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Management of Rodent Populations by Anticoagulant Rodenticides: Toward Third-Generation Anticoagulant Rodenticides

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ABSTRACT

Second-generation anticoagulant rodenticides (SGARs) have been used since the 1980s for pest management. They are highly efficient even in warfarin-resistant rodents. Nevertheless, because of their tissue persistence, nontarget poisoning by SGARs is commonly described in wildlife. Due to this major problem, a new generation of anticoagulants must be developed to limit this risk. This study proposes a method of developing a new generation of anticoagulant rodenticides by revisiting the old SGARs based on the concept of stereochemistry. Each current SGAR is a mixture of diastereomers. Diastereomers of each compound were purified, and their biologic properties were compared by determining their ability to inhibit vitamin K epoxide reductase (VKOR) activity involved in the activation of vitamin K-dependent clotting factors and their toxicokinetic properties. Systematically, for each SGAR, both diastereomers are as effective in inhibiting VKOR activity. However, their toxicokinetic properties are very different, with one of the two diastereomers always more rapidly cleared than the other one. For all SGARs except flocoumafen, the less persistent diastereomer is always the less predominant isomer present in the current mixture. Therefore, the development of baits containing only the less persistent diastereomer would avoid the ecotoxicological risk associated with their use without decreasing their efficacy.

Introduction

Managing populations of commensal rodents is essential. By sharing our environment, they can transmit to humans, directly or indirectly, more than 40 zoonotic pathogens, such as *Yersinia pestis*, Hantavirus, and *Leptospirosis* sp. (Buckle and Smith, 2015). They can cause the destruction or degradation of cereals, accounting for nearly 10% of the grain crops in the world, with considerable variation depending on the country (Buckle and Smith, 2015) [see "Public health policy—can there be an economic imperative? An examination of one such issue" (http://www.cieh.org/jehr/jehr3.aspx?id=11442)]. Rodents also damage substructures and electronic networks.

The management of rodent populations should be based on sanitary and architectural approaches. However, these approaches are often widely associated with the use of rodenticides. The sophisticated social organization of the populations of rats and the neophobic behavior of these animals make the action of toxicity with an immediate effect completely ineffective. The only really effective molecules that can be used to manage such populations are anticoagulant rodenticides (ARs). Their delayed action, with mortality occurring several days after consumption, makes them effective in controlling neophobic species. Indeed, by inhibiting the VKORC1-dependent vitamin K epoxide reductase (VKOR) activity, ARs lead to the progressive reduction of the pool of vitamin K necessary for the activation of clotting factors II, VII, IX, and X (Suttie, 1985; Furie and Furie, 1988). Therefore, prolonged or repeated exposure to ARs leads to rodent death by hemorrhage within 3–7 days. ARs are usually classified into two generations. The first generation of ARs includes diphacinone, warfarin, coumatetralyl, and chlorophacinone. The second generation of ARs includes bromadiolone, difenacoum, brodifacoum, flocoumafen, and difethialone. All ARs are 4-hydroxycoumarin derivatives except chlorophacinone and diphacinone, which are a derivative of indane-1,3-dione (Hadler and Buckle, 1992).

First-generation ARs (FGARs) generally require several days to cause rodent death. The activity of these FGARs is now largely limited by the genetic resistance phenomenon. The first case of resistance to FGARs in brown rats was observed in 1958 in the United Kingdom (Boyle, 1960). In subsequent years, resistance was reported everywhere in rats and mice in Europe (Dodsworth, 1961), the United States (Jackson and Kaukeinen, 1972), Canada (Siddiq and Blaine, 1982), Australia (Saunders, 1978), and Japan. The most common resistance mechanism is a result of single-nucleotide polymorphisms in the *Vkorc1* gene, leading to an enzyme that is less sensitive to the action of ARs (Li et al., 2004; Rost et al., 2004).

The development of second-generation ARs (SGARs) resulted from the emergence of resistance to FGARs in rodent populations. SGARs have been registered from 1975 to 1984, with difenacoum being used first, followed by brodifacoum, bromadiolone, difethialone, and flocoumafen. They are more potent and active after only one feeding due to their longer half-life (Vandenbroucke et al., 2008). Nevertheless, due to their increased tissue persistency, nontarget poisoning by SGARs is commonly described in wildlife. Different species are affected by this exposition, such as stoats, weasels (Elmeros et al., 2011), red foxes (Sage et al., 2010; Geduhn et al., 2015), European minks (Fournier-Chambrillon et al., 2004), bobcats (Serieys et al., 2015), and various raptors such as red kite, buzzards, kestrels, peregrine falcons, and eagle

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ABBREVIATIONS: AR, anticoagulant rodenticide; AUC, area under the curve; FGAR, first-generation anticoagulant rodenticide; HPLC, high-performance liquid chromatography; SGAR, second-generation anticoagulant rodenticide; VKOR, vitamin K epoxide reductase.

owls (Thomas et al., 2011; Christensen et al., 2012; Hughes et al., 2013; Langford et al., 2013; Ruiz-Suárez et al., 2014). ARs were thus identified by the European Union as candidates for future comparative risk assessment and substitution. However, in the absence of an alternative, AR molecules were included in annex 1 of the European Union Biocidal Products Directive 98/8/EC, and their use is still tolerated until a more appropriate solution is found.

