Reduction of the Growth Rate of the Walker 256 Tumor in Rats by Rhodamine 6G Together with Hypoglycemia


ABSTRACT

Previous attempts to use tumor energy metabolism as a target for antineoplastic therapy have used single agents aimed at inhibiting either glycolysis or oxidative phosphorylation. Since most tumor cells use both pathways for energy production, this approach is unlikely to succeed. The aim of this study was to simultaneously manipulate both sources of intracellular ATP to achieve more selective control of tumor growth.

Rhodamine 6G (R6G) is a fluorochrome mitochondrial dye which inhibits oxidative phosphorylation. 3-Mercaptopicolinic acid inhibits gluconeogenesis and is a potent hypoglycemic agent in the fasting state. Dose-response relationships were established for R6G and 3-mercaptopicolinic acid, and a nontoxic dose of the compounds was selected for subsequent experiments. Thereafter, groups of rats (n = 7 per group) underwent s.c. implantation of Walker 256 carcinosarcoma. Following a 24-h fast each group received either saline, R6G (0.8 mg/kg), 3-mercaptopicolinic acid (40 mg/kg), or the combination given i.p. Seven days after tumor implantation animals were sacrificed, and tumors were excised and weighed.

Administration of R6G during a period of hypoglycemia significantly reduced the tumor growth rate when compared to control experiments (3.6 ± 0.3 g cf. 7.1 ± 0.7 g, mean ± SE; P < 0.05). In contrast, neither R6G nor the period of hypoglycemia alone significantly affected tumor growth.

These results suggest that simultaneous manipulation of oxidative phosphorylation and glycolysis may be used to selectively inhibit tumor growth in vivo.

INTRODUCTION

Current cancer chemotherapy involves the use of cytotoxic agents to inhibit cell division within the tumor. These agents are nonselective since they also inhibit cell division within the tissues of the host. A more selective approach to the treatment of cancer might be based on differences between tumor and the host cells. One such difference is the altered energy metabolism of many tumor cells (1-4).

It has been recognized for over 50 yr that tumors may demonstrate abnormally high rates of glycolysis (1). More recently, it has been shown that a number of tumors are dependent upon glucose as a major energy substrate (4-6). Furthermore, the mitochondria of several tumors have been shown to be abnormal in morphology and enzyme content (2). Since glycolysis and oxidative phosphorylation are the two major pathways of energy production within the cell, it may be that such abnormalities can render tumors vulnerable to selective growth inhibition.

Rhodamine 123 and rhodamine 6G are two related permeant cationic fluorochrome dyes which have been shown to be cytotoxic in vitro (7-9). Both agents are potent inhibitors of mitochondrial oxidative phosphorylation (10-12), and it has been suggested that their cytotoxic activity is related to an inhibition of ATP production (7, 13). Rhodamine 123 is taken up into the mitochondria of both normal and certain carcinoma cells but is retained for much longer in those of the carcinoma cells (14). This retention is associated with the cytotoxic action of the dye. In addition, rhodamine 123 has been shown to be selectively cytotoxic against numerous malignant cell lines (7). This selectivity is thought to be related to a transformation-dependent abnormality of mitochondrial membrane potential (15). If the glucose antimetabolite, 2-deoxyglucose, is administered simultaneously, the two agents have been shown to have synergistic antitumor activities both in vitro (7) and in vivo (16). Thus it would appear that much greater efficacy can be achieved if both glycolysis and oxidative phosphorylation, two major intracellular sources of ATP, are inhibited simultaneously.

An alternative method of reducing glycolysis would be to induce a state of hypoglycemia. This can be achieved by inhibiting gluconeogenesis in the fasting state. The present investigation was undertaken to examine the effects of an antimitochondrial agent, an inhibitor of gluconeogenesis, and the combination of the two on the growth rate of the Walker 256 carcinosarcoma in rats. Rhodamine 6G is a fluorochrome antimitochondrial dye (11) and has been shown to be a more potent inhibitor of tumor cell growth in vitro than rhodamine 123 (9). Hypoglycemia was induced with 3-mercaptopicolinic acid. This compound blocks the synthesis of glucose de novo by inhibiting phosphoenolpyruvate carboxykinase (EC 4.1.1.32) (17), and it has been shown to induce marked hypoglycemia in both fasted rats (17) and humans (18).

