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Induction of human CYP3A4 by huperzine A, ligustrazine and oridonin through pregnane X receptor-mediated pathways

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The pregnane X receptor (PXR) is a key regulator of CYP3A4, which is involved in catalyzing the metabolic conversion of a number of endogenous substrates. In this study, we screened 22 compounds isolated from traditional Chinese herbal medicines using luciferase reporter gene assays for inspecting their capabilities in inducing PXR-mediated transactivation of CYP3A4 expression. In addition, the mRNA and protein expressions of CYP3A4 and PXR as well as the enzymatic activites of CYP3A4 were analyzed by real-time PCR, Western blot analysis and UPLC-MS/MS-based metabolite assay in LS174T cells. Huperzine A, ligustrazine and oridonin were identified to be the inducers of CYP3A4. These compounds induced the CYP3A4 reporter luciferase activity, and up-regulated CYP3A4 mRNA and protein levels significantly. Besides, huperzine A, ligustrazine and oridonin significantly up-regulated enzymatic activities of CYP3A4. However, the three compounds showed no effects on PXR mRNA and protein expression. To our knowledge, it is the first identification of these three compounds as PXR activators to induce CYP3A4. These results indicate that huperzine A, ligustrazine and oridonin induced CYP3A4 expression and activation *via* PXR dependent pathways, and might contribute to drug-drug interactions.

1. Introduction

Herbal medicines are widely used as dietary supplements or therapeutic drugs, which are often co-administered with other clinical drugs (Goldman 2001; Bent and Ko 2004). Many studies have demonstrated that herbal medicines can exert clinical herb-drug interactions by induction or inhibition of drug-metabolizing enzymes (Chen et al. 2012; Hermann and von Richter 2012; Posadzki et al. 2013).

The oxidative cytochrome P450 (CYP) is a superfamily consisting of a series of hemethiolate-containing proteins involved in catalyzing the metabolic conversions of a number of endogenous substrates. CYP3A4 accounts for up to 50% of the total hepatic CYP proteins (Li et al. 1995). Many herbal medicines are known to induce or inhibit human CYP3A4, such as St. John's wort (Di et al. 2008; Peltoniemi et al. 2012), *Ginkgo biloba* (Yale and Glurich 2005), *Schisandra chinensis* (Iwata et al. 2004), *Sophora flavescent* (Wang et al. 2010) and *Glycyrrhiza uralensis* (Hou et al. 2012).

In recent years, the transcriptional regulation of CYP3A4 has been widely studied, and research shows that the induction of CYP3A4 is often governed by activation of several nuclear receptors (Huang et al. 2011; Sachar and Ma 2013). As a member of the nuclear receptor family, PXR can be activated and then form a heterodimer with the retinoid X receptor α. This heterodimer then acts as a co-activator, and subsequently regulates the CYP3A expression and activation by directly binding to the everted or direct repeats of (A/G)G(G/T)TCA spaced by six(ER6) or three(DR3) base pairs, respectively (Bertilsson et al. 1998; Goodwin et al. 1999; Klaassen and Slitt 2005). Many compounds have been reported to regulate CYP3A4 through the PXR-mediated pathways, including ginkgolide A (Lau et al. 2010), ligustilide (Yu et al. 2011), byakangelicin (Yang et al. 2011) and baicalin (Li et al. 2010), but data on other herbal compounds are still limited.

In the present study, we evaluated the induction effects of 22 commonly used herbal compounds (as shown in Table 1) on PXR-mediated CYP3A4 expression and activation. Since these compounds are usually co-administered with other clinical drugs, the present study may provide some information about herb-drug interactions.

