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Abstract

Environmental contamination by chlorotriazines has been evidenced in mother-child cohort suggesting more detailed risk assessment of these compounds in drinking water. Exposure of rodents to atrazine has been associated to alterations of endocrine and reproductive functions by disrupting neuroendocrine control at hypothalamus level. Perinatal exposure to low doses of atrazine has been associated to reproductive dysfunction, and to behavioral abnormalities in adult exposed during embryogenesis. The objectives of the current investigation were to (1) evaluate the influence of physico-chemical properties of chlorotriazines on tissue distribution in pregnant rats and in fetuses, (2) gain a better understanding of fetal distribution of chlorotriazines in specific tissues, particularly in brain.
Serial blood samples were obtained from pregnant rats after administration of atrazine (ATZ), propazine (PRO) and simazine (SIM) via oral route at a dose of 10 mg/kg from day 15 to day 19. Maternal and fetal tissues were harvested at day 20, 24 hrs after the last dosing.
The metabolic extraction ratio was estimated to 87 % suggesting a significant first-pass effect explaining the low oral bioavailability. Blood exposure to parent compounds (ATZ, PRO and SIM) was negligible (lower than 5 %) compared to metabolite exposure. The main metabolite exposure involved diamino-s-chlorotriazine (DACT), ranging from 60 to 90 % depending on the molecules administered. A correlation between tissue-to-blood ratio and physico-chemical descriptors were observed for fat and mammary gland tissues but not for brain in adult rats. A more pronounced distribution in fetal brain was observed for ATZ and PRO, the two most lipophilic compounds.

Introduction

Atrazine (ATZ), simazine (SIM), and propazine (PRO) are triazine herbicides registered for use in the United States (U.S.). ATZ is the second-most widely used herbicide after glyphosate in agricultural sector (Atwood *et al.*, 2017) and the most frequently pesticide contaminant detected in U.S. waters as a result of its high mobility in soil and ground water, and its persistence in the environment (Thurman *et al.*, 1992). ATZ is mainly used as herbicide on crops and grasses, SIM as an herbicide to control broadleaf and grassy weeds mostly on fruits, and PRO is used as an herbicide for indoor greenhouse use and to control broadleaf and grassy weeds on sorghum. Their risk assessment has been reviewed recently by USEPA (US EPA, 2016). Atrazine and plant protection products containing this substance were banned from Europe in 2003 by Commission Decision (European Commission, 2003; Sass and Colangelo, 2006).

Environmental contamination by chlorotriazines (either directly due to residential proximity to crops or indirectly through drinking tap water) has been evidenced in a mother-child cohort suggesting a more detailed risk assessment of triazine compounds in public drinking water (Chevrier *et al.*, 2014). The chlorometabolites are classified as toxicologically equipotent to the parent triazines by the US EPA and as such belong to the common mechanism group triazines while the conjugated derivatives are considered toxicologically inactive metabolites (US EPA, 2016).

In animal models, exposure to atrazine has been shown to alter both endocrine and reproductive functions in rodent by disrupting the neuroendocrine control of ovarian function at the level of the hypothalamus (Eldridge *et al.*, 1999; Cooper *et al.*, 2007). Short-term ATZ exposure in male mice has led to behavioral abnormalities with impairment of motor, cognitive and emotional functions (Lin *et al.*, 2013).
Exposure to endocrine disrupting compounds (EDC) especially in developmental periods may interfere with pathways for behavioral development. Perinatal exposure of rodents to low doses of ATZ has been associated to behavioral abnormalities and neurodevelopmental disorders in mice delivered from dams exposed during gestation and lactation (Belloni *et al.*, 2011). More recently, alterations in dopamine and serotonin homeostasis associated with neurobehavioral effects have been described in off-spring mice after low-dose exposure during gestational and lactational periods (Lin *et al.*, 2014). Reproductive dysfunction and molecular alterations have been observed in adult zebrafish exposed only during embryogenesis (Wirbisky *et al.*, 2016).
Moreover, epidemiologic studies have shown that gestational exposure to environmental ATZ is associated to impair fetal growth (Chevrier *et al.*, 2011), to term low birth weight (Almberg *et al.*, 2018), to male genital abnormalities (Agopian *et al.*, 2013; Winston *et al.*, 2016) and to alteration in the timing of puberty (Namulanda *et al.*, 2017).

Basic knowledge of the biodistribution of xenobiotics including the fetal brain, and factors of variation modifying this exposure, is crucial for a better risk assessment, and for the detection of the factors of vulnerability in sensitive populations.

Several studies have explored the pharmacokinetics of ATZ and its metabolites while few studies have described their tissue distribution in animals, and no data are available for SIM and PRO. Furthermore, studies of the tissue distribution of ATZ have been performed in detail in adult animals, while in the fetus it has been performed only as per whole fetus basis.
Hepatic microsomal metabolism of ATZ, SIM and PRO was extensively different between species (Hanioka *et al.*, 1999). ATZ is metabolized preferentially to desethylatrazine (DEA) than to deisopropylatrazine (DIA), and both are metabolized to diamino-s-chlorotriazine (DACT) (McMullin *et al.*, 2007). ATZ and its chlorometabolites are conjugated via glutathione S-transferases (Timchalk *et al.*, 1990), and 8 to 12 metabolites of ATZ have been identified or postulated (Barr *et al.*, 2007).

After single dose of ATZ (ranging from 5 to 250 mk/kg body weight) in mice, DACT was the most abundant metabolite, and plasma levels of DEA and DIA were around 10-fold lower (Ross *et al.*, 2009). In a physiologically-based pharmacokinetic (PBPK) study in rats, a rate-limiting step in intestinal absorption was suggested (McMullin *et al.*, 2007).
In a PBPK study in rats after administration of 14C-ATZ (90 mg/kg), DACT appeared as about 95 % of the total chlorotriazine exposure, and DIA concentrations were about 3-fold higher than DEA suggesting a preferential attack of the isopropyl group by the CYP450 enzymes (McMullin *et al.*, 2003). In workers exposed to ATZ, urine bio-monitoring showed that DACT was the major metabolite (Catenacci *et al.*, 2003). However, more recent data in humans showed a significant inter-individual variability in DACT concentrations and suggested that both DACT and DEA appear to be the most important metabolites to measure in order to evaluate the exposure to ATZ-related chemicals (Barr *et al.*, 2007).

If few studies have described tissue distribution of ATZ and its metabolites in animals, no published data are available for SIM and PRO.
After single dose in mice (Ross *et al.*, 2009) ATZ and DACT reached high levels in the liver compared to the kidney. Moreover, DACT reached high levels in the brain while ATZ displayed a modest distribution, and DIA and DEA were not detected.
In a study performed in male mice, after oral gavage (from 5 to 250 mg/kg) a PBPK model for ATZ showed that brain concentrations of ATZ and DACT were lower than the corresponding plasma values, especially for DACT (Lin *et al.*, 2011). In the liver, ATZ concentrations were higher than in plasma (at least 10-fold higher) while DACT concentrations in liver were lower than in plasma (less than 5-fold).
A different pattern was observed in rats neonates and in dam after oral exposure to atrazine (5 and 25 mg/kg) during the gestation (Fraites *et al.*, 2011). In neonates, the DACT, DIA and DEA levels in plasma and in brain were similar while ATZ levels in the brain were higher than in plasma (around 30 to 50-fold at 5 and 25 mg/kg dosing). In dam, the DACT, DIA and DEA levels in plasma were higher than in brain (especially for DIA and DEA) while ATZ levels in brain and plasma were similar.

