Biochemical and Biophysical Research Communications xxx (2016) 1–6

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Evaluation of the *in vitro* and *in vivo* metabolic pathway and cytochrome P450 inhibition/induction profile of Huperzine A

Ping-ping Lin, Xue-ning Li, Fei Yuan, Wei-li Chen, Meng-jie Yang, Hong-rong Xu*

Department of Clinical Pharmacology, Zhongshan Hospital, Fudan University, Shanghai, 200032, China

ARTICLE INFO

Article history:
Received 8 October 2016
Accepted 14 October 2016
Available online xxx

Keywords:
Huperzine A
Metabolic pathway
CYP inhibition
CYP induction
Drug interaction

ABSTRACT

Huperzine A (HupA), one of the reversible and selective acetylcholinesterase inhibitors derived from Chinese herb *Huperzia Serrata*, possesses affirmative action of ameliorating cognitive dysfunction of Alzheimer's disease. Up to now, the effects of HupA on human cytochrome P450s (CYPs) have not been fully elucidated. The purpose of the present study was to clarify the metabolic pathway of HupA *in vitro* and *in vivo*, and to evaluate the CYPs inhibition/induction profile of HupA *in vitro*. The catalytic activity of CYP enzymes (CYP1A2, 2A6, 2C9, 2C19, 2D6, 2E1 and 3A4) was measured by the quantification of specific enzyme substrates using validated liquid chromatography-tandem mass spectrometry (LC/MS/MS) methods. The *in vivo* metabolic pathway evaluation was performed in an open, single-dose pharmacokinetic study of HupA in fourteen elderly subjects, with urine collecting at certain intervals. In human liver microsomes, HupA (10 ng/mL) was not metabolized within 90 min, and it showed negligible inhibition against these CYP isoforms within 0.2–100 ng/mL. In human liver hepatocytes, the activities of CYP1A2 and CYP3A4 were not significantly altered when incubated at 2 or 20 ng/mL of HupA. After oral administration of 0.1 mg HupA, the total proportion of HupA excreted through urine was relatively high, accounting to 35± 9% at the limited time period of 48 h. These results suggest that HupA is substantially excreted by kidney unchanged rather than metabolized by human liver, and is unlikely to cause clinically relevant drug-drug interaction (DDI) when co-administrated with drugs that are metabolized by CYP isoenzyme system.

1. Introduction

Huperzine A (HupA), a well-documented agent naturally extracted from Chinese herb *Huperzia Serrata*, is a reversible and selective inhibitor of acetylcholinesterase inhibitors (AChEIs). It has been reported in previous studies that HupA can reverse or attenuate cognitive function, improve behavioral disturbance and slow the progression course of Alzheimer's disease (AD) in animal models as well as in AD patients [1–4]. A large number of clinical trials suggest that HupA demonstrates the favorable safety profiles at a relatively wide dose range and the potent efficacy in AD patients [5–9]. Additionally, compared with other AChEIs, HupA has shown to have a higher oral bioavailability, better potential and longer-lasting effect [10–12] that, if definitely confirmed in clinical practice, would offer another supplementary treatment for AD patients. For a natural drug to be taken by the elderly, who commonly have several chronic disorders, the metabolic pathway, the effects of HupA on the activities of CYP enzymes and its ability to cause drug-drug interactions (DDIs) should be known. A preclinical study [13] has shown that the metabolism of HupA was mediated mainly by CYP1A2 in rat liver microsomes (RLMs) at the therapeutic concentration. However, limited information is available about the inhibition and induction effect of HupA on human liver microsomes (HLMs) to date. Although the inhibition and induction of CYPs activities by HupA have been evaluated in rats [13,14], it is still difficult to extrapolate the results from rats to human beings because of the extensive existence of species diversity [15,16]. Besides, the existing pharmacokinetic parameters of HupA used in the drug instruction were inadequate, as it is barely obtained from animal models, the reference value of which was limited clinically. Therefore, it is imperative to evaluate the effects of HupA on CYPs activities to indicate possible clinically relevant DDI when co-administrated with other drugs.

