In Vitro Assessment of Mitochondrial Dysfunction and Cytotoxicity of Nefazodone, Trazodone and Buspirone

James A. Dykens¹, Joseph D. Jamieson², Lisa D. Marroquin², Sashi Nadanaciva³, Jinghai J. Xu⁴, Margaret C. Dunn⁴, Arthur R. Smith⁴, and Yvonne Will⁵*

¹Drug Safety Research and Development, Pfizer, Inc., Ramsgate Road, Sandwich, England CT139NJ
² Drug Safety Research and Development, Pfizer, Inc., 10646 Science Center Drive, San Diego, CA 92121
³MitoSciences, Inc., 1850 Millrace Drive, Eugene, OR 97403
⁴Systems Biology, Pfizer Research Technology Center, Pfizer, Inc., 620 Memorial Drive, Cambridge, MA 02139.
⁵* Exploratory Safety Differentiation, Pfizer, Inc., Eastern Point, Groton CT 06340

* To whom correspondence should be addressed.
Fax: (860) 441-9637
Phone: (860) -686-2832
E-mail: yvonne.will@pfizer.com.

Abbreviations:

OXPHOS - oxidative phosphorylation
CYP3A4 – cytochrome P450, isoform 3A4
KEY WORDS: nefazodone, trazodone, buspirone, hepatotoxicity, mitochondria, drug toxicity,
Abstract

Mitochondrial toxicity is increasingly implicated in a host of drug-induced organ toxicities, including hepatotoxicity. Nefazodone was withdrawn from the US market in 2004 due to hepatotoxicity. Accordingly, we evaluated nefazodone, another triazolopyridine trazodone, plus the azaspirodecanedione buspirone, for cytotoxicity and effects on mitochondrial function. In accord with its clinical disposition, nefazodone was the most toxic compound of the three, trazodone had relatively modest effects, while buspirone showed the least toxicity. Nefazodone profoundly inhibited mitochondrial respiration in isolated rat liver mitochondria and in intact HepG2 cells where this was accompanied by simultaneous acceleration of glycolysis. Using immunocaptured OXPHOS complexes, we identified Complex 1, and to a lesser amount Complex IV, as the targets of nefazodone toxicity. No inhibition was found for trazodone, and buspirone showed 3.4-fold less inhibition of OXPHOS Complex 1 than nefazodone. In human hepatocytes that express CYP3A4, after 24 hr exposure, nefazodone and trazodone collapsed mitochondrial membrane potential, and imposed oxidative stress, as detected via glutathione depletion, leading to cell death. Our results suggest that the mitochondrial impairment imposed by nefazodone is profound and likely contributes to its hepatotoxicity, especially in patients co-treated with other drugs with mitochondrial liabilities.
Dykens et al., 3/5/2008

Introduction

Nefazodone is a triazolopyridine antidepressant. Its efficacy is based on inhibiting the reuptake of serotonin by antagonizing the 5-HT2 receptor and also $\alpha_1$-adrenergic receptors (Taylor et al., 1995; Eison et al., 1999). Trazodone is also a triazolopyridine derivative that inhibits serotonin re-uptake and also is a 5-HT2 receptor antagonist. The azaspirodecanedione anxiolytic and antidepressant buspirone is a 5-HT1A receptor partial agonist, and a mixed agonist/antagonist on postsynaptic dopamine receptors.

Bristol-Myers Squibb discontinued the sale of nefazodone (“Serzone”) in the United States in 2004 due to hepatotoxicity with an incidence of 28.9/100,000 patients (Aranda-Michel et al., 1999; Lucena et al., 2003; Eloubeidi et al., 2000; Choi, 2003; DeSanty and Amabile, 2007). However, several generic formulations of nefazodone are still available. In contrast, trazodone is associated with lower incidence of liver injury (DeSanty and Amabile, 2007), and adverse event reports on organ toxicity are rare for buspirone. Although the mechanisms of hepatotoxicity for trazodone have been attributed to a hypersensitivity mechanism (Chu et al., 1983; Shiek and Nies, 1983; Longstreth and Hershman, 1985; Beck, 1993; Hull, 1994; Fernandes, 2000), the mechanism of idiosyncratic liver injury for nefazodone is not fully understood.

Nefazodone is metabolized by CYP3A4, but also inhibits the enzyme which can lead to drug-drug interactions with numerous xenobiotics including statins and macrolides, many of which have their own mitochondrial liabilities (Benazzi, 1997; Alderman, 1999; Nadanaciva et al., 2007b). For example, most of the statins, except fluvastatin, are metabolized by CYP3A4 (Karnik and Maldonado, 2005). Trazodone only weakly inhibits CYP3A4 (Caccia, 1998).
Kostrubsky et al. (2006) reported inhibition of canicular transport with nefazodone, but not with trazodone or buspirone. Cmax values of 2-4µg/ml are reported in human plasma, and there are no data documenting the plasma-to-liver ratio of nefazodone, but if nefazodone accumulates in the liver it could potentially inhibit its own elimination (Kostrubsky et al., 2001). Indeed, reduced hepatic clearance has been reported in elderly women taking nefazodone (Caccia, 1998).

