Biochemical Pharmacology, Vol. 38, No. 7, pp. 1115–1120, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

MICROSOMAL WARFARIN BINDING AND VITAMIN K 2,3-EPOXIDE REDUCTASE

H. H. W. THIJSSEN* and L. G. M. BAARS
Department of Pharmacology, University of Limburg, Maastricht, The Netherlands

(*Received* 26 *July* 1988; *accepted* 19 *October* 1988)

Abstract—Rat liver microsomal 4-hydroxycoumarin binding was studied by assaying specific [^{14}C]warfarin binding. Microsomes of warfarin-sensitive rats contained about 40 pmole of specific binding sites per mg of microsomal protein. There was no difference for *R*- or *S*-[^{14}C]warfarin. Neither was there any difference between the enantiomers of acenocoumarol and phenprocoumon to prevent the *in vitro* racemic [^{14}C]warfarin binding. Pretreatment of the microsomes with dithiothreitol, the *in vitro* reductor for microsomal vitamin K epoxide reductase activity, reduced the warfarin binding. Vitamin K epoxide nor vitamin K affected the warfarin binding. Microsomes of the Welsh warfarin resistant genotype showed weak warfarin binding properties. The Scottish resistant variant, on the other hand, did not differ from sensitive microsomes. Warfarin binding was reduced in microsomes of rats to which *S*-warfarin was administered. The reduction in warfarin binding was linear with the inhibition of microsomal vitamin K epoxide reductase activity and was linear with the amount of *S*-warfarin present in the microsomes. The results show the microsomal 4-hydroxycoumarin binding to be related to the target enzyme vitamin K epoxide reductase.

Along several experimental lines it has become clear that liver tissue contains one or more saturable binding sites for 4-hydroxycoumarin oral anticoagulants. Pharmacokinetic analysis of the plasma warfarin elimination in hepatectomized rats led Covell *et al.* [1] to assume a hepatic warfarin binder with a capacity of 2.7 μg/ml liver tissue and a K_d of 1.5 ng/ml. In our laboratory Scatchard analysis of *in vivo* steady state tissue levels of the *S*-enantiomer of acenocoumarol in rat livers showed a single hepatic binding site: B_{max} about 4 nmol/g liver tissue, K_d about 7 nM [2]. This binder was felt to be responsible for the typical dose dependent pharmacokinetic behavior of the 4-hydroxycoumarins in rats.

Thierry *et al.* [3] were the first to report on [^{14}C]warfarin liver distribution in rats and they found a 1% deoxycholate insoluble precipitate enriched in ribosomes to contain a warfarin binder. Later, Lorusso and Suttie [4] and Searcy *et al.* [5] showed that the warfarin binding protein stemmed from the endoplasmatic reticulum and that it was reduced in warfarin-resistant rats. These results suggested the microsomal binding protein to be related to the, at that time unknown, 4-hydroxycoumarin receptor.

Today, it is believed that the 4-hydroxycoumarin anticoagulants exert their activity by blocking the enzyme vitamin K 2,3-epoxide (vitamin KO) reductase. The enzyme is part of the so-called vitamin K cycle which resides in the endoplasmatic reticulum of the hepatocyte and of other tissue cells [6, 7]. In *in vitro* assays the enzyme needs a dithiol (dithiothreitol, DTT) to function and from activity assays in the presence of thiol blockers it is proposed the enzyme to contain two active site sulfhydryl groups which become oxidized during substrate reduction [8, 9]. DTT is needed for reactivation, i.e. reduction of the enzyme. An alternative model suggests the dithiol to function as a substrate [10]. The mechanism of interaction of the 4-hydroxycoumarins with vitamin KO reductase is unknown, except for the recognition that (a) the interaction probably only occurs with the oxidized (inactive) enzyme; or in the absence of the dithiol substrate [10, 11]; (b) the interation is very tight, i.e. the coumarin drug does not dissociate from its binding site [12, 13] unless the enzyme is converted to its active form either by reduction of the disulfide or by binding of the dithiol substrate [14, 15]. The reactivation, however, is hampered due to the bound coumarin derivative.

