Preclinical Studies of Anticancer Efficacy of 5-Fluorouracil when Co-Administered with the 1,4-β-D-Galactomannan

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Soluble 1,4-β-D-galactomannan (GM) was obtained from a plant source by controlled acid hydrolysis and further purification under Good Manufacturing Practice (GMP) conditions. Co-administration of the GM along with 5-fluorouracil (5-FU) by intravenous injection to mice bearing human colon tumors (COLO 205 and HT-29) significantly increased efficacy of the 5-FU. This article describes the principal results of three separate preclinical studies, employing (i) COLO 205-bearing mice at one dose of GM and 5-FU; (ii) COLO 205-bearing mice at escalating GM doses in combination with 5-FU; and (iii) HT-29-bearing mice at two GM doses in combination with 5-FU with and without leucovorin. The studies have shown a GM dose-related effect with a maximum efficacy at 120 mg/kg/dose of GM. Effect of an additional oral administration of leucovorin was minimal. Combination of the GM with 5-FU, compared to 5-FU alone, resulted in the decrease in median tumor volume to 17%–65% and an increase in mean survival time (days) to 150%–190%, respectively.

INTRODUCTION

Some carbohydrates have been viewed for a long time as drugs or drug components acting on tumor-associated targets. Various types of cellular interactions mediated by lectins have been studied for the last 20 years. These studies have resulted in the identification of a good number of compounds (monoclonal antibodies, some polysaccharides, and simple sugars) that allegedly interact with lectins on cancer cell surfaces. Some of these compounds reportedly result in inhibition of tumor cell colony development, as described previously (1–5). Simple sugars such as methyl-α-D-lactoside and lacto-N-tetrose have been shown to inhibit metastasis of B16 melanoma cells, while D-galactose and arabinogalactose inhibited liver metastasis of L-1 sarcoma cells (6).

However, to the best of our knowledge: (*i*) no simple sugar drugs, aimed at interactions with lectins, are available in clinical practice; (*ii*) none of these carbohydrates are able to increase in vivo efficacy of known chemotherapy drugs, such as 5-fluorouracil (5-FU), doxorubicin, or others, widely used in cancer chemotherapy; and (*iii*) none of them are able to increase in vivo efficacy of any other drug when co-administered.

Despite advances in chemotherapeutic regimens for metastatic colorectal cancer as well as other solid tumors, patients refractory to these therapies represent a large unmet medical need. One widely used chemotherapeutic drug is 5-FU. It is used alone or in a combination with many other chemotherapeutic agents for treatment of solid tumors, particularly colorectal, breast, hepatic, and gastric cancers (7,8). These combinations, while marginally improving efficacy, may increase toxicity. It is reasonable to hypothesize that more specific targeting of efficacious chemotherapeutic agents, such as 5-FU, to tumor cells could result in higher response rates along with reduced toxicity.

Compared to many other known polysaccharides, galactomannans have multiple side-chain galactose units that should readily interact with galactose-specific receptors (such as galectins on the tumor cell surface), modulate the tumor surface physiology, and potentially affect delivery of 5-FU to the tumor.

In this report, we describe the principal results of three preclinical efficacy studies that employed a relatively simple polysaccharide, 1,4-β-D-galactomannan (GM). This polysaccharide was obtained by a controlled partial chemical degradation of a high molecular weight galactomannan from *Cyamopsis tetragonoloba*, or guar gum. Intravenous (i.v.) injection of this modified lower molecular weight galactomannan along with 5-FU increased the efficacy of the 5-FU in tumor-bearing mice. The studies

Table 1. Tumor Volumes, Dynamics of Tumor Volumes, and Survival Time for COLO 205 Human Tumor-Bearing Mice Treated with 5-FU, GM, and Their Combination on a q4d × 3 Schedule

	Saline (control)	5-FU (75 mg/kg)	GM (120 mg/kg)	5-FU plus GM (75 + 120 mg/kg)
Median time to quadrupling of tumor volume (days)	12.5	23.7	15.5	56.0
Median tumor volume (mm^3) at the end point (56 days after treatment initiation)	2058	2254	1813	379
Mean survival time (days)	14.2	23.7	19.2	44.2

were performed at Southern Research Institute, Birmingham, AL (studies 1 and 2) and Charles River Laboratories, Wilmington, MA (study 3).

RESULTS

Study 1

Effect of GM on efficacy of 5-FU in NCr-*nu* mice with subcutaneous (s.c.) implants of COLO 205 human colon tumor at one GM dose. This study employed a relatively high dose of 5-FU (75 mg/kg/dose) that exceeded the maximum tolerated dose in mice. The data are shown in Table 1 and Figure 1. Their statistical analysis is illustrated in Table 2.

