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Effects of 5-fluorouracil, leucovorin, and glucarate in rat colon-tumor explants*

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Summary. In a previous study, we showed that 5-fluorouracil (FU) is active against the dimethylhydrazine-induced colon tumor in rats; a 7-day infusion of FU at 30 mg/kg daily produced 85% tumor-free cures. The present study examined the effects of FU alone and in combination with leucovorin (LV) or D-glucarate (GT) using an ex vivo system that maintained the growth of the rat colon-tumor explants on collagen gels. The labeling index (LI) was determined by the incorporation of [^{3}H]-thymidine and autoradiography. The mean LI of the untreated control was 64.8% ± 19.8%. The IC_{50}, IC_{90}, and IC_{95} values following a 7-day exposure to FU were 0.36, 0.75, and 1.22 μM, respectively. In comparison, the steady-state FU concentration required to produce 67% tumor-free cures in rats following a 7-day infusion is 1.54 μM. LV alone did not produce any antiproliferative effect at concentrations as high as 10 μM. The addition of LV at concentrations of 0.001–10 μM did not significantly reduce the IC_{50} of FU. The lack of effect of LV may have been due to tissue saturation with folate provided in the culture medium. GT alone reduced the tumor LI by 20%–30% at concentrations of 0.1–10 μM. GT enhanced the effect of FU. As compared with FU alone, the addition of GT at concentrations of 0.1 and 1.0 μM reduced the IC_{50} of FU by 47% and 60% to 0.21 and 0.16 μM, respectively. Assessment of the potentiation of the inhibitory effect of FU by GT using two-way analysis of variance and the isobologram method indicated a significant synergistic interaction between FU and GT. This interaction occurred within the FU concentration range of 0.08 and 0.4 μM. In summary, these data indicate that (a) the IC values for FU are comparable in tumor explants and in rats, suggesting that the effects in cultured tumors reflect those in intact animals; (b) GT alone showed antitumor activity, albeit relatively minor as compared with FU; (c) FU and GT exhibited synergistic activity, which was most pronounced at FU concentrations that produced submaximal activity (<30% inhibition of tumor LI); and (d) GT and LV had different effects on the growth inhibition by FU, suggesting that GT acts by a mechanism different from the thymidylate synthase-directed effect of FU and LV.

Introduction

One of the agents of choice in the treatment of colorectal carcinoma is 5-fluorouracil (FU). Despite its widespread acceptance, single-agent FU therapy produces a response rate in the range of 10%–25% [5, 9]. Many attempts have been made to enhance its activity by biochemical modulation with normal metabolites and combination chemotherapy with other antitumor agents. Examples include the combination of FU with agents such as leucovorin (LV) [9, 10, 25], thymidine [28], cisplatin [22], levamisole [7], and interferon [29]. Among these combinations, the addition of LV to FU has resulted in significant increases in the response rate to 40%–50% [9]. By increasing the folate pool, LV stabilizes the ternary complex of 5-fluorodeoxyuridine monophosphate, 5,10-methylenetetrahydrofolate, and thymidylate synthase. This results in an enhanced inhibition of thymidylate synthase and DNA synthesis [9]. These improvements suggest that the activity of FU can be enhanced by the appropriate biochemical modulators. The identification of effective treatment modalities for the remaining 50% of patients who do not respond to the FU and LV combination remains a challenge.

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Abbreviations: FU, 5-Fluorouracil; LV, leucovorin; GT, D-glucarate; LI, labeling index; IC, inhibitory concentration; TdR, thymidine

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Dietary calcium glucarate (GT) has been shown to potentiate the in vivo antitumor activity of 13-*cis*-retinoic acid and *N*-(4-hydroxyphenyl)retinamide in a rat mammary tumor model [1, 2]. Similarly, potassium GT has enhanced the activity of retinoid or FU in a human mammary-carcinoma cell line [19]. Calcium GT is a natural food component and an end product of glucuronate metabolism. The in vivo enhancement of the activity of retinoids by GT is tentatively thought to be due to the equilibrium conversion of GT to D-glucaro-1,4-lactone, a potent inhibitor of β-glucuronidase. This would lead to the reduced degradation and enhanced net glucuronidation of retinoids [21]. The exact mechanism of GT enhancement of FU in vitro is not fully understood [19].

