Kidney proximal human tubule HK-2 cell line as a tool for the investigation of P-glycoprotein modulation by natural compounds

[La línea celular de túbulo proximal de riñón humano HK-2 como una herramienta para la investigación de la modulación de P-glicoproteína por compuestos naturales]

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Abstract

ABCB1 (MDR1/P-glycoprotein), the best characterized multidrug transporter belonging to the ABC transporter superfamily, is nowadays acknowledged to have a major impact on drug resistance to chemotherapy and drug bioavailability and disposition. A number of natural compounds, dietary phytochemicals and herbal remedies have the ability to modulate P-glycoprotein function and/or expression and therefore the potentiality to cause food-drug, or herb-drug interactions. The elucidation of these interactions may be important not only to predict possible undesirable effects deriving from the concomitant intake of herbal constituents and conventional drugs, but also for further studies of positive uses of these interactions as a way to increase the bioavailability of drugs that are P-gp substrates. In particular, the study of P-gp inhibition by herbal constituents may provide an approach for the identification of lead compounds for the design of new chemosensitizers to reverse multidrug resistance in tumor cells. This review describes our recent investigations on the potential herb-drug interactions involving P-glycoprotein, using as a model the HK-2 cells, an immortalized line of proximal tubule cell derived from human normal kidney.

Keywords: HK-2 cell line, P-glycoprotein, Calcein-AM, Rhodamine-123, phytochemicals.

Resumen

El ABCB1 (MDR1/P-glicoproteína), el transportador multidroga mejor caracterizado que pertenece a la superfamilia de transportadores ABC, se reconoce hoy día por tener un gran impacto en la resistencia de los fármacos a la quimioterapia y a la biodisponibilidad y disposición de los medicamentos. Varios compuestos naturales, fitoquímicos dietéticos y remedios herbarios tienen la capacidad de modular la función y/o expresión de la glicoproteína P (P-gp) y, por consiguiente, la potencialidad para causar interacciones alimento-fármaco o hierba-fármaco. La elucidación de estas interacciones no sólo es importante para predecir posibles efectos indeseables que derivan de la ingestión concomitante de componentes herbarios y los fármacos convencionales, también se usa para los estudios sobre los usos positivos de estas interacciones como una manera de aumentar el biodisponibilidad de fármacos que son substratos de P-gp. En particular, el estudio de la inhibición de P-gp por los componentes herbarios provee un acercamiento a la identificación de compuestos para el diseño de quimio-sensibilizadores para invertir la resistencia multidroga en células tumorales. Esta revisión describe nuestras recientes investigaciones sobre las interacciones potenciales hierba-fármaco que involucran P-gp mediante un modelo de células HK-2, una línea celular inmortalizada de túbulo proximal derivada del riñón humano normal.

Palabras clave: Línea celular HK-2, P-glicoproteína, Calceína-AM, Rodamina-123, fitoquímicos.

Abbreviation list:

ABC- ATP-binding cassette  ADME- absorption, distribution, metabolism and excretion
CYP- cytochrome P450  MDR- Multidrug resistance
MRP- Multidrug resistance-associated protein  P-gp- P-glycoprotein
INTRODUCTION

Pharmacological interactions possibly derive from the modulation in expression and/or activity of two major pharmacokinetic disposition systems, namely cytochrome P450 (CYP) and the multidrug transporter P-glycoprotein (Aszalos, 2007a and 2007b; Liu et al., 2007; Marchetti et al., 2007; Pal and Mitra, 2006).

The transmembrane efflux pump P-glycoprotein (P-gp) is a member of the ATP-binding cassette (ABC) superfamily transporters, membrane-bound proteins, able to export from cells a variety of chemicals, including drugs, xenobiotics, natural products and peptides. These efflux pumps, which are powered by the energy of ATP hydrolysis, have important physiological, pharmacological and toxicological functions (Linton, 2007; Schinkel and Jonker, 2003).

P-gp, encoded by the MDR1 gene (now ABCB1), is the best known member of the ABC transporters. P-gp was initially discovered in 1970s in tumor cells as responsible for the emergence of the multidrug resistant (MDR) phenotype. The early years of P-gp research have been described in detail in a recent review by Gottesman and Ling (2006).

