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Quantitative characterization of direct P-glycoprotein inhibition by St John's wort constituents hypericin and hyperforin

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Abstract

The ATP-binding cassette transporter P-glycoprotein (P-gp) exerts a critical role in the systemic disposition of, and exposure to, lipophilic and amphipathic drugs, carcinogens, toxins and other xenobiotics. The ability of P-gp to transfer a wide variety of structurally unrelated compounds from the cell interior across the membrane bilayer remains intriguing. Since natural product chemicals in the widely consumed St John's wort appear to exert antidepressant effects by an unknown mechanism, the constituents are frequently studied for interactions with various biomacromolecules as well as cytotoxins or isolated cells. The drug interactions caused by this widely used herbal remedy are under-appreciated. Various clinical interactions have been observed upon the co-administration of St John's wort, and P-gp and CYP3A4 have been indicted as the cause. We characterized several St John's wort constituents for their interaction with P-gp and their specific effects on the P-gp export activity of several marker substrates. Two of these constituents, hyperforin and hypericin, inhibit the active efflux of the fluorescent markers daunorubicin (IC_{50} ~ 30 μM) and calcein-AM. Herein, we show in-vitro results that can both explain the competing clinical observations of initial elevated exposure of P-gp substrate drugs (P-gp inhibition) followed by under-exposure (P-gp induction) when St John's wort is co-administered, and provide a further warning against unchecked co-administration of drugs with St John's wort.

Introduction

Transmembrane transport proteins are critical to the disposition of many xenobiotics. Cellular and tissue exposure can be dependent on the action of ATP-binding cassette (ABC) transporters, in particular P-glycoprotein (P-gp) (Gottesman & Pastan 1993; Ambudkar et al 1999; Johnson 2002). Among ABC transporters, P-gp appears to have the broadest substrate specificity and perhaps the widest tissue distribution. Consequently, it is also the most intensely studied of the energy-dependent multidrug resistance (MDR) enzymes. This transport enzyme actively effluxes or ejects many structurally unrelated drugs from various tissues and organs, including the liver, intestine, brain, kidney and lymphocytes. Accordingly, P-gp has a significant effect on the bioavailability and distribution, and hence efficacy, of many drugs, including chemotherapeutic drugs, natural products, toxicants and peptides.

P-gp is a 170 kDa phosphorylated glycoprotein encoded by the MDR1 gene. It has two homologous halves, each of which contains a transmembrane domain(s) and an ATP-binding domain. This transport enzyme uses ATP (via hydrolysis) as the source of energy for 'translocating' various substrates (Sharom et al 1993), with some allosteric interactions indicated for certain substrate combinations (Wang et al 2000a, b, 2001). The identification and characterization of specific dietary chemicals, and an increased understanding of their biological activity, has elevated the distinction of natural product chemicals (phytochemicals) in disease prevention and treatment. St John's wort (*Hypericum perforatum*, and extract) is a herbal treatment that has been recognized as an antidepressant (Kim et al 1999; Gaster & Holroyd 2000). The flourishing use of 'herbal' medications, particularly St John's wort, in combination with other pharmaceuticals has resulted in many drug interactions with such drugs as warfarin, oral

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contraceptives, ciclosporin and digoxin (Henderson et al 2002; Hennessy et al 2002). Chronic use of St John's wort reduces the bioavailability of a number of drugs, including the HIV protease inhibitor indinavir, the immunosuppressant ciclosporin and the cardiac glycoside digoxin (Johne et al 1999; Piscitelli et al 2000; Ruschitzka et al 2000). These interactions have been explained by the induction and increased levels of P-gp and cytochrome P450 by multiple St John's wort dosing (Durr et al 2000). However, no significant change was observed in digoxin exposure on the first dose of the St John's wort extract (at day 6), yet after 10 days of treatment, digoxin exposure had decreased by about 25% (Johne et al 1999). Furthermore, a single dose of St John's wort *increased* the maximum plasma concentration of fexofenadine by 45%, whereas long-term treatment caused a 35% decrease (Wang et al 2002). St John's wort extract and a purified component, hypericin, have been shown to mildly inhibit some active transport of rhodamine 123 (Rho) in Caco-2 and LS-180V cells (Perloff et al 2001). In this report we show that a major constituent of St John's wort, hyperforin, can inhibit effective function of P-gp-mediated efflux. This reaction could increase the short-term cellular exposure to co-administered xenobiotics (such as drugs), thus causing drug interactions.

