Published in final edited form as:
Pharmacogenet Genomics. 2009 October ; 19(10): 742–750. doi:10.1097/FPC.0b013e32832ee55b.

Consequences of the *Y139F Vkorc1* mutation on resistance to AVKs: in-vivo investigation in a 7th generation of congenic *Y139F* strain of rats

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Abstract

Objectives—In humans, warfarin is used as an anticoagulant to reduce the risk of thromboembolic clinical events. Warfarin derivatives are also used as rodenticides in pest control. The gene encoding the protein targeted by anticoagulants is the Vitamin K-2,3-epoxide reductase subunit 1 (*VKORC1*). Since its discovery in 2004, various amino acid and transcription-regulatory altering *VKORC1* mutations have been identified in patients who required extreme antivitamin K dosages, or wild populations of rodents that were difficult to control with anticoagulant rodenticides. One unresolved question concerns the dependency of the *VKORC1* on the genetic background in humans and rodents that respond weakly or not at all to anticoagulants. Moreover, an important question requiring further analyses concerns the role of the *Vkorc1* gene in mediating resistance to more recently developed warfarin derivatives (superwarfarins).

Methods—In this study, we bred a quasicongenic rat strain by using a wild-caught anticoagulant resistant rat as a donor to introduce the Y > F amino acid change at position 139 in the *Vkorc1* into the genetic background of an anticoagulant susceptible Spraque–Dawley recipient strain.

Results and conclusion—In this manuscript we report the prothrombin times measured in the F7 generation after exposure to chlorophacinone, bromadiolone, difenacoum and difethialone. We observed that the mutation *Y139F* mediates resistance in an otherwise susceptible genetic background when exposed to chlorophacinone and bromadiolone. However, the physiological response to the super-warfarins, difenacoum and difethialone, may be strongly dependent on other genes located outside the congenic interval (28.3 cM) bracketing the *Vkorc1* in our F7 generation congenic strain.

Keywords

anticoagulant; congenic strain; in-vivo experiments; mutation; *Rattus norvegicus*; resistance; *Vkorc1*

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Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's website (http://links.lww.com/FCP/A27)

Introduction

The oral anticoagulant compounds warfarin and acenocoumarol are widely prescribed drugs for the prevention and treatment of arterial and venous thromboembolic events that jeopardize human health. These compounds are referred to as antivitamin K (AVK), or vitamin K antagonists, because they inhibit the vitamin K 2,3-epoxide reductase protein complex, VKOR, of the vitamin K cycle. Such inhibition prolongs blood coagulation times and reduces the ability of blood to form clots. The gene encoding a warfarin sensitive component of the VKOR is the vitamin K 2,3-epoxide reductase complex subunit 1 (*Vkorc1*).

In humans, adverse side-effects of AVKs are of great concern. The estimation of AVK doses required by patients to meet the narrow therapeutic range of the drugs while avoiding adverse side-effects is complicated, time-consuming and expensive. Generally, the inter-individual variability in response to AVK is considered environmental and multifactorial. The former is affected, for example, by nutrition [1], whereas the latter refers to the involvement of multiple genes in the AVK response, including the warfarin metabolizing enzyme cytochrome P450 2C9 (*Cyp2C9*) in humans [2], and potentially other metabolic processes localized in the endoplasmatic reticulum [3].

Warfarin and its derivatives are also used as a rodenticide to control rat and mouse infestations. High doses of warfarin or other more recently developed anticoagulant rodenticides cause fatal bleeding after being orally ingested by the animals. However, numerous rat and mouse populations have developed resistance to warfarin and to some of the second-generation, more potent anticoagulant rodenticides. The first cases of resistance to warfarin were reported in *Rattus norvegicus* in 1958 in Scotland [4]. As a consequence of the spreading of warfarin resistance, the more potent anticoagulants have been used since the 1970's. The earliest second-generation anticoagulant rodenticides employed to control warfarin-resistant rodent populations were difenacoum and bromadiolone. However, resistance to rodenticides has been reported since the 1980s in some wild populations of rodents [5–7]. In response to reports of such highly resistant rodent populations even more potent anticoagulant poisons have been developed, including brodifacoum, flocoumafen and difethialone. These single-feeding compounds are generally believed to be lethal to resistant strains of rats and mice.

