Improvement of the Antitumor Activity of Intraperitoneally and Orally Administered 5,6-Dimethylxanthenone-4-Acetic Acid by Optimal Scheduling

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ABSTRACT

Purpose: 5,6-Dimethylxanthenone-4-acetic acid (DMXAA), a new anticancer drug that has recently completed Phase I clinical trial, is effective against transplantable murine tumors with established vasculature. We wished to determine the relationship between administration schedule and antitumor activity.

Experimental Design: C57Bl/6 mice with s.c. implanted Colon 38 tumors were used for determination of maximal tolerated doses and tumor growth delay. Plasma and tissue DMXAA concentrations were measured by high-performance liquid chromatography.

Results: Continuous infusion (30 mg/kg/day for 3 days) and daily i.p. administration schedules (7.5 mg/kg) were ineffective. A pharmacokinetically guided schedule was developed to increase tumor tissue drug concentrations without increasing the maximal plasma concentration. A schedule comprising a loading dose (25 mg/kg, i.p.) followed by supplementary doses (5 mg/kg after 4 and 8 h) provided a 1.6-fold increase in tumor tissue area under the concentration-time curve, no increased toxicity, and superior antitumor activity (100% cure rate, as compared with 55% for a single i.p. dose of 25 mg/kg). A similar strategy was developed for oral administration with a loading dose (30 mg/kg) and supplementary doses (15 mg/kg after 4 and 8 h). It provided a 90% cure rate, in contrast to a single oral dose (0% cure rate).

Conclusions: The antitumor action of DMXAA is schedule dependent, and the achievement of an adequate tumor tissue DMXAA concentration above a threshold value appears to be critical for activity. The use of a pharmacokinetically guided schedule provides excellent oral activity against Colon 38 tumors and provides a basis for developing more effective administration schedules in clinical trials.

INTRODUCTION

5,6-Dimethylxanthenone-4-acetic acid (DMXAA), a new anticancer agent developed in this laboratory (1), has now completed two Phase I clinical trials (2, 3). DMXAA is thought to exert its antitumor effect through an antivascular action, selectively reducing tumor blood flow in both experimental studies (4, 5) and clinical studies (2, 3, 6). Experiments with knockout mice lacking the gene for either tumor necrosis factor [TNF (7)] or TNF receptor 1 (8) have provided compelling evidence that the DMXAA-induced synthesis of TNF within tumor tissue is a major contributor to its action. A thorough investigation of whether TNF is produced in human tumor tissue in response to DMXAA has not yet been carried out, but preliminary data show increased TNF in a tumor biopsy taken from one patient (9). The in situ production within tumor tissue of TNF and perhaps other inflammatory cytokines and vasoactive molecules could form the basis of a new approach to human antitumor therapy (10, 11).

An important consideration in the further clinical development of DMXAA is to optimize the schedule of administration. Most previous preclinical studies have used single-dose i.p. administration, although one study has shown that repeat dosing after 3 days causes both increased antitumor activity and increased TNF production (12). The question of optimal scheduling is of particular importance to oral administration, which may offer significant patient benefit in the clinic. In mice, DMXAA has reasonable bioavailability but only marginal antitumor activity after single-dose oral administration (13). The optimal schedule of administration of an antivascular agent will depend on its mode of action. If it acts directly and cumulatively on vascular endothelial cells, a repeated daily administration schedule is required for the suppression of endothelial cell proliferation in the tumor bed by antiangiogenic drugs (14, 15), might be beneficial. On the other hand, if a threshold drug concentration is required for the generation of a tissue TNF response, administration schedules could be optimized for this threshold. Previous studies involving the coadministration of thalidomide (16) or cyproheptadine (17) with DMXAA have demonstrated that pharmacokinetic interactions, leading to reduced plasma clearance of DMXAA, rather than an increased C\text{max} (maximum drug concentration) may be responsible for the increased activity. These results raise the question of whether improvement of the antitumor activity of DMXAA might be achieved by optimization of the administration schedule. This could be particularly important for oral administration, where DMXAA has good bioavailability, but a single dose has only marginal antitumor activity (13).

In this study, we have determined the activity of DMXAA,
administered in daily, continuous infusion and intermittent schedules, against the Colon 38 adenocarcinoma in mice. We have also used both plasma and tumor tissue pharmacokinetics to devise i.p. and oral administration schedules for DMXAA that result in greatly improved antitumor activity. The results have implications for the design of administration protocols for DMXAA in clinical trials.

