Pharmacokinetic Studies in Mice of Two New Thioxanthenones (183577 and 232759) That Showed Preferential Solid Tumor Activity

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ABSTRACT

Two new thioxanthenones, 183577 and 232759, have rekindled interest in the development of representatives from this class of structures as useful antitumor agents. Although the mechanism of action is unknown, both compounds demonstrated a similar spectrum of solid tumor selectivity. 232759 was selected for clinical development because it showed no hepatotoxicity in preliminary studies, whereas 183577 showed hepatotoxicity but only at the maximum tolerated dose (MTD). The limiting toxicity for the clinical candidate was myelosuppression in preliminary studies. Plasma and tissue drug levels, as well as protein binding, were studied in mice using optimal administration times at the MTD for each drug (for 183577, this was a 4-h infusion at 1350 mg/m² and for 232759, it was a 5-min injection at 240 mg/m²), as well as at one-half the MTD for the clinical candidate. The drugs were 96–100% bound by plasma proteins. The peak drug concentrations, half-life, and area under the concentration-time curve for plasma 183577 were 3483 ng/ml, 465 min, and 2018 µg/ml · min, respectively. The peak drug concentration, half-life, and area under the concentration-time curve for 232759 were 5257 ng/ml, 44 min, and 276 µg/ml · min, respectively, at the MTD and 2810 ng/ml, 40 min, and 110 µg/ml · min at one-half the MTD. In all instances of simultaneous measurements, drug concentrations were equal or higher in tissues than they were in plasma. Unlike the plasma and kidney concentrations of 183577, the liver concentrations did not show a declining trend over the 8-h observation period. Declines in plasma, liver, kidney, and tumor levels of 232759 were detected over the 8-h observation period. The sustained high 183577 concentration in liver is believed to be responsible for its prolonged half-life and hepatotoxicity. Evidence for metabolism of the parent drugs was based on the finding of additional peaks on the high-pressure liquid chromatography tracings. Future studies will focus on identification and antitumor studies of these presumed metabolites in hopes of a better understanding of the solid tumor activity profiles and toxic effects of these compounds.

INTRODUCTION

The soft agar colony formation disk-diffusion assay developed by Corbett et al. (1, 2) provides a cost-effective means for the evaluation of the abilities of a large number of synthetic compounds and natural products to inhibit the growth of tumor cells. The preferential inhibition of solid tumor cells compared to leukemic cells in the same environment is used as the initial step in selecting compounds of potential interest for further development (3). Solid tumor cells were chosen as the preferred target in hopes of increasing the likelihood that compounds chosen for development from this system would have exploitable antitumor activity in patients with solid tumors.

The thioxanthenones are a class of compounds that contain three aromatic rings, joined together in a chromophore nucleus with a sulfur substitution in the middle ring (Fig. 1, boxed area). Hycanthone, a thioxanthenone marketed as an antischistosomal drug, was evaluated as an antitumor agent in Phase I and II trials sponsored by the National Cancer Institute over a decade ago (4–7). However, interest in the further development of hycanthone waned because of irreversible hepatotoxicity and minimal clinical antitumor activity. During the evaluation of compounds supplied by Sterling/Kodak in the soft agar colony formation disk-diffusion assay, the thioxanthenone 183577 (Fig. 1) demonstrated solid tumor selectivity compared to leukemia and normal cells (8). Thus, although the demonstration of antitumor activity by thioxanthenones in preclinical tumor models was not new, the demonstration of solid tumor selectivity by 183577 and the availability of a large inventory of analogues for similar evaluation was sufficient to rekindle interest in the possible development of thioxanthenones as antitumor agents.

Preliminary studies of 183577 in mice showed an improved efficacy profile (more antitumor activity and less toxic effects) over that of hycanthone (8). The improvements at therapeutic levels included greater efficacy, less deterioration in activity level, less weight loss and mortality during treatment, and more rapid host recovery following treatment (9). There was a high dose requirement for antitumor effect with 183577, which necessitated it being given as an infusion to avoid the acute lethality associated with rapid injection (8). 183577 also showed
subacute evidence of hepatotoxicity at lethal dose levels, manifested as elevations in alanine aminotransferase. Multiple thioxanthenone compounds supplied by Sterling/Kodak were evaluated in the soft agar colony formation disk-diffusion assay, which also demonstrated solid tumor selectivity compared to leukemia and normal cells after discovery of the lead compound (8). One compound, 232759, was more potent, maintained a high level of antitumor activity (8, 10) and was completely devoid of hepatotoxicity in preliminary preclinical evaluations (9). In addition, no acute toxic effects were observed when the drug was given as a 5-min infusion. Thus, 232759 was chosen as the candidate for clinical development from this series of thioxanthenones. In mice, the dose-limiting toxicity of 232759 was leukopenia (9).

