Piperine, a Major Constituent of Black Pepper, Inhibits Human P-glycoprotein and CYP3A4

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ABSTRACT

Several dietary constituents and phytochemicals are now identified as important factors affecting drug disposition (Walter-Sack and Klotz, 1996; Wilkinson, 1997; Evans, 2000). Frequently, the underlying mechanism of altered drug concentrations is induction or inhibition of drug-metabolizing enzymes or transporters (Walter-Sack and Klotz, 1996; Wilkinson, 1997; Evans, 2000; Ayrton and Morgan, 2001). For example, consumption of charcoal-broiled or smoked meat, cruciferous vegetables, and the herbal remedy St. John’s wort leads to induction of several drug-metabolizing enzymes or transporters (Walter-Sack and Klotz, 1996; Wilkinson, 1997; Evans, 2000). Dietary salt has been shown to induce metabolism and/or transport of CYP3A4 and P-glycoprotein substrates (phenytoin and rifampin) in rodents and increases plasma concentrations of several drugs, including P-glycoprotein substrates (phenytoin and rifampin) in humans. However, there are no direct data whether piperine is an inhibitor of human P-glycoprotein and/or CYP3A4. We therefore investigated the influence of piperine on P-glycoprotein-mediated, polarized transport of digoxin and cyclosporine in monolayers of Caco-2 cells. Moreover, by using human liver microsomes we determined the effect of piperine on CYP3A4-mediated formation of the verapamil metabolites D-617 and norverapamil. Piperine inhibited digoxin and cyclosporine A transport in Caco-2 cells with IC\textsubscript{50} values of 15.5 and 74.1 \ \mu M, respectively. CYP3A4-catalyzed formation of D-617 and norverapamil was inhibited in a mixed fashion, with K\textsubscript{i} values of 36 ± 8 (liver 1)/49 ± 6 (liver 2) and 44 ± 10 (liver 1)/77 ± 10 \ \mu M (liver 2), respectively. In summary, we showed that piperine inhibits both the drug transporter P-glycoprotein and the major drug-metabolizing enzyme CYP3A4. Because both proteins are expressed in enterocytes and hepatocytes and contribute to a major extent to first-pass elimination of many drugs, our data indicate that dietary piperine could affect plasma concentrations of P-glycoprotein and CYP3A4 substrates in humans, in particular if these drugs are administered orally.

In addition to dietary constituents leading to reduced plasma concentrations of drugs, there are examples of increased plasma concentrations by nutrients due to inhibition of drug metabolism. Ingestion of grapefruit juice clearly results in pronounced drug interactions, primarily due to inhibition of (intestinal) CYP3A4, resulting in increased plasma concentrations of felodipine, nitrendipine, saquinavir, cyclosporine A, terfenadine, and other compounds (Bailey et al., 1998; Kane and Lipsky, 2000).

Due to high concentrations in the gut lumen, dietary constituents are likely to exert a major effect at the level of intestinal enterocytes. These cells represent the first cell lining limiting entry of orally ingested compounds into the body. Both, P-glycoprotein and CYP3A4 are expressed in enterocytes and determine bioavailability of many drugs such as cyclosporine A, midazolam, verapamil, HIV protease inhibitors, digoxin, or talinolol (Kolars et al., 1991; Fromm et al., 1996; Paine et al., 1996; Kim et al., 1998; Greiner et al., 1999; Westphal et al., 2000). Moreover, induction and inhibition of (intestinal) P-glycoprotein and CYP3A4 have been identified as important mechanisms underlying drug inter-

ABBREVIATIONS: HIV, human immunodeficiency virus; TEER, transepithelial resistance.
actions (Thummel et al., 1996; Fromm et al., 1999; Greiner et al., 1999; Westphal et al., 2000).

The alkaloid piperine is a major component of black (Piper nigrum Linn) and long pepper (Piper longum Linn). Piperine has previously been shown to inhibit several cytochrome P450-mediated pathways and phase II reactions in animal models (Atal et al., 1981; Singh et al., 1986). Accordingly, treatment of rodents with piperine resulted in increased plasma concentrations of several compounds such as theophylline, phenytoin, rifampin, and propranolol (Atal et al., 1981; Velpandian et al., 2001). With regard to the effect of piperine or black pepper on drug disposition in humans, there are only very limited data. Administration of piperine significantly increased plasma concentrations of rifampin, phenytoin, propranolol, and theophylline in humans (Zutshi et al., 1985; Bano et al., 1987, 1991). Very recently, it was shown that a single administration of 1 g of black pepper more than doubled area under the plasma concentration-time curve and elimination half-life of phenytoin (Velpandian et al., 2001). The mechanisms underlying these drug interactions in humans have not been investigated so far.