MATERIALS AND METHODS

Chemicals. 3-Mercaptopicolinic acid (SKF-34288) was a gift from Dr. N. W. Di Tullio, Smith, Kline, and French, Ltd., Philadelphia, PA. Rhodamine 6G was obtained from BDH Chemicals, Ltd., Poole, Dorset, England. This preparation was found to be 98% R6G by high-pressure liquid chromatography.

Animals. Female inbred Wistar rats, aged between 12 and 16 wk and weighing 180 to 220 g, were allowed free access to food (SDS diet; SDS, Ltd., Witham, Essex, United Kingdom) and water except where stated. Animals were kept in conditions of controlled temperature and lighting (20 ± 2°C, 12-hr light-dark cycle).

Tumor. The rapidly growing Walker 256 carcinosarcoma, as described previously (19), was used. Viable tumor fragments (100 mg) were transplanted s.c. into the right flank of rats under aseptic conditions and light ether anesthesia. The tumor doubling time was about 36 h.

Cannulation of Rats for Serial Measurement of Blood Glucose Concentration. The right common carotid artery was cannulated 24 h prior to drug administration under aseptic conditions and halothane-nitrous oxide anesthesia. The cannula (1-mm external diameter Portex polythene tubing; Protex, Ltd., Hythe, Kent, United Kingdom) was heparinized (5000 units/ml) and then externalized onto the dorsal aspect of the animal's neck via a s.c. tunnel. Once outside the animal, the cannula was protected by a tightly coiled stainless steel spring. The spring and cannula were suspended from a pulley system which allowed the animal
to move freely within a wire-bottomed, Perspex metabolism cage.

Serial blood samples (100 μl) were obtained via the cannula, and the total blood volume of the rats was maintained by replacing each blood sample with an equal volume of isotonic saline (0.8% NaCl w/v).

Measurement of Blood Glucose Concentration. The concentration of glucose in blood was determined by an enzymatic method (GOD-Perid; Boehringer Mannheim, Lewes, Sussex, England). Blood samples (100 μl) were immediately deproteinized with 1 ml of uranyl acetate solution (Boehringer Mannheim) and then centrifuged. The supernatant was stored at 4°C and assayed on the same day.

Drug Administration. Animals underwent tumor implantation 48 h prior to drug administration. The hypoglycemic effect of 3MPA was only observed in the fasted state; therefore, rats were fasted for 24 h prior to drug administration except where stated. The animals were then fasted for a further 8 h after drug administration before being allowed access to food.

R6G was dissolved in water and injected in a volume of 0.2 ml. 3MPA was dissolved in 0.15 M HCl. This solution was then titrated to pH 7.4 with 0.15 M NaOH. The total volume injected was 0.2 ml. Both solutions were passed through a 0.22-μm nonpyrogenic filter (Millipore S. A., Molsheim, France) prior to administration by the i.p. route.

Statistical Analysis. Overall statistical significance was determined by an analysis of variance. Dunnett's test (20) was used when multiple comparisons were made within each group.

RESULTS

Effect of 3MPA and R6G on Blood Glucose Concentration of Starved Normal Rats. The blood glucose concentration of control rats fasted for 24 h was approximately 3.5 mmol/liter and remained constant throughout the 7-h sampling period (Fig. 1). Administration of 3MPA (70 mg/kg) to rats fasted for 24 h resulted in a significant decrease in the blood glucose concentration (P < 0.01) within 15 min. This decrease continued and reached a minimum level of 1.4 mmol/liter after 180 min. Thereafter, there was a gradual increase in blood glucose concentration until normal fasting values were achieved 360 min after drug administration (Fig. 1).

Rats given both 3MPA (70 mg/kg) and R6G (0.8 mg/kg) had significantly lower mean blood glucose concentrations at 120 and 180 min compared with rats given 3MPA (70 mg/kg) alone (Fig. 2cf. Fig. 1; P < 0.01). Furthermore, this reduction in blood glucose concentration was irreversible. The combination of a lower dose of 3MPA (40 mg/kg) and the same dose of R6G (0.8 mg/kg) induced a reversible period of hypoglycemia lasting approximately 240 min with a trough blood glucose concentration of 2.2 mmol/liter at 180 min (Fig. 2).

