EFFECTS OF BERGAMOTTIN ON HUMAN AND MONKEY DRUG-METABOLIZING ENZYMES IN PRIMARY CULTURED HEPATOCYTES

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ABSTRACT:

We investigated the effect of bergamottin, a major furanocoumarin in grapefruit juice, on phase I and phase II drug-metabolizing enzymes using cultured human and monkey hepatocytes. Both cultured systems were compared and evaluated for the direct effects of bergamottin as well as control treatments on liver enzymes. Treatment of hepatocytes with 0.1, 1, 5, and 10 μM bergamottin resulted in a concentration-dependent reduction in CYP3A4 activity (40–100%) in both human and monkey cells, as measured by testosterone β6-hydroxylation activity. Bergamottin was potent at eliciting these inhibitory effects at both basal and induced states of CYP3A. Bergamottin (5 μM) completely inhibited α-naphthoflavone-induced ethoxyresorufin O-dealkylase (EROD) and methoxyresorufin O-dealkylase (MROD) activities in human hepatocytes and caused a 100% decrease in EROD activity in monkey hepatocytes. A 48-h exposure of cultured human hepatocytes to bergamottin resulted in increased levels of immunoreactive CYP3A4, CYP1A1, and CYP1A2 proteins, and CYP3A4, CYP1A1, CYP1A2, CYP2B6, and UDP-glucuronosyl transferase mRNAs. There was only a 20 to 30% reduction in glucuronidation and sulfation of 4-methylumbelliferone in human hepatocytes by 10 μM bergamottin and no effect on conjugation in the monkey hepatocytes. These results suggest that bergamottin causes both inhibition of CYP3A and CYP1A1/2 enzymatic activities and induction of correspondent proteins and mRNAs.

Concomitant ingestion of grapefruit juice with medications has been attributed to the transient enhancement of systemic bioavailability for many drugs, in particular orally administered CYP3A4 substrates, such as nifedipine, nisoldipine, nitrendipine, felodipine, and cyclosporine A (Bailey et al., 1991, 1993, 1998a,b; Ameer and Weintrab, 1997; Fuhr, 1998). If administered with grapefruit juice, the peak plasma concentrations (Cmax) and the area under the curve (AUC) of these drugs were dramatically increased resulting in clinically significant drug interactions including an increase in lowering blood pressure and increase in heart rate (for review see Fuhr, 1998). These effects are thought to be primarily due to grapefruit-mediated decreases in intestinal CYP3A4 protein as well as inhibition of P-glycoprotein (Lown et al., 1997; Takanaga et al., 1998; Eagling et al., 2000; Swanson et al., 2000; Seldner et al., 1999; Spahn-Langguth and Langguth, 2001).

This work was supported in part by Grants GM 61393, GM 60346, ESO 5780, and DK 92310 to Stephen C. Strom. 1Abbreviations used are: AUC, area under the curve; P450, cytochrome P-450; 4-MU, 4-methylumbelliferone; SSC, standard saline citrate; TAO, triacetylemycin; β-NF, β-naphthoflavone; HPLC, high-performance liquid chromatography; EROD, ethoxyresorufin O-dealkylase; MROD, methoxyresorufin O-dealkylase; DMSO, dimethyl sulfoxide; 3MC, 3-methylcholanthrene.

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Schmiedlin-Ren et al. (1997) and Bailey et al. (1998b) have indicated that the functional alteration of CYP3A4 in the intestinal mucosa is likely the cause for the grapefruit juice-drug interaction, whereas Takanaga et al. (1998) demonstrated that grapefruit juice specifically inhibits the P-glycoprotein drug efflux transporter in small bowel enterocytes. There were few changes observed in drug pharmacokinetics when grapefruit juice was given orally with intravenous drugs, supporting the findings that grapefruit juice acts primarily by altering intestinal metabolism of drugs (Ducharme et al., 1995; Kupferschmidt et al., 1995; Rashid et al., 1995). In contrast to the acute effect of grapefruit juice, studies in rodents have shown that long-term grapefruit juice administration enhances nifedipine clearance and induces hepatic oxidative enzymes (Dakovic et al., 1999; Mohri et al., 2000). Although several hundred components have been identified in grapefruit juice (Ranganna et al., 1983; Fukuda et al., 1997, 2000), bergamottin and 6, 7′-dihydroxybergamottin are predominant components of grapefruit juice that are mechanism-based inactivators of CYP3A and major human liver microsomal P450s (Schmiedlin-Ren et al., 1997; He et al., 1998; Guo et al., 2000). In rats, administration of bergamottin in duodenum at doses equivalent to that in grapefruit juice resulted in increased AUC of nifedipine, an effect similar to grapefruit juice (Mohri and Uesawa, 2001). Bergamottin administered to dogs either orally or intravenously prior to oral diazepam resulted in a similar increase in plasma levels of diazepam, suggesting the inhibition of hepatic-metabolizing enzymes (Sahi et al., 2002). This study was designed to directly evaluate the effect of bergamottin on
phase I and phase II drug-metabolizing enzymes in human and monkey cultured hepatocytes after acute or prolonged treatments.