Because both rifampin and phenytoin are substrates of the drug transporter P-glycoprotein (Schinkel et al., 1996; Schuetz et al., 1996), we tested the hypothesis whether piperine is an inhibitor of human P-glycoprotein. Moreover, it is not known whether piperine affects human CYP3A4. We therefore also tested by using human liver microsomes the influence of piperine on CYP3A4-mediated formation of the verapamil metabolites D-617 and norverapamil (Kroemer et al., 1992, 1993). Thus, this study investigates the potential influence of a dietary constituent on function of two proteins (P-glycoprotein and CYP3A4), which are of considerable importance for drug disposition and drug interactions in humans (Watkins, 1997; Fromm, 2000).

Materials and Methods

Chemicals. Piperine was obtained from Sabinsa Corporation (Piscataway, NJ). [3H]Cyclosporine A was purchased from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK). Verapamil, its metabolites norverapamil, D-617, D-702, D-703, and internal standards were obtained from Knoll AG (Ludwigshafen, Germany). Unlabeled digoxin was purchased from Sigma Chemie (Deisenhofen, Germany). Unlabeled cyclosporine A was a generous gift from Novartis (Basel, Switzerland).

Transport Studies. Transport studies were carried out using the human colon carcinoma cell line (Caco-2), as described previously (Kim et al., 1998; Pauli-Magnus et al., 2000). In brief, cells were grown as polarized monolayers on semipermeable filters, in which P-glycoprotein is expressed on their apical surface, thereby allowing study of vectorial transepithelial transport, i.e., basal-to-apical and apical-to-basal transport of drugs. Caco-2 cells were obtained from American Type Culture Collection (Manassas, VA). Cells passages 33 to 50 were plated on 0.4-μm polycarbonate Transwell filters (2 × 10⁶ cells/well; Costar, Cambridge, MA). Transport experiments were performed on day 7 after plating. About 1 h before the start of transport experiments, the medium in each compartment was replaced with OptiMEM medium (Invitrogen, Carlsbad, CA). For transport experiments the medium in each compartment was then replaced with 800 μl of OptiMEM medium with addition of the drug (5 and 1 μM for digoxin and cyclosporine A, respectively) on the basal or the apical side of the monolayer. The amount of drug appearing in the opposite compartment (basal or apical) after 1, 2, 3, and 4 h was measured in 25-μl aliquots and drug transport calculated as the percentage of the amount initially added. Net basal-to-apical transport was calculated after 4 h by subtracting the apical-to-basal from the basal-to-apical transport rate. Apparent permeability coefficients (P-app) were also determined according to the following equation:

\[ P_{\text{app}} = \frac{dQ/dt}{A \cdot C_o} \text{[cm/s]} \]

where dQ/dt (micromoles per second) is the transport rate, C_o (micromoles per cubed centimeters) is the initial concentration in the donor chamber, and A (squared centimeters) is the surface area of the monolayer.

Inhibition of Drug Transport. Inhibition of P-glycoprotein-mediated transport across confluent Caco-2 cell monolayers was determined in a similar manner after addition of the putative inhibitor to both compartments. Concentrations ranging from 0.1 to 250 μM were investigated for piperine [final ethanol concentration in each well was below 0.5% (v/v) ethanol]. The corresponding IC₅₀ values for inhibition of P-glycoprotein-mediated digoxin and cyclosporine A transport by piperine were calculated by GraphPad Prism, version 3.02 (GraphPad Software, San Diego, CA).

Experiments were conducted only in those wells that showed a transepithelial resistance (TEER) of >200 Ω after correction for the resistance obtained in control blank wells. TEER was also verified after each transport experiment in all wells to determine the effect of test substances on the monolayer integrity. Moreover, monolayer integrity was also assessed by measuring transepithelial translocation of [125I]insulin in presence of different concentrations of piperine. Transepithelial translocation of insulin was always less than 1%/h. All experiments were conducted at least in triplicate.

Metabolism Studies with Human Liver Microsomes. Microsomes were prepared from five human livers as described previously (Kroemer et al., 1992). The study was approved by the local ethics committee, and written informed consent was obtained from each patient. CYP3A4 content of human liver microsomes was determined according to a method described previously (Glaeser et al., 2002).

