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Elevated difenacoum metabolism is involved in the difenacoum-resistant phenotype observed in Berkshire rats homozygous for the L120Q mutation in the vitamin K epoxide reductase complex subunit 1 (*Vkorc1*) gene

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Abstract

BACKGROUND

Soon after difenacoum began to be used, resistance to this rodenticide was detected in rats in northeast Hampshire and northwest Berkshire in England. Resistance to difenacoum has been reported to be stronger in rats from Berkshire than in rats from Hampshire. Surprisingly, after the discovery of the vitamin K epoxide reductase complex subunit 1 (*Vkorc1*) gene, rats from Berkshire and Hampshire were all shown to be homozygous for the L120Q mutation in *Vkorc1*.

RESULTS

This study aimed to evaluate the resistance of Berkshire rats to confirm their extreme resistance and determine mechanisms supporting this resistance. For this purpose, we created a quasicongenic rat F7 strain by using a Berkshire rat as a donor to introduce the L120Q mutation in *Vkorc1* into the genetic background of an anticoagulant-susceptible recipient strain. The use of F7 rats enabled demonstration of (i) the level of resistance to difenacoum conferred by the L120Q mutation, (ii) co-dominance of the L120 and Q120 alleles, (iii) the extreme resistance of Berkshire rats compared with Q120/Q120 rats as a consequence of additional resistance mechanisms, and (iv) the involvement of cytochrome P 450 (CYP450) enzymes in this extreme resistance.

CONCLUSION

This study demonstrated that elevated CYP450 oxidative metabolism leading to accelerated difenacoum detoxification is involved in the Berkshire phenotype. © 2017 Society of Chemical Industry

1 INTRODUCTION

Difenacoum, a derivative of 4-hydroxycoumarin, is an anticoagulant molecule used as a rodenticide.^{1, 2} Like all the molecules in this family, difenacoum is a non-competitive inhibitor of the vitamin K epoxide reductase complex subunit 1 (VKORC1) enzyme involved in the recycling of vitamin K.³ This enzyme catalyzes vitamin K epoxide reductase (VKOR) activity,

allowing the reduction of vitamin K epoxide to vitamin K. This activity is crucial to activate clotting factors II, VII, IX and X. Indeed, these clotting factors must be gamma-carboxylated to be active and, during this gamma-carboxylation, one molecule of vitamin K is converted to vitamin K epoxide.⁴ Dietary vitamin K intake is insufficient, and thus VKOR activity is essential for the maintenance of the pool of vitamin K. By inhibiting the VKORC1 enzyme, difenacoum prevents normal activation of vitamin K-dependent clotting factors and thus impairs the coagulation process.

While the use of 4-hydroxycoumarin derivatives as rodenticides has been reported since the early 1950s, difenacoum was introduced in England in 1974 by UK-based Sorex Ltd (Widnes, UK).^{1, 2} Its introduction followed a period of intensive use of the first molecules derived from 4-hydroxycoumarin qualified as first-generation anticoagulant rodenticides (FGARs), such as warfarin, coumatetralyl and chlorophacinone.⁵ This intensive use of FGARs was responsible for the emergence of many rodent strains resistant to FGARs in all European countries and in the rest of the world in the late 1950s.⁶⁻⁸ These cases of resistance, which are still found many years after their detection, have been determined to be predominantly associated with *Vkorc1* single nucleotide polymorphisms (SNPs).⁹ In Europe, these polymorphisms in the *Vkorc1* gene have been widely studied in brown rats and three major SNPs were detected, the Y139F, Y139C and L120Q mutations. The Y139F SNP is essentially found in France and Belgium,^{10, 11} Y139C in Germany, the Netherlands and Denmark¹²⁻¹⁴ and L120Q in England^{15, 16}. *In vitro* studies suggested a negative effect of these mutations on vitamin K recycling, leading to an increase in the requirement for vitamin K. Moreover, these mutations were shown to lead to resistance to the action of FGARs.^{17, 18}

The emergence of these resistant strains led to the development of second-generation anticoagulant rodenticides (SGARs) in the 1970s effective against the main strains of rodents resistant to FGARs. Difenacoum was the first SGAR available on the market.^{1, 2} It is now used widely throughout the world.^{15, 19, 20} Its use is permitted throughout Europe to control populations of rats and mice in and around buildings, in waste sites and in sewers, and, depending on the country, in open areas. For example, difenacoum was reported to be used in approximately 45% of agricultural premises in Great Britain.^{15, 19} It was initially reported to be efficient to control all resistant strains.²¹ Nevertheless, some warfarin-resistant rats trapped on a farm in northeast Hampshire in England were rapidly reported also to be resistant to difenacoum.²²⁻²⁵ A derived strain was established and called the homozygous Hampshire strain HH.²² In 1982, rats from a farm in northwest Berkshire, close to the county of Hampshire, were also found to possess resistance to difenacoum. A derived strain was established and called the homozygous Berkshire strain (HB1).¹³ In contrast to the homozygous Hampshire strain, the homozygous Berkshire strain was shown also to possess moderate resistance to brodifacoum, one of the most efficient SGARs currently

available.²⁶ The molecular mechanism supporting resistance in the Berkshire strain was thus assumed to be different from that of the Hampshire strain. Surprisingly, after the discovery of the *Vkorc1* gene in 2004,⁹ difenacoum-resistant rats of the Berkshire and Hampshire strains were all shown to be homozygous for the L120Q mutation,¹³ suggesting the existence of an additional resistance mechanism in Berkshire rats. This study aimed to explore the existence of additional mechanisms of resistance to anticoagulant rodenticides other than pharmacodynamic resistance by characterizing the resistance phenotype of Berkshire rats compared with that of laboratory rats introgressed with the L120Q mutation in the *Vkorc1* gene.

2 MATERIALS AND METHODS

2.1 Chemicals

Difenacoum was supplied by Liphatech (Agen, France). Dimethyl sulfoxide (DMSO), acetonitrile, methanol, acetone, diethyl ether and orthophosphoric acid were obtained from VWR International (Fontenay sous bois, France), and Vetflurane[®] and vitamin K1 were obtained from Alcyon (Miribel, France). High-performance liquid chromatography (HPLC)-grade water, prepared using a milli-Q plus system (Millipore, Saint-Quentin en Yvelines, France), was used for preparation of HPLC eluents. All the chemicals used in this study were handled under institutionally accepted safety standards.

2.2 Origin and husbandry of animals

HB1 Berkshire rats homozygous for Q120 in *Vkorc1* were a generous gift from UK-based Sorex Ltd (Widnes, UK). The founder animals of this strain were initially trapped on an English farm in the Berkshire countryside.¹³ Since then, the HB1 Berkshire rat strain has been maintained in VetAgro Sup by intercrossing HB1 Berkshire male rats with HB1 Berkshire female rats.

