Inhibition of cytochrome P450 by nefazodone *in vitro*: studies of dextromethorphan *O*- and *N*-demethylation

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Nefazodone (NEF), a 5-HT_{2A/2C} antagonist antidepressant, is extensively metabolized in the human body to hydroxy NEF (OH-NEF), *p*-hydroxy NEF (pOH-NEF), a dione metabolite, and via cleavage of the molecule to *m*-chlorophenyl-piperazine (mCPP) and BMY-33604. The latter is further metabolized to BMS-183695-01 (BMSa) and BMS-183562-01 (BMSb). To investigate the potential of NEF and its metabolites to interfere with the metabolism of other drugs, we tested these compounds for their ability to alter dextromethorphan (DMO) *O*-demethylation to dextrorphan (DOP; an index reaction for CYP2D6) and *N*-demethylation to 3-methoxy morphinan (MEM, a recently proposed index reaction of CYP3A3/4). The assay was performed in an *in vitro* system with human liver microsomes from three different donors. NEF, OH-NEF, pOH-NEF, mCPP and BMSb were weak inhibitors of DMO *O* and *N*-demethylation, with average K_i values ranging from 18 to 50 μM for DOP formation, and from 21 to >200 μM for MEM formation. The dione metabolite and BMSa did not produce detectable inhibition of either pathway. The findings for DMO *O*-demethylation, well-established as a CYP2D6-mediated reaction, indicate that NEF and metabolites are weak inhibitors of this reaction, with K_i values at least 100 times higher than fluoxetine ($K_i = 0.1 \mu\text{M} \pm 0.09$). The implications of results on DMO *N*-demethylation are not clear. *In vivo* data, as well as *in vitro* data based on 'pure' CYP3A3/4 substrates, provide evidence for clinically relevant CYP3A3/4 inhibition by NEF, OH-NEF, and pOH-NEF. Thus, formation of MEM by *N*-demethylation of DMO may not constitute a suitable index reaction to probe CYP3A3/4 activity.

Keywords nefazodone *in vitro* human liver microsomes CYP3A3/4 CYP2D6 cytochrome P450 dextromethorphan

Introduction

Nefazodone (NEF), a 5-HT_{2A/2C} antagonist and serotonin reuptake inhibitor, is a phenyl-piperazine derivative with established antidepressive efficacy in acute [1, 2] as well as in long-term prophylactic treatment [3]. It is extensively metabolized by humans, has a presystemic extraction of approximately 86% after oral administration [4], is highly protein bound and exhibits non-linear pharmacokinetics [5–7]. Principal metabolites are hydroxy NEF (OH-NEF) [4, 5, 7–10], *p*-hydroxy NEF (pOH-NEF) [4, 5, 8], a dione metabolite [10] and *m*-chlorophenyl-piperazine (mCPP) formed by cleavage of the molecule [4, 5, 7–10]. Three other

potential metabolites have not yet been identified in human plasma, but are formed *in vitro* by human liver microsomes (data unpublished) and were isolated in human urine [11]; these are BMY-33604, the remaining half of the molecule after cleavage, and two subsequent products formed by further metabolism, BMS-183695-01 (BMSa) and BMS-183562-01 (BMSb; Figure 1). Inhibition of cytochrome P450 activity by the newer antidepressant drugs, in particular the selective serotonin reuptake inhibitors (SSRIs), can lead to clinically important drug interactions. Accordingly, we evaluated the inhibitory capacity of NEF and its metabolites for CYP-enzymes mediating dextromethorphan (DMO) *O*-demethylation, a CYP2D6 mediated reaction, and

Figure 1 Structural formulas of nefazodone and its metabolites

N-demethylation, recently suggested to be mediated by CYP3A3/4 [12, 13].