In the fetus, ATZ and metabolites levels (analyzed on a per whole fetus basis) were similar to that measured in the maternal plasma for both compounds (Fraites *et al.*, 2011). In the whole fetuses, DACT accounted for around 60-90 % of the total chlorotriazines. In the fetuses, the exposure was in the following rank order DACT > DIA > DEA > ATZ.
PBPK modeling incorporating experimental data from different studies and groups was used to develop a model to improve the understanding of fetal and neonates animal exposure especially in the context of developmental ATZ exposure since several studies have suggested that ATZ may cause neurobehavioral deficits (Lin *et al.*, 2013). However, in these studies, distribution of ATZ in fetuses has been performed on a per whole fetus basis (i.e., mean organs concentration) so that levels in specific fetal tissues are not available to our knowledge.

A primary objective of the present investigation was to test the hypothesis that physico-chemical properties of chlorotriazine derivatives influence their biodistribution in maternal and fetal tissues. Indeed, bioanalysis of ATZ, PRO and SIM and their metabolites (DIA, DEA or DACT) after chronic administration of ATZ, PRO and SIM in pregnant rats led to biodistribution data for a homologous series of six compounds in a wide range of lipophilicity and polarity.
A related aim was to gain a better understanding of distribution of chlorotriazines in specific fetal tissues, particularly in brain tissue, given the potential neurodevelopmental issues described in the literature.

Materials and Methods

Chemicals and Reagents

Atrazine (ATZ, purity 99.5%, lot number 90812), propazine (PRO, purity 98.5% , lot number 10114), simazine (SIM, purity 98%, lot number 10408), chlorotriazine metabolites: desethyl-atrazine (DEA, purity 99 %, lot number 21105), deisopropyl-atrazine (DIA, purity 98.7 %, lot number 20423), diamino-chloro-triazine (DACT, purity 98.5 %, lot number 81114) were purchased from CIL Cluzeau (Sainte-Foy-La-Grande, France). For oral administration (via a cannula), ATZ, PRO and SIM were dissolved in Miglyol 812 which is a low viscosity pharmaceutical excipient for lipophilic drug administration.
Acetonitrile, acetone, hexane (pesticide residue grade in each case), anhydrous magnesium sulfate and dimethyl sulfoxide (DMSO - analytical reagent grade) were provided by Fisher Scientific (Illkirch, France). Silica PSA SPE and silica C18 ODS SPE bulk sorbents were obtained from Interchim (Monluçon, France). All reagents were of analytical grade.
Physico-chemical characteristics (i.e., Log P, Log D and percentage of polar surface area TPSA) of the six triazine compounds were estimated from the online platform Chemicalize that is based on ChemAxon's technology (https://chemicalize.com). The chemical structures and the physico-chemical are displayed in Figure 1.

Experimental design

- Animals

The study was performed according to a protocol approved by the local committee of laboratory investigation and animal care of our institution, in accordance with the rules and guidelines concerning the care and use of laboratory animals (n° R-2012 -NB-01).
Non-pregnant (weighting between 200 and 250 g) and pregnant (weighting between 300 and 350 g) Sprague-Dawley rats were obtained from Janvier (Le Genest-Saint-Isle, France). The rats were housed in 12-h light/12-h dark cycle conditions and were supplied with food and water ad libitum.

- Study design

In a preliminary study we performed a pharmacokinetic study of ATZ in 6 non-pregnant Sprague - Dawley rats after single administration via IV (2.5 mg/kg, dissolved in DMSO/0.9% NaCl solution) and oral routes (10 mg/kg, dissolved in Miglyol 812) with blood and maternal tissue collection. Blood samples were drawn at 5, 10, 30, 60, 90, 120 and 180 min after IV and oral dosing. Brain and liver samples were collected at 180 min after dosing.

In a second step we performed a pharmacokinetic study of ATZ in 3 pregnant Sprague - Dawley rats after chronic administration of ATZ (10 mg/kg, dissolved in Miglyol 812) via oral route throughout the gestation (from D1 to D19) with blood and maternal and fetal tissue collection at delivery.

The final pharmacokinetic studies were performed in 12 pregnant Sprague–Dawley rats equipped with a tunnelized jugular catheter after chronic administration of ATZ (n = 6), PRO (n = 3) and SIM (n = 3) at a dose of 10 mg/kg (dissolved in Miglyol 812), via oral route from D15 to D19. Blood profiles as a function of time (2, 5, 10, 30, 60, 120, 180, 480 and 1440

min) were performed at D15 and D19. Intercurrent blood sampling were withdrawn immediately before dosing at D16, D17, D18. Blood and maternal tissues as well as fetal tissues were collected at D20 (i.e., 24 h after the last dosing).

- blood and tissue collection (maternal - fetal)

Maternal (mammary gland, brain, fat, muscle, spleen and liver) and fetal (brain and liver) organs and the liquid amniotic and the placenta were harvested, weighed and stored at -20°C until use for pesticide quantification.
Tissue sampling was performed in triplicate. For each adult animal and in each organ, 3 independent tissue samples were removed and analyzed for chlorotriazines and their metabolites. For each sample, a tissue-to-blood ratio was computed. Six fetuses were harvested randomly per dam, stored and 3 of them were used for determination of chlorotriazines and their metabolites in brain and liver. For each sample a tissue-to-maternal blood ratio was computed.

Sample preparation

Extraction procedure was previously described (Brandhonneur *et al.*, 2015). Briefly, blood samples (200 ul) and organ samples (weighting between 150 and 250 mg) were thawed, dehydrated and homogenized with $MgSO_4$. Acetonitrile, pesticides and internal standards were added to the appropriate control samples. Hexane was added and the samples were incubated for 10 min at room temperature, with shaking, before being transferred to -20°C conditions for 30 minutes. The samples were centrifuged for 5 min at 30,000 g, the hexane was discarded and the organic layer was transferred to a tube containing dispersive solid-phase extraction (dSPE) medium. The mixture was incubated for 10 min at room temperature, with shaking and was then centrifuged at 30, 000 g for 5 minutes. The organic layer was removed and the solvent was evaporated off to dryness under nitrogen. Finally the sample was dissolved and the resulting solution was injected into the GC-MS machine.

GC-MS analytical procedure

We used Thermo Systems Instrument Trace GC and Trace DSQ2 for the simultaneous separation and quantification in samples of the chlorotriazines ATZ, SIM and PRO and their chlorinated metabolites DIA, DEA and DACT (Brandhonneur *et al.*, 2015).

We used an Optima 17 MS (30 m × 0.25 mm x 0.25 µm) analytical chromatographic column. Helium was used as the carrier gas, at a flow rate of 1.0 ml/min. A volume of 1 µl of extract was injected in the PTV split-less mode from 50 °C to 260°C. The oven temperature was programmed as follows: initial temperature 105°C, increase from 105°C to 180°C at a rate of 40°C/min over a period of 2 min, then from 180°C to 220°C at 3°C/min for 13 min, and from 220°C to 280°C at a rate of 40°C/min for 2 min, with a final phase of heating at 280°C.

The mass spectrometer was operated with an electron impact ionization source in the SIM mode. The electron energy was 70 eV. Mass spectrometry for confirmation and

quantification was carried out in the SIM mode, with the selection of two m/z values for each pesticide. We used SIM mode peak location to carry out the analysis and to match GC-MS retention times under RTL conditions for the screening of the target pesticides.

Pharmacokinetic analysis

In the preliminary study in non-pregnant rat after single dosing, non-compartmental analysis was performed (Monolix version 4.2.2, Lixoft Company, 92160 Antony France) on ATZ allowing the determination of the blood clearance after IV dosing, and of the apparent elimination half-life after IV and oral dosing.
Since the animals did not receive both IV and oral ATZ (i.e., the animal were sacrificed at 180 min for tissue sampling), the oral absolute bioavailability (Fpo) was not calculated as the standard methodology using a cross-over protocol. Fpo (%) was calculated from the mean AUC 0-180 min obtained after IV and oral dosing of ATZ.

