METABOLISM AND PHARMACOKINETICS OF EAPB0203 AND EAPB0503, TWO IMIDAZOQUINOXALINE COMPOUNDS PREVIOUSLY SHOWN TO HAVE ANTI-TUMORAL ACTIVITY ON MELANOMA AND T-LYMPHOMAS

Sonia Khier†, Florence Gattacceca†, Safia El Messaoudi, Florian Lafaille, Carine Deleuze-Masquéfa, Jacques Bompart, Jean-François Cooper, Isabelle Solassol, Frédéric Pinguet, Pierre-Antoine Bonnet, and Françoise M.M. Bressolle

Clinical Pharmacokinetic Laboratory (S.K., F.G., S.E.M., F.L., F.M.M.B.) and Pharmacochemistry and Biomolecule Laboratory (C.D.M., J.B., P-A.B.), EA4215, Faculty of Pharmacy, 15 Avenue Ch. Flahault, University Montpellier I, 34093 Montpellier Cedex 5, France; Biochemistry and environment laboratory (J.F.C.), EA 4215, Perpignan University, 66860 Perpignan Cedex 9, France; Oncopharmacology Department (I.S., F.P.), Pharmacy Service, Val d’Aurelle Anticancer Centre, Parc Euromédecine, 34298 Montpellier Cedex 5, France

Copyright 2010 by the American Society for Pharmacology and Experimental Therapeutics.
Running title page

Running title:

METABOLISM AND PHARMACOKINETICS OF EAPB0203 AND EAPB0503

Name, address, telephone and fax numbers, and e-mail address of the corresponding author:

Dr Françoise Bressolle, Laboratoire de Pharmacocinétique Clinique, EA4215, Faculté de Pharmacie, 15 Avenue Ch. Flahault, Université Montpellier I, 34093 Montpellier Cedex 5, France. Tel.: (33) 4 67 54 80 75; fax: (33) 4 67 54 80 75; e-mail: fbressolle@aol.com

Number of text pages: 35

Number of tables: 4

Number of Figures: 7

Number of references: 39

Abstract: 249 words

Introduction: 654 words

Discussion: 1296 words

ABBREVIATIONS: HTLV-I, human T-lymphotropic virus Type I; HSA, human serum albumin; LC/ESI-MS, liquid chromatography/electrospray ionization-mass spectrometry; EAPB0203, N-methyl-1-(2-phenethyl)imidazo[1,2-α]quinoxalin-4-amine; EAPB0202, 1-(2-phenethyl)imidazo[1,2-α]quinoxalin-4-amine; EAPB0503, 1-(3-methoxyphenyl)-N-methylimidazo[1,2-α]quinoxalin-4-amine; EAPB0502, 1-(3-methoxyphenyl)imidazo[1,2-α]quinoxalin-4-amine; EAPB0603, 1-(3-hydroxyphenyl)-N-methylimidazo[1,2-α]quinoxalin-4-amine; EAPB0602, 1-(3-hydroxyphenyl)imidazo[1,2-α]quinoxalin-4-amine;
NMR, nuclear magnetic resonance; DMSO, dimethylsulfoxide; LLOQ, lower limit of quantitation; C\textsubscript{met}, hydroxylated plus demethylated metabolite concentration; C\textsubscript{demet}, demethylated metabolite concentrations; f\textsubscript{B(microsomes)}, nonspecific bindings to human microsomes; CL\textsubscript{int,in vitro} in vitro intrinsic clearances; CL\textsubscript{int,in vivo} scaled in vivo intrinsic clearance; CL\textsubscript{Hp}, human hepatic plasma clearance; f\textsubscript{u}, unbound fraction in plasma; C\textsubscript{blood}, drug concentration in the blood; C\textsubscript{plasma}, plasma drug concentration; f\textsubscript{u(microsomes)}, unbound fraction in the microsomal reaction mixture; Q\textsubscript{H}, hepatic blood flow in human; AUC, area under the mean plasma drug concentration versus time curve extrapolated to infinity; AUMC, area under the first moment of the concentration-time curve; CL, total plasma clearance; V\textsubscript{ss}, steady-state volume of distribution; t\textsubscript{1/2}, terminal half-life; k, rate constant; LC-MS/MS, liquid chromatography/tandem mass spectrometry; P450, cytochrome P450; r.t., retention time; IC\textsubscript{50}, 50% inhibitory concentration.
Abstract:

Since several years, our group develops quinoxalinic compounds. Two of them, EAPB0203 and EAPB0503, have emerged as the most promising anticancer drugs. In the present work, we determined metabolism pathways using liver microsomes from four mammalian species including human. We identified the P450 isoform(s) involved in the metabolism. Then, the pharmacokinetics and metabolism of EAPB0203 and EAPB0503 were investigated in rat after intravenous and intraperitoneal administration. Biotransformation of the compounds involved demethylation and hydroxylation reactions. Rat and dog metabolized the compounds with a higher rate than mouse and human. In all species, CYP1A1/2 and CYP3A isoforms were the predominant enzymes responsible for the metabolism. From human liver microsomes, unbound intrinsic clearances were around 56 ml/min/g protein. EAPB0203 and EAPB0503 were extensively bound to human plasma proteins, mainly serum albumin (HSA) (~98-99.5%). Thus, HSA could act as carrier of these compounds in human plasma. The Scatchard plots showed patterns in which the plots yielded upwardly convex hyperbolic curves. Based on the Hill coefficients, there appears to be interaction between the binding sites of HSA, suggesting positive cooperativity. The main in vitro metabolites were identified in vivo. Total clearances of EAPB0203 and EAPB0503 (3.2 and 2.2 l/h/kg, respectively) were notably lower than the typical cardiac plasma output in rat. The large volumes of distribution of these compounds (4.3 l/kg for EAPB0203 and 2.5 l/kg for EAPB0503) were consistent with extensive tissue binding. After intraperitoneal administration, bioavailability was 22.7% for EAPB0203 and 35% for EAPB0503 and a significant hepatic first-pass effect occurred.
Introduction

Melanoma and T-lymphomas are major public health problems; their incidence and mortality rates increase in the world populations and, despite a major effort in clinical research scrutinizing various treatment regimens, the prognosis for these patients still remains poor. Given the limited effectiveness of a large variety of conventional anti-cancer drugs, novel approaches or drugs are urgently needed.

Since several years, our group develops quinoxalinic compounds. Three chemical series have been synthesized: the imidazo[1,2-α]quinoxalines, the imidazo[1,5-α]quinoxalines, and the pyrazolo[1,5-α]quinoxalines. These compounds are analogs of imiquimod, the first member of the imidazoquinolone family, used in the topical treatment of genital and anal warts by increasing the activity of the body's immune system (Rudy, 2002; Sauder, 2003; Hengge and Cusini, 2003). Imiquimod is also efficacious as a topical therapy for certain types of skin cancers (van Egmond et al., 2007; Kang et al. 2008; Steinmann et al., 2000). Its therapeutic spectrum has been further extended to cutaneous B-cell lymphomas (Spaner et al., 2005).

