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Effects of Spice Constituents on P-Glycoprotein-Mediated Transport and CYP3A4-Mediated Metabolism in Vitro

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ABSTRACT:

The effects of eight components from six commonly consumed spices on P-glycoprotein (P-gp) transport and CYP3A4 metabolism were evaluated in vitro. P-gp-mediated [^{3}H]digoxin fluxes across the L-MDR1 (LLC-PK1 cells transfected with human *MDR1* gene) and Caco-2 (human colon carcinoma) cell monolayers showed a marked asymmetry compared with that in the LLC-PK1 (porcine kidney epithelial cells) cell monolayers. Curcumin (from turmeric) at 30 to 60 μM and 6-gingerol (from ginger) at 100 to 500 μM were observed to inhibit P-gp-mediated [^{3}H]digoxin transport in L-MDR1 and Caco-2 cells. Effects of spices on midazolam (MDZ) 1′-hydroxylation and 4-hydroxylation of CYP3A4 activity were determined in pooled human liver microsomes (HLM). The following IC_{50} values for effects of spices on MDZ 1′-hydroxylation in HLM were obtained : 29 μM for curcumin, 1.17 mM for allyl methyl disulfide (AMD) (from Chinese chive), 1.02 mM for 1,8-cineole (from coriander), and 1.28 mM for β-caryophyllene (from curry leaf). CYP3A4-mediated 4-hydroxylation of MDZ was inhibited by curcumin at 30, 45, and 60 μM (4-hydroxy-MDZ formation was decreased to 52, 30, and 29%, respectively, compared with control), by 6-gingerol at 60, 100, and 500 μM (71, 68, and 38%), by AMD at 1 and 4 mM (29 and 14%), by *d*-limonene (from coriander) at 4 mM (65%), by 1,8-cineole at 0.5, 1, and 4 mM (74, 64, and 59%), and by citral (from lemongrass) at 1 mM (59%). Among the spices that showed inhibitory effect on MDZ metabolism in HLM, only AMD showed a preincubation time-dependent inhibitory effect on MDZ metabolism in HLM, suggesting the AMD as an irreversible CYP3A4 inhibitor.

P-glycoprotein (P-gp)-mediated efflux and CYP3A4-mediated metabolism play important roles in influencing the oral bioavailability of their substrates (Watkins, 1997). Intestinal P-gp and CYP3A4 are further hypothesized to form a coordinately regulated alliance, acting in tandem to limit the absorption of xenobiotics, including drugs (Johnson et al., 2003). Both proteins are localized at the villus tip of mature enterocytes (Watkins, 1997), and they exhibit overwhelming overlaps in substrate, inhibitor, and inducer specificities (Schuetz et al., 1996a). P-gp activity and expression may also have an influence on cellular CYP3A4 levels (Schuetz et al., 1996b), and the expressions of both proteins have been shown to be coordinately regulated by nuclear receptors, an example of which is the steroid and xenobiotic receptor, SXR (Synold et al., 2001).

Given the wide substrate specificities of the intestinal P-gp and CYP3A4, undesirable therapeutic outcomes can result from coadministration of potent drugs with xenobiotics that are substrates, inhibitors, or inducers of either protein. Of concern is the increasing use of complementary and alternative medicines (Eisenberg et al., 1998), particularly herbal medicines (Tindle et al., 2005) with prescription medicines in the United States. Unintended herb-drug interactions have already been documented and are become a growing medical concern (Brazier and Levine, 2003). Drug interactions involving P-gp and CYP3A4 have, for example, been reported for St. John's wort (*Hypericum perforatum*) (Johne et al., 1999) and garlic (*Allium sativum*) (Piscitelli et al., 2002).

On the other hand, a variety of spices are widely consumed on a daily basis by many Asian populations, yet little is known of the influence of spices on P-gp and CYP3A4 mediation of drug bioavailability. Curcumin (from turmeric) was only recently reported to inhibit cellular P-gp function and expression in vitro (Anuchapreeda et al., 2002). Although our laboratory observed that this spice component also inhibited CYP3A function in liver microsomes, subsequent experiments in the rat model showed that coadministered curcumin increased the area under the curve and C_{max} of peroral midazolam (CYP3A substrate) and celiprolol (P-gp substrate) (Zhang et al., 2007). Curcumin did not attenuate the function of CYP3A and P-gp in vivo but affected the pharmacokinetics of midazolam and celiprolol through down-regulation of CYP3A and P-gp protein expression in the small intestine. Similarly, whereas garlic inhibited CYP3A4 function in vitro (Foster et al., 2001), clinical experiments suggested that this spice induced CYP3A4 expression in vivo to decrease the area under the curve and C_{max} of saquinavir, a CYP3A4 substrate (Piscitelli et al., 2002). Clearly, more research is required to provide understanding of the clinical effects of spices on drug transport and metabolism.

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ABBREVIATIONS: P-gp, P-glycoprotein; AMD, allyl methyl disulfide; MDZ, midazolam; HLM, human liver microsomes; *MDR1*, multidrug resistance gene 1; MEM, minimal essential medium; HBSS, Hanks' balanced salt solution; DMSO, dimethylsulfoxide; TEER, transepithelial electrical resistance; AB, apical to basal; BA, basal to apical; HPLC, high-performance liquid chromatography; GI, gastrointestinal.

