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Drug membrane transporters and CYP3A4 are affected by hypericin, hyperforin or aristoforin in colon adenocarcinoma cells

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ABSTRACT

Our previous results have shown that the combination of hypericin-mediated photodynamic therapy (HY-PDT) at sub-optimal dose with hyperforin (HP) (compounds of *Hypericum* sp.), or its stable derivative aristoforin (AR) stimulates generation of reactive oxygen species (ROS) leading to antitumour activity. This enhanced oxidative stress evoked the need for an explanation for HY accumulation in colon cancer cells pretreated with HP or AR. Generally, the therapeutic efficacy of chemotherapeutics is limited by drug resistance related to the overexpression of drug efflux transporters in tumour cells. Therefore, the impact of non-activated hypericin (HY), HY-PDT, HP and AR on cell membrane transporter systems (Multidrug resistance-associated protein 1-MRP1/*ABCC1*, Multidrug resistance-associated protein 2-MRP2/*ABCC2*, Breast cancer resistance protein – BCRP/*ABCG2*, *P*-glycoprotein-*P*-gp/*ABCC1*) and cytochrome P450 3A4 (CYP3A4) was evaluated. The different effects of the three compounds on their expression, protein level and activity was determined under specific PDT light (T0+, T6+) or dark conditions (T0− T6−). We found that HP or AR treatment affected the protein levels of MRP2 and P-gp, whereas HP decreased MRP2 and P-gp expression mostly in the T0+ and T6+ conditions, while AR decreased MRP2 in T0− and T6+. Moreover, HY-PDT treatment induced the expression of *MRP1*. Our data demonstrate that HP or AR treatment in light or dark PDT conditions had an inhibitory effect on the activity of individual membrane transport proteins and significantly decreased CYP3A4 activity in HT-29 cells. We found that HP or AR significantly affected intracellular accumulation of HY in HT-29 colon adenocarcinoma cells. These results suggest that HY, HP and AR might affect the efficiency of anti-cancer drugs, through interaction with membrane transporters and CYP3A4.

1. Introduction

Photodynamic therapy is an anti-cancer approach for the treatment of various types of non-malignant as well as malignant diseases. Hypericin (HY), a naturally occurring photosensitive compound synthesised by *Hypericum* sp., possesses properties suitable for PDT and demonstrates photocytotoxic activity both *in vitro* [1–5] and *in vivo* [6,7]. Hyperforin (HP) is a phloroglucinol-derivative that has emerged as a key player not only in the antidepressant activity of the plant extract but also as a suppressor of bacterial, lymphocyte and tumour cell proliferation [8], and as an inhibitor of matrix proteinases. Aristoforin (AR), one of hyperforin's synthetically prepared derivatives, has proved to be more soluble and is even stable in aqueous solution. Importantly, it retains the antitumour properties of the parental compound without inducing toxicity in experimental animals. These data strongly suggest that AR has even greater potential as an anticancer drug [9].

Two important ABC-transporters (ATP-binding cassette transporter family), ABCG2 (BCRP – breast cancer resistance protein; BMDP – brain multidrug resistance protein) and ABCB1 (MDR1 – multidrug resistance protein 1, *p*-gp – *p*-glycoprotein), play a major role in limiting the penetration of drugs across the blood-brain barrier by mediating the status of multidrug resistance [10]. Medicinal compounds derived from herbal sources including *Hypericum perforatum* (St John's wort) have been shown to activate multidrug resistance, as do pharmaceutical drugs containing HP [11]. Some of the 49 human ABC transporter proteins [12] have been described as being involved in resistance to drugs during tumour therapy. P-gp is an ATP-dependent drug efflux pump for xenobiotic compounds, responsible for decreased drug accumulation in multidrug-resistant cells, and often mediates the development of resistance to anticancer drugs. MRP1 (Multidrug resistance-associated protein 1) works as a multispecific organic

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anion transporter and MRP2 (Multidrug resistance-associated protein 2) works in biliary transport of mainly anionic conjugates and other substrates, including anticancer drugs. BCRP as a xenobiotic transporter may play a major role in multi-drug resistance. The effect of HY on cytochrome P450 3A4 (CYP3A4) and P-gp in specific conditions has been described previously [13]. A study by Komoroski et al. [14] showed that HP treatment increases the mRNA level, protein concentration, and activity of CYP3A4 and CYP2C9, but has no effect on CYP1A2 or CYP2D6 in the human hepatocyte model. However, acute administration of HP 1 h before, or at the same time as the substrate inhibited CYP3A4 activity. Although most research has pointed to the ability of St. John's wort to induce CYP3A4, one report documented the potent inhibition of CYP3A4, CYP2C9, and CYP2D6 by HP and HY *in vitro* [15] and another report showed CYP3A4, CYP2B as undergoing major biotransformations of HP pathway *in vivo* [16].

Therefore as described previously [1], our research in this area is principally focused on studying the mechanisms of anticancer drug efflux in cells affected by non-activated HY, HY-PDT and HP or AR. Additionally, some other metabolites that are present in St. John's wort extracts also demonstrate biologically-relevant activities. Whereas HY needs activation by light to achieve its cytotoxicity, HP/AR action is not light dependent.

Using several methods including Western blot analysis of proteins, mRNA expression by qRT-PCR and flow cytometric analysis of protein activity we demonstrated that HY-PDT, HP and AR change the protein level, as well as the activity of proteins involved in relevant transport mechanisms and drug metabolism in HT-29 colon adenocarcinoma cells. Moreover the activity of CYP3A4 in the resistant cell line HT-29-OxR was also affected. Our results also suggest light-dependent inactivation of membrane transport mechanisms in the presence of HP or AR alone.

