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Differences in teratogenicity of some vitamin K antagonist substances used as human therapeutic or rodenticide are due to major differences in their fate after an oral administration

Thomas Chetot¹, Marjorie Mouette-Bonnet¹, Shira Taufana¹, Isabelle Fourel¹, Sébastien Lefebvre¹, Etienne Benoit¹, Virginie Lattard¹

Abstract

All vitamin K antagonist active substances used as rodenticides were reclassified in 2016 by the European authorities as active substances "toxic for reproduction", using a "read-across" alternative method based on warfarin, a human vitamin K antagonist drug. Recent study suggested that all vitamin K antagonist active substances are not all teratogenic. Using a neonatal exposure protocol, warfarin evokes skeletal deformities in rats, while bromadiolone, a widely used second-generation anticoagulant rodenticide, failed to cause such effects. Herein, using a rat model we investigated the mechanisms that may explain teratogenicity differences between warfarin and bromadiolone, despite their similar vitamin K antagonist mechanism of action. This study also included coumatetralyl, a first-generation active substance rodenticide. Pharmacokinetic studies were conducted in rats to evaluate a potential difference in the transfer of vitamin K antagonists from mother to fetus. The data clearly demonstrate that warfarin is highly transferred from the mother to the fetus during gestation or lactation. In contrast, bromadiolone transfer from dam to the fetus is modest (5% compared to warfarin). This difference appears to be associated to almost complete uptake of bromadiolone by mother's liver, resulting in very low exposure in plasma and eventually in other peripheral tissues. This study suggests that the pharmacokinetic properties of vitamin K antagonists are not identical and could challenge the classification of such active substances as "toxic for reproduction".

Keywords

Antivitamin K anticoagulants; warfarin; rodenticide; Bromadiolone; teratogenicity; Fetal warfarin syndrome; pharmacokinetics; risk assessment

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Introduction

In June 2016, the EU Member States, in line with the recommendation of the European Chemicals Agency (ECHA) reclassified all vitamin K antagonist active substances used as anticoagulant rodenticides (AR) as 'toxic to reproduction'. The active substances concerned by this classification are the first-generation anticoagulant rodenticides (FGAR) i.e., warfarin, chlorophacinone, coumatetralyl, and the second-generation anticoagulant rodenticides (SGAR) i.e., brodifacoum, bromadiolone, difenacoum, difethialone and flocoumafen. War-

farin and brodifacoum have been classified as reprotoxic with proven toxicity for embryo development (reprotoxic category 1A), and the other (chlorophacinone, coumatetralyl, difenacoum, flocoumafen, bromadiolone and difethialone) with supposed toxicity for embryo development (reprotoxic category 1B (UE) 2016/1179). All these active substances, except warfarin which is no longer used as rodenticide but used as human therapeutic, are extensively used worldwide by the oral route for rodent management and are almost the only active substances approved for this application [1].

Classification of these ARs as active substances “toxic to reproduction” has major consequences on the use of these active substances in Europe. Any bait with an AR concentration of 30 µg/g or more has been reclassified as ‘toxic to reproduction’. Since March 1, 2018, no product classed this way can be sold to amateurs and professional use products will have to carry the warning symbol and the ‘May harm the unborn child’ wording on their labels.

This classification of all ARs was decided according to a “read-across” approach based on observations obtained for warfarin. The “Read-across” approach is one of the most commonly used alternative approaches for data gap filling in registrations submitted under the REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) regulation in the European Union. It involves the use of relevant information from analogous substance(s) to predict certain physicochemical properties, mammalian toxicity, environmental fate and ecotoxicity for the ‘target’ substance(s) under consideration. This approach consisting to prevent as much as possible any animal experiment leads ineluctably to some misclassification. Warfarin was first marketed in 1948 as a rodenticide. In 1954, warfarin transitioned into clinical use under the trade name “Coumadin” [2–4] and has become the most commonly used human drug to prevent thromboembolic disorders. Since, warfarin has been the most commonly drug used in human medicine to prevent thromboembolic disorders and is now no longer used as a rodenticide. Unfortunately, the prescription of warfarin to pregnant women has been shown to be sometimes responsible for congenital abnormalities known as “fetal warfarin syndrome”, characterized by skeletal malformations, i.e. nasal hypoplasia (hypoplasia of the maxillary bones) and chondrodysplasia punctata [5, 6].

Warfarin is behind the discovery of all the ARs. Indeed, they all have a common structure, i.e. a 4-hydroxycoumarin ring, with warfarin and/or a mechanism of action similar to that of warfarin, i.e. inhibitor of the vitamin K epoxide reductase involved in the recycling of vitamin K by catalyzing the reduction of the vitamin K epoxide to vitamin K (Stafford, 2005). Vitamin K is essential for the carboxylation of many proteins known as vitamin K-dependent proteins - coagulation factors II, VII, IX and X, matrix Gla protein and osteocalcin. These characteristics justified the classification of all ARs as “toxic to reproduction” according to the read-across method.

