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Pharmacokinetic and inhibition potency of the Four stereoisomers of difethialone in rats–Development of modeling allowing the choice of appropriate stereoisomers ratio

Sébastien Lefebvre¹ Isabelle Fourel¹, Nolan Chatron¹, Hervé Caruel², Etienne Benoit¹, Virginie Lattard¹

Abstract

The current management of rodent pest populations is based on second-generation anticoagulant rodenticides (SGAR). These molecules, of which difethialone is part, are much more efficient than the first generation. Nevertheless, this efficiency comes with a major drawback, SGARs are tissue-persistent that increases the exposure of rodent predators to them. According to its chemical structure, difethialone has four stereoisomers, whose specific inhibition potency and pharmacokinetic have never been described and might be useful to design new eco-friendly rodenticides. The study aimed to investigate the ability to inhibit anticoagulant target enzyme (VKORC1) and the pharmacokinetics in rats of the four difethialone stereoisomers in rats. We show that stereoisomers are all highly efficient to inhibit VKORC1 activity, but they have distinct initial half-life with 6.0h, 25.4h, 69.3h and 82.3h for respectively E4-trans, E2-cis, E1-trans and E3-cis stereoisomer. These results open the way of the development of eco-friendly and efficient rodenticide by mixing some of these stereoisomers. Preferential incorporation of the E4-trans stereoisomer (high inhibitory VKORC1 potency, relatively shorter liver half-life) into difethialone rodenticides baits might produce a more eco-friendly product than current commercially-available difethialone formulations. In addition, we put forward modeling to help design bait according to the circumstance of use (presence of non-target species, food competition, etc) by modulating the theoretical AUC and the theoretical concentration of the product at the death of the rodent pest. Thus, this modeling might allow to diminish the use of laboratory animal in assay.

Keywords

Modeling — Difethialone — Rodents

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Introduction

The use of second generation anticoagulant rodenticides (SGAR) is the cornerstone of the management of the rodent pest population. Indeed, as the first-generation anticoagulant rodenticides (FGARs), SGARs have a delayed action avoiding food aversion due to the association by rodents of bait consumption and death of other rodents, if this occurs quickly after bait consumption. In addition, SGARs like FGARs have an efficient and safe antidote, vitamin K1. A considerable advantage over FGAR, SGAR molecules are also efficient on rodents carrying Vkorc1 mutations leading to resistance to

vitamin K anticoagulant, this is the main reason for the development of these molecules. However, this advantage comes with a dark side, SGARs are tissue-persistent. This last point is a major concern today, considering the ecotoxicology of the SGAR. Indeed, many studies have pinpointed an exposure of rodent predators to SGAR [1–6]

In the two last years, significant progress has been made in the development of new AR. It is based on the stereochemistry of SGAR and more specifically on the modification of cis/trans diastereoisomers proportions. Indeed, for the same AR molecule, both diastereoisomers exhibit always different half-life compared to the other one while keeping their inhibitory potency [7, 8]. Moreover, a recent ecological study found that only trans-bromadiolone is involved in the poisoning of non-targeted species whereas the rodent bait contained both diastereoisomers [9, 10] However, considering difethialone, the diastereoisomers approach failed. The initial half-lives of both diastereoisomers are not sufficiently different. To go through the diastereoisomers concept and to improve again the efficiency and the eco-compatibility of rodenticides, we have separately studied properties of the two enantiomers of each diastereomer of difethialone.

This study opens an innovative way to improve difethialone by producing a new mix of the four stereoisomers instead of the mix currently used in the different formulations containing difethialone, the latter being composed of more than 90% of cis-isomers. This new approach implies to study the pharmacokinetics of the four molecules of difethialone to describe their respective elimination kinetic and inquire about the possibility for improved ecotoxicology. Then, their ability to inhibit VKORC1 activity has to be confirmed as equivalent to actual formulation to validate their efficacy.

The context of rodent pest management can widely differ according to the prevalence of resistant phenotype among rodent populations, the availability of palatable foods that can reduce the interest for the baits or even the presence of non-target species or predators. Hence, it may be interesting to increase or decrease the persistence of formulated products to achieve the best compromise between safety and efficiency appropriate to each ecosystem taking into account resistance, non-target species presence, a food competition, and the environment. Nevertheless, how the choice the mixes to test for each situation among the wide range of mixes that can be produced with four stereoisomers? It is fundamental to rationalize the choice of mix composition by mathematic modeling in order to reduce laboratory animal use in new rodenticide research and homologation tests. Thus, the end of this study proposes a modeling approach to optimize rodenticide formulation before animal testing.

