

**INHIBITORY ACTIVITY ON THE HUMAN CYTOCHROME P450 AND IN VITRO
CYTOTOXIC EFFECTS ON HUMAN HEPATOCYTES OF NEFAZODONE,
TRIAZOLEDIONE, M-CHLOROPHENYLPIPERAZINE AND TRAZODONE**

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Running Title: **NEFAZODONE CYTOTOXICITY AND P450 INHIBITION**

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ABSTRACT

The antidepressant nefazodone (NFZ) determined several cases of acute hepatotoxicity/liver failure. Since the mechanism of hepatotoxicity still remains unknown, NFZ and two of its main metabolites, triazoledione (TZL) and m-chlorophenylpiperazine (mCPP), have been studied for their cytochrome P450 inhibition and cytotoxicity on human hepatocytes. Trazodone (TZD) was also investigated since it shares with nefazodone the mCPP metabolite.

The inhibitory activity on recombinant human cytochrome P450 isoforms (CYP1A2, CYP2A6, CYP3A4, CYP2C8, CYP2C9 and CYP2D6) was determined by a fluorimetric assay whereas cytotoxicity was tested on human hepatocytes primary cultures.

NFZ strongly inhibited CYP 3A4 (IC₅₀ = 1.55 μM) and to a lesser extent CYP 2C9 (IC₅₀ = 5.13 μM). A weak inhibition on CYP 2D6 was exerted by TZD, NFZ and m-CPP (IC₅₀ = 29 μM, 20 μM and 7.78 μM, respectively) while only m-CPP weakly inhibited CYP 2A6 (IC₅₀ = 10 μM). In human hepatocytes primary cultures NFZ resulted highly cytotoxic (IC₅₀ = 44.35 μM and 27.78 μM after 24h and 48h, respectively). On the contrary, TZD and mCPP showed very low cytotoxicity (IC₅₀ ≈ 400 μM) as well as TZL (IC₅₀ > 800 μM).

In conclusion, the present study demonstrates that NFZ is a strong inhibitor of the cytochrome P450 isoenzyme CYP 3A4 and highly cytotoxic on human hepatocytes, while no relevant cytochrome P450 inhibition or cytotoxic effects on human hepatocytes could be ascribed to its metabolites TZL and m-CPP and to the antidepressant drug TZD.

Key words: nefazodone, triazoledione, m-chlorophenylpiperazine, trazodone, cytochrome P450 inhibition, human hepatocytes.

INTRODUCTION

The antidepressant nefazodone (NFZ) has been withdrawn from the market after the occurrence of several cases of acute hepatotoxicity/liver failure (1-6). Since the mechanism of hepatotoxicity still remains unknown, NFZ and two of its main metabolites, triazoledione (TZL) and m-chlorophenylpiperazine (mCPP) (7) have been analyzed to determine their inhibitory activity on the human cytochrome P450 isoforms and their cytotoxic effect on human hepatocytes. The effects of trazodone (TZD) were also investigated since this drug shares with nefazodone the mCPP metabolite (8).

MATERIALS AND METHODS

Drugs

NFZ (Sigma-Aldrich) and TZL (ACRAF S.p.A.) were dissolved in DMSO while TZD (ACRAF S.p.A.) and m-CPP (ACRAF S.p.A.) were dissolved in Tris-phosphate buffer. All compounds were tested up to the maximum soluble concentration.

Cytochrome P450 inhibition tests

Cytochrome P450 inhibition was assessed using the fluorescent High Throughput Method (Gentest, BD Bioscience) as described by Crespi *et al.* (9,10). The assay was performed in 96-well microplates in duplicate and the IC₅₀ were determined for the following isoenzymes: CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP3A4, CYP2D6. As reference compound a standard inhibitor specific for each isoenzyme was used.

Cytotoxic effects on human hepatocytes

Platable human cryopreserved hepatocytes and the relevant culture media were obtained from In Vitro Technologies. The cells (1.400.000 cell/well) diluted in Plating Medium were cultured in collagen coated 48 well plates. After overnight incubation at 37°C with 5% CO₂ to allow attachment of the cells, plates were washed with Incubation Medium and the drugs, properly diluted in culture medium, were added to the cultures. Chlorpromazine HCl (Sigma) was used as positive reference drug. After 24 and 48 hour-incubation cytotoxic effects were determined assessing hepatocytes viability as function of adenosine triphosphate production (ATPlite Kit, Perkin Elmer). The percent of viability was determined comparing the luminescence of treated cells to that of untreated control cells. Sigmoidal regression curves of cell survival and the IC₅₀ (concentration of each compound that kills 50% of cells) were calculated using Graph Pad Prism software.