There appears to be more than one mechanistic reason for the antitumor activity of the series (8). For example, the clinical candidate demonstrated activity linked with topoisomerase II, whereas the lead compound did not (11, 12). Clearly, additional studies are required to determine the mechanism(s) of antitumor activity of the thioxanthenones and to determine whether their mechanism(s) are compound specific.

The pharmacokinetic behavior (drug levels in plasma, tissue distribution, and protein binding) of the lead compound from the series and the clinical candidate were investigated in mice following i.v. treatment prior to initiation of the Phase I trial. The optimal dose and schedule used for these pharmacokinetic studies were determined based on the largest dose that could be given i.v. that was associated with reproducible antitumor effects and no acute lethality and that caused death in less than 10% of the animals treated and observed for over 30 days following treatment. These studies were performed with both compounds in hopes of obtaining information that might aid in understanding their preferential solid tumor activity and their toxicity profile difference. This is a comparative report of the results obtained from these studies.

**MATERIALS AND METHODS**

**Drug.** The thioxanthenones 183577 and 232759 were supplied in powder form (molecular weights of 369 and 433, respectively) by Sterling Winthrop (Rensselaer, NY). The appropriate amount of drug was weighed then dissolved in dilute (1 μM) HCl. The injection solution was then buffered to pH 5.0 with sodium bicarbonate powder. The drug solution was made immediately prior to treatment for each experiment.

**Chemicals and Solvents.** All chemicals and solvents were either analytical reagent or HPLC grade. Methanol and acetonitrile were obtained from J.T. Baker, Inc. (Phillipsburg, NJ). Acetic acid was obtained from Fisher Scientific Company (Fair Lawn, NJ). HCl was obtained from Mallinckrodt (Chesterfield, MO). Ammonium acetate and Tris were obtained from Sigma Chemical Co. (St. Louis, MO).

**Mice.** Female B6D2F1 (C77Bl/6 × DBA2) mice were obtained from the Frederick Cancer Research and Development Center, National Cancer Institute (Frederick, MD). The first-generation (F1) hybrid mice were used because they are cheaper and harder than inbred mice.

Colon adenocarcinoma 38 (or colon 38) was implanted as 30–60-mg fragments of fresh tumor using a 12-gauge trocar. Tumors were allowed to grow until they weighed approximately 0.5–1.0 g. Mice bearing L1210 leukemia were obtained by similarly implanting fragments of this tumor that had been adapted to grow s.c. This L1210 tumor had been adapted by serially implanting and removing the s.c. tumor cells until they consistently formed palpable subcutaneous tumors weighing 0.3–0.5 g, within 5–10 days following implant.

All animals were kept in standard cages and allowed to consume a standard pellet diet and water ad libitum until the time of drug treatment.

**Treatment and Sample Collection.** Each mouse received, based on their weight, 1350 mg/m² (450 mg/kg) 183577 by a 4-h infusion into a tail vein using the technique previously described (13) or either 120 or 240 mg/m² (40 or 80 mg/kg, respectively) 232759 by a 5-min injection into a tail vein. Upon completion of treatment, mice were placed in cages prelabeled

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The abbreviations used are: HPLC, high-pressure liquid chromatography; SPE, solid-phase extraction; AUC, area under the concentration-time curve; Cl, plasma clearance of drug; MTD, maximum tolerated dose.
with the time of blood collection (three or four mice per time point). Blood collection time points were 5, 10, 15, 30, 60, 240, 480, 720, and 1440 min following completion of the 183577 infusion and 5, 10, 15, 30, 60, 120, 240, 360, 480, and 1440 min following completion of the 232759 injection. Blood was collected by open chest intracardial puncture from each mouse and then immediately transferred to a heparinized micro-test tube. Test tubes containing heparinized blood were centrifuged for 10 min in a fixed-angle microcentrifuge. Plasma samples were pooled based on the blood sample collection time point. After blood collection, liver and kidneys were removed at 30, 60, 240, and 480 min following 183577 treatment, and liver, kidneys, and tumors were removed at 30, 60, 360, and 480 min following 232759 treatment. Brain, heart, lung, muscle, pancreas, and spleen were removed at 60 min following treatment with 232759.

