Inhibition of P-Glycoprotein Transport Function by Grapefruit Juice Psoralen

Er-jia Wang,[1] Christopher N. Casciano,[1] Robert P. Clement,[1] and William W. Johnson[1,2]

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Purpose. The grapefruit juice component bergamottin is known to inactivate cytochrome P450 3A4, with grapefruit juice consumption causing increased absorption and enhanced oral bioavailability of many cytochrome P450 3A4 substrates. Many of these substrates are also recognized by the efflux transporter P-glycoprotein. The gene product of MDR1 (multidrug resistance transporter), P-glycoprotein also confers protection against xenobiotics.

Methods. Using a whole cell assay in which the retention of a marker substrate is evaluated and quantified, we studied the ability of grapefruit juice components to inhibit the function of this transporter.

Results. In a cell line presenting an overexpressed amount of the human transporter, the enzyme exhibited a 40 μM IC_{50} for inhibition by bergamottin. Additionally, using the ATP-hydrolysis assay, we showed that bergamottin increases P-gp-mediated ATP hydrolysis by approximately 2.3 fold with a K_m of 8 μM. The concentration for this interaction is similar to that for CYP3A4 inactivation.

Conclusions. These results suggest that observed grapefruit juice – drug pharmacokinetic clinical interactions may be due to P-gp inhibition rather than or in addition to CYP3A4 inhibition. Inhibition of P-gp by citrus psoralens could present ways both to enhance bioavailability of therapies without increasing the dose and to diminish drug resistance in refractory cells.

KEY WORDS: P-glycoprotein; grapefruit; MDR; furanocoumarin; drug interaction.

INTRODUCTION

Cellular resistance to a wide spectrum of xenobiotics and endogenous lipophilic amphiphiles has been a subject of increasing interest to those concerned with the disposition of many therapeutic drugs (1). Of the various forms of resistance, the phenomenon of a multidrug resistance (MDR) transporter has received the most attention, as it plays a major role in the disposition of many drugs (2–5). The most extensively studied MDR transporter, the gene product of MDR1, appears to be a single 130–190 kDa (depending on the degree of glycosylation and/or phosphorylation) membrane glycoprotein known as P-glycoprotein (P-gp). Other members of this family include the multidrug resistance-associated proteins (MRP1, MRP2, MRP3, etc.).

These transport proteins evidently act as ATP-driven pumps which remove xenobiotics from the interior of cells. Expression of P-gp in normal human tissues, particularly within the cellular membranes of the gastrointestinal tract, liver, blood-brain barrier, adrenals, and kidneys, suggests that the enzyme plays a role in cellular protection as well as in secretion (2). While the primary function of this protein is unknown, its ability to confer resistance to a wide variety of structurally and chemically unrelated compounds remains impressive. Indeed, the substrate list for this transporter now appears to share a similar tolerance or acceptance as cytochrome P450 3A4 (CYP3A4), the predominant intestinal and hepatic cytochrome P450 oxygenase enzyme, and may even prove to be more extensive in its substrate recognition.

As a member of the ATP-binding cassette (ABC) superfamily of transporters, P-gp possesses two ATP binding sites and uses ATP (via hydrolysis) as the source of energy for 'translocating' substrates (6–8). The substrates enter from the lipid bilayer, and can bind to two (or more) nonidentical sites (9,10). Moreover, allosteric and perhaps synergistic effects have been observed for certain substrate combinations and conditions (11,12).

