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Chapter 2

Comparative Biology of the Resistance to Vitamin K Antagonists: An Overview of the Resistance Mechanisms

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http://dx.doi.org/10.5772/64204

Abstract

Vitamin K antagonists (VKA) are used in human medicine as well as for the management of rodent populations. In both cases, we have to deal with inter-individual resistances. Many mechanisms of resistances are common in humans and rodents. Moreover, with the large use of vitamin K antagonist rodenticides, the resistant phenotype is overrepresented in some rodent populations. Consequently, some resistance mechanisms with a low prevalence in the human population have a higher prevalence in rodent population; thus, they can be more studied in rodents. The aim of this chapter is to cross knowledge coming from human medicine and rodent research in order to better understand each resistance mechanism. After an overview of the essential knowledge for the understanding of the VKA action, this chapter presents the different methods of VKA resistance studying and then it assesses the current knowledge on VKA resistance in humans and rodents.

Keywords: vitamin K antagonists, warfarin, comparative biology, pharmacogenomics

1. Introduction

Vitamin K antagonists (VKA) are inhibitors of the regeneration cycle of vitamin K. The diminution of the available and usable vitamin K in the body induced by VKA leads indirectly to a hypocoagulable status of the blood. The use of VKA in humans and rodents pursues this status. Nevertheless, the final aim is different for each. In the human case, VKA anticoagu-

lants are used to prevent venous and arterial thrombotic event. In spite of the increasing use of new oral anticoagulants, VKA and, more precisely, warfarin are the most commonly prescribed anticoagulants [1]. Considering rodents, VKA are used in pest population management. In this case, death resulting from hemorrhages is pursued. VKA are currently the most used and one of the best rodenticides for two reasons. First, the delay between VKA administration and death is of several days, which avoids association between bait and death by the other rodents. Second, conversely to other rodenticides, VKA have an antidote: the vitamin K, which increases their safety for the human population and nontarget species.

In humans, VKA anticoagulants have a narrow therapeutic range [2]. Under- and overdoses can have serious consequences by the lack of efficacy or adverse event. Warfarin has been ranked number 9 among primary suspect drugs having serious outcomes in the United States during the beginning of the 2000's decade [3]. Indeed, the VKA dose has to be modulated, reflecting the genotype of patients more than other common drugs. Thus, it is necessary to identify and characterize each gene and mutation which may influence the VKA dose. In this task, the VKA research on pest management can be helpful. Indeed, management of rodent population has to deal with VKA resistances as well as in human medicine. Many mechanisms of resistances are common in humans and rodents. Moreover, with the large use of vitamin K antagonist rodenticides, the resistant phenotype is over-represented in some rodent populations. Consequently, some resistance mechanisms with a low prevalence in the human population have a higher prevalence in rodent population; thus, they can be more studied in rodents.

After a rapid presentation of the basis of vitamin K and VKA mechanisms, this chapter presents the different methods to assess VKA resistance mechanisms. Then, an assessment of the VKA-resistant pathways described in humans and in different rodent species is performed.

2. Basis of vitamin K metabolism

2.1. Vitamin K

The name "vitamin K" gathers a great number of molecules. All vitamin K are based on a naphthoquinone core and are sorted in three classes, numbered from 1 to 3. The substitution on the carbon 3 of the core determinates the class of the vitamin K. Vitamin K1 is composed of only one molecule, the phylloquinone, where the carbon 3 is substituted by a phytyl moiety. It was the first vitamin K described in 1935 by Dam [4], and chemically identified and synthesized by Doisy [5]. Vitamin K2 regroups the menaquinones. The substitute is a chain of prenyl, and the number of prenyls is indicated in the name. For example, the menaquinone 4 side chain is composed of 4 prenyl. Finally, vitamin K3 or menadione is only constituted by the naphthoquinone core (**Figure 1**).

Phylloquinone is synthesized by plants [6]. Menaquinones are synthesized from phylloquinone. The number of prenyls of the synthesized menaquinone depends on the bacteria, fungi, and animals which synthesize them. Mammals are only able to synthesize menaquinone 4 with

http://dx.doi.org/10.5772/64204

the help of the *UBIAD1* gene [7, 8]. Consequently, other menaquinone forms come mainly from fermented alimentation or gut microbial synthesis [9, 10].