The cytotoxic activities of the compounds synthesized by our group have been evaluated, in vitro, against human cancer cell lines (Moarbess et al., 2008a; Deleuze-Masquéfa et al., 2009; Khier et al., 2009; Khier et al., 2010). The highest cytotoxic efficacies were obtained in the imidazo[1,2-α]quinoxaline series. On A375 human melanoma cancer cell line, EAPB0203 (50% inhibitory concentration, IC50, 1.57 µM) had an in vitro activity 110 times higher than fotemustine (IC50, 173 µM) and 45 times higher than imiquimod (IC50, 70 µM) (Moarbess et al., 2008a). In a mice melanoma model, EAPB0203 was more potent than fotemustine (Moarbess et al., 2008a; Khier et al., 2010). This compound also displayed interesting cytotoxic activity with micromolar IC50 values against colon and breast human cancer cell lines (Moarbess et al., 2008a). The recently synthesized compound, EAPB0503, showed in vitro cytotoxic activity on A375 melanoma cancer cell line about 10 times higher than
EAPB0203 (IC_{50}, 150 nM) (Khier et al., 2010). Thus, EAPB0203 and EAPB0503 have emerged as the most promising drugs. EAPB0203 has also been evaluated in T-cell lymphomas and human T-lymphotropic virus Type I (HTLV-I) associated adult T-cell leukaemia/lymphoma (Moarbess et al., 2008b). This compound exhibits an important cytotoxicity in vitro on HTLV-I-infected CD4^{+} T-cell lines HuT-102, MT-2, and C91-PL as well as on HTLV-1-negative malignant CD4^{+} T-cell lines CEM, Jurkat, HuT-78, and MOLT-4.

We have shown that imidazo[1,2-a]quinoxaline derivatives inhibit cyclic nucleotide phosphodiesterase enzymes 4, resulting in increased intracellular cAMP level, and consequently CREB phosphorylation (Deleuze-Masquéfa et al., 2004), and that these compounds activate the p38 MAPK pathway, and inhibit the PI3K pathway (Morjaria et al., 2006). The mechanism of action of EAPB0203 has been studied in T-cell lymphomas and HTLV-I associated adult T-cell leukemia/lymphoma (Moarbess et al., 2008b). This compound exhibits inhibition of cell proliferation, G2/M cell cycle arrest, and induction of apoptosis in HTLV-I transformed and HTLV-I negative malignant T cells while normal resting or activated T lymphocytes are resistant. Furthermore, EAPB0203 almost completely inhibited the growth of freshly isolated ATL cells at concentrations of 1 to 10 µM. EAPB0203 treatment significantly down-regulated the anti-apoptotic proteins c-IAP-1 and Bcl-XL, and resulted in a significant loss of mitochondrial membrane potential, cytoplasmic release of cytochrome c, and caspase dependent apoptosis. In addition, in HTLV-I transformed cells only, EAPB0203 treatment stabilized p21 and p53 proteins but had no effect on NF-kB activation.

In the present paper, we determined the binding of EAPB0203 and EAPB0503 to human plasma proteins and human serum albumin (HSA) solutions in vitro, and we investigated the in vitro metabolism of these two compounds by comparing the metabolite profiles in rat, mouse, dog, and human liver microsomes. Specific chemical inhibitors were used to identify
the CYP enzymes that play a role in the metabolism of these compounds. Metabolites were identified using a liquid chromatography/electrospray ionization-mass spectrometry method (LC/ESI-MS). Moreover, results on the distribution, metabolism, and elimination of EAPB0203 and EAPB0503 in rat, one of the principal species used in preclinical studies, were reported.
Materials and Methods

Chemicals. EAPB0203 (N-methyl-1-(2-phenethyl)imidazo[1,2-a]quinoxalin-4-amine; molecular weight, 302), EAPB0202 (1-(2-phenethyl)imidazo[1,2-a]quinoxalin-4-amine; molecular weight, 288), EAPB0503 (1-(3-methoxyphenyl)-N-methylimidazo[1,2-a]quinoxalin-4-amine; molecular weight, 304), EAPB0603 (1-(3-hydroxyphenyl)-N-methylimidazo[1,2-a]quinoxalin-4-amine; molecular weight, 290), EAPB0502, (1-(3-methoxyphenyl)imidazo[1,2-a]quinoxalin-4-amine; molecular weight, 290) and EAPB0602 (1-(3-hydroxyphenyl)imidazo[1,2-a]quinoxalin-4-amine; molecular weight, 276) (Fig. 1A,B) were synthesized by the Pharmacochemistry and Biomolecule Laboratory (Montpellier I University, France) (Masquéfa et al., 2008). The purity of these standards was evaluated by elemental analysis, LC-MS and nuclear magnetic resonance (NMR). They were stored at 20°C protected from light. The physicochemical properties of these molecules are presented in Table 1. Human plasma was obtained from blood samples collected from healthy volunteers not undergoing drug therapy (Etablissement Français du sang, Montpellier, France). Crystallized HSA, NADH, NADPH, ketoconazole, quercetin, sulfaphenazole, coumarine, α-naphthoflavone, diethyldithiocarbamate, quinidine and dimethylsulfoxide (DMSO) were obtained from Sigma (St Louis, MO, USA). Human, dog, rat, and mouse liver microsomes were purchased from BD Biosciences (Le Pont de Claix, France). All other chemicals and solvents were purchased from commercial suppliers and were of high-performance liquid chromatography grade.

Analytical Methods. EAPB0503, EAPB0203 and their metabolites were quantified in rat plasma using validated LC/ESI-MS methods (Khier et al., 2009; Khier et al., 2010). The LC-MS analysis was performed using an Agilent 1100 quadrupole mass spectrometer coupled to a Hewlett Packard LC system equipped with an autosampler set at 4°C. In brief, the sample-pretreatment procedure involved solid-phase extraction of plasma using Oasis HLB cartridge
after protein precipitation with 10 ml/l trifluoroacetic acid. The internal standard used was of the same chemical series. Chromatography was performed at 20°C on a C8 Zorbax eclipse XDB column with a mobile phase consisting of acetonitrile and formate buffer (pH 3) gradient elution (flow rate, 0.8 ml/min). LC-MS data were acquired in single ion monitoring mode at \(m/z\) values corresponding to the protonated molecules, [M+H]\(^+\). The drug/internal standard peak area ratios were linked via quadratic relationships to plasma concentrations. The methods are precise (precision, \(\leq 14\%\)) and accurate (recovery, 92-113\%). Mean extraction efficiencies > 72% for each analyte were obtained. No significant matrix effects occurred. Dilution had no influence on the performance of the method. The lower limit of quantitation (LLOQ) was 5 µg/l (i.e., \(\sim 0.017 \mu M\)) for each of the analytes.

Animal samples were processed with a standard curve, and quality control samples were included in each analytical sequence to verify the stability of study samples during storage and accuracy and precision of analysis.

Identified metabolites and their parent drugs were quantified in microsomal incubates using the LC-MS conditions described above. Samples were processed with an eight-point standard curve prepared in a mixture of buffer (containing all the components for microsomal incubation, except NADH) and acetonitrile. Calibrators were prepared by spiking appropriate amounts of each compound (EAPB0203 and EAPB0202; or EAPB0503, EAPB0502, EAPB0603 and EAPB0602) to the buffer-acetonitrile mixture to obtain final concentrations in the range of 0.05 to 10 µM. In the absence of analytical standards, the relative amount of each hydroxylated metabolite was calculated using the following formula:

\[
[(C_{\text{met}} (\mu M) - C_{\text{demet}} (\mu M)) \times (\text{peak area of one hydroxylated metabolite} / \text{peak areas of all hydroxylated metabolites})] \times 100 / C_{\text{met}} (\mu M); \text{ where } C_{\text{met}} \text{ is the hydroxylated plus demethylated metabolite concentration calculated by subtracting the unmetabolized concentration after microsomal incubation (µM) to the initial concentration (i.e. 10 µM in the}
\]
buffer-acetonitrile mixture) and $C_{\text{demet}}$ is the sum of all demethylated metabolites quantified in the incubation medium.

In an attempt to identify hydroxylated derivatives, an Agilent 6400 series triple quadrupole LC-MS/MS (Agilent Technologies, Les Ulis, France) was used. Chromatographic conditions were similar to those described above. The mass selective detector was operated in scan mode for the qualitative analyses of microsomal incubates ($m/z$ from 100 to 320). Nebulizer pressure was 35 psi, and the drying gas (nitrogen) was delivered at 12 l/min. Capillary and nozzle voltages were 3000 and 1500 V, respectively. The fragmentor (collision-induced dissociation cell) was set at 35 V.