From *in vivo* hepatic distribution studies of microdoses of radio-labeled warfarin and the effect of the *in vivo* administration of unlabeled 4-hydroxycoumarins thereupon, we recently hypothetized the hepatic 4-hydroxycoumarin binder (see above) and the target enzyme vitamin KO reductase to be one and the same [15].

In order to get futher evidence for this we investigated the *in vitro* microsomal warfarin binding in rat livers at various conditions. The results are reported in the present paper.

MATERIALS AND METHODS

Racemic [^{14}C]warfarin (46 mCi/mmol) was obtained from Amersham Co. (The Netherlands). *R*-/*S*-[^{14}C]warfarin were isolated according to the procedure of Banfield and Rowland [16] with some modifications; *N*-t-BOC-L-proline (Sigma Chemical Co., St Louis, MO) was used instead of *N*-CBZ-L-proline because the diastereoisomers of the former showed a more favorable chromatographic behavior on TLC (Silicagel TLC plate with concentration zone; Merck, Darmstad, F.R.G. Elut-

* To whom correspondence should be addressed.

ing phase: petroleum ether, ethylacetate, methanol; 75, 25, 1; v,v,v). The enantiomers of warfarin and acenocoumarol were prepared according to the procedure of West *et al.* [17]. The enantiomers of phenprocoumon were a gift of Hoffman La Roche (Basel, Switzerland).

Rat (male Wistar, 270–300 g) liver microsomes were prepared as follows: under light ether anesthesia blood was withdrawn via the abdominal aorta. The liver was perfused *in situ* with ice-cold saline via the portal vein. The liver was excized, rinsed and after mincing in three times its volume of ice-cold 0.02 M Tris–HCl buffer pH 7.4, containing 0.25 M sucrose and 0.15 M KCl by a blender, it was homogenized (Potter-Elvehjem technique). All subsequent manipulations were performed in the cold. Microsomes were pelleted from the 10,000 *g* homogenate supernatant by centrifugation at 100,000 *g*, 60 min. The microsomes were washed by resuspending them in the original volume of buffer (Tris buffer without sucrose) followed by the centrifugation step. The wash step was repeated with a 0.02 M Tris buffer containing 1 M NaCl. Finally, the microsomes were resuspended in the latter buffer to contain about 15 mg protein per ml microsomal suspension. The microsomes were stored at −70°. Microsomal protein was assayed by the Lowry method.

Warfarin binding to microsomes was assayed as follows: the microsomal suspension was diluted 5-fold in 0.02 M Tris–HCl buffer pH 7.4. Of this mixture 0.5 ml was pipetted in two rows of polycarbonate reaction vessels, one of which contained 20 μg (about 0.66 μmole) unlabeled racemic warfarin. The reaction mixtures were incubated for 3 min at 30° whereafter [^{14}C]warfarin was added in increasing amounts, i.e. 3×10^3–10^5 dpm. After 10 min incubation (shaking waterbath), 2 ml ice-cold Tris buffer was added and the reaction mixture was filtered immediately over a Millipore^R HA filter, pore size 0.45 μm, using a Millipore^R 1225 sampling manifold. The filters were rinsed with an additional 2 ml of ice-cold buffer and after suction to dryness they were brought into counting vials containing 10 ml of liquid scintillation cocktail (Formulat 989, Du Pont). The bound warfarin was obtained by subtracting the counts recovered of the mixtures containing the unlabeled warfarin (blanks) from those without warfarin (samples). The amount of unbound, free exchangeable, warfarin was obtained by subtracting the counts of the samples from the counts initially added. The binding experiments were performed in duplo. The relationship bound vs. unbound was analyzed by a non-lin curve fitting program (CRISP^R, Crunch Software Co, San Francisco, CA) assuming one binding site.

Microsomal vitamin KO reductase activity was assayed as described previously [14]. Microsomal warfarin content was assayed by HPLC procedure [18].

