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TITLE PAGE

**DEVELOPMENT OF AN ECOFRIENDLY ANTICOAGULANT RODENTICIDE BASED ON
THE STEREOCHEMISTRY OF DIFENACOUM**

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RUNNING TITLE PAGE

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List of nonstandard abbreviations:

ARs, anticoagulant rodenticides

FGAR, first-generation of anticoagulant rodenticides

SGAR, second-generation of anticoagulant rodenticides

SNP, Single-Nucleotide Polymorphisms

LOD, limit of detection

LOQ, limit of quantification

VKORC1, vitamin K epoxide reductase enzyme

VKPD, Vitamin K protein dependent

ABSTRACT

Difenacoum, an antivitamin K anticoagulant, has been widely used as rodenticide to manage populations of rodents. Difenacoum belongs to the second generation of anticoagulant and as all the molecules belonging to the second generation of anticoagulant, difenacoum is often involved in primary poisonings of domestic animals and secondary poisonings of wildlife by feeding contaminated rodents. To develop a new and ecofriendly difenacoum, we explored in this study the differences in properties between diastereomers of difenacoum. Indeed, the currently commercial difenacoum is a mixture of 57% of cis-isomers and 43% of trans-isomers. Cis- and trans-isomers were thus purified on a C18-column and their respective pharmacokinetic properties and their efficiency to inhibit the coagulation of rodents were explored. Tissue-persistence of trans-isomers was shown to be shorter than that of cis-isomers with an half-life 5-fold shorter. Efficiency to inhibit the vitamin K epoxide reductase activity involved in the coagulation process was shown to be similar between cis- and trans-isomers. The use of trans-isomers of difenacoum allowed to drastically reduce difenacoum residues in liver and other tissues of rodents when the rodent is moribund. Therefore, secondary poisonings of wildlife should be decreased by the use of difenacoum largely enriched in trans-isomers.

INTRODUCTION

Anticoagulant rodenticides (ARs) are used worldwide since the 1940s to control infestations of rats and mice which cause important agricultural and structural damage and associated to public health issues. The death of rodents occurring 3 to 7 days after consumption of baits. This delay of action eludes the alimentary aversion problem which is a very important behavioral trait among rodents. The first commercial rodenticide was dicoumarin replaced a couple of years later by warfarin, a more efficient molecule (Hadler and Buckle, 1992). These products with coumatetralyl, chlorophacinone and other molecules developed from 1950s compose the first-generation of ARs (FGARs), which require multiple ingestions to cause death of rodents (Rattner *et al.*, 2014).

ARs, derivatives of either 4-hydroxycoumarin or indane-1,3-dione are non-competitive inhibitors of the vitamin K epoxide reductase enzyme (VKORC1) described for the first time in 2004 (Li *et al.*, 2004), (Rost *et al.*, 2004). VKORC1 is essential to reduce vitamin K 2,3 epoxide to vitamin K hydroquinone. Vitamin K hydroquinone is the essential cofactor for the γ -glutamyl carboxylase which catalyses the post-translational modifications of the clotting factors II, VII, IX and X and other vitamin K dependent proteins (Furie and Furie, 1988), (Suttie, 1985). ARs acting as VKORC1 inhibitors stop the vitamin K-dependent clotting factors activation impairing the coagulation function and thus lead to the death of rodents by haemorrhages.

After 10 years using FGARs, the first case of apparent resistance to FGARs was discovered in the United Kingdom in 1958 (Boyle, 1960). In the following years, resistant rodents have been described everywhere in Europe (Dodsworth, 1961) and also in the United States (Jackson and Kaukeinen, 1972), Canada (Siddiq and Blaine, 1982), Australia (Saunders, 1978) and Japan. Since then, two main resistance mechanisms have been described, a metabolic resistance due to an overexpression of CYP450 and a target resistance due to a decrease of the susceptibility of the VKOR activity to ARs. This target resistance was definitively associated with SNPs (Single-Nucleotide Polymorphisms) in the *Vkorc1* gene in 2004 after the discovery of this gene (Pelz *et al.*, 2005), (Rost *et al.*, 2009). The occurrence of resistance in all the major commensal species to all the FGARs led to the development of a second-generation of ARs (SGARs) in the 1970s. SGARs are usually more toxic and more

persistent in animal tissues than FGARs and they are thus active after a single bait feeding (Rattner *et al.*, 2014). Because of this increased persistence in animal tissues, especially in liver (Langford *et al.*, 2013), the use of SGARs is associated with an increased risk of secondary poisoning for predators and scavengers feeding on contaminated rodents. Evidence of secondary poisoning was confirmed in predatory bird species such as barn owl (*Tyto alba*), buzzard (*B. buteo*), kestrel (*Falco tinnunculus*), Red kite (*Milvus milvus*), and tawny owl (*Strix aluco*) (Christensen *et al.*, 2012 ; Hughes *et al.*, 2013 ; Geduhn *et al.*, 2015), in predatory mammals such as red foxes (*Vulpes vulpes*) (Sage *et al.*, 2010), European mink (*Mustela lutreola*) (Fournier-Chambrillon *et al.*, 2004), weasels (*Mustela nivalis*) (Elmeros *et al.*, 2011). Therefore, ARs were identified by the European Union as candidates for future comparative risk assessment and substitution in view of their unacceptable risk of secondary poisoning for wildlife. However, in the absence of an alternative, ARs molecules were included on Annex 1 of EU Biocidal Products Directive 98/8/EC, and their use is still tolerated until a more appropriate solution is found.

Difenacoum (*i.e.*, 2-hydroxy-3-[3-(4-phenylphenyl)-1-tetralinyl]-4-chromenone) described for the first time in 1975 belongs to SGAR. It is efficient to control warfarin-resistant rodent populations (Hadler *et al.*, 1975), (Hadler and Shadbolt, 1975), (RRAC, 2015) and would be less toxic for non-target species than for target species (Buckle and Smith eds, 2015). This molecule is widely used worldwide (Buckle *et al.*, 2013), (Atterby *et al.*, 2005), (Hughes *et al.*, 2013). For example difenacoum was reported to be used on approximately 45% of agricultural premises in Great Britain (Buckle *et al.*, 2013), (Atterby *et al.*, 2005). It is used to control rodent pests in and around buildings, in waste sites and in sewers (European Parliament, 2009) but, according to the European countries, not always authorized in open areas because of its associated ecotoxicity. Difenacoum has two asymmetric carbons in its chemical structure (Buckle and Smith eds, 2015), (Kelly *et al.*, 1993) conferring this product to exist in two diastereomeric forms (1R,3R)(1S,3S) or (1R,3S)(1S,3R) with potentially different chemical and physical properties. The difenacoum currently available on the market is a mixture of both diastereomers reported to contain from 50 to 80% of one major diastereomer.

This study aims to develop a new difenacoum based on the concepts of stereochemistry. Indeed, diastereomers of difenacoum might have different tissue-persistence. The change of the composition of the difenacoum currently available, either by decreasing or by increasing the proportion of the major diastereomer, might result in a way to diminish its tissue persistence and thus its ecotoxicity, while keeping its inhibiting activity.