P-gp has been retrieved also in many normal cells and tissues where, as well as many others ABC transporters, is believed to play a significant role in detoxification and protection against xenobiotics. In fact, P-gp is physiologically expressed at the apical surface of epithelial cells of several organs, like liver, intestine and kidney, devoted to secretion/excretion of a variety of metabolites, xenobiotics and drugs. Other strategical locations are the brain blood-barrier, blood–testis and blood–ovarian barriers, and placenta (Fromm, 2004; Marchetti et al., 2007).

In the normal liver, P-gp is believed to play a significant role in the secretory processes of endo- and xenobiotics across the canalicular membrane into the bile and, like other transport systems localized to the canalicular membrane, it may be considered as one of the complex multistep process which ensures the vectorial transport of a variety of chemicals across the hepatocyte (LeBlanc, 1994). Accordingly, P-gp located at the luminal side of renal proximal tubule appears involved in the active secretion of many drugs and nephrotoxins (Ernest and Bello-Reuss, 1998), while enterocyte P-gp activity is important in the absorption process of a series of chemicals (refs. in Berggren et al., 2007).

Nowadays, P-gp is considered a crucial molecule involved in all the pharmacokinetic processes: absorption, distribution, metabolism and excretion (ADME) (Tanigawara, 2000; Varma et al. 2003) and a number of single-nucleotide polymorphisms (SNPs) in ABCB1 gene has been correlated with different responsiveness to many drugs (Marzolini et al., 2004). P-gp shows broad substrate specificity with a prevalent affinity for hydrophobic compounds; so many natural and synthetic xenobiotics and endogenous molecules are substrates/modulators of its activity (Chan et al., 2004). It is generally accepted that coadministration of compounds that interact with this transporter (as a substrate, inhibitor, or inducer) can result in interactions that affect the pharmacokinetics and pharmacodynamics of the co-administered drugs (Aszalos, 2007a; Aszalos, 2007b; Endres et al., 2006; Keogh and Kunta, 2006; Lin, 2007; Lin, 2003; Yu, 1999). Among these chemicals a number of vegetables and related phytochemicals have been reported to inhibit P-gp including garlic, black pepper, green tea polyphenols, flavonoids, curcumin, genistein, naringin, capsacin, and others (Han et al., 2006; Hu et al., 2005; Izzo and Ernst, 2001; Izzo, 2005; Jodoin et al., 2002; Meijerman et al., 2006; Molnar et al., 2006; Nabekura et al., 2005; Nabekura et al., 2007; Pal and Mitra, 2006; Rodriguez-Proteau et al., 2006; Zhou et al., 2004; Zhou et al., 2007). It is therefore crucial to rapidly identify chemical entities -including herbal remedies, nutraceuticals, and food derived compounds- able to modulate P-gp, in order to predict possible herb-drug interactions. This review will primarily focus on the recent efforts of our laboratory devoted to investigate in vitro these interactions by using, as a model, the human cell line HK-2 derived from the proximal convoluted tubule of the kidney.

Assessment of P-gp modulation by chemicals

Various screening models and methodologies have been evolved for the identification and characterization of P-gp modulators (Perloff et al., 2003; Rautio et al., 2006; Shirasaka et al., 2006; Taub et al., 2005; Varma et al., 2003). Each technique has its own advantages and limitations. The most commonly in vitro methods used to evaluate whether a chemical compound is a modulator of the P-gp efflux transporter are three: i) Bi-directional transport assay with a probe P-gp substrate in functionally polarized epithelial cell monolayer. The preferred cells lines include Caco-2, transfected LLC-PK1-
MDR1, and transfected MDCK-MDR1. LLC-PK1 and MDCK wild type cells are used as negative controls; ii) ATPase activity assay that measure substrate stimulated-ATPase activity in the presence of substrates, which interact on different P-gp sites, with and without test compounds, allowing identification of P-gp inhibitors. ATPase activity can be determined using cell membranes, cultured cells and membrane vesicles; iii) Drug accumulation and efflux assays where the amount of a substrate, generally a fluorescent probe, taken up by cells expressing P-gp, is compared in the presence and absence of inhibitors. This last method, also if cannot distinguish substrate from inhibitors, can screen compounds rapidly and could suggest further investigations employing more complex methodologies.

In our laboratory, for the study of possible interactions between chemicals and P-gp, we utilized a cell line from human kidney proximal tubule and uptake/efflux assays. Furthermore, because the expression of P-gp may be influenced by chemicals, we used this cell line also for the in vitro evaluation of P-gp modulation at protein (immunoblotting) and at mRNA (RT-PCR) levels.