F7-introgressed rats homozygous for Q120, F7-introgressed rats homozygous for L120 and heterozygous F7-introgressed rats were derived from backcrossings and intercrossings between Sprague-Dawley rats and rats from the Berkshire wild rat strain (Fig. 1). The Berkshire rat strain with a L>Q mutation at position 120 of the VKORC1 protein was used as a donor. Recipient strain Sprague-Dawley rats were purchased from Charles River Laboratories (St Germain sur l'Arbresle, France). To generate congenic strains, four L120Q homozygous Berkshire males were crossed with four Sprague-Dawley females to create the F1 hybrid generation. F1 male rats were backcrossed to Sprague-Dawley females to produce the F2 generation. The genotype of young F2 rats was determined by the allele-specific polymerase chain reaction (PCR) method, as described below. Heterozygous females were backcrossed to the recipient Sprague-Dawley strain for five additional generations to yield the F7 generation. Finally, an F7 intercross of males heterozygous for L120Q with females heterozygous for

L120Q was carried out to obtain strains of F7-introgressed rats homozygous for Q120 (Q120/Q120), F7-introgressed rats homozygous for L120 (L120/L120) and F7-introgressed rats heterozygous for L120Q (L120/Q120).

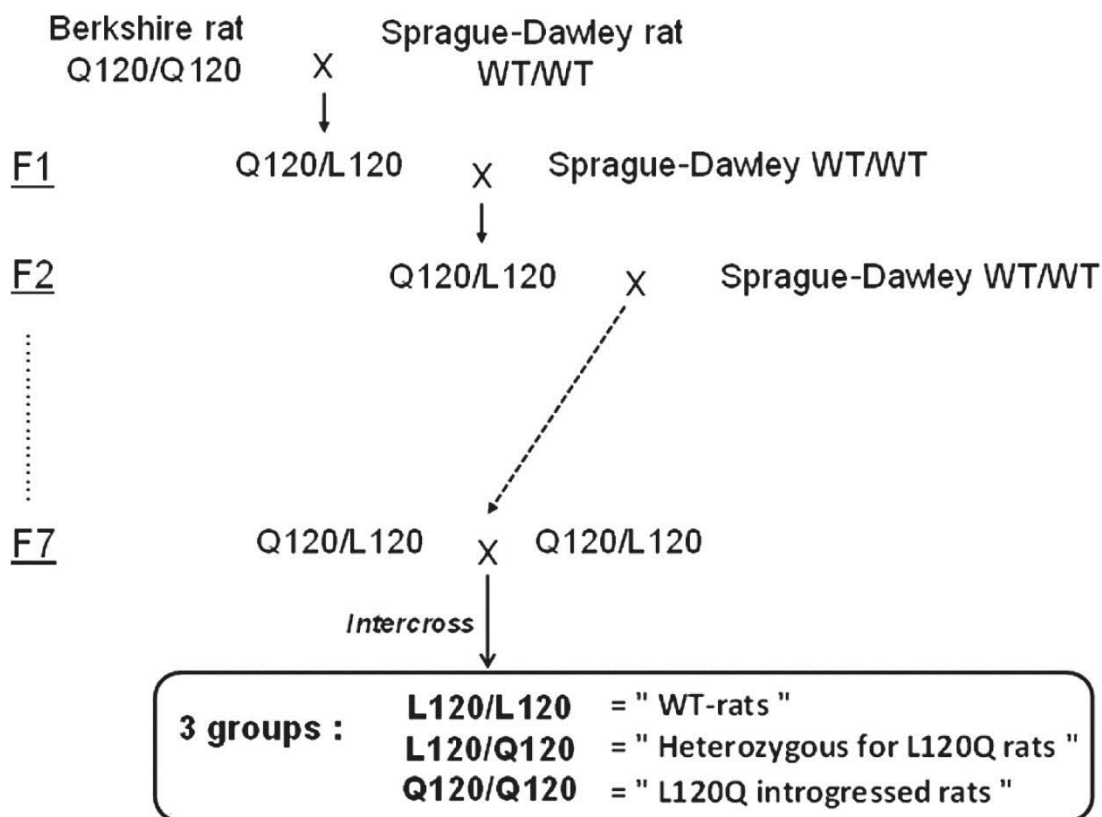


Figure 1 Schema of introgression of the L120Q mutation into the Sprague-Dawley *Rattus norvegicus* strain. WT, wild type.

Animals were kept in standard cages (Eurostandard, Type IV; Tecniplast, Limonest, France), and received standard feed (SAFE A04, Scientific Animal Food and Engineering, Augy, FRANCE) and water *ad libitum*.

2.3 Genetic characterization of animals

Genetic characterization of animals was performed using an allele-specific PCR method, as described in Grandemange *et al*¹⁷ and modified to specifically detect the L120Q mutation in the *Vkorc1* gene. Genomic DNA was extracted from tail tissue using the Nucleo-spin Tissue extraction kit (Macherey-Nagel, Hoerd, France). The allele-specific PCR method employed two pairs of primers purchased from Eurogentec (Angers, France). The forward primer was one of the two primers in which the 3' nucleotide matched the *Vkorc1* sequence of the SD strain (i.e. the rat genome sequence of *Vkorc1*; L120 primer: 5'-tgtcgctggttctctgtacct-3') or the 3' nucleotide matched the mutation in the *Vkorc1* sequence of the donor strain (120Q-primer: 5'-tgtcgctggttctctgtacca-3'). The reverse primers matched the *Vkorc1* sequence of the rat genome (5'-tcagggcttttgaccttg-3'). We amplified 135 base pairs of the *Vkorc1* gene using

quantitative real-time PCR (qPCR) in a Thermocycler Mx3000P (Stratagene, Massy, France). The reaction mixture contained 5X HOT BIOAmp EvaGreen (Biofidal, Vaulx-en-Velin, France) qPCR Mix (no ROX). The 15- μ L reactions contained 1 μ L of genomic DNA, 0.5 pmol μ L⁻¹ of each forward and reverse primer, 5 \times EvaGreen qPCR buffer including dNTPs, HOT BIOAmp DNA polymerase, MgCl₂ at 12.5 mM, and EvaGreen dye. The cycling was initiated by one denaturation step at 95°C for 5 min, followed by 40 cycles of 95°C for 15 s (denaturation), 60°C for 20 s (annealing step) and 72°C for 20 s (elongation). Fluorescence from both the EvaGreen dyes was recorded at the annealing step. The point at which the PCR product was first detected above a fixed threshold, the thermal cycle threshold (C_t), was determined for each sample in duplicate, and the average C_t of duplicate samples was calculated. The three possible genotypes L120/L120, L120/Q120 and Q120/Q120 were deduced from the differences between their characteristic cycle threshold values (ΔC_t), as described by Grandemange *et al.*¹⁷; that is, the difference in C_t values between the matched and the mismatched primer extensions for homozygous rats, and the absence of such a difference in C_t values for heterozygous animals.