The long-lasting effort to identify the molecular genetic basis of warfarin resistance in rodents has culminated in the identification of the *Vkorc1* gene [8,9]. It has also been established that the variation in responsiveness of human patients to warfarin, at least partially, is explained by the same genetic mechanisms involving a mutant *Vkorc1* gene. Numerous reports have now associated genetic polymorphisms in the *Vkorc1* gene with the response of humans and rodents to anticoagulants [9–14]. Currently, it is widely acknowledged that mutations in *Vkorc1* seem to form one essential genetic change affecting resistance to anticoagulant rodenticides in rats and mice, and the dosage requirements of AVKs in humans during oral anticoagulant therapy. However, it remains difficult to fully explain all the aspects of resistance to warfarin and, in particular, the genetic mechanisms affecting resistance in rodents to the recently developed, highly potent anticoagulants bromadiolone, difenacoum, and others. It is generally recognized that the pharmacokinetic response to anticoagulant compounds involves genes of the cytochrome P450 group, notably the *Cyp2C9* gene in humans [2], several Cyp450 genes in *R. norvegicus* [15] and *Cyp3a2* in *R. rattus* [16,17]. A unique resistance mechanism involving the calumenin gene has been proposed to mediate resistance to warfarin in a strain *R. norvegicus* isolated from Chicago, USA [3]. Here we used warfarin-resistant *R. norvegicus* initially trapped on French farms in the 1980's as a donor strain to introduce a tyrosine (Y) to phenylalanine (F) change at

position 139 in the amino acid sequence of the VKORC1 into a warfarin-susceptible inbred recipient Sprague–Dawley strain (SD). We used this newly developed quasicongenic strain to investigate open questions regarding the in-vivo interaction between the *Vkorc1* gene and anticoagulants by measuring prothrombin time (PT) to establish the level of blood coagulation. Specifically, we examined the ability of the *Vkorc1* gene carrying the *Y139F* mutation to maintain PT at normal levels after in-vivo exposure to four anticoagulants on its own, that is, when placed in the otherwise anticoagulant susceptible genetic background of the SD strain. This design enabled us to evaluate the dependency of the *Vkorc1* gene on genetic factors located outside the introgressed wild-derived genomic region on chromosome 1 carrying the *Vkorc1* gene or elsewhere in the genome.

Methods

Origin and husbandry of animals

Two *R. norvegicus* strains were used in this study. First, a wild-derived strain resistant to warfarin owing a Y > F mutation at position 139 founding of the amino acid sequence of the VKORC1 protein was used as donor [13]. The founder animals of this donor strain were initially trapped on French farms in the 1980s and have since been maintained at the Lyon College of Veterinary Medicine, France. Recipient strain SD rats were purchased from the Charles River Laboratories (St. Germain sur l'Arbresle, France). To form a congenic strain one Y > F homozygous mutant male was crossed with two SD females to create the F2 hybrid generation. The F2 males were backcrossed to SD females to give the F3 generation. F3 individuals carrying the *Y139F* mutation, determined by the allele-specific polymerase chain reaction (PCR) genotyping method as described below, were backcrossed to the recipient SD strain for four additional generations yielding F7 generation. Finally, an F7 intercross of males with females was carried out to obtain the three segregating genotypes SD/SD, SD/Y139F, Y139F/Y139F; SD denoting the nonmutant allele present in the recipient strain SD.

All rats were bred in the Lyon College of Veterinary Medicine, France, following protocols approved by the Ethics Committee of the Lyon College of Veterinary Medicine, France, and the guidelines described in the US National Health Institute for the Care and Use of Laboratory animals. 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 libidum*. Crosses were conducted by placing single males in individual cages each with 2–3 females until female(s) were pregnant, as was judged by visual inspection. The date of birth of pups was designated as the day pups were first observed and is thus a slight underestimate of their ages. Pups were weaned between the ages of 20–21 days.

