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Combination therapy with 5-fluorouracil and L-canavanine: *in vitro* and *in vivo* studies

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L-Canavanine (CAV) is a potent L-arginine antagonist, produced by legumes such as the jack bean, *Canavalia ensiformis*. CAV is cytotoxic to MIA PaCa-2 human pancreatic cancer cells. We sought to determine whether CAV's efficacy as an anticancer agent might be increased in combination with 5-fluorouracil (5-FU), a pyrimidine antimetabolite with activity against solid tumors. Using optimal conditions for the expression of CAV's cytotoxicity against MIA PaCa-2 cells, CAV was more cytotoxic to the cells than 5-FU. The combination of both drugs at a fixed molar ratio of 1:1 exhibited synergistic effects in the cells as determined by combination index analysis. The combination of 5-FU:CAV was tested at a ratio of 5:1 and exhibited antagonism at lower effect levels, additivity at 50% effect levels and slight synergism at higher effect levels. A 10:1 combination of both drugs (5-FU:CAV) exhibited antagonistic effects at all levels. When the drugs were combined at a molar ratio of 20:1, increased antagonism was observed. When CAV (1.0 or 2.0 g/kg daily) and/or 5-FU (35 mg/kg daily) was administered to colonic tumor-bearing rats for five consecutive days, the antitumor activity of the drug combination was significantly greater than the combined effects of either drug alone. However, the body weight loss experienced by CAV-treated rats was increased in those rats exposed to a combination of both drugs. These studies using different tumors provide *in vitro* and *in vivo* evidence that combination therapy offers a viable means of improving CAV's intrinsic efficacy while decreasing the concentration of 5-FU required to produce the same cytotoxic effect. On the other hand, greater success is needed in further reducing body weight loss. Current efforts at CAV analog development are focusing on ways to amplify its antineoplastic activity while reducing significant body weight loss in order to produce a clinically useful therapeutic agent for the treatment of human pancreatic cancer.

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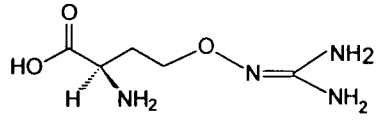
Introduction

L-Canavanine (CAV), L-2-amino-4-(guanidinooxy)-butyric acid, is a potent antimetabolite and structural analog of L-arginine (ARG) characterized by the replacement of the terminal methylene group of ARG with an oxygen.[1]



L-canavanine

CAV's deleterious biological effects are revealed dramatically by the striking developmental aberrations resulting from larval consumption of this toxicant by a CAV-sensitive insect such as the tobacco hornworm, *Manduca sexta*.[2] Insectan studies with *M. sexta* have established that CAV is activated via aminoacylation by arginyl-tRNA synthetase to generate structurally anomalous, canavanyl proteins[3] in which CAV has replaced ARG residues. CAV-containing proteins exhibit altered conformation; typically they also suffer lost function (reviewed in Rosenthal[4]). Recent experiments have linked canavanyl protein formation to the developmental aberrations observed in CAV-treated *M. sexta* larvae.[5]

In the earliest report of CAV's antitumor properties, Green *et al.*[6] observed that CAV-treated mice, bearing L1210 leukemic cells, had an increase in lifespan of 44% compared with control animals. CAV also amplified the palliative effects of gamma-irradiation on a human tumor cell line.[7] More recently, Thomas *et al.*[8] provided the first demonstration that CAV can attenuate significantly solid rat colonic

tumor growth in male Fischer F433 rats. For example, in this study administration of CAV at 2.0 g/kg for 5 days produced a tumor versus control growth value of −13% after 5 days. The negative values indicate regression of the tumor. However, CAV's therapeutic potential was compromised by its cumulative toxicity as expressed by a weight loss of 19% in animals dosed with 3.0 g/kg CAV for 5 days.[8]

In vivo studies with radiolabeled CAV administered orally or by subcutaneous injection to rats by Thomas and Rosenthal,[9] showed that CAV was incorporated preferentially into newly synthesized pancreatic proteins. Swaffar *et al.*[10] recently reported that CAV exhibited cytotoxicity against the human pancreatic adenocarcinoma cell line, MIA PaCa-2, and that ARG dramatically reversed the deleterious effects of CAV in this cell line; after 18 h this inhibition became irreversible.