Samples of control plasma and tissues were obtained from mice of the same strain, sex, and age that had not received treatment with either thioxanthenone. All samples from both controls and treated mice were frozen immediately after collection and stored (−20°C) until the time of sample preparation and analysis. Samples were stored for up to 6 months, and these thioxanthenones have demonstrated stability under similar storage conditions for more than 1 year. This stability is based on the analysis of control plasma samples immediately after the addition of a known quantity of each thioxanthenone followed by analysis at regular intervals (3–4 months) under storage conditions of −20°C for up to 17 months.

Sample Preparation. Plasma samples were slowly thawed in an ice-water bath. The protein was precipitated from a 0.5-ml aliquot using chilled methanol (1:2, v/v). The tissues were allowed to thaw, and then they were weighed. Tissue homogenates (1:3, w/v) were then made using buffer (0.1 M Tris-HCl, pH 7.4). The protein was precipitated from the tissue homogenates in a manner similar to that used to separate plasma from four healthy volunteers, or buffer (0.25 M ammonium acetate, pH 4.0), at concentrations of 0.5, 1.0, 5.0, 10.0, and 50 μg/ml and 0.5, 1.0, 10.0, 40.0, and 80 μg/ml for 183577 and 232759, respectively. The concentrations in buffer solution were used both as nonfiltered controls and to determine the degree of nonspecific binding of drug to the membrane of the separation system. A 1-ml aliquot of each solution was incubated at 37°C for 1-h, and then the entire volume of each was placed into an Amicon Centrifree micropartition system (Amicon Division, W.R. Grace and Co., Beverly, MA) and centrifuged for 30 min at 3000 × g using a fixed-angle rotor. Aliquots (50 μl) of the ultrafiltrate and unfiltered buffer concentrations were assayed directly (without sample preparation as described above) using the HPLC assay described below. The percent of thioxanthenone bound to protein was determined by comparing the peak area of the plasma ultrafiltrate to the peak area of the unfiltered buffer control using the equation:

\[
\text{% protein bound} = 1 - \frac{\text{area of ultrafiltrate sample}}{\text{area of unfiltered sample}} \times 100.
\]

Means of 2% of the 183577 and 5% of the 232759 showed nonspecific drug binding to the membrane of the separation system.

**HPLC Apparatus and Assay.** The HPLC system consisted of a Waters Maxima Workstation and chromatography program, a 710B Wisp autosampler (Waters), two 510B solvent pumps (Waters), and a 773 Spectraflow UV-visible detector (Kratos, Inc., Westwood, NJ) set at 254 nm. The analytical
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The mobile phase was 75% 0.25 M column fitted with a J1Bondapak C,5 precolumn cartridge in a 0.2-1.6% for 183577 and 232759, respectively. Drug recovery curve linear regression range was 0.996-0.999 for both thioxanthenones in control matrix (plasma or tissue-concentrations) constructed from the results of dissolving the concentration was quantified using a standard curve (five or six quantitation were 100 and 65 ng/ml for 183577 and 232759, respectively. The lower limits of these conditions, the thioxanthenone peaks eluted near 8 and 13 min for 183577 and 232759.