This study proposes a method of developing a new generation of ARs by improving the old SGARs based on the concept of stereochemistry. A previous study (Damin-Pernik et al., 2016) demonstrated that difenacoum, the first developed SGAR, is a mixture of two diastereomeric forms, namely, 43% of the composition is *trans*-isomers (with a short half-life) and 57% of the composition is *cis*-isomers (with a long half-life). The production of a new difenacoum containing exclusively *trans*-isomers could significantly reduce its tissue persistency and thus avoid secondary poisoning associated with its use. This method of producing an ecofriendly difenacoum may be applicable to all SGARs. Indeed, all SGARs have two asymmetric carbons (Kelly et al., 1993; Buckle and Smith, 2015). Therefore, all SGARs are a mixture of two diastereomeric forms that could have different biologic properties. This study aimed to explore the differences in the biologic properties of the diastereomers of SGARs and to propose the best diastereomer of each SGAR mixture to use as a rodenticide.

Materials and Methods

Chemicals. Bromadiolone (3-[3-[4-(4-bromophenyl)phenyl]-3-hydroxy-1-phenylpropyl]-4-hydroxychromen-2-one) and difethialone (3-[3-[4-(4-bromophenyl)phenyl]-1,2,3,4-tetrahydronaphthalen-1-yl]-2-hydroxythiochromen-4-one) were supplied by Liphatech (Agen, France). Difenacoum (2-hydroxy-3-[3-(4-phenylphenyl)-1,2,3,4-tetrahydronaphthalen-1-yl]chromen-4-one), brodifacoum (3-[3-[4-(4-bromophenyl)phenyl]-1,2,3,4-tetrahydronaphthalen-1-yl]-4-hydroxychromen-2-one), and flocoumafen (4-hydroxy-3-[3-[4-[[4-(trifluoromethyl)phenyl]methoxy]phenyl]-1,2,3,4-tetrahydronaphthalen-1-yl]chromen-2-one) were purchased from Hangzhou Ich Biofarm Co. (Hangzhou, China).

The (1R,3R) and (1S,3S) isomers (called *trans*-isomers) and (1R,3S) and (1S,3R) isomers (called *cis*-isomers) of difenacoum, difethialone, flocoumafen, and brodifacoum were separated and purified in a silica gel column in our laboratory and assigned according to information available in published articles (Damin-Pernik et al., 2016), regulatory documents (European Parliament, 2007, 2009), and patents (Caruel et al., 2015). The (1R,3S) and (1S,3R) *trans*-isomers and (1R,3R) and (1S,3S) *cis*-isomers of bromadiolone were also separated and purified in a silica gel column in our laboratory. Dimethylsulfoxide, acetonitrile, methanol, acetone, diethyl ether, hexane, and orthophosphoric acid were obtained from VWR International (Fontenay-sous-Bois, France), and vetflurane and vitamin K_1 were obtained from Alcyon (Miribel, France). Vitamin K_1 was converted to vitamin K epoxide according to the method described in Tishler et al. (1940). Purity was estimated by liquid chromatography/mass spectrometry and was higher than 99%. High-performance liquid chromatography (HPLC)–grade water, which was prepared using a Milli-Q Plus system (Millipore, Saint-Quentin-en-Yvelines, France), was used for the preparation of the HPLC eluents.

Animals. Eight-week-old male Oncins France strain A 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 (reference A04, Scientific Animal Food and Engineering Limoges, France) and water ad libitum. Experimental research on the rats was performed according to an experimental protocol following international guidelines and with approval from the ethics committee of the Veterinary School of Lyon.

Pharmacokinetic Study. Male Oncins France strain A Sprague-Dawley rats received, by oral administration, *cis*- or *trans*-isomers of difenacoum, bromadiolone, flocoumafen, brodifacoum, and difethialone. Anticoagulants were dissolved in 10% dimethylsulfoxide and 90% vegetable oil and were administered by force feeding. The animals were given a daily subcutaneous injection of vitamin K_1

