Evaluation of the Tg.AC Assay: Specificity Testing with Three Noncarcinogenic Pharmaceuticals that Induce Selected Stress Gene Promoters in Vitro and the Inhibitory Effects of Solvent Components

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Understanding the strengths and limitations of alternative models, such as the Tg.AC assay, for evaluation of the potential carcinogenicity of pharmaceuticals requires assessment of assay specificity through studies that specifically target biologically active compounds that are known to not be carcinogens in rodents. To identify drugs that might provoke a false positive response in the Tg.AC assay, we screened pharmaceuticals for in vitro induction of the gadd153 promoter and the E-globin promoter. We have previously found a high correlation between induction of the gadd153 promoter in HepG2 cells and activity in the Tg.AC assay. The three drugs selected through screening 99 noncarcinogenic pharmaceuticals were amiloride, dipyridamole, and pyrimethamine. A 26-week skin paint study was conducted in hemizygous Tg.AC mice with the three drugs at two doses selected by a 4-week dose range finding study. Evidence of systemic toxicity was observed in animals dosed chronically with pyrimethamine or amiloride, but no skin papillomas were observed in mice treated with amiloride, dipyridamole, or pyrimethamine for 26 weeks. All male mice and 80% of female mice treated with 12-O-tetradecanoylphorbol-13-acetate (TPA) in acetone developed a maximal tumor burden. However, mice treated with TPA in a vehicle containing 2.4% DMSO had greatly reduced incidences of papillomas. In summary, the correct negative response was shown in the Tg.AC assay for three noncarcinogenic pharmaceuticals, which adds further favorable evidence of appropriate specificity of this model system. However, vehicle composition must be carefully selected because the outcome of this assay can be confounded by certain commonly used solvents.

Key Words: Tg.AC; gadd153; TPA; DMSO; solvent.

The proposal agreed to by the International Conference on Harmonization Expert Working Group on Safety to consider alternative models of carcinogenicity testing in the evaluation of the safety of human pharmaceuticals in place of a second bioassay provided the impetus for an in-depth consortial evaluation of these assays organized by the International Life Sciences Institute (ILSI; Robinson and MacDonald, 2001). The neonatal mouse and four lines of genetically altered mice (Tg.AC, rasH2, p53+/–, and XPA−/−p53+/–) that had been suggested for possible use in routine carcinogenicity testing as complements to conventional life-time feeding studies in rodents were evaluated by this ILSI-sponsored collaborative research program. The genetic alterations in these four lines are all in genes involved in human cancer (e.g., the ras oncogene, the p53 tumor suppressor gene, the XPA nucleotide excision repair gene) that result in accelerated development of treatment-induced tumors in a relatively short time frame (6–9 months). The Tg.AC mouse, which carries an activated v-Ha-ras gene driven by a E-globin promoter, has phenotypic similarity in skin paint studies to genetically initiated mouse skin, incurring skin papillomas upon dermal treatment with tumor promoters or complete carcinogens (Leder et al., 1990; Tennant et al., 2001). The E-globin promoter sequence in the Tg.AC transgene appears to play a role in the restricted tissue-specificity of transgene expression and tumor occurrence (Sistare et al., 2002). Evidence suggests that papilloma formation in the Tg.AC model is also dependent on factors such as the structure of Line-1 element sequence on either side of the integration site (Leder et al., 2002), transgene methylation status (Cannon et al., 1998), and the palindromic orientation of two copies of the transgene within the multicycopy tandem array of transgene sequence (Honchel et al., 2001; Thompson et al., 1998). Of the spectrum of carcinogens that the Tg.AC model can detect, the best predictivity has been observed with non-genotoxic carcinogens. This assay is not as sensitive in detecting direct-acting carcinogens such as alkylating agents (Sistare et al., 2002).

