Comparison of the inhibition effect of different anticoaguants on vitamin K epoxide reductase activity from warfarin-susceptible and resistant rat

R. Lasseur, A. Grandemange, C. Longin-Sauvageon, P. Berny, E. Benoit *  
UMR 1233 INRA/DGER, Métabolisme des Xénobiotiques et Mycotoxines, Lyon College of Veterinary Medicine,  
1 av. Bourgelat, 69280 Marcy l’Etoile, France  
Received 3 August 2006; accepted 15 November 2006  
Available online 22 November 2006

Abstract

Anti-vitamin K drugs are widely used as anticoagulant in human thromboembolic diseases. Similar compounds have also been used as rodenticides to control rodent population since 1950s. Massive use of first generation anticoagulants, especially warfarin, has lead to the development of genetic resistances in rodents. Similar resistances have been reported in human. In both cases, polymorphisms in VKORC1 (Vitamin K epoxide reductase subunit 1), the subunit 1 of the VKOR (Vitamin K epoxide reductase) complex, were involved. In rats (Rattus norvegicus), the Y139F mutation confers a high degree of resistance to warfarin. Little is known about the in vitro consequences of Y139F mutation on inhibitory effect of different anticoagulants available. A warfarin-susceptible and a warfarin-resistant Y139F strain of wild rats (Rattus norvegicus) are maintained in enclosures of the Lyon College of Veterinary Medicine (France). Using liver microsomes from susceptible or resistant rats, we studied inhibition parameters by warfarin ($K_i = 0.72 \pm 0.1 \mu M$; $29 \pm 4.1 \mu M$), chlorophacinone ($K_i = 0.08 \pm 0.01 \mu M$; $1.6 \pm 0.1 \mu M$), diphacinone ($K_i = 0.07 \pm 0.01 \mu M$; $5.0 \pm 0.8 \mu M$), coumachlor ($K_i = 0.12 \pm 0.02 \mu M$; $1.9 \pm 0.2 \mu M$), coumatetralyl ($K_i = 0.13 \pm 0.02 \mu M$; $3.1 \pm 0.4 \mu M$), difenacoum ($K_i = 0.07 \pm 0.01 \mu M$; $0.26 \pm 0.02 \mu M$), bromadiolone ($K_i = 0.03 \pm 0.02 \mu M$; $0.91 \pm 0.07 \mu M$), and brodifacoum ($K_i = 0.04 \pm 0.01 \mu M$; $0.09 \pm 0.01 \mu M$) on VKOR activity. Analysis of the results leads us to highlight different anticoagulant structural elements, which influence inhibition parameters in both susceptible and Y139F resistant rats.

© 2006 Elsevier Inc. All rights reserved.

Keywords: Vitamin K epoxide reductase; Liver microsomes; Warfarin resistance; Inhibition parameters; Chlorophacinone; Diphacinone; Bromadiolone; Difenacoum; Brodifacoum; Coumatetralyl; Coumachlor; Rattus norvegicus

1. Introduction

Coumarin derivatives such as warfarin are used as anticoagulant drugs in human thromboembolic diseases. They have also been widely used since 1950s in the control of rodent populations. These drugs belong to the anti-vitamin K group (AVK). They inhibit the vitamin K epoxide (K>O) reduction activity (VKOR), which is part of the vitamin K cycle and as a consequence they block the vitamin K dependent γ-carboxylation of the coagulation factors. Finally, the suppression of VKOR activity by AVK compromises the coagulation process [1]. In humans and rodents, genetic warfarin resistances are observed [2–4]. In these patients or in these animals, anticoagulant activity of warfarin is lowered and the warfarin inhibiting activity towards liver microsomal VKOR is reduced [2,3,5,6].

In rats, a large-scale utilization for 50 years of these chemicals has created an intense selection of resistant individuals. Nowadays, some places count up to 75% of resistant animals [7]. Control of such populations leads to the utilization of much more potent chemicals, such as difenacoum, bromadiolone or brodifacoum [8]. Nevertheless,
resistances to difenacoum and bromadiolone are now observed, which confirms the evolution of resistance mechanisms and rodent adaptation. Unfortunately, because of their increased activity, these rodenticides are much more threatening for non-target species [9].

