Identification of Enzymes Responsible for Primary and Sequential Oxygenation Reactions of Capravirine in Human Liver Microsomes

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ABSTRACT: Capravirine, a new non-nucleoside reverse transcriptase inhibitor, undergoes extensive oxygenation reactions, including \( N \)-oxidation, sulfoxidation, sulfonation, and hydroxylation in humans. Numerous primary (mono-oxygenated) and sequential (di-, tri-, and tetra-oxygenated) metabolites of capravirine are formed via the individual or combined oxygenation pathways. In this study, cytochrome P450 enzymes responsible for the primary and sequential oxygenation reactions of capravirine in human liver microsomes were identified at the specific pathway level. The total oxygenation of capravirine is mediated predominantly (>90%) by CYP3A4 and marginally (<10%) by CYP2C8, 2C9, and 2C19 in humans.
FIG. 1. P450 enzyme(s) responsible for each of the primary and sequential oxygenation reactions of capravirine in human liver microsomes. Arrows indicate definitive pathways. The percentage at the end of an arrow indicates the sequential contribution, while the percentage at the head of an arrow indicates the precursor contribution. No data shown at the end or head of an arrow indicates that the sequential or precursor contribution is 100% (i.e., a single pathway is responsible for the sequential oxygenation or formation of a metabolite).
Dual Inhibition. [14C]Capravirine (2 μM) was coinubated with both ritonavir (2 μM) and one of the three chemical inhibitors, quercetin (for CYP2C8 at 0 or 30 μM; Marilly et al., 2002), sulfaphenazole (for CYP2C9 at 0 or 2 μM; Back et al., 1988), and ticlopidine (for CYP2C19 at 0 or 10 μM; Donahue et al., 1997) in human liver microsomes for 30 min at 37°C. All other incubation conditions and sample preparation procedures were the same as described above (Microsomal Metabolism).

Inhibition of Sequential Metabolism. In separate incubations, each of the seven authentic metabolites (2 μM) of capravirine was coincubated with ritonavir (2 μM) in human liver microsomes for 0 and 10 min at 37°C. All other incubation conditions and sample preparation procedures were the same as described above (Microsomal Metabolism).

Supersomal Metabolism. [14C]Capravirine (2 μM) was incubated with each of the seven P450 Supersomes (CYP1A2, 3A4, 2C8, 2C9, 2C19, 2D6, and 2E1) at 20 nM or with one of the three FMO Supersomes (FMO1, FMO3, and FMO5) at 200 μg/ml for 0 or 20 min at 37°C. All other incubation conditions and sample preparation procedures were the same as described above (Microsomal Metabolism).

Metabolite Profiling. Metabolite profiling was performed on an Agilent (Wilmington, DE) 1100 HPLC system coupled with an IN/US (Tampa, FL) model 3 β-RAM radioprobe detector and a Finnigan LCQ-Deca ion-trap mass spectrometer (Thermo Electron Corporation, San Jose, CA). Separation was achieved using a Phenomenex (Torrance, CA) Aqua C18 column (150 x 4.6 mm, 5 μ) at a flow rate of 1.0 ml/min with an injection volume of 100 μl for all extracted samples. The effluent was split to allow 20% to the mass spectrometer via the supplied electrospray ionization source and 80% to mix with the Packard BioScience (Meriden, CT) ULTIMA FLO-M scintillation cocktail at 2.4 ml/min and then flow through the radiodetector. A mobile phase gradient of 20 mM ammonium acetate (pH 4) (A) and methanol (B) was programmed as follows: initiated with 100% A for 10 min, changed to 60% A from 10 to 30 min, changed to 35% A from 30 to 35 min, held at 35% A from 35 to 60 min, changed to 40% A from 60 to 70 min, changed to 10% A from 70 to 80 min, held at 10% A from 80 to 90 min, and held at 100% A from 90 to 92 min, and held at 100% A from 92 to 100 min for the column to be equilibrated. All above gradient changes were linear. Major operating parameters for the ion-trap electrospray ionization-MS method are as follows: positive ion mode with a spray voltage of 4.5 kV, capillary temperature of 200°C, sheath gas flow rate of 20 (arbitrary), and an auxiliary gas flow rate of 20 (arbitrary). Laura 3 V3.0 (IN/US Systems) and Xcalibur V1.4 (Thermo Electron Corp.) were used to control the β-RAM detector and the liquid chromatography-MS system for data acquisition and processing.

