</tr>
<tr>
<td>600,000</td>
<td>100</td>
<td>6</td>
</tr>
</tbody>
</table>

**Survival was determined within the first 3 weeks after completion of therapy.**

Regressions and inhibition of tumor growth. Responses were considerably greater than those achieved with FUra combined with either LV or rIFN-α2a as single modulators. The drug interaction among FUra, LV, and rIFN-α2a was dependent on reduced ability to salvage dThd, thereby confirming previous data obtained using cultured GC3/c1 cells (1, 2).

We then evaluated the influence of the rIFN-α2a dose or systemic exposure on FUra activity. When FUra was combined with rIFN-α2a as a single modulator, the greatest effect was seen at the 600,000-IU dose level. However, this would constitute a suprapharmacological exposure not achievable for clinical studies. In contrast to results with FUra-rIFN-α2a, potentiation of FUra-LV-induced growth inhibition was independent of the rIFN-α2a dose over the dose range examined, as was the extent of volume regressions at the 25,000- and 200,000-IU dose levels. The lowest rIFN-α2a dose (25,000 IU) yielded significant potentiation of FUra-LV-induced growth inhibition, as high as achieved with the 600,000-IU rIFN-α2a combined with FUra ± LV. Thus, in combination with LV, lower pharmaceutically relevant doses of rIFN-α2a were effective in modulating FUra.

As recently reviewed by Galpin and Evans (32), many studies have shown systemic exposure to anticancer drugs to be related to both toxicity and efficacy. Different approaches have been used to estimate systemic exposure, including AUC, peak plasma concentration, and time above a minimum effective concentration. Studies of the antiviral properties of rIFN-α2a showed that 2',5'-oligoadenylate synthetase activity was related to dose and route of rIFN-α2a administration (33). At similar dosages, 2',5'-oligoadenylate synthetase activity in peripheral blood mononuclear cells was greater when rIFN-α2a was given i.m. compared with i.v. administration, paralleling prolonged systemic exposure at low concentration from the i.m. route and short exposure with an initial high peak level from the i.v. route. This would suggest antiviral activity is associated with prolonging rIFN-α2a exposure above a minimum effective concentration for some period of time rather than association with a high peak concentration. Additionally, Wills et al. (28), studying the pharmacokinetics of rIFN-α2a in volunteers, observed that adverse effects from rIFN-α2a were related to exceeding and maintaining serum concentrations above a threshold level.

For comparison with rIFN-α2a systemic exposure determined in mice, we simulated the rIFN-α2a plasma concentra-
tions achievable in humans following s.c. administration at the rIFN-α2a dose levels used in clinical trials of FUrα-rIFN-α2a (11-14) or FUrα-LV-rIFN-α2a (15). Simulations were constructed for 5 × 10^6 IU/m^2 rIFN-α2a administered daily for 5 days (15) or 9 × 10^6 IU administered on alternate days for 3 doses (11-14). The total AUC measured in mice following 25,000 IU rIFN-α2a administered daily for 5 consecutive days was 41 and 66% of that determined from pharmacokinetic simulations of the two clinical dosing schedules. Similarly, estimated peak plasma concentrations of rIFN-α2a were lower in both clinical studies (~90 IU/ml) than determined in mice (~4000 IU/ml). The reduced effect of rIFN-α2a dose on tumor volume regressions and the lack of a dose-response relationship for rIFN-α2a when combined with FUrα and LV in inducing growth inhibition in the xenograft studies suggested that time above a minimum effective concentration may be more important than the peak plasma concentration in inducing a modulatory response to FUrα-LV activity.

Simulations of dosing schedules used clinically indicated plasma rIFN-α2a concentrations would be maintained above 50 IU/ml each day for a period of 8 h, and with the alternate day schedule, for 8 h three times per week. Of interest from this study was that the 25,000 IU rIFN-α2a dose level in mice maintained a rIFN-α2a plasma concentration of ≥50 IU/ml for 6–8 h. This concentration of rIFN-α2a for the durations obtained provided in vivo modulation of FUrα-LV activity in mice. Thus, when combined with LV it may be possible that intermittent and repeated exposure to lower dosages (i.e., concentrations) of rIFN-α2a is sufficient to modulate and potentiate the activity of FUrα.

Clearly, intermittent exposure to rIFN-α2a has been modulatory in the xenograft studies, and maintenance of critical rIFN-α2a levels for 6- to 8-h periods daily appears to be sufficient to modulate the activity of FUrα in the presence of LV. Since the window of intermittent rIFN-α2a exposure is very similar between mice and humans, it is conceivable from the simulations presented that the rIFN-α2a doses used clinically will modulate FUrα-LV activity. It is also evident from previous studies with cultured cells (1, 2) and from the xenograft studies that lower rIFN-α2a doses may be used to modulate FUrα activity in the presence of LV. However, the optimal schedule remains to be elucidated.

In patients, an additional effect of interferons has been to increase FUrα systemic exposure, thereby increasing FUrα-associated toxicity. Most of these investigations have been conducted with an alternative IFN-α subtype (IFN-α2b; Ref. 34), although effects appear to be similar with rIFN-α2a (15). In these studies pharmacokinetic analyses indicated that FUrα CLs decreased by 20–35% (34). Grem et al. (15) determined that reduction in FUrα CLs was related to the dose of rIFN-α2a, resulting in an increase of FUrα AUC of 30 and 50% in patients receiving 5 × 10^6 and 10 × 10^6 IU/m^2, respectively, of rIFN-α2a. However, the effects of interferons are species specific, and for the xenograft studies, a human IFN-α subtype was used in a murine model system bearing human tumors. Thus, as anticipated, no increase in FUrα-associated toxicity was observed in these studies. It is unlikely therefore that suitable data on the influence of rIFN-α2a on FUrα toxicity will be forthcoming from studies conducted in mice. In this regard, it is noteworthy that even at the highest rIFN-α2a dose, the toxicity of FUrα or FUrα-LV was not enhanced. Consequently, this type of information will be more readily gained from clinical studies.

Modulation of the activity of FUrα by rIFN-α2a has been possible in an in vivo model system, and has been considerably greater at a more clinically relevant lower dose level of rIFN-α2a in the presence of LV. Plasma levels of rIFN-α2a achieved in mice have been maintained above critical concentrations for periods of time achievable clinically. It is feasible that dual modulation may enhance the clinical activity of FUrα in patients with colorectal cancer, although whether responses and survival are increased significantly over the use of single modulators will await results from randomized phase III clinical trials in this population.

REFERENCES


Potentiation of 5-fluorouracil-leucovorin activity by alpha2a-interferon in colon adenocarcinoma xenografts.
