Effect of Intermittent Dosing Regimens of Erlotinib on Methylnitrosourea-Induced Mammary Carcinogenesis

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Abstract

EGF receptor (EGFR) inhibitors are used in the therapy of lung and pancreatic cancers and effectively prevent cancers in multiple animal models. Although daily dosing with erlotinib is effective, weekly dosing may reduce toxicity and have advantages, particularly for prevention. We tested alternative dosing regimens for preventive/therapeutic efficacy in a rat mammary cancer model. For prevention, erlotinib was administered by gavage beginning 5 days after methylnitrosourea (MNU). For therapy and biomarker studies, rats with palpable mammary cancers were treated for six weeks or for six days, respectively. Experiment A, erlotinib (6 mg/kg body weight/day, intragastric): daily (7 times/week); one day on/one day off; and two days on/two days off. All regimens reduced tumor incidence, increased tumor latency, and decreased cancer multiplicity versus controls \((P < 0.01)\). However, intermittent dosing was less effective than daily dosing \((P < 0.05)\). Experiment B, erlotinib (6 mg/kg body weight/day) daily or two days on/two days off or one time per week at 42 mg/kg body weight. All regimens reduced cancer incidence and multiplicity versus controls \((P < 0.01)\). Interestingly, daily and weekly dosing were equally effective \((P > 0.5)\). Experiment C, erlotinib administered at 42 or 21 mg/kg body weight 1 time per week, decreased tumor incidence and multiplicity \((P < 0.01)\). Erlotinib had a serum half-life of \(<8\) hours and weekly treatment yielded effective serum levels for \(\leq 48\) hours. Daily or weekly treatment of cancer bearing rats reduced mammary tumor size 25% to 35%, whereas control cancers increased >250%. Levels of phosphorylated extracellular signal-regulated kinase (ERK) were strongly decreased in rats treated daily/weekly with erlotinib. Thus, altering the dose of erlotinib retained most of its preventive and therapeutic efficacy, and based on prior clinical studies, is likely to reduce its toxicity.

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Introduction

Rat mammary cancer models have been used for several decades to evaluate potential chemopreventive agents. Chemically induced models of mammary carcinogenesis were initially developed by Huggins and colleagues (1). Subsequently, female Sprague–Dawley rats treated with methylnitrosourea (MNU) were shown to develop multiple hormonally responsive mammary cancers starting within 5 weeks after carcinogen administration (2). These cancers were histologically and by gene expression similar to well-differentiated estrogen receptor (ER) human breast cancers (3). As expected, treatments that altered the hormonal axis (e.g., SERMS, aromatase inhibitors) were strong chemopreventive agents in this model (4, 5). In addition, the cancers were responsive to various agents, including a variety of RXR agonists and farnesyltransferase inhibitors that do not act directly on the hormonal axis (6, 7).

The EGF receptor (EGFR) pathway was defined more than 20 years ago, and was quickly shown to be associated with a variety of important cellular pathways (8, 9). These included cellular proliferation and the cell-cycle pathway. Given its integral role in the cell cycle and that EGFR was overexpressed in a variety of cancers (head and neck, lung, etc), it was immediately recognized as a potential target for cancer therapy (10). The EGFR inhibitors are approved for the treatment of lung (11) and pancreatic cancers (in conjunction with standard therapy in an advanced setting). In addition, the EGFR inhibitors have shown some efficacy in a variety of cancers in small phase II trials in early settings. In the treatment of ER+ breast cancer (although it is not

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Inhibitor gefitinib was highly effective in both a preventive and therapeutic setting (16, 17). We previously reported that the EGFR inhibitor gefitinib was highly effective in both a preventive and therapeutic setting in the MNU-induced model of ER+ breast cancer in rats (18). In that study, it was further observed that gefitinib strongly inhibited phosphorylation of the target molecule EGFR as well as the immediate downstream proteins AKT and extracellular signal-regulated kinase (ERK).

