Title page

N-Oxygenation of Oxycodone and Retro-reduction of Oxycodone N-Oxide[a]

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Running Title Page

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Introduction 408

Discussion 1041

Abbreviations

AO, aldehyde oxidase; DETAPAC, diethylenetriaminepentaacetic acid; DPT,

dimethylaminoalkyl phenothiazine; FMO, Flavin-containing monooxygenase, MMI, N-methyl

mercapto imidazole; TAO (troleandomycin), TMA, trimethylamine

2

Abstract

Oxycodone is used as a potent analgesic medication. Oxycodone is extensively metabolized. To fully describe its metabolism, the oxygenation of oxycodone to oxycodone N-oxide was investigated in hepatic preparations. The hypothesis tested was that oxycodone *N*-oxygenation was enzymatic and the amount of N-oxide detected was a consequence of both oxygenation and retro-reduction. Methods for testing the hypothesis included both in vitro and in vivo studies. Results indicated that oxycodone was *N*-oxygenated by the flavin-containing monooxygenase. Oxycodone N-oxide is chemically quite stable but in the presence of hepatic preparations and NADPH was retro-reduced to its parent compound, oxycodone. Subsequently, oxycodone was metabolized to other metabolites including noroxycodone, noroxymorphone and oxymorphone via cytochrome P-450. Retro-reduction of oxycodone N-oxide to oxycodone was facilitated by quinone reductase, aldehyde oxidase and hemoglobin but not to a great extent by cytochrome P-450 or the flavin-containing monooxygenase. To confirm the in vitro observations, oxycodone was administered to rats and humans. In good agreement with in vitro results, substantial oxycodone N-oxide was observed in urine after oxycodone administration to rats and humans. Administration of oxycodone N-oxide to rats showed substantial amount of recovered oxycodone N-oxide. In vivo, noroxycodone was formed as a major rat urinary metabolite from oxycodone N-oxide presumably after retro-reduction to oxycodone and oxidative N-demethylation. To a lesser extent, oxycodone, noroxymorphone and oxymorphone were observed as urinary metabolites.

DMD # 89300

Significance

This manuscript describes the *N*-oxygenation of oxycodone in vitro as well as in small animals

and humans. A new metabolite was quantified as oxycodone N-oxide. Oxycodone N-oxide

undergoes extensive retro-reduction to oxycodone. This re-establishes the metabolic profile of

oxycodone and introduces new concepts about a metabolic futile cycle related to oxycodone

metabolism.

Introduction

Oxycodone is a potent medication used as an analgesic and antitussive. Oxycodone is also used

for severe pain associated with arthritis, disc disease and cancer (Leow, et al., 1992). The

potential beneficial properties of oxycodone for pain is somewhat confounded by its incidence of

addiction liability. Oxycodone and its metabolite oxymorphone are potent mu opioid receptor

agonists (Ahtee and Attila, 1980) and exert their pharmacological activity through this receptor.

Oxycodone pharmacokinetics have been studied (Hoskin et al., 1989; Poyhia et al., 1992).

Oxycodone is extensively metabolized. Oxycodone is metabolized by cytochrome P450 (CYP)

enzymes (Guengerich, 2008) to desmethyl metabolites noroxycodone and oxymorphone

(Baldacci et al., 2004; Ishida et al., 1982; Lalovic et al., 2004). Once *O*-demethylated, oxycodols

form and can be conjugated in Phase II metabolism.

Tertiary amines such as oxycodone could also in principle be *N*-oxygenated by hepatic flavin-

containing monooxygenases (FMOs) (Cashman, 2003; Cashman, 2002; Cashman and Zhang,

2006; Ziegler, 1990). FMOs accept nucleophilic, lipophilic tertiary amine substrates and

4

generally convert them to polar, tertiary amine N-oxides (Cashman, 2000). In certain cases,

prochiral tertiary amines can be stereoselectively *N*-oxygenated by FMO (Cashman, et al., 1992;

Park et al., 1993). Generally, tertiary amine N-oxides are stable although in certain cases, Cope-

type elimination reactions have been observed (Cashman, 1991). Previously, definitive evidence

for metabolic formation of oxycodone N-oxide was not reported. Certain reports suggested the

presence of oxycodone N-oxide as a metabolite (Ishida et al., 1979; Moore et al., 2003; Sonar et

al., 2012) but no unambiguous evidence was reported.

Tertiary amine N-oxides can also be metabolically retro-reduced to their parent amine (Kitamura

et al., 1999). For example, tamoxifen N-oxide is retro-reduced by CYPs and hemoglobin (Parte

and Kupfer, 2005). There was one report of the retro-reduction of aliphatic hydroxylamines by

pig FMO1 (Poulsen et al., 1986) but this was not observed for amphetamine or

methamphetamine hydroxylamine with human FMO3 (Cashman et al., 1999).

Herein, we report that human liver microsomes form oxycodone N-oxide from oxycodone as

determined by LCMS-MS. Oxycodone N-oxide is chemically stable but in the presence of

hepatic microsomes or cytosol, oxycodone N-oxide is rapidly retro-reduced to oxycodone by at

least three distinct hepatic protein systems (i.e., quinone reductase, aldehyde oxidase and

hemoglobin). To confirm in vitro observations, oxycodone was administered to rats and humans.

In both cases, significant amounts of oxycodone N-oxide was formed. Administration of

oxycodone N-oxide to rats showed significant amounts of urinary oxycodone and its metabolites

and confirmed retro-reduction in vivo.

Methods

General

Reagents, starting materials and solvents were purchased in the highest purity available from commercial suppliers and used as received. Human clinical grade oxycodone, oxycodone N-oxide, d^6oxycodone, oxymorphone, noroxycodone, noroxymorphone and (α and β)-oxycodols in >99% pure (based on GLP certificates of analysis) was obtained from Noramco (Athens, GA). Morphine-6-glucuronide was obtained from NIDA drug repository (NIDA, NIH). Naltrexone was purchased from Mallincrodt. Trimethyl amine (TMA), 2-Mercapto-1-methylimidazole (MMI), cotinine, nicotine, menadione, KCN, sodium arsenite, hemoglobin, alpha naphthylflavone, quinidine, $NADP^+$, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and other components of the NADPH-generating system were commercially available from VWR (San Diego, CA). Troleandomycin (TAO) was from Enzo Biochemical, Inc., (Farmingdale, New York). N,N-Dimethyl-alkyl-phenothiazines (i.e., 3-DPT, 5-DPT and 8-DPT) were synthesized as reported previously (Brunelle et al., 1997). Compounds were tested as hydrochloride salts unless otherwise noted. Phosphate buffer, diethylenetriaminepentaacetic acid, catalase and other reagents in the microsome or highly purified enzyme assays were purchased from Life Technologies (Carlsbad, CA).

Photometric Incubations. Human liver microsomes and cytochrome P-450 (CYP1A1, 2A6, 2D6, 3A4) (CYP) were purchased from BD Gentest (now Corning, Corning, NY). Human liver microsomes possessed FMO3 activity (e.g., HH18 or HK37, 2800 and 3600 pmol of methyl *p*-tolyl sulfide S-oxide formed per (mg protein x min), respectively. Human liver microsomes possessed CYP activity (e.g., HH18 and HK37, 12000 and 2500 pmol of 6β-hydroxy testosterone formed per (mg protein x min), respectively. Human liver S-9 was from Cellz Direct

DMD # 89300

(Durham, NC). Human liver S-9 was from Xenotech (Kansas City, KS). Highly purified mouse FMO1, FMO3 and FMO5 was prepared as described previously (Brunelle, et al., 1997, Motika, et al., 2012). A typical photometric incubation mixture contained 5-10 µg recombinant FMO protein and NADPH (0.2 mM) in 0.05 M potassium phosphate buffer (pH 7.4 or pH 10) containing 0.3 mM diethylenetriaminepentaacetic acid (DETAPAC, as an anti-oxidant) placed on ice. Test compounds (10-100 µM) were added to initiate the incubations at 37 ℃. The consumption of NADPH was monitored by UV-vis and initial rate measurements obtained. Time- and protein-dependent studies were done to establish conditions for optimal substrate-dependent studies.