The present study examined the growth-inhibitory effects of FU used alone and in combination with LV or GT on explants of a dimethylhydrazine-induced rat colon tumor. The rat colon tumor is sensitive to FU; a 7-day infusion of FU at a daily dose of 30 mg/kg produced a tumor-free cure rate of 80% in rats after 90 days, whereas all of the untreated animals died within 80 days [3]. In the present ex vivo model, tumor fragments (about 1 mm^3) were maintained in short-term culture and the drug-effect studies were completed within 30 days. The tumor-explant culture has several advantages over suspension and monolayer tissue cultures. The three-dimensional architecture, cell-to-cell interactions, and cell heterogeneity are maintained. Furthermore, such culture obviates the need for mechanical or enzymatic disaggregation of the tumor into a single-cell suspension, thus minimizing potential cellular damage. Drug penetration into the three-dimensional tumor explant is more similar to penetration into a solid tumor in vivo than into a monolayer. The explant system also offers some advantages over the in vivo animal model. The drug concentration and the exposure period are well controlled in a tissue-explant system, whereas these parameters are subjected to pharmacokinetic variability in animals. The use of rat colon-tumor explants to define the drug concentration-effect relationship is more convenient and cost-effective than animal experimentation.

Materials and methods

Chemicals and supplies. Sterile pigskin collagen (Spongostan Standard) was purchased from Health Designs Industries (Rochester, N. Y.); cefotaxime sodium, from Hoechst-Roussel (Somerville, N. J.); NTB-2 nuclear track emulsion, from Eastman Kodak (Rochester, N. Y.); [methyl-^{3}H]-thymidine (TdR), from ICN Biomedicals (Irvine, Calif.); FU, from Sigma Chemical Co., (St. Louis, Mo.); and potassium GT, from Gallard-Schlesinger (Carle-Place, N. Y.). LV was a gift from Burroughs Wellcome Company (Research Triangle Park, N. C.). All other reagents, tissue-culture medium, and supplies were purchased from Gibco Laboratories (Grand Island, N. Y.).

Transplantation of the rat colon tumor. Dimethylhydrazine-induced colon tumors were transplanted into 2- to 3-month-old female Fischer rats (Charles River Breeding Laboratories, Wilmington, Mass.). The animals were anesthetized with ether, and 100 mg nonnecrotic tumor fragments was transplanted subcutaneously into the ventral side of both hind limbs [30]. Tumor size was calculated as one-half of the product of the tumor length and the square width. When the tumor weight had reached approximately 1 g, the tumor was removed and either transplanted into another host or placed in culture. Tumors between the third and the tenth generations were used.

Tumor-explant culture. The method used to culture the rat colon tumor was similar to that described for other human solid tumors [6, 26]. Briefly, the necrotic portions of the tumor were trimmed off and the nonnecrotic portions were dissected into 1-mm^3 fragments. The fragments were mixed to ascertain randomization. Four to five tumor fragments were placed on a 1-cm^2 piece of collagen gel and cultured in six-well plates in a humidified atmosphere comprising 95% air and 5% CO_2 at 37° C. The culture medium consisted of Eagle's minimal essential medium supplemented with 9% heat-inactivated fetal bovine serum, 0.1 mM nonessential amino acids and antibiotics, gentamicin (100 μg/ml), and cefotaxime sodium (95 μg/ml). The cultures were fed with fresh medium twice weekly. The pH of the medium was 7.4. The proliferative activity of the tumors was determined by [^{3}H]-TdR labeling and autoradiography. Cultures were incubated for 6 h with medium containing 1 μCi [^{3}H]-TdR/ml (sp. act., 60 Ci/mmol). Afterwards, the tissues were rinsed three times with 4 ml culture medium to wash off residual [^{3}H]-TdR. The tissues were then fixed overnight in 10% buffered formalin, dehydrated, and embedded into paraffin blocks. The blocks were cut into 4-μm sections using a microtome and the sections were fixed onto microscope slides. The slides were deparaffinized, coated with Kodak NTB-2 autoradiographic emulsion for 7 days, developed, and stained with hematoxylin and eosin.

