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Inhibitory Effects of Silibinin on Cytochrome P-450 Enzymes in Human Liver Microsomes

Svane Beckmann-Knopp[1], Stephan Rietbrock[1], Roland Weyhenmeyer[2], Ronald H. Böcker[3], K. Tobias Beckurts[4], Werner Lang[2], Miriam Hunz[1] and Uwe Fuhr[1]

[1]Institute for Pharmacology, Clinical Pharmacology, University of Köln; [2]Madaus AG, Köln; [3]Department of Experimental and Clinical Pharmacology and Toxicology, University of Erlangen; [4]Department of Visceral and Vascular Surgery, University of Köln, Germany

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Abstract: Silibinin, the main constituent of silymarin, a flavonoid drug from *silybum marianum* used in liver disease, was tested for inhibition of human cytochrome P-450 enzymes. Metabolic activities were determined in liver microsomes from two donors using selective substrates. With each substrate, incubations were carried out with and without silibinin (concentrations 3.7–300 μM) at 37° in 0.1 M KH_2PO_4 buffer containing up to 3% DMSO. Metabolite concentrations were determined by HPLC or direct spectroscopy. First, silibinin IC_{50} values were determined for each substrate at respective K_M concentrations. Silibinin had little effect (IC_{50}>200 μM) on the metabolism of erythromycin (CYP3A4), chlorzoxazone (CYP2E1), S(+)-mephenytoin (CYP2C19), caffeine (CYP1A2) or coumarin (CYP2A6). A moderate effect was observed for high affinity dextromethorphan metabolism (CYP2D6) in one of the microsomes samples tested only (IC_{50}=173 μM). Clear inhibition was found for denitronifedipine oxidation (CYP3A4; IC_{50}=29 μM and 46 μM) and S(−)-warfarin 7-hydroxylation (CYP2C9; IC_{50}=43 μM and 45 μM). When additional substrate concentrations were tested to assess enzyme kinetics, silibinin was a potent competitive inhibitor of dextromethorphan metabolism at the low affinity site, which is not CYP2D6 ($K_{i,c}$=2.3 μM and 2.4 μM). Inhibition was competitive for S(−)-warfarin 7-hydroxylation ($K_{i,c}$=18 μM and 19 μM) and mainly non-competitive for denitronifedipine oxidation ($K_{i,n}$=9 μM and 12 μM). With therapeutic silibinin peak plasma concentrations of 0.6 μM and biliary concentrations up to 200 μM, metabolic interactions with xenobiotics metabolised by CYP3A4 or CYP2C9 cannot be excluded.

Silibinin (fig. 1) is the main isomer of a group of flavonoids summarised as silymarin. It is obtained from the seeds of the milk thistle *silybum marianum.* Silidianin, isosilibinin and silicristin are the other isomers present in seeds. As a standardised extract, silymarin is therapeutically used for the treatment of acute and chronic liver injury (Leng-Peschlow & Strenge-Hesse 1991; Flora *et al.* 1998).

Mean maximum plasma concentration after an oral dose of 700 mg silymarin, containing 254 mg of silibinin, is 317 ng/ml or 0.6 μM. Accumulation in plasma during three daily medications is negligible. Plasma protein binding is reported to reach about 90–95%. Elimination occurs predominantly via the bile, where accumulation of silibinin may result in maximum concentrations of 150 μM. Mean elimination half-life of silibinin is about 6 hr (Lorenz *et al.* 1982; Weyhenmeyer *et al.* 1992).

Both silibinin and silymarin have protective properties for hepatocytes in several *in vitro* studies and in animal models. In these investigations, such flavonoids reduced hepatocyte damage caused by administration of various chemicals including, among others, allyl alcohol (Miguez *et al.* 1994), carbon tetrachloride (Mourelle *et al.* 1989), halothane (Siegers *et al.* 1983), acetaminophen (Muriel *et al.* 1992) and phenylhydrazine (Valenzuela *et al.* 1985). Several mechanisms of action were discussed: (1) stimulation of protein synthesis in the hepatocytes (Machicao & Sonnenbichler 1977), (2) radical scavenging and antioxidant properties (Ubeda *et al.* 1995), (3) prevention of penetration of toxins into the cell (Münter *et al.* 1986), and (4) inhibition of 5-lipoxygenase with reduced formation of leucotriens (Dehmlow *et al.* 1996).

