Drug Treatments for Metastasis of the Lewis Lung Carcinoma: Lack of Correlation between Inhibition of Lung Metastasis and Survival

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ABSTRACT

The abilities of the Eli Lilly compounds LY150310, LY189332, and LY135305 to inhibit spontaneous metastasis and to increase animal survival were evaluated. These compounds represent widely varied structures and were evaluated because they have been found to inhibit thromboxane synthetase, cyclooxygenase, and thrombin activation, respectively. These biochemical processes have been proposed in the literature as targets for antimetastatic drugs. The purpose of this investigation was twofold: (a) to compare the antimetastatic activities of the Eli Lilly compounds to those of the reference antimetastatic compounds nafazatrom and RA233, and (b) to examine the correlation between inhibition of spontaneous lung metastasis and survival. Spontaneous metastasis of the Lewis lung carcinoma was used to evaluate the antimetastatic activity of the compounds. In this model 5 x 10^6 tumor cells were implanted into the gastrocnemius muscle, the primary tumor was resected on Day 14, and metastatic lung lesions were counted on Day 25. Compounds were administered every 12 h on Days 5 through 19. Nafazatrom, LY150310, LY189332, and LY135305 were found to inhibit spontaneous lung metastasis in a dose-dependent manner. The ED50 values for the respective inhibitions with these compounds were 50, 0.5, 2, and 0.35 mg/kg/day; the respective therapeutic indexes (LD50/ED50) were 7, 180, 255, and 511. To evaluate the effect of nafazatrom, LY150310, LY189332, and LY135305 on animal survival, the compounds were given at maximally antimetastatic doses of 200, 60, 20, and 6 mg/kg/day, respectively. Two dosing schedules were used: (a) on Days 5 through 19 and (b) on Day 5 until death. Neither the median survival times nor the numbers of long-term survivors were significantly changed with any of the compounds at any dosing schedule. RA233, given to a maximally tolerated dose of 200 mg/kg/day on Day 5 until death, did not inhibit lung metastasis and did not increase median survival time. Postmortem examination of animals dosed with nafazatrom, LY150310, LY189332, and LY135305 showed complete inhibition in lung lesions and the appearance of lesions in the liver, kidney, spleen, and brain. The results of this investigation show that the effect a compound has on the number of metastatic lesions in a target organ may not be predictive of its effect on survival. To successfully translate laboratory data into the clinic, survival should be considered as a predictor of a compound's potential clinical utility.

INTRODUCTION

Metastasis, the hallmark of malignant cancers, is responsible for major problems in the diagnosis, staging, and treatment of cancers (1). This has resulted in a considerable amount of research on the biological processes involved in metastasis (for reviews on this research see Refs. 2–5). The ultimate goal of this research is to develop better therapies for malignant diseases.

Since Wood's original observations (6) in vivo on the involvement of thrombi in the metastasis of V2 rabbit carcinoma cells, there have been numerous studies implicating the involvement of fibrin and platelets in metastasis (7–9). Because of these studies, it is not surprising that anticoagulants and platelet aggregation inhibitors have been examined as antimetastatic agents. The results of these numerous studies have been conflicting. Some investigators found an inhibition of metastasis, others an increase, and still others found no effect. Part of the controversy, especially in the older investigations, may be due to poor experimental design because of an incomplete understanding of the pharmacology of the agents studied. Several investigators, however, have pointed out that a major reason for confusion in the literature is due to the metastasis models used to evaluate an agent. In many studies the antimetastatic action of anticoagulants or platelet aggregation inhibitors has been observed in models where the tumor cells are administered i.v. Useful insights have been gained from these "artificial" metastasis models. However, these models can be criticized as assays of tumor cell arrest that occur under abnormally high cell burdens. In some cases, the results obtained in "artificial" models cannot extrapolate to those obtained in "spontaneous" metastasis models where all the steps of the metastatic cascade are in place (10). A more important flaw in the experimental design of most reported antimetastasis studies is that they do not evaluate an agent's effect on survival. As discussed later, survival data may be a more relevant measure of an experimental drug's potential clinical utility.

