Enhancement of the Antitumor Effect of Flavone Acetic Acid by the Bioreductive Cytotoxic Drug SR 4233 in a Murine Carcinoma

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ABSTRACT

Flavone acetic acid (FAA, NSC 347512) is a new anticancer drug currently undergoing clinical investigation. Although the precise mechanism for its broad spectrum of activity against transplanted murine solid tumors is unknown, it has been reported that FAA reduces tumor blood flow and produces hemorrhagic necrosis. We have confirmed this finding with the murine transplanted carcinoma SCCVII: 200 mg/kg FAA reduced tumor blood flow to 20-30% of normal for 1-2 days as determined by rubidium 86 extraction. In an attempt to exploit the tumor hypoxia produced by FAA, we have combined it with the novel bioreductive drug SR 4233, a benzotriazine dioxide with high selective toxicity for hypoxic cells. Marked enhancement of the antitumor effect of FAA (200 mg/kg) was observed when it was combined with SR 4233 (0.1 and 0.2 mmol/kg). This was seen using tumor cell survival, regrowth delay, and histological endpoints, with the best results obtained when the two agents were injected simultaneously. These data suggest that targeting bioreductive cytotoxic agents to tumors by producing tumor hypoxia may be a valid way of increasing the tumor cell killing of these agents.

INTRODUCTION

FAA is a new anticancer agent whose antitumor activity was discovered by the National Cancer Institute screening program (1). This drug is currently in clinical studies, both in Europe and in the U.S. (2-4), and it has a number of properties which make it of great interest. First, its chemical structure does not resemble that of any known anticancer agents. Also, unlike other cancer agents, it shows no toxicity against normal human bone marrow progenitor cells in vitro at physiological concentrations (5). Perhaps its most interesting feature, however, is its high activity in mice against s.c. implanted slowly growing tumors (1, 6, 7). The fact that treated tumors rapidly develop hemorrhagic necrosis within a few hours after FAA administration and that the FAA levels achieved in tumors are lower than those necessary for cytotoxic activity of FAA against the same cells in vitro has led to the suggestion that the mechanism of action of the drug is an indirect one (8, 9).

Although the majority of investigators have focused on the possibility that this indirect mechanism of action of FAA may be mediated by natural killer cells or other components of the immune system (4, 9-11), the finding of Evelhoch and colleagues (12) that FAA produces a dramatic loss of ATP and a large reduction in tumor blood flow, this would produce an increase in tumor hypoxia and hence make the tumor cells susceptible to the cytotoxic action of SR 4233. We have examined the antitumor effect of both compounds in three different ways: by clonogenic cell survival, by tumor growth delay, and by quantitative histology. Our results demonstrate that the antitumor effect of FAA can be markedly enhanced by SR 4233, both in the SCCVII tumor and also in the more refractory RIF-1 transplanted tumor.

MATERIALS AND METHODS

Drugs. Both FAA (LM975, NSC 347512) and SR 4233 (3-aminobenzotriazine-1,4-dioxide) were generously provided by the Division of Cancer Treatment, National Cancer Institute. FAA was dissolved in sterile saline solution (0.9% NaCl) at a concentration of 20 mg/ml immediately prior to use and was injected i.p. usually at a dose of 200 mg/kg body weight. SR 4233 was dissolved in physiological saline (0.8 mg/ml) by sonication immediately prior to use and injected i.p. at a dose of 0.2 mmol/kg (35.64 mg/kg) body weight in the majority of the experiments.

Animals and Tumors. The animals used in this work were 3-4-month-old C3H female mice which were bred and maintained under defined flora conditions in our Radiation Biology Mouse Facility. Food and water were available ad libitum. Both the SCCVII carcinomas and RIF-1 sarcomas were produced by intradermal injection of 2 x 10^7 tumor cells in a volume of 0.05 ml of Waymouth's medium intradermally at a site approximately 5 mm proximal to the base of the tail on the shaved dorsum of the C3H mice. The tumors reached a mean diameter of 5-6.5 mm 12-14 days after injection of the cell suspension and were treated at this size. Details of the derivation and maintenance of the tumors have been described previously (15).

