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Dose-response Assessment of the Dermal Toxicity of Triclosan in B6C3F1 Mice

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The views presented in this article do not necessarily reflect those of the U.S. Food and Drug Administration.

ABSTRACT

Triclosan [5-chloro-2-(2, 4-dichlorophenoxy)phenol] is a widely used antimicrobial agent in personal care products, household items, medical devices, and clinical settings. Due to its extensive use, there is potential for humans in all age groups to receive life-time exposures to triclosan, yet data on the chronic dermal toxicity/carcinogenicity of triclosan are still lacking. The present study evaluated the toxicity of triclosan administered dermally to B6C3F1 mice for 13 weeks. Groups of 10 male and 10 female B6C3F1 mice received dermal applications of 0, 5.8, 12.5, 27, 58, or 125 mg triclosan/kg body weight (bw) daily for 13 weeks. The doses were administered in 1 ml ethanol/kg bw. All mice survived the 13-week treatment period. Body weights of female mice receiving 125 mg triclosan/kg bw/day weighed 94% ($p < 0.05$) of the female control mice; male mice administered 58 and 125 mg triclosan/kg bw/day weighed 91% ($p < 0.05$) and 82% ($p < 0.01$) of the control male mice. Liver weights were significantly increased in females receiving 58 and 125 mg triclosan/kg bw/day and in males in the 125 mg triclosan/kg bw/day dose group. A significant dose-dependent decrease in the levels of thyroxine and cholesterol was observed in both sexes. The highest dose of triclosan increased the percentage of reticulocytes in both sexes; in addition, the 58 mg triclosan/kg bw/day dose increased the percentage of reticulocytes in females. Skin lesions (dermal fibrosis and inflammation; epidermal hyperplasia, inflammation, necrosis, and ulceration, and parakeratosis) were observed in both sexes, with a dose-dependent increase in severity and incidence.

Keywords: triclosan; dermal toxicity; skin lesions.

INTRODUCTION

Triclosan [5-chloro-2-(2, 4-dichlorophenoxy)phenol] is widely used as an antimicrobial agent in personal care products, household items, medical devices, and clinical settings.[1-3] The general population is exposed to triclosan through ingestion or dermal contact with daily hygiene products or through the consumption of food and drinking water contaminated with triclosan. In addition, workers may be exposed to triclosan in the manufacturing environment by dermal contact and inhalation.[3]

Due to its extensive use, triclosan has been found in drinking water, surface water, wastewater, and environmental sediments[4-8] and has been identified in human breast milk, plasma, and urine.[4,9-13]

Triclosan reaches the systemic circulation via absorption through the mucous membranes of the oral cavity[14] and gastrointestinal tract after oral exposure[11,14,15] and the skin after dermal contact.[16,17] After absorption, triclosan is readily metabolized to glucuronide and sulfate conjugates.[17-19] In addition, 2,4-dichlorophenol, hydroxytriclosan, and 4-chlorocatechol have been detected in the urine and/or feces of rats and mice, indicating the occurrence of both aromatic hydroxylation and cleavage of the ether bond of triclosan.[19,20]

Triclosan is excreted in the feces and urine. Rats and mice show predominantly biliary excretion into the feces, whereas guinea pigs excrete the majority of the dose via the kidney. In humans, urinary excretion is the major route of elimination.[11,15,19,21]

The carcinogenicity of triclosan has been investigated in male and female CD-1 mice administered triclosan in the diet at doses of 10, 30, 100, or 200 mg/kg/day for 18 months. Doses of 30 mg/kg/day and above resulted in a statistically significant increase in hepatocellular neoplasms (adenoma or carcinoma combined) (summarized by Rodricks *et al.*[1]). The carcinogenicity of triclosan following dermal application is less clear. Lyman and Furia[22] treated an unspecified number of Swiss mice dermally three times per week for 18 months with acetone solutions containing 0.5 and 1% triclosan (approximately 20 and 40 mg triclosan/kg bw/application). The authors reported that the findings in the triclosan-treated mice were similar

to those in the control mice and that triclosan was not carcinogenic; however, specific details were not provided.

Due to the extensive use of triclosan, a re-assessment of the dermal carcinogenicity is important. As a prelude to a 2-year chronic dermal toxicity/carcinogenicity study, we conducted a 13-week dermal study of triclosan in B6C3F1 mice. The toxicological characterization included assessments of gross and histopathological organ toxicity (the skin at the site of application and liver), hematology, and clinical chemistry. The potential effects of triclosan on reproduction were assessed by the evaluation of testicular and spermatozoal parameters and by characterization of estrous cycle alterations. In addition, the induction of micronuclei in peripheral blood was determined.

MATERIALS AND METHODS

Chemicals

Triclosan [5-chloro-2-(2, 4-dichlorophenoxy) phenol] was obtained from Alfa Aesar (Ward Hill, MA). The purity, as assessed by high performance liquid chromatography and nuclear magnetic resonance spectroscopy, was 99.0%. Ethanol (95%; 190 proof, ACS/USP grade) was obtained from Pharmaco-AAPER Alcohol (Shelbyville, KY).