RESULTS

To retain microsomes by filtration, pore widths less than 1 μm were needed. Control filtrations with buffer only showed about 5–10% of the [^{14}C]warfarin

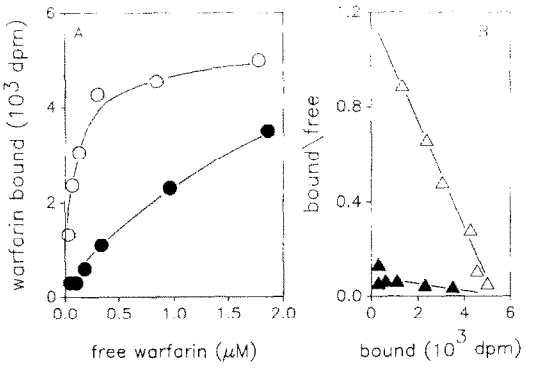

Fig. 1. Microsomal *rac.* [^{14}C]warfarin binding. (A) Plot of microsomal bound warfarin per reaction mixture vs. free warfarin concentration. (B) Scatchard plot of the data; the ratio bound/free is calculated from the data in dpm. Open symbols are native microsomes of male Wistar rats. Closed symbols are microsomes pre-incubated (3 min, 30°) with 5 mM DTT before the addition of [^{14}C]warfarin.

soluted in the buffer to be adsorbed to the filter. As binding data were obtained by subtracting blanks (see Materials and Methods) filter adsorption was not corrected for. Figure 1 shows a plot of microsomal bound racemic [^{14}C]warfarin vs its free concentration. The plot suggests saturable warfarin binding which is also apparent from a Scatchard plot (Fig. 1). Data analysis of this typical experiment, assuming one binding site showed a total binding capacity of 35 pmole (standard error 3%) of racemic warfarin per mg of microsomal protein. The free warfarin concentration where half of the binding sites were occupied was 77 nM (standard error 10%). In general, the standard error of the parameter estimate of the maximal binding capacity was less than 10%. The estimate of the concentration of half-maximal binding, however, showed errors ranging between 5 and 100%.

Pretreatment of the microsomes with dithiothreitol (DTT, 5 mM) reduced the microsomal specific warfarin binding (Fig. 1). Binding, however, was not completely abolished, but the binding curve seemed to be shifted to the right.

There were no differences between *R*- and *S*-warfarin in their microsomal binding. The following values were obtained for the binding capacity: 44 ± 5, 38 ± 6, 40 ± 1 (mean ± SD, N = 3) pmole/mg protein for *R*-, *S*- and racemic warfarin. The free warfarin concentration for half maximal occupation were: 210 ± 50, 170 ± 26, and 160 ± 25 nM, respectively. All further experiments, therefore, were performed with racemic [^{14}C]warfarin.

Warfarin binding was investigated in liver microsomes of warfarin resistant rats. Two genetic strains of warfarin resistance were compared: the Welsh and the Scottish genotype. As can be seen from the plots in Fig. 2, microsomes of the Welsh type warfarin resistant (HW) rats showed weak warfarin binding in comparison to the control (TAS) and to the Scottish resistant (HS) ones. Binding capacities for the TAS and HS microsomes were about 40 and 34 pmole/mg microsomal protein with half maximal saturation at about 80 and 50 nM free warfarin, respectively. Reli-

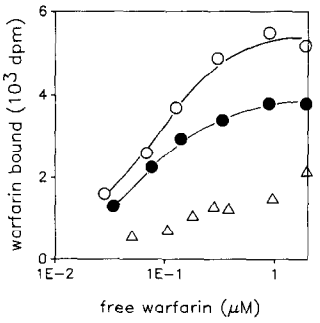

Fig. 2. Comparative microsomal *rac.* [^{14}C]warfarin binding in a sensitive rat strain (TAS, ○—○), and in warfarin resistant rat strains; the Scottish genotype (HS, ●—●) and the Welsh genotype (HW, △—△). The livers of these strains were a gift of Dr. A. D. MacNicoll, Ministry of Agriculture, Fisheries and Food, Tolworth Laboratory, U.K. Abscissa:log concentration of free warfarin; ordinate: bound warfarin per reaction mixture.

able data for binding to HW microsomes could not be obtained from these experiments. The observed vitamin KO reductase activities for the respective microsomes were 390, 420 and 180 pmole of vitamin K formed per min per mg protein for TAS, HS and HW.