The teratogenic effect of warfarin was experimentally re-

produced using a rat model, whereas no effect was observed when bromadiolone was used under the same experimental conditions. Indeed, warfarin administered orally daily at high doses [5, 7] or at therapeutic doses [7] to newborn pups leads to a shortening of the growth of facial bones and to a lesser extent long bones. Daily administration of the same dose of bromadiolone under the same conditions does not result in impaired bone growth [7]. These differences challenge the classification of all ARs as “toxic to reproduction” based on he read-across alternative method applied in the absence of data. The aim of this study was to decipher the origin of this observed difference between warfarin, a first-generation vitamin K antagonist, and bromadiolone, a second-generation vitamin K antagonist widely used as rodenticide. Since warfarin is no longer used as rodenticide, this study includes coumatetralyl, a first-generation vitamin K antagonist like warfarin, but still used as rodenticide. As the origin of the difference does not seem to be explained by the pharmacodynamic properties of the active substances, the potential role of pharmacokinetics in explaining the dichotomy was investigated in this study, the differences in pharmacokinetic properties being at the origin of the classification of ARs into two generations, the second generation including highly efficient in a single dose and highly tissue-persistent active substances [8–11]. Herein, we analyzed the differences in distribution among the three active substances during gestation and lactation period.

1. Material and methods

1.1 Chemicals

Warfarin and coumatetralyl were purchased from Sigma-Aldrich (l’Isle d’Abeau, Chesnes, France). Bromadiolone (85% trans/15% cis) were supplied by Liphatech (Agen, France). Dimethylsulfoxide, acetonitrile, methanol, acetone, diethyl ether, and orthophosphoric acid were obtained from VWR International (Fontenay sous bois, France). Vitamin K1 was obtained from TVM (Lempdes, France), and oxytocin 10 UI/mL from Alcyon (Paris, France). High-performance liquid chromatography (HPLC) grade water was prepared using a milli-Q plus system (Millipore, Saint-Quentin en Yvelines, France) and used for the preparation of HPLC eluents. Warfarin, bromadiolone and coumatetralyl were dissolved in 5% of DMSO and 95% of corn oil (Sigma-Aldrich) for per os administration.

1.2 Ethics statement and animals

Animal experiment was reviewed and approved by France government under the European Communities Council Directive of 24 November 1986 (86/609/EEC) and experimental procedures involving animals were performed according to an experimental protocol approved (Authorization n°2017041812474440) from the ethics committee of the Veterinary School of Lyon. Environment, housing and management of rats were in compliance with rat animal welfare and ARRIVE guidelines. Adult female OFA Sprague-Dawley rats (8-weeks old, 175-200g) were obtained from a commercial

breeder (Charles Rivers, L'Arbresle, France) and were acclimated for a minimum of 10 days. They were housed four per cage under a constant photoperiod and ambient temperature. Animals were kept in standard cages, Eurostandard, Type IV (Tecniplast, Limonest, France) and received standard feed (Scientific Animal Food and Engineering, reference A04) and water ad libitum.

1.3 Fetal exposure to vitamin K antagonists

Female OFA Sprague Dawley rats (10 weeks old) were mated with male OFA Sprague Dawley rats. The date of fertilization was determined by eosine/thiazine stained cervical smears (Aerospray, EliTechGroup), with the presence of sperm corresponding to the first day of gestation. From the day D+17 of gestation and for three consecutive days, the rats received daily oral administration of 0.5 mg/kg body weight of bromadiolone, warfarin or coumatetralyl (4 rats per anticoagulant). The dose of 0.5 mg/kg corresponded to the estimated ED50 of bromadiolone (the effective dose capable of inducing a 5-fold increase in prothrombin time in 50% of the animals 24 hours after dosing), so the rats received a cumulative dose for all active substances of 1.5 mg/kg (vehicle volume (corn oil) did not exceed 1 mL/kg of rat body weight). Rats received a co-injection of vitamin K1 (10 mg/kg body weight) to protect them from the hemorrhagic effect of the anticoagulant. At day D+20 of gestation, the rats were euthanized. Fetuses were removed from the placenta. Livers from the dam and fetuses, and the remainder of the fetuses were collected and frozen to determine the concentrations of anticoagulant.

1.4 Exposure during the suckling period

Female OFA Sprague Dawley rats (10 weeks old) were mated with male OFA Sprague Dawley rats. After parturition, from day D+3 of the lactation period and for three consecutive days, suckling Sprague Dawley rats (10-weeks old) received daily oral administration of 0.5 mg/kg of bromadiolone, warfarin or coumatetralyl (7 rats per anticoagulant), so the rats received the same cumulative dose for all anticoagulants (1.5 mg/kg body weight - vehicle volume (corn oil) did not exceed 1 mL/kg of rat body weight).

Twenty-four hours after administration of the initial AR dose, milk from lactating females was recovered. For this, lactating females were separated from their litters for up to 60 minutes and then received an intramuscular injection of oxytocin (2 IU/animal). Ten minutes after injection, 100 μ l of milk was recovered by capillary aspiration under volatile anaesthesia (isoflu vet, Alcyon, France) and frozen for subsequent determination of anticoagulant concentration.