Dose selection

Doses of 0, 5.8, 12.5, 27, 58, and 125 mg triclosan/kg bw/day in ethanol were selected for the 13-week dermal study because these doses encompassed the dose range (including the no-observed-adverse-effect level, NOAEL) used in previous dermal toxicity studies in mice.[23,24] Ethanol was selected as the vehicle because in a study comparing ethanol, propylene glycol, and a generic cream, the absorption of triclosan was more uniform as a function of dose with ethanol.[19] Furthermore, the extent of oral ingestion due to grooming behavior was the least with ethanol as the vehicle.[19]

Study design

Male and female B6C3F1/Nctr mice were obtained from the NCTR breeding colony and tail-tattooed for identification. Treatment was initiated when the animals were six weeks of age. The animals were housed individually and had *ad libitum* access to NIH-41 irradiated pellets and

Millipore-filtered drinking water. The animal room was maintained on a 12-hour light/dark cycle, with 10-15 air changes per hour. Environmental controls were set to maintain the temperature at 22 ± 4°C, with a relative humidity of 40-70%. Animal use was in accordance with the Institute for Laboratory Animal Resources Guide for the Care and Use of Laboratory Animals. All experimental protocols were reviewed and approved by the Animal Care and Use Committee at the National Center for Toxicological Research.

Each dose group consisted of 10 randomly assigned mice per sex. The dosage groups were 0, 5.8, 12.5, 27, 58, and 125 mg triclosan/kg bw/day in 95% ethanol. Doses were applied seven days/week for 13 weeks to a shaved dorsal area posterior to the scapulae to the base of the tail. The dosing volume was 1.0 ml/kg bw. Dosing was conducted in the morning and completed for each mouse within 2 hours of treatment time on the previous day. All animals were observed for mortality or morbidity twice daily and clinical observations were conducted daily. Body weights were measured on the first treatment day prior to the initial treatment, weekly thereafter until study termination. The doses were adjusted weekly based on these body weight measurements.

Clinical pathology

Blood was drawn by cardiac puncture for hematology and clinical chemistry analyses after animals were euthanized by exposure to carbon dioxide. Aliquots of blood were placed into an EDTA tube for hematological analyses and a serum-separating tube for serum preparation. Blood samples in serum-separating tubes were allowed to clot and then centrifuged. The serum was removed and an aliquot was stored at -80°C until clinical chemistry measurements.

Leukocyte differential and count, erythrocyte count and morphologic assessment, hemoglobin, platelet and morphologic assessment, hematocrit, mean cell volume, mean cell hemoglobin, and mean cell hemoglobin concentration were determined on whole blood using an ABX Pentra 60 C+ analyzer (ABX, Irvine CA). Packed cell volume was determined by manual reading after the blood sample was centrifuged for 2 min in a CritSpin Microhematocrit centrifuge (Iris Sample Processing, Inc., Westwood, MA). Reticulocyte counts were determined from the microscopic analysis of blood smears stained with a freshly prepared solution of methylene blue.

Clinical chemistry analyses were conducted on an Alfa Wassermann ALERA chemistry system (West Caldwell, NJ). Reagents for assays of glucose, alkaline phosphatase, alanine aminotransferase, total protein, albumin, cholesterol, triglycerides, blood urea nitrogen, creatine kinase, and creatinine were obtained from Alfa Wassermann. Reagents for assays of sorbitol dehydrogenase and total bile acids were obtained from Catachem (Bridgeport, CT). Analyses of triiodothyronine (T3), thyroxine (T4), and testosterone were conducted using "Coat-a-Count" radioimmunoassay techniques (Siemens Medical Solutions Diagnostics, Tarrytown, NY). The tests were conducted on a Wizard 2400 series gamma counter (PerkinElmer, Shelton, CT).

All testing was completed on the day of sample collection and the procedures followed the manufacturers' protocol.

Sperm and vaginal cytology evaluation

At the end of the study, samples were collected for sperm motility and vaginal cytology evaluations on mice exposed to 0, 27, 58, and 125 mg triclosan/kg bw/day. Male mice were evaluated for testicular spermatid head counts, epididymal sperm counts, and morphology and motility as previously described.[25] For the last sixteen consecutive days prior to scheduled terminal sacrifice, samples of vaginal fluid and cells were obtained and stained. The treatment effects on estrous cyclicity were evaluated as previously described.[25]

Peripheral blood micronucleus analyses

Micronucleus analyses were conducted using a MicroFlow^{Plus} mouse kit from Litron Laboratories (Rochester, NY) according to the manufacturer's instructions. Flow cytometry was performed on a BD FACSCanto^{TM} II (Becton-Dickinson, San Jose, CA). Data were acquired and processed using BD FACSCanto^{TM} clinical v2.4 and the templates supplied by Litron with the MicroFlow^{Plus} kit. At the beginning of each day of flow cytometric analysis, instrumentation and acquisition/analysis software parameters were calibrated according to the Litron protocol using the biological standards provided with the kit. Approximately 20,000 reticulocytes (RETs, CD71-positive cells) were analyzed for each sample to maximize the discrimination between RETs and normochromatic erythrocytes (NCEs).[26] Adjusted cutoff values for the bivariate plots

were established and used in the analyses of the samples. The cutoff values were determined using assays performed with and without RNase digestion.[27,28]

