INHIBITORY ANTI-PEPTIDE ANTIBODY AGAINST HUMAN CYP3A4

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ABSTRACT:

An inhibitory anti-peptide antibody was raised against a 21-amino acid peptide (VKRMKESRLDQTQHRVDFLQ) corresponding to residues 253–273 of human cytochrome P450 3A4. High titer antibodies were produced by rabbits immunized with this peptide coupled to keyhole limpet hemocyanin, as judged by ELISA. Anti-peptide antibody recognized a single protein band in microsomes prepared from cells expressing recombinant human CYP3A4 in immunoblotting analysis. No immunodetectable proteins were found in microsomes containing other cytochrome P450 isoforms. In addition, the antibody did not recognize CYP3A5, a closely related isoform in the CYP3A family. In human liver microsomes, only one protein band which comigrated with human CYP3A4 was recognized by this antibody and the relative blotting intensity of this protein band correlated significantly with human CYP3A4-catalyzed testosterone 6β-hydroxylase activities (r = 0.96). More importantly, this antibody exhibited greater than 90–95% inhibition of testosterone 6β-hydroxylation, while other cytochrome P450-mediated reactions in human liver microsomes were not inhibited. Because of its specificity and inhibitory potency, this anti-peptide antibody should be a valuable tool in evaluating the role of CYP3A in mediating in vitro metabolism of therapeutic agents.

Significant progress has been made in recent years in the use of in vitro metabolism of therapeutic agents to address the potential in vivo induction, inhibition, drug-drug interaction, and individual variability issues (1–5). Central to these studies is the unambiguous identification of specific drug-metabolizing enzyme(s), particularly human cytochrome P450 P450 isoform(s) responsible for the metabolism of drugs. This objective can be achieved by using selective cytochrome P450 inhibitors, antibodies, recombinant cytochrome P450s, and correlation analysis (2).

Polyconal or monoclonal antibodies produced against purified cytochrome P450 or specific peptide sequence unique to individual cytochrome P450 isoforms have been used widely to study the regulation, structure, and function of cytochrome P450s (6–10). Specific and inhibitory antibodies are extremely useful tools for the identification of specific cytochrome P450 involvement in the in vitro metabolism of therapeutic agents. However, because of a limited supply of purified single cytochrome P450s used for immunization, antibodies against specific human cytochrome P450s are not always available for such studies. In addition, antibodies raised against specific cytochrome P450 isolated from human liver tissues potentially could cross react with other cytochrome P450 isoforms (11). The nonspecificity is possibly a result of contamination with structurally related cytochrome P450s which are difficult to separate. Cross reactivity has also been reported even when purified recombinant cytochrome P450 was used as an immunogen (12, 13). This is because some of these antibodies can recognize regions of high sequence homology in related cytochrome P450s. By using a peptide that targets a specific region of the protein as an immunogen, one may overcome these problems and hopefully generate antibodies with less potential for cross reactivity. To this end, we have attempted to generate antibodies against specific peptide sequences for different cytochrome P450 isoforms. Since many of the peptide antibodies reported in the literature are either noninhibitory or only partially inhibitory (14–17), we have focused on the production of highly specific and highly inhibitory antibodies against cytochrome P450s. Partially inhibitory antibodies are not useful for the identification of the specific cytochrome P450s responsible for the metabolism of therapeutic agents since one cannot determine whether partial inhibition is a result of the involvement of additional cytochrome P450 isoforms or the weak inhibitory property of the antibodies. In this paper we report the production of antibodies that are inhibitory against a 21-amino acid peptide corresponding to amino acid 253–273 of human CYP3A4. To our knowledge, this is the first peptide antibody against CYP3A4 that shows greater than 90–95% inhibition on CYP3A4-mediated reactions.

Materials and Methods

Materials. Testosterone, 6β-hydroxytestosterone, phenacetin, tolbutamide, chloroxazone, corticosterone, glucose 6-phosphate, NADP, and glucose 6-phosphate dehydrogenase were purchased from Sigma Chemical Co. (St. Louis, MO). Acetaminophen was obtained from Aldrich Chemical Co. (Milwaukee, WI). Methylhydroxytolbutamide and 6-hydroxychloroxazone were obtained from Research Biochemical International (Natick, MA). Bufuralol and 1′-hydroxybufuralol were purchased from Gentest Corp. (Woburn, MA). All other reagents and solvents were of high analytical grade supplied by Fisher Scientific (Fair Lawn, NJ).