Although this class of agents still retains significant promise, questions of toxicity and potential dosing regimens (19) inhibit their use in a prevention setting. In an attempt to address whether one might alter the dose scheduling of this class of agents while maintaining efficacy, we determined: (i) the preventive efficacy of erlotinib by daily dosing, dosing every other day, dosing 2 days on/2 days off, and weekly dosing at higher doses; (ii) the therapeutic efficacy of daily and weekly dosing; (iii) the effects of daily and weekly dosing with erlotinib on the phosphorylation of ERK; and (iv) the pharmacokinetics of erlotinib and an active metabolite OSI 420 in rat serum following daily or weekly dosing.

Materials and Methods

Chemicals and animals

Treatment of female Sprague–Dawley rats for the prevention and therapy studies were as previously described (7, 18) In brief, MNU was obtained from the National Cancer Institute (NCI) Chemical Carcinogen Repository, and was injected intravenously (75 mg/kg body weight) via the jugular vein when the animals were 50 days of age. Teklad diet and rats were obtained from Harlan Sprague–Dawley, Inc. Erlotinib was supplied by OSI Pharmaceuticals, LLC and was administered by gavage either on a daily or weekly basis. Agents were administered in a volume of 0.5 mL/gavage. The vehicle for erlotinib was ethanol: polyethylene glycol 400 (10:90; v/v).

Data collection and analyses

In all studies, rats were palpated for mammary tumors twice each week and weighed 1 time per week. Body weights of the rats did not differ more than 5% in either of the prevention or therapeutic studies. Statistical analyses of cancer incidence and latency as well as final tumor multiplicity were determined using log-rank analysis as previously described (5).

Erlotinib prevention studies

In the prevention studies, treatment of rats with erlotinib was initiated 5 days after MNU administration (or at 55 days of age). The number of rats per group was 15. At the end of the experiment (4 months after MNU treatment), animals were sacrificed and tumors were weighed and submitted for histologic evaluation. In experiment A (Fig. 1A), erlotinib (6 mg/kg body weight) was administered daily, one day on/one day off or 2 days on/2 days off. In experiment B (Fig. 1B), erlotinib (6 mg/kg body weight) was administered daily, 2 days on/2 days off or once per week (42 mg/kg body weight). In experiment C (Fig. 1C), erlotinib was administered as a weekly dose at either 42 or 21 mg/kg body weight.

Erlotinib therapeutic study

Rats received MNU at 50 days of age and were observed for the development of mammary cancers. When an animal developed a cancer of approximately 100 to 200 mm2, the rat received erlotinib (6 mg/kg body weight/day or 42 mg/kg body weight once per week) for 6 weeks (Fig. 2). Tumor size was determined with calipers before the initiation of treatment and twice each week during the course of treatment. The largest diameter of the cancer was measured and this value multiplied by the perpendicular diameter (size expressed in mm2). The erlotinib-treated weekly group had 16, whereas the control and erlotinib daily groups had 10 rats.