Absolute blood exposure was assessed by the AUC (10^3 nmol.h/L) calculated at steady state at D19. Relative blood exposure (%) between the different compounds were calculated from their respective absolute blood exposure.

The tissue analysis was performed on D20 that is 24h after the last dosing (D19). Usually tissue distribution studies are performed at a time corresponding to the Cmax of the drug. In the present case, compounds studied had different Cmax with DACT known to peak quite lately. Moreover, given the fact that animals were sacrificed, and that most of the organs were removed, we wanted to avoid any risk cross-contamination from residual compounds present in the GI tract.

Results

Blood exposure

Oral and IV blood profiles of ATZ and metabolites in non-pregnant rats are illustrated in Figure 2 a-b. After IV dosing, ATZ displayed a rather rapid elimination with an apparent elimination half-life was 36.8 ± 3.2 min and a systemic clearance of 0.724 L/h. After oral dosing, the apparent elimination half-life was calculated only in 3 animals (33.3 ± 4.6 min) since the remaining animals showed a plateau.
The systemic exposure to ATZ metabolites was in the following order DACT >> DIA > DEA. The variability in ATZ blood exposure was 18 % after IV dosing, and 36 % after oral dosing. After single dosing in normal rats, blood exposure to ATZ was quite significant after IV dosing (32 %) but negligible (2 %) after oral dosing (Table 1).

Blood exposure to ATZ, PRO, SIM and their respective metabolites from first dosing at D15 to D19 are illustrated in Fig 3 a-c (individual concentration data are provided as Supplementary Materials at https://doi.org/10.5061/dryad.8931zcrkh). A double-peak phenomenon was apparent in several blood profiles after either ATZ, PRO or SIM administration for both parent drugs and metabolites.

After repeated oral dosing in pregnant rats, blood exposure to ATZ, PRO and SIM was negligible compared to metabolite exposure with a contribution lower than 5 % in most of the cases for parent compounds (Table 2). The metabolite exposure was in the rank order DACT >> DIA > DEA as observed after IV and oral dosing in non-pregnant rats (Table 1).

Tissue exposure

Tissue exposure to ATZ, DACT, DIA and DEA after single dosing (IV and oral routes) in non-pregnant rats are summarized in Table 3. Liver and brain tissue levels at 3h post-dosing were highly dependent on the route of administration for ATZ but not for the metabolites.

Tissue exposure to ATZ, PRO, SIM and their respective metabolites at 24h after the last dosing in pregnant rats are summarized in Table 4.
It clearly appears that concentrations of ATZ, PRO and SIM in mammary gland and especially fat tissue were higher than in the other tissues, while it was the reverse for DACT, and DIA and DEA had quite similar concentrations (Table 4).

A clear correlation was observed between tissue-to-blood ratios and logP, logD and % TPSA for mammary gland and fat tissues (Figure 4 a-f). There were no significant correlations for the other tissues between tissue-to-blood ratios and these physico-chemical parameters.

It appeared that the distribution of the chlorotriazines and of their metabolites in the brain were not influenced by their differences in physico-chemical properties. On the whole, exposure to DACT contributed to 97 %; 98 % and 88 % to the total exposure after ATZ, PRO and SIM administration.
Brain concentrations of ATZ were higher than the corresponding blood values (both in normal and in pregnant rats) while there were no apparent difference for the metabolites (Table 3-4). PRO and SIM brain levels (and of their corresponding metabolites) were of the same order than blood levels.

Tissue exposure of fetuses

In the fetal tissues (brain and liver), DACT contributed to around 99 % of the exposure after ATZ and PRO administration, and to around 90 % after SIM administration (Table 3-4).

Comparison of brain-to-blood ratio between dam and fetus showed that ATZ and PRO distributed more in dam's brain than in the fetal brain. No difference was apparent for SIM, DEA and DIA while the more lipophilic compound DACT showed a more pronounced fetal brain distribution after ATZ administration or no difference after SIM and PRO administration.

Discussion

Blood exposure

Our preliminary IV-oral study in non-pregnant rat showed that ATZ had a low oral absolute bioavailability (6.2 %) after oral dosing at 10 mg/kg. It was estimated at 5.0 % after oral administration at 50 mg/kg (data not shown). Eventhough the oral absolute bioavailability was not calculated according to a standard methodology (cf experimental section), we are confident in the value reported because the variability in AUC after IV and oral dosing were quite small.
Given the systemic clearance and a liver blood flow of 0.828 L/h (Davies and Morris, 1993), the liver extraction ratio was estimated at 87 % suggesting a significant first-pass effect explaining the low oral bioavailability.
This low oral bioavailability is consistent with a model-prediction of intestine first-pass elimination (64 %) reported previously after ATZ administration in rats at a dose of 150 mg/kg (McMullin *et al.*, 2007). A pharmacokinetic study of 14C-atrazine has been performed in non-human primates after IV (0.26 mg) and oral dosing (from 1 to 100 mg) (Hui *et al.*, 2011). This study showed a higher oral bioavailability of around 60 % in line with an estimated extraction ratio of around 40 % calculated from their systemic clearance and a liver blood flow of 2.62 L/h.kg (Catenacci *et al.*, 1993). This suggests that oral bioavailability may differ between animal species depending on the extent of first-pass effect. However, caution should be taken in interpreting bioavailability and elimination half-life data in rhesus-monkey since the metabolites were not analyzed separately and the measurement corresponded to total radioactivity in plasma.
As a result of its high clearance, ATZ disappeared rapidly from blood with an apparent half-life of around 30 min after IV dosing, and also after oral dosing in animals in which it could be determined. Comparison cannot easily be made with data in the literature since ATZ has not been administered previously by IV route in rats. An apparent elimination half-life of approximately 11h has been reported previously after oral dosing at a dose of 30 mg/kg (Timchalk *et al.*, 1990). The much higher half-life value may result from the measurement of total radioactivity in plasma and not from the selective measurement of ATZ.

After repeated administration in pregnant rats, a double-peak phenomenon was noticed after either ATZ, PRO or SIM administration for both parent drugs and metabolites (Figure 3a-c). Such a phenomenon has already been described in previous studies with ATZ. After single dose ATZ (ranging from 5 to 250 mk/kg body weight) in mice, peak plasma ATZ was detected 1h post-dosing followed by a rapid decline. A second peak in plasma ATZ was noted at around 12h attributed to a discontinuity in the GI tract absorption (Ross *et al.*, 2009). Similarly, after single dose of ATZ (30 mg/kg body weight) in rats, peak plasma ATZ was detected 10h post-dosing followed by a rapid decline (Timchalk *et al.*, 1990). A rather slow absorption was also noticed in rats dosed at 90 mg/kg in rats (Tmax around 15 h post-dosing) and was attributed to either a slow transport across gut epithelium or to a slow dissolution from the ATZ suspension used for dosing (McMullin *et al.*, 2007). A PBPK study after administration of ATZ and of its chlorometabolites in rats confirmed the double-peak phenomenon with a longer plateau of compound in plasma suggesting a rate-limiting step for most the intestinal absorption (McMullin *et al.*, 2007).
In our study, the compounds were administered as a solution so that a slow-dissolution from solid particles is unlikely unless a precipitation occurred in the GI tract of the animals.

The blood exposure to parent compounds (ATZ, PRO and SIM) was negligible compared to metabolite exposure (lower than 5 %, Table 2). The main metabolite exposure involved DACT, ranging from 60 to 90 % depending on the molecules administered. The relative exposure to ATZ and metabolites were very closed after single and repeated oral dosing in normal and pregnant rats, respectively, and different of that observed after IV dosing.