**Materials and Methods**

**Chemicals.** Hepatocyte maintenance (modified Williams E) culture medium, dexamethasone, and insulin were obtained from BioWhittaker (Walkersville, MD). Penicillin G/streptomycin was acquired from Invitrogen (Carlsbad, CA). Bergamottin was purchased from Indofine Chemical Co. (Somerville, NJ). Testosterone and testosterone metabolites were from Steraloids Inc. (Newport, RI). Trolox (1,2,4-tri-tolylamine, 50 µM) was used as a selective probe to measure glucuronidation and sulfation (Pohl and Fouts, 1980). Resorufin (Pohl and Fouts, 1980). The product resorufin was measured in intact cells by conversion rate of EROD and MROD to 4-methylumbelliferone, 3-methylcholanthrene, ethoxy- and methoxy-resorufin and resorufin, 4-methylumbelliferone, 4-methylumbelliferone sulfates were purchased from Sigma-Aldrich (St. Louis, MO). Type I (rat-tail) collagen was purchased from Upstate Biotechnology (Waltham, MA). Expressed forms of CYP3A4, CYP1A1, and CYP1A2 and primary anti-CYP1A1 antibody were purchased from BD Gentest (Woburn, MA).

**Hepatocyte Culture and Treatment Protocol.** Human hepatocytes were prepared from livers not used for whole organ transplant. Monkey hepatocytes were prepared from untreated cynomolgus monkey (Macaca fascicularis) at age 37°/H9252 cytes by the 6 under light microscopy that could be attributed to cell toxicity. Hepatocytes were treated with 10 to attach for 4 to 6 h. At this time, the medium was replaced with serum-free penicillin G, 100 M. Penicillin G/streptomycin was acquired from Invitrogen (Carlsbad, CA). Three oligonucleotides per gene designed to detect cDNA representing the P450 isoforms in human and amino-modified 50mer oligos were spotted onto SuModic slides at 20 µM in 150 µM sodium phosphate buffer (Kane et al., 2001). For quality control and normalization purposes, 16 control sequences were analyzed for cross-hybridization potential. A mixture of synthetic transcripts, each mRNA at a specific copy per cell, was spiked into experimental RNA. To generate fluorescent-labeled cDNA targets for microarray hybridization, reverse transcription (SuperScript II; Invitrogen) in the presence of random primers (3.75 µM) was carried out using 10 µg of RNA isolated from hepatocytes (control and treated) and Cy3- or Cy5-dCTP (0.16 mM) in the cDNA synthesis (42°C for 2 h). To obtain a mixture of synthetic transcripts, each mRNA at a specific copy per cell was spiked into reverse transcription reaction. RNA was hydrolyzed (1.5 mM EDTA and 30 mM NaOH 10 min at 70°C), and control and treated cDNAs were mixed and purified (Concert PCR purification system; Invitrogen). Purified cDNA was mixed with buffer and formamide to a final hybridization volume of 250 µl (4.1% Denhardt’s solution, 43.5% SSC, 50% formamide). Samples were placed on microarrays overnight at 42°C, washed with 1× SSC/0.2% SDS, 0.1× SSC/0.2% SDS, and 0.1× SSC (no SDS), dried, and scanned for Cy3 and Cy5 signal intensity (Molecular Dynamics Gen III scanner). Data was normalized based upon intensity values between the Cy3 and Cy5 channel of control transcripts spiked at a 1:1 ratio.