Incubations were carried out as described previously (Kroemer et al., 1992). For the inhibition studies, 250 μM verapamil, with and without different concentrations of piperine [dissolved in 1% (v/v) dimethyl sulfoxide] or 0.5 μM ketoconazole as a positive control, 50 μg of microsomal protein, 30 mM MgCl₂, and 50 mM KH₂PO₄, pH 7.4, were preincubated for 2 min at 37°C. The reaction was started by the addition of 4.8 mM NADPH and terminated after 5 min with 1.7 ml of ice-cold ethanol. The IC₅₀ values for the inhibition of formation of D-617 and norverapamil by piperine were determined using GraphPad Prism, version 3.02.

To determine the enzyme kinetic parameters, different concentrations of verapamil (10–200 μM) were incubated with and without 50 μM piperine. The experimental conditions were kept identical as described above except for the increase of microsomal protein content and incubation time to 100 μg and 10 min, respectively, to allow detection of metabolites at the lower substrate concentrations. Vmax and Km values were calculated with the program Leonora 1.0 (Oxford Science, Oxford, UK). The apparent dissociation constants of the enzyme-inhibitor complex (Kᵢ) of piperine for formation of D-617 and norverapamil were determined by nonlinear regression curve fitting (Leonora 1.0; Oxford Science). Both competitive and noncompetitive models were tested. The type of inhibition was determined by the enzymatic model that gave the smallest standard error for the estimation of Kᵢ. Parameter. Piperine (50 μM) was chosen for this study after determining piperine’s IC₅₀ values for inhibition of the formation of D-617 and norverapamil. In addition, the effect of piperine preincubation (50 μM for 10 min) in the presence of NADPH followed by incubation with verapamil (200 μM) for additional 10 min was compared with vehicle preincubation. All the metabolism experiments were performed in triplicate.
Drug Analyses. Aliquots (25 μl) containing radiolabeled digoxin, cyclosporine A, and insulin were analyzed by liquid scintillation counting (LS1800; Beckmann, Unterschleissheim, Germany) after the addition of 5 ml of Aquasafe 300 Plus (Zinsser Analytic, Frankfurt, Germany).

Concentrations of norverapamil, D-617, D-702, and D-703 were determined by a high-performance liquid chromatography-electrospray mass spectrometry assay (von Richter et al., 2000). In brief, to each reaction mixture containing ice-cold ethanol, 25 μl of internal standard (containing 50 pmol of D-832 and 3H2-norverapamil) was added. After centrifugation (10,000g for 10 min), supernatant was removed and evaporated under a stream of nitrogen. The residue was reconstituted with 150 μl of 5 mM ammonium acetate buffer (pH 4.2)-acetonitrile (70:30), and 5 μl was injected into liquid chromatography/mass spectrometry system.

Statistical Analysis. All data are presented as mean ± S.D. Differences in \( P_{\text{app}} \) were analyzed for statistical significance by repeated measures analysis of variance with subsequent Bonferroni multiple comparisons tests.

Results

Transport Studies. TEER measured before (0 h) and after (4 h) completion of transport studies showed no difference and was found to be higher than 200 Ω. The transport rate of \(^{3}H\text{I}nulin\) was not changed in the presence of different concentrations of piperine. The inhibition studies for digoxin and cyclosporine A transport showed a dose-dependent decrease in basal-to-apical and increase in apical-to-basal transport (Table 1). The vehicle used for dissolving piperine did not alter digoxin or cyclosporine A transport (data not shown). The basal-to-apical and apical-to-basal transport of digoxin and cyclosporine A in the presence of different concentrations of piperine is shown in Figs. 1 and 2. The IC\(_{50}\) values calculated for piperine-induced inhibition of digoxin and cyclosporine A transport are 15.5 (Fig. 3) and 74.1 μM, respectively.

Metabolism Studies with Human Liver Microsomes.

As expected, formation of D-617 and norverapamil correlated with CYP3A4 content of the five individual livers tested (D-617: \( r = 0.95, P = 0.01 \); norverapamil: \( r = 0.94, P = 0.01 \)). Piperine showed a dose-dependent inhibition of D-617 and norverapamil formation. The effect of piperine on inhibition of D-617 formation in the five different microsomes preparations is shown in Fig. 4. The IC\(_{50}\) values for inhibition of D-617 and norverapamil formation by piperine averaged 53.8 and 64.4 μM, respectively.