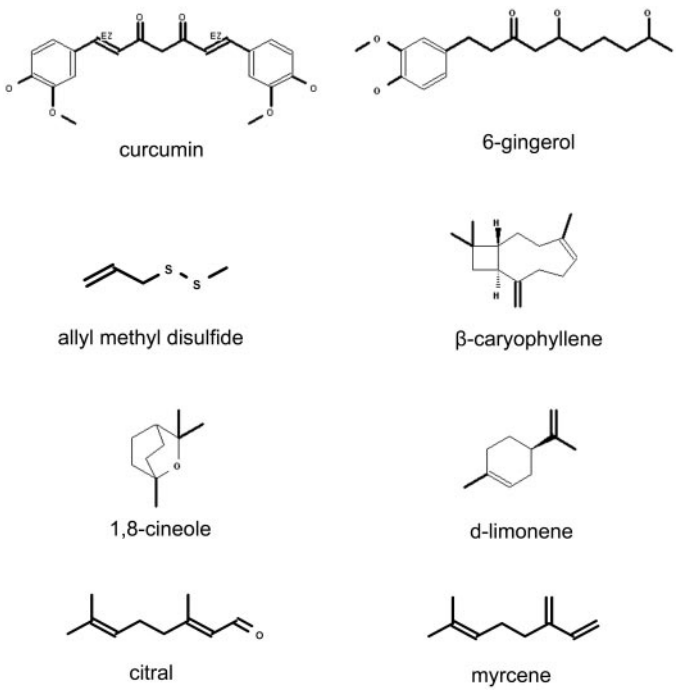

FIG. 1. Chemical structures of the components of spices.

In the present study, we screened eight components from six commonly consumed spices for their modulating activities on the P-gp and CYP3A4 with a view to isolating promising components for further experimentation. Pure components were preferred to whole spices as the compositions of the latter could be highly variable, being influenced by plant species, as well as the conditions of growth, postharvest processing, and storage. The spice components were allyl methyl disulfide (AMD), present in both garlic (*Allium sativum*) and Chinese chive (*Allium tuberosum*), curcumin from turmeric (*Curcuma longa*), 6-gingerol from ginger (*Zingiber officinale*), myrcene and citral from lemongrass (*Cymbopogon citratus*), 1,8-cineole and *d*-limonene from coriander (*Coriandrum sativum*), and β-caryophyllene from curry leaf (*Murraya koenigii*). Their chemical structures are shown in Fig. 1. Of these, curcumin, garlic, and turmeric have in recent years attracted significant research interest as potential anticancer adjuvants (Aggarwal and Shishodia, 2006). To evaluate P-gp efflux activity, bidirectional transport studies of [^{3}H]digoxin were carried out using the LLC-PK1, L-MDR1, and Caco-2 cell monolayers. CYP3A4-mediated metabolism of midazolam (MDZ) was evaluated using pooled human liver microsomes (HLM).

Materials and Methods

Cell Culture. LLC-PK1 is a porcine kidney-derived cell line that expresses very low levels of P-gp (Weiss et al., 2003), whereas L-MDR1 cells are LLC-PK1 cells stably transfected with the human *MDR1* gene (Schinkel et al., 1996). Caco-2 is a human colon carcinoma cell line that constitutively expresses P-gp and has been widely used as a surrogate for the human intestinal epithelium. L-MDR1 cells (passage 220) were kindly provided by Dr. A. H. Schinkel (Division of Experimental Therapy, Netherlands Cancer Institute, Amsterdam, The Netherlands), LLC-PK1 cells (passage 230) were obtained from the American Type Culture Collection (Rockville, MD), and Caco-2 cells (passage 40) were from the Riken Cell Bank (Tsukuba, Ibaraki, Japan).

LLC-PK1 (passage 232–240) and L-MDR1 (passage 223–230) cells were cultured in M199 medium (Invitrogen, Grand Island, NY) supplemented with 50 U/ml penicillin (Sigma-Aldrich, St. Louis, MO), 50 μg/ml streptomycin (Sigma-Aldrich), and 10% (v/v) of fetal bovine serum (Invitrogen). Caco-2 cells at passages 50 to 60 were cultured in MEM (Invitrogen) supplemented with 10% fetal bovine serum, 1% nonessential amino acids (Sigma-Aldrich), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air (NuAire US autoflow, NuAire Inc., MN), with medium exchange on alternate days. Cells were subcultured every 3 days after trypsinization.

In Vitro Cytotoxicity Studies. Cytotoxicity of spices was determined by the 3–(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay (Scudiero et al., 1988). L-MDR1 and LLC-PK1 cells were cultured on 96-well plates at a seeding density of 1×10^4 cells/well in 100 μl of M199 medium. Caco-2 cells were similarly seeded at a density of 8×10^3 cells/well in MEM medium. Cells were cultured in 5% CO_2/95% air at 37°C for 48 h after which the culture medium was exchanged for 100 μl of spice samples (0–4 mM in HBSS-HEPES transport medium containing 0.5% DMSO, pH 7.4), and the cells further incubated for 4 h at 37°C. All spice components were obtained from Sigma-Aldrich, except for 6-gingerol, which was from Wako Pure Chemicals (Osaka, Japan), and AMD, which was a gift from Flavor Consultants (Las Vegas, NV). Control samples comprised HBSS-HEPES, 1% sodium lauryl sulfate in HBSS-HEPES (positive control), and 1% dextran in HBSS-HEPES (negative control), all of which were supplemented with 0.5% DMSO. To determine cell viability, the spice or control samples were decanted, and the cells were incubated with 100 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium solution (1 mg/ml in HBSS-HEPES, pH 7.4) for 4 h at 37°C. Intracellular formazan crystals were extracted into 100 μl of DMSO and quantified by measuring the absorbance of the cell lysate at 590 nm (Spectra Fluor plate reader; Tecan, Grödig; Austria). Cell viability was calculated as a percentage based on the absorbance measured relative to the absorbance obtained from cells exposed only to the HBSS-HEPES transport medium containing 0.5% DMSO.

