Identification of the Pharmacogenetic Determinants of Alfentanil Metabolism: Cytochrome P-450 3A4

An Explanation of the Variable Elimination Clearance


There is considerable variability in the elimination clearance of the opioid analgesic alfentanil. It has been shown previously that alfentanil clearance is independent of the polymorphic debrisoquine hydroxylase (P-450 2D6), and it is therefore of interest to identify the human cytochrome P-450 enzymes involved in noralfentanil formation, the primary reaction involved in the oxidative N-dealkylation at the piperidine nitrogen. Purified human P-450 3A4 showed appreciable catalytic activity, and yeast recombinant P-450 3A4 also showed alfentanil oxidation activity. When microsomes prepared from different human liver samples were compared, nor-alfentanil formation activity was well correlated (r = 0.95, P < 0.005) with nifedipine oxidation (a P-450 3A4 marker) but not with markers of other P-450s, including phenacetin O-deethylation (P-450 1A2), chlorzoxazone 6-hydroxylation (P-450 2E1), and (S)-mephenytoin 4'-hydroxylation (a P-450 2C enzyme). Using antibodies that recognize specific human P-450 enzymes (immunoinhibition techniques), it was possible to demonstrate that anti-P-450 3A4 nearly completely inhibited alfentanil oxidation activity in the human liver microsomes, but no other antibodies showed a measurable inhibitory effect. Selective chemical inhibitors of P-450 3A4, gestodene and troleandomycin, inhibited as much as 90% of the microsomal nor-alfentanil formation activity, but other chemical inhibitors did not show a detectable inhibitory effect. 7,8-Benzoflavone inhibited as much as 90% of the alfentanil oxidation activity of the microsomal or reconstituted P-450 3A4 system. This work indicates that P-450 3A4 contributes significantly to human liver microsomal alfentanil oxidation, whereas P-450 2D6 does not contribute. Human P-450 3A4 metabolizes many clinically important substrates, including nifedipine, cyclosporin, midazolam, lidocaine, and quindine, and is induced by barbiturates and some antibiotics. Therefore, the possibility exists for pharmacokinetic interactions between alfentanil and other concomitantly administered drugs that are also substrates for, or inducers of, P-450 3A4. (Key words: Analgesics: alfentanil. Anesthetics, intravenous: alfentanil. Biotransformation: alfentanil. Liver: microsomes. Metabolism: cytochromes P-450, P-450 3A4, P-450 2D6; genetic factors; immunoinhibition.)

Although the use of the synthetic opioid alfentanil has become well established in anesthetic practice, there is considerable interindividual variability in the plasma concentration time profile among patients, resulting in marked difference among individuals in the rate of alfentanil infusion required.1 Using alfentanil population pharmacokinetic parameters, Maitre et al. determined that after taking into account age, weight, and gender, 48% of the variability in alfentanil clearance remained unexplained.2 Although intersubject variability in drug metabolism has been known for many years, it is only recently that the pharmacogenetic determinants of drug oxidation have been defined.3 A defect in the ability to hydroxylate the antihypertensive agent debrisoquine is present in 5–10% of Caucasians,4 and individuals can be classified as either extensive metabolizers or poor metabolizers because of genetic polymorphism.

The clinical importance of this genetically determined impairment of debrisoquine oxidation stems from the wide spectrum of drugs the metabolism of which is also impaired in poor metabolizers of debrisoquine. However, Henthorn et al.5 demonstrated that alfentanil clearance is not altered in poor metabolizers of debrisoquine and is thus independent of the polymorphic enzyme debri-
soquine 4-hydroxylase (cytochrome P-450 2D6). In contrast to the bimodal distribution of enzyme function associated with debrisoquine genetic polymorphism, it appears that the wide interindividual activity of the enzyme cytochrome P-450 3A4, which has been shown to be pivotal for the metabolism of a wide variety of drugs, including nifedipine, midazolam, lidocaine, cyclosporin and erythromycin, exhibits a broad unimodal distribution of enzyme activity without a distinct subgroup.