Methods

Chemicals

NEF and its metabolites were kindly provided by Bristol Myers Squibb, Princeton NJ, USA. The cofactors NADP, ($\pm$)-isocitric acid, MgCl_2 , and isocitrate dehydrogenase were purchased from Sigma, St Louis, MO, USA, as was the potassium phosphate salt used in buffer solution and quinidine. Dextromethorphan (DMO) and dextrorphan (DOP) were purchased from RBI, Natick, MA, USA. 3-methoxy-morphinan (MEM)

was a generous gift from Dr Edward M. Sellers, University of Toronto, Canada. Ketoconazole (KET) was kindly provided by the Janssen Research Foundation, Beerse, Belgium. All organic solvents were reagent grade.

Liver samples and preparation of microsomes

Liver samples, obtained from the International Institute for the Advancement of Medicine (Exton, PA, USA), were from three different transplant donors with no known liver disease. The tissue was partitioned and kept at -80°C until the time of microsome preparation, as described previously [14, 15].

Incubations

Incubation mixtures contained 50 mM potassium phosphate buffer (pH adjusted to 7.5 at 25°C). DMO (10–600 μM), 0.5 mM NADP, 3.75 mM ($\pm$)-isocitric acid, 1 unit/ml of isocitrate dehydrogenase and 5 mM Mg^{++} . Substrate concentrations for the control curve (without inhibitors) ranged from 1 to 1000 μM . Inhibition data points were obtained by incubating DMO (10, 50 and 100 μM) with KET (0.25 and 0.5 μM), quinidine (QUIN; 2.5 and 5 μM), fluoxetine (FLU; 5 and 10 μM), NEF, OH-NEF, pOH-NEF, DIONE, mCPP, BMSa or BMSb (50 and 100 μM). The metabolite BMY-33604 could not be tested because of assay interference. Final volumes were 250 μl , with a microsomal protein concentration of 200 $\mu\text{g ml}^{-1}$ (linear range: 40–1000 μg). Solutions of all compounds were prepared in methanol. The solvent was evaporated to dryness prior to addition of cofactors and buffer. Substrate, inhibitors, incubation buffer, and cofactors were preincubated at 37°C for 5 min, and reactions were initiated by the addition of 50 μg microsomal protein. Incubations were performed for 20 min (linear range: 10–60 min) at 37°C, then stopped by the addition of 50 μl of acetonitrile and cooling on ice. Pronethalol was then added as an internal standard, and the mixture was spun at 16000 g for 5 min in a Micro-MB centrifuge. Supernatants were injected into the h.p.l.c. All incubations were done in duplicate.

Analysis of dextrorphan and 3-methoxy-morphinan by h.p.l.c.

Concentrations of DOP and MEM were determined using h.p.l.c. (Waters Associates, Milford, MA, USA). A 30 cm $\times$ 3.9 mm steel C₁₈ μ Bondapak column was used for separation. A Perkin-Elmer 650-10S fluorescence spectrophotometer was set at an excitation and emission wavelength of 280 nm and 310 nm, respectively. The mobile phase consisted of 25% acetonitrile and 75% 50 mM potassium phosphate buffer (pH 6); the flow rate was 1.5 ml min⁻¹. Standard curves were prepared by adding incubation buffer and internal standard (prone-thalol) to known amounts of DOP and MEM, yielding a final volume of 250 μl . Samples were injected directly for assay. With the exception of BMY-33604, the inhibitors did not interfere with the assay. Repeated injections of identical samples over 48 h varied less than 3%. The limits of detection were 0.03 nmol DOP and 0.1 nmol MEM, respectively. Average coefficient of variation for duplicate control curve data points was 4.8% (s.d. 4.2) and for inhibitor data points 7.0% (s.d. 6.3).

Identification of metabolites

Incubations without cofactor were used to verify that formation of the observed metabolic products were NADPH dependent. The identity of the metabolite was verified by comparing retention times with those of authentic standards.

