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# THE *IN VITRO* KETONE REDUCTION OF WARFARIN AND ANALOGUES

## SUBSTRATE STEREOSELECTIVITY, PRODUCT STEREOSELECTIVITY AND SPECIES DIFFERENCES

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**Abstract**—The *in vitro* metabolic ketone reduction of warfarin and its 4'-analogues acenocoumarol (4'-nitrowarfarin) and 4'-chlorowarfarin has been investigated using microsomal and cytosolic fractions of several species. Both subcellular fractions showed ketone reductase activity. The cytosolic fractions, in most species, exhibited strong substrate stereoselectivity as well as product stereoselectivity, i.e. the R(+)enantiomer was preferred as a substrate to be reduced mainly to the RS alcohol. Phenobarbital and methylcholanthrene induced cytosolic ketone reductase activity in the rat 5- to 11-fold and 3- to 7.4-fold, respectively. The microsomal fractions also showed substrate and product stereoselectivity. Contrary to the cytosolic fractions a general pattern for substrate and product stereoselectivity could not be seen. Stereoselectivity seemed species dependent (e.g. sheep vs bovine and pig). Rat liver microsomes showed practically no ketone reductase activity. Induction by phenobarbital or methylcholanthrene resulted in only a slight rise, if any, in rat microsomal ketone reductase activity. Both cytosolic and microsomal ketone reductases proved to be NADPH dependent. Substitution of the 4'-hydrogen of warfarin resulted in a change in reduction rates and in some cases, even in a change in substrate or product stereoselectivity. The data indicate that microsomal ketone reductases are different from cytosolic ketone reductases. Prelog's rule for product stereoselectivity of metabolic ketone reduction, when applied to the ketone reduction of the warfarin analogues did not agree with all data presented here.

Reduction of aldehydes or ketones to their corresponding alcohols plays a role in the metabolism of both endogenous (e.g. glucuronic acid, steroids) and exogenous compounds (e.g. *p*-nitrobenzaldehyde, daunorubicine, metyrapone, etc.) in various species [1–6]. The enzymes, responsible for this type of reaction are found in microsomal and cytosolic fractions of various tissues [4–9], but mainly in the liver.

The *in vivo* ketone reduction of the acetonyl sidechain of the oral anticoagulant warfarin to its alcohols in man was first described by Trager *et al.* [10]. As warfarin possesses a stereocenter, two optical active enantiomers exist: The R(+)enantiomer and its antipode the S(−)enantiomer. Therefore, from racemic warfarin, four diastereomeric alcohols will be formed by (aselective) reduction. By the use of thin-layer chromatography two pairs of alcohols have been separated, alcohol 1 being the RS or SR alcohols and alcohol 2 being the RR or SS alcohols [11].

The *in vivo* and *in vitro* metabolic reduction of warfarin in the rat and in man proved to be stereoselective for both substrate preference and product formation [11–15]. In *in-vitro* systems, both species prefer the R-enantiomer as a substrate, reducing it mainly to the RS-alcohol. S-warfarin is mainly reduced to the SS alcohol. In the *in-vivo* reduction in man the same substrate and product stereoselectivity has been observed [14]. In the rat, a discrepancy between the *in vivo* and *in vitro* reduction exists in that the *in vivo* reduction is stereoselective for the S-enantiomer which is mainly reduced to the SS alcohol [13].

The 4'-nitro analogue of warfarin, acenocoumarol (see Fig. 1) has also been shown to be reduced to its alcohols in man [16]. However, information on the stereochemistry of its metabolic ketone reduction is absent. Acenocoumarol displays great differences in the pharmacokinetics of its enantiomers [17, 18]. To study whether these differences can be ascribed to stereoselective metabolism, the *in vitro* cytochrome P-450 dependent acenocoumarol metabolism in the rat has been investigated previously in our laboratory (data to be published). However, this metabolic pathway could only partly explain the pharmacokinetic differences observed. Therefore, the ketone reductase mediated pathway has reached our attention.

