Short-Term \textit{In Vitro} and \textit{In Vivo} Analyses for Assessing the Tumor-Promoting Potentials of Cigarette Smoke Condensates

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Previous studies found that repeated application of smoke condensate from tobacco-burning reference cigarettes to chemically initiated SENCAR mouse skin promoted the development of tumors in a statistically significant and dose-dependent manner, while condensate from prototype cigarettes that primarily heat tobacco promoted statistically fewer tumors. Based on the recognized correlation between sustained, potentiated epidermal hyperplasia and tumor promotion, we conducted tests to examine the utility of selected short-term analyses for discriminating between condensates exhibiting significantly different promotion activities. \textit{In vitro} analyses assessing the potential for inducing cytotoxicity (ATP bioluminescence) or free radical production (cytochrome \(c\) reduction, salicylate trapping) demonstrated significant reductions when comparing condensate collected from prototype cigarettes to reference condensate. Short-term \textit{in vivo} analyses conducted within the context of a mouse skin, tumor-promotion protocol (i.e., comparative measures of epidermal thickness, proliferative index, myeloperoxidase activity, leukocyte invasion, mutation of \(c\)-H\(a\)-ras, and formation of modified DNA bases) provided similar results. Reference condensate induced statistically significant and dose-dependent increases (relative to vehicle control) for nearly all indices examined, while prototype condensate possessed a significantly reduced potential for inducing changes that we regarded as consistent with sustained epidermal hyperplasia and/or inflammation. Collectively, these data support the contention that selected short-term analyses associated with sustained hyperplasia and/or inflammation are capable of discriminating between smoke condensates with dissimilar tumor-promotion potentials. Moreover, our results suggest that comparative measures of proliferative index and myeloperoxidase activity, both possessing favorable correlation coefficients relative to tumor formation (i.e., \(r > 0.95\) after 8 or 12 weeks of promotion), may constitute reasonable endpoints for further investigation.

Key Words: tumor promotion; short-term analyses; sustained epidermal hyperplasia; inflammation.

Mouse skin provides an effective means for examining multistage carcinogenesis, contributing significantly to the delineation of three seemingly distinct but complementary stages of initiation, promotion, and progression (DiGiovanni, 1992; Slaga \textit{et al}., 1987; Yuspa and Poirier, 1988). Previous reports have supported the notion that cellular evolution to malignancy involves the sequential alteration of proto-oncogenes and/or tumor suppressor genes, whose products participate in critical pathways for the regulation of signal transduction and/or gene expression (Bishop, 1991; Marshall, 1991; Yuspa \textit{et al}., 1994).

Skin tumor initiation by chemical carcinogens (e.g., 7,12-dimethylbenz[\(a\)]anthracene, DMBA) constitutes an irreversible stage likely involving a somatic mutation of the \(H\)\(a\)-\(ras\) oncogene (Slaga \textit{et al}., 1987; Yuspa and Poirier, 1988). Experimental data have revealed a reasonable correlation between the carcinogenicity of numerous chemical agents and their mutagenic potential (Slaga \textit{et al}., 1987). Most tumor-initiating agents either generate or are metabolically converted to electrophilic reactants, which in turn bind covalently to cellular DNA (DiGiovanni, 1992; Slaga \textit{et al}., 1987). Free radicals and the modified DNA bases generated by these radicals have been directly implicated in the carcinogenic process (Ames \textit{et al}., 1993; Malins, 1993; Perchellet and Perchellet, 1989).

Evidence from several laboratories indicates that activation of the \(H\)\(a\)-\(ras\) gene occurs early in the process of mouse skin carcinogenesis and is likely equivalent to the initiation event (Balmain \textit{et al}., 1984; Bizub \textit{et al}., 1986; Brown \textit{et al}., 1990; Quintanilla \textit{et al}., 1986). Quintanilla \textit{et al}., 1986 demonstrated the presence of an activated \(c\)-\(H\)\(a\)-\(ras\) gene in mouse skin papillomas and carcinomas induced by DMBA; activation was associated with a high frequency of A-T transversions at codon 61. Subsequent studies revealed that the mutation type depended on the chemical initiator but was independent of the promoter, suggesting a direct effect of the initiator on \(c\)-\(H\)\(a\)-\(ras\) (Bizub \textit{et al}., 1986; Brown \textit{et al}., 1990). Furthermore, infection of mouse skin by a virally activated \(H\)\(a\)-\(ras\) gene (v-\(H\)\(a\)-\(ras\)) was shown to effectively serve as the initiating event in two-stage carcinogenesis (Spalding \textit{et al}., 1993).

Sustained epidermal hyperplasia and/or inflammation, altered cellular differentiation, and genetic instability all lead to the selective expansion of initiated cells into papillomas and carcinomas, and are characteristic of the tumor-promotion and
progression stages (DiGiovanni, 1992; Slaga et al., 1987; Yuspa and Poirier, 1988). Phorbol esters (e.g., 12-O-tetradecanoylphorbol-13-acetate, TPA), indole alkaloids, and polycatate-type promoters appear to function through a membrane receptor protein kinase C, whereas benzo[e]pyrene, chryrsarobin, and certain peroxides appear to act via a free radical mechanism (DiGiovanni, 1992; Slaga et al., 1987).

Histological observations of mice treated topically with tumor promoters have demonstrated that sustained epidermal hyperplasia plays a critical role in tumor promotion (Klein-Szanto and Slaga, 1981, 1982; Naito et al., 1987; Slaga et al., 1976; Spalding et al., 1993). Indeed, a qualitative correlation between sustained epidermal hyperplasia and tumor promotion in mouse skin has been established for numerous structurally diverse chemicals (Argyris, 1985, 1989; DiGiovanni, 1992; Kruszewski et al., 1989; Naito et al., 1987; Walborg et al., 1998). An important aspect of carcinogenesis, especially at the tumor-promotion stage, is that carcinogens and tumor promoters induce the formation of free radicals (e.g., superoxide anions and hydroxyl radicals) through both direct and indirect mechanisms.

