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Specific Inhibition of Human CYP1A2 Using a Targeted Antibody

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ABSTRACT. The structural similarity of related forms of P450 makes selective immunoinhibition of individual forms notoriously difficult to achieve. We report the use of a targeted antibody to overcome this problem. An antibody was raised against the synthetic peptide, Ser-Lys-Lys-Gly-Pro-Arg-Ala-Ser-Gly-Asn-Leu-Ile, corresponding to residues 291–302 of human CYP1A2. This sequence of human CYP1A2 is located in a similar position to a proinhibitory region previously identified in rat CYP1A1 and CYP1A2. The antibody bound strongly and specifically to CYP1A2 in human hepatic microsomal fraction. Binding was unaffected by denaturation of the protein. The specificity of the antibody was demonstrated by immunoblotting of human hepatic microsomal fraction where a single immunoreactive band was identified at M_r 54,000. The intensity of this band correlated strongly with high-affinity phenacetin O-deethylase activity of the microsomal fractions. In addition, the antibody bound to a single protein at M_r 54,000 in the microsomal fraction of lymphoblastoid cells expressing human CYP1A2, but not to any other recombinant P450 enzyme. CYP1A2-dependent activity (high-affinity phenacetin O-deethylase) of human hepatic microsomal fraction was inhibited >90% by whole antiserum or purified immunoglobulin. This decrease in activity represents complete inhibition of CYP1A2 activity, residual phenacetin O-deethylase activity being due to low-affinity enzymes. In contrast, the antibody, which does not bind to rat CYP1A2, had no effect on CYP1A2-dependent activity (high-affinity phenacetin O-deethylase) of rat hepatic microsomal fraction. The antiserum also had no effect on human hepatic microsomal debrisoquine 4-hydroxylase (CYP2D6) or coumarin 7-hydroxylase (CYP2A6) activities, indicating that inhibition was specific to human CYP1A2. These results demonstrate the importance of the region comprising residues 291–302 of human CYP1A2 in the catalytic activity of this enzyme. BIOCHEM PHARMACOL 54;1:189–197, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. cytochrome P450, CYP1A2, antipeptide antibody, human, inhibition, phenacetin O-deethylase

P450 enzymes are haemoproteins that catalyse the oxidative biotransformation of a wide range of endogenous and exogenous substances [1]. The structure of the apoprotein moiety determines which substrates are metabolised, the rate of the reaction, and the position of oxidation. P450 enzymes display unique yet overlapping substrate specificities, with many forms metabolising the same compounds but at different rates [2]. Although useful information can be gained from studying individual purified or expressed enzymes, determination of the contribution of each P450 enzyme to the metabolism of a compound is best performed using an intact system, such as the microsomal fraction, in which there is a full complement of P450 enzymes and associated proteins. This may be achieved by using chemicals or antibodies as specific inhibitors [3, 4].

The P450 enzyme CYP1A2 is constitutively expressed in human liver and is inducible by a wide variety of compounds present in the environment, including constituents of cooked meat, cruciferous vegetables, cigarette smoke, and also by exposure to polycyclic aromatic hydrocarbons and polychlorinated biphenyls [5]. Amongst the numerous reactions catalysed by CYP1A2, the ability to activate pro-mutagenic compounds including heterocyclic amines present in cooked food is of current concern [6].

Antibodies have been used extensively to identify, localise, and quantify specific P450 enzymes [4]. In the past, antiprotein antibodies, both polyclonal and monoclonal, have been used, but more recently the use of antipeptide antibodies has increased [7–11]. This has been made possible by the publication of cDNA sequences of numerous P450 genes from which the primary structures of the various enzymes are predicted [12]. Antipeptide antibodies have the advantage that there is no need to purify the protein for use as an immunogen. This is particularly important when studying human P450 enzymes, where restrictions of tissue availability may make purification of sufficient material difficult, although the use of recombinant technology to express high levels of enzymes is increasingly an alternative [13]. Antipeptide antibodies also have the advantage that they are directed to a predetermined region on the target protein. By comparison of the sequences of related forms of

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P450, antibodies can be targeted to unique sequences thereby ensuring that they bind to a specific P450 enzyme. In addition, antipeptide antibodies can be directed against regions of the enzyme involved in catalytic activity [14]. This approach should permit a single enzyme to be inhibited specifically in the presence of a full complement of P450 enzymes, providing a powerful *in vitro* tool for determining the contribution of the target enzyme to the metabolism of a compound.

A proinhibitory region on the surface of rat CYP1A2 has previously been identified using an antipeptide antibody targeted against residues 290–296 of the enzyme [14]. The aim of the present study was to produce an antibody that specifically and potently inhibits human CYP1A2 activity, by targeting a specific region of the enzyme.