Recently it was reported that nefazodone preferentially inhibits Complex I of the electron transport system (Nadanaciva et al., 2007a). Here we expand upon this work by evaluating nefazodone, trazodone and buspirone effects on isolated rat liver mitochondria, and on cytotoxicity to HepG-2 cells grown with only galactose as respiratory substrate to render them more reliant on OXPHOS (Marroquin et al., 2007). Because HepG-2 cells have little or no CYP3A4 activity, and because they are transformed cells derived from a tumor, the three drugs were also evaluated in primary human hepatocytes that express CYP3A4 (Wilkening et al., 2003) by monitoring mitochondrial membrane potential, cytotoxicity and cellular glutathione levels.
Materials and Methods

Materials

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and Toronto
Research Chemicals (Toronto, Canada) and were of the highest purity available.
Phosphorescent oxygen-sensitive probe, type A65N-1, was from Luxcel Biosciences
(Cork, Ireland). The BCA kit for protein determination was from Pierce (Rockford, IL).
Black body clear bottom 96-well plates (Costar 3631) were purchased through VWR
(Westchester, PA). Nunc Maxisorp clear bottom 96-well plates were purchased from
Fisher. Protein G plates were from Pierce (Rockford, IL). All monoclonal antibodies
were from MitoSciences Inc. (Eugene, OR). Cell culture media and supplements were
purchased from Invitrogen (Carlsbad, CA), except for fetal bovine serum (FBS) which
was purchased from Tissue Culture Biologics (Los Alamitos, CA). Culture flasks (BD
Biocoat) and 96-well plates (BD Biocoat) were purchased from VWR (Westchester, PA).
CellTiter-Glo Luminescent Cell Viability Assay kits were purchased from Promega
(Madison, WI). Culture plates for metabolic profiling were obtained from Seahorse
Biosciences (Chicopee, MA).

Animals

Care and Maintenance were in accordance with the principles described in the
Guide for Care and Use of Laboratory Animals (NIH Publication 85-23, 1985). Male
Sprague-Dawley Rats (150-180g) were purchased from Charles River (Wilmington,
MA). Animals were housed in pairs in a controlled environment with constant
temperature (21 ±2 °C) and a 12-h light/dark cycle. Food and water were available ad
Dykens et al., 3/5/2008

libitum. Animals were euthanized with an overdose of carbon dioxide. Organs were rapidly excised and placed into ice-cold mitochondrial isolation buffers (see below).

Cell Culture Conditions for HEPG2 cells

High-glucose media: high-glucose Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen 11995-065) containing 25mM glucose and 1mM sodium pyruvate and supplemented with 5mM N-2-hydroxyethylpiperazine-N\#-2-ethanesulfonic acid (HEPES), 10% FBS, and penicillin-streptomycin (pen-strep; 500 µg/ml final concentration).

Galactose media: DMEM deprived of glucose (Invitrogen 11966-025) supplemented with 10mM galactose, 2mM glutamine (plus 4mM in media prior to supplementation to yield total of 6mM), 5mM HEPES, 10% FBS, 1mM sodium pyruvate, and pen-strep as above.

HepG2 cells (ATTC, Manassas, VA) were either grown in glucose or galactose-containing media and kept in 5% CO₂ at 37°C. Cells were maintained on collagen-coated 150 cm² flasks (356486, BD Biocoat) and seeded onto 96-well plates for individual experiments.

Measurement of Cellular ATP Content in HepG2 cells

Cells were plated at 40,000 cells/ml on collagen-coated, clear 96-well plates. The final media volume was 100 µl. Cellular ATP concentrations were assessed by using the CellTiter-GloCell Viability Assay as per manufacturer’s instructions. For drug treatments, compound stock solutions were prepared in DMSO and added to the wells to
Dykens et al., 3/5/2008

give the indicated final drug concentrations. Final DMSO concentration was 0.5%. Drugs were added 24 h before measurements.

**Metabolic profiling**

*Growth medium:* Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen 11995-065) containing 25mM glucose and 1mM sodium pyruvate and supplemented with 5mM N-2-hydroxyethylpiperazine-N\#-2-ethanesulfonic acid (HEPES), 10% FBS, and penicillin-streptomycin (pen-strep; 500 µg/ml final concentration).

*Unbuffered medium:* Dulbecco’s modified Eagle’s medium Base 8.3g/L (Sigma-Aldrich D5030) supplemented with 2mM GlutaMax-1, 1mM Sodium Pyruvate, 25mM Glucose, 31.58mM NaCl, and 15mg of Phenol Red. Media was warmed to 37°C and the pH was adjusted to 7.4 using 5M KOH.