Concomitant with the experiments intended to study the effect of vitamin K or vitamin KO on the microsomal warfarin binding, the effect of the vehiculum used to dissolve these vitamins, i.e. Triton X-100, was investigated (Table 1). The results showed the detergent (0.05%; w,v) to reduce the microsomal binding capacity. Apparently, the presence of vitamin K or vitamin KO (0.2 mM) counteracted this effect. To make the findings more conclusive, the effect of vitamin KO on B_{max} was assayed using isopropanol as the vehiculum of the vitamin. Isopropanol (5%, final concentration) appeared not to affect warfarin binding nor did vitamin KO. B_{max} for warfarin binding in: control 44 ± 5; isopropanol, 42 ± 8; vitamin KO, 41 ± 7 (mean ± SD for 3 experiments in triplicate) pmole/mg protein.

The effect of the *in vivo* *S*-warfarin administration on microsomal warfarin binding, on microsomal vitamin KO reductase activity and on the microsomal warfarin content was investigated (Table 2 and Fig. 3). The experiments showed the microsomal warfarin binding capacity to decrease to about 10% of control with increasing warfarin dose (0.01–0.3 mg/kg). Beyond the 0.3 mg/kg dose no further suppression was observed (Table 2). Microsomal vitamin KO reductase activity decreased concomitantly and when plotted vs the microsomal warfarin binding capacity

Table 1. Microsomal *rac.* [^{14}C]warfarin binding. The effects of vitamin K/vitamin KO and of Triton X-100

Conditions*		B_{max} (pmole/mg protein)	Free warfarin (nM) for half max. occupation
Control	(N = 4)	35.7 ± 2.5	60 ± 13
Vitamin K/vitamin KO	(N = 5)	34 ± 6	30 ± 14
Triton X-100	(N = 3)	18 ± 5	37 ± 6

* Control conditions were as described in the Materials and Methods section. Vitamin K (N = 2) or vitamin KO (N = 3), 25 μl of a stock solution in 1% Triton X-100 in Tris–KCl buffer, were added before the addition of *rac.* [^{14}C]warfarin. The final concentrations were 0.2 mM for vitamin K and vitamin KO, 0.05% for Triton X-100. The effect of 0.05% Triton X-100 was assayed by the addition of 25 μl of 1% Triton X-100 in Tris–KCl buffer.

Table 2. *Rac.* [^{14}C]Warfarin binding, vitamin KO reductase activity and warfarin content in liver microsomes of S-warfarin treated rats*

Dose (mg/kg)	N	B_{max}† (pmole/mg protein)	% Vitamin KO‡ reductase	*S*-warfarin‡ (pmole/mg protein)
Control	4	44 ± 6	100 ± 9‖	0
0.01	3	38 ± 9	89 ± 3‖	6.6 ± 0.2‖
0.03	3	27 ± 1.3	51 ± 9‖	15 ± 0.4‖
0.1	3	16 ± 5	20	36.3
0.3	3	13.5 ± 2	12	46
1	3	7.5 ± 3	18	49
10	3	11 ± 10	11	44.5

* S-warfarin was administered subcutaneously 20 hr before the isolation of the liver microsomes. Control rats received saline.

† *Rac.* [^{14}C]warfarin binding was assayed in the pooled microsomes; data are the mean ± SD for at least 3 assays.

‡ Vitamin KO reductase assays and *S*-warfarin analysis were performed in duplo for the pooled microsomes except for

‖ Where the individual microsomes were assayed.

