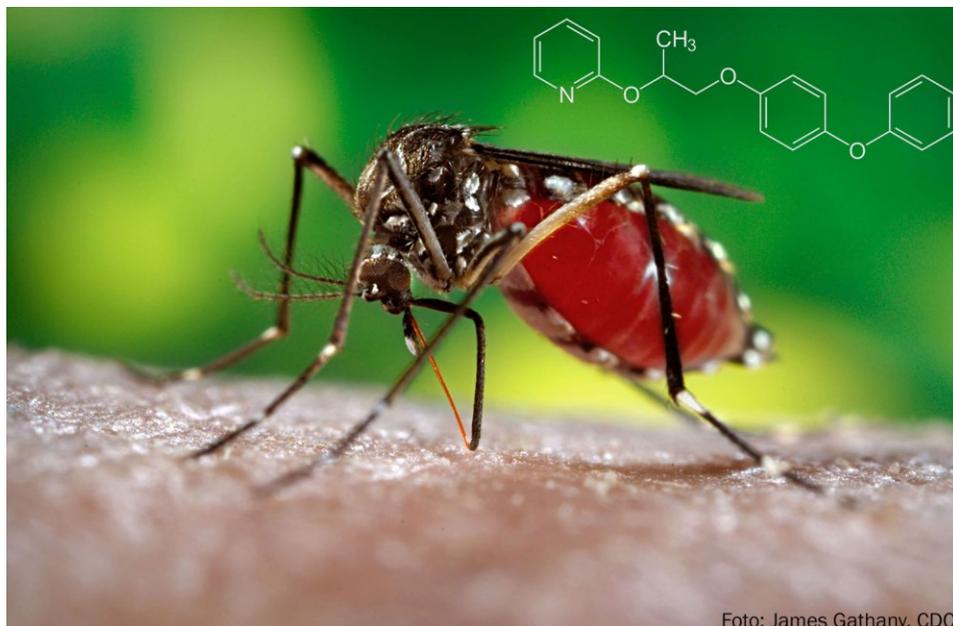




Swedish Toxicology Sciences
Research Center



Pyriproxifen and microcephaly: an investigation of potential ties to the ongoing “Zika epidemic”

REPORT FROM A WORKING GROUP
SWETOX, MARCH 2016

Pyriproxifen and microcephaly: an investigation of potential ties to the ongoing “Zika epidemic”

BACKGROUND TO THE REPORT

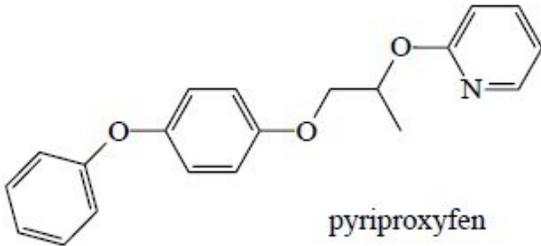
Against the back-drop of the debate around a causative relationship between an escalating mosquito-bourne Zika virus infection epidemic and the rise in incidence of microcephaly in new-borns (1), groups of scientists and doctors related to the environmental movement in Brazil and Argentina have raised the possibility that exposure to an insecticide, pyriporoxifen (PPF), may be involved. A change in the pattern of usage of this approved pesticide from the beginning of 2014 was claimed to coincide with a rise in the incidence of children born with microcephaly in affected areas of Brazil (2). This association have been denied by The Brazilian Ministry of Health (3)

The causal relation between Zika virus and microcephaly and other severe developmental effects is supported by several studies including epidemiological reports (4) and case-reports where the infection and the virus have been found in mothers and affected fetuses. Indeed a very recent report provides molecular links between Zika infection and detrimental effects on neuronal progenitor cells early in brain development (5). The relation between Zika virus and microcephaly will not be further investigated in this report.

OVERVIEW OF THE REPORT

As part of the Swetox mission to react to emerging concerns in chemical health and environmental safety, a preliminary litterature investigation was undertaken to gather all readily available scientific information on PPF with respect to safety assessment, in order to better understand potential links between chemical exposure and the devopment of microcephaly in affected areas. Therefore the contents of the report do not constitute an attempt at either questioning the use of existing regulatory data in the manner prescribed by international regulatory procededures, or as a new risk assessment, based on the scientific information and concepts discussed. Here we report our findings, with particular emphasis on exisiting regulatory information, potential for lack of translation of results from regulatory animal testing to humans, lack of human exposure data and suggestions on plausible mode(s) of action of PPF in human neurodevelopmental adversities such as microcephaly.

