Metabolism of alfentanil by cytochrome P4503A (CYP3A) enzymes

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Nonstandard abbreviations:

AMX, N-phenylpropionamide; TAO, troleandomycin
Abstract

The synthetic opioid alfentanil is an analgesic and an *in vivo* probe for hepatic and first-pass CYP3A activity. Alfentanil is a particularly useful CYP3A probe because pupil diameter change is a surrogate for plasma concentrations, thereby affording noninvasive assessment of CYP3A. Alfentanil undergoes extensive CYP3A4 metabolism via two major pathways, forming noralfentanil and N-phenylpropionamide. This investigation evaluated alfentanil metabolism in vitro to noralfentanil and N-phenylpropionamide, by expressed CYP3A5 and CYP3A7 in addition to CYP3A4, with and without co-expressed or exogenous cytochrome \( b_5 \). Effects of the CYP3A inhibitors troleandomycin and ketoconazole were also determined. Rates of noralfentanil and N-phenylpropionamide formation by CYPs 3A4 and 3A5 in the absence of \( b_5 \) were generally equivalent, although the metabolite formation ratio differed, while those by CYP3A7 were substantially less. CYPs 3A4 and 3A5 were equipotently inhibited by troleandomycin, whereas ketoconazole was an order of magnitude more potent towards CYP3A4. Cytochrome \( b_5 \) qualitatively and quantitatively altered alfentanil metabolism, with \( b_5 \) co-expression having a greater effect than exogenous addition. Addition or co-expression of \( b_5 \) markedly stimulated the formation of both metabolites, and changed the formation of noralfentanil but not N-phenylpropionamide from apparent single-site to multi-site Michaelis-Menten kinetics. These results demonstrate that alfentanil is a substrate for CYP3A5 in addition to CYP3A4, and the effects of the CYP3A inhibitors troleandomycin and ketoconazole are CYP3A enzyme-selective. Alfentanil is one of the few CYP3A substrates which is metabolized in vitro as avidly by both CYPs 3A4 and 3A5. Polymorphic CYP3A5 expression may contribute to interindividual variability in alfentanil metabolism.
Introduction

Alfentanil is a short-acting synthetic opioid used most commonly for short surgical procedures. Alfentanil is a low- to intermediate extraction drug with low clearance (<5 ml•kg\(^{-1}\)•min\(^{-1}\)), which exhibits considerable interindividual variability and is proportional to alfentanil hepatic metabolism (Kharasch et al., 2004c). Interindividual variability in alfentanil clearance is therefore attributed to variability in alfentanil metabolism (Meuldermans et al., 1988). Nevertheless, interindividual variability in alfentanil clearance is otherwise incompletely understood (Kharasch et al., 1997; Kharasch et al., 2004c).

Alfentanil undergoes piperidine and amide N-dealkylation, respectively, to two major metabolites; noralfentanil and N-phenylpropionamide (AMX) (Fig 1), catalyzed predominantly in vitro by two independent hepatic CYP3A4-catalyzed pathways (Yun et al., 1992; Kharasch and Thummel, 1993; Labroo et al., 1995). Subsequent clinical investigations confirmed that alfentanil metabolism and clearance in vivo are also determined primarily by CYP3A activity (Kharasch et al., 1997; Kharasch et al., 2004c). Urine noralfentanil accounts for approximately 30% of the dose and 40% of metabolites recovered over 24 h. AMX formation in vivo has not been reported, due possibly to further metabolism to N-(4-hydroxyphenyl)propionamide and N-(4-hydroxyphenyl) acetamide, but amide N-dealkylation accounts for 20-25% of the dose and 30% of the 24 hr urinary metabolites, indicating the significance of this pathway in vivo as well as in vitro (Meuldermans et al., 1988).

Alfentanil has been used as an in vivo probe for hepatic and first-pass CYP3A activity and drug interactions, due to the considerable dependence of alfentanil clearance on CYP3A activity (Kharasch et al., 1997; Phimmasone and Kharasch, 2001; Kharasch et al., 2003; Kharasch et al., 2004c; Kharasch et al., 2004b). In addition, alfentanil causes pupil constriction (miosis) that is essentially log-linear between 10 and 100 ng/ml, and the time course of alfentanil-dependent miosis mirrors that of alfentanil plasma concentrations. Thus miosis is a surrogate for plasma concentration, and hence alfentanil miosis is an noninvasive in vivo probe for hepatic and first-pass CYP3A activity (Phimmasone and Kharasch, 2001; Kharasch et al., 2003; Kharasch et al., 2004c; Kharasch et al., 2004b).
The catalytically important members of the human CYP3A subfamily include CYP3A4 (the most quantitatively abundant CYP in human liver and intestine, accounting for 30-50% and 70% of total CYP, respectively), CYP3A5, and CYP3A7 (Burk and Wojnowski, 2004). CYP3A5 shares considerable sequence homology and qualitatively similar substrate selectivity with CYP3A4, and is polymorphically expressed (Xie et al., 2004). Quantitatively, however, the relative metabolic activities of CYPs 3A4 and 3A5 are substrate- and regio-selective. For most CYP3A substrates, *in vitro* intrinsic clearance (Vmax/Km) values are greater for CYP3A4 than CYP3A5 (Cook et al., 2002; Williams et al., 2002; Kalgutkar et al., 2003; Patki et al., 2003; Shen et al., 2004), although these CYPs may have equivalent specific activity for testosterone 6ß-hydroxylation (Kamdem et al., 2004). In contrast, midazolam is avidly metabolized by CYP3A5 in vitro, and 1'-hydroxylation by CYP3A5 can be 2 to 3-fold greater than by CYP3A4 (Gorski et al., 1994; Wandel et al., 1994; Williams et al., 2002; Yamaori et al., 2003). CYP3A7 was long considered to be expressed predominantly and exclusively in fetal liver, however significant and polymorphic CYP3A7 mRNA expression in adult human liver and intestine has recently been demonstrated, although the presence and significance of functional CYP3A7 expression in adult liver remains unknown (Burk and Wojnowski, 2004). For all or most CYP3A substrates, *in vitro* intrinsic clearance or Vmax values are much lower for CYP3A7 than CYP3A4 (Williams et al., 2002).