For this, an appropriate analytical method was developed in order to separate diastereomers of difenacoum. After characterization and assignment of each diastereomer of difenacoum by RMN, pharmacokinetics studies were performed in order to evaluate the tissue-persistence of each diastereomer. Thus, comparative evaluation of efficacy of each diastereomer as inhibitor of the VKOR activity were performed *in vitro* and *in vivo* in order to compare their anticoagulant activity.

MATERIALS AND METHODS

Chemicals

Difenacoum was supplied by Hangzhou Ich Biofarm Co (China). (1*R*,3*R*)(1*S*,3*S*)-isomers of difenacoum (called in the following trans-isomers) and (1*R*,3*S*)(1*S*,3*R*) isomers of difenacoum (called in the following cis-isomers) were separated and purified by mean of flash column chromatography on silica gel in our laboratory. Brodifacoum was obtained from Sigma (l'Isle d'Abeau, Chesnes, France), dimethyl sulfoxide (DMSO), acetonitrile, methanol, acetone, diethyl ether, orthophosphoric acid were obtained from VWR International, (Fontenay sous bois, France) and Vetflurane[®] and vitamin K1 from Alcyon, (Miribel, France). Vitamin K1 was converted to vitamin K epoxide according to the method described by Tishler et al. (1940). Purity was estimated by LC/MS using an analytical standard (Cayman chemical, MI, USA) and was higher than 99%. HPLC grade water was prepared using a milli-Q plus system, (Millipore, Saint-Quentin en Yvelines, France) and used for preparation of HPLC eluents.

Animals

Experimental research on animals was performed according to an experimental protocol following international guidelines and with approval from the ethics committee of the Veterinary School of Lyon.

Eight-week old male OFA-Sprague Dawley rats (each weighing 175-200 g) were obtained from a commercial breeder (Charles Rivers, l'Arbresle, France) and were acclimated for a minimum period of 5 days. The rats were housed four per cage under a constant photoperiod and ambient temperature. The 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*. Male OFA-Sprague Dawley rats received through *peros* administration either commercial difenacoum (*i.e.*, corresponding to a mixture of 57% of cis-isomers and 43% of trans-isomers) or silica gel column-purified cis-isomers or silica gel column-purified trans-isomers. Anticoagulants were dissolved in 10% DMSO and 90% vegetable oil and were administered by force-feeding. Rats were

maintained in life by daily subcutaneous administration of vitamin K1 (5 mg.kg⁻¹). Finally, rats were anesthetized with isoflurane and blood was taken by cardiac puncture into citrated tubes. Finally, rats were euthanized with CO₂ and organs of each rat were immediately collected and stored at -20°C until analysis.

Prothrombin Time (PT) determination

After blood collection into citrated tubes, blood sample was centrifuged immediately at 3000 rpm for 10 min. The PT (in seconds) was assessed immediately in duplicate using a Biomerieux Option 2 Plus (Behnk Electronick, Norderstedt, Germany) with the Neoplastin CI, INR Determination kit (Diagnostica Stago, Asniere, France). The PT was determined in 100 µl samples of plasma with thromboplastin, according to the manufacturer's instructions. PT was determined as the clotting time of a citrated plasma sample to which thromboplastin had been added. Each value was determined as the mean of 2 measurements.

Extraction of difenacoum in plasma

An aliquot of 1 ml of sample supplemented with 1.9 mM of an internal standard (*i.e.*, brodifacoum) was placed in a tube with 8 ml of diethyl ether. The extract was mixed and centrifuged at 3000 rpm for 5 min. The diethyl ether layer was evaporated to dryness under a gentle nitrogen flow at ambient temperature. The final purified sample was dissolved in 200 µl of methanol and difenacoum was analysed by HPLC as described in the paragraph “difenacoum analysis by HPLC”.

Extraction of difenacoum in tissues

The samples (1 g of tissue) were extracted with 40 ml of acetone using an Ultra Turrax tissue disperser (IKA Labortechnik®, VWR International, Strasbourg, France) for 1 min after addition of 1.9 mM of an internal standard (*i.e.*, brodifacoum). The extract was centrifuged at 3000 rpm for 5 min. The supernatant was transferred in a flask and evaporated at 50°C with a rotary evaporator. The flask was rinsed three times with 1 ml of methanol which were put together and evaporated to dryness under a

gentle nitrogen flow. The final purified sample was dissolved in 200 μ l of methanol and difenacoum was analysed by HPLC as described in the next paragraph “difenacoum analysis by HPLC”.

Difenacoum analysis by HPLC

Analysis of cis- and trans-difenacoum was determined by HPLC. Brodifacoum was used as internal standard. The HPLC system (JASCO LC-Net II/ADC [®]) consists of a PU2089 JASCO pump, an AS2051 sampler, a UV2075 detector (258 nm), and a LACHROM start software [®] (VWR International, Strasbourg, France). 50 μ l of extracted sample or standard solution were injected in a LIChrospher[®] 100RP18 encapped column (5 μ m particle size, 250 \times 4.6mm) (VWR International, Strasbourg, France). A gradient elution system was used with a flow rate of 1 ml/min as follows: from 60% acetonitrile/40% water acidified with 0.2% H₃PO₄ to 80% acetonitrile/20% water acidified with 0.2% H₃PO₄ at 5 min.

The method was fully validated according to the guideline on Bioanalytical Method Validation published by the European Medicines Agency (2011) with respect to specificity, carry-over, Limit Of Detection (LOD) (i.e., 65, 119, 56, 228, 80, 64 and 34 ng/g for liver, small intestine, kidney, feces, colon, plasma and urine, respectively), Limit Of Quantification (LOQ) (i.e., 162, 298, 140, 570, 201, 161 and 107 ng/g for liver, small intestine, kidney, feces, colon, plasma and urine, respectively), calibration curve, accuracy, precision, dilution integrity, matrix effect and stability. The recovery rate of diastereomers of difenacoum from tissues were between 74 to 90 % in liver and 80 to 100 % in plasma with a precision below 15%.

Preparation of liver microsomes

Liver microsomes were prepared from fresh livers by differential centrifugation. Briefly, livers were resuspended in 50 mM phosphate buffer (pH 7.4) containing 1.15 % (w/v) of KCl. Liver cells were ~~broken~~ homogenized in buffer by using a motor-driven Potter glass homogenizer and further submitted to differential centrifugation at 4°C. The 100,000 g pellet corresponding to the membrane fraction was resuspended by Potter homogenization in HEPES glycerol buffer (50 mM HEPES, 20%

glycerol, pH 7.4). Protein concentrations were evaluated by the method of Bradford using bovine serum albumin as a standard. Microsomes were frozen at -80°C and used for kinetic analysis.

VKOR activity assay and kinetics

Microsomal vitamin K epoxide reductase (VKOR) activity was assayed according to the protocol described by Hodroge et al (2011, 2012). The inhibiting effect of the silica gel-column-purified cis- or trans-isomers of difenacoum was evaluated by the determination of K_i . K_i was determined after addition of various concentrations of the anticoagulant to the standard reaction in the presence of increasing amounts of vitamin K epoxide (from 0.001 to 0.2 mM) using anticoagulant concentrations from about 0.05 to 20 x K_i (K_i of commercial difenacoum is 0.03 μM (Hodroge *et al.*, 2011)). The K_i values were obtained from at least three separate experiments performed on two different batches of proteins.