Human plasma protein binding. Plasma protein binding of EAPB0203 and EAPB0503 was determined by ultracentrifugation, since preliminary studies showed that these compounds were both subject to unacceptable levels of nonspecific binding when analyzed by ultrafiltration or equilibrium dialysis. The lack of proteins in the supernatant was assessed using a bicinchoninic acid protein assay kit (Sigma). The time necessary for achievement of equilibrium was determined in a preliminary study at concentrations of 50, 100 and 1000 µg/l (i.e., ~0.16, 0.33 and 3.3 µM). For this, multiple spiked plasma samples (10 ml each) in polypropylene tubes were placed in an incubator at 37°C for 0.5 to 3 h. At selected time intervals, one tube was removed; a 8-ml aliquot plasma was introduced in a polycarbonate tube then centrifuged in a pre-cooled rotor at 100,000 g for 14 h at +4°C using a Beckman Optima LE70 ultracentrifuge (Fullerton, CA). The free fraction in the supernatant taken from halfway between the supernatant surface and the pellet was analyzed. The remaining 2 ml were used to quantify the total drug concentration. Using optimized conditions (i.e., 2 h at 37°C), the percentage of bound drugs in plasma was determined from plasma spiked samples at concentrations of 62.5, 100, 500, 1000 and 2000 µg/l (i.e., ~0.21 to 6.6 µM). Samples were
placed at 37°C for two hours then centrifuged according to the same procedure as described above.

In a second phase of this study, EAPB0203 and EAPB0503 bindings by HSA at approximately physiologic concentration of this protein (40 g/l in pH 7.4 phosphate buffer) were studied. The percentage of bound drug was determined at concentrations of 62.5, 100, 250, 500 and 1000 µg/l (i.e.,~ 0.21 to 3.3 µM). Thereafter, the association (Kₐ) and dissociation (Kᵣ=1/Kₐ) constants were determined. For this, each compound, at concentrations of 62.5, 250 and 1000 µg/l, was added to albumin solutions at concentrations ranging from 0.25 to 40 g/l (0.25, 0.5, 1, 2.5, 5, 10 and 40 g/l). The following Scatchard equation (Scatchard, 1949) was used:

$$\frac{r}{[D_f]} = K_a n - K_a r$$

where n is the number of binding sites per albumin molecule, r is the number of moles of bound drug per mole of protein and [Dᵢ] is the molar concentration of free drug. By plotting r/[Dᵢ] against r, the association constant is calculated.

The nonspecific binding of EAPB0203 and EAPB0503 to human liver microsomes (fᵢₙ(microsomes)) was also evaluated. Binding experiments were conducted at 37°C to mimic conditions used in in vitro microsomal metabolism studies but were conducted in the absence of NADH so that metabolism of the compounds would not occur. The methodology used was similar to that described above for HSA.

All determinations were performed in triplicate.

**In vitro metabolism assays with liver microsomes.** These studies were conducted in accordance with the EMA’s guidance document on the conduct of in vitro metabolism studies (EMA, 2009) and the principles promulgated by Jia and Liu (2007) and Huang et al. (2008). All determinations were performed in replicates (n=3-4).
General protocol for microsomal incubations. Control incubations were carried out to ensure that metabolite formation required microsomal enzymes and cofactor. Pilot experiments were performed to obtain optimum metabolic activity. The cofactor (NADH or NADPH), the pH (6.3 or 7.4) and the time of incubation were optimized. Incubations were carried out under aerobic conditions at 37°C in a shaking incubator. The best conditions were obtained when the substrates, dissolved in DMSO then diluted in phosphate buffer (pH 6.3, final DMSO concentration < 1%, v/v) containing 1% bovine serum albumin, were incubated 4 h with pooled liver microsomes (1 g protein/l) in 0.25-ml incubation mixtures containing potassium phosphate buffer (pH 6.3). In the incubation medium, concentrations of EAPB0203 and EAPB0503 were 20 µM. Moreover, using these optimal conditions, experiments ensured that metabolite formation was linear with time (15 to 240 min) and protein concentration (from 0.2 to 4.8 g/l). Zero-time, zero-substrate, zero-protein and zero-cofactor served as controls. After an initial preincubation of 15 min with substrate, reactions were initiated by the addition of NADH (10 mM). The reaction was terminated by addition of 250 µl of cold acetonitrile containing 1% formic acid and the internal standard. Precipitated proteins were removed by centrifugation (14,000 g for 20 min at 4°C), and the supernatant fractions were analyzed by LC-MS.

Enzyme kinetics in human liver microsomes and estimation of intrinsic clearance. For both EAPB0203 and EAPB0503, intrinsic clearances (CL_{int,in vitro}) were calculated from substrate disappearance rate in presence of human liver microsomes (1 g/l). The substrate disappearance with time (0, 5, 10, 30, 60, 90, 120, 180 and 240 min) followed a first-order reaction, thus, the unchanged drug profile as a function of time was described as follows: 
\[ C_t = C_0 e^{-k_e x t}, \]
where \( C_0 \) is the initial concentration of the drug and \( k_e \) is the disappearance rate constant. CL_{int,in vitro} was calculated from the following equation: 
\[ k_e / D_{cell} \]
where \( D_{cell} \) is the cell density (g/ml) (Naritomi et al., 2003). Using scaling factors published by Carlile et al.
(1997) and Obach (1999): 45 mg of microsomal protein per gram of liver, and 20 g of liver per kilogram of body weight, the in vivo intrinsic clearance (CL_{int,in \text{vivo}}) was calculated.

For EAPB0203, CL_{int,in \text{vivo}} was also calculated from the Michaelis-Menten parameters, by incubating increasing concentrations of EAPB0203 (0.3-50 µM) in human microsomal preparations according to the protocol described above. CL_{int,in \text{vivo}} was calculated as the ratio of V_{max} over K_m. (Houston and Kenworthy, 2000).

From the CL_{int,in \text{vivo}} values, the human hepatic plasma clearance (CL_{Hp}) was calculated using the well-stirred model as follows (Yang et al., 2007):

$$
\text{CL}_{Hp} = \frac{Q_H \times f_u \times \text{CL}_{int,in \text{vivo}}/f_u(\text{microsomes})}{Q_H + f_u \times (\text{CL}_{int,in \text{vivo}}/f_u(\text{microsomes}))/\left(\text{C}_{\text{blood}}/\text{C}_{\text{plasma}}\right)}
$$

where $f_u$ is the unbound fraction in plasma, $\text{C}_{\text{blood}}$ is the drug concentration in the blood, $\text{C}_{\text{plasma}}$ is the plasma drug concentration, $f_u(\text{microsomes})$ is the unbound fraction in the microsomal reaction mixture and $Q_H$ the hepatic blood flow in human (21 ml/min/kg). The blood to plasma concentration ratio was evaluated in vitro after incubation of one-milliliter aliquots of blood for 1 h at 37°C with the analytes (2 and 20 µM for each compound).

*Inhibition studies.* The choice of inhibitors and their concentrations were based on literature data (Reidy et al., 1989; Halpert et al., 1994; Rodrigues, 1994; Baldwin et al., 1995; Bourrie et al., 1996). EAPB0203 and EAPB0503 were incubated in human, dog, rat, and mouse liver microsomes in the presence and absence of the following selective inhibitors: ketoconazole (CYP3A; Eagling et al., 1998); quercertin (CYP2C8; Rahman et al., 1994); sulfaphenazole (CYP2C9; Newton et al., 1995); coumarine (CYP2A6; Pearce et al., 1992); α-naphthoflavone (CYP1A1/2; Rendic and Di Carlo, 1997); diethyldithiocarbamate (CYP2E1 and CYP2A; Rendic and Di Carlo, 1997]) and quinidine (CYP2D6; ; Newton et al., 1995]). α-naphthoflavone was also an activator of CYP3A activity. Two concentrations of each
inhibitor were used; inhibitor concentrations are shown in Fig. 2 for EAPB0203 and in Fig. 3 for EAPB0503; concentrations of α-naphthoflavone were 100 and 500 µM. Stock solutions of inhibitors were prepared in desionized water (diethyldithiocarbamate), methanol (α-naphtoflavone, ketoconazole, sulfaphenazole, coumarine), DMSO (quinidine) or 0.1M sodium hydroxide (quercertin). They were extemporaneously diluted to the appropriate concentrations with phosphate buffer (pH 6.3). All inhibitors and substrates were preincubated prior to the addition of NADH, as described above.