Another possible mechanism of hepatoprotection may be the inhibition of the cytochrome P-450 enzymes responsible for the bioactivation of hepatotoxins such as ethanol (Albano *et al.* 1999), halothane (Kharasch *et al.* 1996) and aflatoxin B_1 (Guengerich & Kim 1990). Indeed, inhibitory silibinin/silymarin effects have been observed on oxidation of various cytochrome P-450 substrates in rat and mouse, including aminopyrine, ethanol, benzo(a)pyrene, hexobarbital, and 7-ethoxycoumarin (Down *et al.* 1974; Valenzuela *et al.* 1989; Lettéron *et al.* 1990; Ubeda *et al.* 1995). In one report however, induction of the metabolism of aminopyrine and nitroanisole by silymarin was found in rat, whereas elimination half-life of aminopyrine or phenylbutazone examined in 16 healthy volunteers each was not affected by long-term administration of 70 mg of silymarin three times daily (Leber & Knauff 1976). Silibinin at concentrations of 50 μM or 500 μM had no protective effects in isolated human hepatocytes treated with tetrachlormethan or acet-

Author for correspondence: Uwe Fuhr, Institute of Pharmacology, Clinical Pharmacology, University of Köln, Gleueler Str. 24, D-50931 Köln, Germany (fax +49 221 478 5022, e-mail uwe.fuhr@medizin.uniko eln.de).

aminophen which are activated by CYP2E1. The conclusion of the authors was that silibinin does not inhibit this enzyme (Miguez *et al.* 1994).

Besides these equivocal data, there is indirect evidence that silibinin may inhibit human cytochrome P-450 enzymes other than CYP2E1. For many flavonoids, inhibition of human CYP3A4 has been shown (Fuhr 1998), giving rise to metabolic drug–drug interactions, as CYP3A4 plays a predominant role in the metabolism of many important drugs like calcium channel antagonists, cyclosporine, antihistamines, midazolam, erythromycin and quinidine (Kinirons *et al.* 1993). Indeed, CYP3A4 is also a major enzyme in activating toxic and carcinogenic chemicals (Guengerich & Shimada 1991). Additional cytochrome P-450 enzymes such as CYP1A2 or CYP2A6 may also be affected by flavonoids (Ubeda *et al.* 1995; Dai *et al.* 1997; Fuhr 1998, Lee *et al.* 1998).

Thus, it is important to characterise the effect of silibinin on cytochrome P-450 enzymes to further elucidate the mechanism of action of the flavonoid (see above) and to be aware of possible metabolic interactions with other drugs administered concomitantly (Committee for Proprietary Medicinal Products 1997). In the present investigation the inhibitory effect of silibinin on the activity of the seven most important human cytochrome P-450 enzymes in drug metabolism, including CYP2D6, 3A4, 1A2, 2E1, 2C19, 2C9, and 2A6, was studied in human liver microsomal incubations using selective substrates (Fuhr *et al.* 1996a; Bertz & Grannemann 1997). Although Miguez *et al.* (1994) had already published data related to silibinin effects on human CYP2E1, we also included CYP2E1 in our studies for comparison. In the case of CYP3A4, two model substrates were used, i.e. denitronifedipine (Böcker *et al.* 1986) and erythromycin (Riley & Howbrook 1997; Wang *et al.* 1997), because for this enzyme multiple conformers with distinct substrate specificity may exist (Koley *et al.* 1996 & 1997).

Materials and Methods

Erythromycin was purchased from Sigma Biochemikalien und Reagenzien, Deisenhofen, Germany. Silibinin (batch # 881122/ICH, purity 93.4%) was provided by the Department of Chemistry, Madaus AG, Germany. For the sources of all other chemicals see Beckmann-Knopp *et al.* (1999).