Based on previous investigations, we sought to determine if CAV might be more efficacious when combined with other antineoplastic agents. Chemotherapy of pancreatic carcinoma has centered around 5-fluorouracil (5-FU). However, about 80% of patients receiving this drug do not experience significant tumor response (i.e. significant reduction in tumor size). Indeed, survival rates are not enhanced with chemotherapy (reviewed in Arbuck[11]). Combinations of potentially synergistic chemotherapeutic agents such as cisplatin, doxorubicin, streptozotocin or mitomycin C with 5-FU do not result in an improved outcome.[11] 5-FU plus radiation is associated with modest improvement in patient outcome. 5-FU is also the foundation of treatment for colorectal carcinoma, and since CAV is active against a rat colon tumor, we tested these drugs in combination with this animal model.

We are also actively synthesizing novel analogs of CAV in order to enhance drug potency while decreasing cumulative toxicity. As part of our ongoing assessment of CAV as a chemotherapeutic agent, we have now determined the cytotoxicity of 5-FU and CAV in an *in vitro* system employing MIA PaCa-2 cells and the antitumor activity of this drug combination in an *in vivo* system that used a solid rat colonic tumor. We report that the combination of 5-FU and CAV has enhanced activity in an *in vivo* system and in an *in vitro* system when combined in a molar ratio of 1 compared with the combined effects of each drug given alone. As the molar ratio of 5-FU:CAV was increased, the combination became less synergistic. At a molar ratio of 20:1, effects of the two drugs were purely antagonistic.

Materials and methods

Drugs and chemical reagents

CAV was isolated from acetone-defatted jack bean seeds, *Canavalia ensiformis* (Leguminosae), purified by ion-exchange chromatography and crystallized from ethanol–water.[12] The crystallized CAV was treated with decolorizing charcoal and recrystallized as described. Elemental analysis, melting point, automated amino acid analysis of a highly concentrated solution of CAV and NMR evaluations were conducted periodically. These determinations confirmed that our preparative procedures consistently yielded material of at least 99% purity that was free of detectable contaminant amino acid. Doses for injection were prepared by dissolving the appropriate drug in 0.9% (w/v) NaCl (pH 8.1). For the *in vitro* cytotoxicity assays, CAV was dissolved in sterile phosphate-buffered saline (PBS). MTT reagent and 5-FU were purchased from Sigma (St Louis, MO). Media reagents were purchased from Gibco/BRL (Grand Island, NY).

Animals and tumor cell lines

MIA PaCa-2 cells were grown in Dulbecco's modified eagle's medium (D-MEM) (Gibco/BRL) containing 4.5 g glucose/l. This medium was supplemented with a penicillin/streptomycin solution (100 units/ml and 100 μg/ml, respectively), 10% calf serum [consisting of 10 parts fetal bovine serum and 90 parts newborn calf seum (Gibco)], 0.25 μg/ml Fungizone and 1% (w/v) L-glutamine. The cells were maintained at 37°C in a humidified 5% CO_2 atmosphere and subcultured every 4–5 days. An arginine-reduced medium (ARM), containing 0.4 μM ARG, was prepared as described previously.[10]

Male Fischer rats, weighing 150–175 g, were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Rats were housed five to a cage in polycarbonate cages with sawdust bedding. They received Purina Rodent Laboratory Chow no. 5001 and tap water *ad libitum* throughout the experiments. The rat colon carcinoma was obtained from Dr Jerrold Ward (NIH, Bethesda, MD) and has been described elsewhere.[8]

In vitro cytotoxicity versus MIA PaCa-2 cells

The protocol used for the MTT assay with several modifications was similar to that reported pre-

viously by Swaffar *et al.*[13,14] MIA PaCa-2 cells in ARM were incubated on microtiter plates as previously described.[10] Cells were treated with CAV alone, 5-FU alone and in combination. When used individually, the stock solution of each drug was six times the final desired concentration. An aliquot of 20 μl of this stock solution was then added to 100 μl of cell suspension in wells to yield the desired (1×) concentration.

