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Identification of human liver cytochrome P450 isoforms mediating secondary omeprazole metabolism

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1 The *in vitro* metabolism of omeprazole was studied in human liver microsomes in order to define the secondary metabolic pathways and identify the cytochrome P450 (CYP) isoforms responsible for the formation of the secondary metabolites of omeprazole.

2 The major secondary omeprazole metabolite was the hydroxysulphone, which was formed during incubation with both hydroxyomeprazole and omeprazole sulphone. A second metabolite, tentatively identified as pyridine-*N*-oxide omeprazole sulphone, was also formed during incubation with omeprazole sulphone. The formation kinetics of these two metabolites from omeprazole sulphone were biphasic suggesting the involvement of multiple CYP isoforms in each case. In contrast, the formation kinetics of hydroxysulphone from hydroxyomeprazole were linear.

3 Inhibition studies, performed with omeprazole sulphone as substrate at concentrations at which the high affinity activities predominated, with a series of isoform selective inhibitors as well as with an anti-CYP2C3 antibody suggested a dominant role of S-mephenytoin hydroxylase in the formation of hydroxysulphone from omeprazole sulphone. By contrast, CYP3A activities were predominant in the formation of hydroxysulphone from hydroxyomeprazole as well as in the formation of pyridine-*N*-oxide omeprazole sulphone from omeprazole sulphone.

Keywords omeprazole human microsomal metabolism kinetics CYP isoforms

Introduction

Omeprazole binds to the gastric proton pump, H^+/K^+-ATPase, in the parietal cell secretory membrane resulting in suppression of gastric acid secretion [1–3]. Results from studies on the healing of peptic ulcers and reflux oesophagitis have shown that omeprazole heals these acid related diseases significantly more rapidly than histamine H_2-receptor blockers [4–6].

Omeprazole is extensively and rapidly (half-life usually < 1 h) metabolised by the liver, and the major metabolites found in plasma are hydroxyomeprazole and omeprazole sulphone [7]. Eighty per cent of a given dose is excreted in urine as metabolites, primarily hydroxyomeprazole and its corresponding carboxylic acid, and the rest is found in faeces as a result of biliary secretion [8–10]. Omeprazole sulphone is, like omeprazole itself, metabolised extensively to secondary metabolites since this metabolite was not detected in urine in significant amounts. Previous *in vivo* studies, which indicated that the formation of hydroxyomeprazole is mediated by S-mephenytoin hydroxylase [7, 11–14], have recently found support in an *in vitro* study performed in this laboratory [15]. Apart from showing that hydroxyomeprazole is formed mainly by S-mephenytoin hydroxylase we have also demonstrated that the formation of omeprazole sulphone is mediated almost exclusively by a cytochrome P450 (CYP) 3A isoform(s). Previous *in vivo* studies have also indicated that the further metabolism of omeprazole sulphone is mediated by S-mephenytoin hydroxylase while that of hydroxyomeprazole is not [7, 8]. It was shown further in our recent study that the main secondary metabolite formed *in vitro* from both hydroxyomeprazole and omeprazole sulphone is hydroxysulphone [15].

The present study was performed in order to characterise the secondary metabolism of omeprazole

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in human liver microsomes. This report presents results on the formation kinetics of secondary metabolites and also investigates which CYP isoforms perform the further transformation of the main omeprazole metabolites, hydroxyomeprazole and omeprazole sulphone. Results from effects of chemical and antibody inhibitors selective for various CYP isoforms are presented[1].