Earlier studies using a standardized (30-week) mouse skin, tumor-promotion protocol (Meckley et al., 2004b) demonstrated a statistically significant decrease for both total tumors and percentage of tumor-bearing animals in response to promotion with ECLIPSE prototype 7-026A condensate compared to the response elicited by condensate collected from 1R4F reference cigarettes (Meckley et al., 2004a). The aim of this study was to evaluate the utility of selected short-term analyses for discriminating between test articles (i.e., smoke condensates) exhibiting significantly different tumor-promoting potentials. Selected analyses examined several aspects of tumor promotion, focusing on the relative epidermal hyperplastic and inflammatory potentials of these condensates.

Invitro analyses included ATP bioluminescence, cytochrome c reduction, and salicylate trapping assays to assess the relative cytotoxic, superoxide anion and hydroxyl radical production potentials of the reference and prototype condensates; these analyses were intended to examine the potential mechanism of action (for the condensates) during tumor promotion. Tissues collected as a result of the short-term mouse skin protocol were subjected to an evaluation of indices associated with sustained epidermal hyperplasia and inflammation; specifically, endpoints include epidermal thickness, proliferative index, myeloperoxidase (MPO) activity, and leukocyte invasion. Ha-ras mutation (codon 61) and modified DNA base (8-hydroxydeoxyguanosine, 8-OH-dG) formation were similarly examined. Our results show that selected short-term analyses associated with sustained hyperplasia and/or inflammation effectively discriminate between smoke condensates with dissimilar tumor-promotion potentials.

MATERIALS AND METHODS

Participating laboratories. R. J. Reynolds Tobacco Co. (RJRT, Winston-Salem, NC) performed the generation, collection, and chemical characterization of cigarette smoke condensates (CSCs) as well as the dermal tumor-promotion portion of the study. Veritas Laboratories, Inc. (Burlington, NC) performed the collection and preparation of skin specimens used during subsequent in vivo analyses. Finally, in vitro and in vivo analyses were conducted in laboratories of the AMC Cancer Research Center (Denver, CO); this entailed transfer of the same CSCs subsequently used during the dermal tumor-promotion effort for in vitro analyses as well as paraffin-embedded skin sections (DiGiovanni, 1992; Slaga et al., 1987).

CSC preparation. ECLIPSE 7-026A prototype and Kentucky 1R4F reference cigarettes were supplied by RJRT. An extensive analysis of mainstream constituents for each cigarette type has been previously published (Borgering et al., 1997). The ECLIPSE prototype uses a unique cigarette design to produce smoke primarily by heating rather than burning tobacco; consequently, this cigarette is designed to burn approximately 3% as much tobacco as conventional tobacco-burning cigarettes (e.g., 1R4F reference).

Cigarettes were smoked and the corresponding CSCs collected and characterized as previously described (Meckley et al., 2004a,b). CSC dosing solutions were prepared by serial dilution of the pooled CSC to predetermined ‘tar’ concentrations (‘tar’ defined as mass of wet total particulate matter minus the mass of nicotine and water) using 8% water in high purity acetone. High-dose CSC solutions were transferred (via overnight delivery and packaged with dry ice) for subsequent in vitro analyses.

DMBA, used as a chemical initiator for control and experimental groups within the initiation-promotion protocol, was obtained from Sigma Chemical Co. (St. Louis, MO). The dosing solution was prepared using high-purity acetone and subject to concentration analysis prior to use. OPTIMA grade acetone was purchased from Fisher Scientific (Fair Lawn, NJ).

ATP bioluminescence assay. Selection of the luciferin-luciferase bioluminescent assay for examining intracellular ATP content was based on the specificity and sensitivity provided by this method (Crouch et al., 1993). ATP levels were examined within both 3PC keratinocytes and J774A.1 macrophages following CSC exposure. The 3PC cell line was established as a consequence of in vitro exposure of adult SENCAR mouse primary keratinocytes to DMBA (Klann et al., 1989), while the J774A.1 cell line (ATCC, Rockville, MD) was adapted to culture from a tumor-bearing BALB/c mouse.

Cultures were established by plating 5000 cells per well using Dulbecco’s modified Eagle medium (2.5 ml) supplemented with 4 mM L-glutamine and 10% bovine serum; cells were allowed to attach and grow for approximately 48 h in a 5% CO2/air atmosphere at 37°C. Following exposure to the test articles (i.e., reference or prototype CSC, at concentrations ranging from 22.5 to 360 mg tar/l cell culture medium, for 24–72 h), the cells were harvested. Suspended populations of cells were lysed, and the ATP was stabilized during orbital shaking. Following the addition of substrate solution, solubilized samples were subjected to luminescence determinations using a Packard Spectra Count (Meriden, CT).

Cytochrome c reduction assay. Superoxide dismutase (SOD) protects cells and tissues from injury induced by superoxide anion radicals through its ability to catalyze dismutation of the radicals to hydrogen peroxide and oxygen gas. Relative abilities of the reference and prototype CSCs to modulate the superoxide anion–scavenging activity of SOD were assessed using the cytochrome c reduction assay, as previously described by Bagchi et al. (1997). More specifically, formation of superoxide anion was assayed by measuring the amount of reduced cytochrome c that was produced following the reaction of oxidized cytochrome c with superoxide radical.

Briefly, a reaction mixture [216 mM potassium phosphate buffer containing 10.7 mM EDTA, 1.1 mM cytochrome c, and 0.108 mM xanthine (as a component of the xanthine/xanthine oxidase system that generates the superoxide radical)] was adjusted to pH 7.8 and warmed to 25°C. Absorbance of the solution was monitored at 550 nm using a Beckman thermostated spectrophotometer (Beckman Coulter, Inc., Fullerton, CA). Once a constant absorbance was achieved, deionized water, xanthine oxidase enzyme (8 mU/ml), SOD (13 mU/ml), and different concentrations of CSC were added; both enzymes were obtained from Sigma Chemical Co. The solution was immediately mixed by inversion, and absorbance (at 550 nm) was monitored for 360 s. Reaction
mixtures lacking CSC, with and without SOD, served as positive and negative controls, respectively.