HepG2 cells were seeded in XF 24-well cell culture microplates at 7.5×10^4 cells/well in 250µl growth medium and then incubated at 37°C/5% CO2 for 20–24 h. Assays were initiated by removing the growth medium from each well and replacing it with 750µl of unbuffered medium prewarmed to 37°C. Cells were incubated at 37°C for 30 min to allow media temperature and pH to reach equilibrium before the first rate measurement. Prior to the rate measurements, the XF24 Analyzer gently mixed the assay media in each well for 10 min to allow the oxygen partial pressure to reach equilibrium. Following the mixing, the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured simultaneously three times to establish a baseline rate. For each measurement there was a mix/wait time of about 5 minutes to restore normal oxygen tension and pH in the microenvironment surrounding the cells. After the baseline
measurement, 75µl of a testing agent prepared in assay medium was then injected into each well to reach the desired final concentration as indicated on the graphs. Multiple measurements as well as compound injections were made at the time points indicated. The values of OCR and ECAR reflect both the metabolic activities of the cells and the number of cells being measured. For relative measurements comparing metabolic rate after compound exposure to a pre-exposure baseline, that is, when data are expressed as a percentage of OCR or ECAR change over baseline, the number of cells present in a well does not confound analysis since the same cell population is assayed at each time interval, i.e., paired comparison design.

**Measurement of Respiration in Isolated Rat Liver Mitochondria**

Liver mitochondria were isolated and oxygen consumption was monitored in 96-well plate format using a phosphorescent oxygen sensitive probe as previously described (Hynes et al., 2006; Will et al., 2006; Nadanaciva et al., 2007b) with minor modifications. Briefly, A65N-1 oxygen probe was reconstituted in 10.5 ml of mitochondrial incubation buffer to a concentration of approximately 100 nM. 100 µl of this solution were pipetted into each well of a 96 well plate (10 pmol of probe per well). For drug treatments, compound stock solutions were prepared in DMSO and added to the wells to give the indicated final concentrations (final DMSO <0.5%). All drug concentrations are presented as nmol/mg of mitochondrial protein. After drug or vehicle addition, 50 µl of mitochondria stock solutions were added to each well giving the desired final concentration of mitochondria, followed by 50 µl of substrate (12.5/12.5 mM glutamate/malate/malate final concentration) without or with ADP (1.65 mM final
concentration) in incubation buffer. Finally, 100 µl of heavy mineral oil was added to each well to seal the samples from ambient oxygen, and the plate was placed in a fluorescence plate reader (Safire² Tecan, Innsbruck, Austria) equilibrated at 30°C and monitored over a period of 20 minutes measuring probe fluorescence signal in each well every 1.5 minutes in kinetic mode. Instrument settings were: 380/650nm excitation/emission, filters, a delay time of 30 µs and a measurement window of 100 µs and active temperature control of the microplate compartment at 30°C. To ensure gas and temperature equilibration of samples at the start of the assay, all the dispensing steps were carried out at 30°C using pre-warmed solutions and a Multi-Blok® heater (Barnstead/LabLine, Melrose Park, IL) holding the microplate. After completion of fluorescence measurements, time profiles of fluorescence intensity in each well were analyzed using Magellan® (Tecan) and Excel® (Microsoft) software, to determine the rates of oxygen consumption based on the known relationship between probe fluorescence and oxygen concentration (Will et al., 2006). Rates of change of dissolved oxygen were subsequently determined from the slopes of these concentration profiles, over the initial 8 min.

Measurement of Activities of Individual OXPHOS Complexes

Bovine heart mitochondria were isolated according to Smith (1967). Activities of Complex I (NADH-Ubiquinone oxidoreductase), Complex II + III (Succinate-cytochrome c oxidoreductase), Complex IV (Cytochrome c oxidase), and Complex V (F₁F₀-ATPase) were all performed as previously described (Nadanaciva et al, 2007a, b).
For drug treatments, compound stock solutions were prepared in DMSO, added to multichannel Dilux™ Dilution Reservoirs (ISC BioExpress, Kaysville, UT) containing the appropriate assay solution and then dispensed into each 96 well plate in quadruplicate wells: in the Complex I, IV and V activity assays, measurements for a compound at a given concentration were done in triplicate wells coated with the appropriate immunocapture monoclonal antibody and a single well containing a null capture antibody (negative control); in the Complex II + III activity assay, measurements for a compound at a given concentration were done in triplicate wells with bovine heart mitochondria and a single well with no mitochondria (negative control). The final DMSO concentration in all these activity assays was 1.5% (v/v). This concentration of DMSO did not have an inhibitory effect in any of the assays. Each assay was read in a SpectraMax Plus™ plate reader immediately after addition of the assay solution (containing the drugs) to the 96-well plates. Absorbance values obtained during all activity assays on the SpectraMax Plus™ plate reader were exported from SoftmaxPro to either Excel or SigmaPlot. 100% activity (i.e. no inhibition) for each complex was determined as the mean of the triplicate measurement in absence of compound – negative control value in absence of compound. IC_{50} values were generated using a four-parameter logistic equation.