In this report we present the results of our investigation on the antimetastatic action of the Eli Lilly compounds LY150310, LY189332, and LY135305. These compounds were discovered in screening assays designed to identify inhibitors of thromboxane synthetase, cyclooxygenase, and thrombin activation, respectively. The purpose of this investigation was twofold. First, the antimetastatic actions of compounds LY150310, LY189332, and LY135305 were compared to those of RA233 and nafazatrom. RA233, an inhibitor of platelet aggregation (see citations in Ref. 11), is marketed in West Germany as an antimetastatic agent under the trade name Rapenton. It is being evaluated in the U.S. as an antimetastatic agent (12). Nafazatrom (Bay g 6575), an antithrombotic agent (see citations in Refs. 13, 14), has been reported by Honn et al. to inhibit the artificial and spontaneous metastasis of B16 melanoma and Lewis lung tumors (14). It has also been evaluated clinically as an antimetastatic agent (15 and citations in Ref. 16). The second purpose of this investigation was to examine the correlation between the inhibition of metastasis and animal survival.

MATERIALS AND METHODS

Drugs. The structures of the drugs used in this study are shown in Fig. 1. RA233 (Rapenton, mopidamole) was purchased in West Germany from Boehringer Ingleheim. Nafazatrom, LY150310, and LY135305 were made as described in the patent literature (17–19). Compound LY189332 was prepared from the intermediate, 4'-amino-4,4''-dimethoxy-1,1':2',1''-terphenyl, as described in a patent by Matsumoto et al. (20). All drugs were dissolved in a sterile vehicle composed of 1 part Emulphor 620 in 39 parts 0.9% saline. Emulphor 620 (polyoxyethylated vegetable oil) was purchased from General Aniline and Film, Wayne, NJ. The antimetastatic action of these Lilly compounds were discovered in screens looking for agents that inhibit tumor...
Fig. 1. Structures of the drugs evaluated.

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After this initial use of trypsin, the cells were passed using a solution of collagenase (24 units/cm²) and DNase (7 units/cm²). The collagenase used in this investigation. The tumor was used between in vitro passages of 5 to 7. Single cell suspensions were prepared for implantation into the mice using the collagenase/DNase solution described above. All culture manipulations were done under gold fluorescent light.

Animals and Surgical Procedure. C57BL/6N female mice were obtained from the Portage, MI, breeding facilities of Charles River Laboratories, Inc. Like other investigators (22), we found an increase in the variability and a decrease in the number of metastatic lung colonies as the age of the host animals increased. Therefore, only mice between 4 and 6 weeks old were used in this investigation. A group of five randomly chosen animals from each shipment was evaluated upon arrival, and at the end of each experiment, for murine pathogens. These evaluations are important because we have found that the variability increases and the number of lung metastases decreases when animals are infected with a pathogen, such as murine hepatitis virus. Animals were housed in plastic shoebox cages covered with a microisolator filter top. All animal feed, water, bedding, and cages were sterilized. The animals were provided ad libitum water (pH 3.0) and a 4% fat mouse diet manufactured by Teklad, Madison, WI. All animal manipulations were done aseptically in a HEPA, 100% exhaust, vertical laminar flow hood.

Surgical procedures were done with sterile instruments and aseptic technique. A 100% oxygen atmosphere saturated with methoxyflurane (metofane) and water was used to obtain surgical anesthesia (23). Body temperature was maintained at 37°C with an incandescent heat source. A skin incision was made completely around the leg just above the tumor and the skin was then pulled toward the calcanei tendon. Vessels were ligated with 4-0 surgical thread and the leg was amputated just below the head of the femur. The wound was closed with stainless steel surgical clips. In animal survival studies, the surgical clips were removed after 14 days to prevent ulceration and infection of the wound. Using this procedure recurrence of the tumor was rarely seen at the incision site. After amputation the animals were injected s.c. with 1 ml of sterile 0.9% saline to prevent dehydration. Sterile saline was also used to irrigate the eyes and prevent blindness. During postanesthetic recovery the animals were kept at 37°C.

Spontaneous Metastasis Model and Drug Treatments. The following procedure was used to evaluate the effect of drugs on spontaneous lung metastasis of Lewis lung carcinoma. On Day 0, 5 x 10⁶ tumor cells (in a volume of 25 µl) were implanted into the gastrocnemius muscle and the animals were then randomly divided into treatment groups (10 animals per group). In agreement with others (see citations in Ref. 24), we have found that a small amount of artificial seeding occurs when a tumor cell suspension is implanted intramuscularly. To determine the amount of artificial seeding, the tumor bearing leg in a group of animals was amputated on Day 1. These animals were also treated with drug vehicle. On Day 14, the primary tumor was resected in the vehicle-treated and drug-treatment groups. Day 14 was the maximum time that surgery could be delayed and still remove all the primary tumor. The corrected mean number of spontaneous metastases was determined by subtracting the number of tumor colonies in the Day 1 amputation group from the number of colonies in animals that had the primary tumor resected on Day 14 and were treated with vehicle or drug. Legs were amputated on Day 14 in one group of nontumor bearing animals. These animals were used as surgical controls for a normal tissue background in evaluation of metastatic colonies in the organs of the vehicle-treated and drug-treated animals. These nontumor bearing animals were also treated with drug vehicle. All animals were sacrificed on Day 25 by CO₂ asphyxiation and the number of macroscopic lung colonies were determined after fixation in Bouin’s fixative. The animals were sacrificed on Day 25 because our initial studies had shown that the animals would begin to die on Day 28.