Transport Experiments. LLC-PK1 and L-MDR1 cells were separately cultured in M199 medium (apical 0.5 ml; basal 1.5 ml) at a seeding density of 4×10^5 cells/12-mm well on 3.0-μm polycarbonate membrane filters (Transwell 3402; Costar, Bedford, MA). Cells were supplemented with fresh media every 2 days and used for transport experiments on the 6th or 7th day after plating when they registered a transepithelial electrical resistance (TEER) of 200 Ω·cm^2 or greater (Millicell ERS ohmmeter; Millipore Corporation, Bedford, MA). Caco-2 cells for transport experiments were cultured in MEM medium at a seeding density of 1×10^5 cells/well on 0.4-μm polycarbonate membrane filters (Transwell 3401; Costar), with medium exchange on alternate days. On days 21 to 25, cell monolayers with TEER values >300 Ω·cm^2 (corrected for blank) were used for transport experiments. The dosing solutions comprised [^{3}H]digoxin (5 μM, 0.51 μCi/ml) or [^{14}C]mannitol (10 μM, 0.54 μCi/ml) (PerkinElmer Life and Analytical Sciences, Boston, MA) dissolved in the respective transport medium (Opti-MEM for L-MDR1 and LLC-PK1 cells or HBSS-HEPES at pH 7.4 for Caco-2 cells) supplemented with 0.5% DMSO and containing 0 to 60 μM curcumin, 0 to 500 μM 6-gingerol, 0 to 1 mM citral, or 0 to 4 mM AMD, *d*-limonene, 1,8-cineole, β-caryophyllene, or myrcene. Dosing solutions without spices were spiked with 100 μM verapamil HCl to serve as positive controls.

Transport experiments were initiated by replacing the culture medium in the apical and basal chambers with 700 μl of transport medium. After 1 h of equilibration, the medium in the donor compartment was replaced with 700 μl of the respective dosing solution and that in the receiver compartment with 700 μl of corresponding blank transport medium (containing the same DMSO and spice concentrations as the dosing solution in the donor compartment). The amount of [^{3}H]digoxin or [^{14}C]mannitol appearing in the receptor compartment after 1, 2, 3, and 4 h was measured in 50-μl aliquots sampled from each receiver compartment and expressed as a percentage of the initial loaded dose. Solute concentration was determined by measuring the radioactivity (LS 3801; Beckman Instruments, Fullerton, CA) of the aliquot samples incubated overnight with 3 ml of scintillation fluid. The receiver chamber was replenished with 50 μl of the corresponding blank transport medium after each sampling. At the end of the transport experiment, the cell monolayers were reincubated with the transport medium for 30 min at 37°C before the measurement of TEER to assess the integrity of the cell monolayers.

The apparent permeability (P_{app} in centimeters per second) was calculated using the equation $P_{app} = (dQ/dt)/(A \cdot C_0)$ (cm/s), where dQ/dt (micromoles per second) is the initial transport rate, C_0 (millimolar concentration) is the initial drug concentration in the donor chamber, and A (square centimeters) is

the surface of the cell monolayer. Transport was conducted in the apical to basal (AB), and basal to apical (BA) directions. Net efflux was expressed as the quotient of $P_{app\ (BA)}$ to $P_{app\ (AB)}$. Data are presented as means ± S.D. ($n = 4$).

CYP3A4-Mediated MDZ Biotransformation in HLM. The assay of MDZ 1′-hydroxylase and 4-hydroxylase activities of human CYP3A4 was performed according to the method of Kim et al. (2006), using the same microsome protein and MDZ concentrations as well as incubation time. A preliminary study showed that, at the protein concentration of 0.5 mg/ml, the formation of 1′-OH-MDZ and 4-OH-MDZ increased linearly with incubation time to reach maximum concentrations at 15 to 20 min. Thus, a reaction time of 15 min, which was within the range of values (5–20 min) reported in the literature for similar studies (Perloff et al., 2005), was chosen for subsequent experiments. Pooled HLM (H452161; BD Gentest, San Jose, CA) were reconstituted at a concentration of 0.5 mg/ml in 100 mM phosphate buffer (pH 7.4) to a final volume of 250 μl and preincubated with 10 μM MDZ (Dormicum; Roche Diagnostics, Basel, Switzerland) for 5 min at 37°C. Metabolic reactions were initiated by adding 15 μl of NADPH-regenerating system (3.3 mM glucose 6-phosphate, 1.3 mM β-$NADP^+$, 3.3 mM $MgCl_2$, and 0.4 U/ml glucose-6-phosphate dehydrogenase, BD Gentest) and terminated after 15 min by placing the incubation mixtures on ice and adding 250 μl of ice-cold acetonitrile. Norclomipramine (Sigma-Aldrich) as internal standard was added to a final concentration of 2 μg/ml, and the incubation mixtures were centrifuged at 20,000*g* for 10 min at 4°C (MIKRO 22R; Andreas Hettich GmbH and Co. KG, Tuttlingen, Germany). The supernatants (20 μl) were subjected to HPLC analysis.

The effects of curcumin (0–60 μM), 6-gingerol (0–500 μM), citral (0–1 mM), AMD (0–4 mM), *d*-limonene (0–4 mM), 1,8-cineole (0–4 mM), β-caryophyllene (0–4 mM), and myrcene (0–4 mM) on CYP3A4-mediated metabolism of MDZ were investigated. Spices dissolved in DMSO were added into the HLM and MDZ mixture to give final DMSO concentration of 0.5%, and the enzyme reaction was initiated by adding the NADPH-regenerating system. Control experiments were performed in parallel using ketoconazole (5 μM) as an inhibitor of CYP3A4-mediated MDZ 1′-hydroxylation and 4-hydroxylation (Perloff et al., 2005), α-naphthoflavone (3 μM; Sigma-Aldrich) as an activator of MDZ 1′-hydroxylation (Fujita et al., 2005), and testosterone (50 μM; Sigma-Aldrich) as an activator of MDZ 4-hydroxylation (Cameron et al., 2005). Ketoconazole, α-naphthoflavone, and testosterone were also dissolved in DMSO and added to the enzyme mixture to give DMSO final concentration of 0.5%. The rates of formation of 1′-OH-MDZ and 4-OH-MDZ in the presence of the spices were expressed as the corresponding rates of metabolite formation in the absence of an effector. The IC_{50} values for spices that exhibited inhibitory effects on 1′-OH-MDZ formation in the HLM system were determined by linear regression analysis using GraphPad Prism 4 software (GraphPad Software Inc., San Diego, CA). All experiments were performed in triplicate.

