# *In vitro* inhibition and induction of human liver cytochrome P450 enzymes by NTBC and its metabolism in human liver microsomes.

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## ABSTRACT

2-(2-Nitro-4-trifluoromethylbenzoyl)-1, 3-cyclohexanedione (NTBC, also known as nitisinone and marketed as Orfadin®) is an inhibitor of 4-hydroxyphenylpyruvate dioxgenase (HPPD) that is used to prevent the liver and kidney toxicity associated with tyrosinemia type 1, a metabolic disorder in the tyrosine catabolism caused by fumarylacetoacetate hydrolase (FAH) deficiency. Genetically modified mice deficient in FAH [Fah-/-/Il2rg-/-Rag2-/- (FRG) mouse strain] can be repopulated with human hepatocytes to support, among other applications, studies of drug metabolism and disposition. To sustain mouse hepatocellular function prior to (and during) the repopulation with human hepatocytes, FRG mice are treated with NTBC to inhibit the formation of hepatotoxic levels of fumarylacetoacetate (FAA). In the present study, we investigated the metabolism of NTBC in human liver microsomes (HLM) and the potential for NTBC to inhibit or induce human cytochrome P450 (CYP) enzymes. NTBC (1, 10 and 100 µM) was incubated with multiple concentrations of NADPH-fortified pooled HLM (0.5, 1 and 2 mg /mL) for multiple incubation times (0, 30, 60, 120 and 240 min). Little-to-no loss of NTBC was observed, suggesting that NTBC undergoes little or no oxidative metabolism by human liver CYP enzymes and little-to-no ketone reduction by microsomal carbonyl reductase. These results are consistent with the long clinical plasma half-life ($t_{1/2}$ ~ 52 –54 h) reported for NTBC. In CYP inhibition experiments, performed with pooled HLM (0.1 mg/mL), NTBC caused direct inhibition of CYP2C9 ($IC_{50}$ 11 µM). NTBC caused no direct inhibition of CYP1A2, 2B6, CYP2C8, 2C19, 2D6 and 3A4/5. Furthermore, NTBC caused no metabolism dependent inhibition of any of the CYP enzymes evaluated. To evaluate CYP induction, freshly isolated human hepatocytes (n = 3) were cultured and treated once daily for three consecutive days with NTBC (1, 10, and 100 µM), after which microsomal CYP activities and mRNA expression were measured. NTBC (100 µM), had negligible effects (< twofold) on CYP2B6 and 3A4/5 activity but caused a 7.60-fold increase in CYP1A2 activity. However, as a CYP1A2 inducer, NTBC (100 µM) was only 9% as effective as omeprazole. In summary, NTBC has no capacity to inhibit or induce human CYP enzymes which suggests that repopulated FRG mice undergoing NTBC treatment are suitable for studies of drug metabolism involving human CYP enzymes.

XENOTECH

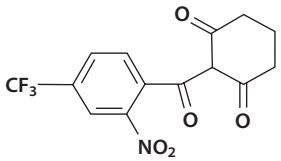
# INTRODUCTION

The results of studies conducted with new drug candidates in nonclinical species like mouse, rat, dog and monkey are always of questionable relevance to humans because of well documented species differences in the function of drug-metabolizing enzymes, such as cytochrome P450 (CYP) enzymes, and their regulation, as reflected, for example, in species differences in the xenosensors (like AhR, CAR and PXR) that mediate the induction of CYP enzymes. For this reason, regulatory agencies advocate the use of *in vitro* studies with human-derived test systems and their nonclinical counterparts (such as hepatocytes or liver subcellular fractions) that permit, for example, an evaluation of species differences in the routes of metabolism of a drug candidate and the potential for drug-drug interactions due to CYP inhibition or induction.

Transgenic mice in which one or more murine genes encoding a drug-metabolizing enzyme or xenosensor have been replaced with their human counterparts provide an *in vivo* test system with which to study selected aspects of drug disposition in a more clinically relevant manner. For example, in mice that have been "humanized" with respect to the xenosensor PXR, the expression of CYP3A is regulated by enzyme-inducing drugs in a more clinically relevant manner than occurs in wild-type mice (Gonzalez and Yu, 2006).