The animals were weighed at the start of an experiment and on Day 25 to determine if the compounds had any effect on animal weight. The tumor bearing legs amputated on Day 14 were used to determine if a compound had any effect on the growth of the primary tumor. To estimate tumor weight the amputated legs were first dried in a vacuum oven. Tumor weight was then estimated by subtracting the mean leg weights for the nontumor bearing animals from the mean leg weights for the vehicle-treated and drug-treated tumor bearing animals. To evaluate antimetastatic activity, the drugs (or vehicle) were given i.p. every 12 h on Days 5 through 19. This treatment period was chosen from our initial studies that determined the time-course for lung metastasis of the Lewis lung carcinoma after intramuscular implantation (data not shown). In these studies the Lewis lung tumor was found to start metastasizing on Day 6. By Day 10, 100% of the animals had metastatic disease. Treatment was continued through Day 19 to allow for the clearance of any tumor cells released from the primary tumor on Day 4. On Day 19 treatment was stopped to allow for the growth of tumor cells and, thus, determine whether the antimetastatic activity was because of a cytostatic activity of the compounds.

Animal Survival Studies. In these studies 5 x 10⁶ cells were implanted into the gastrocnemius muscle on Day 0 and the animals were randomly divided into treatment groups. For the studies with nafazatrom, LY189332, LY150310, and LY135305 there were 20 animals in each group. For the study with RA233 there were 10 mice in each group. Amputations were done on Days 1 and 14 as discussed above. Except for RA233, animals were treated with a compound at the dose found to maximally inhibit spontaneous lung metastasis. Two dosing sched-
Effects on Spontaneous Lung Metastasis. In Table 1 is shown the effect of nafazatrom on the number of metastatic lesions for the individual animals in each treatment group. The number of lesions for the individual animals in each control group represent the number that were observed in the control groups for the other compounds. Nafazatrom inhibited spontaneous lung metastasis in a dose-dependent manner (Fig. 2). This inhibition was significant \((P < 0.05)\) at 200 mg/kg/day. However, the curve that described the inhibition was steep. A 54 and a 100% inhibition in the number of metastatic lung lesions was obtained at doses of 60 and 200 mg/kg/day, respectively. The \(ED_{50}\) for the antimitastatic action of nafazatrom was approximately 50 mg/kg/day. Some toxicity that resulted in a 10% mortality was also seen at 200 mg/kg/day. At concentrations greater than this, nafazatrom was more toxic and had a \(LD_{50}\) value of 335 mg/kg/day. Nafazatrom was estimated to have a therapeutic index \((LD_{50}/ED_{50})\) of 7.

In Fig. 2 the antimitastatic action of LY150310 is also shown. At 2 mg/kg/day or higher, this compound significantly \((P < 0.05)\) inhibited spontaneous lung metastasis in a dose-dependent manner. However, the curve that described the inhibition was not as steep as that for nafazatrom. A plateau of 100% inhibition in lung metastasis was obtained at doses of 20 and 60 mg/kg/day. The \(ED_{50}\) for LY150310 was 0.5 mg/kg/day. The doses of LY150310 that inhibited lung metastasis by 50 and 100% were 1/10th and 1/100th, respectively, of those for nafazatrom. At doses from 0.6 to 60 mg/kg/day there was no effect on animal weights or growth of the primary tumor. At doses greater than 60 mg/kg/day LY150310 was toxic and had an \(LD_{50}\) value of 90 mg/kg/day. However, the antimitastatic action of LY150310 did not decrease at toxic doses. The therapeutic index of LY150310 was approximately 180, which is 26 times greater than that for nafazatrom.