The overlapping substrate specificity for CYP3A enzymes suggests that alfentanil might be a substrate for CYP3A5, potentially explaining some of the considerable interindividual variability in alfentanil metabolism and clearance. Subsequent to the initial identification of CYP3A4 as the major catalyst of alfentanil metabolism, which did not evaluate CYP3A5 (Yun et al., 1992; Kharasch and Thummel, 1993; Labroo et al., 1995), an investigation with yeast-expressed CYP3A5 suggested that CYP3A5 did not catalyze alfentanil metabolism (Guitton et al., 1997). Nonetheless, the increasing interest in alfentanil as a CYP3A probe suggests that this merits reevaluation. The purpose of this investigation was to test the hypothesis that alfentanil is a substrate for CYP3A5 (and possibly CYP3A7), in addition to CYP3A4 and to characterize the kinetic parameters for CYP3A-catalyzed alfentanil metabolism using expressed CYP3A enzymes in vitro. Additional experiments elucidated the role of
cytochrome \( b_5 \) in alfentanil metabolism, and the CYP3A form-selective effect of the CYP3A inhibitors ketoconazole and troleandomycin (TAO).
Methods

Chemicals. Noralfentanil was a gift from Janssen Research Foundation (Piscataway, NJ). R38527 (used as an internal standard for noralfentanil and alfentanil assay) was purchased from Research Diagnostics (Flanders, NJ). Alfentanil was provided by the National Institute of Drug Abuse through Research Triangle Institute (Research Triangle Park, NC). AMX and ring-labeled d5-AMX were synthesized as described previously (Labroo et al., 1995). Ketoconazole was obtained from Biomol Research Laboratories, Inc (Plymouth Meeting, PA) All other chemicals were ACS grade or better, obtained from Sigma-Aldrich (St Louis, MO) or Fisher Scientific, Inc. (Pittsburgh, PA) or J.T. Baker (Phillipsburg, NJ), and all buffers and reagents were prepared with high-purity (18.2MΩ • cm) water (Milli-Q; Millipore, Bedford MA). Baculovirus-insect cell microsomes (Supersomes®) containing expressed CYP3A enzymes, co-expressed human cytochrome P-450 reductase, (and where indicated, co-expressed human cytochrome \( b_5 \)) were purchased from BD Gentest Corporation (Woburn, MA). These included CYP3A4, CYP3A4 with co-expressed \( b_5 \), CYP3A5, and CYP3A7 with co-expressed \( b_5 \) . Human cytochrome \( b_5 \) overexpressed in \( E. \ coli \) and purified to homogeneity was obtained from PanVera (Madison, WI).

Enzyme incubation conditions. Incubations contained either CYPs 3A4 or 3A5, with or without exogenously added cytochrome \( b_5 \), or CYPs 3A4 or 3A7 with co-expressed cytochrome \( b_5 \). Incubations (final volume 1.0 ml) contained 100 mM potassium phosphate buffer (pH 7.4), CYP3A (15 pmol/ml CYP3A4, CYP3A5 or CYP3A7; 7.5 pmol/ml CYP3A4 or CYP3A5 with added cytochrome \( b_5 \), or 2.5 pmol/ml CYP3A4 with co-expressed \( b_5 \)), and alfentanil, were preincubated at 37 °C for 3 min, and the reaction initiated by adding an NADPH regenerating system (final concentrations: 10 mM glucose-6-phosphate, 1 mM \( \beta \)-NADP, 1 U glucose-6-phosphate dehydrogenase and 5 mM magnesium chloride, preincubated at 37°C for 10 min to preform NADPH). Incubations containing exogenous \( b_5 \) added to CYP3A4 and CYP3A5 were allowed to interact at room temperature for 15 min prior to addition of substrate (Evert et al., 1997), followed by preincubation at 37 °C for 3 min, and addition of the NADPH generating system. The molar ratio of exogenously added \( b_5 \) to CYP3A was 7:1, to mirror the \( b_5 \): CYP3A molar ratio of CYP3A4 + coexpressed cytochrome \( b_5 \) (7- to 8:1 in various lots). Control incubations
substituted 100 mM potassium phosphate buffer for the NADPH regenerating system, and were performed for all experiments. Incubations were terminated after 12 min (CYP3A4 and CYP3A5), 9 min (CYP3A4 and CYP3A5 with added b5), 30 min (CYP3A7 with co-expressed b5) or 5 min (CYP3A4 with co-expressed b5) with 20 µl phosphoric acid and placed on ice.

The influence of ketoconazole and TAO on alfentanil (1 µM) metabolism was determined in incubations containing CYP3A4 and CYP3A5. TAO and ketoconazole were added in methanol (final concentration of 0.5%), which was also added to uninhibited controls. TAO was preincubated for 15 min with enzyme and NADPH regenerating system and the reaction started by addition of alfentanil (1 µM). Ketoconazole was included with the buffer, enzyme and alfentanil for the 3 min preincubation prior to initiating the reaction by adding the NADPH regenerating system.