**Human Hepatocyte Imaging Assay**

Cryopreserved human hepatocytes were obtained commercially (CellzDirect [http://www.cellzdirect.com/]). The cells were plated on collagen I-coated BD BioCoat 96-well plates in hepatocyte plating medium (Dulbecco’s Minimal Essential Medium with 5% fetal bovine serum; all media obtained from CellzDirect). Upon cell attachment,
the medium was changed to hepatocyte culturing medium (Williams E medium, from CellzDirect). On the 2nd day, the Matrigel overlay was applied according to CellzDirect recommended protocol. On the 3rd day, the cells underwent a medium change with hepatocyte culturing medium. On the 4th day, the cells were treated overnight with the compound of interest or vehicle (0.1% DMSO). All compounds were initially solubilized in DMSO and diluted in culturing medium containing 5% fetal bovine serum to a final DMSO concentration of 0.1%. After 24 hours of incubation (37°C, 5% CO₂, 100% humidity), media were removed and the cells were stained by fluorescent probes in the same culturing medium lacking serum. The fluorescent probes were: tetramethyl rhodamine methyl ester for mitochondrial membrane potential (TMRM; 0.02 uM, 1hr), 1,5-Bis[(2-(dimethylamino)ethyl)amino]-4,8-dihydroxyanthracene-9,10-dione for nuclei and lipids DNA (DRAQ5; 45 uM, 30 min), 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester for reactive oxygen species (CM-H₂DCFDA; 10 uM, 30 min), and finally monochlorobimane for glutathione (mBCl; 80 uM, 5 min). Automated live-cell multi-spectral image acquisition was performed on a Kinetic Scan Reader (Cellomics (http://www.cellomics.com/)) using a 20x objective and an XF93 filter. The mBCl probe was added live on the deck of the instrument. The fluorescence images were captured according to the excitation and emission wavelengths of each probe:

- $655 \pm 15$ and $730 \pm 25$ nm for DRAQ5.
- $475 \pm 20$ and $515 \pm 10$ nm for CM-H₂DCFDA
- $549 \pm 4$ and $600 \pm 12.5$ nm for TMRM
- $365 \pm 25$ and $515 \pm 10$ nm for mBCl
To capture enough cells (>500) for analysis, six image fields starting from the center of a well were collected from each well. Image analysis was performed using ImagePro Plus software (Media Cybernetics, Bethesda, MD).

**Statistics**

For mitochondrial respiration measurements, the slopes of O₂ consumption during the initial 8 min of the reaction were analyzed via one-way ANOVA, followed by Tukey’s *post hoc* test (GraphPad Prism 4). For ATP depletion and isolated mitochondrial respiration assays, IC₅₀ values were generated using GraphPad Prism 4 or Sigma Plot.
Results

Effect on ATP content in glucose and galactose grown HepG2 cells.

Recently we have developed a cell model where HepG2 cells are cultured in media where glucose is replaced by galactose. Under these conditions the cells are more reliant on OXPHOS, thereby becoming susceptible to mitochondrial toxicants and to drugs with known mitochondrial liabilities (Marroquin et al., 2007). As such, these cells are better suited for assessments of drug-induced mitochondrial dysfunction. Here we evaluated Nefazodone, Buspirone and Trazodone in both cell lines to examine possible mitochondrial effects of the three compounds.

HepG2 cells grown in glucose and galactose-containing media were treated with 200µM of either nefazodone, trazodone or buspirone for 24 hours (Figure 1). At this concentration, nefazodone was the most toxic drug, depleting 100% of ATP in both, glucose and galactose-grown cells, trazodone showed severe toxicity in galactose-grown cells (>90% depletion), but only showed moderate toxicity (<50%) in glucose-grown cells. Buspirone was even less toxic; in galactose cells it exhibited moderate toxicity (60%) and it glucose-grown cells it exhibited no toxicity (Figure 1). Due to nefazodone’s detrimental effect at 200µM, we generated a full dose response curve for this compound in both glucose- and galactose-grown cells (Figure 2). As already shown for trazodone and buspirone, galactose-grown cells were much more susceptible to nefazodone treatment, but at much lower concentration than with the other two drugs. The IC50 values were 38.4µM and 9µM for glucose- and galactose-grown cells, respectively (Figure 2). The rank order of toxicity for the three compounds was therefore nefazodone > trazodone > buspirone.
Metabolic Profiling

The notion that the cytotoxicity exhibited by these molecules could be attributed to mitochondrial toxicity was further examined via simultaneously monitoring oxygen consumption and extracellular acidification rates using Seahorse BioSciences technology as described in materials and methods (Figure 3). Rotenone, a potent respiratory Complex I inhibitor, was included as a positive control, and a DMSO vehicle treatment as a negative control. At 6.25uM (first injection), nefazodone profoundly inhibited oxygen consumption (OCR, Figure 3A), accompanied by a compensatory increase in extracellular acidification rate (ECAR, Figure 3B). These responses were worsened by the second injection, yielding 12.5uM concentration, but less of a response was noted with subsequent injections of drug. Similar, but less severe, responses were observed with trazodone and buspirone (25uM after each injection; 100µM after final injection).

In accord with the viability data of galactose-grown cells (Fig 1), trazodone is slightly more toxic than buspirone.