Untreated control tumors grew well in all mice, with the median number of days for quadrupling the tumor volume equal to 12.5 days. There was no tumor regression after 56 days of the study, and there was practically no tumor reduction. Median tumor volume increased from 111 mm^3 at treatment initiation (in this case with saline only) to 2058 mm^3 after 5–8 weeks. Mean survival time was equal to 14.2 days.

A dosage of 75 mg/kg/dose of 5-FU (that is, 225 mg/kg total dose over 8 days) was in excess of the maximum tolerated dosage and produced treatment-related deaths for three mice out of ten often within 2 weeks. The treatment caused a delay in the median number of days to quadrupling the tumor volume from 12.5 to 23.7 days. Again, there was no tumor regression after 56 days of the study; however, two relatively small tumors were observed that grew from 75 mm^3 each, at initiation of treatment, to 126 and 567 mm^3 by the end of the study. Median tumor volume increased from 101 mm^3 at treatment initiation to 2254 mm^3 after 56 days of the study. Mean survival time shifted from 14.2 days (untreated control animals) to 23.7 days.

GM, at a dosage of 120 mg/kg/dose administered alone on a q4d (once every 4 days) × 3 schedule, was well-tolerated. No deaths or body weight loss was observed. The median number of days to quadrupling the tumor volume equaled 15.5 days, which is slightly longer than the value for untreated animals (12.5 days). There was no tumor regression after 56 days of study, however, two relatively small tumors (compared to median tumor volume)

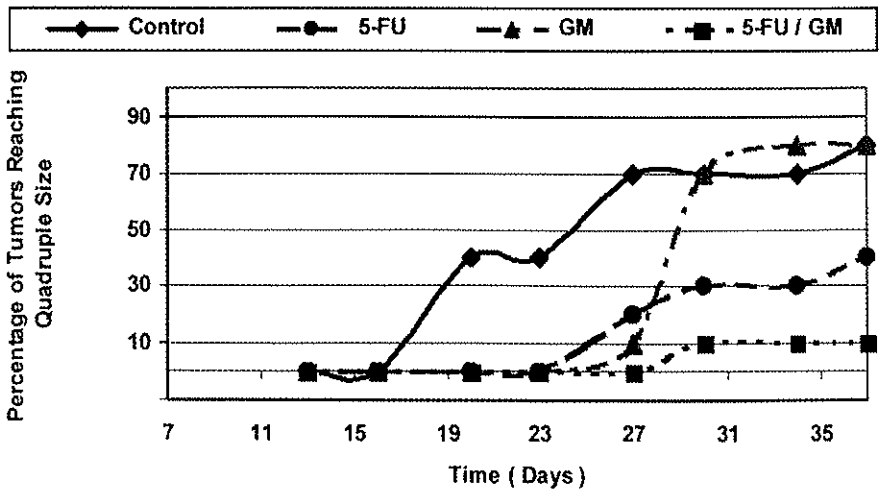


Figure 1. Effect of GM (120 mg/kg/dose), 5-FU (75 mg/kg/dose), and their combination in a q4d × 3 regimen on tumor volume in NCr-*nu* mice with s.c. implants of COLO 205 human colon tumor (study 1).

Table 2. Statistical Analysis of Tumor Volumes for COLO 205 Human Tumor-Bearing Mice Treated with 5-FU[a], GM[b], and Their Combination on a q4d × 3 Schedule, for all Data Points from the Day of Treatment Initiation Onward

Treatment group	Observations (n)	Mean (x̄)	P Value[c]	P Value[d]
Control	146	1174	—	<0.0001
GM	162	962	0.0237	<0.0001
5-FU	118	652	<0.0001	0.0004
5-FU plus GM	103	358	<0.0001	—

[a]75 mg/kg/dose.

[b]120 mg/kg/dose.

[c]One-sided Student's *t* test *P* value, compared to the control (untreated animals).

[d]One-sided Student's *t* test *P* value, compared to the 5-FU plus GM treatment.

were observed that grew from 100 and 126 mm^3, at initiation of treatment, to 270 mm^3 and 729 mm^3, respectively, by the end of the study. Median tumor volume increased from 100 mm^3, at treatment initiation, to 1813 mm^3 after 56 days of the study, which is noticeably less compared to 2058 mm^3 for untreated animals and 2254 mm^3 for 5-FU (75 mg/kg/dose)-treated animals. Mean survival time was prolonged from 14.2 days (untreated control animals) to 19.2 days.