Because the variability of alfentanil pharmacokinetics demonstrated by Maître et al. also did not appear to be polymorphic, we hypothesized that alfentanil oxidation was more likely to be catalyzed by the enzyme cytochrome P-450 3A4. With the use of antibodies that recognize different human cytochrome P-450 enzymes, it is possible to identify the specific enzymes involved in reactions of alfentanil metabolism. Thus, the goal of the present study was to define the enzymes in human liver that are responsible for alfentanil oxidation by using immunoinhibition and recombinant techniques, including expression of the cytochrome P-450 of interest in yeast.

Materials and Methods

CHEMICALS

[3H]-Alfentanil (12.3 Ci/mmol), labeled with 3H at the 3-position of the phenyl ring, and its unlabeled metabolites were obtained from Janssen Research Foundation (Beerse, Belgium); [3H]-alfentanil was > 99.9% radiochemically pure as judged by reverse-phase high performance liquid chromatography (HPLC) and was used without further purification. 7,8-Benzoflavone and quinidine were purchased from the Aldrich Chemical Co. (Milwaukee, WI). Gestodene was provided by Professor H. Kuhl (University of Frankfurt). Troleandomycin was a gift of Professor P. H. Beaune (Necker Institut National de la Santé et de la Recherche Médicale, Paris). Sulfinpyrazone was obtained as a gift from the Meiji Yakuhin Co. Ltd. (Tokyo).

†The nomenclature used here for the P-450 enzymes is that described by Nebert et al. For reviews of human P-450 enzymes see references 51–57. Four different human P-450 enzymes in the P-450 3A family have been described, and all are probably recognized by the antibody used in the current study. There is evidence that P-450 3A4 is the major one of these enzymes expressed in adult human liver: P-450 3A7 appears to be restricted primarily to fetal liver, and P-450 3A5 is expressed in only about 25% of the population and usually at levels approximately one-third that of P-450 3A4.57 and P-450 3A5 was shown to be expressed at a level <5% of that of P-450 3A4 in 12 different liver samples. The number of genes in the human P-450 3A family is probably not greater, since only three genomic fragments were recognized in common by long, nonoverlapping complementary DNA probes. The total size of the hybridizing gene fragments is in the range of 50–60 kb, and Kamataki and associates recently have characterized the P-450 3A4 and 3A7 genes and found both to be 27 kb in length (personal communication).

Other chemicals were of the highest grade commercially available.

ENZYME PREPARATIONS AND ANTIBODIES

Human liver samples were obtained from organ donors through Tennessee Donor Services (Nashville, TN; denoted “HL” for human liver and including a code number). Microsomes were prepared as described elsewhere.7 Human P-450 3A4,8 P-450 2C8,9 P-450 2C9 (a P-450 2C enzyme with (S)-mephentoyin 4'-hydroxylase),10 P-450 2E1,11 P-450 1A2,12 and P-450 2D6,12 were prepared as described elsewhere. Rabbit liver NADPH-P-450 reductase was purified essentially as described elsewhere.13 Rabbit anti-P-450 3A4,8 anti-P-450 2C14,15 anti-P-450 2E1,15 anti-P-450 1A2,12 and anti-P-450 2UT (2D1)16 preparations have been characterized previously.