LD_50 Assay. The systemic toxicity of the drug combinations was measured using LD_50 as the endpoint. Nontumor-bearing mice were injected i.p. with different concentration of FAA and FAA combined with SR 4233 (0.1 mmol/kg) or (0.2 mmol/kg). Four to eight mice were used for each group. The number of mice dying over a 7-day period after injection was recorded, and the drug concentration (with 95% confidence limits) necessary to kill 50% of mice (LD_50) was determined with a computer program using the logit method of analysis (16).

Tumor Cell Survival Assay. The surviving fraction of tumor cells after administration was assayed by an in vivo-in vitro excision method as described previously (17). Briefly, unanesthetized tumor-bearing mice were injected i.p. with either of FAA, SR 4233, or a combination of the drugs. Two mice were used for each treatment group in each of the experiments with a series of three different FAA and SR 4233 concentrations. CL 200 mg/kg FAA was also tested. This concentration of FAA was the highest that did not kill the tumor-bearing mice on the day of sacrifice. Other tumor-bearing mice injected with saline were used as controls.

Received 3/24/89; revised 7/7/89; accepted 7/20/89.

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1 This investigation was supported by Grant CA15201 from the National Cancer Institute, Department of Health and Human Services.

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3 The abbreviations used are: FAA, flavone acetic acid [2-phenyl-8-(carboxymethyl)]benzopyran-4-one, LM975, NSC347512; LD_50, dosage level resulting in 50% lethality; HPLC, high-performance liquid chromatography.
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The mice were sacrificed 24 h later, and the tumors were excised, minced, and enzymatically disaggregated to produce a single cell suspension. This was filtered and centrifuged (1000 rpm, 10 min) and the cells were resuspended, counted, serially diluted, and plated into petri dishes to assay for colony formation with Waymouth's medium + 15% fetal calf serum. Fourteen days later the colonies were stained and counted. Survival was expressed as relative clonogenic cells/tumor. This is the product of the plating efficiency and cell yield of the treated tumors compared to that of the untreated control tumors.

Regrowth Delay. Growth delay was determined using groups of six mice by measuring three orthogonal diameters three times weekly following drug treatment, until the tumors reached at least four times their original volume or 100 days (in the case of cured tumors). The time taken for each individual tumor to reach four times its diameter at treatment was determined and the mean values (± SE) for each group calculated. Analysis of the significance of the difference between

Fig. 1. Effect of FAA (200 mg/kg) on blood flow in the SCCVII tumor as determined by extraction of "Kb. Mean values (± 1 SE) of three mice per point.

Table 1 Effect of timing of SR 4233 and FAA on growth delay in SCCVII carcinoma

<table>
<thead>
<tr>
<th>Gp</th>
<th>Treatment</th>
<th>Days to reach 4× treatment volume (± SE)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Saline</td>
<td>7.0 ± 0.7</td>
<td>0.2</td>
</tr>
<tr>
<td>2</td>
<td>SR 4233 (0.2 mmol/kg)</td>
<td>10.6 ± 0.7</td>
<td>0.01</td>
</tr>
<tr>
<td>3</td>
<td>FAA (200 mg/kg)</td>
<td>13.5 ± 2.7</td>
<td>0.06</td>
</tr>
<tr>
<td>4</td>
<td>SR 4233 - 2 h - FAA</td>
<td>&gt;17.7 ± 3.3*</td>
<td>0.00</td>
</tr>
<tr>
<td>5</td>
<td>SR 4233 - 0 h - FAA</td>
<td>&gt;22.7 ± 2.3*</td>
<td>0.01</td>
</tr>
<tr>
<td>6</td>
<td>FAA - 2 h - SR 4233</td>
<td>13.9 ± 1.4</td>
<td>0.46</td>
</tr>
<tr>
<td>7</td>
<td>FAA - 6 h - SR 4233</td>
<td>&gt;18.7 ± 3.3**</td>
<td>0.06</td>
</tr>
<tr>
<td>8</td>
<td>FAA - 12 h - SR 4233</td>
<td>15.5 ± 1.3</td>
<td>0.04</td>
</tr>
<tr>
<td>9</td>
<td>FAA - 24 h - SR 4233</td>
<td>12.5 ± 1.8</td>
<td>0.40</td>
</tr>
<tr>
<td>10</td>
<td>FAA - 48 h - SR 4233</td>
<td>13.6 ± 3.6</td>
<td>0.49</td>
</tr>
</tbody>
</table>