Various drug metabolizing enzymes have been associated with clinical interactions between drugs and other xenobiotics or food and herbal components. Grapefruit juice consumption has been shown to significantly increase oral bioavailability of various drugs metabolized by cytochrome P450 3A4 (CYP3A4) (dihydropyridines (13), cyclosporine A (14), midazolam (15), triazolam (16), terfenadine (17), saquinavir (18), lovastatin (19), simvastatin (20), atorvastatin (21) and ethinylestradiol (22)). The observed interactions are purported to have been caused by grapefruit juice inhibiting the activity and decreasing the content of intestinal and hepatic CYP3A4. Of the several furanocoumarins in grapefruit juice extract, bergamottin (BG) is the most abundant (≈ 23 μM) (23), and was recently shown to rapidly inactivate CYP3A4 with a rate of 0.3 min^{-1} and a K_i of 7.7 μM (23). In addition to BG, other constituents include the flavonoids naringenin, naringin, quercetin, and kaempferol, which have been shown to competitively inhibit CYP3A4 (24,25).

Because the substrates for P-gp and CYP3A4 are very similar, it is difficult to discern whether the observed drug interactions thought to be mediated by CYP3A4 substrates/inhibitors are due in part to P-gp. Indeed, recent *in vitro* results indicate that an extract of grapefruit juice can inhibit the transport of vinblastine (26). Additionally, the grapefruit juice components naringin and 6′,7′-dihydroxybergamottin were suggested to be moderate inhibitors of P-gp-mediated saquinavir transport in Caco-2 cell monolayers (27). This report, therefore, presents an evaluation of several grapefruit juice constituents for their ability to inhibit the function of P-gp active transport and quantifies this effect. These observations demonstrate that bergamottin is a potent competitive inhibitor of P-gp.

MATERIALS AND METHODS

Chemicals

Bergamottin and kaempferol were purchased from Indofine Chemical Company (Somerville, NJ). Daunorubicin (DNR), verapamil, colchicine, cyclosporin A, quercetin, naringin, naringenin, mannitol, dithiothreitol, ATP disodium, ammonium molybdate, ascorbic acid, sodium meta-arsenite, aprotinin, leupeptin, EGTA, EDTA, HEPES, ouabain,

[1] Drug Metabolism and Pharmacokinetics, Schering-Plough Research Institute, Lafayette, New Jersey 07848.

[2] To whom correspondence should be addressed. (e-mail: William.w.johnson@spcorp.com)

ABBREVIATIONS: P-gp, P-glycoprotein; ABC, ATP-binding cassette; MDR, multidrug resistance; BG, bergamottin; DNR, daunorubicin.

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phenylmethylsulfonyl fluoride, and TRIZMA base were purchased from Sigma Chemical Co. (St. Louis, MO). Hanks' balanced salt solution, Alpha Minimum Essential Medium, DMEM, penicillin/streptomycin, fetal bovine serum (FBS), and trypsin-EDTA were obtained from Life Technologies, Inc. (Rockville, MD). Microplates (Costar 96-well), plastic tubes, and cell culture flasks (75 cm^2) were purchased from Corning Inc. (Corning, NY). All other reagents were of the highest grade commercially available.

Cell Lines

CR1R12 cell line, provided by Dr. Alan Senior (Univ. of Rochester), was maintained in complete α-minimum essential medium (α-MEM) supplemented with 10% FBS, penicillin/streptomycin (50 units/50 μg/ml) in a 5% CO_2-95% air atmosphere at 37°C. Colchicine (0.5 μg/ml) was added to the culture medium. Cells were grown to 80–90% confluency and treated with trypsin-EDTA before subculturing. The 3T3 G185 cell line presenting the gene product of human MDR1 was licensed from NIH and maintained in DMEM.

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 argon laser (excitation at 488 nm, emission at 530/30 and 570/30 nm band-pass filters). Analysis was gated to include single 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 direct functional assay for the P-gp efflux pump in MDR cells was performed with the flow cytometer (28).