Human liver microsomal preparations were kindly provided by Dr. Jerome Lasker (Mount Sinai Medical Center, New York, NY).

Peptide Synthesis and Conjugation to Carrier Protein. The peptide (VKRMKESRLDQTQHRVDFLQ) was synthesized on an Applied Biosystems 430 A peptide synthesizer using solid phase chemistry by AnaSpec, Inc. (San Jose, CA). A cysteine residue was introduced to the N-terminus for use in
conjugation to the carrier protein. The peptide was coupled to keyhole limpet hemocyanin (KLH)\(^1\) using N-succinimidyl bromoacetate as a cross-linking reagent (21).

**Antibody Production and Purification.** The immunization was performed at HRP Inc. (Denver, PA) according to the method of Vaitukaitis et al. (22). All animal protocols in this study were conducted in accordance with Merck Institutional Animal Care and Use Committee Guidelines. Rabbits were initially immunized with 100 \(\mu\)g of the peptide conjugated to KLH in complete Freund’s adjuvant by intranodal injection and then by sc injection with 250 \(\mu\)g in Freund’s incomplete adjuvant on day 20. Rabbits were continuously boosted at 3-week intervals with 125 \(\mu\)g of antigen in Freund’s incomplete adjuvant. Sera were collected 10 days after each injection. IgG fractions were purified from rabbit sera by caprylic acid precipitation and ammonium sulfate fractionation (23). The titers of the antisera were determined by ELISA as described (24).

**Western Blots.** Human liver microsomes (15 \(\mu\)g) or microsomes from cells containing recombinant human cytochrome P450 (50 \(\mu\)g) were subjected to 10% SDS-polyacrylamide gel electrophoresis according to Laemmli (25) and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) using the method of Towbin et al. (26). The nitrocellulose sheets were blocked with nonfat dry milk, incubated with antibody and then treated with \(^{125}\)I-Protein A (Amersham Corp.). The immunoblotted intensity was quantified by the pdi Imaging Densitometer and Quantity One Software (pdi Inc., Huntington Station, NY).

**Immunoinhibition.** Immunoinhibition was conducted by preincubating microsomes for 30 min at room temperature with various amounts of rabbit pre-immune IgG or anti-peptide IgG. Reaction was started by addition of buffer, NADPH-generating system and substrate as described for the following enzyme assays.

**Enzyme Assays.** Testosterone 6\(\beta\)-hydroxylation was determined as described (27). Microsomal samples were incubated with 100 \(\mu\)M testosterone in 100 mM potassium phosphate buffer (pH 7.4) with 1 mM EDTA, 6 mM MgCl\(_2\), and a NADPH-generating system consisting of 10 mM glucose 6-phosphate, 1 mM NADP and 0.35 units glucose 6-phosphate dehydrogenase in a total volume of 0.5 ml. Reactions were performed at 37°C for 10 min with 0.34 mg of human liver microsomes containing 0.1 nmol cytochrome P450, and at 37°C for 20 min with 0.25 mg of microsomes prepared from human B-lymphoblastoid cells. After reactions were stopped by adding 5 ml of CH\(_2\)Cl\(_2\), the samples were spiked with 20 \(\mu\)l of 1 mM corticosterone as internal standard, vortexed and centrifuged at 3,000 \(g\) for 10 min. The organic layer was removed and evaporated to dryness under nitrogen stream. Samples were dissolved in 0.2 ml of methanol and analyzed by HPLC. Phenacetin O-deethylation (28), tolbutamide methylhydroxylation (29), bufuralol 1\''-hydroxylation (30), and chloroazoxone 6-hydroxylation (31) were determined for CYP1A2, CYP2C9/10, CYP2D6, and CYP2E1-mediated reactions, respectively. The substrate concentrations and incubation time used for each assay were 100 \(\mu\)M phenacetin for 20 min, 200 \(\mu\)M tolbutamide for 60 min, 100 \(\mu\)M bufuralol for 10 min, or 500 \(\mu\)M chloroazoxone for 20 min. Reactions were quenched by adding 0.05 ml of 85% H\(_2\)PO\(_4\). Samples were centrifuged at 14,000 \(\times\) \(g\) for 10 min, and the supernatants were directly injected for HPLC analysis.