**Renal P-glycoprotein**

Together with the liver, the kidney is the most important detoxifying organ (Inui et al., 2000). The kidney tubular cell has a large spectrum of functions, which modify the ultrafiltrate before it’s turning into final urine. The modification regards solutes secretion or solutes absorption. Many of these movements of substances between blood and tubular duct occur by different carrier work. The proximal tubular cells have a transport activity higher than all the other renal segments and, furthermore, are exposed to high concentrations of xenobiotics. These cells express a lot of membrane transporters, some of which belonging to the ABC superfamily, in particular P-gp (ABCB1) (Kruidering et al., 1994; Lee and Kim, 2004; Schlatter et al., 2006). P-gp is localized together other multidrug resistance-associated proteins (MRPs) on apical surface (brush border) and is implicated in endogenous substrate transport and tubular secretion of toxic substances (Ernest and Bello-Reuss, 1998).

A scheme of the main ABC transporters present in the proximal tubular cell is shown in Fig. 1.

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**Figure 1.** Localization of P-gp and other ABC transporters in the kidney proximal tubule.

*Left:* Immunohistochemical localization of P-gp in human kidney (polyclonal anti mdr Ab-1).

*Right:* P-gp/MDR1 (ABCB1), MRP2 (ABCC2), MRP4 (ABCC4) and BCRP (ABCG2) are expressed on the apical (luminal) membrane of renal epithelial cells and export compounds from the cytoplasm of tubule cell to the urine. MRP1 (ABCC1) and MRP6 (ABCC6) are expressed on the basolateral membrane. (Inui et al., 2000; Huls et al., 2008; Lee and Kim, 2004).
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P-glycoprotein modulation by natural compounds in HK-2 cells

The HK-2 cell line

HK-2 cell line is an immortalized line derived from the human epithelial renal proximal tubule. Zager and coll. utilized a kidney from a male normal adult considered unsuitable for transplantation (Ryan et al., 1994). The cortical proximal tubule segment was isolated, cultured and exposed to a recombinant virus containing the E6 and E7 genes of HPV16. A cell clone, in which PCR analysis confirmed the incorporation of HPV16 E6/E7 construct in genomic DNA, was designed as HK-2 and was able to continuously grow for more than a year. At the molecular level the products of E6 and E7 genes bind to the DNA regulatory proteins, resulting in a cell facilitated proliferation.

Phenotypically, the cell line HK-2 has the same characteristics of adult normal tubular cells, as demonstrated by a series of biochemical tests. In particular, it was shown that the HK-2 cells maintain the brush border typical enzymatic activities (acid and alkaline phosphatase, leucine aminopeptidase, gammaglutamyltranspeptidase).

Therefore, this new line has been proposed as a valuable tool for the study of physiological and physiopathological human renal tubule, as well as the mechanisms of damage and repair at the level of tubular cell and has been widely employed in research. In our laboratory we showed, for the first time, the presence of a functional MDR1 (ABCB1)-encoded P-gp in HK-2 cells (Tramonti et al., 2001) and thereafter we characterized such a line for a series of parameters regarding this transporter, including the presence of MDR1, but not MDR3 (ABCB4) transcripts, expression at protein level on cell membranes, immunocytochemical localization and the responsiveness to a series of modulators including acknowledged P-gp inhibitors, like verapamil and cyclosporin A, as well as some drugs, like dexamethasone and calcitriol (Romiti et al., 2002). On the whole, the results suggested that HK-2 cells could be suitable as a tool to investigate the role of P-gp in renal physiopathology. Further characterization have indicated that HK-2 line, while expressing a robust amount of P-gp, that resulted not only quite stable in the time, passage number, culture conditions, but also responsive to chemical modulation, apparently lacks expression of the apical ABC transporter MRP2 (Fig. 2, Chieli and Romiti, unpublished results).

Figure 2. RT-PCR and Western blot analysis of ABCB1/P-gp and ABCC2/MRP2 in HK-2 or Caco-2 cell lines.

Left: RT-PCR analysis shows that HK-2 express MDR1 (ABCB1) mRNA only, while Caco-2 cell line expresses both MDR1 (ABCB1) and MRP2 (ABCC2) mRNAs. M.W.,123-bp DNA ladder. Right: Western blot analysis of crude membranes from HK-2 or Caco-2 cells shows that both cell lines express, also if to a different degree, P-gp, while the band corresponding to MRP2, well represented in Caco-2, is undetectable in HK-2 cell membranes (lane 3). For immunoblotting monoclonal anti-P-gp or polyclonal anti-MRP2 antibodies and peroxydase-conjugated anti-mouse or anti-rabbit secondary antibodies were used. Blots were developed with the ECL detection system.