Effect of 3MPA and R6G on Blood Glucose Concentration of Fed and Starved Tumor-bearing Rats. The mean blood glucose concentration of 24-h-fasted tumor-bearing rats was 2.3 ± 0.3 mmol/liter (Table 1) 180 min after injection of 3MPA (40 mg/kg) plus R6G (0.8 mg/kg). In contrast, the mean blood glucose concentration of rats fed ad libitum and given 3MPA (40 mg/kg) plus R6G (0.8 mg/kg) was 6.7 ± 0.3 mmol/liter (Table 1). This was not significantly different from controls fed ad libitum.

The fasting blood glucose concentration of tumor-bearing rats was not significantly different from that of 24-h-starved non-tumor-bearing rats (Table 2). Moreover, the blood glucose concentration in fasted tumor-bearing rats 180 min after administration of 3MPA (70 mg/kg) or 3MPA (40 mg/kg) plus R6G (0.8 mg/kg) was not significantly different from that of non-tumor-bearing rats given the same drug regimen (Table 2).

Effect of R6G on Growth Rate of Walker 256 Tumor in Rats. The effect of R6G administration 48 h after tumor implantation...
SELECTIVE MANIPULATION OF TUMOR ENERGY METABOLISM

Table 2 Effect of 3MPA alone or 3MPA plus R6G on blood glucose of starved tumor-bearing or non-tumor-bearing rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Feeding regimen</th>
<th>Tumor wt (g)</th>
<th>Wt loss (%)</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>Starved (24 h)</td>
<td>7.1 ± 0.7a</td>
<td>2 ± 0</td>
<td>0</td>
</tr>
<tr>
<td>R6G (0.8 mg/kg)</td>
<td>Starved (24 h)</td>
<td>5.6 ± 0.5</td>
<td>5 ± 0</td>
<td>0</td>
</tr>
<tr>
<td>R6G (4.6 mg/kg)</td>
<td>Starved (24 h)</td>
<td>5.0 ± 0.5</td>
<td>9 ± 0</td>
<td>0</td>
</tr>
<tr>
<td>R6G (2.4 mg/kg)</td>
<td>Starved (24 h)</td>
<td>1.8 ± 0.5b</td>
<td>8 ± 0.7a</td>
<td>14</td>
</tr>
<tr>
<td>R6G (3.0 mg/kg)</td>
<td>Starved (24 h)</td>
<td>0.5 ± 0.2a</td>
<td>13 ± 0.7a</td>
<td>43</td>
</tr>
<tr>
<td>R6G (4.6 mg/kg)</td>
<td>Starved (24 h)</td>
<td>0.5 ± 0.2a</td>
<td>13 ± 0.7a</td>
<td>43</td>
</tr>
</tbody>
</table>

* Mean ± SE.

Table 3 Effect of increasing doses of R6G on growth rate of Walker 256 tumor in rats

R6G was administered i.p. at the doses shown 48 h after tumor implantation. Five days after drug administration, the animals (n = 7 per group) were sacrificed, and the tumors were excised and weighed. The percentages of weight loss and mortality of the animals over the 7 days of the study are also shown.

<table>
<thead>
<tr>
<th>Group</th>
<th>Feeding regimen</th>
<th>Tumor wt (g)</th>
<th>Wt loss (%)</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>Starved (24 h)</td>
<td>7.1 ± 0.7</td>
<td>2 ± 0</td>
<td>0</td>
</tr>
<tr>
<td>R6G (0.8 mg/kg)</td>
<td>Starved (24 h)</td>
<td>5.6 ± 0.5</td>
<td>5 ± 0</td>
<td>0</td>
</tr>
<tr>
<td>R6G (4.6 mg/kg)</td>
<td>Starved (24 h)</td>
<td>5.0 ± 0.5</td>
<td>9 ± 0</td>
<td>0</td>
</tr>
<tr>
<td>R6G (2.4 mg/kg)</td>
<td>Starved (24 h)</td>
<td>1.8 ± 0.5</td>
<td>8 ± 0.7</td>
<td>14</td>
</tr>
<tr>
<td>R6G (3.0 mg/kg)</td>
<td>Starved (24 h)</td>
<td>0.5 ± 0.2</td>
<td>13 ± 0.7</td>
<td>43</td>
</tr>
<tr>
<td>R6G (4.6 mg/kg)</td>
<td>Starved (24 h)</td>
<td>0.5 ± 0.2</td>
<td>13 ± 0.7</td>
<td>43</td>
</tr>
</tbody>
</table>

* Mean ± SE.

Table 4 Effect of 3MPA plus R6G on growth rate of the Walker 256 tumor in rats fed ad libitum or starved for 24 h prior to, and for 8 h after, drug administration i.p.