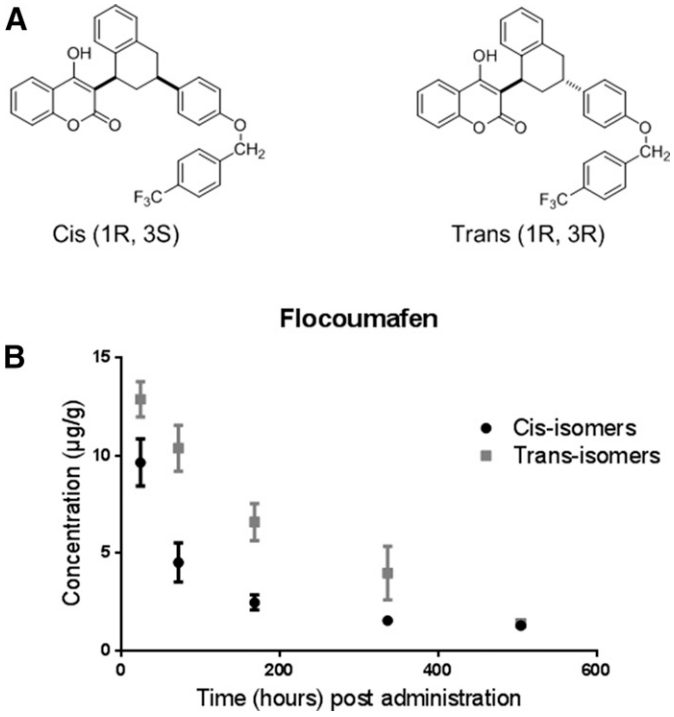

Fig. 1. Structures of the *cis*- and *trans*-isomers of flocoumafen (A). The *cis*- and *trans*-isomers referenced in the text arise from the alternative orientations of the substituents on the tetralin ring system. Time-dependent concentrations of the *cis*- and *trans*-isomers of flocoumafen after oral administration of 3 $mg \cdot kg^{-1}$ of silica gel column-purified *cis*- or *trans*-isomers of flocoumafen to susceptible rats. The results are the mean ± S.D. of four rats. The time points are 24, 72, 168, 336, and 504 hours, which correspond to 1, 3, 7, 14, and 21 days, respectively (B).

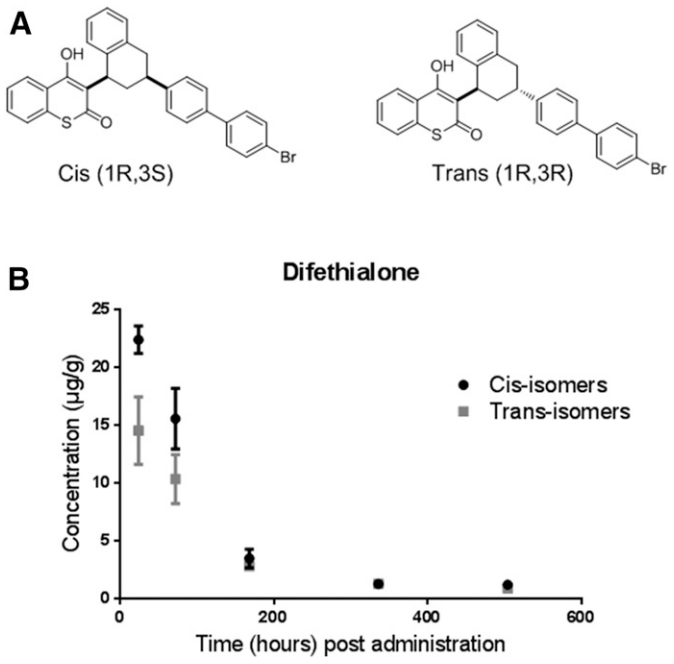

Fig. 2. Structures of the *cis*- and *trans*-isomers of difethialone (A). The *cis*- and *trans*-isomers referenced in the text arise from the alternative orientations of the substituents on the tetralin ring system. Time-dependent concentrations of *cis*- and *trans*-isomers of difethialone after oral administration of 3 $mg \cdot kg^{-1}$ of purified *cis*- or *trans*-isomers of difethialone to susceptible rats. The results are the mean ± S.D. of four rats. The time points are 24, 72, 168, 336, and 504 hours, which correspond to 1, 3, 7, 14, and 21 days, respectively (B).

(5 mg/kg). Finally, the rats were anesthetized with vetflurane. Blood samples were obtained via terminal blood collection by cardiac puncture in citrated tubes. The rats were euthanized with CO_2, and the livers were then collected and stored at −20°C until analysis.

No-Choice Feeding Test with *Cis*- or *Trans*-Isomers of Bromadiolone and Difethialone. Before testing, all of the animals were maintained on diet A04 (Scientific Animal Food and Engineering) and water ad libitum. Individuals were weighed before the start of the test. No-choice feeding tests were performed on groups of 10 rats. The rats were fed bait containing 0.005% *cis*- or *trans*-isomers of bromadiolone or 0.0015% *cis*- or *trans*-isomers of difethialone. *Cis*- and *trans*-isomers of bromadiolone and difethialone are pure at 93%, 98%, 99%, and 94% (the remaining part is the other stereoisomer). Feeding periods of 4 days were used. The contents of food pots were weighed daily, and the intake of bait was recorded. The mortality of rats was also observed daily. At the end of the feeding period with toxic baits, the animals were given diet A04 again and were observed for an additional period of 17 days.