RESULTS**Cytochrome P450 inhibition**

As reported in Table 1, no inhibition of CYP 1A2 and CYP 2C8 was observed with test compounds; in the same experiments the IC₅₀ of the positive control furafylline was 2.92 µM and the IC₅₀ of quercitine was 0.77 µM. No inhibition of CYP 2A6 was induced by TZD, NFZ and TZL, while the IC₅₀ of m-CPP was 10 µM.

The positive control tranlycypromine showed a IC₅₀ = 0.46 µM. TZD, NFZ and m-CPP exerted a weak inhibition on CYP 2D6 with IC₅₀ equals to 29 µM, 20 µM and 7.78 µM,

respectively, while TZL had no effects. In the same experiments the IC₅₀ of quinidine was 0.03 μM. NFZ strongly inhibited CYP 3A4 (IC₅₀ = 1.55 μM) and to a lesser extent CYP 2C9 (IC₅₀ = 5.13 μM), while no inhibition of these two isoenzymes was induced by TZL, m-CPP and TZD. In the same experiments the positive controls ketoconazole showed an IC₅₀ of 0.114 μM on CYP 3A4 and sulfaphenazole an IC₅₀ of 0.46 μM on CYP 2C9.

Table 1. Cytochrome P450 inhibition

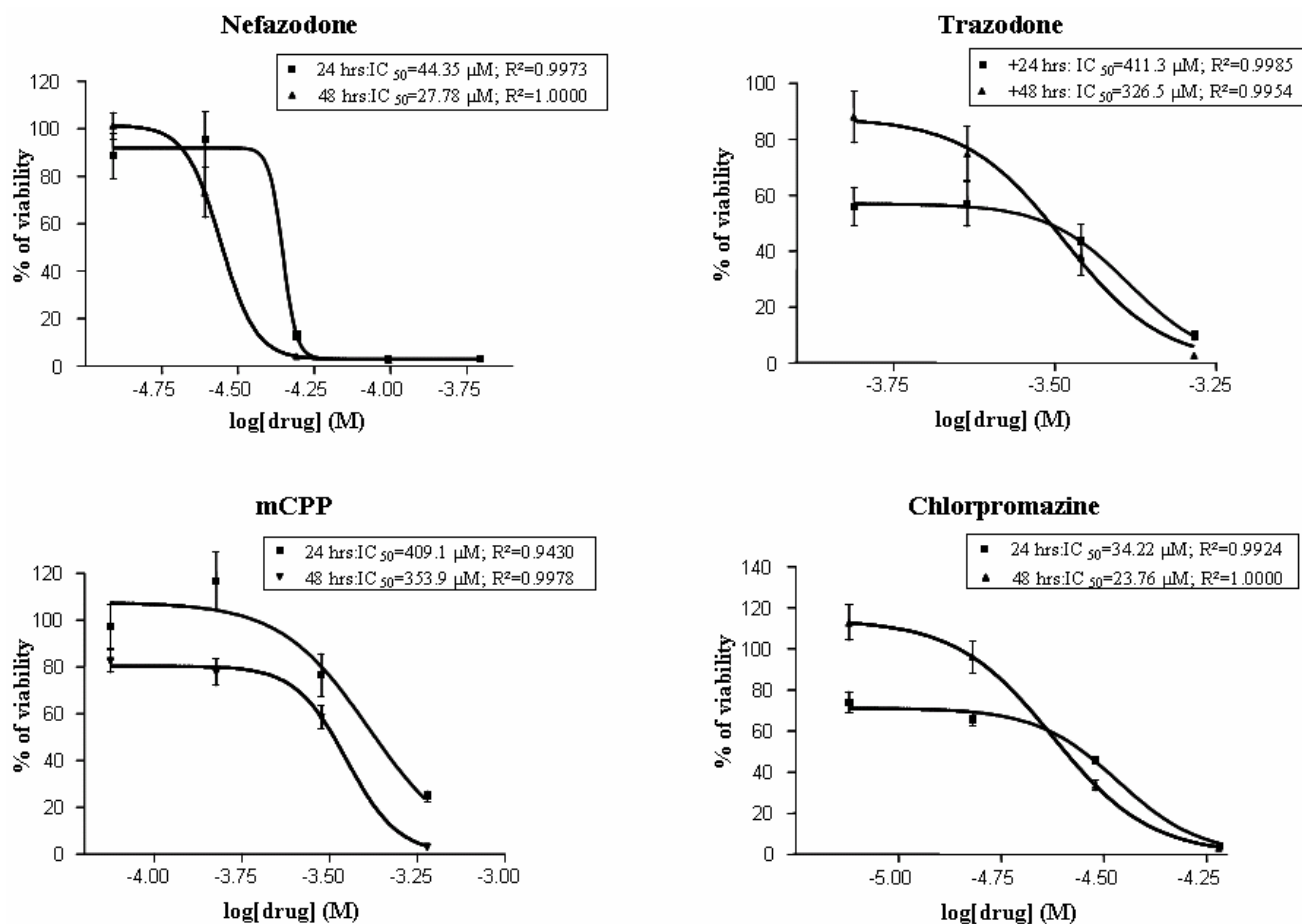
	IC ₅₀ (μM)				
	Nefazodone	Triazoledione	m-CPP	Trazodone	Standard inhibitor
CYP 1A2	>197	>202	129.5	>490	Furafylline 2.92
CYP 2A6	>197	>202	10.03	>490	Tranlycypromine 0.46
CYP 2C8	110.66	>202	>858	>490	Quercitine 0.77
CYP 2C9	5.13	120	>858	374	Sulfaphenazole 0.46
CYP 2D6	20.72	184	7.78	29.02	Quinidine 0.03
CYP 3A4	1.55	117.82	>858	395	Ketoconazole 0.114