MRP2 together with P-gp are known as the main apical transporters in tubule cell, devoted to excretion of drugs and xenobiotics in the tubule lumen (refs. in Huls et al., 2008). The absence of MRP2 expression and, in parallel, the observed lack of effect of probenecid, an inhibitor of MRPs, in P-gp functional tests (Nieri et al., 2006), suggest the HK-2 cells suitable to assay chemical effects selectively on P-gp, rather than cells in which also MRP2 is appreciably expressed, e.g. Caco-2 (Prime-Chapman et al., 2004).

Assessment of chemical-P-gp interactions: methodological notes

The HK-2 model has been used by us to check if chemical compounds (in particular natural compounds from vegetables) present in food or employed in phytotherapy, may modify the in vitro expression and function of P-gp. While P-gp expression at protein level (immunoblotting) or at mRNA level (RT-PCR) doesn’t pose difficulties in interpretation, functional assays for P-gp merit some comments.

Throughout our studies we have used two different tests to evaluate the influence of chemicals on P-gp activity, i.e. the Calcein-AM and the Rhodamine-123 accumulation assays. The principle of these assays are schematically shown in Figs. 3 and 4.

**Figure 3.** Scheme of the Calcein-AM assay for the assessment of P-glycoprotein activity.

The P-gp substrate nonfluorescent Calcein-AM is hydrolysed by cellular esterase to the fluorescent Calcein. Calcein is not a substrate for P-gp and it cannot leave the cell via the plasma membrane, whereas the nonfluorescent Calcein-AM is extruded from the MDR-1 expressing cells. The intracellular accumulation of fluorescent Calcein is inversely proportional to the transport capacity of P-gp. A low expression of P-gp or the presence of P-gp inhibitors produces a rise in cell fluorescence.

**Figure 4.** Scheme of the rhodamine-123 assay for the assessment of P-glycoprotein activity.

Left: scheme of the rhodamine-123 test (Rho-123) for the measurement of P-gp activity. The fluorescent mitochondrial probe Rho-123 enters into the cells and accumulates in cytoplasm and mitochondria. Because Rho-123 is a substrate for P-gp and it is extruded actively form the cells, the intracellular amount of Rho-123 is inversely proportional to P-gp activity. Inhibitors of P-gp cause an increase in cell fluorescence.

Right: effects of two acknowledged inhibitors of P-gp, Verapamil and Cyclosporine A on Rho-123 accumulation by HK-2 cells. Cells were incubated in medium containing 5 μg/ml Rho-123 for two hours; thereafter cell plates were washed and extracted with n-butanol. Rho-123 was quantified by spectrophotofluorimetry. At the top: the relative fluorescence microscope images.
Calcein-AM test: This assay is largely employed (Eneroth et al., 2001; Holló et al., 1994; Tiberghien and Loo, 1996). At least two main cautions in the measurement of P-gp activity by this method must be considered. First, it is known that whereas Calcein-AM is a substrate of P-gp, the fluorescent calcein is transported, out of the cell, by MRPs, therefore, this test couldn’t be strictly specific for P-gp. Anyway, in HK-2 this possibility may be reasonably excluded, due to the fact that, as yet recalled, the major apical MRP transporter, MRP2, is apparently not expressed; furthermore, the MRPs inhibitor probenecid did not influence, in HK-2 cells, the results of Calcein-AM test (Nieri et al., 2006). Secondly, it must be taken into account that, independently from P-gp activity, the fluorescent calcein derives from the action of esterase on the nonfluorescent precursor Calcein-AM (Fig. 3). This occurrence -also reflected by the fact that Calcein-AM test is also employed as an index of cell viability- alerts on possible interferences by compounds able to influence cell esterase activity. In this regard it is suggestive that some flavonoids have been described recently to inhibit esterase activity (Li et al., 2007). Effects of this type (or even opposite) could influence the results of Calcein-AM assay causing under/overestimation of the test compound influence on the P-gp activity.