Methods

Chemicals

Omeprazole (5-methoxy-2[[(4-methoxy-3,5-dimethyl-2-pyridinyl)-methyl]sulphinyl]-1H -benzimidazole), omeprazole sulphone (5-methoxy-2-[[(4-methoxy-3,5-dimethyl-2-pyridinyl)methyl]sulphonyl]-1H-benzimidazole), hydroxyomeprazole (5-methoxy-2-[[(4-methoxy-3-methyl-5-hydroxymethyl-2-pyridinyl)-methyl]sulphinyl]-1H-benzimidazole), H215/02 or 5-*O*-desmethylomeprazole (5-hydroxy-2-[[(4-methoxy-3,5-dimethyl-2-pyridinyl)-methyl] sulphinyl]-1H-benzimidazole), H 195/77 or hydroxysulphone (5-methoxy-2-[[(4-methoxy-3-methyl-5-hydroxymethyl-2-pyridinyl)methyl]sulphonyl]-1H-benzimidazole), (Figure 1), and the assay internal standard (4,6-dimethyl-2-[[(4-methoxy-2-pyridinyl) methyl]sulphinyl]-1H-benzimidazole) were obtained from Astra Hässle AB (Mölndal, Sweden). Coumarin, diethyldithiocarbamate and troleandomycin were purchased from Sigma Chemical Co (St Louis, MO). Other drugs were obtained from the following sources: α-naphthoflavone from Aldrich Chemical Co (Milwaukee, WI), sulphaphenazole from Ciba-Geigy Aust (Sydney, Australia), R,S-mephenytoin from Sandoz Ltd (Basel, Switzerland) and quinidine from Burroughs Wellcome Aust (Sydney, Australia). Furafylline was a kind gift from Dr Rodolpho Gasser, Hoffmann La Roche (Basel, Switzerland).

$NADP^+$, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were purchased from Sigma Chemical Co (St Louis, MO). All other reagents and solvents were of analytical grade.

Liver samples

Human liver samples were obtained from renal transplant donors and relevant details of the donors of livers used in the present study (H8 and H9) have been published elsewhere [16]. Liver samples were stored at −18° C until used. Microsomes were prepared by differential centrifugation as previously described [17] and microsomal protein concentration was measured by the procedure of Lowry *et al.* [18] using crystalline bovine serum albumin as standard. Ethical approval was obtained from the Flinders Medical Centre Committee on Clinical Investigation to use these livers for drug metabolism studies.

[1]For information on cytochrome P450 nomenclature, see Nebert *et al.*, DNA 1993; **12**: 1–51.

Measurement of hydroxysulphone and pyridine-N-oxide omeprazole in human liver microsomes

The method used for determination of hydroxysulphone and another secondary metabolite, tentatively identified as pyridine-*N*-oxide omeprazole sulphone (see Results), was similar to the method used for analysis of hydroxyomeprazole and omeprazole sulphone [19], except that in the present study the incubation time was set to 30 min instead of 15 min. The formation of hydroxysulphone from both hydroxyomeprazole and omeprazole sulphone was linear up to at least 60 min. The formation of pyridine-*N*-oxide omeprazole sulphone from omeprazole sulphone was also linear up to at least 60 min incubation time.

Briefly, reaction mixtures contained human liver microsomes 1 mg (experiments on protein dependence showed linearity up to 1.5 mg), hydroxyomeprazole (0.5–200 μM) or omeprazole sulphone (0.5–200 μM), and NADPH-generating system in a final volume of 1.0 ml 0.1 M potassium dihydrogen phosphate buffer (pH 7.4). After 30 min incubation at 37° C the samples were extracted with dichloromethane:butanol (99:1) and the organic phase was evaporated to dryness under nitrogen. The residue was reconstituted in the h.p.l.c. mobile phase (dichloromethane:5% NH_4OH/MeOH: 2-propanol (191:8:1)) and an aliquot injected onto the chromatograph. Using a Supersphere SI-60 column (4 μm particle size, 125 × 4 mm ; E. Merck, Darmstadt, Germany) and an Aquapore Silica guard column (7 μm particle size, 15 × 3 mm; Brownlee laboratories, CA) and a mobile phase flow rate 1.5 ml min^{-1}, retention times for omeprazole sulphone, hydroxyomeprazole, 5-*O*-desmethylomeprazole, hydroxysulphone, pyridine-*N*-oxide omeprazole sulphone and internal standard were 2, 13.5, 11, 9, 4 and 3.3 min, respectively (see also ref. 19). The standard curve for hydroxysulphone was linear over the concentration range studied (0.5– 4.0 μM).

After incubation with hydroxyomeprazole in the absence of NADPH generating system a small peak corresponding to the hydroxysulphone was seen. The amount found (0–15% of that obtained in the presence of NADPH generating system) of hydroxysulphone was dependent on substrate concentration and was not detected after direct injection of hydroxyomeprazole; under these conditions the compound formed probably arises from degradation of substrate during incubation or extraction. The amounts of hydroxysulphone formed in the absence of NADPH generating system were measured at different concentrations of hydroxyomeprazole and after construction of a standard curve in each experiment a correction was applied. Neither pyridine-*N*-oxide omeprazole sulphone nor hydroxysulphone were formed during incubation with omeprazole sulphone in the absence of NADPH generating system.