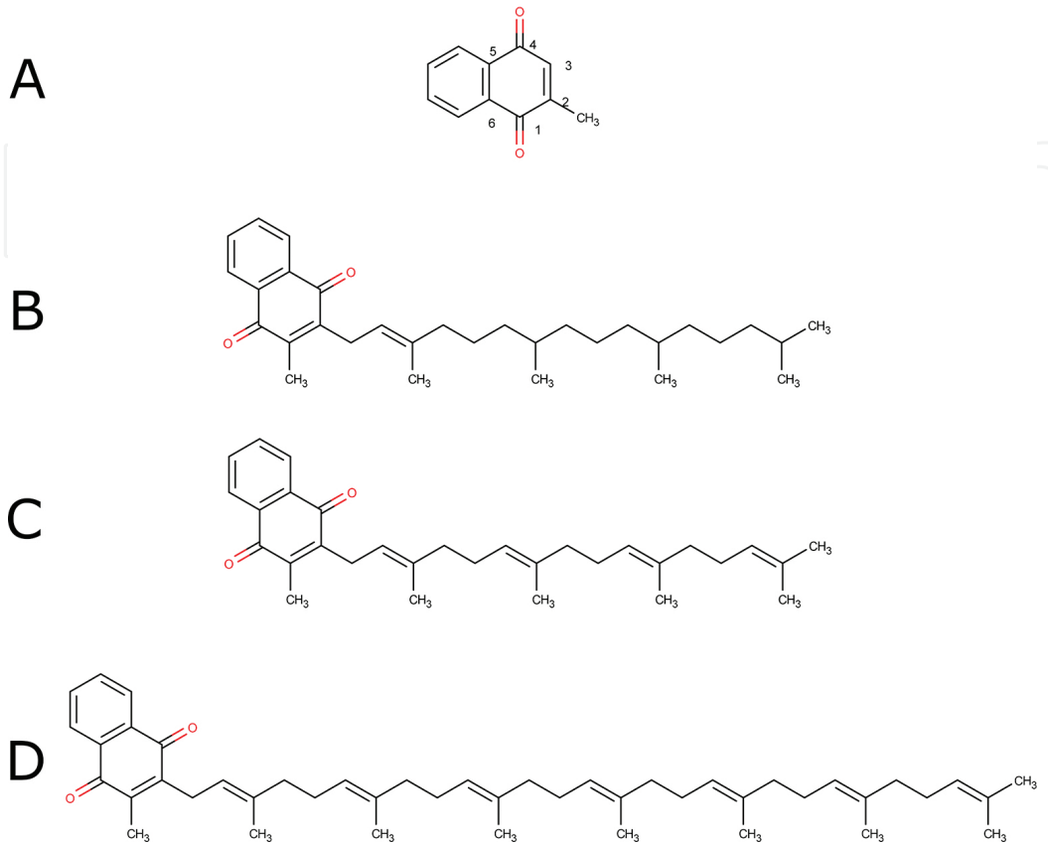

Figure 1. Examples of vitamin K: (A) menadione; (B) phylloquinone; (C) menaquinone 4; (D) menaquinone 7.

As many other fat-soluble vitamins, vitamin K's absorption increases with fatty intake [11]. The absorption of vitamin K occurs in the gut, nevertheless its mechanism has been unclear during many years [12]. Recently, a study suggested that the cholesterol transporter, the Niemann-Pick C1-like 1 protein, would be responsible for the vitamin K's absorption [13].

2.2. Vitamin K roles

The name of the vitamin K comes from the German word "koagulation." Indeed, when vitamin K was discovered, its deficiency involved bleeding [4]. Nevertheless, it is only in the 1970s that we began to understand the vitamin K's mechanism of action. Vitamin K is a cofactor of a post-transcriptional gamma-carboxylation which activates vitamin-K dependent proteins (VKDP) [14]. Four clotting factors of the coagulation cascade are the VKDPs, factors II, VII, IX, and X, which explain the bleeding issues observed in case of deficiency. Proteins C, S, and Z are also VKDPs involved in coagulation, but they have an antithrombotic effect. Nevertheless, the main effect of VKA is anticoagulation, even though the rapid decrease of these antithrombotic molecules can lead to a transient hypercoagulable state at the beginning or at the end of treatment with possible adverse events [15, 16].

The second great role of vitamin K is bone regulation with two VKDPs: osteocalcin and matrix GLA protein [17, 18]. This last protein is also involved in the protection against tissue calcification [19]. Vitamin K is involved in many other biological functions which are reviewed in Refs. [20, 21].

2.3. Vitamin K regeneration cycle

2.3.1. Vitamin K cycle

In spite of the low vitamin K level in food, vitamin K deficiency is rare [22]. Indeed, vitamin K is recycled by cells. The cycle is composed of two great steps: the use of vitamin K hydroquinone by the GGCX enzyme to activate VKDPs and the regeneration of the vitamin K hydroquinone from the epoxide form by VKORC1 (**Figure 2**).

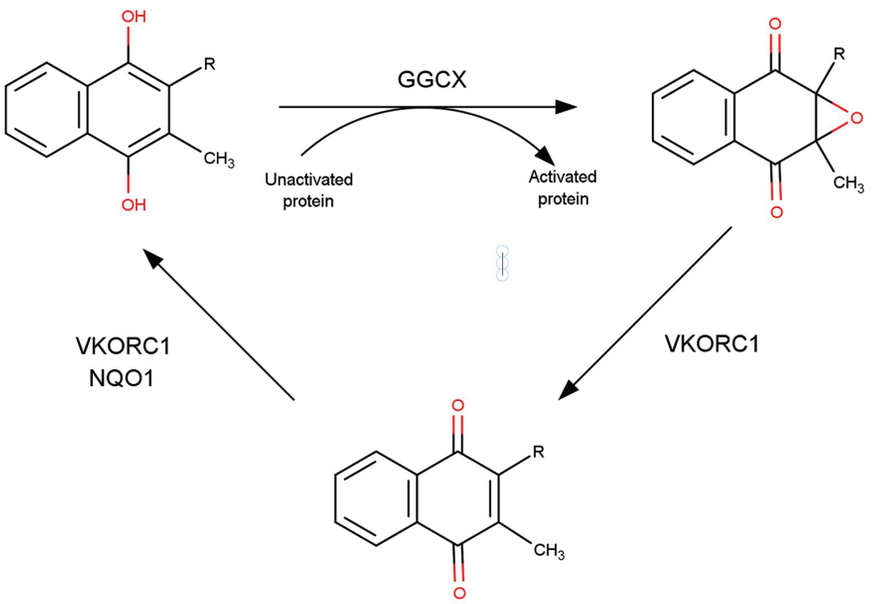

Figure 2. Vitamin K regeneration cycle.

