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In vitro assessment of competitive and time-dependent inhibition of the nevirapine metabolism by nortriptyline in rats

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ABSTRACT

Nevirapine (NVP) is a non-nucleoside reverse transcriptase inhibitor of human immunodeficiency virus type 1 (HIV-1) widely used as a component of High Active Antiretroviral Therapy (HAART) since it is inexpensive, readily absorbed after oral administration and non-teratogenic. In the present work, the mechanism of a previously described pharmacokinetic interaction between NVP and the antidepressant drug nortriptyline (NT) was studied using rat hepatic microsomes. The obtained results showed a competitive inhibition of the NVP metabolism by NT. The three main NVP metabolites (2-OH-NVP, 3-OH-NVP and 12-OH-NVP) where competitively inhibited with similar inhibitory constant values (K_i = 4.01, 3.97 and 4.40 μM, respectively). Time-dependent inhibition of the NVP metabolism was also detected, with a 2.5-fold reduction in the IC_{50} values of NT for 2-, 3-, and 12-OH-NVP formation when NT was preincubated with the microsomal suspension in the presence of an NADPH-generating system. A concentration-dependent inhibition of the formation of NVP metabolites by the main NT metabolite (10-OH-NT) was also observed, however, the inhibitory potency of 10-OH-NT was much lower than that of the parent drug. The apparent hepatic intrinsic clearance of NVP determined in these *in vitro* experiments was used to predict the *in vivo* clearance of NVP using the "well-stirred" and the "parallel-tube" models, resulting in values close to those previously observed *in vivo* clearance. Finally, a good prediction of the increase in the plasma concentrations of NVP when co-administered with NT was obtained employing the inhibitory constant of NT determined *in vitro* and the estimated plasma concentration of NT entering the liver.

1. Introduction

The Acquired immunodeficiency syndrome (AIDS) remains, at present, an incurable disease. However, the introduction of highly active antiretroviral therapy (HAART) in the mid-1990s has dramatically decreased morbidity and mortality among patients infected with the human immunodeficiency virus (HIV) [1]. The goal of HAART is to prevent the immune system from deteriorating to the point that opportunistic infections become more likely. Successful long-term HAART results in a gradual recovery of CD4 T-cell numbers and an improvement of immune responses. HAART regimens are usually maintained during several months, until a dosage change or an antiretroviral drug replacement in the regimen is applied due to toxicity-associated events, poor adherence or virologic failure [2].

At least three active drugs of different classes are used in HAART, with nevirapine (NVP) being one of these drugs in some HAART protocols. NVP is a non-nucleoside reverse transcriptase inhibitor (NNRTI) of human immunodeficiency virus type-1 (HIV-1) widely used as it is inexpensive, readily absorbed after oral administration and non-teratogenic. For this reason, NVP is widely prescribed for HIV-infected pregnant women instead of other NNRTIs to prevent mother-to-child transmission [3]. Despite its major therapeutic benefits, treatment with

Abbreviations: AIDS, acquired immunodeficiency syndrome; AUC, area under the plasma concentration versus time curve of NVP in absence of NT; AUC_I, area under the plasma concentration versus time curve of NVP in presence of NT; AUC_{ratio}, ratio between AUC_I and AUC (AUC_I/AUC); C/C_b, ratio between total plasma and total blood NVP concentrations; $CL_{app, int}$, apparent hepatic intrinsic clearance based on the total drug concentration; CL_H, hepatic plasma clearance; CL_{int}, intrinsic hepatic clearance based on the unbound drug concentration; C_{max}, maximum plasma concentration; CYP, cytochrome P450; f_u, free fraction of NVP in plasma; $f_{u, m}$, free fraction of NVP in the microsomal incubations; HAART, highly active antiretroviral therapy; HIV, human immunodeficiency virus; HIV-1, human immunodeficiency virus type-1; HPLC, high-performance liquid chromatography; [I], plasma concentration of the inhibitor (NT); IC_{50}, concentration of inhibitor required for 50% inhibition of substrate (NVP) metabolism; K_i, inhibitory constant; K_m, Michaelis-Menten constant; LLOQ, lower limit of quantification; β-$NADP^+$, β-nicotinamide adenine dinucleotide phosphate; NNRTI, non-nucleoside reverse transcriptase inhibitor; NT, nortriptyline; NVP, nevirapine; 10-OH-NT, 10-hydroxynortriptyline; 2-, 3-, 8- and 12-OH-NVP, 2-, 3-, 8- and 12-hydroxynevirapine; Q_H, hepatic blood flow rate; RLM, rat liver microsomes; TCA, tricyclic antidepressant; TDI, time-dependent inhibition; V_{max}, maximum rate