Kinetics of secondary omeprazole metabolism

Ten different concentrations (0.5–200 μM) of hydroxyomeprazole or omeprazole sulphone were used in the

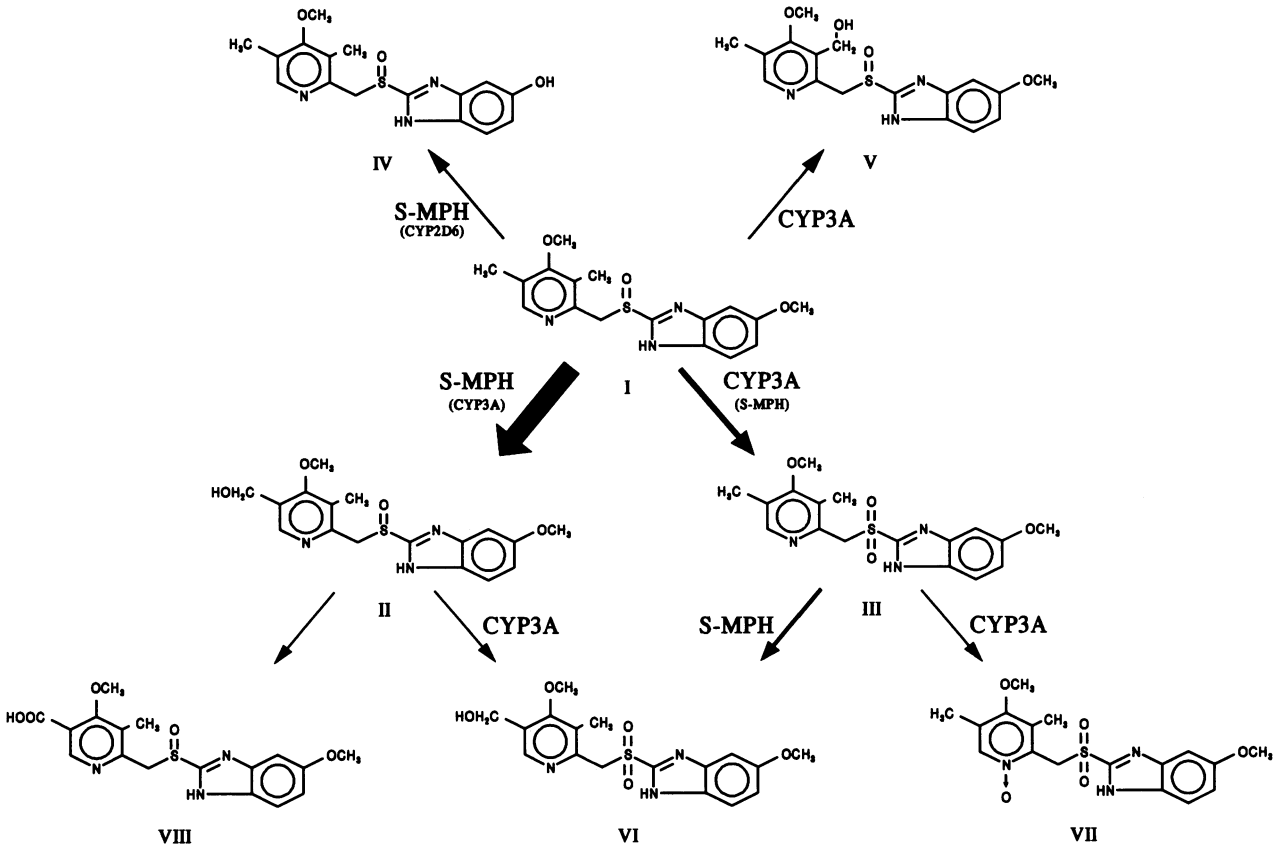

Figure 1 Proposed scheme for omeprazole metabolism in humans. Structures: I = omeprazole, II = hydroxyomeprazole, III = omeprazole sulphone, IV = 5-*O*-desmethylomeprazole, V = 3-hydroxyomeprazole, VI = hydroxysulphone, VII = pyridine-*N*-oxide omeprazole sulphone, VIII = carboxyomeprazole. (S-MPH = S-mephenytoin hydroxylase).