MATERIALS AND METHODS

Mice and Tumor Models. All procedures were approved by the University of Auckland Animal Ethics Committee. C57Bl/6 mice used in these studies were between 10 and 12 weeks of age, obtained from Animal Resources Unit, University of Auckland, and housed under constant temperature and humidity using sterile bedding, water, and food. Mice were anesthetized with sodium pentobarbitone (87 mg/kg, i.p.), and fragments (1 mm3) of the murine Colon 38 adenocarcinoma were implanted s.c. in the left flank. DMXAA treatment was initiated when tumor volumes were approximately 60 mm3 (10–14 days after implantation). Tumor size was measured thrice weekly using calipers, and volumes were calculated as 0.52\(a^2b\), where \(a\) and \(b\) were the minor and major tumor axes. The arithmetic means and SE of the means were calculated for each time point, counting cured animals as having zero tumor volume, and expressed as fractions of the pretreatment tumor volume. Growth delay was determined as the difference in the number of days required for the untreated and treated tumors to reach \(4\times\) the pretreatment volume.

Drug Administration. The administration routes were i.p. bolus injection, oral gavage, and s.c. continuous infusion. Infusion schedules were performed using Alzet micro-osmotic pumps (model 1003D; Alza Corp., Palo Alto, CA), which were implanted s.c. into right flanks of mice under anesthesia (pentobarbitone, 87 mg/kg) and used to deliver drug for 3 days. The pumps were then removed and checked to ensure they were empty by transferring them into a vial containing 1 ml of Milli-Q water and measuring any residual DMXAA by high-performance liquid chromatography assay (see below). The postinfusion release was within the manufacturer’s margins (<5%).

Pharmacokinetic Studies. Tumor-bearing mice (3 mice/group) were treated i.p. or orally with DMXAA. After the indicated times, mice were anesthetized with halothane, and blood samples (700–800 \(\mu\)l) were collected from the ocular sinus into heparinized tubes and centrifuged (1000 \(g\), 10 min). Plasma was collected and stored at –20°C until analysis. Imme-

<table>
<thead>
<tr>
<th>No</th>
<th>Schedule</th>
<th>C57Bl/6 mice</th>
<th>Route</th>
<th>Toxicity</th>
<th>Weight loss*</th>
<th>Deaths</th>
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<tbody>
<tr>
<td>1</td>
<td>Daily 5 mg/kg/day (28 days)</td>
<td>No tumor</td>
<td>i.p.</td>
<td>%</td>
<td>Day</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Daily 10 mg/kg/day (10 days)</td>
<td>No tumor</td>
<td>i.p.</td>
<td>0/5</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Daily 5 mg/kg/day</td>
<td>Tumor</td>
<td>i.p.</td>
<td>0/5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Daily 7.5 mg/kg/day</td>
<td>Tumor</td>
<td>i.p.</td>
<td>0/5</td>
<td></td>
<td></td>
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<tr>
<td>5</td>
<td>7 mg/kg/day (3d pump)</td>
<td>Tumor</td>
<td>s.c.</td>
<td>11, 5</td>
<td>0/1</td>
<td></td>
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<tr>
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<td>10 mg/kg/day (3d pump)</td>
<td>No tumor</td>
<td>s.c.</td>
<td>5, 2</td>
<td>0/1</td>
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</tr>
<tr>
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<td>s.c.</td>
<td>15, 8</td>
<td>0/1</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>20 mg/kg/day (3d pump)</td>
<td>No tumor</td>
<td>s.c.</td>
<td>16, 8</td>
<td>0/1</td>
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</tr>
<tr>
<td>9</td>
<td>30 mg/kg/day (3d pump)</td>
<td>No tumor</td>
<td>s.c.</td>
<td>18, 7</td>
<td>0/1</td>
<td></td>
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<tr>
<td>10</td>
<td>30 mg/kg/day (3d pump)</td>
<td>Tumor</td>
<td>s.c.</td>
<td>18, 6</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>25 mg/kg, 5 mg/kg at 4 h</td>
<td>No tumor</td>
<td>i.p.</td>
<td>8, 1</td>
<td>0/2</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>25 mg/kg, 5 mg/kg, 5 mg/kg at 4 h</td>
<td>No tumor</td>
<td>i.p.</td>
<td>12, 2</td>
<td>0/2</td>
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<td>13</td>
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<td>Oral</td>
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<tr>
<td>14</td>
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<td>No tumor</td>
<td>Oral</td>
<td>12, 1</td>
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<tr>
<td>15</td>
<td>30 mg/kg, 15 mg/kg, 15 mg/kg at 4 h</td>
<td>Tumor</td>
<td>Oral</td>
<td>5, 1</td>
<td>0/5</td>
<td></td>
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</table>

*Maximal weight loss (percentage) as compared with the first day of treatment (day 0), including the day after first treatment at which this was observed.