For combination studies, the two drugs were first used at a fixed molar ratio of 1:1. Aliquots of 100 μl of the 6× stock solutions of individual drugs were then mixed together to yield a solution containing 3× concentration of each compound. Then, 20 μl of this mixture was added to 100 μl of the cell suspension. When incubated with cells in ARM, CAV was at least 10 times as potent as 5-FU. For drug combinations in ARM, three additional constant molar ratios of 5-FU:CAV were used: 5:1, 10:1 and 20:1. A volume of 10 μl of varying concentrations of each drug was pipetted to quadruplicate drug combination wells to give the desired 1× concentration. Single drug wells contained 10 μl of PBS and 10 μl of drug to yield the desired 1× concentration. (For each molar ratio, a comparative set of single drug wells was always run.) Control wells contained 20 μl of PBS.

After a 72 h exposure, absorbances were measured spectrophotometrically as described previously.[10] Results from three or four independent experiments were expressed as a percentage of control absorbance ± SEM. As suggested by Chou and Talalay,[15] survival of cells was plotted on the y-axis as a function of drug concentration used individually or in combination, plotted on the x-axis. For example, for the drug combination, the point on the plots corresponding to the 1 mM combination actually represented 0.5 mM 5-FU + 0.5 mM CAV.

The survival curves were then analyzed by plotting median effect plots in order to calculate IC_{50} values. Combination index (CI) plots, as described below, were then constructed. The survival curves by themselves do not describe the nature of the drug interaction. The nature of interaction (additive, synergistic or antagonistic) is derived only from the final CI plots and not the survival plots.

Median effect analysis and CI plots

Median effect plots, used to determine IC_{50} values, were based on the median effect principle of the mass action law as reported by Chou and Talalay.[15] These plots linearize dose–response curves by taking the logarithms of the median effect equation to give:

$$\log(F_a/F_u) = m\log(D) - m\log(D_m)$$

where F_a is the fraction of cells affected by the dose (D) of the drug, F_u is the fraction of cells unaffected by D, D_m is the dose of drug required to inhibit the growth of 50% of the cells (the IC_{50}) and m is the Hill-type coefficient which determines the curve's sigmoidicity (i.e. the slope). Log F_a/F_u is plotted versus log(D) to give the log(D_m), i.e. the IC_{50}, which is where the dose–effect plot intersects the median effect axis (where $F_a = F_u$, hence, log $F_a/F_u = 0$). Whenever R, the correlation coefficient for the regression line, was greater than 0.9, the above equation was considered valid for the dose–effect relationship.

A CI was determined using computer analysis[16] to graph CI with respect to F_a as described in detail by Chou and Talalay.[15] These investigators have defined CI by the following relationship:

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} + \frac{\alpha(D)_1(D)_2}{(D_x)_1(D_x)_2}$$

where $(D_x)_1$ is the dose of drug 1 required to inhibit cell growth by x%. This plot allowed the determination of whether the combination of the two drugs was antagonistic (CI > 1), additive (CI = 1) or synergistic (CI < 1).[15] Drug effects were considered to be mutually exclusive (acting by the same mechanism) whenever $m_1 = m_2$ and was equal to $m_{1,2}$. In this case, $\alpha = 0$. Mutually nonexclusive effects (drugs are acting independently by different mechanisms) were indicated whenever $m_1 = m_2$ but is $< m_{1,2}$. In this situation, $\alpha = 1$. When m_1 and m_2 were not equal, exclusiveness of drug effects could not be unambiguously determined; use of both $\alpha = 0$ and $\alpha = 1$ were required to calculate the CI.