Erlotinib biomarker study

When an animal developed a palpable cancer, it was randomized into one of 3 groups: daily administration of erlotinib (6 mg/kg body weight/day) for 7 days, administration of a single dose of erlotinib (42 mg/kg body weight) on day 0 followed by sacrifice 7 days later, and vehicle control for 7 days (Fig. 3). Frozen erlotinib-treated or vehicle-treated mammary cancer samples were lysed using liquid nitrogen and a pestle and mortar. A protease inhibitor cocktail tablet was dissolved in 50 mL modified radioimmunoprecipitation assay buffer (RIPA) buffer (pH 7.4) and added to the samples. Samples were incubated on ice for 30 minutes and then centrifuged twice at 13,000 rpm for 10 minutes at 4°C, keeping the supernatant fraction each time. Protein concentration was measured. Samples were prepared using lysis buffer pH 8.0 (50 mmol/L Tris Base, 1% NP-40, 150 mmol/L NaCl) and 6 × SDS sample buffer and denatured by heating at 95°C for 5 minutes. Samples (50 µg) were loaded onto a 10% SDS gel and run using constant current (45 mA/2 gels) for 1 hour. Then, the proteins were transferred for 1.5 hours with a constant voltage of 100 V. The membrane was blocked using 5% nonfat skim milk for 1 hour at room temperature and then washed with TBS-T (Tris Base Solution-0.1% Tween 20). The primary antibody [p-ERKs T202/Y204 diluted 1:1,000 in 5% bovine serum albumin (BSA)-1X TBS-0.1% Tween 20] was incubated overnight at 4°C (Cell Signaling, Cat#9101). The membrane was then washed with TBS-T 3 times for 10 minutes and incubated for 1 hour at room temperature with a goat antirabbit horse radish peroxidase (HRP) secondary antibody diluted 1:5,000 in 5% nonfat skim milk (Santa Cruz Biotechnologies SC-2004). The membrane was washed 3 × 10 minutes with TBS-T and exposed in the dark room. Exposure time was 8 minutes to detect phosphorylated ERKs.
After exposure, the membrane was washed with TBS-T and then stripped with 1/C2 Millipore Re-Blot Plus Strong Solution (Cat #2504) at room temperature for 50 minutes. The stripping buffer was removed by washing 3/C2 10 minutes with TBS-T. The membrane was then blocked using 5% nonfat skim milk and washed using TBS-T to remove milk residue. A total ERKs antibody, diluted 1:1,000 in 5% BSA-1X TBS-0.1% Tween 20, was added and the membrane incubated at 4°C overnight (Cell Signaling, Cat #9102). The blots were then washed with TBS-T 3 times for 10 minutes and incubated with a goat anti-rabbit HRP secondary antibody diluted 1:5,000 in 5% nonfat skim milk for 1 hour at room temperature. Finally, the membranes were washed 3 times for 10 minutes in TBS-T and exposed in the dark room. Exposure time was 1 to 4 minutes to detect ERKs.

Pharmacokinetic studies
Serum samples were collected from 5 rats per group administered erlotinib either daily (6 mg/kg body weight/day) or weekly (42 mg/kg body weight). Serum was collected at time 0, 2, 4, 8, 24, and 48 hours after the last gavage dose of erlotinib. Analysis of erlotinib and an active demethylated metabolite (OSI 420) using isocratic reverse phase/tandem mass spectrometry methods were conducted as previously described (20, 21). In brief, diluted plasma in buffer was extracted with methyl tertiary butyl ether. The dried product was resuspended and separated on a Waters Symmetry C18 column using ammonium formate buffer and methanol as the mobile phase. The eluate were ionized by a heated nebulizer and the mass transitions monitored at 393.4/277.8, 379.3/277.9, and 407.4/292.1 m/z for erlotinib, OSI 420, and a standard, respectively. Noncompartmental pharmacokinetic parameters were calculated using linear-log trapezoid rule with WinNonlin version 5.2.
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Results

Prevention

In our initial studies, the preventive activity of various daily doses of erlotinib was examined (data not shown). It was found that a dose of 6 mg/kg body weight/day was highly effective, reducing both tumor incidence and multiplicity. In the present studies, we altered the dose by implementing intermittent dosing schedules (Fig. 1A–C). In the first study, erlotinib (6 mg/kg body weight) was administered using the following schedules: daily, one day on/one day off or 2 days on/2 days off. This resulted in reductions of tumor incidence and increases in latency for all 3 regimens versus controls (P < 0.01). However, the intermittent dosings were less effective than daily dosing: (P < 0.06, 1 day on/1 day off; P < 0.065, 2 days on/2 days off). We have determined tumor multiplicity at various time points (Supplementary Fig S1A) and found that daily, 1 day on/1 day off, and 2 days on/2 days off treatments reduced final tumor multiplicity by 90%, 65%, and 75%, respectively. All 3 regimens greatly reduced final tumor multiplicity compared with vehicle controls (P < 0.001), whereas daily dosing decreased final tumor multiplicity more than either of the intermittent dosing schedules (P < 0.05).

In Fig. 1B, rats were treated with erlotinib (6 mg/kg body weight) daily, intermittent erlotinib (6 mg/kg body weight) 2 days on/2 days off, or erlotinib weekly at 42 mg/kg body weight (the equivalent daily dose more than a one-week period). All 3 treatment regimens reduced tumor incidence and increased tumor latency compared with controls (P < 0.01). The intermittent dosings were marginally less effective than daily dosing (P < 0.125), whereas the efficacy of daily or weekly dosing was comparable. As shown in Supplementary Fig. S1B, we found daily, 2 days on/2 days off, and weekly dosing reduced multiplicity 85%, 69%, and 89%, respectively, all highly significant relative to controls (P < 0.005). However, based on final multiplicity the dosing of 2 days on/2 days off was less effective than daily dosing (P < 0.05). Weekly dosing at either 42 or 21 mg/kg body weight strongly inhibited tumor incidence (Fig. 1C) and tumor multiplicity (Supplementary Fig. S1C).