**Salicylate trapping assay.** Salicylate trapping of hydroxyl free radicals has been used as a method to examine free radical formation in biochemical (Floyd et al., 1984) and biological (Floyd et al., 1986) systems. When exposed to hydroxyl radicals, salicylate (2-hydroxybenzoic acid) is converted to either 2, 3- or 2,5-dihydroxybenzoic acid (DHBA); in the absence of hydroxyl radicals, salicylate is relatively stable with very little spontaneous production of DHBA. According to the (cell-free) in vitro assay employed (Ghiselli, 1998), 1 mM OH2 and 2 mM salicylate were added to a solution of 10 mM sodium phosphate buffer (pH 7) containing 104 mM EDTA and 1 mM FeCl3. To reduce FeCl3 to the Fe2+ form and begin the reaction, 1 mM ascorbate was added to the reaction mixture, with CSC solutions added in place of the iron reagents (at different concentrations). Reactions were incubated at 37°C for 1.5 min and terminated by acidification with 70% perchloric acid. Samples were filtered through a 0.22-mm syringe filter and analyzed for DHBA production using an isocratic high-pressure liquid chromatography method with electrochemical detection (LC-600/SPD-6A-CR4A HPLC system; Shimadzu Corp., Columbia, MD). Data were analyzed using CoulArray software (ESA, Inc., Chelmsford, MA) and expressed in terms of pmol DHBA per nmol salicylate.

**Experimental animals and tumor-promotion protocol.** Prior to the conduct of any effort involving animals, the study-specific protocol is reviewed by the RIRBT Institutional Animal Care and Use Committee. This committee evaluated all animal procedures associated with the present study and ensured that all proposed methods were appropriate.

Female SENCAR mice (5–7 weeks old) were received from the National Cancer Institute, Frederick Cancer Research and Development Center (Frederick, MD) and placed into quarantine for 2 weeks prior to the initiation of animal dosing. Procedures for animal quarantine (including health screens), identification, and assignment to control or experimental groups have been described previously (Meckley et al., 2004a,b). Mice were housed and cared for in accordance with the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council document, “Guide for the Care and Use of Laboratory Animals” (1996), as detailed by Meckley et al. (2004a,b).

Animals were provided ad libitum access to certified PMI Rodent Diet 5002 feed (Purina Mills, Inc., St. Louis, MO) in pellet form; each feed lot was analyzed (by the supplier) for trace components and contaminants. Water originated from the municipal supply and was subsequently filtered through activated carbon and 5-μm particulate filters prior to animal delivery. Water was provided ad libitum via an automatic system, with analysis for trace components and contaminants performed semiannually.

Experimental groups (n = 30) were initiated with a single, 200-ml application of 75 μg DMBA in acetone, followed by three times per week promotion (for 4, 8, or 12 weeks) using either 9, 18, 27, or 36 mg tar (i.e., collected from either prototype or reference cigarettes) in acetone per 200-ml application. CSC exposure levels were based on results from previous tumor-promotion studies, with serum levels based on results from previous tumor-promotion studies, with control groups being initiated with DMBA, 2-week acclimation period for the highest dose of CSC employed. Among the dosing performed as described by Meckley et al. (2004a,b); this included a 2-week acclimation period for the highest dose of CSC employed. Among the control groups were vehicle control animals that were initiated with DMBA, followed by repetitive promotion with acetone (vehicle).

Procedures for daily observations (morbidity/mortality checks) and the collection of clinical signs, body weights, and mass tracking were performed as previously described (Meckley et al., 2004a,b).

**Collection of tissue from the chemical application site.** Tissue (skin) samples were collected from each animal (chemical exposure area) at the time of necropsy, i.e., ~48 h following the last scheduled application of test article; approximately 10 animals per group were sacrificed per treatment interval (at 4, 8, or 12 weeks of promotion). Sections specified for either DNA or MPO analysis were snap-frozen using liquid nitrogen and placed in the freezer (~80 ± 5°C); sections collected for histological examination were fixed in 10% neutral-buffered formalin. A minimum of seven animals per group were used during assessments of epidermal hyperplasia, Ha-ras mutation frequency, and modified DNA-based formation; evaluation of proliferative index, MPO activity, and leukocyte invasion was accomplished using a minimum of five animals per group.

To ensure the collection of tissue sections devoid of animal hair (i.e., a potential confounder during subsequent DNA and/or RNA analysis), the chemical application site of each animal was treated with a hair-removal agent (Nair, Church and Dwight Co., Inc., Princeton, NJ). A sufficient amount of water was used to remove all residual chemical.

**Quantitative evaluation of epidermal hyperplasia.** Tissues used for histological evaluation were prepared using conventional paraffin sections and hematoxylin-eosin staining. Epidermal thickness was evaluated using an Olympus BX45 microscope (Melville, NY), with a minimum of 20 randomly selected sites (per animal) averaged.

**Measurement of proliferative index.** For proliferative index analyses, mice were subjected to intraperitoneal injection of 5-bromo-2′-deoxyuridine (BrdU; 100 μL, 15 mg/ml 0.9% NaCl) 60 min prior to sacrifice. Tissue sections were immunostained with anti-BrdU antibody (100 μg IgG/100 μl) using the InnoGenex Mouse-on-Mouse Iso-IHC Kit (San Ramon, CA). Following deparaffinization and dehydration, slides were incubated in 1 mg/ml pepsin/0.1 N HCl for 10 min at 37°C to enhance staining intensity; slides were then counterstained with hematoxylin and mounted in glycerol. The percentage of BrdU-positive epidermal cells was determined, with an average of 500–1000 cells counted for each tissue section.

**Assay of MPO activity.** Quantification of MPO activity was carried out using methods described previously (Bradley et al., 1982; Trush et al., 1994). Briefly, MPO was extracted from 10-mm-diameter punches of skin. Punches were then minced and homogenized for 20 s in potassium phosphate buffer containing 0.5% hexadecyltrimethyl-ammonium bromide (pH 6.0), with homogenized solutions freeze-thawed and sonicated three times. Following final sonication, suspensions were centrifuged at 15,000 × g and 4°C for 20 min. The resulting supernatant was assayed for MPO activity at 460 nm on a Packard SpectraCount 96-well plate reader. MPO activity was calculated by comparison with curves from known concentrations of MPO standard, 0.3–10 mU.

**Quantitative evaluation of leukocyte invasion.** Generally speaking, skin carcinogens and tumor promoters increase dermal thickness and cellularity, with the latter considered an important measure of inflammation induced by test article application. The relative proportion of inflammatory cells (polymorphonuclear leukocytes and lymphocytes, macrophages, fibroblasts, and mast cells) within the dermis is determined by measuring the number of cells per square micrometer in 10 high-magnification fields (×1000) per animal (Gimenez-Conti et al., 1998).