Materials and Methods

Chemicals

Daunorubicin (DNR), Rho, verapamil, hyperforin, hypericin and pseudo-hypericin were purchased from Sigma Chemical Co. (St Louis, MO). LDS (LDS 751) was purchased from Molecular Probes (Eugene, OR). Ciclosporin A was purchased from Alexis Biochemicals (San Diego, CA). Hanks' balanced salt solution, Alpha Minimum Essential Medium, Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin, fetal bovine serum (FBS), and trypsin-EDTA were obtained from Life Technologies, Inc. (Rockville, MD). All other reagents were of the highest grade commercially available.

Cell lines

The NIH-3T3-G185 cell line presenting the gene product of human MDR1 was licensed from NIH and maintained in DMEM as described previously (Wang et al 2000c).

FACS flow cytometry

Fluorescence measurements of individual cells were performed using a Becton–Dickinson FACScalibur fluorescence-activated cell sorter (San Jose, CA) equipped with an ultraviolet argon laser (excitation at 488 nm, emission at 530/30, 570/30 and 650/30 nm band-pass filters). Analysis was gated to include single viable cells on the basis of forward and side light-scatter and was based on acquisition of data from 10 000 cells. Log fluorescence was collected and displayed as single-parameter histograms. A modified inhibition assay for the P-gp efflux pump in viable transfected cells was performed with the flow cytometer as previously described (Wang et al 2000b). The efflux incubation was 15 min at 37 °C and the marker substrate concentrations of LDS and Rho were 0.2 μM and DNR was used at 2 μM.

Cell viability test

Cell viability was assessed using propidium iodide staining. Dead cells in which propidium iodide was bound to double strands of DNA or RNA were detected in certain regions of the cytometry dot plots and not included in the final data calculations.

Calculation of relative fluorescence

The fluorescence intensity of individual cells was recorded as histograms. The mean fluorescence intensity of 10 000 cells was used for comparison among different conditions. Ciclosporin A or verapamil was selected as a positive control because it can significantly inhibit the P-gp-mediated active efflux of fluorescent substrate markers. Relative fluorescence was used for quantitation and comparison among different compounds.

ATP hydrolysis and phosphate release

The consumption of ATP was quantified by determining the amount of liberated inorganic orthophosphate, which forms a colour complex with molybdate. The ATP hydrolysis assay based on phosphate-release determination using membrane microsome preparations (human P-gp; Gentest, Woburn, MA) was carried out in a 96-well microplate (Wang et al 2000a). The microsomes were thawed at ~ 37 °C in a water bath and then placed on ice before diluting to 0.6 mg mL^{-1} (15 μg protein per well) in ice-cold ATPase buffer (KCl, 50 mM; dithiothreitol, 2 mM; MOPS-Tris buffer, 50 mM; pH 7.0) containing 0.1 mM EGTA-Tris (to inhibit Ca-ATPase), 1 mM ouabain (to inhibit the Na/K-ATPase) and 5 mM sodium azide (to inhibit the mitochondrial ATPase). The total incubation volume including the various inhibitors was 51 μL. After pre-warming the solutions above to 37 °C, the incubation reaction was initiated by adding Mg-ATP to a final concentration of 3.2 mM. The plate was then incubated for 20 min at 37 °C. The reaction was terminated by the addition of 40 μL 5% sodium dodecyl sulfate solution at room temperature. This was followed by the addition of 200 μL of a 8.75 mM ammonium molybdate/7.5% ascorbic acid (pH ~5.0) solution. The colour reaction was allowed to develop for 20 min at 37 °C, and the released phosphate was quantified by absorbance using a microplate reader (Tecan SpectraFluor Plus, RTP, NC) at 830 nm. By comparison to a standard curve, the amount of phosphate released – and hence ATP hydrolyzed – was quantified. The results were plotted as activity versus substrate concentration using GraphPad Prism software V.3.00 for Windows (San Diego, CA). Water-insoluble drugs were dissolved in methanol or DMSO; the maximum methanol or DMSO concentration (2% v/v) was shown not to affect the ATPase activity.