Gross pathology and histopathology

A complete necropsy was performed on all animals, and digital images of representative skin gross lesions were acquired. The pituitary, liver, thymus, left and right kidney, left and right testis, left and right epididymis, left and right ovary, heart, and lungs of each animal were dissected and weighed. The bilateral organs were weighed separately. Tissues for microscopic examination were fixed and preserved in 10% neutral buffered formalin (except for the eyes and testes, which were fixed in modified Davidson's solution), processed and trimmed, embedded in infiltrating media (Formula R®), sectioned to a thickness of approximately 5 μm, and stained with hematoxylin and eosin. Complete histopathological examinations were performed on all animals in the control and the highest dose group as well as select treatment related tissues in lower dose groups. The skin at the application site was examined in all mice. When applicable, non-neoplastic lesions were graded for severity as 1 (minimal), 2 (mild), 3 (moderate), or 4 (marked).

Statistics

Analyses were conducted at the 0.05 significance level as two-sided comparisons of dosed groups to control using Dunnett's method[29] of adjusted contrasts (unless otherwise noted).

Body weights were analyzed by a repeated measures, mixed model analysis of covariance (ANOCOVA) adjusted for baseline. Within-group correlations were modeled using a heterogeneous first-order autoregressive correlation structure, which allowed for correlated differences in variability across time points. Organ weights were analyzed by ANOCOVA adjusted for receiving body weight.

Hematology and clinical chemistry data were analyzed by an analysis of variance using a nonparametric method with midranks for ties and an unstructured covariance.[30]

Sperm morphology data were analyzed using a generalized linear model with a Poisson

distribution and a log link function. Adjustment for multiple comparisons was performed using Hochberg's method.[31] Percent sperm motility, testes sperm counts, and cauda sperm counts were analyzed by an analysis of variance using Kenward-Roger estimated degrees of freedom.[32]

Analyses of estrous stages (estrus, diestrus, or proestrus) were performed using a generalized logit model and maximum likelihood estimation for nominal outcomes. Adjustment for multiple comparisons was performed using Holm's method.[33]

RETs, micronucleated reticulocytes (MN-RETs), and micronucleated normochromatic erythrocytes (MN-NCEs) were analyzed by an analysis of variance using an arcsin square root transformation for percent data.

The Poly-3 method[34,35] was used to test for an increasing trend in non-neoplastic incidence with increasing dose and to compare treatments to control. The Poly-3 tests were performed as one-sided, continuity-corrected tests at the 0.05 significance level per National Toxicology Program guidelines. If the variance could not be estimated (*i.e.*: 0% or 100% incidence), incidences were compared using the exact Cochran-Armitage trend test.[36] Lesion severity scores were analyzed as one-sided using the Jonckheere-Terpstra test.[37,38] Comparisons of lesion severity between treated groups and the control group were performed by Shirley's method,[39] modified by Williams.[40]

Benchmark doses (BMD) and the lower 95% confidence limits (BMDL) were calculated using Environmental Protection Agency Benchmark Dose Software (version 2.4.0.70; http://www.epa.gov/ncea/bmds). The calculations were conducted using gamma, logistic, log-logistic, log-probit, multistage, probit, and Weibull models to fit the non-neoplastic skin lesion incidences and the doses of triclosan administered dermally. The BMD was defined as the dose that caused a 10% excess risk of the specified adverse effect over that observed in the appropriate control group.

RESULTS

Survival and body weights

Groups of 10 male and 10 female mice were dermally administered 0, 5.8, 12.5, 27, 58, or 125 mg triclosan/kg bw/day in 95% ethanol for 13 weeks. All animals exposed to triclosan survived until the end of study. Treatment-related clinical findings occurred in the skin at the site of application in all 58 and 125 mg triclosan/kg bw/day female and male mice and included dermal irritation, thickened skin, crust formation, and ulceration. These finding were also noted in some females and males exposed to 27 mg triclosan/kg bw/day, but not in controls or the two lowest dose groups. Dermal irritation was observed within 72 h after the first skin application of triclosan to the highest dose groups and started to progress to skin ulceration within 2 weeks. The severity of the skin lesions persisted over the 13-week period; nonetheless, the lesions were not considered sufficiently severe to require early termination of any of the mice in the study.

The final mean body weights of 125 mg triclosan/kg bw/day female mice were significantly lower than that of the controls at the end of the 13-week period (**Table 1; Figure 1S, Supplementary Data**). In male mice, treatment with 125 mg triclosan/kg bw/day resulted in significant decreases in mean body weights from weeks 4-13 (with the exception of week 6), with the final mean body weights being 82% of the control group. Male mice treated with 58 mg triclosan/kg bw/day had significant decreases in mean body weights from weeks 9-13, with the final mean body weights being 91% of the control group (**Table 1; Figure 1S, Supplementary Data**).

