



The Soap and Detergent Association

May 5, 2009

Cynthia Oshita
Office of Environmental Health Hazard Assessment
Proposition 65 Implementation
P.O. Box 4010
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Submitted by e-mail: coshita@oehha.ca.gov

RE: Prioritization: Chemicals for Consultation by the Carcinogen Identification Committee

Dear Ms. Oshita:

The Soap and Detergent Association (SDA) appreciates this opportunity to provide input into the prioritization of chemicals for consultation by the Carcinogen Identification Committee (CIC), as announced by the California Environmental Protection Agency's Office of Environmental Health Hazards Assessment (OEHHA) on March 5, 2009. SDA is a one-hundred plus member trade association representing the \$30 billion U.S. cleaning products market. SDA members include the formulators of soaps, detergents, and general cleaning products used in household, commercial, industrial and institutional settings; companies that supply ingredients and finished packaging for these products; and oleochemical producers.

As reviewed by USEPA, triclosan should not be considered a human carcinogen for the following reasons: 1) positive results in mice but not rats or hamster; 2) the absence of any mutagenic or genotoxic effects; 3) difference in pharmacokinetics in mice compared to hamsters and humans; and 4) clear evidence that triclosan produced liver tumors in mice by a mode of action that is not relevant to humans (USEPA 2008). Therefore, OEHHA should rank triclosan as no priority for further consideration as a human carcinogen.

SDA has attached a detailed review of the scientific studies that are relevant to OEHHA's deliberations on this substance. Please let us know if you have any questions or wish to discuss this matter.

Sincerely,

A handwritten signature in black ink that reads "Richard Sedlak". The signature is written in a cursive, flowing style.

Richard Sedlak
Senior Vice President, Technical & International Affairs

Enclosure: Triclosan: Comments on Carcinogenicity Studies and Other Relevant Data.

cc: Gene Livingston, Greenburg Traurig

TRICLOSAN

Comments on Carcinogenicity Studies and Other Relevant Data

Submitted to:

California Environmental Protection Agency
Office of Environmental Health Hazard Assessment
Carcinogen Identification Committee

Submitted by:

The Soap and Detergent Association

May 5, 2009

BACKGROUND

Triclosan (2,4,4'-trichloro-2'-hydroxy-diphenyl ether) is an antibacterial ingredient also known as Irgasan DP300, FAT 80'023, CH 3565, GP41-353, and Irgacare® MP (the pharmaceutical grade of triclosan that is >99% pure). Triclosan has been used in consumer products since 1968 and in dental products since the 1980s in Europe and the mid-1990s in the United States (US) following approval by the US Food and Drug Administration (FDA) (FDA 1974; FDA 2006). The general population can and has come in contact with triclosan when using a variety of these consumer and dental care products.

On Thursday, March 5th 2009, the California Environmental Protection Agency's Office of Environmental Health Hazard Assessment (OEHHA) released a Prioritization Notice where it proposed a series of chemicals, including triclosan, for consideration and consultation by the Carcinogen Identification Committee (CIC) under Proposition 65. A preliminary toxicological assessment was conducted by OEHHA of the relevant studies for triclosan was based primarily on an evaluation of the carcinogenicity of triclosan conducted by the United States Environmental Protection Agency (USEPA 2008).

The USEPA (2008) Cancer Assessment Review Committee (CARC) concluded after a review of the relevant data that,

“In accordance with the EPA Final Guidelines for Carcinogen Risk Assessment (March 19, 2005), the CARC classified Triclosan as Not likely to be Carcinogenic to Humans”.

This classification is the most favorable category among those considered by USEPA (2005) in their guidelines for developing a narrative as to potential carcinogenicity in humans when all of the data are considered.

We concur with the USEPA's conclusion and are submitting these comments in support of that conclusion. USEPA's review of the triclosan data concluded that triclosan should not be considered a human carcinogen for the following reasons: 1) positive results in mice but not rats or hamster; 2) the absence of any mutagenic or genotoxic effects; 3) difference in pharmacokinetics in mice compared to hamsters and humans; and 4) clear evidence that triclosan produced liver tumors in mice by a mode of action that is not relevant to humans (USEPA 2008). Therefore, OEHHA should not include triclosan in their prioritization process as a chemical classified as a human carcinogen.

This submission provides comments and additional details on the studies identified by OEHHA used in the preliminary toxicological evaluation for triclosan as part of the listing process. Our comments on the triclosan data relied primarily on reviews by the USEPA (2008) and Rodricks et al. (2009). The organization of these comments is consistent with the recommended approach to human health cancer assessment, as outlined in USEPA guidelines (2005).

CARCINOGENICITY DATA

Epidemiological Data

No typical epidemiological studies, e.g., long-term exposure and follow-up, of potential chronic or carcinogenic effects have been conducted either in workers, both those producing triclosan and those manufacturing consumer products containing triclosan, or the general population who use triclosan-containing products.

Other studies have been conducted to specifically evaluate human safety and tolerability of triclosan in oral care products (DeSalva et al. 1989; Lucker et al. 1990; Safford 1991; Barnes 1991a; Barnes 1991b; Fishman 1993) and noted that:

- No overt signs of toxicity were observed in more than 2500 subjects following the daily use of toothpaste containing 0.01% to 0.6% triclosan for periods ranging from 12 weeks up to 4 years; and,
- No treatment-related changes in biochemical or hematological parameters or changes in indices of liver or kidney function were observed.

While these studies are informative, i.e., changes in biochemical parameters that could be indicative of early signs of liver or kidney damage were absent, these studies were not adequate to assess, even qualitatively, chronic, longer term effects, including cancer. Therefore, with regard to epidemiological data, there are not epidemiological studies involving triclosan that are appropriate for application of OEHHA's Epidemiology Data Screens.

Carcinogenicity Studies in Animals

Chronic bioassays in mice, rats, and hamsters treated with triclosan in the diet for 18, 24 or 21 months, respectively, were conducted to evaluate the chronic toxicity and carcinogenic potential of triclosan (USEPA 2008; Rodricks et al. 2009), all of which met GLP and/or OEDC

guidelines (Rodricks et al. 2009). The only significant effect noted was the increased incidence of liver tumors in mice, but not in rats or hamsters. The major findings related to potential for carcinogenicity noted in these studies are presented below.