Yeast (Saccharomyces cerevisiae) expressing P-450 3A4 came from previous work,17 and the protein was partially purified using slight modifications of a method described elsewhere.8,17

ASSAYS

Protein was assayed using a bicinchoninic acid procedure according to the manufacturer’s directions (Pierce Chemical Co., Rockford, IL). P-450 concentrations were determined by Fe²⁺-CO versus Fe²⁺ difference spectroscopy.18

Noralfanil formation was analyzed with the procedure of Lavrijsen et al.19 with slight modifications. Each of the purified P-450s (10–50 pmol) was reconstituted immediately before use in the enzyme assays in 0.5 ml of 50 mM potassium phosphate buffer (pH 7.4) containing 30 μM L-α,1,2-diarnyl-sn-glycerol-3-phosphocholine and a 2-fold molar excess of rabbit liver NADPH-P-450 reductase. In the case of microsomal assays, the microsomal protein (100 μg) was incubated in 0.5 ml 50 mM potassium phosphate buffer (pH 7.4). After a 3-min preincubation in the presence of alfentanil (10 μM), the reactions were initiated by the addition of an NADPH-generating system using open glass vials in a shaking water bath at 37°C. The reactions were stopped (usually after 5–10 min) by the addition of 1.0 ml CH₂Cl₂ with mixing, using a vortex device.

After separation and concentration of the organic phase to dryness under N₂ gas, the residue was dissolved in 50 μl CH₃OH and aliquots (20 μl) were analyzed immediately by HPLC. Metabolite analysis was performed on a Zorbax octadecylsilane (C₁₈) HPLC column (4.6 × 150 mm; MacMudd, Chadds Ford, PA). The solvents used were 0.1 M ammonium acetate (pH 6.2) (solvent A) and a mixture of 1.0 M ammonium acetate (pH 6.2)/acetonitrile/methanol/tetrahydrofuran (10:30:20:40, v/v/v/v) (sol-
vent B) with a linear gradient running from 75% solvent A and 25% solvent B to 50% solvent A and 50% solvent B (v/v) over 20 min. The flow rate was 1 ml/min. The eluent was monitored at 230 nm. Quantitation of products
was done using a Radiometric Flo-one counter (Radmatic,
Tampa, FL) after the effluent was mixed with liquid scin
tillation cocktail (Flo-scint II, Radomatic).

INHIBITION STUDIES

Immunoinhibition studies were done by incubating
human liver microsomes with varying amounts of an
tibodies (immunoglobulin G fraction) for 20 min at 23°C
before addition of other components required for catalytic
activity. In control experiments, varying levels of the
preimmune immunoglobulin G were used.

Inhibition of noralfentanil formation by gestodene,
troleandomycin, sulfaphenazole, diethylthiocarbamate,
and quinidine were determined using human liver microsomes; the general methods have been presented else
derwhere. Briefly, microsomes were incubated with the
compound under consideration and an NADPH-generat
ing system in a small volume (50 μl) for 10 min and
then diluted 10-fold for assay of catalytic activity. The
same approach was used with 7,8-benzoflavone, which
can either inhibit or stimulate different P-450 en
zymes.

Results

ALFENTANIL OXIDATION ACTIVITIES OF PURIFIED
HUMAN LIVER P-450s AND COMPARISON TO HUMAN
LIVER MICROSOMES AND RECOMBINANT P-450 3A4

Alfentanil was rapidly metabolized in human liver mi
crosomes, yielding noralfentanil as the main metabolite,
as reported previously. The human liver microsomes
prepared from sample HL 110, known to be high in P-
450 3A4, had the highest alfentanil oxidation activity (ta
ble 1). The catalytic activities of several purified human
liver P-450s were measured and compared to the most
active microsomal preparation (from HL 110) (table 1).
Only the P-450 3A4 preparation had alfentanil oxidation
activity. Also, yeast microsomes expressing P-450 3A4
showed catalytic activity. Purified P-450 3A4 from these
yeast also showed activity. Neither purified P-450 1A2,
P-450 2C8, P-450 2D6, P-450 2E1, nor P-450 2CMP cata
lyzed alfentanil oxidation (data not shown).