Rhodamine-123 test: This test is also widely used (Perloff et al., 2003; Wang et al., 2006). However, this also requires some cautions. First, Rho-123 is a fluorescent mitochondrial dye, therefore it accumulates in cell and mitochondrial uptake accumulation may depend on mitochondrial transmembrane potential (MTP) and cell membrane potential. Moreover, even if Rho-123 has always been considered to enter into the cells in a passive way, Troutman and Thakker (2003) have suggested the presence of a membrane influx carrier for this fluorescent probe. These things must be taken into account especially when a lack of correlation is observed between the results obtained with different methods, making difficult the interpretation.

From some time, routinely, in our laboratory, always we use both Calcein-AM and Rho-123 tests, which in most situations gave congruent results.

Table 1 summarize our studies about the interactions between herbal constituents and P-gp in the HK-2 model.

Flavonoids

The plant flavonoids are a wide class of natural compounds commonly present in fruits, vegetables and beverages obtained from plants, like tea, red wine, and beer. Consumption of a diet rich in flavonoids is believed to produce a variety of health benefits, including antioxidant, anti-inflammatory, antiallergic effects, as well as protection in cardiovascular diseases and cancer (Yao et al., 2004). Flavonoids have been recognized as P-glycoprotein modulators for a long time, also if contradictory effects were reported in studies using different multidrug-resistant cell lines. Anyway, because flavonoids were shown to be modulators with bi-functional interactions at vicinal ATP-binding site and steroid-interacting region of P-gp, the controversial reports about flavonoid-P-gp interactions are now generally ascribed to the different binding of the model substrates to multiple binding sites of P-gp (Shapiro and Ling, 1997; Conseil et al., 1998). The effects of flavonoids on ABC transporters, including P-gp, have been recently reviewed and, from the majority of the more recent studies, the indication emerges that flavonoids are on average inhibitors of P-gp-mediated transport (Di Pietro et al., 2002; Morris and Zhang, 2006).

In our previous studies employing primary rat hepatocytes as a model of cells expressing mdr1b P-gp we found that the flavonols quercetin, kaempferol and galangin were able, also if at a different degree, and with differences related to the used substrate, to modulate P-gp activity (Chieli et al., 1995). Other flavonoids, like the flavone apigenin, were also shown to inhibit P-gp activity, and, more consistently, to significantly downregulate P-gp expression in primary rat hepatocytes (Chieli et al., 1998). These results have been confirmed also in the HK-2 model (Fig. 5, unpublished results).

The effects of apigenin appear very interesting by considering that it is a common flavonoid, present in a variety of vegetables, as well as in their extracts, marketed as diet and herbal supplements. Apigenin has demonstrated anticancer activities and chemopreventive potential towards prostate cancer (Patel et al., 2007).
Apigenin, and to a minor extent, kaempferol, downregulate Pgp amounts (top) and mdr1 transcripts (bottom) in HK-2 cells and in rat hepatocytes.

Cont- Control, Kaem- Kaempferol, Apig- Apigenin, Curc- Curcumin, Resv- Resveratrol

Like many other flavonoids, apigenin is poorly bioavailable and little is known about its absorption and metabolism. Apigenin disposition has been studied in Caco-2 cells by Hu et al. (2003). The downregulation of P-gp expression, observed by us (in two different models) coupled with the inhibition of transport function, could theoretically lead to pharmacokinetic interactions with drugs that are transported by P-gp by enhancing their oral bioavailability. Of course, the relative contribute of CYP3A4 ought to be investigated. Our results are in agreement with a recent study of Lohner et al. (2007) in which apigenin, unlike many other flavonoids, was found unable to induce P-gp expression in Caco-2 cells and intestinal cells in vivo.

Some flavonoids belonging to different subclasses, i.e. epigallocatechin gallate (EGCG) the most abundant flavanol found in green tea, the flavanone naringenin, abundant in grapefruit juice and the chalcone xanthohumol, a flavonoid abundant in hop (Humulus lupulus), a beer ingredient, were also tested in HK-2 cells. All of three phytochemicals were able, with different potencies, to decrease the activity of P-gp (Table 1), according to literature data obtained in the Caco-2 transport model (Jodoin et al., 2002; Rodriguez-Proteau, 2006) or in other cell line, like the MDR overexpressing KB-C2 cells in which P-gp activity inhibition was assessed by the Rho-123 accumulation test (Kitagawa et al., 2005). Furthermore, besides the inhibition of P-gp function, we observed also a significant decrease of P-gp expression in immunoblots of HK-2 cells cultured in the presence of these compounds.