Genetically modified mice whose livers have been repopulated with human hepatocytes potentially allow for the global expression of human hepatic function. These so-called chimeric or humanized mice are immunocompromised to prevent rejection of human hepatocytes, and they harbor other genetic modifications to promote the growth of inoculated human hepatocytes over that of mouse hepatocytes. The PXB mouse, for example, is a urokinase-type plasminogen activator (uPA)$^{+/+}$/severe combined immunodeficient mouse (uPA/SCID mouse) that can be repopulated with human hepatocytes (in some cases to more than 90%). Such mice have been used to support a number of ADME-Tox applications, (Katoh and Yokoi, 2007; Katoh *et al.*, 2007; Schultz *et al.*, 2007). However, uPA$^{+/+}$ mice must be inoculated with hepatocytes at a young age (within two weeks of birth) and are prone to bleeding disorders. They are also prone to a relatively high rate of spontaneous reversion, which allows for the expansion and repopulation of the liver with mouse hepatocytes.

The Yecuris FRG mouse (or Hepatomouse) can also be repopulated with human hepatocytes to support ADME-Tox studies (Azuma *et al.*, 2007; Strom *et al.*, 2010). The Hepatomouse is an immunocompromised mouse (due to genetic deficiencies in Rag2 and Il2rg) with a genetic deficiency in fumarylacetoacetate hydrolase (FAH) that leads to the accumulation of hepatotoxic levels of fumarylacetoacetate (a catabolite of tyrosine) in mouse hepatocytes. The build-up of this toxic intermediate can be controlled throughout the life of the FRG mouse with either a low tyrosine diet or, more conveniently, with NTBC (2-(2-nitro-4-triuoromethylbenzoyl)-1,3-cyclohexanedione, also known as nitisinone). NTBC inhibits 4-hydroxyphenylpyruvate dioxgenase (HPPD), another enzyme involved in tyrosine metabolism, and is marketed under the brand name Orfadin® for the treatment of tyrosinemia type 1 (which is caused by FAH deficiency).




NTBC (2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione

Because NTBC is used to treat FRG mice at various times during the repopulation of their livers with human hepatocytes and control the rate of cell death in mouse hepatocytes, we conducted the present study to ascertain whether NTBC has the potential to interact with human CYP enzymes either as a substrate, inhibitor or inducer.

# MATERIALS & METHODS

### Chemicals and reagents

The sources of reagents used in this study have been described previously (Robertson *et al.*, 2000; Madan *et al.*, 2003; Ogilvie *et al.*, 2006; Paris *et al.*, 2009). NTBC was obtained by Yecuris Corporation through license under Swedish Orphan AB (Stockholm, Sweden).

### Test systems

Cultured human hepatocytes and pooled human liver microsomes (HLM) were prepared at XenoTech, LLC (Lenexa, KS, USA).

### Stability of NTBC in HLM

### Analytical method set-up

To study the metabolism of NTBC based on substrate loss, an LC/MS/MS method was developed and NTBC (analyte) quantitation was based on the following mass transitions listed in **Table 1:**

**Table 1.**

**Mass transitions**

| Compound | Ion Transition (amu) |
|---|---|
| NTBC (substrate) | 328 → 281 |
| 4-Hydroxydiclofenac-$d_4$(internal standard) | 314 → 270 |

### Incubation of NTBC with human liver microsomes (HLM)

NTBC (1, 10 and 100 µM) was incubated with pooled human liver microsomes (0.5, 1 and 2 mg protein/mL) at 37 ± 1°C in 0.2-mL incubation mixtures containing potassium phosphate buffer (50 mM, pH 7.4), $MgCl_2$ (3 mM) and EDTA (1 mM, pH 7.4) with and without cofactor (NADPH-generating system), at the final concentrations indicated. Reactions were started by addition of the cofactor, and were stopped at designated times (0, 30, 60, 120 and 240 min) by the addition of an equal volume of acetonitrile containing internal standard. Zero-time, zero-cofactor (no NADPH), zero-substrate and zero-protein incubations served as blanks. Samples were subjected to centrifugation (920 x g for 10 min at 10°C) to remove precipitated protein. The supernatant fractions were analyzed by LC/MS/MS to quantify the amount of unchanged NTBC.

