ALFENTANIL *N*-DEALKYLATION: MONITORING THE FORMATION OF *N*-PHENYLPROPIONAMIDE (AMX) TO DETERMINE CYP3A4 ACTIVITY *IN VITRO*

Phyllis Yerino, Paul Toren and Andrew Parkinson
XenoTech LLC | 16825 West 116th Street | Lenexa KS 66219 | USA

INTRODUCTION

Alfentanil is a synthetic analgesic cleared exclusively by hepatic metabolism. Alfentanil undergoes oxidative *N*-dealkylation at the piperidine nitrogen to noralfentanil and *N*-dealkylation at an amide linkage to *N*-phenylpropionamide (AMX) and is catalyzed predominantly *in vitro* by CYP3A4 enzymes.[1] Due to the highly linear correlation between alfentanil systemic clearance and CYP3A4 activity, as well as the direct pharmacologic effects on pupil size, it has been used as non-invasive metabolic probe for CYP3A4 activity *in vivo*.[2-3]

Since AMX is a direct metabolite of alfentanil[4], not a secondary metabolite of noralfentanil, and since noralfentanil is not commercially available, we set out to evaluate the potential for solely monitoring the formation of AMX as direct measurement of CYP3A4 activity *in vitro*.

MATERIALS & METHODS

Chemicals and Reagents

Alfentanil was purchased from US Pharmacopoeia (Rockville, MD). *N*-Phenylpropionamide (propioanalide, AMX), testosterone, ketoconzaole, midazolam, troleandomycin, propionic anhydride, and aniline-d_5 were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Deuterated *N*-phenylpropionamide was synthesized as described below. All other reagents were obtained from commercial sources.

Test Systems

Human liver microsomes (pool of 16) and various animal liver microsomes used in this study were prepared at the XenoTech LLC (Lenexa, KS) and characterized with respect to the activities of various CYP enzymes. Recombinant human CYP enzymes expressed in insect cells (Supersomes™) were purchased from BD Biosciences (Woburn, MA).

Synthesis of *N*-phenylpropionamide-d_5 (AMX-d_5, internal standard)

To a reaction mixture containing 5 mL of water and 200 µL of aniline-d_5 was added a few drops of 6 N hydrochloric acid until the solution was homogeneous. The mixture was then placed in an ice bath followed by the addition of 200 µL of propionic anhydride and a few drops of saturated sodium carbonate solution until formation of bubbles ceased. An additional 2 mL of water was added to precipitate the AMX-d_5, which was recrystalized from hexane. Purity check of an aliquot of an AMX-d_5 solution by LC/MS/MS (multiple reaction monitoring mode) showed no contamination with AMX-d_0.

Metabolism of alfentanil

All incubations were performed with an automated liquid handling system (Tecan Genesis RSP150). Pooled human liver microsomes (0.05 – 0.1 mg/mL) were incubated in a total volume of 0.2 mL containing high purity water, potassium phosphate buffer (50 mM, pH 7.4), $MgCl_2$ (3 mM), EDTA (1 mM), and alfentanil (5 – 500 µM) at the final concentrations indicated. The reactions were started with the addition of NADPH-regenerating system: NADP (1 mM), glucose 6 phosphate (5 mM), glucose-6-phosphate dehydrogenase (1 Unit/mL) at the final concentrations indicated. The reactions were stopped by the addition of 175 µL of acetonitrile containing 2% formic acid and approximately 200 ng/mL of the deuterated internal standard, AMX-d_5.

Determination of Kinetic Constants (K_m and V_{max})

Substrate concentration dependent *N*-dealkylation of alfentanil by human liver microsomes was determined in triplicate incubations of alfentanil (5 – 500 µM) for 5 minutes with protein concentration of 0.05 mg/mL.

Recombinant CYP enzymes

Alfentanil (400 µM) was incubated in triplicate at 37 ± 1 °C for 10 minutes with recombinant human CYP enzymes (rCYP1A1, rCYP1A2, rCYP2A6+b_5, rCYP2B6+b_5, rCYP2C8+b_5, rCYP2C9+b_5, rCYP2C18, rCYP2C19+b_5, rCYP2D6, rCYP2E1+b_5, rCYP2J2+b_5, rCYP3A4+b_5, rCYP3A5+b_5, rCYP4A11, rCYP4F2+b_5 and rCYP4F3B at 5 pmol P450 per incubation, normalized to 0.085 mg protein/incubation with microsomes from insect cell control+b_5.

Sample-to-sample variation in alfentanil *N*-dealkylation

Alfentanil (100 µM) was incubated with a bank of 16 samples of human liver microsomes to determine the inter-individual differences in metabolite formation. This bank of human liver microsomes had been characterized with marker substrates to determine the activity of the major CYP enzymes (namely CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4/5 and CYP4A11). The sample-to-sample variation in the rates of AMX formation from alfentanil were compared by correlation analysis with the sample-to-sample variation from CYP3A4 substrate markers midazolam and testosterone.