Chemical information of Pyriproxyfen

Compound name	Pyridine, 2-[1-methyl-2-(4-phenoxyphenoxy)ethoxy]-
CAS RN	95737-68-1
Name and abbreviation applied herein	Pyriproxyfen (PPF)
Chemical Structure	 <p style="text-align: center;">pyriproxyfen</p>
Usage	Insecticide An insecticidal juvenile hormone analog that perturbs insect and tick development.
Other names	2-[1-Methyl-2-(4-phenoxyphenoxy)ethoxy]pyridine; Admiral; Archer IGR; BCP 8702; Distance; Distance IGR; Esteem; Juventox; Juvinal; Knack; Lano Tape; Nemesis; NyGuard IGR; Nylar; OMS 3019; Pluto MC; Pyriproxyfen ; S 31183; S 71639; S 9138; SK 591; Seize; Sumilarv; Tiger; Tiger (insecticide); Tiger 10EC
Molecular weight	321.37
log K _{ow}	5.37 at 25 ± 1 °C; 5.498 (Scifinder, CA database, modeled value)
Water solubility	0.367 ± 0.004 mg/l at 25 ± 1 °C; 2.9 mg/L (Scifinder, CA database, modeled value)
pK _a	3.62±0.12 as the ammonium compound (Scifinder, CA database, modeled value)
Vapour pressure	2.83 10 ⁻⁸ Torr (Scifinder, CA database, modeled value)
Databases	US NIH TOXNET http://toxnet.nlm.nih.gov/cgi-bin/sis/search2/r?dbs+hsdb:@term+@DOCNO+7053 Pesticide Action Network (PAN) Pesticide Database http://www.pesticideinfo.org/Detail_Chemical.jsp?Rec_Id=PC35792 PPDB: Pesticide Properties DataBase, University of Hertfordshire http://sitem.herts.ac.uk/aeru/ppdb/en/Reports/574.htm Scifinder: http://www.cas.org/products/scifinder

REGULATORY DOCUMENTATION AND PUBLISHED TOXICITY FINDINGS

Repeat dose animal toxicity and developmental and reproductive toxicity

Since its introduction to use, several regulatory documents have been issued by the WHO (6,7), EU-EFSA (8), EU-ECHA (9) and the US-EPA (10), all essentially citing the findings of the animal experimentation performed by Monsanto/Sumimoto in the original submission for marketing licence. Examination of the documentation reveals evidence of low toxicity in repeat dose GLP animal toxicity studies, with the lowest NOAEL being noted in a 52 week dog study (10 mg/kg/day, based on increased liver weights, histopathological changes (hypertrophy) and altered cholesterol metabolism). In the rat the corresponding NOAELs range from 29 to 23 mg/kg/day for 28 day and 6 month oral exposures, respectively, again set on liver findings. Low toxicity was noted in the rat by inhalation and dermal application for one month. No evidence of genotoxicity or carcinogenicity potential were reported. Standard teratogenicity and

developmental toxicity in rats and rabbits define NOAELs at 100 mg/kg/day, based solely on maternal toxicity. A standard 2-generation reproductive toxicity study in rats revealed a NOAEL for parental toxicity at 13.3 mg/kg/day and 66.7 mg/kg/day based on F1/F2 losses of body weight gain, in the absence of impaired fertility.

A survey of available literature in the SciFinder data base (www.scifinder.cas.org) revealed that, of the 232 entries under "Adversity and toxicity" PPFs toxicity has been tested in a variety of marine species including *Daphnia magna* (11,12), and insects (13,14). There is one report of reproductive toxicity in a mollusc 2-generation study (15) and clear effects on fecundity reported in *Daphnia magna* at pM concentrations (16). No evidence of neurotoxicity, developmental or otherwise, has been reported in any species.