kinetic experiments. Two different livers (H8 and H9) were studied and apparent K_m and V_{max} values for the formation of hydroxysulphone were determined. These parameters were initially estimated from graphical analysis of Eadie-Hofstee plots to provide first estimates for MK Model, an extended least squares modelling programme [20]. The apparent K_m value for the formation of the other secondary metabolite, tentatively identified as pyridine-*N*-oxide omeprazole sulphone, was estimated using peak height ratios (PHR) as an index of reaction velocity.

Inhibition experiments

The effects of various CYP isoform-specific inhibitors or substrates (i.e. compounds acting as competitive inhibitors) on the formation of hydroxysulphone (from hydroxyomeprazole) and on the formation of hydroxysulphone and pyridine-*N*-oxide omeprazole sulphone (from omeprazole sulphone) were studied. The isoform selective inhibitors or alternative substrates were α-naphthoflavone (CYP1A inhibition at low concentrations and normally CYP 3A activation at higher concentration [16]), furafylline (CYP1A2; [21]), coumarin (CYP2A6; [22, 23]), sulphaphenazole (CYP2C9/10; [24]), R,S-mephenytoin (S-mephenytoin hydroxylase; [25]), quinidine (CYP2D6; [26, 27]), diethyldithiocarbamate (CYP2E1; [28]), and troleandomycin (CYP3A; [29]). The putative inhibitors were studied at a concentration chosen to be selective for the respective CYP isoforms on the basis of published IC_{50}, K_i or K_m values. Except for diethyldithiocarbamate, which was dissolved in water, inhibitors were dissolved in DMSO with a volume of 5 μl added to the incubation mixture. In all cases, inhibited activities were compared with activities of control incubations containing 5 μl (0.5%) DMSO or, 5 μl water as appropriate. Troleandomycin was preincubated with microsomes and NADPH generating system for 10 min before the reaction was started by addition of the hydroxyomeprazole or omeprazole sulphone [29]. Otherwise, there was no preincubation with inhibitor and incubations were started by adding NADPH generating system. Two different livers (H8 and H9) were investigated for all inhibitors. The concentration of omeprazole sulphone utilised in the inhibition experiments was 10 μM to allow assessment of the high affinity activity, while that of hydroxyomeprazole was 100 μM (see Results). These concentrations were used also in the antibody inhibition experiments (see below).

Immunoinhibition experiments with an anti-rabbit CYP2C3 antibody [30] were carried out by preincubating antibody with microsomes at room temperature for 15 min, before initiating reactions by the addition of substrate and NADPH generating system. The total amount of protein (in the form of antibody) added to each incubation was kept constant by the addition of preimmune antibody, and the proportion

of antibody to microsomal content in reaction mixtures was 15:1. The effect of the antibody on the primary metabolism of omeprazole using the same conditions was also studied (i.e. the effect of the antibody on the formation of hydroxyomeprazole, omeprazole sulphone and 5-*O*-desmethylomeprazole). This latter experiment was performed in order to verify further the findings reported in our earlier study [15]. The omeprazole concentration was 5 μM in this experiment.

In a complementary study omeprazole sulphone (10–120 μM) was tested as a potential inhibitor of the formation of hydroxyomeprazole as well as on the formation of 5-*O*-desmethylomeprazole. The substrate (omeprazole) concentration was 5 μM also in this experiment.

Results

Microsomes from both livers converted hydroxyomeprazole to hydroxysulphone and converted omeprazole sulphone to hydroxysulphone and another metabolite. Aliquots of this second metabolite were collected for mass spectrometric analysis performed at Astra Hässle AB (Mölndal, Sweden); its structure was determined tentatively as pyridine-*N*-oxide omeprazole sulphone (Weidolf *et al.*, unpublished results).