Metabolic activities of cytochrome P-450 enzymes were determined in liver microsomes from two donors each using the following selective substrates: dextromethorphan (CYP2D6), erythromycin or denitronifedipine (CYP3A4), caffeine (CYP1A2), chlorzoxazone (CYP2E1), S-(+)-mephenytoin (CYP2C19), S-(−)-warfarin (CYP2C9) or coumarin (CYP2A6) (see table 1). With each substrate except caffeine, incubations were carried out without a possible inhibitor and in presence of silibinin at five different concentrations (3.7, 11.1, 33.3, 100 or 300 μM) at 37° in 0.1 M KH_2PO_4 buffer containing up to 3% DMSO. For caffeine incubations, silibinin concentrations were 9, 30, 90, and 300 μM, and DMSO was not used in this case. First, silibinin IC_{50} values were determined for each substrate at respective K_M concentrations. If IC_{50} values were below 200 μM, additional substrate concentrations were tested to fully assess enzyme kinetics. These were 1.9, 5.6, 16.7, 50 and 100 μM for denitronifedipine, 0.2, 1, 5, 40, 200, 1000, and 2000 μM for dextromethorphan, and 0.3, 1, 3, and 10 μM for S-(−)-warfarin, respectively. With the exception of erythromycin metabolism, the procedures used to assess individual enzyme activity are fully described elsewhere (Beckmann-Knopp *et al.* 1999). Incubations were usually carried out in duplicate, and means given in all cases.

To monitor erythromycin metabolism, the formation of formaldehyde was measured (Nash 1953, Riley & Howbrook 1997; Wang *et al.* 1997). Described K_M values for erythromycin metabolism by CYP3A4 range between 33 and 88 μM. Incubations (2 mg protein/ml) were carried out for 30 min. as described (Beckmann-Knopp *et al.* 1999) and stopped by addition of 100 μl of 20% trichloracetic acid. After centrifugation, 350 μl of the supernatant was diluted with 150 μl Nash reagent (0.15 mol/l ammonium acetate and 0.2 mol/l acetylacetone in 3% acetic acid), and transferred into a water bath of 53° for 30 min. Colorimetric quantification of the Hantzsch reaction solution was done directly by absorbance (405 nm) at room temperature using a MAXline Microplate Reader (Sunnyvale, California, USA). The limit of quantification was 7.8 nM, corresponding to a formaldehyde formation rate of 0.1 pmol/min/mg protein.

Evaluation of the enzyme kinetic parameters was done based on extended Michaelis – Menten equations as described, and best models were determined using log-likelyhood tests (Beckmann-Knopp *et al.* 1999).

Results

In the absence of any inhibitor, substrate turn-over was clearly above the limits of detection for all human cytochrome P-450 enzymes tested (CYP3A4, 2D6, 2E1, 2C19,

Table 1.

Effect of silibinin on activities of human cytochrome P-450 enzymes at K_M substrate concentrations.

Cytochrome P-450 enzyme	Substrates and concentrations used/(μM)		V_0*/(pmol/min/mg protein)	IC_{50} of silibinin*/μM
CYP3A4	Denitronifedipine	50	409 (P10)/279 (P14)	29 (P10)/46 (P14)
CYP3A4	Erythromycin	37	277 (P13)/509 (HO12)	>200 (P13)/>200 (HO12)[#]
CYP2D6	Dextromethorphan high affinity site	5	79 (P10)/49 (P20)	173 (P10)/∞(P20)
Unknown	Dextromethorphan low affinity site	2000	232 (P10)/141 (P20)	384 (P10)/∞ (P20)
CYP2E1	Chlorzoxazone	50	115 (P11)/101 (P13)	799 (P11)/460 (P13)
CYP2C19	S-(+)-Mephenytoin	20	13 (P11)/15 (P13)	309 (P11)/424 (P13)
CYP2C9	S-(−)-Warfarin	5	0.39 (P13)/0.54 (HO12)	43 (P13)/45 (HO12)
CYP1A2	Caffeine	500	26 (P13)/65 (P17)	20000 (P13)/108500 (P17)
CYP2A6	Coumarin	2	468 (P13)/494 (HO12)	541 (P13)/688 (HO12)

v_0=metabolite formation rates in absence of an inhibitor; IC_{50}=inhibitor concentration reducing metabolite formation rate by 50% at the given substrate concentration (Beckmann-Knopp *et al.* 1999); *data pairs were obtained using human liver microsomes from two different donors each, for characterisation of donors given in parentheses see Fuhr *et al.* (1996b); [#]proper calculation not possible because of activation at low inhibitor concentrations.

Table 2.

Enzyme kinetic parameters obtained for dextromethorphan O-demethylation and inhibitory effect of silibinin in human liver microsomes.

Binding site	Microsome sample*	V_{max}/(pmol/min./mg protein)	K_M/(μM)	$K_{i,c}$/(μM)
High affinity site	P10	201	7.0	112
(CYP2D6)	P20	110	4.6	135
Low affinity site	P10	66	501	2.3
(not known)	P20	75	703	2.4

parameters were estimated using equation 1; *for identification of microsome samples, see Fuhr *et al.* (1996b); V_{max}=maximal metabolite formation rate; K_M=Michaelis-Menten constant; $K_{i,c}$=inhibition constant for competitive inhibition.