Cell Viability Test

Cell viability was assessed using exclusion of 0.4% trypan blue as well as 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 DNR fluorescence intensity of individual cells was recorded as histograms. The mean fluorescence intensity of 10,000 cells was used for comparison among different conditions. Verapamil was selected as a positive control to normalize the measurements because it can extensively inhibit the P-gp efflux pump. Relative fluorescence was used for quantitation and comparison among different compounds. The relative fluorescence (% inhibition) represents a ratio obtained through the following formula: the geometric mean fluorescence of a discrete sample divided by the geometric mean fluorescence in the presence of 10 μM verapamil, times 100 or expressed as:

$$\text{Relative fluorescence} = \frac{\text{Fluorscence of sample geometric mean}}{\text{Fluorescence of verapamil geometric mean}} \times 100$$

Membrane Microsome Preparations

CR1R12 cell membranes enriched with the MDR1 gene product transport enzyme were used for preparation of membrane microsomes. Cells were washed with complete Hanks' buffer before being resuspended in 10 ml lysis buffer (Tris-HCl, 50 mM; mannitol, 50 mM; EGTA, 2 mM; and dithiothreitol, 2 mM; pH 7.0 at 25°C) containing protease inhibitors (phenylmethylsulfonyl fluoride, 1 mM; aprotinin, 10 μg/ml; leupeptin, 10 μg/ml). All subsequent steps were performed at 4°C. The cells were lysed by nitrogen cavitation (Parr Instrument Co., Moline, IL) at 500 psi for 15 min twice. Nuclei and mitochondria were sedimented by centrifugation at 4000 ×g for 10 min. The microsomal membrane fraction was then sedimented by centrifugation at 100,000 ×g for 60 min. The pellet was resuspended in 0.25 M sucrose buffer (10 mM Tris-HCl, 1 mM EDTA, pH7.5) and homogenized using a Potter-Elvehjem homogenizer. Aliquots of membrane microsomes were rapidly frozen and stored at −80°C until analysis.

ATP Hydrolysis and Phosphate Release

The consumption of ATP was determined by the liberated inorganic orthophosphate, which forms a color complex with molybdate (29). We have developed an ATP hydrolysis assay based on phosphate-release determination using membrane microsome preparations (30,31). The method was modified to be carried out in a 96-well microplate. The microsomes were thawed on ice prior to diluting to 3.5 μg protein per well in ice-cold ATPase buffer (sodium ATP, 3 mM; KCl, 10 mM; MgSO4, 10 mM; dithiothreitol, 3 mM; Tris-HCl, 50 mM; pH 7.0) containing 0.5 mM EGTA (to inhibit Ca-ATPase), 0.5 mM ouabain (to inhibit the Na/K-ATPase), and 3 mM sodium azide (to inhibit the mitochondrial ATPase). The total incubation volume including the various inhibitors was 100 μl. The incubation reaction was initiated by transferring the plate from ice to 37°C, incubated for 30 min, and then terminated by the addition of 50 μl 12% SDS solution at room temperature, followed by the addition of 50 μl of a mixture solution of (equal volumes) of 18% fresh ascorbic acid in 1N HCl and 3% ammonium molybdate in 1 N HCl. After 4 min, 100 μl of a solution of 2% sodium citrate and 2% sodium meta-arsenite in 2% acetic acid was added to fix the color formation. After 30 min incubation at room temperature, the fixed released phosphate was quantitated colorimetrically in a microplate reader (Bio-Tek FL600, VT) at 750 nm. The respective values for background with ATPase assay buffer alone were obtained in parallel and subtracted from the values for experimental samples. By comparison to a standard curve, the amount of phosphate released, and hence ATP consumed, was quantified. Water-insoluble drugs were dissolved in methanol; the maximum methanol concentration (2% v/v) was shown not to affect the ATPase activity. The data was analyzed similar to previous models (10).