Eadie-Hofstee plots for the formation of hydroxysulphone from both hydroxyomeprazole and omeprazole sulphone in liver H8 (representative of both livers) are presented in Figure 2. The plot for the formation of hydroxysulphone from hydroxyomeprazole was linear, while that for hydroxysulphone from omeprazole sulphone was biphasic. This latter finding indicates that the formation of this metabolite from omeprazole sulphone could be mediated by multiple CYP isoforms. The Eadie-Hofstee plot for the formation of pyridine-*N*-oxide omeprazole sulphone from omeprazole sulphone was also biphasic. In the case of hydroxysulphone and pyridine-*N*-oxide omeprazole sulphone formation from omeprazole sulphone, the data were best fitted by the Michaelis-Menten expression for a two-enzyme system, whereas the data for hydroxysulphone formation from hydroxyomeprazole were best fitted by a one-enzyme system. The derived K_m and V_{max} values are presented in Table 1. The K_m for hydroxysulphone formation from hydroxyomeprazole was approximately 100 μM for both livers. The mean K_m values for the high and low affinity component of hydroxysulphone formation from omeprazole sulphone was 7.6 μM and 427 μM, respectively. Corresponding values for formation of pyridine-*N*-oxide omeprazole sulphone were 40 μM and 620 μM.

The Michaelis-Menten expression for a two-enzyme system with the values (Table 1) for hydroxysulphone formation from omeprazole sulphone was used to derive an omeprazole sulphone concentration (10 μM) that would result primarily in a contribution of the high affinity activity to total reaction velocity in further experiments. Values were 87%

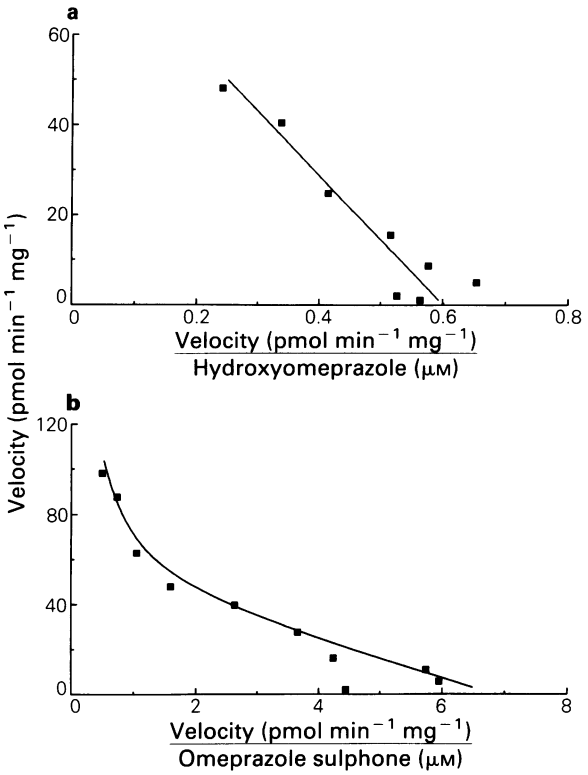

Figure 2 Eadie-Hofstee plots for formation of hydroxysulphone from hydroxyomeprazole (a) and from omeprazole sulphone (b) by microsomes from liver H8. Points are experimentally determined values while solid lines are the computer generated curves of best fit.

Table 1 Computer derived Michaelis-Menten parameters for formation of hydroxysulphone from hydroxyomeprazole and for the formation of hydroxysulphone and pyridine-*N*-oxide omeprazole sulphone from omeprazole sulphone in human liver microsomes from two livers (H8 and H9)

Liver number	*Hydroxysulphone from hydroxyomeprazole*		*Hydroxysulphone from omeprazole sulphone*				*Pyridine-N-oxide omeprazole sulphone from omeprazole sulphone*	
	K_m	V_{max}	K_{m1}	K_{m2}	V_{max1}	V_{max2}	K_{m1}	K_{m2}
H8	142	0.09	7.8	341	0.05	0.15	27	660
H9	120	0.11	7.3	513	0.09	0.30	52	579
Mean	131	0.10	7.6	427	0.07	0.22	40	620

Note: K_m expressed as μM and V_{max} expressed as nmol mg^{-1} min^{-1}. V_{max} values for formation of pyridine-*N*-oxide omeprazole sulphone could not be determined as activity was assessed only by peak height ratios (see Methods).

and 90% for liver H8 and H9, respectively. For pyridine-*N*-oxide omeprazole sulphone the corresponding values were 90% and 93%, respectively. The concentration of hydroxyomeprazole used was 100 μM, i.e. close to the K_m value.