Data analysis

Data points consisting of MEM reaction velocities at varying concentrations of the substrate DMO without inhibitors were fitted by nonlinear least-squares regression [16] to the Michaelis Menten equation. Because DOP formation rates displayed a biphasic pattern in the Eadie-Hofstee plot, DOP reaction velocities were fitted to a double Michaelis Menten equation. The inhibition data were then fitted to the (double) Michaelis Menten equation assuming competitive inhibition at the high affinity site utilizing the predetermined V_{max} and K_m .

Results and Discussion

K_i values for NEF, OH-NEF, pOH-NEF, mCPP and BMSb against DOP formation were more than two orders of magnitude higher (Table 1) compared with QUIN and FLU. The dione metabolite and BMSa did not impair this reaction at all. Thus, NEF and its metabolites appear to be weak inhibitors of CYP2D6 *in vitro* and are therefore unlikely to cause clinically relevant drug interactions with other drugs that are substrates for CYP2D6. In a recent study conducted by van Laar *et al.* [10] subjects received either 100 or 200 mg of oral NEF daily resulting in steady state plasma levels varying between 100 and 600 ng ml⁻¹ (0.2 to 1.2 μM). Metabolites detected in plasma were OH-NEF (0.1 to 0.62 μM), mCPP (0.07 to 0.2 μM) and DIONE (1.3 to 3.7 μM). The liver/plasma partition ratio of NEF and its metabolites is not known. However, assuming partition ratio similar to that of diazepam (11.5) [17], K_i values of NEF and its metabolites for HL 1 and 2 (Table 1) are considerably higher than expected intrahepatic concentrations during usual therapeutic use. Although the estimated liver concentrations of NEF and metabolites might exceed K_i values for HL 3, inhibition by these compounds is considerably less than with FLU, a proven CYP2D6 inhibitor *in vivo* and with a much smaller K_i *in vitro*. It is not clear why inhibition constants for the *O*-demethylation pathway in HL 3 were so different from the other two samples. DOP formation rates in the control condition in all livers (without inhibitors) were consistent with the extensive metabolizer phenotype. V_{max} and K_m values for the high affinity site of HL 1-3 were: 1.3 nmol min⁻¹ mg⁻¹ and 8.53 μM ; 2.54 nmol min⁻¹ mg⁻¹ and 18.5 μM ; and 0.38 nmol min⁻¹ mg⁻¹ and 0.69 μM , respectively. Metabolic capacities, expressed as intrinsic clearance (V_{max}/K_m ratio), were: 0.15, 0.14, and 0.55 nmol min⁻¹ mg⁻¹ μM^{-1} . Coefficients of variation for iterated parameters for both pathways in HL 1, HL 2, and for *N*-demethylation in HL 3 were in a similar range, with a mean of 17.0%. However, kinetic parameters for *O*-demethylation by HL 3 revealed a much higher variation, with a mean coefficient of variation of 125%. The reason for this variation is not known, but might be clarified by investigation of the CYP2D6

Table 1 Inhibition constants (K_i in μM) for quinidine, ketoconazole, fluoxetine, and for nefazodone and its metabolites with respect to the *O*-demethylation (high affinity site) and *N*-demethylation of dextromethorphan by human liver microsomes

	O-demethylation (dextrophan formation)			N-demethylation (3-methoxy-morphinan formation)		
	HL1	HL2	HL3	HL1	HL2	HL3
QUIN	0.04	0.25	0.0016	*	*	*
KET	*	*	*	0.45	0.35	0.31
FLU	0.20	0.03	0.07	*	*	*
NEF	25	50	1.6	20	22	21
OH-NEF	19	34	0.57	23	26	24
pOH-NEF	*	91	7.9	28.96	32.44	35.30
DIONE	*	*	*	*	*	*
mCPP	18	66	1.9	234	365	131
BMS-695	*	*	*	*	*	*
BMS-562	*	39	12	157	214	275

* Because essentially no inhibition was observed, a K_i value could not be determined.

genotype of HL 3, which is clearly from a very extensive metabolizer.