Among the goals of the ILSI-sponsored collaboration was the evaluation of the response of the alternative assays to species-specific carcinogens and to noncarcinogens (Robinson and MacDonald, 2001). Sulfisoxazole was the only noncarcinogen tested in the Tg.AC mouse by the ILSI consortium. Our study was designed to complement the ILSI effort through
the additional testing of noncarcinogens chosen by screening for potential "false positive" activity in the Tg.AC assay. To develop an in vitro assay system that could detect gene induction patterns correlative with in vivo activity in the Tg.AC assay, 24 Tg.AC tested compounds were analyzed in four rapid throughput in vitro reporter gene assays (Thompson et al., 2000). These assays were three CAT-Tox (L) assays that measured induction of the gadd153 promoter, c-fos promoter, and p53 response elements in HepG2 cells and a fourth assay that measured induction of the ζ-globin promoter in K562 cells. Of the four assays, the gadd153-CAT assay showed the strongest overall concordance (81%) with activity in the Tg.AC assay. The ζ-globin promoter assay correctly classified only 64% of Tg.AC positive and 58% of Tg.AC negative compounds.

To prioritize selection of drugs for testing Tg.AC assay specificity, 99 pharmaceuticals that had tested negative for carcinogenic activity in male and female rats and mice, and were available from commercial or internal sources were screened for their ability to induce the gadd153 promoter in vitro (see accompanying article). Approximately 10% of the screened drugs induced the gadd153 promoter by four-fold or more. Several criteria were used to select among this subset of nine drugs for the three best candidates for subsequent in vivo testing in a Tg.AC assay: whether drug solubility in acetone or ethanol was sufficient to elicit systemic toxicity; the level of gadd153, ζ-globin, and c-fos promoter inductions by the drug; the potency of the drug in the in vitro assays; and the cost of the drug required for six months of dosing. Based on these criteria, amiloride, dipyridamole, and pyrimethamine were selected for testing the specificity of the Tg.AC assay towards pharmacologically active, noncarcinogenic drugs.

**MATERIALS AND METHODS**

**Experimental animals.** Male and female FVB/N or Tg.AC hemizygous mice were received from Taconic Farms (Germantown, NY). The in-life study was performed at Therimmune Research Corp., Gaithersburg, MD. The animals were held 11 days before dosing commenced at 4–5 weeks of age. The animals were randomly distributed into study groups. Males were housed singly and females were housed five per cage in polycarbonate shoebox cages with microisolator lids and Sani-chip hardwood bedding. The animals were fed Purina Pico Rodent Chow (5058) and had access to house water (reverse osmosis deionized), both available ad libitum. The animal room was kept at a temperature range of 64–79°F. The application site was prepared by closely shaving the back of the animals once a week as needed with Wahl mustache clippers (Sterling, IL). The dosing region was from the intrascapular area to about 1.5 cm from the base of the tail.

**Chemicals and solvents.** 12-O-tetradecanoylphorbol-13-acetate (TPA; ~99% purity) was from Alexis Corp., San Diego, CA. Pyrimethamine, amiloride hydrochloride hydrate, and dipyridamole were all purchased from Sigma, St. Louis, MO. A.C.S. reagent grade ethanol (99.5%, Aldrich), molecular biology grade dimethyl sulfoxide (DMSO; >99.9%, Sigma), HPLC grade acetone (Thomas Scientific), and Millipore-filtered house distilled H2O were used as solvents.

**Dose range finding study.** Groups of 10 FVB/N mice of each sex were dosed with each of the test solutions or vehicle alone 5 days per week for 4 weeks. Doses were extrapolated from maximum tolerated doses (MTDs) in 2-year bioassays. The MTD was converted from mg/kg/day to mg/mouse/day, assuming a 25 g mouse, multiplied by 7 to derive the total weekly exposure, and then divided by 5 to derive the daily skin paint high dose. The daily skin paint target dose was divided by 200 μl (the constant skin paint delivery volume) to calculate the concentration of the high-dose application solution needed.

Stability studies indicated that the dosing solutions could be prepared weekly and stored at 4°C (data not shown). Dipyridamole was prepared as a 25 mg/ml solution in ethanol and applied once or twice daily. For the low dose group, the dipyridamole stock solution was diluted 1:1 in ethanol. To prepare the high dose solution, amiloride was dissolved in DMSO to 150 mg/ml and diluted 1:33 with ethanol. The middle and low dose solutions were prepared by diluting the high dose solution 1:2.5 or 1:1.5 with ethanol. The high-dose pyrimethamine solution was prepared by first adding 70% ethanol and then acidifying with 1N HCl until dissolved. This solution was applied once or twice a day for the middle and high dose groups, respectively. The high-dose pyrimethamine solution was diluted 1:3 in ethanol to prepare the low dose formulation.