**Table 1: Inhibition of spontaneous lung metastasis of the Lewis lung carcinoma by nafazatrom.**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>mg/kg/day</th>
<th>Number toxic deaths/number evaluated</th>
<th>Metastatic lung lesions per mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1 control</td>
<td>Vehicle</td>
<td>0/10</td>
<td>0, 1, 3, 4, 5, 5, 6, 6</td>
</tr>
<tr>
<td>Day 14 control</td>
<td>Vehicle</td>
<td>0/10</td>
<td>9, 10, 11, 12, 16, 22, 23, 26, 31</td>
</tr>
<tr>
<td>Nafazatrom</td>
<td>20</td>
<td>0/10</td>
<td>0, 1, 5, 6, 7, 9, 16, 27, 31, 33</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0/9</td>
<td>0, 6, 1, 2, 12, 16, 17, 20, 21</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>1/10</td>
<td>0, 0, 0, 0, 0, 2, 6, 17</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>10/10</td>
<td>Toxic</td>
</tr>
</tbody>
</table>

**Fig. 2. Dose-response relationships for the inhibition by nafazatrom (CTR), LY150310 (•), LY189332 (A), and LY135305 (◊) of spontaneous metastases of the Lewis lung carcinoma.** Animals were implanted with \(5 \times 10^5\) cells on Day 0 and randomly divided into treatment groups as described in “Materials and Methods.” Drugs were administered i.p. at one-half the daily dose every 12 h. Treatment was given on Days 5 through 19. The number of metastatic lung lesions was determined on Day 25.
Inhibition of Lung Metastasis and Survival

In the Table 2, animals that were dosed with nafazatrom on Days 5 through 19, or on Day 5 until death, died sooner than the control animals. The decrease in survival for the animals dosed on Days 5 through 19 was statistically significant ($P = 0.031$). There were no 90-day survivors in the animals that were dosed with nafazatrom at either dosing schedule. The median survival time was not significantly changed, and there were no 90-day survivors for the animals dosed with LY189332 at either dosing schedule. The median survival times were also not significantly changed for the animals dosed with LY150310 and LY135305 at either dosing schedule. Seventeen percent of the animals survived 90 days when they were dosed with LY150310 on Days 5 through 19; when given on Day 5 until death the surviving fraction was only 10%. Similar to LY150310, only 24% of the animals survived 90 days when they were dosed with LY135305 on Days 5 through 19, and this decreased to 13% in the animals dosed on Day 5 until death. The number of 90-day survivors observed with LY150310 or with LY135305 was not statistically significant ($P > 0.10$). In these studies the antimetastatic agent RA233 was also evaluated. RA233 was given at 200 mg/kg/day (its maximally tolerated dose) and at 60 mg/kg/day on Day 5 until death. This compound had no effect on median survival time or the number of 90-day survivors (Table 2). Postmortem examination showed no inhibition in lung metastasis in animals treated with either dose of RA233 (data not shown).

Effects on Anatomical Distribution of Metastatic Lesions. As discussed above, treatment with antimetastatic agents at their maximally effective doses did not increase mean survival times or produce a high proportion of 90-day survivors (Table 2). To better understand this paradox, major organs from animals treated with vehicle or with antimetastatic drugs were evaluated for the presence of metastatic colonies. In Table 3 is shown the...
Table 2 Summary of effect of treatment with nafazatrom, LY150310, LY189332, and RA233 on survival of mice bearing Lewis lung carcinoma

On Day 0 animals were implanted with 5 x 10^6 Lewis lung carcinoma cells into the gastrocnemius muscle and randomly divided into treatment groups. The primary tumor was resected in the control and drug treated animals on Day 14. Except for RA233, animals were treated every 12 h with a dose of drug that was found to maximally inhibit spontaneous lung metastasis. Two dosing schedules were used: (a) on Days 5 through 19 and (b) on Day 5 until death. RA233 was dosed at 60 and 200 (maximally tolerated dose) on Day 5 until death. Animals were checked every 4 to 12 h for survival.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>mg/kg/day</th>
<th>Treatment days</th>
<th>Median days survival (range)</th>
<th>% 90-day survivors</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1 (n = 20)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 14 control</td>
<td>Vehicle</td>
<td>5–death</td>
<td>30.0 (25.0–37.0)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Nafazatrom</td>
<td>200</td>
<td>5–19</td>
<td>27.5 (24.0–31.0)</td>
<td>0</td>
<td>0.031</td>
</tr>
<tr>
<td>LY189332</td>
<td>20</td>
<td>5–19</td>
<td>26.0 (24.0–36.0)</td>
<td>0</td>
<td>0.093</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>29.5 (25.0–39.0)</td>
<td>0</td>
<td>0.971</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>33.0 (24.0–38.0)</td>
<td>0</td>
<td>0.575</td>
</tr>
<tr>
<td>Experiment 2 (n = 20)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 14 control</td>
<td>Vehicle</td>
<td>5–death</td>
<td>33.0 (28.0–53.0)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>LY150310</td>
<td>60</td>
<td>5–19</td>
<td>34.5 (29.0–113.0^)</td>
<td>17</td>
<td>0.310</td>
</tr>
<tr>
<td>LY135305</td>
<td>6</td>
<td>5–19</td>
<td>35.5 (31.1–112.0^)</td>
<td>10</td>
<td>0.294</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>38.0 (28.0–113.0^)</td>
<td>24</td>
<td>0.187</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>32.0 (26.0–113.0^)</td>
<td>13</td>
<td>0.952</td>
</tr>
<tr>
<td>Experiment 3 (n = 10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 14 control</td>
<td>Vehicle</td>
<td>5–death</td>
<td>30.5 (25.0–32.0)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>RA233</td>
<td>60</td>
<td>5–death</td>
<td>28.5 (24.0–33.0)</td>
<td>0</td>
<td>0.594</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>5–death</td>
<td>32.0 (25.0–34.0)</td>
<td>0</td>
<td>0.266</td>
</tr>
</tbody>
</table>