2.4 Phenotypic characterization of animals

Experimental research on the animals was performed according to an experimental protocol following international guidelines (directive 2010/63/EU) and with approval from the ethics committee of the Veterinary School of Lyon. All animals tested were between the age of 10 and 12 weeks. The difenacoum administered was a mix of diastereomers composed of 57% of (1R,3S)(1S,3R) (designed as *cis*) and 43% of (1R,3R)(1S,3S) (designed as *trans*).²⁷ The amount of difenacoum administered *per os* was expressed as a factor of the acute (i.e. single dose) ED₅₀ values. ED₅₀ is the dose that creates an increase of the prothrombin time, expressed as the international normalized ratio, higher than 50% compared to the normal population. The ED₅₀ in response to antivitamin Ks (AVKs) in rats differs between females and males. To account for this, as recommended by the technical monograph of the Rodenticide Resistance Action Committee,²⁸ the doses administered were multiples of ED₅₀ adjusted for males and females. Difenacoum was dissolved in the vehicle solution 5% DMSO, ethanolamine and sunflower oil and administered by force-feeding. Experiments were performed on female and male rats. Each dosage was assayed in a group of four animals.

Forty-eight hours after difenacoum administration, 3 ml of blood was taken by cardiac puncture into citrate tubes (3.2%; 1:9 v/v) and PT was measured immediately, as previously described by Damin *et al.*²⁷

2.5 Determination of difenacoum concentration in liver

Liver tissue (1 g) was extracted with acetone using an Ultra Turrax tissue disperser from IKA Labortechnik® (VWR International, Strasbourg, France) according to the method previously described by Damin-Pernik *et al.*²⁷ and validated according to the guideline on Bioanalytical Method Validation published by the European Medicines Agency.²⁹ The extract was centrifuged at 5000 *g* for 10 min, and the supernatant was evaporated at 50°C under a gentle nitrogen flow. The 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 concentrations of difenacoum and its metabolites were analyzed by HPLC on a LICHrospher® 100RP18 encapped column (5µm particle size; 250 × 4.6 mm) (VWR International) at detection wavelengths of 258 nm. 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 recovery rate of difenacoum from tissues was between 75 and 95% in liver with a precision < 15%.

2.6 Preparation of liver microsomes

Liver microsomes were prepared from fresh livers by differential centrifugation, as described by Moroni *et al.*³⁰ Briefly, livers were resuspended in 50 mM phosphate buffer (pH 7.4) containing 1.15 % (w/v) of KCl. Liver cells were homogenized in buffer using a motor-driven Potter glass homogenizer (VWR International, Fontenay-sous-bois, France and further subjected 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 and 20% glycerol, pH 7.4). Protein concentrations were evaluated by the method of Bradford³¹ using bovine serum albumin as a standard. Microsomes were frozen at -80°C and used for kinetic analysis.

2.7 CYP450-dependent oxidation assay

Oxidation of difenacoum by liver microsomes was assessed using the amount of hydroxy-difenacoum produced in the presence of an Nicotinamide adenine dinucleotide phosphate (NADPH) regenerating system. In a final volume of 1 mL, a typical reaction mixture contained phosphate buffer (100 mM, pH 7.4), 0.3 to 0.5 mg of protein, 12.5 µM of difenacoum as a substrate and a regenerating system containing NADP (1 mM), glucose 6-phosphate (10 mM), glucose 6-phosphate dehydrogenase (1 unit), and MgCl₂ (10 mM). Incubations were conducted in glass vials at 37°C for 30 min in air and were performed in duplicate. Reactions were stopped by the addition of 2 ml of ethyl acetate. The ethyl acetate layer was dried under nitrogen. The dry residue was immediately dissolved in 0.2 mL of methanol and reaction products were analyzed by HPLC-UV according to the method as described above.

2.8 Statistical analysis

Data are presented as the mean \pm standard deviation obtained from four to five individual rats. Comparisons between groups, when more than two groups were present, were performed using the Kruskal–Wallis test or Tukey's multiple comparisons test, with GRAPHPAD PRISM 6 software (GraphPad, San Diego, CA, USA). Comparison between two groups was performed using the Mann–Whitney test. $P < 0.05$ was the accepted level of significance.

3 RESULTS

3.1 Analysis of prothrombin time of rats belonging to the different strains

The PT of our control groups, that is, the basal levels of PT when not exposed to AVK, were 17.9 ± 2.5 and 15.8 ± 1.4 s, respectively, for male and female F7-introgressed rats homozygous for L120; 19.3 ± 0.5 and 16.0 ± 2.1 s, respectively, for male and female F7-introgressed rats homozygous for Q120; 17.0 ± 0.9 and 18.2 ± 2.3 s, respectively, for male and female F7-introgressed heterozygous rats, and 17.4 ± 1.4 and 16.5 ± 1.0 s, respectively, for male and female Berkshire rats. Therefore, no significant differences in PT were found between groups when no AVK was administered (Kruskal–Wallis test; $P > 0.05$).

3.2 *In vivo* determination of the level of resistance to difenacoum associated with the L120Q mutation of the *Vkorc1* gene

For the L120/L120 genotype, 48 h after difenacoum administration, PT times increased dramatically with even the smallest dose of difenacoum (i.e. corresponding to $2 \text{ ED}_{50} = 1.3$ mg/kg for males and 1.58 mg/kg for females) for males (368 ± 42 s) (Fig. 2) and females (213 ± 50 s) (Fig. 2). For the Q120/Q120 and L120/Q120 genotypes, PT increased only slightly at 2.6 mg/kg (i.e. corresponding to 4 ED_{50}) in males. No significant differences in PT were found between Q120/Q120 and L120/Q120 males 48 h after administration of 2.6 mg/kg of difenacoum. In contrast, 48 h after administration of 5.2 mg/kg of difenacoum, Q120/Q120 male rats showed a PT of 101 ± 32 s, higher than that measured for L120/Q120 male rats (181 ± 30 s) (Fig. 2). For the Q120/Q120 and L120/Q120 females, PT increased only slightly (116 ± 25 s) at 6.32 mg/kg (i.e. corresponding to 8 ED_{50}) for heterozygous females, while no increase in PT (21 ± 3 s) was observed for the Q120/Q120 females after the same administration (Fig. 2). Therefore, the PT of the three genotypes L120/L120, L120/Q120 and Q120/Q120 differed significantly 48 h after administration of 8 ED_{50} of difenacoum (Kruskal–Wallis test; $P < 0.01$).