Cytotoxic effects on human hepatocytes

Human hepatocytes monolayers, without any added test compound, maintained viability over the 72-hour culture period. Addition of the test compounds resulted in decreased viability, which correlated with the increase of concentrations and with the length of incubation.

Results of cytotoxic effects induced by test compounds after 24 hours and 48 hours of incubation are summarized in Figure1.

Figure 1. Cytotoxicity on human hepatocytes



The positive control drug, chlorpromazine, showed a high cytotoxic effect with IC_{50} equal to $34.22 \mu\text{M}$ and $23.76 \mu\text{M}$ after 24 hr and 48, respectively. NFZ showed a high cytotoxic effect, similar to chlorpromazine, with IC_{50} equal to $44.35 \mu\text{M}$ and $27.78 \mu\text{M}$, respectively after 24 hours and 48 hours. On the contrary, TZD and mCPP showed very low cytotoxicity ($IC_{50} \approx 400 \mu\text{M}$) and TZL had no effect up to the maximum soluble concentration ($IC_{50} > 800 \mu\text{M}$).

DISCUSSION

The present work shows that the combined use of the Cytochrome P450 inhibition test and the cytotoxicity test on human hepatocytes primary cultures can give important information and reliable prevision about the potential hepatotoxicity and the potential drug-drug interactions of a compound.

Data on Cytochrome P450 inhibition obtained with a fluorescent High Throughput Method, which can be used at the early stages of preclinical drug development, confirm data from literature about the strong inhibition induced by NFZ on the CYP3A4 and the weaker inhibitions of CYP 2C9 and CYP2D6 (11).

Similarities in non linear pharmacokinetic profile, plasma levels, clinical efficacy and metabolism of NFZ and hydroxynefazodone (OH-NFZ) have been extensively described in literature (12-17). Moreover, similarly to NFZ, OH-NFZ is metabolized by CYP3A4 to TZL and m-CPP (18). Thus in the present study we focused our attention on the metabolites TZL and m-CPP. Although the metabolite TZL reaches plasma levels approximately 10 times higher than those of NFZ and shows a long elimination half life (11), no inhibition of Cytochrome P450 was detected in our study, even at high concentrations.

m-CPP is a metabolite of NFZ and of TDZ, it is formed by N-dealkylation of NFZ or OH-NFZ and it shows a linear pharmacokinetic (7). However m-CPP accumulates at levels one-tenth to one-hundredth those of nefazodone and it is a substrate for CYP2D6 (12,13). In our experiment a weak interaction of m-CPP with CYP2A6 and CYP2D6 was observed.

TZD is an antidepressive drug that undergoes extensive hepatic metabolism with the formation of m-CPP (11). CYP3A4 (8,20), CYP2D6 (8) and CYP1A2 (19) are implicated in the metabolism of TZD and the formation of its metabolites. No inhibition of Cytochrome P450 was observed in the present work.

Although hypothesis about the formation of possible intermediates responsible for the hepatotoxicity have been reported for NFZ and TZD (21,22) no cytotoxicity studies using hepatocytes have been reported in the literature with these two drugs and their metabolites. The cytotoxicity test performed using human hepatocytes indicate that NFZ is highly cytotoxic, at concentrations similar to those of the positive reference compound. Considering that the hepatic metabolism catalyzed by cytochrome P450 CYP3A4 represents the principal clearance mechanism of NFZ in humans and considering the strong inhibition induced by NFZ on the CYP3A4 (7,17), drug concentrations close to the IC_{50} values are likely to be reached in the liver after repeated administrations. On the contrary, TZD and m-CPP resulted at least ten times less cytotoxic; considering that these two compounds are metabolized by CYP3A4, CYP2D6 and CYP1A2 but they are not cytochrome P450 inhibitors, cytotoxic concentrations in the liver are very unlikely to be reached.

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