* In the case of the groups which included cured tumors a value equal to the longest growth delay observed in the experiment (28.8 days) was assigned to the mice with the cured tumors.

Regrowth Delay. The level of SR 4233 and its two-electron reduction metabolite SR 4317 in SCCVII tumors. SR 4233 (0.2 mmol/kg) was injected (i.p.) into tumor-bearing mice at intervals after FAA injection and sacrificed 20 min later. Plasma and tumor levels of SR 4233 and SR 4317 were measured by HPLC. ■, tumor level; □, blood level; △, tumor level control; △, blood level control. Mean values (± 1 SE) of three mice per point.

Fig. 2. The levels of SR 4233 and its two-electron reduction metabolite SR 4317 in SCCVII tumors. SR 4233 (0.2 mmol/kg) was injected (i.p.) into tumor-bearing mice at intervals after FAA injection and sacrificed 20 min later. Plasma and tumor levels of SR 4233 and SR 4317 were measured by HPLC. ■, tumor level; □, blood level; △, tumor level control; △, blood level control. Mean values (± 1 SE) of three mice per point.

Histology. Mice bearing SCCVII or RIF-1 tumor were treated with a single injection of FAA (200 mg/kg), SR 4233 (0.2 mmol/kg), or FAA + SR 4233. The mice were killed by cervical dislocation and their tumors excised from 1 to 72 h after the injection. They were fixed in 10% formalin, embedded in paraffin wax, sectioned, and stained with hematoxylin & eosin by standard methods. The sections were examined...
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Fig. 5. Photomicrographs of the RIF-1 tumor at 24 h after injection of saline (a), SR 4233 (0.2 mmol/kg) (b), FAA (200 mg/kg) (c), and FAA + SR 4233 given together (d). Hematoxylin & eosin staining and photographed at 100× magnification. No obvious morphological changes are seen with SR 4233 alone. With FAA focal areas of pyknotic cells are seen surrounded by normal regions (c). With the two drugs combined these focal areas of pyknotic cells occupy essentially the whole tumor section (d).

at 100× magnification and the percentage necrosis per tumor was estimated by dividing the section into approximately 1-mm squares using an eyepiece grid. Thirty predetermined spots on the grid were scored as viable tumor or necrosis and at least two different sections for each tumor (differing in depth by a minimum of 1 mm) were scored until a total of at least 400 points per tumor had been reached. Two tumors for each time point were scored for the three different drug treatments (giving at least 800 points scored per time point per drug treatment). All the sections were scored by one observer.

Tumor Blood Flow Measurements. The effect of FAA on tumor blood flow was assessed using the ^86Rb extraction technique of Sapirstein (18). A volume of 0.1 ml ^86RbCl (specific activity, 1–8 mCi/mg, Amersham International plc) was injected i.v. into each mouse. Two min later the mice were sacrificed, the tumors quickly excised, and transferred to preweighed counting tubes. Care was taken to exclude normal tissue and to wipe off any excess blood. The tails of injected mice were also excised and counted to check on residual activity at the site of injection. Samples were rejected if the tail counts were greater than 10% of the injected solution. Radioactive determinations were made on a Packard Gamma counter. Radioactive counts per minute were expressed as % injected/g of tumor. Preliminary experiments were performed to establish 2 min as an optimum time for excision. These studies showed that tumor radioactivity reached a maximum by 1 min after injection and remained at this plateau level for at least 3 min.