Other Incubations. A typical incubation was conducted in a total volume of 0.25 mL. Incubation mixtures contained 0.4 mg protein, an NADPH-generating system consisting of $NADP^+$ (0.5 mM), glucose-6-phosphate (0.5 mM), glucose-6-phosphate dehydrogenase (1 IU), diethylenetriaminepentaacetic acid (0.3 mM, DETAPAC, as an anti-oxidant) and 0.05 M potassium phosphate buffer (pH 7.4 or pH 10) placed on ice. Incubations were initiated by the addition of test compounds to the ice-cold incubation and placed at 37 ℃ with constant shaking. Incubations were stopped by flash-freezing in dry ice with or without the addition of 0.5 mL 0.5% ammonium hydroxide. Before LCMS analysis, a cold acetonitrile solution containing an internal standard (oxycodone-d[6]) was added to the sample or calibration standard. For anaerobic incubations, screw cap culture tubes were used and microsome incubations were bubbled with Argon gas and sealed under an atmosphere of Argon.

Analytical. Prior to analysis, frozen incubations were combined with 0.5% ammonium hydroxide in acetonitrile (0.5 ml), thawed, mixed thoroughly and centrifuged (15,000 rpm, 7 mins). Analysis of selected in vitro samples was conducted using a multiple reaction

monitoring/information dependent acquisition/enhanced product ion (MRM/IDA/EPI) analysis method. The aqueous-organic material (20 µL) was reconstituted for LC/MS/MS analysis. The mass spectrometer was an Applied Biosystems 4000 QTrap^R with a Turbo Spray source (Applied Biosystems, Waltham, MA). LC pumps were Shimadzu/LC-10AD VP (Shimadzu, Columbia, MD). LC was run on a RP column (i.e., Thermo BetaBasic^{TM} phenyl 3 µm, 150 mm x 2.1 mm i.d.) equilibrated with 93% Solvent A (0.02 M ammonium formate, 0.2% formic acid in water) and 7% solvent B (0.2% formic acid in acetonitrile:isopropanol (95:5)) and the eluant was detected by ESI MS. Typical LCMS retention times for this system were: noroxymorphone (2.35 mins), oxymorphone (2.72 mins), noroxycodone (4.81 mins), d^6-Oxycodone (5.37 mins), Oxycodone (5.44 mins), oxycodone-N-oxide (6.86 mins) and were all simultaneously analyzed by ESI LCMS/MS via multiple reaction monitoring using d^6oxycodone as an internal standard.

Heat Inactivation Studies

Microsomes were placed in buffer and DETAPAC (0.3 mM) and 2 IU of catalase in a screw cap culture tube and purged with argon gas, sealed with a Teflon screw-tight cap placed on ice. The incubation tube was removed from ice and placed at 55 ℃ for 1.5 min. Thereafter, the incubation was placed on ice, the cap removed and the NADPH-generating system was added. The incubation was initiated by the addition of substrate at 37 ℃ with shaking. Generally, under these conditions 60-85% of FMO3 activity was lost while at the same time decreasing CYP only marginally (Cashman, 1999).

Aldehyde Oxidase or Quinone Reductase Assays.

Cotinine formation was a convenient method for verifying hepatic aldehyde oxidase (AO) formation. Conversion of CYP2A6-generated nicotine iminium ion to cotinine was used to determine AO activity (Cashman et al., 1992). Incubations were as above but human liver

cytosol or human liver S-9 (0.5 mg protein) was used in the presence of Tris buffer (50 mM, pH 7.4) containing 3 mM $MgCl_2$. At the termination of the incubation, cold dichloromethane (1 mL) and sodium carbonate (10 mg) was added followed by thorough mixing and centrifugation (4,000 rpm). The organic layer was separated, evaporated to dryness and reconstituted in MeOH for analysis of nicotine and cotinine (Cashman et al., 1992). Quinone reductase assays were conducted as above. For anaerobic incubations, screw cap culture tubes were used and cytosol or human liver S-9 incubations were bubbled with Argon gas and sealed under an atmosphere of Argon.

Data Analysis

Statistics including mean, standard deviation, relative standard deviation and linear regression analysis where conducted when appropriate. Values were compiled using Microsoft Excel[R] and Prism[R] software.

Animal Studies

Animal work followed the Guide for Care and Use of Animals as adopted by NIH. Formal - approval was obtained from the IACUC of HBRI. Five male and five female seven week-old rats (Charles River, Hollister, CA) were administered compounds (oral) and placed in stainless steel metabolic chambers. Urine was collected and food and water consumption was monitored. Two animals of each gender were administered oxycodone N-oxide (20 mg/kg, oral) in sterile water. Three animals of each gender were administered oxycodone HCl (20 mg/kg, oral) in sterile water.

Human Studies

Urine for quantification of oxycodone, noroxycodone, oxymorphone, and oxycodone N-oxide

was collected as part of a Phase 1, three-way crossover, active controlled, pharmacokinetic study

to determine the pharmacokinetics of PTI-821, a slow release form of oxycodone, in healthy

volunteers. The study protocol was approved by IntegReview IRB (Austin, TX). Subjects

underwent an overnight 10-hour fast prior to drug administration. Subjects received an oral dose

of 50 mg naltrexone HCl the evening prior to as well as 30 minutes prior to drug administration.

After administration of 40 mg oxycodone, urine was collected from 0-12, 12-24, 24-36, 36-48,

48-60, 60-72, 72-84, and 94-96 hours after dosing. Urine data was used to calculate the total

amount of each analyte excreted in the urine and to estimate the percentage of the administered

dose of oxycodone recovered in the urine. Only subjects with complete urine collection through

96 hours were included in the calculations. After collection, urine was frozen and shipped to

Advion BioSciences, Inc., Ithica, NY, for bioanalysis by LCMS-MS. Urine samples were

thawed and acetonitrile containing internal standard (oxycodone-d6) was added to the sample

(0.25 mL). Thereafter, 0.5 mL of 0.5% ammonium hydroxide in acetonitrile was added to the

sample, mixed thoroughly for 3 minutes and centrifuged at 15,000 rpm for 7 min. The organic

fraction was injected onto the LCMS-MS system for analysis as described above.

Results

For oxycodone, microsomal metabolism studies showed that in addition to metabolites

previously reported formed (i.e., oxymorphone, noroxycodone, and noroxymorphone),

oxycodone N-oxide was also observed (**Figure 1**). After administration of oxycodone, we also

found oxycodone N-oxide in the urine of rats and relatively high levels in the urine of humans.

Accordingly, we examined the *N*-oxygenation of oxycodone and other related compounds in some detail.

In Vitro *N*-Oxygenation with FMOs.