The results of D-617 formation in the absence or presence of 50 μM piperine are illustrated in Fig. 5. The respective

![Figure 1](image1.png)

**Fig. 1.** Effect of piperine on P-glycoprotein-mediated digoxin transport (5 μM) in monolayers of Caco-2 cells (a, digoxin alone; b, digoxin +1 μM piperine; c, digoxin + 10 μM piperine; and d, digoxin + 50 μM piperine).

![Figure 2](image2.png)

**Fig. 2.** Effect of piperine on P-glycoprotein-mediated cyclosporine A transport (1 μM) in monolayers of Caco-2 cells (a, cyclosporine A alone; b, cyclosporine A + 1 μM piperine; c, cyclosporine A + 10 μM piperine; and d, cyclosporine A + 250 μM piperine). 

\( V_{\text{max}} \), \( K_{\text{m}} \), and \( K_{i} \) for the metabolism studies are summarized in Table 2. Using nonlinear regression fitting, we found that piperine was a mixed type inhibitor of D-617 and norverapamil formation. In contrast, CYP2C-mediated formation of

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>( P_{\text{app}} ) (cm/s) \times 10^{-6}</th>
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<th>( P_{\text{app}} ) (cm/s) \times 10^{-6}</th>
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<tr>
<td></td>
<td>(basal-apical)</td>
<td></td>
<td>(apical-basal)</td>
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<tr>
<td>Control</td>
<td>11.66 ± 1.34</td>
<td></td>
<td>1.37 ± 0.33</td>
</tr>
<tr>
<td>+ 0.1 μM piperine</td>
<td>11.44 ± 0.48</td>
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<td>3.06 ± 0.86</td>
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<tr>
<td>+ 1 μM piperine</td>
<td>12.58 ± 1.03</td>
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<td>2.46 ± 0.36</td>
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<tr>
<td>+ 10 μM piperine</td>
<td>11.13 ± 0.02</td>
<td></td>
<td>4.40 ± 0.35</td>
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<tr>
<td>+ 25 μM piperine</td>
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<td></td>
<td>5.09 ± 0.05*</td>
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<tr>
<td>+ 50 μM piperine</td>
<td>7.05 ± 0.73*</td>
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<td>5.09 ± 0.25*</td>
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<tr>
<td>+ 100 μM piperine</td>
<td>7.57 ± 0.67*</td>
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<td>6.74 ± 1.54*</td>
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<tr>
<td>+ 250 μM piperine</td>
<td>7.52 ± 0.19*</td>
<td></td>
<td>6.95 ± 1.38*</td>
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N.D., not determined.

\* \( P < 0.05 \) control versus piperine (additional statistically significant differences between the different piperine concentrations are not shown).
the verapamil metabolites D-702 and D-703 (Busse et al., 1995) was only modestly inhibited by piperine (inhibition at 250 μM piperine of D-702, 47.8% and of D-703, 54.2%). Preincubation of piperine for 10 min resulted in a further 31 and 28% decrease of D-617 and norverapamil formation, respectively, compared with experiments with preincubation of vehicle only.

Discussion

In this study we identified piperine, which is a major constituent of black and long pepper, as an inhibitor of both human P-glycoprotein and CYP3A4. Piperine inhibited transport of the P-glycoprotein substrates digoxin and cyclosporine A with IC50 values of 15.5 and 74.1 μM, respectively. Moreover, piperine was also an inhibitor of human CYP3A4. CYP3A4-catalyzed formation of the verapamil metabolites D-617 and norverapamil was inhibited by piperine with Ki values of 36 ± 8 (liver 1) and 49 ± 6 (liver 2) μM and 44 ± 10 (liver 1) and 77 ± 10 (liver 2) μM, respectively. For the following reasons we believe that these observations are relevant for drug therapy in humans. First, many drugs are substrates of P-glycoprotein and/or CYP3A4, among them several with a small therapeutic index (e.g., digoxin, phenytoin, and cyclosporine A). Second, administration of black pepper (1 g, single dose) or piperine (single or multiple doses) resulted in an approximately 2-fold increase in plasma concentrations of the P-glycoprotein substrates phenytoin and rifampin (Zutshi et al., 1985; Bano et al., 1987; Velpandian et al., 2001). Finally, a 200-ml soup containing 1 g of black pepper (Velpandian et al., 2001) will contain about 1.1 mM piperine (Jensen-Jarolim et al., 1998) and local (intestinal) concentration might be in the range of our obtained IC50 and Ki values and will most likely result in inhibition of intestinal P-glycoprotein and CYP3A4. In addition, maximum piperine plasma concentrations after oral administration of 1 g of black pepper are in the low micromolar range (Velpandian et al., 2001) and might contribute to additional systemic effects, e.g., in liver. Indeed, we observed that preincubation of piperine before CYP3A4 substrate addition led to a further 30% decrease of CYP3A4 activity compared with vehicle preincubation. This observation suggests either that a piperine metabolite has a stronger inhibitory potency than piperine itself, or a suicide inhibition by piperine. The latter hypothesis is supported by previous results obtained with rats in which piperine administration resulted in 50% decrease of the total cytochrome P450 content (Dalvi and Dalvi, 1991).