**Analysis of Ha-ras codon 61 mutations.** PCR amplification and subsequent analysis of Ha-ras mutations occurring in the dosed skin were performed as previously described (Hanausek et al., 2001). Signal analysis was accomplished using Kodak Digital Science 1D Image Analysis Software (Eastman Kodak Co., Rochester, NY), with the bioimage software and its associated camera providing digitized autoradiograms.

**Detection of oxidative damage.** Analysis of modified DNA base 8-Oh-dG was performed as previously described (Hanausek et al., 2001) using HPLC (Shimadzu Corp.) in series with an electrochemical detection unit consisting of an array of eight electrodes (ESA, Inc.); normal bases were quantified using an in-line UV detector. All tissue analyses were performed in triplicate, and appropriate standard curves were used to correlate area units with concentration. Data are expressed as the ratio of 8-Oh-dG/105 G (pmol/pmol).

**Statistical analysis.** In general, statistical analysis was based on ANOVA. Contrast tests among treatment means (post hoc F-tests for sets of treatment means using error estimates from the ANOVA) were used to make most comparisons of interest. In some instances, logarithms of measured values were analyzed to provide more consistent variation for groups receiving different treatments. Dunnett’s test, frequently used to compare a set of treatments to control, is a post hoc procedure (ANOVA) used for the particular situation of comparing a set of treatments to a single control. This test recognizes the increased chance of a
false positive with more than one comparison and uses more stringent criteria (which depend on the number of treatments and the certainty of the standard deviation estimate) to declare significance. Otherwise, p values less than 0.05 were deemed statistically significant.

ANOVA was performed on the log of ATP bioluminescence and was conducted separately for each exposure interval and cell type. Dunnett’s test was applied to contrast comparisons of treatment means to control means. Contrast comparisons of the means of the two CSCs to each other were made based on the average across all doses and separately at each dose.

Regarding cytochrome c data, ANOVA was performed separately for each dose level, followed by pair-wise contrast comparisons of the treatments, with p < 0.05 required for significance. Also, the data set providing absorbance measurements as a function of time (only available at a single dose) was analyzed to compare the slopes of regression lines and fit to predict change in absorbance from expired time. These slopes were compared using contrast tests based on an ANOVA model that fit separate lines for each treatment.

ANOVA was performed on the logs of measurements provided by the salicylate trapping data; this was to provide more consistent levels of within-group variability. CSC-related comparisons were made for the only dose level in common (for both CSCs).

Pearson correlation coefficients were calculated as measures of predictability for tumor end points (i.e., number of total tumors and tumor-bearing animals) and measurements from each of the in vivo analyses. The latter measurements were correlated among themselves, with data from only seven treatment groups being available. Accordingly, the calculated coefficients may not represent precise values but do provide indications of relationships between the in vivo measurements and tumor end points (including rank ordering in terms of predictability).

RESULTS

ATP Bioluminescence Assay for Assessing Cytotoxic Potential

Relative cytotoxic potentials for CSCs collected from the reference and prototype cigarettes were determined using both 3PC keratinocytes and J774A.1 macrophages, with exposure times ranging from 24 to 72 h (Table 1). In nearly all instances, regardless of the chemical exposure interval, reference and prototype CSCs yielded statistically significant reductions (Dunnett’s, p < 0.006) for ATP bioluminescence (in both 3PC and J774A.1 cells) compared to their respective controls. Generally, reductions were more prevalent for the reference CSC than for the prototype CSC.

Addition of reference CSC to the culture medium of 3PC cells for 24 h, at concentrations of 22.5, 45, 90, and 180 mg tar per liter (mg tar/l), reduced ATP bioluminescence 1.8, 17.1, 34.8, and 95.8%, respectively. Decreased ATP bioluminescence values were consistent with results obtained during the counting of viable cells; for example, reference CSC concentrations of 90 and 360 mg tar/l yielded reductions of 27.1 and 94.5%, respectively. Comparatively, the addition of prototype CSC at identical concentrations yielded corresponding reductions of 10.3, 5.5, 6.8, and 24.8%, respectively. Direct comparison of CSC-induced cytotoxic potentials demonstrated that significant differences (p < 0.05) were observed for the prototype CSC (relative to the reference CSC) at concentrations ≥ 90 mg tar/l. Our finding that prototype CSC exhibited a significantly reduced potential for inducing cytotoxicity was similarly evident at 48 and 72 h postexposure.

Employing a second cell type, we found that the addition of reference CSC to the culture medium of J774A.1 cells for 24 h, at concentrations of 45, 90, and 180 mg tar/l, reduced ATP bioluminescence 39.3, 62.4, and 95.6%, respectively. Comparatively, the addition of prototype CSC at the same concentrations yielded corresponding reductions of 13.2, 18.8, and 30.8%. Direct comparison of CSC-induced cytotoxic potentials demonstrated that significant differences (p < 0.05) were observed for the prototype CSC (relative to the reference CSC) at concentrations ≥ 45 mg tar/l. Our finding that prototype CSC exhibited a significantly reduced potential for inducing cytotoxicity was similarly evident at 48 and 72 h postexposure.