FMO *N*-oxygenates nucleophilic tertiary amines and converts them to polar, readily excreted tertiary amine N-oxides (Cashman, 2004). We examined the *N*-oxygenation of selected tertiary amine opioids, metabolites and prototypical substrates with highly purified human FMO3 and FMO5. For comparison, we also examined N-oxygenation of selected tertiary amine opioids with highly purified recombinant mouse FMO1, FMO3 and FMO5 (see **Table 1**). Recombinant FMO enzyme was expressed and purified to near homogeneity and used in photometric assays (i.e., NADPH consumption). For FMO3, oxygenation of prototypical substrates MMI and TMA occurred at normal rates (i.e., 18.3 and 20.2 nmol/min/mg of protein). Oxycodone and oxymorphone showed even greater specific rates of *N*-oxygenation (**Table 1**). Interestingly, neither the N-cyclopropyl methyl opioid, naltrexone, nor the 6-glucuronide of morphine (i.e., M-6-G) was detectably *N*-oxygenated by human FMO3. As discussed previously (Cashman, 2000; Ziegler, 1990), FMO3 is sensitive to steric bulk around the tertiary amine moiety and it is not surprising that naltrexone was not a good substrate for human FMO3. The polar nature of the glucuronide, morphine-6-glucuronide, probably precluded it as a substrate for FMO3. Highly purified mouse FMO3 *N*-oxygenated oxymorphone and oxycodone (**Table 1**). Somewhat surprisingly, naltrexone was *N*-oxygenated by mouse FMO. It may be that mouse FMO3 tolerates substrates with larger steric bulk about the tertiary amine. Mouse FMO1 also *N*-oxygenated all three substrates. Unfortunately, an active hFMO1 prep was not available for comparison. The data was in agreement with the literature that suggested FMO1 has a slightly

more accommodating substrate binding domain than FMO3 (Cashman, 2000; Ziegler, 1990). The *N*-oxygenation of oxycodone, oxymorphone and naltrexone by mouse and human FMO5 was examined. Little has been reported on the FMO5 enzyme system (Motika, 2012). Both FMO5 enzymes *N*-oxygenated oxymorphone and oxycodone albeit at relatively similarly low rates. In contrast to human FMO5, mouse FMO5 did not *N*-oxygenate naltrexone. The similarity of mouse and human FMOs to *N*-oxygenate the tertiary amines examined was striking. We have previously reported that tertiary amines were substrates for mouse FMO5 and this data is in agreement with that report (Motika et al., 2012). Overall, the data is in agreement with reports that show a reasonable agreement between the *N*-oxygenation of selective functional substrates by mouse and human FMOs (Motika et al., 2012). This data is shown in **Table 2**.

Table 2 shows that mouse and human FMOs show a similar profile of *N*-oxygenation for selective functional substrates of FMO1, 3 and 5. It is notable that, compared with human FMO1, mouse FMO1 more efficiently *N*-oxygenated 3-, 5-, and 8-DPT. This was confirmed by a comparison of the catalytic efficiency (V_{max}/K_m) of MMI for mouse FMO1 (3.9) and human FMO1 (0.4) affording about a 10-fold difference. This is likely due to the stability and inherent robustness of the mouse recombinant enzyme compared with human FMO1 but the structure activity relationship (SAR) closely paralleled human FMO1. Mouse FMO3 and human FMO3 showed virtually identical DPT *N*-oxygenation SAR. Likewise, mouse and human FMO5 appeared to have similar DPT *N*-oxygenation SAR. In summary, on the basis of recombinant mouse and human FMO *N*-oxygenation of selected opioids (**Table 1**) and dimethylaminoalkyl phenothiazines (DPTs) (**Table 2**), there was a general agreement in the SAR for *N*-oxygenation. Together, the data points to a prominent role of FMO3 in oxycodone metabolism. Accordingly,

we examined the microsomal FMO- and CYP-dependent metabolism of oxycodone in greater

detail.

Microsomal Metabolism of Oxycodone.

In the presence of human liver microsomes enriched with FMO3 (preparation HK37), pH 7.4,

and supplemented with NADPH, oxycodone (200 µM) was converted to noroxycodone and

oxymorphone. Significant amounts of oxycodone N-oxide were observed but no

noroxymorphone was detected (**Table 3**). In the presence of human liver microsomes enriched

in CYP3A4 (preparation HH18), pH 7.4, significantly less noroxycodone and oxymorphone was

formed. Less oxycodone N-oxide was formed compared to microsomes enriched in FMO3 and

no noroxymorphone was detectable (**Table 3**). The time course for product formation was quite

rapid and linear for only 10 mins.

To explore a possible role of FMO3 in metabolite formation, incubations were repeated at pH 10.

In contrast to CYP (pH optima of 7.4), FMO3 has a pH optima of 10 (Cashman, 2005). In the

presence of human liver microsomes enriched with FMO3 (pH 10) noroxycodone and

oxymorphone formation was significantly increased 3- and 1.5-fold, respectively, compared with

incubations run at pH 7.4. At pH 10, oxycodone N-oxide was increased almost 2-fold but

oxymorphone formation was not detected (**Table 4**). In contrast, in the presence of human liver

microsomes (pH 10) enriched in CYP3A4, less noroxycodone and oxymorphone were formed

(**Table 4**). Again, no detectable amount of noroxymorphone was observed. As expected,

compared with incubations at pH 7.4, an increase in formation of oxycodone N-oxide was

observed at pH 10 for both microsome preparations but microsomes enriched in FMO3 showed the greatest N-oxide product formation (**Table 4**).

Microsome Heat Inactivation.

Heat inactivation studies under conditions that preserved ~85% of CYP activity but significantly decreased FMO3 activity were done to further determine a role of FMO3 versus CYP in the formation of oxycodone N-oxide and other oxycodone metabolites (Cashman, 2004). In the presence of heat-inactivated microsomes enriched in FMO3 activity (preparation HK37), pH 7.4, a 3.5- and 2.7-fold decrease in noroxycodone and oxymorphone, respectively, was observed compared with non-heat-inactivated microsomes (**Table 5**). In the presence of heat-inactivated microsomes, formation of oxycodone N-oxide was decreased but not as much as observed for the other oxycodone metabolites observed. Non-enzymatic oxidation of oxycodone (i.e., zero time point, data not shown) was a surprisingly facile process. To assess the relative role of FMO3 and CYP in the metabolism of oxycodone in heat-inactivated microsomes, microsome preparations enriched for CYP3A4 were examined. In the presence of heat-inactivated microsomes enriched in CYP3A4 (preparation HH18), pH 7.4, formation of noroxycodone and oxymorphone was decreased 2- and 1.6-fold, respectively (**Table 6**). In the presence of heat-inactivated microsomes, oxycodone N-oxide formation was decreased 2-fold (**Table 6**). Overall, total oxycodone metabolites formed in microsome preparation HK37 (elevated FMO3) was 75% less than HH18 (low FMO3) but heat inactivation decreased total oxycodone metabolite formation similarly (i.e., 60% and 53% in HK37 and HH18, respectively). On the basis of pH studies and heat inactivation work, we interpreted this data to suggest that FMO3 plays a significant role in the conversion of oxycodone to oxycodone N-oxide (and after retro-reduction of oxycodone N-oxide, in indirectly facilitating oxymorphone and noroxycodone formation).

14

Highly Purified FMO3.

The ability of highly purified human FMO3 to catalyze the *N*-oxygenation of oxycodone was examined. As shown in **Table 7**, oxycodone was *N*-oxygenated by human FMO3 supplemented with NADPH. This result was in agreement with the data of **Table 1** showing oxycodone was a substrate for human FMO3. An extremely small amount of noroxycodone was also detected in the incubation with human FMO3 but no oxymorphone or noroxycodone was observed. Because certain tertiary amine N-oxides are unstable (Cashman, 1991), we examined the chemical and metabolic stability of oxycodone N-oxide in the presence of microsomes and purified enzymes.