Effect on Oxygen Consumption by Isolated Rat Liver Mitochondria

The higher toxicity of all compounds in galactose-grown cells, plus decreased oxygen consumption and increased acidification, strongly suggest mitochondrial inhibition as a possible mechanism of toxicity. To further test this hypothesis, the three compounds were evaluated by monitoring respiration of isolated rat liver mitochondria. Mitochondrial oxygen consumption measurements were performed in two different states: State 2, where oxidizable substrates (glutamate/malate or succinate) are provided in the absence of exogenous ADP, and State 3, where both substrates and ADP are
Dykens et al., 3/5/2008

provided. At 200nmol/mg protein, nefazodone profoundly and significantly inhibited glutamate/malate driven basal State 2, and even more potently State 3, respiration (Figure 4). In contrast, at the same concentration, trazodone showed a significant, but relatively modest, inhibition of State 2 respiration. Buspirone was indistinguishable from controls. The rank order of mitochondrial impairment for the three compounds is: nefazodone >> trazodone > buspirone. Basal State 2 respiration fueled by succinate, a substrate that provides electrons to Complex II thereby bypassing Complex I, was also inhibited by nefazodone, but only at the two highest concentrations (data not shown). Such inhibition independent of Complex I suggests a second, less potent, mechanism of inhibition. Neither trazodone nor buspirone had any effect on succinate-fueled respiration.

In light of the effect of nefazodone, dose response curves were generated for nefazodone starting at 100nmol/mg protein (Figure 5). Figure 5A shows the dose dependent effect on basal respiration, whereas figure 5B corroborates the more potent effect on ADP-driven State 3 respiration noted above. The IC$_{50}$ values were 16.7 nmol/mg protein and 9.8 nmol/mg protein for glutamate/malate basal and ADP-driven respiration, respectively, and the IC$_{50}$ for basal succinate respiration is >100nmol/mg mitochondrial protein.

**Target Identification**

The potency of nefazodone to impair respiration fueled by glutamate/malate versus succinate suggests that Complex I could be a likely target, or perhaps adenine nucleotide translocase. Accordingly, we evaluated the effects of the drugs on the
individual OXPHOS complexes. This was initially determined at 150 µM, and a 9-point dose response, using 2-fold dilutions, was performed if an effect was observed.

When tested for inhibition of Complexes I, II/III, IV and V activity, nefazodone potently inhibited Complex I, with an IC$_{50}$ of 14µM (Table 1, figure 6). Buspirone showed 3.4 fold less inhibition resulting in an IC$_{50}$ of 48µM for Complex I. Trazodone had no effect on Complex I activity. No Complex II/III inhibition was observed for any of the three antidepressants (Table 1). Complex IV was inhibited by nefazodone (IC$_{50}$ 70µM), but not by trazodone or buspirone (Figure 7), rendering this a likely candidate for the respiratory inhibition with succinate observed in isolated mitochondria. No complex V inhibition was observed for any of the three compounds (Table 1). The rank order of potency for the three drugs is: nefazodone > buspirone > trazodone.

**Effects on Primary Cultures of Human Hepatocytes**

The potential cytotoxic effects of the three antidepressants were tested in sandwich-cultured primary human hepatocytes for 24 hours at 100x their single-dose therapeutic Cmax values (average systemic serum Cmax after a single oral dosing; Table 2, bottom row). This yields concentrations of 92µM for nefazodone, 505µM for trazodone, and 0.5µM for buspirone. Figure 8 shows representative images of the same field of hepatocytes stained by DRAQ5 for nuclei and intracellular lipids (first column), CM-H$_2$DCFDA for reactive oxygen species (second column), TMRM for mitochondrial membrane potential (third column), and mBCl for intracellular glutathione (fourth column). Under this treatment condition, primary human hepatocytes treated with 0.1% DMSO vehicle (Fig 8, top row) exhibited normal round-shaped nuclear morphology, a
basal level of reactive oxygen species (a basal level of oxidized DCF signal sequestered into the bile canaliculi compartment with very little intracellular fluorescence), healthy mitochondria with normal mitochondrial membrane potential (peri-nuclear mitochondrial accumulation and intensity of TMRM signals), and normal intracellular reduced glutathione (mBCl fluorescence throughout the cytoplasm). Primary human hepatocytes treated with buspirone produced similar imaging results as the DMSO vehicle (Fig. 8, bottom row). On the other hand, hepatocytes treated with nefazodone resulted in significant cell loss (Fig. 8, first column, second row), increased reactive oxygen species in some remaining cells (Fig. 8, second column, second row), near complete abolishment of the mitochondrial membrane potential even in the remaining cells (Fig. 8, third column, second row), and dramatic depletion of intracellular reduced glutathione (Fig. 8, last column, second row). Interestingly, trazodone also induced significant cell loss, mitochondrial damage, and loss of glutathione, although induction of reactive oxygen species was not observed (Fig. 8, third row).
Of the three antidepressants evaluated, the data indicate that nefazodone is cytotoxic to HepG2 cells grown in either glucose or galactose, although the latter are significantly more susceptible. This was also the case for trazodone, and to a lesser extent buspirone which had only modest toxicity to glucose-grown cells. This potency rank order was corroborated via metabolic profiling and via monitoring respiration of isolated rat liver mitochondria. When respiration was fueled by succinate, inhibition by nefazodone was apparent at only the two highest concentrations, suggesting that Complex I is a primary site of inhibition, but that there is probably another mechanism of inhibition. This was confirmed using immunocaptured OXPHOS complexes, where nefazodone potently inhibited not only Complex I, but also, albeit less potently, Complex IV. In primary human hepatocytes that express CYP3A4, 24 hr exposure to nefazodone and trazodone collapses mitochondrial membrane potential, depletes glutathione and kills the cells.