Organ weights

Treatment with triclosan had a significant dose-response trend on mean kidney and liver weights in both female and male mice and on mean ovary weights in female mice (**Table 2**).

Liver weights were significantly increased in the two highest dose groups of female mice and in the highest dose group of male mice. In females, respective liver weights of 58 and 125 mg triclosan/kg bw/day were 10% and 38% greater than the controls, while liver weights in the 125 mg triclosan/kg bw/day group of male mice were 10% greater than the control male mice (**Table 2**).

Kidney weights of 125 mg triclosan/kg bw/day female mice were 13% greater than the female

control mice. In contrast, triclosan significant decreased kidney weights of 125 mg triclosan/kg bw/day male mice, with the value being 82% of the control male mice (**Table 2**). Similarly, ovary weights in the highest dose group of female mice were 81% of the control female mice (**Table 2**).

There were no dose-related changes in heart, lung, pituitary gland, thymus, epididymis, or testes weights.

Clinical pathology

The hematology and clinical chemistry data are listed in **Table 1S** (**Supplementary Data**). The amount of blood collected from some mice was not sufficient to analyze all the parameters.

The monocyte count in the highest dose group in both sexes was approximately two times greater than that in the controls, with the difference being significant in female mice (**Table 1S, Supplementary Data**). There were significant declines in the percentage of eosinophils in both sexes. The value in the highest dose group of female mice was only 32% of the control level. In male mice, the values for the 58 and 125 mg triclosan/kg bw/day dose groups were 9 and 17% of the control value, with the significant being observed only at 58 mg triclosan/kg bw/day dose group. These changes are probably a reflection of the inflammation and necrosis in the skin at the site of application.

Platelet counts increased in both female and male mice treated with triclosan (**Table 1S, Supplementary Data**); however, they were still within the normal range in mice ($3 \sim 10 \times 10^5$ /mm^3).[41]

The levels of total cholesterol decreased at a dose-dependent manner (**Table 1S, Supplementary Data**). The levels of total cholesterol in the female mice treated with 5.8, 12.5, 27, 58, and 125 mg triclosan/kg bw/day were, respectively, 33, 52, 67, 70, and 76% lower than those in the controls. Male mice at those doses had levels of total cholesterol that were 7, 25, 38, 51, and 71% lower, respectively, than those in the controls. In female mice, there was a dose-dependent increase in triglyceride concentrations, which were significant at 12.5 mg triclosan/kg bw/day and higher. The increase was approximately two-fold greater than the controls.

A decline in the levels of T4 was observed in both sexes of mice (**Table 1S, Supplementary Data**). The levels of T4 in female mice exposed to 27, 58, and 125 mg triclosan/kg/day were 21, 30, and 58% lower than those in the female control mice. For the male mice, a significant decrease in the levels of T4 occurred only in the highest dose group, with a value being 40% less than those in the male control mice. Although an insufficient number of serum samples lowered power to detect differences between groups for the levels of T3, especially in female mice, the levels of T3 were 11% lower than control for female mice in the 58 mg triclosan/kg bw/day group and 25% lower than control for male mice in the 125 mg triclosan/kg bw/day group (**Table 1S, Supplementary Data**).

Estrous status

Vaginal cytology evaluations were performed on samples collected from for the last sixteen consecutive days of the 13-week study. Triclosan treatment did not cause any changes in the percentage of mice at various stages of the estrous cycle (**Table 2S, Supplementary Data**).

Sperm morphology and count

Triclosan treatment did not alter testis and epididymis weights. There were no changes in sperm morphology, sperm motility, cauda sperm counts, or testes sperm counts in male mice at any of the triclosan exposure levels (**Table 3S and 4S, Supplementary Data**).

Induction of micronuclei

The percentage of RETs, micronucleated RETs, and micronucleated NCEs was determined at the end of the study. Treatment of mice daily for 13 weeks with triclosan (0-125 mg triclosan/kg bw/day) resulted in an increase in the percentage of RETs in both sexes and the percentage of micronucleated NCEs in female mice (**Table 3**). In female mice, the percentage of RETs in the 58 and 125 mg triclosan/kg bw/day treatment groups was 64% and 203% greater, respectively, than that in the control group. For the male mice, the percentage of RETs was 78% greater than the control group at the highest dose. Female mice treated with 125 mg triclosan/kg bw/day had a 34% increase in the percentage of micronucleated NCEs. Since a similar change was not observed in male mice, the significance of this finding is uncertain.

Gross pathology and histopathology

One male mouse from the 58 mg triclosan/kg bw/day group had a gross liver lesion that was diagnosed as a hepatocellular adenoma.

Treatment-related gross observations were mainly skin lesions at the site of application which were described as nodules and/or crust varying in size and color. These observations were noted in all of the mice administered 125 mg triclosan/kg bw/day and most of the mice exposed to 58 mg triclosan/kg bw/day. Lower incidences of skin lesions were observed in the 27 and 12.5 mg triclosan/kg bw/day groups. Skin lesions were not present in the lowest dose (5.8 mg triclosan/kg bw/day) or control groups.