The effects of various inhibitors/substrates on the formation of hydroxysulphone from hydroxyomeprazole as well as on the formation of hydroxysulphone and pyridine-*N*-oxide omeprazole sulphone from omeprazole sulphone are shown in Figures 3 and 4. Inhibitors selective for CYP1A2 (furafylline), CYP2A6 (coumarin), CYP2C9/10 (sulphaphenazole), CYP2D6 (quinidine), and CYP2E1 (diethyldithiocarbamate) had essentially no effect on any of the pathways studied. Troleandomycin (CYP3A inhibitor) inhibited almost completely (>90%) the formation of hydroxysulphone from hydroxyomeprazole as well as the formation of pyridine-*N*-oxide omeprazole sulphone from omeprazole sulphone, but only pyridine-*N*-oxide omeprazole sulphone formation was activated (60%) by 10 μM of α-naphthoflavone. There was a negligible inhibition of these pathways by R,S-mephenytoin. The formation of hydroxysulphone from omeprazole sulphone, on the other hand, was substantially inhibited (45%) by R,S-mephenytoin, while troleandomycin exhibited minor inhibition (20%) only of this pathway.

The results from the anti-CYP2C3-antibody inhibition experiments, presented in Figure 5, show that hydroxysulphone formation from omeprazole sulphone was reduced by 70%, consistent with the inhibitory effect of R,S-mephenytoin presented above. There was also a slight inhibition (23%) of pyridine-*N*-oxide omeprazole sulphone formation from the omeprazole sulphone while negligible effect was obtained for hydroxysulphone formation from hydroxyomeprazole. Furthermore, the results on primary metabolic pathways for omeprazole show that hydroxyomeprazole and 5-*O*-desmethylomeprazole formation was inhibited 75% by the antibody while little or no effect was observed for omeprazole sulphone formation.

The results from the inhibition experiment with omeprazole sulphone (Figure 6) show that the formation of both hydroxyomeprazole and 5-*O*-desmethylomeprazole was inhibited ~80% by this primary omeprazole metabolite.

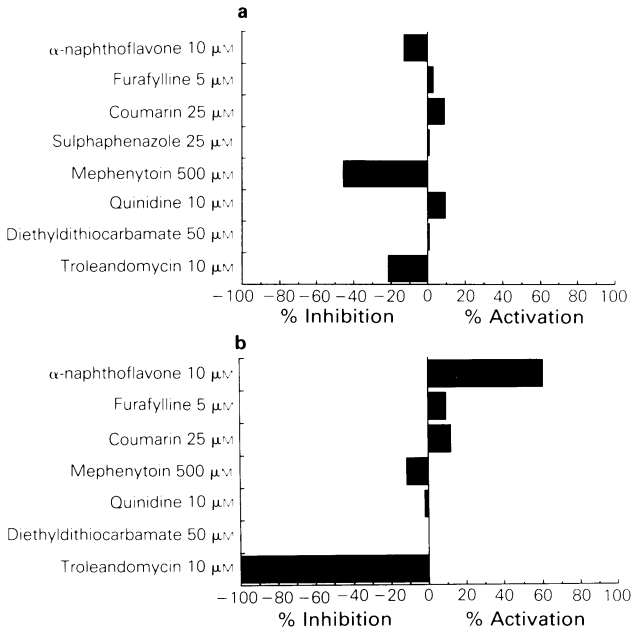

Figure 4 Effects of various specific CYP inhibitors and/or substrates on the formation of hydroxysulphone (a) and pyridine-*N*-oxide omeprazole sulphone (b) from omeprazole sulphone by human liver microsomes. Each bar represents the mean of two different livers (H8 and H9).