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NVP may cause severe hepatotoxicity and/or skin rash in some patients. Studies suggest that reactive metabolites of this drug, rather than the levels of the parent drug, should be responsible for its adverse effects [4,5].

In humans as well as in rats, NVP undergoes hepatic biotransformation into several hydroxylated metabolites: 2-, 3-, 8-, and 12-hydroxynevirapine (2-, 3-, 8-, and 12-OH-NVP) by cytochrome P450 (CYP), being eliminated mainly as glucuronide metabolites in urine. Less than 3% of an administered dose is excreted in urine as the parent compound [6]. The main CYP isoenzymes implicated in NVP metabolite formation are CYP3A4 (responsible of 2-, 8-, and 12-OH-NVP formation) and CYP2B6 (responsible of 3- and 8-OH-NVP formation). It has been suggested that CYP2D6 and CYP2C9 could also be involved in the formation of 12-OH-NVP [7], the major metabolite described both in humans and rats [8,9], to which skin rash has been attributed [10].

One of the most frequent comorbidities in individuals with HIV infection is depression, with a lifetime prevalence of 22–45% [11]. Nortriptyline (NT) is a tricyclic antidepressant (TCA) that is more frequently used than others in this group because of its superior pharmacological properties and lower toxicity [12]. The main routes of NT metabolism are E-10-hydroxylation (the major route) and *N*-demethylation (a minor route) [13,14]. *E*-10-Hydroxylation is mediated mainly by CYP2D6, although *in vitro* studies have shown that CYP3A4 is also involved [15]. Like most TCAs, NT is a competitive inhibitor of CYP2D6 [16] and a weak inhibitor of 2C19 [17]. Additionally, several TCAs, including NT, have been implicated as time-dependent inhibitors of rat CYP [18].

In a previous study [19], an *in vivo* pharmacokinetic interaction between NVP and NT was detected in rats. The use of liver microsomes confirmed *in vitro* the inhibitory effect of NT on the NVP metabolism; this effect being more intense in rat than in human microsomes [19]. However, the mechanism of such an inhibition was not studied. The aim of the present work was to characterize the mechanism of NVP metabolism inhibition by NT in rat hepatic microsomes, studying competitive and time-dependent inhibition. Furthermore, the *in vivo* clearance of NVP was predicted by means of the "well-stirred" and the "parallel-tube" models using the apparent hepatic intrinsic clearance determined *in vitro* and it was compared to the previously observed *in vivo* clearance in order to evaluate the predictive capacity of these models. Finally, the inhibitory constant of NT determined *in vitro* was used to predict the *in vivo* increase of NVP plasma concentrations.

2. Materials and methods

2.1. Chemicals

NVP (Viramune®) was obtained from Boehringer Ingelheim (Barcelona, Spain). 2-, 3-and 12-OH-NVP and 10-OH-NT were purchased from Toronto Research Chemicals (North York, Canada), while NT (hydrochloride salt), β-nicotinamide adenine dinucleotide phosphate (β-$NADP^+$), glucose-6-phosphate, glucose-6-phosphate dehydrogenase, magnesium chloride ($MgCl_2$), methanol, potassium phosphate dibasic (K_2HPO_4), trichloroacetic acid, acetonitrile, sodium phosphate monobasic (NaH_2PO_4) and trimethylamine were purchased from Sigma-Aldrich Corporation (Madrid, Spain).