As the diastereomeric alcohols of warfarin can be quantitated easily by HPLC [19], the enantiomers of warfarin and its 4'-analogues acenocoumarol and 4'-chlorowarfarin have been used to study the substrate and product stereoselectivity of the *in vitro* ketone reduction of the acetonyl sidechain. Furthermore, the effect of species differences has been investigated, using subcellular fractions of seven species; also the effect of enzyme induction (in rats) has been looked upon.

### MATERIALS AND METHODS

All chemicals were of analytical grade and were

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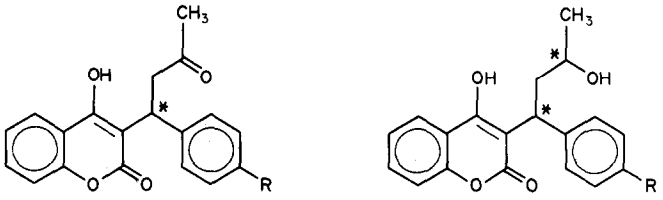

Fig. 1. The structure of the warfarin analogues and the corresponding alcohols. −R = H: warfarin, −R = $NO_2$: acenocoumarol, −R = Cl: 4′-chlorowarfarin.

obtained from Merck (Darmstadt, F.R.G.) unless stated otherwise.

*Preparation of microsomal and cytosolic fractions.* Livers of the (female) bovine (N = 1), pig (N = 1), horse (N = 1) and sheep (N = 2) were obtained from the regional abbatoir and stored on ice immediately. Rat livers (N = 4; male Wistar rats) and rabbit (N = 2) livers were removed directly after the animals were killed (by bleeding to death under ether anesthesia).

Human liver (N = 1) was obtained via the department of Pathology of the Academic Hospital Maastricht (The Netherlands) from autopsy of a young female that died of brain damage.

If more livers from one species were used, these livers were pooled for preparation of microsomal and cytosolic fractions. Livers were homogenized in 3 vol. of ice-cold Tris/KCl/sucrose buffer, pH = 7.4 (0.02 M Tris, 0.15 M KCl and 0.25 M sucrose, brought to pH with 6 N HCl solution), using a potter at 1500 rpm. The homogenate was then centrifuged at 10,000 *g* for 30 min. The pellet was discarded and the supernatant was centrifuged at 110,000 *g* for 50 min. The 110,000 *g* supernatant was used as the cytosolic fraction in the ketone reduction assay. The 110,000 *g* pellet was washed twice by resuspension in the original amount of Tris/KCl buffer, then homogenized and centrifuged at 110,000 *g* for 50 min. The final microsomal pellet was resuspended in Tris/KCl/NaCl (0.02 M, 0.15 M and 1.0 M, respectively) to contain about 20 mg of protein per ml homogenate. Rats were induced by phenobarbital (0.1% w/v in drinking water for 6 days; N = 4) or by methylcholanthrene (25 mg/kg i.p. for 3 days; N = 4). The effect of administration of enzyme inducers was tested, measuring cytochrome P-450 content and 7-ethoxycoumarin deethylase activity.

The protein content of the fractions was determined by the method of Lowry *et al.* [20] using bovine serum albumin as standard.

All fractions were stored at −80° until used in the assay.

*Preparation of the R- and S-enantiomers of warfarin and its analogues.* The respective enantiomers were isolated by the method of West *et al.* [21]. Racemic warfarin was obtained from Sigma Chemical Co. (St Louis, MO). 4′-Chlorowarfarin was from Aldrich Chemicals and acenocoumarol was a gift of Ciba-Geigy (Basel, Switzerland).

Purity of the enantiomers was tested by the formation of their *t*-BOC-L-proline diastereomers [22] and subsequent analysis by HPLC (Lichrosorb Si 60; *n*-hexane/ethylacetate/methanol 75/25/0.2). The purity of the enantiomers was higher than 97%.

*Assay for the ketone reduction.* Microsomal and 110,000 *g* fractions were incubated in 55 × 11/12 test tubes for 10 min at 37° at aerobic conditions. The incubation mixture contained (in Tris/KCl, pH = 7.4, final vol. 360 μl): 110 μl microsomal or supernatant fraction, 0.2 mM substrate, 1.6 mM NADPH, 20.2 mM glucose-6-phosphate (G-6-P), 5.8 mM magnesium chloride and 2.8 M units of G-6-P dehydrogenase (Sigma).