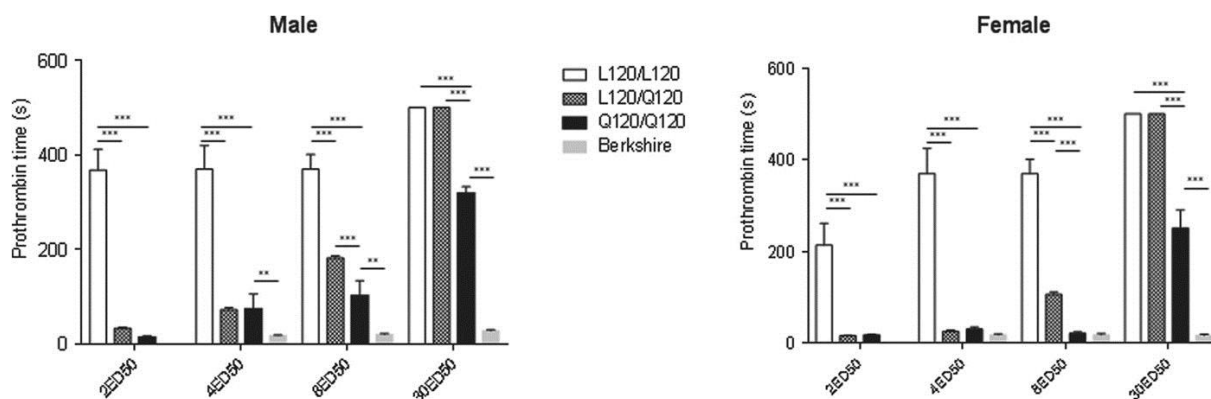


Figure 2 Prothrombin times (in seconds) of male and female F7-introgressed rats homozygous for Q120 (Q120/Q120), homozygous for L120 (L120/L120) or heterozygous (L120/Q120) or belonging to the Berkshire strain, 48 h after *per os* administration of difenacoum. Values are represented as mean + SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

3.3 *In vivo* determination of the level of resistance to difenacoum of rats belonging to the Berkshire wild rat strain

For Q120/Q120 males and females, PT increased dramatically 48 h after administration of 30 ED₅₀ (i.e. 19.5 mg/kg for males and 23.7 mg/kg for females) of difenacoum (i.e. 319 ± 34 s for males and 251 ± 39 s for females) (Fig. 2). Surprisingly, male and female rats belonging to the Berkshire wild rat strain showed a normal PT at 28 ± 2 s for males and 17 ± 2 s for females, 48 h after administration of 30 ED₅₀ of difenacoum. Statistical analysis using Tukey's multiple comparisons test highlighted a significant increase in PT for Q120/Q120 rats compared with rats belonging to the Berkshire wild rat strain ($P < 0.001$) for males and females.

3.4 Determination of difenacoum concentration in the liver 48 h after administration of 30 ED₅₀ of difenacoum

For Berkshire rats or F7-introgressed rats homozygous for Q120 (Q120/Q120), the concentration of difenacoum in the liver was significantly higher in females than in males 48 h after *per os* administration of 30 ED₅₀ (Fig. 3). Moreover, concentrations of difenacoum in the livers of Berkshire rats were significantly lower than those measured in the livers of Q120/Q120 rats. Difenacoum concentrations in the livers of Berkshire rats were 1.1 ± 0.1 and 1.9 ± 0.4 µg/kg for males and females, respectively, while in the livers of Q120/Q120 rats, concentrations were 3.2 ± 0.2 and 5.4 ± 1.2 µg/kg for males and females, respectively. Whatever the strain and the sex of the rat were, > 95% of the hepatic difenacoum present in the liver 48 h after administration was *cis*-isomers (Fig. 3), while the difenacoum administered corresponded to a mixture of 57% *cis*-isomers and 43% *trans*-isomers.

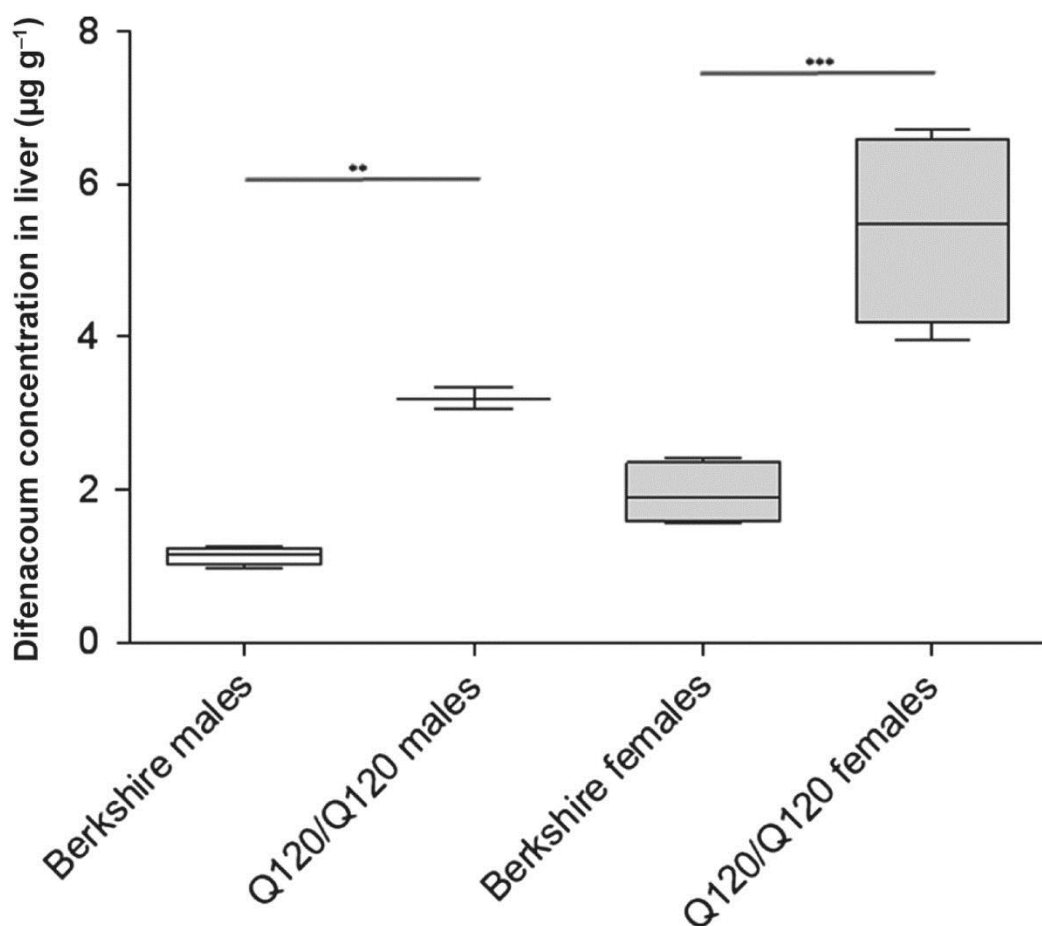


Figure 3 Difenacoum concentration in livers of male and female rats of the Berkshire strain and the F7-introgressed strain homozygous for L120Q (Q120/Q120), 48 h after *per os* administration of 30 ED₅₀ of commercial difenacoum composed of 57% *cis*-isomer and 43% *trans*-isomer, with the relative proportions of *cis* and *trans* isomers. Values are represented as mean ± SD. **P*<0.05; ***P*<0.01; ****P*<0.001.

