Life Sciences 144 (2016) 30–36

Contents lists available at ScienceDirect

Life Sciences

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

Effects of *Hypericum perforatum* extract and its main bioactive compounds on the cytotoxicity and expression of CYP1A2 and CYP2D6 in hepatic cells

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ARTICLE INFO

Article history:
Received 13 March 2015
Received in revised form 19 October 2015
Accepted 4 November 2015
Available online 21 November 2015

Keywords:
Hypericum perforatum
Hypericin
Hyperforin
CYP1A2
CYP2D6
Hepatic cells
Herb-drug interactions

ABSTRACT

Aims: Hypericum perforatum (*H. perforatum*) is one of the most used medicinal plants. However, it has been associated with relevant interactions with several drugs. This situation is probably mediated by cytochrome P450 enzymes (CYP450), namely the 1A2 (CYP1A2) and 2D6 (CYP2D6) isoforms This study aims to assess the cytotoxic and CYP1A2 and CYP2D6 inductive and/or inhibitory effects of a *H. perforatum* extract and its main bioactive components in hepatic cell lines.

Main methods: A MTT proliferation assay was performed in WRL-68, HepG2 and HepaRG cells after exposition to different concentrations of *H. perforatum* extract, hypericin and hyperforin for 24 and 72 h. Then, a real-time PCR analysis was accomplished after incubating the cells with these products evaluating the relative CYP1A2 and CYP2D6 expression.

Key findings: These products have relevant cytotoxicity at a 10 μM concentration and it was also demonstrated for the first time that *H. perforatum* can lead to a significant CYP1A2 and CYP2D6 induction in all cell lines. Moreover, hypericin seems to induce CYP1A2 in HepG2 cells and to inhibit its expression in HepaRG cells while hyperforin induced CYP1A2 in HepG2 and in WRL-68 cells. Additionally, hypericin and hyperforin induce CYP2D6 in HepG2 cells but inhibits its expression in HepaRG and in WRL-68 cells.

Significance: This study not only evidenced that *H. perforatum* extract and two of its bioactive components can have toxic effects in hepatic cell lines but also emphasized the potential risk of the consumption of *H. perforatum* with CYP1A2- and CYP2D6-metabolized drugs.

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1. Introduction

Herbal medicines are being widely used worldwide and some of them are becoming an important part of clinical routine. An example is *Hypericum perforatum* L. (Hypericaceae), commonly known as St. John's wort, a perennial plant widely distributed in Europe, Asia and North Africa [6], which has been reported to be effective for the treatment of depressive disorders [28]. It has also other properties, namely antioxidant, cytotoxic, antimicrobial, anticonvulsant, anxiolytic and wound healing [12,20,53–55,58], due to its main bioactive compounds [10,48], as hypericin and hyperforin. While hypericin is a potent natural photosensitizer, with potential application in photodynamic cancer therapy [1,34], hyperforin is usually indicated as the one responsible for the observed antidepressant effects [39].

Several studies showed that some phytochemicals have a significant capability of affecting the pharmacology and toxicology of some drugs [59], particularly by inducing or inhibiting the cytochrome P450 enzymes (CYP450), resulting in eventual interactions.

The CYP450 family is responsible for the metabolism of drugs [62,66], mainly through the isoforms 1A2, 2D6, 3A4, 2C9, 2C19, 2A6 and 2E1 [56]. Thus, the kinetic disposition of several drugs can be changed by exposure to CYP450 inducers or inhibitors [36]. A relevant example is *H. perforatum*, which has been described over the years as a CYP450 inductive agent. Despite the fact that this effect has been mainly associated with CYP3A4, other isoforms, including CYP1A2 and CYP2D6, which are involved in the metabolism of several drugs [13,18,29], can also be involved and are far less studied than CYP3A4. In fact, previous studies showed that CYP1A2 plays an important role in the metabolism of several drugs including theophylline, clozapine and tacrine [13,18], and the interaction of St. John's wort with clozapine has also been described [60]. An increased CYP1A2 protein level was also observed after an extended treatment with *H. perforatum* [57], while others observed no alterations [3,65].