**Pharmacokinetic studies.** This research adhered to the “Principles of Laboratory Animal Care” (NIH publication #85-23, revised 1985). The animal studies were approved by the local Animal Use Committee.

*Animals.* Sprague-Dawley male rats from Charles River (L’Arbresle Cedex, France) were housed in standard wire-bottom cages (maximum of 6 rats per cage) and allowed free access to standard rodent diet (UAR sterile food, Usine d’Alimentation Rationnelle, Villemoisson, Epinay s/Orge, France) and water. They were kept under conditions of constant temperature (21-25°C) and relative humidity of approximately 40-65% with standard light/dark cycle. Body weights at the time of dosing were 200 to 250 g. All animals underwent a period of one week of observation and acclimatization before treatment. Rats were fasted overnight (12 h) before drug administration and then weighed.

*Dosing.* Rats were randomly distributed in four experiment groups. In the first two groups, rats received single 5 mg/kg intravenous (i.v.) and intraperitoneal (i.p.) doses of EAPB0503; in the third and fourth groups, rats received single 2.5 mg/kg i.v. and i.p. doses of EAPB0203. Doses were chosen according to the maximum tolerated doses in rat. All dose formulations were freshly prepared on the day of administration in dimethylsulfoxide. Injection volumes
were limited to 100 µl. Intravenous doses were administered into the penis vein; before administration, each animal was anaesthetized with sevofluran.

**Blood sampling.** After single dose administration, blood (one sample per rat, 3 animals per time-point) was obtained at 5, 10, 30 minutes, and 1, 2, 4, 8, 12, 24 and 36 h postdose. Samples were collected after sacrifice of the animals by section of the carotid artery. Two minutes prior to sampling, animals were anaesthetized with sevoflurana. Blood samples were collected in heparinized polypropylene tubes (0.1 ml lithium heparinate per tube), immediately placed on ice, then centrifuged (4000 g) at 4°C for 10 min. The plasma was separated then immediately stored at –80°C until assay.

**Pharmacokinetic data processing.** After single administration, pharmacokinetic parameters were calculated by non-compartmental analysis from the average concentration values at each time point using the Pk-fit software (Farenc et al., 2000). When the concentrations were below the quantitation level, the mean values were obtained from several values at, or above, the LLOQ with the other values taken as zero. The area under the mean plasma drug concentration versus time curve extrapolated to infinity (AUC) was estimated using the log/linear trapezoidal method. The total plasma clearance (CL) and steady-state volume of distribution (Vss) of the parent drug were calculated from the relationships: CL= dose/AUC and Vss = CL x (AUMC/AUC), respectively; where AUMC is the area under the first moment of the concentration-time curve. The terminal half-life (t1/2) was calculated as \( \ln 2/k \), where k is the rate constant determined from the slope of the log linear part curves. The absolute bioavailability (F) was calculated from the AUC values obtained for both the parent drug and its active metabolite(s) exhibiting similar in vitro cytotoxicity as the parent compound (i.e., EAPB0202 for EAPB0203; EAPB0603 and EAPB0502 for EAPB0503) as follows: 

\[
F = \frac{(\text{AUC}_{\text{unchanged compound}+\text{active metabolite(s)}})_{\text{i.p.}}}{(\text{AUC}_{\text{unchanged compound}+\text{active metabolite(s)}})_{\text{i.v.}}}
\]
Results

**Plasma Protein Binding.** EAPB0203 and EAPB0503 bound strongly to plasma proteins and HSA (about 98 to 99.5%). For both compounds, only a small fraction was present in free form. For drug concentrations between 62.5 and 1000 µg/l, the percentage of EAPB0203 and EAPB0503 bound at 40 g/l HSA is presented in Table 2. Although a few differences were observed, the percentage of bound drug appeared to increase with the drug concentration over the range of concentration studied. Similar results were obtained with human plasma (data not shown). However, using the Kruskal-Wallis test, the difference was not statistically significant ($p = 0.0769$ for EAPB0203 and $p = 0.13$ for EAPB0503). The extent of binding of EAPB0203 and EAPB0503 with different concentrations of HSA is shown in supplemental data Fig 1 that accompanies this paper. The binding was reduced to 65-70% at very low concentration of albumin (0.25 g/l). For EAPB0203 and EAPB0503, the Scatchard plots showed patterns in which the plots yielded upwardly convex hyperbolic curves (Fig. 4). Using the Hill equation, the Hill coefficients ($n$), calculated from natural logarithm of $r/(1 – r)$ versus the logarithm of free drug concentration, were $n = 1.42$ (95% confidence interval: 1.26-1.57) for EAPB0203 and $n =1.32$ (95% confidence interval: 1.14-1.50) for EAPB0503. These values, greater than 1, could indicate positive cooperativity of binding. The dissociation constants, $K_D$, were $0.18 \times 10^{-6}$ M for EAPB0203 and $0.46 \times 10^{-6}$ M for EAPB0503. The corresponding association constants were $5.55 \times 10^6$ M and $2.18 \times 10^6$ M, respectively. Estimated $K_D$ values were similar to the lowest concentrations tested (0.21 µM), but in the experimental conditions used, we cannot evaluate lower drug concentrations as we are limited by the LLOQ of the free fraction. Thus, the estimation of $K_D$ was repeated from lower drug concentrations (33, 50, 100, 165, 200 and 210 nM) but using a single albumin concentration of 0.25 g/l. Estimated $K_D$ values were of the same order of magnitude: $0.26 \times 10^{-6}$ M for EAPB0203 and $0.23 \times 10^{-6}$ M for EAPB0503.
**In Vitro Metabolism Studies.** Metabolic identification. Quantitatively, rat and dog metabolized the compounds with a higher rate than mouse and human (see supplemental data Fig. 2 that accompanies this paper). When EAPB0203 was incubated with rat, dog, mouse and human liver microsomes in the presence of NADH for 4 h, 55, 68, 27.5 and 34% of the drug were metabolized, respectively. The LC-MS analysis of liver microsomal incubates allowed to identify eight metabolites related to an oxidative process (hydroxylation and N-demethylation) that did not appear when NADH was omitted from the mixture. These compounds were more polar than EAPB0203 (retention time, 6.5 min) and eluted at 3.6 min (M1), 4.0 min (M2), 4.1 min (M3), 4.2 min (M4), 4.5 min (M5), 4.5 min (M6), 4.8 min (M7) and 5.8 min (M8) (Fig. 5). The first metabolism route in all species studied consisted in an oxidative N-demethylation of EAPB0203 yielding EAPB0202 (M8, protonated molecule at m/z 289). The structure of EAPB0202 was proposed based on the LC-MS spectra and on the basis of coelution with a reference compound. Microsomes from human were the most effective N-demethylating system (39% of total metabolite levels), followed by those of mouse (22%) (Table 3). A second pathway resulted in the formation of four hydroxylated derivatives of EAPB0203 (M2, M4, M6 and M7; protonated molecules at m/z 319). They were detected in all the species except the dog. Human and mouse microsomes predominantly formed M2 and M7, while rat microsomes favored the production of M2 and M6, and dog microsomes the production of M2 (supplemental data Fig. 2). Other hydroxylated metabolites (M1, M3 and M5) showed the protonated molecules at m/z 305. These metabolites could correspond to the hydroxylated derivatives of EAPB0202. Most of them were formed at low levels. Table 3 summarizes the relative abundance of the metabolites identified in liver microsomes from animal species and human.
In an attempt to identify hydroxylated derivatives, microsomal incubates were analyzed by LC-MS/MS. On the basis of fragmentation patterns deduced from the obtained spectra, two hydroxyl groups could be positioned on the imidazoquinoxaline cycle (M6, M7) and the others (M2 and M4) on the phenyl cycle of the phenethyl group. M5 should be the demethylated compound of M7; M1 that of M2, and M3 that of M6. However, in the absence of standards, the precise oxidation position could not be elucidated further in view of the mass spectrometry data available. For the exact identification of these unidentified metabolites, more experimental work should be performed. The metabolic scheme is presented in Fig. 1A.