* The log rank test was used to determine P values for the difference between the survival curves for drug treated animals relative to the curves for vehicle treated animals. The statistical analysis was done as described in "Materials and Methods."

** Experiments were ended on these days.

Table 3 Effect of treatment with nafazatrom, LY189332, LY150310, and LY135305 on organ distribution of Lewis lung carcinoma cells

On Day 0 animals were implanted with 5 x 10^6 Lewis lung carcinoma cells into the gastrocnemius muscle and randomly divided into treatment groups. The primary tumor was resected in the control (vehicle) and drug treated animals on Day 14. Animals were treated every 12 h from Day 5 until death with a dose of drug that was found to maximally inhibit spontaneous lung metastasis. Animals were checked every 4 to 12 h, organs were removed as close to death as possible, and evaluated for macroscopic metastatic lesions.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Lung</th>
<th>Liver</th>
<th>Kidney</th>
<th>Spleen</th>
<th>Brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 14 control</td>
<td>Vehicle</td>
<td>36 (10–80)</td>
<td>0 (0–0)</td>
<td>0 (0–0)</td>
<td>0 (0–0)</td>
<td>0 (0–0)</td>
</tr>
<tr>
<td>Nafazatrom</td>
<td>200 mg/kg/day</td>
<td>0 (0–0)^*</td>
<td>6 (2–25)^*</td>
<td>2 (0–6)^*</td>
<td>1 (0–2)^*</td>
<td>0 (0–2)^*</td>
</tr>
<tr>
<td>LY189332</td>
<td>20 mg/kg/day</td>
<td>1 (0–3)^f</td>
<td>10 (2–20)^f</td>
<td>1 (0–3)^f</td>
<td>0 (0–0)^f</td>
<td>1 (0–2)^f</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 14 control</td>
<td>Vehicle</td>
<td>24 (16–51)</td>
<td>0 (0–1)</td>
<td>0 (0–0)</td>
<td>0 (0–0)</td>
<td>0 (0–0)</td>
</tr>
<tr>
<td>LY150310</td>
<td>60 mg/kg/day</td>
<td>0 (0–1)^f</td>
<td>6 (5–20)^f</td>
<td>1 (0–5)^f</td>
<td>0 (0–5)^f</td>
<td>1 (0–4)^f</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Day 14 control</td>
<td>Vehicle</td>
<td>40 (20–89)</td>
<td>0 (0–1)</td>
<td>0 (0–1)</td>
<td>0 (0–0)</td>
<td>0 (0–0)</td>
</tr>
<tr>
<td>LY135305</td>
<td>6 mg/kg/day</td>
<td>2 (0–7)^f</td>
<td>8 (4–19)^f</td>
<td>3 (0–6)^f</td>
<td>0 (0–0)^f</td>
<td>0 (0–4)^f</td>
</tr>
</tbody>
</table>

The following P-values comparing the number of lesions in the drug treated animals relative to the number in the Day 14 control groups were computed by the Wilcoxon rank-sum test as described in "Materials and Methods": * P < 0.001, ‡ P = 0.005, § P = 0.006, ¶ P = 0.015, ʃ P = 0.035, † P = 0.078, and ‡ P > 0.100.