CORRELATION OF RATES OF NORALFENTANIL
FORMATION WITH OTHER ENZYMIC ACTIVITIES
IN HUMAN LIVER MICROSOMES

Noralfentanil formation rates ranged from 0.14 to 4.38
(nmol product formed)·min⁻¹·(mg microsomal pro
tein)⁻¹ in microsomes prepared from 15 different human
liver samples, a difference of 31-fold (fig. 1). Importantly,
good correlation was observed between rates of noralfen
tanil formation and nifedipine oxidation, a marker of P-
450 3A4 in different human liver microsomes (r = 0.95,
P < 0.0005; fig. 1A).

No significant correlation (P > 0.10) of rates of nor
alfentanil formation was seen with rates of phenacetin O-
deethylation (P-450 1A2) (fig. 1B), chlorzoxazone 6-hy
droxylation (P-450 2E1) (fig. 1C), or (S)-mephentoin 4'-
hydroxylation (P-450 2CMP) (fig. 1D). Previous studies
had shown no correlation with P-450 2D6.

IMMUNOINHIBITION OF ALFENTANIL OXIDATION
ACTIVITY BY ANTIBODIES IN HUMAN
LIVER MICROSOMES

To determine which P-450 is primarily responsible for
alfentanil oxidation, immunoinhibition studies with anti-
P-450 3A4, anti-P-450 2C, anti-P-450 2D1, anti-P-450
1A2, and anti-P-450 2E1 were carried out. Only anti-
P-450 3A4 inhibited the alfentanil oxidation activity in
human liver sample HL 110 microsomes (fig. 2). None of
the other antibodies inhibited the alfentanil oxidation
activity. These results strongly suggest that P-450 3A4 is
responsible for almost all of the alfentanil oxidation ac
tivity in human liver.

INHIBITION OF ALFENTANIL OXIDATION ACTIVITY
BY CHEMICALS IN HUMAN LIVER MICROSOMES

Gestodene and troleandomycin are effective inhibi
tors of P-450 3A enzymes, whereas sulfaphenazole inhibits tolbutamide hydroxylation (P-450 2C9 and 2C10)
in human liver microsomes and in vivo. Diethylthio
 carbamate and quinidine are selective inhibitors of P-450 2E1 and P-450 2D6, respectively. Neither sul
faphenazole, diethylthiocarbamate, nor quinidine had

<table>
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<tr>
<th>Sample</th>
<th>Noralfentanil Formation (nmol product formed·min⁻¹·nmol P-450⁻¹)</th>
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<tbody>
<tr>
<td>Human liver microsomes</td>
<td></td>
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<tr>
<td>HL 110</td>
<td>5.15</td>
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<tr>
<td>Purified human liver P-450s</td>
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<tr>
<td>1A2</td>
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<td>2C8</td>
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<td>2CMP</td>
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<td>2D6</td>
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<tr>
<td>2E1</td>
<td>&lt;0.01</td>
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<tr>
<td>3A4</td>
<td>0.86</td>
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<tr>
<td>Yeast recombinant P-450s</td>
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<td>3A4 (yeast microsomes)</td>
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<tr>
<td>3A4 (partially purified from yeast)</td>
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any measurable effect. In contrast, gestodene and troleandomycin (50 µM), inhibitors of P-450 3A enzymes, inhibited 69–88% of the alfentanil oxidation activity in the most active human liver microsomal sample (HL 110) among 15 microsomal preparations (fig. 3). This finding is consistent with the results of the antibody work described in the previous section, which suggest that the major P-450 enzymes catalyzing alfentanil oxidation are P-450 3A4 or other enzymes in the P-450 3A subfamily.

**EFFECT OF 7,8-BENZOFLAVONE ON OXIDATION ACTIVITY CATALYZED BY HUMAN LIVER MICROSONES OF PURIFIED P-450 3A4**

7,8-Benzoflavone was added to the human liver microsomal samples from HL 110 to examine its effect on the alfentanil oxidation activity of P-450 3A4, since some activities of this enzyme are stimulated by this compound.28,29 At a concentration of 50 µM 7,8-benzoflavone, the alfentanil oxidation activity in the microsomal system from HL 110 was inhibited by 89% (fig. 4). In the case of reconstituted purified P-450 3A4, 7,8-benzoflavone (50 µM) inhibited 78% of the alfentanil oxidation activity.