2C9, 2A6) in liver microsomes preparations each from two donors.

Silibinin had only little effect on the metabolism of five of the eight substrates investigated. For erythromycin metabolism, IC_{50} could not be determined reliably because of activation (<20%) at low silibinin concentrations, but it was clearly above 200 μM. Also, the effects of silibinin on the metabolism of chlorzoxazone, S-(+)-mephenytoin, caffeine and coumarin were only minor, with IC_{50}>200 μM in all cases (table 1).

A moderate effect was observed for dextromethorphan metabolism at the high affinity site in one microsomal sample only (IC_{50}=173μM and ∞ μM), whereas the effect on metabolism at the low affinity site appeared to be only minor (table 1). Clear inhibition was found for denitronifedipine metabolism (IC_{50}=29 μM and 46 μM) and S(−)-warfarin metabolism (IC_{50}=43 μM and 45 μM). Therefore, additional concentrations of dextromethorphan, S(−)-warfarin, and denitronifedipine were tested to fully describe extent and type of inhibitory action of silibinin in these cases.

Dextromethorphan formation rates in both microsomal samples could be fitted best to a Michaelis Menten equation with two binding sites, each with a competitive inhibition constant $K_{i,c(1)}$ and $K_{i,c(2)}$ (equation 1).

Equation 1: Best model describing dextromethophan metabolism by human liver microsomes

$$V=\frac{V_{max(1)}\times[S]}{K_{M(1)}\times(1+\frac{[I]}{K_{i,c(1)}})+[S]}+\frac{V_{max(2)}\times[S]}{K_{M(2)}\times(1+\frac{[I]}{K_{i,c(2)}})+[S]}$$

numerals=indices for the respective binding site; v=measured metabolite formation rate; V_{max}=maximal metabolite formation rate; [S]=substrate concentration; K_M=Michaelis-Menten constant; [I]=inhibitor concentration; $K_{i,c}$, $K_{i,n}$=inhibitor constants for competitive (c) and non-competitive (n) inhibition, respectively.

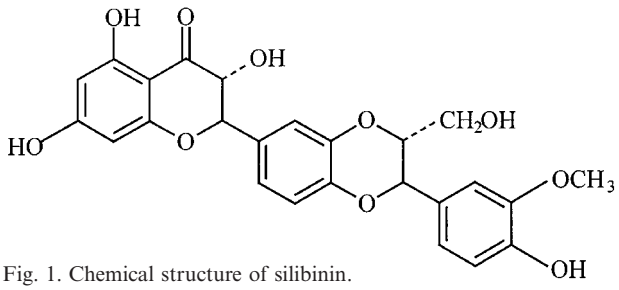

Fig. 1. Chemical structure of silibinin.

Thus, silibinin was characterised as a competitive inhibitor of dextromethorphan metabolism, but a high inhibition potency was only observed for the low affinity binding site, with K_i values of 2.3 and 2.4 μM, respectively (table 2). The graphical display of data points and the curve fitting achieved using equation 1 is presented in fig. 2.