Effect of treatment with nafazatrom, LY189332, LY150310, and LY135305 on the organ distribution of Lewis lung carcinoma cells. Only macroscopic, pulmonary metastases were observed in animals that were treated with vehicle and had their primary tumor removed on Day 14. (Note, organs were not histologically evaluated for the presence of microscopic metastases). Consistent with previous studies, there was a significant (P < 0.001) decrease in the number of pulmonary metastases in animals whose primary tumor was resected on Day 14 and were treated with either nafazatrom, LY189332, LY150310, or LY135305 on Day 5 until death. In animals treated with these compounds, however, there was also a marked increase in extrapulmonary metastases. The most significant number of metastatic lesions were observed in the liver (P < 0.001) and kidney (P < 0.006). Fewer lesions were observed in the spleen and brain, and only occasionally was their number significant.

**DISCUSSION**

In this investigation the antimetastatic action of several different drugs, representing widely varied structures and different mechanisms of action, were evaluated. The purposes of this investigation were twofold. First, the antimetastatic actions of compounds LY135305, LY150310, and LY189332, were compared to those of RA233 and nafazatrom. Second, the correlation between the inhibition of spontaneous lung metastasis and animal survival was examined. This research was done as part of a program to discover noncytotoxic drugs to treat cancer metastasis.

RA233 was used as a reference antimetastatic drug. RA233 did not inhibit spontaneous lung metastasis nor did it have any effect on median survival time. These results have also been observed by other investigators using the Lewis lung carcinoma (see citations in Ref. 7). In contrast, Maniglia et al. have observed an inhibition in both the incidence and the frequency of spontaneous lung metastases of the B16 melanoma (27). RA233 is an inhibitor of platelet aggregation (see citations in Ref. 11). Its mechanism of action appears to be the inhibition of cyclic AMP phosphodiesterase. Maniglia et al. concluded, however, that the inhibition of metastasis of the B16 melanoma was not related to inhibition of platelet aggregation. In a small clinical study by Gastpar et al. (28), RA233 was found to
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increase survival in patients with sarcoma or lymphoma of the head and neck when used as an adjunct to surgery or radiotherapy. Currently, RA233 is being studied in a larger clinical trial by Zacharski et al. (12). It will be interesting to see if RA233 is found to have clinical activity in this larger study.

Nafazatrom was also used as a reference antimetastatic drug. In agreement with other studies (14, 29), nafazatrom was found to inhibit the spontaneous formation of lung metastases. However, other investigators, using the same and different tumor types, have not observed an antimetastatic action with nafazatrom (29–31). The dose of nafazatrom for 100% inhibition was 25 times greater than that reported by Honn et al. (14). Many factors could explain this difference in the therapeutic efficacy for nafazatrom. For example, in the study by Honn et al. the Lewis lung carcinoma was grown s.c. and the drug was given s.c.; whereas, in our investigation the tumor was grown intramuscularly and the drug was given i.p. Intraperitoneal administration was used because it approximates oral administration (32). For nafazatrom this could be important because after oral administration it is extensively metabolized during first passage in the liver (33). Other factors, such as drug vehicle and treatment duration, could also be important.

Nafazatrom's ability to increase levels of prostacyclin in the vascular endothelium has been proposed as a mechanism for its antimetastatic action (34). This increase is thought to result from both a stimulation in synthesis and from an inhibition in degradation of prostacyclin. Nafazatrom's prostacyclin enhancing action is thought to inhibit tumor cell-platelet interactions by changing the balance between the proaggregating and vasodilative actions of TXA₂ and the antiaggregating and vasodilative actions of PGI₂.

LY150310 was evaluated as an antimetastatic agent because it had been found to inhibit TXA₂ synthetase (18). Agents that inhibit TXA₂ synthetase have been proposed as antimetastatic agents because of their ability to change the ratio of TXA₂ to PGI₂ and thus inhibit tumor cell-platelet interactions (34). Honn et al. have reported that TXA₂ synthetase inhibitors have antimetastatic activity in vivo (34). However, other investigators have not found a correlation between inhibition of TXA₂ synthetase and inhibition of spontaneous metastasis (35).

LY189332 was evaluated as an antimetastatic agent because it had been found to inhibit cyclooxygenase (20). In some tumors a significant elevation in the levels of prostaglandins has been observed (see citations in Refs. 35 and 36). This has led to the concept that prostaglandins may be important in tumor dissemination. Some prostanooids are thought to be important in tumor cell-platelet interactions, in modulating the function of the immune system, and possibly in the growth of tumor cells (36, 37). It should be noted, however, that the relative amounts and ratios of different prostanooids can vary greatly among tumors. Thus, in some tumors prostaglandin metabolism may not play an important role in their growth and dissemination. The Lewis lung carcinoma used in this study has been shown to make high amounts of prostanooids, especially prostaglandin E₂ and prostaglandin F₂α (38). The importance of this to the metastatic activity of this tumor is still in question (39). Several experimental studies have examined the effect of cyclooxygenase inhibitors on metastasis (see citations in Ref. 7). The results of these studies can be summarized as inconclusive. The reasons for this confusion may be due to differences in procedures used to evaluate antimetastatic action, or because tumors in which prostaglandins have no role in metastasis may have been used.