Other polyphenols

Another phenolic compound, which has received much attention in recent years, resveratrol, has been investigated in the HK-2 model. Resveratrol (trans-3,5,4′-trihydroxystilbene), a natural product derived from grapes, has been shown to have a series of beneficial properties, including anti-inflammatory, antioxidant and chemopreventive activities (Das and Maulik, 2006; Holme and Pervaiz, 2007; Shankar et al., 2007). Resveratrol was found to inhibit significantly P-gp-dependent Rho-123 efflux from rat hepatocytes (Chieli et al., 1998) with the same potency of the P-gp inhibitor verapamil, but in the HK-2 cells it was unable to produce significant effects on P-gp activity, also if able to slightly increase the P-gp immunoblottable amount in cells cultured for three days in its presence (Table 1). This result suggests that pharmacokinetic interactions of resveratrol with P-gp substrates are improbable. On the other hand, red wine, assayed in our model, produced a substantial increase in P-gp expression at protein level, possibly due to the phenolic mix present in this beverage.

Curcumin, (diferuloylmethane), is a natural phenolic yellow pigment present in the roots of Curcuma longa that has attracted great interest for its biological activities, potentially useful for clinical use, including antioxidant, anticarcinogenic and hepatoprotective properties (refs. in Romiti et al., 1998). Our previous studies employing the model of primary rat hepatocytes showed that curcumin was able to lower the increase of mdr1b P-gp that spontaneously occurs during culture, and to inhibit
| Table 1. Interactions of various chemicals with P-glycoprotein investigated on the HK-2 cell model. |
|-------------------------------------------------|------------------|-----------------|------------------|-----------------|------------------|
|                                                 | Inhibition of P-gp activity | P-gp expression | MDR-1 expression | References       |
|                                                 | Rho-123 | Calcein-AM | WB |         | RT-PCR |         |         |         |
| Pure phytochemical compounds                    |         |           |    |         |         |         |         |         |
| Apigenin                                        | ++      | +         | decreased | decreased |         |         |         |         |
| Crocin                                          | none    | none      | n.d. | n.d.    |         |         |         |         |
| Curcumin                                        | +       |           | =    | =       |         |         |         |         |
| Resveratrol                                     | none    | n.d.      | ≥    | =       |         |         |         |         |
| Kaempferol                                      | ++      | ++        | decreased | n.d.    |         |         |         |         |
| Naringenin                                      | n.d.    | ++        | decreased | n.d.    |         |         |         |         |
| EGCG                                            | + + +   | +         | decreased | =       |         |         |         |         |
| Xanthohumol                                     | + + +   | +         | decreased | decreased |         |         |         |         |
| Polyacetylenes & polyenes from *Echinacea pallida* | + +     | +        | n.d. | n.d.    |         |         |         |         |
| Crude extracts                                  |         |           |    |         |         |         |         |         |
| Hypericum p.                                    | + + +   | +         | decreased | decreased | unpublished results |
| Humulus l.                                      | + +    | +         | decreased | =       |         |         |         |         |
| Neem oil                                        | +       | + +       | n.d. | n.d.    |         |         |         |         |
| Saffron                                         | ≥  | ≥       | increased | =       |         |         |         |         |
| Propolis                                        | n.d.    | n.d.      | decreased | n.d.    |         |         |         |         |
| Echinacea spp. n-hexane extracts                | + +    | +        | n.d. | n.d.    |         |         |         |         |
| Beverages                                       |         |           |    |         |         |         |         |         |
| Grapefruit                                      | +       | + +       | decreased | decreased |         |         |         |         |
| Red wine                                        | n.d.    | n.d.      | increased | n.d.    | unpublished results |
| Drugs                                            |         |           |    |         |         |         |         |         |
| Calcitriol                                      | + + + + | +         | increased | increased |         |         |         |         |
| Cimetidine                                      | none    | +         | =    | =       |         |         |         |         |
| Cyclosporin A                                   | + + + + | +         | increased | increased |         |         |         |         |
| Dexamethasone                                   | + +    | +         | increased | increased |         |         |         |         |
| Verapamil                                       | + +    | + + +     | =    | =       |         |         |         |         |
| Simvastatin                                     | +       | + + +     | increased | n.d.    |         |         |         |         |
| Cannabinoids                                    | + +    | + + +     | ≥    | n.d.    |         |         |         |         |