3.5 Determination of the metabolites of difenacoum in the liver 48 h after administration of 30 ED₅₀ of difenacoum

Three different metabolites were detected with retention times of 6.4, 6.9 and 7.4 min, respectively, and a UV spectrum similar to that obtained for difenacoum (data not shown). These metabolites were detected in the livers of Berkshire rats, but also in the livers of Q120/Q120 rats. The molecular mass of these metabolites, investigated by mass spectrometry, was 459 uma (data not shown). This molecular mass corresponded to the molecular mass of difenacoum added of 16 amu, corresponding to the molecular mass of a hydroxylated metabolite. Quantification of hydroxylated metabolites was performed by UV-HPLC by comparison to a range of difenacoum. The results are presented in Figure 4. The results correspond to the amount of hydroxylated metabolites measured in the liver 48 h after the administration of 30 ED₅₀ of difenacoum. In the livers of female Berkshire rats, the concentration of hydroxylated metabolites (i.e. 9.4 ± 0.5 µg/g of liver) was significantly higher

(30%) than that measured in the livers of female Q120/Q120 rats (i.e. $7.0 \pm 0.1 \mu\text{g/g}$ of liver) (Mann–Whitney test; $P < 0.01$). In the livers of male Berkshire rats, the concentration of hydroxylated metabolites (i.e. $5.5 \pm 0.8 \mu\text{g/g}$ of liver) was not statistically different from that of male Q120/Q120 rats (i.e. $4.3 \pm 0.7 \mu\text{g/g}$ of liver).

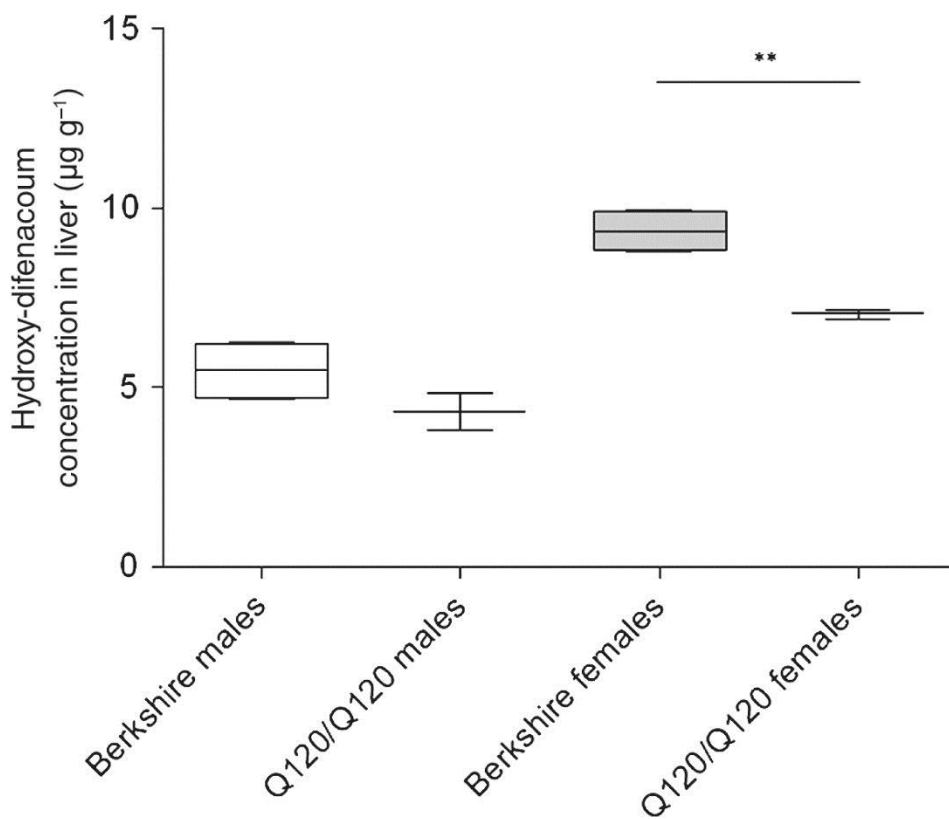


Figure 4 Concentration of hydroxylated metabolites of difenacoum, 48 h after *per os* administration of 30 ED₅₀ of difenacoum in male and female rats of the Berkshire strain and the F7-introgressed strain homozygous for L120Q (Q120/Q120). Values are represented as mean \pm SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

3.6 Determination of CYP450-dependent oxidative activity in the liver

Incubation of liver microsomes of male Sprague-Dawley rats with difenacoum led to the production of only one detectable hydroxylated metabolite by UV-HPLC at 258 nm with a retention time of 6.9 min, corresponding to the major metabolites detected in the liver after *per os* administration of difenacoum to rats. In the presence of a saturating concentration of difenacoum (i.e. $12.5 \mu\text{M}$ of difenacoum), the specific activity obtained to produce this hydroxylated metabolite in the presence of liver microsomes of male Sprague-Dawley rats was $27.9 \pm 3.2 \text{ nmol/min/mg}$ of total protein. For Berkshire and Q120/Q120 rats, the results obtained are presented in Figure 5. The specific activity obtained to produce hydroxylated metabolites was 1.5-fold higher in Berkshire rats compared with Q120/Q120 rats

and this difference was statistically significant (Mann–Whitney test; $P < 0.01$ for males and $P < 0.05$ for females).