To evaluate the effect of preincubation on CYP3A4 activity, the spices were added to the HLM and NADPH regenerating system in the absence of MDZ, and after preincubation periods of 5, 10, 15, or 20 min, the enzyme reaction was initiated by the addition of MDZ (10 μM). Ketoconazole (1 μM) served as the negative control (Perloff et al., 2005) and verapamil (50 μM) a mechanism-based inhibitor of CYP3A4 (Wang et al., 2005) as the positive control.

Quantification of 1′-Hydroxymidazolam and 4-Hydroxymidazolam in HLM. 1′-OH-MDZ and 4-OH-MDZ in the HLM mixture were quantified by HPLC assay (Good et al., 2004). The HPLC system consisted of an Agilent 1100 system (Agilent Technologies, Palo Alto, CA) with a diode array detector. Drugs and internal standard (norclomipramine) were separated on a Symmetry C_{18} column (200 mm × 4.6 mm, 5 μm; Waters, Milford, MA), preceded by a Symmetry C_{18} guard column. Gradient elution was performed at a flow rate of 1.0 ml/min. The mobile phase was maintained at 45% acetonitrile, 10% methanol, and 45% KH_2PO_4 buffer (20 mM, pH 7.4) for 8 min before a linear gradient run from 8 to 20 min was performed to give final concentrations of 56% acetonitrile, 10% methanol, and 34% buffer. The detection wavelength was 220 nm. HPLC peaks were recorded and integrated using Agilent data analysis software.

The HPLC was calibrated with 1′-OH-MDZ and 4-OH-MDZ in the concentration range of 0.05 to 3.2 μg/ml. Stock solutions, including those of the internal standard, norclomipramine, were prepared in methanol at 1 mg/ml and stored at −20°C. Standard solutions were prepared from corresponding stock solutions with the heat-inactivated HLM mixture as solvent. The intraday and interday coefficients of variation were <15% for 1′-OH-MDZ and 4-OH-MDZ at the test concentrations of 0.1, 0.4, and 0.8 μg/ml. The lower limit of quantification was 25 ng/ml for both metabolites. Typical retention times recorded for 1′-OH-MDZ, 4-OH-MDZ and the internal standard were 6.1, 5.4, and 19.3 min, respectively.

Statistical Analysis. Data were analyzed by one-way ANOVA with Tukey's test (SPSS 10.0; SPSS Inc., Chicago, IL) applied for paired comparisons of mean values. A p value ≤0.05 was considered statistically significant.

Results

In Vitro Cytotoxicity of Spices. In vitro cytotoxicity experiments were conducted with HBSS as the control medium and 1% SDS and 1% dextran as positive and negative controls, respectively. Based on the dextran data, the spices were considered to be cytotoxic when they reduced mean cell viability by 20% or more relative to HBSS. On this basis, the viabilities of LLC-PK1, L-MDR1, and Caco-2 cells were considered to be unaffected by 4 h of coincubation with up to 60 μM curcumin, 500 μM 6-gingerol, 4 mM AMD, 4 mM *d*-limonene, 4 mM 1,8-cineole, 4 mM β-caryophyllene, 4 mM myrcene, and 1 mM citral. Cell viability was, however, adversely affected by the addition of 90 μM curcumin and 1.5 mM citral. The Caco-2 cells were more resilient (75% viability) than the L-MDR1 (21%) and LLC-PK1 (36%) cells to 90 μM curcumin, although all three cell types exhibited equally poor viabilities in 120 μM curcumin. Similarly, although the Caco-2 cells were more sensitive (50% viability) to 1.5 mM citral than the L-MDR1 and LLC-PK1 cells (>80%), all three cell types showed comparable reductions in viability when the citral concentration was raised to 3 mM. To avoid confounding the drug transport data, subsequent experiments were performed with spice concentrations that yielded cell viability ≥80%.

Effects of Spices on P-gp-Mediated [^{3}H]Digoxin Transport. Transepithelial [^{3}H]digoxin fluxes across the L-MDR1 cell monolayers showed a marked asymmetry, yielding a net efflux value of 6.36. The polarized permeability is characteristic of an efflux system that assists in the transfer of intracellular digoxin back to the apical chamber (Cavet et al., 1996). The unidirectional transport was abolished in the presence of 100 μM verapamil HCl, a well-documented P-gp inhibitor (Greiner et al., 1999) (Table 1), which reduced the net efflux to 1.19 by increasing the AB permeability and reducing the BA permeability of [^{3}H]digoxin across the L-MDR1 cell monolayers. By comparison, transepithelial [^{3}H]digoxin fluxes across the LLC-PK1 cells, the parent cell line of the L-MDR1 cells, were much less polarized (net efflux of 1.88) (Table 1), whereas those across the Caco-2 cell monolayers exhibited intermediate asymmetry, the net efflux value being 4.62 (Table 2). For both cell lines, the coadministration of verapamil HCl (100 μM) also abolished the polarity of digoxin transport (Tables 1 and 2). These data, which corresponded to those reported in the literature (Kim et al., 1998; Bhardwaj et al., 2002), suggested a ranking of P-gp efflux activity on the order of L-MDR1 > Caco-2 > LLC-PK1. Whereas the high level of P-gp functionality in the transfected L-MDR1 cells was expected, the data implied expression of low levels of porcine P-gp in the parental LLC-PK1 cells and a significant level of P-gp constitutively expressed in the Caco-2 cell monolayers.