Many studies on resistance mechanisms have been conducted and recently a protein of the VKOR complex has been identified and named VKORC1 (Vitamin K epoxide reductase subunit 1) [2,10]. The heterologous expression of this protein in SF9 (insect cells) or HEK (Human Embryonic Kidney) cells was found to be able to catalyze vitamin K\(\rightarrow\)O reduction, this reaction being sensitive to inhibition by warfarin. An important step was achieved in the understanding of resistance mechanisms, identifying different SNPs (Single Nucleotide Polymorphism) in warfarin resistant human and rodent VKORC1 sequences. In rodents, mutated VKORC1 showed a reduced enzyme activity and a partial resistance towards warfarin inhibition [3].

The study by Pelz et al. [3] comparing different resistant laboratory strains and wild-caught rats for mutations in the VKORC1 gene showed that the mutation Y139F confers resistance in laboratory strains and wild-caught rats for mutations in the VKORC1 (Vitamin K epoxide reductase subunit 1) [2,10]. The Y139F mutation is highly conserved (from 0.7 to 29\(\mu\)M) in the Y139F rats. In view of rapid resistance evolution in rodents, it was interesting to understand the behavior if inhibitors on the pharmacological target VKOR.

We screened different anticoagulants (warfarin, chlorophacinone, diphacinone, coumachlor, coumatetralyl, bromadiolone, difenacoum and brodifacoum) on VKOR activity from wild-type and Y139F rat liver microsomes. The aim of this study was to observe the consequences of this mutation on the activity of the different AVK available.

2. Materials and methods

2.1. Animals

Warfarin-susceptible and warfarin-resistant rats bred in the Lyon College of Veterinary Medicine (ENVL) were initially trapped on French farms in the 1980s. Each strain received the same feed and water ad libitum. Animals were not supplemented in vitamin K and received a standard feed. The warfarin-resistant rats survived a 6-day no-choice feeding test of 0.025% warfarin-containing oats [12]. Susceptibility was determined at 100% for the susceptible rat strain. All experimental protocols were approved by the ethics committee of the Lyon College of Veterinary Medicine and followed the guidelines described in the National Health Institute for the Care and Use of Laboratory Animals. VKORC1 was genotyped according to [11]. Only rats homozygous for the VKORC1 Y139F SNP or VKORC1 wild type were used for the study of vitamin K epoxide reductase activity (VKOR). Rats were euthanized by decapitation under isoflurane anaesthesia, 3–4 months after the termination of warfarin exposure. The liver from 10 warfarin resistant and 10 warfarin susceptible rats were removed and stored in liquid nitrogen until assay.

2.2. Tissue preparation from rat livers

Microsomes were prepared from thawed livers by differential centrifugation as described by [13]. Protein concentrations were determined by the method of Bradford [14] using bovine serum albumin as a standard. Microsomes were frozen at \(-80^\circ\)C until analysis.

2.3. Kinetics studies on vitamin K epoxide reductase

Microsomal vitamin K epoxide reductase (VKOR) activity was assayed as described by [6,15,16], except that the reaction solution contained 2 mM dithiothreitol (DTT) and buffer solution was 200 mM Tris–HCl/0, 15 M KCl buffer (pH 7.4); therefore 1.5 mg of protein was added for assay of liver microsomes in a final volume of 1 ml. VKOR activity was assayed at 30 \(^\circ\)C. The reaction was started by the addition of vitamin K\(\rightarrow\)O solution in 1% Triton X-100 (10\(\mu\)l volume). The incubation time was 20 min and the reaction was stopped by adding of 4 ml of iced 1:1 isopropanol/hexane solution. After centrifugation (5000 \(\times\) g, 5 min), the hexane layer was removed and was dried under nitrogen. The dry residue was immediately dissolved in 0.2 ml of isopropanol. Vitamin K was measured by HPLC (D7000 HS Hitachi AS 2000 automatic injector, injection volume of 50 \(\mu\)L, L 7100 pump, L 4000 UV detector). Separation was achieved on a LiChrospher 100 RP18e, 150 x 4 mm, 5 \(\mu\)m analytical column run at 1.5 ml/min in 100% methanol HPLC grade. Internal standard (Vitamin D3) was added and quantified at 254 nm. In order to determine apparent \(K_m\) values, eight different concentrations of the substrate vitamin KO were used ranging from 12.5 to 100 \(\mu\)M. Incubations were performed in triplicate. Inhibition parameters were first determined with anticoagulant concentrations of 0.01; 1; 10 and 30 \(\mu\)M and further more precisely characterized using anticoagulant concentrations from about 0.05 \(\times\) \(K_i\) to 20 \(\times\) \(K_i\). A non-linear model using the Rpackage was fitted to the data (free access on internet).