Determination of AR Concentration in the Liver. Liver tissue (1 g) was extracted with acetone using an Ultra Turrax tissue disperser from IKA Labortechnick (VWR International, Strasbourg, France) according to the method previously published in Damin-Pernik et al. (2016) and validated according to the guideline on bioanalytical method validation published by the European Medicines Agency (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf). The extract was centrifuged at 3000 rpm for 10 minutes, and the supernatant was evaporated at 60°C under a gentle nitrogen flow. Dry extract was resuspended in acetonitrile/hexane (50%/50%). The hexane layer was eliminated, and the remainder was dried at 60°C under a gentle nitrogen flow. The final dry extract was dissolved in methanol, and the AR concentrations were analyzed by HPLC on a reverse phase C-18 column (4.6 × 150 mm, 5 μm; Waters, Milford, MA) at detection wavelengths of 258, 260, 265, 267, and 272 nM for difenacoum, difethialone, bromadiolone, brodifacoum, and flocoumafen, respectively. The C-18 column was heated at 40°C for difenacoum, bromadiolone, flocoumafen, and brodifacoum and at 30°C for difethialone. A gradient elution system was used with a flow rate of 1 ml/min as follows: from 30% methanol/70% water (acidified with 0.2% H_3PO_4) to 70% methanol/30% water at 10 minutes for difenacoum; from 30% acetonitrile/70% water (acidified with 0.1% H_3PO_4) to 90 acetonitrile/10% water at 14 minutes for brodifacoum; from 60% methanol/40% water (acidified with 0.2% H_3PO_4) to 80% methanol/20% water at 20 minutes for bromadiolone; from 60% methanol/40% water (acidified with 0.2% H_3PO_4) to 90% methanol/10% water at 22 minutes for flocoumafen; and from 60% acetonitrile/40% water (acidified with 0.2% H_3PO_4) to 75% acetonitrile/25% water at 10 minutes for difethialone. The recovery rate of diastereomers of SGAR from tissues were between 75% and 95% in liver and the CV% values for precision of the different ARs were less than 15%.

VKOR Activity Assay and Kinetics. Liver microsomes were prepared from fresh livers by differential centrifugation according to the protocol described in Hodroge et al. (2011). Microsomal VKOR activity was assayed according to the protocol described in Hodroge et al. (2011, 2012). The inhibiting effect of the silica gel column-purified *cis*- or *trans*-isomers of ARs was evaluated by determining the K_i value after the 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 approximately 0.05 to 20 × K_i.

Data Analysis. Pharmacokinetic calculations were performed using the noncompartmental approach on the mean results per group. The elimination half-life ($t_{1/2(el)}$) was calculated using linear regression in the GraphPad Prism 6 software (GraphPad, La Jolla, CA). 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 → ∞). Statistical analyses of the pharmacokinetic parameters were done using GraphPad Prism 6. A Mann-Withney test was used with $\alpha < 0.05$ to compare statistically the results between the two groups. For kinetic analysis of the VKOR activity, data were fitted by nonlinear regression to the noncompetitive or competitive inhibition model using GraphPad Prism 6. The choice of the best model was based on the corrected Akaike information criterion.

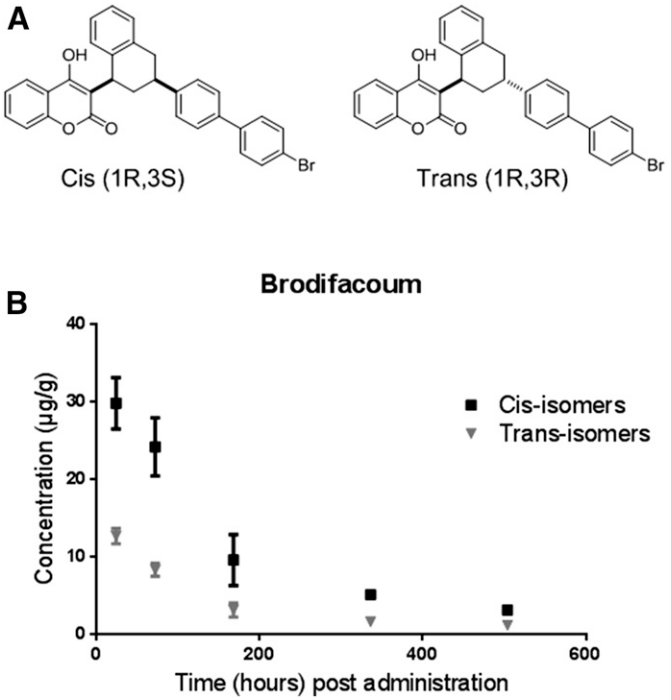

Fig. 3. Structures of the *cis*- and *trans*-isomers of brodifacoum (A). The *cis*- and *trans*-isomers referenced in the text arise from the alternative orientations of the substituents on the tetralin ring system. Time-dependent concentrations of *cis*- and *trans*-isomers of brodifacoum after oral administration of 3 mg·kg^{-1} of silica gel column-purified *cis*- or *trans*-isomers of brodifacoum to susceptible rats. The results are the mean ± S.D. of four rats. The time points are 24, 72, 168, 336, and 504 hours, which correspond to 1, 3, 7, 14, and 21 days, respectively (B).