Genetic characterization of animals

Five warfarin-resistant F7 rats (three males and two females) carrying the *Vkorc1* gene of the donor strain and five susceptible F7 rats (three males and two females) carrying the *Vkorc1* sequence of the recipient strain were used to estimate the size of the genomic segment introgressed from the anticoagulant resistant wild-derived donor strain into the SD strain. This involved two steps. First, the single nucleotide polymorphism (SNP) of A to T underlying the *Y139F* mutation in the *Vkorc1* gene of the donor strain was assayed by using an allele-specific PCR. After that, the size of the genomic segment introgressed was estimated by genotyping of a panel of 25 microsatellites following a protocol employed by the Charles River Laboratory (Animal Genetic Monitoring branch, Troy, New York). For SNP typing, genomic DNA was prepared from tail clips. Genomic DNA was extracted using the Macherey-Nagel Nucleo-spin Tissue extraction kit (Hoerdt, 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 which the three prime nucleotide of the first one matched the *Vkorc1* sequence of the SD strain (i.e. the rat genome sequence of the *Vkorc1*; SD-primer: 5′-cat tgt ttg cat cac cac cta-3′) and the three prime nucleotide of the other matched the mutation in the *Vkorc1* sequence of the donor strain (mut-primer: 5′-cat tgt ttg cat cac cac ctt-3′). The reverse primers matched the *Vkorc1* sequence of the rat genome sequence for the *Vkorc1* (5′-tca ggg ctt ttt gac ctt gtg-3′). We amplified 91 base pairs of *Vkorc1* gene using quantitative real-time PCR (qPCR) in a Thermocycler Mx3000P Stratagene (Massy, France). The reaction mixture contained MESA Blue qPCR Master Mix Plus for SYBER Assay Low ROX sample (EUROGENTEC). The 20 μl reactions contained 10 ng of genomic DNA, 50 nmol/l of each forward and reverse primers, 2x reaction buffer including dNTPs, Meteor Taq DNA polymerase, $MgCl_2$ at 4 mmol/l final concentration, blue dye, ROX passive reference and stabilizers. The cycling was initiated by one denaturation step at 95°C for 5 min; followed by 40 cycles of 95°C for 15 s, 60°C for 1 min and 72°C for 10 s each. Fluorescence from both the SYBER and ROX dyes was recorded at the annealing step.

The genotyping results were used to evaluate the size of the introgressed region as follows. The microsatellite genotypes for rats were transformed to reflect the origin of alleles from the recipient strain (SD) and the alleles from the donor strain (Y139F). Microsatellite markers for which the donor and recipient strains had identical alleles were discarded (*DlRatl*, *DlRat39*, *D1MU7*, and *D1MU32*) (Supplementary Table). To obtain a measure for the probability of obtaining any particular configuration of *Y139F* associated alleles in five warfarin-resistant F7 rats carrying the *Vkorc1* gene of the donor strain and five susceptible F7 rats carrying the *Vkorc1* sequence of the recipient strain we applied a resampling with replacement procedure to generate a larger genotyping matrix that could be used for statistical analysis. Specifically, we sampled *Y139F* and SD alleles from the empirical genotypes in Supplementary Table (excluding non-informative markers), separately for the group of five susceptible rats and five resistant rats to account for the uneven allele frequencies in each group. In effect, we simulated the sampling of a crossing-scheme involving 75 susceptible and 75 resistant rats. The probabilities of observing individual genotypes at each microsatellite marker for five rats (e.g. observing the wild-derived microsatellite alleles associated with the *Y139F* resistance allele in all five rats of the resistant group) were tabulated from the random data. We also tabulated the frequency of observing runs of two (or more) wild-derived micro-satellite alleles associated with the *Y139F* resistance allele, that is, the probability of observing such runs of the *Y139F* allele in five resistant rats or runs of the SD allele in five susceptible rats.

Phenotypic characterization of animals

All animals tested were between the ages of 10 and 12 weeks. Blood was collected and PT was measured 24 h after the administration of the AVKs. For PT measurements, 3 ml of blood was collected in citrate tubes (3.2%; 1:9 v/v) 24 h after anticoagulant injection. Isofluran was used as the anaesthetic. PT times were measured in duplicate using a Biomerieux Option 2 Plus (Behnk Electronick, Norderstedt, Germany) with the Neoplastin CI Determination of Prothrombin Time kit (Diagnostica Stago, Asniere, France). All measurements were performed twice.

The amounts of AVKs administered were expressed as multiples of effective dose 50 (ED_{50}). ED_{50} is the dose that creates an increase in the international normalized ratio higher than 5 for 50 percent of the tested population. The ED_{50} in response to AVKs in rats differs between female and male (Table 1). To account for this, as recommended by the technical monograph of 2003 [18], the doses administered were multiples of ED_{50} for each anticoagulant adjusted for males and females. The anticoagulants chlorophacinone,

bromadiolone, difenacoum and difethialone were dissolved in the vehicle solution dimethyl sulfoxide and administered by intraperitoneal injection. Experiments on chlorophacinone and difethialone were performed on female rats and experiments on bromadiolone and difenacoum were performed on male rats. Each dosage was assayed on a group of six animals. At the basal levels, blood clotting easily takes place in the tube. Results of such assays were discarded. Because the ED_{50} values were adjusted for sex in this study such comparisons between males and females are justified [18] (Table 1). This strategy enabled us to minimize the number of animals needed for this study by using all the offsprings.