Plasma and Tumor Pharmacokinetics. In a parallel series of experiments, SR 4233 (0.2 mmol/kg) was injected i.p. into tumor-bearing mice at intervals after FAA injection and the mice sacrificed 20 min later. We have shown previously that by 20 min after injection the combined total of SR 4233 and SR 4317 in these tumors has reached a maximum value (14). Plasma and tumor levels of SR 4233 and its two-electron reduction product SR 4317 were measured by HPLC as described earlier (14). Three mice were used for each point and the results averaged. Plasma samples were prepared for assay by precipitation of proteins with methanol (one part plasma to four parts methanol). Tumor specimens were removed as quickly as possible (in less than 30 s) from killed animals and plunged into liquid nitrogen (for later processing) in order to prevent any further metabolism of SR 4233 as tumors became hypoxic on removal. Tumor samples were subsequently thawed and homogenized with an equal volume of saline before addition of methanol (one part tumor to four parts methanol). The sample for assay was taken from the supernatant following centrifugation to remove protein precipitates. HPLC was performed by reversed phase using a C18 Bondapak column (Waters Association, Milford, CT) with a mobile phase of 25% methanol and 10% acetic acid in water. Drugs were detected by absorbance at 263 nm.

RESULTS

Blood Flow and Drug Pharmacokinetics. Fig. 1 shows the effect of FAA (200 mg/kg) on blood flow in the SCCVII tumor as determined by extraction of rubidium 86 injected 2 min prior to removal of the tumors. By 1 h after injection, tumor blood flow was reduced to approximately 40% of control and remained below 30% of control values for roughly 24 h. Thereafter, there was a gradual return towards normal levels by 48 h after injection. We have confirmed this time course independently by measuring the concentration of SR 4233 and its
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Fig. 6. Photomicrographs of the SCCVII tumor (100× magnification) at 3 days after the injection of saline (a), SR 4233 (0.2 mmol/kg) (b), FAA (200 mg/kg) (c) and FAA + SR 4233 given together (d). Hematoxylin & eosin staining and photographed at 100× magnification. No obvious morphological changes are seen with SR 4233 alone. With FAA focal areas of necrosis are seen surrounded by normal regions (c). With the two drugs combined these focal areas of necrosis occupy essentially the whole tumor section (d).

Metabolite SR 4317 in the tumors (by HPLC) 20 min after giving a single injection of SR 4233 (0.2 mmol/kg) at different times after a single dose of FAA. Tumor levels of SR 4233 + SR 4317 remained low for 24 h, but returned to control levels by 48 h after FAA injection (Fig. 2, top). In Fig. 2, bottom, we have plotted the ratio of SR 4317 (the two-electron reduction product) to the parent drug SR 4233 in both the blood and tumor as a function of time after the injection of FAA. In all cases the mice were sacrificed at 20 min after the injection of SR 4233. It can be seen that the ratio of SR 4317/SR 4233 in the blood remained constant at approximately 0.7 throughout the experiment, but the ratio of the two drugs in the tumor peaked rapidly after FAA and slowly returned to control levels (of approximately 2) by 24–48 h after injection. Since SR 4233 is metabolized to SR 4317 at least 50× faster under hypoxic than aerobic conditions (19), and the levels of SR 4317 build up rapidly as SR 4233 falls when SR 4233 bearing tumors are clamped to occlude their blood supply (14), we interpret this rise in the SR 4317/SR 4233 ratio as being an indication of tumor hypoxia as a result of the occlusion of blood flow by FAA.