Co-administration of GM (120 mg/kg/dose) and 5-FU (75 mg/kg/dose) on a q4d × 3 schedule resulted in a remarkable effect, which caused a significant delay in quadrupling of the tumor volume from 12.5 days for untreated animals (control) and 23.7 and 15.5 days for 5-FU alone and GM alone, respectively, to 56.0 days for their combination. There was one tumor that completely disappeared by the end of the study. Two more tumors were relatively small in size, less than 20% that of the control value, by the end of the study. Overall, median tumor volume increased from 111 mm^3 at treatment initiation to only 379 mm^3 after 56 days of study, a value significantly less than that for untreated animals or animals treated with 5-FU alone. Mean survival time increased from 14.2 days (untreated control animals) and 23.7 days (5-FU treatment) to 44.2 days for the combination treatment.

Study 2

Effect of GM on efficacy of 5-FU in NCr-nu mice with s.c. implants of COLO 205 human colon tumor at an escalated GM dose. The principal differences from the first study were: (*i*) there were four consecutive injections, not three; (*ii*) there were four doses of the galactomannan tested, not one; and (*iii*) 5-FU was administered (i.v.) at a more well-tolerated dose of 5-FU (48 mg/kg), compared to that used in the first study (see above). The data are shown in Table 3 and Figure 2. Results of their statistical analysis are provided in Table 4.

No mice died in this study. As in the preceding study, untreated control tumors grew well in all mice. The median number of days to quadrupling the tumor volume equaled 7.2 days. No tumor regression or reduction occurred after 13 days of the study. Median tumor volume increased from 162 mm^3, at treatment initiation (in this case with saline only), to 1288 mm^3 after 13 days.

A dosage of 48 mg/kg/dose of 5-FU (192 mg/kg total dose over 12 days) was well tolerated and produced some growth delay in the median number of days to quadrupling the tumor volume, increasing it from 7.2 to 8.7 days. Two tumors in the group of 10 mice were significantly (three times or more) smaller, compared with the median tumor size, after 13 days of treatment, growing from 100 and 163 mm^3 at initiation of treatment to 270 and 138 mm^3, respectively, by the end of the study. Median tumor volume increased from 172 mm^3 at treatment initiation to 800 mm^3 after 13 days of the study, which was less than the control value 1288 mm^3.

Co-administration of 5-FU (48 mg/kg/dose) and GM (6, 30, 120, and 600 mg/kg/dose) on

Table 3. Dynamics of Tumor Volumes for COLO 205 Human Tumor-Bearing Mice Treated with 5-FU, GM, and Their Combination on q4d × 4 Treatment Schedule

	Saline (control)	5-FU (48 mg/kg)	GM (120 mg/kg)	5-FU plus GM (48 + 6 mg/kg)	5-FU plus GM (48 + 30 mg/kg)	5-FU plus GM (48 + 120 mg/kg)	5-FU plus GM (48 + 600 mg/kg)
Median time to quadrupling of tumor volume (days)	7.2	8.7	6.9	14.8	13.5	16.5	16.2
Median tumor volume (mm^3) at the end point (13 days after treatment initiation)	1288	800	1152	715	695	540	588

a q4d × 4 schedule was well-tolerated at all dosages tested and caused a significant delay in quadrupling of the tumor volume, from 7.2 days for untreated animals (control) and 8.7 and 6.9 days for 5-FU alone and GM alone, to 14.8, 13.5, 16.5, and 16.2 days for 5-FU and GM combined, respectively (Table 3). The best results were obtained with a combination of 5-FU and 120 mg/kg/dose GM, which resulted in a median tumor volume of 540 mm^3 at day 13, the day after the final day of treatment, compared with that of 800 mm^3 for 5-FU treatment alone. Also, the median number of days for quadrupling the tumor volume was almost twice as much for the 5-FU plus GM 120 mg/kg/dose than for the 5-FU alone (Table 3).