### Cytochrome P450 inhibition in human liver microsomes

NTBC was assessed for its ability to cause three types of CYP inhibition:

1. Direct inhibition, in which case NTBC and the CYP marker substrate were added simultaneously and incubated for 5 min to determine CYP activity.
2. Time-dependent inhibition (TDI), in which case NTBC was incubated for 30 min with HLM in the absence of NADPH prior to the addition of CYP marker substrate (followed by a 5-min incubation to determine CYP activity).
3. Metabolism-dependent inhibition (MDI) in which case NTBC was incubated for 30 min with HLM in the presence of NADPH prior to the addition of CYP marker substrate (followed by a 5-min incubation to determine CYP activity).

CYP inhibition experiments were conducted with conditions as previously described (Ogilvie *et al.*, 2006; Ogilvie *et al.*, 2008, Paris *et al.*, 2009).

### Enzyme induction and toxicity in human hepatocytes

Cultured human hepatocytes from three donors were treated according to a standard CYP induction protocol (Robertson *et al.*, 2000; Paris *et al.*, 2009). Briefly, after a two-to-three day adaptation period, hepatocytes were treated once daily for three consecutive days with DMSO, (0.1% v/v; vehicle control) one of three concentrations of NTBC (1, 10 and 100 µM) or one of three known prototypical inducers, namely, omeprazole (100 µM), phenobarbital (750 µM) and rifampin (10 µM). During treatment, medium was collected at 0, 24, 48 and 72 hours post treatment and analyzed for lactate dehydrogenase release, an indicator of cell toxicity (loss of membrane integrity) as described in the Roche Applied Science Cytotoxicity Detection Kit (Catalog #1644793). Following treatment, hepatocytes were either harvested for the preparation of microsomes as described by Paris *et al.*, 2009 for subsequent analysis of enzymatic activity or lysed with TRIzol reagent to isolate RNA for analysis of mRNA expression by qRT-PCR as described by Neat *et al.*, 2009.

Microsomes isolated from human hepatocytes at the end of the treatment period were assayed for the following CYP activities: CYP1A2 (phenacetin *O*-dealkylation), CYP2B6 (bupropion hydroxylation) and CYP3A4/5 (testosterone 6β-hydroxylation). Incubation conditions and the analytical methods to measure metabolite formation by LC/MS/MS were those described by Paris *et al.*, 2009.

# RESULTS

**Figure 1** shows the effect of incubation time on the loss of NTBC (1, 10 and 100 µM) from incubations with human liver microsomes (1 mg/mL). Under the conditions evaluated, little to no loss of NTBC was observed even when 1 µM NTBC was incubated with 1 mg/mL HLM for 4 hours. These results suggest that NTBC undergoes little or no metabolism by human liver CYP enzymes (or does so at a very low rate) and that NTBC does not undergo ketone reduction by microsomal carbonyl reductase. These results are consistent with the long clinical plasma half-life ($t_{1/2}$ ~ 52 – 54 h) reported for NTBC (Hall *et al.*, 2001).

**Table 2** shows a summary of the evaluation of NTBC as a direct, time-dependent and metabolism-dependent inhibitor of human CYP enzymes in HLM.

NTBC caused direct inhibition of CYP2C9 with an $IC_{50}$ value of 11 µM as shown in **Figure 2**. There was little or no evidence that NTBC caused direct inhibition of CYP1A2, CYP2B6, CYP2C8, CYP2C19, CYP2D6 and CYP3A4/5 (measured by testosterone 6β-hydroxylation and midazolam 1´-hydroxylation), and the $IC_{50}$ values were reported to be greater than the highest concentration of NTBC studied (*i.e.*, 100 µM). Furthermore, there was no compelling evidence that NTBC caused time-dependent or metabolism-dependent inhibition of any of the CYP enzymes evaluated.