Studies in Mice

A study has been conducted in male and female CD-1 mice to evaluate the effects of chronic exposure to triclosan (Auletta 1995), which was reviewed in detail in USEPA (2008) and Rodricks et al. (2009). Groups of mice were given triclosan in the diet at concentrations that resulted in doses of 0, 10, 30, 100, or 200 mg/kg/day for 18 months (50 animals per sex per dose group). The major findings were changes indicative of adverse liver effects, the only target organ identified to be affected, and included:

Increases in absolute liver weights and liver weights relative to brain and body weights in male and female mice (30 mg/kg/day group and higher);

Alterations in clinical chemistry parameters associated with liver function (increased ALT and ALKP levels and decreased serum cholesterol levels) at 30 (females only), 100 and 200 mg/kg/day (both sexes);

Increased incidence of enlarged livers and an increase in hepatic nodules/masses and/or discolorations in males in all treatment groups and in females treated with 100 or 200 mg/kg/day;

Minimal to moderately severe hepatocellular hypertrophy that increased in both incidence and severity with dose in males in all treatment groups and in females in the 30, 100, and 200 mg/kg/day treatment groups;

Increase in incidence of a brown pigment, identified as lipofuscin, in the two highest treatment groups in both sexes; and,

Increase in the incidence of benign and malignant hepatocellular neoplasms (adenomas and/or carcinomas) in males and females in the 30 mg/kg/day group and at higher doses.

According to USEPA (2008) and Rodricks et al. (2009), triclosan administration resulted in an increase in hepatocellular carcinomas and adenomas in both male and female mice. As discussed in the following sections, the USEPA (2008) determined that the liver tumors in mice were produced by a rodent-specific mode of action (PPAR α -mediated process) that is not relevant to or predictive of human health outcomes.

Studies in Rats

Male and female Sprague-Dawley rats were fed triclosan in the diet at concentrations of 0, 300, 1,000, or 3,000 ppm for 13, 26, 52, 78 and 104 weeks (Yau and Green 1986), which was reviewed in detail in USEPA (2008) and Rodricks et al. (2009). The 3,000 ppm exposure level was considered by the authors to be the maximum tolerated dose (MTD). An additional group of rats was fed 6,000 ppm (approximately 300 mg/kg/day) triclosan in the diet for 52 weeks. This was considered to be a toxic dose. No treatment-related, biologically significant changes that were consistent with time and dose were found. Observations included:

There were slight and transient differences between treated groups and controls were noted in some clinical chemistry parameters indicative of liver effects; however, these changes were not consistent with either time of examination or dose.

Average absolute and relative liver weights of all treatment groups at 13, 26, 78, and 104 weeks were comparable to control means.

There were no treatment-related gross or macroscopic lesions in any rats from any treatment group throughout the study.

According to USEPA (2008) and Rodricks et al. (2009), there was no evidence of tumors or pre-neoplastic lesions in rats treated with triclosan up to 300 mg/kg/day in the diet for 52 weeks or 150 mg/kg/day for 104 weeks.

Studies in Hamsters

Three groups of 60 male and 60 female Bio F1D Alexander Syrian hamsters were treated with triclosan at doses of 12.5, 75 or 250 mg/kg/day for 90 weeks (females) or 95 weeks (males) (Chambers 1999), which was reviewed in detail in USEPA (2008) and Rodricks et al. (2009).

The results included:

No changes in hematological and clinical chemistry parameters were considered to be treatment-related.

Treatment-related, non-neoplastic effects were noted in the kidney, testes and stomach.

No effects on the liver were seen in either the macroscopic or microscopic examinations.

According to USEPA (2008) and Rodricks et al. (2009), there was no evidence of carcinogenicity of triclosan seen at the doses given in this study.

Conclusions Based on the Carcinogenicity Studies

Triclosan produced an increase in liver tumors in male and female mice given triclosan in the diet for 18 months (Auletta 1995) but not in rats (Yau and Green 1986) or hamsters (Chambers 1999) in either males or females given triclosan in the diet at comparable doses for 24 and 21 months, respectively.

SUPPORTING STUDIES

Subacute, Subchronic and Chronic Studies

As detailed in USEPA (2008) and Rodricks et al. (2009), triclosan has been tested in repeated dose studies (30 days or less) by the:

oral route in mice (Thevenaz 1987; Molitor et al. 1992), rats (Molitor and Persohn 1993), baboons (Noel et al. 1969), or hamsters (Thomas 1994);

dermal route in mice (Burns 1996; Burns 1997a) or rats (Burns 1997b); or, inhalation route in rats (Leutkemeier et al. 1974).

Triclosan has also been tested in subchronic (approximately 45 or 90 days) and chronic studies by the:

oral route in mice (Trutter 1993; Auletta 1995), rats (Goldsmith and Craig 1983; Yau and Green 1986), hamsters (Schmid et al. 1994; Chambers 1999), rabbits (Paterson 1969; Leuschner et al. 1970a), dogs (Paterson 1967; Leuschner et al. 1970b), and baboons (Noel et al. 1969; Drake 1975); or,

dermal route in rats (Trimmer 1994), dogs (Dorner 1973) or Rhesus monkeys (Dalgard 1979; Parkes 1979).

Adverse effects that could be related to hepatic tumor formation were seen in the liver of some species tested included:

Changes indicative of adaptive responses (changes in liver weight or liver enzymes or increases in hypertrophy without accompanying alterations in histopathology), as seen in mice at doses greater than 10 mg/kg/day (Thevenaz 1987; Molitor et al. 1992; Auletta 1995; Burns 1997a), rats at doses greater than 300 mg/kg/day (Goldsmith and Craig 1983; Yau and Green 1986; Trimmer 1994), and hamsters at doses greater than 350 mg/kg/day (Schmid et al. 1994; Thomas 1994).

Changes, such as hepatocellular hypertrophy, increases in peroxisome number and size, and increases in smooth endoplasmic reticulum (SER), that were associated with the

neoplastic effects in the liver of mice (Thevenaz 1987; Molitor et al. 1992; Trutter 1993; Auletta 1995).