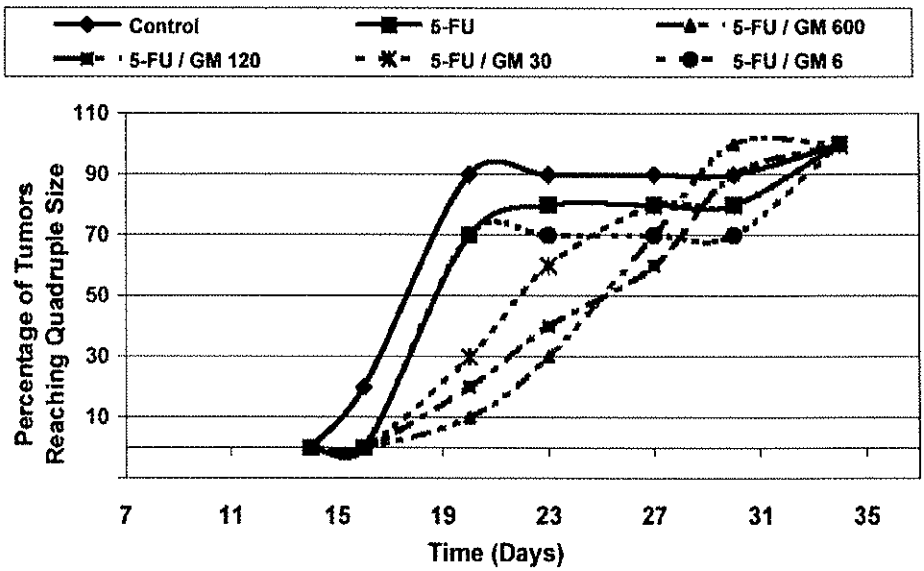


Figure 2. Effect of GM at escalating doses (6, 30, 120, and 600 mg/kg/dose), 5-FU (48 mg/kg/dose), and their combination in a q4d × 4 regimen on tumor volume in NCr-*nu* mice with s.c. implants of COLO 205 human colon tumor (study 2).

Study 3

Effect of GM on efficacy of 5-FU in NU/NU-nuBR mice with s.c. implants of HT-29 human colon tumor. The principal differences from the second study were: (*i*) another tumor (HT-29) was used; and (*ii*) leucovorin was added to the treatment regimen (see Methods and Materials). The data are shown in Table 5 and Figure 4. Their statistical analysis is provided in Table 6.

As in the two preceding studies, control (untreated) tumors grew well in all mice, with a median of 13.3 days for quadrupling of the tumor volume. Median tumor volume increased from 196 mm^3, at treatment initiation (day 7 after tumor implantation), to 1318 mm^3 after 26 days.

A dosage of 48 mg/kg/dose of 5-FU (192 mg/kg total dose over 12 days of the treatment) along with an oral administration of leucovorin as described above was within the maximum-tolerated dosage, producing no treatment-related deaths in the group of 10 mice within 3 weeks. The treatment caused a delay of 2 days for the quadrupling of the tumor volume (from 13.3 to 15.3 days). Median tumor volume increased from 179 mm^3, at treatment initiation, to 1120 mm^3 on study day 26. Co-administration of

Table 4. Statistical Analysis of Tumor Volume for COLO 205 Human Tumor-Bearing Mice Treated with 5-FU[a], GM, and Their Combination on a q4d × 4 Schedule, on Day 13 after Treatment Initiation and from the Day of Treatment Initiation Onward

Treatment Group	Mean (x̄) at Day 13 ($n = 10$)	P Value[b]	Mean (x̄) for all Data Points ($n = 50$)	P Value[c]
Control	1232	0.0055	652	0.00359
5-FU	736	—	419	—
5-FU plus GM (6 mg/kg/dose)	646	0.2496	392	0.3149
5-FU plus GM (30 mg/kg/dose)	594	0.1259	356	0.1201
5-FU plus GM (120 mg/kg/dose)	544	0.0773	342	0.0740
5-FU plus GM (600 mg/kg/dose)	576	0.0904	335	0.0509

[a]48 mg/kg/dose.

[b]One-sided Student's t test P value, compared to the 5-FU treatment alone on day 13.

[c]One-sided Student's t test P value, compared to the 5-FU treatment alone, for all data points.

Table 5. Tumor Volumes and Dynamics of Tumor Volumes for HT-29 Human Tumor-Bearing Mice Treated with 5-FU, Leucovorin, GM, and Their Combination on a q4d × 4 Treatment Schedule

	Saline (control)	GM plus Leucovorin (120 + 25 mg/kg)	5-FU plus Leucovorin (48 + 25 mg/kg)	5-FU plus GM plus Leucovorin (48 + 30 + 25 mg/kg)	5-FU plus GM plus Leucovorin (48 + 120 + 25 mg/kg)
Median time to quadrupling of tumor volume (days)	13.3	12.5	15.3	18.1	23.5
Median tumor volume (mm^3) at the end point (26 days after treatment initiation)	1318	1595	1120	1521	729

5-FU with GM (30 mg/kg/dose), along with an oral dose of leucovorin as described above, brought further delay in tumor growth, particularly in the first half of the study: quadrupling of the tumor volume occurred from 15.3 days without GM to 18.1 days with GM (Table 5).

Increasing the GM dose to 120 mg/kg/dose in co-administration with 5-FU on a q4d × 4 schedule along with an oral administration of leucovorin, as described above, again produced a significant delay in the quadrupling of the tumor volume: from 13.3 days for untreated control animals and 15.3 days for 5-FU/leucovorin-treated animals to 23.5 days for animals treated with all three drugs. Furthermore, when all three drugs were used in combination, one tumor completely disappeared 4 weeks after treatment initiation, two more tumors were of a relatively small size (269 and 352 mm^3) by the end of the study, and three additional tumors were practically stabilized at a volume well below 1000 mm^3. Overall, median tumor volume increased from 176 mm^3 at treatment initiation to only 729 mm^3 at study day 26 (significantly less than the 1318 mm^3 for untreated animals and 1120 mm^3 for 5-FU plus leucovorin-treated animals).