2. Investigations and results

2.1. Effects of herbal compounds on PXR-mediated activation of CYP3A4 in HepG-2 cells

Reporter gene assays were performed to determine whether the 22 herbal compounds tested could activate the PXR-mediated pathways in the induction of CYP3A4. As shown in Fig. 1, CYP3A4 luciferase activity was strongly induced by rifampin, which acted as the positive control (12.27-fold at 10 μM). Compared to the control group, huperzine A, ligustrazine and oridonin significantly increased the luciferase of CYP3A4 reporter gene. Meanwhile, other herbal compounds did not influence the CYP3A4 reporter gene expression. Since the CYP3A4 reporter gene is constructed *via* PXR, the previous results imply that the effects of huperzine A, ligustrazine and oridonin be

Table 1: Herbal compounds investigated in this study

Name of herbal compounds	Herbal source	Molecular weight
Andrographolide	*Andrographitis paniculata*	350.44
Apigenin	*Apium graveolens*	270.24
Breviscapine	*Erigeron breviscapus*	908.72
Chrysin	*Oroxylum indicum*	254.23
Echinacoside	*Cistanche deserticola*	786.72
Evodiamine	*Tetradium ruticarpum*	303.36
Ferulic acid	*Ligusticum chuanxiong*	194.18
Ginsenoside Rc	*Panax notoginseng*	1079.27
Ginsenoside Rd	*Panax notoginseng*	947.15
Ginsenoside Rf	*Panax notoginseng*	801.01
Huperzine A	*Erigeron annuus*	242.32
Jasminoidin	*Gardenia jasminoides*	388.37
Ligustrazine	*Ligusticum chuanxiong*	136.19
Oleanolic acid	*Olea europaea*	456.7
Oridonin	*Rabdosia rubescens*	364.42
Palmatine	*Fibraurea recisa*	352.41
Peoniflorin	*Paeonia lactiflora*	480.47
Polydatin	*Polygonum cuspidatum Sieb*	390.39
Protopanaxatriol	*Panax notoginseng*	476.73
Resveratrol	*Polygonum cuspidatum Sieb*	228.24
Rutin	*Ruta graveolens*	610.52
Tectoridin	*Belamcanda chinensis*	300.26

attained through PXR-mediated pathways. Further studies were then undertaken to test these three compounds at various concentrations. Fig. 2 illustrates the effects of huperzine A, ligustrazine and oridonin on *CYP3A4* luciferase reporter gene expression. Compared to the control group, huperzine A and ligustrazine significantly induced luciferase activity to 2.71- and 2.56-fold at the maximum dosage of 20 μM, respectively ($p < 0.05$). Oridonin at concentrations of 10 and 20 μM significantly induced *CYP3A4* luciferase reporter activity to 2.88- and 4.12-fold ($p < 0.05$, $p < 0.01$).

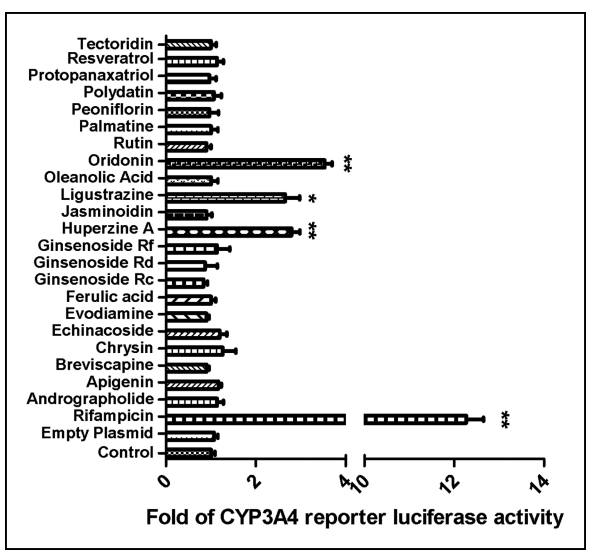

Fig. 1: CYP3A4 reporter gene construct transactivation by herbal compounds in transiently transfected HepG2 cells. HepG2 cells were transiently transfected with PXR expression plasmid and the CYP3A4 reporter plasmid, empty vectors were transfected as the empty plasmid control. Six hours later, the cells were treated with the tested compounds for 24 h. Luciferase activity was determined and normalized against *Renilla* luciferase activity. The results are shown as the mean fold increase in activity compared with control from more than three independent experiments. All values are presented as mean ± S.D.; $*p < 0.05$, $**p < 0.01$.