In vivo combination effects of 5-FU and CAV

Preparation of the tumors, their inoculation and tumor evaluations were conducted as described previously.[8] The initial tumor volume was estimated by the formula.

$$Vol = l \times w^2/2$$

where l is the length and w is the width of the tumor (in mm).

For each experiment, the tumor-bearing rats, ran-

domized into groups of five animals, were given daily s.c. injections of drug(s) at the specified dose daily for five consecutive days. The control groups received a 0.9% (w/v) NaCl solution using the same dosing schedules as the treated animals. To prevent direct contact of the drug with the tumor, the drugs were injected into the right flank of all experimental animals. At the indicated time interval, tumor volume was determined. Two days after receiving the final treatment, animals were sacrificed, and tumors were excised and weighed individually. Tumor response to drug treatment was calculated as:

$$T/C = \frac{\text{Final tumor weight} - \text{initial tumor weight of treated tumors}}{\text{Final tumor weight} - \text{initial tumor weight of control animals}} \times 100$$

In experiments where tumor regression was observed, the percentage of regression was calculated by the formula:

$$\% \text{ regression} = 1 - \frac{\text{final tumor weight}}{\text{initial tumor weight}} \times 100$$

All other experimental methods and protocols have been described fully elsewhere.[8]

Results

In vitro combination effects

In an attempt to enhance the efficacy of CAV against MIA PaCa-2 cells, the drug was tested in combination with 5-FU. Our *in vitro* studies were performed with MIA PaCa-2 cells in ARM, since this medium was found to be optimal for the expression of CAV's cellular toxicity.[10] The sensitivity of MIA PaCa-2 cells to CAV alone, to 5-FU alone and to a combination of the two drugs at a fixed molar ratio of 1:1 after a 72 h exposure in ARM is illustrated in Figure 1. Under these conditions, the cells were more sensitive to CAV than to 5-FU.

Median effect plots[15] were used to calculate IC_{50} values for the drug combination and for each drug alone. As shown in Figure 2, the IC_{50} of the combination of both drugs was lower than the IC_{50} value with either drug alone. Parameters for this combination are shown in Table 1. Since R values were >0.9, this method of linearization was valid. The nature of the interaction between CAV and 5-FU was evaluated by CI analysis.[15] This graphical representation was obtained by plotting CI values versus fraction affected (F_a). From the median effect plot

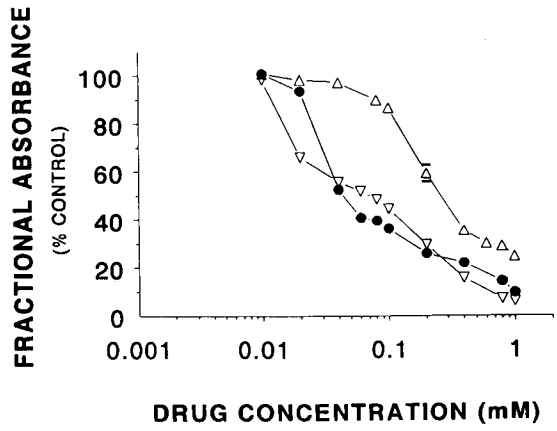


Figure 1. Dose–response curves for cytotoxicity of CAV, 5-FU and a 1:1 combination of the two drugs towards MIA PaCa-2 cells incubated in ARM for 72 h. Cell survival was determined by the MTT assay as described in the text. ▽, CAV; △, 5-FU; ●, 1:1 CAV:5-FU. The mean values were obtained from four independent experiments. SEM is shown when it exceeded the area occupied by the data point.

in Figure 2, it is readily seen that m_1 and m_2 differ (i.e. the regression lines were not parallel). Therefore, both $\alpha = 0$ and $\alpha = 1$ were used to calculate the CI values. Less synergism or more antagonism is predicted by a mutually non-exclusive assumption than with the mutually exclusive assumption.[15] As seen in Figure 3, the CI values were less than 1, a value indicative of a synergistic interaction.[15] However, when more than 90% of the cells were affected (i.e. at higher F^a values), the drug combination became less synergistic.