Studies with Oxycodone N-Oxide

Incubation of oxycodone N-oxide in phosphate buffer over extended periods of time (24-48 hours) showed no indication of degradation as judged by HPLC. We concluded oxycodone N-oxide was sufficiently stable for metabolism studies. In the presence of human liver microsomes enriched with FMO3 (pH 7.4), a very fast conversion of oxycodone N-oxide (200 µM) to its retro-reduced metabolite oxycodone was observed (**Table 8**). Significant amounts of noroxycodone and oxymorphone were also formed. However, in the presence of a lower oxycodone N-oxide concentration (20 µM) an even greater percent conversion of oxycodone N-oxide to oxycodone and noroxycodone was observed (**Table 8**). In the presence of relatively low oxycodone N-oxide substrate conditions, no oxymorphone or noroxymorphone was detected (**Table 8**). To examine whether the pH optima for FMO3 (i.e., pH 10) influenced the rate of apparent retro-reduction, the study was repeated at pH 10. Compared with incubations done at pH 7.4, in the presence of oxycodone N-oxide (200 µM) and human liver microsomes (pH 10), considerably less oxycodone, noroxycodone and oxymorphone was observed (**Table 9**). When

15

the study was repeated at 20 µM oxycodone N-oxide, the results observed were similar to the

results obtained at a substrate concentration of 200 µM. We interpret the results to suggest a

very high affinity and highly efficient reductase system (or systems) was operating optimally in

human liver microsomes at pH 7.4. The results suggested that the retro-reduction of oxycodone

N-oxide was at least in part dependent on a non-FMO3-dependent process because retro-

reduction was not enhanced at pH 10.

In the presence of heat-inactivated human liver microsomes enriched with FMO3 (pH 7.4), the

retro-reduction of oxycodone N-oxide (20 µM) to oxycodone and formation of noroxycodone

was not significantly different than non-heat inactivated microsomes (**Table 10**). No detectable

oxymorphone or noroxymorphone was formed in the presence of low oxycodone N-oxide

substrate conditions (**Table 10**). The stability of oxycodone N-oxide in the presence of highly

purified human FMO3 supplemented with NADPH or buffer alone was examined. Oxycodone

N-oxide was completely stable under the incubation conditions employed. The results indicated

that oxycodone N-oxide was stable in the presence of FMO3 and buffer but a very efficient

NADPH-dependent reductase system(s) was present in human liver microsomes to afford retro-

reduction. To examine this in greater detail, we examined a role of quinine reductase and other

hemoprotein systems in the retro-reduction of oxycodone N-oxide.

Oxycodone N-Oxide Retro-Reduction

We observed retro-reduction of oxycodone N-oxide in vitro (**Figure 1**). Because of the apparent

rapid oxycodone N-oxide retro-reduction and oxycodone metabolism observed, *N*-oxygenation

was likely underestimated both in human liver microsomes and other hepatic preparations. To

explore this point further, we investigated the retro-reduction of oxycodone N-oxide.

16

The conversion of oxycodone N-oxide to oxycodone in the presence of human liver microsomes, S-9 and purified enzymes was examined to identify the reductase(s) responsible for retro-reduction. Incubations were done under anaerobic conditions and aerobic conditions. As shown in **Table 8**, in the presence of human liver microsomes, anaerobic retro-reduction of oxycodone N-oxide was observed (pH 7.4). The ratio of oxycodone formed vs. oxycodone N-oxide (53% vs. 41%) was not strongly dependent on microsome samples enriched in CYP or FMO3. The ratio of noroxycodone formed vs. oxycodone N-oxide (6.5% vs. 7%) was also not strongly dependent on the presence of CYP or FMO3. To further examine a role of either CYP or FMO3 in oxycodone N-oxide retro-reduction the incubation was repeated at pH 10 (the pH optima for FMO3).

As shown in **Table 9**, the ratio of oxycodone formed vs. oxycodone N-oxide (29% vs. 74%) after 20 mins was greater at pH 10 in the presence of microsome samples enriched in CYP. The ratio of noroxycodone formed vs. oxycodone N-oxide (4.4% vs. 11%) after 20 mins was also greater at pH 10 in the presence of microsomes enriched in CYP. We interpreted this result to suggest both CYP and FMO3 may be contributing to microsomal retro-reduction. To explore this point, heat inactivation studies were done.

As shown in **Table 10**, the ratio of oxycodone formed vs. oxycodone N-oxide (22% vs. 38%) after 20 min incubation was less in the presence of heat-inactivated microsome samples enriched in FMO3 at pH 7.4. The ratio of noroxycodone formed vs. oxycodone N-oxide (3.5% vs. 5.6%) after 20 mins was also less in the presence of microsomes enriched in FMO3. Highly purified human FMO3 did not have a marked effect on the retro-reduction of oxycodone N-oxide and the alternate competitive substrate, MMI did not have any apparent effect on retro-reduction (data

17

not shown). The results suggested that FMO3 may have contributed to microsomal retro-reduction but the contribution was very minor or non-catalytic and relatively rapid based on the amount of product formed at the zero time point. To confirm this, oxycodone N-oxide was incubated with highly purified human FMO3 at pH 7.4 (**Table 11**). Both oxycodone and noroxycodone were detected albeit at low levels. Accordingly, we examined for the involvement of other retro-reduction systems in human liver microsomes.

Retro-reduction of oxycodone N-oxide in human liver microsomes was strongly dependent on NADPH (**Table 12**). Compared to the complete system, in the absence of NADPH or in the absence of NADPH plus menadione, inhibition of retro-reduction to oxycodone was 93% and 92%, respectively (**Table 12**). Likewise, compared to the complete system, in the absence of NADPH or in the absence of NADPH plus menadione, inhibition of formation of noroxycodone was 95% and 98%, respectively. In the presence of menadione alone, inhibition of retro-reduction to oxycodone N-oxide was 73%. In the presence menadione alone, inhibition of formation of noroxycodone was 95%. The results suggested a contribution from quinone reductase in the conversion of oxycodone N-oxide to oxycodone because: a) anaerobic retro-reduction of oxycodone N-oxide was NADPH dependent, and b) retro-reduction activity was not enhanced by other quinones (Kitamura et al., 1999).

Another reductase system present in cytosolic hepatic preparations is aldehyde oxidase (AO) (Pryde et al., 2010). Menadione is a potent inhibitor of AO and is used to eliminate AO so other reductases can be detected. The study was repeated with human liver cytosol (20 µM, pH 7.4, 30 mins) that had been characterized for AO functional activity. The results paralleled the results observed in human liver microsomes although less dramatic. To study this aspect in greater

detail, human liver S-9 was examined because it contained both cytosol and components that

comprise microsomes.

Oxycodone N-oxide retro-reduction in the presence of S-9.

As shown in **Table 13**, in the presence of S-9, oxycodone N-oxide retro-reduction was not

inhibited by KCN or sodium arsenite. Addition of hemoglobin markedly increased the rate of

oxycodone N-oxide retro-reduction and noroxycodone formation.   The results suggested a

contribution from quinone reductase and other hemoproteins such as hemoglobin in the retro-

reduction of oxycodone N-oxide. We examined other hemoproteins such as highly purified

CYPs (i.e., human CYP1A1, CYP2A6, CYP2D6 and CYP3A4) supplemented with NADPH in

the reduction of oxycodone N-oxide.

Metabolism of Oxycodone N-oxide by Highly purified CYPs and FMO3

cDNA-expressed human CYP1A1, CYP2A6, CYP2D6, CYP3A4 and FMO3 were examined for

their ability to retro-reduce oxycodone N-oxide (**Table 14**). In general, robust CYP-mediated

retro-reduction of oxycodone N-oxide was not observed. NADPH alone did not retro-reduce

oxycodone N-oxide suggesting that reducing equivalents alone were not sufficient to support

reduction of the N-oxide. Based on use of selective functional inhibitors, CYP1A1 and CYP2D6

may have contributed to slight retro-reduction of oxycodone N-oxide but the amount of retro-

reduction was very small. In the presence of alpha naphthylflavone, apparent inhibition of

CYP1A1-mediated reduction of oxycodone N-oxide was observed. Similarly, in the presence of

quinidine, apparent inhibition of CYP2D6-mediated oxycodone N-oxide reduction was observed.