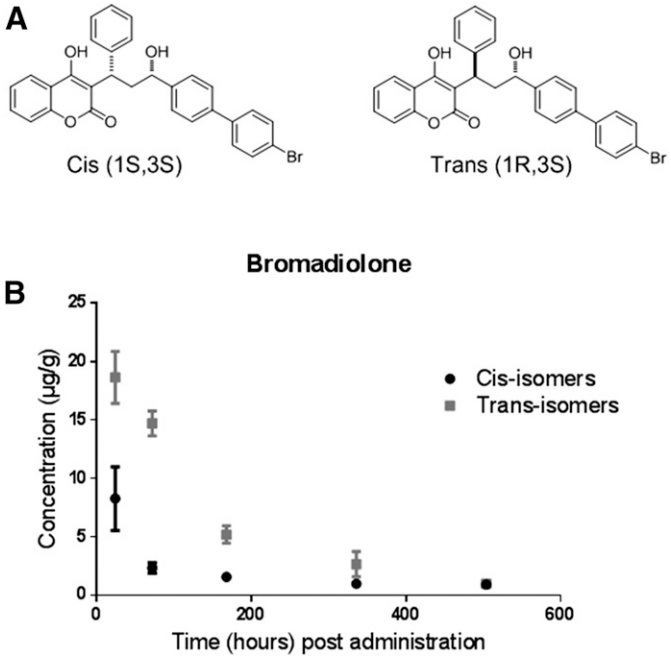

Fig. 4. Structures of the *cis*- and *trans*-isomers of bromadiolone (A). The *cis*- and *trans*-isomers referenced in the text arise from the alternative orientations of the substituents on the tetralin ring system. Time-dependent concentrations of *cis*- and *trans*-isomers of bromadiolone after oral administration of 3 mg·kg^{-1} of purified *cis*- or *trans*-isomers of bromadiolone to susceptible rats. The results are the mean ± S.D. of four rats. The time points are 24, 72, 168, 336, and 504 hours, which correspond to 1, 3, 7, 14, and 21 days, respectively (B).

Results

Separation of the Diastereoisomers of SGARs. The analysis of SGARs by HPLC on a C18 reverse phase column as described in the experimental procedure showed, for each compound, two different peaks corresponding to the *cis*- and *trans*-diastereomer forms. The structures of the *cis*- and *trans*-isomers of flocoumafen, difethialone, brodifacoum, bromadiolone, and difenacoum are shown in Figs. 1A, 2A, 3A, 4A, and 5A, respectively. The *cis*- and *trans*-isomers are shown in only one alternative orientation of substituents. Both peaks of each compound presented the same absorption spectrum, with maximum absorbances at 258, 265, 272, 267, and 260 nm for difenacoum, bromadiolone, flocoumafen, brodifacoum, and difethialone, respectively. The retention times were 11.3 and 12.5 minutes for the *cis*- and *trans*-isomers of difenacoum, 15.0 and 16.2 minutes for the *cis*- and *trans*-isomers of brodifacoum, 20.1 and 20.7 minutes for the *cis*- and *trans*-isomers of flocoumafen, 17.1 and 17.9 minutes for the *cis*- and *trans*-isomers of bromadiolone, and 20.2 and 21.3 minutes for the *cis*- and *trans*-isomers of difethialone, respectively.

Comparative Analysis of the Tissue Persistence of the *Trans*- and *Cis*-Isomers of the Five SGARs in the Liver. To study the hepatic persistence of the *trans*- and *cis*-isomers of SGARs, male Oncins France strain A Sprague-Dawley rats were divided into groups of four rats that received, by oral administration, 3.0 $mg \cdot kg^{-1}$ of *trans*- or *cis*-isomers of flocoumafen, difethialone, brodifacoum, bromadiolone, or difenacoum. The rats were killed 24, 72, 168, 336, or 504 hours after the administration, and the concentrations of the *trans*- and *cis*-isomers of SGARs were determined in the liver for each rat. The time-dependent evolution of the *cis*- and *trans*-isomers in the liver is presented in Fig. 1B for flocoumafen, Fig. 2B for difethialone, Fig. 3B for brodifacoum, Fig. 4B for bromadiolone, and Fig. 5B for difenacoum.

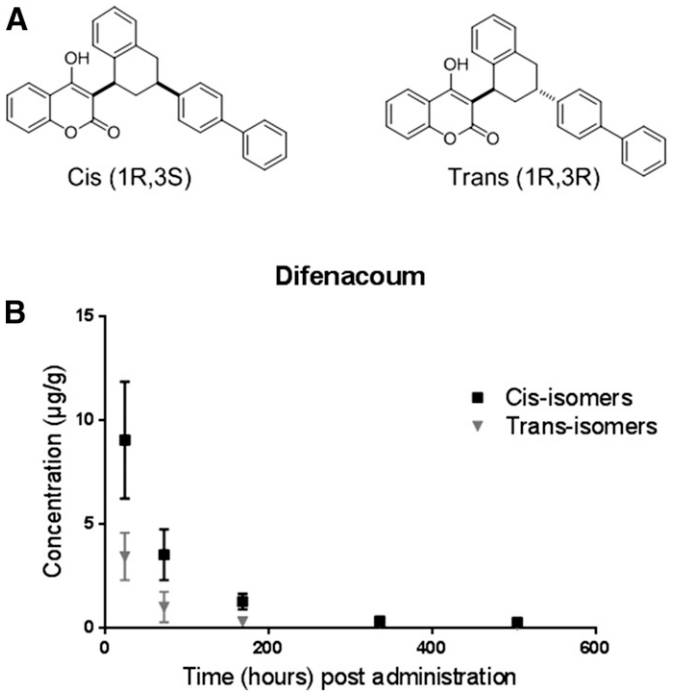

Fig. 5. Structures of the *cis*- and *trans*-isomers of difenacoum (A). The *cis*- and *trans*-isomers referenced in the text arise from the alternative orientations of the substituents on the tetralin ring system. The time-dependent concentrations of the *cis*- and *trans*-isomers of difenacoum after oral administration of 3 $mg \cdot kg^{-1}$ of purified *cis*- or *trans*-isomers of difenacoum to susceptible rats. The results are the mean ± S.D. of four rats. The time points are 24, 72, 168, 336, and 504 hours, which correspond to 1, 3, 7, 14, and 21 days, respectively (B).