Effect of SR 4233 on Systemic Toxicity of FAA. The LD₅₀ of FAA with or without SR 4233 was measured over a 7-day period following injection. However, all the mice died within the first 2–3 days. The LD₅₀ was found to be 576 mg/kg (range, 520–639; 95% confidence intervals) for FAA alone, 462 mg/kg (range, 429–498) for FAA plus SR 4233 (0.1 mmol/kg), and 317 mg/kg (range 297–338) for FAA plus SR 4233 (0.2 mmol/kg) respectively. None of mice injected with FAA (200 mg/kg) with or without SR 4233 (0.2 mmol/kg) in the regrowth delay or histology studies died or showed signs of major discomfort.

Effect of Timing of SR 4233 with FAA. Fig. 3 shows the effect of varying the time between a single injection of FAA (200 mg/kg) and a single injection of SR 4233 (0.2 mmol/kg) when the tumors were excised 24 h after FAA injection. These data show that the combination of FAA with SR 4233 produces a greater than additive cytotoxicity of the tumor cells, but only when the two agents are separated by 2 h or less. The greatest effect was seen when SR 4233 was injected immediately prior to FAA injection. A difficulty with these experiments was the very low cell yield obtainable from the tumors treated with SR 4233 and FAA at separations of 1 h or less between the two drugs and the data for simultaneous injection (time 0 in Fig. 3) are an upper limit because no tumor cells were counted in the cell suspension from this time period in two of the three experiments.

In order to confirm whether the optimum timing of the two drugs was to administer them simultaneously as indicated from the cell survival experiments, we performed an experiment with similar timing between the drugs, but using growth delay as an endpoint. Table 1 shows the data. Again, it is clear that the greatest effect is produced when the two drugs are given to-
to determine whether the enhancement of FAA antitumor effect by SR 4233 could be monitored by a quantitative histological examination of the amount of necrosis in the treated tumors. C3H mice bearing the SCCVII or the RIF-1 tumor were injected with SR 4233 (0.2 mmol/kg) or FAA (200 mg/kg) or the two drugs given together, and the mice sacrificed and their tumors prepared for histology at varying times from 1 h to 3 days after treatment. Figs. 5 and 6 show representative photomicrographs of the RIF-1 and SCCVII tumors at 1 and 3 days after injection. Figs. 7 and 8 show to-scale drawings of sections from these tumors to show the focal nature and distribution of the necrosis, and Figs. 9 and 10 show the results of quantitative scoring of

Fig. 7. Representative drawing of sections (H & E staining) of the RIF-1 tumor at 1 and 3 days after the injection of FAA (200 mg/kg) alone or combined with SR 4233 (0.2 mmol/kg).

Fig. 8. Representative drawing of sections (H & E staining) of the SCCVII tumor at 1 and 3 days after injection of FAA (200 mg/kg) alone or combined with SR 4233 (0.2 mmol/kg).

Fig. 9. Means (± 1 SE) of the scores of the percentage necrosis in sections of RIF-1 tumors taken from mice sacrificed at various times after FAA (200 mg/kg), SR 4233 (0.2 mmol/kg), or the two drugs given together. Infiltrating muscle and blood vessels were not counted in the analysis. Two tumors each with two nonadjacent sections were scored per point.
the sections. The data show that SR 4233 alone produced little or no tumor cell pyknosis or necrosis (Figs. 5, 6, 9, and 10). However, by 3 h after FAA or FAA + SR 4233 hemorrhagic areas and regions of pyknotic cells could be distinguished. By 24 h these areas were large (Fig. 5) and occupied close to 100% of the tumor in the case of mice injected with the combined treatment (Figs. 7–10). By 3 days after injection there was less necrosis in the mice treated with FAA alone (Figs. 7–10), probably as a result of proliferation of the viable regions. On the other hand, at 3 days after injection of the combination both the SCCVII and RIF-1 tumors had experienced extensive shrinkage (Figs. 7 and 8), and 100% or very close to 100% of the tumor sections showed necrosis.