The reaction mixture was pre-incubated at 37° for 2 min; then the substrate was added. The reaction was stopped after 10 min by the addition of 500 μl ice-cold acetonitrile, containing an internal standard. Warfarin was used as internal standard for the assay of acenocoumarol and 4′-chlorowarfarin; acenocoumarol was used as the internal standard for the assay of warfarin. The mixture was centrifuged at 2500 rpm for 5 min to precipitate the protein. The supernatant was evaporated under a stream of nitrogen at 37°. To the residue 0.5 ml 0.1 N HCl solution was added and the warfarin analogues and their alcohols were extracted with 4 ml of a 1/1 (v/v) mixture of petroleum ether and methylene chloride. The organic phase was evaporated to dryness at 37° under a stream of nitrogen. The residue was taken up in a 1/1 (v/v) mixture of acetonitrile and water and was analysed by HPLC.

Blank incubations were run in parallel; all incubations were carried out in triplicate.

The HPLC system consisted of Chromspher C-18 (200 × 3 mm, Chrompack, The Netherlands) as the stationary phase and a mixture of 0.1% acetic acid and acetonitrile (7/3 v/v brought to pH 4.76 with ammonia) as the mobile phase. UV absorption at 303 nm was measured on a Kratos spectroflow 783 and areas were calculated on Hitachi D-2000 integrator.

*Identification of the alcohols.* The alcohols of the warfarin analogues for reference purpose were formed by reduction with sodium-boronhydride in methanol [13]. The alcohols were purified by the use of a two-dimensional thin-layer (silica) chromatography system. In the first dimension the non-reduced warfarin analogue was separated from the diastereomeric alcohols, using a mixture of acetone and dichlorethane (90/10 v/v) as the mobile phase. The RR and SS diastereomers were separated from the RS and the SR diastereomers in the second dimension, using diethylether/acetic acid (99/1.5 v/v) as the mobile phase. On the thin-layer system the RS/SR alcohols (therefore also called alcohol 1) travelled faster than the RR/SS alcohols (alcohol 2). On the reversed phase HPLC system, the opposite was true.