These data suggest a significant pre-systemic metabolism of both compounds as previously shown for ATZ in rats. The higher exposure to DACT compared to ATZ, DIA and DEA may result from a more rapid clearance. Plasma levels in the same rank order were observed for ATZ in rats after oral gavage (from 90 mg/kg) (24). A model with re-parameterization including new *in vivo* data in rats and *in vitro* data in human hepatocytes (Campbell *et al.*, 2016) and indicated that the plasma exposure was in the following rank order DACT >> DIA > DEA >> ATZ. Similar results were observed in mice after oral gavage (from 5 to 250 mg/kg) with the plasma levels in the same rank order: DACT >> DIA > DEA >> ATZ (Lin *et al.*, 2011; Lin *et al.*, 2013).

Total blood exposure (measured by the AUC) increased of around 50-60% from D15 to D19 suggesting that steady-state was not reached (Table 2). However, the exposure to ATZ, SIM, PRO, DIA and DEA were similar at D15 and D20 suggesting that steady-state accumulation was reached for these compounds at D20 while the exposure to DACT still increased. Such phenomenon may be attributable mainly to DACT given its longer half-life (Figure 2 a-c) which is responsible of its accumulation in the body, and to its prevalent contribution to blood exposure.
The preferential dealkylation of the iso-propyl-group compared to the ethyl-group was suggested by the preferential formation of DIA over DEA after ATZ dosing (5-fold blood exposure). This was confirmed after PRO and SIM dosing, leading to DIA and DEA respectively with a 2 to 3-fold difference in blood exposure (Table 2).

Tissue exposure of pregnant rats

Given that the driving force to tissue distribution depends on the blood concentration and on permeability characteristics of the compounds, tissue-to-blood ratios were computed, and a correlation was checked with descriptors of lipophilicity and polarity.
A clear correlation was observed between mammary gland-to-blood and fat-to-blood ratios and physico-chemical descriptors (Figure 4 a-f) while no significant correlations was observed for the other tissues. This indicated that for triazine derivatives difference in lipophilicity and/or in the polarity of the molecules apparently played only a major role in the distribution in the most lipoidic tissues. Based on these data, fat tissues may act as a reservoir for the most lipophilic pesticides.

It appeared that the distribution of chlorotriazines and of their metabolites in the brain were not influenced by their differences in physico-chemical properties. On the whole, exposure to DACT contributed to 97 %, 98 % and 88 % to the total exposure after ATZ, PRO and SIM administration. Brain concentrations of ATZ were higher than the corresponding blood values (both in normal and pregnant-rats) while there were no apparent difference for the metabolites (Table 4). PRO and SIM brain levels (and of their corresponding metabolites)

were of the same order than blood levels. In a previous study in mice after ATZ administration (Ross *et al.*, 2009), DIA and DEA had modest concentrations in the liver, and were not detected in the brain. Such a different pattern in metabolite distribution compared to our study in rats, where DEA and particularly DIA reached quite high levels in blood and in tissues, may likely result from a more rapid conversion of DEA and DIA to DACT in mice leading to low systemic levels rather that a true difference in distribution.

Tissue exposure after oral administration in non pregnant rats has to be compared with caution with those in pregnant rats because the interval from last dosing is different (3h versus 24h) so that tissue-to-blood ratio may differ according to the blood and tissue elimination half-life of the different compounds. However, it appeared that brain and liver concentrations of DACT, DIA and DEA in rats were closed the corresponding blood values as in pregnant rats. Difference appeared in the liver for the parent compound ATZ that displayed higher exposure than blood in rats (with a high inter-animal variability). On the whole, these data suggest that pregnancy in rats does not seem to affect significantly ATZ and metabolites distribution in brain and liver.

Comparison with data of the literature is not easy and has to be done with caution because the study protocols differed in sampling time that influences greatly the ratio if the compound has different blood and tissue elimination half-lives, in animal species (mice or rats) and in matrices used (blood or plasma).
We have chosen to use blood levels in reporting the systemic exposure of the different compounds because ATZ is a high clearance drug so that blood concentrations better reflect the PK than plasma concentration. Furthermore this was more relevant for the comparison of the systemic exposure between the different compounds that have different lipophilicity and may have different red blood cells partitioning. For purpose of comparison to previous studies where systemic exposure is reported as plasma levels a correction with a red blood cell partitioning ratio should be performed for the different compounds. Red blood cell binding parameters for ATZ and DACT have been used in previous studies dealing with physiologically based pharmacokinetics modeling (Lin *et al.*, 2011; Lin *et al.*, 2013). Additionally, blood and plasma concentrations of radiolabeled ATZ as a function of time and dose administered have been reported indicating that blood concentration were approximately 10-times higher than plasma concentration (McMullin *et al.*, 2003). Hence, when comparing systemic exposure to ATZ an order of magnitude has to be roughly considered for between blood and plasma levels.

However, our brain distribution pattern can be compared with some data in the literature. Differences clearly appeared between previous studies carried out in mice and rats (Ross *et al.*, 2009; Lin *et al.*, 2011; Lin *et al.*, 2013). In mice, plasma levels higher than brain levels were reported for both ATZ and DACT (DEA and DIA were not detected in mice brain (Ross *et al.*, 2009; Lin *et al.*, 2011). However, plasma levels lower than in brain were measured in a study in rats reported by Lin *et al.* 2013. In the latter, brain to plasma ratio of ATZ and DACT (determined at 2h and 6h after the last dosing) were shown to be time-dependent for ATZ (1.4 and 4.3 at 2h and 6h respectively) and not for DACT (close to 1 at both 2h and 6h). The increase in the brain to plasma ratio as the delay after dosing increased may result from a slower elimination half-life of ATZ from brain compared to its elimination from the systemic circulation. The fact that DACT has a longer elimination half-life than ATZ may explained that

tissue-to-plasma ratio for DACT were less-dependent on the sampling time. This highlights that sampling time may be a confounding factor when comparing tissue-to-blood or plasma ratio between species, more especially for compounds having relatively short elimination half-life. It is a limitation of our study that the evaluation of the tissue distribution was only performed at 24 hour post-dosing (without intermediate samplings), and hence the tissue exposure should be considered as such. Depending on the respective elimination half-life of the compounds in the different tissues, tissue-to-blood ratios should vary, especially in early times after administration where variations in concentrations are quite significant so that tissue-to-blood ratios are very sensitive to sampling times.

In adult rats, plasma and brain levels have been shown to be quite close for ATZ and DACT (Fraites *et al.*, 2011; Lin *et al.*, 2013). Considering a blood to plasma ratio for ATZ of approximately 10, this suggests that blood levels should exceed brain levels which is exactly the opposite of what we found (Table 4).
Our results are more comparable to those reported in rat neonates (Fraites *et al.*, 2011) after oral exposure to ATZ (5 and 25 mg/kg) during the gestation. Indeed, in neonates, the DACT, DIA and DEA levels in plasma and in brain were quite similar while ATZ levels in the brain were higher than in plasma (around 30 to 50-fold at 5 and 25 mg/kg dosing). In dam, plasma and brain levels of ATZ and DACT were close while brain levels of DIA and DEA were lower than in plasma.

Tissue exposure of fetuses

To our knowledge, this is the first report of fetal tissue of chlorotriazines in animals. Previous studies have reported fetus distribution of ATZ and DACT on a per whole fetus basis in rats (Fraites *et al.*, 2011).
It appeared that DACT accounted for at least 99 % of the total triazine exposure in fetal brain and liver after ATZ and PRO administration, and accounted for 90 % of total exposure after SIM administration. Indeed as a result of the preferential dealkylation of the iso-propyl-group leading to DIA after administration of SIM, exposure to DIA was around 10 %. This contribution of DACT to total exposure appeared higher than that reported previously in rat fetuses (between 60 and 90 % based on whole fetus concentration, Fraites *et al.*, 2011).