Of the spices evaluated, *d*-limonene and myrcene at the concentrations tested did not affect [^{3}H]digoxin transport in the L-MDR1 cells ($p > 0.05$) (Table 1). For spices that did modulate the [^{3}H]digoxin transport profiles in these cells, only curcumin and 6-gingerol produced transport profiles suggestive of P-gp inhibition. Curcumin at 30 and 60 μM significantly increased the AB [^{3}H]digoxin flux with a

TABLE 1

Effects of spices on [^{3}H]digoxin transport in the AB and BA directions across confluent L-MDR1 and LLC-PK1 cell monolayers

Data are shown as mean ± S.D. $n = 4$.

Sample	Conc.	L-MDR1			LLC-PK1		
		P_{app}		Net Efflux	P_{app}		Net Efflux
		AB	BA		AB	BA	
	μM	$\times 10^{-6}$ cm/s			$\times 10^{-6}$ cm/s		
Control	0	1.03 ± 0.13	6.60 ± 0.18	6.36	1.78 ± 0.27	3.34 ± 0.48	1.88
Verapamil	100	2.72 ± 0.05*	3.24 ± 0.04*	1.19	1.95 ± 0.07	2.03 ± 0.03*	1.04
Curcumin	15	0.84 ± 0.03	6.39 ± 0.04	7.59	1.40 ± 0.03	2.88 ± 0.31	2.05
	30	1.43 ± 0.14*	4.98 ± 0.15*	3.49	1.67 ± 0.06	2.98 ± 0.13	1.78
	60	2.14 ± 0.02*	4.31 ± 0.02*	2.01	2.60 ± 0.30*	3.28 ± 0.17	1.26
6-Gingerol	100	2.11 ± 0.27*	5.70 ± 0.19*	2.70	1.89 ± 0.06	2.93 ± 0.08	1.55
	250	2.34 ± 0.06*	5.49 ± 0.35*	2.34	1.94 ± 0.05	2.45 ± 0.10*	1.26
	500	2.70 ± 0.14*	4.49 ± 0.31*	1.66	2.30 ± 0.01*	2.30 ± 0.08*	1.00
AMD	1000	1.12 ± 0.02	5.71 ± 0.23*	5.11	1.89 ± 0.20	3.66 ± 0.19	1.93
	4000	1.33 ± 0.05	5.24 ± 0.21*	3.94	1.73 ± 0.14	3.62 ± 0.35	2.09
d-Limonene	1000	1.16 ± 0.04	6.54 ± 0.28	5.64	1.59 ± 0.11	3.20 ± 0.32	2.02
	4000	1.38 ± 0.04	6.47 ± 0.21	4.70	2.64 ± 0.04	3.55 ± 0.26	1.35
1,8-Cineole	1000	1.61 ± 0.04	7.18 ± 0.27	4.59	1.82 ± 0.14	3.42 ± 0.40	1.88
	4000	2.77 ± 0.07*	6.95 ± 0.39	2.62	2.61 ± 0.15*	3.61 ± 0.28	1.38
β-Caryophyllene	1000	1.15 ± 0.01	5.54 ± 0.19*	4.79	1.69 ± 0.16	3.23 ± 0.29	1.92
	4000	1.33 ± 0.05	5.81 ± 0.11*	4.36	1.71 ± 0.24	3.64 ± 0.24	2.13
Myrcene	1000	1.16 ± 0.06	6.28 ± 0.13	5.42	1.40 ± 0.15	3.13 ± 0.11	2.23
	4000	1.60 ± 0.04	6.75 ± 0.12	4.22	1.32 ± 0.07	3.63 ± 0.11	2.75
Citral	250	1.49 ± 0.01	6.04 ± 0.13	4.04	1.46 ± 0.08	2.80 ± 0.23	1.92
	1000	2.52 ± 0.08*	6.70 ± 0.21	2.66	3.29 ± 0.07*	3.35 ± 0.06	1.02

* $P \leq 0.05$ compared with control. Net efflux is expressed as the quotient of P_{app} (BA) to P_{app} (AB).

TABLE 2

Effects of spices on [^{3}H]digoxin and [^{14}C]mannitol transport in the AB and BA directions across confluent Caco-2, L-MDR1, and LLC-PK1 cell monolayers

Data are expressed as mean ± S.D. $n = 4$.

Sample	Conc.	[^{3}H]Digoxin P_{app}			[^{14}C]Mannitol P_{app}					
		Caco-2			Caco-2		L-MDR1		LLC-PK1	
		AB	BA	Net efflux	AB	BA	AB	BA	AB	BA
	μM	$\times 10^{-6}$ cm/s			$\times 10^{-7}$ cm/s					
Control	0	1.52 ± 0.07	7.01 ± 0.18	4.62	4.77 ± 0.63	4.86 ± 0.77	2.76 ± 0.34	2.70 ± 0.40	2.89 ± 0.24	2.93 ± 0.35
Verapamil	100	2.70 ± 0.07*	3.32 ± 0.01*	1.23						
Curcumin	15	1.77 ± 0.07	6.79 ± 0.55	3.84	5.21 ± 0.72	5.00 ± 0.69	2.17 ± 0.33	2.39 ± 0.38	3.08 ± 0.26	2.85 ± 0.30
	30	2.09 ± 0.04*	5.81 ± 0.57*	2.78	5.43 ± 0.67	5.26 ± 0.62	2.24 ± 0.37	2.46 ± 0.41	2.98 ± 0.23	2.79 ± 0.34
	60	2.16 ± 0.02*	5.31 ± 0.32*	2.46	5.77 ± 0.83	5.05 ± 0.63	2.53 ± 0.08	2.30 ± 0.11	2.58 ± 0.31	2.64 ± 0.36
6-Gingerol	100	2.21 ± 0.11*	5.93 ± 0.31*	2.68	5.23 ± 0.63	4.72 ± 0.21	2.29 ± 0.31	1.78 ± 0.29	1.45 ± 0.08	1.38 ± 0.07
	250	2.39 ± 0.17*	5.19 ± 0.27*	2.17	5.77 ± 0.83	5.05 ± 0.63	2.04 ± 0.39	1.90 ± 0.33	1.33 ± 0.11	1.30 ± 0.04
	500	2.58 ± 0.25*	4.83 ± 0.23*	1.87	5.21 ± 0.57	6.28 ± 0.57	2.13 ± 0.23	2.35 ± 0.27	1.43 ± 0.17	1.37 ± 0.20