MATERIALS AND METHODS

Materials

N-α-9-Fluorenylmethoxycarbonyl protected amino acids linked to polydimethylacrylamide/Polyhipe resin, *N*-α-9-fluorenylmethoxycarbonyl amino acid pentafluorophenyl, and 3-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine esters were purchased from Calbiochem-Novabiochem (Nottingham, Notts, UK) as was keyhole limpet haemocyanin (KLH).† Acetonitrile (grade S) was from Rathburn Chemicals (Walkerburn, Peebleshire, UK). Polystyrene 96-well microtitre plates were from Dynatech Laboratories (Billinghurst, W. Sussex, UK). All SDS-polyacrylamide gel electrophoresis reagents were from National Diagnostics (Aylesbury, Bucks, UK). Hybond-C nitrocellulose hybridisation membrane, enhanced chemiluminescent reagents, and Hyperfilm were purchased from Amersham (Aylesbury, Bucks, UK). Microsomal fractions prepared from human lymphoblastoid cells expressing various human P450 enzymes were purchased from Gentest Corporation (Woburn, MA). Antirabbit immunoglobulin conjugated to horseradish peroxidase was from Sigma (Poole, Dorset, UK). Recombinant truncated protein G (GammaBind G) coupled to agarose was purchased from Merck-BDH (Lutterworth, Leicestershire, UK). All other chemicals were purchased from Sigma or Merck-BDH and were of analytical grade or the best equivalent.

Preparation of Microsomal Fractions From Human Liver

Morphologically normal human liver samples from renal transplant donors were obtained from the human tissue bank at the Royal Postgraduate Medical School [15]. Local Ethics Committee approval and Coroner's permission were obtained for their use in these studies. The preparation of the microsomal fraction was as described previously [15].

† *Abbreviations:* ELISA, enzyme-linked immunosorbent assay; KLH, keyhole limpet haemocyanin; MC, 3-methylcholanthrene; N.S., not significant; POD, phenacetin *O*-deethylase; r_s, Spearman's Rank Correlation Coefficient; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

Treatment of Animals

Male Wistar rats (200–250 g) were obtained from Harlan Olac Ltd (Bicester, Oxon, UK). Male New Zealand White rabbits (3 kg) were from Froxfield Farms Ltd. (Petersfield, Hampshire, UK). Male Balb/c mice (6–10 weeks) and male Dunkin-Hartley guinea pigs (250–300 g) were from A Tuck & Son Ltd. (Battlebridge, Essex, UK). Male Golden Syrian hamsters (90–110 g) were supplied by Belgrave Trading Ltd. (Lambourn, Berks, UK). These animals were left untreated or were administered 3-methylcholanthrene (MC) (80 mg/kg) in corn oil as a single intraperitoneal injection and killed 48 hr later. All animals were killed humanely in accordance with approved Home Office procedures appropriate to the species, the livers were rapidly removed, and microsomal fractions prepared as described previously [15].

Hepatic microsomal fraction from a pool of five untreated male marmosets and a pool of five marmosets treated with a single subcutaneous dose of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (1 μg/kg body weight) dissolved in dimethylsulphoxide/toluene (2:1 v/v) and killed 7 days later were prepared as described previously [16] by Dr. T. Schulz, Department of Clinical Pharmacology, R.P.M.S. The marmosets were housed and maintained at the Institut für Toxikologie und Embryopharmakologie, FU Berlin, Germany, and were not treated specifically for this study.

Liver tissue was also obtained from male cynomolgus monkeys treated orally with a single dose of TCDD (1 μg/kg body weight) dissolved in polyethyleneglycol 300 (1 mL/kg body weight) or with polyethyleneglycol 300 only, and were killed 3 days later. The monkeys were housed and maintained at Hazleton Laboratories, Vienna, MA, under conditions described elsewhere [17]. Liver samples from these monkeys were kindly donated by Dr. S. S. Thorgeirsson, Laboratory of Experimental Carcinogenesis, National Cancer Institute, Bethesda, MD.

Peptide Synthesis, Conjugation to Carrier Protein, and Production of Antibodies

Synthesis of peptides, their conjugation to a carrier protein, KLH, and the immunisation of rabbits were as described previously [18]. The peptides synthesised were Cys-Ser-Lys-Lys-Gly-Pro-Arg-Ala-Ser-Gly-Asn-Leu-Ile, Cys-Ser-Lys-Lys-Gly-Pro-Arg-Ala-Ser, Cys-Ser-Lys-Lys-Gly-Pro-Arg, Cys-Lys-Gly-Pro-Arg-Ala-Ser-Gly, and Cys-Arg-Ala-Ser-Gly-Asn-Leu-Ile, which represent residues 291–302, 291–298, 291–296, 293–299, and 296–302 of human CYP1A2, respectively (Table 1). In all cases cysteine was incorporated at the *N*-terminus of the peptides for the purpose of conjugation to KLH as described previously [18]. A null conjugate (KLH-Cys) was produced by substituting cysteine for the thiolated peptide. All peptides were >95% pure as determined by reversed-phase high-pressure liquid chromatography under the conditions described previously [18], and from their expected molecular