The profile of elimination was the same for the five molecules studied; specifically, one isomer was eliminated more quickly than the other, and the decrease followed a clear biphasic profile. For difenacoum, difethialone, and brodifacoum, the *cis*-isomer was eliminated less rapidly than the *trans*-isomer. However, for bromadiolone and flocoumafen, the *cis*-isomer was eliminated more quickly. The half-lives and AUCs of the *trans*- and *cis*-isomers in the liver were calculated using a linear regression model and are presented in Table 1.

Analysis of the Inhibiting Effect of the *Trans*- and *Cis*-Isomers of SGARs on VKOR Activity. The ability of the *cis*- and *trans*-isomers of each SGAR to inhibit the VKOR activity catalyzed by warfarin-susceptible rat liver microsomes was evaluated by determining the K_i constants. The results are presented in Table 2. The *cis*- and *trans*-isomers of each SGAR were all able to inhibit VKOR activity. For each SGAR, the K_i constants were similar between the diastereomers.

No-Choice Feeding Test with the *Cis*- or *Trans*-Isomers of Bromadiolone or Difethialone. A no-choice feeding test was performed to confirm the efficiency of baits enriched with the less persistent pair of diastereomers and to determine the benefits in terms of liver residues. Assays were performed with bait containing mainly one isomer of bromadiolone or difethialone at concentrations of 50 and 15 ppm for bromadiolone and difethialone, respectively (Table 3). Bromadiolone was chosen because it is the SGAR with the greatest difference of half-life between *cis*- and *trans*-isomers. Difethialone was chosen because it is the SGAR with the smallest difference of half-life between *cis*- and *trans*-isomers. The baits were composed of 93% *cis*-isomers with 7% *trans*-isomers or 2% *cis*-isomers with 98% *trans*-isomers for bromadiolone and 6% *cis*-isomers with 94% *trans*-isomers or 99% *cis*-isomers with 1% *trans*-isomers for difethialone. The daily consumption of baits was similar for the first 3 days between treatments, with an important decrease on day 4. A 100% mortality rate was observed for all of the baits used. Regardless of the bait used, the rats died in an average of 5 days, with mortality observed between days 3 and 9. The concentrations of the AR residues were determined after the death of the rats. The residues of bromadiolone in the liver following the death of the rats were 4-fold reduced using the bait containing principally the *cis*-isomers compared with the bait containing the *trans*-isomers of bromadiolone. The residues of difethialone in the liver were only 1.5-fold reduced using the *trans*-isomers compared with the *cis*-isomers.

Discussion

ARs are crucial for controlling rodent populations. Indeed, trapping can be effective if there is a low density of rodents; however, in the

TABLE 1

Pharmacokinetic parameters in the liver after single oral administration of the *cis*- or *trans*-isomers of ARs in susceptible rats at a dose of 3 $mg \cdot kg^{-1}$

Molecule	AUC	$t_{1/2}$ (95% Confidence Interval)	
	$\mu g \cdot h \cdot g^{-1}$	*hour*	
DFC *cis*-isomers	709.4 ± 206.10[a]	78.33 (64.66–99.33)	$P = 0.0044$
DFC *trans*-isomers	236.8 ± 87.37[a]	24.18 (14.67–68.56)	$P = 0.0044$
BDF *cis*-isomers	4827 ± 830.40[a]	120.82 (110.74–732.89)	$P < 0.0001$
BDF *trans*-isomers	1665 ± 242.80[a]	68.73 (57.28–85.84)	$P < 0.0001$
FLO *cis*-isomers	1247 ± 176.00	76.73 (60.48–104.91)	$P < 0.0001$
FLO *trans*-isomers	2713 ± 446.00	177.45 (145.13–228.31)	$P < 0.0001$
BDL *cis*-isomers	822.7 ± 131.40[a]	26.89 (19.65–42.55)	$P < 0.0001$
BDL *trans*-isomers	2733 ± 379.90[a]	75.62 (64.72–90.96)	$P < 0.0001$
DFT *cis*-isomers	2410 ± 308.00[a]	71.60 (44.52–61.78)	$P = 0.0310$
DFT *trans*-isomers	1894 ± 459.00[a]	52.93 (46.36–61.67)	$P = 0.0310$

BDF, brodifacoum; BDL, bromadiolone; DFC, difenacoum; DFT, difethialone; FLO, flocoumafen.