Drug recovery from the SPE column was determined by comparing the peak areas obtained from standard curve concentrations of each thioxanthenone dissolved in control matrix, prepared as described previously. The assay was linear to 50,000 ng/ml for 232759 and greater than 90% over the range of 100-32,000 ng/ml for 183577. The thioxanthenone peaks eluted near 8 and 13 min for 183577 and 232759, respectively. The lower limits of quantitation were 100 and 65 ng/ml for 183577 and 232759, respectively, and the assay was linear to 50,000 ng/ml for 183577 and to 32,000 ng/ml for 232759. The thioxanthenone concentration was quantified using a standard curve (five or six concentrations) constructed from the results of dissolving the appropriate thioxanthenone in control matrix (plasma or tissue-specific homogenate) and extracted in a manner similar to that used to extract the samples from treated mice. The standard curve linear regression range was 0.996–0.999 for both thioxanthenones. Assay validations were accomplished using run standards (drug dissolved in control matrix and processed in a similar manner) and analyzed after every four samples. The intraassay coefficient of variation ranges were 0.5–1.8% and 0.2–1.6% for 183577 and 232759, respectively. Drug recovery from the SPE column was determined by comparing the peak areas obtained from standard curve concentrations of each thioxanthenone dissolved in control matrix, prepared as described above to peak areas of the same concentration of each drug dissolved in methanol, and then similarly dried and reconstituted for injection. SPE column drug recovery was greater than 80% over the range of 500–50,000 ng/ml for 183577 and greater than 90% over the range of 100–32,000 ng/ml for 232759.

Pharmacokinetic Data Analysis. The pharmacokinetic data from plasma were analyzed by a computer-generated nonlinear least squares regression analysis with a weighting of \(1/\sqrt{y + \hat{y}^2}\), where \(y\) equals the observed plasma concentration and \(\hat{y}\) equals the predicted plasma concentration (14, 15). The computer program CRVFIT (kindly provided by Dr. L. Hart, Institute of Cancer Research, Sutton, Surrey, United Kingdom) was used to facilitate these calculations. The data points were fitted to a monoexponential model to facilitate comparisons using the equation:

\[
C = Ae^{-at},
\]

where \(C\) is the plasma thioxanthenone concentration at time \(t\), \(A\) is the concentration constant, and \(a\) is the first-order rate constant. The AUC was determined using the trapezoidal rule (16). The CI was calculated using the equation:

\[
CI = \text{Dose/AUC}.
\]

The \(t_{1/2}\) was calculated from the first-order rate constant using the equation:

\[
t_{1/2} = 0.693/\text{first-order rate constant}.
\]

The computer program PK2 (kindly provided by Dr. D. Newell, Newcastle University, Newcastle-Upon-Tyne, United Kingdom) was used to facilitate calculations of CI and \(t_{1/2}\).

RESULTS

Pharmacokinetics. A graph of the plasma drug concentrations in mice following i.v. treatment with either 183577 or 232759 at their MTDs of 1350 and 240 mg/m², respectively, is shown in Fig. 2. The corresponding monoexponential (linear regression) line for each compound is also shown. Plasma concentrations of 232759 were below the limit of detection after 240 min, whereas 183577 remained detectable over the entire sampling period. The slope of the 183577 line was much flatter than that of the 232759 line. A comparative summary of the pharmacokinetic parameters of each drug in mice following treatment with their MTDs and with half the MTD (120 mg/m²) for the compound chosen for clinical trial (232759)

Table 3  232759 concentrations in nontumored and tumored mice following a slow i.v. injection of 240 mg/m²

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Plasma (μg/ml)</th>
<th>Plasma* (μg/ml)</th>
<th>Liver (μg/g)</th>
<th>Kidney (μg/g)</th>
<th>Tumor (μg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>2.7</td>
<td>2.7</td>
<td>10</td>
<td>26</td>
<td>6</td>
</tr>
<tr>
<td>60</td>
<td>1.4</td>
<td>2.2</td>
<td>8</td>
<td>19</td>
<td>8</td>
</tr>
<tr>
<td>360</td>
<td>BLQ*</td>
<td>BLQ</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>1</td>
</tr>
<tr>
<td>480</td>
<td>BLQ</td>
<td>BLQ</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>1</td>
</tr>
</tbody>
</table>

* Plasma results are from pooled plasma (three or four mice per time point), and tissue results are the mean values of tissues from three or four mice per time point.