Drug-effect studies. Cultures were maintained for at least 5 days prior to drug exposure. Cultures were exposed for 7 days to the following drugs: FU, at concentration range of 0.01–1.0 μg/ml (0.077–7.7 μM); LV, at 10, 0.1, or 0.001 μM; or GT, at 10, 1.0, 0.1, or 0.01 μM. In some experiments, LV or GT was used in combination with FU. Each concentration was repeated in triplicate wells, and each well contained 4–5 tumor fragments; therefore, a total of 12–15 tumor replicates were used for each drug concentration. Following exposure, the drug was removed and the gel was rinsed three times with 4 ml drug-free medium. After a 6-h incubation with [^{3}H]-TdR, the samples were rinsed and processed for autoradiography.

The drug effect was measured as the inhibition of the cell labeling index (LI). Tumor cells but not stromal cells were quantitated. Tumor cells accounted for approximately 90% of the total cell population. Samples were counted using a Zeiss Axiovert 35 microscope (Carl Zeiss, Thornwood, N. Y.) equipped with a 7×7-mm disc micrometer in the right eyepiece (Thomas Scientific, Swedesboro, N. J.). The LI was defined as the number of labeled nuclei divided by the total number of nuclei found within this grid at a magnification of × 400. Approximately 100–200 cells/explant were counted. Tumor specimens that contained <10 total cells within the grid were discarded from the analysis. The microscopic section was scanned at a magnification of × 100 to select the most proliferative region, and the LI was determined in this region. This procedure standardized the selection procedure for evaluation of different samples. This method overestimates the tumor LI. The drug effect is established by comparing the LI in the drug-treated tissue with that in the untreated control. In a separate study evaluating the antiproliferative effects of mitomycin C in human bladder-tumor explants, we compared the manual counting method with an automated cell-counting method using an image analysis system that counts the entire tissue section (Schmittgen and Au, unpublished data). The data show that the automated counting method gave an LI value of 33% for the untreated control, which is lower than the 53% value obtained by the manual counting method. The higher LI resulting from the manual counting method was attributable to preselection of the most proliferative area. When the data were expressed as a percentage of the control value, the concentration-effect relationship curves obtained by the two methods were superimposable, exhibiting identical inhibitory concentration (IC) values.

Data analysis. The concentration-effect relationship was analyzed by the following equation:

$$E = E_0 \times \left(1 - \frac{C^n}{K^n + C^n}\right), \quad (1)$$

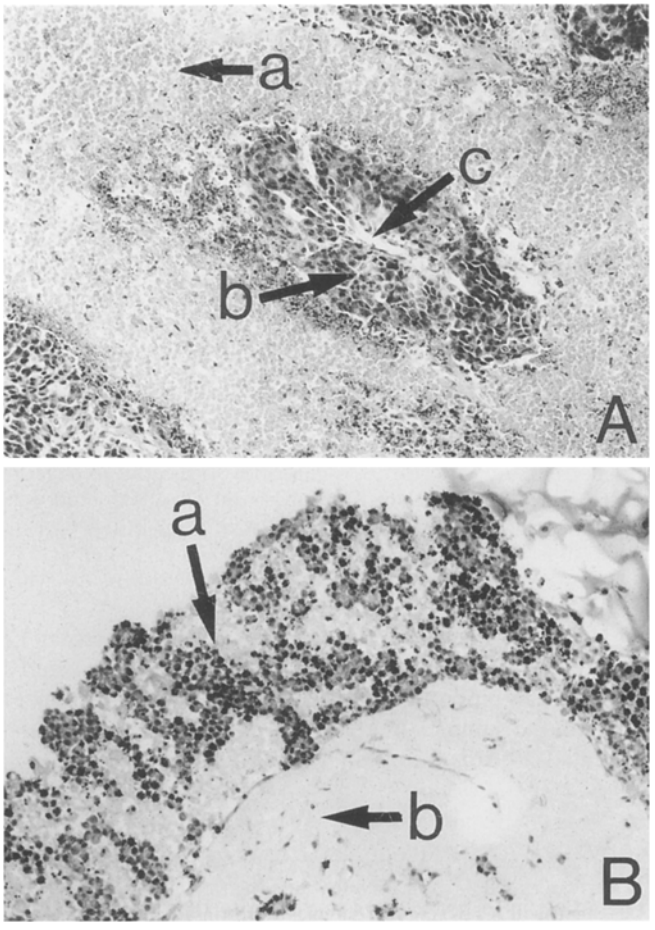

Fig. 1 A, B. Histopathology of rat colon tumor prior to and after culture on collagen gel. **A** Original tumor specimen (uncultured) following its removal from the rat. Note the portions of necrotic (*a*) and proliferative (*b*) cells surrounding a small blood vessel (*c*). **B** Autoradiograph of a rat colon-tumor explant following 14 days' culture on collagen gel. Note the degree of proliferation in the tumor periphery as indicated by the black grains over the stained nuclei (*a*). The central region contains necrotic and degenerating tumor tissue along with scattered viable fibroblasts (*b*). Hematoxylin and eosin counterstain. × 200

where E is the LI expressed as a percentage of the control value, C is the drug concentration, E_0 is the baseline effect in the absence of drug, K is the concentration at one-half E_0, and n is a curve-shape parameter [11]. The concentration-effect relationship was analyzed by computer-fitting the data to Eq. 1 using nonlinear least-squares regression (NONLIN84; Statistical Consultants Inc., Lexington, Ky.). LI as a percentage of the control value was plotted versus the FU concentration, and the IC values were determined from this fit. The interaction between FU and LV or GT was examined using the isobologram method [24]. The isobologram is a plot of the equieffective concentrations of each drug, as used alone and in combination. The line connecting the IC values for each single agent represents the theoretical additivity between agents. Values that fall above or below this line indicate an antagonistic or synergistic effect, respectively.

Statistical analysis. The effect of LV or GT on the FU-induced reduction in the tumor LI was determined by two-way analysis of variance, whereby LI was the dependent variable. Follow-up Tukey multiple comparisons were made between the different concentrations of FU at each concentration of GT. Levels of significance were determined using a Student's *t*-test. A 95% confidence interval was used.

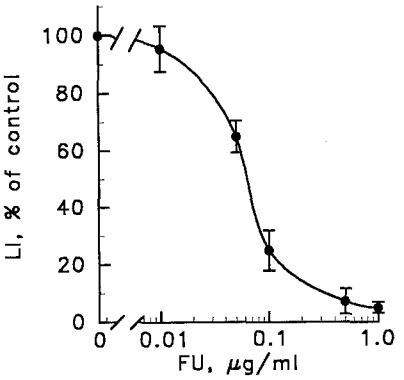

Fig. 2. Antiproliferative effects of FU. A rat colon-tumor explant was exposed to FU for 7 days. The LI was determined by [^{3}H]-TdR incorporation and autoradiography. *Points*, Mean experimental data; *curve*, nonlinear least-squares fit according to Eq. 1; *bars*, SEM. FU concentration: 1 μg/ml is equivalent to 7.7 μM

Results

Tumor explants

The tumor histopathology prior to culture was compared with that after culture (Fig. 1). The primary rat colon-tumor carcinoma grew in vivo as a subcutaneous nodule to a solid tumor weighing about 1 g. The tumor cells were undifferentiated and were characterized by polygonal, basophilic cells exhibiting vesicular nuclei and prominent nucleoli. There was a high mitotic rate. The primary tumor displayed an alternating pattern of viable and necrotic tissue: the most viable tumor cells were found surrounding small blood vessels, whereas tumor cells lying more distant from the vasculature were necrotic. The explants showed an identical pattern after 2 weeks in culture: the tumor cells were most viable near the perimeter of the explants, whereas the center of the explants were predominantly necrotic, exhibiting scattered fibroblasts and a few degenerate tumor cells. The explants showed cords of viable tumor cells interspersed between necrotic tissue. Necrotic tumor cells and tumor cells undergoing ballooning vascular degeneration were observed adjacent to the viable cells. The morphology of the viable cells was similar to that of the primary tumor. There was also a mild degree of fibroblast proliferation in the explants that was not associated with the tumor cells.