Incubations of pooled rat, dog, mouse and human liver microsomes with EAPB0503 (4 h at 37°C in the presence of NADH as a cofactor) were analyzed by LC-MS; 50, 68, 26 and 39% of the drug have been transformed, respectively (supplemental data Fig. 2). In a typical HPLC separation, EAPB0503 eluted at 6.2 min. Seven more polar EAPB0503 metabolites were separated from the extracted mass chromatograms. They were named accordingly: M'1 (retention time, r.t., 3.0 min), M'2 (r.t., 3.2 min), M'3 (r.t., 3.5 min), M'4 (r.t., 3.7 min), M'5 (r.t., 4.0 min), M'6 (r.t., 4.1 min) and M'7 (r.t., 5.3 min) (Fig. 6). The structures of M'2 (EAPB0602, protonated molecule at m/z 277), M'5 (EAPB0603, protonated molecule at m/z 291) and M'7 (EAPB0502, protonated molecule at m/z 291) were identified based on LC-MS spectra and on the basis of coelution with available standards. At 20 µM substrate concentrations, 4-7 metabolites were formed by liver microsomes depending on the species (supplemental data Fig. 2). EAPB0603 was predominant in human and rat microsomes: (39 and 33% of the total metabolite levels, respectively). In contrast, in microsomes from dog and mouse, EAPB0603 is formed to a much lesser extent (<3% and 8% of total EAPB0503 disappearance, respectively), and the N-demethylated EAPB0502 is the predominant metabolite; it accounts for 56 and 27% of total metabolism, respectively. Products eluting with retention times of 3.7 and 4.1 min (M'4 and M'6) showed the protonated molecule at m/z
321, indicating the molecular mass of hydroxylated derivatives of EAPB0503. Metabolite M’4 was detected in all species (from 19% of the total metabolite levels in human and dog to 39% in rat). Metabolite M’6 was not detected in human and rat microsomes, while it was produced at high level in microsomes from mouse. M’1 and M’3 showed the protonated molecule at m/z 307, indicating the molecular mass of demethylated derivatives of M’4 and M’6, respectively. Metabolite M’1 was only detected in dog microsomes. Metabolite M’3 was not detected in human and rat and remained very low in dog microsomes. Table 3 reports the relative abundance of the identified metabolites. Based on fragmentation patterns deducted from the obtained spectra by LC-MS/MS, one hydroxyl group should be positioned on the imidazoquinoxaline cycle (M’4) and the other on the phenyl group linked to the imidazole cycle (M’6). M’1 should be the demethylated compound of M’4; and M’3 that of M’6. However, as reported for EAPB0203, the precise oxidation position was not elucidated.

Biotransformations by cytochrome P450-mediated EAPB0503 metabolism are presented in Fig. 1B.

**In vitro intrinsic clearance.** Supplemental data Fig 3 that accompanies this paper illustrates the time courses of EAPB0203 and the formation of the main metabolites in human microsomal incubation. Mean intrinsic metabolic clearances (CL_{int,in vitro}) calculated from substrate disappearance rate were 2.37 ± 0.05 ml/min/g protein for EAPB0203 and 2.70 ± 0.038 ml/min/g protein for EAPB0503. For EAPB0203, similar intrinsic clearance was estimated using the Michaelis-Menten parameters. K_m and V_max values were 20 µM and 54.8 pmol/min/mg protein, respectively, yielding a CL_{int,in vitro} of 2.74 ml/min/g protein. The apparent K_m value of 20 µM indicates that enzymes metabolizing EAPB0203 into EAPB0202 have a modest affinity for the substrate. According to the classification for categorizing compounds (Nassar et al., 2009), CL_{int,in vitro} could be considered as low to intermediary values. Scaling-up of the CL_{int,in vitro} values to reflect intrinsic clearances on a per kilogram
body weight basis for human (CL<sub>int,in vivo</sub>) yielded mean values of 2.13 ml/min/kg for EAPB0203 and 2.43 ml/min/kg for EAPB0503. The nonspecific bindings of EAPB0203 and EAPB0503 to human microsomes (f<sub>B(microsomes)</sub>) calculated from 1.0 g protein/l and drug concentrations at 20 µM were 95.5%. Thus, unbound CL<sub>int,in vivo</sub> values, calculated from CL<sub>int,in vivo</sub> and f<sub>U(microsomes)</sub>, were 47.1 ml/min/kg for EAPB0203 and 54.0 ml/min/kg for EAPB0503. Predictions for the human hepatic plasma clearances with the “well-stirred” model incorporating f<sub>U</sub>, f<sub>U(microsomes)</sub> and a factor C<sub>blood</sub>/C<sub>plasma</sub> of 0.6 for both EAPB0203 and EAPB0503 gave the following results: 0.92 ml/min/kg for EAPB0203 and 0.74 ml/min/kg for EAPB0503.

**Inhibition study.** The relative contributions of CYP1A1/2, CYP2A, CYP3A, CYP2C8/9, CYP2D6 and CYP2E1 to EAPB0203 and EAPB0503 N- and O-demethylations in liver microsomes were investigated. Studies to identify the CYP450 isoform(s) involved in EAPB0203 and EAPB0503 hydroxylations were also undertaken. As shown in Fig. 2, for all species, CYP3A was found to play the major role in EAPB0203 N-demethylation. At high and low concentrations, ketoconazole decreased EAPB202 formation by about 50% in human liver microsomes and by about 60% in mouse liver microsomes. In dog and rat liver microsomes, coincubation of EAPB0203 with high ketoconazole concentration resulted in about 40% (dog) and 60% (rat) inhibition of EAPB202 formation; less than 20% were inhibited at low concentration. CYP2A6 and CYP2C9 in human, CYP2E1 in dog and mouse and CYP2C9 at high concentration of sulfaphenazole in rat also contributed significantly to EAPB0202 formation (Fig. 2). CYP1A1/2 and CYP3A predominantly generated hydroxylated metabolites of EAPB0203. At high and low concentrations, α-naphthoflavone inhibited formation of all hydroxylated metabolites of EAPB0203 in mouse hepatic microsomes, with similar 46% inhibition. This compound decreased formation of M2 and M7 by 40% in dog microsomes; but it had a much weaker inhibitory effect in human and rat...
microsomes (about 6-10%). At the two concentrations studied, α-naphthoflavone also inhibited completely the formation of M4 in rat microsomes and inhibited the formation of this metabolite by 49% in human microsomes. Except in mouse, no effect of this compound was observable on M6 formation. In all species, we observed a stimulatory effect of α-naphthoflavone on the formation of EAPB0202 (5-fold in human and mouse, 3-fold in rat and dog). The inhibitor of CYP3A, ketoconazole, had also an important inhibitory effect on the formation of M2 (48% in human, 12% in mouse), M6 (52% in human, 33% in dog, and 10% in rat and mouse microsomes) and M7 (95% in human, 34% in rat, 64% in mouse and 11% in dog microsomes). CYPs of CYP2A and CYP2E1 subfamilies also participated in formation of hydroxylated metabolites of EAPB203 (M4 in human, M6 in rat and dog, and M7 in dog microsomes) but only to a lesser extent (18-23%).