The alternate substrate competitive inhibitor MMI had no apparent affect on oxycodone N-oxide

19

retro-reduction (**Table 14**). The overall contribution of CYP1A1, CYP2D6 or FMO3 to oxycodone N-oxide retro-reduction was very small in comparison to other reductase systems. In the presence of CYP1A1, CYP2A6, CYP2D6, CYP3A4 and FMO3, no other products (i.e., noroxycodone, oxymorphone or noroxymorphone) were formed from oxycodone N-oxide (**Table 14**).

Oxycodone Administration to Rats: Oxidation of Oxycodone In Vivo.

After administration of oxycodone (20 mg/kg, oral) to rats the urinary metabolic profile showed that oxycodone was converted to oxycodone N-oxide, noroxycodone, oxymorphone and noroxymorphone as determined by LCMS-MS. The amount of oxycodone N-oxide recovered in the urine was relatively modest. In male rats, the amount of oxycodone N-oxide excreted over 24 hrs was approximately 0.14% of the total mass of parent and characterized metabolites excreted. In female rats, the amount of oxycodone N-oxide excreted over 24 hrs was approximately 2% of the total mass of parent oxycodone and characterized metabolites excreted. The gender difference may be due to the fact that the amount of FMO3 in young male rats is much lower than that in young female rats (Lattard et al., 2001; Lattard et al., 2002).

Oxycodone N-oxide administration to rats in vivo.

After administration of oxycodone N-oxide (20 mg/kg, oral), a large amount of unchanged oxycodone N-oxide was observed in both male and female rats. The predominant metabolite by far was noroxycodone, followed in descending order by oxycodone, noroxymorphone and oxymorphone. Retro-reduction of oxycodone N-oxide to oxycodone was efficient as evidenced by a significant amount of oxycodone and oxycodone metabolites present in the urine.

Oxycodone Administered to Humans

In humans, a total of 9.6 mg of oxycodone and metabolites were recovered in urine after administration of 40 mg PTI-821 (a slow release form of oxycodone). The order of prevalence and percent recovery based on total free drug and metabolites excreted (i.e., 27.3%) included noroxycodone (66%) followed by intact oxycodone (27.1%), oxycodone N-oxide (5.5%) and oxymorphone (0.77%) as determined by LCMS-MS. The greatest amount of oxycodone and metabolites recovered was during the first 12 hours of urine collection.

Discussion

CYP-dependent *O*- and *N*-demethylation are well-recognized metabolic processes (Guengerich, 2008). Oxycodone is converted to its N-desmethyl metabolite, noroxycodone, by the action of oxycodone *O*-demethylase. *O*-Demethylation is also involved in the conversion of oxycodone to oxymorphone. Both oxymorphone and noroxycodone are metabolized to noroxymorphone by the action of CYP3A4 and CYP2D6, respectively (Lalovic, 2004). In addition to CYP-mediated metabolism, cytosolic aldo-keto reductase also contributes to the reduction of the ketone functionality of oxycodone to afford alpha and beta oxycodol diastereomers (**Figure 1**). During the course of this study, we found evidence for extensive formation of noroxycodone and oxymorphone from oxycodone both in vitro and in vivo. Formation of noroxymorphone was observable but it was not formed to the same extent as noroxycodone and oxymorphone. We also observed significant amounts of oxycodone N-oxide formed. We did not examine for the formation of oxycodols.

Previously, the existence of oxycodone N-oxide as a metabolite of oxycodone was postulated (Baldacci, 2004; Moore et al., 2003). One report suggested oxycodone N-oxide was formed in

21

small animals but its structure was not confirmed (Ishida, et al., 1979). The report herein presents

data for the first time that quantitatively confirms the presence of oxycodone N-oxide as a

metabolite of oxycodone both in vitro and in vivo and presents verification of its presence by

LCMS-MS. In addition, robust retro-reduction of oxycodone N-oxide to oxycodone was

observed. The combined data presented in the report herein appears to, for the first time,

complete the description of the Phase I metabolic pathway for oxycodone.

NADPH-dependent formation of oxycodone N-oxide in human liver microsomes was linked to

the presence of human FMO3. Human liver microsomes enriched in FMO3 formed more

oxycodone N-oxide than microsomes enriched in CYP. Heat inactivation of human liver

microsomes under conditions that preserved CYP but destroyed FMO3 afforded microsomes that

formed less oxycodone N-oxide. The difference between findings reported herein and other

previous work (Lalovic, et al., 2004), is likely due to the nature of the metabolite, the thermal

instability of FMO3 and the way metabolism studies were conducted. In the absence of NADPH,

FMO3 is thermally labile (Cashman, 2005). Typically, about 65-80% of enzyme activity is lost

by treating microsomes or other sources of FMO3 with heat (50-60 ℃) for as little as 1-2 mins.

Traditionally, metabolism studies have been conducted by pre-heating human liver microsomes

at 37 ℃ in the absence of NADPH. Often, incubations have been initiated by addition of

NADPH. Under these conditions, most of human FMO3 functional activity is lost. In addition,

analytical methods for quantifying tertiary amine N-oxides can be challenging because N-oxide

metabolites are polar and not easy to isolate. The analytical methodology used herein was robust

and very sensitive.

22

DMD # 89300

In rats, oral administration of oxycodone afforded urinary FMO3 metabolite oxycodone N-oxide

and the CYP-mediated metabolites noroxycodone, oxymorphone and noroxymorphone.

Compared to male rats, female rats excreted approximately 13-fold more oxycodone N-oxide.

The gender difference may be due to the fact that the amount of FMO3 in young male rats is

much lower than that in young female rats (Lattard et al., 2001; Lattard et al., 2002). There may

also be contributions to gender-specific differences in oxycodone N-oxide reductases (e.g.,

aldehyde oxidase).

In the presence of liver preparations and hemoproteins, oxycodone N-oxide is very efficiently

retro-reduced to oxycodone. Retro-reduction of the N-oxide to oxycodone is a pseudo-futile

cycle. In the presence of hepatic preparations, oxycodone is *N*-oxygenated and retro-reduced

many times thus appearing to be a futile cycle. However, NADPH is consumed with each cycle.

Therefore, this is not a pure futile cycle. Because of its polar nature, oxycodone N-oxide tends

not to cross cell membranes and could serve as a reservoir in the tissues where it is formed to

provide a sustained source of material to be converted to oxycodone and in turn, other

metabolites.

Data presented herein point to at least three prominent systems responsible for retro-reduction of

oxycodone N-oxide. Aldehyde oxidase makes a contribution to oxycodone N-oxide retro-

reduction. Secondly, similar to studies of other molecules (Kitamura et al., 1999) quinone

reductase and hemoglobin makes strong contributions to oxycodone N-oxide retro-reduction. In

contrast to other studies (Parte and Kupfer, 2005), however, other hemoproteins such as CYP do

not make a significant contribution to oxycodone N-oxide retro-reduction. If FMO3 contributes

23

to retro-reduction, it is likely via a non-catalytic, protein template-type effect previously reported (Cashman et al., 1999). Thus, CYP and FMO3 have a very strong preference for oxidation of oxycodone and not retro-reduction.

Results from in vitro retro-reduction studies described above were consistent with in vivo studies. In rats, after administration of oxycodone N-oxide, the quantitative order of urinary excretion was as follows: oxycodone N-oxide > noroxycodone > noroxymorphone > oxycodone > oxymorphone. Administration of oxycodone N-oxide to rats showed a gender effect: females excreted a greater amount of oxycodone N-oxide than males on the basis of total mass of oxycodone N-oxide, oxycodone and oxymorphone. Males excreted a higher percentage of noroxycodone and noroxymorphone than females. Again, this may be due to relative functional differences in oxygenases and reductases present in male versus female rats.