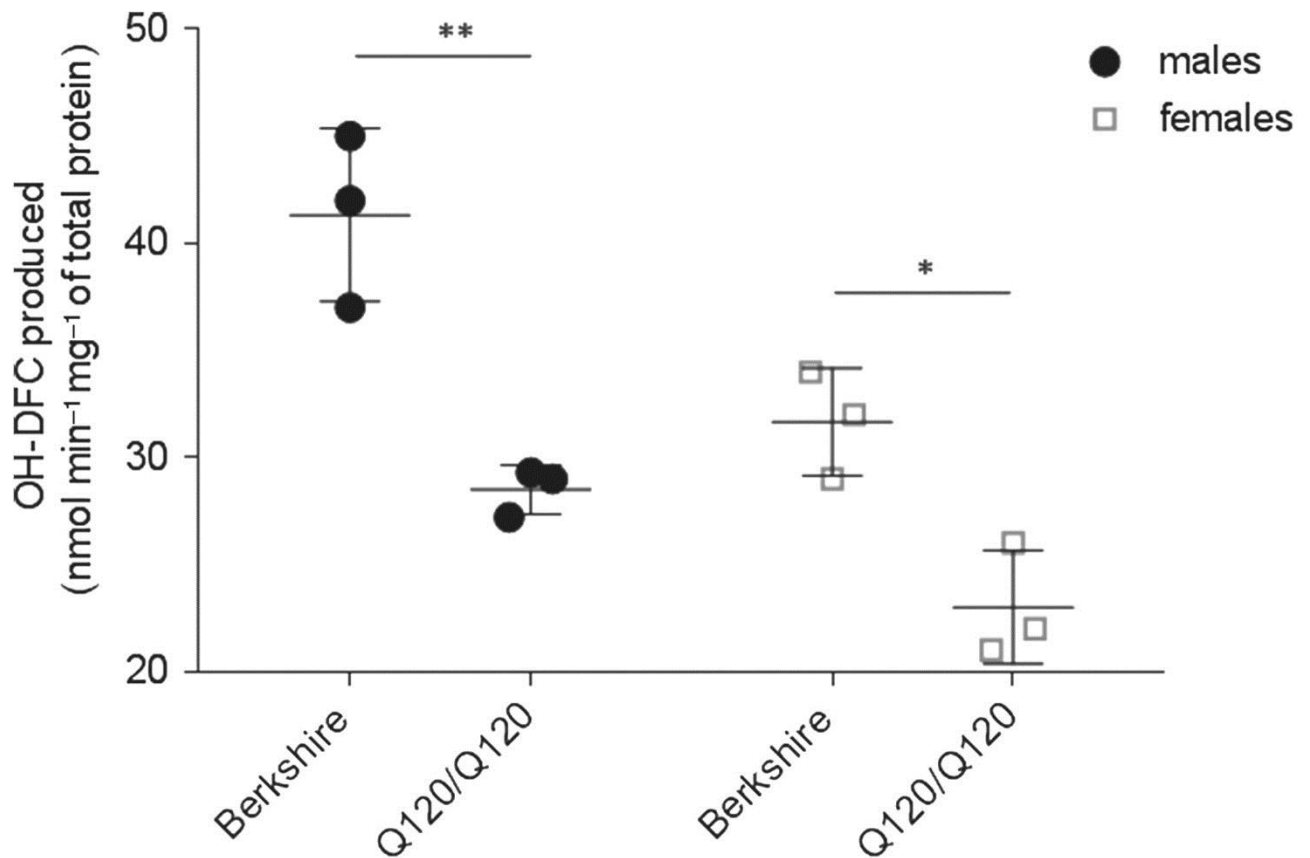


Figure 5 CYP450-dependent oxidative metabolism of difenacoum by liver microsomes of male Berkshire rats or male F7-introgressed rats homozygous for L120Q (Q120/Q120). Values are represented as mean \pm SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

4 DISCUSSION

Since the description of wild Norway rats resistant to second-generation AVK rodenticides in the counties of Hampshire and Berkshire, the degree of resistance in Hampshire and Berkshire rats has been widely discussed because of (i) inconsistencies in results between laboratory studies and field trials and (ii) detection of the same mutation in the *Vkorc1* gene associated with differences in the degree of resistance.^{13, 26}

To advance the understanding of Berkshire/Hampshire resistance, we tried first to define the degree of resistance induced by the unique L120Q mutation by studying the relationship between genotype and phenotype. For this, we introgressed the L120Q mutation present in Berkshire rats into a laboratory rat strain through a series of seven successive backcrosses in order to remove all additional genetically based mechanisms that may contribute to the physiological response to AVK exposure. Indeed, Greaves and Cullen-Ayres²² postulated that resistance to difenacoum might be multigenic. After these seven repeated backcrosses, the

genome of the original wild Berkshire rat is so diluted in the genome of the laboratory rat that its influence on the phenotype is negligible. Indeed, after seven backcrosses, the percentage of the genome of the original wild rat may be < 0.8%. However, as a consequence of our method of selection based on the selection of the L120Q mutation, there is still a fragment of chromosome 1 of the wild Berkshire rat around the introgressed gene, which was in a similar experiment estimated at 20 megabases or about 10% of chromosome 1 (250 megabases).¹⁷

The newly generated quasicongenic strain enabled us to observe the physiological response mediated by the L120Q mutation in the *Vkorc1* gene. Unsurprisingly, the L120Q mutation induces a severe difenacoum-resistant phenotype. For introgressed rats homozygous for the L120Q mutation, an 8-fold increase of the normal dose of difenacoum necessary to increase the PT in warfarin-susceptible rats was required to impair the coagulation function. These results are consistent with the *in vitro* results obtained previously with recombinant VKORC1 enzymes expressed in the yeast *Pichia pastoris*,¹⁸ although the resistance factor induced by the L120Q mutation determined *in vitro* (×30) was higher than that determined *in vivo* (×8). This study is thus a supplementary demonstration^{17, 32} revealing that *in vitro* characterization of recombinant VKORC1 enzymes expressed in yeast by the DTT-driven VKOR assay is a perfect tool to characterize the *in vivo* phenotype when no mutation other than the mutation in the *Vkorc1* gene is involved in the resistance.

Moreover, the use of rats of the same F7 strain, but with different *Vkorc1* genotypes generated by intercrosses (i.e. L120/L120, L120/Q120 and Q120/Q120), enabled establishment of the codominance of the Q120 and L120 alleles. This codominant property was also demonstrated for the F139 allele which is also widely distributed among the population of Norway rats in Europe.¹⁷ The codominance of the L120 and Q120 alleles is a crucial point for the selection of the L120Q mutation in the field. In practice, during rodent control efforts, the presence of the mutation even in the heterozygous state and with low frequency in the population can lead to resistance even at high dosages of difenacoum. Because of this resistance, the heterozygotes, which are usually most frequent in the initial phase of the selection, survive better in the control treatment than the susceptible animals. When the proportion of heterozygotes increases, homozygotes for the L120Q mutation appear and are then selected by difenacoum treatment because they survive better.

Furthermore, the use of rats of the F7 introgressed strain enabled us also to demonstrate the extreme resistance of Berkshire rats compared with rats homozygous for the L120Q mutation. Indeed, after administration of almost 20 mg/kg of difenacoum (i.e. 30-fold the dose of difenacoum required to increase PT in warfarin-susceptible rats), PT appeared unchanged in Berkshire rats, while for an 8-fold increased dose, PT was already clearly increased for F7-introgressed rats homozygous for the L120Q mutation. The resistance of the Berkshire strain

is the most potent resistance described until now very severe. In the field, the use of bait containing currently 50 ppm of difenacoum cannot cause death of such rats and is thus certainly inefficient to manage such populations of rats. However, such baits might be still effective to manage populations homozygous for the L120Q mutation, although repeated consumption of this bait is certainly required. Considering the feeding behavior and the neophobia of rats, repeated consumption of baits by rats is quite possible.