As presented in Fig. 3, in all species, ketoconazole inhibited N-demethylation of EAPB0503 by about 40-65%, followed by coumarine (~20%) and sulfaphenazole (~10%) in human, diethydithiocarbamate (~18%) in dog and quercertin (~18%) in rat. Ketoconazole also inhibited O-demethylation of EAPB0503 by about 20-45% in human, rat and dog microsomes. The contribution of CYP3A to the formation of EAPB0603 was less important in mouse microsomes (~5%). Other isoforms were implicated in the O-demethylation of EAPB0503: CYP2A, CYP2C8, CYP2C9 and CYP2E1 in human, and CYP2C8 and CYP2E1 in rat. Except for quercertin and diethydithiocarbamate in rat, there was little influence of inhibitor concentrations on the percentage of inhibition. In microsomes of all species, α-naphthoflavone inhibited formation of M′4 (40 to 50% in human, rat and dog; 90% in mouse) and this compound inhibited completely the formation of M′6 in mouse and dog microsomes. In all species, we observed a stimulatory effect of α-naphthoflavone on the formation of EAPB0502 (1.3 to 2-fold). CYP3A was also involved in the formation of M′4 in rat microsomes (inhibition by ketoconazole, 26%); the contribution of this isoform was less
important in human and mouse microsomes (inhibition by ketoconazole of about 10%). CYP2C9 also contributed significantly to M’4 and M’6 formation in rat microsomes (inhibition by sulfaphenazole of about 40%).

**Pharmacokinetics.** Some results presented here have been previously published in our earlier works (Khier et al., 2009; Khier et al., 2010). In this part of the manuscript we summarized all available data on the pharmacokinetics of the two lead compounds. In vivo, EAPB0203 was metabolized to give EAPB0202 and four hydroxylated metabolites, M1, M2, M3 and M6. Biotransformation of EAPB0503 involved two demethylation steps leading to the formation of EAPB0603, EAPB0502 and EAPB0602, and one hydroxylation step leading to the formation of M’4. EAPB0603 was the main metabolite. Unfortunately, in absence of standards, the hydroxylated metabolites identified in vivo have not been quantified.

Both after i.v. and i.p. administrations, the declines in the EAPB0203 and EAPB0202 plasma concentrations take place in two phases, an initial phase in which the rate of decline is rapid followed by a prolonged terminal phase (Khier et al., 2010). After EAPB0503 administrations, plasma concentrations versus time curves are presented in Fig. 7. EAPB0503 and EAPB0603 exhibited a two-phase decline in plasma concentration while for EAPB0502 and EAPB0602, a monoexponential decline was observed. Basic pharmacokinetic parameters for EAPB0203 and EAPB0503, and their metabolites are presented in Table 4. The pharmacokinetic parameters showed a 72% greater steady-state volume of distribution (4.3 l/kg vs 2.5 l/kg) for EAPB0203 relative to EAPB0503. However, the terminal half-lives of the two parent compounds were similar, ~ 2 h. After intraperitoneal administration, systemic bioavailability was 22.7% for EAPB0203 and 35% for EAPB0503. The metabolic ratios, AUC metabolite/AUC parent drug, were higher after i.p. administration than after i.v. administration suggesting a hepatic first pass effect after i.p. administration. The terminal
half-lives of EAPB0202, EAPB0502 and EAPB0602 were similar to those of the parent drugs, while that of EAPB0603 was delayed compared with the elimination of EAPB0503.
Discussion

From the many structurally related compounds, analogs of imiquimod, that have been synthesized and tested to date, two compounds, EAPB0203 and EAPB0503, have emerged as the most promising based on their in vitro and in vivo activities on melanoma and T lymphomas (Moarbess et al., 2008a,b; Khier et al., 2010). Some data on the mechanism of action of these two compounds have been published (Morjaria S et al., 2006; Moarbess et al., 2008b). However, the specific cellular targets of these two compounds remain to be identified.

Drug-albumin interaction is a major challenge in distribution-metabolism-elimination prediction. The results from this study demonstrate that, over the concentration range 62.5 to 2000 µg/l, EAPB0203 and EAPB0503 are extensively bound to human plasma proteins and HSA at physiologic concentration of this protein (~98-99.5%). Thus, HSA could act as carriers of these compounds in human plasma. These values appear compatible with the ionization constants and lipophilicity of EAPB0203 and EAPB0503 (Table 1), showing that these drugs are acting like weak bases (Ermondi et al., 2004). The in vitro study also demonstrated an unusual concentration dependence of EAPB0203 and EAPB0503 binding in human plasma and HSA. We can see that the binding of EAPB0203 and EAPB0503 in HSA slightly increased with increasing drug concentration (Table 2). Similar results were obtained in human plasma. However, the difference was not statistically significant. One possible explanation for this behavior could be that the concentration of unbound drugs is limited by their low solubility, while the capacity of proteins to bind EAPB0203 and EAPB0503 is much larger. However, such variations did not exceed 2% and are within the range of precision authorized in bioanalysis. The binding status of the drugs and HSA were assessed in vitro by the Scatchard method. Obtained results could suggest the presence of positive cooperativity in which affinity between the unbound drug and the sites to which the drug is bound increases as
the drug binds to the binding sites. These results could also be explained by the presence of albumin aggregates. Indeed, Lin et al. (2000) have reported that the lyophilized protein exhibits a higher level of aggregation upon dissolution. Calculated $K_D$ values were similar for the two compounds: $0.26 \times 10^{-6}$ M for EAPB0203 and $0.23 \times 10^{-6}$ M for EAPB0503.

In this study, hepatic cytochrome P450-dependent EAPB0203 and EAPB0503 metabolisms have been extensively compared in microsomes representing four mammalian species including human, and qualitative and quantitative interspecies differences were observed. Quantitatively, rat and dog metabolized the compounds with a higher rate than mouse and human. A proposed scheme for the metabolism of EAPB0203 is shown in Fig. 1A. EAPB0203 was metabolized via two main metabolic pathways: first, $N$-demethylation leading to the formation of EAPB0202; second, arousing through a series of hydroxylation. Human microsomes have a greater capacity than rat, dog and mouse microsomes to $N$-demethylate EAPB0203. The cytotoxicity of EAPB0202 against A375 melanoma cancer cell line was similar to that of the parent compound (Moarbess et al., 2008a). Thus, this compound had a significant role in the antitumoral activity of EAPB0203. The second pathway appeared to be the most important in all studied species, forming one to two major hydroxylated metabolites of EAPB0203 (Table 3). Hydroxylated metabolites of EAPB0202 were detected to a lesser extent. EAPB0503 was metabolized via three main metabolic pathways as shown in Fig. 1B. From seven detected metabolites, three of them, EAPB0603, EAPB0502 and EAPB0602, were identified. In human, rat and dog microsomes, the main metabolite routes (accounting for at least 60% of total EAPB0503 disappearance) consisted in $N$- and $O$-demethylations, leading to the formation of EAPB0603, EAPB0502 and EAPB0602. EAPB0603 (formed by $O$-demethylation) and EAPB0502 (formed by $N$-demethylation) were as active as EAPB0503 on A375 human cancer cell line, while EAPB0602 was found less effective (52-fold according to its $IC_{50}$ value). Thus, EAPB0603 and EAPB0502 are significantly involved in
the global antimitotic activity of EAPB0503. EAPB0603 was one of the main metabolite in human and rat; EAPB0502 was one of the main metabolite in dog and mouse. Another pathway resulted in the formation of two EAPB0503 hydroxylated derivatives (M’4 and M’6) and their corresponding demethylated compounds. M’4 was detected in all hepatic microsomal incubates while M’6 was not detected in human and rat microsomes. The highest percentage of hydroxylated compounds (64%) was observed in mouse microsomes. Only rat microsomes generated the pattern of EAPB0503 metabolites reproducing that formed by human microsomes. Unfortunately, for the hydroxylated metabolites, the precise oxidation position could not be elucidated further in view of the mass spectrometry data available. The present study suggested that CYP3A isoform was the predominant enzyme responsible for N- and O-demethylations of EAPB0203 and EAPB0503 in human, rat, dog and mouse microsomes. Inhibition results indicated that other CYP isoforms were involved in the demethylation: CYP2A, CYP2C9, CYP2E1 and CYP2C8 in human; CYP2C9 and CYP2C8 in rat; and CYP2E1 in dog and mouse. CYP1A1/2 and CYP3A were found to be the principal enzymes generating hydroxylated metabolites.