In humans, after administration of 40 mg PTI-821 (a slow release formulation of oxycodone) the prevalence (and percent recovery based on total free drug) showed: noroxycodone (27.3%), noroxycodone (66%), oxycodone (27.1%), oxycodone N-oxide (5.5%) and oxymorphone (0.8%) in the first 12 hours. The percent of the dose of oxycodone excreted in human urine as oxycodone N-oxide is consistent with expectations from in vitro studies. That significant oxycodone N-oxide is detected as urinary metabolites in humans suggest that human FMO3 plays a role. As an extension, it may be that gene polymorphisms may play a role in the overall metabolism and distribution of oxycodone (Cashman et al., 2001). Because human FMO3 expression is also developmentally regulated (Koukouritaki et al., 2002), this may also impact the metabolism of oxycodone in the neonate.

24

In summary, this study showed oxycodone was *N*-oxygenated by human FMO3. Oxycodone N-oxide was shown to be retro-reduced to oxycodone by at least three enzymatic systems including AO, quinone reductase and hemoglobin. Oxycodone N-oxide possesses some novel properties in being polar yet sufficiently lipophilic to remain in the endoplasmic reticulum to be converted to oxycodone. Thus, oxycodone N-oxide may serve in a depot effect for metabolic conversion to oxycodone.

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Authorship Contributions

Participated in research design: JC, AdK, GS

Conducted experiments: JC, MG

Contributed new reagents or analytical tools: MG

Performed data analysis: MG, AdK, GS

Wrote or contributed to the writing of the manuscript: JC, AdK, GS

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DMD # 89300

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Footnotes:

Legend for Figure

Figure 1. Metabolic Scheme for Phase I metabolism of Oxycodone and Metabolites.

Table 1. *N*-Oxygenation of Oxymorphone, Oxycodone and Naltrexone by Highly Purified Human and Mouse FMOs

Enzyme	Oxymorphone	Oxycodone	Naltrexone
(nmol N-oxide formed / min / mg of protein)			
m FMO1[a]	27.9[c]	18.3	20.9
h FMO3[b]	30.9	26.8	ND[d]
m FMO3	11.9	11.8	9.4
m FMO5	1.3	0.7	ND
h FMO5	0.9	0.6	0.6

[a]mFMO = Mouse FMO; [b]hFMO = Human FMO; [c]Specific activity for NADPH consumption; [d]ND, Not Detectable. The number of incubations was n = 3 or 4.

Table 2. ***N*-Oxygenation of Selective Functional substrates by mouse and human FMOs.**

Substrate	mFMO1	hFMO1	mFMO3	hFMO3	mFMO5	hFMO5
(nmol product/min/mg of protein)						
3-DPT	195±18	20±1	2±0	6±0	ND[c]	NA[e]
5-DPT	407±21	35±3	41±3	34±1	1.5±0	NA
8-DPT	294±35	24±2	69±1	55±8	11±0	7±0

[a]DPT = 10-(Dimethylamino alkyl)-(3-trifluoromethyl)-phenothiazine where 3-DPT, 5-DPT and 8-DPT has a 3-carbon, 5-carbon and 8-carbon alkyl group, respectively; [b]m = mouse; [c]h = human; [d]ND = Not Detectable
[e]NA = Not Attempted

Table 3. Concentrations of Oxycodone HCl Metabolites in Human Liver Microsomal Samples Incubated with 200 µM Oxycodone HCl at pH 7.4

Human Liver Microsome	Time (min)	Oxycodone[a,b]	Oxycodone-N-Oxide [b,c]	Noroxycodone [b,c]	Oxymorphone [b,c]	Noroxymorphone [b,c,d]
HK37 [e]	0	197.45 ± 5.16	0.20 ± 0.11	0.25 ± 0.01	0.04 ± 0.01	0.011 ± 0.015
	10	196.95 ± 7.14	0.33 ± 0.003	1.73 ± 0.05	0.67 ± 0.05	ND
	20	195.45 ± 7.14	0.36 ± 0.07	1.70 ± 0.03	0.63 ± 0.04	0.013 ± 0.018
HH18 [f]	0	178.93 ± 9.68	0.24 ± 0.09	0.24 ± 0.07	0.02 ± 0.003	0.014 ± 0.02
	10	165.60 ± 7.66	0.29 ± 0.05	0.69 ± 0.08	0.06 ± 0.003	ND
	20	163.70 ± 5.07	0.29 ± 0.07	1.69 ± 0.35	0.13 ± 0.01	ND

a.) The values are expressed as the mean nmoles of oxycodone per mL.

b.) ± standard deviation from the mean.

c.) These values are expressed as the mean nmoles of compound per mL per mg of protein.

d.) ND = Not detected.

e.) CYP3A4 activity was 2.5 nmoles of product per minute per mg of protein in the Testosterone 6β-hydroxylase assay. FMO activity was 3.6 nmoles of product per minute per mg of protein in the methyl *p*-Tolyl sulfide oxidase assay.

f.) CYP3A4 activity was 12.0 nmoles of product per minute per mg of protein in the Testosterone 6β-hydroxylase assay. FMO activity was 2.8 nmoles of product per minute per mg of protein in the methyl *p*-Tolyl sulfide oxidase assay.

Table 4. **Concentration of Oxycodone HCl Metabolites in Human Liver Microsomal Samples Incubated with 200 µM Oxycodone HCl at pH 10**

Human Liver Microsome	Time (min)	Oxycodone[a,b]	Oxycodone-N-Oxide [b,c]	Noroxycodone [b,c]	Oxymorphone [b,c]	Noroxymorphone [b,c,d]
HK37 [e]	0	210.50 ± 1.70	0.32 ± 0.001	0.42 ± 0.09	0.04 ± 0.01	0.013 ± 0.018
	10	196.70 ± 10.61	0.63 ± 0.01	5.08 ± 0.18	0.97 ± 0.02	ND
	20	205.35 ± 0.64	0.70 ± 0.05	5.89 ± 0.11	1.12 ± 0.06	0.09 ± 0.08
HH18 [f]	0	166.03 ± 4.08	0.27 ± 0.05	0.45 ± 0.09	0.02 ± 0.004	ND
	10	170.33 ± 9.08	0.37 ± 0.08	1.07 ± 0.12	0.07 ± 0.004	ND
	20	163.73 ± 6.25	0.32 ± 0.001	0.42 ± 0.09	0.14 ± 0.02	ND

a.) The values are expressed as nmoles of oxycodone per mL.

b.) ± standard deviation from the mean.

c.) These values are expressed as nmoles of compound per mL per mg of protein.

d.) ND = Not detected.

e.) CYP3A4 activity was 2.5 nmoles of product per minute per mg of protein in the Testosterone 6β-hydroxylase assay. FMO activity was 3.6 nmoles of product per minute per mg of protein in the methyl *p*-Tolyl sulfide oxidase assay.

f.) CYP3A4 activity was 12.0 nmoles of product per minute per mg of protein in the Testosterone 6β-hydroxylase assay. FMO activity was 2.8 nmoles of product per minute per mg of protein in the methyl *p*-Tolyl sulfide oxidase assay.