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http://dx.doi.org/10.1016/j.lfs.2015.11.004
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Furthermore, *in vitro* data reported an inhibitory effect of the hypericin and hyperforin on CYP1A2 activity [50].

Regarding CYP2D6, previous studies suggested that its activity can be inhibited *in vitro* by *H. perforatum* [19,27,50,68], but it was not a potent *in vivo* modulator of this enzyme [22].

Since the effects of the extract, hypericin and hyperforin on CYP1A2 and CYP2D6 expression are still unclear [3,19,22,24,27,57,65,68], the aim of the present study was to evaluate the actions of these compounds on cellular viability of different hepatic cell lines, and to determine if they affect the expression of CYP1A2 and CYP2D6 enzyme isoforms.

2. Materials and methods

2.1. Cell cultures

WRL-68 and HepG2 cells were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA), and HepaRG cells from Life Technologies — Invitrogen™ (through Alfagene, Portugal). All these cell lines are of human origin. WRL-68 and HepG2 cells were cultured in 75 or 175 cm^2 culture flasks in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, Inc.), 100 U/mL penicillin (Sigma-Aldrich, Inc.) and 100 μg/mL streptomycin (Sigma-Aldrich, Inc.) at 37 °C in a humidified air incubator with 5% CO_2. HepaRG cells were also cultured in 75 or 175 cm^2 flasks in William's E medium supplemented with 10% FBS, 100 U/mL penicillin (Sigma-Aldrich, Inc.) and 100 μg/mL streptomycin (Sigma-Aldrich, Inc.), 5 μg/mL insulin (Sigma-Aldrich, Inc.) and 5×10^{-5} M hydrocortisone hemisuccinate (Sigma-Aldrich, Inc.) at 37 °C in a humidified air incubator with 5% CO_2. For all cell types, the medium was renewed every 2–3 days until cells reached confluence.

2.2. Preparation of the solutions of the compounds under study

H. perforatum dry hydroalcoholic (30/70) extract, containing 0.3% hypericin (EPO, S.r.L., Milan, Italy), was dissolved in aqueous 5% dimethyl sulfoxide (DMSO; Sigma-Aldrich, Inc., St. Louis, MO, USA) and 0.1% HCl 0.1 M (Merck Millipore, Billerica, MA, USA). This mixture was sonicated for approximately 3 h at 40 °C, filtered and the resulting supernatant was stored at 4 °C. The concentration of the extract was established considering its hypericin percentage..

Hypericin (HWI Analytik, Gmhb, Ruelzheim, Germany) and hyperforin (Sigma-Aldrich, Inc.) were also dissolved in aqueous 5% DMSO (Sigma-Aldrich, Inc., St. Louis, MO, USA) and 0.1% HCl 0.1 M (Merck Millipore, Billerica, MA, USA), at a concentration of 0.01 M (stock solution), and stored at 4 °C.

2.3. Cell treatments with the compounds

When cells reached approximately 90–95% confluence, they were detached by gentle trypsinization, quantified by the trypan-blue assay and seeded in quadruplicated in 96- (MTT assays) or 6-well culture plates (real-time PCR experiments) (1×10^4 or 2×10^4 cells/well, respectively; Nunc, Apogent, Denmark) and left to adhere for 24–48 h in the appropriate growth medium. Then, after reaching 80–90% confluence, the medium was replaced with the correspondent serum-free medium and, after 12 h, cells were incubated in the absence and in the presence of 1 and 10 μM of *H. perforatum* extract, hypericin or hyperforin for 24 or 72 h, again in the appropriate serum-free medium. These concentrations were selected attending to the fact that plasma maximum hyperforin concentrations after oral administration of formulations containing *H. perforatum* extract can achieve values near 1 μM [11]. The concentration of DMSO was kept below 0.1% to prevent the cytotoxicity induced by this solvent. Untreated cells were used as the control. Each experiment was independently repeated at least three times.