Since the IC_{50} of 5-FU appeared to be approximately 10 times that of CAV, the effects of a 10:1 combination of 5-FU:CAV were also assessed, and

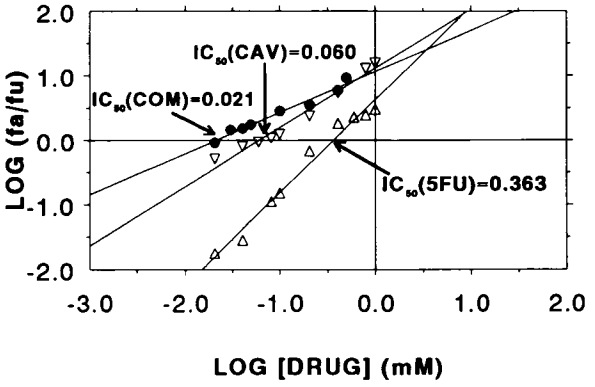


Figure 2. Median effect plots used to calculate IC_{50} values. Dose–response curves from Figure 1 were linearized by the median effect principle as described elsewhere. Points and SEM are the same as in Figure 1.

Table 1. Parameters of median effect plots for 5-FU : CAV combinations

5-FU:CAV ratio	m	D_m (mM)	r
1:1	0.639	0.042	0.987
5:1	1.391	0.145	0.966
10:1	1.504	0.258	0.937
20:1	1.496	0.713	0.936

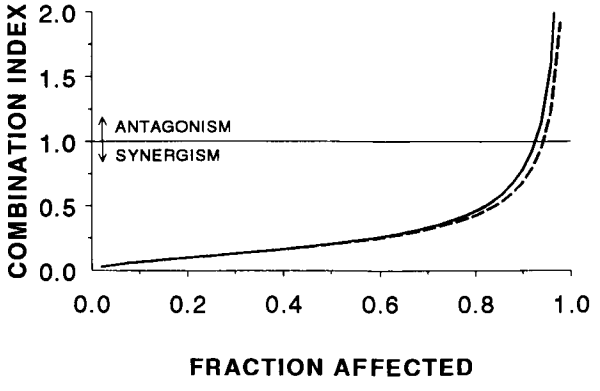


Figure 3. CI plot for a 1:1 ratio of CAV:5-FU for MIA PaCa-2 cells in ARM. Since the regression lines from the median effect plot were not parallel, both $\alpha = 0$ (mutually exclusive) (dashed line) and $\alpha = 1$ (mutually non-exclusive) (solid line) were used to calculate CIs. The curves represent the means obtained from four independent experiments.

the additional fixed ratios of 5:1 and 20:1 were chosen. Dose–response curves for the 5:1 combination were constructed and linearized by median effect plots (data not shown). Results for this combination gave the parameters shown in Table 1. Again, both $\alpha = 0$ and $\alpha = 1$ were used to calculate the CI. The CI plot is shown in Figure 4. The combination index

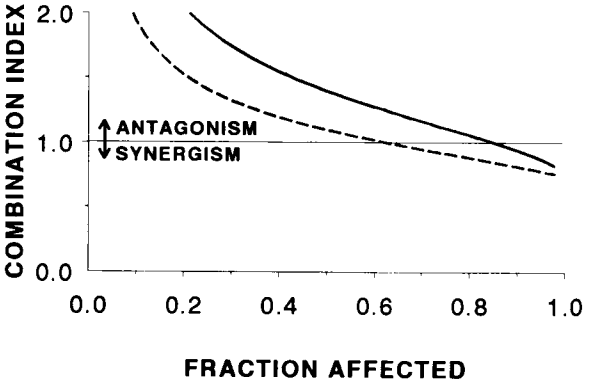


Figure 4. CI plot for a 5:1 molar ratio of 5-FU:CAV for MIA PaCa-2 cells in ARM. $\alpha = 0$ (dashed line) and $\alpha = 1$ (solid line). The curves represent the means obtained from four independent experiments.