1. Material and methods

1.1 Chemicals

Difethialone with the diastereoisomers ratio of 45% of cis-isomers (corresponding to the racemic mixture of 1S,3R and 1R,3S isomers) and 55% of trans-isomers (corresponding to

the racemic mixture of 1S,3S and 1R,3R isomers) and purity above 98% was provided by Liphatech (Pont de Casse, France). The ratio was confirmed by reversed-phase LC-MS/MS [11]. Individual stereoisomers of difethialone were provided by Liphatech by separation of the stereoisomers contained in difethialone mixture by the chiral chromatography method described below.

HPLC-grade acetonitrile and methanol, acetone, dichloromethane and hexane for analysis were supplied by Merck (Darmstadt, Germany). The cartridges used for solid-phase extraction were Oasis® HLB (Hydrophilic-Lipophilic-Balanced) (1mL) purchased from Waters (Milford, Massachusetts, USA). Isoflurane® and vitamin K1 were from Alcyon, (Miribel, France). Vitamin K1 was converted to vitamin K epoxide according to the method described by Tishler et al. [12]. 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.

1.2 Animals

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 7 days. The rats were housed three per cage under a constant photoperiod (12h of light per day) and ambient temperature ($20^{\circ}\text{C} \pm 2$). 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 Pharmacokinetic study

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 (authorization n°201704190941578).

Male OFA-Sprague Dawley rats received through per os administration of 3.4 mg/kg of difethialone composed of 45% of cis- and 55% of trans-isomers dissolved in 10% DMSO and 90% vegetable oil. Rats were maintained in life by daily subcutaneous administration of vitamin K1 (5 mg.kg⁻¹). 4, 9, 24, 48, 120, 168 and 216 hours after difethialone administration, 3 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.

1.4 VKOR activity assay and kinetics

Liver microsomes were prepared from fresh livers of Sprague Dawley rats by differential centrifugation according to the protocol described by Hodroge et al.[13]. Then, the microsomes are pooled from five individuals. Microsomal vitamin K epoxide reductase (VKOR) activity was assayed according to the protocol described by Hodroge et al. [13, 14]. The inhibiting effect of the silica gel column-purified cis- or trans-isomers

of difethialone was evaluated by the determination of K_i 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 . Three replications were performed for each stereoisomer.

1.5 Dosages

Difethialone stereoisomers are dosed as described in Fouré et al. [15]

1.6 Data analysis

The VKA elimination pharmacokinetics are composed of two distinct phases, with two different half-lives, an initial phase during a few days with a half-life of the order of some hours [8] and a terminal-phase may last several months with a half-life of the order of some days considering molecule [16]. Currently, the underlying mechanism is not understood. Considering ecotoxicological and efficacy studies, only the initial half-life is relevant as rodents die between five to ten days. Thus, the initial half-life determines part of the molecule's effectiveness in killing rodents and the remnant concentration that can be consumed by a predator. The terminal half-life is only observed when animals survive (often experimentally by co-administration of vitamin K). Thus, in this study, we only consider the initial half-life of the difethialone stereoisomers. Half-lives are calculated for each stereoisomer with all values from the time to peak to 216h.

Statistical analyses were performed with R v. 3.1.2. Results were expressed as mean values \pm SD or fitted value and confidence interval. Constant of inhibitions or regression curve parameters were compared with Fisher's test. Areas under the curves are compared with a Student's test corrected by Bonferroni correction.

2. Results

2.1 Inhibiting activity of the four individual stereoisomers of difethialone

Prior to our study, the constant of inhibition (K_i) of each stereoisomer was evaluated in vitro on rat liver microsomes expressing wild type VKORC1 (Sprague Dawley rats), in order to confirm their action on vitamin K regeneration cycle. Values are represented in figure 1. Even though, the K_i of E2-cis stereoisomer was significantly greater than the three others ($P < 0.05$) with a K_i about twice as high as the others, all K_i were in range between 22.37 and 51.98 nM comparable to those of effective molecules used in the field. As an example, the K_i value of warfarin, which is ineffective in the field, is 500 nM.