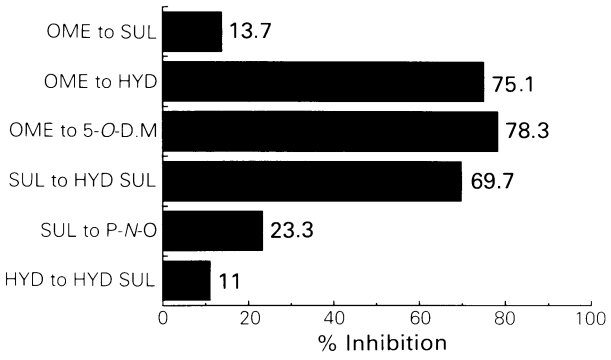

Figure 5 Effects of a CYP2C3-antibody on different metabolic pathways (primary and secondary) of omeprazole metabolism. Each bar represents duplicate samples in one liver (H8). Abbreviations: OME = omeprazole, SUL = omeprazole sulphone, HYD = hydroxyomeprazole, 5-*O*-DM = 5-*O*-desmethylomeprazole, HYD SUL = hydroxysulphone, P-*N*-O = pyridine-*N*-oxide omeprazole sulphone.

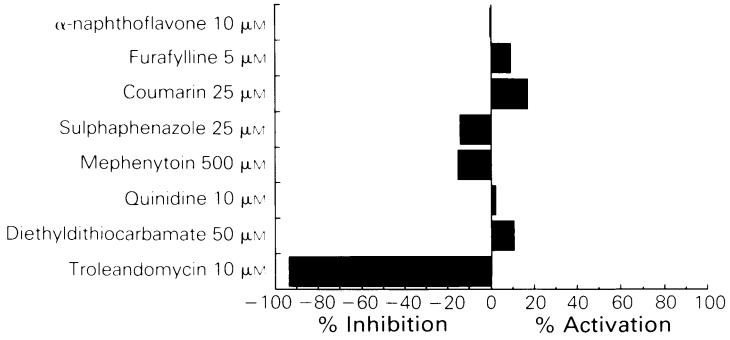

Figure 3 Effects of various specific CYP inhibitors and/or substrates on the formation of hydroxysulphone from hydroxyomeprazole by human liver microsomes. Each bar represents the mean of two different livers (H8 and H9).

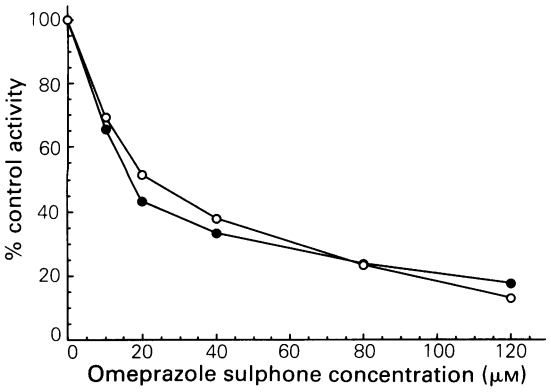

Figure 6 Effects of omeprazole sulphone on the formation of hydroxyomeprazole (●) and 5-*O*-desmethylomeprazole (○) from omeprazole by human microsomes in one liver (H8).

Discussion

Secondary metabolic pathways are common *in vivo* but they are not usually seen at saturating substrate concentrations *in vitro*. In the case of omeprazole, the sulphone is not seen in urine but the hydroxysulphone does appear. Interpretation of sequential metabolic formation clearances *in vivo* is complex as both formation and elimination mechanisms are occurring at the same time. It is, therefore, hard to delineate environmental and genetic influences. This has been clarified in the current instance by mapping the metabolic pathways to the particular CYPs involved. It confirms the suggestions from *in vivo* data that the clearance of the sulphone may also be linked to the S-mephenytoin polymorphism. While the inhibition of the H^+/K^+-ATPase is probably irreversible, the effect of the drug will be related to the area under its concentration-time curve in plasma. It is, therefore, relevant to have some indication of the pharmacokinetic and environmental influences that will effect its clearance *in vivo*.

An updated proposed scheme for both the primary and secondary metabolism of omeprazole is presented in Figure 1. The data are based on results from the present study together with previously reported results [15]. The primary metabolism of omeprazole has been discussed extensively in a previous paper [15], except that the structure of 3-hydroxyomeprazole (V) at that stage was still unknown and this compound was referred to simply as metabolite X. Mass spectrometry experiments on collected aliquots of metabolite X, performed at Astra Hässle AB, revealed the structure of this metabolite (Weidolf *et al.*, unpublished results). However, this discussion will deal mainly with the secondary metabolism of omeprazole. Pyridine-*N*-oxide omeprazole sulphone, one of the two secondary metabolites presented in this study, was also identified tentatively by mass spectrometry performed by Dr Lars Weidolf (Weidolf *et al.*, unpublished results).