**Discussion**

This study has clearly demonstrated that the liver enzyme principally responsible for the oxidation of alfentanil is P-450 3A4. The main metabolic pathway for alfentanil is oxidation (N-dealkylation) with the formation of the metabolite noralfentanil.19,21 We determined the extent of oxidation of alfentanil to its major metabolite noralfentanil by an HPLC assay in human liver microsomes. Antibody specific to P-450 3A enzymes completely inhibited alfentanil oxidation to noralfentanil, whereas the antibodies inhibitory to other P-450 enzymes (including P-450 2D6, the debrisoquine 4-hydroxylase) exhibited no measurable effect. In addition, the selective inhibitors of P-450 3A4 gestodene and troleandomycin inhibited as
much as 90% of microsomal noralfentanil formation. Thus, our data indicate that P-450 3A4 catalyzes the metabolism of alfentanil to noralfentanil.

We suspected that 7,8-benzoflavone might enhance the formation of noralfentanil, because some P-450 3A4 reactions are stimulated by this compound. However, inhibition was observed (fig. 4). This observation is not inconsistent with a role for P-450 3A4, because we recently have demonstrated that the enzyme can oxidize aflatoxin B₁ to both the 8,9-exopoxide and the 3α-alcohol: the former reaction is stimulated by 7,8-benzoflavone, whereas the latter reaction is inhibited. These observations were made with the yeast recombinant enzyme as well as in human liver microsomes and have been interpreted in terms of an allosteric mechanism. Thus, caution is needed in the interpretation of effects of selective modifiers of P-450–catalyzed reactions.

Genetic polymorphism for the pathways of acetylation and hydrolysis (e.g., succinylcholine) have been recognized for many years; however, only a small number of drugs are metabolized by either of these pathways. Most drugs are metabolized by oxidative pathways, and increased knowledge of genetic polymorphism for the oxidative pathways has been one of the most important recent advances in understanding interindividual variability in drug metabolism and pharmacokinetics. Genetic defects in the metabolism of sparteine and debrisoquine were the first oxidation defects to be identified. Subsequently, these were shown to reflect alterations in the activity of the same P-450 enzyme. Multiple genetically determined P-450 cytochrome enzymes exist, and each enzyme is assigned to families and subfamilies according to the degree of similarity of the amino acid sequence of the P-450 isozyme. The locus of the debrisoquine drug oxidation polymorphism, is perhaps the most well known subfamily with regard to genetic polymorphism, and the enzyme has been shown to exhibit marked stereoselective metabolism and a bimodal distribution. However, many commonly administered drugs have now been identified as substrates for another important enzyme, P-450 3A4, namely nifedipine, cyclosporin, erythromycin, midazolam, quinidine, and lidocaine.

Fig. 3. Effects of gestodene (open circles), troleandomycin (filled circles), sulfaphenazole (open squares), quinidine (filled squares), diethylthiocarbamate (triangles), and inhibit of P-450 2E1 (inhibitor of P-450 3A4 enzymes) on rates of noralfentanil formation by human liver microsomal sample HL 110.

Fig. 4. Effect of 7,8-benzoflavone on noralfentanil formation activities catalyzed by human liver microsomal sample HL 110 (open circles) and purified P-450 3A4 (filled circles).
The current study has now shown that P-450 3A is also responsible for the oxidation of the intravenous opioid alfentanil. There appears to be a broad unimodal distribution of enzyme activity for P-450 3A with a wide interindividual variability (more than one order of magnitude), and a distinct subgroup has not been identified. This results in marked interindividual variability in the pharmacokinetics of drugs metabolized by this enzyme, as indeed has been shown to be the case for alfentanil. P-450 3A4 is the enzyme principally responsible for the metabolism of the immunosuppressive drug cyclosporin, and the renal toxicity of cyclosporin is well recognized. To minimize toxicity by better predicting cyclosporin pharmacokinetics, the 14C-erythromycin breath test has been used as a measure of P-450 3A activity in vivo because P-450 3A enzymes also catalyze the N-de-methylation of erythromycin. Thus, the production of 14CO2 in the expired breath has been shown to predict cyclosporin blood levels.