Hormone disruption potential

In view of the hormone disrupting function of PPF, the EPA has recently reported data on Tier 1 investigation of the compounds ability to disrupt human hormonal systems. In the male pubertal assay, there was evidence for anti-androgenicity with decreases in serum testosterone levels and decreases in weights of the androgen sensitive organs/tissues (testes, epididymides, LABC [levator ani-bulbocavernosus muscle], seminal vesicles, ventral prostate and dorsal prostate). No histopathological changes were seen in the testes or epididymides. However, alterations in the hormone levels and tissue weights were seen at a dose that also caused increases in liver enzymes liver weight and hepatocellular hypertrophy. Furthermore, in a mechanistic study conducted to evaluate the effects of pyriproxyfen on testosterone levels in pubertal male rats, administration of pyriproxyfen at the same doses as the Tier 1 pubertal male rat assay resulted in decreases in testosterone levels and of LABC weight. However, in this study, the administered doses resulted in overt toxicity (>10% decrease in body weight). These doses also resulted in liver enzyme induction (CYP2B, CYP3A, CYP4A and UGT), liver weight and hepatocellular hypertrophy. There were no treatment-related effects on 17 β -HSD activity in the testes in this study. The available data suggest that the potential anti-androgenic effects on the male reproductive system seen in these studies may be secondary due to the increased metabolism of the testosterone by the liver.

There was no convincing evidence for potential interaction of pyriproxyfen with the estrogen pathway.

In male pubertal rat assay, histological changes were noted in the thyroid in the male pubertal assay and consisted of reduced colloid area and increased follicular cell height at both the low and high doses. No treatment-related effects were noted on thyroid weight or serum T4 or TSH levels in the male or female pubertal assay at doses up to 1000 mg/kg/day, and no histological changes were noted in the thyroid in the female pubertal assay. In addition, a 14% increase in pituitary weight was noted in the female assay but not in the male assay.

A non-guideline mechanistic study was conducted at the same dose levels as the pubertal assays to investigate potential effects of pyriproxyfen on the thyroid and liver function in pubertal male rats. In this study mean T4 levels were decreased by 25% at 1000 mg/kg/day.

In the chronic dog study, females had non-linear increases of 82 and 63% in thyroid weight at the mid-high and high doses, but similar changes were not observed in the males. However, these effects were considered to be secondary to liver enzyme induction (CYP2B, CYP3A, CYP4A and UGT), increased liver weights and hepatocellular hypertrophy. The findings suggest potential interaction with the androgen pathway in fish. There is no convincing evidence of potential interaction with the thyroid pathway in amphibians.

In summary, pyriproxyfen has been evaluated for its potential as an endocrine disruptor by the EPA. These studies did not find convincing evidence of interaction with the estrogen pathway, while there was some evidence of interaction with the androgen and thyroid pathways. Using a “weight of evidence” approach, no recommendation of Tier-2 testing was issued by the EPA (10).

Overall exposure limits

An assessment factor of 100 was applied to the NOAEL in the 52 week dog study to obtain the Accepted Operator Exposure Level (AEL) AEL of 0.12 mg/kg/day (3-6). Pyriproxyfen has also been considered by WHO under its Pesticides Evaluation Scheme at a recommended dosage of 0.01 mg/l for controlling disease-carrying mosquitoes in drinking-water containers (6)

Human toxicity data

Human toxicity/adversity data are very scarce and there is only one report of human adversity coupled to potential reproductive and/developmental toxicity effects, where prenatal exposure to PPF has been associated with development of rare congenital abnormalities, e.g. bladder exstrophy (17). No other reports were detected in the above mentioned SciFinder search within CAS.

ENVIRONMENTAL ACCUMULATION AND RISK CLASSIFICATION

Under the ICSC Environmental classification, PPF is very toxic to aquatic organisms and bioaccumulation of this chemical may occur in aquatic organisms. The substance may cause long-term effects in the aquatic environment (Multiple “N”-classed in the EU) (8,9).

Recommendations exist to avoid release to the environment in circumstances different to normal use. If released to soil, PPF is expected to have no mobility based upon an estimated Koc of 405,000, and is not expected to volatilize from dry soil surfaces (SRC) based upon its vapor pressure. If released into water, PPF is expected to adsorb to suspended solids and sediment based upon the estimated Koc. Hydrolysis is not expected to be an important environmental fate. The potential for bioconcentration in aquatic organisms is very high.

