Combination Chemotherapy of Mouse Tumors with 8-Azaguanine and Flavotin*

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Treatment of a tumor with more than one chemotherapeutic agent is based on the possibility that different agents might inhibit different metabolic activities of the tumor, resulting in greater inhibition of its growth than might be true when either chemotherapeutic agent is used alone. After it had been shown (5) that the growth of a number of tumors could be inhibited by use of the guanine analog, 8-azaguanine, many investigators found that the combination of this carcinostatic agent with certain other compounds was usually more effective than the use of any one of the drugs alone (1–4, 7, 9–19). The choice of drugs to be used has been guided, in part at least, by an attempt to select chemicals which might be related to metabolites whose concentration in tumor tissue is different from their concentration in normal tissue. For example, it was demonstrated by Pollack et al. (8) that many tumors have somewhat smaller concentrations of riboflavin than normal tissues and considerably less than those tissues especially rich in riboflavin (liver, heart, and kidney).

Because of this fact, Shapiro and Fugmann (9) tested the riboflavin analog, flavotin, on mouse tumor 755, both alone and in combination with 8-azaguanine. They found definite evidence of potentiation as judged by inhibition of tumor growth. However, this finding was restricted to the growth of tumors in female mice. Using riboflavin-5-phosphate, they were able partially or completely to block the potentiating influence of flavotin on 8-azaguanine. Dietrich and Shapiro (1) found that measurable amounts of xanthine oxidase could be found in tumors grown in animals not treated with flavotin. However, no xanthine oxidase activity could be detected in tumors from animals which had received flavotin. In contrast to this, flavotin appeared to have no effect on the xanthine oxidase activity of mouse liver tissue.

In an attempt to add to our knowledge of the mechanism of potentiation, we have tested the activity of flavotin and 8-azaguanine, alone and in combination, on a mouse leukemia and on a mouse mammary carcinoma.

MATERIALS AND METHODS

The solid tumor used in these experiments was mammary adenocarcinoma E 0771 in C57BL/6 mice inoculated by the Snell cytosieve method (18). In Experiment 802, 80 mice of both sexes were used, being divided into five groups of fifteen or twenty mice, each containing approximately half males and half females. Treatment was started on the 4th day after the inoculation of the tumor. Group A served as untreated controls; Group B received flavotin (60 mg/kg/day); Group C, 8-azaguanine (50 mg/kg/day); Group D, flavotin plus 8-azaguanine (same dosages as above, 8-azaguanine injected 1 hour after injection of flavotin); Group E, riboflavin-5-phosphate (100 mg/kg/day) plus flavotin, plus 8-azaguanine, dosages as above (flavotin injected 15 minutes after riboflavin). At the time of the death of the second control mouse, five mice in each group were injected with colchicine (3 mg/kg) and sacrificed 6 hours later. All tumors were weighed, and lungs and tumors were fixed in Vandergrift's fluid for histological examination (14). Daily treatment was continued in each group and survival times recorded.

Experiments 801, 803, and 804 made use of lymphoid leukemia, lymphoma II, inoculated by cytosieve into CAF1 mice. In each of the three experiments there were five groups of mice, twenty in each group. All mice in Experiment 801 (except ten of the controls) were males; all mice in Experiment 803 were females; half of the mice in Experiment 804 were males and half females. In each experiment treatment was begun 24 hours after the inoculation of the tumor. The mice in all experiments were weighed daily throughout, and records of these weights were kept in Experiments 803 and 804. At the time of the death of the second con-
trol, five animals in each group were treated with colchicine (3 mg/kg) and sacrificed 6 hours later. The spleens were weighed (because, in leukemia, spleen size is a better indication of tumor activity than the rather diffuse tumor mass itself [6]). Tumors, spleens, and femurs were fixed in Vander-griff's fluid. The remaining mice were then studied for survival time. In Experiment 801, treatment was discontinued; in Experiments 803 and 804, treatment was continued.

was dissolved by heating in 100 per cent propylene glycol (2.5 gm. flavotin in 100 ml.), then diluted with sterile distilled water to 1000 ml.

Animals were housed in an air-conditioned laboratory, in plastic cages, ten per cage, with wood-shaving bedding, and food and water were offered ad libitum.

RESULTS

Mean tumor weights and mean survival times of tumor E 0771 (Exp. 802) are shown in Table 1.