At day D+6 of the lactation period, female rats and their newborns were euthanized. Liver from the dam and the newborns, and the remaining body of the pups were then collected and frozen to determine anticoagulant concentration.

1.5 Pharmacokinetics study

Female OFA Sprague-Dawley rats (10 weeks-old) received under standard feed either a unique per os administration

of warfarin (2.8 mg/kg body weight; n=36 rats, 4 rats per timeframe) or bromadiolone (0.6 mg/kg body weight; n=40 rats, 4 rats per timeframe). Warfarin and bromadiolone were solubilized in corn oil with 5% of dimethyl sulfoxide (DMSO) (vehicle volume was 1 ml/kg body weight). Pharmacokinetics studies were carried out during 5 days for warfarin and 21 days for bromadiolone. Animals were given a daily subcutaneous injection of vitamin K1 (10 mg/kg) to prevent hemorrhaging. One, 2, 4, 6, 824, 48/72, 72/168, 96/336, and 504 hours after warfarin or bromadiolone administration, four rats were anesthetized with isoflurane and blood was taken by cardiac puncture into citrated tubes. Finally, rats were euthanized with CO₂ and the liver of each rat was immediately collected and stored at -20°C until analysis.

1.6 Anticoagulant molecule quantification

Anticoagulant residues in plasma, milk, liver or body were quantified as previously described [12, 13]. The limit of quantification (LOQ) of warfarin and bromadiolone was 20 and 0.2 ng/mL for plasma, 1 and 2 ng/g for liver, fetus and newborn body, and 1 ng/mL for milk respectively.

1.7 Statistical analysis

A non-compartmental analysis was carried out using Phoenix®WinNonlin®8.0; Pharsight corporation St Louis MO USA. Linear trapezoidal rule was selected for calculations and the estimation of the terminal slope for extrapolation to infinity was done by regression with the best-fit option of Phoenix. A weighting factor of 1/Y was used to estimate the slopes. Area Under the Curve (AUC) and the Mean Residence Time (MRT) for plasma and liver with and without extrapolation to infinity were computed according to Gibaldi and Perrier [14]. The apparent plasma clearance (Cl/F(0-infinity)) was computed by dividing the administered dose by AUC(0-infinity). The apparent steady-state volume of distribution (V_{ss}/F(0-infinity)) was estimated by the product of the mean Cl/F and the mean MRT(0-inf). Descriptive statistics (arithmetic mean, SD...) were obtained with the statistical tool of Phoenix.

Notwithstanding pharmacokinetic parameters, statistical analysis was done using GraphPad Prism 6 software (CA, USA). A Dunn's multiple comparison test was used with $\alpha;0.05$ in order to compare statistically the results among groups.

2. Results

2.1 Assessment of the fetal exposure to vitamin K antagonists

After 3 successive oral administrations of warfarin, coumatetralyl or bromadiolone (0.5 mg/kg/day for 3 days) at gestation days D+17, D+18 and D+19, pregnant OFA-Sprague Dawley females showed no signs of hemorrhage until 24 hours after the last administration. Liver weights of pregnant females were between 10 and 15g; number of pups per litter between 11 and 13; average fetal weight between 2.1 and 5.2g depending on

the litter; average fetal liver weight between 0.15 and 0.33g depending on the litter. Anticoagulant concentrations were determined in liver of females and in liver and extrahepatic tissues of fetuses (Figure 1). Bromadiolone concentration was significantly lower in fetal liver or extrahepatic tissues and was significantly higher in female liver compared to warfarin and coumatetralyl. To assess transplacental passage, the total amounts of AR present in the mother's liver and in all fetuses of the litter were calculated by taking into account the number of pups in the litter, the weight of the livers and the bodies of the fetuses. A placental transfer index corresponding to the ratio of the quantities found in the mother to the quantities found in all of these fetuses was calculated for the three active substances. The results are presented in Figure 1D. This index varies from 1.1 to 2.9 for warfarin, from 0.6 to 1.7 for coumatetralyl, and from 0.05 to 0.16 for bromadiolone. At the same initial dose, this placental transfer index is thus twice as high for warfarin compared to coumatetralyl, and 20 times as high for warfarin compared to bromadiolone.