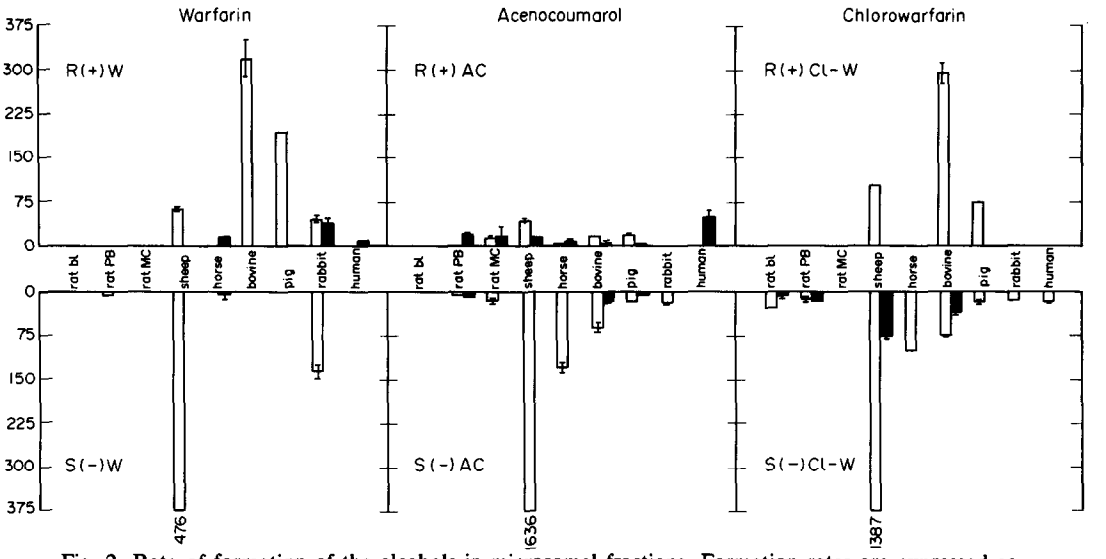


Fig. 2. Rate of formation of the alcohols in microsomal fractions. Formation rates are expressed as pmoles of alcohol formed per min per mg of protein. Blank boxes indicate the formation of the RR- or SS-alcohols (alcohol 2). Filled boxes indicate the formation of the RS- or SR-alcohols (alcohol 1). All incubations were performed in triplicate. Microsomal fractions from the pig, bovine, horse and human were obtained from 1 liver. Microsomal fractions of rat (N = 4 for each group), rabbit (N = 2) and sheep (N = 2) were obtained from pooled livers.

## RESULTS

The extent of the ketone reduction of the acetonyl side-chain of the warfarin analogues by liver microsomal fractions is presented in Fig. 2. As is seen, species differences in substrate selectivity and differences in product stereoselectivity are apparent. For instance, microsomes of untreated rats showed practically no ketone reductase activity although minor activity was observed for S-4′-chlorowarfarin (about 30 pmole alcohol 2 formed per mg × min). The stereochemical selectivity of microsomes from induced rats seemed to vary; phenobarbital induced microsomes formed alcohol 1 (10 pmol/mg × min) as well as alcohol 2 (5 pmol/mg × min) of S-acenocoumarol, whereas with R-acenocoumarol only alcohol 1 was formed (21 pmol/mg × min). Phenobarbital also stimulated the formation of alcohol 1 of S-4′-chlorowarfarin (15 pmole formed vs 5 pmole in native rat microsomes) but decelerated the formation of alcohol 2 (10 pmole vs 30 pmole/mg × min). Methylcholanthrene appeared to potentiate the formation of alcohol 1 of S-acenocoumarol, whereas with R-acenocoumarol the formation of both alcohols was stimulated. No ketone reductase activity was observed for 4′-chlorowarfarin in methylcholanthrene induced rat liver microsomes.

Liver microsomes of the other species investigated in general showed higher reductase activities. The substrate stereoselectivity varied widely, depending on the species and on the warfarin analogue. Generally, the product formed is alcohol 2. Sheep microsomes showed a high selectivity for the S-enantiomer of all analogues; this enantiomer mainly being reduced to the SS-alcohol (alcohol 2). The same was observed for horse microsomes for acenocoumarol and 4′-chlorowarfarin. The activity for warfarin was low, showing a preference for the R-enantiomer of warfarin. Microsomal fractions of the bovine and pig showed a preference for the R-enantiomer of warfarin and its 4′-chloroanalogue which was reduced to alcohol 2. Remarkably, R-acenocoumarol was a poor substrate. Rabbit liver microsomes showed the highest activity for warfarin, with a preference for the S-enantiomer to be reduced to alcohol 2. Both alcohols were found for R-warfarin. Of the analogues, only the S-enantiomer was reduced by rabbit microsomes, resulting solely in the formation of alcohol 2.

Human microsomes appeared to have a preference for the R-enantiomer of warfarin and acenocoumarol to form alcohol 1. The S-enantiomer of 4′-chlorowarfarin was converted to alcohol 2. The microsomal ketone reductases proved to be NADPH-dependent. No activity was observed when NADH, sodium dithionite or dithiothreitol (DTT) were used as reducing agents.

Figure 3 shows the reduction of the warfarin analogues in the cytosolic fractions. In contrast to the microsomal fractions, in all species but the sheep, there is a clear selectivity for the R-enantiomer, which is reduced to alcohol 1: the ratios in conversion rates between the R- and S-enantiomers ranged from 2.2 (4′-chlorowarfarin in the rat) to >10,000 (acenocoumarol in the rabbit). Inversion of this product stereoselectivity was seen in the bovine cytosolic fraction for the R-enantiomer of warfarin. Sheep cytosolic fraction showed selectivity for the S-enantiomer of acenocoumarol and 4′-chlorowarfarin to be reduced to alcohol 2.