Results and Discussion

Inhibition by Ritonavir of Capravirine Oxygenation. The coadministration of ritonavir, both an HIV protease inhibitor (De Clercq, 2001) and a CYP3A4 inhibitor (von Moltke et al., 1998), led to a significant alteration in the disposition of capravirine in healthy volunteers (Bu et al., 2004). In the current study, the influence of ritonavir on the metabolism of capravirine was investigated in vitro to provide some mechanistic insight into the in vivo capravirine-ritonavir interaction in humans. The effect of ritonavir on the formation of capravirine metabolites in human liver microsomes is shown in Fig. 2. The formation of the two monohydroxylated metabolites C19 and C20 remained at residual levels and did not show a clear concentration-dependent inhibition by ritonavir. In practice, the radiochemical analysis of C19 and C20 was semiquantitative because of the extremely low levels and partial separation of the two metabolites. The formation of the sulfoxide C23 and the N-oxide C26 was inhibited by ritonavir in a typical concentration-dependent manner. Overall, ritonavir (up to 2 μM) did not completely inhibit the formation of C19, C20, C23, and C26 (Fig. 2), indicating that other enzymes might be involved in the formation of the four mono-oxygenated metabolites. However, the formation of all sequential metabolites was inhibited by ritonavir in a concentration-dependent manner and was completely inhibited by ritonavir at 2 μM, suggesting that all sequential oxygenations of capravirine appeared to be mediated exclusively by CYP3A4 (Fig. 1). This finding was supported by results of the study involving inhibition of further sequential metabolism of the seven available authentic metabolites. The high percentage remaining of the authentic metabolites in the presence of 2 μM ritonavir (i.e., 99, 103, 103, 111, 107, 92, and 107% for the metabolites C15, C19, C20, C22, C23, C25b, and C26, respectively) suggests that all the sequential reactions were also completely inhibited by ritonavir.

The above inhibition results suggest that capravirine is oxygenated predominantly by CYP3A4, with minor contributions by other enzymes. Ritonavir at 2 μM appeared to achieve the maximal inhibition of CYP3A4 activity (Fig. 2). Therefore, the percentage contribution of capravirine metabolism by CYP3A4 was estimated using the following equation: % (by CYP3A4) = 100 × (M1 − M0)/M1, where M1 and M0 represent the percentage of capravirine metabolized in the presence or absence of ritonavir, respectively. The percentage of capravirine metabolized in the presence of ritonavir at 2 μM was estimated to be >90% (91.7%, n = 3). The minor contribution (<10%) by other enzymes was only to the mono-oxygenation reactions. From the inhibition data of ritonavir at 2 μM (Fig. 2), it can be visually estimated that each of the two major mono-oxygenated metabolites C23 and C26 was inhibited primarily (>90%) by CYP3A4 and secondarily (<10%) by other enzymes. However, it was not possible to estimate the percentage contributions of CYP3A4 and other enzymes to the formation of the two minor mono-oxygenated metabolites C19 and C20 (each <0.5% of the total radioactivity).
Other Enzymes Responsible for Capravirine Oxygenation.

The predominant role of CYP3A4 in the metabolism of capravirine was also confirmed via chemical inhibition by ketoconazole and immunoinhibition by anti-human CYP3A4 immunoglobulin (data not shown). Further phenotyping experiments were conducted to identify enzymes other than CYP3A4 that may contribute (<10%) to the mono-oxygenation reactions of capravirine. The minor contribution by other enzymes was also NADPH-dependent because capravirine mono-oxygenation reactions of capravirine. The minor contribution by other enzymes that may contribute (<10%) to the mono-oxygenation reactions of capravirine. The major contribution by other enzymes that may contribute (<10%) to the mono-oxygenation reactions of capravirine. The minor contribution by other enzymes that may contribute (<10%) to the mono-oxygenation reactions of capravirine. The major contribution by other enzymes that may contribute (<10%) to the mono-oxygenation reactions of capravirine. 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