2.4. Chemicals

Vitamin K (Phylloquinone) was converted to vitamin K\(\rightarrow\)O according to [18]. Vitamin K, DTT, Tris buffer, isopropanol, hexane and methanol were purchased from Sigma–Aldrich (France). Sodium warfarin, coumachlor, difenacoum and brodifacoum were purchased from Sigma–Aldrich (France). Coumatetralyl and diphacinone were from Laboratory Dr. Ehrenstorfer-Schäfers (Germany). Chlorophacinone and bromadiolone were supplied by
LiphaTech (Pont du Casse, France) and of the highest purity available (>99.7%). Primers for VKORC1 genotyping were synthesized by Proligo (Paris, France). DNA genomic Isolation System was from Promega (Charbonnières les Bains, France).

3. Results

3.1. Preliminary investigations

Preliminary experiments showed that enzymatic activity had a linear relationship with protein content and time of incubation (data not shown). Kinetic parameters of VKOR found were similar to the ones published previously [11], $K_m$ was $79.7 \pm 20.6 \, \mu M$ for susceptible rats and was $20.9 \pm 12.4 \, \mu M$ for warfarin resistant rats. Chemical structure of the anticoagulants studied are presented in Fig. 1.

3.2. Warfarin inhibition

Warfarin inhibition of VKOR was studied using microsomes from susceptible rats and from resistant rats. Data were fitted to the Michaelis-Menten non-competitive inhibitor model. $K_i$ obtained was $0.72 \pm 0.1 \, \mu M$ for susceptible rats and $29.0 \pm 4.1 \, \mu M$ for resistant ones. Such results are closely similar to results obtained in [11].

3.3. Coumachlor inhibition

Coumachlor is structurally very close to warfarin except for the chlorine atom. $K_i$ values was $0.12 \pm 0.02 \, \mu M$ for susceptible rats and $1.9 \pm 0.2 \, \mu M$ for resistant rats. These data show that VKOR in microsomes from (genetic) resistant rats is more inhibited by a chlorinated molecule than by warfarin.

3.4. Bromadiolone inhibition

$K_i$ was lower than warfarin $K_i$, values was $0.13 \pm 0.02 \, \mu M$ for susceptible rats and $0.91 \pm 0.07 \, \mu M$ for resistant ones. These results show that the genetic resistance is also responsible for a bromadiolone resistance in a liver microsomal system.

3.5. Difenacoum and brodifacoum inhibition

Brodifacoum has the same type of inhibiting effect as difenacoum, except that a higher $K_i$ for difenacoum was observed in microsomes from resistant rats. $K_i$ for
susceptible rats was 0.07 ± 0.01 μM for difenacoum and 0.04 ± 0.01 μM for brodifacoum. $K_i$ for resistant rats was 0.26 ± 0.02 μM for difenacoum and 0.09 ± 0.01 μM for brodifacoum.

3.6. Diphacinone inhibition

Diphacinone is a first generation inhibitor (such as warfarin) but not from the same chemical group. Warfarin is a coumarin derivated product while diphacinone is an indane-dione one. We observed a resistance phenomenon to this product (like in vivo observations (internal data)). $K_i$ was 0.07 ± 0.01 μM for susceptible rats and 5.0 ± 0.8 μM for resistant ones.

3.7. Chlorophacinone inhibition

Chlorophacinone is structurally very close to diphacinone except for a chlorine atom. We observed $K_i$ of 0.08 ± 0.01 μM for susceptible rats and 1.6 ± 0.1 μM for resistant ones (Fig. 2).