7-Hydroxywarfarin formation rates in both microsomal

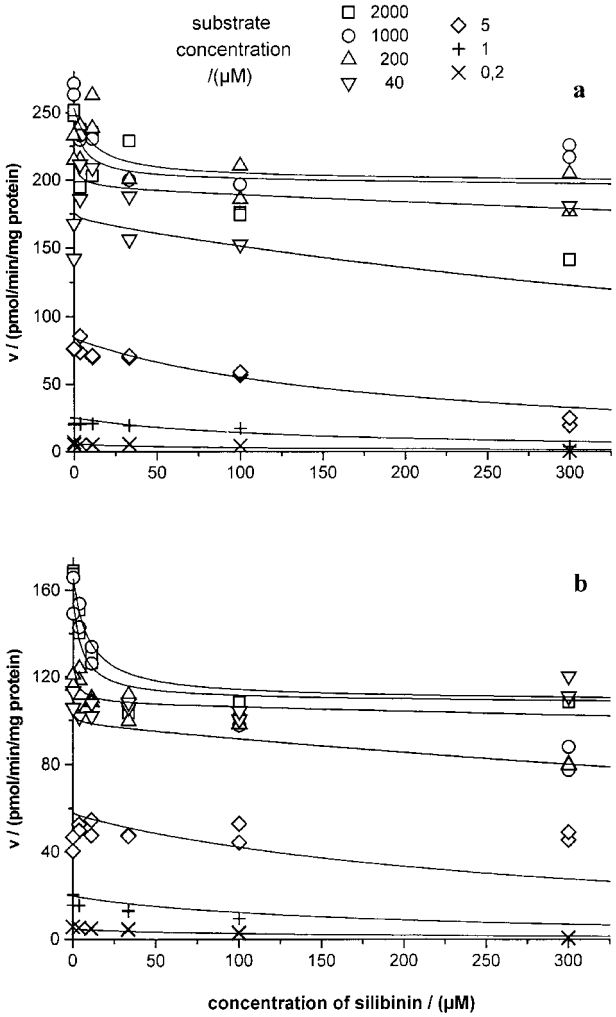

Fig. 2. Linear plots: Inhibitory effect of silibinin on dextromethorphan O-demethylation. **a:** P10 liver microsomes; **b:** P20 liver microsomes.

For characterisation of microsome samples, see Fuhr *et al.* (1996b). Lines were fitted according to equation 1.

Table 3.

Enzyme kinetic parameters obtained for S(−)-warfarin 7-hydroxylation and inhibitory effect of silibinin in human liver microsomes.

Microsome sample*	V_{max} /(pmol/min./mg protein)	K_M /(μM)	$K_{i,c}$ /(μM)
P13	0.550	2.8	18
HO12	0.760	2.3	19

parameters were estimated using equation 2; *for identification of microsome samples, see (Fuhr *et al.* 1996b); for explanation of symbols see legend to table 2.

samples (fig. 3a and b) were described best by a Michaelis Menten equation with one binding site and a competitive inhibition constant (equation 2).

Equation 2: Best model describing S(−)-warfarin metabolism by human liver microsomes

$$v=\frac{V_{max}\times[S]}{K_M\times(1+\frac{[I]}{K_{i,c}})}$$

for explanation of symbols see legend to equation 1.

In vitro inhibition of S(−)-warfarin metabolism by silibinin clearly followed a competitive mechanism of inhibition, and the extent of inhibition was characterised by competitive inhibition constants $K_{i,c}$ of 18 and 19 μM, respectively, for the two microsomes samples tested (table 3).

Denitronifedipine turn-over rates in both microsomal samples was the reaction most affected by silibinin (fig. 4a and b) and could be fitted best to a mixed-type Michaelis Menten equation, including one binding site with both a competitive and a non-competitive inhibition constant $K_{i,c}$ and $K_{i,n}$, and a second linear term, a×[S], accounting for metabolite formation by a process that was not saturated at the substrate concentrations tested (equation 3):

Equation 3: Best model describing S(−)-denitronifedipine oxidation by human liver microsomes

$$v=\frac{V_{max}\times[S]}{K_M\times(1+\frac{[I]}{K_{i,c}})+[S]\times(1+\frac{[I]}{K_{i,n}})}+a\times[S]$$

a; factor for non-saturated metabolite formation; for explanation of further symbols see legend to equation 1.

Table 4.

Enzyme kinetic parameters obtained for denitronifedipine oxidation and inhibitory effect of silibinin in human liver microsomes.

Microsome sample*	V_{max} /(pmol/min./mg protein)	K_{max} /(μM)	$K_{i,c}$ /(μM)	$K_{i,n}$ /(μM)
P10	1282	134	105	9
P14	619	81	104	12

parameters were estimated using equation 3; *for identification of microsome samples, see (Fuhr *et al.* 1996b); $K_{i,n}$=inhibition constant for non-competitive inhibition; for explanation of other symbols see legend to table 2.