Mutagenicity and Genotoxicity Studies

A battery of 24 *in vitro* and *in vivo* mutagenicity and genotoxicity tests designed to evaluate the full range of potential to produce mutagenic or genotoxic effects in prokaryotic and eukaryotic systems indicated that neither triclosan nor its metabolites was mutagenic or genotoxic. (USEPA 2008; Rodricks et al. 2009). The following briefly describes the key findings.

No indication of a mutagenic response was found in any non-mammalian gene mutation assays in *Salmonella typhimurium* strains TA1535, TA1537, TA98 or TA100 tested both with and without increasing concentrations of S9 (Jones and Wilson 1988), or with increasing concentrations of triclosan (up to 5000 µg/plate without S9 and 5.0 µg/plate with S9) (Arni and Muller 1978a; Stankowski 1993). A weak mutagenic response was seen in *Saccharomyces cerevisiae* at 200 µg/mL (Fahrig 1978a), which was not repeated in a similar study (Arni and Muller 1978b).

No increase in mutant frequency at the thymidine kinase (*tk*) locus in mouse lymphoma L5178Y cells with and without metabolic activation was seen at concentrations that did not produce cytotoxicity (Strasser and Muller 1978; Henderson et al. 1988a).

No increases in chromosomal aberrations in Chinese hamster ovary (CHO) cells, with and without S9 metabolic activation at concentrations up to 1 µg/ml (without S9) or 30 µg/ml (with S9) (Brooker et al. 1988). In contrast, concentration-related increases in chromosomal aberrations were noted in Chinese hamster V79 cells exposed to the highest concentration of triclosan tested (3.0 µg/mL), which was near levels causing cytotoxicity (Heidemann 1990). These authors stated that results of genotoxicity studies with V79 cells “must be interpreted with caution as a result of the disruption of normal DNA damage response pathway.”

Triclosan did not induce chromosome aberrations at any dose tested in any of following *in vivo* studies: peripheral bone marrow cells of hamsters following repeated doses of up to 600 mg/kg (Strasser and Muller 1973; Strasser and Muller 1979), rats following a single dose of 4000 mg/kg (Volkner 1991), and in mouse spermatocytes following repeated doses up to 1512 mg/kg (Hool et al. 1978; Hool et al. 1979).

Clastogenic effects were not seen in *in vivo* micronucleus assays using erythrocytes from hamsters following repeated doses of up to 600 mg/kg (Langauer and Muller 1974; Langauer and Muller 1978; Henderson et al. 1988b) or mice following a single dose of 5000 mg/kg (Langauer and Muller 1974; Langauer and Muller 1978; Henderson et al. 1988b).

Triclosan did not induce DNA damage by measuring unscheduled DNA synthesis (UDS) in primary hepatocytes from male F344 rats at concentrations up to 10 µg/L (Riach 1988; SanSebastian 1993).

No evidence of dominant lethality was reported in female mice mated to triclosan-treated males compared to control males (Fritz 1971).

Fahrig (1978b) reported a significant increase in recessive spots in the offspring of females treated with doses of triclosan of up to 50 mg/kg. In contrast, Russell and Montgomery (1980) reported no increase in the frequency of recessive spots in the offspring of females treated with doses of triclosan up to 25 mg/kg (dissolved in 60% methanol). Russell and Montgomery (1980) suggested that the triclosan dose reported in the Fahrig (1978b) study would result in maternal toxicity that precluded evaluation in this assay.

In summary, of these 24 experiments, only three yielded weakly positive responses for the endpoints evaluated (Fahrig 1978a; Fahrig 1978b; Heidemann 1990). Two of these assays used in vitro systems (Fahrig 1978a; Heidemann 1990), while the third was an in vivo test (Fahrig 1978b). As described above, the few weakly positive results are not consistent with respect to type of genetic alterations observed nor have the observations been duplicated in the same or equivalent assays. Accordingly, the overall weight-of-evidence from these experiments indicates that triclosan is not mutagenic or genotoxic.

Pharmacokinetic Studies

The pharmacokinetics of triclosan have been evaluated in animals and humans exposed by the oral and dermal routes (Stierlin 1972a; Stierlin 1972b; Parkes 1978a; Parkes 1978b; Kanetoshi et al. 1988; Lin 1988; Lucker et al. 1990; Van Dijk 1994; Van Dijk 1995; Van Dijk 1996; Habucky 1997a; Chasseaud et al. 1999; Lin 2000; Moss et al. 2000). The pharmacokinetic studies in humans and in multiple animal species (rats, mice, hamsters, dogs, and monkeys) following both single and repeated oral and dermal exposures to triclosan have been reviewed in detail in USEPA (2008) and Rodricks et al. (2009). As reported in Rodricks et al. (2009), there are distinctive differences in the metabolism and distribution across species to include:

Triclosan glucuronide conjugates (parent and/or non-parent) are the major metabolites identified in the hamster, monkey and the human, while the majority of the triclosan detected is in the form of the parent sulfate conjugate in the mouse and the dog.

Similar distribution patterns were noted in the hamster and the rat, with no evidence of accumulation in tissues; in the mouse there was evidence of accumulation in the liver observed.

In the rat and the mouse, enterohepatic recirculation and biliary excretion play a much larger role in excretion than in the hamster or the human. In humans, glucuronidation of the parent and excretion in the urine appears to be the preferred clearance pathway for triclosan, resulting in limited free parent remaining for delivery to target organs.

Biochemical and Mechanistic Studies

Studies have been conducted in mice (Molitor et al. 1992; Eldridge 1993), rats (Molitor and Persohn 1993; Persohn and Molitor 1993) and hamsters (Persohn 1994; Thomas 1994). Similar protocols were used in the cell proliferation studies and in the studies of changes in liver morphology and biochemical parameters. These studies were reviewed in detail in Rodricks et al. (2009). These studies found the following.

Hepatocyte cell proliferation, as measured by increases in proliferating cell nuclear antigen (PCNA), was significantly increased in a dose-related manner in both male and female mice after 45 days and 90 days of treatment at the two highest dose groups (350 and 900 mg/kg/day) and in males after treatment with 200 mg/kg/day at both time points (Eldridge 1993). No increases in hepatic cell proliferation were seen in rats after either 45 or 90 days of treatment at doses as high 300 mg/kg/day (Persohn and Molitor 1993) or in hamsters treated at doses up to 900 mg/kg/day for the same period of time (Persohn 1994).