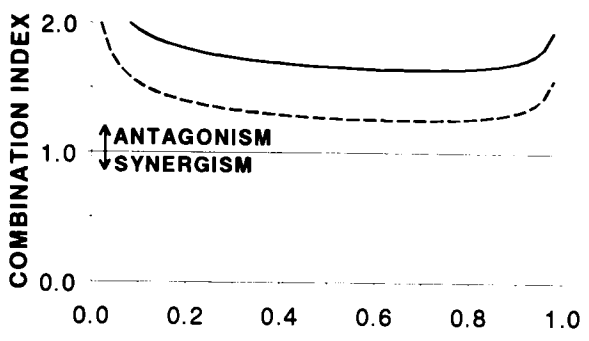


Figure 5. CI plot for 10:1 molar ratio of 5-FU:CAV. $\alpha = 0$ (dashed line) and $\alpha = 1$ (solid line). Curves represent the means obtained from four independent experiments.

indicated antagonism that became slightly synergistic at higher F_a values. When over 50% of the cells were affected, the combination became almost additive.

Analysis of a median effect plot for a constant molar ratio of 10:1 5-FU:CAV gave the parameters shown in Table 1. The CI plot (Figure 5) indicated antagonism at all effect levels. Antagonism increased slightly at higher F_a values.

The highest molar ratio (20:1) of 5-FU:CAV that was tested gave the parameters shown in Table 1. Figure 6 shows the CI plot for this combination which was antagonistic at all effect levels. Greater antagonism was observed at lower effect values.

In vivo combination effects

CAV's *in vivo* effects on a rat colon carcinoma were compared to the drug's effect when used in combination with 5-FU. Five daily s.c. injections of CAV, administered at 2 g/kg, or 35 mg/kg 5-FU were equally effective in inhibiting tumor development. The T/C values for these treatments were 6 or 5%, respectively (Table 2). When 2 g/kg CAV plus 35 mg/kg 5-FU were given as a drug combination, the tumor lost one-half of its initial weight. Thus, CAV and 5-FU administered jointly exhibited greater antineoplastic activity than the combined effects of either drug alone (Figure 7).

Decreasing the CAV concentration to 1 g/kg for 5 days alleviated animal weight loss, but this dose lacked tumor-inhibiting activity. Administration of 5-FU (35 mg/kg) in combination with the lower CAV dose (1 g/kg) reduced the body weight loss to 13% but repression of tumor growth was limited.

In our prior study of this solid rat colon tumor, we

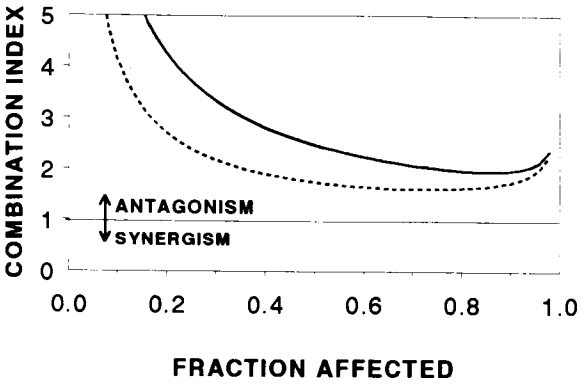


Figure 6. Combination index plot for a 20:1 molar ratio of 5-FU:CAV. $\alpha = 0$ (dashed line) and $\alpha = 1$ (solid line). Curves represent the means obtained from three independent experiments.