A dose-dependent inhibition in spontaneous lung metastases was seen with LY189332. Its dose-response relationship, however, was different from that for LY150310 in two aspects. At doses producing less than 100% inhibition, the antimetastatic action of LY189332 decreased more sharply than that for LY150310. More important, the metastatic action of LY189332 decreased at high doses. The reason for the biphasic dose-response curve of LY189332 is not known. A cumulative toxicity at high dose levels could be one explanation for the decrease in antimetastatic action. This is not supported, however, by the observation that no difference was seen between the animal weights in the control and high dose groups. Another possible explanation is that this drug has different pharmacological actions at different dose levels. For example, aspirin has a dual effect on the formation of thrombosis; low doses are antithrombotic whereas higher doses are thrombogenic (40). This may be explained by aspirin's multiple effects on arachidonic acid metabolism. Low concentrations inhibit the formation of TXA₂ from arachidonic acid while high concentrations inhibit PGI₂ formation.

LY135305 was evaluated as an antimetastatic agent because it was found to inhibit the activation of factor VII by tissue factor (factor III) in the extrinsic coagulation cascade (19). There is considerable experimental and clinical evidence that supports the concept of an association between coagulation and malignant disease (2–4, 7, 8). The generation of thrombin by tumor cells, and the subsequent formation of fibrin, is considered important in the formation of emboli and in the protection of tumor cells from the immune system. Since thrombin has been shown to be a mitogen, it may also stimulate tumor cell growth. The Lewis lung carcinoma has been shown to have high procoagulant activity (41 and citations in Ref. 42). Studies have shown that the spontaneous metastasis of this tumor is associated with a low grade disseminated intravascular coagulation (see citations in Ref. 42). The hemostatic abnormalities seen with the Lewis lung carcinoma are similar to the laboratory abnormalities seen in patients with solid, metastasizing tumors.

The dose-response relationship for the antimetastatic action of LY135305 was similar to that for LY189332. At doses producing less than 100% inhibition, the antimetastatic action decreased sharply. A decrease in antimetastatic action was also seen at high doses. The reason for the biphasic dose-response curve for LY135305 is not known, but it may be the same as that proposed for LY189332.

Numerous studies have been conducted to evaluate the antimetastatic efficacy of anticoagulants. An inhibition in tumor growth and metastasis have been observed with agents, such as heparin, warfarin, and proteinase inhibitors, that prevent thrombus formation (43, 44). Other studies, however, have not found an antimetastatic action with these agents (43, 44 and citations in Ref. 2). As stated in the introduction, this controversy may be due to the different metastasis models used. Of the anticoagulant agents tested, warfarin and related compounds are the most potent. Anticoagulation may not totally explain its mechanism of action, however, since some coumarins that do not have anticoagulant activity have been found to inhibit metastasis (see citations in Ref. 2).

Although the Lilly compounds, LY150310, LY189332, and LY135305 were found to inhibit spontaneous metastasis to the lung, the mechanisms for their antimetastatic actions have not been definitively shown. Studies on the biochemical pharmacology of these agents are currently in progress and will be reported in a later paper.

As stated earlier, numerous studies have examined the effect of a variety of agents on the metastasis of experimental tumors.
These studies have measured the effect of an agent by counting the number of metastatic lesions in a target organ; with few exceptions, have studies measured an antimitotic effect by determining survival. Although it could be argued that evaluation of tumor burden is appropriate for cytoreductive agents, this is not adequate for antimitotic agents. For several reasons, survival data is a more important parameter for the evaluation of these agents. First, survival data in animal models are needed to show that an antimitotic agent has the potential to increase survival in patients that already have metastatic disease. In clinical oncology it is generally held that as many as 50% of patients will have disseminated disease when a cancer is diagnosed (45). Several studies have also established that for most human cancers generalized disease does not occur directly from the primary cancer (see citations in Ref. 2). For a given primary cancer there may be several initial sites of metastasis. Generalized disease is produced by the sequential metastasis from these second sites to third and fourth sites, et cetera. An effective antimitotic agent would prevent this sequence, sometimes called “metastasis from metastasis,” and in effect freeze the metastatic process. In combination with other therapeutic modalities, such an agent could increase survival in patients that have metastatic disease when therapy is started, or aid in the cure of patients treated before metastasis begins (46).