To further minimize the number of animals used for this study, PT was first measured only after exposure to the lowest and highest experimental dosages for the SD/SD animals. Second, for homozygous resistant rats (Y139F/Y139F) PT measurements were determined at the high end of possible concentrations of anticoagulant because at lower concentrations no responsewas expected. Finally, for heterozygous resistant rats (SD/Y139F) PT was measured at all concentrations except for bromadiolone, where it was clear that PT at × l(one time the ED_{50}) was notexpected to be different from × 4 (four times the ED_{50}).

PTwas measured 24 h after the administration of the AVKs to obtain the phenotypes of each genotype. This protocol is well adapted to the detection of a pharmacodynamically-based resistance (i.e. altered enzyme biochemistry), but is not suitable to detect a pharmacokinetic resistance (i.e. a decrease in the half life of the administrated AVK) [19]. Such a short protocol also prevents the animals from dying of haemorrhage before blood sampling.

Chemicals

The suppliers for the anticoagulants used in this study were Liphatech, Pont du Casse, France; for dimethyl sulfoxide, Sigma-Aldrich (Schnelldorf, Germany); for Isofluran, Isofluran Belamont, Nicholas Pimaral, London UK.

Statistical analyses

The results were computed using functions implemented in R-Software [20].

Results

Validation of the genotyping method to detect the *Vkorc1 Y139F* mutation

The *Vkorc1* genotypes of the rats used in this study were determined using a qPCR genotyping method. Before the routine use of this method, we validated the approach for 11 samples of each *Vkorc1* genotype (SD/SD, SD/Y139F, Y139F/Y139F) by DNA sequencing (Biofidal, Vaulx-en-Velin, France) of the *Vkorc1* gene and subsequent comparison to qPCR results. Moreover, both the primer pairs, that is, the primer matching the *Vkorc1* gene sequence as given in the rat genome sequence and the primer matching the *Vkorc1* sequence underlying the *Y139F* mutation were tested separately using the same reverse primer on each DNA sample. The three possible genotypes SD/SD, Y139F/SD and Y139F/Y139F were deduced from the differences between their characteristic cycle threshold values (ΔCt), that is the difference of Ct values between the matched and the mismatched primer extension for homozygous rats, and the absence of such a difference in Ct values for heterozygous animals. An example of such qPCR genotyping results for the SD/SD, SD/Y139F, Y139F/Y139F is shown in Figure 1. Differences in Ct values between genotypes were significant (analysis of variance, $P < 2.2e\text{-}16$, *c.f.* Figure 2).

Size and location of the congenic segment

Microsatellite genotypes were used to infer the boundaries of the introgressed genomic region carrying the *Vkorc1* gene with the *Y139F* mutation of the wild-derived warfarin-

resistant donor strain. We refer to the SD strain that carries this introgressed region as quasicongenic, because, strictly speaking, after six generations of back-crossing it would require another four additional generations (or more) to render this strain into a legitimate congenic strain, generally said to require 10 generations or more of backcrossing. However, as shown below, after six generations we already observed the reduction in size of the introgression, reduction of wild-derived alleles across the genome and differences in the physiological responses of animals to AVK administration in the course of our analysis. Thus, to minimize the number of study animals, we terminated the experiment at backcrossed generation number six. All 10 rats included in the panel for microsatellite genotyping were homozygous for the *Y139F* mutation or the SD allele at the *Vkorc1* (Supplementary Table). After visual inspection of the genotyping results, we placed the region of homozygosity between *DlRat55* on the q arm of rat chromosome 1 at the proximal (with respect to the centromer) position 170.49 mega-bases (Mb) in the ENSEMBL genome assembly of the rat (release 52, December 2008), and *DlRat159* at position 198.79 Mb to the distal end. The introgressed segment contains the *Vkorc1* gene (187.18 Mb) and the makers *DlMgh9*, *DlRat437*, *DlGotl64*, and *DlRatl59* (Table 2, Supplementary Table).

From the analysis of the random resampling of data (see Methods section) we obtained the highest individual significance for the genotypes at the microsatellite markers *DlMgh9*, *DlRat437* and *DlGotl64* ($P = 0.0127$ each), and runs of two or more of these ($P = 0.00l$) (Table 2). Thus, *DlMgh9* and the *Vkorc1* define the minimum size of the congenic segment carrying the *Y139F* mutation. Among individual rats, on average, the locations of the two recombination boundaries in this strain are respectively located between *DlRat55* and *DlMgh9* and between the *Vkorc1* and *DIRat 159*. This size of this interval corresponds to 28.3 Mb or less.