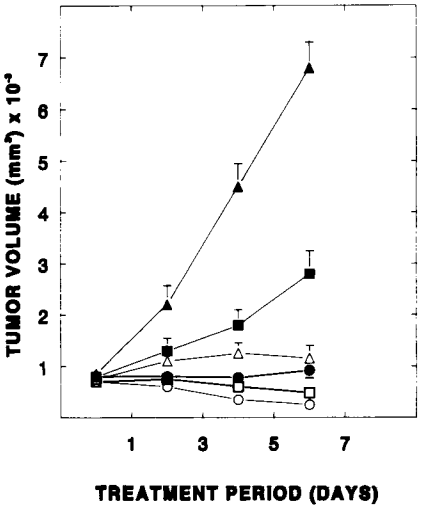


Figure 7. Tumor growth in male Fischer rats following five daily s.c. injections of: ●, 35 mg/kg 5-FU; Δ, 2.0 g/kg CAV; ■, 1.0 g/kg CAV; ○, 35 mg/kg 5-FU + 2.0 g/kg CAV; □, 35 mg/kg 5-FU + 1.0 g/kg CAV. ▲, controls received 0.9% NaCl solution. Points, the means of five rats; bars, SE (shown where larger than the symbol).

addressed the question of whether the reduced tumor growth observed in CAV-treated animals was in fact caused by the reduced food intake of these animals.[8] These experiments established that animal weight loss caused intentionally by food deprivation and equivalent to that observed in our CAV-treated animals had no adverse effect on tumor development.[8] None of the animals died during the *in vivo* experiments.

Discussion

The biochemical basis for the synergism seen with the combination of 2 g/kg CAV and 35 mg/kg 5-FU (a ratio of 57:1 CAV:5-FU) in the *in vivo* rat colon tumor model but not in the MIA PaCa-2 cells when higher molar ratios are provided concurrently is not yet understood. 5-FU is a pyrimidine antimetabolite which, after activation to the phosphorylated nucleotide form, becomes incorporated into RNA and can affect protein translation. 5-FU can also be converted to an alternate active form, 5-FdUMP, which covalently binds to thymidylate synthase (TS). A covalent ternary complex is formed between 5-FU, TS, and the folate cofactor, 5,10-methylenetetrahydrofolate (CH_2THF). This complex, preventing formation of dTMP from dUMP, inhibits thymidine incorporation into DNA. CAV is an ARG antimetabolite which is incorporated into cellular protein in lieu of ARG. Depending on the contribution of a

Table 2. Growth inhibition of rat colon tumor by CAV and 5-FU

Treatment	Initial tumor weight[a] (mg)	Final tumor weight[b] (mg)	T/C (%)[c]	Body weight net change (%)
Control	857 ± 104[d]	5430 ± 722	—	+14 ± 1
35 mg/kg 5-FU	783 ± 146	994 ± 89	+5	−13 ± 0.2
2 g/kg CAV	880 ± 110	1150 ± 130	+6	−15 ± 1
2 g/kg CAV + 35 mg/kg 5-FU	835 ± 17	287 ± 23	−12	+18 ± 1
1 g/kg CAV	826 ± 146	3028 ± 594	+48	−1 ± 1
1 g/kg CAV + 35 mg/kg 5-FU	687 ± 50	586 ± 70	−2	−13 ± 2

Fisher male rats bearing s.c. colon tumors received s.c. injections of CAV and/or 5-FU when tumors reached a size of 500–1000 mm^3. Tumor dimensions were measured daily. Rats were sacrificed and tumors excised and weighed at the conclusion of the experiments. None of the rats died during these experiments.

[a] Estimated from caliper measurements using the formula Vol $= l \times w^2/2$.

[b] Actual weight of dissected tumor.

[c] See Materials and methods.

[d] Mean ± SE.

particular ARG residue to the requisite conformation, this substitution can disrupt protein configuration and impair catalytic activity. In many instances, non-functional, aberrant proteins are produced. It is not yet understood how the mode of action of these antimetabolites interacts to create this synergistic effect.