The skin at the application site was examined microscopically in progressively lower dose groups until a NOAEL was reached. The histopathological changes included dermal fibrosis and inflammation; epidermal hyperplasia, inflammation, necrosis, and ulceration, and parakeratosis (**Figure 1**). The incidence and severity of these microscopic changes increased in a dose-dependent manner in both sexes (**Table 4**). All mice exposed to 125 mg triclosan/kg bw/day displayed the above listed skin changes in both sexes. Most males and females exposed to 58 mg triclosan/kg bw/day had similar skin changes with a lower incidence of epidermal ulceration. The administration of 27 mg triclosan/kg bw/day also resulted in significant increases in the incidence of epidermal hyperplasia (all female mice and 70% of male mice) and parakeratosis (60% of both female and male mice). There was also a significantly increased incidence of epidermal inflammation (60%), dermal inflammation (70%), and dermal fibrosis (40%) in female mice at this dose, with the incidence of epidermal and dermal inflammation being significantly greater in females compared to males. For the 12.5 mg triclosan/kg bw/day group, there were significant increased incidences of epidermal hyperplasia in female (70%) and male (40%) mice and parakeratosis in female mice (50%), with the incidence of parakeratosis being significantly greater in females compared to males. The microscopic changes in the skin at the site of application in the lowest dose group (5.8 mg triclosan/kg bw/day) were similar to those of the control mice.

Epidermal hyperplasia was characterized by a thickening of the squamous epithelium due to increased layers of nucleated cells in the epidermis: one to two cell layers was considered normal, three to four layers as minimal, five to six layers as mild, seven to eight layers as moderate, and more than eight layers as marked. Parakeratosis was characterized by a persistence of the nuclei in keratinocytes as they arise from the stratum corneum. Epidermal necrosis had a loss of cellular and nuclear detail with some retention of normal architecture in addition to an occasional subepidermal cleft filled with cellular debris and intact and/or degenerate neutrophils. A serocellular crust composed of similar material, but lying on the surface of the lesion, was often associated with the necrotic areas. Extension into the underlying dermis was sometimes evident and featured necrotic debris with inflammatory cell infiltrates. Epidermal ulceration was defined by the complete loss of the epidermis with an overlying serocellular crust with epidermal inflammation being characterized by an increased number of polymorphonuclear infiltrating inflammatory cells. Dermal fibrosis featured an increase in collagenous connective tissue while dermal inflammation was evident by an increased number of mononuclear and polymorphonuclear infiltrating inflammatory cells.

As a result of a direct response to the inflammation and necrosis in the skin at the site of triclosan application, there were histopathological changes in the hematopoietic system, such as bone marrow, spleen, and lymph node (**Table 4**). Histopathological examination of these tissues was performed on all animals in the control and the highest dose groups as well as in lower dose groups until a NOAEL was reached. All mice in the highest dose group had a marked increase in hematopoietic cell proliferation in the bone marrow and spleen while most female (80%) and male (70%) mice at this dose displayed a mild to moderate increase in lymphoid cell hyperplasia involving the mandibular lymph node.

The highest doses of triclosan caused an increase in polymorphonuclear cell infiltrates in the kidney of female mice and minimal hepatocellular centrilobular hypertrophy in male mice (**Table 4**). The incidence of these changes was 90% and the severity of these changes was minimal to mild. These changes were not observed at 58 mg triclosan/kg bw/day. The remaining non-neoplastic changes were not significant due to their low incidence levels or were considered as background changes.

BMD calculations

BMD modeling was conducted on skin lesions induced by triclosan (dermal fibrosis and inflammation; epidermal hyperplasia, inflammation, necrosis, and ulceration, and parakeratosis; Supplementary table). In female mice, the most sensitive endpoint was parakeratosis, with BMDL values of 1.8-2.5 mg triclosan/kg bw/day, followed by epidermal hyperplasia epidermis (2.3-5.7 mg triclosan/kg bw/day) and dermal inflammation (2.6-5.7 mg triclosan/kg bw/day). In male mice, the most sensitive endpoint was epidermal hyperplasia, with BMDL values of 3.0-6.3 mg triclosan/kg bw/day, followed by parakeratosis (5.2-8.5 mg triclosan/kg bw/day) (**Table 5S, Supplementary Data**).

DISCUSSION

Our recent toxicokinetic study of triclosan in B6C3F1 mice demonstrated that the dermal application of triclosan results in systemic distribution of the compound and that approximately 20% more triclosan was absorbed without the protection of an Elizabethan collar, suggesting that there was some oral ingestion due to the normal behavior grooming.[19] In the present 13-week dermal toxicity study, no additional procedures were implemented to prevent such oral ingestion.

Several studies have shown that the blood levels of total triclosan in humans following use in either mouth rinses or dentifrices vary from 1.4 nM to 1.4 μM.[9,11,15] Mice treated dermally with 10 – 100 mg triclosan/kg bw/day have plasma levels of 5 – 50 μM.[19] Therefore, the doses used in our study result in blood levels that approach those found in humans.