At necropsy, blood was collected by cardiac puncture after CO2 anesthesia for hematology and clinical chemistry measurements. Kidney, liver, spleen, thymus, and skin were collected for histopathology.

**Twenty-six-week carcinogenesis study.** Each study group contained equal numbers of male and female mice. The negative control groups had 15 animals per sex, the positive control groups had 10 animals per sex, and the test compound groups had 15 animals per sex. All doses were applied in a volume of 200 μl. The following doses and dosing regimens were used for the 26-week study. The solvent control vehicle consisted of 87% ethanol, 2.4% DMSO, 10.6% H2O, and was applied three times/week. One positive control group received 2 μg TPA in acetone three times/week. The second positive control group received 2 μg TPA in solvent control vehicle three times/week. High (3.6 mg/ml) and low (1.8 mg/ml) doses of amiloride were applied five times/week. Amiloride was solubilized in 100% DMSO at 150 mg/ml and diluted with ethanol to make a 3.6 mg/ml solution in 2.4% DMSO, 97.6% ethanol. The low-dose amiloride solution was prepared by diluting the high dose solution 1:1 with ethanol. Initially, pyrimethamine (5 or 10 mg/ml in 87% ethanol, 13% acidified H2O) was applied once daily to the low and high dose groups, respectively. Dipyridamole was prepared as a 25 mg/ml solution in 100% ethanol and applied once or twice daily to the low and high dose groups, respectively. All dosing solutions were prepared and stored in the dark in amber vials to minimize light exposure.

Animals were observed twice daily for mortality and moribundity; body weights and detailed clinical findings including test site papilloma counts were recorded weekly. Papillomas were counted as present if observed for three consecutive weeks. The upper limit of papillomas counted per animal was 24. Necropsy was performed at the end of the study on all animals. Organ weights were taken at necropsy for brain, heart, kidney, liver, and ovaries or testis with epididymis. A complete selection of tissues from negative control, all high dose groups, and the low-dose pyrimethamine group were paraffin-embedded, sectioned, and examined microscopically.

**Statistical analyses.** Pairwise comparisons using two-sample nonparametric statistical procedures (Wilcoxon rank-sum and Mann-Whitney tests) were used for testing significance of differences in weekly skin papilloma counts between pairs of treatment groups. Statistical significance of clinical chemistry parameters, hematology parameters, and mean body and organ weights between vehicle control and treatment groups were determined by analysis of variance using Dunnett’s method.

**RESULTS**

**Dose Range Finding Study.**

Dose selection for the 26-week Tg.AC skin paint study was determined by conducting a 4-week dose range finding study in
would allow delivery of up to 0.9 mg amiloride per dosing with
in DMSO and diluted with ethanol to 4.5 mg/ml. This solution
Evoy, 1998), a 150 mg/ml solution of amiloride was prepared
dosage (20 –25 times the maximum daily human dose; Mc-
apply amounts of drug that meet or exceed the 2-year study
studies (Eastin
1:1 with water had been used in previous Tg.AC skin paint
ethanol/32% water was prepared. A vehicle of ethanol diluted
middle doses, a 15 mg/ml solution of pyrimethamine in 68%
dilution with acidified water. For the 4-week study high and
ubility of pyrimethamine in ethanol was increased through
Tg.AC assays (Tennant
apo in 16 September 2017