Triclosan produced significant, dose-dependent increases in a number of biochemical parameters in mice after 14 days of treatment and a 28-day recovery period (at doses up to 950 mg/kg/day) (Molitor et al. 1992) but not in rats (at doses up to 518 mg/kg/day) (Molitor and Persohn 1993) or hamsters (at doses up to 799 mg/kg/day) (Thomas 1994). These changes included:

Significant increases in cyanide-insensitive peroxisomal fatty acid β -oxidation (palmitoyl CoA oxidation) were seen in mice but not in rats or hamsters.

Significantly increased lauric acid 11-hydroxylation was seen in mice only, while 12-hydroxylation activity was significantly increased in mice at doses of 50 mg/kg/day or higher and in the highest dose groups in rats and hamsters.

In mice, CYP3A and CYP4A were elevated in all dose groups but no change over baseline was noted in CYP1A activity, while in rats CYP1A and CYP2B were strongly elevated (4.2 and 24.7-fold increases, respectively) but only marginal changes were seen in CYP3A and CYP4A and effectively no changes in CYP1A, CYP3A, or CYP4A were seen in hamsters.

Dose-related increases in smooth endoplasmic reticulum (SER), reductions in rough endoplasmic reticulum (RER) and an increase in both the number and size of peroxisomes were observed in mice. In rats, only a moderate increase in (SER) was seen in the high dose group and no changes in these parameters were seen in hamsters.

Structure Activity Considerations

USEPA (2008) classified triclosan as a member of the diphenyl ether class of chemicals and stated that no appropriate analogs were available for comparison. However, in the preliminary review of data conducted by OEHHA, the document mentions two substances, namely polychlorinated biphenyls (PCBs) and decabrominated diphenyl ether (DBDE), as structural analogs. The text of the OEHHA memo also mentions studies of di(ethylhexyl)phthalate (DEHP) as a mechanistic “analog” in the context of PPAR α -mediated hepatocarcinogenicity. We concur with USEPA’s assessment that there is no appropriate structural analog, as discussed in the following paragraphs.

Polychlorinated biphenyls (PCBs)

Polychlorinated biphenyl (PCBs) congeners belong to a family of compounds some of which are considered to be rodent liver carcinogens. The PCB congeners that bind to the Ah receptor, one of a family of nuclear receptors, are those whose configuration, because of the placement and orientation of chloride atoms, holds the molecule in a planar structure that binds to the Ah receptor. Compounds of this family are not an appropriate structural analog from which to infer the potential carcinogenicity of triclosan for the following reasons.

The orientation in space that provides the best fit to the Ah receptor is facilitated by the carbon-carbon bond on the two phenyl rings of PCB congeners when the chlorides are in the appropriate position. Triclosan has an oxygen between the two phenyl rings, i.e., its classification as an ether, which allows greater flexibility of the molecule and would make binding to the Ah receptor less likely.

The most efficient structure for receptor binding for PCBs congeners is to have chlorines in the 2,3,7,8 positions on the biphenyl rings. Congeners with fewer than four chlorines attached to the biphenyl rings or have more than four chlorines but not attached at the 2,3,7,8 positions are not considered rodent carcinogens and are not assigned a toxicity equivalent value when conducting a cancer risk assessment. Triclosan has only three chlorines attached and not in the standard configuration that is considered to be required

to be a possible rodent carcinogens through activation of the Ah receptor. Consequently, PCBs would not be considered an appropriate surrogate based on structure activity.

Additional discussion of the differences between PCBs and other Phenobarbital-acting compounds and triclosan is provided in the section on mode of action.

Decabrominated diphenyl ether (DBDE)

The decabrominated diphenyl ether (DBDE) shares the phenyl ring-oxygen-phenyl ring structure, i.e., a diphenyl ether structure, with triclosan. Its structure, however, contains ten bromines, which are larger and bulkier than the three chlorines in the triclosan molecule and, therefore, likely to have different binding characteristics. DBDE has been tested in two-year bioassays in rats and mice (NTP 1986) and the following should be considered.

According to the NTP, there was “some evidence of carcinogenicity” in male and female Fisher 344 (F344) rats based on the increased incidence of neoplastic nodules but not carcinomas in both dose groups (approximately 3,120 and 6,500 mg/kg/day in the diet). This study was published in 1986 but the actual bioassay conducted earlier.

At that time, the classification “neoplastic nodules” was broadly applied to both hyperplasia and to adenomas (Maronpot et al. 1986). In 1986, a pathology working group re-evaluated and redefined the diagnostic criteria for hepatoproliferative lesion of F344 rats. Hepatocellular hyperplasia was defined as proliferative lesions that are secondary, nonneoplastic responses to degenerative changes in the liver. Foci of cellular alteration, hepatocellular adenomas, and hepatocellular carcinomas are thought to comprise the progression to neoplasia. It is not possible with the data as presented in the NTP bioassay to discern with confidence that DBDE increased hepatic tumors (adenomas and carcinomas) rather than hepatocellular hyperplasia, and therefore, these data can not be extrapolated to be predictive of triclosan.

NTP also stated that there was “equivocal evidence of carcinogenicity” in male mice and “no evidence of carcinogenicity” in female mice. The evidence in male mice was the “increased” incidence of hepatocellular adenomas and carcinomas; however, as noted in Table 18, page 46 of the NTP report, the increased incidence was not statistically significant by pair-wise comparisons or by trend tests, except for the increase of combined adenomas and carcinomas in the low dose (3120 mg/kg/day) but not in the high dose group (6240 mg/kg/day). Decreased survival in the control group (34% v. 57% in the high dose group) confounds interpretation of these results.

Consideration of Other Peroxisome Proliferating Compounds

The preliminary review of data conducted by OEHHA cites the USEPA’s Cancer Assessment Document for Triclosan (USEPA 2008) as well as three other articles included under the heading of “mode of action studies on other peroxisome proliferators” (Ito et al. 2007; Yang

et al. 2007; Takashima et al. 2008). These three articles are discussed first, followed by key information presented in USEPA (2008), and additional insights from the review article prepared by Rodricks et al. (2009).