Analytical Methods. Alfentanil and the metabolites noralfentanil and AMX were quantified by LC-MS following solid phase extraction. To quenched reaction mixtures were added the internal standards (5.6 ng R38527 and 2.8 ng d5-AMX), which were then applied to Oasis MCX (1cc, 30mg, 30µm) solid phase extraction cartridges (Waters Corp., Milford, MA) previously conditioned with 1 ml methanol and 1 ml deionized water. Cartridges were washed with 1 ml 0.1 N hydrochloric acid, eluted into polypropylene tubes (used because AMX recovery was better than with glass) with 1 ml 5% ammonium hydroxide in methanol, and the eluent evaporated to dryness under nitrogen at 65 °C. Samples were stored for <24 hr at 4 °C then reconstituted with 50 µl of 18% acetonitrile in 20 mM formic acid. Samples from incubations containing high (25-100 µM) alfentanil concentrations were further diluted with an additional 100 µl of 18% acetonitrile in 20 mM formic acid to maintain chromatographic peak shape and response at high analyte concentrations.

Noralfentanil, AMX and alfentanil concentrations were measured by LC-MS using selected ion monitoring. The instrument was an Agilent 1100 LC-MSD using a Zorbax Eclipse XBD column (2.1 x 50mm, 5µm) with a Zorbax Eclipse C8 guard column (2.1 x 12.5mm, 5µm) (Agilent). The mobile phase (0.25 ml/min) gradient started at 18% acetonitrile in 20 mM formic acid for 30 sec, increased to 24% (4 min) and 75% (7 min) acetonitrile, and was held at 75% for 1 min before re-equilibrating at 18%
acetonitrile. Injections were 12 µl. Under these conditions d5-AMX, AMX, noralfentanil, alfentanil, and R28527 eluted at 4.2, 4.3, 3.7, 6.2, and 6.5 min and were monitored at m/z 155, 150, 277, 417, and 431, respectively. The nitrogen drying gas was at 325 °C and 6 l/min, fragmentor at 70V, nebulizer pressure 25 psi, and the capillary at 2500 V.

Analytes were quantified using standard curves of peak area ratios. Standard curves were linear (average r² >0.99) over the range of 0.3-500 ng/ml noralfentanil, 0.3-100 ng/ml N-phenylpropionamide, and 0.2-1µM alfentanil.

**Data Analysis.** Incubations with expressed enzymes and inhibitors were performed in triplicate and controls in quadruplicate for each enzyme and inhibitor used. Any background amount of metabolites in controls were subtracted from incubations containing NADPH. Results are expressed as the mean ± SD.

Microsomal velocity vs substrate concentration data were analyzed using several models, based on the assumption, supported by recent data on the crystal structure of CYP3A4, that this enzyme contains at least two binding sites (Korzekwa et al., 1998; Shou et al., 2001; Yano et al., 2004), as described previously (Oda and Kharasch, 2001a; Oda and Kharasch, 2001b). Choice of model was guided by the Eadie-Hofstee curves. When these were linear, velocity vs substrate data were analyzed using a simple Michaelis-Menten model:

\[ V = \frac{V_{\text{max}} \cdot S}{K_m + S} \]  

(eq. 1)

When Eadie-Hofstee curves were hyperbolic and concave, data were analyzed using a dual-enzyme Michaelis-Menten model:

\[ V = \frac{V_{\text{max}1} \cdot S}{K_{m1} + S} + \frac{V_{\text{max}2} \cdot S}{K_{m2} + S} \]  

(eq. 2)

in which S is the substrate concentration; \( K_{m1} \) and \( K_{m2} \), high- and low-affinity Michaelis-Menten constants, respectively; \( V_{\text{max}1} \) and \( V_{\text{max}2} \), high- and low-affinity maximum metabolic velocity, respectively. Data were also analyzed using a cooperative single-enzyme model with two binding sites in which
product can be formed either from the single-substrate-bound form (ES) or from the two-substrate-bound form (ESS) of the enzyme was described by Korzekwa et al. (Korzekwa et al., 1998)

\[
V = \frac{V_{\text{max1}} \cdot S + V_{\text{max2}} \cdot S^2}{\frac{S}{K_{m1}} + \frac{S^2}{K_{m1} \cdot K_{m2}}} \quad (\text{eq. 3})
\]

IC₅₀ values for enzyme inhibition were obtained by fitting to the sigmoid equation:

\[
V = 1 - \frac{S^\gamma}{S^\gamma + IC_{50}^\gamma} \quad (\text{eq. 4})
\]

All data were modeled by non-weighted non-linear regression analysis using SigmaPlot 8.02 (Systat, Point Richmond, CA). Results are expressed as the parameter estimate ± standard error of the estimate.
Results

Preliminary experiments  Initial experiments were conducted to establish reaction conditions that would ensure linearity of product formation with incubation time. Figure 2 shows noralfentanil and AMX formation by CYPs 3A4, 3A5, and 3A7, which were linear over time at all substrate concentrations evaluated. CYP3A concentrations were selected to ensure linearity. When CYP3A4 incubations contained coexpressed cytochrome \( b_5 \), the CYP3A4 content was decreased to ensure linearity with time, and shorter incubation times were used.

Alfentanil metabolism at therapeutic concentrations  Alfentanil metabolism by CYPs 3A4, 3A5, and 3A7 with or without co-expressed or exogenously added cytochrome \( b_5 \) was first evaluated at therapeutic (1 \( \mu \)M) substrate concentrations (Table 1). Rates of noralfentanil formation by CYP3A5 were similar to those for CYP3A4, while AMX formation by CYP3A5 was one-third that by CYP3A4. This resulted in a substantial difference in the noralfentanil:AMX formation ratio (9 vs 21 for CYPs 3A4 and 3A5).