EGCG-epigallocatechin gallate; n.d.- non determined; WB- Western blot
the P-gp-dependent efflux of Rho-123 from hepatocytes in a dose-dependent manner. These observations suggested curcumin as a compound endowed with chemosensitizing properties on MDR phenotype. Recently, such properties of curcumin have been confirmed by various studies (Chearwae et al., 2004; Limtrakul, 2007; Zhang et al., 2007) and the mechanism by which curcumin downregulates the multidrug-resistance mdr1b gene of rat, i.e. the inhibition of PI3K/Akt/NfkappaB pathway, has been identified (Choi et al., 2008). In spite of these results in the HK-2 model curcumin did not modify P-gp expression (Table 1). Furthermore, also interference on Rho-123 accumulation, as an index of P-gp activity inhibition, was very weak. However, these negative results coming from few and preliminary studies need confirmation.

St. John’s wort

The traditional herb St. John’s wort (Hypericum perforatum) has been used since ancient times as a medicinal herb. Today St. John’s wort is widely used for the treatment of mild/moderate depressive disorders (Schulz, 2006). Self-medication with commercial preparations of this herb is acknowledged to cause important interactions with prescribed drugs (Dasgupta et al., 2007; Zhou et al., 2004). The mechanism behind these adverse reactions is believed to be the induction of drug metabolizing enzymes, in particular CYP3A4, the CYP isozyme most abundant and/or the multidrug transporter P-gp (Dürr et al., 2000).

We have tested a crude extract of a commercial preparation of St. John’s wort in our cell model. Western blot of HK-2 cell membranes cultured for three days in the presence of various concentrations of the extract showed a weak, but significant increase in P-gp immunoblottable amount, suggesting P-gp induction (Fig. 6). This result is in agreement with the hypothesis that P-gp induction by St. John’s wort may lead to the decrease in the bioavailability of drugs that are P-gp substrates by increasing their efflux. On the other hand, both Rho-123 and Calcein-AM functional assays indicated a significant inhibition of P-gp pump. The reason for this result is unknown, yet, but it could be ascribed to the effects of hypericum active principles e.g. hypericin, acting either as inducer as well as inhibitor of P-gp (Pal and Mitra, 2006). Furthermore, the commercial preparations of hypericum contain a wide range of flavonoid amount (Gödtel-Armbrust et al., 2007) that could contribute to the observed inhibition of P-gp activity.

Figure 6. Effects of Hypericum perforatum extracts on HK-2 P-gp expression and P-gp activity.
Grapefruit juice

Grapefruit juice (GFJ)-drug interactions, firstly reported in 1990’s, have been demonstrated by many in vitro and in vivo studies (Kane and Lipsky, 2000). GFJ has proven to alter the pharmacokinetics of a variety of drugs, including calcium channel blockers, immunomodulators, statins and others. The major mechanism of GFJ-drug interactions appeared to be the inhibition of CYP enzymes, in particular the CYP3A4 isoform, causing the reduction of the “first-pass” metabolism. However, other mechanisms have been demonstrated, in particular modulation by GFJ of P-gp-mediated efflux transport in intestinal cells. Since CYP3A4 and P-gp share a variety of substrates, their function could modulate in concert the bioavailability of drugs, by affecting their pre-systemic clearance (Kiani and Imam, 2007; Mertens–Talcott et al., 2006).

Although the literature findings about GFJ-P-gp interactions are sometimes in conflict, reporting both activation and inhibition of P-gp transporter by GFJ, recent in vivo and in vitro observations suggest that GFJ is able to inhibit the efflux of P-gp substrates (de Castro et al., 2007).

In our investigations on possible interactions between grapefruit and P-gp in the HK-2 model, we showed that GFJ, as well as its main phenolic constituents, kaempferol and naringenin, were able to inhibit P-gp activity in HK-2 cells in a concentration-dependent fashion (Romiti et al., 2004). In the same year, Honda et al., (2004), observed the same inhibitory effect of GFJ and some GFJ components, on P-gp activity of Caco-2 cells and demonstrated that GFJ was able to interact with not only P-gp but also with MRP2. Because HK-2 cells apparently lack MRP2 (as yet mentioned), the transport inhibitory effects observed in this cell line may be referred more selectively to P-gp. Besides the Calcein-AM assay, employed in our study, the Rho-123 test, even if to a lesser extent, also suggests a modulation of P-gp activity by GFJ (Fig. 7).