**TABLE 1**

### Relative Cytotoxic Potentials for Reference and Prototype CSCs

<table>
<thead>
<tr>
<th>Test article (dose)</th>
<th>ATP bioluminescence (RLU) per 3PC cells</th>
<th>ATP bioluminescence (RLU) per J774A.1 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hours 48 hours 72 hours</td>
<td>24 hours 48 hours 72 hours</td>
</tr>
<tr>
<td>Control</td>
<td>69048 ± 4696 88438 ± 6813 85902 ± 9248</td>
<td>55413 ± 2813 59690 ± 8841 89343 ± 9198</td>
</tr>
<tr>
<td>1R4F (22.5 mg/L)</td>
<td>67815 ± 8014 95744 ± 7369 75828 ± 5646</td>
<td>33636 ± 4749 17566 ± 2502 13452 ± 3105</td>
</tr>
<tr>
<td>1R4F (45 mg/L)</td>
<td>57251 ± 7960 67775 ± 7673 80470 ± 8416</td>
<td>20841 ± 1407 9852 ± 321 5281 ± 979</td>
</tr>
<tr>
<td>1R4F (90 mg/L)</td>
<td>45038 ± 4011 31994 ± 1832 29177 ± 5415</td>
<td>2459 ± 244 247 ± 36 94 ± 82</td>
</tr>
<tr>
<td>1R4F (180 mg/L)</td>
<td>2910 ± 386 693 ± 75 327 ± 89</td>
<td>1036 ± 262 47 ± 18 44 ± 28</td>
</tr>
<tr>
<td>1R4F (360 mg/L)</td>
<td>513 ± 27</td>
<td>376 ± 7</td>
</tr>
<tr>
<td>7-026A (22.5 mg/L)</td>
<td>61861 ± 3629 87811 ± 5542 69714 ± 6160</td>
<td>48088 ± 4512 51904 ± 5009 89003 ± 16304</td>
</tr>
<tr>
<td>7-026A (45 mg/L)</td>
<td>65251 ± 3918 75503 ± 6089 77803 ± 9879</td>
<td>45020 ± 5029 50326 ± 2156 79902 ± 8582</td>
</tr>
<tr>
<td>7-026A (90 mg/L)</td>
<td>64346 ± 2819 73806 ± 3196 67484 ± 10215</td>
<td>38366 ± 2361 36162 ± 1279 55054 ± 2731</td>
</tr>
<tr>
<td>7-026A (180 mg/L)</td>
<td>51894 ± 4886 52117 ± 3067 66236 ± 6099</td>
<td>23491 ± 6416 15267 ± 1162 19887 ± 6842</td>
</tr>
<tr>
<td>7-026A (360 mg/L)</td>
<td>34150 ± 851</td>
<td>21155 ± 1410</td>
</tr>
</tbody>
</table>

*Note. Data presented as means ± standard deviation; RLU, relative luminescence unit.

*aStatistically significant difference compared to vehicle control (Dunnett’s, p < 0.006).

*bStatistically significant difference compared to 1R4F reference CSC (p < 0.05).*
Cytochrome c Reduction Assay for Assessing the Potential of Superoxide Anion Generation

Cells and tissues are protected from injury induced by superoxide anion radicals primarily by SOD, a copper-containing intracellular enzyme that catalyzes dismutation of free radicals to hydrogen peroxide and oxygen gas. Relative abilities of the reference and prototype CSCs to modulate the antioxidant activity of SOD (responsible for scavenging superoxide anions) were assessed using the cytochrome c reduction assay. This was accomplished by examining the time course for modulatory effects of the respective CSCs on cytochrome c reduction in the presence of SOD (Fig. 1), as well as analyzing for concentration-dependent effects at a time of maximal inhibition of cytochrome c reduction by SOD (Fig. 2).

Time-course analysis of the inhibitory effects of reference and prototype CSCs (18 mg tar/l) demonstrated statistically significant increases ($p < 0.05$) in the reduction of cytochrome c relative to the control (13 mU/ml SOD). Direct comparison of the reference and prototype CSCs (based on the slopes of regression lines fit to predict change in absorbance from expired time) revealed that the ability of prototype CSC to increase the reduction of cytochrome c, normally suppressed by SOD, was significantly reduced ($p < 0.05$) compared to reference CSC.

Similar results were obtained for analyses related to the concentration-dependent reduction of cytochrome c activity in response to the reference and prototype CSCs. Specifically, reference CSC demonstrated statistically significant increases in cytochrome c reduction ($p < 0.05$) compared to control (13 mU/ml SOD) at concentrations of 6, 9, and 18 mg tar/l. In contrast, no statistically significant increases were observed for prototype CSC, regardless of concentration. Direct comparison of the reference and prototype CSCs revealed significant differences ($p < 0.05$) at all concentrations tested.

Salicylate Trapping Assay for Assessing the Potential of Hydroxyl Radical Generation

The salicylate trapping assay was used to assess the relative potentials for CSC-mediated production of hydroxyl radicals via the Fenton reaction (Fig. 3). Results from these tests clearly showed that the prototype CSC possessed a significantly reduced (1.8 mg tar/l; $p < 0.05$) potential for the generation of hydroxyl radicals compared to the reference CSC; a 10-fold higher concentration of the prototype CSC was required to elicit a response (expressed as pmol DHBA/nmol salicylate) comparable to reference CSC.
Clinical Observations and Body Weights for Control and Experimental Animals

Major abnormalities of the dosed skin, i.e., abnormalities observed within an experimental group at a frequency exceeding 10%, included desquamation and scabbing. The occurrence of desquamation was limited to animal groups promoted with reference CSC at concentrations ≥27 milligrams tar per application (mg tar/app), while scabbing was limited to animals promoted with reference CSC at 36 mg tar/app.

There were no significant differences observed when comparing the mean body weights of animal groups promoted with either reference or prototype CSC to acetone-promoted (vehicle) controls; hence, reference CSC– and prototype CSC–promoted groups were not directly compared.

Epidermal Thickness for Assessing the Relative Hyperplastic Potential

Previous studies employing a standardized (30-week) mouse skin, initiation-promotion protocol to assess relative tumor-promoting potentials yielded statistically significant differences between the reference and prototype CSCs (Meckley et al., 2004a). Promotion of DMBA-initiated SENCAR mouse skin with reference CSC at concentrations of 9, 18, and 36 mg tar/app for 29 weeks yielded 11, 184, and 242 total tumors, respectively. Similarly initiated animals promoted with prototype CSC at identical tar concentrations exhibited 1, 11, and 31 total tumors, respectively.

Epidermal thickness was used to assess the relative hyperplastic potentials of reference and prototype CSCs following topical application of the respective test articles for periods of 4, 8, and 12 weeks (Table 2). The reference CSC, applied three times per week for 4 weeks, induced statistically significant increases relative to control. Direct comparison of the prototype to the reference CSC demonstrated that the former induced epidermal hyperplasia to a level that was significantly reduced (p < 0.05) compared to the latter; reductions were noted at each of the concentrations tested. The 8- and 12-week exposure regimens yielded similar results.