In toto, the rank order of cyto- and mito-toxicity is nefazodone > trazodone > buspirone. This is in accord with the clinical disposition of these drugs, with nefazodone associated with more frequent and serious instances of hepatotoxicity (reviewed by DeSanty and Amabile, 2007). However, this inference, although justified, must also be informed by bioavailability of the three drugs, which varies widely: at the same oral dose, Cmax of nefazodone is in a similar range as trazodone, and more than 100-fold higher than buspirone (Table 2). Thus, tissue exposure to nefazodone can approach 10µM, rendering the cytotoxicity and other deleterious effects observed here more pathologically germane than those observed with buspirone that were primarily detected at non-
pharmacological concentrations. This is particularly the case given the acute exposures used in some of our studies (see below). In this context, the effects of buspirone on immunocaptured respiratory Complex I may not be relevant to cytotoxicity given its lower dose and exposures in vivo (Table 2). It is interesting to note that at both 100-fold the orally-administered single-dose human therapeutic Cmax values, both nefazodone and trazodone caused collapse of mitochondrial membrane potential, intracellular glutathione depletion, and significant cytotoxicity.

It should be noted that drug exposures in both the HepG2 and human hepatocyte studies were conducted in the presence of serum, so that potential tight protein binding appears not to limit availability. Similarly, the parent molecules (nefazodone and/or trazodone) inhibited isolated mitochondria and isolated respiratory complexes, and were cytotoxic to HepG2 cells lacking CYP3A4, and to human hepatocytes that express it Wilkening et al., 2003). As such, metabolism by CYP3A4 is apparently not required for toxicity. Interestingly, trazodone was generally less toxic than nefazodone in all the models used here except the human hepatocytes where it was equally as toxic as nefazodone. Although admittedly a surrogate, this model more closely approximates clinical reality in that drug metabolism can occur. It bears reiteration that idiosyncratic liver injuries have been reported for trazodone (DeSanty and Amabile, 2007), and the data from the human hepatocytes suggest that drug metabolism may contribute to such toxicity. However, because the drugs were evaluated in the human hepatocyte model at 100-fold over Cmax, trazodone was tested at a dose at least 5 times higher than in the other assays, which could account for its toxicity being more apparent in this model.
The requirement for an elevated exposure in the *in vitro* setting to identify deleterious hepatic effects of these drugs may be due to a combination of: a) liver exposure to an orally-dosed drug can be an order of magnitude higher than its systemic exposure; b) population pharmacokinetic variability due to age, genetics (including metabolism), and drug-drug interactions could further exacerbate local liver exposure and toxicity; c) idiosyncratic organ history (including disease and previous drug exposures); d) onset of hepatotoxicity in vivo is typically much longer than in vitro (see below), fostering dose escalation in most in vitro systems. Indeed, even the systemic Cmax values can vary widely for nefazodone based on dose administered (Table 2) and patients’ age groups (Barbhaiya *et al*., 1995).

There is growing evidence that drugs associated with organ toxicities have disproportionate extents of mitochondrial liabilities (Wallach and Starkov, 2000; Chan *et al*., 2005). For many antivirals and antibiotics, such toxicity is attributable to long-term inhibition of mitochondrial replication and gene expression (Wallace and Starkov, 2000). However, drugs with known organ toxicities are increasingly being found to directly inhibit and/or uncouple mitochondrial respiration (Wallace and Starkov 2000; Szewczyk and Wojtczak, 2002; Chan *et al*., 2005; Hynes *et al*., 2006; Nandanaciva *et al*., 2007b; Dykens *et al*., 2007a,b). For example, the thiazolidinediones variously inhibit and/or uncouple mitochondrial respiration in accord with their toxicity profiles (Nandanaciva *et al*., 2007b). Similarly, the rhabdomyolysis that limits the use of statins also parallels their acute and direct inhibition of mitochondrial respiration (Nandanaciva *et al*., 2007b).

The model that is emerging is one where “off target” impairment of mitochondrial function precipitates cytotoxicity once a bioenergetic threshold is crossed (Boelsterli,
Most cells have robust energetic reserves, and can accelerate ATP production in response to a host of normal or noxious stimuli. That ability to respond to stressors is gradually eroded by drugs that impair mitochondrial function until a point where the cell can no longer generate the ATP needed for survival, and it dies. It should be noted in this context that many drugs induce elevations of serum alanine aminotransferase (ALT), a direct reflection of hepatocellular death, and many of these drugs have mitochondrial liabilities (Boelsterli, 2003; Dykens et al., 2007a).