2.2 Assessment of the exposure of newborns to AR by the mother's milk

After three successive oral administrations of warfarin, coumatetralyl or bromadiolone (0.5 mg/kg body weight/day for three days) at days D+3, D+4 and D+5 post-partum, lactating OFA-Sprague Dawley females (7 females per AR) showed no signs of hemorrhage until 24 hours after the last administration. AR exposure of newborns via milk was determined and compared with the exposure of the lactating females. Liver weights of lactating females were between 10 and 15g; number of pups per litter between, 8 and 15; average newborn weight, 6.1g; average newborn liver weight, 0.39g depending on the litter. AR concentrations were determined in liver of females and in liver and extrahepatic tissues of newborns (Figure 2). Bromadiolone concentration was significantly lower in newborns liver or extrahepatic tissues and was significantly higher in female liver compared to warfarin and coumatetralyl. To assess the exposure of newborns through milk, the total amounts of AR present in the mother's liver and in all newborns of the litter were calculated by taking into account the number of newborns in the litter, the weight of the livers and the bodies of the newborns. A milk transfer index corresponding to the ratio of the quantities found in the mother to the quantities found in all of these newborns was calculated for the three active substances. The results are presented in Figure 2D. At the same initial dose, the warfarin milk transfer index is 2.7 times higher than that obtained for coumatetralyl and 31.6 times higher than that obtained for bromadiolone. AR concentrations in milk were also determined. At the same AR dose administered to lactating females (0.5 mg/kg body weight), concentrations of bromadiolone in milk were approximately 8 times lower than that of warfarin 24 hours after administration, with coumatetralyl being intermediate.

2.3 Comparative pharmacokinetics study

To compare the pharmacokinetics of warfarin and bromadiolone in female rats, these active substances were administered orally to female rats. The dose administered corresponded to the estimated ED₅₀, i.e. the dose resulting in a 5-fold increase in prothrombin time in 50% of the animals 24 hours after dosing (estimated ED₅₀ for warfarin and bromadiolone are, respectively, 2.8 mg/kg and 0.6 mg/kg body weight). The change in AR concentrations was then determined in plasma (in blue) and liver (in red) and are presented in Figure 3A for warfarin and Figure 3B and 3B1 for bromadiolone, figure 3B1 corresponding to an enlargement of figure 3B. The concentrations of warfarin and bromadiolone measured in liver and plasma were assessed using a non-compartmental analysis which enabled determination of pharmacokinetic parameters of warfarin and bromadiolone in liver and plasma without performing assumption on the pharmacokinetic model of these compounds (Table 1). The area under the curve for warfarin in liver was 6-times lower than that calculated for bromadiolone. Area under the curve for warfarin in plasma was 60-times higher than that calculated for bromadiolone while the plasmatic MRT of bromadiolone is significantly greater than that of warfarin.

3. Discussion

The fetal warfarin syndrome observed in humans can be reproduced in newborn rats [5] by daily administrations of warfarin for one month at the human therapeutic dose to lactating rats just after birth of their offspring. The same protocol performed with bromadiolone, a SGAR, does not lead to this fetal syndrome [7]. However, pharmacological properties (including pharmacodynamics and pharmacokinetics properties) of ARs are considered equivalent. According to these results, pharmacokinetics properties of ARs are clearly not equivalent. A pharmacodynamic origin leading to this difference in teratogenicity can be excluded, the mechanism of action i.e., the inhibition of VKORC1, being the same [15]. A pharmacokinetic origin leading to a difference in fetal/newborn exposure seems more likely.

In order to explore this hypothesis, we first evaluated the fetal exposure during gestation and the newborn exposure via its mother's milk. This evaluation concerned warfarin (drug at the origin of the reprotoxic classification of ARs), coumatetralyl (first generation AR, moderately toxic and not very persistent in the liver) and bromadiolone (second generation AR, highly toxic and highly persistent in the liver). Exposure of fetuses or newborns was assessed by measuring the amount of AR found in the liver of the exposed mother (in adults, ARs are preferentially located in the liver and are almost absent from other tissues) compared to the amount found in newborns/fetuses. The 3 ARs considered in this study have been detected in fetuses and newborns. These three active substances are thus able to cross the placental barrier and are excreted in milk, as suggested previously [16–18]. However, the transfer from dam to fetus varies dramatically among the