TABLE 1
Dose Groups for the 4-Week Dose-Finding Study in FVB/N Mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Sex</th>
<th>Test article</th>
<th>Conc. × frequency</th>
<th>Daily dose (mg)</th>
<th>Dose equivalent*</th>
<th>2-year high dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M &amp; F</td>
<td>3% DMSO in ethanol</td>
<td>0.2 ml × 1/day</td>
<td>ND*</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>M &amp; F</td>
<td>Amiloride</td>
<td>1 mg/ml × 1/day</td>
<td>0.2</td>
<td>5.7 mg/kg/day</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>M &amp; F</td>
<td>Amiloride</td>
<td>2 mg/ml × 1/day</td>
<td>0.4</td>
<td>11.4 mg/kg/day</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>M &amp; F</td>
<td>Amiloride</td>
<td>4.5 mg/ml × 1/day</td>
<td>0.9</td>
<td>25.7 mg/kg/day 10 mg/kg/day</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>M &amp; F</td>
<td>Pyrimethamine</td>
<td>5 mg/ml × 1/day</td>
<td>1</td>
<td>28.6 mg/kg/day</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>M &amp; F</td>
<td>Pyrimethamine</td>
<td>15 mg/ml × 1/day</td>
<td>3</td>
<td>85.7 mg/kg/day</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>M &amp; F</td>
<td>Pyrimethamine</td>
<td>15 mg/ml × 2/day</td>
<td>6</td>
<td>171.4 mg/kg/day 150 mg/kg/day</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>M &amp; F</td>
<td>Dipryridamole</td>
<td>12.5 mg/ml × 1/day</td>
<td>2.5</td>
<td>71.4 mg/kg/day</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>M &amp; F</td>
<td>Dipryridamole</td>
<td>25 mg/ml × 1/day</td>
<td>5</td>
<td>143 mg/kg/day</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>M &amp; F</td>
<td>Dipryridamole</td>
<td>25 mg/ml × 2/day</td>
<td>10</td>
<td>286 mg/kg/day 75 mg/kg/day</td>
<td></td>
</tr>
</tbody>
</table>

*The total weekly skin paint dose, which was administered five days per week, was normalized over seven days.
*ND: not determined.
*Amiloride vehicle: 97% ethanol, 3% DMSO.
*Pyrimethamine vehicle: 68% ethanol, 32% H2O.
*Dipryridamole vehicle: 100% ethanol.

FVB/N mice (Table 1). In order to identify an MTD for each
drug, it was first determined if a dose equivalent to the highest
daily dose administered orally in the 2-year bioassay could be
delivered topically (see Materials and Methods). Drug solubility
is a limiting factor in dose selection for skin paint studies, because the dose needs to be delivered in a vehicle
compatible with skin paint application. Acetone, ethanol, and
methanol, as pure solvents or mixtures with water or DMSO,
had been the most commonly used skin paint solvents in
Tg.AC assays (Tennant et al., 2001). The use of DMSO in skin
paint vehicles for the 26-week study was minimized because DMSO has shown a concentration-dependent inhibition of
papilloma induction by TPA at concentrations of 17–100% in
skin carcinogenicity assays in SENCAR mice (Slaga and Fischer, 1983).

The high dose of dipryridamole used in a 2-year carcinogenicity study (75 mg/kg/day; McEvoy, 1998) was the starting
dose for our 4-week study. The 4-week high dose (280 mg/kg/
day) was selected based on the maximal solubility of dipryridamole in ethanol (25 mg/ml) and twice daily dosings. To achieve a dose equivalent to the 2-year bioassay high dose (150
mg/kg/day; NTP) in a skin paint-compatible solution, the solubility of pyrimethamine in ethanol was increased through dilution with acidified water. For the 4-week study high and
middle doses, a 15 mg/ml solution of pyrimethamine in 68%
ethanol/32% water was prepared. A vehicle of ethanol diluted
1:1 with water had been used in previous Tg.AC skin paint
studies (Eastin et al., 2001). Amiloride is highly soluble in
DMSO, but solubility is reduced upon dilution with ethanol. To apply amounts of drug that meet or exceed the 2-year study dosage (20–25 times the maximum daily human dose; McEvoy, 1998), a 150 mg/ml solution of amiloride was prepared in DMSO and diluted with ethanol to 4.5 mg/ml. This solution would allow delivery of up to 0.9 mg amiloride per dosing with
the use of a minimal amount (3%) of DMSO. The animals in
the negative control group received 97% ethanol/3% DMSO.