### TABLE 1

**EFFECT OF FLAVOTIN, 8-AZAGUANINE, AND RIBOFLAVIN ON TUMOR E 0771 IN C57BL/6 MICE***

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Both sexes Mean tumor weight (gm)</th>
<th>Males Mean survival time (days)</th>
<th>Females Mean survival time (days)</th>
<th>No. deaths prior to sacrifice Date/Total no. mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control untreated</td>
<td>7.75±0.88</td>
<td>21.7±1.3</td>
<td>22.4±3.5</td>
<td>20.0±4.1</td>
</tr>
<tr>
<td>Flavotin (60 mg/kg/day)</td>
<td>6.52±0.62</td>
<td>18.5±1.5</td>
<td>19.0±5.7</td>
<td>17.5±5.5</td>
</tr>
<tr>
<td>8-Azaguanine (50 mg/kg/day)</td>
<td>1.77±0.12</td>
<td>26.2±2.0</td>
<td>22.3±2.5</td>
<td>18.3±2.5</td>
</tr>
<tr>
<td>Flavotin and 8-azaguanine</td>
<td>0.33±0.05</td>
<td>16.5±0.5</td>
<td>15.5±1.5</td>
<td>17.5±1.5</td>
</tr>
<tr>
<td>Flavotin, riboflavin, and 8-azaguanine</td>
<td>0.37±0.16</td>
<td>14.6±0.2</td>
<td>14.5±0.5</td>
<td>14.8±0.4</td>
</tr>
</tbody>
</table>

* Experiment 802.

### TABLE 2

**EFFECT OF FLAVOTIN, 8-AZAGUANINE, AND RIBOFLAVIN ON LYMPHOMA II IN CAP1 MICE***

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Both sexes Mean spleen weight (gm)</th>
<th>Males Mean survival time (days)</th>
<th>Females Mean survival time (days)</th>
<th>No. deaths prior to sacrifice Date/Total no. mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control untreated</td>
<td>0.24±0.00</td>
<td>17.4±0.7</td>
<td>14.4±0.9</td>
<td>18.6±2.2</td>
</tr>
<tr>
<td>Flavotin (60 mg/kg/day)</td>
<td>0.25±0.00</td>
<td>13.6±0.8</td>
<td>13.0±0.9</td>
<td>15.6±0.8</td>
</tr>
<tr>
<td>8-Azaguanine (50 mg/kg/day)</td>
<td>0.14±0.01</td>
<td>23.6±0.8</td>
<td>16.0±1.9</td>
<td>20.8±2.0</td>
</tr>
<tr>
<td>Flavotin and 8-azaguanine</td>
<td>0.08±0.01</td>
<td>20.3±0.8</td>
<td>15.0±3.3</td>
<td>17.8±2.5</td>
</tr>
<tr>
<td>Flavotin, riboflavin, and 8-azaguanine</td>
<td>0.07±0.01</td>
<td>21.3±2.1</td>
<td>13.3±2.9</td>
<td>17.4±4.4</td>
</tr>
</tbody>
</table>

* Combined results of Experiments 801, 803, and 804.
† Experiments 803 and 804.
‡ Experiments 803 and 804.

The 8-azaguanine1 was dissolved with the aid of dilute NAOH and was adjusted to pH 7.8-8.0 with HCl. Riboflavin-5-phosphate2 was used from ampules (which contained 10 mg/ml) or was made fresh daily from crystalline riboflavin-5-phosphate which was kept in the dark. The crystals were dissolved in sterile distilled water in proportion to make a solution containing 10 mg/ml. Flavotin3 was obtained through the kindness of Dr. J. H. Williams, Lederle Laboratories Division, American Cyanamid Co., Pearl River, N.Y.

The combination of flavotin and 8-azaguanine caused significantly lower mean tumor weight than did treatment with 8-azaguanine alone. Table 2 presents mean spleen weights and mean survival times of the lymphoma II experiments. The combination of 8-azaguanine and flavotin caused a significant decrease in spleen size compared with that following 8-azaguanine treatment alone but was accompanied by a decrease in survival time.

Riboflavin treatment did not cause reversal of the flavotin-potentiating effects in any of the experiments. The potentiation of 8-azaguanine by flavotin was equally effective in both sexes.

Because of the possibility that the carcinostatic effect of 8-azaguanine and/or flavotin might be in...
fluenced by toxicity, records were kept of the daily weights of all mice in Experiments 802, 803, and 804. The results of Experiments 802 and 804 are shown in Charts 1 and 2. In an effort to learn whether the presence of a tumor might influence the effect of the combination of the three drugs on mouse weight, twenty tumor-free CAP1 mice were so treated. Chart 2 includes the results of this experiment.