Six tumors were removed from hosts and used in the drug-effect studies. The average length of culture was 16 days. The explants of all six tumors appeared viable. The mean LI value obtained for the untreated controls following a 6-h exposure to [^{3}H]-TdR in six different experiments was 64.8% ± 19.8%. The doubling time of the rat colon tumor in Fischer rats is between 3 and 4 days [4]. These data indicate that the rapid proliferation of the rat colon tumor was maintained under ex vivo conditions.

We used [^{3}H]-TdR to measure the LI. TdR is converted via the salvage pathway to thymidine triphosphate (dTTP), which is incorporated into DNA. Inhibition of thymidylate synthase by FU will decrease the de novo synthesis of dTTP. The specific activity of [^{3}H]-dTTP in the FU-treated sample will be higher than that in the untreated control.

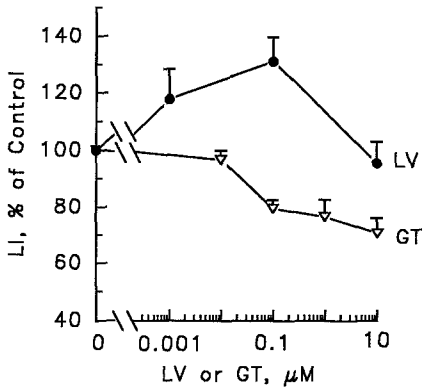

Fig. 3. Effects of LV or GT. A rat colon-tumor explant was exposed to LV or GT for 7 days. The LI was determined by [^{3}H]-TdR incorporation and autoradiography. The data represent mean values ± SEM (10–30 tumor replicates per data point). The differences between the samples treated with 0.1–10 μM GT and the untreated controls were statistically significant (P <0.001)

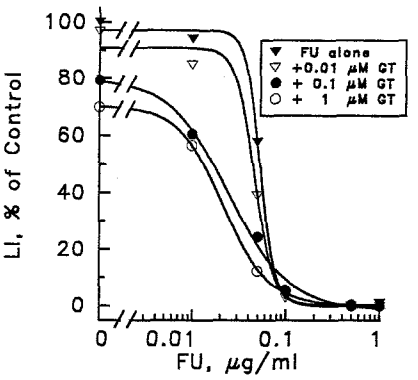

Fig. 4. Antiproliferative effects of FU and GT combinations. A rat colon-tumor explant was exposed to FU and GT for 7 days. The LI was determined by [^{3}H]-TdR incorporation and autoradiography. *Symbols*, Mean experimental data; *curves*, nonlinear least-squares fit according to Eq. 1. FU concentration: 1 μg/ml is equivalent to 7.7 μM. Error bars were omitted for purposes of clarity (SEM, approx. 3%)

However, as our method determines the number of labeled nuclei, a change in specific activity does not alter the LI. TdR has been shown to reverse the cytotoxicity of FU in vitro; the rescue occurs at low (<10 μM) but not at high FU concentrations [8]. The [^{3}H]-TdR concentration used in the present study was only 17 nM, or <0.5% of the 10-μM TdR concentration that is often required to rescue cells from FU toxicity [23].

Effects of single agents

The cultured rat colon tumor responded to FU in a dose-dependent manner; the LI decreased as the FU concentration increased. Figure 2 illustrates a representative concentration-effect relationship from an experiment following a 7-day exposure to FU. The curve is sigmoid and indicates a maximal effect approaching 100% inhibition of the LI. Each data point represents the mean of 10–15 tumor specimens; the SEM was approximately 3% per data point in all tumors studied. The mean IC_{50} value found for the FU-treated samples in six different experiments was 0.355±0.106 μM. LV alone did not reduce the tumor LI (Fig. 3). At concentrations of 0.01–10 μM, GT produced a maximal inhibition of about 30% (Fig. 3). The differences between the samples treated with 0.1–10 μM GT and the untreated controls were statistically significant (P <0.001).