Toxicity after four weeks of drug treatment was assessed
using histopathology and standard clinical chemistry and hematology indicators. No treatment-related effects on mortality,
body weight, major organ weight, or gross pathology were observed in any of the treatment groups. Female mice in the
high-dose amiloride group had elevated mean blood urinary nitrogen (BUN) levels (36.9 ± 12.1 vs. 20.8 ± 5.3 for the
control group, p < 0.05) and elevated serum potassium levels
(p < 0.05). Hyperkalemia and transient elevations in BUN are
reported side effects of amiloride therapy (McEvoy, 1998).
Moderate to severe renal lesions were observed in 5 of 10 males in the high-dose amiloride group. In the dipryridamole
treatment groups, the only indication of an adverse effect was a statistically significant elevation in mean serum glucose levels in female mice receiving the middle dose (5 mg/day) of
ipryridamole for four weeks. Alterations in several hematologic parameters were observed in male mice in the high-dose
pyrimethamine group. The mice in this group had decreased albumin to globulin ratios, decreased white blood cell counts,
and elevated mean corpuscular volume and hemoglobin content (MCV and MCH) values (p < 0.05). Although toxicity
was observed in the high dose group, the middle and high doses
of pyrimethamine may still have been incompletely delivered. It was discovered late into the dose range finding study that the vehicle originally chosen to deliver 15 mg/ml of
pyrimethamine (68% ethanol/32% acidified H2O; pH 5.0) did
not uniformly wet the surface of the skin upon application (see
Fig. 1). With subsequent testing, we observed that 14% was
the maximal concentration of water that could be added to ethanol
and applied to shaved mouse skin in a uniformly wettable
manner. A vehicle containing 70% ethanol/30% water did not
spread evenly over the application site.
Solvent Selection for 26-Week Study

Based on the 4-week dose range finding study results, two doses per drug were selected for the 26-week skin paint study (Table 2). The high dose was the MTD or maximum feasible dose, and the low dose was selected to be about 50% of the high dose. A high dose of 0.72 mg/day amiloride was chosen for the 26-week study because signs of renal toxicity were observed with a 0.9 mg/day dose in the 4-week study. The incomplete delivery of pyrimethamine due to suboptimal solvent wettability in the 4-week study confounded dose selection for this drug. Despite partial delivery of the full dose, toxicity was observed with the 6 mg daily dose in the 4-week study. Therefore, the 26 week high dose was selected to be 2 mg/day, which was twice the 4-week low dose. Another consideration was that changing the solvent to a more wettable formulation lowered the maximal solubility of pyrimethamine in a skin paint solution from 15 mg/ml to 10 mg/ml. For dipyridamole, the maximal feasible dose was selected as the high dose for the 26-week component of the study.

To maximize the dosage applied so that an MTD could be achieved in the Tg.AC studies, the solvent composition was optimized for each drug. A composite of the customized solvents (87% ethanol, 10.6% H2O, 2.4% DMSO) was chosen as the solvent control vehicle for positive and negative control groups. The positive control compound (TPA) was

### Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Sex</th>
<th>Test article</th>
<th>Concentration (mg/ml)</th>
<th>Days/week dosed x doses/day</th>
<th>Dosage per week (mg)</th>
<th>Avg. dose (mg/kg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M &amp; F</td>
<td>Solvent control vehicle</td>
<td>ND</td>
<td>5 x 1</td>
<td>5 x 1</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>M &amp; F</td>
<td>TPA in acetone</td>
<td>0.010</td>
<td>3 x 1</td>
<td>3 x 1</td>
<td>0.006</td>
</tr>
<tr>
<td>3</td>
<td>M &amp; F</td>
<td>TPA in solvent control vehicle</td>
<td>0.010</td>
<td>3 x 1</td>
<td>3 x 1</td>
<td>0.006</td>
</tr>
<tr>
<td>4</td>
<td>M &amp; F</td>
<td>Amiloride</td>
<td>1.8</td>
<td>5 x 1</td>
<td>5 x 1</td>
<td>1.8</td>
</tr>
<tr>
<td>5</td>
<td>M &amp; F</td>
<td>Amiloride</td>
<td>3.6</td>
<td>5 x 1</td>
<td>5 x 1</td>
<td>3.6</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>Pyrimethamine</td>
<td>5</td>
<td>5 x 1</td>
<td>3 x 1</td>
<td>0.75</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>Pyrimethamine</td>
<td>5</td>
<td>5 x 1</td>
<td>5 x 1</td>
<td>5</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>Pyrimethamine</td>
<td>10</td>
<td>5 x 1</td>
<td>3 x 1</td>
<td>1.5</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>Pyrimethamine</td>
<td>10</td>
<td>5 x 1</td>
<td>5 x 1</td>
<td>7.5</td>
</tr>
<tr>
<td>8</td>
<td>M &amp; F</td>
<td>Dipyridamole</td>
<td>25</td>
<td>5 x 1</td>
<td>5 x 1</td>
<td>25</td>
</tr>
<tr>
<td>9</td>
<td>M &amp; F</td>
<td>Dipyridamole</td>
<td>25</td>
<td>5 x 2</td>
<td>5 x 2</td>
<td>50</td>
</tr>
</tbody>
</table>

*The average daily dose was calculated by dividing the weekly dose by seven and assuming a 25 g mouse.