Table 4  232759 concentrations in tumored mice following a slow i.v. injection of 240 mg/m²

<table>
<thead>
<tr>
<th>Mice</th>
<th>Plasma* (μg/ml)</th>
<th>Brain* (μg/g)</th>
<th>Muscle (μg/g)</th>
<th>Spleen (μg/g)</th>
<th>Heart (μg/g)</th>
<th>Lungs (μg/g)</th>
<th>Pancreas (μg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1210 bearing</td>
<td>2.2</td>
<td>4</td>
<td>4</td>
<td>34</td>
<td>6</td>
<td>26</td>
<td>20</td>
</tr>
<tr>
<td>Colon 38 bearing</td>
<td>1.3</td>
<td>1</td>
<td>1</td>
<td>13</td>
<td>1</td>
<td>15</td>
<td>12</td>
</tr>
</tbody>
</table>
is shown in Table 1. Although the MTD of 183577 is slightly over 5 times that of 232759, the 183577 peak plasma concentration is lower than that of 232759 because 183577 requires 4-h infusion to avoid acute lethality, whereas 232759 is tolerated as a slow i.v. injection. The drug half-life obtained following treatment with 183577 was approximately 10 times longer than that obtained following treatment with 232759. The 232759 half-lives obtained following treatment at the MTD and at one-half the MTD were similar. In addition, comparisons of the two peak 232759 concentrations and AUC levels in plasma were proportional to dose. As would be expected based on their half-lives, comparisons of Cs showed that 183577 had a lower value than that of 232759.

**Tissue Distribution.** Summaries of simultaneous plasma and tissue levels in mice following treatment with the thioxanththenones are shown in Tables 2-4. In all instances, the tissue
Pharmacokinetic Studies in Mice

Table 5 Thioxanthenone protein binding in fresh plasma

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration (µg/ml)</th>
<th>% bound to plasma proteins from Mice</th>
<th>Men*</th>
<th>Women*</th>
</tr>
</thead>
<tbody>
<tr>
<td>183577</td>
<td>0.5</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>50.0</td>
<td>99</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>232759</td>
<td>0.5</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>97</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>40.0</td>
<td>96</td>
<td>99</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>80.0</td>
<td>99</td>
<td>98</td>
<td>98</td>
</tr>
</tbody>
</table>

*Human values are the mean of duplicate samples from two healthy volunteers.

Pharmacokinetic studies in mice showed that the lead compound from this inventory (183577) had a half-life that was >10 times longer than that of the analogue chosen for clinical development (232759). The two compounds showed high protein binding (96–100%) in mice and human plasma. Therefore, exposure to similar levels of free drug is predicted to occur in both species.

Rapid tissue distribution was observed with both compounds. High and sustained tissue concentrations were observed with 183577 and clearly contributed to its prolonged plasma half-life. This rapid and high tissue affinity is thought to be responsible for the acute lethality observed when 183577 is given as a rapid i.v. injection. In addition, because concentrations in the liver showed an equilibrium that was not observed in plasma or kidney levels, the hepatotoxicity observed at the high treatment doses (9) may result from sustained high liver drug concentrations. Comparisons of the HPLC tracings obtained from liver and kidney following treatment with 183577 did not show obvious qualitative differences to suggest that the liver produced a locally toxic substance. However, liver production of a potent toxic metabolite below the detection limit cannot be ruled out. The 232759 concentration in all tissues declined over the observation period. Interestingly, no significant concentration differences of 232759 were observed between L1210 and colon 38 tumors. However, it was noted that tracings from treated mice bearing colon 38 were different from those from mice bearing L1210. Although this latter point seems small in visual comparisons of the tracings (Fig. 3), whether these small differences contribute to the observed preferential solid tumor activity of the thioxanthenones should be explored.

Comparisons of potency and in vivo antitumor effect showed poor correlation for the thioxanthenone series, i.e., the most potent agents (lowest dose requirements to reach toxicity) showed the least efficacy (8). The ratios obtained comparing the in vitro µg/disk to the MTD (expressed in mg/kg total dose) in a tumor (Pan 03) that showed equal cytotoxic effects were 11 for 183577 and 7.8 for 232759, indicating no clear potency derived efficacy advantage of one compound over the other (8). In vitro levels (50–125 µg/disk for 183577 and 3–16 µg/disk for 232759) required for antitumor effect in a variety of tumors (8) were achieved in mouse tissues for both compounds and tissues and plasma for 232759 following treatment at the MTD, using the optimal dose and schedule.

Future studies will isolate, identify, and test the metabolites of 232759 for antitumor activity. In addition, pharmacokinetic studies will be performed to assess exposure to parent drug and evaluate metabolism during the Phase I trial of 232759. These results observed in mice may serve as a basis for interspecies comparisons of the pharmacokinetic behavior, including metabolism, of this compound.

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REFERENCES


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