Ito et al. (2007) reported a significant dose-related trend in total liver tumors in PPAR α null male mice (Sv/129 strain) compared with wild type controls following exposure to 0.05% (500 ppm) DEHP in the diet for 22 months. These authors also reported increases in 8-hydroxydeoxyguanosine (8-OHdG) levels in PPAR α -null mice, when compared with wild type, as well as other changes in gene transcription and translation associated with oxidative stress responses. The authors concluded that these results “suggest the possibility of DEHP tumorigenesis via a non-PPAR α pathway.”

Takashima et al. (2008) attempted to extend the findings of Ito et al. (2007) by conducting microarray transcriptional profile analyses on a liver adenoma from a PPAR α null mouse and a liver adenoma from a wild type mouse from the Ito et al. (2007) study. The authors also conducted real-time quantitative PCR analyses on several liver adenomas from PPAR α -null and wild type mice from the Ito et al. (2007) study. The authors noted a variety of different changes in RNA transcription in both the PPAR α null and wild type mice exposed to 0 and 0.05% DEHP. However, they focused on an upregulation of genes associated with mitosis (including Cyclin B2 and Cdc25b) in PPAR α -null mice and an upregulation of genes associated with cell cycle arrest (including Myt1, p21/cip, and Gadd45a) in wild type mice, concluding that these cell cycle regulatory genes may be important in the development of liver tumors in the PPAR α -null mice.

The results of these two studies should not be over-interpreted as these data in the null mouse do not weaken the conclusion that DEHP and other PPAR α agonists, including triclosan, induce rodent liver tumors in wild-type mice through PPAR α -mediated mechanisms. Several points should be considered with respect to these two studies:

The results reported in studies by Ito et al. (2007) and Takishima et al. (2008) conflict with other carcinogenicity studies in wild type and PPAR α -null mice (Peters et al. 1997; Hays et al. 2005; Shah et al. 2007). Shah et al. (2007) and Peters et al. (1997) tested the PPAR α agonist, WY-14,643, while Hays et al. (2005) evaluated bezafibrate, another PPAR α agonist. In each of these studies, liver tumors were produced in the wild type mouse but not in the PPAR α -null mouse. Further, evidence of liver toxicity that was present in wild type mice treated with DEHP was not seen the PPAR α -null mouse (Ward et al. 1998)

Ito et al. (2007) indicated that their experimental design was based on a previous study (David et al. 1999); in which wild type B6C3F1 mice were exposed to DEHP. However, at the same dietary exposure levels, the tumor incidence rate in the wild type mice in the Ito et al. (2007) study were much lower than in the David et al. (1999) study (2/20 vs. 21/65 at the 0.05% exposure level). The reasons for this apparent difference in responsiveness is unclear, but it may be related to differences in the B6C3F1 strain used in the David et al. (1999) study compared with the Sv/129 strain used in the Ito et al. (2007) study. This differential response in the wild type strains used in these two studies introduces some uncertainty in the interpretation of the Ito et al. (2007) study.

As described above, Ito et al. (2007) note a “significant trend between control and 0.05% DEHP-treated group in PPAR α -null mice.” The authors reached this conclusion by comparing the tumor incidence in unexposed PPAR α -null mice (1 hepatocellular carcinoma) with that in the PPAR α -null mice exposed to 0.5% DEHP (6 hepatocellular adenomas, 1 hepatocellular carcinoma, and 1 cholangiocellular carcinoma). However, the guidelines established by McConnell et al. (1986) state that the incidence of hepatocellular and cholangiocellular carcinomas should not be combined. In addition, it is impossible to determine if the single hepatocellular carcinoma occurred in an animal that also had an adenoma. Pairwise Fisher’s exact tests on these same data did not show a statistically significant difference in tumor incidence between unexposed PPAR α -null mice (1/25) and PPAR α null mice exposed to 0.5% DEHP (either 6/31 or 7/31). More importantly, pairwise Fisher’s exact tests on the tumor incidence in the PPAR α wild type (2/20) and PPAR α -null mice (either 6/31 or 7/31) exposed to 0.05% DEHP revealed no statistically significant differences. Given this, it is difficult to determine if exposure of PPAR α -null mice to 0.5% DEHP is really associated with an increased tumor incidence or if the apparent increase is simply due to normal variability in response.

Other biological and physiological differences exist between wild type and PPAR α -null mice (Howroyd et al. 2004; Atherton et al. 2009). In PPAR α -null mice, a number of defects in lipid metabolism occur, and lipids accumulate in the liver. With aging, PPAR α -null mice exhibited a higher incidence, shorter latency, or increased severity of various non-neoplastic, spontaneous lesions. Further, in a comparison of unexposed PPAR α -null and wild type mice of the same strain, SV129, PPAR α -null mice had decreased longevity and an increase in carcinomas and multiple adenomas in the liver. It raises the question if the increase in adenomas seen in the liver of PPAR α -null mice in the Ito et al. (2007) compared to the incidence in the wild type is actually DEHP-related.

If one accepts the proposition that tumor incidence is increased in PPAR α -null mice when compared with wild type mice, it is possible that the absence of an active PPAR α gene may have given rise to an alternate pathway involving another PPAR isoform that would not be favored in the presence of active PPAR α . Significant cross-talk exists between different PPAR isoforms and other nuclear receptors (Shipley and Waxman 2004), and the activation of PPAR γ by the DEHP metabolite MEHP has been demonstrated (Hurst and Waxman 2003). Further, Rodricks et al (2009) discuss a likely role for PPAR γ in Kupffer cells to contribute to hepatocellular proliferation following exposure to PPAR α agonists.

The paper by Takashima et al. (2008) paper offers, at best, a simplistic snapshot of gene transcription in wild type and PPAR α null mice exposed to 0.05% DEHP. It is certainly not surprising that the transcriptional profiles differ between these groups. However, the authors only conducted microarray analyses on one wild type and one PPAR α null mouse and only at one time point (23 months). These severe limitations make it impossible to draw meaningful conclusions from this experiment.