Cytochrome \( b_5 \) substantially increased CYP3A4- and 3A5-catalyzed alfentanil metabolism, and the degree of stimulation depended on the mode of cytochrome \( b_5 \) supplementation. Noralfentanil and AMX formation by CYP3A4 were both increased 5-fold by coexpression of cytochrome \( b_5 \) with CYP3A4. In contrast, noralfentanil and AMX formation by CYP3A4 were increased only 30-40% by exogenously added cytochrome \( b_5 \). The noralfentanil:AMX formation ratio for CYP3A4 was not changed by either coexpressed or exogenously added cytochrome \( b_5 \). Noralfentanil and AMX formation by CYP3A5 were both increased 2-fold by exogenously added cytochrome \( b_5 \). The noralfentanil:AMX formation ratio for CYP3A5 was not changed by addition of exogenous cytochrome \( b_5 \).

CYP3A7 was a relatively poor catalyst of alfentanil metabolism. Even with co-expressed cytochrome \( b_5 \), rates of alfentanil metabolism by CYP3A7 were 35 to 60-fold less than by CYP3A4 with co-expressed \( b_5 \). The noralfentanil:AMX formation ratio for CYP3A7 was intermediate to that of CYPs 3A4 and 3A5.

Kinetics of alfentanil metabolism by CYP3A enzymes  Alfentanil metabolism by CYPs 3A4, 3A5, and 3A7 with or without co-expressed or exogenously added cytochrome \( b_5 \) was next evaluated over a range
of substrate concentrations (0.5 µM-100 µM) (Figures 3 and 4). Kinetic parameters for the formation of noralfentanil and AMX were obtained by nonlinear regression analysis of metabolite formation rate versus substrate concentration data, using single-enzyme, dual-enzyme, and single-enzyme dual-site Michaelis-Menten models (Table 2).

Alfentanil metabolism by CYPs 3A4 and 3A5 in the absence of cytochrome b₅ showed saturable, hyperbolic kinetics (Figure 4). Eadie-Hofstee plot for noralfentanil formation appeared somewhat biphassic and concave hyperbolic (vs parabolic) (insets, Figure 4). Although this typically indicates apparent multienzyme or multi-site kinetics, dual-enzyme or dual-site models (Eq 2 and 3) did not improve the fit of the data, based on regression coefficients and standard errors of the estimates, hence data were analyzed using a single-enzyme Michaelis-Menten model. Eadie-Hofstee plots for AMX formation by CYPs 3A4 and 3A5 were linear, indicating apparent single enzyme (site) kinetics (insets, Figure 3), and data were analyzed using a single-enzyme Michaelis-Menten model. Kₘ and Vₘₐₓ values for noralfentanil formation by CYPs 3A4 and 3A5 were similar, as were the in vitro intrinsic clearances (Vₘₐₓ/Kₘ). Kₘ values for AMX formation by CYPs 3A4 and 3A5 were similar, although Vₘₐₓ and Vₘₐₓ/Kₘ for CYP3A5 were lower. The in vitro intrinsic clearances were 8- to 25-fold greater for noralfentanil than AMX formation, suggesting a greater role for piperidone N-dealkylation in alfentanil metabolic inactivation, and consistent with clinical data (Meuldermans et al., 1988).

Cytochrome b₅ substantially altered the kinetics of alfentanil metabolism, and the effects depended on the metabolite formed, the CYP3A form, and the mode of cytochrome b₅ supplementation (Figure 4). The presence of b₅ increased turnover (noralfentanil more than AMX, 3A5 more than 3A4, and co-expression more than exogenous addition). For both CYPs 3A4 and 3A5, b₅ (via co-expression or exogenous addition) increased the biphasic nature of the Eadie-Hofstee plots for noralfentanil formation, although Eadie-Hofstee plots for AMX formation remained monophasic for both CYPs. Data from these experiments were analyzed using dual-enzyme and dual-site models (Eq 2 and 3), although the results were equivalent and only the former are presented (Table 2). Adding exogenous b₅ decreased the high-affinity Kₘ for noralfentanil formation 10- to 14-fold for CYPs 3A4 and 3A5, and increased Vₘₐₓ/Kₘ 2-
to 3-fold, but had no major effect on the $K_m$ for AMX formation. In contrast, cytochrome $b_5$ coexpression with CYP3A4 not only decreased the high-affinity $K_m$ for noralfentanil formation, but increased the $V_{max}/K_m$ for both noralfentanil and AMX approximately 5-fold. CYP3A7 with coexpressed cytochrome $b_5$ showed monophasic kinetics with $K_m$ values similar to those for CYPs 3A4 and 3A5 without $b_5$, but substantially lower $V_{max}$ values.

Noralfentanil/AMX metabolite ratios were substrate concentration-dependent. This concentration-dependence was unaffected by the presence of cytochrome $b_5$ (Figure 5).

**Inhibition of alfentanil metabolism** Effects of the CYP3A inhibitors troleandomycin (TAO) and ketoconazole on alfentanil metabolism were determined using expressed CYPs 3A4 and 3A5. The mechanism-based inhibitor TAO, which is generally considered to be selective for CYP3A enzymes (Ono et al., 1996; Sai et al., 2000), inhibited noralfentanil and AMX formation by both CYP3A4 and CYP3A5 (Figure 6). The $IC_{50}$s for TAO inhibition of alfentanil metabolism by CYPs 3A4 and CYP3A5 were 0.28 and 0.62 µM for noralfentanil formation, and 0.24 and 0.75 µM respectively for AMX formation (Table 3). The noncompetitive inhibitor ketoconazole, which is nonselective at high concentrations but relatively selective for CYP3A enzymes at lower concentrations (Ono et al., 1996; Sai et al., 2000), inhibited noralfentanil and AMX formation by both CYP3A4 and CYP3A5 (Figure 7). Both noralfentanil and AMX formation were inhibited by ketoconazole, and ketoconazole was a more potent inhibitor of CYP3A4 than CYP3A5. The $IC_{50}$s for ketoconazole inhibition of alfentanil metabolism by CYPs 3A4 and 3A5 were 0.01 and 0.11 µM for noralfentanil formation, and 0.017 and 0.091 µM for AMX formation (Table 3).
Discussion