[a]Denotes a significant difference between two groups (AUC of *cis*- or *trans*-isomers in isolated administration) with $\alpha < 0.05$ by using a Mann-Whitney test.

TABLE 2

The K_i values of the VKOR activity catalyzed by warfarin-susceptible rat liver microsomes for the *cis*- and *trans*-isomers of each SGAR

To determine the VKOR activity, standard reactions were performed in 200 mM Hepes buffer (pH 7.4) containing 150 mM KCl and 0.5 $g \cdot L^{-1}$ of microsomal proteins. Each data point represents the mean ± S.D. of three individual analyses. Comparison between two groups was performed using the Mann-Whitney test with $P < 0.05$.

	K_i Value of VKOR Activity Toward	
	Cis-Isomer	*Trans*-Isomer
nM		
Brodifacoum	18 ± 2	22 ± 1
Bromadiolone	31 ± 2	26 ± 2
Difenacoum	17 ± 1	21 ± 2
Difethialone	27 ± 1	22 ± 1
Flocoumafen	47 ± 2	55 ± 5

presence of a large population, trapping is time-consuming and ineffective (Buckle and Smith, 2015). Efficient repellents/attractants and ultra-sound seem limited because of the habituation of rodents (Berny et al., 2014). Other chemical products (alphachloralose, cholecalciferol, zinc phosphide, and bromethalin) present major drawbacks, making their use or their approval difficult (Rodenticide Resistance Action Committee, 2015). Nevertheless, despite the importance of ARs in the management of rodent populations, their use must take into account different issues: the resistance problem associated with the use of FGARs (Hadler and Buckle, 1992; Rost et al., 2009) and the secondary poisoning of wildlife essentially associated with the use of SGARs (Rattner et al., 2014). Therefore, third-generation ARs are urgently needed. The stereochemistry-based solution proposed recently to produce a new and eco-friendly difenacoum (Damin-Pernik et al., 2016) seems to be a method of rapidly generating this third-generation of ARs at a reduced cost. Indeed, the five commercially available SGARs are a mixture of diastereomeric forms (Huckle et al., 1988; Hunter et al., 1988; Kelly et al., 1993; European Parliament, 2007; Cort et al., 2012), which are generally easy to separate and purify. To rapidly generate the third-generation ARs, it is necessary to revisit the SGARs to identify the diastereomer of each SGAR with the smallest half-life and increase its amount in each mixture.

The data found in the scientific literature, in the regulatory documents, allowed the systematic identification of the major and minor diastereomers in the commercial preparation of each SGAR. Flocoumafen, difenacoum, and brodifacoum are a mixture of 50%–80% *cis*-isomers and 20%–50% *trans*-isomers (Swaine, 1985; Kelly et al., 1993; European Parliament, 2009; Damin-Pernik et al., 2016); bromadiolone is a mixture of more than 70% of *trans*-isomers and less than 30% of *cis*-isomers; and difethialone is a mixture of more than 70% of *cis*-isomers and less than 30% of *trans*-isomers (Caruel et al., 2015). The original reasons for the development of SGARs with such ratios between diastereomers have never been mentioned previously in the literature. We could assume that such ratios resulted from the need to kill the rodent after a single administration when these SGARs were developed (Hadler and Buckle, 1992) by enriching the mix with either the most potent inhibitor or the most persistent pair of diastereomers. Another possibility is that the SGAR synthesis process does not give a racemate but gives a mixture of isomers enriched in one of them.

To characterize the inhibiting properties, the ability to inhibit VKOR activity of *cis*- or *trans*-isomers of SGARs was determined. The K_i results obtained in this study demonstrated that *cis*- and *trans*-isomers of the same SGAR could similarly inhibit VKOR activity (Fig. 6; Table 2). Therefore, at identical hepatic concentrations, diastereomers of the same SGAR have the same anticoagulant properties when used in warfarin-susceptible rats.

To characterize the tissue persistence, toxicokinetic analyses were done. These toxicokinetic studies were conducted in rats after a single oral administration of the AR. This administration was performed by force feeding to better control the dose and time of ingestion. The same dose was used between SGARs regardless of the LD_{50} to be able to compare the molecules. Only the concentrations of ARs in the liver could be followed for 21 days because plasma and other tissue concentrations rapidly decrease below the limit of quantification. Two toxicokinetic parameters were calculated in this study, the AUC and half-life (Fig. 6; Table 1). The AUC is the integrated area under the hepatic AR concentration versus time curve. The more the AUC is elevated, the greater the importance of the risk of secondary poisoning of wild predators or scavengers. The results obtained herein demonstrated that in each mixture of SGAR there is systematically one diastereomeric form with a lower AUC. The ratios of the AUCs between diastereomers of the same SGAR were 1.5 for difethialone and 3–3.5 for difenacoum, bromadiolone, and brodifacoum. The lowest AUC for all of them was systematically observed for the minor diastereoisomer, except for flocoumafen, in which the lowest AUC was observed for the major diastereomer of the current commercial preparation. Because AUCs could be affected by absorption or clearance, the half-life of diastereomers in the liver was also calculated. Interestingly, the diastereomer with the lowest AUC systematically corresponded to the diastereomer with the lowest half-life in the liver, suggesting a faster elimination of this diastereomer than of the other one. This result has already been demonstrated for difenacoum (Damin-Pernik et al., 2016).