Pharmacokinetics

Table 1 shows the plasma concentrations of erlotinib and OSI 420 (an active metabolite). These data indicate that a dose of 42 mg/kg body weight erlotinib in rats was similar to a human dose of 150 mg. As shown, the Cmax for erlotinib at 6 mg/kg body weight was <50% of human levels, whereas the Cmax for the 42 mg dose was similar to that achieved in humans. This difference is even more apparent in the respective area under the curve (AUC) where the 6 mg/kg body weight dose is <20% of the human dose, whereas the 42 mg dose is similar to the human (Table 2). This large decrease in AUC for the rat is reflective of a half-life of 5 to 8 hours in the rat and approximately 24 hours in humans. It is generally thought that serum levels <25 ng/mL are sub-optimal based on cell culture results showing efficacy at doses ≥100 nmol/L in vitro. Thus, therapeutic levels were achieved for approximately 18 hours following a daily dose. However, therapeutic serum levels were achieved for less than 48 hours after the weekly dosing.

Therapy

The MNU cancer model was also used to examine the therapeutic efficacy of erlotinib by allowing palpable tumors to develop before initiating treatment (Fig. 2). Ten rats with tumors were treated with erlotinib (6 mg/kg body weight) on a daily basis, 16 rats were treated with erlotinib (42 mg/kg body weight) on a weekly basis, and 10 rats received the vehicle. Daily or weekly treatment reduced average tumor size by 40% and 25%, respectively, whereas

<table>
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<tr>
<th>Table 1. Serum levels of erlotinib and its major metabolite OSI-420 in female Sprague-Dawley rats</th>
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<tr>
<td>(i) Erlotinib, 6 mg/kg body weight/day</td>
</tr>
<tr>
<td>Hours after final gavage</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>8</td>
</tr>
<tr>
<td>24</td>
</tr>
<tr>
<td>48</td>
</tr>
<tr>
<td>(ii) Erlotinib, 42 mg/kg body weight, 1×/week</td>
</tr>
<tr>
<td>Hours after final gavage</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>4</td>
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<tr>
<td>8</td>
</tr>
<tr>
<td>24</td>
</tr>
<tr>
<td>48</td>
</tr>
<tr>
<td>(iii) Controls</td>
</tr>
<tr>
<td>None detected</td>
</tr>
</tbody>
</table>

*Values are ng/mL of serum; mean ± SEM.
cancers in the vehicle-treated group increased in size by 250% over the 6 weeks period. With either daily or weekly dosing, at least 50% of the mammary tumors decreased by 60% in size. We consider a 60% decrease in size to be a highly significant regression. Thus, daily and weekly dosing were extremely effective.

Biomarker study

A biomarker directly related to the mechanism of action of erlotinib was measured; specifically, the phosphorylation of the downstream effector molecule ERK (22). A striking decrease in phosphorylation of ERK in cancers from animals treated on a daily basis for 7 days with erlotinib was observed in a Western blot analysis (Fig. 3). Surprisingly, a strong decrease in phosphorylated ERK was similarly observed in animals given a single weekly dose of erlotinib and examined 6 days following the treatment.

Discussion

EGF was discovered approximately 30 years ago and its associated receptor EGFR was clearly defined as a relevant target for tumor cell therapy more than 15 years ago (9). EGFR was shown to be integral to cell signaling and cell replication. It has also been shown to be overexpressed in a wide variety of tumors including lung, head and neck, urinary bladder, and basal cell subtype of breast cancer (10). EGFR inhibitors progressed into the clinics and based on testing in advanced cancers have proven highly effective against a subset of non–small cell lung cancer (NSCLC) with particularly striking and extended responses in adenocarcinomas which have mutations in the EGFR gene (11). Lung adenocarcinomas with EGFR mutations are more prevalent in nonsmokers and persons of Asian descent.