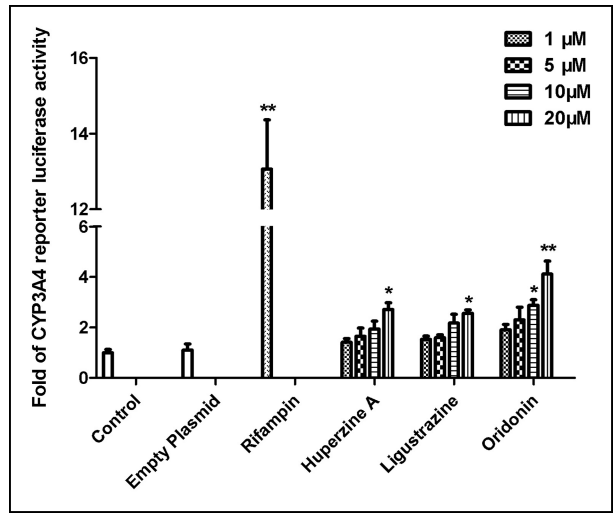

Fig. 2: CYP3A4 reporter gene construct transactivation by Huperzine A, Ligustrazine and Oridonin in transiently transfected HepG2 cells. HepG2 cells were transiently transfected with PXR expression plasmid and the CYP3A4 reporter plasmid, empty vectors were transfected as the empty plasmid control. Six hours later, the cells were treated with vehicle control (0.1% DMSO); rifampin (10 μM); or huperzine A, ligustrazine and oridonin at various concentrations (1, 5,10 and 20 μM), respectively. Following 24 h treatment, luciferase activity was determined and normalized against *Renilla* luciferase activity.The results are shown as the mean fold increase in activity compare with control from more than three independent experiments. All values are presented as mean ± S.D.; $*p < 0.05$, $**p < 0.01$.

2.2. Effects of huperzine A, ligustrazine and oridonin on the expression of CYP3A4 and PXR mRNA in LS174T cells

As presented in Fig. 3A, rifampin, as a positive control, significantly increased the level of *CYP3A4* mRNA compared with control (5.04-fold at 10 μM, $p < 0.01$). huperzine A and ligustrazine significantly induce *CYP3A4* mRNA expression to 2.45- and 1.98-fold at 20 μM, respectively($p < 0.05$, $p < 0.01$). Oridonin at concentrations of 5, 10 and 20 μM significantly induced *CYP3A4* mRNA expression to 2.01-, 2.22- and 3.38-fold, respectively ($p < 0.05$, $p < 0.01$). In Fig. 3B, the results indicated that huperzine A, ligustrazine and oridonin up-regulate the mRNA level of CYP3A4 in a dose-dependent manner, with the maximal induction observed at the concentration of 20 μM. However, in our study, no marked changes on *PXR* mRNA have been observed in LS174T cells after treatment with the test compounds.

2.3. Effects of huperzine A, ligustrazine and oridonin on the expression of CYP3A4 and PXR protein in LS174T cells

The results are shown in Fig. 4, rifampin, the positive control, displayed the greatest CYP3A4 protein induction compared with DMSO control. Consistent with the results of mRNA expression, huperzine A, ligustrazine and oridonin achieved 2.46-, 2.05-, and 2.74-fold induction of the CYP3A4 protein levels at the concentration of 20 μM, respectively ($p < 0.05$, $p < 0.01$). However, the levels of PXR protein did not show any significantly induction after huperzine A, ligustrazine and oridonin treatments.