<table>
<thead>
<tr>
<th>Group</th>
<th>Feeding regimen</th>
<th>Tumor wt (g)</th>
<th>Wt loss (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>Starved (24 h)</td>
<td>7.1 ± 0.7</td>
<td>2 ± 0</td>
</tr>
<tr>
<td>R6G (0.8 mg/kg)</td>
<td>Starved (24 h)</td>
<td>7.4 ± 0.5</td>
<td>4 ± 0.7</td>
</tr>
<tr>
<td>3MPA (70 mg/kg)</td>
<td>Starved (24 h)</td>
<td>5.6 ± 0.5</td>
<td>5 ± 0.5</td>
</tr>
<tr>
<td>3MPA (40 mg/kg)/R6G (0.8 mg/kg)</td>
<td>Starved (24 h)</td>
<td>6.2 ± 0.5</td>
<td>3 ± 0.5</td>
</tr>
</tbody>
</table>

* P < 0.01, a versus c.

DISCUSSION

This study demonstrates clearly that the growth rate of the Walker 256 tumor in rats is reduced significantly when R6G is administered during a period of hypoglycemia. Neither hypoglycemia or R6G (0.8 mg/kg) alone caused a significant inhibition of tumor growth. A significant reduction in tumor growth rate could be achieved with R6G alone but only at a dose associated with significant host weight loss and mortality. In contrast, the combination regimen was well tolerated and reduced tumor growth rate with minimal host toxicity.

When 2-deoxyglucose (an inhibitor of glycolysis) is administered at the same time as rhodamine 123, the combination has synergistic antitumor activity both in vitro (7) and in vivo (16). This suggests that, when combined with selective inhibition of mitochondrial function, a reduction in glycolysis may have a potent antitumor effect. In this study increasing doses of rhodamine 6G were associated with decreased rates of tumor growth. Unfortunately, such high doses of R6G were also associated with increasing host weight loss and mortality (Table 3). However, when the circulating concentration of glucose was reduced with 3MPA, tumor growth rate was inhibited by a dose of R6G which was not toxic to the host (Table 4).

The hypoglycemic effect of 3MPA is due to the inhibition of glucose synthesis de novo (17). Thus hypoglycemia is only observed in the fasting state, because if the animals have free access to food, there is usually an adequate supply of glucose from the diet to maintain blood glucose levels. It might be argued that it was not the hypoglycemic effect of 3MPA which was important in making the R6G-3MPA combination effective. However, the blood glucose concentration of tumor-bearing rats starved for 24 h and given 3MPA (40 mg/kg) plus R6G (0.8 mg/kg) was significantly reduced compared with fasted controls (Table 2). In contrast, there was no reduction of blood glucose concentration (Table 1) or of tumor growth rate (Table 4) in rats given the same combination but fed ad libitum. Thus, a reduction in blood glucose concentration would appear to have been necessary for the combination to be effective.

The effects of starvation on tumor growth rate in vivo appear to vary from one animal model to another. Previous animal studies have demonstrated either no change (21) or a slight reduction of tumor growth rate (22) following variable periods and degrees of caloric restriction. In this study, a period of starvation lasting 32 h had no effect on final tumor weight (Table 4). Moreover, the administration of 3MPA (40 mg/kg) on tumor weight 5 days after drug administration is shown in Table 3. Host weight loss and mortality in each group are also shown. Administration of R6G (0.8 mg/kg) led to a small but not significant reduction in tumor weight. This dose was associated with a weight loss of 5% compared with 2% in the controls. When the dose of R6G was increased to 1.6 mg/kg, tumor weight was reduced by 30%, but again this decrease was not statistically significant. Host weight loss increased to 9.2%, but there was no mortality. At a dose of 2.4 mg/kg, tumor weight was reduced by 75%, but only one of seven animals died. At a dose of 3.0 mg/kg, three of seven animals died, and at 4.0 mg/kg, all the animals died within 3 days.

Effect of 3MPA plus R6G on Growth Rate of Walker 256 Tumor in Fed and Fasted Rats. When the combination of 3MPA (40 mg/kg) and R6G (0.8 mg/kg) was administered 48 h after tumor implantation to rats starved for 24 h, the mean tumor weight 5 days after drug administration was 49% less than that of controls (3.6 ± 0.3 cf. 7.1 ± 0.7 g; P < 0.05). Host weight loss was only 3% in rats receiving combination therapy and was not statistically significant. When 2-deoxyglucose (an inhibitor of glycolysis) is administered 48 h after tumor implantation to rats fed ad libitum, the mean tumor weight was not significantly different from that of untreated tumor-bearing animals (Table 4).