Comparison of these strains has enabled us to confirm without any possible ambiguity that, for the same L120Q mutation of the *Vkorc1* gene, two different phenotypes exist. Thus, Berkshire resistance involves an additional mechanism compared with Hampshire resistance. In addition to the *Vkorc1* gene, different additional genetically based mechanisms have been proposed to be able to contribute to the physiological response to AVK exposure. Modified expression of calumenin³³ and mutation of epoxyde hydrolase^{1,34} both proteins suggested to be components of the VKOR complex, have been proposed to mediate resistance, but the contributions of such mechanisms have never been confirmed. Mutations of CYP4F2, a vitamin K oxidase,³⁵ have been shown to influence the vitamin K1 plasma concentration in humans, and thus the dose of anticoagulant required to maintain an anticoagulation status.^{36, 37} Nevertheless, the extreme degree of resistance observed in the Berkshire strain seems to exclude such a mechanism. In addition to these various mechanisms, an increase in the ability to eliminate a particular xenobiotic can result in resistance to this xenobiotic. In insects, this mechanism has been widely described and allows them to adapt to an insecticide-contaminated environment.³⁸ In humans, it has been demonstrated that CYP3A induction increases the tolerance for higher doses of warfarin in humans.³⁹ Such a mechanism has also been previously suggested for warfarin-resistant wild roof rats in the Tokyo area of Japan.^{40, 41} It is thus possible that modified expression or activity of the drug metabolism enzymes in Berkshire rats accelerates difenacoum detoxification. To explore this hypothesis, difenacoum residues were quantified in the livers of Berkshire and F7-introgressed rats after oral administration and were shown to be 2.5-fold lower in Berkshire rats compared with F7-introgressed rats. Elevated difenacoum metabolism is thus involved in the difenacoum-resistant phenotype observed in Berkshire rats. Metabolic pathways of anticoagulant rodenticides have not yet really been explored, except for warfarin. Warfarin has been shown in rats to be principally oxidized by different CYP450 enzymes.⁴² Therefore, we monitored the production of hydroxylated metabolites. Effectively, three different hydroxylated metabolites were observed after difenacoum administration, suggesting the involvement of phase I metabolism enzymes in difenacoum metabolism. These metabolites were found in Berkshire and F7-introgressed rats and no additional metabolites have been identified in Berkshire rats. Nevertheless, the production of hydroxylated metabolites has been shown to be higher in male Berkshire rats compared with male F7-introgressed rats, suggesting overexpression or an increase in the activity of CYP450 enzymes, although the difference in production was not

statistically significant in females. The involvement of other metabolic pathways such as glucuronidation leading to the elimination of hydroxylated metabolites may explain the absence of hydroxylated metabolite accumulation. To confirm the involvement of CYP450-dependent oxidative metabolism in the Berkshire resistance, *in vitro* assays were performed. The results obtained confirmed the increase in the CYP450 oxidative metabolism of difenacoum in Berkshire rats. Further study of the metabolic pathway of difenacoum will be required to identify the specific isoform involved.

5 CONCLUSIONS

This study is the first demonstration of resistance caused by increased metabolism of anticoagulant rodenticides in brown rats. Markussen *et al*⁴³ had previously suggested that changes in the expression of CYP450 could coexist in a bromadiolone-resistant rat strain carrying the Y139C mutation in the *Vkorc1* gene, but without any apparent consequences. In Berkshire rats, the presence of this metabolic component, in addition to the L120Q mutation in the *Vkorc1* gene, appears to substantially enhance the resistance phenotype. However, it is unclear whether only these two mechanisms are involved or if another mechanism is required to explain the extreme resistance to difenacoum of Berkshire rats.

In practical terms, it is essential to determine the limits of the resistance of Berkshire rats to optimize management methods. Because CYP450-dependent metabolism pathways are modified in Berkshire rats, metabolism of other AVKs must be characterized in these rats. AVK molecules able to control rats homozygous for the L120Q mutation (i.e. brodifacoum, difethialone and flocoumafen)¹⁸ with a different metabolism pathway from difenacoum should be used to control such rats and avoid selection of Berkshire rats.⁴⁴ Alternation of active substances should also be considered to avoid development of other metabolic resistance. Moreover, if Berkshire resistance is associated with a biological cost, the suspension of the use of AVK rodenticides for a while could be recommended to decrease the frequencies of the alleles that confer resistance to difenacoum. It would be also essential to develop a simple tool to detect metabolic resistance to difenacoum to be able to distinguish in the field the different phenotypes revealed in this study.

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REFERENCES

- 1 Hadler MR, Redfern R and Rowe FP, Laboratory evaluation of difenacoum as a rodenticide. *J Hyg***74**:441–448 (1975).

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- 2.Rennison BD and Hadler R, Field trials of difenacoum against warfarin-resistant infestations of *Rattus norvegicus*. *J Hyg* **74**:449–455 (1975).
 - 3.Wallin R, Wajih N and Hutson SM, VKORC1: a warfarin-sensitive enzyme in vitamin K metabolism and biosynthesis of vitamin K-dependent blood coagulation factors. *Vitam Horm***78**:227–246 (2008).
 - 4.Stafford DW, The vitamin K cycle. *J Thromb Haemost* **3**:1873–1878 (2005).
 - 5.Hadler MR and Buckle A, Forty five years of anticoagulant rodenticides - past, present and future trends. *Proc Vert Pest Conf* **15**:149–155 (1992).
 - 6.Boyle CM, Case of Apparent Resistance of *Rattus norvegicus* Berkenhout to Anticoagulant Poisons. *Nature* **188**:517–517 (1960).
 - 7.Dodsworth E, Mice and spreading despite such poisons as warfarin. *Munic Eng Lond* **3746**:1668 (1961).
 - 8.Jackson WB and Kaukeinen D, Resistance of wild Norway rats in North Carolina to warfarin rodenticide. *Science* **176**:1343–1344 (1972).
 - 9.Rost S, Fregin A, Ivaskevicius V, Conzelmann E, Hortnagel K, Pelz H-J *et al.*, Mutations in VKORC1 cause warfarin resistance and multiple coagulation factor deficiency type 2. *Nature* **427**:537–541 (2004).
 - 10.Baert K, Stuyck J, Breyne P, Maes D and Casaer J, Distribution of anticoagulant resistance in the brown rat in Belgium. *Belg. J Zool* **142**:39–48 (2012).
 - 11.Grandemange A, Lasseur R, Longin-Sauvageon C, Benoit E and Berny P, Distribution of VKORC1 single nucleotide polymorphism in wild *rattus norvegicus* in France. *Pest Manag Sci***66**:270–276 (2010).
 - 12.Meerburg BG, Pe Van Gent-Pelzer M, Schoelitsz B and Aj Van Der Lee T, Distribution of anticoagulant rodenticide resistance in *Rattus norvegicus* in the Netherlands according to *Vkorc1* mutations. *Pest Manag Sci* **70**:1767 (2014).
 - 13.Pelz H-J, Rost S, Hünenberg M, Fregin A, Heiberg A-C, Baert K *et al.*, The genetic basis of resistance to anticoagulants in rodents. *Genetics* **170**:1839–1847 (2005).
 - 14.Pelz H-J, Rost S, Müller E, Esther A, Ulrich RG and Müller CR, Distribution and frequency of VKORC1 sequence variants conferring resistance to anticoagulants in *Mus musculus*. *Pest Manag Sci* **68**:254–259 (2012).
 - 15.Buckle A, Anticoagulant resistance in the United Kingdom and a new guideline for the management of resistant infestations of Norway rats (*Rattus norvegicus* Berk.). *Pest Manag Sci***69**:334–341 (2013).
 - 16.Haniza MZH, Adams S, Jones EP, MacNicoll A, Mallon EB, Smith RH *et al.*, Large-scale structure of brown rat (*Rattus norvegicus*) populations in England: effects on rodenticide resistance. *PeerJ***3**:e1458 (2015).
 - 17.Grandemange A, Kohn MH, Lasseur R, Longin-Sauvageon C, Berny P and Benoit E, Consequences of the Y139F *Vkorc1* mutation on resistance to AVKs: in-vivo investigation in a 7th generation of congenic Y139F strain of rats. *Pharmacogenet Genomics* **19**:742–750 (2009).
 - 18.Hodroge A, Longin-Sauvageon C, Fourel I, Benoit E and Lattard V, Biochemical characterization of spontaneous mutants of rat VKORC1 involved in the resistance to antivitamin K anticoagulants. *Arch Biochem Biophys* **515**:14–20 (2011).