Analysis of prothrombin time measurements

The PT of our control groups, that is, the basal levels of PT when not exposed to the AVKs, were as follows: 14.5 ± 1.4 s for SD/SD genotype, 15.8 ± 1.9 s for SD/Y139F genotype and 15.5 ± 1.4 s for Y139F/Y139F genotype. To determine the effects of AVK on each genotype, multiples of ED_{50} of each anticoagulant were administered as recommended [18].

Chlorophacinone—Chlorophacinone (Fig. 3a) doses tested were 0.67, 8.04 and 10.72 mg/kg (corresponding to one, 12, and 16 times the ED_{50}). For the SD/SD genotype, PT times increased dramatically at both 0.67 and 10.72 mg/kg (65.3 ± 18.1 and 126.8 ± 10.1 s). For the Y139F/Y139F genotype PT time increased only slightly at 10.72 mg/kg (22.0 ± 9.7 s). For the heterozygous rats, PT time was highly dependent on the dosage (PT values of 13.4 ± 0.6, 25.8 ± 3.4 and 64.5 ± 24.7 s for 0.67, 8.04 and 10.72 mg/kg, respectively) and increased significantly from one dosage to another (Kruskal–Wallis test, $P = 0.0013$). The PT of the three genotypes SD/SD, SD/Y139F and Y139F/Y139F differed significantly at × l6 ED_{50} of chlorophacinone (Kruskal– Wallis test, $P = 0.0010$).

Bromadiolone—Bromadiolone (Fig. 3b) doses tested were 0.47, 1.88, 2.82 and 3.76 mg/kg (corresponding to one, four, six and eight times the ED_{50}). For the SD/SD genotype there was a high increase in PT times at 0.47 and 3.76 mg/kg (54.4 ± 16.9 and 56.8 ± 8.2 s). For the Y139F/Y139F genotype, PT times showed a very limited increase at 2.82 mg/kg (21.4 ± 3.2 s) but a slightly higher increase at 3.76 mg/kg (45.1 ± 23.0 s) with no significant difference between the two dosages (Kruskal–Wallis test, $P = 0.1416$). For the heterozygous rats, PT time was highly dependent on the dosage (PT values of 21.2 ± 5.8, 48.9 ± 17.4 and 76.6 ± 13.8 s for 1.88, 2.82 and 3.76 mg/kg, respectively) and increased significantly from one dosage to another (Kruskal–Wallis test, $P = 0.0014$).

Difenacoum—Difenacoum (Fig. 3c) doses tested were 0.52 and 1.3 mg/kg (corresponding to 0.8 and two times the ED_{50}). For the SD/SD genotype there was a high increase in PT times at both 0.52 and 1.3 mg/kg (81.0 ± 12.7 and 75.3 ± 18.9 s). For the Y139F/SD genotype and for the Y139F/Y139F genotype, PT times increased with the dosage, 47.9 ± 20.3 and 29.8 ± 14.2 s for the lower dosage and 58.6 ± 10.4 and 73.0 ± 15.9 s for the higher dosage, respectively. We observed a significant difference between the three genotypes for 0.8 times the ED_{50} (Kruskal–Wallis test, $P = 0.0090$) but the difference between the three genotypes for the double dosage of ED_{50} was not significant ($P = 0.2113$).

Difethialone—The PT values at 0.39 mg/kg (corresponding to 0.8 times the ED_{50}) of the SD/SD, Y139F/Y139F and Y139F/SD groups were 49.32 ± 3.2, 57.2 ± 6.5 and 61.6 ± 10.2 s, respectively (Fig. 3d). However, the differences between these genotypes were not significant (Kruskal–Wallis test, $P = 0.4060$).

Discussion

Genetic basis of prothrombin time response to anticoagulants

The main pharmaceutical target of AVKs is the VKOR. The *Vkorc1* gene encodes the warfarin-sensitive component of the VKOR. The inhibitory effect of AVKs on the VKOR is markedly decreased in AVK-resistant rats trapped in the field and tested in the laboratory. The effort to genetically map the resistance in rats was accomplished by localizing the locus with respect to phenotypic markers on chromosome 1 [21], and with respect to microsatellite markers [22,23] and sequence-tagged sites [24]. The gene encoding for *Vkorc1* was discovered in 2004 [8,9].