**HPLC Analysis.** The HPLC used was a Shimadzu SCL 10A system controller consisting of two LC 10AS pumps, a SIL 10A automatic sample injector, SPD10A UV-VIS spectrophotometric detector, and RF10A spectrofluorometric detector. Aliquots of 50 \(\mu\)l samples from the testosterone 6\(\beta\)-hydroxylation incubation were injected onto a Zorbax ODS C18 column (4.6 mm \(\times\) 250 mm, 5 \(\mu\)m, Sigma-Aldrich, Milwaukee, WI). Substrate and metabolite were eluted from the column with methanol (7.5% tetrahydrofuran): H\(_2\)O (7.5% tetrahydrofuran) by a linear gradient from 35% to 60% in 35 min at a flow rate of 1 ml/min and monitored at 254 nm. The retention times for 6\(\beta\)-hydroxytestosterone, corticosterone, and testosterone were 8.9, 17.5, and 25.2 min, respectively. Chromatographic assays were carried out for phenacetin O-deethylation, tolbutamide methylhydroxylation, bufuralol 1\''-hydroxylation and chloroazoxone 6-hydroxylation on a Zorbax SB C8 column.

\(^1\) Abbreviations used are: CYP, cytochrome P450; ELISA, enzyme-linked immunosorbent assay; KLH, keyhole limpet haemocyanin; LKM-1 autoantibodies; liver-kidney microsomal-1 autoantibodies.
co-migrated with recombinant human CYP3A4. In liver microsome UC9402, no immunoreactive band was detected, consistent with the observation that this particular microsomal preparation had very little detectable testosterone 6β-hydroxylase activity. The quantity of immunoblotted protein in human liver microsomes correlated significantly with testosterone 6β-hydroxylase activity (fig. 4).

The specificity of this anti-peptide antibody was also evaluated against CYP3A5, a closely related isoform found in human liver. In this 21-amino acid sequence, CYP3A5 differs from CYP3A4 by only five amino acids. When examined by immunoblotting, this antibody did not recognize CYP3A5 (fig. 5A) whereas an antibody prepared against purified CYP3A4 recognized both CYP3A4 and CYP3A5 (fig. 5B). Thus, the specificity of this anti-peptide antibody is quite remarkable. Consistent with this observed specificity, CYP3A5-catalyzed testosterone 6β-hydroxylation was not inhibited by this antibody (data not shown).

**Effect of the Anti-peptide Antibody against CYP-Mediated Reactions.** Purified IgG strongly inhibited testosterone 6β-hydroxylation, a CYP3A4-catalyzed reaction, in human liver microsomes (fig. 6A) and microsome prepared from human B-lymphoblast cells expressing recombinant human CYP3A4 and P450 reductase (fig. 6B). Greater than 90% of the activities were inhibited at an IgG to cytochrome P450 ratio of 2.5 in human liver microsomes. In contrast, the purified IgG showed little or no inhibition toward other human cytochrome P450-mediated reactions, namely, phenacetin O-deethylation, tolbutamide methyldihydroxylation, bufuralol 1′-hydroxylation, and chlorzoxazone 6-hydroxylation (fig. 7).
Discussion

To select a peptide to produce inhibitory antibody with high specificity for a single isoform of cytochrome P450, many criteria need to be considered. These include structural characteristics of the peptide, degree of sequence homology of the targeted peptide compared with the sequence of other isoforms, and the location of the regions in the enzyme involved in substrate binding and recognition. Because three-dimensional structures of mammalian cytochrome P450s are not available, the proposed substrate recognition sites have been located by using amino acid sequence alignment with bacterial cytochrome P450s (32, 36, 37). Many cytochrome P450 epitopes in various regions of the target cytochrome P450s have been identified by the preparation of antibodies against selected peptides (9, 13, 38 – 41). For example, Leeders et al. (42) identified a minimum antibody-binding sequence in CYP3A1 which located in the K-helix of the protein by epitope mapping studies.

The peptide used in this study is hydrophilic, with high surface probability and located in the loop region between G helix and H helix which is near to but not in any substrate recognition sites proposed previously. Manns et al. (43, 44) reported the presence of inhibitory antibodies against CYP2D6 in certain individuals and identified a 33-amino acid sequence in this region containing the epitope(s) recognized by these autoantibodies. In addition, the common epitope of LKM-1 autoantibodies was identified as a 8-amino acid peptide (DPAQPPRD) corresponding to the residues 263–270 of CYP2D6 (44). Furthermore, inhibitory peptide antibodies against human CYP2D6 were produced previously by selecting a 20-amino acid peptide (residues 254 –273) in this region as immunogen (35).