* Net efflux is expressed as the quotient of P_{app} (BA) to P_{app} (AB).

concomitant reduction of BA [^{3}H]digoxin flux to lower the net efflux ratios to 3.49 and 2.01, respectively (Table 1). 6-Gingerol at concentrations of 100, 250, and 500 μM produced similar effects, yielding net [^{3}H]digoxin efflux ratios of 2.77, 2.34, and 1.66, respectively (Table 1). In contrast, AMD and β-caryophyllene at 1 and 4 mM were not regarded as P-gp inhibitors even though they reduced the [^{3}H]digoxin efflux ratio (Table 1) because both spices lowered the BA [^{3}H]digoxin flux without affecting its transport in the AB direction. On the other hand, 1,8-cineole (4 mM) and citral (1 mM) lowered the [^{3}H]digoxin efflux ratio by increasing transport in the AB direction without changing the BA flux. These two spices were therefore also unlikely to be P-gp inhibitors.

[^{3}H]Digoxin transport across the parent LLC-PK1 cell monolayers was less affected by the spices. Of the spices studied, *d*-limonene, myrcene, AMD, and β-caryophyllene did not change the [^{3}H]digoxin transport profiles of the LLC-PK1 cells when used at the specified concentration ranges. Curcumin at up to 30 μM also did not affect the [^{3}H]digoxin transport profiles, although it increased the AB flux at 60 μM. In the case of 6-gingerol, [^{3}H]digoxin transport profiles reflecting effective P-gp inhibition were apparent only at the highest concentration of 500 μM. Only 1,8-cineole (4 mM) and citral (1 mM) changed the [^{3}H]digoxin transport profiles of the LLC-PK1 cells in a manner consistent with those observed in the L-MDR1 cells.

The P-gp inhibitory activities of curcumin and 6-gingerol were further examined in the Caco-2 cells which, unlike the L-MDR1 cells, constitutively expressed the human P-gp. The data are presented in Table 2. Digoxin transport profiles obtained for the Caco-2 cells in the presence of curcumin and 6-gingerol correlated very well with those obtained for the L-MDR1 cells. Curcumin did not affect digoxin transport across the Caco-2 cells at the low concentration of 15 μM but produced digoxin transport profiles reminiscent of P-gp inhibition at 30 and 60 μM (Table 2). The net digoxin efflux ratio was reduced to 2.78 and 2.46 at 30 and 60 μM curcumin, respectively. 6-Gingerol also increased the AB digoxin flux in the Caco-2 cells with a concomitant reduction of digoxin transport in the BA direction at the concentrations of 100, 250, and 500 μM (Table 2). The corresponding

net efflux ratios were 2.68, 2.17, and 1.87, comparable with those obtained for the L-MDR1 cell monolayers.

Effects of Spices on [^{14}C]Mannitol Transport. [^{14}C]Mannitol is a radiolabeled hydrophilic marker widely used to evaluate the paracellular transport pathway. It is not taken up by absorptive cells in significant amounts but is highly permeable through the tight junctions of cell monolayers (Artursson et al., 1996). In the absence of spices, P_{app} values of 2.76×10^{-7} and 2.70×10^{-7} cm/s were obtained for [^{14}C]mannitol transport in the AB and BA directions, respectively, across the L-MDR1 cell monolayers (Table 2). Corresponding P_{app} values for the LLC-PK1 cells were 2.89×10^{-7} and 2.93×10^{-7} cm/s, whereas those for the Caco-2 cells were 4.77×10^{-7} and 4.86×10^{-7} cm/s. The P_{app} values obtained for the Caco-2 cells corresponded well with values reported in the literature (Demirbas and Stavchansky, 2003). [^{14}C]Mannitol P_{app} values for the L-MDR1 and LLC-PK1 cell monolayers have not been reported; nevertheless, given the lower P_{app} values obtained for [^{14}C]mannitol transport across the L-MDR1 and LLC-PK1 cell monolayers, it was assumed that these cell monolayers also possessed acceptable integrity. Exposure to curcumin (15–60 μM) or 6-gingerol (100–500 μM) did not significantly alter the AB and BA [^{14}C]-mannitol P_{app} values ($p > 0.05$) (Table 2), suggesting that the two spices at the concentrations used did not modulate the paracellular transport pathway in all three cell monolayers

Effects of Spices on CYP3A4-Mediated Metabolism in HLM. Experiments to investigate the effects of the spices on CYP3A4 activity were performed using 125 μg of pooled HLM as the enzyme source and 0.8 μg of MDZ as the model substrate. After 15 min of enzymatic conversion, 0.39 and 0.04 μg, respectively, of 1′-OH-MDZ and 4-OH-MDZ were detected using the HPLC assay. Ketoconazole, a known inhibitor of CYP3A4-mediated 1′- and 4-hydroxylation of MDZ (Perloff et al., 2005), was observed to inhibit 96% of the 1′-OH-MDZ and 100% of the 4-OH-MDZ production when administered at 5 μM. On the other hand, coincubation with 3 μM α-naphthoflavone, a stimulator of MDZ 1′-hydroxylation (Fujita et al., 2005), caused a 1.68-fold increase in 1′-OH-MDZ production. Likewise, the addition of 50 μM testosterone, a stimulator of MDZ 4-hydroxylation (Cameron et al., 2005), raised the production of 4-OH-MDZ by 1.63-fold. These control experiments indicated the presence of functional CYP3A4 in the HLM system that was amenable to inhibition and enhancement.