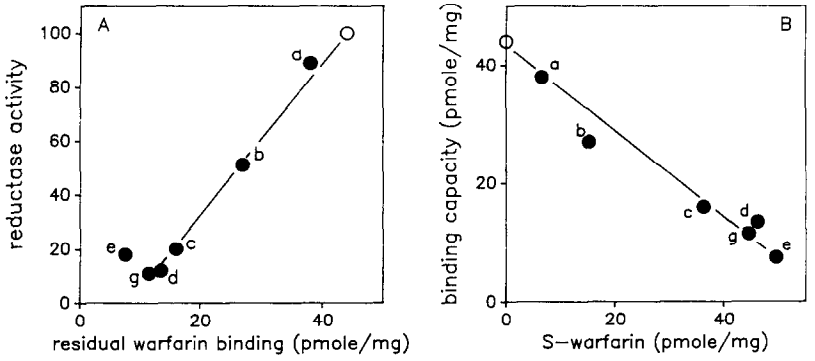

Fig. 3. (A) Plot of vitamin KO reductase activity vs. the residual warfarin binding capacity in microsomes of *S*-warfarin pretreated rats (see Table 2 for details). (B) Plot of the residual warfarin binding capacity vs the microsomal *S*-warfarin content. Characters to the symbols refer to the *S*-warfarin dose: a=0.01, b=0.03, c=0.1, d=0.3, e=1, and g=10 mg/kg.

the reductase activity from beyond 20% of control activity showed a linear relationship to the microsomal warfarin binding (Fig. 3a). The microsomal *S*-warfarin content following the *in vivo* *S*-warfarin administration increased up to a plateau of about 14 ng (46.2 pmole)/mg microsomal protein which was reached after 0.3 mg/kg *S*-warfarin. The *in vitro* microsomal warfarin binding was inversely related to the microsomal *S*-warfarin content (Fig. 3b).

All the results strongly support the idea that the specific microsomal 4-hydroxycoumarin binding site is identical with the enzyme vitamin KO reductase.

As, with hindsight, it was understandable that no differences in microsomal binding between *R*- and *S*-warfarin were observed (see also Discussion section) we looked for differences in the rates of association of the 4-hydroxycoumarin isomers and the microsomal binding site. As the binding of 4-hydroxycoumarins to the microsomes, i.e. to vitamin KO reductase, *in vitro* is irreversible [15], the binding curves do not represent an equilibrium process but rather the rate of association in a titration process. Therefore, it was reasoned that should there be any difference in rate of association, the more potent *S*-enantiomers would inhibit [^{14}C]warfarin binding by 50% at lower concentrations than the *R*-enantiomers. As can be seen from the results in Table 3 no differences between the *R*- and *S*-enantiomers were observed. The results suggest, however, the association or reaction rate to be higher for acenocoumarol and phenprocoumon than for warfarin. Statistical analysis (unpaired Student's *t*-test), however, showed significant differences only between *S*-warfarin and *S*-phenprocoumon.

DISCUSSION

The results confirm the presence of a specific warfarin (= 4-hydroxycoumarin) binding site in rat liver microsomes. Microsomal warfarin binding has been the subject of study already in 1970 by the group of Suttie [3, 4] and by Searcy *et al.* [5]. At that time one was unaware of the biochemical systems for vitamin K function, but the differences in microsomal warfarin binding between warfarin sensitive and warfarin resistant (Welsh type) rats were seen as an indication for the involvement of a vitamin K related system [5]. From *in vivo* [^{14}C]warfarin liver distribution and *in vivo* displacement studies we recently suggested the microsomal 4-hydroxycoumarin binding component to be related to the enzyme vitamin KO reductase [15]. The present results give further support for this.