RESULTS

As a fluorescent substrate transported by P-gp, DNR serves as a marker for active transport function simply by measurement of fluorescence per cell (28). Herein we show that BG can effectively inhibit the P-gp-mediated transport of DNR. The IC_{50} (concentration at half maximum inhibition) can be determined from a simple function as shown in Fig. 1,

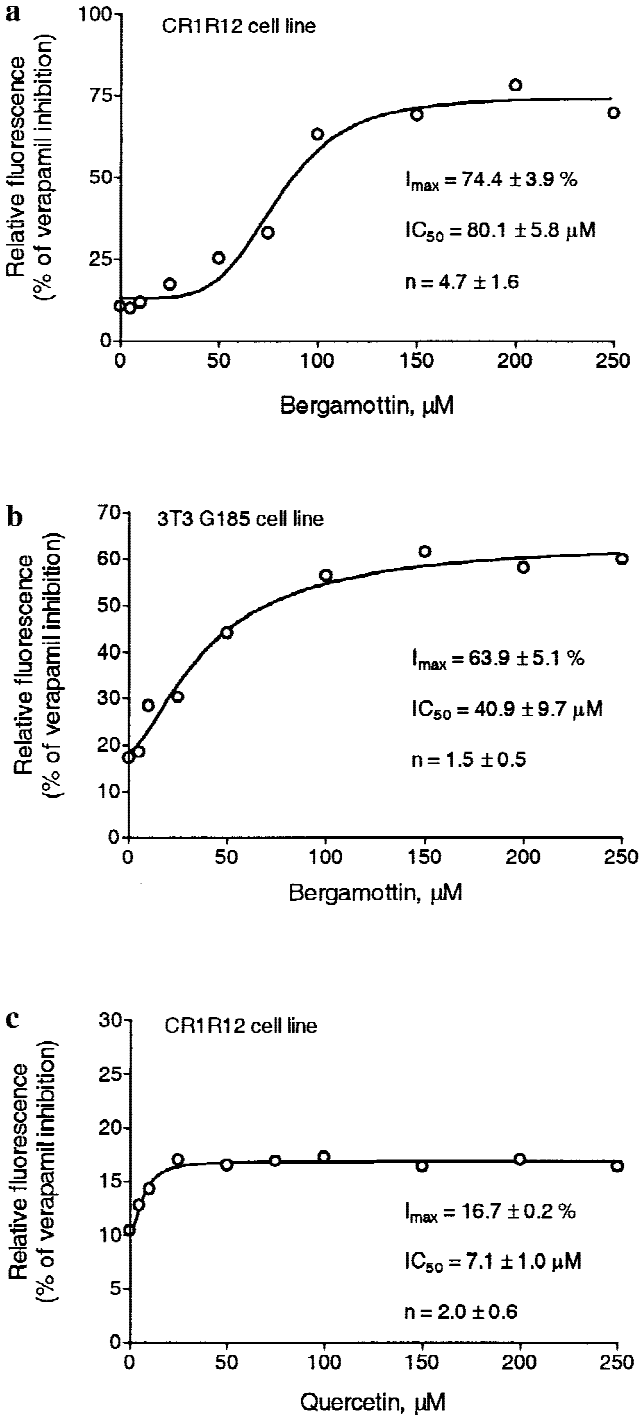

Fig. 1. Intracellular retention of daunorubicin in CR1R12 cells (a, c) and G185 cells (b) versus competing substrate concentration. DNR fluorescence intensity is expressed as relative fluorescence. The efflux phase or incubation was 30 min in all cases. The average number of cells per assay was 10,000. The function for the line through the data is the Hill equation: $v = V_{max}S^n/(K'+S^n)$. The parameters IC_{50} and the Hill coefficient along with the standard deviation are shown on the respective graph.

where the retained fluorescence is measured for samples of viable cells by a flow cytometer at varying concentrations of BG. The concentration dependency of inhibition displayed a sigmoidal response curve (Figure 1a), a consequence of co-