The purpose of this study was to investigate the metabolic stability of HupA in HLMs, as well as evaluate the inhibition and induction effects on human liver CYP isoenzymes. In addition, we collected urine of elder volunteers administered with HupA to

* Corresponding author.
E-mail address: xu.hongrong@zs-hospital.sh.cn (H.-r. Xu).

http://dx.doi.org/10.1016/j.bbrc.2016.10.039
0006-291X/

evaluate the excretion ratio from kidney and to further verify the metabolic pathway of HupA. Our findings may provide helpful information for understanding the metabolic nature of HupA. It was also expected that potential drug interactions might be predicted to ensure the safety medication of HupA for AD patients.

2. Materials and methods

2.1. Chemicals and reagents

Huperzine A reference standard (purity, >99%) was kindly provided by the Chinese Academy of Sciences Shanghai Institute of Materia Medica (Shanghai, China). Disodium hydrogen phosphate and sodium dihydrogen phosphate (analytical reagent) were obtained from Sinopharm Chemical Reagent Co.,Ltd (Shanghai, China). Acetonitrile was obtained from Scharlau chemie S.A and methanol was purchased from Merck. KGaA (Darmstadt, Germany). Ultra-pure water (resistance >18.2 mΩ) was prepared by the Millipore Milli-Q purification system.

For determination of CYP activities, phenacetin (1A2), coumarin (2A6), diclofenac (2C9), S-mephenytoin (2C19), dextromethorphan (2D6), chlorzoxazone (2E1) as well as respective metabolites (acetaminophen, 7-hydroxycoumarin, 4'-hydroxydiclofenac, 4'-hydroxymephenytoin, dextrorphan, and 6-hydroxychlorzoxazone) were purchased from Sigma-Aldrich (St. Louis, MO, USA); Testosterone (3A4) was from Sigma Fluka Company, and its metabolite (6β-hydroxytestosterone) was obtained from Cerilliant Corporation (Texas, United States). Osalmid (IS) was purchased from Shanghai JinYi Fine Chemical Co., Ltd (Shanghai, China). Reserpine (IS) and chloramphenicol (IS) were purchased from Alfa Aesar (Ward Hill, United States). β-NADPH was supplied from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Human liver microsomes and human primary hepatocytes preparation

The mixture of HLMs and hepatocytes was obtained from the abandoned livers or hepatic lobes in transplantations, under the approval of the Ethic Committee of Xuhui central hospital, shanghai, China. Human microsomes were prepared through differential centrifugation and the protein quantitation was performed by the method of modified Lowry [17]. The prepared HLMs were then stored at −80 °C. Human primary hepatocytes were first isolated by collagenase digestion, and then cultured at 37 °C in a humidified atmosphere with 5% CO_2. A terminal density of 7×10^5 cells/mL was obtained with viability and attachment efficiency above 85%. The prepared hepatocytes were frozen in liquid nitrogen pending using.

2.3. Metabolic stability assessment in HLMs

HLMs (0.5 mg/mL) were incubated with 10 ng/mL of HupA (treatment group) or 0.5 μmol/L of testosterone (positive control), and β-NADPH (1 mmol/L, positive control and treatment group) or phosphate buffer (50 mmol/L, vehicle control) at 37 °C in a humidified atmosphere with 5% CO_2. In treatment group, samples (100 μL) were collected at 0, 15, 30, 45, 60, 90 min; in vehicle control and positive control group, the samples were collected at 0 and 60 min. A volume of 400 μL precooled methanol (containing 160 ng/mL osalmid as the internal standard) was added to terminate the reaction. Collected samples were then centrifuged at 16000 g for 5 min at 4 °C. Supernatants were subsequently collected and stored at −80 °C prior to LC/MS/MS analysis (described in the section of "LC/MS/MS assay"). All experiments were performed in triplicate.