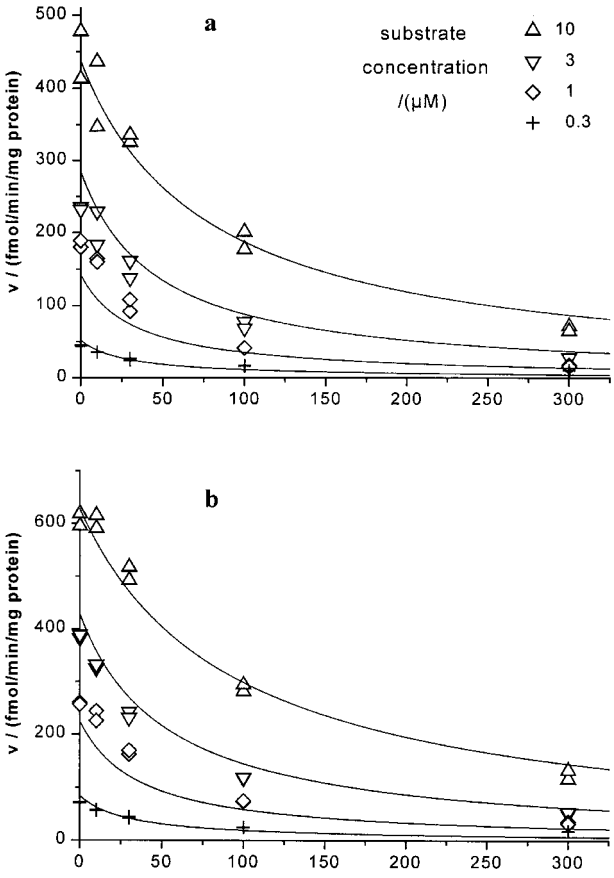

Fig. 3. Linear plots: inhibitory effect of silibinin on S(−)-warfarin 7-hydroxylation **a:** P13 liver microsomes; **b:** HO12 liver microsomes.

For characterisation of microsome samples, see Fuhr *et al.* (1996b). Lines were fitted to data according to equation 2.

The main mechanism by which silibinin affected denitronifedipine metabolism was non-competitive inhibition with $K_{i,c}$ values of 9 and 12 μM (table 4).

Discussion

The observed metabolite formation rates were within the range of variation observed in other studies, taking the lower activities caused by addition of DMSO into account (Beckmann-Knopp *et al.* 1999). This indicates that no poor metaboliser of one of the genetically polymorphic enzymes was among the donors of the samples. Metabolite concentrations measured after incubations sufficiently exceeded the lower limit of quantification of the respective assays in all cases in order to allow reliable evaluation of IC_{50} values (table 1). The role of DMSO effects on enzyme activity has been discussed (Beckmann-Knopp *et al.* 1999).

The lack of relevant inhibitory silibinin effects on human cytochrome CYP2E1 confirms a previous study, however carried out with different methods (Miguez *et al.* 1994).

The findings obtained with dextromethorphan as a substrate require some comments. The substance has two binding sites in human liver microsomes, a low and a high affin-

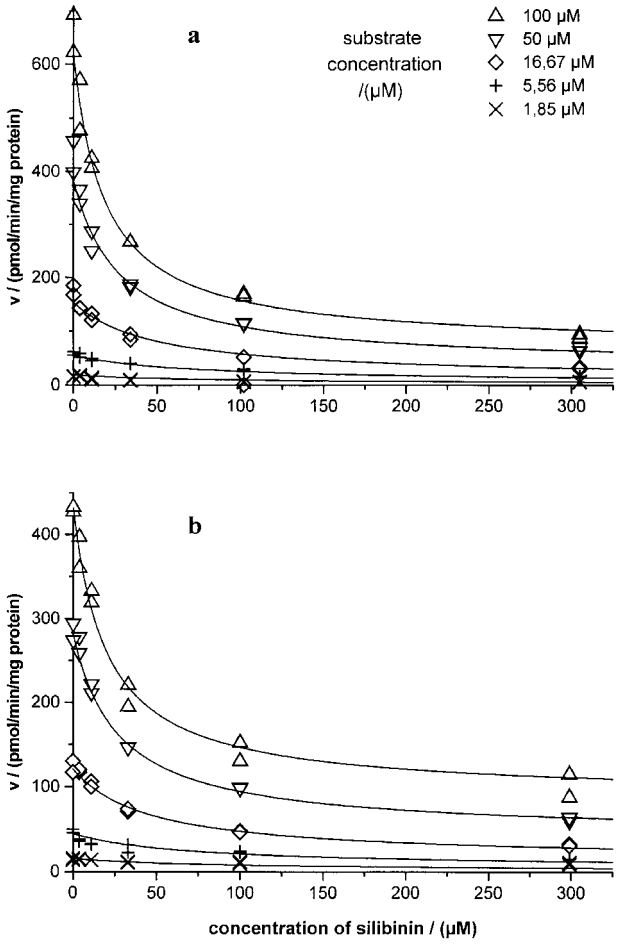

Fig. 4. Linear plots: Inhibitory effect of silibinin on denitronifedipin oxidation **a:** P10 liver microsomes; **b**: P14 liver microsomes.