In summary, statistical evaluation of the data indicates the following. In the first study (COLO 205 tumors treated with a high 5-FU dose), there was a significant advantage for 5-FU plus GM versus the control ($P < 0.0001$) and versus GM alone

Table 6. Statistical Analysis of Tumor Volume for HT-29 Human Tumor-Bearing Mice Treated with Combinations of 5-FU[a], Leucovorin[b], and GM on a q4d × 4 Schedule, on Day 26 after Treatment Initiation and for All Data Points from the Day of Treatment Initiation Onward

Treatment group	Mean (X) at Day 26 (n = 7–10)	Mean (X) for all data points (n = 21–80)	P value for All Data Points
Control	1624	801	0.0778[c] 0.4559[d]
5-FU plus leucovorin	1438	635	0.0778[c]
5-FU plus leucovorin plus GM (30 mg/kg/dose)	1519	540	0.1796[c]
5-FU plus leucovorin plus GM (120 mg/kg/dose)	516	455	0.0255[c] 0.0051[d]

[a]48 mg/kg/dose.

[b]25 mg/kg/dose.

[c]One-sided Student's t test P value, compared to the 5-FU plus leucovorin treatment.

[d]Two-sided Student's t test P value, compared to the galactomannan plus leucovorin treatment.

[e]One-sided Student's t test P value, compared to control (untreated animals).

($P < 0.0001$). The difference between 5-FU plus GM and 5-FU was significant at a confidence level higher than 99%. In the second study (COLO 205 tumors treated with a low 5-FU dose), there was a significant advantage for 5-FU plus GM 120 mg/kg/dose versus 5-FU alone (with a 93%–95% confidence level), which numerically favored the combination of 5-FU with the GM. In the third study (HT-29 tumors), there was a significant advantage for 5-FU/leucovorin plus GM 120 mg/kg/dose versus 5-FU/leucovorin with a 97% confidence level, which again numerically favored the combination of 5-FU/leucovorin with GM.

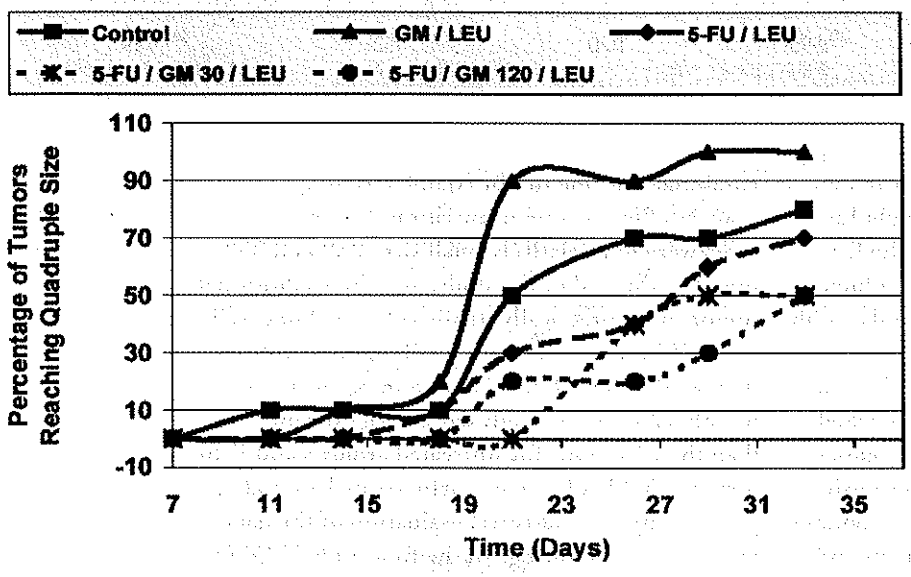


Figure 3. Effect of GM (30 and 120 mg/kg/dose), 5-FU (48 mg/kg/dose), and leucovorin (p.o., 25 mg/kg/dose), and their combination in a q4d × 4 regimen on tumor volume in NU/NU-*nu*BR mice with s.c. implants of HT-29 human colon tumor carcinoma (study 3).

DISCUSSION

The described studies form an experimental basis for our long-range goal of discovering mechanism-based chemotherapeutic agents that reduce the toxicity and increase the efficacy of co-administered antineoplastic agents. Co-administration of 5-FU with GMs with certain chemical structures (including a specific mannose/galactose ratio and molecular size/weight), which specifically interact with galactose-specific lectins (galectins) on the tumor cell surface, apparently facilitates delivery of 5-FU into cancer cells. While details of this mechanism are not

within the scope of this report, the current observations were basic for determining the logistics and the experimental design of the preclinical efficacy studies described in this paper.