The primary objective of this investigation was to test the hypothesis that alfentanil is metabolized in vitro by CYP3As other than CYP3A4. Previous evaluation of microsomal and CYP3A4-catalyzed alfentanil metabolism in vitro showed that noralfentanil and AMX are the major metabolites, arising from two separate CYP3A-catalyzed pathways (Meuldermans et al., 1988; Labroo et al., 1995), hence this study focused on these major metabolites. The present results showed that alfentanil is a substrate in vitro for CYP3A5 and CYP3A7, as well as CYP3A4, confirming the above hypothesis. Like CYP3A4, both CYP3A5 and CYP3A7 catalyzed both primary routes of alfentanil metabolism, piperidine N-dealkylation and amide N-dealkylation.

Clear evidence for alfentanil metabolism by CYP3A5 contrasts with a previous report which concluded that metabolism is catalyzed solely by CYP3A4 (Guitton et al., 1997). That conclusion was based on experiments with yeast-expressed CYPs, including CYP3A5, and on a positive correlation between alfentanil disappearance and hepatic microsomal CYP3A4 content. An explanation for the difference between the present and previous results not apparent, but may relate to the different CYP expressions systems used.

There are notable quantitative similarities and differences between CYP3A enzymes in alfentanil metabolism. CYP3A5 was at least as active as CYP3A4. At therapeutic alfentanil concentrations, metabolism by CYP3A5 was comparable to that by CYP3A4, and even greater with exogenously added $b_5$. In vitro intrinsic clearances for CYP3A5 were comparable to those for CYP3A4, and even greater with exogenous $b_5$. In contrast, CYP3A7, even with co-expressed cytochrome $b_5$, was comparatively inactive, with 20- to 30-fold lower rates of metabolism and intrinsic clearances than CYP3A4 (despite equivalent $K_m$ values). Thus CYP3A7 is unlikely to participate significantly in human liver alfentanil metabolism. Alfentanil, like numerous other CYP3A substrates such as benzodiazepines, steroids, and calcium channel blockers, shows substantially less metabolism ($V_{max}$ and/or in vitro intrinsic clearance) by CYP3A7 compared with CYPs 3A4 or 3A5 (Williams et al., 2002; Yamaori et al., 2003; Kamdem et al., 2004; Miller et al., 2004; Shen et al., 2004). Alfentanil is relatively distinctive among CYP3A
substrates, in that the metabolic capacity (in vitro intrinsic clearance) of CYP3A5 equaled or exceeded that of CYP3A4. Like alfentanil, the metabolic capacity of CYP3A5 exceeded that of CYP3A4 for oxycodone N-demethylation (Lalovic et al., 2004) and midazolam 1'- and 4-hydroxylation (Gorski et al., 1994; Wandel et al., 1994; Williams et al., 2002; Yamaori et al., 2003). In contrast, for most CYP3A substrates, such as the calcium channel blockers, steroids, benzodiazepines, amitriptyline, and haloperidol, the metabolic capacity of CYP3A4 exceeded that of CYP3A5 (Hirota et al., 2001; Williams et al., 2002; Kalgutkar et al., 2003; Patki et al., 2003; Yamaori et al., 2003; Miller et al., 2004; Shen et al., 2004). Nevertheless, it should be cautioned that these conclusions are sensitive to reaction conditions, specifically the presence of (and mode of adding) $b_5$. For example, the in vitro intrinsic clearance for testosterone 6ß-hydroxylation was 40-fold greater for CYP3A4 with coexpressed $b_5$ than for 3A5 with added $b_5$ (Williams et al., 2002). In contrast, testosterone metabolism by CYPs 3A4 and 3A5 with added $b_5$ was equivalent (and much greater when $b_5$ was coexpressed with CYP3A4) (Kamdem et al., 2004). Similar results were obtained for alprazolam (Hirota et al., 2001). We compared alfentanil metabolism by CYPs 3A4 and 3A5 both in the absence or presence of added $b_5$, to ensure equivalent conditions for the comparison. Under these conditions CYP3A5 was at least as active as CYP3A4.

A second major purpose of this investigation was to characterize the kinetics of CYP3A-catalyzed alfentanil metabolism, and to elucidate the role of cytochrome $b_5$. Differences in alfentanil metabolism between CYP3A enzymes were due predominantly to $V_{max}$, since the $K_m$ was relatively invariant. These were also manifest as differences in the noralfentanil:AMX product ratio. CYP3A enzyme differences in metabolite formation ratios have been observed previously, for midazolam and other substrates (Kronbach et al., 1989; Gorski et al., 1994; Wandel et al., 1994; Kuehl et al., 2001; Lin et al., 2002; Williams et al., 2002). The 2- to 3-fold difference between CYPs 3A4 and 3A5 in the alfentanil metabolite ratio is similar to that for midazolam, but less than that for alprazolam (Williams et al., 2002). CYP3A can exhibit atypical kinetics, with different substrates variably showing autoactivation or homotropic positive cooperativity (sigmoidal substrate saturation curves and horizontally convex Eadie-Hofstee curves), heteroactivation by additional effectors or heterotropic positive cooperativity, substrate
inhibition (with a hook in the upper quadrant of the Eadie-Hofstee curves), partial inhibition, or negative cooperativity (hyperbolic substrate saturation curves but biphasic concave Eadie-Hofstee curves), in addition to classical Michaelis-Menten kinetics, which is generally accepted to denote multiple substrate (and effector) binding sites (Korzekwa et al., 1998; Shou et al., 2001; Galetin et al., 2003; He et al., 2003). For example, midazolam shows substrate activation or substrate inhibition (Kronbach et al., 1989; Perloff et al., 2000; Schrag and Wienkers, 2001; Khan et al., 2002; Yamaori et al., 2003). In contrast, alfentanil metabolism by CYPs 3A4 and 3A5 showed predominantly classical single-site Michaelis-Menten kinetics in the absence of cytochrome b₅, and biphasic kinetics (negative cooperativity) in the presence of b₅. Thus alfentanil, in addition to l-α-acetylmethadol (LAAM), methadone, naphthalene, and the antiarrhythmic agent BRL32872, is one of the few substrates which exhibits negative cooperativity in CYP3A4-catalyzed metabolism (Clarke, 1998; Korzekwa et al., 1998; Oda and Kharasch, 2001a; Oda and Kharasch, 2001b; Kharasch et al., 2004a).