The data in Table 4 suggest that there is no difference in the distribution of the different compounds in the fetal tissues after administration of ATZ (in brain and liver), SIM and PRO (in brain and liver).
The data in Table 4 also showed the placental barrier may not play a significant protective role as a limiting factor because the fetal tissue concentrations were close to maternal blood concentrations. Furthermore, placenta concentrations were either close of higher than blood concentrations (Table 4). These findings are consistent with previous studies based on whole fetus concentrations suggesting that the placental barrier did not appear to limit the transfer of ATZ and its metabolites in rats (Fraites *et al.*, 2011; Lin *et al.*, 2013).
More precisely, our data showed that only ATZ and PRO distributed significantly more in maternal brain than in the fetal brain with maternal-to-fetal brain ratios of 4.3 and 2,2, respectively (Table 4). Differences in distribution of xenobiotics in fetal brain may result from the influence of the two protective barriers, blood brain barrier and placental barrier, and/or

from the affinity for brain tissue. Differences in brain distribution of xenobiotics between adult and fetuses may arise from differences in brain lipid content. Indeed, brain contains a complex mixture of lipids with uniform lipid-rich structures (revealed by magnetic resonance imaging) with changes in major lipids during the developmental and the aging process (Dawson, 2015). Hence, as a high lipid content tissue, differences in brain distribution between adult and fetus may be more easily evidenced for compounds of high lipophilicity such as ATZ and PRO.
Bias in the brain distribution study of DACT should be considered since previous studies have shown that DACT can form stable protein adducts in brain of adult rats (Dooley *et al.*, 2010). Hence, free DACT concentrations measured in brain may not reflect the total DACT concentrations.

In conclusion, our study of ATZ pharmacokinetics suggested a significant first-pass effect explaining the low oral bioavailability. A correlation between tissue-to-blood ratio and physico-chemical descriptors of the 6 chlorotriazine derivatives was observed for fat and mammary gland tissues but not for brain in adult rats indicating that lipophilicity is not the main distribution driver for all tissues. Furthermore, our specific study of the distribution in fetal tissues showed a more pronounced distribution in fetal brain for ATZ and PRO, the two most lipophilic compounds. These data could be used to improve existing rat PBPK models, especially for the fetal internal distribution and internal (target organ) dosimetry prediction in fetal tissues.

Acknowledgements

This work was partially supported by the French Ministry for the Environment and Sustainable Development (Antiopes, project EXPIOMARK, INERIS). We thank the animal house platform ARCHE (SFR Biosit, Rennes, France) and Laurence Bernard-Touami for her assistance. We thank also Yu Augagneur for her technical assistance.

FIGURE LEGEND

Figure 1: Chemical structures and physico-chemical characteristics of atrazine (ATZ), propazine (PRO), simazine (SIM) and their metabolites DIA, DEA and DACT.

Figure 2a-b: Blood concentration-time profile of atrazine (ATZ) in non-pregnant rats after IV (2a, n = 6) and oral dosing (2b, n = 6) at a dose of 2.5 and 10 mg/kg, respectively.

Figure 3a-c: Blood concentration-time profile of atrazine, propazine, simazine (ATZ, PRO and SIM) and their respective metabolites (DEA, DIA and DACT) in pregnant rats after daily oral dosing from D15 to D19 (1b) at a dose 10 mg/kg of atrazine (3a, n = 6), propazine (3b, n = 3) and simazine (3c, n = 3), respectively.

Figure 4a-f: Scatter plots illustrating the relationships between mammary gland-to-blood ratios (4a-c) and fat -to-blood ratios (4d-f) and some physico-chemical properties of triazines (Log P, log D and % TPSA, percentage of polar surface area).

TABLE LEGEND

Table 1: Mean absolute (10^3 nmol.h/L) and relative (%) maternal blood exposure to atrazine (ATZ), DEA, DIA and DACT after single IV (2.5 mg/kg, n = 6) and oral (10 mg/kg, n = 6) dosing in rats.

Footnote : The absolute exposure is expressed by the AUC computed from time zero to the end of the experiment at 180 min.

Table 2: Mean absolute (10^3 nmol.h/L) and relative (%) maternal blood exposure to atrazine, propazine, simazine (ATZ, PRO and SIM) and their respective metabolites (DEA, DIA and DACT) at days of first (D15) and last (D19) administration after daily oral dosing from D15 to D19 of atrazine (n = 6), propazine (n = 3) and simazine (n = 3) at a dose 10 mg/kg in pregnant rats.

Footnote : The absolute exposure is expressed by the AUC (10^3 nmol.h/L) computed from time zero to 24 h at D15 and D19.

Table 3: Blood, liver and brain concentrations (nM, n = 6) of atrazine (ATZ), DEA, DIA and DACT after single IV (2.5 mg/kg, n = 6) and oral (10 mg/kg, n = 6) dosing in adult rats. Samples were collected at 180 min after dosing.

Table 4: Maternal blood, maternal tissue and foetal tissue concentrations (nM) to atrazine, propazine, simazine (ATZ, PRO and SIM) and their respective metabolites (DEA, DIA and DACT) at D20 after daily oral dosing of atrazine (from D15 to D19, n = 6 and from D1 to D19, n = 3), propazine (from D15 to D19, n = 3) and simazine (from D15 to D19, n = 3) at a dose 10 mg/kg in pregnant rats.

Footnote : Maternal concentrations were calculated from tissues of 9 (ATZ) or 3 (SIM and PRO) animals. In each animal and in each organ, 3 independent tissue samples were analyzed. For each sample a tissue-to-blood ratio was computed. Fetal concentrations were calculated from tissues of 3 independent fetuses. For each individual sample a tissue-to-maternal blood ratio was computed.

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Table 1

		Absolute exposure					Relative exposure			
Compound administered		ATZ	DACT	DEA	DIA	Total Exposure	ATZ	DACT	DEA	DIA
ATZ	IV	4.79	5.12	1.17	3.97	15.04	32	34	8	26
	VO	0.65	21.16	1.60	4.33	27.73	2	76	6	16

Table 2

		Absolute exposure							Relative exposure			
Compound administered	Day of study	ATZ	PRO	SIM	DACT	DEA	DIA	Total Exposure	DACT	DEA	DIA	Compound administered
ATZ	D15	4.3			107.0	7.3	33.0	151.6	70.6	4.8	21.8	2.8
	D19	3.3			193.9	7.3	44.2	248.7	78.0	2.9	17.8	1.3
PRO	D15		2.4		147.5	20.0		169.9	86.8	11.8		1.4
	D19		3.1		223.0	34.2		260.3	85.7	13.1		1.2
SIM	D15			11.2	118.9		66.7	196.8	60.4		33.9	5.7
	D19			8.0	225.5		66.9	300.5	75.1		22.3	2.7

Table 3

		ATZ	DACT	DEA	DIA
IV	Blood	3.4	22.3	0.5	3.2
	Liver	0.1	64.4	1.1	3.2
	Brain	0.1	81.1	1.0	4.2
VO	Blood	1.2	97.3	5.1	17.3
	Liver	24.3	92.2	5.0	14.8
	Brain	1.7	88.5	5.0	14.8