The Materials and Methods section describes purification, controlled partial hydrolysis, and characterization of the GM from guar gum, *C. tetragonoloba*. A physical combination of a GM with 5-FU was filed with the FDA as a part of a specific Investigational New Drug (IND) and was approved for clinical trials. Summarized in Figure 4, GM increases the efficacy of 5-FU, in terms of decreasing median tumor volume and increasing mean survival time. The GM co-administered along with 5-FU by i.v. once every 4 days for a total of three injections (study 1) or four injections (studies 2 and 3) results in the decrease of median tumor volume to 18%, 42%, and 55% compared to control (untreated animals) and to 17%, 68%, and 65%, respectively, compared to 5-FU alone. Furthermore, the co-administration of GM and 5-FU compared to 5-FU alone results in the increase of mean survival time (days) to 190%, 190%, and 150%, for the three studies, respectively. The significance of these findings is increased because the effects were reproduced in kind using two different human colon tumors (COLO 205 and HT-29), as documented by two independent commercial testing facilities (Southern Research Institute and Charles River Laboratories).

The GM used here was thoroughly characterized using high-performance liquid chromatography (HPLC), multi-angle laser light scattering (MALLS), nuclear magnetic resonance (NMR), and quantitative chemical analysis. All of the solutions for i.v. injections were subjected to a tight quality control, using HPLC analysis in a linear range of GM and 5-FU concentrations. In addition, leucovorin did not interfere with GM when both compounds were combined with 5-FU (study 3). This nonclinical data has important clinical implications, because GM/5-FU may be administered in the future to patients treated with the 5-FU/leucovorin combinations.

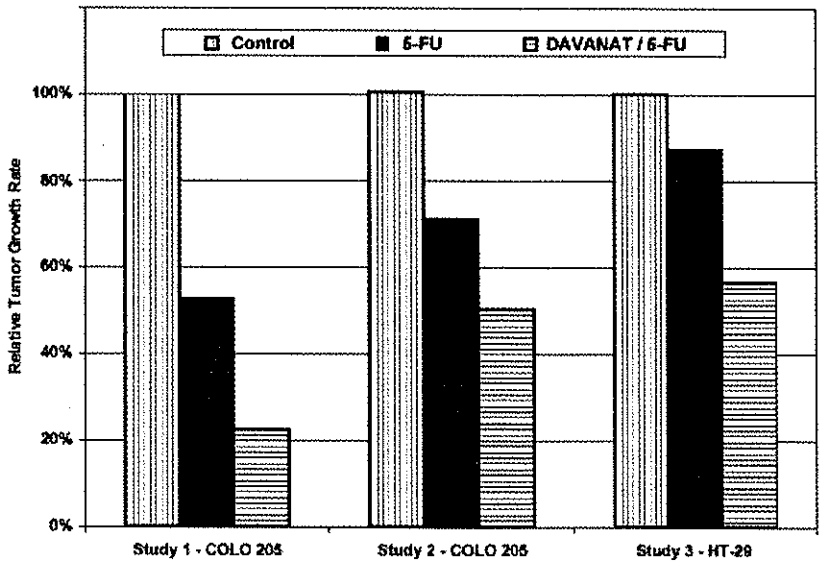
MATERIALS AND METHODS

Purification, Modification, and Characterization of the GM from *C. tetragonoloba*

GM was isolated from *C. tetragonoloba* (guar gum) using a procedure of water extraction and ethanol precipitation of GM, followed by purification via Cu^{2+} complex (9). The purified GM was partially hydrolyzed with HCl (pH 2.0–2.3) at controlled conditions, washed with ethanol, and dried. The final yield was 50% from the weight of guar gum flour. Molecular weight of the GM from *C. tetragonoloba* was 54 kDa (MALLS) and 95 kDa (viscometry). Mannose/galactose ratio (by the NMR and chemical analysis after complete acid hydrolysis) of the GM was 1.7.

Quantitative Determination of the GM and 5-FU

The compounds were analyzed in 0.9% saline, pH 9.2, using HPLC with UV detection at 254 nm (for 5-FU) and refractive index detection (for GM).



□, Untreated control animals; ■, 5-FU alone or in the presence of leucovorin (study 3); ▤, 5-FU plus GM (studies 1 and 2) or 5-FU plus GM plus leucovorin (study 3).