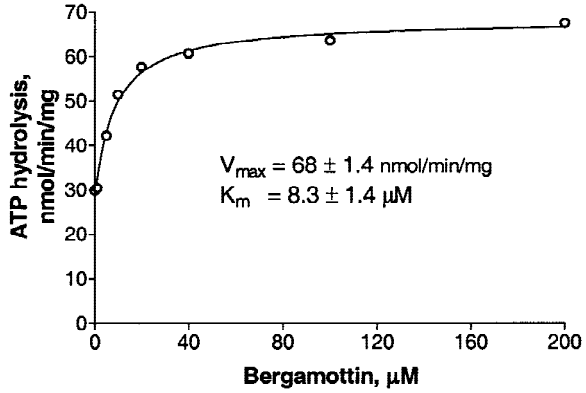

Fig. 2. P-gp-mediated ATP hydrolysis rates in the presence of bergamottin. The data is fit to a hyperbola and the V_{max} = 68 ± 1.4 nmol/min/mg membrane protein with a K_m = 8.3 ± 1.4 μM.

operativity, and the Hill equation for allosteric interaction enzymes is therefore the appropriate function for fitting to the data: $v = V_{max}S^n/(K'+S^n)$ (12). The IC_{50} in the CR1R12 cell line is ≈ 80 μM, and BG can achieve about 75% of inhibition caused by the positive control verapamil. As shown in Figure 1b, the IC_{50} for BG in the G185 cells (which express the gene product of human MDR1) is ≈ 41 μM, which is twice as potent as the inhibition observed in the CR1R12 cell line overexpressing the rodent enzyme. Because these cell lines overexpress the respective transporter enzymes, the IC_{50} would be expected to be higher than under *in vivo* conditions where far fewer copies of the enzyme would be contained per cell. The efficacy of ~ 64 % of verapamil-induced inhibition is similar to many known P-gp substrates/inhibitors (28).

The flavonoid components—naringin, naringenin, and kaempferol—had no significant effect on the active P-gp-mediated transport of the substrate DNR in either the CR1R12 or G185 cell lines (data not shown), whereas quercetin slightly inhibited function but at a low concentration with an IC_{50} of about 6 μM (Fig. 1c) in the CR1R12 cell line. It should be noted, however, that the absence of an effect on a particular transport marker in these cell lines does not necessarily mean that the test compounds do not interact with the transporter and ATP hydrolysis can reveal binding site recognition.

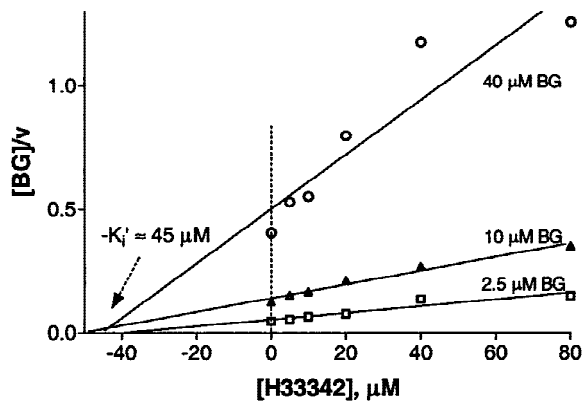

Fig. 3. Cornish-Bowden plot at various fixed concentrations of BG. The intersection below the [H33342] axis is characteristic of mixed-type inhibition. The corresponding [H33342] at the line intercept represents $-K_i'$, as shown on the graph, and is about 45 μM.