Effects of drug combinations

The addition of LV did not have a significant effect on the antiproliferative activity of FU. Over a concentration span of 0.001–10 μM, LV did not reduce the IC_{50} value for FU (Table 1). The addition of GT to FU produced a shift in the concentration-effect curve; a representative curve is shown in Fig. 4. The IC_{50} value for FU decreased with increasing GT concentration. At concentrations of 0.01 to 10 μM, GT reduced the IC_{50} value for FU by 10%–60% (Table 1). GT concentrations of 0.1 and 1.0 μM reduced the IC_{50} value for FU by 47% and 60% to 0.21 and 0.16 μM, respectively. The effect of GT was most pronounced at FU concentrations that caused <30% inhibition of the tumor LI. The IC_{30} value for FU was decreased by 79% from 0.317 to

Table 1. Effect of LV or GT on the antiproliferative activity of FU

Substance added (μM)	IC_{30} of FU (μM)	% of FU alone	IC_{50} of FU (μM)	% of FU alone	IC_{90} of FU (μM)	% of FU alone
LV:						
0	0.230±0.116	100	0.292±0.116	100	0.560±0.059	100
0.001	0.150±0.027	65.2	0.204±0.033	69.9	0.527±0.031	94.1
0.1	0.206±0.082	89.6	0.265±0.102	90.8	0.557±0.237	99.5
10	0.302±0.023	131.3	0.349±0.048	119.5	0.503±0.169	89.8
GT:						
0	0.317±0.040	100	0.390±0.027	100	0.670±0.064	100
0.01[a]	0.272	85.8	0.344	88.2	0.584	87.2
0.1	0.114±0.099	36.0	0.205±0.108	52.6	0.710±0.039	106.0
1.0	0.066±0.079	20.9	0.155±0.081	39.7	0.570±0.137	85.1
10[a]	0.063	19.9	0.205	52.6	0.605	90.3

Mean IC values ± SD for FU in the presence or absence of LV or GT are shown

[a] Data represent the results of 1 experiment using 50–60 tumor replicates. All other data are from triplicate (LV) or duplicate (GT) experiments

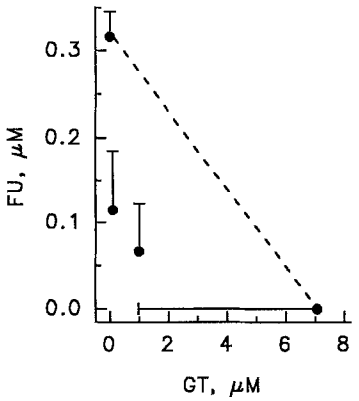

Fig. 5. Evaluation of FU and GT interaction. Isobologram for 30% inhibition of tumor LI by FU and GT used a single agents or in combination. The *dashed line* represents the theoretical antiproliferative additivity between agents. The *points*, representing mean experimental data from duplicate experiments (50–60 tumor replicates per experiment), fall below the dashed line, indicating synergy. *Bars*, SEM. Results of single experiments from Table 1 and Fig. 3 are not included

0.066 μM by 1.0 μM GT. The effect of GT diminished at high FU concentrations that produced >90% inhibition; the IC_{90} value for FU was decreased slightly (15%) from 0.670 to 0.570 μM by 1.0 μM GT.

Evaluation of drug interaction

To determine whether the antiproliferative effect of the GT and FU combination was additive or synergistic, we constructed an isobologram using the GT or FU concentrations required to reduce the tumor LI by 30% (Fig. 5). The maximal inhibitory effect produced by GT alone was 30%. The data points fell below the dashed line, indicating that GT and FU interacted synergistically [24]. Two-way analysis of variance showed a significant synergistic interaction between GT and FU ($P < 0.0001$); this interaction occurred at FU concentrations ranging between 0.08 and 0.4 μM.

Discussion

The rat colon-tumor explants were successfully cultured using the native-state tissue-culture method. This method was first described by Hoffman and co-workers [6] for cultivation of a variety of human solid tumors. The tumor explants are more in vivo-like than are traditional monolayer, suspension, or soft agar cultures. Histopathological examination revealed the maintenance of tissue structural organization and cell morphology in the cultured rat colon tumor. The tumor-cell morphology was maintained for at least 2 weeks in culture. The rat colon tumor grows rapidly in rats, exhibiting a doubling time of 3–4 days [4]. The LI of the rat colon-tumor explants following a 6-h exposure to [^{3}H]-TdR was about 65%, indicating that the high rate of proliferation was maintained under ex vivo conditions. An additional advantage of the explant cultures is the large number of sample replicates that can be studied. In our studies, a single drug-combination experiment used as many as 300 individual tumor replicates. This type of repetition would be prohibitively costly and labor-intensive for an analogous in vivo rodent study. The mean IC_{90} and IC_{95} values obtained following a 7-day exposure to FU in our ex vivo study were 0.748 ± 0.359 μM (range, 0.511–1.47 μM) and 1.22 ± 1.14 μM (range, 0.606–3.54 μM), respectively. In comparison, the FU concentration producing a 67% tumor-free cure rate in rats following a 7-day infusion is 1.54 μM [3]. The comparable effective FU concentrations under in vivo and ex vivo conditions support the use of tissue explants for pharmacological studies.