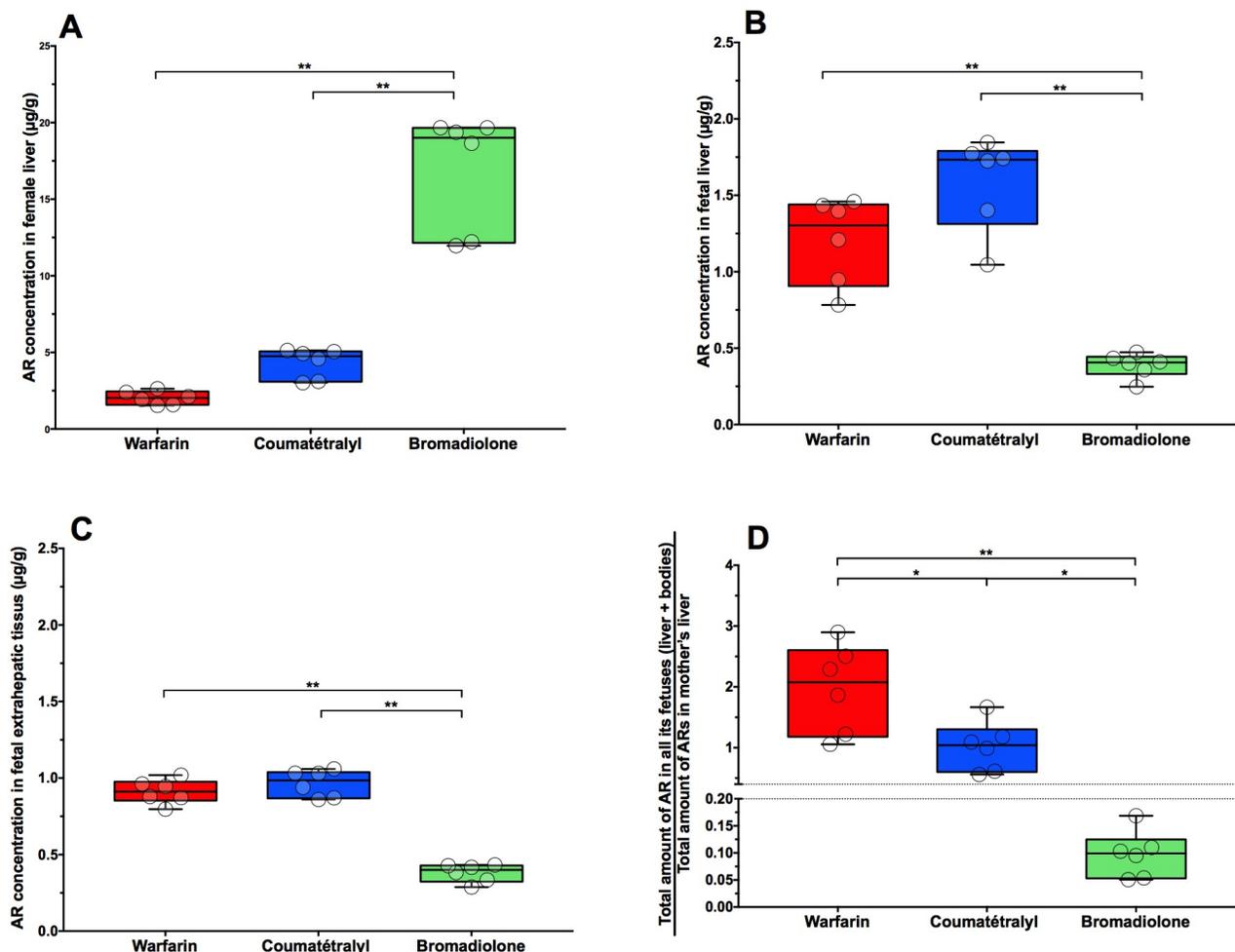


Figure 1. AR distribution between pregnant rats and their fetuses after three days of oral administration (0.5 mg/kg/day) of warfarin, coumatétralyl and bromadiolone to pregnant rat (n = 4) at gestation days D+17, D+18 and D+19. A/ AR concentration in mother's livers, B/ AR concentration in fetal livers, C/ AR concentration in fetal bodies, D/ Ratio between AR found in mother's liver and AR distributed in all its fetuses. Individual values and mean ± standard deviation with 95% confidence interval are presented. * p-value < 0.05 and ** p-value < 0.01.

ARs evaluated in this study. In fact, warfarin and coumatétralyl are roughly half-distributed between the liver of the mother and the fetus while bromadiolone is found almost exclusively in the liver of the mother and in very small quantities in fetus.

Why such a difference among vitamin K antagonists? May liver uptake of bromadiolone prevent its distribution to peripheral tissues? Does the placental barrier prevent the passage of bromadiolone to the fetus? In order to explore these hypotheses, pharmacokinetic studies were performed for warfarin and bromadiolone in female rats, the active substances for which the greatest differences in distribution between mother and fetus are observed. These pharmacokinetic studies were conducted at the 50% effective dose (ED 50) for each compound. Indeed, this ED 50 is the dose that allows an increase by a factor of 5 in the prothrombin time in half of the animals 24 hours after administration. The warfarin and the bromadiolone doses used in this study both evoke the same magnitude

of biological effect.

Warfarin, after oral administration of the estimated ED50 dose, is widely found in the systemic circulation (Figure 4A). At the absorption peak, plasma warfarin represents ≈30% of the warfarin administered, i.e., 160 µg out of the 560 µg administered (considering a plasma volume of 8.0 mL for a 200-g female [19–21] with a plasma concentration reaching ≈20 µg/ml (Figure 4B)). Only 16% of the administered amount are found into the liver, the remainder quantities are either distributed to peripheral tissues or metabolized; the hydroxylated metabolites of warfarin [22] have not been quantified in this study. By contrast, bromadiolone, after oral administration of the ED50 dose, is almost exclusively found in the liver (Figure 4A). At the absorption peak, hepatic bromadiolone accounts for more than 80% of the administered bromadiolone. Only 0.65% is found in the plasma, i.e. only 0.8µg out of the 120µg administered with a maximum concentration lower than 0.1