*Solvent control vehicle: 87% ethanol, 10.6% H2O, 2.4% DMSO.

*ND: not determined.
tested both in acetone and in the test vehicle to control for possible effects of a nonstandard solvent mixture on papilloma formation.

Twenty-Six-Week Skin Paint Study

The 26-week skin paint study was conducted in groups of male and female hemizygous Tg.AC mice with amiloride, dipyridamole, and pyrimethamine at two dose groups per drug. Amiloride was administered five times per week at doses of 0.9 or 1.8 mg in 97.6% ethanol/2.4% DMSO. Dipyridamole was administered five days a week at a dose of 5 mg once or twice a day in 100% ethanol. Initially, pyrimethamine was given five times per week at doses of 1 or 2 mg in 87% ethanol/9.75% H2O/3.25% 1N HCl, pH 5. During the second week of the study, a sudden onset of mortality was observed among male mice in the low and high-dose pyrimethamine groups and in female mice in the high-dose pyrimethamine (see Fig. 2). Male mice were more sensitive to pyrimethamine toxicity than female mice. Necropsy failed to reveal the cause of death. Doses of pyrimethamine were lowered until mortality stabilized (at week 6). From weeks 6 to 26, male mice received pyrimethamine at doses of 0.15 or 0.3 mg five days per week and female mice received 1 or 1.5 mg five times per week (see Table 2).

After 26 weeks of treatment, no skin papillomas were observed in mice treated with amiloride, dipyridamole, or pyrimethamine (Table 3). No gross or microscopic lesions attributable to exposure to the test article were observed. The most common spontaneous tumor observed at sacrifice were odontomas of the temporomandibular joint. These tumors were observed in 4/20 control animals and in 7/73 animals in the treatment groups. These incidences are similar to the sponta-

![FIG. 2. Weekly cumulative mortality in pyrimethamine dose groups in the 26-week study. High dose males (filled circles) received 2 mg/day, 3×/week at week 1. The dose was lowered initially to 2 mg/day, 3×/week at week 3 and then adjusted to 0.5 mg/day, 3×/week at week 7 for the remainder of the study. High dose females (filled boxes) received 2 mg/day until week 6, when the dose was lowered to 1.5 mg/day, 3×/week. Low dose males (open circles) initially received 1 mg/day, 3×/week. At week 4, the dosage was lowered to 0.25 mg/day, 3×/week for the remainder of the study. Low dose females (open boxes) received 1 mg/day throughout the study.](image-url)

**TABLE 3**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor incidence (%)</th>
<th>Mean time (weeks ± SD)</th>
<th>Mean tumors (± SD)</th>
<th>Mean time (weeks ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M/F</td>
<td>M/F</td>
<td>M/F</td>
<td>M/F</td>
</tr>
<tr>
<td>Solvent control vehicle</td>
<td>0/15 (0%) 1/15 (6.7%)</td>
<td>16</td>
<td>4</td>
<td>0.27 ± 1</td>
</tr>
<tr>
<td>TPA in acetone</td>
<td>10/10 (100%) 8/10 (80%)</td>
<td>8.7 ± 1.8</td>
<td>9.4 ± 2.9</td>
<td>14.1 ± 3.4 18.75 ± 4.4</td>
</tr>
<tr>
<td>TPA in solvent control</td>
<td>4/10 (40%) 3/10 (30%)</td>
<td>17 ± 1.4 17.7 ± 2.5</td>
<td>2.7 ± 0.5 1.3 ± 0.6</td>
<td>0.3 ± 0.48</td>
</tr>
<tr>
<td>Amiloride, low</td>
<td>0/15 (0%) 0/15 (0%)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Amiloride, high</td>
<td>0/15 (0%) 0/15 (0%)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Dipyridamole, low</td>
<td>0/15 (0%) 0/15 (0%)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Dipyridamole, high</td>
<td>0/15 (0%) 0/15 (0%)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Pyrimethamine, low</td>
<td>0/8 (0%) 0/15 (0%)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Pyrimethamine, high</td>
<td>0/6 (0%) 0/11 (0%)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

*NA: not applicable.
neous rate of 13–17% reported for jaw tumors in Tg.AC mice (Mahler et al., 1998). Odontomas in the nasal turbinates were observed in 3/25 control animals and in none of the treatment groups. Foreestomach squamous papillomas were observed in most treatment and control groups and occurred at an overall incidence of 9%, which is within the reported historical spontaneous incidence range of 7–10% (Tennant et al., 2001).