When rabbits were immunized with the same KLH-conjugated peptide, high titer antibodies against CYP3A4 were produced in all three rabbits as judged by ELISA. However, antibodies produced by two rabbits were not inhibitory. Thus, only the highly inhibitory antisera were purified and characterized in this study. The reason for this animal dependent variation in inhibitory property of antibodies is unknown. In a previous study (35), we also noted that all rabbits produced high titer antibodies against a peptide (residues 254 –273) of CYP2D6 and antibodies have variable degree of inhibitions against CYP2D6-mediated dextromethophan O-demethylation (44). Furthermore, inhibitory peptide antibodies against human CYP2D6 were produced previously by selecting a 20-amino acid peptide (residues 254–273) in this region as immunogen (35).

When rabbits were immunized with the same KLH-conjugated peptide, high titer antibodies against CYP3A4 were produced in all three rabbits as judged by ELISA. However, antibodies produced by two rabbits were not inhibitory. Thus, only the highly inhibitory antisera were purified and characterized in this study. The reason for this animal dependent variation in inhibitory property of antibodies is unknown. In a previous study (35), we also noted that all rabbits produced high titer antibodies against a peptide (residues 254–273) of CYP2D6 and antibodies have variable degree of inhibitions against CYP2D6-mediated dextromethophan O-demethylation in human liver microsomes. Such observed variable results are probably not just limited to the use of peptide as immunogen. Other investigators have reported that antibodies against purified, recombinant human cytochrome P450s exhibit different degrees of inhibition on metabolism.

![Western Blot detection of CYP3A4 and CYP3A5 in microsomes prepared from baculovirus infected insect cells.](image1)

**Fig. 5.** Western Blot detection of CYP3A4 and CYP3A5 in microsomes prepared from baculovirus infected insect cells.

Microsomes prepared from control insect cells or cells containing expressed CYP3A4 and CYP3A5 were immunoblotted with anti-peptide antibody (panel A) or antibody against purified CYP3A4 (panel B).

**Fig. 6.** Inhibition of testosterone 6β-hydroxylation by anti-peptide IgG.

Human liver microsomes containing 0.1 nmol cytochrome P450 (A) or microsomes (0.25 mg) prepared from human B-lymphoblastoid cells expressing human CYP3A4 and P450 reductase (B) were preincubated with 0.05–1.5 mg of preimmune IgG (■) or anti-peptide IgG (○). The control activities of testosterone 6β-hydroxylation were 3.15 nmol/min/nmol P450 for human liver microsome and 1.08 nmol/min/mg protein for microsome from cells expressing human CYP3A4 and P450 reductase.

![Inhibition of testosterone 6β-hydroxylation by anti-peptide IgG.](image2)

**Fig. 7.** Effect of anti-peptide antibody on CYP-mediated reactions.

CYP1A2, CYP2C9/10, CYP2D6, and CYP2E1-mediated reactions were determined after 30 min preincubation of human liver microsomes containing 0.1 nmol cytochrome P450 with 0.05–1 mg of preimmune IgG or anti-peptide IgG. The control activities were 2.39, 0.19, 3.46, and 7.35 nmol/min/nmol P450 for phenacetin O-deethylation (●), tolbutamide methylhydroxylation (■), bufuralol 1′-hydroxylation (▲), and chlorzoxazone 6-hydroxylation (▼), respectively. There were no significant inhibitions on all reactions when preimmune serum was used in the incubations.
(12). Soucek et al. (13) reported that polyclonal antibodies raised against recombinant human P450s varied in specificity, depending upon the individual rabbits used. More studies are needed to investigate the individual variability on the production of inhibitory antibodies in animals. Before we can consistently generate inhibitory antibodies in all animals, it is important to screen each animal individually to determine the inhibitory potency of the antibody on metabolism.

On the basis of the results from western blots and inhibition studies, the anti-peptide antibody can bind to both native and denatured forms of human CYP3A4. The binding of this antibody to CYP3A4, but not other cytochrome P450 isoforms including the closely related CYP3A5, confirms that the amino acid sequence in this loop region is unique for CYP3A4. Production of inhibitory antibodies against CYP3A4 peptide provides a valuable tool for evaluating the role of this important cytochrome P450 in mediating in vitro metabolism of new therapeutic agents. In addition, noninhibitory antibodies can be used for cytochrome P450 epitope investigation, gene expression and regulation, tissue localization, and many other studies. In conclusion, the amino acid sequence near the loop region between G helix and H helix seems to be an excellent target for the design of inhibitory antibodies against various forms of cytochrome P450.

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References