In order to chelate calcium and to be active, the glutamate residues (Glu) of VKDPs have to be carboxylated to carboxyglutamic acid (Gla). This reaction is mediated by gamma-glutamyl carboxylase (GGCX). GGCX recognizes the VKDPs with the the help of their propeptide [23, 24]. Then, GGCX removes the gamma-hydrogen of Glu residues and adds CO_2; the oxidation of vitamin K hydroquinone (KH_2) to vitamin K epoxide (K > 0) provides the required energy [25]. This reaction is performed in the endoplasmic reticulum [26].

2.3.2. VKORC1 structure

The *VKORC1* gene was located in 1969 on the chromosome 1 of rat by discovering a link between the coat color heritage and the resistance to warfarin [27]. Then it was located on the chromosome 7 of mice in 1976 [28] and on the human chromosome 16 in 2002 [29]. The first

characterization of the *VKORC1* activity was performed in 1974; nevertheless, at this time the responsible protein of this activity was unknown [30]. The identification of the *VKORC1* coding gene has only been performed in 2004 by two teams: one used short interference RNA functional screening [31] and the other used interspecies genetic linkage analysis [32]. Once identified and sequenced, the VKORC1's structure and function study begun.

A complete review of the current knowledge on the VKORC1 structure has been recently done [33]. VKORC1 is a membrane protein of the endoplasmic reticulum. The activity of VKORC1 seems to be due to CXXC patterns. VKORC1 presents two CXXC patterns, with cysteines positioned at Cys43, Cys51, Cys132, and Cys135 in human VKORC1. These cysteines are widely conserved through species, which might indicate that they have a key role in VKOR activity [34]. Cys132 and Cys135 are located in a transmembrane domain. They seem to be essential for the VKORC1 activity and might lead the nucleophilic attack supposed by a biochemical model of the VKOR reaction [34, 35]. Mutation of one of them to serine abolishes the enzymatic VKOR activity [36]. To reduce vitamin K, VKORC1 needs to be itself reduced by aphysiological partner. This partner and its mechanism of action are currently unknown. Schulman et al. proposed that the partner might reduce the loop cysteines (Cys43 and Cys51). Then the loop cysteines would transfer the reducing power to Cys132 and Cys135 of the active site [37]. However, other studies have reported that the mutation of one of these cysteines to serine has no consequence on the VKORC1 activity [38–40]. Moreover, the conformation of VKORC1 is still under debate between a topology with three transmembrane domains or four [41]. This last point is determining for the comprehension of the possible role of the loop cysteines. Indeed, they are either in the cytosol for the three transmembrane model or in the endoplasmic reticulum's lumen for the other model.

VKORC1 presents a strong homology between rodents and humans, allowing to compare the mutation between them. Mammals and bacterial VKORs are homologs, but their conformation and reaction seem different [42].

Mammals have another enzyme able to reduce the vitamin K epoxide, the VKORC1L1. It is mainly expressed in the extrahepatic tissues [43–45] and has a great similarity with VKORC1 [46]. Its inhibition by VKA is lower than its homolog [43]. Nevertheless, due to its low hepatic expression, its influence on the anticoagulant resistance is negligible. However, it might explain that the other vitamin K functions are not significantly impacted by VKA treatments.

2.4. Vitamin K antagonists

First VKA has been discovered in 1941 and then isolated in spoiled sweet clover by Hueber and Link [47]. Sweet clover (*Melilotus officinalis*) contains coumarin. In a poorly preserved silage or hay, this coumarin can be changed to dicoumarol by *Penicillium* species commonly present in soil [48, 49]. The dicoumarol contained in spoiled forage causes a hemorrhagic condition called sweet clover disease. Some years after this discovery, Coumadin, a synthetic VKA also called warfarin, was synthesized [50]. This last molecule is still the most commonly prescribed anticoagulant. Concerning rodents, many new VKA have been developed to reply to resistance emergence.

Three VKA families are used: the 4-hydroxycoumarin derivatives, the 4-hydroxy-thiocoumarin derivatives, and the indane-1,3-dione derivatives (**Figure 3**). All derivatives are used against rodents. Conversely, in human medicine, only the 4-hydroxycoumarin derivatives (for instance, warfarin) and the indane-1,3-dione derivatives (for instance, fluindione) are used. Moreover, in order to deal with rodent resistances, second generation of 4-hydroxycoumarin derivatives and of 4-hydroxy-thiocoumarin derivatives have been designed with complex radicals (**Figure 3C** and **D**).

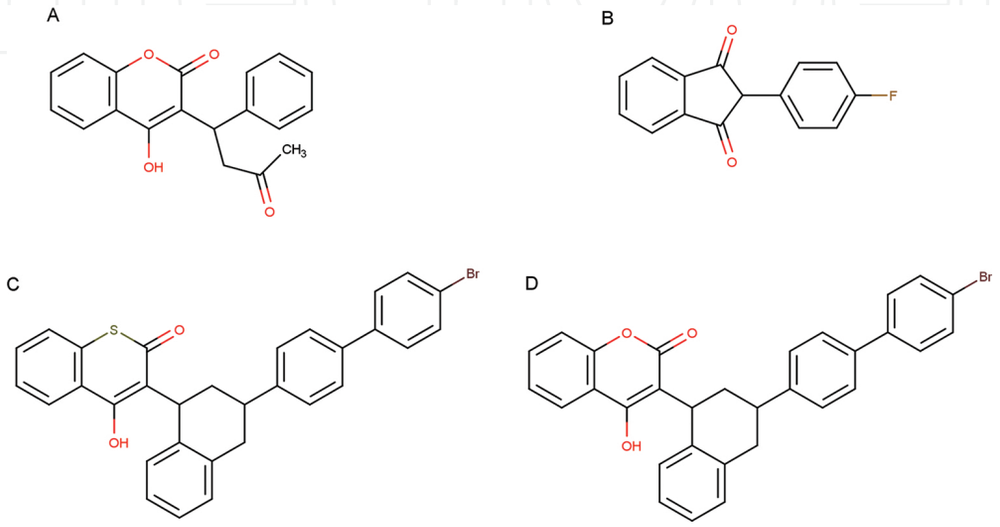

Figure 3. Examples of some VKA: (A) warfarin; (B) fluindione; (C) difethialone; (D) brodifacoum.