All mice survived the 13-week dermal administration of triclosan. Treatment-related lesions of the skin were limited to the site of application and included dermal fibrosis, and inflammation; epidermal hyperplasia, inflammation, necrosis, and ulceration, and parakeratosis. These skin lesions are consistent with a rat 90-day dermal toxicity study conducted by Trimmer *et al*.[42] They dermally administered triclosan at doses of 0, 10, 40, or 80 mg triclosan/kg bw/day in propylene glycol with the application site being covered with gauze for at least 6 h. Dermal erythema and/or edema were observed in all treatment groups, especially in the high-dose group. Histopathology examinations indicated the presence of eschar and desquamation,

hyperplasia/hyperkeratosis of epidermis, dermal inflammation, and focal necrosis at the application site in treated animals. The presence of epidermal hyperplasia suggests that triclosan may have a proliferative potential in rodent skin. Recently, we found that triclosan stimulated mouse epidermis-derived JB6 Cl 41-5a cell growth in a concentration- and time-dependent manner and that this involved the activation of a serine/threonine-specific protein kinase B (also known as Akt) and extracellular signal-regulated kinases 1 and 2.[43]

In our study, there were limited adverse effects on body and liver weights in male and female mice exposed to 58 or 125 mg triclosan/kg bw/day. There were significant decreases in body weight in male mice at the two highest doses (58 and 125 mg triclosan/kg bw/day) and in female mice at the highest dose (125 mg triclosan/kg bw/day). Liver weights were significantly greater in females receiving the two highest doses and in males in the highest dose group. The increases in liver weights in male mice were accompanied by an increase in hepatic centrilobular hypertrophy. These data indicate that in addition to the site of application, the liver could also be a potential target for topically applied triclosan. An increased incidence of liver tumors induced by triclosan has been reported in an oral 18-month carcinogenicity bioassay with triclosan in mice, an effect attributed to the activation of peroxisome proliferator-activated receptor alpha (PPARα).[1] Recently, we demonstrated that triclosan increased PPARα activity in a mouse PPARα receptor reporter assay. Triclosan also increased the level of acyl-coenzyme A oxidase, a PPARα target, enhanced DNA synthesis, and suppressed transforming growth factor beta-mediated apoptosis.[44] Additional studies are necessary to determine if these effects occur *in vivo*.

Triclosan has structural similarity to thyroid hormones; as such, subchronic exposure to triclosan may disrupt thyroid hormone homeostasis. Pinto *et al.*[45] reported that exposure of adult zebrafish to triclosan for 21 days led to an increase in follicle number and area, a reduction in thyrocyte height, and an up-regulation of thyroid-stimulating hormone and the sodium–iodide symporter genes, suggesting an inhibition effect of triclosan in the thyroid hormone synthesis process. Several studies have documented that triclosan can disturb the thyroid system in rats.[46-50] Exposure to triclosan by oral gavage markedly decreased the serum T4 level in weanling female Long-Evans rats and male Wistar rats.[46,49,50] Perinatal exposure to triclosan by oral gavage decreased both maternal and neonatal serum T4 levels in Long-Evans rats.[47,48] In our 13-

week dermal study in B6C3F1 mice, a similar reduction in serum T4 levels was observed, with the dose-dependent response being stronger in female mice. Although the observed systemic T4 decreases have been associated with the up-regulation of hepatic catabolism of thyroid hormones,[47-49] further studies to investigate the effects of triclosan on thyroid hormone homeostasis, including thyroid hormone synthesis, transport, and metabolism, are clearly warranted.

Triclosan is an effective antimicrobial agent and inhibits bacterial lipid biosynthesis by blocking the enzymatic activity of the type II fat acid synthase, enoyl reductase.[51] In addition, triclosan has been demonstrated to inhibit human type I fat acid synthase, with an IC_{50} between 10 and 50 μM in human breast cancer cell lines MCF-7 and SK-BR-3.[52] Fatty acids are important metabolic molecules for adipocytes. In pre-adipocytes, they are also potent signals for the expression of genes involved in fatty acid metabolism and adipocyte differentiation. As a result, the inhibition of fatty acid synthesis and impairment of fatty acid metabolism may prevent pre-adipocyte differentiation in mouse 3T3-L1 cells[53] and adipocyte differentiation from human mesenchymal stem cells.[54] Our study demonstrated that triclosan caused a significant dose-related decrease in blood levels of cholesterol, with the decreases being significant at all dose levels. However, the mechanism underlying the decrease in blood levels of cholesterol by triclosan is not clear.

There is no evidence of genotoxic or mutagenic activity of triclosan in a battery of *in vitro* and *in vivo* studies, including micronucleus test in Chinese hamster bone marrow.[1,3,21] Our 13-week dermal study confirmed the lack of micronucleus induction. Peripheral blood micronucleus analyses did indicate a significant increase in the percentage of RETs in females at the two highest doses and in males at the highest dose. The increase was accompanied with a decrease in red blood cells and hemoglobin concentration (**Table 1S, Supplementary Data**); however, the decrease was minimal and probably biologically not significant.