The following spices were found to inhibit CYP3A4-mediated 1′-hydroxylation of MDZ in the HLM: curcumin at 45 and 60 μM (73 and 82% inhibition, respectively, compared with control values); AMD at 1 and 4 mM (68 and 76%); 1,8-cineole at 1 and 4 mM (55 and 71%); and β-caryophyllene at 1 and 4 mM (51 and 71%). Corresponding IC_{50} values were 29 μM for curcumin, 1.02 mM for 1,8-cineole, 1.17 mM for AMD, and 1.28 mM for β-caryophyllene. Compared with ketoconazole, which had a reported IC_{50} of 0.04 μM (Perloff et al., 2000), these spices might be regarded as weak CYP3A4 inhibitors. CYP3A4-mediated 1′-hydroxylation of MDZ was enhanced by the addition of 6-gingerol at 100 μM (1.66-fold increase), *d*-limonene at 1 and 4 mM (1.55- and 1.57-fold, respectively), myrcene at 4 mM (1.38-fold), and citral at 60 μM (1.59-fold). None of these spices was as potent as α-naphthoflavone in stimulating CYP3A4-mediated 1-OH-MDZ (Fig. 2A).

CYP3A4-mediated 4-hydroxylation of MDZ was inhibited by a greater number of spices, including curcumin at 30, 45, and 60 μM (4-OH-MDZ formation was decreased to 52, 30, and 29%, respectively, compared with control), 6-gingerol at 60, 100, and 500 μM (71, 68, and 38%), AMD at 1 and 4 mM (29 and 14%), *d*-limonene at 4 mM (65%), 1,8-cineole at 0.5, 1, and 4 mM (74, 64, and 59%), and citral at 1 mM (59%). Again, none of the spices was more potent than

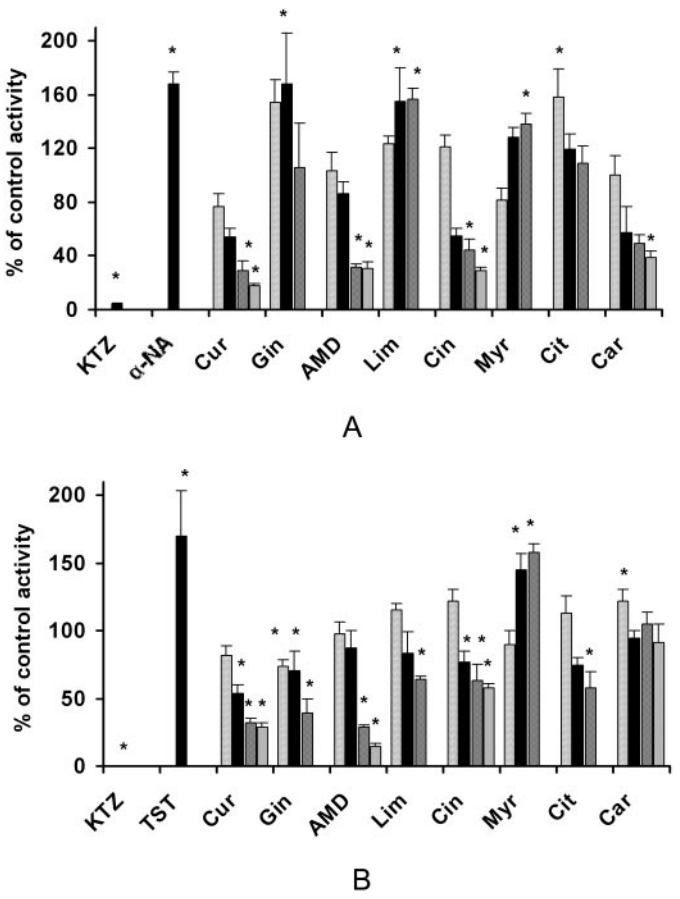

FIG. 2. Effects of spices on the activity of CYP3A4 in human liver microsomes. A, midazolam 1′-hydroxylation. B, midazolam 4-hydroxylation. Concentrations are as follows: ketoconazole (KTZ), 5 μM; testosterone (TST), 50 μM; α-naphthoflavone (α-NA), 3 μM; curcumin (Cur), 15, 30, 45, and 60 μM; 6-gingerol (Gin), 60, 100, and 500 μM; allyl methyl disulfide (AMD), *d*-limonene (Lim), and β-caryophyllene (Car), 60 μM, 500 μM, 1 mM, and 4 mM; 1,8-cineole (Cin) and myrcene (Myr), 60 μM, 1 and 4 mM; and citral (Cit): 60, 250, and 1000 μM. The corresponding control activities of midazolam 1′-hydroxylation and 4-hydroxylation by human liver microsomes were 2.31 and 0.243 nmol/min/mg of protein, respectively. Data are shown as averages of triplicate experiments. *, $p \leq 0.05$ significant difference compared with control.

ketoconazole in inhibiting the formation of 4-OH-MDZ. Two of the spices, myrcene at 1 and 4 mM and β-caryophyllene at 60 μM, were observed to enhance the formation of 4-OH-MDZ, although the increase in 4-OH-MDZ concentration was not as high as that seen with 50 μM testosterone (Fig. 2B).