2.4.1. Mechanism of VKA action

Vitamin K antagonists stop the vitamin K recycling by performing noncompetitive inhibition of the VKORC1 enzyme [51, 52]. Nevertheless, the binding of VKA with VKORC1 enzyme is still a gray area. The reversibility of the binding is presently unknown [52–54], as well as the binding site. First, the binding site has been located at the level of the TYA motif (residues 138–140 in human VKORC1) close to the CXXC active site. Indeed, the mutation of the 139 tyrosine of this motif is associated with warfarin resistance in humans and rodents [55, 56], and by analogy the dicoumarol's binding site on NQO1 is also a TYA motif [57]. However, these mutations are moderately susceptible to second generation VKAs in rats [55, 58], which can suggest that other amino acids might be involved in this binding. Recently, Czogalla et al. have proposed a model involving three binding interfaces between warfarin and human VKORC1 [59].

2.4.2. VKA elimination pathway

The VKA treatments are established on the long term. Consequently, their elimination is a key factor which determines their liver concentration and finally their efficiency. The elimination pathway seems to depend on the molecule and on its enantiomeric form. Indeed, enantiomers of warfarin are eliminated differently. The (S)-enantiomer is metabolized exclusively by the

http://dx.doi.org/10.5772/64204

hepatic cytochrome P450 isoform 2C9 (CYP2C9), while (R)-enantiomer is metabolized by isoforms CYP1A2, CYP2C19, CYP3A, and hepatic ketoreductase [60, 61]. Although the (R)-enantiomer has a longer half-life, it is less efficient, and the modulation of its elimination has no significant impact on the coagulation [62–64]. However, the activity of CYP2C9 is critical in the determination of the warfarin dose. Indeed, CYP2C9 activity is influenced by many drug interactions [65–68], and polymorphism of CYP2C9 can also modulate the sensitivity to warfarin.

3. Assess the resistances

Since the discovery of rats that are resistant to warfarin in 1960 by Boyle [69], the assessment and the study of resistance mechanisms have become a key issue for the rodent population management and in human medicine. Many methods have been developed to study these resistance mechanisms. Their purpose is to isolate the possible origins of the resistance in a standardized model and to evaluate if the induced resistance factor is of the same order as that one observed *in vivo*. The resistance factor is the factor by which the dose or concentration of VKA required for a susceptible population must be multiplied to achieve the same effect within a resistant population. We present below the main methods, and for each we pinpoint its advantages and limits.

3.1. *In vivo* animal studies

In vivo studies were commonly used in rodent VKA research in order to evaluate the efficiency of an anticoagulant or the consequences of *VKORC1* mutations. The first resistance case in rodents was assessed by Boyle. He fed suspected resistant with containing VKA food and control rats during 5 days. Then, the death rate between the two groups was compared [69]. This kind of survival challenge is efficient to qualify a resistance state and remains the gold standard to test commercial rodenticides. Nevertheless, these tests are long and have many shortcomings concerning animal welfare and the repeatability. Moreover, it is difficult with this test to compare two VKAs and to assess the dose–response law.

Later, new methods based on blood clotting test have been developed [70–72]. They were standardized by the Rodenticide Resistance Action Committee in order to obtain a discriminating dose for each VKA [73]. These doses of anticoagulants, called effective doses 50 (ED50), were determined to quantify the susceptibility of rats to anticoagulants. ED50 is the dose leading to 50% of animals tested with a fivefold increase of the international normalized ratio 24 h after the administration of anticoagulants. This technique is more precise and rapid. Nevertheless, this method gives few information on the mechanism of resistance.

The origins of resistances in wild strain can be multifactorial. For example, they can involve VKORC1-linked resistance and cytochrome-linked resistance. It is possible to refine *in vivo* methods by introgressing the studied wild mutation in laboratory stain. With this method, it is possible to isolate the resistant mutation in a susceptible genetic background [55].

If the animal *in vivo* studies are still the gold standard to the understanding of the complex effect of VKAs and of resistant mutations, they are expensive and laborious in the preparation and care of the strain and in the realization of experiments. Moreover, it may be interesting to study more precisely each mechanism by *in vitro* methods [74].

3.2. *In vitro* enzymatic study

The VKOR activity can be reproduced *in vitro*. These methods are based on the kinetics of vitamin K quinone production by an enzyme system from vitamin K > O. To perform this reaction, the substrate (vitamin K > O) is beforehand synthesized according to the method described by Tishler et al. [75]. The evaluated enzyme can come from animal tissues or heterologous expression culture (cell, bacteria, yeast, etc.). Finally, dithiothreitol (DTT) is commonly used to transfer the reduction power to the enzyme. The studied VKOR-specific activity and the Michaelis constant are calculated [76]. It is also possible to add VKA to study its inhibition efficiency. In this case, an inhibition constant can be obtained for each couple of enzymatic system and VKA. Methods to perform these experiments are described in Refs. [43, 58, 76].