Results

As fluorescent substrates transported by mammalian P-gp, DNR, Rho and others serve as markers for active transport function simply by measurement of retained fluorescence per cell (Wang et al 2001). The IC_{50} (concentration at half-maximum inhibition) could be determined from a simple function, as shown in Figure 1, where the retained fluorescence was measured for samples of viable cells by a flow cytometer at varying concentrations of compound (Wang et al 2000c). The concentration dependency of inhibition displayed a sigmoidal response curve (Figure 1), a consequence of cooperativity (Wang et al 2000b), with the Hill equation for allosteric interaction enzymes therefore being the appropriate function for fitting to the data: $I = I_{max}S^n/(IC_{50} + S^n)$. The IC_{50} for hyperforin against DNR active efflux is $\sim 30\,\mu$M in the NIH3T3-G185 cell line (which over-expresses the gene product of human MDR1), and hyperforin achieved about 400% of relative retention (Figure 1A). The well-known P-gp inhibitor ciclosporin impeded P-gp efflux in this system of over-abundant membrane transporter with an $IC_{50} \sim 2\,\mu$M. Hyperforin also inhibited calcein-AM efflux at $5\,\mu$M, but a plateau was not achieved at even $100\,\mu$M (Figure 1B). Hypericin, pseudo-hypericin and hyperforin all inhibited P-gp mediated transport of calcein-AM at $10\,\mu$M, but again a plateau was not reached at even $100\,\mu$M, thus precluding an IC_{50} quantification with this marker (Figure 2A). It is clear from the control data in the figures that efflux inhibition effects on the parent (non-transfected) cell line are insignificant and therefore do not contribute to the results (for the DNR and calcein-AM markers).