**Figure 3** shows the mean fold induction for both CYP activity and mRNA following treatment of human hepatocytes with NTBC, daily, for three consecutive days.

Under the conditions of this study, where the positive controls caused anticipated increases in CYP enzyme activities and mRNA levels, NTBC at 1 and 10 µM concentrations caused no increase in either CYP1A2, CYP2B6 and CYP3A4/5 activity, or CYP1A2, CYP2B6 and CYP3A4 mRNA levels. In contrast, 100 µM NTBC caused an increase in CYP1A2 activity and in CYP1A2 and CYP3A4 mRNA levels; however, these increases were less than 40% of the positive controls, omeprazole (CYP1A2) and rifampin (CYP3A4). At the highest concentration tested (100 µM) and at the last time point (72 h) NTBC caused an increase in LDH release in two of three cultures (data not shown). Additionally, the highest concentration of NTBC (100 µM) caused a decrease in CYP2B6 and CYP3A4/5 activity (individual data not shown) in two of three hepatocyte preparations, which was associated with cell toxicity (based off LDH release).

## CONCLUSIONS

- Little-to-no substrate loss of NTBC was observed when NTBC was incubated for up to 4 hours with NADPH-fortified pooled HLM (1 mg/mL), suggesting that NTBC undergoes little or no oxidative metabolism by human liver CYP enzymes and little-to-no ketone reduction by microsomal carbonyl reductase. These results are consistent with the long clinical plasma half-life ($t_{1/2}$ ~ 52 –54 h) reported for NTBC.
- In human liver microsomes, NTBC was a direct inhibitor of CYP2C9 ($IC_{50}$ 11 µM) but, at concentrations up to 100 µM, NTBC caused no direct inhibition of CYP1A2, 2B6, CYP2C8, 2C19, 2D6 or 3A4/5. Furthermore, NTBC caused no metabolism-dependent inhibition of any of the CYP enzymes evaluated.
- In cultured human hepatocytes NTBC had negligible effects (< twofold) on CYP2B6 and 3A4/5 activity but, at the highest concentration tested (100 µM) NTBC caused a 7.6-fold increase in CYP1A2 activity. However, as a CYP1A2 inducer, NTBC (100 µM) was only 9% as effective as omeprazole.
- In summary, with the exception of CYP2C9 inhibition, NTBC has little effect on human CYP enzymes either as an inhibitor or inducer. Furthermore, NTBC is not rapidly or extensively metabolized by microsomal CYP enzymes. The results suggest that FRG mice repopulated with human hepatocytes and undergoing (or having undergone) NTBC treatment are suitable for studies of drug metabolism involving human CYP enzymes.

**Table 2.**

**Summary of *in vitro* evaluation of NTBC as an inhibitor of human CYP enzymes in HLM**

| | | Direct inhibition | | Time-dependent inhibition | | Metabolism-dependent inhibition | | Potential for time-dependent or metabolism-dependent inhibition[c] |
|---|---|---|---|---|---|---|---|---|
| | | Zero-minute preincubation | | 30-minute preincubation without NADPH | | 30-minute preincubation with NADPH | | |
| Enzyme | Enzyme reaction | $IC_{50}$ (µM)[a] | Maximum inhibition at 100 µM (%)[b] | $IC_{50}$ (µM)[a] | Maximum inhibition at 100 µM (%)[b] | $IC_{50}$ (µM)[a] | Maximum inhibition at 100 µM (%)[b] | |
| CYP1A2 | Phenacetin *O*-dealkylation | >100 | NA | >100 | NA | >100 | 2.3 | Little or no |
| CYP2B6 | Efavirenz 8-hydroxylation | >100 | 2.9 | >100 | 7.9 | >100 | 8.3 | Little or no |
| CYP2C8 | Amodiaquine *N*-dealkylation | >100 | 6.1 | >100 | 5.4 | >100 | 26 | Little or no |
| CYP2C9 | Diclofenac 4´-hydroxylation | 11 | 90 | 10 | 90 | 9.1 | 91 | Little or no |
| CYP2C19 | *S*-Mephenytoin 4´-hydroxylation | >100 | 9.7 | >100 | 11 | >100 | 12 | Little or no |
| CYP2D6 | Dextromethorphan *O*-demethylation | >100 | 0.68 | >100 | 2.1 | >100 | 6.2 | Little or no |
| CYP3A4/5 | Testosterone 6β-hydroxylation | >100 | 2.3 | >100 | NA | >100 | 5.6 | Little or no |
| CYP3A4/5 | Midazolam 1´-hydroxylation | >100 | NA | >100 | NA | >100 | 9.6 | Little or no |