The metabolic stability of HupA and testosterone was calculated as the percentage of the remaining concentration *versus* the initial concentration of the parent compound. Metabolic stability was estimated according to the following formula: percent remaining (%) = Parent of T_x (Concentration or AreaRatio)/Parent of T_0 (Concentration or AreaRatio) × 100%. The half-life of HupA was calculated as $T_{1/2} = -0.693/K$, of which "K" was the rate constant of Log [the concentration of parent drug]/incubation time.

2.4. Inhibition studies

The incubation mixtures for direct inhibitions were in a final volume of 150 μL and consisted of 0.9 mg/mL HLMs, 100 mM phosphate buffer saline, specific CYP isoenzyme substrates in the absence or presence of HupA at various concentrations. HupA was diluted with DMSO to prepare a series of stock solutions, and subsequently diluted with 0.1 mmol/L PBS to prepare a series of standard solutions. A volume of 50 μL of the standard solution of HupA or PBS buffer (vehicle control) was spiked into 50 μL HLMs (0.9 mg/mL) for pre-incubation at 37 °C for 15 min. The reaction was subsequently initiated by the addition of 25 μL of classical substrates of CYP isoenzymes and 25 μL β-NADPH to make a final volume of 150 μL, with the terminal HupA concentration of 0.2, 1, 2, 10, 20 and 100 ng/mL in the reaction mixture. After further incubation for 30 min under the same conditions, the reaction was terminated by adding 400 μL precooled methanol containing the corresponding internal standard (IS). Osalmid was used as the IS, except for CYP1A2 and CYP2E1 assay, in which, reserpine and chloramphenicol were used as IS, respectively. The reaction mixtures were then centrifuged at 16000 g for 5 min to collect the supernatants, which were first dried by nitrogen and then analyzed for the metabolites concentrations according to the method described in the section of "LC/MS/MS assay". All experiments were performed in triplicate, and its mean value was taken as the experimental result. The specific substrate concentrations, specific conversions and metabolites for each CYP isoenzyme were showed in Table 1.

The inhibition effect of HupA on CYP isoenzymes was determined by the change of the metabolites of each specific substrate. Relative enzyme activity was estimated as the percentage of metabolite concentration in the treatment group *versus* the corresponding value in the vehicle control group. The half maximal inhibitory concentration (IC_{50}) by HupA was estimated from nonlinear regression of inhibition plots where relative activity of CYP isoenzyme (Y value, the normalized response, 100% down to 0%) plotted against the logarithm of HupA concentration (X value) according to the following equation: $Y = 100/[1 + 10^{\wedge}(LogIC_{50} - X) * HillSlope]$ (GraphPad Prism version 6.0).

2.5. Induction studies

Human primary hepatocytes were respectively incubated with HupA, omeprazole (CYP1A2 inducer), rifampcin (CYP3A4 inducer) or PBS buffer (vehicle control) for 3 days at 37 °C in an atmosphere of 5% CO_2, after which phenacetin (CYP1A2 substrate) or testosterone (CYP3A4 substrate) was added for another incubation of 60 min. Final concentrations of the tested compound and substrates in the reaction mixtures were as follows: HupA (2 and 20 ng/mL); omeprazole (100 μM); rifampcin (25 μM); phenacetin (50 μM); testosterone (100 μM). After further incubation, the reaction was terminated by adding precooled methanol containing reserpine or osalmid as the IS. Supernatants were then collected and analyzed for metabolite concentrations (described in the section of "LC/MS/MS assay"). All experiments were performed in triplicate.

The induction effect of HupA on CYP isoenzymes was determined by the variation of the metabolites of each specific substrate.

Please cite this article in press as: P.-p. Lin, et al., Evaluation of the *in vitro* and *in vivo* metabolic pathway and cytochrome P450 inhibition/induction profile of Huperzine A, Biochemical and Biophysical Research Communications (2016), http://dx.doi.org/10.1016/j.bbrc.2016.10.039