Before discussing the results in more detail it has to be emphasized that the specific microsomal 4-hydroxycoumarin binding essentially is irreversible, i.e. *in vitro* the bound 4-hydroxycoumarin cannot be washed out or dialyzed [12, 13], nor can it be dis-

Table 3. Comparative *in vitro* potencies of the enantiomers of acenocoumarol, phenprocoumon and warfarin to inhibit the microsomal *rac.* [^{14}C]warfarin binding*

Acenocoumarol		μM for 50% inhibition Phenprocoumon		Warfarin	
R	*S*	*R*	*S*	*R*	*S*
1.2 ± 0.4	1.0 ± 0.3	1.2 ± 0.5	0.9 ± 0.2†	2.1 ± 0.9	1.7 ± 0.8

* The microsomal *rac.* [^{14}C]warfarin binding was assayed in 0.5 ml reaction mixtures (see Materials and Methods section) for one [^{14}C]warfarin concentration, i.e. 2 μM = 10^5 dpm. Dilutions of the 4-hydroxycoumarin enantiomers were prepared in the *rac.* [^{14}C]warfarin stock solution and aliquots, containing 10^5 dpm, were added to the incubation mixture. 50% inhibition value was obtained from the bound (dpm) vs. log [enantiomer] curve. The data are the mean ± SD for 4 assays.

† Different from S-warfarin; $P < 0.01$ (unpaired Student's *t*-test).

placed [4, 15]. Hence, the binding curves although suggestive for receptor ligand interactions, in fact are titration curves [4], and what seems to be the apparent dissociation constant is the point where at the test conditions half of the binding sites were titrated. Nevertheless, a one site ligand binding model was fitted to the data to estimate the total amount of binding sites and the free warfarin concentration at the condition of 50% titration. Obviously, the latter parameter has no physical meaning. One would expect this parameter to be inversely dependent on the incubation time. However, in an experiment where we compared 10 and 30 min incubation times no differences were observed (results not shown). In any case, we believe that the wide ranges observed in values for 50% titration is because we are not dealing with a true receptor ligand equilibrium. Our discussion therefore mainly will deal with the data on the specific warfarin binding capacity.

The models proposed in literature for the mechanistic function of vitamin KO reductase contain (1) two active site sulfhydryl groups which after substrate reduction have to be reactivated by a dithiol [8], or (2) a dithiol substrate binding site [10]. For both the models, it is proposed that only in the inactive stage, i.e. the oxidized form (model 1) or in the absence of the dithiol substrate (model 2), the enzyme binds 4-hydroxycoumarins [10]. The observed microsomal warfarin binding following DTT pretreatment is not fully compatible with this postulate; no complete abolishment of the binding was seen (Fig. 1). The residual binding might be due to enzyme titration as soon it got oxidized (by air) or as soon DTT dissociated. Alternatively, the binding curve depicts a binding shift to the right rather than a slowed titration, suggesting a DTT-dependent affinity of warfarin for the enzyme. This explanation, however, is not compatible with the observation, own and that of others [10, 19], that upon pretreatment with DTT, the warfarin inhibition of the microsomal vitamin KO reductase initially is zero but increases in time. If, on the other hand, warfarin is added to reductase assays before DTT, a potent inhibition is observed which remains constant.

The absence of stereoselectivity in total warfarin binding merely shows that stoichiometrically both isomers bind equally to the enzyme. The absence of significant differences between the warfarin isomers for the concentration where half of the enzyme was titrated suggests that their rates of association with the enzyme do not differ grossly. Neither were there differences between the isomers of acenocoumarol and phenprocoumon in their potency to inhibit [^{14}C]warfarin binding (Table 3). This would indicate that this step is not the base for the stereoselectivity in 4-hydroxycoumarin anticoagulant activity. Contrary to these findings, Searcy *et al.* [5] reported the warfarin binding protein they isolated by cosedimentation with ribosomes, to be selective for the *S*-enantiomer. Warfarin binding to this protein also was reported to compete with vitamin K. In this study, however, a competition between the *in vitro* microsomal warfarin binding and vitamin K or vitamin KO was not observed (Table 1). The absence of any competition is in agreement with the proposed interaction mechanisms [10, 20]. The differences of our observations with those reported by Searcy *et al.* [5] suggest that the warfarin binding protein isolated by the latter group might be unrelated to vitamin KO reductase. Concerning the stereoselectivity for anticoagulant activity of the 4-hydroxycoumarins, we recently presented evidence that the reactivation of the enzyme–anticoagulant complex is the ultimate stereosensitive step [20].