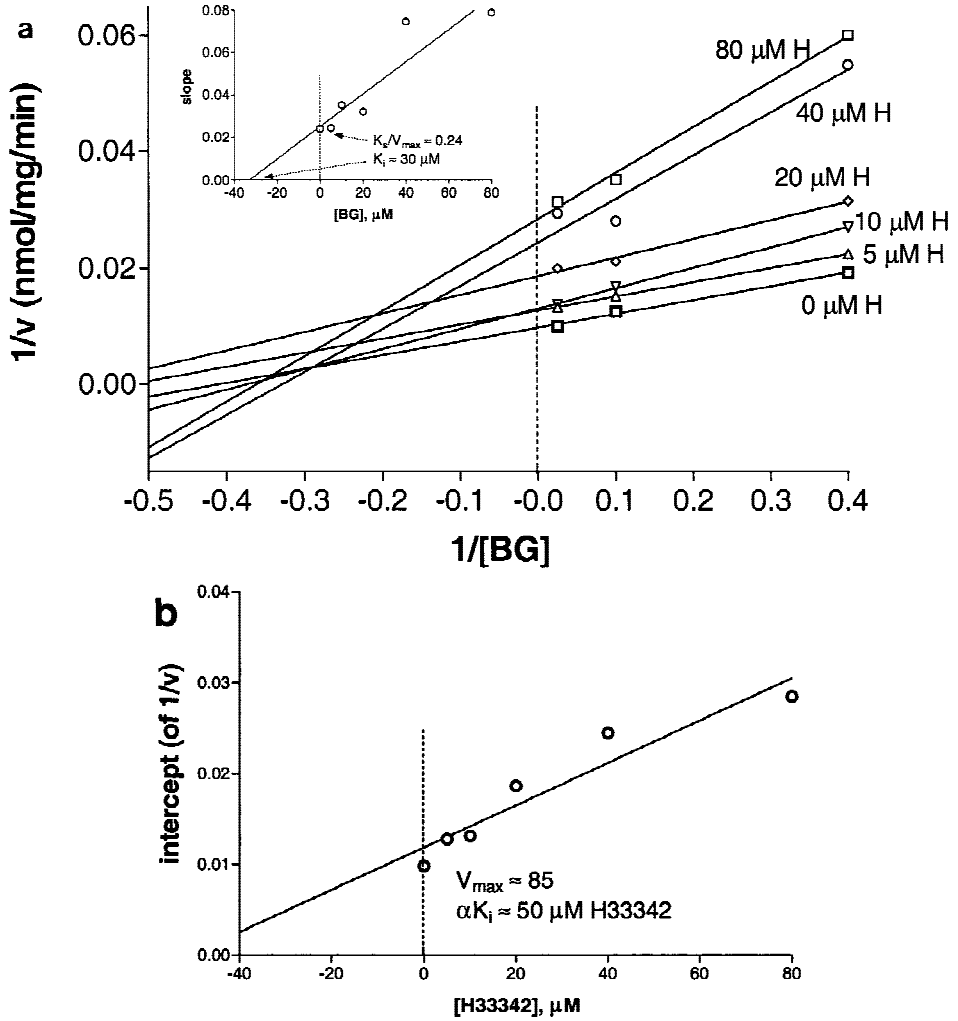

Fig. 4. (a). The reciprocal 1/v versus 1/[BG] plot in the presence of different fixed concentrations of H33342. The characteristics of the intersection are of a mixed-type inhibitor. Inset: Slope replot of reciprocal plot data revealing a K_i of about 30 μM. A K_s was estimated from the double reciprocal plot at about 2–3 μM. (b) Intercept of 1/v axis replot of the reciprocal plot data. Characteristic of mixed-type or noncompetitive inhibition, this analysis indicates an $\alpha \approx 1.5$. The V_{max} is estimated at about 85 (nmol/min/mg) by the intersection of the line with the vertical axis and αK_i is estimated by the intersection with the [H33342] axis to be about 50 μM.