In the same study, we observed also a down-regulation of P-gp in cells cultured for few days in presence of GFJ and its main phenolics, in the absence of any toxicity. As a functional consequence, the decreased amount of P-gp protein made cells more susceptible to toxic effects of P-gp drug substrates, like cyclosporine A or vinblastine. These findings suggested to us that GFJ could alter the handling of P-gp substrates not only by modulating P-gp dependent transport, but also P-gp amount. Such interactions might play additive roles in modify pharmacokinetic of P-gp substrates.

Figure 7. Effects of grapefruit juice on HK-2 P-gp activity

HK-2 P-gp activity modulation by concentrated grapefruit juice obtained by a commercial source (GFJ), or juice obtained by hand-squeezed fresh fruit (GF). Final concentration of both juices in culture medium was 5%. Verapamil (Vp) was used as internal control. Bars are mean ± SD of a representative experiment with five replicates. Either the Calcein-AM assay or - also if to a lesser extent - the Rho-123 test, show an increase in cell fluorescence in treated cells as compared with controls, suggesting an inhibition of P-gp pump function.

Echinacea

One of the most frequently used medicinal plants both in the United States and in Europe, is today represented by echinacea, a popular herb used for centuries by Native Americans primarily to reduce the symptoms and duration of colds and flu-like illness. A variety of health benefits have been attributed to echinacea, including the increase in non specific activity of the immune system, facilitation of wound healing, anti-inflammatory and antioxidant effects due to the presence in the medicinal echinacea species (E. angustifolia, E. purpurea, E. pallida) of several classes of active constituents in particular polyphenols, polysaccharides and alkylamides (Barnes et al., 2005, Woelkart et al., 2006; Woelkart and Bauer, 2007). In spite of the impressive record of laboratory and clinical research, many aspects of echinacea properties are still lacking. In particular, very few studies have been devoted to investigate possible interactions between echinacea and the major pharmacokinetic disposition systems. While the effects of echinacea on CYP have been recognized (Yale and Glurich, 2005; Modarai et al., 2007), the
influence of this plant on P-gp is largely unknown. A very recent in vivo study (Gurley et al., 2008) suggests that echinacea supplementation probably should not be a cause of drug interactions dependent on P-gp, being unable to significantly modify the pharmacokinetic profile of digoxin, a P-gp substrate. However, at the same time, in our in vitro model, we have shown for the first time that lipophilic extracts of echinacea roots are able to inhibit P-gp activity in HK-2 cells (Romiti et al., 2008). Furthermore, isolated components from E. pallida, like poly-acetylenes and polyenes (Pellati et al., 2006), were also able to inhibit P-gp-dependent transport (Table I and Fig. 8). The observed inhibitory effects on HK-2 P-gp by echinacea extracts or by the lipophilic components of E. pallida are not, on the whole, dramatical. However, at the same time, in our in vitro model, we have shown for the first time that lipophilic extracts of echinacea roots are able to inhibit P-gp activity in HK-2 cells (Romiti et al., 2008). Furthermore, isolated components from E. pallida, like poly-acetylenes and polyenes (Pellati et al., 2006), were also able to inhibit P-gp-dependent transport (Table1 and Fig. 8). The observed inhibitory effects on HK-2 P-gp by echinacea extracts or by the lipophilic components of E. pallida are not, on the whole, dramatical. However, by considering the widespread and increasing use of echinacea preparations in complementary and alternative medicine, this feature of the plant constituents requires additional investigation and suggests some caution in the use of echinacea herbal products for conventional therapy. On the other hand, also the potentiality of echinacea phytochemicals as chemosensitizers under P-gp mediated resistance in cancer therapy deserves further consideration.

**CONCLUSION**

Recently, many important advances have been made in the knowledge of the roles played by drug metabolizing enzymes (phase I, like CYP), phase II (various conjugating enzymes), or phase III (several ABC transporters, including P-gp), in the ADME process involving xenobiotics, drugs and natural compounds. In parallel, evidences for the likely interactions drug-drug, drug-herb, herb-herb, are also dramatically increased. Against this very complex background, the importance emerges of a rapid individualization of chemicals potentially able to cause such adverse reactions. In addition to sophisticated methods, expensive, time-consuming, and sometimes not well reproducible, it may be useful to have simple in vitro assays to screen compounds for interactions with the major biological determinants of drug disposition. Our results show that HK-2 cell line may be conveniently employed as a sensitive, versatile, reproducible tool to investigate the effects of various chemicals on P-gp, providing indications that can represent a starting point for more refined and detailed studies.

**REFERENCES**


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