For characterisation of microsome samples, see Fuhr *et al.* (1996b). Lines were fitted according to equation 3.

ity site (see Beckmann-Knopp *et al.* 1999). It appears that silibinin had some effect on the turn-over of dextromethorphan in our system (fig. 2). From a formal point of view, this effect was described best assuming a minor competitive inhibition of CYP2D6, i.e. the high affinity site, and a potent competitive inhibition of the low affinity site (see equation 1 and table 2). The physical identity of the latter is unknown. However, as the curve fit does not fully describe the data (fig. 2) and the net effect of silibinin on dextromethorphan demethylation does not increase markedly with higher silibinin concentrations, it appears that this result is not very important.

Results for inhibition by silibinin were contradictory for denitronifedipine and erythromycin, the two CYP3A4 substrates tested. While the effect on erythromycin demethylation was not uniform and showed activation at low and minor inhibition at high silibinin concentrations, the effect of silibinin on oxidiation of the dihydropyridine derivative was a clear and pronounced non-competitive inhibition. A possible explanation for these findings comes from the very complex behaviour of human CYP3A4. It appears that for this enzyme multiple conformers with distinct substrate specificities may exist (Koley *et al.* 1996 & 1997). Futhermore, cooperativity in oxidations catalysed by the enzyme was described and attributed to the existence of more than one binding site at the CYP3A4 molecule (Ueng *et al.* 1997). Finally, the specificity of the two substrates used with respect to CYP3A5 and CYP3A7 is not clearly defined (Daly *et al.* 1995). Both enzymes are present in human liver, although usually at much lower levels than CYP3A4 (Tateishi *et al.* 1999). Our results suggest that silibinin may be useful in further characterising human CYP3A enzymes. Indeed, structure–activity relationships of flavonoids with respect to effects on other cytochrome P450 enzymes have been established (Siess *et al.* 1995; Edenharder *et al.* 1997; Lee *et al.* 1998), but these data have not yet been used to obtain information on the binding site characteristics of the enzymes.

An important question is whether the inhibitory effects of silibinin observed in the microsomal system will become relevant when the drug is administrated to man. For S(−)-warfarin 7-hxdroxylation and for denitronifedipine oxidation, estimated K_i values are only one order of magnitude above maximal plasma concentrations of 0.6 µM (Weyhenmeyer *et al.* 1992). As the concentration of silibinin at the binding site of the enzyme is not known and may well exceed plasma concentrations, it cannot be excluded that silibinin affects the activity of hepatic CYP2C9 and/or CYP3A4, thereby causing pharmacokinetic drug interactions with substrates for these enzymes. Accumulation of the lipophilic silibinin in the bile with peak concentrations of 150 µM indeed suggests that also in the hepatocytes concentrations relevant for enzyme activity may be reached. Furthermore, the presence of high concentrations of silibinin in the gut just after oral intake and/or as the result of an enterohepatic recirculation may affect gut wall metabolism. CYP3A4 is a major drug metabolising enzyme in the gut wall and makes an important contribution to first pass metabolism of many orally administered CYP3A4 substrates (Fromm *et al.* 1996; Fuhr 1998). Inhibition of intestinal CYP3A4 is the reason for grapefruit juice interactions, which may cause a more than 100% increase in bioavailability of drugs undergoing pronounced first-pass metabolism by gut wall CYP3A4 (Fuhr 1998). For the grapefruit juice interactions, another flavonoid, i.e. naringenin, has been discussed as one of the causal agents (Fuhr 1998).

In conclusion, the minor inhibitory effects of silibinin *in vitro* on marker substrates of CYP2E1, CYP2D6, CYP2C19, CYP1A2 or CYP2A6 suggest that there is no clinically relevant inhibition of these enzymes to be expected by silibinin upon its administration in man. However, from our results we cannot exclude that comedication of silibinin with CYP2C9 or CYP3A4 substrates may result in increased concentrations of such drugs. Clinical studies are required to clarify this issue. On the other hand, a possible *in vivo* inhibition of CYP3A4 by silibinin might contribute to protection against prohepatotoxins such as aflatoxin

B_1 that are activated by this enzyme, as postulated for other flavonoids (Guengerich & Kim 1990).

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