**Results**

**Effect of Bergamottin on CYP3A Activity in Cultured Hepatocytes.** To examine the effect of bergamottin on CYP3A activity, human and monkey hepatocytes were incubated with increasing concentrations of bergamottin. As shown in Figure 1A, treatment of cultured human hepatocytes with bergamottin resulted in a concentration-dependent reduction in formation of 6β-hydroxytestosterone. CYP3A4 activities were undetectable at 5 µM bergamottin in human cells. In a separate experiment, human hepatocytes prepared from a different donor were induced for CYP3A with rifampicin (10 M) resulting in a 50-fold increase in CYP3A4 activity as compared with uninduced hepatocytes (20 versus 1000 pmol/min/mg of protein) (Fig. 1B). Bergamottin (1 M) decreased CYP3A4 activity by 75%. At a concentration of 5 µM, bergamottin decreased CYP3A4 activity to the basal level. The extent of this inhibition was comparable with the known CYP3A inhibitor, TAO at 10 and 50 M. As with human hepatocytes, treatment of monkey hepatocytes with increasing concentrations of bergamottin resulted in a marked inhibition of basal CYP3A activity in a dose-dependent pattern (Fig. 2A). In hepatocytes prepared from a different monkey, treatment with rifampicin doubled CYP3A8 activity (Fig. 2B) over control cells, where the basal CYP3A8 activity was about 20 times greater than that in human cells, a finding common for monkeys. Rifampicin-induced activity was inhibited at basal level after treatment with 1 µM bergamottin and was further decreased to about 10% of the induced level with 5 µM bergamottin. The level of inhibition of CYP3A8 activity by 5 µM bergamottin was comparable with that observed with 50 M TAO.

**Effect of Bergamottin on CYP1A1/2 Activity.** As shown in Figure 3A, both EROD and MROD activities catalyzed by CYP1A1/2 were below the detectable limit in untreated cultured human hepatocytes.
cytes but were induced by 50 μM β-NF. Bergamottin (0.5 μM) decreased EROD and MROD activity by 80% and >95% at 1 μM. Bergamottin at 5 and 10 μM completely eliminated CYP1A1/2 activity in cells induced with β-NF. Similarly, α-NF, a selective inhibitor of CYP1A, inhibited both EROD and MROD by 85 and 100% at 1 and 10 μM, respectively. Similar to human hepatocytes, the basal levels of EROD and MROD were not detectable in untreated monkey hepatocytes (Fig. 3B), but both activities were substantially elevated in cells treated with 50 μM β-NF. In the presence of increasing concentrations of bergamottin, the inhibition of MROD was shown to be moderate, amounting to approximately 2-fold loss of the induced activity. There were no appreciable differences in inhibition of

![Graph A](image1.png)

**Fig. 1.** Effect of bergamottin on testosterone CYP3A4 activity in human hepatocytes.

A, cultured human hepatocytes were treated with 0, 0.1, 1, 5, and 25 μM bergamottin along with 200 μM testosterone for 30 min. B, hepatocytes from a different donor were pretreated for 48 h with 10 μM rifampicin. At the end of 48 h, the culture medium was discarded, and fresh medium containing indicated concentrations of bergamottin or TAO (μM) and 200 μM testosterone was added to the cells (B). TAO was added 1 h before bergamottin. After 30 min of incubation, aliquots of medium were taken, and 6β-hydroxytestosterone was measured by HPLC as described under Materials and Methods. Each value represents the mean of duplicate treatments with the range indicated by the vertical bars. UN, control cells; n.d., not detected; Ber, bergamottin; Rif, rifampicin.
MROD in response to bergamottin concentrations of 0.5 to 10 μM. However, induced EROD activity was completely inhibited by 0.5 μM bergamottin, indicating that bergamottin is a more effective inhibitor of EROD than MROD activity. In contrast, a concentration of 1 μM α-NF only slightly inhibited both EROD and MROD induced by β-NF, whereas a concentration of 10 μM α-NF completely abolished the induced levels of both activities.

**Effect of Bergamottin on CYP3A4 and CYP1A1/2 Proteins.**
Human hepatocytes were treated with increasing concentrations of bergamottin (0.1 to 25 μM) for 48 h, and CYP3A4 and CYP1A1/2 proteins were measured. There were increases in CYP3A4 proteins (1.3- to 2-fold) at 1 to 10 μM in two different donors (Fig. 4A). The level of immunoreactive CYP3A in untreated cells from donor 1 was more than 2 times greater than that in donor 2 (Fig. 4A). Bergamottin...
was more potent in inducing CYP3A4 in hepatocytes from donor 2, compared with hepatocytes from donor 1, probably due to the lower basal expression. There was a 2-fold increase in CYP1A2 protein at 1 /H9262 M bergamottin and an increase in CYP1A1 at 5 and 10 /H9262 M (Fig. 4B).