2.4. Effects of huperzine A, ligustrazine and oridonin on the activity of CYP3A4 in LS174T cells

The functions of CYP3A4 in LS174T cells were then investigated by determination of specific CYP3A4-mediated midazolam hydroxylation activity. Consistent with the changes

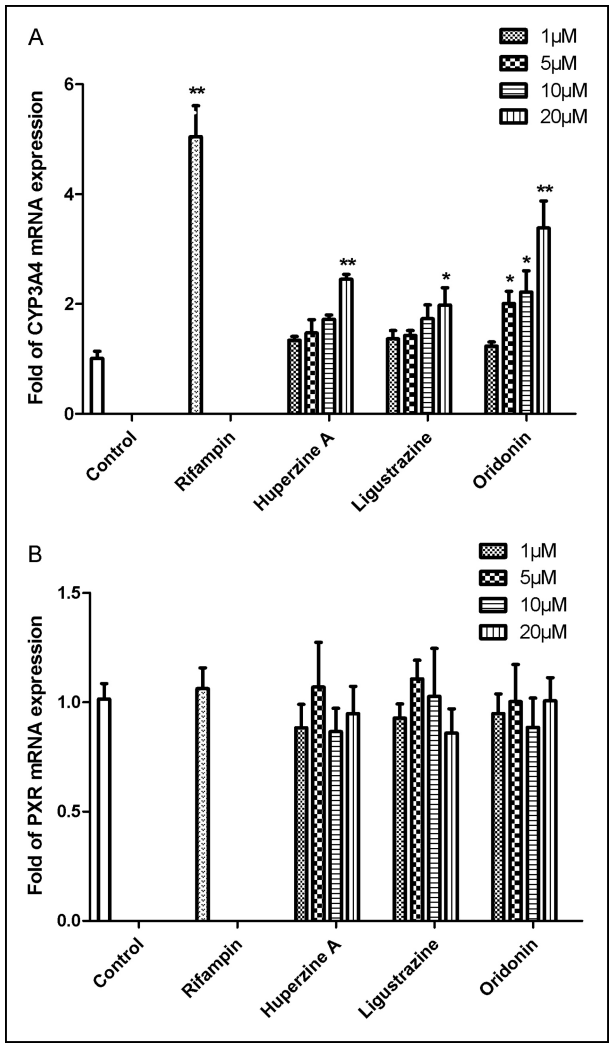

Fig. 3: Effect of huperzine A, ligustrazine and oridonin on the expression of CYP3A4 mRNA and PXR mRNA in LS174T cells. LS174T cells were treated with vehicle control (0.1% DMSO), rifampin (10 μM), or huperzine A, ligustrazine and oridonin at various concentrations (1, 5,10 and 20 μM) for 48 h.Total RNA was isolated, reversely transcribed and analyzed by real-time PCR. **(A)**The effects of the tested compounds on mRNA expression for CYP3A4 was normalized against *β-actin* and relative folds over vehicle control (0.1% DMSO). **(B)**The effect of the tested compounds on mRNA expression for PXR was normalized against *β-actin* and relative folds over vehicle control.The data are presented as mean ± S.D. of three experiments; $*p < 0.05$, $**p < 0.01$.

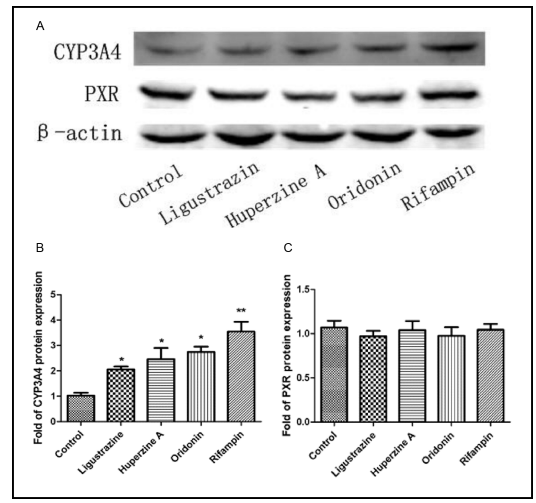

Fig. 4: Effect of huperzine A, ligustrazine and oridonin on the expression of CYP3A4 and PXR protein in LS174T cells. LS174T cells were pretreated with vehicle control(0.1% DMSO), rifampin(10 μM).or huperzine A, ligustrazine and oridonin at 20 μM for 72 h. The cell homogenates were subject to western blot(**A**). The data are expressed as relative folds over vehicle control(**B,C**). The results are expressed as relative folds of activity to vehicle control. All data are presented as mean ± S.D. of three experiments; $*p < 0.05$, $**p < 0.01$.

of CYP3A4 mRNA and protein expression levels, CYP3A4 activity was increased in LS174T cells after huperzine A, ligustrazine and oridonin treatments. As shown in Fig. 5, compared with the control, huperzine A, ligustrazine and oridonin significantly induced the enzymatic activity of CYP3A4 to 1.65, 1.45- and 1.95-fold, respectively ($p < 0.05$).