This simple experiment pattern allows us to assess the efficiency of large origins of enzymatic system. As the VKOR proteins are located in the membrane of the endoplasmic reticulum, they are present in the microsomal fraction of tissues. The commonly used microsomal preparation methods are described in Ref. [76]. Microsomes are prepared from other cell components by differential centrifugation. Microsomal enzymatic activity evaluates the enzymatic efficiency of whole microsomal VKOR activity and not only of the VKORC1 enzyme activity. Indeed, Hammed et al. have pinpointed that a modeling with two enzymes (VKORC1L1 and VKORC1) is necessary to explain the inhibition of the testis microsomal activity by warfarin [43]. Moreover, as for *in vivo* experiment, the strains with interesting mutations have to be selected and preserved.

To isolate the activity of one enzyme or to perform enzymatic activity with human-like enzyme or mutated enzyme, it is possible to perform a heterologous expression in yeast (*Pichia pastoris*) of VKOR enzymes and then extract the yeast's microsomes. The method is described in Ref. [58]. Yeast has neither endogenous VKORC1 nor VKORC1L1. Thus, this method allows the study of the influence of VKORC1 and/or VKORC1L1 mutation on their respective specific activities and VKA resistance without interaction. The yeast model has been validated for some mutations as Y139F for which the resistance rate of rat's liver microsomes is the same as that of yeast microsomes. Moreover, it is also possible to test some hypothetic mutations. The yeast expression method is more focused on the enzyme activity than tissue microsomes and opens many experimental opportunities. However, the studied activity is still the microsome activity. With the previous methods, enzymes are in their microsomal environment. In order to study more precisely VKORC1-linked resistances, it would be interesting to purify VKORC1.

The purification of VKORC1 was for many years a big business [77, 78]. Indeed, this enzyme loses its activity during the solubilization. It was only in 2006 than Chu et al. have purified the recombinant human VKORC1 produced from a baculovirus in insect cells which was still active after purification [79]. In this experiment, microsomes were washed from non-VKORC1

http://dx.doi.org/10.5772/64204

proteins. The artificial membrane inclusion of VKORC1 after heterologous expression in *Escherichia coli*, its extraction, and its purification have recently been described [80]. Briefly, the *hVKORC1* gene has been expressed in *E. coli* BL21(DE3) in a KSI–hVKORC1–His_6 construct [81]. The ketosteroid isomerase (KSI) part promotes the expression of the protein in insoluble inclusion bodies. KSI part was linked with hVKORC1 by a formic acid cleavage site. Finally, the construct was hexahistidine-tagged. After the expression, proteins were preserved under denaturing conditions and purified with a HisTrap Ni-NTA column. Then the KSI part is removed with formic acid. The refolding of hVKORC1 was performed with pulsated renaturation and "artificial chaperone." Then the proteins were incorporated in a lipid layer to form a liposome. Jaeneke reported that the obtained hVKORC1 liposome had only 10% of the specific activity of the enzyme obtained with HEK293 cell [80]. These new purification methods are not currently used in the resistance study and need improvement. Nevertheless, they open the way for a better understanding of the VKORC1 topology and the influence of mutations on VKOR activity.

The enzymology studies are the basis of our current understanding of VKORC1 mutations and interaction with VKA. It is currently the technique with the best reproducibility and more reliability. Nevertheless, some issues are still pending with these methods. The major issue comes from the DTT which gives the reducing power to VKORC1 in experiments. DTT is a powerful reductor which might bypass some dysfunctional mechanisms of mutated enzyme by directly activating the active site [37]. Moreover, high concentration of DTT (>1 mM) add a background level. To limit it, Krettler et al. proposed the use of tris(3-hydroxypropyl)phosphine instead of DTT [82]. Nevertheless, there is not enough knowledge currently on this new methods and its VKORC1 interaction to change the method. Moreover, tris(3-hydroxypropyl)phosphine is less efficient in VKORC1 enzyme reduction than DTT at usual concentration.

Another issue might be that these methods study only the impact of VKA and resistant VKORC1 enzyme on the vitamin K regeneration cycle and not on the vitamin K-dependent protein gamma carboxylation. The required lipid environment and detergents are different for the activity between GGCX and VKORC1. Thus, it is currently difficult to study the interaction between both enzymes in an *in vitro* enzymatic assay [83]. These discrepancies imply that enzymology results have to be analyzed in view of the *in vivo* results.

3.3. *In vitro* cell study

In order to evaluate the complete reaction from vitamin K reduction to gamma-carboxylation and to solve some enzymatic study issues, the functional study of the vitamin K cycle in mammalian cells has been recently developed by Tie et al. [83], which was then adapted by other teams [84, 85]. In these studies, the production of a complete carboxylated protein by cell culture was assessed. The cells were HEK 293 cells transfected with the studied *VKORC1* gene and a VKDP. Then, cells were cultured in an environment containing vitamin K epoxide and the studied VKA. All methods have followed the gamma-carboxylation of factor IX gla domain by its monoclonal antibodies recognition [86] or by measuring its activity (only for complete factor IX). Three different vitamin K-dependent proteins have been used: the simple factor IX protein (FIX) [59, 84, 87]; the chimeric protein FactIXGLA, which was protein C with its gla

domain replaced by the factor IX gla domain [40, 83]; the chimeric protein FIXgla-PRGP2, as previously the gla domain of the proline-rich gla protein was replaced by FIXgla domain [85]. Concerning the method using FIX [84, 87], the gamma-carboxylation was assessed by measuring the clotting activity of the concentrated culture medium mixed with factor IX-depleted plasma. For the FIXgla-PC method [40, 83], quantification is performed on the culture medium by an enzyme-linked immunosorbent assay (ELISA) with anti-FIXgla domain antibodies as coating antibodies. Although Jamil also used a chimeric protein transformed with a FIXgla domain for its quantification, in this case, it was the membrane protein, the proline-rich Gla protein 2, which was used. With this modification, carboxylated protein gla domains were presented at the surface of the cell, then recognized by monoclonal antibody conjugated to allophycocyanin and quantified by flow cytometer [85].