2.2 Determination of the pharmacokinetics of the four individual stereoisomers of difethialone

The first step of our study of the four stereoisomers of difethialone was to compare their respective pharmacokinetic parameters in rats. At $t=0$ h, 3.4 mg/kg of difethialone has been

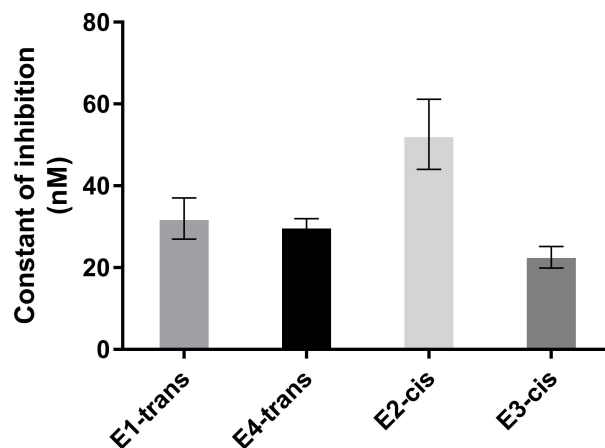


Figure 1. Constant of inhibition of each difethialone stereoisomer for wild Vkorc1 from liver microsomes of laboratory rats. Values are presented with 95% confidence interval. E2-cis K_i is significantly higher than those of three other difethialone stereoisomer ($P < 0.05$).

administered to each rat, then at 4, 9, 24, 48, 120, 168 and 216 hours a group of three rats was euthanized and their liver was collected. The hepatic difethialone concentrations of each stereoisomer versus time are shown in Figure 2 A, B, C and D for respectively the trans isomers (E1-trans and E4-trans) and the cis-isomers (E2-cis and E3-cis). The pharmacokinetic parameters of each stereoisomer were reported in Table 1. To calculate the initial half-life of each stereoisomer concentration we used only values after the peak concentration time in an exponential decay model. The peak concentration time occurred at 9h for E1-trans, E3-cis and E2-cis stereoisomers and at 4h for E4-trans stereoisomer.

Half-life of the E4-trans stereoisomer (6.0 h) is significantly lower than the one of the three others ($P < 0.0001$). Likewise, E2-cis half-life is significantly lower compared to E1-trans and E3-cis stereoisomers ($P < 0.05$). Considering the area under the curve, stereoisomer AUCs are significantly different between them ($P < 0.001$).

2.3 Development of a pharmacokinetic model able to predict the half-life of a mixture

Finally, we tested the ability of a simple model made with elimination constants previously estimated to predict the difethialone liver concentration versus time after administration of a stereoisomers mix. In order to build this model, we assume that (i) the absorption constant of each of the four stereoisomers is common (ii) after 24h post oral administration, the variation of liver difethialone concentration only depends on the elimination rate (iii) the elimination rate of each stereoisomer is not influenced by the presence of the other stereoisomer. Thus, the equation of the difethialone concentration versus time can be defined as in equation 1. In this equation, f_1 to f_4 are the fractions of each stereoisomer

Stereoisomer	Elimination coefficient (h ⁻¹)	Initial Half-life (h)	R ²	Area under the curve (h x μg/g of liver)
E1-trans	0.009 [0.006-0.014]	69.3 [48.2-123.4]	0.793	458.1 [381.1-535.2]
E4-trans	0.114 [0.083-0.146]	6.0 [4.8-8.3]	0.959	54.5 [43.5-65.6]
E2-cis	0.027 [0.015-0.039]	25.4 [17.5-46.1]	0.871	226.9 [166.1-287.6]
E3-cis	0.008 [0.004-0.012]	82.3 [57.7-143.8]	0.777	952.9 [806.2-1,099.8]

Table 1. Pharmacokinetic parameters of each difethialone stereoisomer liver concentration after a single per os administration of 3.4 mg/kg of difethialone composed of 45% of cis- and 55% of trans-isomer. Values are presented with 95% confidence interval.