CYP Enzyme Inhibition: IC_{50} determinations

To evaluate the potential for direct or metabolism-dependent inhibition, ketoconazole (0.0005 – 2 µM), troleandomycin (0.01 – 50 µM), midazolam (0.5 – 25 µM) and testosterone (5 – 250 µM) were preincubated with NADPH fortified human liver microsomes for 0 or 30 min at 37 °C. After the preincubation period, alfentanil (at a concentration approximately K_m) was added to the incubation mixture and the incubation was continued for 5 minutes to measure the residual rate of alfentanil *N*-dealkylation.

Analytical methods

All analyses were performed using either Applied Biosystems / Sciex API2000 or API3000 HPLC-MS/MS systems equipped with an electrospray (Turbo IonSpray) ionization source (Applied Biosystems, Foster City, CA), two LC-10ADvp pumps with a SCL-10Advp controller, SIL-HTA autosampler and DGU-14 solvent degasser (Shimadzu, Columbia, MD). The HPLC column was a Waters Atlantis dC18, 5 µm, 100 × 2.1 mm column (Torrance, CA), which was preceded by a direct connection guard column with a C8, 4.0 mm × 2.0 mm cartridge (Phenomenex, Torrance, CA).

Figure 1 **Major pathways of alfentanil metabolism**

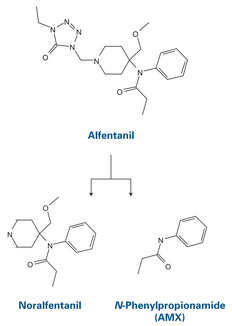


Figure 2 **Concentration-dependent alfentanil metabolism by human liver microsomes**

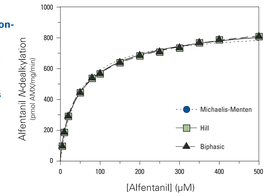


TABLE 1

Kinetic data for alfentanil metabolism to AMX by human liver microsomes K_{m1} and V_{max1} represent the high-affinity site; K_{m2} and V_{max2} represent the low-affinity site.

Kinetic Parameter	Single enzyme Hill coefficient = 1.0 (Michaelis-Menten)	Single enzyme Hill coefficient = 0.8 (Allosteric)	Two enzymes Hill coefficient = 1.0 for both
K_{m1} (µM)	44.5	26.2	25.8
K_{m2} (µM)	—	—	396
V_{max1} (pmol/mg/min)	852	946	614
V_{max2} (pmol/mg/min)	—	—	407

Figure 3 **Alfentanil *N*-dealkylation to AMX and noralfentanil by a bank of human liver microsomes (n = 16)** In the absence of an authentic standard for noralfentanil, metabolite formation was estimated based on peak AUC.

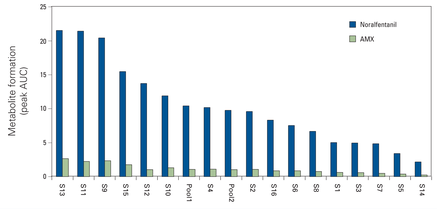


Figure 4 **Correlation of alfentanil *N*-dealkylation, testosterone 6β-hydroxylation and midazolam 1'-hydroxylation by human liver microsomes (n = 16 samples)**

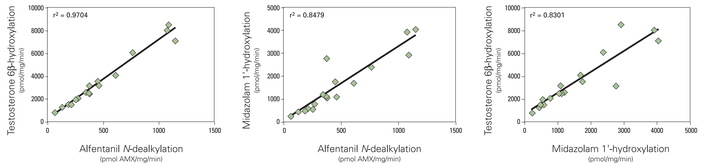


Figure 5 **Alfentanil *N*-dealkylation to AMX by a panel of recombinant human cytochrome P450 (CYP) enzymes**

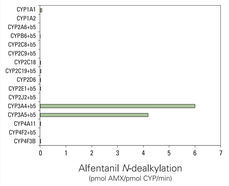


Figure 7 **Comparison of alfentanil *N*-dealkylation and testosterone 6β-hydroxylation activities in liver microsomes from various species**

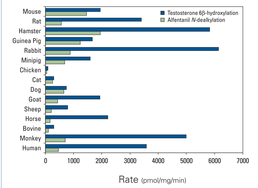


Figure 8 **Correlation analysis of alfentanil *N*-dealkylation and testosterone 6β-hydroxylation by liver microsomes from various animal species**

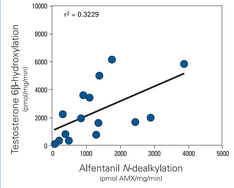


Figure 6 **Direct and metabolism-dependent inhibitory effects of ketaconazole, testosterone, midazolam and troleandomycin towards alfentanil *N*-dealkylation by human liver microsomes** Alfentanil concentration was 40 µM (≈K_m) and protein concentration was 0.1 mg/ML.