Effect of Bergamottin on Phase II Conjugating Enzymes. To investigate the effects of bergamottin treatment on conjugation, we measured the glucuronidation and sulfation rates of 4-MU. Both rates showed a slight, 20 to 30% decline in human cells treated with 10 /H9262 M bergamottin (Fig. 5A). The formation rates of 4-MU conjugates in monkey hepatocytes remained unchanged after treatment with 5 or 10 /H9262 M bergamottin (Fig. 5B). The rates of glucuronidation and sulfation were 2.6- and 3-times higher in monkey hepatocytes as compared with human, respectively.

Effect of Bergamottin on Human Hepatocyte mRNA. To assess the effect of bergamottin on mRNA expression in cultured human hepatocytes, cells were treated with bergamottin (5 μM) and four positive controls: rifampicin (10 μM), 3MC (8 μM), β-NF (50 μM), and phenobarbital (2 mM). As shown in Table 1, treatment with bergamottin for 48 h resulted in an 8-fold increase in CYP3A4 mRNA, an increase similar to that observed with phenobarbital, and one-third of that induced by rifampicin. Bergamottin caused increases in CYP1A1 (53-fold) and CYP1A2 (12-fold) mRNA levels. These increases were comparable with those observed with 3MC and β-NF, indicating that bergamottin is a potent inducer of CYP1A mRNAs and proteins (Fig. 4B). A small increase was observed in UDP-glucuronosyl transferase mRNA in cells treated with bergamottin. This mRNA also was increased by phenobarbital, 3MC and β-NF, treatments known to induce UDP-glucuronosyl transferase. Both bergamottin and phenobarbital increased CYP2B6 mRNA with no effect by 3MC or β-NF treatments. Thus, it appears that bergamottin is an inducer of a variety of enzymatic mRNAs that are associated with increased CYP3A4, CYP1A1, and CYP1A2 proteins (Fig. 4).

Discussion

Flavonoids, such as narirutin, quercitin, and several prevalent furanocoumarines, especially 6',7'-dihydroxybergamottin, were regarded to be potent components responsible for the clinical effects of grapefruit juice (Edwards et al., 1996; Bellevue et al., 1997). However, direct experimental evidence did not support these conclusions (Edwards and Bernier, 1996; Bailey et al., 1998b; Edwards et al., 1999; Guo et al., 2000). Bergamottin is one of the key compounds causing the drug-grapefruit juice pharmacokinetic interactions (Schmiedlin-Ren et al., 1997; He et al., 1998; Sahi et al., 2002). Some
Evidence to support this assertion is that bergamottin is more potent than 6',7'-dihydroxybergamottin, with the values of maximal rate constant (k_{inactivation}) and the concentration of inactivator required for half-maximal rate of inactivation (K_i) being 0.3 min^{-1} and 7.7 μM, respectively, for bergamottin (He et al., 1998) and 0.16 min^{-1} and 59 μM, respectively, for 6',7'-dihydroxybergamottin (Schmiedlin-Ren et al., 1997) in the reconstituted CYP3A4 system. In addition, bergamottin inhibits other P450s, such as CYP1A2, 2A6, 2B1, 2C9, 2C19, 2D6, and 2E1 in human liver microsomes (Cai et al., 1996; He et al., 1998; Tassaneeyakul et al., 2000). However, Guo et al. (2000) and Tassaneeyakul et al. (2000) recently showed that two furanocoumarin dimers, GF-I-1 and GF-I-4, caused the most potent inhibition of CYP3A4 in human microsomes, but their presence in the grapefruit is of minor quantity (Fukuda et al., 2000; Guo et al., 2000). A combined action of many furanocoumarins, including bergamottin is likely to be responsible for the overall potent inhibitory effect of grapefruit juice (Guo et al., 2000).