Figure 4. Enhancement of 5-FU efficacy with GM on two human colon cancer tumors in mice in three separate efficacy studies: study 1, mice bearing COLO 205 human colon tumor (5-FU 75 mg/kg/dose, GM 120 mg/kg/dose); study 2, mice bearing the same tumor (5-FU 48 mg/kg/dose, GM 120 mg/kg/dose); and study 3, mice bearing HT-29 human colon carcinoma [5-FU 48 mg/kg/dose, GM 120 mg/kg/dose, leucovorin (p.o.) 25 mg/kg/dose].

Stock solutions for 5-FU and GM were in the range of 0.64–5.5 and 0.8–8.0 mg/mL, respectively. The procedure was applicable (after dilution for the analysis) at concentrations of 5-FU from 0.0128 to 0.128 mg/mL and GM from 0.8 to 8.0 mg/mL. At these concentration ranges, both of the analyses were linear, reproducible, and highly accurate.

Efficacy Experiments on Tumor-Bearing Mice: GM, 5-FU, Leucovorin (in Study 3), and Their Combinations

In the first two studies, male NCr-*nu* athymic nude mice (Frederick Cancer Research and Development Center, Frederick, MD, and Taconic Farms, Germantown, NY, respectively) were acclimated in the laboratory 1 week prior to experimentation. The animals were housed in microisolator cages, five per cage, and using a 12-hour light/dark cycle. Weight of the animals was in the range of (*i*) 25–34 g on day 13 of the first study (day of treatment initiation); (*ii*) 21–33 g on day 14 of the second study (day of treatment initiation); and (*iii*) 24–25 g on day 7 of the third study (day of treatment initiation). In the third study, female NU/NU-*nu*BR athymic nude mice were housed as in the previous two studies. All animals in the three studies received sterilized tap water and sterile rodent food ad libitum. The animals were observed daily, and clinical signs were noted. The mice were healthy and had not been previously used in other experimental procedures. The mice were randomized and were comparable at the initiation of treatment in all three studies.

The first two efficacy studies were conducted as follows. Thirty- to forty-milligram fragments from an in vivo passage of COLO 205 human colon tumor were implanted s.c. in mice near the right axillary area using a 12-gauge trocar needle. Tumors were allowed to reach 75–198 mm^3 in size/volume before the start of treatment. A sufficient number of mice were implanted, so that tumors in a volume range as narrow as possible were selected for the trial on the day of treatment initiation (day 13 or 14 after tumor implantation in the first and second study, respectively). Those animals selected with tumors in the proper size range were divided into the various treatment groups of 10 mice in each. The median tumor volumes in each treatment group ranged from 94 to 117 mm^3 in the first study and from 150 to 172 mm^3 in the second study.

In the third efficacy study, human colon carcinoma (HT-29) cells were injected (with 5×10^6 cells) into the right lateral thorax of 70 female nude mice, of which 50 tumor-bearing mice were used on the study. Tumors were allowed to reach 100–200 mm^3 in volume before the start of treatment. Those animals selected with tumors in the proper size range were divided into five treatment groups of 10 mice in each. The median tumor volumes in each treatment group ranged from 172 to 196 mm^3.

Study duration was 70 days after tumor implantation or 56 days after treatment initiation in the first study; 27 days after tumor implantation or 13 days after treatment initiation in the second study; and 33 days after tumor implantation or 26 days after treatment initiation in the third study.

The s.c. tumors were measured, and the animals were weighed twice weekly starting with the first day of treatment. Tumor volume was determined by caliper measurements (mm) and using the formula for an ellipsoid sphere: $L \times W^2/2 = mm^3$, where L and W refer to the dimensions for length and width collected at each measurement.

Drug Formulation and Administration

Study 1. There was a total of four groups of 10 animals each, s.c.-implanted with COLO 205 human colon tumor xenografts. The groups were treated by i.v. on day 13 after tumor implantation on a q4d × 3 schedule as follows: (*i*) saline (NaCl, 0.9%); (*ii*) 5-FU, 75 mg/kg/dose (225 mg/m^2/dose); (*iii*) GM, 120 mg/kg/dose (360 mg/m^2/dose); and (*iv*) 5-FU (75 mg/kg/dose) plus GM (120 mg/kg/dose).

5-FU was formulated in fresh saline on each day of treatment at a concentration of 3.75 mg/mL, pH 9.2. In the groups where GM and 5-FU were coadministered, GM powder was dissolved in the 5-FU solution to yield the GM concentration of 6 mg/mL and 5-FU concentration of 3.75 mg/mL. Both individual compounds and their mixture were administered according to exact body weight, with the injection volume being 0.2 mL/10 g body weight.