Our results suggest that LV has no effect in modulating the antiproliferative activity of FU in the rat colon-tumor explants. Rustum [25] has reported preliminary data suggesting an enhancement of the antiproliferative activity of FU by LV against the rat colon tumor in vivo. One explanation for the lack of modulation by LV in the present study may be related to the formation of the ternary complex. Correlations have been reported between the sensitivity of human colorectal xenografts to FU and ternary-complex formation [12]. In xenografts that are resistant to FU, the addition of reduced folates is necessary for the formation of the ternary complex. In xenografts that are sensitive to FU, maximal ternary-complex formation is obtained without the addition of folates. Hence, LV is not expected to enhance the activity of FU in sensitive tumors. It is possible that maximal inhibition of thymidylate synthase was achieved in the FU-sensitive rat colon tumor without the additional LV. Second, the minimal essential medium used to culture the rat colon-tumor explants contained 1 μg/Ml folic acid/ml. Houghton et al. [13, 14] have shown a diminished uptake of folate in the presence of pharmacological folate concentrations. Folate receptors and folate transport are affected by the folate content in the culture medium; the amount of receptor activity increases in folate-depleted medium [15, 16]. It is not clear whether the folate in the culture medium affected the response of the rat colon tumor to the additional LV in vitro. Further studies using folate-free culture medium are needed to define the role of folate in the rat colon-tumor model.

GT has been shown to produce antiproliferative activity and is effective in potentiating the antitumor effect of retinoids against a rat mammary tumor [1, 2]. In the present study, GT alone inhibited the tumor LI and synergistically enhanced the antitumor effect of FU. The mechanisms underlying these effects are not well understood. Several possibilities were considered as follows. First, GT is converted to D-glucaro-1,4,-lactone, an inhibitor of β-glucuronidase [21]. It was tentatively postulated that this active GT metabolite is responsible for the enhancement of the activity of retinoic acid under in vivo conditions [2], but other mechanisms are possible. FU has been shown to undergo glucuronidation in rat hepatocytes in vitro [27]. Although the significance of this pathway for FU elimination in cultured tumor cells has not been established, it is not likely to be a major pathway. Hence, it is unlikely that the lactone metabolite contributed significantly to the potentiation of FU activity. Second, GT is structurally similar to D-glucose, which has been shown to potentiate the effect of FU in vivo [20]. It has been postulated that by

decreasing the extracellular pH from 7.4 to 6.3, glucose may enhance the intracellular entrapment of the active nucleotides. In our experiment, the extracellular pH was not altered by GT. It is likely that the intracellular pH also remained unchanged. The addition of ribose donors in the form of glucose or inosine enhances the conversion of FU to ribonucleotides [17]. Because the capacity for nucleotide formation is an important determinant of sensitivity to FU, the enhanced nucleotide formation may potentiate the effect of FU [18]. The effect of GT on the formation of FU nucleotides needs to be studied.

In summary, the mechanism underlying the activity of GT against the rat colon tumor is not clear. It is interesting that the potentiation of FU activity by GT was most pronounced in the range within which the effect of FU is suboptimal, i.e., <30% inhibition of the tumor LI. The effect of GT was diminished when the FU activity approached its maximum, i.e., >90% inhibition of the tumor LI. The different effects of GT and LV on growth inhibition by FU suggest that GT acts by a mechanism different from the thymidylate synthase-directed effect of LV. These data suggest that the combination of GT with FU will be most advantageous in tumors against which FU shows suboptimal activity or in situations in which the dose of FU is kept at a suboptimal level to minimize toxicity. Further investigations of the mechanism underlying the activity of GT used alone and in combination with FU in FU-sensitive and -resistant tumors are warranted.

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