However, additional genetically based mechanisms are known to be able to contribute to the physiological response to AVK exposure. For example, Wajih *et al*. [3] in 2004 observed that a rat strain carrying a *Vkorc1*, which was expected to be sensitive appeared to be resistant. Presumably modified expression of calumenin mediated this resistance. Furthermore, Ishizuka *et al*. [16] and Sugano *et al*. [17] reported altered expression levels of the *Cyp3a* gene in resistant *R. rattus*. Similarly, Markussen *et al*. [15] in 2008 reported the expression changes of several cytochromes (*Cyp2a, Cyp2c, Cyp2e* and *Cyp3a*)ina bromadiolone resistant *R. norvegicus* strain carrying a *Vkorc1 Y139C* mutation. Finally, Greaves and Cullen-Ayres [25] inferred that resistance to difenacoum might be multigenic. The strain they studied carried a *L120Q* mutation in the *Vkorc1* gene. In rats, this mutation is known to confer resistance to first generation anticoagulants, such as warfarin, but mutations in additional genes might be required to mediate resistance to second-generation anticoagulants [25].

Thus, important genes that seem to contribute to the physiological response to AVK exposure have been identified, but more genes remain to be mapped and cloned. Given that wild rat populations have experienced intense selection with various anticoagulant rodenticides at various separate locations and regions of the world for more than 60–70 years, it is conceivable, if not likely, that a potential diverse array of resistance mechanisms has evolved. Some of these unidentified genes may have major effect but so far experiments have failed to identify a single Mendelian locus for resistance to difenacoum. Such resistance seems to require mutations in the *Vkorc1* gene in addition to mutations in other unknown genes [25]. Part of the originality of our study is that the newly generated quasicongenic strain enabled us to observe the physiological response mediated by the *Vkorc1* with the *Y139F* mutation in isolation. Thus, we were able to deduce any such dependencies of AVK resistance mediated by the *Vkorc1* on other genes and genetic interactions in the genome.

A new resource for the study of physiological parameters relevant to the interaction between the *Vkorc1* and anticoagulant compounds

In this study, we introduced a new *R. norvegicus* strain that strictly speaking should be termed quasicongenic. The new strain carries the genomic region of rat chromosome 1 containing the *Vkorc1* gene with the *Y139F* mutation. After six rounds of backcrossing, the fraction of loci that is expected to remain heterozygous across the genomic background for wild-derived alleles in any given F7 rat was estimated to be about 1.56% following the approximation given as $[(1/2)^{N-1}]$ [26].

The strain is maintained by the intercrossing of F7 animals, from which the three genotypes SD/SD, SD/Y139F and Y139F/Y139F can be obtained for the study. After six rounds of backcrossing, the congenic interval is expected to span about 28.3 cM, or 28.3 Mb, when the approximation $[200 \times (1 - 2^{-N})/N]$ is applied, where N is the number of generations and when it is assumed that 1 cM corresponds to 1 Mb of genomic sequence [26]. Given the stochastic and the sex and strain-dependent nature of recombination, this theoretical expectation corresponds well with our statistical inference regarding the size of the introgressed segment, which we considered to be between the microsatellite markers *DlRat55* and *DIRat 159*, corresponding to a length of approximately between 10.85 and 28.3 Mb (Table 2).

Here, the use of this strain enabled us to examine the ability of the wild-derived *Y139F* mutation in the *Vkorc1* gene to mediate the in-vivo physiological response (in-vivo PT) to AVK exposure independently of the wild-derived genetic background. This is a significant advance over our earlier studies of this gene and mutation, which were carried out in a heterogeneous wild genetic background.

Consequences of the *Vkorc1 Y139F* homozygous mutation

Our results indicated that homozygosity for the *Y139F* mutation in the *Vkorc1* gene is able to modify the responses to high doses of the first generation AVKs chlorophacinone, bromadiolone, and to low doses of difenacoum, even when placed in an AVK-sensitive genetic background of the SD strain. This modified response to AVK exposure manifested itself as PTs that remained below those of the SD/SD genotype and at or near basal levels (chlorophacinone, bromadiolone) or statistically below those of the SD/SD genotype (difenacoum).