ATP Hydrolysis

As ATP is consumed at a purported rate of about one or two per transport event, the hydrolysis of ATP represents transport function turnover rate or activity assay (32–35). The presence of BG causes a concentration-dependent increase in the rate of ATP hydrolysis relative to baseline rate, which indicates that it is a comparatively rapid substrate for P-gp (Fig. 2). The K_m is ~ 8 μM, and the V_{max} is ~ 2.3 fold above baseline. A few known substrates, such as H33342, cause ATP hydrolysis rates that are lower than baseline ATP consumption. This effect is ostensibly due to competition with an endogenous substrate with a rate of transport approximately that of the baseline ATP hydrolysis rate. If BG is a substrate for P-gp, then it will compete with other substrates for the active transport site and act as an inhibitor (10). To test this hypothesis, an experiment was performed at various fixed concentrations of bergamottin and the substrate H33342 and a K_i determined for the interaction with the substrate, H33342 (36). As shown by the Cornish-Bowden plot analysis of the data (Figure 3) a K_i' (or αK_i noncompetitive or mixed-type inhibition) of ≈ 45 μM was estimated which indicated that H33342 inhibited BG transport activity. The Lineweaver-Burk plots in Figure 4 also show a pattern of mixed-type inhibition (or nearly noncompetitive) rather than competitive (mutually exclusive binding) at the same site (10). The nonlinearity is due to expected complications from multiple substrate binding sites with unique affinities. A replot of slope versus [H33343] (Fig. 4, inset) indicates an estimated K_i of ≈ 30 μM. The intercept replot (Fig. 4b) indicates an αK_i of about 50 μM. The Dixon plot (Fig. 5) resembles that for mixed-type inhibition, and the slope replot shows an intercept representing $-1/\alpha K_s$, where α is a factor by which the binding of BG affects the K_s of H33342 binding, and vice versa. This graphical analysis results in an estimated K_i for H33342 of approximately 30–40 μM. These rough estimates are in reasonable agreement with the other two graphical analyses described above. The K_i estimated here is for the interaction of

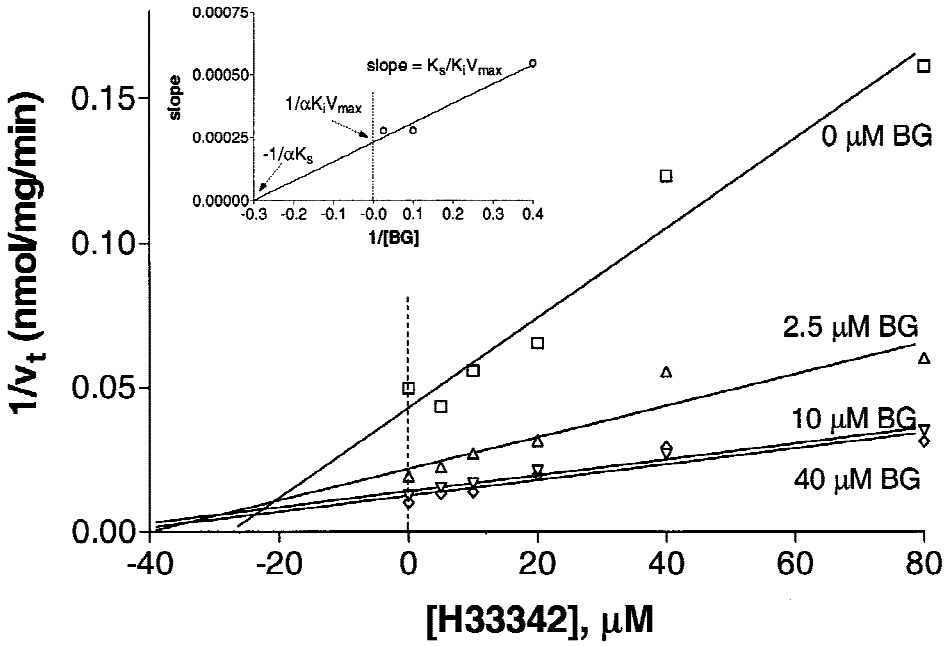

Fig. 5. Dixon plot: 1/v versus [H33342] at different fixed concentrations of BG. The intersection point is lower than $1/V_{max}$, a characteristic of mixed-type inhibition. V_{max} can be estimated to be about 80–90 nmol/min/mg. Inset. Slope replot of Dixon plot. The line does not go through the origin as does the replot for pure competitive inhibition, indicating mixed-type inhibition. The coefficient α represents the degree to which BG binding affects the K_s of H33342 binding, and vice versa. From this graphical analysis, αK_i is roughly 50 μM, and a K_s can be estimated from the reciprocal plot of about 2 - 3 μM, hence permitting an estimate of the affinity perturbation α of about 1.3 and K_i (by H33342) of about 38μM.