Analysis of data

Pharmacokinetic calculations were performed using the noncompartmental approach on the mean results per group. The cis-difenacoum and total difenacoum i.e., cis-isomers + trans-isomers) concentrations used to calculate the elimination rate constant and half-life were selected from the 12- to 336-h time points. For trans-difenacoum, the concentrations used to calculate the elimination rate constant and half life were selected from 12 to 72h, because concentration 72h and more after administration were too weak to be accurately quantify (<0.25 $\mu\text{g}/\text{kg}$). The half-life of elimination ($t_{1/2(\text{el})}$) was calculated using a linear regression linear in GraphPad Prism 6 (CA, USA). The total area under the curve (AUC) was calculated using the linear trapezoidal method and adding the estimated terminal portion of the curve (AUC $0 \rightarrow \infty$). The three models were drawn using Berkeley Madonna Software.

Statistical analysis of pharmacokinetic parameters and prothrombin time were done using GraphPad Prism 6 software (CA, USA). A Mann-Whitney test was used with $\alpha < 0.01$ in order to compare statistically the results between the two groups.

For kinetic analysis of the VKOR activity, data were fitted by non-linear regression to the non-competitive or competitive inhibition model using GraphPad Prism 6. The choice of the best model was based on the Corrected Akaike Information Criterion.

RESULTS

Separation of diastereomers of difenacoum and assignment of the stereochemistry

When pure difenacoum was analyzed by HPLC on a C18 reversed phase column as described in the experimental procedure, two different peaks were obtained with respective retention time of 11.2 ± 0.1 min and 12.1 ± 0.1 min (Fig. 1A). Both peaks presented the same absorption spectrum with a maximal absorbance at 258 nm. Both peaks were thus identified as difenacoum existing in two diastereomeric forms, cis and trans (Fig. 2A).

In order to assign the stereochemistry of diastereomers, they were purified on silica column and analyzed by NMR. Assignment of the stereochemistry was validated in accordance with NMR experiments already reported in the literature for H¹ proton of decalin in C₆D₆ (Fig. 2B) (Van Heerden, *et al.*, 1997a), (Van Heerden *et al.*, 1997b). Molecules eluted at 12.1 min corresponded to trans-isomers of difenacoum with δ 4.85 ppm (dd, $J = 6$ Hz and $J = 2$ Hz). Molecules eluted at 11.2 min corresponded to cis-isomers of difenacoum with δ 4.96 ppm (broad singlet). In CDCl₃, the results provided slightly different signals at 300 MHz : trans-isomers, δ 4.56 ppm (t, $J = 7.0$ Hz); cis-isomers δ 4.84 ppm (dd, $J = 6.0$ and $J = 11.3$ Hz) but also confirmed the assignment described in this solvent (Fig. 2C) (Kelly *et al.*, 1993).

Comparative analysis of the persistence of trans-isomers and cis-isomers of difenacoum in liver

In order to study the hepatic and plasma persistence of trans- and cis-isomers of difenacoum, 42 male OFA-Sprague Dawley rats received by *per os* administration 5.2 mg.kg^{-1} (i.e., $8 \times \text{ED}_{50}$ of difenacoum determined for *Rattus norvegicus*) of commercial difenacoum corresponding to a mixture of 57% of cis-isomers of difenacoum and 43% of trans-isomers of difenacoum. Rats were killed 1, 2, 4, 6, 8, 10, 12, 24, 72, 120, 168, 240, 336, 504 hours after administration of difenacoum and liver concentrations of cis-isomers (Fig. 3B and 3C), trans-isomers (Fig. 3B and 3C), but also total difenacoum (by adding cis- and trans-isomers) were determined for each rat. Representative UV chromatograms of the analysis of difenacoum at different time points of the pharmacokinetics (i.e., 24h and 120h after the administration of 5.2 mg.kg^{-1} of commercial difenacoum) are shown in figure 1B and 1C with elution

of cis-isomers at 11.2 min and trans-isomer at 12.1 min. 120h after the administration, trans-isomers disappeared. Moreover, production of two major metabolites were detected with retention time of 6.8 and 7.3 min, respectively and a UV spectrum similar to that obtained for difenacoum, proportion of these metabolites varying during the pharmacokinetics.

The pharmacokinetic profile of total difenacoum liver concentration (calculated by addition of concentrations of cis- and trans-isomers) is presented in Fig. 3A. It increased rapidly in liver, reached a maximum (C_{max}) in 6 h after the administration and then decreased. Pharmacokinetic parameters of total difenacoum in liver was calculated using a noncompartmental approach and is presented in Table 1. The pharmacokinetic profile of cis- and trans-isomers in liver is presented in Fig. 3B. C_{max} were reached 6 h after the administration for both isomers. When C_{max} were reached, ratio between cis- and trans-isomers was similar to that of the commercial difenacoum (i.e., mix of 57% cis- and 43% trans-isomers) (Fig. 3C). Trans-isomers of difenacoum were more rapidly eliminated than cis-isomers. AUC of trans-isomers was 7-fold smaller than that of cis-isomers. Half-lives of cis-isomers and trans-isomers in liver calculated were about 83 hours and 14 hours, respectively. The cis-isomers represented 76% of the total amount of difenacoum present in liver 24 hours after the administration of the commercial difenacoum (i.e., mix of 57% cis- and 43% trans-isomers) and 99%, 168 hours after the administration.

To avoid competitive effects between cis- and trans-isomers, pharmacokinetic studies were repeated with silica gel column-purified trans-isomers or silica gel column-purified cis-isomers. Male OFA-Sprague Dawley rats received by *per os* administration 3.0 mg.kg^{-1} of trans-isomers or cis-isomers. The pharmacokinetic profile of cis-isomers concentration and trans-isomers in liver after administration of purified isomers is presented in Fig. 4. Trans-isomers of difenacoum were still more rapidly eliminated than cis-isomers. Half-lives of cis-isomers and trans-isomers in liver were calculated using a linear regression model and were similar to those calculated after administration of a commercial mixture of difenacoum (Table 1).

Comparative analysis of the persistence of trans-isomers and cis-isomers of difenacoum in rat tissues

A comparative analysis of persistence of isomers was performed in different tissues of rat to confirm that the liver is the major organ of storage for both isomers and to evaluate the pharmacokinetics of cis- or trans-isomers in extra-hepatic tissues. For that, three days after the administration of 3.0 mg.kg⁻¹ of cis- or trans-isomers, concentration of cis- and trans-isomers were determined in liver, but also in plasma, feces, intestine, kidney, mesenteric fat and urine of rats. Results are presented in Fig. 5. Concentrations of difenacoum after administration of cis-isomers were systematically similar or higher than those obtained after administration of trans-isomers in all the tissues investigated in this study. After administration of trans-isomers, difenacoum was detected almost only in liver, while when cis-isomers were administered, difenacoum was found in liver, plasma, intestine and kidney. Nevertheless, whatever the isomers used, liver was the major tissue of storage.