Yang et al. (2007) used the same strain of Sv/129 PPAR α null mice described by Ito et al. (2007), but further genetically modified such that PPAR α -responsive genes were constitutively activated (in the absence of ligand binding) in hepatocytes, but not in non-parenchymal cells. Using this animal model, these authors demonstrated that while the hepatocyte-specific PPAR α activation resulted in many of the classic PPAR α -related pleiotropic responses (e.g., decreased serum fatty acids, induction of PPAR α target genes, peroxisome proliferation, and hepatocyte proliferation), these mice did not develop liver tumors and the expression of catalase, an enzyme normally associated with peroxisome proliferation, was inhibited.

This study also raises new questions about the detailed mechanisms of PPAR α -induced hepatocarcinogenesis; however it confirms the importance of non-parenchymal cells, such as Kupffer cells, to the carcinogenic process. As described above, Rodricks et al (2009) provide information about the significant role of Kupffer cells in mediating the hepatocellular responses to PPAR α agonists.

MODE OF ACTION IN MOUSE HEPATIC TUMORS AND RELEVANCE OF THESE TO HUMAN HEALTH

Evaluation of the mode of action (MOA) of triclosan in the production of liver tumors in mice has been reviewed by USEPA (2008) and Rodricks et al. (2009) using the Human Relevance Framework (HRF), as described by Cohen et al. (2003), Meek et al. (2003), Cohen (2004), IPCS (2005)), and Boobis et al. (2006). Application of this HRF has demonstrated the lack of human relevance of the animal MOA for some compounds that are rodent liver carcinogens. Holsapple et al. (2006) applied this framework to demonstrate that the MOA of phenobarbital (PB)-like P450 inducers was not relevant in humans, while Klaunig et al. (2003) applied this framework to demonstrate the lack of human relevance of peroxisomal proliferating (PPAR α) chemicals. Activation of the peroxisome proliferation (PPAR α) receptor is a well-

characterized, nongenotoxic MOA specific to the induction of rodent liver tumors (Bentley et al. 1993; Ashby et al. 1994; Cattley et al. 1998; Chevalier and Roberts 1998; Klaunig et al. 2003; USEPA 2003; USEPA 2005). The following sections summarize the data used by USEPA (2008) and Rodricks et al. (2009) in the context of the HRF used to conclude that the data support a MOA for triclosan as a PPAR-agonist, and, therefore, not relevant to human health outcomes.

Postulated Mode of Action for Triclosan

Klaunig et al. (2003) organized the various types of observations reported with PPAR α agonists into a logical framework for the key events representative of the biological cascade from receptor activation to tumor induction. These include: a well-characterized set of biochemical and cellular events has been identified in susceptible rodent strains that include the following: 1) PPAR α activation; 2) alteration in the transcription of genes involved in peroxisome proliferation, cell cycle/apoptosis, and lipid metabolism; 3) increases in fatty acid β -oxidation leading to oxidative stress; 4) stimulation of non-parenchymal cells (NPCs) and inhibition of gap junction intercellular communication, both of which could contribute to the induction of cell proliferation; and, 6) increased cell proliferation and decreased apoptosis leading to proliferation of DNA-damaged cells resulting in hyperplasia and hepatic tumors (Cattley et al. 1998; Klaunig et al. 2003; USEPA 2003; Cattley 2004). The data for triclosan were organized into those categories by USEPA (2008) and Rodricks et al. (2009) as presented in Table 1, and taken together in a weight-of-evidence context provide evidence that the MOA for triclosan in the induction of mouse liver tumors was through PPAR α activation.

Table 1. Triclosan Data Supportive of a MOA in the Induction of Mouse Liver Tumors

#	Event	Evidence	Key Reference
1	Activation of PPAR α	No direct data	
2a	PPAR α -dependent regulation of genes encoding for peroxisomal enzymes	<i>In vivo</i> increases in CYP3A and CYP4A, increased selected testosterone hydroxylation and lauric acid 11-12, hydroxylation	(Molitor et al. 1992)
2b	PPAR α -dependent expression of cell cycle growth and apoptosis	No direct data (not considered necessary data)	(USEPA 2008)
2c	PPAR α -dependent expression of nonperoxisomal fatty acid metabolism genes	Dose-related increases in cyanide-independent palmitoyl CoA oxidation	(Molitor et al. 1992)
3a	Peroxisome proliferation	Increases in liver weight resulting from hypertrophy due to increase in number and size of peroxisomes; increases in smooth endoplasmic reticulum	(Thevenaz 1987; Molitor et al. 1992; Trutter 1993; Auletta 1995)
3b	Perturbation of cell proliferation and/or apoptosis	Dose-dependent increases in PNCA labeling index following 45 and 90 days of treatment	(Eldridge 1993; Trutter 1993)
5	Hepatocyte oxidative stress	Dose-related increases in lipofuscin in region of Kupffer cells	(Trutter 1993)
6	Kupffer cell-mediated events	Kupffer-cell activation present	(Eldridge 1993; Trutter 1993)
7	Selective clonal expansion	Promoted hepatic adenomas and carcinomas in mice	(Auletta 1995)

Key Events

Table 1 presents the triclosan data in the key event paradigm for the PPAR α MOA. When evaluated in its entirety, the weight-of-evidence clearly shows that triclosan is a PPAR α agonist in the mouse with limited, if any, PPAR α activity in the rat and hamster. These studies provide evidence to characterize these key events and to demonstrate that triclosan has PPAR α agonist activity. These data include:

Hepatic cell proliferation (as indicated by the PCNA labeling data) was significantly increased in male and female mice in a dose-dependent manner (Eldridge 1993) but was not increased in male or female rats (Persohn and Molitor 1993) or hamsters (Persohn 1994) given comparable doses of triclosan for approximately the same durations.

Triclosan induced significant changes in biochemical parameters indicative of PPAR α activation in mice at doses as low as 50 mg/kg/day to include significant increases in fatty acid β -oxidation (Palmitoyl CoA oxidation), lauric acid 11-hydroxylase, lauric acid 12-hydroxylase, and CYP4A (Molitor et al. 1992). Increases in lauric acid 12-hydroxylase and CYP4A were also seen in rats and hamsters but only at the highest dose in rats (518 mg/kg/day) (Molitor and Persohn 1993) and two highest doses in hamsters (309.8 and 799 mg/kg/day) (Thomas 1994).