Table 5. Concentration of Oxycodone Metabolites in Human Liver Microsomal (Lot HK37) Samples Incubated with 200 μM Oxycodone HCl at pH 7.4

Human Liver Microsome HK37 [e]	Time (min)	Oxycodone [a,b]	Oxycodone-N-Oxide [b,c]	Noroxycodone [b,c]	Oxymorphone [b,c]	Noroxymorphone [b,c,d]
No Heat treatment	0	165.48 ± 13.54	0.21 ± 0.02	0.09 ± 0.01	0.01 ± 0.05	ND
	20	153.48 ± 11.54	0.23 ± 0.04	1.47 ± 0.17	0.69 ± 0.04	ND
Heat treated	0	160.28 ± 2.27	0.10 ± 0.02	0.04 ± 0.01	0.02 ± 0.01	ND
	20	159.78 ± 5.50	0.15 ± 0.03	0.44 ± 0.04	0.26 ± 0.01	ND

a.) The values are expressed as nmoles of oxycodone per mL.

b.) ± standard deviation from the mean.

c.) These values are expressed as nmoles of compound per mL per mg of protein.

d.) ND = Not detected.

e.) CYP3A4 activity was 2.5 nmoles of product per minute per mg of protein in the Testosterone 6β-hydroxylase assay. FMO activity was 3.6 nmoles of product per minute per mg of protein in the methyl p-tolyl sulfide oxidase assay.

Table 6. Concentration of Oxycodone Metabolites in Human Liver Microsomal (Lot HH18) Samples Incubated with 200 µM Oxycodone HCl at pH 7.4

Human Liver Microsome HH18 [e]	Time (min)	Oxycodone[a,b]	Oxycodone-N-Oxide [b,c]	Noroxycodone [b,c]	Oxymorphone [b,c]	Noroxymorphone [b,c,d]
No Heat treatment	0	176.90 ± 4.88	0.18 ± 0.009	0.45 ± 0.04	0.02 ± 0.001	ND
	20	135.45 ± 4.32	0.87 ± 0.036	8.08 ± 0.36	0.28 ± 0.004	ND
Heat treated	0	155.95 ± 7.04	0.11 ± 0.006	0.14 ± 0.01	0.01 ± 0.040	ND
	20	16.21 ± 1.14	0.42 ± 0.033	4.17 ± 0.42	0.16 ± 0.012	ND

a.) The values are expressed as nmoles of oxycodone per mL.

b.) ± standard deviation from the mean.

c.) These values are expressed as nmoles of compound per mL per mg of protein.

d.) ND = Not detected.

e.) CYP3A4 activity was 12.0 nmoles of product per minute per mg of protein in the Testosterone 6β-hydroxylase assay. FMO activity was 2.8 nmoles of product per minute per mg of protein in the methyl *p*-Tolyl sulfide oxidase assay.

DMD # 89300

Table 7. Concentration of Oxycodone Metabolites in the presence of Purified FM03 Samples Incubated with 200 μM Oxycodone HCl at pH 7.4

Time (min)	Oxycodone[a,b]	Oxycodone N-Oxide[b,c]	Noroxycodone [b,c]	Oxymorphone [b,c]	Noroxymorphone [b,c,d]
20	152.88 ± 8.10	2.09 ± 0.68	0.008 ± 0.002	ND	ND

a.) The values are expressed as nmoles of oxycodone per mL.

b.) ± standard deviation from the mean.

c.) These values are expressed as nmoles of metabolite per mL per minute per mg of protein.

d.) ND = Not detected.

Table 8. Retro-Reduction of Oxycodone N-Oxide by human liver microsomes (pH 7.4).

Human liver microsomes	Oxycodone[b]	Oxycodone N-oxide[a]	Noroxycodone[b]	Oxymorphone[b]	Noroxymorphone[b]
HK37[d]	48.6 ± 3.1	115.9 ± 5.8	7.5 ± 0.5	0.2 ± 0.01	ND[c]
HH18[e]	5.0 ± 0.2	9.4 ± 0.5	0.6 ± 0.02	ND[c]	ND[c]

[a]Values were expressed as the mean $\pm$ std dev. nmols of Oxcodone N-oxide per mL, mean of 4 determinations, pH 7.4. 10 min incubation.

[b]Values were expressed as the mean $\pm$ std dev. nmols of analyte per mL per mg of protein, mean of 4 determinations, pH 7.4. 10 min incubation.

[c]ND, Not detected.

[d]Oxycodone N-oxide was present at 200 µM.

[e]Oxycodone N-oxide was present at 20 µM.

DMD # 89300

Table 9. Retro-Reduction of Oxycodone N-oxide by Human Liver Microsomes (pH 10).

Human liver microsome	Time (min)	Oxycodone[b]	Oxycodone N-oxide[a]	Nor oxy Codone[b]	Oxymorphone[b]	NorOxymorphone[b]
HK37[d]	0	38.8 ± 2.2	147.9 ± 2.5	5.3 ± 0.3	0.1 ± 0.001	ND[c]
	10	42.7 ± 4.5	168.6 ± 14.6	6.4 ± 0.8	0.2 ± 0.04	ND
	20	42.6 ± 1.9	142.4 ± 8.3	6.2 ± 0.04	0.2 ± 0.01	ND
HH18[e]	0	6.5 ± 0.8	13.3 ± 2.2	0.9 ± 0.3	ND[c]	ND
	10	6.8 ± 0.6	12.5 ± 2.2	1.0 ± 0.1	ND	ND
	20	9.4 ± 0.7	12.6 ± 5.5	1.4 ± 0.3	ND	ND

[a]Values were expressed as the mean + std. dev. nmols of Oxcodone N-oxide per mL, mean of 4 determinations, pH 10.

[b]Values were expressed as the mean + std. dev. nmols of analyte per mL per mg of protein, mean of 4 determinations, pH 10.

[c]ND, Not Detected

[d]Oxycodone N-oxide was present at 200 µM.

[e]Oxycodone N-oxide was present at 20 µM.

DMD # 89300

Table 10. Retro-Reduction of Oxycodone N-Oxide by human liver microsomes with or without Heat Inactivation

Human liver microsomes HK37[d]	Time (min)	Oxycodone[b]	Oxycodone N-oxide[a]	Noroxycodone[b]	Oxymorphone[b]	Noroxymorphone[b]
No Heat Treatment	0	2.5 ± 0.2	11.8 ± 0.5	0.3 ± 0.02	ND[c]	ND[c]
	20	4.4 ± 0.2	11.3 ± 2.0	0.6 ± 0.1	ND[c]	ND[c]
Heat Treatment	0	2.9 ± 0.4	12.0 ± 1.3	0.4 ± 0.06	ND[c]	ND[c]
	20	3.5 ± 0.3	15.8 ± 5.2	0.6 ± 0.2	ND[c]	ND[c]

[a]Values were expressed as the mean ± std dev. nmols of Oxcodone N-oxide per mL, mean of 4 determinations, pH 7.4.

[b]Values were expressed as the mean ± std dev. nmols of analyte per mL per mg of protein, mean of 4 determinations, pH 7.4.

[c]ND, Not detected.

[d]Oxycodone N-oxide was present at 20 µM.

DMD # 89300

Table 11. Effect of highly purified human FMO3 on Oxycodone N-Oxide metabolism[a].

Time (min)	Oxycodone[c]	Oxycodone N-oxide[b]	Noroxycodone[c]	Oxymorphone	Noroxymorphone
20	0.001 ± 0.0002	2.4 ± 0.05	0.002 ± 0.0004	ND[d]	ND[d]

[a]Oxycodone N-oxide was present at 20 µM.

[b]Values were expressed as the mean ± std dev. as nmols of Oxcodone N-oxide per mL, mean of 4 determinations, pH 7.4.

[c]Values were expressed as the mean ± std dev. nmols of analyte per mL per mg of protein, mean of 4 determinations, pH 7.4.

[d]ND, Not detected.