Analysis of the *in vitro* inhibiting effect of trans-isomers and cis-isomers of difenacoum on the vitamin K epoxide reductase activity

The ability to inhibit the VKOR activity catalyzed by susceptible rat liver microsomes was evaluated for cis- and trans-isomer by determination of inhibition constants (K_i). The plots of the velocity of the VKOR activity catalysed by rat liver microsomes, versus the substrate concentration in the presence of different concentrations of trans-isomers are presented in Figure 6. Trans-isomers of difenacoum were able to inhibit VKOR activity. Apparent K_m was not modified by the addition of trans-isomers. On the contrary apparent V_{max} was lowered by the use of trans-isomers. This result revealed that trans-isomers inhibited VKOR activity catalysed by rat liver microsomes in a non-competitive manner, as previously reported for commercial difenacoum corresponding to a mixture of cis- and trans-isomers (57%/43%) (A. Hodroge *et al.*, 2011). Data were fitted to the Michaelis–Menten model, which takes into account the presence of competitive, non-competitive, or uncompetitive inhibitor by non-linear regression. The best fit, the higher R^2 and the lower corrected Akaike information criterion were obtained when the model that takes into account a non-competitive inhibitor was used. Finally, K_i towards trans-isomers

for rat liver microsomes was 20.9 ± 1.56 nM. Same experiment was performed with cis-isomers. Cis-isomers were also able to inhibit VKOR activity catalysed by rat liver microsomes in a non-competitive manner with K_i similar to that obtained with trans-isomers (17.4 ± 7.9 nM).

Analysis of the ability of trans-isomers and cis-isomers of difenacoum to inhibit *in vivo* blood coagulation

The ability of cis-isomers or trans-isomers of difenacoum to inhibit the blood coagulation was determined by their respective ability to increase *in vivo* the prothrombin time. The normal prothrombin time was determined to be 17.9 ± 2.5 sec in male OFA-Sprague Dawley rats. Effect of cis- or trans-isomers on prothrombin time was monitored 1, 3 and 5 days after *peros* administration to male OFA-Sprague Dawley rats of 4 ED₅₀ (i.e., 2.6 mg.kg^{-1} for *Rattus norvegicus*) of difenacoum containing only cis-isomers or only trans-isomers. ED₅₀ is the dose that creates an increase of prothrombin time in the international normalized ratio higher than 50 percent of the tested population. Results corresponding to the mean of 4 rats are presented in Fig. 7A. Oral administration of 2.6 mg.kg^{-1} of trans-isomers of difenacoum led to significant increase of the prothrombin time for 24 hours. Three days later it was almost normal again. On the contrary, oral administration of 2.6 mg.kg^{-1} of cis-isomers of difenacoum resulted in a considerable increase in prothrombin time for at least 5 days.

In order to maintain the effect of trans-isomers for at least 3 days, the dose was increased to 3.9 mg.kg^{-1} corresponding to 6ED₅₀). This dose was administered as a single dose on day 0 or in 3 successive doses of $1.3 \text{ mg.kg}^{-1}/\text{day}$ on day 0, 1 and 2. The prothrombin time was thus evaluated on day 4. Results are presented in Fig. 7B. Administration of 3.9 mg.kg^{-1} of trans-isomers as a single dose did not lead to significant increase of the prothrombin time 3 days later. On the contrary, the administration of the same total dose of trans-isomers (3.9 mg.kg^{-1}) in 3 times ($1.3 \text{ mg.kg}^{-1}/\text{day}$ during 3 days) led to drastic increase of the prothrombin time associated with a major anticoagulant effect.

DISCUSSION

Development of SGAR able to kill rodents after a unique administration was achieved by increasing the tissue-persistence of AR enabling the inhibition of VKOR activity and thus blood clotting for several weeks instead of several hours for FGAR. However this increased persistence led to molecules with excessive half-lives while they kill rodents in only 3 to 7 days. For example, half-life of brodifacoum has been reported as being 307 days in mice (Vandenbroucke *et al.*, 2008). While these molecules have been used without any report of cases of secondary poisoning for more than 30 years after the beginning of their marketing (Vandenbroucke *et al.*, 2008), (Kaukeinen, 1982), the ecological impact of these molecules on wildlife is now obvious. In spite of this obvious ecological impact, there has been no improvement of these AR molecules since the patent of difethialone in 1986.

A new generation of ARs is nowadays required to overcome the ecotoxicity associated with the SGARs. In this study, we explored a way to develop an ecofriendly anticoagulant rodenticide able to efficiently inhibit VKOR activity and less persistent into animal tissues to avoid secondary poisoning. The explored solution is an improvement of the existing difenacoum based on the concept of the stereochemistry.–Indeed, many studies report differences in the pharmacological, toxicological or pharmacokinetic properties between stereoisomers of the same molecule (Cort *et al.*, 2012), (Nguyen *et al.*, 2006), (Chhabra *et al.*, 2013).

The pharmacokinetic studies performed herein showed that cis- and trans-isomers of difenacoum are similarly absorbed after oral administration. Indeed, after administration of a mixture of 57% of cis- and 43% of trans-isomers, C_{max} for cis- and trans-isomers are reached at the same time and proportions of cis- and trans-isomers in the liver at T_{max} remains unchanged compared to the initial proportions of the administered mixture. On the other side, these studies demonstrate a clear difference in hepatic half-life between isomers with a 6-fold shorter half-life for the trans-isomers compared to the cis-isomers. This difference in hepatic half-life results in an almost absence of trans-residues in liver 72h after a *peros* administration of 5.2 mg.kg^{-1} of the current difenacoum.

This difference in half-life does not result from a difference in storage between diastereoisomers. Indeed, the determination of concentrations of residues of difenacoum 72h after the administration of

the current difenacoum did not show a preferential localization of trans-isomers in an extrahepatic tissue. In all the tissues analyzed, the concentrations of trans-isomers were systematically lower than those of cis-isomers. This difference in half-life is neither due to a chiral inversion of trans-isomers in cis-isomers. Indeed, when only trans-isomers are administered to rats, no appearance of cis-isomers was detected in any tissue. This difference seems to result from a difference in elimination with an elimination rate constant of trans-isomers (i.e., calculated k_e of 0.044 h^{-1}) 6-fold greater than that of cis-isomers (i.e., calculated k_e of 0.0071 h^{-1}).

The accelerated elimination of trans-isomers could be due to an accelerated metabolism of the trans-isomers compared to cis-isomers. Indeed, production of two metabolites with a UV spectrum similar to difenacoum was observed after administration of difenacoum. Because anticoagulants have been described to be oxidized by CYP450 (Moreau *et al.*, 2011), these metabolites could result from the oxidation of cis- and trans-isomers. These metabolites are then either excreted as hydroxy metabolites or could undergo conjugation into sulfates or glucuronides as reported for warfarin in humans (Jones and Miller, 2011). The absence of other detected metabolites during the pharmacokinetic study suggests that both metabolites are excreted as unchanged. Nevertheless, both metabolites seem to persist in tissues 120 hours after administration even if the proportion of one of the two metabolites clearly decreased. This persistence could be problematic if these metabolites retained anticoagulant activity. However 72 hours after administration of 2.6 mg.kg^{-1} of trans-difenacoum, prothrombin time becomes normal which suggests that metabolites produced from trans-isomers are no longer active. To better understand the role of metabolism in the different elimination observed between cis- and trans-isomers, complete metabolic studies will be necessary.