Ketone reduction in the rat cytosolic fractions proved to be higher in phenobarbital treated (5- to 11-fold, depending on the substrate used) and in

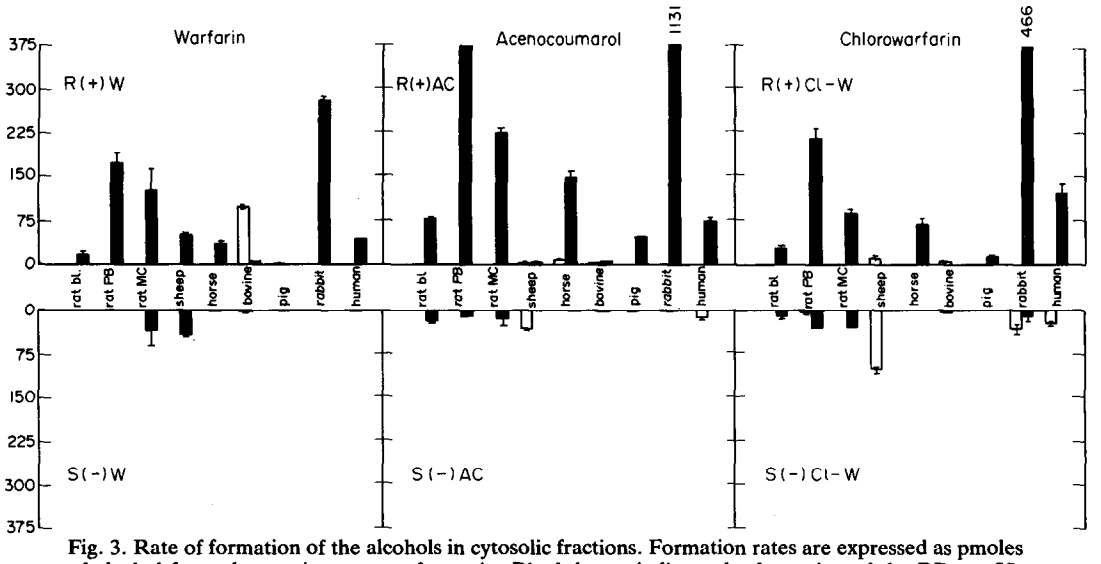


Fig. 3. Rate of formation of the alcohols in cytosolic fractions. Formation rates are expressed as pmoles of alcohol formed per min per mg of protein. Blank boxes indicate the formation of the RR- or SS-alcohols (alcohol 2). Filled boxes indicate the formation of the RS- or SR-alcohols (alcohol 1). All incubations were performed in triplicate. Cytosolic fractions from the pig, bovine, horse and human were obtained from 1 liver. Cytosolic fractions of rat (N = 4 for each group), rabbit (N = 2) and sheep ($N = 2$) were obtained from pooled livers.

methylcholanthrene treated rats (3- to 7.4-fold) than in untreated rats. In general, ketone reductase activity was higher for the warfarin analogues than for warfarin itself; in most cases, acenocoumarol was the best substrate. The cytosolic ketone reductases also proved to be NADPH-dependent, although very small activity was observed with NADH as cosubstrate. Microsomal and cytosolic fractions, denaturated by the addition of acetronitrile did not show any reductase activity.

## DISCUSSION

Ketone reductase and aldehyde reductase activities have been observed in various tissues. The enzymes have been reported to show a broad substrate specificity and their physiological function, among others, is thought to be the reduction of steroidal ketones [1]. In comparison to other metabolic routes of xenobiotic biotransformation, ketone and aldehyde reductases up to now have obtained less attention. Because ketone reduction eliminates reactive carbonyl groups by forming alcohols [5], this metabolic pathway is not likely to share the toxicological problems that can be encountered by cytochrome P-450 mediated reactions or by conjugation reactions. However, the stereochemistry of the reaction in case we are dealing with asymmetric ketones makes the study of this system worthwhile. By studying ketone substrates possessing chirality in one of its side-chains, product selectivity can be followed easily by chromatographic separation of the diastereomeric products.