In agreement with the results of Lorusso and Suttie [4] and Searcy *et al.* [5], we found the microsomal binding capacity for warfarin resistant rats of the Welsh genotype to be reduced (Fig. 2). The microsomes of the Scottish warfarin resistant strain, on the other hand showed "normal" warfarin binding behavior. These findings are in accordance with the properties of the vitamin KO reductases of these rats: Welsh type resistant vitamin KO reductase shows weak affinity for warfarin thus explaining its resistance to 4-hydroxycoumarins [21]; in addition, the amount of enzyme is less. The Scottish and the sensitive enzyme do not differ in their affinity for warfarin [22]. The resistance of the Scottish strain is suggested to be due to a facilitated reactivation of the enzyme–4-hydroxycoumarin complex [14]. The reactivation of the enzyme of the sensitive rat is slow: *in vivo* it takes about 4 hr to reactivate 50% of the vitamin KO reductase–*S*-warfarin complex [20]. Apparently, as soon the liver structure is disrupted the system responsible for the reactivation of the enzyme and/or the enzyme–4-hydroxycoumarin complex is lost, and the coumarin remains irreversible bound to the enzyme. This allows the *in vitro* assay of the remaining enzyme either by reductase assay, i.e. substrate conversion, or by [^{14}C]warfarin binding. This was demonstrated by the experiments described in Table 2 and Fig. 3. The linearity between the microsomal vitamin KO reductase activity and the microsomal warfarin binding capacity (Fig. 3a) once more indicates their mutual relationship. Assuming a one-to-one stoichiometry for the 4-hydroxycoumarin and vitamin KO reductase interaction, the results indicate about 40 pmole (39.5 ± 6.5, mean of nine different livers; each binding assay was performed at least in duplo) of enzyme to be present per mg of microsomal preparation. A warfarin binding capacity of 40 pmol/mg microsomal protein was also observed by Lorusso and Suttie [4]. Searcy *et al.* [5] reported somewhat higher values; about 50–60 pmole. Assuming 50% recovery for liver microsomes [23], liver tissue would contain about 1.5 nmole of vitamin KO reductase per g wet weight. This is lower than the reported *in vivo* hepatic binding capacity; about 9 nmole for warfarin [1] or 4 nmole for *S*-acenocoumarol [2]. This indicates either the loss of enzyme during the isolation procedure of the microsomes, or the presence of additional binding sites not associated with the microsomal fraction. We previously reported the mitochondrial fraction to contain specific warfarin binding capacity but ascribed this to microsomal contamination [15].

In conclusion, the results show the specific and saturable microsomal 4-hydroxycoumarin binding to be related with the target system of these drugs, i.e. vitamin KO reductase. The binding site need not to

be the enzyme *per se*, but might be a subunit structure the enzyme has to interact with to function. Enzyme purification is needed to elucidate the unique interaction with the 4-hydroxycoumarins and the role of the dithiol reductor.