2.4. MTT cell viability assay

The *in vitro* cytotoxicity was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, Inc.) assay. After the incubation period (24 and 72 h) the medium was removed, 200 μL of phosphate buffer saline (PBS) was used to wash the cells and then 100 μL of MTT solution (0.5 mg/mL) prepared in the appropriate serum-free medium was added to each well, followed by incubation for nearly 3 h at 37 °C. Then, the MTT containing medium was removed and the formazan crystals were dissolved with 150 μL of 40 mM HCl solution in isopropanol. The contents were transferred to a reading plate and the absorbance was measured at 570 nm using a microplate reader Anthos 2020 (Alfagene). Cell viability values were expressed as a percentage relative to the absorbance determined in the cells used as controls. Each experiment was repeated three independent times.

2.5. Total RNA extraction and real-time PCR

These assays were performed according to Martinho et al. [44]. The total RNA was extracted from cells using TRI Reagent (Sigma-Aldrich, Inc.), following the manufacturer's instructions. Total RNA of each sample was quantified using UV spectrophotometry at 260 nm (Pharmacia Biotech, Ultrospec3000, Denmark), and its integrity was assessed by ethidium bromide agarose gel (1%) electrophoresis. Total RNA (1 μg) was reverse transcribed for 1 h at 37 °C in a 20-μL reaction containing First Strand 5× buffer (50 mM Tris–HCl, 75 mM KCl, 3 mM $MgCl_2$; Invitrogen, Carlsbad, CA, USA), 10 mM of dithiothreitol (DTT), 0.5 mM of each dNTP (dATP, dCTP, dGTP and dTTP; Nzytech, Lisboa, Portugal), 20 U of RNAse Out (Invitrogen), 25 pmol of random hexamer primers (Nzytech), and 200 U of M-MLV reverse transcriptase (Invitrogen). The mRNA levels were analyzed by RT-PCR in cells incubated in the absence and in the presence of a 1 μM concentration of the compounds included during two time periods (24 and 72 h) and human beta actin (β-actin) was used as endogenous control gene. To analyze CYP1A2 and CYP2D6 expression in each cell line, reactions were carried out using 1 μL of cDNA for WRL-68 and HepG2 cells and 1.5 μL of cDNA for HepaRG cells in a 20-μL reaction containing 10 μL of SYBR Green supermix (Fermentas, Thermo Fisher Scientific, Ontario, Canada), and 3 pmol of β-actin or CYP1A2 in HepG2 and HepaRG or 4 pmol of CYP1A2 in WRL-68, and 3 pmol of β-actin or CYP2D6 in HepG2 and WRL-68 or 4 pmol of CYP2D6 in HepaRG (Table 1). The amplification conditions for HepG2, WRL-68 and HepaRG cells were: 95 °C for 3 min and 40 cycles at 95 °C for 15 s, 63 °C for 30 s, and 72 °C for 30 s. Amplified PCR fragments were checked by melting curves: reactions were heated from 55 to 95 °C with 10 s hold at each temperature (0.05 °C/s). All primers were validated by quantitative *real-time* PCR reactions with decreasing cDNA concentrations (1; 1:10; 1:100; 1:1000), and the reaction efficiencies were calculated. Real-time PCR was carried out using the iCycler IQTM System (Bio-Rad) and fluorescence was measured after each cycle. Every reaction was done in triplicate and each experiment was repeated three independent times. In all cases, amplification conditions were: 95 °C for 3 min and 40 cycles at 95 °C for 15 s, 63 °C for 30 s and 72 °C for 30 s. Amplified PCR fragments were checked by melting curves: reactions were heated from 55 °C to 95 °C with 10-s

Table 1
Primer sequences of human β-actin (hβ-actin) and CYP1A2 and CYP2D6 used in real-time PCR experiments.

Designation	Sequence 5′–3′
hβ-actin fw	cat gta cgt tgc tat cca ggc
hβ-actin rv	ctc ctt aat gtc acg cac gat
hCYP1A2 fw	ctg ggc act tcg acc ctt ac
hCYP1A2 rv	gca gga acc aca gga acc tc
hCYP2D6 fw	tct ctt gga caa agc cgt ga
hCYP2D6 rv	gct ggg ata tgc agg agg ac

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