EXPOSURES AND EXPOSURE MEASUREMENTS

Exposures to PPF have occurred over many parts of the world, with hitherto no alarming relationships to the induction of microcephaly being evident. However, the methods of use of the insecticide, and therefore the pattern and extent of exposure, may be variant. The original Argentinian and Brazilian reports (1,2) draw parallels between introduction of PPF directly into drinking water in areas where mosquito infestation is rising, in parallel to increases in Zika infections and reports of microcephaly. This differs from those exposures resulting from spraying crops or the use of impregnated nets over water sources (18-21). It is also interesting to note that the areas worst affected, are also those worst affected by el Nino and resultant draughts over the last 3 years (22).

At the current time there are no exposure/toxicokinetic (TK) data readily available to us for this review from the regulatory animal repeat dose toxicity and reproductive toxicity studies supporting the regulatory acceptance of PPF (23). Further scrutiny of the original submissions is therefore required. This normally limits any attempts to extrapolate to risk from human exposure. This is further compounded by an almost total lack of published data on human exposures, ranging from actual concentrations in water sources used for drinking, to systemic levels in exposed individuals in areas where PPF is used. The only literature available is limited to workers exposure assessment, where PPF has been assessed as one of many contaminants (24,25). Taken together, the lack of specific exposure data precludes current attempts to perform an appropriate risk assessment of the potential role of PPF in the induction of neurodevelopmental adversities such as microcephaly.

There are no data concerning the ability of PPF to cross the placenta of rats, rabbits or humans. Analysis of the structure reveals that the compound might freely traverse the placenta. Additionally, there is a considerable body of literature indicating species-specific differences in transplacental transfer of organic compounds (26).

METABOLISM

All current data is largely restricted to rodents used in the original regulatory studies. However, there have been some studies performed in lactating goats and laying hens. In rats, pyriproxyfen exhibits a relatively poor bioavailability of circa 40% and is rapidly metabolised via hydroxylation in the liver to 4'-OH Pyriproxyfen and 5',4'-OH Pyriproxyfen, which are further converted to the respective sulfate conjugates (27-29, Figure below), which are mainly excreted via the bile. Up to 93% of the administered oral dose is thus eliminated via the faeces. Metabolism in male rats was shown to be faster than in females. The rapid metabolic clearance and short half-life suggest a considerable first-pass metabolism of the compound in the liver. Little variability in the metabolic profile of PPF has been noted between rats and mice.

There are no data available for the human metabolic disposition of PPF.

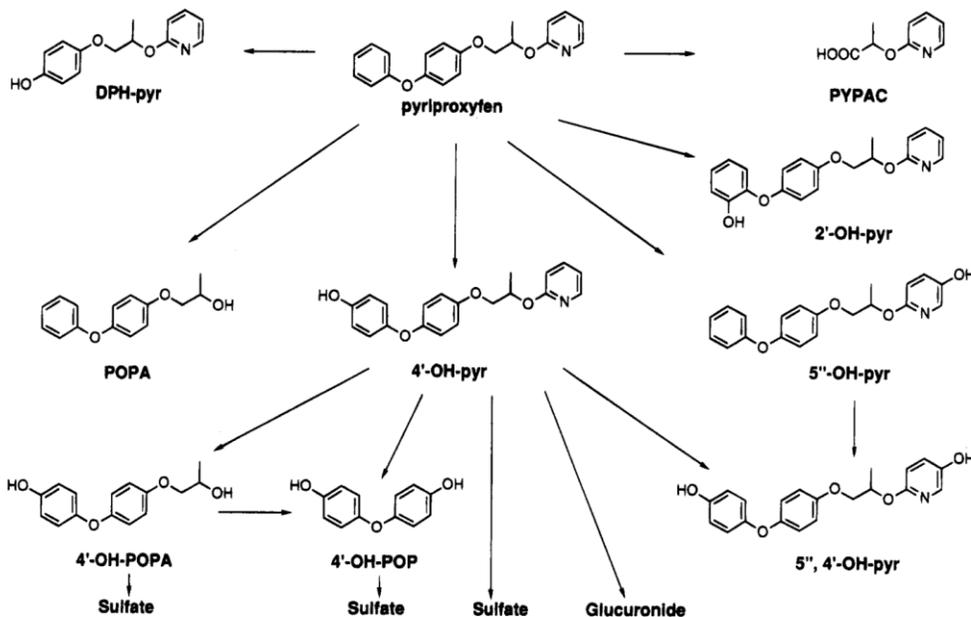


Figure 4. Metabolic pathways of pyriproxyfen in rats and mice proposed on the basis of results obtained in the previous (Matsunaga et al., 1995) and present studies.