H33342 with BG transport activity and not the reverse. The most reasonable approximation of the concentration with which bergamottin interacts with the transport enzyme is the K_m for the bergamottin-induced ATP hydrolysis activity of about 8 μM. Naringin, naringenin, and kaempferol had no significant effect on ATP hydrolysis (data not shown).

DISCUSSION

BG is the furanocoumarin found in the highest concentration in the ethyl acetate extract of grapefruit juice (≈ 23 μM) (23). Our results demonstrate the inhibition of P-gp transport function by BG in a concentration-dependent manner with an IC_{50} of 40 μM in the G185 cell line expressing far above normal quantities of the human transporter enzyme. ATP hydrolysis kinetics show that BG interacts with P-gp with a half maximal saturation concentration of about 8 μM. The concentration for this interaction is similar to that for CYP3A4 inactivation (23). Since BG is known to bind to CYP3A4, and both CYP3A4 and P-gp share a similar substrate definition (diverse lipophilic structures of ~300–1000 MW), and hence share most substrates, it is not surprising that BG would bind to the substrate binding site of P-gp. Indeed, the psoralen moiety typifies the "type II unit" of 3 electron donor groups with a spatial separation of 4.6 ± 0.6Å suggested by Seelig (37) as one of two general patterns for substrate recognition by P-gp (constructed from a structure activity relationship study of known substrates of P-gp).

Extracts of grapefruit juice have been suggested to inhibit P-gp function based on permeability in Caco-2 cell monolayers (26), and the results indicate that the cause may be components other than naringin or naringenin. Additionally, the citrus flavonoid quercetin appeared to inhibit the active efflux of the fluorescent substrate rhodamine 123 in MCF-7 ADR-resistant cells (38). In Caco-2 monolayers, however, only naringin and 6′,7′-dihydroxybergamottin had any effect on the saquinavir transport ratio (27). However, Caco-2 cells present a variety of ABC transporters and exhibit various non-native permeabilities as well as other attributes that confuse interpretation. Also, in Caco-2 monolayers talinolol permeability was increased by grapefruit juice, and binding data suggest that some grapefruit juice components affect the binding of substrates to P-gp (39). Circumstantial evidence includes the clinically significant interaction of grapefruit juice with felodipine and nitrendipine (40), both of which have been shown to interact with P-gp (41,42).

Many CYP3A4 inhibitors (substrates) have exhibited clinical pharmacokinetic interactions with other CYP3A4 substrates (see introduction). The notable overlap of substrates of CYP3A4 and P-gp, however, greatly obscures interpretation of the enzyme responsible for these interactions. BG is yet another example of an effective inhibitor of both CYP3A4 and P-gp. Since P-gp has a significant effect on absorption and disposition of orally administered drugs, its role might often be more significant than intestinal CYP3A4 (43). Many, if not all, of the known clinical interactions with grapefruit juice are with compounds that are substrates of P-gp (2–5). An effective inhibitor of the transport function of P-gp, BG therefore may be a major cause (or contributor) of the known clinical interactions previously suspected to be

CYP3A4 mediated. Moreover, BG interacts with both P-gp and CYP3A4 (23) at a similar concentration, and intestinal P-gp rather than CYP3A4 is a primary determinant of oral cyclosporin A availability (43). However, CYP3A4 can be inactivated (23) by BG, further complicating discernment of the mechanism of impact by BG *in vivo*.

Inhibition of P-gp by citrus psoralens may therefore represent a means to enhance bioavailability of therapies without increasing the dose. Indeed, psoralen analogues may provide candidates for cancer adjuvant therapy via reversal of drug resistance. Concomitant use of drugs with narrow therapeutic indexes and at least large amounts of grapefruit juice should, however, be avoided.

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