Table 4

	ATZ		PRO		SIM		DACT		DEA		DIA	
	Concentration (nM)	SD	Concentration (nM)	SD	Concentration (nM)	SD	Concentration (nM)	SD	Concentration (nM)	SD	Concentration (nM)	SD
Brain (maternal)	**23**	13					**4141**	2494	**15**	19	**99**	163
Brain (fetus)	**6**	6					**7538**	1199	**15**	17	**80**	122
Liver (maternal)	**6**	7					**3914**	1915	**7**	10	**67**	55
Liver (fetus)	**2**	1					**8991**	3211	**23**	17	**68**	129
Amniotic liquid	**8**	8					**7462**	2005	**28**	19	**76**	109
Placenta	**5**	6					**15372**	4155	**31**	65	**107**	143
Mammary gland	**36**	39					**4699**	930	**57**	74	**119**	210
Fat	**235**	31 5					**854**	245	**56**	68	**87**	117
Muscle	**13**	9					**11610**	2615	**27**	30	**112**	131
Spleen	**43**	92					**7861**	1543	**109**	127	**118**	137
blood	**7**	7					**4286**	1998	**23**	22	**88**	153
Brain (maternal)			**26**	20			**7837**	945	**94**	60		
Brain (fetus)			**12**	2			**6745**	994	**63**	39		
Liver (maternal)			**11**	5			**1885**	379	**15**	7		
Liver (fetus)			**12**	3			**7375**	1148	**65**	42		
Amniotic liquid			**13**	3			**7564**	1729	**64**	39		
Placenta			**17**	8			**8408**	851	**93**	59		
Mammary gland			**141**	31			**5606**	1014	**164**	105		
Fat			**1373**	400			**809**	131	**270**	145		
Muscle			**31**	11			**10541**	1642	**112**	74		
Spleen			**34**	23			**6663**	841	**77**	30		
blood			**22**	7			**7483**	723	**113**	52		

Brain (maternal)	**102**	65	**6859**	1180	**811**	450
Brain (fetus)	**82**	49	**7062**	1813	**714**	382
Liver (maternal)	**163**	74	**2495**	672	**114**	58
Liver (fetus)	**106**	66	**7801**	1878	**606**	345
Amniotic liquid	**137**	106	**7609**	2047	**1039**	496
Placenta	**149**	90	**9774**	2350	**1195**	652
Mammary gland	**669**	490	**4893**	1540	**936**	502
Fat	**1322**	725	**900**	245	**759**	396
Muscle	**200**	175	**9917**	1990	**1059**	577
Spleen	**390**	284	**6759**	1387	**505**	284
blood	**172**	107	**7970**	2391	**1321**	809

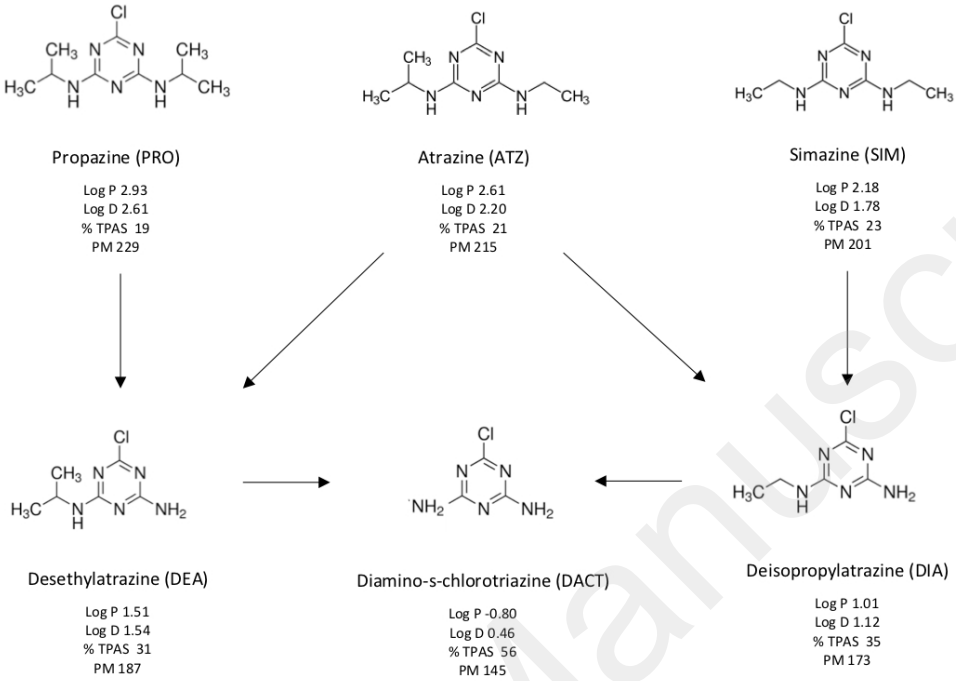

Figure 1: Chemical structures and physico-chemical characteristics of atrazine (ATZ), propazine (PRO), simazine (SIM) and their metabolites DIA, DEA and DACT.

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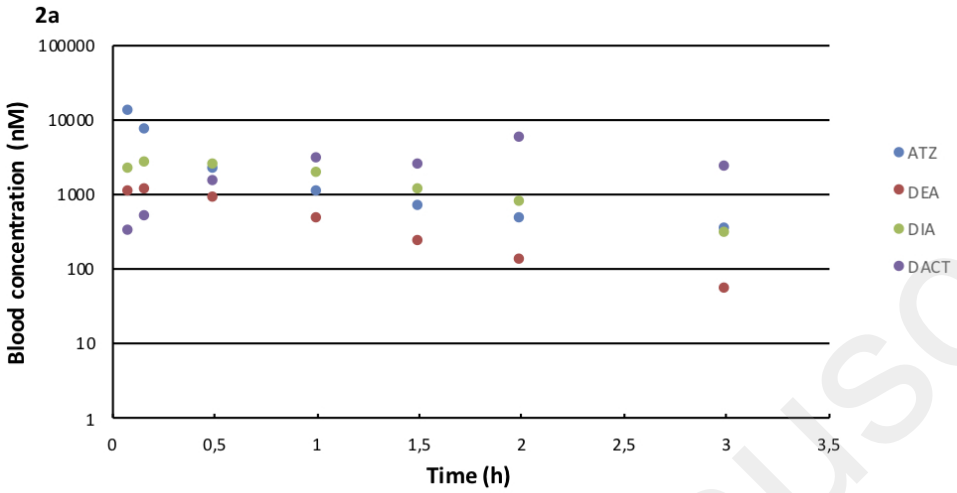

Figure 2a-b: Blood concentration-time profile of atrazine (ATZ) in non-pregnant rats after IV (2a, n = 6) and oral dosing (2b, n = 6) at a dose of 2.5 and 10 mg/kg, respectively.

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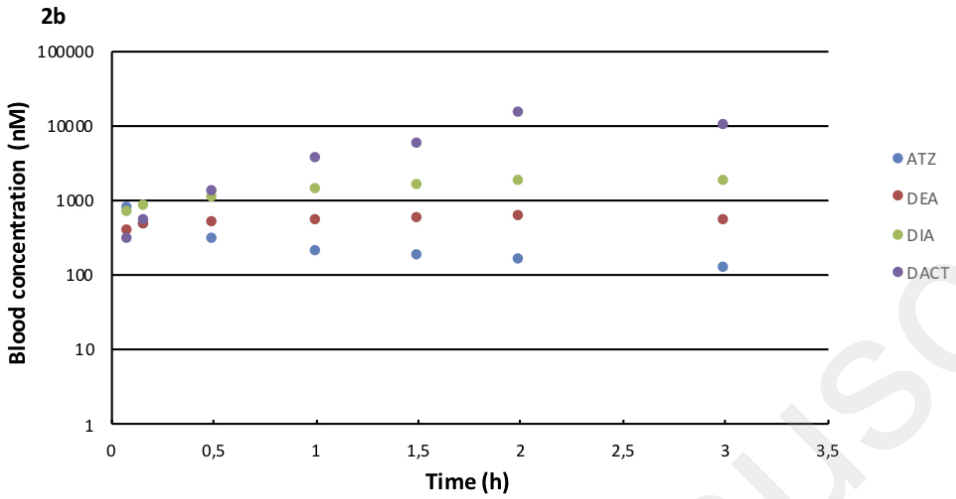