Fig. 1 Antitumor activity of DMXAA using low-dose schedules. Mice with Colon 38 tumors were either untreated (E) or treated daily i.p. with DMXAA at doses of 5 (○) or 7.5 mg/kg (▲). Tumor volumes are depicted as mean values ± SE from five mice.
Table 2 Pharmacokinetic parameters using different dose schedules and routes of administration

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dose schedule</th>
<th>Route</th>
<th>C_{max}^{a} (μmol/liter or μmol/kg)</th>
<th>AUC_{0–24h}^{b} (μmol-h/liter)</th>
<th>AUC ratio^{c}</th>
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<tr>
<td>Plasma</td>
<td>DMXAA (25 mg/kg)</td>
<td>i.p.</td>
<td>397 ± 61</td>
<td>1777</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DMXAA (25 mg/kg) + thalidomide (100 mg/kg)^{d}</td>
<td>i.p.</td>
<td>425 ± 44</td>
<td>2300</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>DMXAA (25 mg/kg followed by 5 mg/kg at 4 h and 8 h)</td>
<td>i.p.</td>
<td>394 ± 23</td>
<td>3070</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>DMXAA (30 mg/kg)^{e}</td>
<td>Oral</td>
<td>271 ± 70</td>
<td>1977</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DMXAA (30 mg/kg followed by 15 mg/kg at 4 h and 8 h)</td>
<td>Oral</td>
<td>279 ± 66</td>
<td>2880</td>
<td>1.4</td>
</tr>
<tr>
<td>Tumor</td>
<td>DMXAA (25 mg/kg)</td>
<td>i.p.</td>
<td>72 ± 1.4</td>
<td>772</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DMXAA (25 mg/kg) + thalidomide (100 mg/kg)^{e}</td>
<td>i.p.</td>
<td>63 ± 6.1</td>
<td>1020</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>DMXAA (25 mg/kg followed by 5 mg/kg at 4 h and 8 h)</td>
<td>i.p.</td>
<td>77 ± 1.3</td>
<td>1200</td>
<td>1.6</td>
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<td></td>
<td>DMXAA (30 mg/kg)^{e}</td>
<td>Oral</td>
<td>54 ± 9.6</td>
<td>484</td>
<td></td>
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<tr>
<td></td>
<td>DMXAA (30 mg/kg followed by 15 mg/kg at 4 h and 8 h)</td>
<td>Oral</td>
<td>161 ± 23</td>
<td>1673</td>
<td>3.5</td>
</tr>
</tbody>
</table>

^{a} Mean ± SE from three mice.
^{b} AUC, area under the concentration-time curve.
^{c} Ratio = AUC_{0–24h}^{b} DMXAA/AUC_{0–24h}^{b} DMXAA (single dose).
^{d} Data from a previously published study (16).
^{e} Data from a previously published study (13).

diately after blood collection, mice were killed by cervical dislocation. The tumor was removed, weighed, and homogenized in 1 ml of 10 mM ammonium acetate buffer (pH 5.5) using a tissue homogenizer (S/N TH-71; Omni International), and the homogenate was frozen and stored at -20°C. Plasma and tumor tissue homogenate samples were assayed using automated solid phase extraction and high-performance liquid chromatography as described previously (16).

Analysis. The area under the concentration-time curve (AUC) was calculated using the log trapezoidal rule, with MK-MODEL software (18). Differences between plasma concentrations in different groups were assessed by Student’s t test.