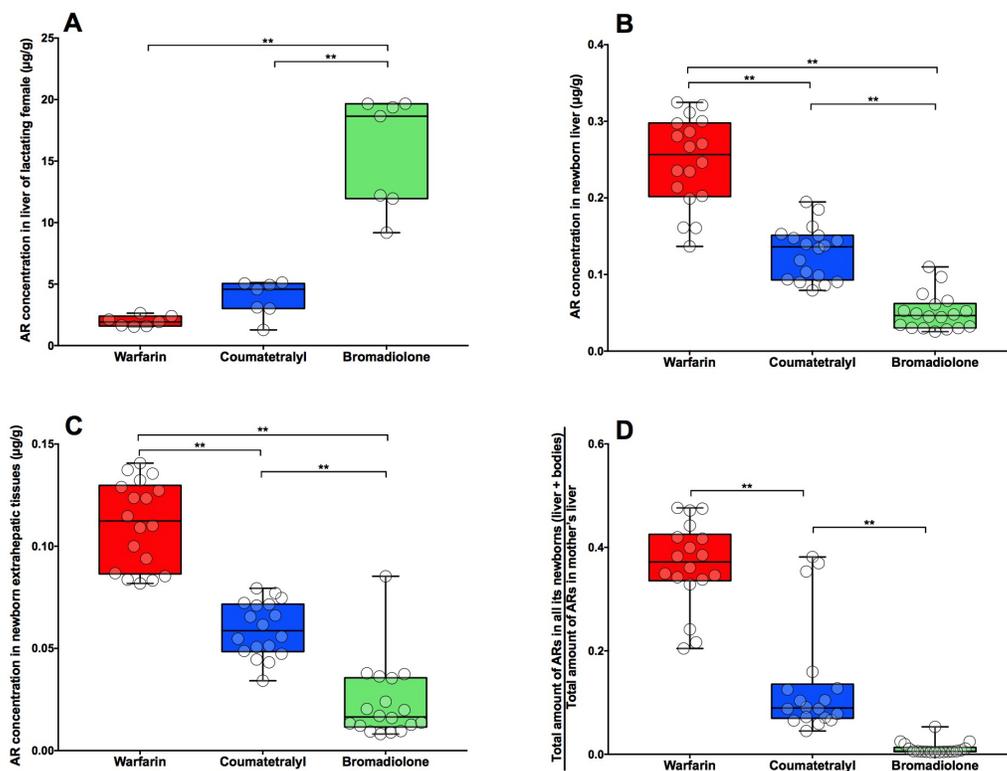


Figure 2. AR distribution between lactating rats and their newborns after three successive oral administration (0.5 mg/kg body weight/day for three days) of warfarin, coumatetralyl or bromadiolone to lactating rat at lactation days D+3, D+4 and D+5. A/ AR concentration in mother's livers, B/ AR concentration in newborn livers, C/ AR concentration in newborn bodies, D/ Ratio between AR found in mother's liver and AR distributed in all its newborns. Individual values and mean \pm standard deviation with 95% confidence interval are presented. * p-value < 0.05 and ** p-value < 0.01.

$\mu\text{g/ml}$ (Figure 4B). In addition, although the plasma MRT of bromadiolone is higher than that of warfarin, its plasma AUC is lower. These findings suggest that bromadiolone may have a greater affinity for hepatic tissue.

Considering the quantity of hepatic bromadiolone at the absorption peak and the available literature on the both ARs [9], it is reasonable to assume that the bioavailability of warfarin and bromadiolone are almost complete ($F \approx 1$). Thus, the apparent clearance (CL/F) and the apparent volume of distribution (V_{ss}/F) are good approximations of the clearance and of the volume of distribution respectively. Volume of distribution of warfarin in rats was found practically equal to one reported in man (140mL/kg) [23], and consistent with previous experiments [24].

A minimal model was used to interpret V_{ss}/F . Equation of the minimal model is: $V_{ss} = V_p + fu_P/fu_T \times V_t$ [25], where V_p is the plasma volume, V_t the extravascular space (tissular volume), fu_P the unbound fraction in plasma and fu_T the unbound fraction in tissue. fu_P and fu_T reflect the fraction of unbound compounds in plasma or tissue respectively. For warfarin, V_{ss} is on the same order of magnitude as the extracellular fluid of rat body, suggesting that warfarin is mainly located in the extracellular space due to its extensive plasma protein binding previously described [10, 26, 27]. The sit-

uation for bromadiolone is the opposite. Here V_{ss} is large and V_p can be ignored in minimal model equation. Thus, the ratio fu_P/fu_T is about 13, which indicate higher affinity of bromadiolone for tissue than for plasma. This difference of affinity does not appear to be due to a lower affinity of bromadiolone for plasma proteins compared to warfarin. Indeed, in vitro studies have highlighted that bromadiolone has a greater affinity for albumin than warfarin [28]. The table 2 present the theatrical distribution of both ARs calculated with their respective V_{ss} , in accordance with the model proposed by Øie and Tozer [29]. This model illustrates bromadiolone's greater affinity for tissue despite its plasma protein binding.