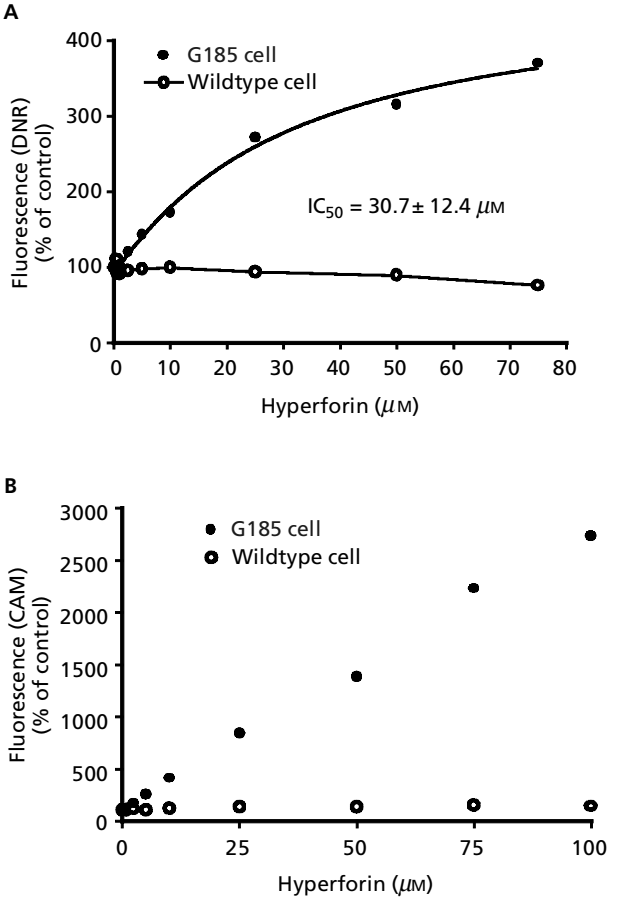

Figure 1 Intracellular retention of DNR (A) and calcein-AM (CAM) (B) in NIH3T3-G185 cells vs competing hyperforin concentration. Fluorescence intensity is expressed as relative fluorescence. The average number of cells per assay was 10 000. The function for the line through the data is the Hill equation: $I = I_{max}S^n/(IC_{50} + S^n)$. At $1\,\mu$M hyperforin concentration the Kolmogorov–Smirnov test resulted in $0.01 < P < 0.05$, and at $\geq 2\,\mu$M $P < 0.01$, indicating statistical significance.

Notably, the control data demonstrates clearly that Rho efflux inhibition effects on the parent (non-transfected) cell line must be accounted for in the analysis (Figure 2B). With all of the constituents tested able to inhibit Rho efflux at concentrations of $10\,\mu$M in the control cell, these results indicate that the St John's wort constituents can inhibit other efflux transporters that can move Rho.

With the observation that various St John's wort constituents were able to affect the ability of P-gp to transport some fluorescent P-gp substrates out of a viable cell, we characterized several of these for transport kinetic parameters. As ATP is consumed at a purported rate of about one or two per transport event, the rate of hydrolysis of ATP represents the transport rate or activity assay of function (Eytan et al 1996; Ambudkar et al 1997; Stein 1997; Shapiro & Ling 1998; Sauna & Ambudkar 2000, 2001; Wang et al 2000a). In the absence of exogenous substrate, the enzyme is still able to hydrolyze ATP to produce a basal level of activity, a reaction that is probably due to the transport of endogenous substrates. The activity data are therefore presented as a percentage of the basal or control activity, as any change in the rate of ATP hydrolysis represents the sum of the basal activity and the contribution of the exogenous substrate to ATP hydrolysis. The presence of hypericin causes a concentration-dependent decrease in the rate of ATP hydrolysis relative to the baseline rate, which indicates that it is a comparatively slower substrate for P-gp than the apparent endogenous constituent causing the baseline activity (Figure 3). The K_m is $\sim 0.2\,\mu$M and the V_{max} is about half of baseline, as indicated in Figure 3.

P-gp commonly exhibits a basal activity that is purported to be caused by endogenous substrates (Shapiro & Ling 1998) such as membrane lipids that may be unfavourable to the cell interior or inner leaflet of the membrane (van Helvoort et al 1996). Not all P-gp transport substrates increase the rate of ATP hydrolysis; some substrates, such as PSC833 and gramicidin (Borgnia et al 1996), cause a decrease in the baseline ATPase activity with a hyperbolic relationship to concentration. As this response is consistent with a rate of transport that is even slower than that of putative endogenous substrates, this reaction pathway supplants the basal activity at sufficient concentrations as it apparently competes with an endogenous substrate in the lipid milieu and is transported at a slower rate (Ramachandra et al 1996; Kerr et al 2001). The