Most of the drugs affected by grapefruit juice are primarily metabolized by CYP3A4, which is the most abundant drug-metabolizing enzyme in both liver and intestine. There is a clear and inverse relationship between bioavailability of individual drugs depending on the first-pass metabolism and the effect of grapefruit juice on AUC and C_{max} parameters (reviewed in Fuhr, 1998). Lown et al. (1997) demonstrated that intestinal CYP3A was selectively and post-transcriptionally down-regulated by grapefruit juice. Recurrent grapefruit juice consumption for 6 days resulted in a 62% decrease in the enterocyte CYP3A4 immunoreactive protein concentrations in healthy volunteers, whereas small intestinal CYP3A4 mRNA was unchanged. Schmiedlin-Ren et al. (1997) also observed a 47% reduction in intestinal CYP3A4 content in a healthy volunteer within 4 h after consuming grapefruit juice. In comparison, the intravenous pharmacokinetics of drugs were not significantly altered by oral grapefruit juice (Ducharme et al., 1995; Kupferschmidt et al., 1995; Rashid et al., 1995). However, purified bergamottin given to dogs, either orally or i.v., produced a similar increase in AUC and C_{max} of orally administered diazepam indicating that bergamottin can also inhibit the liver-metabolizing enzymes (Sahi et al., 2002). In agreement with this data, we found potent inhibition of CYP3A4- and CYP1A1/2-mediated activities by bergamottin in both human and monkey hepatocytes. Bergamottin at 5 μM acutely reduced testosterone 6β-hydroxylase activity by 90% in both species compared with the induced level. Bergamottin dose-dependently decreased basal CYP3A activity as well. Notably the basal activity of testosterone 6β-hydroxylase in monkey is approximately 20 times greater than seen in humans. Consequently, treatment with rifampicin, a strong inducer of CYP3A, resulted only in a 2-fold increase in activity suggesting the limited effect on CYP3A induction in monkey. We further characterized the effects of bergamottin on CYP1A1/2. Bergamottin was a potent inhibitor of CYP1A1/2-mediated EROD and MROD activities in human hepatocytes. Bergamottin at 5 μM completely inhibited EROD and MROD activities in human cells, similar to the response achieved with 10 μM α-NF. These data are in agreement with the inhibition of CYP1A1/2 enzyme activity by bergamottin in human liver microsomes (He et al., 1998; Tassaneeyakul et al., 2000). CYP1A1 was inhibited by 92% with 1 μM bergamottin as measured by inhibition of phenacetin O-deethylation (He et al., 1998). In addition, bergamottin has been proposed to cause a mechanism-based inactivation of CYP1A2 (Cai et al., 1996). Although the levels of EROD and MROD were similar in human and monkey cells induced with β-NF, only EROD was inhibited in monkey cells (Fig. 3). In contrast, 10 μM α-NF blocked both activities, suggesting that MROD is catalyzed by other enzyme(s) than CYP1A, which are not inhibited with bergamottin in monkey.

Western blot analysis of bergamottin-treated human cultures revealed a small increase in CYP3A4 and CYP1A2 proteins (Fig. 4). In addition, a slight increase in CYP1A1 protein also was observed. This associated with correspondent increases in CYP3A4, CYP1A1, and CYP1A2 (Table 1) mRNAs suggesting that both CYP3A4 and CYP1A1/2 proteins were induced at the transcriptional level. It is well established that potent P450 inhibitors including macroline antibiotics, protease inhibitors, and imidazole antimiycotics can also be inducers of CYP3A protein and mRNA (Wrighton et al., 1985; Hostetler et al., 1989). Thus it appears that bergamottin falls under this category.

We found little effect by bergamottin on conjugation of 4-MU (Fig. 5), a nonspecific substrate for glucuronol- and sulfotransferase activities. The lack of inhibitory effect of bergamottin on uridine diphosphate glucuronosyltransferases and sulfotransferases suggests that either bergamottin is not a substrate for these enzymes or that the affinity for bergamottin is lower than that for 4-MU.

The results from our studies strongly support the hypothesis that when acutely administered, bergamottin contributes to the grapefruit juice-drug interactions by inhibiting drug-metabolizing enzymes. The minimal effect of grapefruit juice on the liver-metabolizing capacity in human could in part be explained by intestinal metabolism of bergamottin. However, if delivered to the liver it would inhibit phase I enzymes, as was recently demonstrated in dogs (Sahi et al., 2002).

In conclusion, the data presented in this study demonstrate that bergamottin is a potent acute inhibitor of human and monkey hepatic CYP3A and CYP1A activities. A long-term incubation of bergamottin with primary cultured human hepatocytes produced a small increase in immunoreactive CYP3A4, CYP1A1, and CYP1A2 and corresponding increases in their mRNAs. These results suggest that bergamottin causes both inhibition of P450s activities and induction of P450 proteins and mRNAs.

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References