K_i values for NEF, OH-NEF, and pOH-NEF as inhibitors of MEM formation ranged from 21 to 32 μM , about 100-fold higher than the K_i of KET (0.37 μM) for this reaction (Table 1). The other metabolites were even weaker inhibitors. These findings might suggest that NEF and its metabolites would not be important *in vivo* inhibitors of CYP3A3/4, but a recent clinical study indicated large inhibition of clearance of alprazolam and triazolam (both predominantly metabolized by CYP3A3/4) by coadministration of NEF [18, 19]. A study of inhibition of alprazolam hydroxylation by NEF and metabolites *in vitro* indicated that NEF, OH-NEF, and pOH-NEF were significant inhibitors (though weaker than KET), whereas other NEF metabolites again produced minimal inhibition (von Moltke *et al.*, unpublished data). Thus *in vivo* data, as well as *in vitro* studies using relatively pure CYP3A3/4 substrates, indicate that NEF, and probably also OH-NEF and pOH-NEF, are significant CYP3A3/4 inhibitors. It is possible that DMO *N*-demethylation is not solely a CYP3A3/4 mediated reaction as has recently been proposed [12, 13]. MEM formation was reduced only to about 40% of control (unpublished data) at concentrations of KET known to inhibit the CYP3A4 mediated 6- β -hydroxylation of testosterone almost completely [20]. In the present study the mean K_i of KET for DMO *N*-demethylation was an order of magnitude higher than that for the alprazolam 4-hydroxylation ($K_i=0.046 \mu\text{M}$ [21]). A polyclonal rabbit rat liver CYP3A1 antibody, which inhibits subfamily specific CYP3A isoforms in human liver microsomes, inhibited DMO *N*-demethylation only by 50% (data unpublished), whereas it inhibited alprazolam 4-hydroxylation by more than 70% [22]. Therefore, weak inhibition of DMO *N*-demethylation does not necessarily indicate weak CYP3A3/4 inhibition.

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References

- Fontaine R, Ontiveros A, Elie R, *et al.* A double-blind comparison of nefazodone, imipramine, and placebo in major depression. *J Clin Psychiat* 1994; **55**: 234–241.
- Rickels K, Schweizer E, Clary C, Fox I, Weise C. Nefazodone and imipramine in major depression: a placebo-controlled trial. *Br J Psychiat* 1994; **164**: 802–805.
- Anton SF, Robinson DS, Roberts DL, Kensler TT, English PA, Archibald DG. Long-term treatment of depression with nefazodone. *Psychopharmacol Bull* 1994; **30**: 165–169.
- Shukla UA, Marathe PH, Pittman KA, Barbhuiya RH. Pharmacokinetics, absolute bioavailability, and disposition of [^{14}C]nefazodone in the dog. *Drug Metab Dispos* 1993; **21**: 502–507.
- Shukla UA, Kaul S, Marathe PH, Pittman KA, Barbhuiya RH. Pharmacokinetics of nefazodone following multiple escalating oral doses in the dog. *Eur J Drug Metab Pharmacokin* 1992; **17**: 309–318.
- Shukla UA, Marathe PH, Labudde JA, Pittman KA, Barbhuiya RH. Pharmacokinetics of nefazodone in the dog following single oral administration. *Eur J Drug Metab Pharmacokin* 1992; **17**: 301–308.
- Ferry N, Bernard N, Cuisinaud G, Rougier P, Trepo C, Sassard J. Influence of hepatic impairment on the pharmacokinetics of nefazodone and two of its metabolites after single and multiple oral doses. *Fundam Clin Pharmacol* 1994; **8**: 463–473.
- Franc JE, Duncan GF, Farmen RH, Pittman KA. High-performance liquid chromatographic method for the determination of nefazodone and its metabolites in human plasma using laboratory robotics. *J Chromatogr* 1991; **570**: 129–138.
- Walsh AE, Cowen PJ. Attenuation of the prolactin-stimulating and hyperthermic effects of nefazodone after subacute treatment. *J Clin Psychopharmacol* 1994; **14**: 268–273.
- van Laar MW, van Willigenburg APP, Volkerts ER. Acute and subchronic effects of nefazodone and imipramine on highway driving, cognitive functions, and daytime