Because of the slow elimination rate of cis-isomers, the ecotoxicity associated with the difenacoum currently commercially available is due to its composition rich in cis-isomers. Indeed, it is when rodent is dying that it becomes an easy prey for predators. During difenacoum poisoning, rodent is weakened as it presents a hemorrhagic syndrome - i.e. 3 to 7 days after consumption of difenacoum. At this moment, rodent tissues and especially rodent liver contain only cis-isomers, trans-isomers having been eliminated. Consequently, the use of difenacoum containing only the trans-isomers would

greatly reduce the tissue-persistence of difenacoum and therefore the ecotoxicity associated to this product. Indeed, 3 and 7 days after consumption of 3 mg.kg^{-1} of trans-isomers, there are 3.5 and 4.5-fold less residue in the liver than when rodent consumes 3 mg.kg^{-1} of cis-isomers. Moreover, this decrease of persistence would facilitate the management of primary intoxication in domestic animals if the pharmacokinetic properties of cis- and trans-isomers are identical than those observed in rats. Currently, to treat dogs or cats poisoned with difenacoum currently commercially available, a daily injection of vitamin K1 for 2 to 5 weeks is required (Pouliquen, 2001). The decrease of persistence of difenacoum would certainly reduce the duration of treatment, and therefore the cost associated with a primary poisoning with difenacoum.

To develop the trans-isomers of difenacoum as rodenticide, it must be an inhibitor of the VKOR activity at least as effective as the current difenacoum and inhibit the hepatic VKOR activity for at least 3 consecutive days to deplete the pool of activated clotting factors. This is why the ability of the trans-isomers of difenacoum to inhibit the VKOR activity was assessed *in vitro* and *in vivo*. The inhibition constant (i.e., 20.9 nM) obtained for trans-isomers of difenacoum using rat liver microsomes was similar to that of cis-isomers or difenacoum currently available. This inhibition constant was even lower than those obtained under the same conditions for the other SGARs (70 nM, 30 nM and 40 nM for bromadiolone, brodifacoum, difethialone respectively) (Hodroge *et al.*, 2011). Even if the inhibition constant obtained for trans-isomers is compatible with the use of trans-isomers of difenacoum as rodenticide, a single administration of 2.6 mg.kg^{-1} of trans-isomers (which would correspond to an ingestion of 20 g of baits, the normal amount generally ingested by a 200 g-weighted rat, dosed at 25 ppm) failed *in vivo* in this study to maintain a prolonged inhibition of the VKOR activity. This is due to the low-persistence of trans-isomers leading to liver concentration too low 3 days after the administration. Nevertheless, rodents consume usually several times the same bait when the bait is given in sufficient quantity. In this way the inhibition of the VKOR activity by trans-isomers persists several days and enables to cause death of rodents.

Trans-isomers of difenacoum could thus be considered as an alternative to the use of SGAR for avoiding secondary poisoning problems. If a single-feeding use is necessary for such products, adding

a small proportion of cis-isomers would certainly maintain a prolonged inhibition of the VKOR activity to lead to animal death while maintaining a limited quantity of residues. Further studies will be needed to optimize the most favorable proportions between trans- and cis-isomers to develop a product with a good balance between efficiency and tissue-persistence.

AUTHORSHIP CONTRIBUTIONS

Participated in research design: EB, SB, VL

Conducted experiments: BE, EB, IF, MDP, SB, VL

Contributed new reagents or analytic tools: BE, HC, FP, SB

Performed data analysis: BE, EB, MDP, VL

Wrote or contributed to the writing of the manuscript: EB, IF, FP, MDP, VL

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FOOTNOTES

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FIGURES LEGENDS

Fig. 1 Chromatograms of cis- and trans-isomers of difenacoum, in HPLC on a C18 reversed phase column. Chromatogram A corresponds to the commercial difenacoum at 10 μM with cis-isomers at 11.2 minutes and trans-isomers at 12.1 minutes. Chromatograms B and C are residues on the liver after a single oral administration of 5.2 $\text{mg}\cdot\text{kg}^{-1}$ of difenacoum (ratio 57/43) in warfarin-susceptible rats at 24 and 120 hours respectively

Fig. 2 Chemical structure of trans-isomers and cis-isomers of difenacoum (A) and the assignment of the stereochemistry with NMR experiments using H1' proton of decalin in C_6D_6 (B) or in CDCl_3 (C). The ^1H NMR spectra in C_6D_6 (B) or in CDCl_3 (C) of molecules eluted at 10.9 min (on the right) and at 11.7 min (on the left) are presented plotted as signal intensity (vertical axis) vs. chemical shift (in ppm on the horizontal axis)

Fig. 3 Liver concentration time profiles of total difenacoum (A) and cis- or trans-isomers (B) after a single oral administration of 5.2 $\text{mg}\cdot\text{kg}^{-1}$ of difenacoum (ratio 57/43) in warfarin-susceptible rats. The results of each time are the mean \pm SD of three rats per time. The total difenacoum is the sum of concentration of cis- and trans-isomers of difenacoum at each times. Modellings of the experimental points corresponding to the cis-isomers (modelling cis-isomers), to trans-isomers (modelling trans-isomers) and total difenacoum (modelling total difenacoum) were obtained using a mono-compartmentally model (with the software Berkeley Madonna). The different times are 1, 2, 4, 6, 8, 10, 12, 24, 72, 120, 168, 240, 336, and 504 hours. Ratio of cis- and trans-isomers of difenacoum are shown in figure C

Fig. 4 Time-dependent concentrations of cis- and trans-isomers of difenacoum after *per os* administration of, respectively, 3 $\text{mg}\cdot\text{kg}^{-1}$ of silica gel column-purified cis- or trans-isomers of difenacoum to warfarin-susceptible rats. Results are the mean \pm SD of four rats. The different times are 24, 72, 168, 336 and 504 hours

Fig. 5 Concentrations of difenacoum in different tissues 3 days after a single oral administration of 3 mg.kg⁻¹ of silica gel column purified of cis-isomers or trans-isomers of difenacoum in warfarin-susceptible rats. Results are the mean \pm SD of four rats. * denotes a significant difference between cis- and trans-isomers administration with $\alpha < 0.01$ by using a Mann-Whitney test

Fig. 6 Plots of vitamin K epoxide reductase activity versus vitamin K epoxide (from 1.25 to 200 μ M), in the presence of 0, 10, 20 and 50 nM of trans-isomers of difenacoum incubated with microsomes from warfarin-susceptible rats (A). Each data point represents the mean \pm SD of two determinations. The experimental results were fitted by non-linear regression using the non-competitive inhibition model

Fig. 7 Prothrombin time after single or multiple oral administrations of cis- or trans-isomers of difenacoum in warfarin-susceptible rats. In (A), a comparison of prothrombin time between cis- and trans-isomers was performed. For this, 2.6 mg.kg⁻¹ of cis- or trans-isomers was administered at day 0 and prothrombin time was evaluated 1, 3 or 5 days after the oral administration. In (B), a comparison of prothrombin time after a single or a multiple oral administration of trans-isomers of difenacoum was performed. For this, 3.9 mg.kg⁻¹ of trans-isomers was administered in a single oral administration at day 0 or in three oral administrations (1.3 mg per day) at day 0, 1 and 2 and prothrombin time was evaluated at day 3. Each point is the mean \pm SD of 4 rats. * denotes a significant between two groups with $\alpha < 0.01$ by using a Mann-Whitney test. Value > 500 sec = value out of range