4. Discussion

In human beings, resistances to anti-vitamin K molecules are observed. Some of these resistances are due to mutations in the VKORC1 gene [2–4,10]. At present, about 10 mutations are described and are responsible for an important part of the AVK dosages variability [4,19]. Mutations on the same gene are also observed in wild rat (Rattus norvegicus) and are responsible for resistances to first generation chemicals [3]. Yet, a massive utilization of AVK since 1950 has created a strong selection of these resistant animals [20]. The control of these resistant strains is complicated by the loss of efficiency of available anticoagulants. So it’s of great importance to understand inhibition effect of AVK on VKOR carrying different mutations identified in the field. We hold two strains of wild rats, maintained in enclosures, one of which is a resistant strain homozygous for the mutation Y139F. Consequently, we tested various AVK and analyzed their effect on VKOR activity from both susceptible and resistant rats.

Among AVKs, we can distinguish two main families. The ones derived from 4-OH-coumarin, the ones from indanedione. Concerning $K_i$ values, in susceptible rat, warfarin has a singular position. Indeed, its $K_i$ is quite high (0.72 μM). Coumachlor’s $K_i$ is about 0.12 μM while its only difference with warfarin is a chlorine atom on a lateral phenyl chain. The importance of this halogen is not observed with chlorophacinone/diphacinone $K_i$ values. The only difference between these two chemicals (indanedione structure) is also a chlorine atom on the lateral chain. Nevertheless, $K_i$ values for these active compounds are so low that such an effect of the chlorine atom would be difficult to observe.

Hydrophobia of the lateral chain on the hydroxycoumarin structure seems to be in favor of a decrease in $K_i$ values.
Indeed, coumatetralyl’s $K_i$ (0.13 ± 0.02 μM) is actually higher than difenacoum’s one (0.07 ± 0.01 μM) (cf Fig. 1). Moreover, a bromide at the end of the lateral chain decreases $K_i$ as shown by the couple difenacoum/brodifacoum.

For Y139F (resistant) rats, $K_i$ of hydroxycoumarin and indanedione derivatives systematically increase as compared to susceptible rats. Nevertheless, the observations are not in the same range of values depending on anticoagulants studied. Warfarin’s $K_i$ is highly increased (from 0.72 to 29 μM for resistant rats). For the structurally related molecules such as coumachlor or bromadiolone, $K_i$ is also increased 1.9 ($\times 16$) and 0.91 μM, ($\times 7$), respectively. In other derivatives, mutation effect is limited ($\times 3.9$ and $\times 2$ for difenacoum and brodifacoum, respectively). For these chemicals, $K_i$ observed remain very low, and should be compatible with an in vivo toxic effect.

Hydrophobicity and length of lateral chain increase the interaction of anticoagulant as much in susceptible animal as in resistant animal. With increasing hydrophobicity, there is a decreased destabilizing effect of this mutation on the interaction with anticoagulant (Fig. 3). Moreover, the effect of the chlorine atom on the lateral phenyl chain (coulmachlor) is observed in resistant rats for both coumachlor and chlorophacinone in comparison with diphacinone and warfarin, respectively. Indanedione structure seems to interact with VKOR more efficiently than 4-OH coumarine.

This study was conducted with the Y139F mutation, which is involved in anticoagulant resistance. Catalytic consequences of this mutation are affinity changes of vitamin KO and warfarin for VKOR [11]. This paper describes affinity changes of VKOR activity for all AVK compounds used and highlights the importance of the OH-coumarine vs indanedione nucleus, the chlorine atom and the hydrophobicity of the lateral chain. Nevertheless, some other mutations in VKORC1 have no catalytic consequences on VKOR activity [21]. It would be interesting to study consequences of all identified VKORC1 mutations in rodents on the susceptibility of the mutated enzyme to anticoagulants.

This work concerns the inhibitory potential of different anticoagulants on VKOR. This study does not take into account metabolism and kinetics that have a real part in the in vivo toxicity of these chemicals, and in inter-species variability [22]. Nevertheless, knowing the resistance status of a given rat population would certainly be of great use, in order to use the most adapted anticoagulant compound, with a high efficacy on VKOR. It would also allow us to select one with less dramatic consequences on non-target species.

Acknowledgments

This research was supported by the INRA (Institut National de la Recherche Agronomique) and the DGER (Direction Generale de l’Enseignement et de la Recherche). The authors thank A. Bourret and F. Peigneaux for their technical assistance.
References