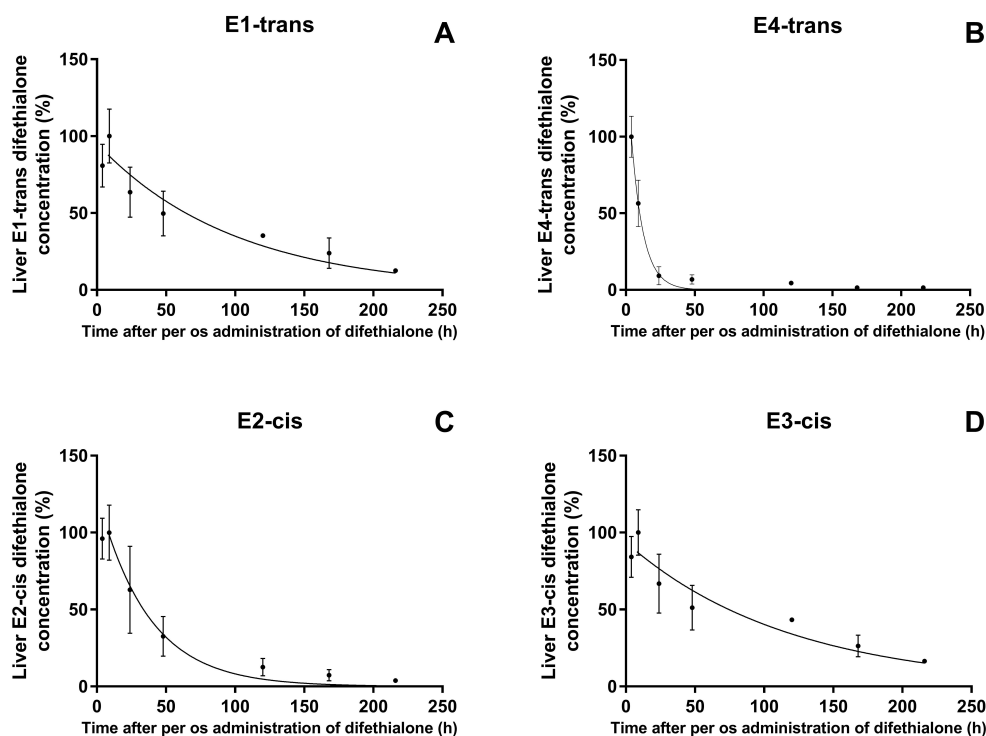


Figure 2. Difethialone stereoisomer liver concentration over time after a single per os administration of 3.4 mg/kg of difethialone composed of 45% of cis- and 55% of trans-isomers. Values are represented as mean of concentrations ± standard deviation, 100% is set as the max mean of each stereoisomer. Parameters of fitting curves are presented in table 1.

in the product administered per os, and C₀ is the theoretical concentration if the mix would have been administered by intravenous. To verify this model's ability for liver concentration prediction of stereoisomers mix, we used two pharmacokinetic data published in [8]. One of these mixes mainly contains trans stereoisomers: 47% of E4-trans and 47% of E1-trans with 3% of each two other stereoisomers, and a cis mix that is a racemic composition of E3-cis and E2-cis stereoisomers. In this validation, only the 24h concentration is used to fit the C₀ value. Then, fitting indicators were calculating only with other point concentrations (figure 3). Models have R_p² of 0.9184 for trans mix and of 0.9100 for cis mix.

$$C(t) = C_0 * (f4 * e^{-0.1147*t} + f1 * e^{-0.009998*t} + f3 * e^{-0.008419*t} + f2 * e^{-0.0273*t})$$

1. Equation predicting the concentration of difethialone in rat liver as a function of the time. The constants f1, f2, f3 and f4 are respectively the fraction of E1-trans, E2-cis, E3-cis and E4-trans in the product.).

3. Discussion

The management of rodent populations is based on the use of anticoagulant rodenticides. Indeed, physical methods as