The fraction of AR circulating in the blood is the only fraction available to the fetus or newborn via the placental barrier or via excretion through milk. Because the blood fraction of bromadiolone is very low, fetuses or newborns are not or barely exposed when the dam is administered bromadiolone. Because the blood fraction of warfarin is high, the warfarin available to the fetus or newborn is high. These results are fully consistent with AR quantifications performed in fetal/newborn tissues and would largely explain the differences in teratogenicity observed in vivo [7]. Nevertheless, bromadiolone persists longer in the mother's liver and might cause chronic exposure of the fetus or newborn with regular

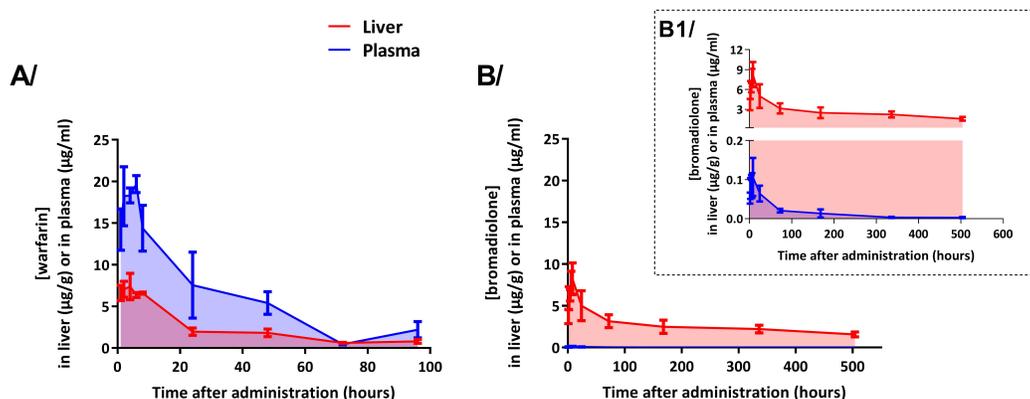


Figure 3. Pharmacokinetic profiles of A/ warfarin (2.8 mg/kg) and B/ bromadiolone (0.6 mg/kg) after single oral administration. Each point represents the mean \pm standard deviation of four individuals. Curves were obtained for plasma (blue line) and liver concentration (red line). B1 is an enlargement of B to observe plasma pharmacokinetic of bromadiolone.

	Warfarin		Bromadiolone	
	Liver	Plasma	Liver	Plasma
Cmax ($\mu\text{g/mL}$ or g)	8.1 \pm 0.88	20.1 \pm 2.3 ^b	8.49 \pm 1.64	0.127 \pm 0.027 ^b
λ_z (h ₋₁)	0.0214 \pm 0.0026 ^a	0.0313 \pm 0.0084 ^b	0.0016 \pm 0.0006 ^a	0.0041 \pm 0.0017 ^b
Terminal half-life calculated with λ_z (h)	32.8 \pm 4.1	23.2 \pm 5.7	460 \pm 198	205 \pm 132
AUC 0-last time (h. $\mu\text{g/mL}$ or g)	212 \pm 15.2 ^a	557 \pm 62.2 ^b	1337 \pm 283 ^a	7.69 \pm 2.63 ^b
AUC 0-infinity (h. $\mu\text{g/mL}$ or g)	251 \pm 22.82	618 \pm 63.7	2410 \pm 475	8.55 \pm 2.90
CL/F (mL/Kg/h)		4.57 \pm 0.47 ^b		77.0 \pm 27.9 ^b
MRT 0-last time (h)	26.8 \pm 1.73 ^a	25.5 \pm 3.28 ^b	202 \pm 10.98 ^a	99.6 \pm 17.5
MRT 0-infinity (h)	44.9 \pm 7.41	35.7 \pm 10.7	648 \pm 310	175 \pm 52.36
Vss/F 0-last time (mL/kg)		116		845
Vss/F 0-infinity (mL/kg)		163		13502

Table 1. Pharmacokinetic parameters of warfarin and bromadiolone liver and plasma concentrations after a single per os administration of 2.8 mg/kg of warfarin or 0.6 mg/kg of bromadiolone. Values are presented with standard deviation. P-value < 0.05, a, between warfarin and bromadiolone in liver; b, between warfarin and bromadiolone in plasma. AUC, area under the curve. Considering MRT, AUC and Vss/F, the last time is 96h or 504h for warfarin and bromadiolone, respectively.

	Plasma	Extracellular outside plasma	Intracellular
Warfarin	31%	46%	23%
Bromadiolone	0.4%	0.6%	99%

Table 2. Distribution of drug in the body at the steady state according to the model of Øie and Tozer [29] with a protein binding of 99% for both compounds.

release from the liver to the systemic circulation. The comparison of the plasma AUC of warfarin and bromadiolone demonstrates this phenomenon. Indeed, the AUC can be considered as a reliable index of the plasma fraction over time. The plasma AUC of warfarin is 70-fold higher than that of bromadiolone, with plasma concentrations above 1 $\mu\text{g/mL}$ for at least 72 hours after administration. Plasma concentrations of bromadiolone never exceeded 0.1 $\mu\text{g/mL}$ and 72 hours after initial administration, they dropped below 0.02 $\mu\text{g/mL}$ with a plasma half-life of bromadiolone in the same order of magnitude as that of warfarin.