In practice, during rodent control efforts in the field, this should manifest itself as resistance of the homozygous mutant genotypes even when exposed to high dosage of chlorophacinone (up to 16 times ED_{50}) and bromadiolone (up to six times ED_{50}). Indeed, empirical data (Liphatech proprietary data) showed that the use of chlorophacinone bait for 7 days would not result in any measurable mortality in the parental wild-derived outbred strain, thereby indicating that in-vivo measurements of PT in our strain enable predictions of control outcomes in the field with anticoagulant poisons. Such predictions regarding difenacoum are less clear, but it is possible that the mutation alone, when homozygous, could hamper control efforts with this compound, in particular, when the intake of the bait is low, that is, only a low-dose treatment regime of exposure is achieved (0.8 times ED_{50}). Difethialone seems to be highly toxic to our strain, indicating that this super-warfarin would be effective against strains carrying our mutation, or, alternatively, if control problems are observed, that other mutations in the *Vkorc1* and/or additional genes outside of the introgressed region studied by us mediate such a response.

Other data on homozygous mutations in the *Vkorc1* are available, enabling us to compare our new in-vivo results with in-vitro results published previously. Specifically, heterologous expression of *Y139F Vkorc1* in HEK cells produces a VKOR that is less susceptible to the

inhibition by warfarin than the nonmutated *Vkorc1* [14]. Catalytic parameters (K_m, K_i, $V_{\text{max app}}$) were highly altered when VKOR activity was assayed using homozygous Y139F rats in comparison with nonmutated rats [13]. K_i for warfarin and chlorophacinone were increased 40-fold and 20-fold, respectively. In contrast, the increase in K_i was only 7-fold and 3.7-fold, respectively, when bromadiolone or difenacoum were tested [27]. This observation is consistent with the involvement of the *Y139F* mutation being a major cause for the phenotype observed.

Comparing our study with other previous studies shows our results to be consistent with a hypothesis that posits that the *Vkorc1 Y139F* mutation mediates resistance to chlorophacinone, bromadiolone and low-dose difenacoum, and are inconsistent with a hypothesis that posits that the *Y139F* mutation is merely associated with resistance to these compounds through genetic linkage. However, at extreme doses of bromadiolone, high doses of difenacoum, or any dose of difethialone, resistance would need to be mediated by other mutations in the *Vkorc1* than *Y139F*, and/or additional genes.

Implications of the study in the detection and management of AVKs rodenticide resistance in the field

Up until now, resistance in wild populations of *R. norvegicus* has been mainly detected in wild caught rats studied subsequently in the laboratory for their PTs or by means of no-choice feeding tests. These tests are time-consuming, expensive, may present risks to the handlers of animals and to colonies of inbred strains kept at the same facility. The detection of resistance in rat populations based on genetic testing would reduce the cost and risk associated with the laboratory testing of wild-derived animals.

We showed that the *Y139F* mutation engenders resistance to first generation AVKs including bromadiolone. But the presence of this mutation does not provide any information about the presence of other genetic phenomena, which can increase or decrease the resistance to these AVKs or increase the resistance to other AVKs such as high-dose difenacoum or difethialone. Nevertheless, our qPCR assay of the SNP underlying the *Y139F* mutation should provide a useful new means to detect the resistance in the field, and thus, enables us to devise a better control strategy.

For example, well-established population genetic models predicting the evolution of pesticide resistance posit that, initially, mutations conferring resistance are often recessive and detrimental to their carrier. Thus, only under intense selection and in the homozygous state is resistance manifest and poses problems to control efforts. In natural populations it requires substantial amounts of time to attain high levels of homozygosity for recessive mutations. However, if the doses of the pesticide used are too low to be effective on homozygous mutants, these dynamics are predicted to be different because weak selection with pesticide favours the heterozygous geno-type also.

Here, our results on the heterozygous (SD/Y139F) genotype are relevant. Resistance that is due to the SD/Y139F genotype predicts that even when the resistance allelic frequency is low, and consequently the frequency of homozygous resistant rats is low, the use of first generation AVKs will selectively favour heterozygous rats, thereby dramatically increasing the frequency of resistance alleles. This is of great interest with regards to the mechanism of the further spread of resistance alleles. However, this phenomenon will be limited for difenacoum and will not exist for difethialone. Thus, it would seem advisable to abstain from the use of first generation AVKs because they promote the build-up of the *Y139F* allele in a population, thereby increasing the frequency of homozygous animals from initially rare to ultimately very common. Owing to the high frequency of the resistance allele, and large abundance of homozygous rats, subsequent control with first generation AVKs would be of

no or little effect; a situation that usually prompts the use of potentially ecotoxic super-warfarins. Thus, two antagonistic risks need to be managed. Although first generation AVKs actively select resistant rats, recently developed more toxic AVKs could present a higher ecotoxicological risk.