The implementation of assays about the metabolic behavior, enabling reliable prediction of hepatic clearance in human of new chemical compounds, is a major component of lead optimization in drug discovery. Human liver microsomes are the most useful high-throughput tool for identifying high-clearance compounds. Thus, in this study, intrinsic clearance was calculated from substrate depletion approach and the nonspecific binding of the drugs to human microsomes was determined by ultracentrifugation. Indeed, for in vitro-in vivo scaling, correction with $f_u(microsomes)$ in the reaction mixture has been reported to be important (Obach, 1999). EAPB0203 and EAPB0503 were metabolized in human liver microsomes with in vitro unbound intrinsic clearances of 52.7 and 60.0 ml/min/g protein, respectively. The extrapolation of in vivo metabolic drug clearance from in vitro data was performed using the
most commonly used scaling factors (Carlile et al., 1997; Obach, 1999). By doing this, in vivo intrinsic clearances for unbound EAPB0203 and EAPB0503 were 47.1 and 54.0 ml/min/kg, respectively. From these values in combination with the well-stirred model, the hepatic plasma clearances were estimated to be 0.92 ml/min/kg for EAPB0203 and 0.74 ml/min/kg for EAPB0503.

Most of the metabolites identified in vitro in rat hepatic microsomes were also found in vivo. These results confirm in vitro studies. In absence of standards, hydroxylated metabolites have not been quantified in rat plasma. Except for EAPB0603, the terminal half-lives of the metabolites were similar to those of the parent drugs. In this case, the real metabolite half-life could not be estimated. EAPB0603 was excreted much more slowly than the parent compound. The delayed elimination of this metabolite may involve a rate driven by the elimination process. Total systemic clearances of EAPB0203 and EAPB0503 (3.2 and 2.2 l/h/kg, respectively) were notably less than the typical cardiac plasma output in rat (9.6 l/h/kg, Davies and Morris, 1993). Plasma clearance of EAPB0503 was comparable to the average hepatic and renal plasma flows in rat (1.8 l/h/kg, Davies and Morris, 1993). The steady-state volumes of distribution of 4.3 l/kg for EAPB0203 and 2.5 l/kg for EAPB0503 were well in excess of the total body water volume in rat (0.67 l/kg, Davies and Morris, 1993), suggesting that tissue binding was extensive and greater than plasma binding. The differences in the volume of distribution between the two drugs were in agreement with the higher lipophilicity of EAPB0203 (logP, 4.91) compared to EAPB0503 (logP, 3.45). After intraperitoneal administration a significant hepatic first-pass effect was observed. The absolute bioavailability of the two compounds did not exceed 40%. These results could be due to low intraperitoneal absorption, large first-pass metabolism (formation of inactive or poorly active metabolites (as EAPB0602)), or a combination of both.
In conclusion, this study generated substantial information about the distribution, metabolism and elimination of two compounds, EAPB0203 and EAPB0503, belonging to a new series of promising anticancer drugs: the imidazo[1,2-α]quinoxalines.
References


EMA. Guideline on studies to evaluate the metabolism and residue kinetics of veterinary drugs in food-producing animals: comparative metabolism studies in laboratory animals. Ref. EMEA/CVMP/VICH/463104/2009-CONSULTATION


chromatography-electrospray mass spectrometry. Application to a Pharmacokinetic Study.


Footnotes

Part of this work was supported by the Ligue Régionale de Lutte contre le Cancer [Grant 5FI8308INNG]

† Sonia Khier and Florence Gattacceca contributed equally to this work.
Legends for Figures

FIG. 1. Chemical structures of EAPB0203 (a) and EAPB0503 (b) and proposed biotransformation pathways

*OH could be positioned on the tricyclic cycle

FIG. 2. Chemical inhibition of EAPB0202 formation by selective P450 inhibitors.
KET, ketoconazole; QUE, quercertin; SPZ, sulfaphenazole; COU, coumarine; QUI, quinidine; DDTC, diethyldithiocarbamate

Effects of $\alpha$-naphthoflavone are not presented

FIG. 3. Chemical inhibition of EAPB0603 and EAPB0502 formations by selective P450 inhibitors.
KET, ketoconazole; QUE, quercertin; SPZ, sulfaphenazole; COU, coumarine; QUI, quinidine; DDC, diethyldithiocarbamate

Effects of $\alpha$-naphthoflavone are not presented

FIG. 4. Plots by Scatchard method of the binding between human serum albumin and drugs.
Insert: Hill plot. (A) EAPB0203; (B) EAPB0503

FIG. 5. Representative LC-MS chromatograms of EAPB0203 metabolites formed by human hepatic microsomes (SIM mode)

FIG. 6. Representative LC-MS chromatograms of EAPB0503 metabolites formed by mouse and dog hepatic microsomes (SIM mode)

FIG. 7. Plasma concentration-time curves after single 5 mg/kg intravenous and intraperitoneal doses of EAPB0503 to male Sprague-Dawlay rats (mean ± SD; three rats sampled per sampling time).

•, EAPB0503; ■, EAPB0603; ▲, EAPB0502, ▼EAPB0602
TABLE 1

Physicochemical properties of the standards

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Melting point</th>
<th>pKa$^a$</th>
<th>Log P$^b$</th>
<th>Physicochemical properties</th>
<th>Solubility</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAPB0203</td>
<td>150°C</td>
<td>5.61 ± 0.4</td>
<td>4.91 ± 1.42</td>
<td>Yellow solid</td>
<td>Low aqueous solubility; good solubility in DMSO (~ 20 mg/ml)</td>
</tr>
<tr>
<td>EAPB0202</td>
<td>186°C</td>
<td>5.13 ± 0.4</td>
<td>4.59 ± 1.42</td>
<td>Yellow solid</td>
<td></td>
</tr>
<tr>
<td>EAPB0503</td>
<td>168°C</td>
<td>5.10 ± 0.4</td>
<td>3.45 ± 1.43</td>
<td>White solid</td>
<td></td>
</tr>
<tr>
<td>EAPB0502</td>
<td>258°C</td>
<td>4.62 ± 0.4</td>
<td>3.52 ± 1.43</td>
<td>White solid</td>
<td></td>
</tr>
<tr>
<td>EAPB0603</td>
<td>&gt;260°C</td>
<td>5.14 ± 0.4</td>
<td>2.94 ± 1.43</td>
<td>White solid</td>
<td></td>
</tr>
<tr>
<td>EAPB0602$^b$</td>
<td>&gt; 260°C</td>
<td>9.44 ± 0.1</td>
<td>3.00 ± 1.42</td>
<td>White solid</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.66 ± 0.4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ calculated using the ACD/Chem Sketch software; $^b$ Amphoteric compound
**TABLE 2**

*Extent of protein binding of EAPB0203 and EAPB0503 in human serum albumin solution*

(40 g/l)