In the same studies, morphological changes indicative of PPAR α were seen in mice but not in rats and hamsters. In mice, electron microscopy revealed an increase in smooth endoplasmic reticulum, a reduction in rough endoplasmic reticulum, and an increase in both peroxisome numbers and size.

Strength and Consistency of the Data

Temporal and Dose-Response Concordance

With triclosan, clear patterns for both temporal and dose-response concordance were evident across the toxicity and mechanistic studies in mice. With regard to the dose-response, induction of liver tumors in mice did not occur at doses lower than those that produced effects that were considered to be precursor events in shorter-term studies.

Hepatic hypertrophy in male mice increased in incidence and severity with increasing dose.

Proliferation of the smooth endoplasmic reticulum membranes and peroxisomal number and size increased in intensity with increasing dose (Thevenaz 1987; Molitor et al. 1992).

Dose-dependent increases in liver weight (relative to body weight), microsomal protein, total cytochrome P-450 content, lauric acid 11-, 12-hydroxylation, peroxisomal fatty acid β -oxidation, and CYP4A activity occurred following 14 days of treatment (Molitor et al. 1992).

A significant increase in hypertrophy was seen at 10 mg/kg/day in males in the 78-week study (Auletta 1995); however, the incidence of hepatic tumors was not significantly increased at that dose but was increased at the next highest dose, 30 mg/kg/day.

Signs of hepatic hypertrophy were seen after only 14 days of treatment (Molitor et al. 1992).

The incidence and severity increased not only with increasing dose but also with increasing duration of exposure (Thevenaz 1987; Trutter 1993; Auletta 1995).

All of the key events tested, preceded the formation of hepatic tumors in male and female mice (Auletta 1995).

Differences in Species

Some PPAR α agonists have produced hepatic tumors in mice and rats, while others have produced tumors in only one species. Differences in response between rats and mice have been attributed to toxicokinetic differences, i.e., differences in metabolism to the active component or differences in elimination (Klaunig et al. 2003). Triclosan produced an increase in the incidence

of hepatic adenomas and carcinomas in mice but not in rats or hamsters. With triclosan, rats did show signs of PPAR α activation, e.g., significant increases in some parameters that reflect some of the key events, but the magnitude of the changes normalized by dose was lower than in the mouse. Differences in toxicokinetics provide the likely explanation for the lack of liver tumors in rats. While mice and rats exhibit similar absorption and metabolism patterns, distribution and elimination was quite different. Triclosan was reported to accumulate in the liver in mice (Van Dijk 1995), resulting in higher tissue concentrations in the liver than in plasma, which was not observed in rats (Van Dijk 1996). This differential distribution and retention results in a larger effective target tissue dose of triclosan in the mouse compared to the rat and is the likely explanation for the species differences in PPAR α receptor activity and subsequent tumor formation.

Concordance with Other PPAR α Agonists

The biochemical and mechanistic data for triclosan are consistent with the results of a number of studies for chemicals identified as PPAR α agonists (Klaunig et al. 2003). When compared to both data rich chemicals, such as DEHP, and to data poor chemicals, such as oxidiazone, triclosan has a sufficient data base illustrating that the results of biochemical, mechanistic, and toxicological studies provide clear evidence that triclosan is a PPAR α agonist.

Alternative Modes of Action

Consideration of a Mutagenic or Genotoxic Mode of Action

As detailed previously, the genotoxic potential of triclosan has been evaluated in a battery of 24 experiments using *in vitro* and *in vivo* assays designed to evaluate the full range of potential genotoxic mechanisms in prokaryotic and eukaryotic systems. The consistency of the negative findings from the comprehensive set of studies indicates that neither triclosan nor its metabolites are genotoxic. Accordingly, the overall weight-of-evidence from these experiments suggests that a genotoxic MOA is not responsible for triclosan-induced mouse liver tumors.

Consideration of a Cytotoxic Mode of Action

Chemically-induced cytotoxicity is another nongenotoxic MOA leading to the formation of tumors in rats and mice as a result of the production of continual hepatic cell death (necrosis)

resulting in restorative hyperplasia during which pre-initiated cells, i.e., existing mutations, would proliferate yielding pre-neoplastic foci leading to tumors due to further clonal expansion (Holsapple et al. 2006). Importantly, significant increases in necrosis would need to proceed both in time and in dose before the appearance of hepatic tumors (Holsapple et al. 2006). The key non-cancer findings in mice were induction of dose- and time-related increases in centrilobular hepatocellular hypertrophy, Kupffer cell activation and necrosis, as indicated by the following (Rodricks et al. 2009).

After 13 weeks of treatment, the incidence of hypertrophy and Kupffer cell activation was significantly increased at doses of 75 mg/kg/day and above, while necrosis, when present, was not increased at doses below 350 mg/kg/day and higher.

After 78 weeks, the incidence of hypertrophy was significantly increased at doses of 10 mg/kg/day and Kupffer cell activation was significantly elevated at doses of 30 mg/kg/day, the lowest dose at which liver tumors were seen in male mice. In contrast, the incidence of necrosis was significantly increased only in two highest dose groups, 100 and 200 mg/kg/day.

The incidence of necrosis did not show the same pattern as either the observed hypertrophy or Kupffer cell activation of increasing incidence with increasing exposure duration or an increase in severity with increasing dose.

According to the USEPA (2008), the cell proliferation data from the 45- and 90-day studies are not consistent with a cytotoxic mode of action.

Hepatocellular hypertrophy was consistent with responses to peroxisome proliferating compounds – increased induction of smooth endoplasmic reticulum membranes and an increase in the size and number of peroxisomes (Thevenaz 1987; Molitor et al. 1992; Trutter 1993) and increased relative and absolute liver weights, rather than non-PPAR α associated cytotoxicity. Kupffer cell activation has been associated with the proliferation of hepatocytes in response to PPAR α compounds (Klaunig et al. 2003; Rusyn et al. 2006). Kupffer cells were shown to be activated by treatment with PPAR α agonists *in vivo* (Bojes and Thurman 1996). According to Roberts et al. (2007),

“Overall, these data support a role for Kupffer cells in facilitating a response of hepatocytes to PP via a mechanism that remains to be determined but is ultimately dependent on the presence of PPAR α receptors”.