REFERENCES

1. Covell DG, Abbrecht PH and Berman M, The effect of hepatic uptake on the disappearance of warfarin from the plasma of rats: A kinetic analysis. *J Pharmacokinet Biopharm* **11**: 127–145, 1983.
2. Daemen MJAP, Vervoort-Peters HTM and Thijssen HHW, Apparent dose-dependency of the hepatic (S)-acenocoumarol clearance in the rat: A study using open-liver biopsies. *J Pharm Sci* **75**: 238–240, 1986.
3. Thierry JM, Hermodson MA and Suttie JW, Vitamin K and warfarin distribution and metabolism in the warfarin-resistant rat. *Am J Physiol* **219**: 854–859, 1970.
4. Lorusso DJ and Suttie JW, Warfarin binding to microsomes isolated from normal and warfarin-resistant rat liver. *Mol Pharmacol* **8**: 197–203, 1972.
5. Searcy MT, Graves CB and Olson RE, Isolation of a warfarin binding protein from liver endoplasmatic reticulum of Sprague–Dawley and warfarin-resistant rats. *J Biol Chem* **252**: 6220–6267, 1977.
6. Suttie JW, The metabolic role of vitamin K. *Fed Proc* **39**: 2730–2735, 1980.
7. Suttie JW and Preusch PC, Studies of the vitamin K dependent carboxylase and vitamin K epoxide reductase in rat liver. *Haemostasis* **16**: 193–215, 1986.
8. Lee JJ and Fasco MJ, Metabolism of vitamin K and vitamin K 2,3-epoxide via interaction with a common disulfide. *Biochemistry* **23**: 2240–2252, 1984.
9. Lee JJ, Principe LM and Fasco MJ, Identification of a warfarin-sensitive protein component in a 200 S rat liver microsomal fraction catalyzing vitamin K and vitamin K 2,3-epoxide reduction. *Biochemistry* **24**: 7063–7070, 1985.
10. Silverman RB, Mukharji I and Nandt DL, Solubilization, partial purification, mechanism, and inactivation of vitamin K epoxide reductase. In: *Current Advances in Vitamin K Research* (Ed. Suttie JW, pp. 65–74, 1988.
11. Fasco MJ, Principe LM, Walsh WA and Friedman PA, Warfarin inhibition of vitamin K 2,3-epoxide reductase in rat liver microsomes. *Biochemistry* **22**: 5655–5660, 1983.
12. Fasco MJ and Principe LM, *R*- and *S*-warfarin inhibition of vitamin K and vitamin K 2,3-epoxide reductase activities in the rat. *J Biol Chem* **257**: 4894–4901, 1982.
13. Thijssen HHW, Janssen CAT and Drittij-Reijnders JM, The effect of S-warfarin administration on vitamin K 2,3-epoxide reductase activity in liver, kidney, and testis of the rat. *Biochem Pharmacol* **35**: 3277–3282, 1986.
14. Thijssen HHW, Warfarin-resistance: Vitamin K epoxide reductase of Scottish resistance genes is not irreversible blocked by warfarin. *Biochem Pharmacol* **36**: 2753–2757, 1987.
15. Thijssen HHW and Baars LGM, Hepatic uptake and storage of warfarin. The relation with the target enzyme vitamin K 2,3-epoxide reductase. *J Pharmacol Exp Ther* **243**: 1082–1088, 1987.
16. Banfield C and Rowland M, Stereospecific high performance liquid chromatographic analysis of warfarin in plasma. *J Pharm Sci* **72**: 921–924, 1983.
17. West BD, Preis S, Schroeder CH and Link KP, Studies on the 4-hydroxycoumarins. The resolution and absolute configurations of warfarin. *J Am Chem Soc* **83**: 2676–2679, 1961.
18. Thijssen HHW, Baars LG and Reijnders MJ, Analysis of aceonocoumarine and its amino and acetamido metabolites in body fluids by high performance liquid chromatography. *J Chromatogr* **274**: 231–238, 1983.
19. Wallin R and Martin LF, Vitamin K-dependent carboxylation and vitamin K metabolism in liver. Effects of warfarin. *J Clin Invest* **76**: 1879–1884, 1985.
20. Thijssen HHW, Baars LGM and Vervoort-Peters HTM, Vitamin K 2,3-epoxide reductase: the basis for the stereoselectivity of 4-hydroxycoumarin anticoagulant activity. *Br J Pharmacol* **95**: 675–682, 1988.
21. Whitlon DS, Sadowski JA and Suttie JW, Mechanism of coumarin action: Significance of vitamin K epoxide reductase inhibition. *Biochemistry* **17**: 1371–1377, 1978.
22. MacNicoll AD, A comparison of warfarin resistance and liver microsomal vitamin K epoxide reductase activity in rats. *Biochem Biophys Acta* **840**, 13–20, 1985.
23. Procacini RL and Klunk LJ, The subcellular distribution of ^{14}C-lidamidine and its metabolites. *Drug Metab Dispos* **9**: 202–206, 1981.