Starvation for a period of 52 h commencing 48 h after tumor implantation had no effect on tumor weight 7 days after tumor implantation. Similarly, administration of 3MPA (70 mg/kg) 48 h after tumor implantation to rats starved for 24 h prior to and for 8 h after drug administration had no effect on tumor weight 7 days after implantation (Table 4). Administration of R6G (0.8 mg/kg) 48 h after tumor implantation to rats starved for 24 h prior to and 8 h after drug administration resulted in a slight but not significant reduction in tumor weight 7 days after implantation (Table 4).
or R6G (0.8 mg/kg) to tumor-bearing rats starved for 24 h before drug administration and for 8 h thereafter did not reduce final tumor weight (Table 4). However, when the combination of R6G plus 3MPA was administered to rats starved for a total of 32 h, final tumor weight was reduced by nearly 50% (Table 4). This supports the conclusion that it was the combination of drugs administered in the fasting state which was important in reducing tumor growth rate.

Several malignant or transformed cell types, when deprived of a glucose source, suffer a dramatic lowering of ATP levels within the first hour of starvation (5). However, normal cells are able to maintain their ATP content for 12 to 24 h at levels essentially similar to those of cells grown in the presence of glucose. This differential effect probably arises from the requirement of malignant cells for glucose as an energy source.

Previously it has been demonstrated that the growth rate of the Walker 256 tumor in rats can be reduced by administration of the glucose antimetabolite, 2-deoxyglucose (6), or by administration of hydrazine sulfate, which inhibits gluconeogenesis (23). This suggests that the Walker 256 tumor is largely dependent on glucose as an energy substrate. Walker 256 cells have also been shown to possess few mitochondria, and those that are present have abnormal morphology and a reduced complement of enzymes (2). It is possible that the synergy between R6G and hypoglycemia was the result of R6G inhibiting an already restricted capacity for ATP production via oxidative phosphorylation. Thus, the tumor would be even more dependent on glycolysis for energy production. In this situation even a small decrease in the substrate available for glycolysis might lead to a substantial reduction in intracellular ATP concentration, thereby reducing cell viability.

Under normal circumstances when 1 mol of glucose is fully oxidized to carbon dioxide and water, 2 mol of ATP are produced via glycolysis, and 36 mol are produced from oxidative phosphorylation. Therefore, in terms of ATP production it is much more efficient if glucose is fully oxidized via oxidative phosphorylation rather than partially oxidized to lactate via glycolysis. The AS-30D hepatoma is similar to the Walker 256 tumor in that it is a rapidly growing rodent tumor which is known to exhibit high rates of aerobic glycolysis and lactate production in vitro (2). It has been demonstrated that, when glucose is supplied to AS-30D cells as the exogenous energy source, 60% of total ATP production is derived from glycolysis, and only 40% from oxidative phosphorylation (4). However, in the absence of added glucose, glutamine alone can maintain the same ATP production rates. Thus, although these hepatoma cells rely predominantly on the inefficient glycolytic mode for energy production in vitro, in the absence of glucose other substrates which enter the Krebs cycle directly are able to maintain ATP production.

In the present study, a selective antineoplastic effect was demonstrated by administration of an inhibitor of oxidative phosphorylation (R6G) during a period of hypoglycemia. Neither treatment was effective on its own. This suggests that, for the Walker 256 tumor in vivo, both glycolysis and oxidative phosphorylation are important in the maintenance of cellular ATP levels. Furthermore, cancer cell lines exhibit a broad spectrum of aerobic glycolytic activity. Slow-growing, well-differentiated tumors resemble normal cells in that they exhibit low rates of glycolysis and high rates of oxidative metabolism for energy production (2, 24). Previous attempts to use tumor energy metabolism as a target for antineoplastic therapy have used single agents aimed at inhibiting either glycolysis or oxidative phosphorylation (6, 25). Since most tumor cells use both pathways for energy production, this approach is unlikely to succeed. The results of this and of other studies (7, 16) indicate that simultaneous manipulation of both sources of intracellular ATP may be used to achieve a more selective control of tumor growth.

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REFERENCES

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