Enhancement by cytochrome b₅ of CYP3A catalytic activity is well-established, although the mechanism remains incompletely elucidated (Yamazaki et al., 1996; Yamazaki et al., 1999; Hirota et al., 2001; Yamaori et al., 2003). Alfentanil (1 µM) metabolism and intrinsic clearance by CYP3A4 with co-expressed P450 reductase were increased by 1.5- to 2-fold by exogenously added b₅, and increased 5- to 7-fold (due to an increased V_max) with co-expression of b₅. Effects on CYP3A5 were slightly greater, with addition of b₅ increasing metabolism and intrinsic clearance 2- to 3-fold. Unfortunately CYP3A5 with co-expressed b₅ was not available to enable a complete comparison. Differences between CYP3A4 with added vs coexpressed b₅ cannot be attributed to differences in b₅ content, since this was made equivalent in the two systems (7:1), and well in excess of the b₅ content needed for optimal turnover (typically ≤ 2:1 b₅:P450) (Shet et al., 1993; Yamaori et al., 2003). These results highlight the importance of using equivalent systems (whether no b₅, b₅ addition, or b₅ coexpression) when comparing CYP3A4 vs CYP3A5-catalyzed metabolism, as described above and previously (Kamdem et al., 2004).

More interestingly, b₅ altered the kinetics of alfentanil metabolism, and this effect was regioselective. Adding b₅ changed piperidine N-demethylation to noralfentanil by both CYPs 3A4 and
3A5 from exclusively or predominantly monophasic, to clearly biphasic, based on Eadie-Hofstee plots, revealing a high-affinity component. In contrast, \( b_5 \) had no effect on the kinetics of alfentanil amide N-dealkylation to AMX, or on the noralfentanil:AMX product ratio. Analogous effects of \( b_5 \) on CYP3A have also recently been observed (Yamaori et al., 2003). Addition of \( b_5 \) changed CYP3A4-catalyzed midazolam 1’-hydroxylation from autoactivation (convex Eadie-Hofstee curves) to negatively cooperative (biphasic Eadie-Hofstee curves) kinetics, although there was no such effect on CYP3A5 (Yamaori et al., 2003). Unfortunately, the effect of \( b_5 \) on CYP3A-catalyzed midazolam 4-hydroxylation were not evaluated in this previous investigation. In general, \( b_5 \) effects on P450-catalyzed metabolism are form-selective (Yamazaki et al., 2002), and \( b_5 \) stimulation of CYP3A-catalyzed metabolism is known to be substrate-selective, seen with only approximately one-third of substrates (Yamaori et al., 2003). However alfentanil may be the first example of regioselectivity in \( b_5 \) effects on CYP3A-catalyzed metabolism. The mechanism by which \( b_5 \) alters CYP3A-catalyzed alfentanil kinetics, and the substrate- and region-selectivity of \( b_5 \) effects on CYP3A remain unknown.

The third major objective of this investigation was to assess the effect of the CYP3A inhibitors ketoconazole and troleandomycin (TAO) on CYP3A-catalyzed alfentanil metabolism. Both inhibitors diminished alfentanil piperidine N-demethylation to noralfentanil and amide N-dealkylation, with each inhibitor having similar IC\(_{50}\) values for the two pathways. Both inhibitors showed differential potency for CYPs 3A4 and 3A5, with the difference greater for CYP3A5. This result is consistent with previous observations that TAO inhibits 3A5 as well as 3A4, although it appears more effective towards the latter. For example, TAO (20 uM) caused 90 and 75\% inhibition of testosterone 6ß-hydroxylation catalyzed by expressed human CYPs 3A4 and 3A5, respectively (Chang et al., 1994), and TAO (100 µM) inhibited 90\% of HepG2-expressed CYP3A4 activity but only 50\% of CYP3A5 activity (Ono et al., 1996). The current result that the IC\(_{50}\) for ketoconazole was nearly an order of magnitude lower for CYP3A4 than CYP3A5 is also consistent with previous observations. For example, the K\(_i\) for ketoconazole inhibition of midazolam hydroxylation was 4- to 10-fold lower for CYP3A4 than CYP3A5 (Gibbs et al., 1999). These
results are entirely consistent with the general pattern that the potency of CYP3A inhibitors is CYP3A4 > CYP3A5 > CYP3A7 (Ekins et al., 2002).