TABLE 1. Aligned sequences of CYP1A2 from rat and human in the region of the sequences of human CYP1A2 chosen for synthesis

	Sequence	Position
Human CYP1A2:	-Lys-His-Ser-Lys-Lys-Gly-Pro-Arg-Ala-Ser-Gly-Asn-Leu-Ile-Pro-Gln-	289–304
Synthetic peptides:	Ser-Lys-Lys-Gly-Pro-Arg-Ala-Ser-Gly-Asn-Leu-Ile	291–302
	Ser-Lys-Lys-Gly-Pro-Arg-Ala-Ser	291–298
	Ser-Lys-Lys-Gly-Pro-Arg	291–296
	Lys-Gly-Pro-Arg-Ala-Ser-Gly	293–299
	Arg-Ala-Ser-Gly-Asn-Leu-Ile	296–302
Rat CYP1A2:	-Lys-His-**Ser-Glu- - Asn-Tyr-Lys-Asp-Asn**-Gly-Gly-Leu-Ile-Pro-Gln-	288–302

The amino acid sequences are predicted from their cDNA sequences and are numbered from the *N*-terminal methionine [19, 20]. The synthetic peptides are numbered with respect to human CYP1A2. A space in rat CYP1A2 has been included for optimum alignment. Text in bold typeface indicates the proinhibitory region identified in rat CYP1A2 [14].

weight determined by electrospray mass spectrometry. Male New Zealand White rabbits (3 kg) from Froxfield Farms Ltd. (Petersfield, Hampshire, UK) were immunised with peptides coupled to KLH as described previously [18].

Immunoglobulin Preparation

The IgG fraction of antiserum was purified using protein G-Agarose. Protein G-Agarose G-Agarose (5 mL) was packed into a small chromatography column and washed with 0.1 M sodium phosphate buffer containing 0.15 M sodium chloride, pH 7.0 (loading buffer). Whole serum (5 mL) was mixed with 10 mL of loading buffer and passed through the column at a flow rate of 1 mL/min, followed by loading buffer until the UV absorbance of the eluent declined to zero. The IgG fraction was eluted in 0.5 M acetic acid, pH 3.0, neutralised immediately with 0.3 volumes of 3 M Tris-HCl, pH 8.8, and then dialysed against three changes of PBS.

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was performed as described previously [18]. Antibodies against carrier protein and coupling reagent were depleted from antiserum by affinity adsorption as described before [21].

Immunoblotting

Immunoblotting was performed using microsomal fraction from liver or lymphoblastoid cells containing recombinant CYP1A2. Details of the conditions used have been reported previously [18, 22]. Briefly, blots were developed with antiserum diluted 1:4000, antibody binding was detected using goat antirabbit IgG coupled to horseradish peroxidase diluted 1:20000, and visualised using enhanced chemiluminescence, the result being recorded on Hyperfilm. The relative intensity of the bands was determined by laser densitometry using an LKB UltroScan XL Enhanced Laser Densitometer (Pharmacia LKB Biotechnology, St Albans, Herts, UK).

Measurement of Cytochrome P450-Dependent Monooxygenase Activities

PHENACETIN O-DEETHYLASE (POD) ACTIVITY. High-affinity POD activity of hepatic microsomal fraction from human and untreated rats was determined by the method of Murray and Boobis [23], using a final phenacetin concentration of 4 μM. Hepatic microsomal fraction (0.1 mg protein for humans and either 0.1 or 0.3 mg for rats) was incubated for 10 min at 37°C with varying amounts of antiserum or purified IgG in a total volume of 0.89 mL. Buffer and cofactors were then added, before initiation of the reaction (final volume 1 mL), by addition of substrate. After 20 min the reaction was stopped by addition of 0.3 mL of ice-cold 0.5 M sodium hydroxide and 50 ng deuterated paracetamol added. Excess phenacetin was removed by extraction into 10 mL diethyl ether. The pH of the aqueous layer was brought to 6.5 by addition of 0.1 mL of 75% (w/v) trichloroacetic acid, followed by 1 mL of 1 M potassium phosphate buffer, pH 7.0 before quantitative extraction of paracetamol into 10 mL of ethyl acetate. Measurement by gas chromatography-mass spectrometry was as described by Murray and Boobis [23].