TABLES

Table 1 Pharmacokinetics parameters of difenacoum in liver after a single oral administration of difenacoum (ratio 57/43 cis-/trans-isomers) at 5.2 mg.kg⁻¹ or after administration of cis- or trans-isomers purified in silica gel column at 3 mg.kg⁻¹. * denotes a significant difference between two groups (cis- or trans-isomers in a concomitant administration or cis- or trans-isomers in isolated administration) with $\alpha < 0.01$ by using a Mann-Whitney test

<i>Peros</i> administration of	molecules	AUC ($\mu\text{g}\cdot\text{h/g}$)	Cmax ($\mu\text{g/g}$)	t _{1/2} (h) [95% CI]	Ke [95% CI]
	Cis- + trans-isomers	969.6 ± 232.7	14.35 ± 2.71	75.8 [66.5-88.3]	0,0091 [0.0078-0.0104]
5.2 mg.kg ⁻¹ of commercial difenacoum (57% cis/43% trans)	cis-isomers	872.1 ± 181.2 *	8.82 ± 1.71	97.2* [84.9-113.8]	0.0071 * [0.0061-0.0082]
	trans-isomers	118.3 ± 24.7 *	5.96 ± 0.77	15.6* [14.0-17.6]	0.0444 * [0.0393-0.0495]
3 mg.kg ⁻¹ of cis-isomers	cis isomers	709.4 ± 87.7 *	9.05 ± 2,83 *	70.6* [60.6-84.4]	0.0098 * [0.0082-0.0114]
3 mg.kg ⁻¹ of trans-isomers	trans-isomers	236.4 ± 87.7 *	3.43 ± 1.14 *	24.2* [14.7-68.6]	0.0287 * [0.0101-0.0472]

Fig. 1

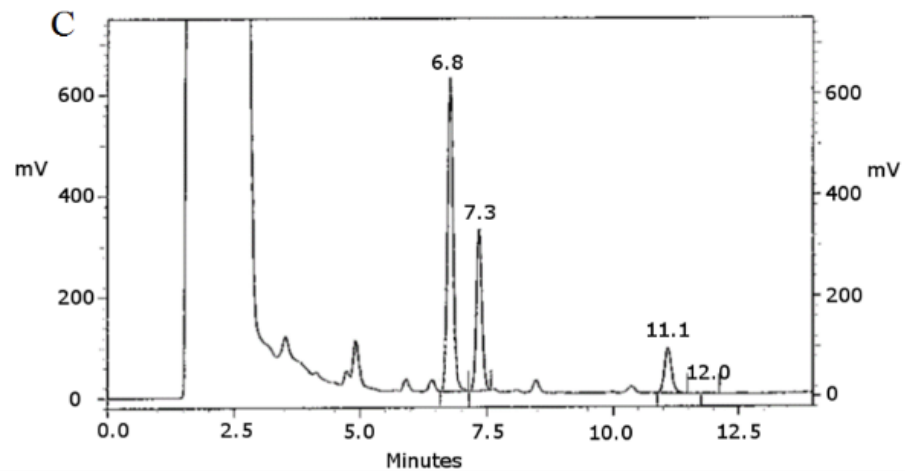
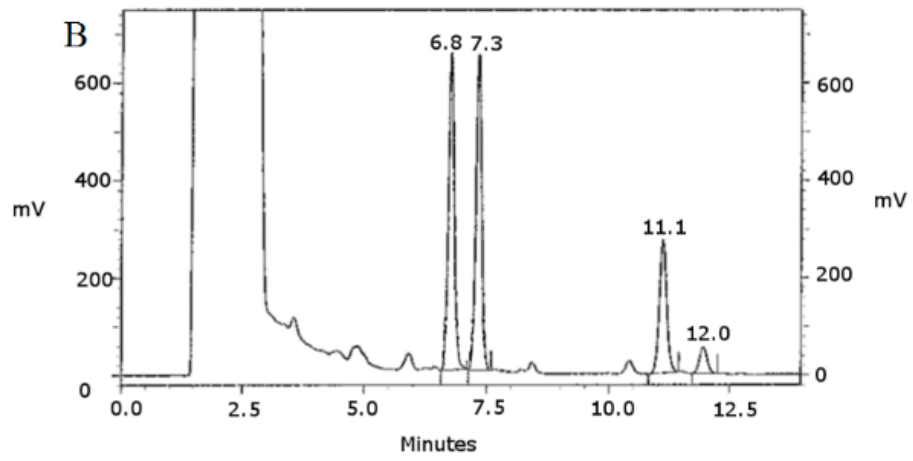
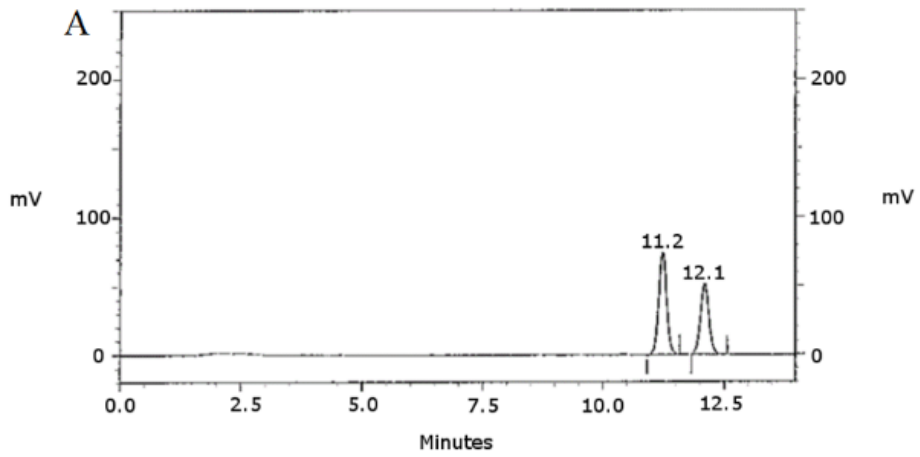
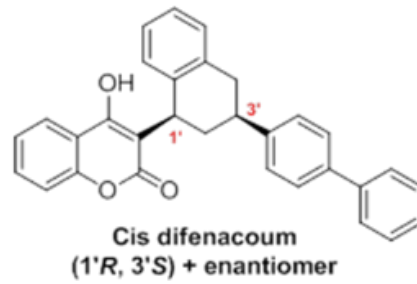
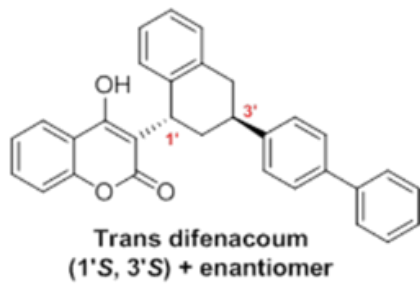
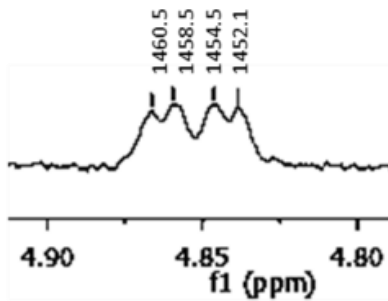