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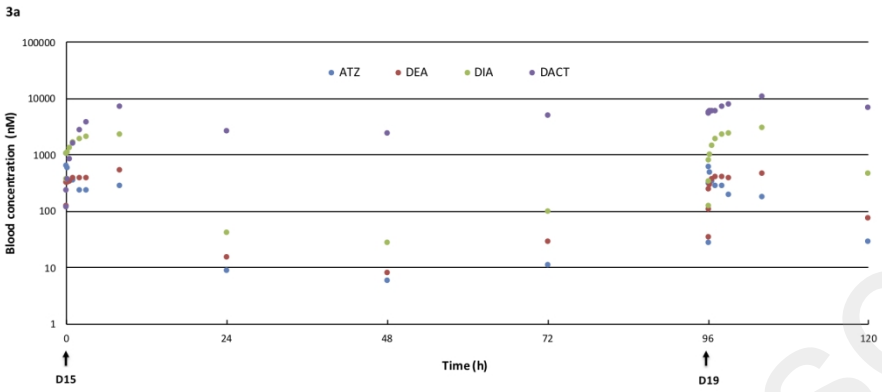

Figure 3a-c: Blood concentration-time profile of atrazine, propazine, simazine (ATZ, PRO and SIM) and their respective metabolites (DEA, DIA and DACT) in pregnant rats after daily oral dosing from D15 to D19 (1b) at a dose 10 mg/kg of atrazine (3a, n = 6), propazine (3b, n = 3) and simazine (3c, n = 3), respectively.

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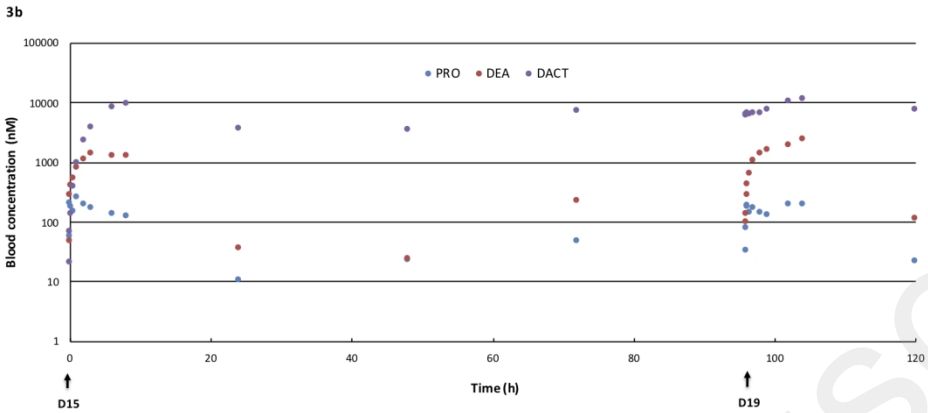

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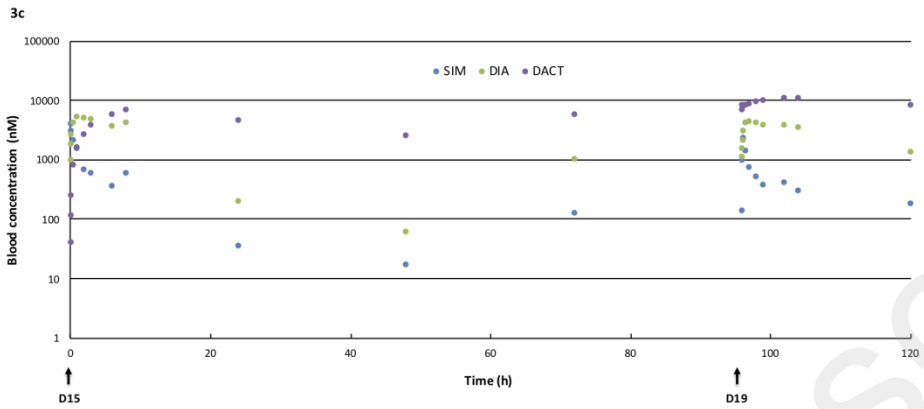

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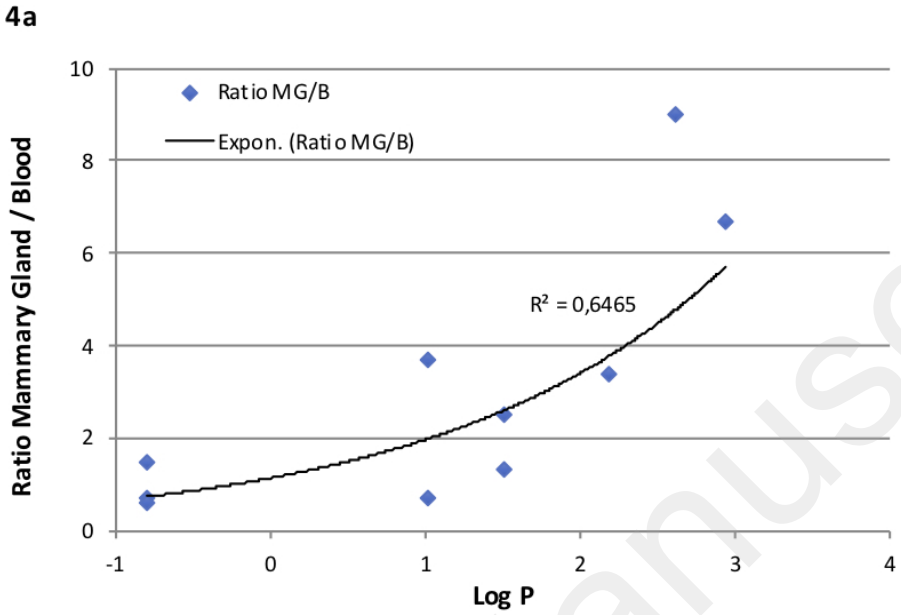

Figure 4a-f: Scatter plots illustrating the relationships between mammary gland-to-blood ratios (4a-c) and fat -to-blood ratios (4d-f) and some physico-chemical properties of triazines (Log P, log D and % TPSA, percentage of polar surface area).

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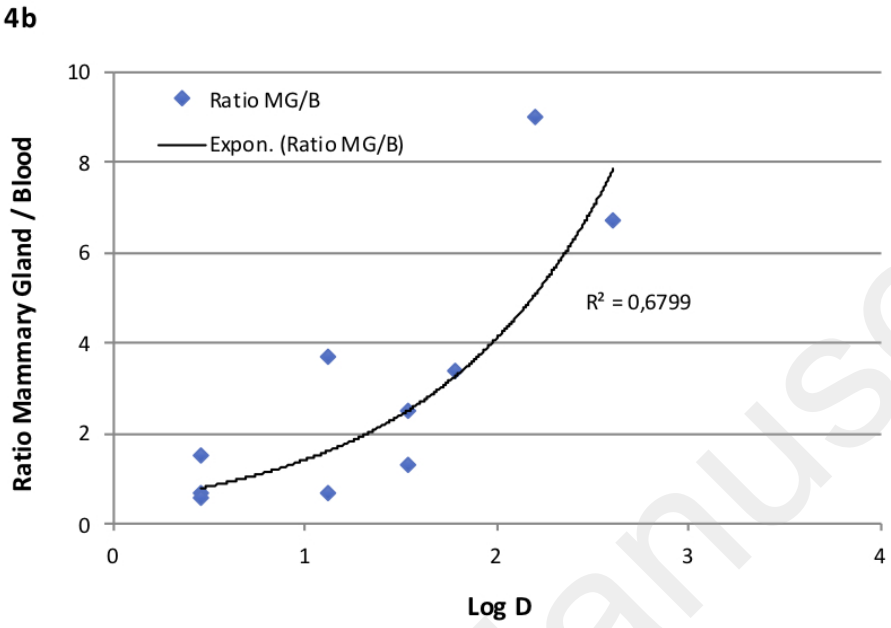