BrdU-Labeling Index for Assessing the Relative Hyperplastic Potential

BrdU-labeling (proliferative) index was also used to assess the relative hyperplastic potentials of reference and prototype CSCs

![FIG. 4. Comparison of relative epidermal thickness (dashed lines) and proliferative index (solid lines) in response to three times per week promotion of DMBA-initiated mouse skin with reference (triangles) and prototype (squares) CSCs for 4 weeks. Data are presented as means ± standard deviations (refer to Table 2 for statistically significant differences).](image)

**TABLE 2**
Relative Hyperplastic Potentials for Reference and Prototype CSCs

<table>
<thead>
<tr>
<th>Test article (dose)</th>
<th>4 weeks</th>
<th>8 weeks</th>
<th>12 weeks</th>
<th>4 weeks</th>
<th>8 weeks</th>
<th>12 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone control</td>
<td>15.2 ± 3.6</td>
<td>17.3 ± 0.9</td>
<td>18.6 ± 1.5</td>
<td>27 ± 6.7</td>
<td>30 ± 5</td>
<td>25 ± 3.5</td>
</tr>
<tr>
<td>1R4F (9mg/app)</td>
<td>30.4 ± 4.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.9 ± 1.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25 ± 6.7</td>
<td>40 ± 5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30 ± 7.1</td>
<td>25 ± 6.1</td>
</tr>
<tr>
<td>1R4F (18mg/app)</td>
<td>33.7 ± 7.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29.2 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.2 ± 3.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>74 ± 7.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>53 ± 8.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>43 ± 5.7&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>1R4F (27mg/app)</td>
<td>33.5 ± 2.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>35 ± 3.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39.5 ± 13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>84 ± 7.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>65 ± 3.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>45 ± 7.9&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>1R4F (36mg/app)</td>
<td>52.6 ± 3.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>50.3 ± 0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>51.1 ± 7.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>150 ± 11.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>78 ± 8.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>61 ± 11.4&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>7-026A (9mg/app)</td>
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<td>16.3 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.7 ± 3.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31 ± 4.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25 ± 6.1</td>
<td>21 ± 4.2</td>
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<tr>
<td>7-026A (18mg/app)</td>
<td>16.7 ± 3.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.6 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20.7 ± 1.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29 ± 4.2&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>22 ± 5.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
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<td>14.5 ± 1.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.8 ± 1.3&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>7-026A (36mg/app)</td>
<td>16.4 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>23.9 ± 3.5&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>30 ± 7.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>28 ± 4.5&lt;sup&gt;b&lt;/sup&gt;</td>
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Note. Data presented as means ± standard deviation; graphical representation of 4-week data provided in Fig. 4 with representative microphotographs provided in Fig. 5.

<sup>a</sup>Statistically significant difference compared to vehicle control (Dunnett’s, p < 0.006).

<sup>b</sup>Statistically significant difference compared to 1R4F reference CSC (p < 0.05).
following topical application of the respective test articles for periods of 4, 8, and 12 weeks (Table 2). The reference CSC, applied three times per week for 4 weeks, induced statistically significant increases (Dunnett’s, $p < 0.006$) in BrdU labeling (within the chemical application site) compared to vehicle control at concentrations of 9, 18, 27, and 36 mg tar/appl (Fig. 4). While the prototype CSC likewise induced a statistically significant increase relative to vehicle control at 27 mg tar/appl, the level of BrdU incorporation was significantly reduced ($p < 0.05$) compared to the reference CSC at each of the exposure concentrations tested.

Analysis of the BrdU labeling induced as a consequence of test article exposure for 8 and 12 weeks yielded similar results. Following 8 and 12 weeks of promotion with the reference CSC, BrdU labeling was significantly increased (Dunnett’s, $p < 0.006$) relative to control at concentrations ≥18 mg tar/appl; the prototype CSC failed to induce statistically significant increases at any of the concentrations tested. Direct comparison of reference and prototype CSCs demonstrated statistically significant reductions ($p < 0.05$) for the latter at concentrations ≥18 mg tar/appl.

**MPO Activity for Assessing the Relative Inflammatory Potential**

MPO activity was used to assess the relative inflammatory potentials of reference and prototype CSCs following topical application of the respective test articles for periods of 4, 8, and 12 weeks (Table 3). The reference CSC, applied three times per week for 4 weeks, induced statistically significant increases (Dunnett’s, $p < 0.006$) in MPO activity (within the chemical application site) compared to vehicle control at concentrations of 18, 27, and 36 mg tar/appl. Conversely, the prototype CSC failed to induce statistically significant increases at any of the concentrations tested. Direct comparison of the reference and prototype CSCs demonstrated significant reductions ($p < 0.05$) for the latter at exposure concentrations ≥18 mg tar/appl.

Analysis of MPO activity induced as a consequence of test article exposure for 8 and 12 weeks yielded similar results. Following 8 and 12 weeks of promotion with reference CSC, MPO activity was found to be significantly increased (Dunnett’s, $p < 0.006$) relative to vehicle control at concentrations ≥18 mg tar/appl. The prototype CSC failed to induce significant changes relative to control, with the level of MPO activity being significantly reduced ($p < 0.05$) compared to reference CSC at concentrations ≥18 mg tar/appl for 8 weeks and at all concentrations for 12 weeks of promotion.

**Leukocyte Invasion for Assessing the Relative Inflammatory Potential**

Leukocyte invasion (i.e., the number of nucleated cells within the dermal layer) was also used to assess the relative inflammatory potentials of reference and prototype CSCs following topical application of the respective test articles for periods of 4, 8, and 12 weeks (Table 3). The reference CSC, applied three times...
per week for 4 weeks, induced statistically significant increases (Dunnett’s, \(p < 0.006\)) in leukocyte invasion (within the chemical application site) compared to vehicle control at concentrations of 18, 27, and 36 mg tar/appl. The prototype CSC induced statistically significant increases at concentrations \(\geq 27\) mg tar/appl; however, the level of leukocyte invasion induced was significantly reduced \((p < 0.05)\) compared to the reference CSC at the same concentrations.

Analysis of leukocyte invasion induced as a consequence of test article exposure for 8 and 12 weeks yielded similar results. Following 8 and 12 weeks of promotion with reference CSC, leukocyte invasion was found to be significantly increased (Dunnett’s, \(p < 0.006\)) relative to control at concentrations \(\geq 27\) mg tar/appl; a significant increase was also observed at 18 mg tar/appl following 8 weeks of promotion. While the prototype CSC did induce statistically significant increases at concentrations \(\geq 27\) mg tar/appl (relative to control), the level of leukocyte invasion was consistently reduced \((p < 0.05)\) compared to the reference CSC applied at the same concentrations.