Ketone reduction of the acetonyl side-chain of warfarin has been described before and the substrate and product selectivity has been emphasized *in vivo* [11, 12, 14] and *in vitro* [15]. The present results are in agreement with these studies showing in rat and human liver cytosolic fraction substrate preference for R-warfarin and product selectivity for the RS-alcohol (= alcohol 1). With the exception of the bovine and sheep, liver cytosolic ketone reductase of the other species also showed substrate preference for the R-enantiomer leading to the formation of the RS-alcohol, suggesting an interspecies relationship of the reductases. The order of sensitivity was R-acenocoumarol > R-4′-chlorowarfarin > R-warfarin. Moreland and Hewick [15] have reported the substrate preference for R-warfarin of the ketone reductase activity in human and rat liver and kidney cytosolic fraction to be a matter of affinity rather than $V_{max}$. Referring to the apparent interspecies relationship of the cytosolic activities, it is likely that this observation also holds for the acenocoumarol and 4′-chlorowarfarin substrates.

Next to the cytosolic fraction, liver microsomes also exhibited ketone reductase activity. Substrate preference and product selectivity (mainly alcohol 2) were different from that of the cytosol. Solubilization of the microsomal enzyme did not change these features (own observations), excluding the possibility that the stereoselectivity is an enviromental (microsomal membranes) phenomenon. Therefore, these data indicate the microsomal and cytosolic reductases to be different enzyme systems.

In enzymatic ketone reduction, product stereoselectivity is a well known phenomenon [23–26]. In studying the reduction of ketones by bacteriological systems, Prelog [24] formulated a rule to predict the configuration of the resulting alcohol; if a ketone is projected in plane with the large group at the left, the resulting alcohol predominantly will have the configuration with the hydroxyl group above the plane (Fig. 4). Prelusky *et al.* [26], by studying the *in*

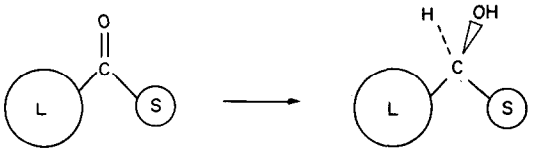


Fig. 4. Prelog's rule for product stereoselectivity of enzymatic ketone reduction.

*vivo* and *in vitro* reduction of some selected arylalkyl ketones in rabbits and rats affirmed the validity of Prelog's rule. For the warfarin analogues the rule would imply the formation of the RS- and the SS-alcohols from the R- and S-enantiomers, respectively. In the cytosolic systems generally this appeared to be true for the R- but not for the S-enantiomers (Fig. 3). For the microsomal systems the S- but less the R-enantiomers followed the rule (Fig. 2). Taking our findings together, one might raise the question whether Prelog's rule is valid for predicting product stereoselectivity of mammalian ketone reductases.

In agreement with the observation of Moreland and Hewick [15], we found the cytosolic ketone reductase activity to be sensitive for inducing agents.

Attaching a meaning to the differences in reductase activities between the species is difficult. For human, bovine, pig and horse only one liver could be used. This implies that for these species the data presented here are only indicative for species differences as intra-species variation can not be ruled out. Furthermore, Moreland and Hewick [15] showed the recovery of R-warfarin reductase activity from rat liver to decrease with post mortem time. Only rat and rabbit livers were obtained immediately after death of the animals and the livers were perfused *in situ* with ice-cold saline before excision. Livers from the slaughter house were processed about 1–2 hr after death. The human liver was removed 3 hr after death. However, except for the human liver, all livers were put directly on ice to minimize loss of enzymatic activity. Reduction rates in both rat and human cytosolic fractions in our study were higher than in the study presented by Moreland and Hewick [15].

Our *in vitro* data on warfarin ketone reduction in man are in accordance to the *in vivo* observations, i.e. the formation of the RS-alcohol mainly. If the *in vitro* data are also predictive for the *in vivo* reduction of acenocoumarol, the data suggest that this metabolic route is not responsible for the great differences in pharmacokinetics between R- and S-acenocoumarol [17, 18].

In conclusion, although it is claimed that ketone reductases have broad substrate specificity, there is a clear substrate stereoselectivity. Also strikingly substituent dependencies are observed, suggesting the involvement of the benzyl moiety of the warfarin analogues in the reaction mechanism. Experiments using purified enzyme preparations are in progress for further characterization of this metabolic route.

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