<table>
<thead>
<tr>
<th>Drug concentration (µg/l)</th>
<th>EAPB0203</th>
<th>EAPB0503</th>
</tr>
</thead>
<tbody>
<tr>
<td>62.5</td>
<td>97.6 ± 0.2</td>
<td>98.8 ± 0.2</td>
</tr>
<tr>
<td>100</td>
<td>98.0 ± 0.3</td>
<td>99.0 ± 0.3</td>
</tr>
<tr>
<td>250</td>
<td>98.6 ± 0.05</td>
<td>99.2 ± 0.3</td>
</tr>
<tr>
<td>500</td>
<td>99.4 ± 0.01</td>
<td>99.5 ± 0.3</td>
</tr>
<tr>
<td>1000</td>
<td>99.4 ± 0.2</td>
<td>99.5 ± 0.3</td>
</tr>
</tbody>
</table>

n, number of replicates
TABLE 3

Evaluation of the relative amounts of EAPB0203 and EAPB0503 metabolites based on responses observed in animal and human microsomal incubations

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Human</th>
<th>Rat</th>
<th>Mouse</th>
<th>Dog</th>
<th>Metabolites</th>
<th>Human</th>
<th>Rat</th>
<th>Mouse</th>
<th>Dog</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>11</td>
<td>8</td>
<td>+</td>
<td>20</td>
<td>M'1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>11</td>
</tr>
<tr>
<td>(r.t., 3.6 min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(r.t., 3.0 min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M2</td>
<td>20</td>
<td>24</td>
<td>26</td>
<td>51</td>
<td>M'2, EAPB0602</td>
<td>17</td>
<td>8</td>
<td>+</td>
<td>10</td>
</tr>
<tr>
<td>(r.t., 4.0 min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(r.t., 3.2 min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M3</td>
<td>5</td>
<td>12</td>
<td>+</td>
<td>-</td>
<td>M'3</td>
<td>-</td>
<td>-</td>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td>(r.t., 4.1 min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(r.t., 3.5 min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M4</td>
<td>+</td>
<td>+</td>
<td>11</td>
<td>-</td>
<td>M'4</td>
<td>19</td>
<td>39</td>
<td>28</td>
<td>19</td>
</tr>
<tr>
<td>(r.t., 4.2 min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(r.t., 3.7 min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M5</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>M'5, EAPB0603</td>
<td>39</td>
<td>33</td>
<td>8</td>
<td>+</td>
</tr>
<tr>
<td>(r.t., 4.5 min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(r.t., 4.0 min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M6</td>
<td>+</td>
<td>32</td>
<td>10</td>
<td>-</td>
<td>M'6</td>
<td>-</td>
<td>-</td>
<td>31</td>
<td>3</td>
</tr>
<tr>
<td>(r.t., 4.5 min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(r.t., 4.1 min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M7</td>
<td>15</td>
<td>10</td>
<td>31</td>
<td>11</td>
<td>M'7, EAPB0502</td>
<td>24</td>
<td>19</td>
<td>27</td>
<td>56</td>
</tr>
<tr>
<td>(r.t., 4.8 min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(r.t., 5.3 min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M8, EAPB0202</td>
<td>39</td>
<td>13</td>
<td>22</td>
<td>14</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(r.t., 5.8 min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

r.t., retention time; not detected, (-); < 3%, (+).
### TABLE 4
Pharmacokinetic parameters of EAPB0203, EAPB0503 and their metabolites following intravenous (i.v.) and intraperitoneal (i.p.) administrations in rats.

<table>
<thead>
<tr>
<th></th>
<th>EAPB0203 i.v. / i.p.</th>
<th>EAPB0503 i.v. / i.p.</th>
<th>EAPB0603 i.v. / i.p.</th>
<th>EAPB0502 i.v. / i.p.</th>
<th>EAPB0602 i.v. / i.p.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL (l/h/kg)</td>
<td>3.2</td>
<td>2.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$V_{ss}$ (l/kg)</td>
<td>4.3</td>
<td>2.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>2.4 / 1.5</td>
<td>2.0 / 0.91</td>
<td>1.76 / 1.95</td>
<td>4.7 / 1.6</td>
<td>1.0 / 0.75</td>
</tr>
<tr>
<td>AUC, mg x h/l</td>
<td>0.78 / 0.15</td>
<td>0.029/0.032</td>
<td>2.31 / 0.59</td>
<td>0.44/0.25</td>
<td>0.029 / 0.12</td>
</tr>
<tr>
<td>$\mu$M x h</td>
<td>2.58 / 0.50</td>
<td>0.094 / 0.11</td>
<td>7.6 / 1.94</td>
<td>1.52 / 0.86</td>
<td>0.1 / 0.41</td>
</tr>
<tr>
<td>Metabolic ratio$^a$</td>
<td>-</td>
<td>0.036 / 0.22</td>
<td>-</td>
<td>0.20 / 0.44</td>
<td>0.013 / 0.21</td>
</tr>
</tbody>
</table>

$^a$AUC metabolite/AUC parent drug

CL, total plasma clearance; $V_{ss}$, steady-state volume of distribution; $t_{1/2}$, half-life of the terminal part of the curve; AUC, area under the plasma concentration-time curve extrapolated to infinity.
Fig. 1A

EAPB203, MW 302

N-demethylation

M8
EAPB202, MW 288

Hydroxylation

M2, M4, MW 318

M6, M7, MW 318

M1, MW 304

M3, M5, MW 304
Fig. 1B

EAPB0503, MW 304

EAPB0502, MW 290

EAPB0603, MW 290

EAPB0602, MW 276

M'1, MW 307

M'4, MW 320

M'5

M'7

M'2

M'3

M'6, MW 320

Demethylation

Hydroxylation

N-demethylation

Demethylation

Hydroxylation

EAPB0502, MW 290

EAPB0603, MW 290

M'7

M'5

M'2

M'3

M'6, MW 320

M'1, MW 307
Fig. 3

Human

Percent inhibition of EAPB0603 and EAPB0502 formation (Mean +/- SD; n=3)

DDC 1000µM
DDC 500µM
QUI 5µM
QUI 1µM
COU 50 µM
COU 10 µM
SPZ 40 µM
SPZ 20 µM
QUE 10 µM
QUE 5µM
KET 2 µM
KET 0.2 µM

Rat

Percent inhibition of EAPB0603 and EAPB0502 formation (Mean +/- SD; n=3)

DDC 1000µM
DDC 500µM
COU 50 µM
COU 10 µM
SPZ 40 µM
SPZ 20 µM
QUE 10 µM
QUE 5µM
KET 2 µM
KET 0.2 µM

Dog

Percent inhibition of EAPB0603 and EAPB0503 formation (Mean +/- SD; n=3)

DDC 1000µM
DDC 500µM
KET 2 µM
KET 0.2 µM

Mouse

Percent inhibition of EAPB0603 and EAPB0502 formation (Mean +/- SD; n=3)

DDC 1000µM
DDC 500µM
KET 2 µM
KET 0.2 µM
Fig. 4

A

\[ \frac{r}{[M]} \]

\[ \ln \left[ \frac{r}{1-r} \right] \]

\[ \ln [M] \]

B

\[ \frac{r}{[M]} \]

\[ \ln \left[ \frac{r}{1-r} \right] \]

\[ \ln [M] \]
Fig. 5

**Human microsomes**

- **m/z 303**
- **m/z 289**
- **m/z 319**
- **m/z 305**

Counts vs. time graph showing various peaks labeled as M1, M2, M3, M4, M5, M6, M7, and Endogenous peak.
Fig. 6

Mouse microsomes

Counts

$m/z$ 305

Counts

$m/z$ 291

Counts

$m/z$ 277
m/z 321  Mouse microsomes

m/z 307  Dog microsomes

Endogenous compound
Fig. 7

Intravenous administration

Intraperitoneal administration.

Concentrations, µg/l vs. Time (hours)