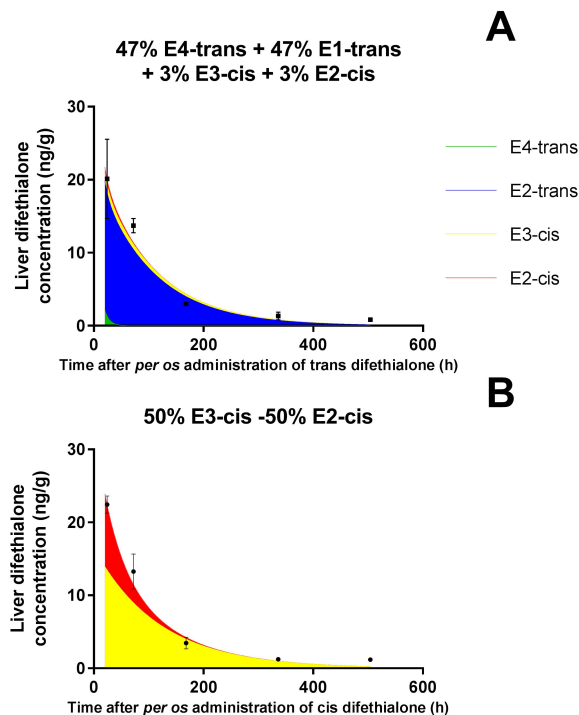


Figure 3. Liver concentration of difethialone over time after administration of two difethialone mixes: (A) trans mix with 47% of E4-trans, 47% of E1-trans, 3% of E3-cis and 3% of E2-cis and (B) cis mix with 50% of E3-cis and 50% of E2-cis. Points represent mean of experimental values \pm standard deviation. Green, blue, yellow and red areas represent respectively the proportion of E4-Trans, E1-trans, E3-cis and E4-trans stereoisomers liver concentrations obtained with the model. The R^2 of the model are 0.9184 for trans mix and 0.9100 for cis mix.

trapping are limited to small invasions of rodents [17]. In addition, there are many problems with the effectiveness of other chemical rodenticides. For example, due to their rapid action, their use results in an association between the bait and its toxicity, leading for the rodent to an aversion to the consumption of the bait [18]. Furthermore, in the case of human or animal intoxication, other chemical rodenticides have no antidote, unlike anticoagulant rodenticides [18]. The FGARs are few remnant, but also hardly efficient when many rodent populations with a resistant mutation of Vkorc1 have emerged in the pas [19]. Conversely, since they were discovered, the SGARs have been categorized as highly tissue persistent and effective in a single administration at lower concentrations compared to FGARs, including against rodents with resistant mutation [13]. These characteristics have established the renown of second-generation molecules in the management of rodent populations. Nevertheless, their reluctance is both an advantage for their efficacy and a drawback for their ecotoxicology. The new approach proposed recently by

Damin-Pernik et al. [7, 8], based on the diastereomers of existing SGARs to reduce their ecotoxicology, is here expanded to the four stereoisomers of difethialone.

The capacity of each difethialone stereoisomer to inhibit the wild rat Vkorc1 enzyme activity has been determined in vitro on a fresh rat liver microsome. Each stereoisomer inhibits the enzyme with a K_i within a range of 20 to 60 nM. These constant of inhibition are similar to those of the SGARs and chlorophacinone which are used in the field. Indeed, K_i of difenacoum, chlorophacinone and bromadiolone are respectively of 30, 40 and 70 nM [13].

Our results show that each stereoisomer has its own initial half-life, which is included between 6.0h and 82.3h. Thus, difethialone E4-trans stereoisomer has a comparable half-life to warfarin enantiomers. Indeed, E4-trans half-life is 6.0h compared to 7.6h and 11h half-life of both warfarin enantiomers [20]. However, the four difethialone stereoisomers reveal a lower K_i than warfarin (500 nM)[13]. Regarding E2-cis and E3-cis, which compose the commercial difethialone currently used, E3-cis half-life is three times greater than E2-cis half-life. This important difference may explain the biphasic elimination described when commercial difethialone liver elimination was studied [16] E2-cis would be early eliminated, constituting the first elimination stage, followed by E3-cis late efflux leading to the second elimination step. These results push us toward questioning the assessment of composed products half-life. Indeed, the half-life of the stereoisomers mix depends on the chosen range of measurement. Thus, for the first time, the global half-life of a mix is linked to the ratio and the half-life of the less remnant stereoisomer. Consequently, as the global half-life of a stereoisomer mix is increasing over time, the mix should not only be assessed by its final half-life. Indeed, with this unique indicator, it is not possible to estimate what is the amount of product remaining in the rodent when it dies and being at risk for the environment. Moreover, if the only aim is to reduce the persistence of the product, the choice of E4-trans stereoisomer is obvious since it is the less remnant difethialone stereoisomer (Figure 2 and Table 1). However, the area under the curve of such products would be so low that its efficiency would be marred.