Previous clinical breast cancer studies using EGFR inhibitors are particularly interesting and partially conflicting. In patients with advanced breast cancer, which had undergone prior therapies, EGFR inhibitors used as monotherapies yielded minimal activity (16, 17). These studies used mixed populations of patients who had been heavily pretreated. In advanced breast cancer, EGFR inhibitors alone or EGFR inhibitors administered with an aromatase inhibitor have proven effective in the subgroup of ER+, tamoxifen-resistant breast cancers (14, 15). These tumors tended to overexpress various growth factors. Interestingly, there are at least 2 reports implying the efficacy of EGFR inhibitors in earlier stages of ER+ breast cancer. One was a true neoadjuvant study in which gefitinib was administered for 12 to 16 weeks (12). Roughly 40% of ER+ tumors were shown to have EGFR expression based on immunohistochemistry. These tumors were treated with gefitinib alone for a period of up to 16 weeks and approximately 40% showed a complete response. A subsequent study comparing anastrozole alone versus anastrozole plus gefitinib revealed no significant improvement in early-stage breast cancer. However, in this case, anastrozole alone was profoundly effective (23).

In the present experiments, our earlier studies were expanded using the EGFR inhibitor erlotinib. We initially showed that erlotinib induced a dose-dependent decrease in tumor incidence and multiplicity. The highest dose of erlotinib (6 mg/kg body weight/day) administered would equate to a human dose of approximately 75 mg or half the

### Table 2. Erlotinib noncompartmental pharmacokinetic parameters in female Sprague-Dawley rats and humans (steady state)

<table>
<thead>
<tr>
<th>Species</th>
<th>Dose, mg/kg</th>
<th>T&lt;sub&gt;max&lt;/sub&gt; hours</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; ng/mL</th>
<th>AUC&lt;sub&gt;0-24&lt;/sub&gt; ng h/mL</th>
<th>T&lt;sub&gt;1/2&lt;/sub&gt; hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>6</td>
<td>2.00</td>
<td>708</td>
<td>4,680</td>
<td>4.61</td>
</tr>
<tr>
<td>Rat</td>
<td>42</td>
<td>8.00</td>
<td>1,590</td>
<td>28,400</td>
<td>8.06</td>
</tr>
<tr>
<td>Human</td>
<td>150 (mg)</td>
<td>2.44</td>
<td>1,740</td>
<td>26,500</td>
<td>24.4</td>
</tr>
</tbody>
</table>
standard human dose of 150 mg. This is based on standard U.S. Food and Drug Administration (FDA) scaling factors where the rat dose in mg/kg is divided by 6 to get the human dose and then multiplied by kg to get the mg equivalent. The effects of altering the dosing schedule to one day on/one day off or dosing 2 days/2 days off was conducted in the first study. Both the daily dosing and intermittent dosing reduced tumor incidence and increased tumor latency (Fig 1A), whereas simultaneously reducing final multiplicity (65%–75% by intermittent dosing and 90% by daily dosing; Supplementary Fig S1A). As indicated, intermittent dosings were less effective than daily dosing when comparing tumor incidence and tumor latency (1 day on/1 day off vs. daily, \( P < 0.06 \); 2 days on/2 days off vs. daily, \( P < 0.065 \) or using final tumor multiplicity (\( P < 0.05 \); intermittent vs. daily dosing). We had previously observed less efficacy with gefitinib when comparing intermittent vs. daily dosing \( (18) \). Based in part on these results, we substantially altered the dosing protocol using weekly dosing with erlotinib as compared with daily dosing or 2 days on/2 days off schedule. The studies (Figs 1B and C, and Supplementary Fig. S1B and S1C) showed that weekly dosing was highly effective because even the lower weekly dose (21 mg/kg body weight), which is one-half the total dose administered by daily gavage of 6 mg/kg body weight, decreased tumor multiplicity by approximately 85%. Given the relatively short half-life of erlotinib in rats (Tables 1 and 2) and the fact that apparently subtherapeutic serum levels were reached in approximately 48 hours, the preventive efficacy was surprising. When standard pharmacokinetic parameters were examined (Table 2), it was observed that the effective daily dose in a rat is substantially lower than that in humans; yielding a \( C_{\text{max}} \) that is approximately 60% lower than in the human and an AUC which is 5 times less. These results reflect the shorter half-life of daily erlotinib in the rat versus the human (5–6 hours vs. 16–24 hours). The weekly dosing regimen was also effective in a therapeutic setting (Fig. 2). It should be noted, however, that the cancers in this model are not as genetically advanced as a typical human breast cancer.