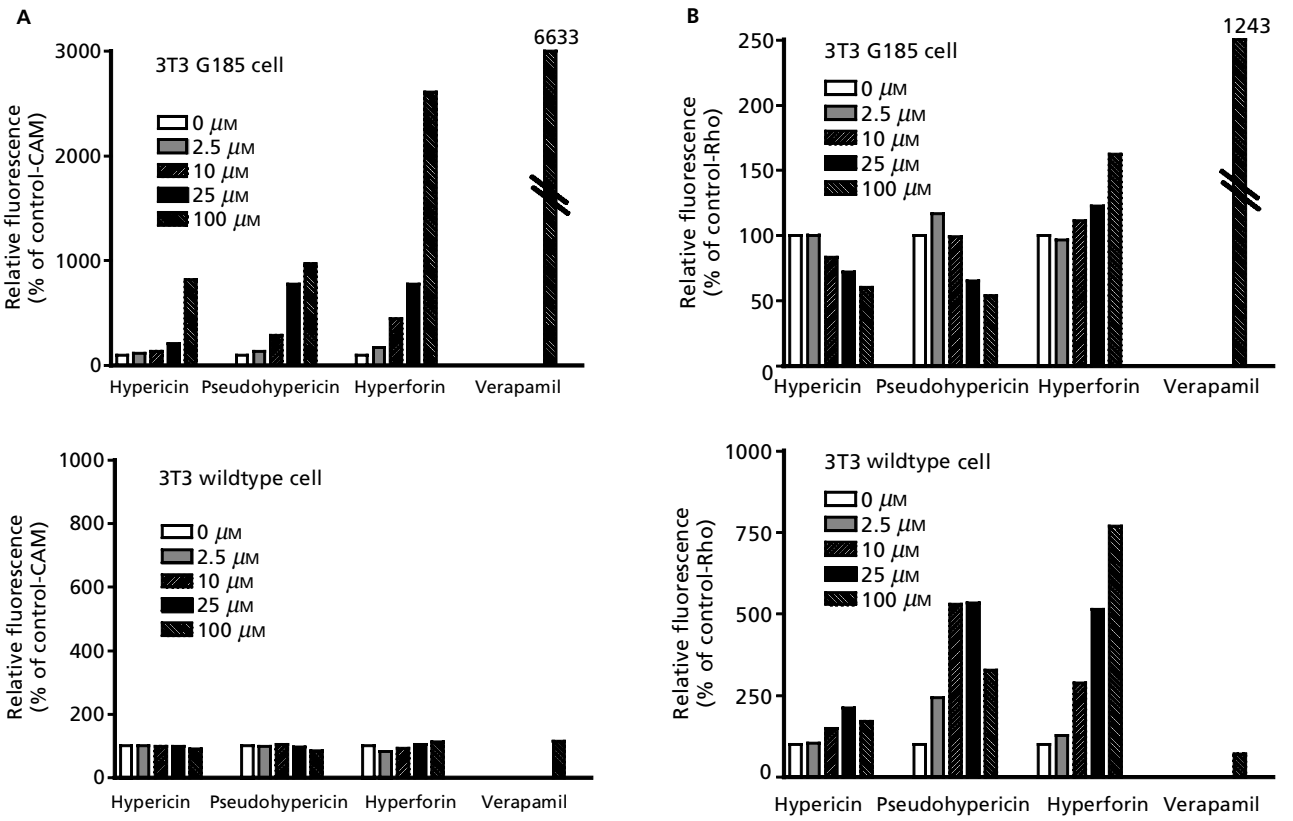

Figure 2 Intracellular retention of calcein-AM (CAM) (A) and Rho (B) in NIH3T3-G185 cells vs competing St John's wort constituent concentration. Fluorescence intensity is expressed as relative fluorescence. The average number of cells per assay was 10 000. At concentrations of 2.5 μM the Kolmogorov–Smirnov test resulted in $P < 0.01$, indicating statistical significance. The results with Rho suggest that this is not a useful marker for this transporter in this system.