The high hepatic extraction of bromadiolone could be explained: 1/ by its high partition coefficient ($\log P = 3.8\text{-}4.1$, pH 6-7) (European Chem Agency, 2012), higher than that of warfarin ($\log P = 2.7$, pH unspecified) [30] or coumatetralyl ($\log P = 3.46$, pH unspecified), or 2/ by a different active transport among vitamin K antagonists, which has already been suggested by recent studies [31]. Considering the first hypothesis, the higher lipophilicity of bromadiolone could lead to greater association to lipoproteins in the enterocytes. The lipoprotein bound compounds have a greater plasmatic clearance that, in this case, is mainly performed by the liver [32]. Thus, the first pass effect of bromadiolone would be much more important than that of warfarin or coumatetralyl. Notwithstanding the first pass effect that reduce the quantity of bromadiolone reaching the placenta, it would be worthwhile to study the specific abilities and modalities of compounds to cross the placental barrier.

Nevertheless, regardless of the underlying mechanisms, this difference in pharmacokinetics appears to be responsible for the difference in the observed teratogenic effect. The risk

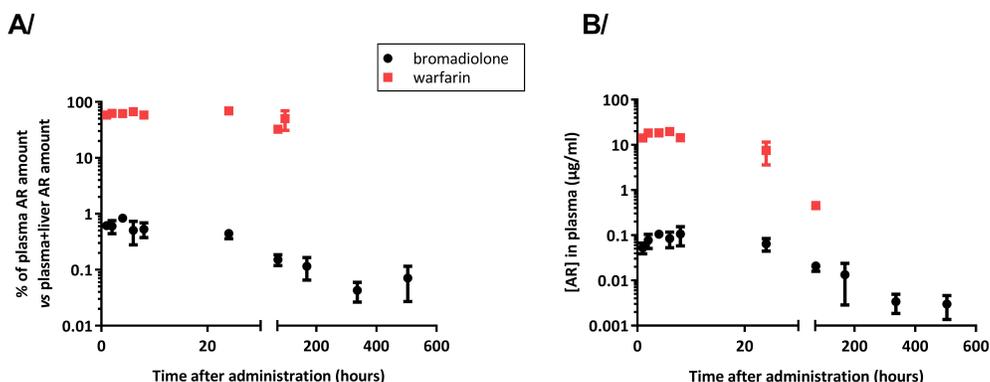


Figure 4. AR concentrations in plasma in female rats after single oral administration of warfarin (2.8 mg/kg) or bromadiolone (0.6 mg/kg). A/ shows the relative AR plasma fraction over time, B/ shows the plasma AR concentration over time.

to the fetus cannot, therefore, be considered identical among vitamin K antagonists. If the mechanism of action of these vitamin K antagonists is identical, the concentration on the site of action, i.e. the fetus, is absolutely not the same among active substances for a comparable exposure of the mother. This assessment should be carried out for each vitamin K antagonist.

The risk assessment in humans is of course based on the assessment of the hazard to the fetus, but also on the assessment of the likelihood of exposure of the mother to vitamin K antagonist. Bromadiolone is an active substance used exclusively in rodent management and formulated in baits (except during the manufacturing of these baits). Moreover, in Europe, these baits have to be used in bait boxes for biocidal use which prevents direct contact between people and baits. Considering warfarin, it is one of the most prescribed drugs in the world and people on such treatment receive it on a daily basis. Moreover, warfarin has been widely prescribed since the 1950s for pregnant women with thromboembolic problems due to its low-cost. The uses of these active substances are therefore different and certainly lead to a different frequency of exposure. In the USA, the incidence of human exposure to second-generation ARs has been reported to be 0.004% per year (315,951 cases of exposure over 25 years, with only 100,000 requiring treatment) according to the annual report of the American Association of Poison Control Centers' National Poison, most of these exposures are unintentional and 90% involve children [33, 34]. In China[35] and Europe, the incidence of this exposure appears to be lower, but still with a predominance of cases in children. In view of these data, the likelihood of a pregnant woman being exposed to ARs via baits appears to be very low. Exposure to warfarin which is used in human medicine seems much more likely.

All pharmacokinetics concerns presented in this work could challenge the read-across approach applied to classify all ARs as toxic to reproduction, which has major consequences on the use of these products in Europe. As no data available for each AR, further work is needed to evaluate

those active substances individually. Indeed, the 30 µg/g-concentration limit defined by the regulations beyond which baits are classified as toxic to reproduction is nowadays resulting in a reduction of the active substance concentration in bait. Could this reduction have consequences on the effectiveness of rodent management? A decrease in concentration at 30 µg/g of brodifacoum would have no consequence on the effectiveness of baits on non-resistant rodent populations [36]. What about the efficacy of low concentrated baits on resistant rodent populations? Moreover, the decrease in concentration of active substance could have serious consequences related to rodent resistance selection in Europe where numerous VKORC1 mutations have been described in rodents [37, 38].

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