In an attempt to understand the downstream effects of EGFR inhibition in this model, we examined the levels of phosphorylated ERK in tumors of rats treated daily or weekly with erlotinib. We had previously shown daily dosing with gefitinib inhibited phosphorylation of EGFR, AKT, and ERK as determined by immunohistochemistry \( (18) \). In the present study, it was observed that a decrease occurred in ERK phosphorylation (as determined by Western blotting) even one week following a large bolus dose of erlotinib. We similarly observed the efficacy of both dosing regimens in the prevention and therapy for tumors in rats. This biomarker result was unexpected given the finding that effective serum levels of erlotinib were probably maintained for less than 48 hours, even following a single weekly dosing with erlotinib. The specific mechanism responsible remains to be determined.

There are a number of important questions derived from the results in these studies. First, could the results have been due to chance? The reproducibility of the effect in 2 separate experiments suggests otherwise. Second, is this effect generally applicable to all EGFR inhibitors? Similar results have been seen with 2 additional EGFR inhibitors in the same model system \( (27) \). Third, will the altered dosing regimen be applicable to a wide range of cancers in different organs? We have tested weekly erlotinib treatment in both a transgenic model of ER-negative breast cancer and a carcinogen-induced urinary bladder model; in both models, strong efficacy was observed (Lubet and Grubbs, unpublished data). Fourth, will this greatly altered dosing regimen be applicable to a wider range of kinase inhibitors? We have observed efficacy of weekly dosing with an allosteric AKT inhibitor, although this may reflect in part the pharmacokinetics of this specific agent.

The most important question, however, is whether the altered dosing regimen will alter the toxicity profile favorably enough to enable the testing (and ultimate use if shown to be effective) of erlotinib in a prevention setting. Milton and colleagues \( (28) \) examined the effects of weekly dosing of erlotinib in a phase I/II study conducted in patients with previously treated NSCLC. They showed with doses up to 2,000 mg/week (more than 13-fold greater than the FDA-approved single daily dose of 150 mg) that the toxicity profile was generally modest with no severe \( (\geq 3) \) rash or diarrhea at any dose level, and moderate \( (\geq 2) \) rash in 17% or 42% of patients treated at the 1,200/1,600 mg/week or 2,000 mg/week dose levels, respectively. Similarly, a study of 9 patients with brain metastases who were treated with a median dose of 1,500 mg weekly also showed a low incidence of rash, with mild to moderate \( (\geq 1 < 2) \) rash in only 2 patients \( (29) \); significant efficacy was observed at this dose. As with all preventive interventions, the balance between benefit, risk, and tolerability will need to be established before this class of drugs can be used in this setting.

The results presented show for the first time that a greatly altered dosing regimen (consisting of once weekly high doses of erlotinib) is effective in the prevention and treatment of carcinogen-induced mammary cancers. Given the human data showing diminished toxicity with weekly dosing \( (28, 29) \), these data provide a scientific rationale for testing such regimens in human cancer prevention trials in appropriately high-risk individuals.

Disclosure of Potential Conflicts of Interest

K.K. Iwata is an employee of Astellas. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Tucker, A. Bode, C.J. Grubbs

Analysis and interpretation of data (e.g., statistical analysis, biosimulation, computational analysis): R.A. Lubet, S.C. Gill, A. Bode, V.E. Steele, M.M. Juliana, H.L. Nicastro

Writing, review, and/or revision of the manuscript: R.A. Lubet, E. Szabo, K.K. Iwata, S.C. Gill, A. Bode, H.L. Nicastro, C.J. Grubbs

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): V.E. Steele, C.J. Grubbs

Study supervision: R.A. Lubet, V.E. Steele, C.J. Grubbs

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