3. Discussion

As herbal medicines or supplements are widely used, the safety and efficacy of herb-drug combinations have received increasing attentions. The difference in expressions and activities of CYP3A4 then lead to different pharmacokinetic properties of CYP3A4 substrate drugs, and may influence the therapeutic responses or toxicity of these drugs. PXR is the dominant activator of CYP3A4 transcription. Many xenobiotics have been reported to regulate the transcription of CYP3A4 gene through PXR-mediated pathways, such as rifampin (Savas et al. 2000; Tolson and Wang 2010), polycyclic aromatic hydrocarbons (Kumagai et al. 2012) and diethylstilbestrol (Kuzbari et al. 2013). Although a large number of studies have reported the effects of xenobiotics like synthetic drugs on CYP3A4, data on the effects of herbal compounds on this enzyme and the underlying mechanism are still limited.

We thus examined 22 commonly used herbal compounds for their capabilities of activating PXR-mediated CYP3A4 expression using a luciferase reporter gene assay. The results showed that huperzine A, ligustrazine and oridonin significantly enhanced CYP3A4 reporter luciferase activity. Previous studies (Yu et al. 2011) demonstrated that the ethanol extracts of Rhizoma Chuanxiong could activate PXR signalling. Our data suggest that ligustrazine, as a main component of Rhizoma Chuanxiong, might contribute to the activation of PXR-mediate CYP3A4 expression by the ethanol extracts. In the current work, the tested compounds ginsenoside Rc, ginsenoside Rd, ginsenoside Rf and protopanaxatriol are the main bioactive components

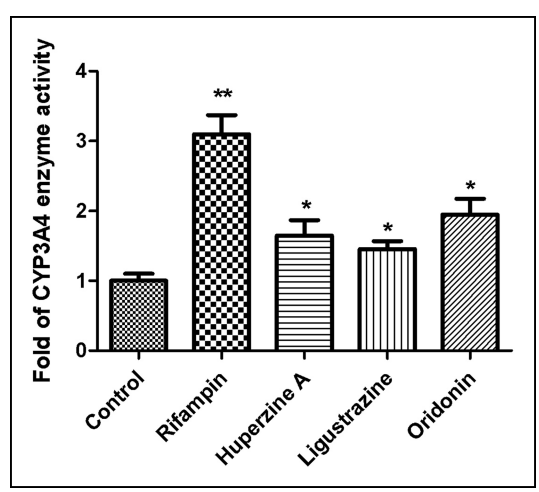

Fig. 5: Effect of huperzine A, ligustrazine and oridonin on the activity of CYP3A4 in LS174T cells. LS174T cells were pretreated with vehicle control(0.1% DMSO), rifampin(10 μM), or huperzine A, ligustrazine and oridonin at 20 μM for 72 h. CYP3A4 activity was measured metabolite concentrations of specific CYP3A4-mediated midazolam hydroxylation. The results are expressed as relative folds of activity to vehicle control. All data are presented as mean ± S.D. of tthree experiments; $*p < 0.05$, $**p < 0.01$.

of *Panax ginseng* or *Panax notoginseng* which did not produce any activation of PXR. This observation might give a partial mechanistic explanation to the previous findings (Liu et al. 2012) that *Panax notoginseng* does not change CYP3A4 activity significantly, but can up-regulate the protein expression of CYP1A2 which is not regulated by PXR. In addition, andrographolide (Ooi et al. 2011), apigenin (Kimura et al. 2010), oleanolic acid (Kim et al. 2004), palmatine (Su et al. 2007) and resveratrol (Liu et al. 2012) are found having no activity of PXR, which is also in accordance with previous studies. We further tested the effects of huperzine A, ligustrazine and oridonin on luciferase reporter gene expression at various concentrations, and the data suggested an inductive effects on PXR-mediated CYP3A4 reporter gene expression in a dose-dependent manner.