Considered together, the current results suggest that CYP3A5 may be important in human alfentanil metabolism. There are widely varying estimates of hepatic CYP3A5 expression levels, ranging from 2 to 60% of total hepatic CYP3A (Williams et al., 2003). Nevertheless, additional evidence for alfentanil metabolism by CYP3A5 has been obtained using human liver microsomes (Klees et al., 2004). In microsomes from two groups of livers, matched for CYP3A4 protein content but differing in CYP3A5 content (2-5% vs 46-76% of total CYP3A, respectively), noralfentanil and AMX formation were both 3-fold greater in CYP3A5*1 livers expressing higher amounts of CYP3A5 protein (Klees et al., 2004). Furthermore, there was a relatively poor correlation between alfentanil metabolism and CYP3A4 content \( (r^2=0.30) \), but an excellent correlation when CYP3A5 (i.e. total CYP3A content) was considered \( (r^2=0.81, p<0.0001) \). The metabolite formation ratio (noralfentanil/AMX) in low CYP3A5 livers \( (10 \pm 1) \) was close to that for expressed CYP3A4 in the current investigation \( (9 \pm 1) \), while that in high CYP3A5 livers \( (13 \pm 1) \) was intermediate to that observed currently for expressed CYP3A4 \( (9 \pm 1) \) and CYP3A5 \( (21 \pm 1) \) (Klees et al., 2004). Finally, inhibition by TAO was similar in the low and high CYP3A5 livers, while ketoconazole inhibition was greater in the high CYP3A5 livers (Klees et al., 2004). Alfentanil metabolism by CYP3A in vitro bears several resemblances to that of midazolam. Midazolam is metabolized at least as well by CYP3A5 as 3A4 (Gorski et al., 1994; Wandel et al., 1994; Williams et al., 2002; Yamaori et al., 2003), microsomal metabolism was substantially higher in livers and intestines expressing CYP3A5 in addition to CYP3A4, and the ratio of 1'-hydroxymidazolam to 4-hydroxymidazolam was greater in livers expressing CYP3A5 compared with those expressing only CYP3A4 (Kronbach et al., 1989; Gorski et al., 1994; Kuehl et al., 2001; Lin et al., 2002). In contrast to midazolam and alfentanil, based on a survey of substrates metabolism by expressed CYP3As, CYP3A4 rather than CYP3A5 content was considered to be the major determinant of hepatic metabolism (Williams et al., 2002). Based on the well-stirred model, the low clearance of alfentanil, and the activity of CYP3A5 toward alfentanil metabolism, the CYP3A5 polymorphism would be expected to influence
alfentanil disposition. The role of CYP3A5 in alfentanil metabolism and clearance in humans in vivo, and the pharmacogenetic influence of CYP3A5 polymorphisms, merit evaluation. Indeed, the role of CYP3A5 in drug disposition in general remains the subject of considerable discussion (Thummel, 2003; Wilkinson, 2004; Xie et al., 2004).

In summary, the present investigation demonstrates that multiple CYP3A enzymes catalyze the metabolism of alfentanil, with the relative activity of CYP3A4 ≥ CYP3A5 >> CYP3A7. The CYP3A inhibitors troleandomycin and ketoconazole both inhibit alfentanil metabolism, albeit with differential selectivity for CYP3A4 and CYP3A5. These results, together with others, clearly show that CYP3A5 plays a significant role in human liver alfentanil metabolism. Clinical investigations to assess the role of CYP3A5 in human alfentanil metabolism are warranted, particularly since alfentanil is an excellent in vivo probe for CYP3A activity.
References


Footnotes

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Figure legends

Figure 1. Major pathways of alfentanil metabolism in vitro; piperidine N-dealkylation to noralfentanil and amide N-dealkylation to N-phenylpropionamide (AMX).

Figure 2. Alfentanil metabolism by expressed CYP3A enzymes. Incubations contained 15 pmol/ml CYP3A4 (■), 15 pmol/ml CYP3A5 (▲) and 15 pmol/ml CYP3A7 with coexpressed cytochrome b₅ (●). Alfentanil concentrations were 0.5 µM (panels A and B) and 100 µM (panels C and D). Metabolite formation was typically linear for 15 min with CYP3A4 and CYP3A5 and for 50 min with CYP3A7. Metabolite formation by CYPs 3A4 and 3A5 (7.5 pmol/ml) with added cytochrome b₅ was linear for 10 min, and by CYP3A4 (2.5 pmol/ml) containing co-expressed cytochrome b₅ was linear for 6 min (data not shown).

Figure 3. Concentration-dependent alfentanil metabolism by expressed CYP3A4 (A) and CYP3A5 (B). Results show the formation of noralfentanil (●) and AMX (▲). Reaction mixtures contained 15 pmol/ml CYP3A4 or CYP3A5 (without cytochrome b₅) and were incubated for 12 min. Data points are the mean ± SD (n=3). Lines represent rates predicted using Michaelis-Menten kinetic parameters derived from nonlinear regression analysis of the data. The insets show Eadie-Hofstee plots.

Figure 4. Concentration-dependent alfentanil metabolism by expressed CYP3A with co-expressed or exogenously added cytochrome b₅. Incubations contained (A) CYP3A4 (7.5 pmol/ml) with added cytochrome b₅ (9 min incubation), (B) CYP3A5 (7.5 pmol/ml) with added cytochrome b₅ (9 min incubation), (C) CYP3A4 (2.5 pmol/ml) with co-expressed cytochrome b₅ (5 min incubation), (D) CYP3A7 (15 pmol/ml) with co-expressed cytochrome b₅ (30 min incubation). Results show the formation of noralfentanil (●) and AMX (▲) Data points are the mean ± SD (n=3). Lines represent rates predicted using Michaelis-Menten kinetic parameters derived from nonlinear regression analysis of the data. The insets show Eadie-Hofstee plots.

Figure 5. Noralfentanil/AMX metabolite ratios. Results are for CYP3A4 (○, 15 pmol/ml, 12 min incubation), CYP3A4 with added cytochrome b₅ (●, 7.5 pmol/ml, 9 min incubation), CYP3A4 with co-expressed cytochrome b₅ (▲, 2.5 pmol/ml, 5 min incubation), CYP3A5 (□, 15 pmol/ml, 12 min incubation).
incubation), CYP3A5 with added cytochrome \( b_5 \) (■, 7.5 pmol/ml, 9 min incubation), and CYP3A7 with co-expressed cytochrome \( b_5 \) (▲, 15 pmol/ml, 30 min incubation). Data points are the mean ± SD (n=3).