The data indicate that the response in Kupffer cells is a result of a peroxisomal proliferative MOA and not a cytotoxic MOA acting independently of PPAR α activation (Klaunig et al. 2003; Roberts et al. 2007).

Consideration of Other Receptor-Mediated Modes of Action

The effects of triclosan on microsomal ethoxresorufin *O*-demethylase (EROD) and pentoxyresorufin *O*-deethylase (PROD) activity were also evaluated in the same 14-day studies investigating the cellular and biochemical effects of triclosan in the mouse (Molitor et al. 1992), rat (Molitor and Persohn 1993), and hamster (Thomas 1994), as reviewed in Rodricks et al. (2009). Activation of CYP1A1/2 by PAHs, such as 3-methylcholanthene (3-MC), has been assumed to reflect interaction with the AhR receptor characteristic of dioxin-like compounds with EROD as the model enzyme assay. Activation of the CYP2B family has been assumed to reflect Phenobarbital-like or PCB-like activity and interaction with the CAR receptor with PROD as the model enzyme assay. Activation of Cyp4A is characteristic of induction by PPAR α agonists. A weight-of-evidence analysis concluded that activation by these pathways is not the mode of action in the production of liver tumors in mice based in part on the following (Rodricks et al. 2009).

Triclosan induced significant changes in Cyp4A in mice but only marginal changes in rats and hamsters and only at the highest dose tested.

A number of PPAR α agonists induce CYP2B1/2, as measured directly by P450 protein content, or indirectly by measuring PROD activity to include clofibrate (Shaban et al. 2005), ciprofibrate (Zangar et al. 1996), diclofop-methyl (Palut et al. 2002), nafenopin (Molitor et al. 1992) and cinnamyl anthranilate (Elcombe et al. 2002).

A number of PPAR α agonists induce CYP1A, as measured by EROD activity to include clofibrate (Lake et al. 1984) and nafenopin (Molitor et al. 1992).

Nafenopin, a classic PPAR α agonist, induced an increase in both EROD and PROD activity in mice (Molitor et al. 1992) and hamsters (Thomas 1994); however, liver tumors were only found in mice.

Phenobarbital-like compounds (Cyp1A/1/2 inducers) are ligands for the (CAR), also a member of a nuclear hormone receptor superfamily that, as do PPAR α compounds, form a heterodimer with RXR. Interaction or “cross-talk” between these receptors has been demonstrated (Zangar et al. 1995; Zangar et al. 1996; Shaban et al. 2004a; Shaban et al. 2004b; Shaban et al. 2005). The presence of mutual effects between AhR and PPAR α receptors, i.e., down-regulation of target genes of one of these receptors due to

stimulation by the other receptor has been demonstrated (Shaban et al. 2004a; Shaban et al. 2004b).

Consideration of an Endocrine Mode of Action

There is no evidence of any hormonally-mediated MOA associated with hepatic tumors in mice. Two recent short-term studies have suggested a role for triclosan in the alteration of thyroid hormone levels in female and male rats (Crofton et al. 2007; Zorrilla et al. 2009). Crofton et al. (2007) noted dose-dependent decreases in serum thyroxine (T4) in the three highest dose groups (100 mg/kg/day and higher for 4 days). Zorrilla et al. (2009) noted dose-dependent decreases in T4 at doses of 30 mg/kg/day and above following 28 days of treatment; however, thyroid stimulating hormone (TSH) levels were not affected at any dose and Triiodothyronine (T3) levels were only reduced in rats in the 200 mg/kg/day dose group. Further, these changes were not associated with androgen-dependent tissue weights or preputial separation. Further, thyroid histopathology has been evaluated in several of the subchronic and chronic rodent toxicity studies (Rodricks et al. 2009). No adverse effects on the thyroid have been noted in any species in which the thyroid was evaluated, in particular in the two-year bioassays in mice, rats, or the hamster. The lack of an effect on thyroid weights, gross pathology, or histopathology in these studies suggests that the reductions of serum T4 in the short-term studies by Crofton et al. (2007) and Zorrilla et al. (2009) are not associated with adverse effects following chronic exposure.

Although the disruption of thyroid hormone homeostasis has been associated with the development of thyroid tumors in rodents, humans are generally considered to be refractory to these effects (USEPA 1998). The differences in species sensitivity to these effects are likely due to the narrower homeostatic range in rodents compared with humans.

CONCLUSIONS

Triclosan has been tested in mice in subacute, subchronic and chronic toxicity studies. The results of these studies are consistent. The data in these studies provide clear and compelling evidence for key elements in the mode of action for PPAR α -induced tumors in mice and show dose-dependent changes that are concordant and consistent with this proposed mode of action. As noted above, triclosan induced gene expression of a PPAR α -specific target genes

resulting in the induction of CYP4A and the subsequent induction of lauric acid 11-,12-hydroxylase. Fatty acid β -oxidation, a characteristic of PPAR α activation was also significantly increased following treatment with triclosan. Peroxisomes were increased in size and number along with an increase in smooth endoplasmic epithelium leading to hypertrophy. Significant increases in cell proliferation rates that were correlated with dose and both hepatic adenomas and carcinomas were significantly increased in male and female mice.

Hepatic tumors produced by a PPAR α receptor-mediated MOA, such as triclosan, are not relevant or predictive of human health outcomes (Klaunig et al. 2003; USEPA 2008), and, therefore, not considered quantitatively in the development of relevant toxicological benchmarks (USEPA 2008). There is robust and abundant experimental evidence that the carcinogenic responses of rodents to PPAR α agonists are not shared by humans. This evidence includes data from humans chronically exposed to therapeutic doses of hypolipidemic drugs showing no increased incidence of cancer (Klaunig et al. 2003), as well as data from experiments with rodents carrying “humanized” PPAR α genes (Morimura et al. 2005; Gonzalez and Shah 2008; Yang et al. 2008) and with non-human primates (Klaunig et al. 2003; Cariello et al. 2005) demonstrating that these “humanized” PPAR α rodent models and non-human primates are refractory to the hepatocarcinogenic effects of PPAR α agonists seen in rodents.

As concluded by USEPA (2008), and we concur, there is sufficient evidence that triclosan is a PPAR α agonist and that triclosan is “Not likely to be a human carcinogen”.

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