This new method is not standardized yet, and there is still a gray area in the interpretation of the obtained results. Indeed, some discrepancies exist between enzymatic assay and cell assay results [84, 88] and even between cell assay results [40, 59]. The origin of these discrepancies might be the interference of the cell's endogenous enzymes, in particular VKORC1L1, which is naturally more resistant than the wild VKORC1 enzyme [43]. In this way, standard cell assays have showed that all tested mutations were resistant [59, 84]. Tie et al. have dealt with this by knocking out endogenous *VKORC1* and *VKORC1L1* genes, using the transcription activator-like effector nuclease technology [40]. Thus, Tie et al. have confirmed the interaction of the endogenous enzyme in the cell assay, and they have showed that some mutations previously classed as resistant by Czogalla et al. [59] are in fact not resistant [40].

The second issue for the interpretation of cell assay is the lack of knowledge and control on it. This kind of assay is more complex than simple enzymatic activity; it involves many mechanisms that are currently uncontrolled or unknown: the level of the recombinant VKORC1 enzymes and the recombinant VKDP; the quantity and the efficiency of the physiological partner of VKOR enzymes, which are still unknown; the level of the endogenous GGCX protein; finally, the possible other mechanisms currently undescribed. All these elements determine if the studied enzymes are not bypassed and if the vitamin K reduction is the limiting reaction. These two conditions have to be fulfilled to validate completely the cell assay. However, some studies have pinpointed that the limiting reaction might be the VKORC1 enzyme reduction by its physiological partner and not the vitamin K reduction [89–91].

Although cell assays are recent and still have a gray area in the interpretation of their results, there is no doubt that they will become a key element of the study of VKA resistance mechanisms.

3.4. Pharmacogenomics study

The pharmacogenomics studies are the main source of *in vivo* information on the VKA resistance in humans. These studies assess the correlation between some genetic parameters and the warfarin dose, which allows to keep the patient's INR in the target zone. Among these parameters, the VKORC1 and the CYP2C9 polymorphisms are keys. But many other parameters, which might influence the VKA susceptibility, are unknown and cannot be evaluated by these studies. Pharmacogenomics studies assess the epidemiology of the mutations linked

to resistance and can help in the determination of the treatment's starting dose by taking in account of patient's genome. But, their contribution to the study of the resistance pathway is limited to the confirmation or not of the results obtained with other methods.

4. Resistance pathways

4.1. VKOR-linked resistances

4.1.1. VKORC1 human genotypes

The pharmacogenomic studies and the reports of warfarin treatment failures have pinpointed two kinds of polymorphisms linked to VKORC1 which influence the warfarin dose requirements. The first is linked to the polymorphism in the noncoding region and the second is linked to missense mutations.

The polymorphism in the noncoding region is the origin of the majority of VKA dose variations [92–95]. Indeed, the noncoding region influences the transcription level of VKORC1 [94]. Nevertheless, these variations are minor, few milligrams of increase or decrease. So, it is not really resistance to VKA, but rather a slight modulation of VKA sensibility.

Conversely, the VKORC1 coding mutations are rare, and some involve a real resistance to VKA. They are characterized by a dose to stabilize the anticoagulation, which is higher than the high-dose threshold defined by Watzka: phenprocoumon 3.0 mg/day, acenocoumarol 3.5 mg/day, warfarin (W) 7.1 mg/day, and fluindione 19.8 mg/day, for mean age patients [56]. Please note that the definition of warfarin resistance is not well-defined. Currently, there are more than 27 mutations that have been described in patients with high requirements of VKA dose. They are summarized in **Table 1**.

Mutation	Number of patients	Molecule	Dose (mg /day)	Stable anticoagulation	Ref.	Confirmation of resistance by *in vitro* method[†]
A26P	1	W	20	No	[106]	Yes [88]
		F	100	No		
A26T	1	W	6	No	[56]	No [88]
L27V	1	W	7	Yes	[107]	No [88]
		F	60	No		
H28Q	1	P	10	yes	[56]	No [88]
V29L	2	W	14	?	[32, 56]	
		P	26	Yes		
A34P	1	W	27	Yes	[108]	
D36Y	31	W	16.1 [3–36][*]	~	[56, 96, 105, 106, 109	No [88]
		F	45	No		