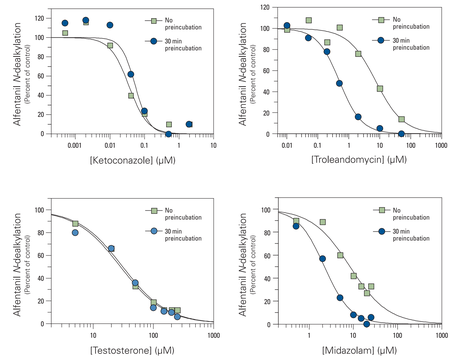
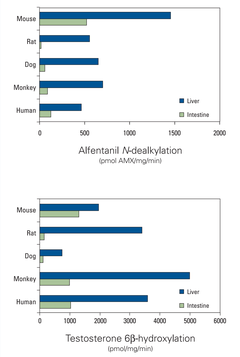


Figure 9 **Comparison of alfentanil *N*-dealkylation and testosterone 6β-hydroxylation activities in liver and intestinal microsomes from mouse, rat, dog, monkey and human**



RESULTS

- **Figure 1** shows that alfentanil is converted to two *N*-dealkylated metabolites, noralfentanil and AMX.
- **Figure 2** and **Table 1** summarize the kinetics of AMX formation by human liver microsomes. Michaelis-Menten analysis indicated a K_m of ~45 µM. Slightly lower estimates were obtained with other types of kinetic analysis.
- **Figure 3** shows that the rate of formation of AMX varied more than 6 fold in a bank of 16 samples of human liver microsomes, and was highly correlated ($r^2 > 0.95$) with the sample-to-sample variation in noralfentanil formation.
- **Figure 4** shows that the sample-to-sample variation in AMX formation correlated well ($r^2 > 0.83$) with two markers of CYP3A4 activity, namely testosterone 6β-hydroxylation and midazolam 1'-hydroxylation.
- **Figure 5** shows that rCYP3A4 and rCYP3A5 were the only recombinant CYP enzymes examined that converted alfentanil to AMX at a high rate.
- **Figure 6** shows that the *N*-dealkylation of alfentanil to AMX was inhibited by the direct-acting CYP3A4 inhibitors testosterone (IC_{50} 31 µM) and ketoconazole (IC_{50} 0.04 µM) and by the metabolism-dependent inhibitors midazolam (IC_{50} 2.2 µM) and troleandomycin (IC_{50} 0.5 µM).
- **Figures 8** and **9** show the relationship between species differences in liver microsomal testosterone 6β-hydroxylation and the *N*-dealkylation of alfentanil to AMX. The two activities tended to vary in parallel from one species to the next, but the overall correlation was not high (r^2 = 0.32).
- **Figure 9** shows that liver and intestinal microsomes from mouse, rat, dog, Cynomolgus monkey and human converted alfentanil to AMX at rates that corresponded reasonably well with the 6β-hydroxylation of testosterone.

CONCLUSION

AMX is one of two metabolites of alfentanil formed by CYP3A4. Although it is the minor metabolite, it has the advantage of being commercially available, and a deuterated analog of AMX can easily besynthesized.

The results of the present study suggest that the *N*-dealkylation of alfentanil to AMX (*N*-phenylpropionamide) is a reliable marker of CYP3A4 activity in human liver and intestinal microsomes, and that CYP3A enzymes in liver microsomes from several other species likely contribute significantly to AMX formation.

REFERENCES

1. Labroo RB, *et al.* Gas chromatographic-mass spectrometric analysis of alfentanil metabolites: Application to human liver microsomal alfentanil biotransformation. *J Chromatography B*, **660**: 85 – 94 (1994).
2. Kharasch ED, *et al.* Disposition and miotic effects of oral alfentanil: A potential noninvasive probe for first-pass cytochrome P4503A activity. *Clin Pharmacol Ther* **73**: 199 – 208 (2003).
3. Kharasch ED, *et al.* Intravenous and oral alfentanil as *in vivo* probes for hepatic and first-pass cytochrome P450 3A activity: Noninvasive assessment by use of papillary miosis. *Clin Pharmacol Ther* **76**: 452 – 466 (2004b).
4. Klees TM, *et al.* Metabolism of alfentanil by cytochrome P4503A (CYP3A) enzymes. *Drug Metab Dispos* **33**: 303 – 311 (2005).

XENOTECH
Uncommon Science | Uncommon Service