**Figure 6.** Troleandomycin (TAO) inhibition of alfentanil (1 µM) metabolism. (left) Noralfentanil formation (right) AMX formation. Incubations (12 min) included 15 pmol/ml CYP3A4 or 3A5. Results are expressed relative to uninhibited control rates. Data points are the mean ± SD (n=3). Lines represent rates predicted by nonlinear regression analysis using a sigmoidal model.

**Figure 7.** Ketoconazole inhibition of alfentanil (1 µM) metabolism. (left) Noralfentanil formation (right) AMX formation. Incubations (12 min) included 15 pmol/ml CYP3A4 or 3A5. Results are expressed relative to uninhibited control rates. Data points are the mean ± SD (n=3). Lines represent rates predicted by nonlinear regression analysis using a sigmoidal model.
Table 1  Alfentanil metabolism by CYP3A enzymes at therapeutic concentrations.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Metabolite Formation (pmol min(^{-1}) pmol(^{-1}))</th>
<th>Noralfentanil/AMX ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP3A4</td>
<td>0.511 ± 0.008</td>
<td>0.059 ±0.002</td>
</tr>
<tr>
<td>CYP3A5</td>
<td>0.507 ± 0.012</td>
<td>0.024 ±0.002</td>
</tr>
<tr>
<td>CYP3A4 with added cytochrome (b_5)</td>
<td>0.707 ± 0.041</td>
<td>0.075 ±0.007</td>
</tr>
<tr>
<td>CYP3A5 with added cytochrome (b_5)</td>
<td>1.23 ± 0.03</td>
<td>0.062 ±0.005</td>
</tr>
<tr>
<td>CYP3A4 with co-expressed cytochrome (b_5)</td>
<td>2.73 ± 0.13</td>
<td>0.326 ±0.029</td>
</tr>
<tr>
<td>CYP3A7 with co-expressed cytochrome (b_5)</td>
<td>0.078 ± 0.004</td>
<td>0.0057 ±0.0007</td>
</tr>
</tbody>
</table>

Incubations contained 1.0 µM alfentanil and an NADPH regenerating system in 100 mM potassium phosphate (pH 7.4). Enzyme-selective conditions were: CYP3A4 or CYP3A5 (15 pmol/ml, 12 min), CYP3A4 or CYP3A5 (7.5 pmol/ml) with added cytochrome \(b_5\) (9 min), CYP3A4 (2.5 pmol/ml) with co-expressed cytochrome \(b_5\) (5 min), CYP3A7 (15 pmol/ml) with co-expressed cytochrome \(b_5\) (30 min), Results are the mean ± SD (n=3).
Table 2: Kinetic data for expressed CYP3A enzymes.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Noralfentanil</th>
<th>AMX</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$</td>
<td>$V_{max}$</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>14.1 ± 1.4</td>
<td>6.05 ± 0.18</td>
</tr>
<tr>
<td>CYP3A5</td>
<td>10.7 ± 0.7</td>
<td>5.12 ± 0.10</td>
</tr>
<tr>
<td>CYP3A4 with added $b_5^*$</td>
<td>$K_{m1}$ 1.0 ± 1.1</td>
<td>$V_{max1}$ 1.0 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>$K_{m2}$ 37.5 ± 11.2</td>
<td>$V_{max2}$ 8.1 ± 0.5</td>
</tr>
<tr>
<td>CYP3A5 with added $b_5^*$</td>
<td>$K_{m1}$ 1.1 ± 1.1</td>
<td>$V_{max1}$ 1.8 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>$K_{m2}$ 24.2 ± 7.8</td>
<td>$V_{max2}$ 9.8 ± 0.8</td>
</tr>
<tr>
<td>CYP3A4 with co-expressed $b_5^*$</td>
<td>$K_{m1}$ 2.3 ± 1.0</td>
<td>$V_{max1}$ 7.0 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>$K_{m2}$ 71.4 ± 19.3</td>
<td>$V_{max2}$ 40.4 ± 2.6</td>
</tr>
<tr>
<td>CYP3A7 with co-expressed $b_5$</td>
<td>10.1 ± 0.5</td>
<td>0.89 ± 0.01</td>
</tr>
</tbody>
</table>

Results are the parameter estimate ± standard error of the estimate.

*Noralfentanil kinetics were analyzed using a two-enzyme model. $K_{m1}$ and $V_{max1}$ represent the high affinity site, $K_{m2}$ and $V_{max2}$ represent the low affinity site.
Table 3: Inhibition of alfentanil metabolism by expressed CYP3A enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>troleandomycin IC$_{50}$ (uM)</th>
<th></th>
<th>Ketoconazole IC$_{50}$ (uM)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Noralfentanil</td>
<td>AMX</td>
<td>Noralfentanil</td>
<td>AMX</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>0.28 ± 0.02</td>
<td>0.24 ± 0.02</td>
<td>0.010 ± 0.001</td>
<td>0.017 ± 0.002</td>
</tr>
<tr>
<td>CYP3A5</td>
<td>0.62 ± 0.03</td>
<td>0.75 ± 0.05</td>
<td>0.11 ± 0.01</td>
<td>0.091 ± 0.008</td>
</tr>
</tbody>
</table>

Incubations were performed in triplicate. Results are the parameter estimate ± standard error of the estimate.
Figure 2
Figure 4
Figure 5
Figure 6
Figure 7

![Graph showing the effect of ketoconazole on norfentanyl and AMX formation by CYP3A4 and CYP3A5.](graph.png)