DEBRISOQUINE 4-HYDROXYLASE ACTIVITY. This activity was determined using a substrate concentration of 1 mM debrisoquine sulphate as described by Kahn *et al.* [24], except that 0.5 mg of hepatic microsomal fraction was mixed with 0.1 mL of antiserum in a total volume of 0.84 mL for 10 min at 37°C prior to addition of the reaction mixture (final volume 1 mL). After 12 min the reaction was stopped by addition of 0.2 mL of 1 M sodium hydroxide. The product, 4-hydroxydebrisoquine, was extracted as described previously and quantified by gas chromatography-mass spectrometry [24].

COUMARIN 7-HYDROXYLASE ACTIVITY. The reaction conditions for this assay were as described by Edwards *et al.* [22]. Hepatic microsomal fraction (0.1 mg) was mixed with 0.1 mL of antiserum for 10 min at 37°C before initiation of the reaction (final volume 1 mL). After 20 min at 37°C the reaction was stopped by addition of 0.5 mL of 6% (w/v) trichloroacetic acid and the product, 7-hydroxycoumarin, quantified as described by Aitio [25].

All of these monooxygenase reactions were linear with respect to the amount of microsomal protein and duration of incubation.

Statistical Analysis

The relationship between relative apoprotein levels and enzyme activity was assessed by calculation of Spearman's Rank Correlation Coefficient (r_s). Statistical significance was determined using Student's t-test and probability values exceeding 0.05 were considered as not significant (NS).

RESULTS

Antibodies were raised against peptides corresponding to residues 291–302, 291–296, 291–298, 293–299, and 296–302 of human CYP1A2. Antibody binding to human hepatic microsomal fraction was examined in ELISA. The antibody raised against residues 291–302 of human CYP1A2 bound strongly to human hepatic microsomal fraction, whereas none of the other antisera showed any binding (Fig. 1a), although all of the antisera recognised their respective immunising peptides (data not shown). Similar results were obtained when microtitre plate wells were coated with hepatic microsomal fraction denatured by treatment with 8 M urea, only the antibody raised against residues 291–302 of CYP1A2 binding (Fig. 1b).

Immunoblotting of human hepatic microsomal fraction showed that the antibody raised against residues 291–302 of human CYP1A2 bound to a single band at M_r 54,000 (Fig. 2). The intensity of the immunoreactive band varied between donors and correlated with high affinity POD activity ($r_s = 0.87$, $n = 30$, $P < 0.001$), but not debrisoquine 4-hydroxylase activity ($r_s = 0.07$, $n = 29$, NS) or coumarin 7-hydroxylase activity ($r_s = 0.11$, $n = 30$, NS). In contrast, no immunoreactivity was detected using antipeptide antibodies raised against residues 291–298, 291–296, 293–299, or 296–302 of human CYP1A2 (data not shown).

The specificity of the antibody raised against residues 291–302 of human CYP1A2 was also investigated by examining its binding to microsomal fractions prepared from human lymphoblastoid cells expressing various P450 enzymes (Fig. 3). A single immunoreactive band, with M_r 54,000, was detected in lymphoblastoid cells expressing human CYP1A2, similar to the band identified in human hepatic microsomal fraction. No binding to microsomal fractions prepared from control lymphoblastoid cells or from lymphoblastoid cells expressing human CYP1A1, CYP2A6, CYP2B6, CYP2C9-Arg, CYP2C9-Cys, CYP2C19, CYP2D6-Val, CYP2E1, or CYP3A4 was detected. Adequate levels of expression for detection by immunoblotting was confirmed for CYP2A6, CYP2B6, CYP2D6-Val, and CYP3A4 using antibodies directed against these forms (data not shown, other forms not tested).

Binding of the antibody against residues 291–302 of CYP1A2 to peptides representing portions of the immunising peptide was examined in ELISA to determine the location of the major epitope. Antiserum was depleted of antibodies against KLH-Cys and then binding to the various peptides conjugated to KLH was assessed. The antipeptide antibody bound strongly to the immunising peptide (Ser-Lys-Lys-Gly-Pro-Arg-Ala-Ser-Gly-Asn-Leu-Ile) and with slightly reduced affinity to Ser-Lys-Lys-Gly-Pro-Arg-Ala-Ser (residues 291–298), Lys-Gly-Pro-Arg-Ala-Ser-Gly (residues 293–299), and Arg-Ala-Ser-Gly-Asn-Leu-Ile (residues 296–302). However, binding to Ser-Lys-Lys-Gly-Pro-Arg (residues 291–296) was much reduced (Fig. 4).