- sleepiness in healthy adult and elderly subjects. *J Clin Psychopharmacol* 1995; **15**: 30–40.
- 11 Mayol RF, Cole CA, Luke GM, Colson KL, Kerns EH. Characterization of the metabolites of the antidepressant drug nefazodone in human urine and plasma. *Drug Metab Dispos* 1994; **22**: 304–311.
 - 12 Jacqz-Aigrain E, Funck-Brentano C, Cresteil T. CYP2D6- and CYP3A-dependent metabolism of dextromethorphan in humans. *Pharmacogenetics* 1993; **3**: 197–204.
 - 13 Gorski JC, Jones DR, Wrighton SA, Hall SD. Characterization of dextromethorphan N-demethylation by human liver microsomes. Contribution of the cytochrome P450 3A (CYP3A) subfamily. *Biochem Pharmacol* 1994; **48**: 173–182.
 - 14 von Moltke LL, Greenblatt DJ, Harmatz JS, Shader RI. Alprazolam metabolism *in vitro*: studies of human, monkey, mouse, and rat liver microsomes. *Pharmacology* 1993; **47**: 268–276.
 - 15 von Moltke LL, Greenblatt DJ, Cotreau-Bibbo MM, Duan SX, Harmatz JS, Shader RI. Inhibition of desipramine hydroxylation *in vitro* by serotonin-reuptake-inhibitor antidepressants, and by quinidine and ketoconazole: a model system to predict drug interactions *in vivo*. *J Pharmacol Exp Ther* 1994; **268**: 1278–1283.
 - 16 Norby J, Rubenstein S, Tuerke T, Farmer S, Forood R, Bennington J. *SigmaPlot, v. 4.13*. Jandel Corporation 1991.
 - 17 Scavone MJ, Friedman H, Greenblatt DJ, Shader RI. Effect of age, body composition, and lipid solubility on benzodiazepine tissue distribution in rats. *Arzneimittel-Forschung* 1987; **37**: 2–6.
 - 18 Greene DS, Dockens RC, Salazar DE, Barbhuiya RH. Coadministration of nefazodone (NEF) and benzodiazepines I: Pharmacokinetic assessment. *Clin Pharmacol Ther* 1994; **55**: 141.
 - 19 Barbhuiya RH, Shukla UA, Kroboth PD, Greene DS. Coadministration of nefazodone and benzodiazepines: II. A pharmacokinetic interaction study with triazolam. *J Clin Psychopharmacol* 1995; **15**: 320–326.
 - 20 Newton DJ, Wang RW, Lu AYH. Cytochrome P450 inhibitors. Evaluation of specificities in the *in vitro* metabolism of therapeutic agents by human liver microsomes. *Drug Metab Dispos* 1995; **23**: 154–158.
 - 21 von Moltke LL, Greenblatt DJ, Cotreau-Bibbo MM, Harmatz JS, Shader RI. Inhibitors of alprazolam metabolism *in vitro*: effect of serotonin-reuptake-inhibitor antidepressants, ketoconazole and quinidine. *Br J Clin Pharmacol* 1994; **38**: 23–31.
 - 22 von Moltke LL, Greenblatt DJ, Court MH, Duan SX, Harmatz JS, Shader RI. Inhibition of alprazolam and desipramine hydroxylation *in vitro* by paroxetine and fluvoxamine: comparison with other selective serotonin reuptake inhibitor antidepressants. *J Clin Psychopharmacol* 1995; **15**: 125–131.

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