POTENTIAL MODE(S) OF ACTION IN NEURODEVELOPMENTAL DISTURBANCES

This section details initial tentative suggestions around potential molecular ties between those toxicological observations arising from existing studies with PPF, and potential mechanisms related to neurodevelopmental disorders, specifically microcephaly.

Firstly, it must be stated that the induction of microcephaly is a relatively rare side-effect reported in regulatory animal reproductive and developmental toxicity studies. Similarly, although rare, there are clear cases of translational differences in teratogenic potential across species barriers. For instance, several marketed drugs, such as phenytoin (30) and metotrexate (31) carry warnings with respect to use in pregnancy and risks for microcephaly, despite having been through standard regulatory testing in animals. In each case, the mechanism of teratogenesis is the subject of continuing mechanistic study and increasing understanding.

A potential cholesterol/nutrition link

All regulatory animal studies performed revealed NOAELs which in some way were either directly or indirectly linked to liver adversity. One of the observations routinely raised related to disturbed cholesterol disposition. Therefore, it is interesting to note that agents that impair cholesterol biogenesis possesses clear teratogenicity potential, inducing induction of holoprosencephalic brain anomalies such as microcephaly (32, 33), resembling Smith-Lemli-Opitz syndrome, a recessive autosomal disorder with strong relationship to microcephaly (34). Any

relationship to cholesterol metabolism may also be potentiated by variations in nutritional status in affected populations.

Links to mutations in selective genes

There are a growing number of human genes that have been associated with the development of microcephaly, via GWAS (Genome-wide association study) and directed mutational analysis studies. These include KIF11: kinesin-like protein 1, with a point mutation leading to decreased protein associated with microcephaly involved in mitotic spindle formation (35). DYRK1A: additional copy in Down's Syndrome, leading to increased protein associated with microcephaly, a kinase that can lead to inhibition of notch signaling (36). Microcephalin and other MCPH proteins that are important for mitotic spindle formation and centrosome biogenesis (37) and a variety of other genes very recently identified by whole genome sequencing (RAB3GAP1, RNASEH2B, ERCC8, CASK and BRCA2) (38). Any one of these gene products may function as a potential molecular target for PPF, assuming sufficient fetal exposure. Further, it may be that particular genotypes are more susceptible than others and that this may also be related to their geographical and ethnic distribution.

Links to bHLH-PAS "target"-related proteins

One major molecular target for PPF in the mosquito larva has been established to be the Methoprene-tolerant (Met) protein, which is activated upon PPF binding. The Met-protein contains a basic helix-loop-helix (bHLH) motif followed by two Per-Arnt-Sim (PAS) domains, synonymous with other bHLH-PAS proteins (39). Other potential binding partners are also emerging as knowledge of the insect juvenile hormone receptor signal transduction mechanism increases. There are a number of mammalian bHLH-PAS proteins which typically form heterodimeric transcription factors (AhR, ER, RA) that are important for neurodevelopment. bHLH-PAS proteins are expressed in different areas of brain and under different windows of embryogenesis, and some family of proteins (e.g. Sim and NPAS) regulate transcription of genes that are important for neurodevelopment, predominantly the neuro-progenitor cell proliferation and differentiation (40). Therefore, it may be plausible that PPF is able to interfere with these critical potential binding partners during early stages of neurodevelopment.

Links to disruption in thyroid hormone function

The regulatory screening studies reported by the EPA (10) indicated that PPK may interact with the mammalian thyroid hormone system in some manner. Thyroid hormones are essential for brain development through specific time windows influencing neurogenesis, neuronal migration, neuronal and glial cell differentiation, myelination, and synaptogenesis, and there may be significant inter-species differences in these processes. The actions of thyroid hormones are mostly due to interaction of the active hormone T3 with nuclear receptors and regulation of gene expression. T4 and T3 also perform non-genomic actions. The genomically-active T3 in

brain derives in part from the circulation, and in part is formed locally by 5'-deiodination of T4, mediated by Dio2 in the astrocytes, in proportions that depend on the developmental stage. T4 and T3 are degraded by Dio3 present in neurons. Entry of T4 and T3 in brain is facilitated by specific trans-membrane transporters, mainly the monocarboxylate transporter 8 (Mct8) and the organic anion transporter polypeptide 1c1 (Oatp1c1). In rodents Mct8 facilitates the transfer of T4 and T3 through the blood-brain barrier (BBB). Oatp1c1 transports T4 through the BBB and into to the astrocytes facilitating the generation of T3 in these cells. Primates have low amounts of OATP1C1 in the BBB, and depend of MCT8 for thyroid hormone transport. Therefore MCT8 mutations in humans cannot be compensated by T4 transport as in rodents (41).