The antibody against residues 291–302 of CYP1A2 was tested for its ability to bind to hepatic microsomal fractions from other species (Fig. 5). Binding was found only in the TCDD-treated cynomolgus monkey and guinea pig hepatic microsomal fractions. In a hepatic microsomal fraction from the TCDD-treated, but not untreated cynomolgus monkey, the antibody recognised a single protein band that migrated slightly further than human CYP1A2. In untreated guinea pigs a slower migrating immunoreactive

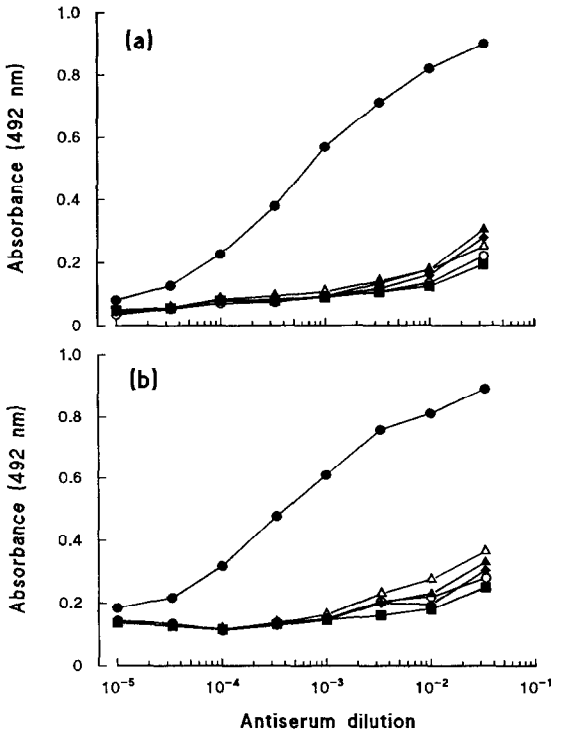

FIG. 1. Relative binding of antipeptide antibodies to human hepatic microsomal fraction. The wells of microtitre plates were coated with human hepatic microsomal fraction suspended in (a) PBS or (b) dissolved in PBS containing 8 M urea. Serial dilutions of antipeptide antibodies raised against residues 291–302 (●), 296–302 (■), 291–298 (▲), 293–299 (◆), and 291–296 (△) of human CYP1A2, and preimmune serum (○) were added. All antisera were depleted of antibodies against KLH-Cys prior to application. Bound antibody was determined as described in Materials and Methods. Each point is the mean of two determinations, and the data shown are from a typical experiment representative of three experiments with similar results.

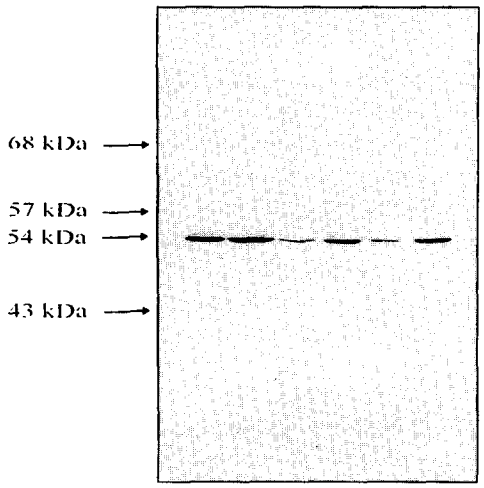

FIG. 2. Binding specificity of the antibody raised against residues 291–302 of CYP1A2 to human hepatic microsomal fraction. Samples of 30 μg of human hepatic microsomal fraction from six donors were subjected to immunoblotting using the antipeptide antiserum raised against residues 291–302 of human CYP1A2 at a dilution of 1:4000. Immunoreactive bands were detected as described in Materials and Methods.

band was detected; the intensity of this band was unaffected by treatment of the animals with MC. However, following MC treatment a second very weak band, migrating to a similar position as human CYP1A2, was observed. No immunoreactivity was detected with hepatic microsomal fractions from untreated rats, mice, rabbits, hamsters, or marmosets, or MC-treated rats, mice, rabbits, or hamsters, or TCDD-treated marmosets.

The effect of the antibody against residues 291–302 of CYP1A2 on enzyme activity was investigated by incubating human hepatic microsomal fraction in the presence of antiserum before measuring high-affinity POD activity, a selective assay for CYP1A2 activity. Enzyme activity was progressively inhibited by increasing volumes of antiserum. Maximum inhibition of 90% was achieved on addition of 0.1 mL antiserum to 0.1 mg of hepatic microsomal protein (Fig. 6). Incubation with 0.6 mg of purified IgG (which is

FIG. 3. Immunoreactivity of recombinant human P450 enzymes. Immunoreactivity of microsomal fraction from a pool of six human liver samples (lanes 1, 4, 7, 11, 14, and 17) compared with that of human lymphoblastoid cells expressing CYP1A1 (lane 2), CYP1A2 (lane 3), CYP2A6 (lane 5), CYP2B6 (lane 6), no P450 (lane 8), CYP2C9-Arg (lane 9), CYP2C9-Cys (lane 10), CYP2C19 (lane 12), CYP2D6-Val (lane 13), CYP2E1 (lane 15), and CYP3A4 (lane 16). Each lane contains 20 μg protein. Immunoblotting was performed using the antipeptide antiserum raised against residues 291–302 of human CYP1A2 at a dilution of 1:4000. Immunoreactive bands were detected as described in Materials and Methods. Only the central section of the blot containing immunoreactive material is shown.