In our opinion, two new pharmacokinetic indicators have to be designed in order to assess both the presumptive efficacy and the presumptive persistence of an anticoagulant. First, to assess the presumptive efficacy, we propose to consider the relative AUC between 24h and 120h. The goal of this indicator is to confirm that, if rodents eat the target concentration of bait, the AUC within 5 days after the ingestion is sufficient to conduct to its death. The second one is the percent of the 24 h concentration remaining at 120h after administration. Indeed, the death of rodents occurs most of the time from this fifth day after administration. Consequently, this indicator should be minimized and the AUC should be maximized.

We created a model based on the half-lives of each stereoisomer (equation 1). This model has been validated with experimental data of pharmacokinetic of difethialone

products with different stereoisomers ratios. Thus, we were able to simulate the elimination pharmacokinetics of different hypothetic difethialone mixes. These simulations allowed us to determine the best theoretical mix composition. While such simulations do not replace pharmacokinetic or efficacy studies, they can help to reduce the number of tests on animals, in accordance with the 3R principle. Once the constraints of efficacy and ecotoxicity have been defined, which is equivalent to defining a minimum AUC (efficacy) and a maximum anticoagulant concentration at the time the rodent pest begins to be symptomatic and die (ecotoxicity). The model makes it possible to target the best stereoisomer mixtures to be tested, which allows sparing laboratory animals by avoiding to test inefficient bait or bait with an ecotoxicity not adapted to the intended use.

To preselect some mixes with our model, we determined the best stereoisomers composition to maximize the AUC, according to a fixed persistence of difethialone at 120h. The results of these simulations are presented in Figure 4. According to this diagram, it is possible to design rodenticides with a wide range of AUC and persistence. The diagram pinpoints the importance of E2-cis stereoisomer in the design of new products. Indeed, with combinations of E2-cis and one of the trans stereoisomers (E1-trans or E4-trans), it is possible to design a large range of products adapted to different situations. In a vulnerable environment with few food competition (rodents eat the bait several times), a rodent population with moderate or no resistance might be controlled with a moderate persistence bait (E2-cis and E1-trans). Conversely, to control resistant populations or in environments where a one-shot bait is mandatory (important food competition), the use of more persistent baits would be more relevant to reach or maintain sufficient hepatic concentrations (E4-trans fraction and E2-cis fraction, but still without E3-cis which is really too persistent and even almost not eliminable). Obviously, the level of actions to prevent non-target poisoning has to be in accordance with the persistence of the used product.

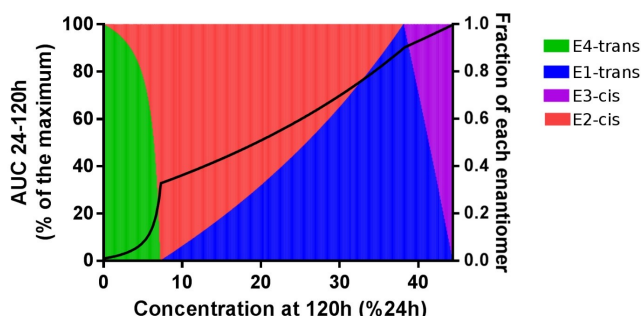


Figure 4. Evolution of difethialone AUC between 24 and 120h (curve and left axis) simulated with the best bait mix of the four difethialone stereoisomers (color area and right axis) for a fixed residual concentration of difethialone at 120h (in percent of the 24h concentration).

However, these kinds of simulation have some limitations and can only be lifted by confirming the efficacy and per-

sistence of the product directly on target species. The first limitation is the conditions of the pharmacokinetics studies, especially the administered dose, limited information is available on the self-induction of AR elimination. If such modulation exists, the pharmacokinetics of AR might be different according to the administered dose. The second main limitation is the behavior of wild rodent regarding to the poisoned bait. All new baits have to be tested in vivo in at least a baits choice feeding efficacy test, to check that the bait palatability is sufficient to ensure an adequate intake of active products in one or more times.

In conclusion, this study shows that four difethialone stereoisomers are able to inhibit the wild *Vkorc1* rat enzyme with the same efficiency. Moreover, the pharmacokinetics of these stereoisomers are widely different which allows the creation of new baits that might assure the efficiency of difethialone and modulate a persistence adapted to the treated environment. Finally, this article presents a model to select better difethialone formulation. The developed model brings a new powerful method to design more efficient ARs and could be applied to other ARs stereoisomers formulation studies.

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