Fig. 2

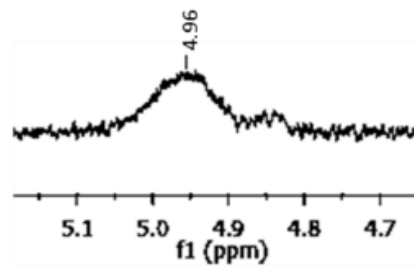
A



B

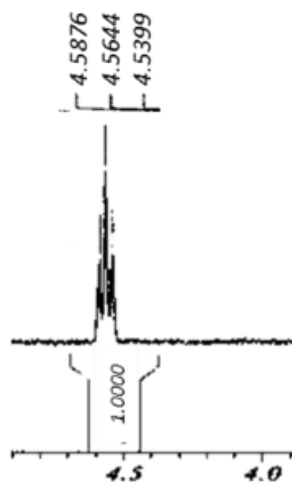


^1H NMR (300 MHz) of $\text{H}_{1'}$ of *trans* difenacoum (*S,S*) and (*R,R*) in C_6D_6

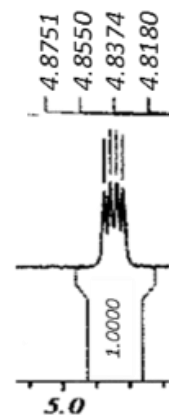


^1H NMR (300 MHz) of $\text{H}_{1'}$ of *cis* difenacoum (*R,S*) and (*S,R*) in C_6D_6