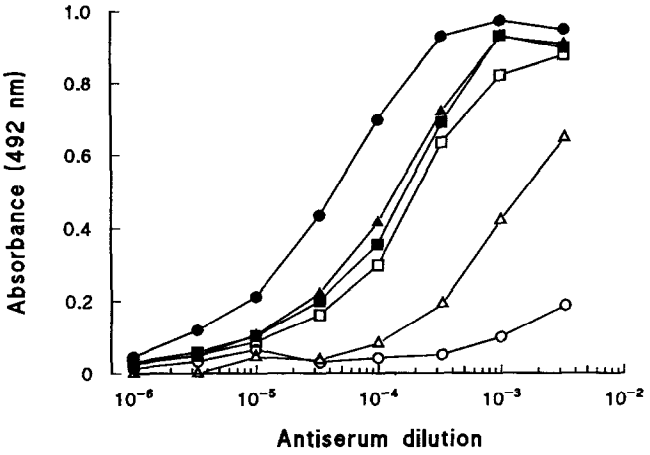

FIG. 4. Relative binding of the antibody raised against residues 291–302 of CYP1A2 to related peptides. The wells of microtitre plates were coated with conjugates of KLH coupled to peptides corresponding to residues 291–302 (●), 291–298 (▲), 291–296 (△), 293–299 (□), and 296–302 (■) of human CYP1A2, and KLH-Cys (○). To each group of wells a series of dilutions of the antibody against human CYP1A2 at residues 291–302, depleted of antibodies against KLH-Cys, was added. Bound antibody was determined as described in Materials and Methods. Each point is the mean of two determinations, and the data shown are typical of two experiments with similar results.

equivalent in IgG content to 0.1 mL of antiserum) produced a similar 85% inhibition of POD activity compared with IgG from preimmune serum when incubated with 0.1 mg of a human hepatic microsomal fraction. None of the other antisera raised here had any appreciable effect on enzyme activity (data not shown).

To rule out the possibility of a nonspecific effect of the antiserum on POD activity through, for example, binding of substrate or product to bring about an apparent inhibition of enzyme activity, the effect of the antiserum on POD activity of rat hepatic microsomal fraction was determined. Hepatic microsomal fraction from untreated rat contains

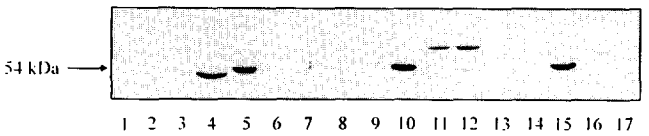

FIG. 5. Immunoreactivity of hepatic microsomal fraction from various species. Samples of 20 μg of hepatic microsomal fraction prepared from a pool of six human liver samples (lanes 5, 10, and 15), untreated hamster (lane 1), MC-treated hamster (lane 2), untreated cynomolgus monkey (lane 3), TCDD-treated cynomolgus monkey (lane 4), untreated rabbit (lane 6), MC-treated rabbit (lane 7), untreated marmoset (lane 8), TCDD-treated marmoset (lane 9), untreated guinea pig (lane 11), MC-treated guinea pig (lane 12), untreated mouse (lane 13), MC-treated mouse (lane 14), untreated rat (lane 16), and MC-treated rat (lane 17) were subjected to immunoblotting using the antipeptide antiserum raised against residues 291–302 of human CYP1A2. Antiserum was diluted 1:4000. Immunoreactive bands were detected as described in Materials and Methods. Only the central section of the blot containing immunoreactive material is shown.

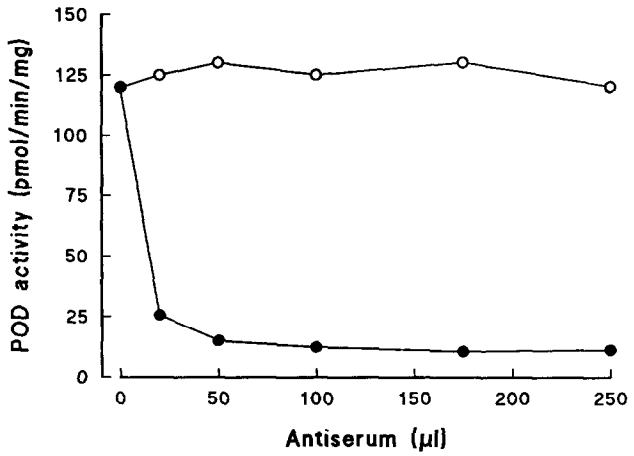

FIG. 6. Inhibition of phenacetin O-deethylase activity of human hepatic microsomal fraction by antipeptide antiserum. Microsomal protein (100 μg), prepared from a pool of seven human liver samples, was incubated for 10 min at 37°C with varying volumes of antipeptide antiserum raised against residues 291–302 of human CYP1A2 (●) or preimmune serum (○). The reaction was then started by addition of 4 μM phenacetin and incubated for 20 min at 37°C. POD activity was measured as described in Materials and Methods.