2. Materials and methods

2.1. Reagents

Hypericin (4,5,7,40,50,70-hexahydroxy-2,20-dimethylnaphtodiantron; CAS No. 548-04-9; HPLC grade, AppliChem GmbH, Darmstadt, Germany), hyperforin (CAS 110 79-53-1; AppliChem) or aristoforin (CAS 849215-53-8; Alexis Biochemicals, Lausen, Switzerland) were dissolved in dimethyl sulphoxide (DMSO) and stored at −80 °C. Mitoxantrone dihydrochloride (MTX; substrate for BCRP; CAS No. 70476-82-3), indomethacin (CAS 53-86-1; MRP1 inhibitor), MK-571 (CAS 115103-85-0; MRP1 and MRP2 inhibitor,), Texas red (CAS 386229-76-1; substrate for MRP2) all from Sigma-Aldrich (MO, USA) and PSC-833 (CAS 121584-18-7; u-gp inhibitor, Tocris Bioscience, Missouri, UK), Ko143 (CAS 461054-93-3; BCRP inhibitor, Santa Cruz Biotechnology, Santa Cruz, CA, USA), JC-1 (CAS 3520-43-2; fluorescent substrate for *p*-gp, Molecular Probes, Inc., Eugene OR, USA), Calcein-AM (CAS 148504-34-1; substrate for MRP1, Molecular Probes, Inc.), stock solutions were prepared in DMSO, except for MTX that was prepared in distilled water, and stored at −20 °C. Working solutions of each of the above-mentioned reagents were freshly prepared immediately before addition to the cell culture. The final concentrations of DMSO and distilled water did not influence the cytokinetic parameters.

2.2. Cell culture

The human colon adenocarcinoma cell line HT-29, the ovarian carcinoma cell line A2780, the lung epithelial adenocarcinoma cell line A549, and the acute myeloid leukaemia cell line UT7 were purchased from ATCC (American Type Culture Collection, Rockville, MD, USA). All cells were cultured in RPMI-1640 supplemented with 10% foetal calf serum and antibiotics (Antibiotic-Antimycotic, Biosera, XC-A4110/100, France). For various analyses, 2×10^4bcells/cm^2 were equally seeded into six-well plates. Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere and always protected from light after HY treatment. The HT-29-OxR cell line stably oxaliplatin resistant (kindly provided by prof. Alois Kozubík and prepared by Dr. Strakova, Institute of Biophysics, Brno, Czech Republic) was exposed to 10 μM oxaliplatin (TEVA, UK), once a week (between two passages) to maintain its resistance. The CDDP-resistant A2780cis cell line (kindly provided by prof. Alois Kozubík (Institute of Biophysics, Brno, Czech Republic) was exposed to 1 μM cisplatin (EBEWE Pharma, Austria), once a week (between two passages) to maintain its resistance.

2.3. Experimental design of hypericin, hyperforin, aristoforin treatment

Cells were incubated for 24 h in the dark and for the next 16 h in growth medium supplemented with given concentrations of HY (50 nM, 100 nM), HP (1 μM, 5 μM, 10 μM) or AR (1 μM, 5 μM, 10 μM), followed by irradiation with a single light dose (described in Section 2.4.). All controls (untreated controls, HY alone, HP alone, AR alone) were irradiated in the same way, with the same light dose administered during the same time period as a combined treatment of HY-PDT with HP or AR (Fig. 1) using the same protocol as our previous study [1]. The description T0+ time represents analysis immediately after PDT, T0− means the same time point without light activation, T6+ analysis was performed 6 h after PDT and T6− means analysis at the same time without light activation.

2.4. Cell photosensitisation

Experimental groups as well as untreated controls were placed on the diffuser glass of the irradiating device in sequence and exposed to irradiation for specific time periods to reach 3.15 J cm^{-2}. The irradiation device consists of eleven L18W/30 (Osram, Berlin, Germany) fluorescent tubes with a maximum emission in the range of λ = 530–620 nm, which covers the maximum absorbance region of HY (590–600 nm). The irradiator fluence rate was measured by TES 1335 luxmeter (Rotronic, Taiwan) at the diffuser surface and the dedicated dose was achieved by multiplication by the irradiation time.

2.5. RNA isolation and qRT-PCR analysis

Total RNA was isolated from the specified cell lines using TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. Total RNA concentration and purity (OD 260/280 ratio and OD 260/230 ratio) were evaluated using a BioSpec-nano Shimadzu spectrophotometer (Life Science). Reverse transcription (RT) was performed at 37 °C in a 20 μl volume using 1 μg of total RNA, 10 mM random hexamer primers and 200U M-MLV reverse transcriptase (Invitrogen) according to the manufacturer's

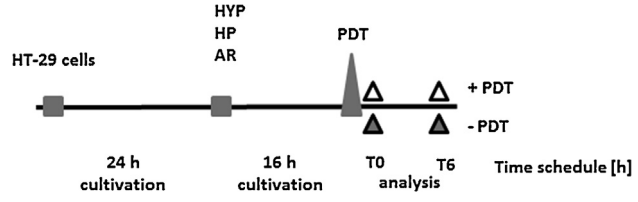

Fig. 1. Experimental design. HT-29 cells were incubated for 24 h before treatment with non-activated HY, HY-PDT, HP or AR. Cells were treated with PDT (3.15 J/cm^2) at 16 h after treatment. Analyses were performed at time: 0 h (- without PDT, + after PDT), 6 h (- without PDT, + after PDT).