A second reason for survival studies in animals is to show that an anticoagulant or a platelet aggregation inhibitor has not exacerbated a cancer by increasing the dissemination of metastases. Most blood-borne human tumor cells produce metastatic colonies in the first capillary bed encountered (47 and citations in Refs. 2 and 48). The same is true for animal tumors, regardless of whether the metastases develop from i.v. injected cells or spontaneously from solid tumors (48). If an agent inhibits metastasis to the organ of first pass clearance, it is possible that it could promote capillary passage and produce a greater dissemination of metastasis. It is assumed that most tumor cells egressing from a capillary bed are not viable (see citations in Ref. 2). However, these studies have been done on a limited number of tumor types. For each tumor and agent being evaluated, survival studies are needed to definitively show this. This information, preferably obtained in several tumors, is needed to decide on whether an agent should be tested in humans.

For the reasons given above the effect of the Lilly compounds LY150310, LY189332, and LY135305, and nafazatrom were evaluated for their effect on survival. These compounds were given at their maximally effective antimitotic doses. RA233 (Rapenton) was also evaluated to its maximally tolerated dose. None of the compounds produced a significant number of long-term survivors or significantly increased the median survival time. These results were surprising, especially for the Lilly compounds, because they were potent inhibitors of spontaneous lung metastasis. The lack of a correlation between inhibition of metastasis and survival was also disappointing because therapy was started on Day 5. This is approximately 1 day before we could detect any metastatic lung colonies. To be relevant to clinical oncology, it is also important that an antimitotic agent increase the life span of animals when therapy is delayed until metastasis has already occurred. Considering the effect on survival when therapy was begun just before metastasis started, it is quite possible that this effect would be even less if therapy were delayed.

To understand why there was not a correlation between the inhibition of spontaneous lung metastasis and animal survival, the anatomic distribution of tumor lesions was evaluated after treatment with the compounds. Inhibition of metastatic lung lesions was observed with nafazatrom, LY150310, LY189332, and LY135305. However, the decrease in pulmonary lesions was associated with an increase in metastatic lesions in extra-pulmonary sites, such as the liver, kidney, spleen, and brain. Thus, the lack of a correlation between inhibition in lung metastasis and an increase in survival appears to result from an increased dissemination to extra-pulmonary sites. To the best of our knowledge, this is the first report of this phenomenon using a spontaneous metastasis model. Several studies have shown that treatment with anticoagulants can lead to a wider distribution of i.v. injected radiolabeled tumor cells (see citations in Ref. 49). Other investigators have also observed a change in the anatomic distribution of metastatic lesions with other agents. With an artificial metastasis model using B16 melanoma, Haggmar and Boeryd found that heparin, at a dose that inhibited lung lesions, increased the colonization of several extra-pulmonary sites (48). In a recent study Willmott et al. examined the effect of RA233 on the organ colonization of sarcoma 180 cells (47). These investigators found that RA233 treatment did not change the survival time or the incidence of pulmonary lesions but it did cause a higher incidence of extra-pulmonary lesions. Although not determined in this investigation, this may be why RA233 did not increase survival in animals bearing the Lewis lung carcinoma.

The goal of cancer drug development is to decrease the mortality or morbidity of cancers. To obtain this goal, the endpoint for an animal tumor model, no matter what the model, should be clinically relevant so that the information obtained in the laboratory can be used to justify further clinical evaluation. In this investigation we found that a variety of antithrombotic agents could inhibit spontaneous lung metastasis of the Lewis lung carcinoma. However, when animal survival was examined, no significant increase was seen in either the number of survivors or in median survival times. An initial conclusion that could be reached from this investigation is that antithrombotic and anticoagulant drugs will not be clinically useful in the prevention of metastasis. This conclusion should be reached with caution, however, because only one tumor type was evaluated. Considering the variability that has been shown to occur among tumors, it is unwise to make generalizations about all tumors based on the results obtained with one tumor. The effects of the agents used in this investigation could be quite different if they were evaluated with a different tumor model. With this caveat aside, however, the results of this investigation show that the effect a compound has on the number of metastatic lesions in a target organ may not be predictive of its effect on survival. To successfully translate laboratory data into the clinic, survival should be considered as a predictor of a compound’s potential clinical utility. It is probably also best to evaluate a compound with more than one tumor model. The use of survival as an endpoint in preclinical studies should be considered important given the high cost of doing clinical trials and the current regulatory requirements for the approval of a new cancer drug (50).

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