similar amounts of CYP1A2 to human (this was confirmed by immunoblotting with an antibody that recognises CYP1A2 in both species), but rat CYP1A2 catalyses the O-deethylation of phenacetin about three times slower than the human enzyme. Therefore, antiserum was mixed with different amounts of a rat hepatic microsomal fraction so that incubations contained either similar amounts of CYP1A2 apoprotein (using 0.1 mg of the microsomal fraction) or activity (using 0.3 mg of the microsomal fraction) to those found in humans. Under these conditions, 0.1 mL of antiserum had essentially no effect on rat POD activity, which was 38 and 36 pmol/min/mg protein in incubations containing 0.1 and 0.3 mg protein, respectively, compared with 40 pmol/min/mg protein in the presence of the preimmune serum.

The antiserum had no effect on human hepatic coumarin 7-hydroxylase activity. Activities were 1.19 and 1.24 nmol/min/mg protein in the presence of the preimmune serum and antiserum (0.1 mL), respectively. Similarly, human hepatic debrisoquine 4-hydroxylase activity was unaffected by the antibody. Activities were 120 and 114 pmol/min/mg protein in the presence of the preimmune serum and antiserum (0.1 mL), respectively.

DISCUSSION

We have previously identified a proinhibitory region present on both rat CYP1A1 and CYP1A2 by directing antipeptide antibodies to surface regions of these P450 enzymes [8, 14]. However, neither of these antibodies was able to inhibit enzyme activity completely. This could have been because the proinhibitory region was not accurately targeted. If so, directing antibodies to adjacent regions might enable more potent inhibitory antibodies to be produced. Although targeting an overlapping region towards the *N*-terminus of rat CYP1A2 resulted in an antibody that bound strongly to native and denatured CYP1A2, this had no significant effect on enzyme activity [26]. A similar result was found when the equivalent region of human CYP1A2 was targeted [27]. In the present study, antibodies were directed against an overlapping region towards the C-terminus of human CYP1A2.

An antibody raised against the synthetic peptide, Ser-Lys-Lys-Gly-Pro-Arg-Ala-Ser-Gly-Asn-Leu-Ile, which represents residues 291–302 of human CYP1A2, bound strongly and specifically to CYP1A2, both as the recombinant enzyme and in human hepatic microsomal fractions. Binding was equally strong to native and denatured CYP1A2. The intensity of binding to CYP1A2 correlated strongly with high-affinity POD activity in microsomal fractions. The antibody did not bind to any recombinant P450 other than CYP1A2. Examination of protein sequence databases confirmed that the targeted sequence occurs only in CYP1A2.

High-affinity POD activity has been shown previously to be catalysed specifically by CYP1A2 in human hepatic microsomal fraction [28, 29]. POD activity at a substrate concentration of 4 μM was inhibited >90% when human hepatic microsomal fraction (0.1 mg) was preincubated with antiserum (0.1 mL). This decrease in activity represents complete inhibition of CYP1A2 activity, residual activity being due to low-affinity enzymes [30]. Similar inhibition was found using purified immunoglobulin fraction from the antiserum. In contrast, the antibody, which does not bind to rat CYP1A2, had no effect on high-affinity POD activity in the rat hepatic microsomal fraction, which is also catalysed by CYP1A2. This suggests that the effect on human hepatic CYP1A2 is due to the inhibition of enzyme function and not by some other mechanism, such as adsorption of substrate. The antiserum had no effect on human hepatic microsomal debrisoquine 4-hydroxylase (CYP2D6) or coumarin 7-hydroxylase (CYP2A6) activities, indicating that, like binding, inhibition was specific to CYP1A2.

In an attempt to define more precisely the proinhibitory region of CYP1A2, antibodies were also raised against smaller peptides within the targeted region. Although the resultant antisera all contained antibodies against the respective peptides, none bound to human CYP1A2 in ELISA or immunoblots (data not shown). Peptides are highly flexible in solution and presumably also when bound *N*-terminally to carrier protein, adopting several different conformations. It is likely that one or more of these conformations is the preferred immunogenic conformation. Obviously, only those peptides adopting such conformations that also occur in the native protein will result in the production of antibodies capable of binding to intact P450. Interestingly, in the present case, the proinhibitory antibody recognised both native and denatured proteins, suggesting that the conformation of this epitope is not subject

TABLE 2. Comparison of known CYP1A2 sequences in the region equivalent to residues 291–302 of human CYP1A2