![Chart 1](chart1.png)

**Chart 1.**—Exp. 802; effect of flavotin, 8-azaguanine, and riboflavin on mouse weight.

![Chart 2](chart2.png)

**Chart 2.**—Exp. 804; effect of flavotin, 8-azaguanine, and riboflavin on mouse weight.

**DISCUSSION**

It is evident that the results of these experiments confirm the work of Shapiro and Fugmann (9) and Dietrich and Shapiro (1) in that flavotin alone is not effective as a carcinostatic agent. Unlike their findings with tumor 755, however, we have demonstrated that with E 0771 and lymphoma II the combination of flavotin and 8-azaguanine is significantly carcinostatic in both males and females (Tables 1 and 2). Mean tumor weights and mean survival times of males and females bearing tumor E 0771 do not differ significantly in any of the five groups (Table 1). Mean spleen weights and mean survival times of males and females bearing lymphoma II were not significantly different in any of the groups except that of mice receiving 8-azaguanine alone (Table 2). Even here, spleen weights were not different, but females did survive significantly longer than males.

Treatment with 8-azaguanine alone may result in an initial weight loss, but in the great majority of cases not only is all this initial loss recovered, but many mice subsequently gain weight (Charts 1 and 2). Shapiro and Gellhorn (10) reported one group of 30 mice, however, which showed a mean weight loss of 3.6 giu. (923.1—19.5 gm.) after 11 days of 8-azaguanine treatment (50 mg/kg/day).

Shapiro and Fugmann (9) reported a maximum weight loss of 6 per cent for the combination of 8-azaguanine, riboflavin, and flavotin and of 8 per cent weight loss for the combination of 8-azaguanine and flavotin. We have found, however, that the combination of two and of three drugs results in a very considerable weight loss even in the absence of a tumor. Attempts to explain the difference in results are made difficult because it is not clear from Shapiro and Fugmann's paper exactly how long the mice in any given experiment were subjected to both or to all three drugs. They state "intraperitoneal therapy was given depending upon the daily weight of the treated animals, injections usually being given daily. Therapy was begun upon well established tumors, varying between experiments from 3 to 18 days old. The duration of tumor growth varied between experiments from 20 to 34 days." In the reversal experiments "daily intraperitoneal therapy was begun upon well established tumors varying between experiments from 5 to 7 days old. The duration of tumor growth varied between experiments from 2 to 24 days."

In the present experiments, all mice were treated daily (including Saturdays and Sundays). Injections were begun 24 hours after the inoculation of lymphoma II and 4 days after the inoculation of E 0771. As shown in Chart 1, mice bearing tumor E 0771 and receiving either flavotin and 8-azaguanine or flavotin, 8-azaguanine, and riboflavin did not start to lose weight seriously until after 9 or 10 days of treatment. Loss of weight began earlier in lymphoma II-bearing mice (Chart...
2), suggesting a possible strain difference, because it was found even in nontumor-bearing mice. Growth characteristics of tumor 755 are more like those of E 0771 than like those of lymphoma II, so it is especially important to know exactly how long Shapiro and Fugmann subjected tumor 755-bearing mice to both and to all three drugs. We found, as they did, that in certain cases a number of mice in a group receiving both (or all three) drugs failed to survive until the end of the experiment (Tables 1 and 2). These are not included in calculating the mean per cent weight change. Usually, mice failing to survive in an experiment of this sort tend to lose weight markedly before they die. If, in the Shapiro and Fugmann work, such mice died before the end of the experiment, the survivors would tend to have less weight loss. Conversely, if many weight-losing mice happened to survive until the end of the experiment, the mean per cent weight loss would be large. It is possible that such circumstances may help to explain the apparent difference in toxicity found by Shapiro and Fugmann in contrast to what we have found.

SUMMARY
The activity of flavotin, a riboflavin analog, alone and in combination with 8-azaguanine, a guanine analog, has been tested on a mouse leukemia, lymphoma II, and on mouse mammary carcinoma E 0771. The weight of tumor-bearing mice receiving either drug alone remained essentially unchanged. Loss of weight occurred when both flavotin and 8-azaguanine were injected, and a similar decrease was observed when riboflavin-5-phosphate was used together with flavotin and 8-azaguanine.

The use of 8-azaguanine alone resulted in smaller spleens and longer survival time than the controls in lymphoma II, and in much smaller tumors than the controls in E 0771. Flavotin alone was ineffective, but in combination with 8-azaguanine it resulted in smaller spleens and longer survival time (lymphoma II) and smaller tumors (E 0771) than those found in untreated controls. The combination resulted in smaller spleens, but shorter survival time (lymphoma II) and smaller tumors (E 0771) than when 8-azaguanine was injected alone.

The potentiation of 8-azaguanine by flavotin was significantly effective in both males and females.

Riboflavin-5-phosphate did not cause reversal of the effects of flavotin in these experiments.

REFERENCES
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