Previous studies proofed that LS174T cells expressed endogenous PXR and were used to assess the induction of CYP3A4. In these cell lines, we investigated the effects of huperzine A, ligustrazine and oridonin on CYP3A4 mRNA expression using real time PCR and evaluated CYP3A4 protein expression by Western blot analysis. Furthermore, we analyzed CYP3A4 enzymatic activity by detection of specific CYP3A4-mediated midazolam hydroxylation activity in LS174T cells. To our knowledge, this is the first report of these three compounds as PXR activators to induce drug-metabolizing enzymes CYP3A4.

In summary, the present study found inductive effects of huperzine A, ligustrazine and oridonin on CYP3A4 mRNA and protein expression, as well as enzyme activity in human LS174T cells. These effects were exerted *via* PXR-mediated pathways. Therefore, these compounds should be used with caution to avoid potential herb-drug interactions. The influences on the pharmacokinetic properties of other co-administered drugs with these compounds still need further exploration.

4. Experimental

4.1. Chemicals and reagents

All herbal compounds tested (Table 1, purity > 99%) were available from Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Rifampicin and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Lipofectamine TM 2000 Transfection Reagent and TRIzol reagent were provided by Invitrogen (Carlsbad, CA, USA). Dual-Luciferase® Reporter Assay System was purchased from Promega (Madison, WI, USA). PrimeScript® RT reagent Kit with gDNA Ereaser and SYBR® Premix DimerEreaser™ were obtained from Takara Biotechnology Co., Ltd (Dalian, China). Rabbit antihuman CYP3A4 antibody and mouse antihuman PXR antibody were purchased from Abcam (Cambridge, UK). The cytotoxicity of the compounds on LS174T cells were detected by MTS cytotoxicity assay. These compounds did not show cytotoxicity on LS174T cells at the maximum dosage (20 μM).

4.2. Cell culture

HepG2 and LS174T cell lines were originally supplied by the American Type Culture Collection (Manassas VA, USA). Both cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), and were cultured in a humidified incubator with a 5% CO_2 atmosphere.

4.3. Construction of plasmids

The pGL4.17-CYP3A4 luciferase reporter was constructed to contain the basal promoter(-362/ + 53) with the distal xenobiotic responsive enhancer module(-7836/-7208) of *CYP3A4* gene 5'-flanking region, and was inserted to the pGL4.17 reporter vector. The pregnane X receptor expression palsmids pcDNA 3.1-hPXR and the retinoid X receptor$_\alpha$ expression palsmids pcDNA 3.1-hRXR were constructed according to the routine reported before (Yu et al. 2009). The pGL-TK control vector and pcDNA 3.1-empty vector were obtained from Promega (Madison, WI, USA).

4.4. Transient transfection and reporter gene assays

For transfection, HepG2 cells were seeded into 24-well plates at a density of 1×10^5 cells/well. After reaching 80–90% confluence in plates, the transfection was performed in DMEM medium at the absence of serum using LipofectamineTM 2000 Transfection Reagent. In brief, cells were transfected with pcDNA3.1-hPXR and pcDNA 3.1- hRXR expression plasmids, pGL4.17-CYP3A4 luciferase reporter and pGL-TK for 6 h. After that, the transfected cells were treated for 24 h with herbal compounds, 10 μM rifampicin (positive control for hPXR assay) and 0.1% DMSO as a control. The concentrations of the herbal compounds were all 20 μM, which was demonstrated to be nontoxic to HepG2 cells by our pilot study. Following treatment, the cells were washed with PBS and lysed by passive lysis buffer. Then luciferase reporter activity was determined using the Dual-Luciferase Reporter Assay System following manufacturer's instructions.