These AUC and half-life differences between diastereomers of the same SGAR could result in major practical consequences. The composition of each SGAR could thus be systematically modified to enrich the mixture with the less persistent diastereomer to obtain a new AR with the same anticoagulant properties but with decreased tissue persistence. Thus, a new bromadiolone containing more than 70% *cis*-isomers and a new brodifacoum, difenacoum, or difethialone containing more than 70% *trans*-isomers may be developed to create the new generation of anticoagulants.

The benefits will be greater for the current ARs 1) containing essentially the most persistent diastereomer and 2) with the most

TABLE 3

No-choice feeding test with the *cis*- or *trans*-isomers of bromadiolone and difethialone

The daily consumption of bait was measured, and the liver of each rat was collected after death. The concentrations of the different isomers were measured in the liver. The results are the mean of 10 rats.

Bait Containing	Number of Deaths	Day of Death	Consumption of			Residue (μg/liver)			% Residue/Total AR Ingested
			Bait	*Cis*-Isomer	*Trans*-Isomer	*Trans*-isomer	*Cis*-isomer	Total Residue	
			g	μg	μg	μg	μg	μg	
BDL, 50 ppm 93% *cis*/7% *trans*	6/6	6	44	2025	152	25	32	57	2.6
BDL, 50 ppm 2% *cis*/98% *trans*	6/6	5	47	47	2292	236	0	236	10.8
DFT, 15 ppm 94% *trans*/6% *cis*	5/5	5	49	44	691	43	4	47	6.4
DFT, 15 ppm 1% *trans*/99% *cis*	5/5	5	47	695	7	0	71	71	10.2

BDL, bromadiolone; DFT, difethialone.

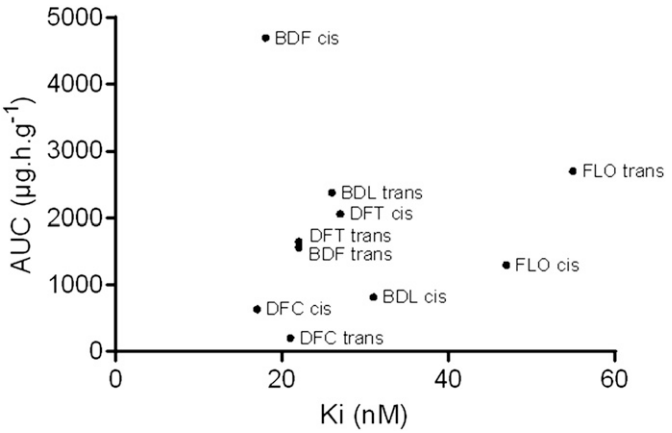

Fig. 6. Two-dimensional representation of the biologic properties of *cis*- and *trans*-isomers of SGAR.

significant differences in half-life between the diastereoisomers. Bromadiolone and difethialone were chosen because bromadiolone is the most favorable example to illustrate the concept (highest difference in AUC and half-life and high percentage of the persistent diastereomer in the current mixture), while difethialone is the most unfavorable example (almost no difference in the AUC between the diastereomers). To illustrate this benefit, the efficiency and persistence of four different types of baits were evaluated: bait containing the current bromadiolone mixture was compared with bait containing a new bromadiolone mixture containing 93% *cis*-isomers, and bait containing the current difethialone mixture was compared with bait containing a new difethialone mixture containing 94% *trans*-isomers. The baits were all effective, with 100% mortality obtained after 4 days of exposure at the current commercial concentration for bromadiolone baits or even below for difethialone baits (concentrations of commercial baits containing bromadiolone or difethialone are currently 50 and 25 ppm, respectively). However, the baits enriched with the lowest persistent diastereomer systematically showed a decrease in the quantity of residues in the liver at the time of death of the animals. The reduction of the residues in the liver was greater for bromadiolone enriched in *cis*-isomers with a 4-fold reduction of the residues compared with the current bromadiolone. For difethialone, the use of *trans*-isomers reduced the liver residues by a factor of approximately 2 compared with the current difethialone.

The development of such baits enriched in the less persistent diastereomer could avoid the ecotoxicological risk associated with their use, especially regarding plant protection. These baits would reduce the amount of residues at the time of death of the rodents. Nevertheless, if these dying rodents with reduced residues are eaten by scavengers or predators, the elimination of such an AR could also be accelerated in the liver of scavengers or predators. Therefore, the ecotoxicological gain could be at both levels, and these third-generation ARs would be a good solution to avoid environmental problems associated with pest management.

Authorship Contributions

Participated in research design: Espana, Lattard.

Conducted experiments: Espana, Benoit, Fourel, Damin-Pernik, Lattard.

Contributed new reagents or analytic tools: Espana, Caruel, Damin-Pernik.

Performed data analysis: Espana, Benoit, Damin-Pernik, Lefebvre, Lattard.

Wrote or contributed to the writing of the manuscript: Espana, Fourel, Damin-Pernik, Lefebvre, Lattard.

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