Pyrimethamine induced a high rate of mortality early in the study before doses were adjusted downward, indicating that the starting dose exceeded the MTD for male Tg.AC mice. Evidence of systemic toxicity was also observed in mice that received chronic dosing with the adjusted high dose of pyrimethamine. After 26 weeks of treatment, both males and females in this group had lower kidney weights (as % body weight; p < 0.01).

All male mice and 80% of female mice treated with TPA in acetone developed a maximal tumor burden (Fig. 3). The two females that developed no papillomas in the positive control group were further analyzed for evidence of alterations in DNA lesions such as fragile sites, structural chromosomal aberrations, and sister chromatid exchanges (Egeil, 1998). Although the Tg.AC assay has reduced sensitivity in detecting clastogenics other than benzene. The negative result seen in this study with maximally tolerated doses of pyrimethamine suggests that noncarcinogenic, bone marrow clastogens do not produce false positive results in the Tg.AC assay.

A vehicle containing 2.4% DMSO had a strongly inhibitory effect on papilloma induction by TPA in this study. The same concentration of DMSO was used in the vehicle used to deliver the high dose of amiloride, which may have confounded interpretation of the true tumorigenic potential of amiloride in the

DISCUSSION

The three noncarcinogenic pharmaceuticals (amiloride, dipyriramole, and pyrimethamine) selected for in vitro activation of the gadd153 promoter and the ζ-globin promoter all tested negative in the Tg.AC assay. By using induction of a stress response gene in vitro as a selection criterion, we were able to identify drugs that induced known cellular signal pathways associated with toxicity from among over 99 noncarcinogenic drugs while minimizing consumption of time and resources. The results from this study lend added assurance that the Tg.AC assay can show correct specificity towards pharmaceuticals administered at maximally tolerated or maximally feasible doses.

Pyrimethamine, administered dermally at doses that produced systemic toxicity, failed to induce papillomas in Tg.AC mice. Although pyrimethamine has a clear genotoxic effect in rodent bone marrow micronucleus tests (Ono et al., 1997; Vijayalaxmi and Vishalakshi, 2000), this drug tested negative in female mice and rats of both sexes in a carcinogenesis bioassay (National Toxicology Program, 1978). Reduced survival in both control and test groups prevented assessment of the carcinogenic potential of this drug to male mice. Inhibition of dihydrofolate reductase by high doses of pyrimethamine can cause depletion of deoxyribonucleotide pools, which increases the error rate during DNA synthesis, promoting the formation of DNA lesions such as fragile sites, structural chromosomal aberrations, and sister chromatid exchanges (Egeil, 1998). Although the Tg.AC assay has reduced sensitivity in detecting mutagenic carcinogens (Sistare et al., 2002), less evidence is available on how this assay responds to clastogens other than benzene. The negative result seen in this study with maximally tolerated doses of pyrimethamine suggests that noncarcinogenic, bone marrow clastogens do not produce false positive results in the Tg.AC assay.
Tg.AC assay. Inhibition by DMSO has been seen in the twostage skin carcinogenesis model in studies with SENCAR mice that examined the effect of solvent on TPA induction of papillomas after initiation with 7,12-dimethylbenz(a)anthracene (DMBA; Slaga and Fischer, 1983). In this system, the concentration of DMSO in the vehicle was directly proportional to the degree of inhibition observed. Based on these results, it was reasonable to assume that the use of small amounts of DMSO would have minimal inhibitory effects on papilloma formation in Tg.AC mice and we designed our study accordingly. However, a recent study of the effects of vehicle composition on the activity of TPA in Tg.AC mice by Stoll et al. has shown that DMSO concentration has an inverse relationship to inhibition of papilloma formation in this model. A vehicle containing 20% DMSO was more inhibitory to papilloma induction by TPA than was 100% DMSO (Stoll et al., 2001). Although the Tg.AC mouse has the phenotype of preinitiated mouse skin, the relationship between dose of DMSO and degree of inhibition is reversed between Tg.AC mice and DMBA-initiated SENCAR mice. In both models, papilloma formation is follicular in origin and associated with activating mutations in the Ha-ras gene (Binder et al., 1998; Hansen and Tennant, 1994), but the results with DMSO suggest that there may be mechanistic differences between these two models. The process by which DMSO is inhibitory is not known but it is not thought to be through altering the delivery of TPA to the skin. Preapplication of DMSO immediately or up to 1 h before promotion with TPA in acetone is inhibitory to papilloma formation in a two-stage model of skin carcinogenesis using CD-1 mice (Jacob and Weiss, 1986). In addition, limited evidence suggests that the inhibitory effect of DMSO on papilloma formation is not specific to TPA, because the use of DMSO as a vehicle also has a dampening effect on methylcholanthrene-induced skin carcinogenesis in hairless mice (Iversen et al., 1981). Therefore, this solvent should best be avoided in Tg.AC studies because of its ability to reduce a papilloma response, which could confound results with weak tumorigens. The use of a vehicle containing 20% DMSO/80% ethanol in the ILSI sponsored evaluation of the Tg.AC assay was not associated with a robust response to treatment (Tennant et al., 2001). It may be possible that a response could have been seen with amiloride if DMSO had not been used in the vehicle.