4.5. Real-time PCR analysis of CYP3A4 mRNA and PXR mRNA

LS174T cells (4.0×10^5 per well) were seeded into 12-well plates and cultured for 24 h. After that, the cells were then treated with 0.1% DMSO, 10 μM rifampicin and the tested herbal compounds for another 48 h, respectively. Total RNA was isolated from cultured LS174T cells using the TRIzol reagent according to manufacturer's instructions, and quantified by the absorbance at 260 nm. The purity of the RNA was assessed by measuring O.D.260 nm /O.D.280 nm ratio, and the integrity was evaluated by electrophoresis on 1% agarose gels. cDNA were synthesized from 2 μg of total RNA using PrimeScript® RT reagent Kit. The primer pairs were specific to CYP3A4 (forward primer 5'-CAGGAGGAAATTGATGCAGTTTT-3', reverse primer 5'-GTCAAGATACTCCATCTGTAGCACAGT-3') and PXR (forward primer 5'- TGCGAGATCACCCGGAAGAC -3', reverse primer 5'- ATGGGAGAAGGTAGTGTCAAAGG -3'). For the control gene β-actin, the forward primer was 5'-TGACTGACTACCTCATGAAGAT-3' and reverse primer 5'-CATGATGGAGTTGAAGGTAGTT-3'. All the PCR reactions were carried out using SYBR® Premix DimerEreaser™ according to manufacturer's instructions. The mRNA expression of *CYP3A4* and *PXR* were normalized against *β-actin*. The real time PCR assays were performed on an LightCycler® 480 (Roche Diagnostics) according to the manufacturer's instructions.

4.6. Western blot analysis

LS174T cells were treated with huperzine A, ligustrazine and oridonin at concentrations of 20 μM for 72 h. Homogenate protein (15 μg per lane) from treated LS174T cells were separated by 12% sodium dodecyl sulfatepolyacrylamide gel (SDS-PAGE) and transferred onto PVDF transfer membranes. After incubation in blocking solution (5% non-fat milk), membranes were incubated with polyclonal antibody against CYP3A4 (1:1000; Abcam), PXR (1:500; Abcam) and β-actin (1:5000; Sigma) for overnight at 4 °C. Membranes were washed and then incubated with a 1:10000 dilution of second antibody for 2 h, and the membranes were detected with the enhanced chemiluminescence system. Relative intensities of protein bands were analyzed by Quantity One® software.

4.7. UPLC-MS/MS Analysis

UPLC-MS/MS is commonly used to detect metabolite concentrations to analyze CYP3A4 catalytic activity. In the present study, the LS174T cells were exposed to the tested compounds or 0.1% DMSO for 72 h, then culturing mediums were removed and mediums with 50 μM midazolam were added. After 4 h of incubation, the media were collected and extracted with methyl *tert*-butyl ether (MTBE), then the MTBE layers were evaporated to dyness with N_2. The residues were reconstituted with 80 μl of the mobile phase (30:70, acetonitrile:water with 0.1% formic acid). Chromatographic separations were achieved using a Waters Acquity BEHC18column (2.1 mm × 50 mm, particle size 1.7 μm, Waters, Wexford, Ireland). The flow rate was 0.2 mL·min^{-1} with isocratic elution. MS/MS was performed in selected reaction monitoring (SRM) mode with positive electrospray ionization (ESI) interface using the $[M+H]^+$ ions, m/z 341.8→m/z 324.2 for 1-OH-midazolam. Ionization conditions were optimized as follows: ion spray voltage was + 4.5 kV, heater gas temperature was 350 °C, and declustering potential (DP) was set at 80 V with collision energy (CE) of 17 eV. For detecting the metabolite, mass chromatograms and mass spectra were acquired by MassLynx software (Waters).

4.8. Data analysis

All data are presented in the statistics of three independent experiments in the form of mean ± S.D. The significance of the difference was analyzed by one-way analysis of variance (ANOVA) or unpaired Student's test. A value of $P < 0.05$ was considered statistically significant.

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