In conclusion, dermal application of triclosan for 13-week resulted in dermal fibrosis and inflammation; epidermal hyperplasia, inflammation, necrosis, and ulceration, and parakeratosis. The incidence of epidermal hyperplasia was significantly elevated in both sexes of mice at 12.5 mg triclosan/kg bw/day; the incidence of parakeratosis was also significantly elevated in female

mice administered this dose. BMD modeling gave BMDL values of 1.8-2.5 mg triclosan/kg bw/day for parakeratosis in female mice, 2.3-5.7 mg triclosan/kg bw/day for epidermal hyperplasia in female mice, 3.0-6.3 mg triclosan/kg bw/day for epidermal hyperplasia in male mice.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

FIGURE LEGENDS

Figure 1. Representative histopathological changes at the site of application in the skin of B6C3F1 mice treated with 0, 5.8, 12.5, 27, 58, or 125 mg triclosan/kg bw/day for 13 weeks. The arrows indicate the histological changes observed including (A) epidermal inflammation, necrosis, and ulceration; (B) epidermal hyperplasia; (C) dermal inflammation and fibrosis; and (D) parakeratosis.

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TABLES

Table 1. Body weights of B6C3F1 mice in the 13-week dermal study of triclosan

Sex	*Dose (mg triclosan/kg bw/day)*	*Mean bw[a] (g)*			*Final weight relative to controls (%)*
		Initial	*Final*	*Change*	
Female mice	0	18.8 ± 0.2	28.7 ± 0.6	9.9 ± 0.6	-
	5.8	19.3 ± 0.3	31.3 ± 1.0	12.0 ± 0.8	109
	12.5	19.5 ± 0.3	29.9 ± 0.6	10.4 ± 0.4	104
	27	18.7 ± 0.3	27.7 ± 0.4	9.0 ± 0.2	97
	58	19.2 ± 0.3	27.9 ± 0.8	8.7 ± 0.7	97
	125	18.9 ± 0.3	26.9 ± 0.6	8.0 ± 0.4	94*
Male mice	0	23.6 ± 0.4	36.3 ± 1.1	12.7 ± 1.0	-
	5.8	24.5 ± 0.3	37.1 ± 0.7	12.6 ± 0.4	102
	12.5	24.3 ± 0.6	37.2 ± 1.4	13.0 ± 1.0	103
	27	23.8 ± 0.6	34.4 ± 1.4	10.6 ± 1.0	95
	58	23.8 ± 0.4	33.1 ± 1.1	9.3 ± 0.9	91*
	125	23.5 ± 0.5	29.9 ± 0.5	6.4 ± 0.4	82***

[a]Body weigts and body weight changes are presented as the mean ± standard error.

[*]Significant pairwise comparison of the dose group to the control group using Dunnett's method of adjusted contrasts (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

Table 2. Organ weights of B6C3F1 mice dermally administered triclosan for 13 weeks[a]

	Dose (mg triclosan/kg bw/day)											
	0		***5.8***		***12.5***		***27***		***58***		***125***	
Organ	***Mean (g)***	***SE***	***Mean (g)***	***SE***	***Mean (g)***	***SE***	***Mean (g)***	***SE***	***Mean (g)***	***SE***	***Mean (g)***	***SE***
						Female mice						
Kidney paired	0.381***	0.008	0.402	0.012	0.383	0.011	0.394	0.011	0.389	0.008	0.429***	0.012
Liver	1.365***	0.024	1.453	0.043	1.428	0.028	1.460	0.040	1.500*	0.053	1.878***	0.066
Ovary paired	0.016**	0.001	0.016	0.000	0.016	0.001	0.015	0.001	0.016	0.001	0.013*	0.001
						Male mice						
Kidney paired	0.637**	0.014	0.634	0.012	0.624	0.018	0.593	0.018	0.563	0.008	0.523*	0.017
Liver	1.716***	0.058	1.713	0.053	1.757	0.089	1.672	0.071	1.627	0.063	1.893***	0.072

[a]Each group consisted of 10 mice/sex. An * in the control (0 mg triclosan/kg bw/day) column indicates a significant dose-response trend; an * in the dose column indicates a significant pairwise comparison of the dose group to the control group using Dunnett's method of adjusted contrasts (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

Table 3. Frequency of RETs, MN-RETs, and MN-NCEs in peripheral blood of B6C3F1 mice dermally administered triclosan for 13 weeks[a]

	Dose (mg triclosan/kg bw/day)											
	0		***5.8***		***12.5***		***27***		***58***		***125***	
	Mean	***SE***	***Mean***	***SE***	***Mean***	***SE***	***Mean***	***SE***	***Mean***	***SE***	***Mean***	***SE***
Female mice												
MN-NCEs (%)	0.096**	0.003	0.113	0.007	0.109	0.004	0.104	0.003	0.112	0.005	0.129**	0.011
MN-RETs (%)	0.209	0.013	0.187	0.011	0.190	0.008	0.194	0.010	0.179	0.008	0.188	0.010
RETs (%)	1.177***	0.101	1.249	0.062	1.414	0.083	1.654	0.148	1.929*	0.168	3.562***	0.455
Male mice												
MN-NCEs (%)	0.141*	0.005	0.141	0.006	0.149	0.011	0.131	0.003	0.135	0.003	0.129	0.006
MN-RETs (%)	0.230***	0.014	0.234	0.010	0.264	0.019	0.227	0.006	0.225	0.009	0.185*	0.014
RETs (%)	1.450***	0.094	1.400	0.042	1.423	0.031	1.547	0.052	1.638	0.077	2.575***	0.182