Study 2. There were a total of seven groups of 10 animals each, implanted s.c. with COLO 205 human colon tumor xenografts. The groups were treated by i.v. on day 14 after tumor implantation on q4d × 4 schedule as follows: (*i*) saline (NaCl,

0.9%); (*ii*) 5-FU (48 mg/kg/dose); (*iii*) GM (120 mg/kg/dose); (*iv*) 5-FU (48 mg/kg/dose) plus GM (6 mg/kg/dose); (*v*) 5-FU (48 mg/kg/dose) plus GM (30 mg/kg/dose); (*vi*) 5-FU (48 mg/kg/dose) plus GM (120 mg/kg/dose); and (*vii*) 5-FU (48 mg/kg/dose) plus GM (600 mg/kg/dose).

5-FU was formulated in fresh saline on each day of treatment at a concentration of 4.8 mg/mL, at pH 9.2. In the groups where GM and 5-FU were co-administered, GM powder was dissolved in the 5-FU solution to yield the GM concentration of 0.6, 3.0, 12, and 60 mg/mL and 5-FU concentration of 4.8 mg/mL.

Study 3. There were a total of five groups of 10 animals each, implanted s.c. with HT-29 human colon carcinoma xenografts. The groups were treated (i.v., except leucovorin) on day 7 after tumor implantation on q4d × 4 schedule as follows: (*i*) saline (NaCl, 0.9%); (*ii*) GM (120 mg/kg/dose) plus leucovorin (by mouth [p.o.], 25 mg/kg/dose); (*iii*) 5-FU (48 mg/kg/dose) plus leucovorin (p.o., 25 mg/kg/dose); (*iv*) 5-FU (48 mg/kg/dose) plus GM (30 mg/kg/dose) plus leucovorin (p.o., 25 mg/kg/dose); and (*v*) 5-FU (48 mg/kg/dose) plus GM (120 mg/kg/dose) plus leucovorin (p.o., 25 mg/kg/dose). Leucovorin was administered by oral gavage (p.o.), 2 hours after the injection (via tail vein), at a dose of 25 mg/kg/dose on the same q4d × 4 schedule.

GM was formulated in 0.9% fresh sterile saline on each day of treatment at a concentration of 12 mg/mL. Leucovorin powder (clinical formulation, leucovorin calcium for injection) was reconstituted with 0.9% sterile saline to yield a concentration of 2.5 mg/mL. 5-FU and combinations of 5-FU with GM were formulated as in study 2. Both individual compounds and their mixture were administered according to exact body weight with the injection volume being 0.1 mL/10 g body weight.

Statistical Methods

Study 1. All available tumor volume data points in all animal groups (control and treatment groups), from the day of treatment initiation onward, were evaluated using unpaired one-sided Student's *t* test. This was justified, since the research hypothesis (and its experimental proof) that combination of 5-FU and GM decreases tumor volumes compared to control, GM alone, and 5-FU alone was directional and permitted a one-tail test of significance. In these calculations, only animals that were alive at the time of the post-baseline evaluation were included, as well as in the calculations of the means and medians. The major comparisons of interest were against the 5-FU plus GM combination. For these comparisons, the combination treatment served as the reference group, while the remaining treatment variables were converted into three indicator variables. The data were modeled using PROC MIXED with the indicator treatment variables, time, and the resulting interaction terms. The CONTRAST and LSMEANS (using Dunnett's adjustment for multiple comparisons) procedures were used to evaluate the differences between the combination treatment group and the three other treatment groups.

Study 2. The median tumor weighs were calculated for day 27, which was the day after treatment was complete, and at each post-baseline time. The serial tumor volumes were each analyzed using a longitudinal growth model to evaluate the post-baseline slopes for each treatment group from day 16 through day 27. The model used all available tumor volume data, since no animals died or were sacrificed before the final visit in this study. The major comparisons of interest were against the 5-FU alone group; comparisons against the control (untreated group of animals) and the GM alone groups are also provided for completeness. Statistical evaluation was performed as described above for the preceding study.

Study 3. The median tumor volumes were calculated for each post-baseline time. In these calculations, only animals that were alive at the time of the post-baseline evaluation were included. The serial tumor volumes were analyzed using a longitudinal growth model to evaluate the post-baseline slopes for each treatment group from day 11 through day 33. The model used all available tumor volume assessments, however, no data was included if animals died or were sacrificed before the final visit. The major comparisons of interest were against the 5-FU plus leucovorin group. The data were modeled using statistical tools as described above.

ACKNOWLEDGMENTS

We thank colleagues from Southern Research Institute (SRI) and Charles River Laboratories for

conducting animal studies, Drs. Yulia Maxuitenko (SRI, Birmingham, AL) and Philip T. Lavin (Averion, Framingham, MA) for conducting statistical analysis of the data, and Drs. Mildred Christian and Robert Diener (Argus International, Horsham, PA) for discussing the data and the manuscript.

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