Species	Sequence	Reference
Human	-Ser-Lys-Lys-Gly-Pro-Arg-Ala-Ser-Gly-Asn-Leu-Ile-	[19]
Cynomolgus	- - - - - - - - - - - -	[41]
Marmoset	-Cys-Glu- -Arg-Ser-Gly- - - -Asp- - -	[41]
Rat	- -Glu-----Asn-Tyr-Lys-Asp-Asn- -Gly- - -	[20]
Mouse	- -Glu-----Asn-Tyr-Lys-Asp-Asn- -Gly- - -	[42]
Rabbit	- -Glu- -Asn-Ser-Lys- -Asn-Ser-Gly- - -	[43]
Hamster	- -Glu-----Asn-Ser-Lys-Asp- - -Gly- - -	[44]
Guinea pig	- -Glu-Gln-Ser- -His-Val- Asn- -Asp- - -	GenEMBL U23501

Where no amino acid is shown, it is identical to that of human CYP1A2.

to denaturation. As found here and previously, antibodies against longer peptides are more likely to crossreact with protein antigens, and it has been suggested that this is because larger peptides adopt structures in solution that better mimic the target regions in proteins [31]. Larger peptides can also produce a greater diversity of antibodies with potentially more immunogenic sites, thereby increasing the chance of an antibody recognising the protein.

The major epitope for the proinhibitory antibody was determined by comparing binding to peptides representing smaller regions of the immunising peptide. Compared with binding to the immunising peptide (residues 291–302 of CYP1A2), binding to the smaller peptides representing residues 291–298, 293–299, and 296–302 of CYP1A2 was only slightly reduced. In contrast, little binding to residues 291–296 was found indicating that few antibodies were produced against the *N*-terminal region, which is the end coupled to carrier protein. Similar observations have been made for other antipeptide antibodies [32–34].

Therefore, the proinhibitory sequence in human CYP1A2 overlaps and is slightly C-terminal to the proinhibitory region first identified in rat CYP1A2 [14]. The more complete inhibition of CYP1A2 by this antibody may be due to more accurate targeting of the proinhibitory region. In relation to the three-dimensional structure of P450, this region occurs in a loop region *N*-terminal to the I-helix [35]. Interestingly, human liver and kidney microsomal type-1 autoantibodies, which bind to CYP2D6 and inhibit its activity, have been mapped to a similar region on this enzyme [36–38]. Crystallographic studies of the structures of CYP101 and CYP102 have shown that this region is highly exposed to solvent and appears to be some distance from the active site [39, 40]. The mechanism by which an antibody binding to this region inhibits activity still has to be determined, but interference of electron transfer from P450 reductase is one possibility that deserves further attention. Clearly, binding does affect catalytic function rather than simply interfering sterically with the enzyme because binding of antibody to an overlapping region [27] has no effect on activity.

Binding of the antibody to CYP1A2 from other species was remarkably limited. Clear binding was seen only to TCDD-treated cynomolgus monkey hepatic microsomal fraction. The electrophoretic migration of the immunoreactive band and its presence in the TCDD-treated, but not untreated cynomolgus monkey hepatic microsomal fraction is consistent with previous observations [22]. Cynomolgus monkey CYP1A2 has recently been sequenced and is identical to human CYP1A2 in this region (Table 2). In contrast, no binding was seen to marmoset monkey CYP1A2, although induction of this enzyme has been shown previously with another anti-CYP1A2 antibody [26]. This is explained by differences in the structure of marmoset monkey CYP1A2 compared with human CYP1A2 in this region (Table 2). Similarly, the lack of binding of the antibody to rat, mouse, rabbit, and hamster CYP1A2 is consistent with substantial differences in the primary structure of these enzymes in the equivalent regions to that targeted in human CYP1A2, particularly C-terminally (Table 2). In the guinea pig hepatic microsomal fraction the antibody bound to a protein with a higher molecular weight than CYP1A2 that was not inducible by treatment with MC. However, a weak, inducible immunoreactive band that migrated to the expected position of CYP1A2 was also seen. As high levels of CYP1A2 are expressed in MC-treated guinea pig liver [26], it would appear that the antibody binds only poorly to guinea pig CYP1A2. This may occur through binding to the C-terminus of the targeted region that contains some structural similarity to human CYP1A2. It would be interesting to raise antibodies that specifically target the equivalent regions of CYP1A2 in other species, where known, to determine if such antibodies have similar inhibitory potency to that found in humans.

In conclusion, an antipeptide antibody against residues 291–302 of human CYP1A2 selectively binds to CYP1A2 and potently inhibits CYP1A2-catalysed activity providing a powerful *in vitro* tool for the identification of the contribution of this enzyme in metabolism studies. These findings also confirm the importance of the region adjacent to the I-helix in the catalytic function of P450.

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