[a]Each group consisted of 10 mice/sex. Measurements of RETs, MN-RETs, and MN-NCEs were made in peripheral blood samples at the terminal sacrifice. The frequency of RETs was expressed as the percentage of RETs (CD71-positive cells) among all cells (CD71-positive cells and CD71-positive cells); the frequency of MN-RETs was expressed as the percentage of MN-RETs among all RETs (CD71-positive cells); the frequency of MN-NCEs was expressed as the percentage of MN-NCEs in the total NCEs (CD71-negative cells). An * in the control (0 mg triclosan/kg bw/day) column indicates a significant dose-response trend; an * in the dose column indicates a significant pairwise comparison of the dose group to the control group using Dunnett's method of adjusted contrasts (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

Table 4. Incidence and severity of non-neoplastic lesions in B6C3F1 mice in the 13-week dermal study of triclosan[a]

Lesion	*Dose (mg triclosan/kg bw/day)*					
	0	***5.8***	***12.5***	***27***	***58***	***125***
			Female mice			
Liver						
Centrilobular, hypertrophy	0/10				0/10	1/10 (1.0)
Bone marrow						
Hyperplasia, lymphoid	0/10					10/10*** (4.0)
Lymph node – Mandibular						
Hyperplasia, lymphoid	2/10 (1.5)					8/10** (2.9)
Spleen						
Hematopoietic cell proliferation	0/10	0/1			1/1 (4.0)	10/10*** (3.9)
Hyperplasia, lymphoid	1/10 (2.0)	1/1 (3.0)			1/1 (2.0)	2/10 (2.0)
Skin, site of application						
Fibrosis, dermis	0/10	0/10	0/10	4/10* (1.3)	8/10*** (1.6)	10/10*** (2.7)
Inflammation, dermis	1/10 (1.0)	0/10	3/10 (1.0)	7/10** (1.3)	9/10*** (1.7)	10/10*** (2.9)
Hyperplasia, epidermis	0/10	0/10	7/10*** (1.0)	10/10*** (1.4)	10/10*** (1.7)	10/10*** (3.0)
Inflammation, epidermis	1/10 (1.0)	1/10 (1.0)	2/10 (1.0)	6/10* (1.2)	9/10*** (1.9)	10/10*** (4.0)
Necrosis, epidermis	1/10 (1.0)	0/10	1/10 (1.0)	4/10 (1.3)	6/10* (2.3)	10/10*** (4.0)
Ulcer, epidermis	1/10 (1.0)	0/10	0/10	0/10	3/10 (2.7)	10/10*** (3.1)
Parakeratosis	0/10	0/10	5/10** (1.0)	6/10** (1.0)	7/10*** (1.7)	10/10*** (3.5)
Kidney						
Infiltration cellular	0/10				0/10	9/10*** (2.1)
			Male mice			
Liver						
Centrilobular, hypertrophy	0/10				1/10 (1.0)	9/10*** (1.2)
Bone Marrow						
Hyperplasia, myeloid cell	0/10					10/10*** (4.0)
Lymph node – Mandibular						
Hyperplasia, lymphoid	0/10					7/10*** (2.4)
Spleen						
Hematopoietic cell proliferation	0/10				1/1 (3.0)	10/10*** (3.8)
Hyperplasia, lymphoid	0/10				1/1 (2.0)	4/10* (2.0)
Skin, site of application						
Fibrosis, dermis	0/10	0/10	0/10	1/10 (1.0)	7/10*** (2.0)	10/10*** (2.6)
Inflammation, dermis	0/10	0/10	0/10	2/10 (1.0)	6/10** (1.8)	10/10** (2.9)
Hyperplasia, epidermis	0/10	0/10	4/10* (1.0)	7/10*** (1.3)	10/10** (1.7)	10/10** (2.9)
Inflammation, epidermis	0/10	0/10	0/10	1/10 (2.0)	6/10** (2.8)	10/10** (3.9)
Necrosis, epidermis	0/10	0/10	1/10 (1.0)	3/10 (1.3)	6/10** (2.8)	10/10** (3.9)
Ulcer, epidermis	0/10	0/10	0/10	0/10	3/10 (1.3)	10/10** (3.3)
Parakeratosis	0/10	0/10	0/10	6/10** (1.2)	7/10*** (1.9)	10/10** (2.9)

[a]Each group consisted of 10 mice/sex. The incidence is reported as the number of animals with lesions observed microscopically. The average severity is given in parentheses. The severity was scored as 1 = minimal, 2 = mild, 3 = moderate, and 4 = marked. An * in the dose column indicates a significant pairwise comparison of the dose group to the control (0 mg triclosan/kg bw/day) group (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).