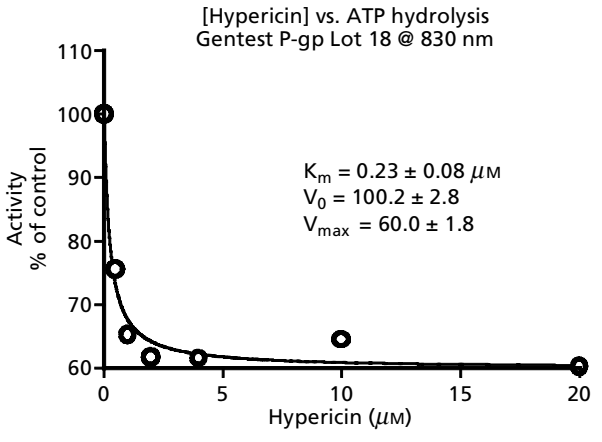

Figure 3 P-gp-mediated ATP hydrolysis rates in the presence of hypericin. The data is fitted to a hyperbola and the $V_{max} = 60\%$ of control with $K_m = 0.23 \pm 0.08\,\mu$M. Enzyme activity from microsomes of NIH-3T3-G185 cells overexpressing hamster P-gp.

other St John's wort constituents similarly reached activity at a V_{max} that was lower than baseline ATP hydrolysis, although with relatively low K_m.

Discussion

The concentrations at which these St John's wort constituents can inhibit P-gp are attainable in the intestine and may therefore cause herb/drug interactions that are the opposite of those observed after chronic dosing. These experiments use the transfected cell line expressing supraphysiological quantities of the P-gp transporter. Hyperforin or hypericin inhibition of P-gp in vivo should therefore be significantly more potent (than this in-vitro IC_{50} of about 30 μM; ciclosporin in this system has an IC_{50} of $\sim$2 μM). Steady-state plasma concentrations of hypericin (at 300 mg St John's wort three times daily) reach more than 0.03 μM, and intraluminal concentrations are most likely much higher (Kerb et al 1996; Johne et al 1999). Chronic use of St John's wort reduces the bioavailability of a number of drugs (Johne et al 1999; Piscitelli et al 2000; Ruschitzka et al 2000), primarily because of the induction and increased levels of P-gp and cytochrome P450 by multiple St John's wort dosing (Durr et al 2000). Hyperforin is a potent ligand for the pregnane X receptor, an orphan nuclear receptor that regulates expression of CYP3A4 and P-gp (Bertilsson et al 1998; Moore et al 2000; Geick et al 2001). However, no significant change

was observed in digoxin exposure on the first dose of the St John's wort extract (at day 6), yet after 10 days of treatment digoxin exposure had decreased by about 25% (Johne et al 1999). Furthermore, a single dose of St John's wort *increased* the maximum plasma concentration of fexofenadine by 45%, whereas long-term treatment caused a 35% decrease (Wang et al 2002). St John's wort and hypericin have been shown to mildly inhibit some active transport of Rho in Caco-2 and LS-180V cells (Perloff et al 2001, although we have shown here that Rho transport can be non-specifically inhibited by the St John's wort constituents). This opposing clinical outcome of initially elevated exposure following by suppressed exposure at longer term dosing could be explained by P-gp inhibition that is eventually overwhelmed by high levels of P-gp and the commensurate efflux activity.

The observation described here has no simple relationship between ATP-hydrolysis stimulation and stimulation of drug transport; rather, it suggests that communication between the hypericin binding site and the substrate/transport binding site does not involve the ATPase domain. Occupation of the allosteric site might affect k_{cat} for certain substrates only (since the effect is observed with only two of the three substrate markers) or increase substrate affinity. However, this result could indicate significantly more potent effects on various transport activities by some of these constituents.

Although St John's wort is widely used for the treatment of depression, the drug interactions caused by administration of this herbal remedy are under-appreciated. Various clinical interactions have been observed on the co-administration of St John's wort, and P-gp and CYP3A4 have been indicted as the cause. Herein, we have shown in-vitro results that can both explain the competing observations of initial elevated exposure of P-gp substrate drugs (P-gp inhibition) followed by under-exposure (P-gp induction) and provide a further warning against unchecked co-administration of drugs with St John's wort.

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