Finally, ineffective doses of AVKs, if used in the field, should promote the evolution of genetic modifiers, which could either affect the resistance profile *per se* (e.g. result in the build-up of resistance to even more toxic compounds) and/or decrease for the detrimental effect of the original mutation, so that homozygous resistant individuals carrying modifiers no longer suffer from a cost of resistance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors want to thank Florian Peigneaux and Maria Olivier for their technical assistance and Timothy Avison for the linguistic revision. This study was supported by the INRA (Institut National de la Recherche Agronomique) and the DGER (Direction Generale de l'Enseignement et de la Recherche).

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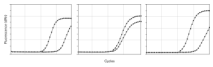

Fig. 1.

Example of the validation process underlying the quantitative real-time PCR genotyping method of the *Vkorc1* gene. The filled black coloured squares are for the mut-type primer and the uncoloured dots are for the SD-primer. The first graph represents the SYBER green fluorescence curves for a SD/SD animal, with a cycle threshold (ΔCt) of – 9.28 cycles, the second graph represents the SYBER green fluorescence curves for a SD/Y139F animal, with a ΔCt of 0.91 cycles and the third graph represents the SYBER green fluorescence curves for a Y139F/Y139F animal, with a ΔCt of 9.83 cycles. dRn, baseline corrected normalized fluorescence.

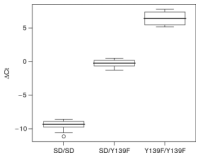

Fig. 2.

Statistical analysis of the validation of the allele-specific genotyping method. Boxplot represents the gap between the cycle threshold (ΔCt) of the three groups of rats of SD/SD, SD/Y139F and Y139F/Y139F genotypes.

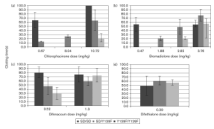

Fig. 3.

Prothrombin time results for the antivitamin K (AVK) compounds chlorophacione (a) bromadiolone (b) difenacoum (c) and difethialone (d). On the x-axis the dosages of AVK injected into the control group of AVK-sensitive rats (SD/SD) and the test groups of SD/Y139F and Y139F/Y139F rats are shown. As explained in the Methods section only those experiments deemed necessary were conducted to minimize the number of animals to be used for the study. As a result, measurements for low to intermediate doses are missing in (a) and (b).

Table 1

ED_{50} values of four anticoagulants used In this study

Anticoagulant	Sex	ED_{50} (mg/kg)
Chlorophacinone	Male	0.54
	Female	0.67
Bromadiolone	Male	0.47
	Female	0.61
Difenacoum	Male	0.65
	Female	0.79
Difethialone	Male	0.43
	Female	0.49

ED_{50} is the dose that creates an increase in international normalized ratio higher than 5 for 50% of the animals tested [18].

Table 2

Delineation of the minimum size of the introgressed segment carrying the *Y139F* mutation in the *Vkorcl* gene (dark grey) and the location of possible recombination breakpoints (light grey)

Marker[a]	Mb[b]	*P* values determined for individual genotypes of susceptible rats	*P* values determined for individual genotypes of resistant rats	*P* value to obtain runs of two or more Y139F/Y139F genotypes
D1Mgh2	22.84	0.2159	0.2222	
D1Rat8	29.68	0.7587	0.0603	
D1Rat19	43.87	0.7587	0.0603	
D1Rat93	63.99	0.7587	0.0603	NS
D1Wox30	78.52	0.2159	0.3841	
D1Rat29	103.15	0.7587	0.3841	
D1Rat34	115.16	0.7587	0.3841	
D1Rat350	135.62	0.7587	0.0603	
D1Rat45	146.19	0.7587	0.2571	
D1MH8	157.36	0.7587	0.2222	NS
D1Rat423	164.74	0.7587	0.2571	
D1Rat55	170.49	0.7587	0.2571	
D1Mgh9	176.85	0.7587	0.0127	
D1Rat437	180.67	0.0254	0.0127	<0.001
D1Got164	187.00	0.2159	0.0127	
Vkorc1	187.70	NA	NA	
D1Rat159	198.79	0.7587	0.0635	
D1Rat70	205.04	0.2159	0.3841	
D1Wox23	212.12	0.7587	0.2222	NS
D1Wox10	220.64	0.7587	0.2222	
D1Rat76	230.41	0.7587	0.0603	

Mb, megabase; NA, not applicable; NS, not significant at $\alpha = 0.05$.

[a] Microsatellite markers included in the 25 marker panel of the Charles river laboratory animal genetic monitoring branch, Troy, New York.

[b] The location of the microsatellite markers, given as megabases.