RESULTS

Daily Dose Schedules. In mice where DMXAA was administered in a daily dosing schedule for 28 days, one death was observed in mice treated at 10 mg/kg/day, but no deaths were observed in mice treated at 5 mg/kg/day (Table 1). DMXAA was administered at doses of 7.5 and 5 mg/kg/day in tumor-bearing mice for 11 days, providing growth delays of 3.5 and 3 days, respectively, with no cures (Fig. 1). Table 2 summarizes the pharmacokinetic data. Based on the pharmacokinetics of DMXAA at a dose of 10 mg/kg (16), a daily low-dose administration schedule (7.5 mg/kg/day i.p. for 11 days) would be expected to provide a C_{max} each day of approximately 95 μM and a total AUC of 4760 μmol-h/liter. Thus, the C_{max} values were well below that provided by a single i.p. dose of 25 mg/kg (397 μM; Table 2), whereas the AUC was well above that for a single dose (AUC, 1777 μmol/h/liter; Table 2).

Continuous Infusion Dose Schedules. Continuous (3-day) schedules were achieved with s.c. implanted osmotic pumps. To exclude the effect of surgery, mice were treated with either DMXAA or saline for 3 days. There was no significant weight loss in control mice. In mice treated with DMXAA, no deaths were recorded at doses up to 30 mg/kg/day, but dose-dependent significant body weight loss was observed (P < 0.01), limiting the dose to 30 mg/kg/day (Table 1), and the weight loss during treatment was followed by rapid body weight gain and corresponding regrowth of tumors when infusion ceased. This administration schedule produced a growth delay of 5 days with no cures (Fig. 2). Drug clearance in a continuous infusion schedule was calculated as 0.0368 liter/h/kg from the plasma concentration (125 μM) achieved after a 6-h infusion at a rate of 6 mg/kg/h. Based on this clearance, the 72-h infusion schedule (1.25 mg/kg/h for 3 days) would be expected to provide a steady-state concentration of 40.2 μM and an AUC of 3014 μmol-h/liter. Thus, the C_{max} values were well below those provided by a single dose of 25 mg/kg, and the AUC values were well above those provided by a single dose of 25 mg/kg.
Loading Dose Schedules with i.p. Drug Administration.
The above results suggested that the maintenance of a high C\text{max}, rather than an increase in AUC, might be required for high antitumor activity. We devised a schedule to maintain a high plasma concentration without exceeding the initial C\text{max}. DMXAA administered i.p. (25 mg/kg) provided a plasma C\text{max} of 400 \mu M and a plasma half-life of 4 h. Supplementary doses of DMXAA (5 mg/kg) were administered after each half-life (i.e., 4 and 8 h) to increase the plasma drug concentration without exceeding the initial C\text{max} (Fig. 3). The antitumor activity of the single dose and supplementary dose administration schedules of DMXAA was compared using the Colon 38 tumor in two independent experiments (Fig. 4). In mice receiving a single dose of 25 mg/kg, 40% of the tumors regressed with an overall 12-day and 16-day growth delays for the two experiments. In mice treated with a loading dose (25 mg/kg) and two supplementary doses (5 mg/kg), 100% of the tumors regressed completely within 20 days in both experiments. With both dose schedules, slight diarrhea, ruffled fur, and some weight loss was observed after 1 day, with full recovery by 2 days. Mice receiving the two supplementary doses alone (5 mg/kg at a 4-h interval) showed no significant tumor growth delays and no cures. The DMXAA supplementary dose schedule increased the plasma AUC by 1.7-fold and the tumor tissue AUC by 1.6-fold, as compared with the single-dose schedule (Table 2). The supplementary doses also increased the tumor tissue C\text{max} from 72 to 77 \mu M, but the change was not significant (Table 2).

Loading Dose Schedules with Oral Drug Administration. We extended the above scheduling strategy to oral administration, where a single dose (30 mg/kg) provided a lower C\text{max} than did an i.p. dose (25 mg/kg) but also produced lower drug clearance (13). To compensate for these changes, we increased the size of each of the supplementary oral doses to 15 mg/kg. The supplementary dosing schedule did not show increased toxicity (Table 1) but produced considerably higher antitumor activity (a 28-day tumor growth delay and 80% cures in the first experiment and 100% cures in the second experiment) as compared with that (6-day growth delay, no cures) using a single oral dose (30 mg/kg; Fig. 5). The schedule did not increase plasma C\text{max} above the initial value but significantly increased (3.5-fold; P < 0.01) the tumor C\text{max} (Fig. 6). This value was also significantly higher (2.1-fold; P < 0.01) than that obtained using the corresponding i.p. schedule (Fig. 3). Administration of loading and supplementary doses also increased the plasma AUC by 1.7-fold and the tumor tissue AUC 3.5-fold, as compared with the single-dose schedule (Table 2).