2.2. Study of the metabolism of NVP and its inhibition by NT

Rat liver microsomes (RLM), obtained from a pool of three livers of male Wistar rats as previously described [20], were used to study the metabolism of NVP in the absence and presence of NT. For this purpose, different volumes of NVP solutions in methanol (50 or 100 μg/ml) were evaporated in glass vials to assay various final concentrations of NVP (19, 38, 94, 376 and 750 μM). Thereafter, the following components were added to the NVP residue: 8 μl of hepatic microsomes (equivalent to 0.1 mg of protein), the NADPH generating system consisting in 5 μl of a 20 mg/ml glucose-6-phosphate solution, 5 μl of a 20 mg/ml β-$NADP^+$ solution and 10 μl of a glucose-6-phosphate dehydrogenase solution (10 IU/ml), and 5 μl of $MgCl_2$ solution (13.4 mg/ml). To evaluate the inhibitory effect of NT, 10 μl of NT solutions were added to the vials to obtain final concentrations of 0, 1.7, 3.3 and 6.7 μM. The volume of content in the vials was adjusted to 0.1 ml by adding 0.1 M phosphate buffer (pH 7.4) and the mixture was incubated at 37 °C for 30 min. At the end of the incubation time, the reaction was terminated by addition of 0.1 ml of 12% trichloroacetic acid/acetonitrile (50/50, v/v) and the concentrations of remaining NVP and formed metabolites (2-, 3- and 12-OH-NVP) were determined as described below. These concentrations were used to calculate the corresponding rates which were used to determine parameters such as the maximum rates (V_{max}) of disappearance of NVP and formation of the metabolites, the corresponding Michaelis-Menten constants (K_m) and the inhibitory constants (K_i).

2.3. Time-dependent inhibition of the NVP metabolism by NT

Single Point and IC_{50} Shift assays were carried out to estimate the time-dependent inhibition (TDI) of NVP by NT in RLM. The procedure was similar to that described above in the enzyme inhibition experiments, with the following modifications: microsomes were preincubated with NT in the presence or absence of the NADPH-generating system for 30 min at 37 °C. After that, 11 μl of the preincubated mixture were added to another glass vial containing NVP and the NADPH-generating system, as previously described, and the mixture was incubated for 5 min at 37 °C.

For the Single Point assay, the NVP and NT concentrations in the incubated mixture were fixed at 750 μM and 33.5 μM, respectively, and the percentage of TDI corresponding to each NVP metabolite was calculated using the equation proposed by Atkinson et al. [21].

$$\%\ TDI = 100 * \left(1 - \left(\left(\frac{R + I^{NADPH}}{R + I^{NO\ NADPH}}\right) / \left(\frac{R - I^{NADPH}}{R - I^{NO\ NADPH}}\right)\right)\right) \quad (1)$$

where $R + I^{NADPH}$ is the formation rate of the metabolism when the preincubation is carried out in the presence of the NADPH-generating system and inhibitor (NT), $R - I^{NADPH}$ is the formation rate when the preincubation is carried out in the presence of the NADPH-generating system but in the absence of inhibitor, $R + I^{NO\ NADPH}$ is the formation rate when the preincubation is carried out in the absence of the NADPH-generating system but in the presence of inhibitor (NT) and $R - I^{NO\ NADPH}$ is the formation rate when the preincubation is carried out in the absence of the NADPH-generating system and inhibitor.

To further explore TDI, an IC_{50} Shift assay was performed with 750 μM of NVP and different concentrations of NT in the incubated mixture (3.3, 6.7, 13.3, 33.5 and 133 μM). The magnitude of the IC_{50} shift was determined from the ratio of the IC_{50} values of NT obtained with and without the NADPH-generating system during the preincubation period. A shift ≥1.5 was considered indicative of TDI [22].

2.4. Inhibition of the NVP metabolism by 10-OH-NT

To compare the inhibition of NT and its main metabolite, 10-OH-NT, on the metabolism of NVP, the experimental procedure was similar to that described above, with the following modifications: the NVP concentration in the final mixture was fixed at 19 μM, the final concentrations of NT or 10-OH-NT were 0.7, 1.7, 3.3, 13.3 and 33.5 μM, and the mixture was incubated at 37 °C for 5 min only. The subsequent processing of the samples to determine the concentrations of formed NVP metabolites was similar to that described above.

2.5. Analytical method

Concentrations of NVP and metabolites (2-, 3- and 12-OH-NVP) in microsome samples were determined by HPLC with ultraviolet