In the *in vitro* pancreatic cancer cell system, the finding of synergism or antagonism was influenced by the molar ratio of the two drugs. In the case of a 1:1 molar ratio with cell incubations in ARM, significant synergism was observed. However, as the ratio of 5-FU:CAV increased, the combination became more antagonistic. This may be related to kinetics of drug uptake. With higher concentrations of 5-FU in the mixture, it is conceivable that 5-FU preferentially entered the cells. 5-FU is known to rapidly enter cells by passive diffusion. Maximal accumulation of free intracellular 5-FU has been shown to occur within 200 s in the Novikoff hepatoma cell line.[17] Longer times are probably required for uptake, activation and aminoacylation of CAV, and this may be dependent on competition with ARG for aminoacylation. Thus, higher concentrations of 5-FU may antagonize effects of CAV.

Several different methods of determining the combination effects of drugs have been reported and considerable controversy exists as to which method is best for detecting synergy. The data of our median effect plots indicate that mutual non-exclusivity can be indicated. We believe that using the combination index plot is the most valid method for interpreting our results. According to Chou and Talalay,[15] the isobologram method would not be valid whenever drug effects are found to be mutually non-exclusive (i.e. it is only valid for drugs whose effects are mutually exclusive). Berenbaum[18] did not concur with the application of mutual non-exclusivity and the extra term used in calculating the combination index (i.e. when $\alpha = 1$); he believes that the isobologram method is the correct approach. However, that method is less popular due to the requirement of numerous experimental data. Recently, Nocentini *et al.*[19] reported a comparison of several methods used to detect synergy, including the isobologram method according to Berenbaum,[20] the modified isobologram method of Steel and Peckham,[21] and the CI method of Chou and Talalay.[15] They concluded that the overall results were similar when any one of the methods was used. Using the CI method of Chou and Talalay,[15] with several different molar ratios, they found synergism at 1:1, increased synergism at 8:1, slightly decreased synergism at 64:1 and antagonism at 256:1. It thus appears that in some situations, molar ratios are important determining factors for synergism, as we have also found. This could also have important implications for clinical applications. Overall, our results suggest that when the antimetabolic activity of CAV becomes insignificant (high 5-FU-CAV molar ratio), antagonism will be observed.

Over the last 30 years more than 30 agents have been tested for treatment of pancreatic cancer and only 5-FU has been reported to have response rate greater than 20%.[11] Although CAV has never been tested in actual clinical trials, our *in vitro* results showed that, on a molar basis, MIA PaCa-2 cells were more sensitive to CAV than to 5-FU under optimal conditions. Several other drugs, in theory, should exhibit synergism in combination with 5-FU, although little benefit has been accrued in clinical trials for pancreatic cancer using this therapeutic approach.[11,22] Reported randomized trials of combination regimens have shown a median survival of 3–6.5 months. Clinical trials of 5-FU plus leucovorin have also not shown significant benefit in pancreatic cancer;[11,23] however, for colon cancer, several randomized clinical trials have shown that this combination is synergistic.[11] Thus far, it appears that combination chemotherpay for pancreatic cancer is not superior to single-agent therapy; only combined modality treatment has been shown to be superior. Our *in vitro* results may justify future clinical trials of 1:1 combinations of CAV and 5-FU in pancreatic cancer patients.

The studies reported in this communication confirm unequivocally the antitumor potentials of CAV and the validity of the premise that its intrinsic efficacy may be enhanced in combination with 5-FU. Since the appearance of our initial paper,[8] considerable insight has been gained into the biochemical basis for CAV's antimetabolic properties. Serving as an effective substrate for arginyl-tRNA synthetase,[1] CAV is activated via aminoacylation and incorporated into *de novo*-synthesized proteins. Once incorporated, CAV affects protein conformation and function.[4] Our experimental efforts also demonstrated marked selectivity for CAV incorporation into pancreatic proteins. These emerging biochemical insights have permitted development of a biorationale for designing ARG and CAV derivatives that are able to produce structurally aberrant proteins that exhibit more desirable antineoplastic effects. It is our hope that analogs presently under development will exhibit enhanced potency with diminished body weight loss in experimental animals.

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