This study shows that hydroxysulphone is the main secondary omeprazole metabolite formed during incubation with both hydroxyomeprazole and omeprazole sulphone. Following incubation with omeprazole sulphone a second metabolite, tentatively identified as pyridine-*N*-oxide omeprazole sulphone, was detected and measured. The kinetics of hydroxysulphone formation from hydroxyomeprazole were linear while the hydroxysulphone formation from omeprazole sulphone exhibited biphasic kinetics, the latter finding suggesting involvement of multiple CYP isoforms. Biphasic kinetics were observed also for the formation of pyridine-*N*-oxide omeprazole sulphone.

The lack of effect on the secondary metabolic pathways by inhibitors and/or substrates specific for CYP1A2, CYP2A6, CYP2C9/10, CYP2D6 and CYP2E1 indicates strongly that none of these isoforms is involved in the various reactions. The complete inhibition of the formation of hydroxysulphone from hydroxyomeprazole and of the formation of pyridine-*N*-oxide omeprazole sulphone from omeprazole sulphone by troleandomycin shows clearly that these secondary metabolic pathways are mainly CYP3A mediated reactions. However, of these two reactions, only the formation of pyridine-*N*-oxide omeprazole sulphone was accompanied by an activation by α-naphthoflavone. Alpha-naphthoflavone is normally activator of CYP3A mediated reactions but exceptions, like that above, have previously been reported for other substrates [31]. By contrast, the substantial inhibition by R,S-mephenytoin of hydroxysulphone formation from omeprazole sulphone indicates that this reaction is S-mephenytoin hydroxylase mediated as previously suggested on the basis of *in vivo* experiments [7, 8]. The weak inhibition by troleandomycin of this pathway suggests that CYP3A could be involved as a minor component in this metabolic transformation.

The results from the CYP2C3 antibody experiments show excellent concordance with previously reported inhibitory data on the primary metabolism of omeprazole, i.e. a major role for S-mephenytoin hydroxylase in the formation of hydroxyomeprazole and 5-*O*-desmethylomeprazole from omeprazole [15]. Furthermore, the antibody experiments are in good agreement with the other inhibitory data presented in this report as regards the formation of secondary metabolites. S-mephenytoin hydroxylase is the dominant (70%) enzyme responsible for the formation of hydroxysulphone from omeprazole sulphone. The inhibition of this pathway by R,S-mephenytoin (45%) and troleandomycin (20%) was not complete. Therefore, a contribution from another isoform to this transformation cannot be excluded. However, inhibition by R,S-mephenytoin is typically weak and, since the inhibitory effect of the CYP2C3 antibody probably affords a better measure of the contribution of the S-mephenytoin hydroxylase, we find it more likely that only two isoforms are involved in this transformation. A negligible influence of the CYP2C3 antibody was observed on the two other secondary metabolic steps.

In the previous paper we showed that formation of hydroxyomeprazole and 5-*O*-desmethylomeprazole from omeprazole were mediated mainly by the S-mephenytoin hydroxylase and that the apparent K_m values for these reactions were in the range 3–16 μM. Omeprazole sulphone would be expected to inhibit

these reactions as data in this paper suggest that the formation of hydroxysulphone from omeprazole sulphone is mediated by the same enzyme. This was confirmed as shown by the data in Figure 6. Further, with omeprazole used at a substrate concentration 5 μM, equal to its apparent K_m, it can be calculated that the IC_{50} for an inhibition is $2K_i$. From Figure 6 it can be estimated that IC_{50} is about 20 μM giving a K_i of about 10 μM which is consistent with the high affinity K_m for omeprazole sulphone (see Table 1), assuming competitive inhibition.

Variation in metabolism between different livers [15] is possible, but the results obtained using our two livers were similar. Thus we conclude that the formation of hydroxysulphone from omeprazole sulphone is mainly mediated by S-mephenytoin hydroxylase while the formation of the same metabolite from hydroxyomeprazole is mainly CYP3A mediated. Pyridine-*N*-oxide omeprazole sulphone formation from omeprazole sulphone also seems to be a CYP3A mediated reaction.

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