In this model of drug toxicity via mitochondrial dysfunction, the deleterious effects on mitochondria are likely a fixed value, occurring in most patients to the same extent. The factors that contribute to idiosyncratic susceptibility are organ history and capacity to compensate for loss of mitochondrial capacity, which in turn has a genetic basis dictated by both the nuclear and mitochondrial genomes. Estimates of mitochondrial half-lives vary, but mitochondrial replacement occurs on a time scale of days-to-weeks. Given this time scale, even mild drug-induced mitochondrial impairment can gradually accumulate, amplifying the deleterious consequences of acute drug exposure. It bears reiteration that the time scales and drug concentrations used in our models reflect experimental expediency much more than the realities of pathophysiology. For example, cytotoxicity was assessed only after 24hr exposures, and it is feasible that longer exposures at lower doses could have exacerbated toxicity. Moreover, many drugs can be bio-accumulated by transmembrane carriers at the plasma membrane and at the mitochondrial membranes. For example, the statins paradoxically induce apoptosis in anaerobically-poised, fast-twitch fibers, and not in mitochondrially-enriched slow-twitch
fibers, because of a selective membrane transporter on the susceptible fibers that takes up the statin (Westwood et al., 2005; Dykens et al., 2007b). Indeed, cations can also bio-accumulate into the mitochondrial matrix because of the strong (approx. 220mV) mitochondrial membrane potential, further exacerbating deleterious consequences (Dykens et al., 2007a,b).

In addition to the bioenergetic threshold, drug-induced mitochondrial impairment can yield toxicity via other routes. For example, respiratory impairment increases the time the various OXPHOS remain in the reduced state, correspondingly increasing the probability of untoward autoxidation and univalent O2 reduction to yield superoxide and other reactive oxygen or nitrogen species (ROS, RNS)(Brown and Borutaite, 2007).

Similarly, under some conditions, uncoupling can also increase mitochondrial ROS formation (Dykens 1994). In our models, depending on the time scales, such increased free radical formation is detected directly, but also secondarily via glutathione depletion which serves as a longer-term index of cellular redox status. The known idiosyncratic hepatotoxin troglitazone very potently impairs mitochondrial respiration (Nadanaciva et al., 2007b; Dykens et al., 2007b), but hepatotoxicity is not apparent in pre-clinical animal models. Only when the mitochondrial form of superoxide dismutase is knocked down by 50% is the hepatotoxicity of troglitazone revealed in vivo (Ong et al., 2005). In this light, organ toxicity with a mitochondrial etiology is also a consequence of the equilibrium between radical formation and antioxidant capacity. In this case, however, the threshold is the antioxidant status of the cell.

Of course, the bioenergetic and antioxidant thresholds are metabolically and genetically connected, but they illustrate that drug-induced mitochondrial impairment can
injure and kill cells in a myriad of ways. And they also underscore the importance of mitochondrial assessments in pre-clinical drug development programs, and the need for more models that better predict potential organ toxicity earlier in the drug development process.
References


   study of Buspirone extended-release tablets with a reference immediate-release 

34. Smith, A.L. (1967). Preparation, properties, and conditions for assay of 


   Wright, R.N., and Yocca, F.D. (1995). Pharmacology and neurochemistry of 


   Statin-induced muscle necrosis in the rat: distribution, development, and fibre 

   hepatocytes and hepatoma cell line HepG2 with regard to their biotransformation 

   mitochondrial function using phosphorescent oxygen-sensitive probes. *Nat. 
   Protoc.* **1**, 2563-2572.
Table 1. IC50 values for inhibition of immunocaptured respiratory Complexes I, II/III, IV and V.

<table>
<thead>
<tr>
<th>Complex</th>
<th>Nefazodone</th>
<th>Trazodone</th>
<th>Buspirone</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>14 uM</td>
<td>No effect</td>
<td>48 uM</td>
</tr>
<tr>
<td>II/III</td>
<td>No effect</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td>IV</td>
<td>70 uM</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td>V</td>
<td>No effect</td>
<td>No effect</td>
<td>No effect</td>
</tr>
</tbody>
</table>
Table 2:
Dosing regimen and Cmax values for nefazodone, trazodone and buspirone. For in vitro-in vivo correlation (IVIVC), we used serum Cmax from healthy volunteers after a single dose of 100 mg nefazodone and trazodone, but 20 mg for buspirone (bottom row).