### Mutated Ha-ras Frequency for Assessing the Relative Genotoxic Potential

Analysis of Ha-ras mutations (codon 61) was intended as a means of assessing the relative genotoxic potentials of reference and prototype CSCs following topical application of the respective test articles for periods of 4, 8, and 12 weeks (Table 4). In the absence of DMBA initiation, repetitive application of reference CSC (36 mg tar/appl, 12 weeks) to mouse skin failed to induce mutations in a manner that was significantly different from vehicle controls (data not shown). Consequently, results from these efforts are most appropriately interpreted in terms of...
expansion of the initiated cell population through the selected propagation of cells expressing mutated Ha-ras as a consequence of DMBA initiation.

The reference CSC, applied three times per week for 12 weeks, induced statistically significant increases (p < 0.05) in the mutant signal intensity, expressed as a percentage of wild-type signal intensity (reflecting in increases in the percentage of cells expressing mutated Ha-ras within the chemical application site), compared to vehicle control at concentrations ≥ 18 mg tar/appl. Conversely, the prototype CSC failed to induce any statistically significant increases. Direct comparison of reference and prototype CSCs demonstrated statistically significant reductions (p < 0.05) for the latter at concentrations ≥ 18 mg tar/appl.

Analysis of Ha-ras mutations induced as a consequence of test article exposure for 4 and 8 weeks yielded similar results. Following 4 and 8 weeks of promotion with the reference CSC, the mutant signal intensity was found to be significantly increased (p < 0.05) relative to control at concentrations ≥ 18 mg tar/appl; a significant increase was also observed at 9 mg tar/appl after 4 weeks of promotion. Conversely, the prototype CSC failed to induce statistically significant changes relative to control, with the exception of 27 mg tar/appl administered for 8 weeks. Mutant signal intensity (reflecting the percentage of cells expressing mutated Ha-ras) induced by the prototype CSC was significantly reduced (p < 0.05) compared to reference CSC at concentrations ≥ 9 mg tar/appl for the 4-week and ≥18 mg tar/appl for the 8-week exposure regimens.

As part of the current study, analysis of Ha-ras mutation was also used to assess the relative potentials of the reference CSC (36 mg tar/appl) and TPA (0.5 μg/appl) to expand the initiated cell population (data not shown). Direct comparison of the mutant signal intensity induced by the reference CSC and TPA demonstrated that the latter was significantly increased (p < 0.05) relative to the former, regardless of dosing duration (i.e., 4, 8, or 12 weeks). Moreover, statistically significant increases (p < 0.05) were observed for TPA (compared to reference CSC) at initiating doses of 2.5, 7.5, and 25 mg DMBA (8- and 12-week promotion).

8-OH-dG Adduct Formation for Assessing the Relative DNA Oxidative Damage

Modified DNA base (8-OH-dG) formation was used to assess the relative DNA oxidative damage induced by the reference and prototype CSCs following topical application of the respective test articles for periods of 4, 8, and 12 weeks (Table 4). The reference and prototype CSCs, applied three times per week for 4 weeks, failed to induce statistically significant differences (Dunnnett’s, p < 0.005) in the 8-OH-dG to dG ratio (within the chemical application site) compared to vehicle control at any of the concentrations tested. Analysis of 8-OH-dG formation following test article exposure for 8 and 12 weeks yielded results best characterized as spurious, with no apparent dose-related trends being evident.

DISCUSSION

Initiation-promotion studies using mouse skin have consistently reported tumor-promoting activity associated with CSC; in contrast, few mouse skin studies have attributed any significant initiating activity to CSC (summarized by Ilgren, 1992). Slaga and coworkers (1981) reported that SENCAR mice initiated with CSC and promoted twice weekly with TPA (50 weeks) exhibited 0.09 papillomas per animal at study termination. Comparing the initiating potentials of diesel extracts, roofing tar, coke oven emissions, and CSC, it was concluded that the initiating properties of CSC (in mouse skin) were, for all practical purposes, negligible. This finding is in good agreement with the work by Nesnow (1989), correlating results of skin carcinogenesis studies with human respiratory cancer using three known human respiratory carcinogens; relative initiating potencies for coke oven emissions, roofing tar, and CSC were found to be 1.0, 3.9, and 0.0024, respectively.

Slaga et al. (1996) recently reported on efforts to correlate sustained epidermal hyperplasia and increased epidermal ornithine decarboxylase activity with tumor-promoting potential. During an examination of more than 50 suspected carcinogens, it was concluded that CSC contributed to tumor formation primarily through the promotion of initiated cells. Recognizing that CSC exhibits weak tumor-initiating activity within mouse skin, a standardized 30-week protocol has been developed to assess the relative tumor-promotion potential of CSCs. Prior initiation of mouse skin using subcarcinogenic doses of DMBA permits the subsequent assessment of tumor-promoting activity, providing both reliable and reproducible dose responses for numbers of total tumors and tumor-bearing animals (Meckley et al., 2004a,b).

As previously stated, histological observations from mouse skin initiation-promotion studies have demonstrated that sustained epidermal hyperplasia plays an essential role during tumor promotion (Klein-Szanto and Slaga, 1981, 1982; Naito et al., 1987; Slaga et al., 1976; Spalding et al., 1993). In fact, a qualitative correlation between sustained, potentiated epidermal hyperplasia and tumor promotion has been established (in mouse skin) for numerous structurally diverse chemicals (Argyris, 1985, 1989; DiGiovanni, 1992; Kruszewski et al., 1989; Naito et al., 1987; Walborg et al., 1998). The aim of the current study was to examine the utility of selected short-term in vitro and in vivo indices associated with sustained hyperplasia and/or inflammation for discriminating between test articles (i.e., CSCs) exhibiting significantly different tumor-promoting potentials. Earlier studies (Meckley et al., 2004a) demonstrated statistically significant decreases for total tumors and percentage of tumor-bearing animals in response to promotion of DMBA-initiated SENCAR mouse skin with condensate from prototype cigarettes that primarily heat tobacco compared to that from tobacco-burning reference cigarettes.