Additionally, it is well established that the onset of the fetal thyroid gland function, which occurs only at mid-gestation in humans, fetal brain relies on maternal TH. TH first crosses the placenta and then the brain-blood barrier. The differential transport of T4 and T3 has an important consequence: maternal hypothyroxinemia, that is, low T4 level in maternal serum with T3 and TSH levels within normal range, is a cause of neurodevelopmental disorders (41). Human mutations of *MCT8* increase rather than suppress TH circulating levels but have dramatic consequences on neurodevelopment, therefore suggesting a predominant function of MCT8 for TH transport across the brain-blood barrier (42). These molecular targets all warrant further investigation as they are not components of the current test battery utilized by the EPA. Here, it is interesting that a preliminary in silico docking exercise utilizing both the structure of PPF and its primary hydroxylated metabolite reveals that the metabolite and not the parent actually shares molecular structure features of a known transthyretin binder. This trans-membrane protein is known to be involved in uptake of the thyroxin hormone across the blood brain barrier (43).

SUMMARY, GAP ANALYSIS AND SUGGESTIONS FOR FURTHER STUDIES

The correlative epidemiological studies pertaining to a potential relationship between a rise in reporting of microcephaly cases and increases in the spread of Zika virus and its host mosquito stimulated considerable public and regulatory unrest around the globe, particularly in affected areas of South America. This has stimulated considerable efforts to understand the epidemiological factors underlying this relationship, as well as the underlying molecular and biological mechanisms. However, the recent claims that increases in the use of the PPF insecticide in affected and/or high risk areas, may contribute in some way to the growing medical problem cannot pass without further investigation, as it may constitute a potentially complex human health risk situation that must be addressed post-haste. This will, therefore, necessarily involve further investigations around a potential contribution from PPF alone, or in combination with Zika viral infection.

The current review presents several arguments supporting further investigation of this chemical exposure in affected populations in Brazil and other countries, where large quantities are being

directly applied to the sole drinking sources of many people. We have identified several areas where sufficient information is lacking in order to perform a suitable risk assessment. These constitute areas where efforts should be placed immediately and in a coordinated manner in order to either support direct or indirect causality, or lift attention from this otherwise important insecticidal agent.

Initial focus should be placed on rapidly filling data gaps via directed environmental epidemiology studies, much in the same way as is being performed with Zika infection. The aims of such studies would be to draw more clear relationships between the increased instances of neurodevelopmental and other toxicities in “affected” and “unaffected areas” of Brazil, in relation to changing patterns in utilization of PPF in areas reporting increased microcephaly and those not. Here initial focus could be placed on families with a verified microcephaly diagnosis. The studies would necessarily encompass attempts to describe any temporal, geographical and population-specific factors involved.

An overall gap analysis of the available literature reveals that the following specific areas of endeavor might be included in support of these timely epidemiological investigations.

Field studies to:

- Provide data on actual dosage practices and concentrations in drinking water at the source.
- Identify any other sources of exposure.
- Identify other potential individual-specific factors (nutritional status, specific disease and metabolism genotypes etc.).
- Provide information on blood levels of PPF and its major metabolites in exposed individuals.

Laboratory studies to:

- Describe the metabolic profile of the compound in humans, as well as variation in this.
- Study the ability of PPF and its major metabolites to cross the human placenta.
- Determine any potential interactions with mammalian proteins/binding partners/pathways which may be related to a human-specific teratogenicity potential in the developing brain, with initial focus on proteins involved in thyroid hormone function.

Literature and in silico studies to:

- Further investigation of raw data contained in regulatory files on PPF, with particular reference to any exposure and mortality data in repeat dose and reproductive/development toxicity studies.
- Support biological mode of action studies in areas relevant to potential human teratogenicity in the developing brain.
- Support TK analysis and predictions from field and laboratory studies.

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