Notice that for nefazodone, higher Cmax values have been reported after b.i.d. vs. single dose. In our attempts to better predict idiosyncratic liver injury in humans, we exposed primary cultures of human hepatocytes to 100 times these Cmax values. Total Cmax values were used in this case, because of the presence of significant amounts of both serum proteins and Matrigel in the hepatocyte culture. Thus, 100 times of these Cmax values approximate maximal hepatic exposures after oral administration in a diverse population.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Nefazodone</th>
<th>Trazodone</th>
<th>Buspirone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daily dose</td>
<td>100-200 mg in 2 divided doses</td>
<td>50-200 mg in 2 divided doses</td>
<td>10-30 mg in 2 divided doses</td>
</tr>
<tr>
<td>Dose /Cmax</td>
<td>50mg bid/270ng/ml</td>
<td>50mg/840 ng/ml</td>
<td>10mg/0.9ng/ml</td>
</tr>
<tr>
<td></td>
<td>100mg bid/730ng/ml</td>
<td>100mg/1880 ng/ml</td>
<td>20mg/1.7ng/ml</td>
</tr>
<tr>
<td></td>
<td>200mg bid/2050ng/ml</td>
<td></td>
<td>40mg/3.0ng/ml</td>
</tr>
<tr>
<td>Single-dose serum Cmax for IVIVC*</td>
<td>434 ng/ml, 0.92 μM (Barbhaiya et al, 1995)</td>
<td>1880 ng/ml, 5.05 μM (Nilsen and Dale, 1992))</td>
<td>1.92 ng/ml, 0.005μM (Skar and Andheria, 2001)</td>
</tr>
</tbody>
</table>

*IVIVC: in vitro in vivo correlation
Figure Legends

Figure 1:
Viability of HepG2 cells grown in either glucose (open bars) or galactose-containing media (black bars) exposed to 200µM nefazodone, trazodone or buspirone for 24 hours. ATP content was measured and expressed as percent of vehicle-treated controls. Data are mean ± SD (n=3 separate experiments). Means not significantly different share superscripts (ANOVA F = 900, P < 0.0001, Bonferroni multiple comparison test; percentage data were transformed prior to ANOVA via Y=(ln)Y).

Figure 2:
HepG2 cells grown in either glucose (■) or galactose (◆) media were incubated with the indicated concentrations of nefazodone. As reflected by the left shift in the curve, galactose-grown cells were significantly more susceptible to nefazodone treatment (IC$_{50}$ = 8.98µM) compared to cells grown in glucose media (IC$_{50}$ = 38.40µM). Data are mean ± SD (n=3).

Figure 3:
Metabolic Profiling reveals a decrease in oxygen consumption (OCR) upon exposure to nefazodone, trazodone, buspirone (Panel A; rotenone is a positive control, and DMSO is vehicle control). This is accompanied by an increase in acidification rate (ECAR) as glycolysis accelerates to compensate for reduced respiration (Panel B). Data are collected simultaneously, and nefazodone, trazodone, buspirone and rotenone were injected to 6.25µM, 25 µM, 25µM, and 1.25 µM final concentrations, respectively, at each of the time points A, B, C and D. For example, after 4 injections, cells are exposed to 25µM nefazodone.

Figure 4
Oxygen consumption by isolated rat liver mitochondria was severely inhibited by nefazodone. Panel A: the effect of nefazodone, trazodone and buspirone (all at 200nmol/mg mitochondrial protein) on basal glutamate/malate-driven State 2 respiration. Panel B: effects on ADP-stimulated State 3 respiration. Letters indicate statistically
significant differences between treatment groups as analyzed by ANOVA. Data are mean ± SE (n=7).

**Figure 5**
Nefazodone inhibits basal and ADP-stimulated respiration in isolated liver mitochondria in a dose depend matter. Panel A: effects on basal glutamate/malate-driven basal State 2 respiration at the drug concentrations indicated. Panel B: effects on ADP-stimulated State 3 respiration. Data are mean ± SE (n=7 separate experiments).

**Figure 6:**
IC$_{50}$ curves for nefazodone (A), trazodone (B) and buspirone (C) on OXPHOS Complex I. Data are mean ± SD (n=3 separate experiments).

**Figure 7:**
IC$_{50}$ curves for nefazodone (A), trazodone (B) and buspirone (C) on OXPHOS complex IV. Data are mean ± SD (n=3 separate experiments).

**Figure 8:**
Representative images of hepatocytes treated 24hr with 0.1% DMSO (top row), nefazodone (second row), trazodone (third row), buspirone (bottom row), all at 100X Cmax (see Table 2) and stained with DRAQ5 for nuclei and intracellular lipids (first column), CM-H2DCFDA for reactive oxygen species (second column), TMRM for mitochondrial membrane potential (third column), and mBCl for intracellular glutathione (fourth column). The images from each row are obtained from the same image field of the same treatment sample.
Figure 2
127x169mm (600 x 600 DPI)
Figure 3
254x190mm (300 x 300 DPI)
Figure 4

190x254mm (96 x 96 DPI)
Figure 5
190x254mm (600 x 600 DPI)
Figure 6a
443x361mm (150 x 150 DPI)
Figure 6b
443x361mm (150 x 150 DPI)
Figure 6c
443x361mm (150 x 150 DPI)
Figure 7a
443x361mm (150 x 150 DPI)
Figure 7b

Complex IV Activity (%) vs. Trazodone (µM)

443x361mm (150 x 150 DPI)
Figure 7c
443x361mm (150 x 150 DPI)
Figure 8
254x190mm (300 x 300 DPI)