In vitro analyses included ATP bioluminescence, cytochrome c reduction, and salicylate trapping assays to assess the
cytotoxic, superoxide anion and hydroxyl radical production potentials of the reference and prototype CSCs, respectively. Efforts to examine the relative cytostatic potentials of reference and prototype CSCs using both 3PC keratinocytes and J774A.1 macrophages demonstrated significant differences between the two test articles. Specifically, reference CSC was observed to exhibit significant cytotoxicity for each of the two cell types at concentrations lower than those required for prototype CSC; in nearly all instances, cytotoxicity induced by prototype CSC was significantly reduced compared to reference CSC.

Examination of the relative potentials for generating superoxide anions and hydroxyl radicals likewise demonstrated significant differences between the two CSCs. Prototype CSC was observed to possess a significantly reduced capacity for modulating reduction of cytochrome c in the presence of SOD compared to reference CSC, indicating perhaps a reduced potential for generating superoxide anions. Similarly, using the salicylate trapping assay, a 10-fold higher concentration of the prototype CSC was required to induce production of hydroxyl radicals to the level induced by reference CSC. Collectively, results from these analyses support the conclusion that the prototype CSC may possess a reduced potential for inducing cytotoxicity and generating reactive oxygen species compared to the reference CSC.

Tissues collected as a consequence of the mouse skin, tumor-promotion protocol were subject to evaluation based on epidermal thickness, BrdU-labeling (proliferative) index, MPO activity, and leukocyte invasion. Based on results from proliferative analyses (i.e., epidermal thickness and BrdU-labeling index), the prototype CSC possessed a significantly reduced potential for inducing epidermal hyperplasia compared to the reference CSC. For both indices, the reference CSC elicited statistically significant increases compared to vehicle control at each of the promotion intervals and at concentrations ≥ 18 mg tar/appl; in several instances, changes were similarly observed at the lowest concentration tested (i.e., 9 mg tar/appl). In contrast, few significant differences were observed for the prototype CSC (relative to vehicle control), with the level of epidermal hyperplasia significantly reduced compared to the reference CSC in nearly all instances regardless of promotion interval or promoter concentration. Generally, proliferative indices provided the greatest discrimination between CSCs at the 4-week promotion interval. These findings are in good agreement with clinical observation data (not shown) that mouse skin begins to acclimate to CSC dosing between 4 and 8 weeks of promotion, with clinical signs progressively abating as dosing duration is increased.

Correlation coefficients (Table 5) were determined by comparing the proliferative indices data for reference and prototype CSCs to the numbers of total tumors and tumor-bearing animals generated by each CSC at the conclusion of a 30-week, dermal tumor-promotion assay (Meckley et al., 2004a). BrdU-labeling index correlated most closely with total tumors and tumor-bearing animals. In relation to total tumors, coefficients were 0.936, 0.971, and 0.975 for BrdU labeling following 4, 8, and 12 weeks of promotion, respectively; correlation coefficients in relation to numbers of tumor-bearing animals were 0.923, 0.95, and 0.964 for 4, 8, and 12 weeks, respectively. Epidermal thickness likewise provided reasonable correlation coefficients, ranging from 0.851 to 0.894 for total tumors and from 0.859 to 0.887 for tumor-bearing animals across the three promotion intervals.

Results from analyses examining MPO activity and leukocyte invasion suggested that the prototype CSC had a significantly reduced potential for inducing inflammation compared to the reference CSC. For both indices, reference CSC elicited statistically significant increases compared to vehicle control at each of the promotion intervals and at concentrations ≥ 18 mg tar/appl, with the exception of number of nucleated cells at 12 weeks. The prototype CSC failed to elicit any statistically significant differences for MPO activity compared to vehicle control; statistically significant reductions were noted, relative to reference CSC, in nearly all instances, except for the 9 mg tar/appl dose at 4 and 8 weeks of promotion. While statistically significant increases for leukocyte invasion were noted in response to promotion with the prototype CSC at concentrations ≥ 27 mg tar/appl, the observed increases were significantly reduced compared to the reference CSC. Correlation coefficients were similarly determined using the inflammation data for the...
and reversibility. CSC-induced tumor promotion. Future studies will have a
favorable correlation coefficients relating these data to num-
bers of total tumors and tumor-bearing animals evident at the end
of the 29-week promotion interval, sufficient justification exists
for this lesion, with its overall contribution to muta-
genesis being relative weak (Hanausek et al., 2003).

Interpretation of analyses examining Ha-ras mutations and
8-OH-dG formation requires additional consideration. The inability of reference CSC (36 mg tar/appl) to induce detectable
Ha-ras mutations in codon 61 following repeated application
(12 weeks) to unininitiated mouse skin suggests that the observed
differences in mutation frequency in the comparative studies
were likely attributable to the hyperplastic potentials of the
respective CSCs. More specifically, expansion of the initiated
cell population was more a function of increased and selective
propagation than of increased mutation. This interpretation is
consistent with the historically low initiating potential demon-
strated in mouse skin for CSCs (summarized by Ilgren, 1992).
Examination of 8-OH-dG formation indicates that the respective
test articles failed to elicit biologically meaningful change
for this end point relative to control. However, direct comparison
of the reference and prototype CSCs showed statistically
significant decreases in 8-OH-dG formation for the latter,
though only at the higher concentrations tested. 8-Hydroxy-2-
deoxyguanosine is efficiently repaired by a DNA glycosylase
specific for this lesion, with its overall contribution to muta-
genesis being relative weak (Hanausek et al., 2003).

In conclusion, several short-term in vitro and in vivo analyses
used to assess the relative potentials for inducing cytotoxicity,
free radical generation, and sustained epidermal hyperplasia
and/or inflammation have successfully demonstrated differ-
ences between CSCs exhibiting dissimilar capacities for promot-
ing mouse skin tumor formation. Particularly intriguing was the
observed ability of reference CSC to induce reasonable dose-
response relationships for indices easily integrated into the stan-
dardized mouse skin, tumor-promotion protocol. Considering
the favorable correlation coefficients relating these data to num-
bers of total tumors and tumor-bearing animals evident at the end
of the 29-week promotion interval, sufficient justification exists
for the continued development of short-term indices (e.g., pro-
liferative index and myeloperoxidase activity) as predictors of
CSC-induced tumor promotion. Future studies will have a
greater emphasis on demonstrating dose response, threshold,
and reversibility.

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