-
- 19. Atterby H, Kerins GM and MacNicoll AD, Whole-carcass residues of the rodenticide difenacoum in anticoagulant-resistant and -susceptible rat strains (*Rattus norvegicus*). *Env Toxicol Chem* **24**:318–23 (2005).
 - 20. Hughes J, Sharp E, Taylor MJ, Melton L and Hartley G, Monitoring agricultural rodenticide use and secondary exposure of raptors in Scotland. *Ecotoxicology* **22**:974–984 (2013).
 - 21. Hadler MR, Redfern R and Rowe FP, Laboratory evaluation of difenacoum as a rodenticide. *J Hyg (Lond)*. **74**:441–448 (1975).
 - 22. Greaves JH and Cullen-Ayres PB, Genetics of difenacoum resistance in the Rat, in *Current Advances in Vitamin K Research*, ed. by JW Suttie. Elsevier, Amsterdam pp. 389–397 (1988).
 - 23. Johnson RA, Performance studies with the new anticoagulant rodenticide, flocoumafen, against *Mus domesticus* and *Rattus norvegicus*. *EPPO Bull* **18**:481–488 (1988).
 - 24. Lund M, Resistance to the second-generation anticoagulant rodenticides resistance to the second-generation anticoagulant rodenticides *Proc Vertebrate Pest Conf* **11**:89–94 (1984).
 - 25. Redfern R and Gill JE, The development and use of a test to identify resistance to the anticoagulant difenacoum in the Norway rat (*Rattus norvegicus*). *J Hyg (Lond)* **81**:427–431 (1978).
 - 26. Gill JE, Kerins GM and MacNicoll AD, Inheritance of Low Grade Brodifacoum Resistance in the Norway Rat. *J Wildl Manage* **56**:809 (1992).
 - 27. Damin- Pernik M, Espana B, Besse S, Fourel I, Caruel H, Popowycz F *et al.*, Development of an Ecofriendly Anticoagulant Rodenticide Based on the Stereochemistry of Difenacoum. *Drug Metab Dispos* **44**:1872–1880 (2016).
 - 28. RRAC *Anticoagulant resistance management strategy for pest management professionals, central and local government and other competent users of rodenticides*. Technical Monograph. Rodenticide Resistance Action Committee, CropLife International, Brussels (2003).
 - 29. European Medicines Agency, Guideline on bioanalytical method validation, EMEA/CHMP/EWP/192217/2009 Rev.1 Corr.* Committee for Medicinal Products for Human Use (CHMP) (2011).
 - 30. Moroni P, Longin-sauvageon C and Benoit E, The Flavin-Containing Monooxygenase in Rat Liver: Evidence for the Expression of a Second Form Different from FMO1. *Biochem Biophys Res Commun*. **212**:820–826 (1995).
 - 31. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**:248–254 (1976).
 - 32. Goulois J, Chapuzet A, Lambert V, Chatron N, Tchertanov L, Legros L *et al.*, Evidence of a target resistance to antivitamin K rodenticides in the roof rat *Rattus rattus*: identification and characterisation of a novel Y25F mutation in the *Vkorc1* gene. *Pest Manag Sci* **72**:544–550 (2016).
 - 33. Wajih N, Sane DC, Hutson SM and Wallin R, The inhibitory effect of calumenin on the vitamin K-dependent gamma-carboxylation system. Characterization of the system in normal and warfarin-resistant rats. *J Biol Chem* **279**:25276–25283 (2004).
 - 34. Vecsler M, Loebstein R, Almog S, Kurnik D, Goldman B, Halkin H *et al.*, Combined genetic profiles of components and regulators of the vitamin K-dependent gamma-carboxylation system affect individual sensitivity to warfarin. *Thromb Haemost* **95**:205–211 (2006).

-
- 35. McDonald MG, Rieder MJ, Nakano M, Hsia CK and Rettie AE, CYP4F2 is a vitamin K1 oxidase: An explanation for altered warfarin dose in carriers of the V433M variant. *Mol Pharmacol* **75**:1337–46 (2009).
 - 36. Caldwell MD, Awad T, Johnson JA, Gage BF, Falkowski M, Gardina P *et al.*, CYP4F2 genetic variant alters required warfarin dose. *Blood* **111**:4106–4112 (2008).
 - 37. Takeuchi F, McGinnis R, Bourgeois S, Barnes C, Eriksson N, Soranzo N *et al.*, A genome-wide association study confirms VKORC1, CYP2C9, and CYP4F2 as principal genetic determinants of warfarin dose. *PLoS Genet* **5**:e1000433 (2009).
 - 38. Scott JG, Cytochromes P450 and insecticide resistance. *Insect Biochemistry and Molecular Biology* **29**:757–777 (1999).
 - 39. Izzo AA, Herb-drug interactions: an overview of the clinical evidence. *Fundam Clin Pharmacol* **19**:1–16 (2005).
 - 40. Ishizuka M, Okajima F, Tanikawa T, Min H, Tanaka KD, Sakamoto KQ *et al.*, Elevated warfarin metabolism in warfarin-resistant roof rats (*Rattus rattus*) in Tokyo. *Drug Metab Dispos* **35**:62–66 (2007).
 - 41. Sugano S, Kobayashi T, Tanikawa T, Kawakami Y, Kojima H, Nakamura K *et al.*, Suppression of CYP3A2 mRNA expression in the warfarin-resistant roof rat, *Rattus rattus*: possible involvement of cytochrome P450 in the warfarin resistance mechanism. *Xenobiotica* **31**:399–407 (2001).
 - 42. Guengerich FP, Dannan GA, Wright ST, Martin MV and Kaminsky LS, Purification and characterization of liver microsomal cytochromes p-450: electrophoretic, spectral, catalytic, and immunochemical properties and inducibility of eight isozymes isolated from rats treated with phenobarbital or beta-naphthoflavone. *Biochemistry* **21**:6019–6030 (1982).
 - 43. Markussen MD, Heiberg A-C, Fredholm M and Kristensen M, Differential expression of cytochrome P450 genes between bromadiolone-resistant and anticoagulant-susceptible Norway rats: a possible role for pharmacokinetics in bromadiolone resistance. *Pest Manag Sci* **64**:239–248 (2008).
 - 44. Espinosa MB, Efficacy of anticoagulant drugs as rodenticides and genetic variation on *Vkorc1* of *Mus musculus* from Buenos Aires province (Argentina). *J Basic Appl Genet* **24**:27–31 (2013).