Mutation	Number of patients	Molecule	Dose (mg /day)	Stable anticoa-gulation	Ref.	Confirmation of resistance by *in vitro* method†
		A	7	No	–112]	
		P	7 [6.9–7.1]	Yes		
D36G	1	W	20	Yes	[56]	No [88]
A41S	1	W	16	?	[94]	No [88]
V45A	1	W	45	No	[32]	No [88]
S52L	1	P	9	No	[113]	Yes [40]
S52W	1	P	10	Yes	[56]	No [40]
V54L	2	W	21 [10–32]	~	[105, 106]	Yes [88]
		F	60	No		
		A	8	No		
S56F	1	P	15	No	[56]	No [88]
R58G	1	W	34	?	[32]	No [88]
W59R	3	A	9.3 [8–12]	No	[113, 114]	Yes [40]
		P	9	No		
W59C	1	P	11	No	[56]	No [88]
W59L	1	P	15	No	[56]	Yes [40]
V66M	15	W	31.5 [20–42]**	Yes	[56, 103, 105,	No [40]
		P	9.5 [9–10]	~	106, 108]	
V66G	1	P	8	Yes	[56]	No [40]
H68Y	1	–	–	–	[115]	Yes [88]
G71A	1	P	6	No	[56]	No [40]
N77S	1	P	9	No	[56]	Yes [40]
N77Y	1	W	25	Yes	[56]	No [40]
I123N	1	P	21	No	[56]	Yes [88]
L128R	16	W	44	No	[32, 105,	Yes [40]
		F	80	No	106, 116]	
		A	13	No		
		P	30	No		
Y139H	1	P	9	No	[56]	Yes [88]

Adapted and completed with permission from Ref. [88].
* A case report, which includes a 2-year-old girl, is not included [117].
** A report of two cases was not used due to its imprecision on the doses [118].
† Results of the Oldenburg's team are not reported [59, 84].
? no data are present on the stabilization of the anticoagulation in study.
~ only some patients have a stabilization of their anticoagulation.

Table 1. Genetic variations in the coding sequence of human VKORC1 in patients requiring high dose of a vitamin K antagonist.

Conversely, another mutation has been described by Rost in two Libyan families, the R98W mutation. Homozygous patients with this mutation have a combined deficiency of vitamin-K-dependent clotting factor type 2 which causes bleeding [32]. This deficiency can be treated by a daily dose of vitamin K. Rost et al. have shown in enzymatic assays that R98W-muted VKORC1, which was expressed in HEK cell, has a VKOR activity diminished of 90% [32].

Some mutations have a higher prevalence in some populations. Thus, the D36Y mutation is relatively well represented in some African populations [96]. The Ethiopian population and the Ashkenazi Jews population have a D36Y allele frequency of respectively 15% and 4% [97–99], while this mutation is absent in South African or in Chinese populations [100, 101]. Concerning the V66M mutation, it has been described in African and African-descent populations [102–104]. Finally, the L128R mutation has been described in different families [32, 105].

Nevertheless, the other reported mutations have been described only one time and sometimes on patient with unstabilized anticoagulation. These elements reduce the possibilities to determine a resistance factor for each. Moreover, the mutations have been often described as fortuitous events of pharmacogenomics studies [94]. Finally, the interactions with other mutations on cytochromes, GGCX, or noncoding part of *VKORC1* genes can also modulate the warfarin dose and enhance or reduce the resistance. Thus, the assessment of these mutations only from case report entails a lot of bias.

In order to deal with the described bias, the *in vitro* results can be useful. The *in vitro* results from enzymology [88] and cell assays [40] have been reported in **Table 1**. Nevertheless, all mutations do not seem to be associated with resistance in *in vitro* assays. Only Oldenburg have found that all the VKORC1 coding mutations were resistant with a cell method assay; his results are not reported in the table [59, 84]. This discrepancy between assays might be due to the *VKORC1L1* gene as afore-explained in the description of the cell assays. Moreover, enzymology assay pinpoints that some mutations drastically diminish the VKOR activity of the enzyme [88], whereas cell assays do not [40]. The study of the paralog mutations in rodents may help to understand the real impact of these mutations on the resistance.

4.1.2. VKORC1 rodent genotypes

Resistances have evolved differently in rodents according to their species. Indeed, the anticoagulant pressure is exerted differently on each. These differences depend on the behavior of the rodents, and more particularly the feeding behavior. Thus, some rodents feed preferentially on one food source (rat), while others feed on many sources (house mice). Thus, rats eat a lot of poisoned grains, whereas mice eat few poisoned baits and dilute them with other food sources.

Resistances have been largely described in house mice (*Mus musculus*) and in Norway rat (*Rattus norvegicus*). VKORC1-linked resistances are due to missense mutations. They have been detected whereupon rodenticide treatment fails. Due to the purpose of the VKA rodenticides, their doses used for management of rodents are important. Consequently, only huge resistances have been pinpointed and then isolated by introgressing the concerned genes in

laboratory strains. Thus, the noncoding mutations, which are involved in the modulation of VKA dose in humans, have not been described in rodents.

Concerning the Norway rat, it has been the first target of the VKA rodenticides and the first to develop resistances [69]. The first description occurred in Scotland, and rapidly the resistance spread all over Great Britain [119, 120]. Denmark has been the second country with warfarin-resistant rats [121]. Entrapments have pinpointed that 24.2% of Denmark's rats were resistant in the 1960s [122]. Since then, resistances have been brought to light from all around the world [123, 124].

Currently, 25 mutations have been described in Norway rats [125]. Nevertheless, only few mutations are widely present and linked with important resistances. The five main mutations in Europe are L120Q, L128Q, Y139C, Y139F, and Y139S [126]. These mutations have different frequencies depending on the geographical areas. Thus, Y139C is the main mutation in Germany and Denmark [126, 127], and Y139F in France [128]. Concerning Great Britain, important discrepancies on the frequencies of mutations have been pinpointed between counties [126, 129, 130]. The frequencies of rat mutations are disproportionate comparatively to human coding mutation frequency. Thus, Y139F mutation is detected in 21% of the French rats [128], but less than 1% of the world human population carries one coding mutation. Moreover, in some areas, the prevalence of the resistant rats is of 100% [131].