Because so many drugs have now been shown to be substrates for P-450 3A, the possibility of important clinical pharmacokinetic drug interactions exist. It is interesting that erythromycin, an antibiotic frequently administered preoperatively, has been shown to inhibit the metabolism of alfentanil. Erythromycin administration for 7 days significantly increased alfentanil elimination half-life and decreased clearance, indicating that erythromycin inhibits the metabolism of alfentanil. Subjects differed in their sensitivity to erythromycin (in keeping with the demonstrated wide interindividual variation in activity of cytochrome P-450 3A4), so that alfentanil elimination is not predictable. A case report has described unconsciousness associated with midazolam and erythromycin in an 8-yr-old boy with a ventricular-septal defect given erythromycin for antibiotic prophylaxis before adenoidectomy. The plasma concentration of midazolam was greater in this patient than in six other children who did not receive erythromycin. Such interactions might also be predicted to occur for alfentanil.

Previous in vitro work, using the dehydroxylation of desmethylpipramine as a probe for debrisoquine hydroxylase activity, had suggested that alfentanil competitively inhibits the enzyme P-450 2D6 (debrisoquine hydroxylase). However, other in vitro studies have shown that debrisoquine is only a weak noncompetitive inhibitor of alfentanil metabolism and of the formation of its major metabolites. Although drugs that are metabolized by the same enzyme both bind to that enzyme, drugs may also bind to the enzyme but not themselves be metabolized by the enzyme. Thus, for example, quinidine binds tightly to P-450 2D6 and inhibits the clearance of drugs that are also metabolized by P-450 2D6, such as debrisoquine and propranolol. However, quinidine itself is not metabolized by this enzyme, so that in vivo, the metabolism of quinidine does not cosegregate with that of debrisoquine because it is metabolized not by P-450 2D6 but by P-450 3A4. There is, therefore, no difference in quinidine metabolism in poor and extensive metabolizers of debrisoquine.

Similarly, for alfentanil, alfentanil clearance in vivo is not altered in poor metabolizers of debrisoquine. We interpret these previous studies to imply that alfentanil binds to P-450 2D6 and hence inhibits the metabolism in vitro of debrisoquine. Because alfentanil is not itself metabolized by P-450 2D6 but rather, as we have shown, by P-450 3A4, debrisoquine will not competitively inhibit alfentanil clearance in vitro. Our demonstration that P-450 3A4 makes a major contribution to human liver microsomal alfentanil oxidation and that P-450 2D6 does not, therefore resolves the previous apparent conflict between the in vitro and in vivo data for alfentanil metabolism.

In summary, we have shown that P-450 2D6 (the debrisoquine 4-hydroxylase) is not responsible for the oxidation of alfentanil to noralfentanil, but that another enzyme, P-450 3A4, has the major role in the oxidation of alfentanil. Human cytochrome P-450 3A enzymes are known to metabolize many clinically important substrates, namely nifedipine, cyclosporin, midazolam, lidocaine, quinidine, and now alfentanil. Thus, the possibility exists for pharmacokinetic interactions between alfentanil and other concomitantly administered drugs that are also substrates for P-450 3A4. Alfentanil oxidation by P-450 3A4 in vivo would in large part explain the wide interindividual variation in alfentanil kinetics demonstrated perioperatively. Further research is required to demonstrate that alfentanil metabolism in vivo correlates with P-450 3A4 functional activity.

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References

ALFENTANIL METABOLISM BY CYTOCHROME P-450 3A4