DISCUSSION
In this study we have compared two alternative strategies for DMXAA administration: the first aimed at maintaining DMXAA plasma concentrations by daily administration or con-
tinuous infusion schedules; and the second aimed at providing a transient high drug concentration within tumor tissue. The results of the first strategy contrast strongly with those of the second, and it is clear that the antitumor activity of DMXAA is not related directly to plasma AUC. High activity is related to achieving high tumor tissue concentrations for a relatively short time. This finding has been exploited to demonstrate for the first time that with the appropriate schedule, DMXAA displays excellent activity after oral administration.

Previous studies using combinations of DMXAA and either thalidomide (16) or cyproheptadine (17) showed that increased antitumor activity was associated with reduced plasma clearance of the drug rather than with increased plasma $C_{\text{max}}$. However, neither host toxicity nor antitumor activity was directly related to plasma AUC. There are several possible interpretations of these results. One is that the increased antitumor activity results from better distribution of DMXAA in tumor tissue. Because tumor vasculature is less efficient than that of normal tissue, plasma concentrations might need to be sustained for some time to allow adequate diffusion of the drug to all tumor cells. Another interpretation is that optimal antitumor activity requires an intracellular DMXAA concentration to exceed a threshold concentration for a certain amount of time. Such a threshold concentration has been hypothesized to be required to phosphorylate and thereby inactivate the inhibitory IκB subunit of the nuclear factor κB transcription factor (19), the main transcription factor required for the synthesis of TNF and other cytokines (20). The requirement for a threshold concentration is consistent with the observed steep dose-response curve of DMXAA in mice (5, 21).

Studies with TNF receptor 1 knockout mice have shown that the host toxicity of DMXAA is linked to the ability of the mouse to synthesize TNF (8), suggesting that a threshold TNF concentration in normal tissue might be associated with host toxicity. The strategy that we have developed aims to increase tumor tissue concentrations of DMXAA without exceeding a threshold concentration in plasma or, by implication, in normal tissue. The rationale for selectively increasing tumor tissue concentrations is based on differential clearance of DMXAA from normal and tumor tissue because previous studies have shown that clearance of DMXAA from tumor tissue is slower than that from hepatic and splenic tissue (16, 17). The loading and supplementary dose schedule thus maintained plasma levels for a longer time, allowing better diffusion of drug to all tumor cells. With this schedule, the plasma and tumor tissue pharmacokinetic profiles were similar to those observed after coadministration of DMXAA with thalidomide (Table 2). The success of this schedule suggested that it might be applied to oral administration, where a single dose (30 mg/kg) was previously found to be ineffective (13). The oral schedule, comprising a loading

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**Fig. 5** Antitumor activity of DMXAA given orally using a loading strategy. *Expt. I* and *Expt. II*, mice implanted with Colon 38 tumors received no drug (○) or DMXAA as a single oral dose (30 mg/kg) followed by two oral doses (15 mg/kg) after 4 and 8 h (●). In *Expt. II*, mice also received DMXAA orally (30 mg/kg) as a single oral dose (●) or as two oral doses (5 mg/kg) administered 4 h apart (▲). Each point represents the mean ± SE from five to six mice.

**Fig. 6** Mean DMXAA concentration-time profile in plasma and Colon 38 tumor of mice measured up to 24 h after treatment with DMXAA, either 30 mg/kg once orally (○) or 30 mg/kg orally followed by 15 mg/kg twice orally at 4 and 8 h after the initial dose (●). Each point represents the mean ± SE from three to five mice.
dose (30 mg/kg) and two supplementary doses (15 mg/kg), differed slightly from the i.p. schedule because the plasma pharmacokinetics of DMXAA after oral administration were different, with a lower Cmax (13). Application of this schedule demonstrated that DMXAA is potentially highly active as an orally administered drug (Fig. 5).

The results imply that the strategy for optimizing the activity of DMXAA differs considerably from that required for cytotoxic agents. Rather than being a direct function of AUC, activity appears to be related to achieving a nontoxic plasma drug concentration and maintaining it for a time sufficient to allow drug diffusion to the poorly diffused areas of tumor tissue. In clinical schedules, this might be achieved by nonlinear i.v. infusion rates to provide a constant plasma concentration for several hours. For oral administration in a clinical setting, the principles used in the present oral study could be applied with appropriate correction for differences between mouse and human plasma pharmacokinetics.

REFERENCES
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