DISCUSSION

The principal goal of the present study was to explore the hypothesis that tumor specific killing can be achieved using agents with high selective cytotoxicity for hypoxic cells if these agents are combined with procedures which produce selective tumor hypoxia. It has been known for a number of years that certain vasoactive drugs can produce selective tumor hypoxia (20, 21) and selective tumor cell killing has been demonstrated using the vasodilating drug hydralazine combined with SR 4233 (22) and RSU 1069 (23). In our own studies with hydralazine, we have found that this drug decreases tumor blood flow in the SCCVII tumor to ~30% of control values, and this decrease is relatively transitory, in that blood flow has returned to control levels by 6 to 8 h after injection.4 This produces a marked, but again transitory, increase in tumor hypoxia. Little or no antitumor effect of hydralazine alone was seen in these studies (22, 23). However, it appears from the data obtained in these studies in which blood flow was significantly reduced for 1 to 2 days after FAA, and from data of Evelhoch et al. (12), which show nucleotide triphosphate and tumor pH depressed for 2 to 3 days following FAA injection, that the antitumor effect of FAA could be simply the result of prolonged hypoxia, decreased pH, lack of nutrients or a combination of all three. This would certainly be consistent with all of the data indicating a unique mechanism of action of FAA against solid tumors, and data which show that the effect is likely to be an indirect one.

The present results show that despite the marked effect of FAA alone, a further enhancement of the antitumor effect of this compound can be achieved with SR 4233. The data of Fig. 3 and Table 1 suggest that this effect is more than that expected from addition of the cytotoxicity of the two compounds acting independently, and the effect of altering the timing between the two drugs is entirely consistent with the postulated mechanism of action that the enhancement is a result of killing of cells by intratumor metabolism of SR 4233 to the toxic free radical as a result of the hypoxia produced by FAA. The plasma half-life of SR 4233 in this strain of mice is ~40 min (14), so that one would expect a decreasing effectiveness of giving SR 4233 prior to FAA with a complete loss of the effect by 2 h after injections. Fig. 3 and Table 1 show this to be the case. The pharmacokinetic data shown in Fig. 2 also reveal the reason for the declining effectiveness of SR 4233 given after FAA injection. Not only does the lower tumor blood flow after FAA begin to restrict the uptake of drug into the tumors (decreasing to 50% of control values by 12 and 24 h after injection), but the level of drug metabolism (indicated by the ratio SR 4317/SR 4233 in the lower panel of Fig. 2) falls steeply after 3 h after injection. Whether this reflects decreasing tumor hypoxia or death of cells so they cannot metabolize SR 4233 is not clear.

The present data also confirm the fact that FAA produces extensive necrosis in transplanted mouse tumors, which is observable 3–4 h after treatment and reaches a peak 1 to 2 days later. The data also show that the enhancement of the antitumor effect of FAA by SR 4233 can be monitored by observing an increase and a prolongation of the extent of tumor necrosis produced by FAA alone. The value of this observation may be more relevant to other methods of monitoring the possible enhancement of FAA by bioreductive drugs, if the two are combined in the clinic. It would seem likely, for example, that an enhancement and/or prolongation of the extent of tumor necrosis would be able to be monitored by measuring nucleoside triphosphate levels with nuclear magnetic resonance spectroscopy in a manner similar to that performed by Evelhoch and colleagues (12).

In conclusion, we have shown that FAA produces a marked and prolonged reduction in tumor blood flow and extensive tumor necrosis. We have also shown that the bioreductive cytotoxic agent SR 4233 can produce a marked enhancement in the antitumor effect of FAA when the two drugs are given together. This effect is more than that predicted on the basis of the cytotoxicity produced by each agent alone. However it is clear from the toxicity data that SR 4233 increases the systemic toxicity of FAA so it is not possible to determine whether the combination produces a therapeutic gain. Moreover, it is yet too early to know whether FAA is likely to be a significant drug in the clinic or whether SR 4233 will be the best bioreductive drug to be combined with FAA. Early indications from clinical studies of FAA are somewhat disappointing in that the drug is not producing the antitumor effect seen in mice (24). It is significant, however, to note that more potent analogues of both FAA and SR 4233 are under development (25, 26).

REFERENCES


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