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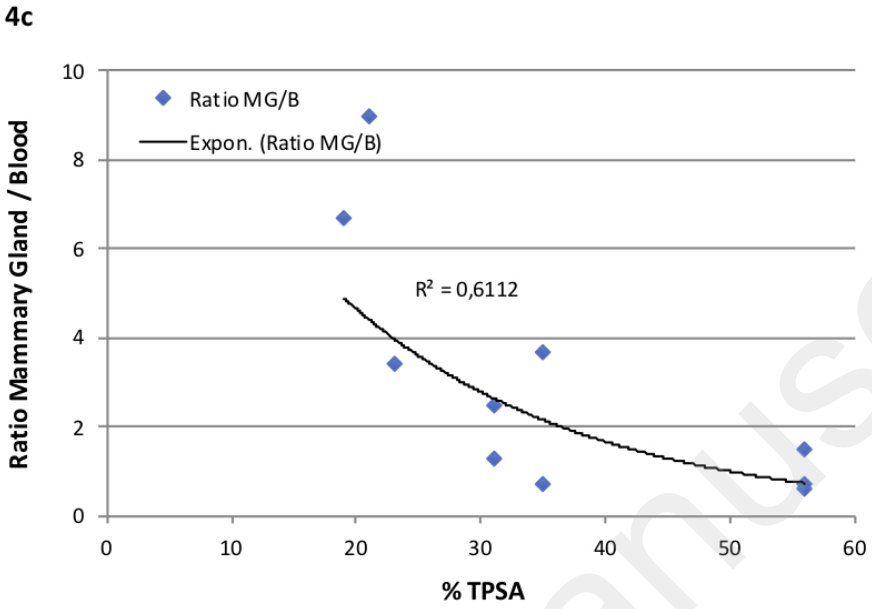

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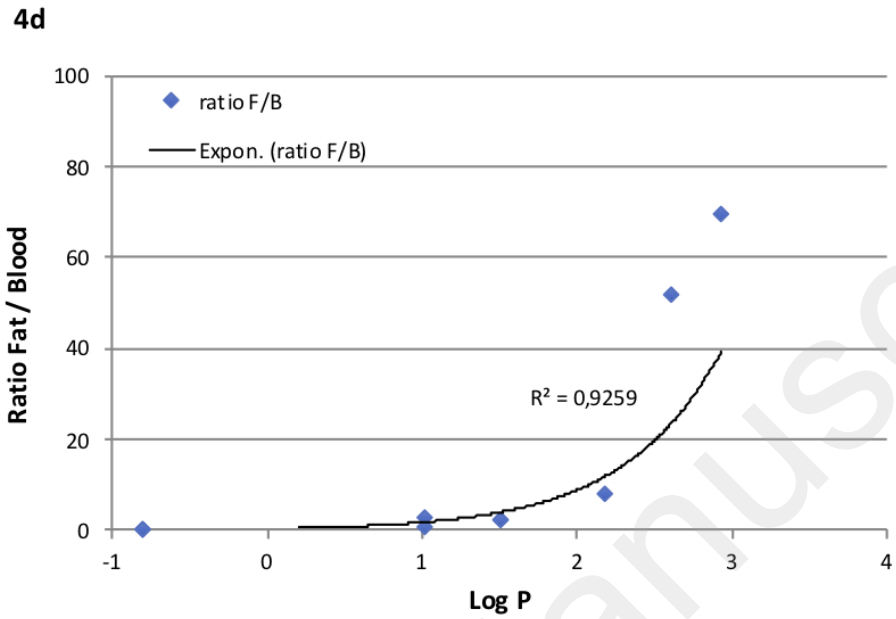

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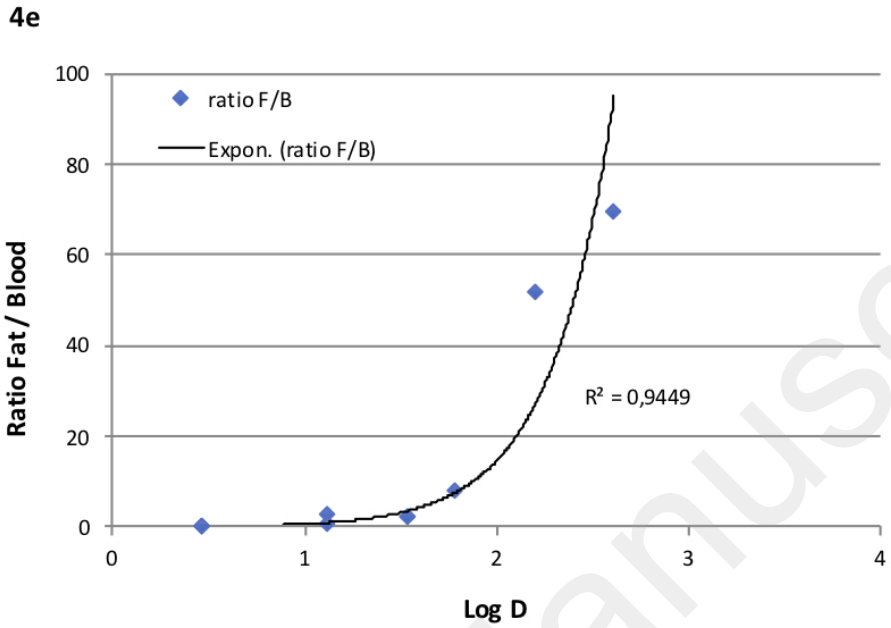
148x96mm (150 x 150 DPI)



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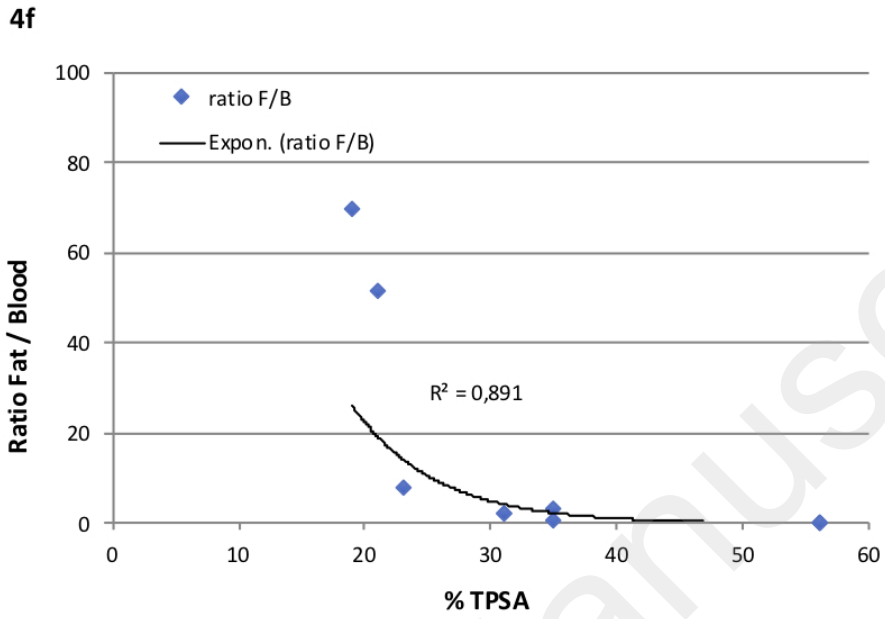

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