Dietary consumption of black pepper varies considerably from one population group to another and even within a population group. Furthermore, precise assessments of black pepper consumption are not widely available. However, Kinnell (1984) did report an average daily consumption of 359 mg of black pepper daily in the United States. Given that the content of piperine in black pepper varies between 5 and 9%,

<table>
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<th>D-617</th>
<th>Norverapamil</th>
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<tr>
<td>Liver 1</td>
<td>Liver 2</td>
</tr>
<tr>
<td>Vmax (pmol/min/mg protein)</td>
<td>728 ± 102</td>
</tr>
<tr>
<td>Km (μM)</td>
<td>90 ± 21</td>
</tr>
<tr>
<td>Ki (μM)</td>
<td>36 ± 8</td>
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this would suggest a daily consumption of approximately 60 to 110 μmol of piperine.

Many drug-drug interactions can be explained by inhibition of P-glycoprotein and/or CYP3A4. Because a broad variety of drugs are substrates for both P-glycoprotein and CYP3A4 and because many compounds are inhibitors of both proteins, elevated plasma concentrations of a drug by a concomitantly administered substance can be due to a dual effect on drug transport and metabolism (Watkins, 1997; Wacher et al., 2001). Since the identification of major drug-metabolizing enzymes in the gut wall mucosa (Watkins et al., 1987), it became increasingly clear that metabolism in the enterocytes can play an important role for low or variable oral bioavailability of drugs (Kolars et al., 1991; Fromm et al., 1996; Paine et al., 1996). In addition, P-glycoprotein expressed in the apical (luminal) membrane of enterocytes determines bioavailability of several drugs, including cyclosporine, HIV protease inhibitors, digoxin, and the β-adrenoceptor antagonist talinolol (Lown et al., 1997; Kim et al., 1998; Greiner et al., 1999; Westphal et al., 2000).

It is now well established that nutrients and phytochemicals can have pronounced impact on drug disposition. Reduced plasma concentrations of different drugs were observed during intake of charcoal-broiled or smoked meat, cruciferous vegetables, and high salt diet (Walter-Sack and Klotz, 1996; Wilkinson, 1997; Darbar et al., 1998). Moreover, the mild antidepressant St. John’s wort induces intestinal CYP3A4 and P-glycoprotein via hyperforin-mediated activation of pregnane X receptor (Dürr et al., 2000) and significantly reduces plasma concentrations of cyclosporine, HIV protease inhibitors, and digoxin. On the other hand, grapefruit juice is the best studied nutrient inhibiting primarily intestinal drug metabolism and causing increased plasma concentrations of CYP3A4 substrates. Interestingly, constituents of grapefruit juice do not seem to have a relevant effect on P-glycoprotein function in humans (Becquemont et al., 2001).

Our data indicate that piperine might affect disposition of drugs that are substrates for both P-glycoprotein and CYP3A4. In addition, we were also able to show that piperine probably has no clinically relevant effect on enzymes of the CYP2C subfamily because there was little effect on formation of the P450 2C subfamily are the major enzymes involved in the O-demethylation of verapamil in humans. Naunyn-Schmiedeberg’s Arch Pharmacol 355:116–121.


It is obvious that there are pronounced differences in drug disposition not only within but also between ethnic groups. In addition to genetic factors, it is likely that interethnic differences in diet composition contribute to these differences. For example, one could speculate that a higher pepper intake in South Asians compared with Caucasians contributed to 3-fold higher plasma concentrations of the CYP3A4 substrate nifedipine, which was observed in the former group (Ahsan et al., 1991).

In summary, we provide strong evidence that a major constituent of pepper inhibits function of human P-glycoprotein and CYP3A4. This is in line with a limited number of clinical studies showing an effect of pepper or piperine intake on drug disposition. Further studies in humans are needed to clarify the impact of this nutrient on disposition of orally administered substrates of P-glycoprotein, CYP3A4, and possible other drug-metabolizing enzymes.

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References


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