Table 12. Effect of Menadione on Human Liver Microsome metabolism of Oxycodone N-Oxide[a, b]

Description	Oxycodone	Oxycodone N-oxide	Noroxycodone	Oxymorphone	Noroxymorphone
Complete	0.06 ± 0.02	0.2 ± 0.01	0.01 ± 0.003	0.001 ± 0.0001	ND[c]
-NADPH	0.01 ± 0.003	0.2 ± 0.04	0.001 ± 0.001	ND[c]	ND[c]
+NADPH +Menadione	0.01 ± 0.001	0.3 ± 0.02	0.001 ± 0.0001	ND[c]	ND[c]
-NADPH +Menadione	0.01 ± 0.002	0.3 ± 0.03	0.0004 ± 0.0004	ND[c]	ND[c]

[a]Oxycodone N-oxide was present at 20 µM. Incubations conducted for 30 mins.

[b]Values were expressed as the mean + std dev. as nmols of analyte per mg of protein, mean of 4 determinations, pH 7.4.

[c]ND, Not detected.

Table 13. Effect of Quinone Reductase inhibitors and Hemoglobin on Human Liver S-9 metabolism of Oxycodone N-Oxide[a, b]

Description	Oxycodone	Oxycodone N-oxide	Noroxycodone	Oxymorphone	Noroxymorphone
Complete	0.09 ± 0.006	0.01 ± 0.001	0.02 ± 0.001	0.001 ± 0.0	ND[c]
+KCN	0.01 ± 0.001	0.004 ± 0.001	0.001 ± 0.0	ND[c]	ND[c]
+Na Arsenite +Menadione	0.01 ± 0.001	0.004 ± 0.001	0.001 ± 0.0	ND[c]	ND[c]
+Hemoglobin +Menadione	0.1 ± 0.002	0.002 ± 0.002	0.005 ± 0.001	0.0004 ± 0.0	ND[c]

[a]Oxycodone N-oxide was present at 20 µM. Incubations conducted for 30 mins.

[b]Values were expressed as the mean ± std dev. as nmols of analyte/min/mg of protein, mean of 4 determinations, pH 7.4.

[c]ND, Not detected.

DMD # 89300

Table 14. Effect of human CYP inhibitors or human FMO3 inhibitor on highly purified enzyme-mediated metabolism of Oxycodone N-Oxide[a, b].

Description	Oxycodone	Oxycodone N-oxide	Noroxycodone	Oxymorphone	Noroxymorphone
None	0.004 ± 0.001	14.6 ± 0.8	ND[c]	ND[c]	ND[c]
Control	0.01 ± 0.002	15.3 ± 1.0	ND	ND[c]	ND[c]
CYP1A1	0.06 ± 0.003	14.3 ± 0.2	ND	ND[c]	ND[c]
CYP1A1 + α-Naphthoflavone	0.04 ± 0.02	14.3 ± 0.2	ND	ND[c]	ND[c]
CYP2A6	0.02 ± 0.001	1.94 ± 0.1	ND	ND	ND
CYP2A6 +Tranylcypromine	0.13 ± 0.001	1.9 ± 0.1	ND	ND	ND
CYP3A4	0.06 ± 0.01	1.8 ± 0.1	ND	ND	ND
CYP3A4 +TAO	0.06	1.9 ± 0.1	ND	ND	ND
CYP2D6	0.04 ± 0.002	14.2 ± 0.5	ND	ND	ND
CYP2D6 +Quinidine	0.02 ± 0.001	13.6 ± 0.8	ND	ND	ND
FMO3[d]	0.002 ± 0.0002	4.4 ± 0.2	ND	ND	ND
FMO3[d] +MMI[e]	0.002 ± 0.0	4.0 ± 0.2	ND	ND	ND

[a]Oxycodone N-oxide was present at 20 µM. Incubations conducted for 10 mins.

[b]Values were expressed as the mean ± std dev. as nmols of analyte/min/nmol of CYP, mean of 4 determinations, pH 7.4.

[c]ND, Not detected.

[d]Values were expressed as the mean ± std dev. as nmols of analyte/min/84.5 µg of FMO3, mean of 4 determinations, pH 7.4.

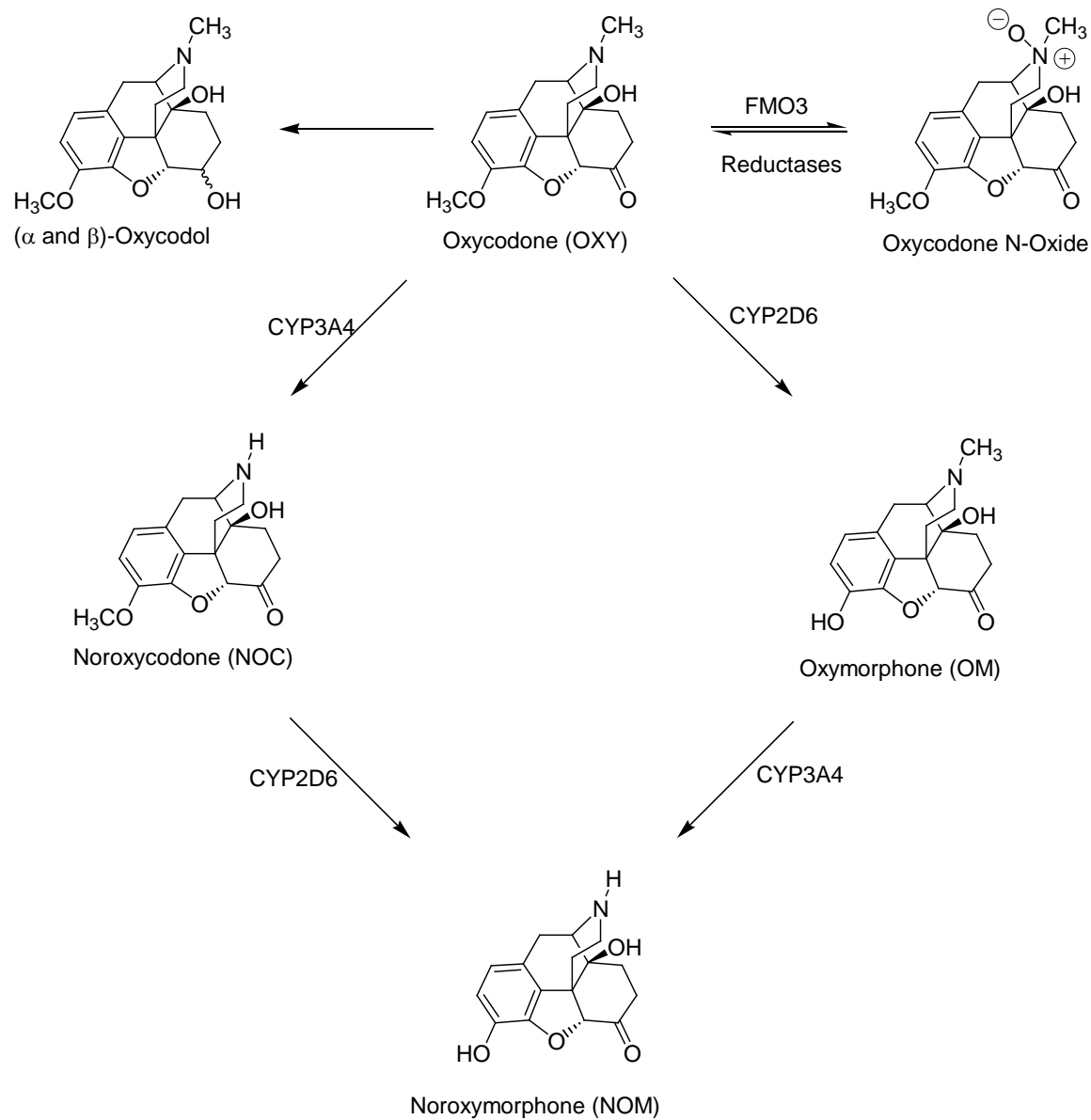[e]MMI, methyl mercapto imidazole, is an alternate substrate competitive inhibitor of FMO3.

Figure 1.