For maximal drug exposure, a skin paint solvent should evaporate or be rapidly absorbed after application. This study clearly shows that using a vehicle with incomplete wettability (67% ethanol/33% H2O) does not deliver a full dose of drug to the animal. Greater toxicity was seen with a 3 μg dose of pyrimethamine in 87% ethanol/13% H2O than with a higher dose (6 μg) that was applied in 67% ethanol/33% H2O. These data emphasize that the wettability of a nonstandard vehicle should be confirmed before the study commences.

The conceptual basis of this project lies in the hypothesis that enhancement of the expression of the v-Ha-ras transgene, particularly through stimulation of the transcriptional activity of the ζ-globin promoter directly or through upstream enhancer elements, can be a mechanism by which positive responses are generated in the Tg.AC assay by noncarcinogens. Based on this theory, a reporter assay measuring activation of the ζ-globin promoter in a permissive cell line was constructed for use in an in vitro screen. However, activation of the ζ-globin promoter in vitro did not have high correlation with the ability to induce papillomas in Tg.AC mice (Thompson et al., 2000). Subsequently, through analysis of Tg.AC mice that were nonresponsive to TPA, we observed that the linear positioning of copies of the transgene in a head-to-head palindromic orientation correlated with induction of transgene expression and papilloma formation in response to tumor promoter treatment (Honchel et al., 2001; Thompson et al., 1998). Evidence to date suggests that the secondary structure of the transgene locus, the nucleotide sequence of the transgene, and the surrounding insertion site are all involved in the regulation of transgene expression. Therefore, this system would be very difficult to model in an in vitro screen. Based on the transgene structure and sequence, it might be thought that the reporter phenotype of Tg.AC mice, i.e., the generation of skin papillomas upon dermal application of carcinogens or tumor promoters, is simply a direct consequence of induction of transgene expression through regulating the activity of the ζ-globin promoter. The reality appears to be much more complex and to involve a series of specific genetic and epigenetic events that are incompletely understood at present. Activation of the ζ-globin promoter may be a necessary component for Tg.AC tumorigenesis but it appears not to be sufficient.

The presence of a nonresponder Tg.AC mouse in our positive control group whose phenotype could not be detected using the standard genotyping protocol emphasizes the importance of monitoring for phenotypic responsiveness among the Tg.AC breeding colony. We are currently investigating whether this mouse carried a deletion in the right hand Line-1 sequence recently associated with loss of responsiveness in Tg.AC mice (Leder et al., 2002). There may be alternative mechanisms in the Tg.AC mouse model besides loss of palindromic transgene sequence or integration site sequence that result in loss of responsiveness and, although these examples may be rare, they require continual vigilance.

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REFERENCES