Concerning the house mice, their mutations have been described in 1961 in many countries [132]. Currently, more than 10 *VKORC1* mutations have been described [133]. Moreover, 80% of the trapped mice carried at least one *VKORC1* mutation in a study in Germany, Switzerland, and Azores [133]. Finally, some population of house mice have multiple mutations, Arg12Trp/Ala26Ser/Ala48Thr/Arg61Leu, which involve VKA resistance [133]. This mutation may come from an interspecific hybridization with *Mus spretus*. But, only little information is available on *Mus spretus*'s resistance to VKA [133].

4.1.3. Overall approach of human and rodent mutations

The presence of *VKORC1* mutations in humans with very low frequency (<1%) and the fast emergence of resistance in rodents indicate that the mutations were present in rodents well before the use of VKA as rodenticide. The large use of these molecules has selected the resistant mutations of *VKORC1* and increased their prevalence in rodent populations. Moreover, the use of excessive dose of VKA has selected the mutations leading to the most severe resistance, especially in *Rattus norvegicus* populations. Indeed, in Europe, while more than 20 mutations were published, only three mutations seem now widely spread. As the roof rat population is less exposed to VKA than brown rats, the prevalence of their *VKORC1* mutations might be close to human prevalence. The roof rat resistance linked to *VKORC1* mutation has not been widely explored. Recently, Goulois has characterized the Y25F mutation in roof rats [134].

Five amino acid positions of the VKORC1 enzyme carry a described mutation in both humans and rodents. They are the positions A26, R58, W59, L128, and Y139. Nevertheless, only the mutations A26T, R58G, and W59R are identical. These three mutations are not common in humans, and the reported cases did not have a stable anticoagulation. The A26T mutation does

not seem to enhance the resistance in the rat [125]. These results were obtained with rat *VKORC1* cell expression, and they agree with the results obtained with human *VKORC1* [88]. Moreover, the frequency of this mutation is low in rats which might suggest that the resistance linked to this mutation is insufficient to be selected by the VKA rodenticide pressure. Concerning the W59R mutation, it diminishes considerably the VKOR activity of the enzyme in rats conversely to in humans [40, 125]. Finally, R58G mutation is described both in humans and mice. This mutation does not seem to involve resistance in mice, and as the W59R mutation in rat, it diminishes the VKOR activity [125]. Thus, the results obtained with VKORC1 enzyme of rodents are in accordance with the *in vitro* results with human *VKORC1* gene or VKORC1 enzyme.

The mutations at positions L128 and Y139 are the major mutations of the Norway rat. Moreover, the studies on the Y139F mutations have shown that the resistance factor of this mutation is conserved between *in vivo* and *in vitro* assays [55, 58]. In rats, these mutations involve resistance factors against warfarin, which are of 8 for the L128Q mutation and greater than 200 for the Y139 mutations [58]. Moreover, in rats, L128Q does not seem to diminish the VKOR activity while Y139F does it. Conversely, in humans, the L128R mutation conducts to a reduction of more than 90% of the VKOR activity in enzymatic assay, with the VKORC1 enzyme produced in yeasts or HEK cells [32, 88]. But, this mutation does not modify the level of gamma-carboxylation in cell assay [40, 84]. Possible origins of these discrepancies are argued in Section 3.3. Consequently, it is currently difficult to conclude on the possible resistance linked with the L128R mutation in humans.

As aforementioned, some mutations decrease the efficiency of the VKOR activity. Matagrin has studied the origins of this loss of efficiency for the Y139 mutation [135]. He showed that Y139 mutations involve a diminution of the VKOR activity and the creation of inactive vitamin K metabolites (3-OH vitamin K) which are eliminated. This induces an increase of the food requirements of vitamin K in rodents carrying these mutations [136, 137]. In humans, the influence of coding mutations on nutritional requirement of vitamin K has not been well studied [22]. However, by analogy with rodent mutations, human mutations might be involved in cardiovascular diseases without VKA treatment [138]. Thus, the rodents carrying these mutations might be use to model and to better understand the possible consequences of these mutations on the human health.

4.2. Cytochrome-linked resistances

Cytochromes are essential elements in the elimination of xenobiotics. Thus, it would be expected that their polymorphism might result in an origin of resistances. But in human medicine, the cytochrome polymorphism is associated with an increase in the patient's VKA sensibility [93, 97]. Moreover, the polymorphism of CYP2C9 is a key element in the prediction of the VKA treatment dose [97]. Thus, no VKA resistance linked to cytochrome has been described in human medicine.

In rodents, expression profiles of cytochromes are different between sensitive and resistant rats carrying a Y139C mutation on VKORC1 [139]. Nevertheless, it is difficult to identify the part of the resistance due to VKORC1 mutation and the one due to the expression profiles of

cytochromes. Nevertheless, one example of warfarin resistance linked to cytochrome has been described without association with VKORC1 mutation in roof rats from Tokyo [140, 141]. This rat population overexpresses the cytochrome 3A2. Thus, the concentration of blood warfarin 1 h after the warfarin administration is eightfold lower in resistant rats. However, it is currently the only reported case of cytochrome-linked resistance.

5. Conclusion

The assessment and the comprehension of the resistance mechanisms are essential in the safety and the efficiency of VKA treatments and in the rodent population management. However, the complexity of the mechanisms and the interactions between them prevent the use of only one studying method. The understanding of the VKA actions and their interaction with individual variability can only be achieved by a multiscale approach which combines humans, rodents, and *in vitro* and *in vivo* knowledge.

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