a. Average data (*i.e.*, percent of control activity) obtained from duplicate samples for each test article concentration were used to calculate $IC_{50}$ values. $IC_{50}$ values were calculated with XLFit.

b. Maximum inhibition (%) is calculated with the following formula and data for the highest concentration of test article evaluated (results are rounded to two significant figures): Maximum inhibition (%) = 100% – Percent solvent control.

c. Potential for time-dependent or metabolism-dependent inhibition was assessed by comparison of $IC_{50}$ values with and without preincubation or NADPH and/or by comparison of the maximum inhibition (%) with and without preincubation or NADPH and by visual inspection of the $IC_{50}$ plot.

NA. Not applicable. No value was obtained as the rates at the highest concentration of NTBC evaluated (100 µM) were higher than the control rates.

**Figure 1.**

**Effect of incubation time on the loss of NTBC (1, 10 and 100 µM) from incubations with human liver microsomes (1 mg/mL)**

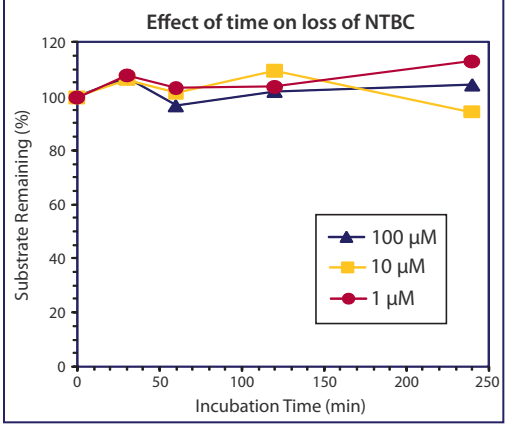



**Figure 2.**

**Inhibition of CYP2C9 (diclofenac 4´-hydroxylation) by NTBC: $IC_{50}$ determination**

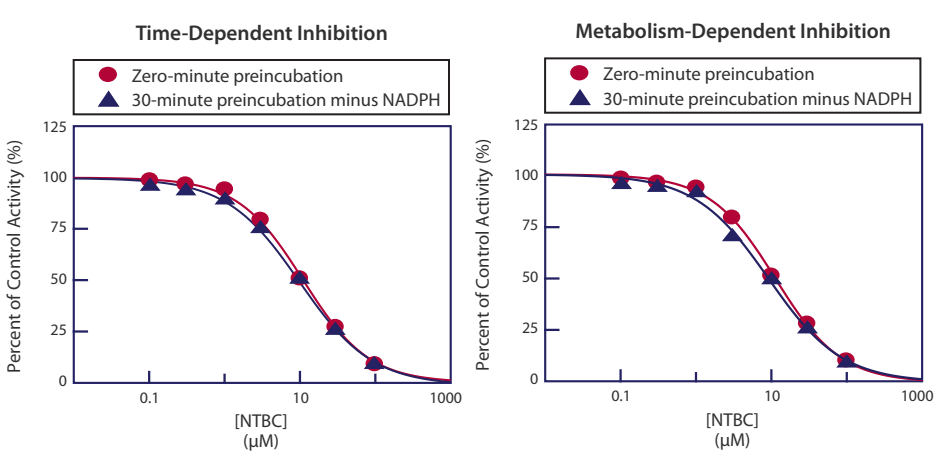
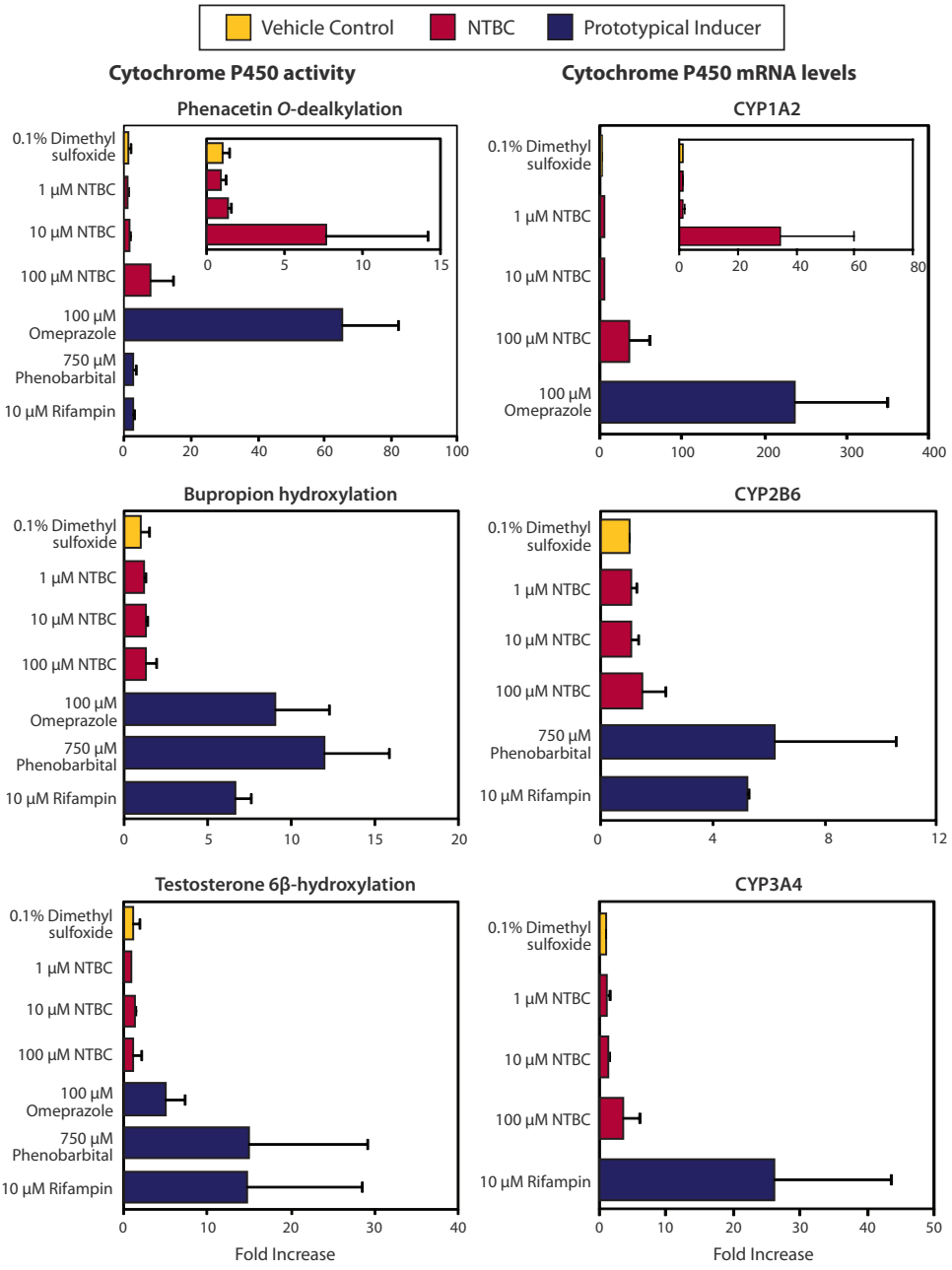


**Figure 3.**

**The effects of treating cultured human hepatocytes with NTBC or prototypical inducers on microsomal cytochrome P450 (CYP) enzyme activity and mRNA levels**




Fold increase = treated / vehicle control

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