C



^1H NMR of $\text{H}_{1'}$ of *trans* difenacoum (*S,S*) and (*R,R*) in CDCl_3



^1H NMR of $\text{H}_{1'}$ of *cis* difenacoum (*R,S*) and (*S,R*) in CDCl_3

Fig. 3

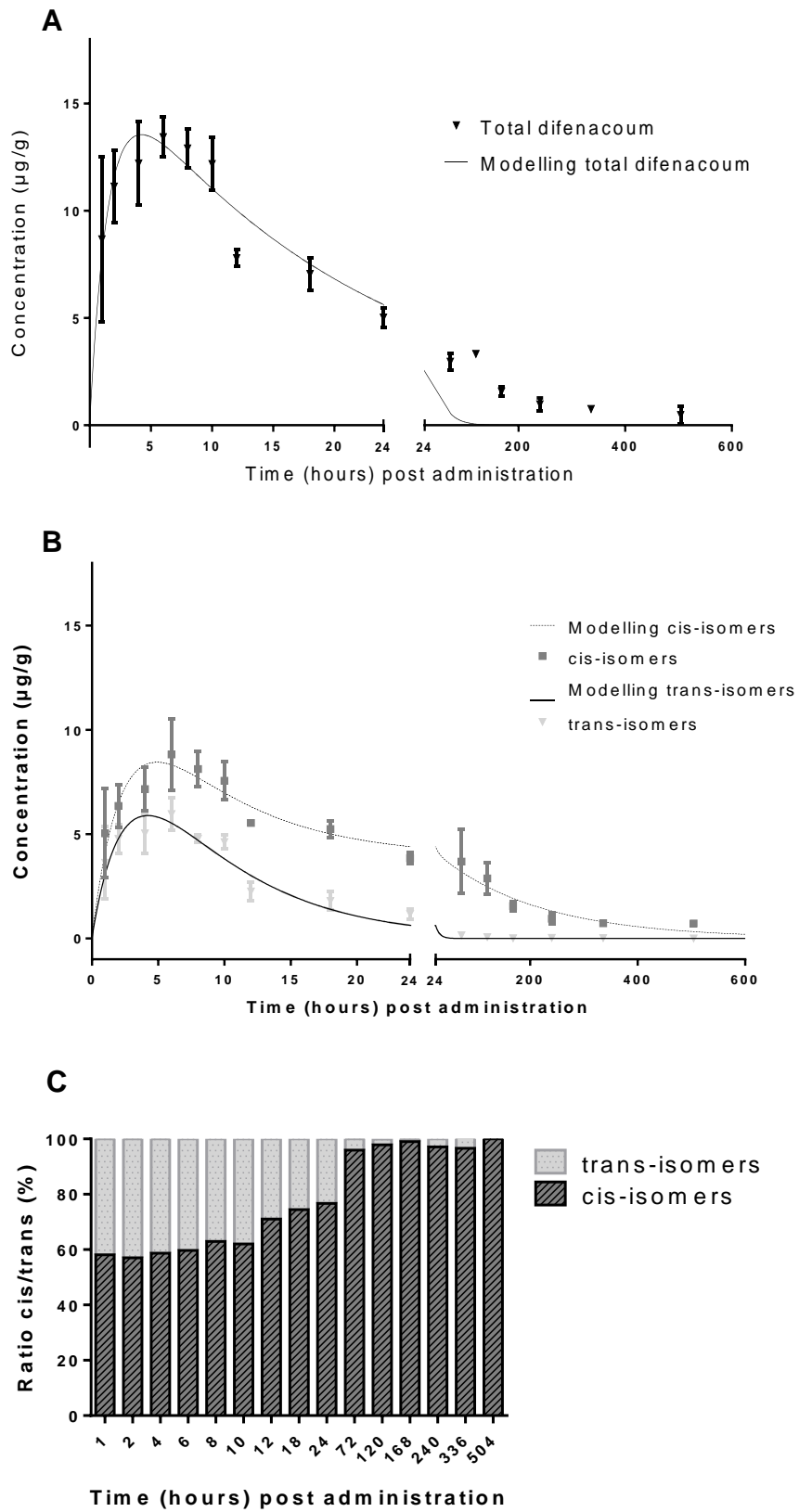


Fig. 4

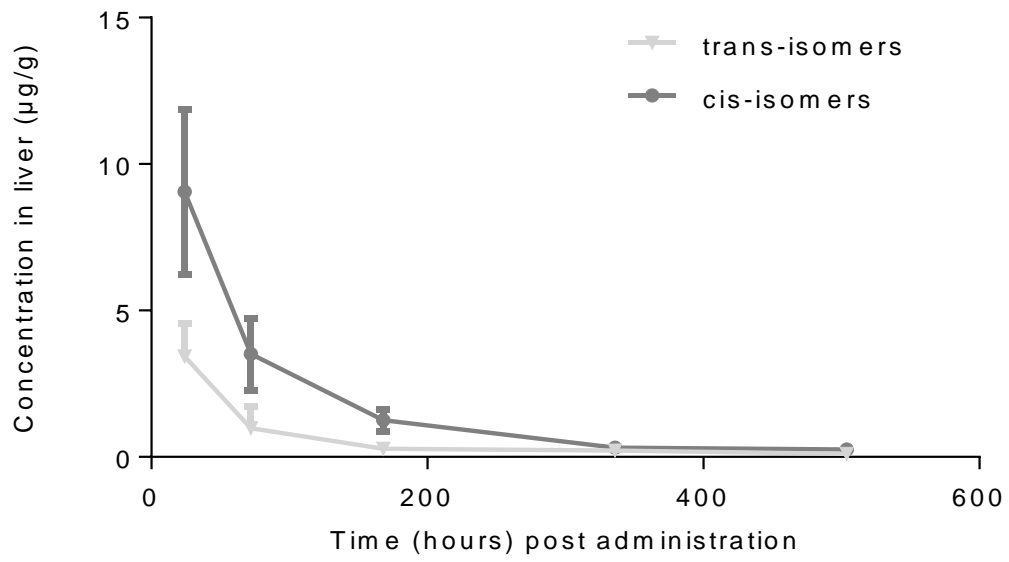


Fig. 5

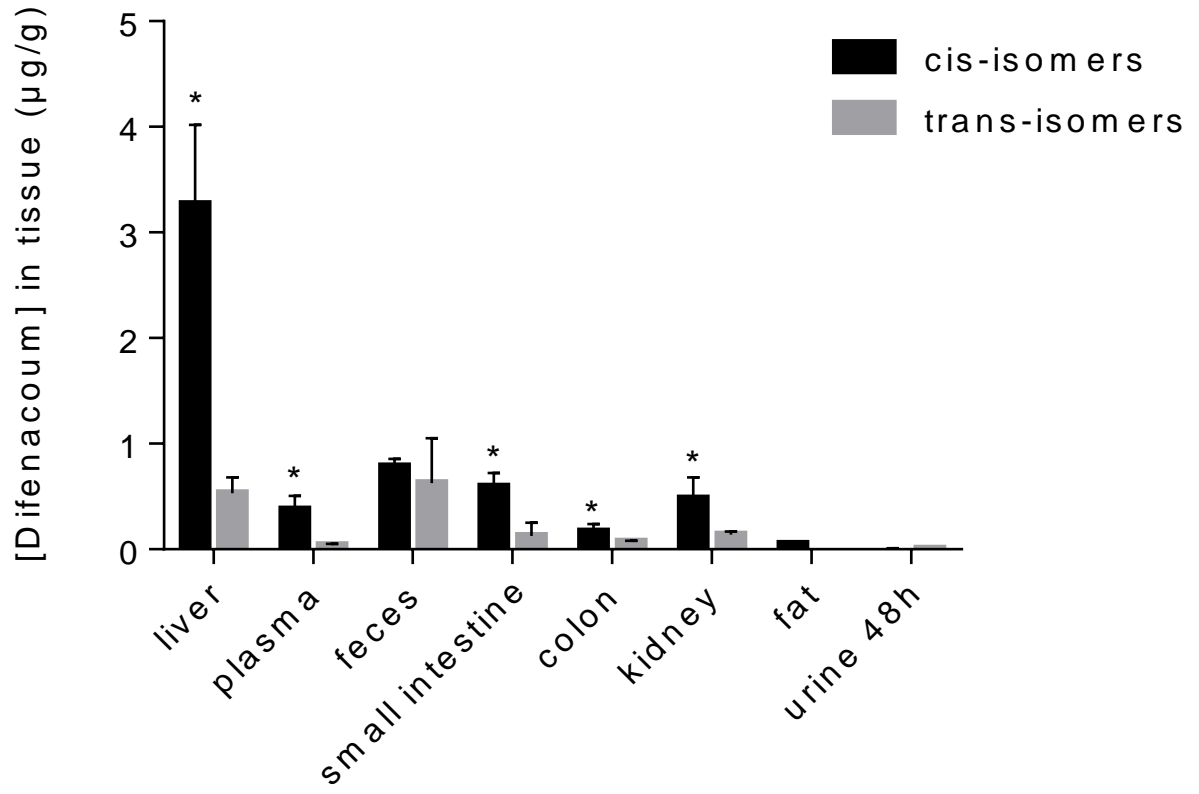


Fig. 6

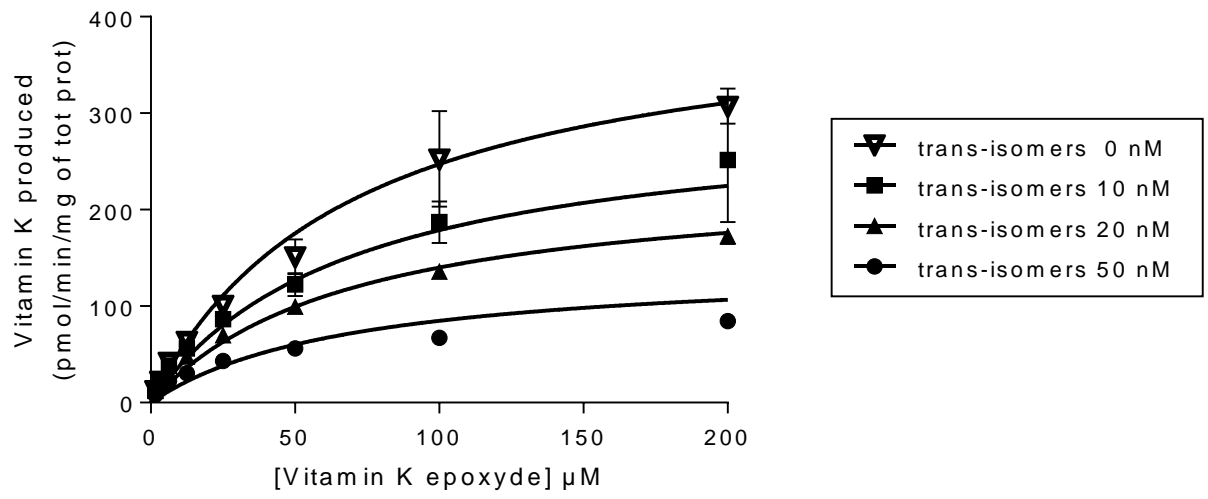
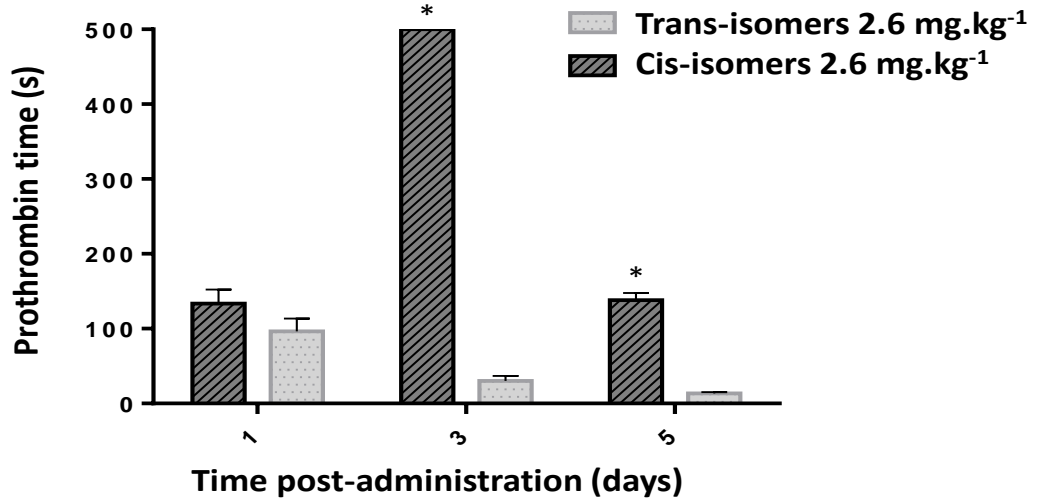


Fig. 7

A/



B/