Because curcumin, AMD, and 1,8-cineole showed inhibitory effects on both MDZ 1′-hydroxylation and MDZ 4-hydroxylation, these spices were further investigated for possible mechanism-based inhibition of CYP3A4 activity in the HLM. Ketoconazole, a known reversible inhibitor of CYP3A4 (Perloff et al., 2005), was used as a negative control and verapamil, a mechanism-based inhibitor of CYP3A (Wang et al., 2005), as the positive control. As expected, the inhibitory effect of ketoconazole on CYP3A4-mediated MDZ metabolism did not increase with prolonged preincubation, whereas that of verapamil increased with prolonged preincubation (Fig. 3). Curcumin and 1,8-cineole did not exhibit any preincubation time-dependent inhibition of 1′-hydroxylation and 4-hydroxylation of MDZ in the HLM (Fig. 3). In contrast, AMD showed increased inhibition of 1′-OH-MDZ and 4-OH-MDZ formation with prolonged preincubation, suggesting that it might be a mechanism-based inhibitor of the CYP3A4.

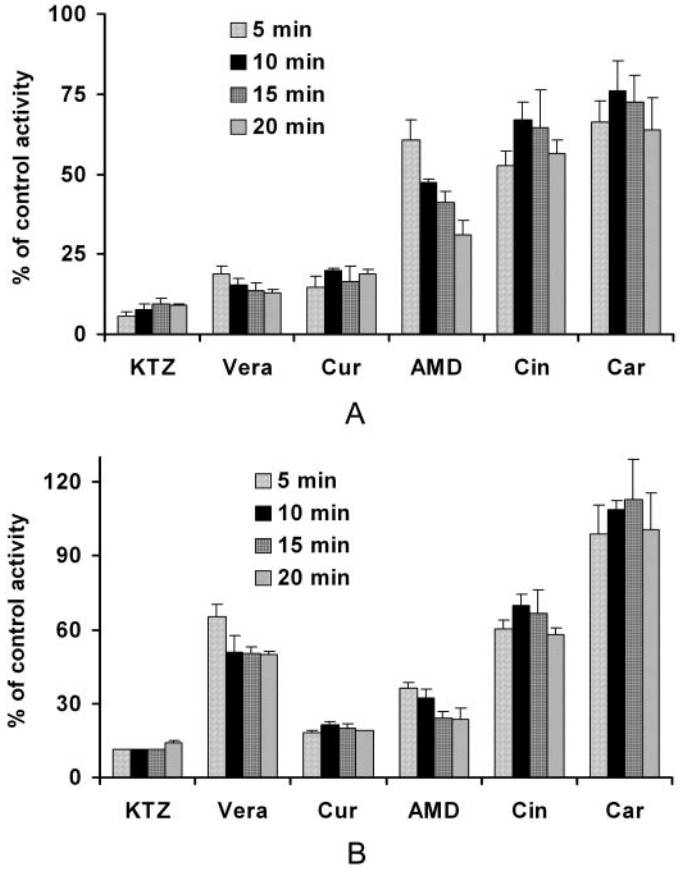

FIG. 3. Effect of preincubation time on the inhibition of midazolam 1′-hydroxylation (A) and 4-hydroxylation (B) by spices. ketoconazole (KTZ), 1 μM; verapamil (Vera), 50 μM; curcumin (Cur), 45 μM; allyl methyl disulfide (AMD), 1,8-cineole (Cin), and β-caryophyllene (Car), 1 mM. Data are shown as averages of triplicate experiments.

Discussion

Food-drug interactions are everyday occurrences that can become clinically important if they involve potent drugs with narrow therapeutic indexes. However, despite the widespread consumption of spices in many populations, there have been few reports on spice-drug interactions. In the present study, eight commercially available, purified components of spices regularly consumed in Southeast Asia were evaluated for their effects on P-gp-mediated [^{3}H]digoxin transport and CYP3A4-mediated MDZ metabolism as a tool to assess their potential to induce food-drug interactions.

Of the eight spice components, curcumin (30–60 μM), 6-gingerol (100–500 μM), AMD (1–4 mM), β-caryophyllene (1–4 mM), 1,8-cineole (4 mM), and citral (1 mM) were found to significantly modulate [^{3}H]digoxin transport in the L-MDR1 and Caco-2 cell monolayers. However, only curcumin and 6-gingerol at the concentrations used produced [^{3}H]digoxin transport profiles characteristic of P-gp inhibition. The mechanisms by which the other spices modulate [^{3}H]digoxin transport across the cell monolayers are not known at present. [^{3}H]Digoxin transport across the cell monolayers could be affected not only by P-gp efflux activity but also by passage through the paracellular and transcellular pathways via passive diffusion (Tanigawara et al., 1992). Modulation of the paracellular or transcellular passive diffusion pathways would, however, lead to comparable changes in [^{3}H]digoxin transport in both the AB and BA directions, with net efflux being unaffected (Tanigawara et al., 1992). This was not observed. Although the effects of AMD, β-caryophyllene, 1,8-cineole, and citral on the paracellular and transcellular transport pathways were not evaluated in this study, all four spices modified the [^{3}H]digoxin efflux ratio by unidirectional modification of [^{3}H]digoxin flux.

Of the eight spice components, only curcumin and 6-gingerol inhibited P-gp activity. There may be a structural basis for this activity, as a comparison of the chemical structures in Fig. 1 indicates similarities in their chemical structures, specifically a 4-hydroxy-3-methoxyphenyl ring and a keto group on the third carbon of the side chain. Both spices were concentration-dependent P-gp inhibitors, which is in agreement with previously published data showing that curcumin and 6-gingerol inhibited the P-gp-mediated cellular accumulation of rhodamine 123 (Anuchapreeda et al., 2002; Nabekura et al., 2005). P_{app} values in Tables 1 and 2 suggest that curcumin is the more efficient P-gp inhibitor on a concentration basis compared with 6-gingerol, although curcumin at the highest applied concentration of 60 μM was also incapable of completely abolishing the vectorial transport of [^{3}H]digoxin. Higher curcumin concentrations were not used in view of its significant cytotoxicity at concentrations $\geq$90 μM. Because the [^{3}H]digoxin transport studies were conducted using subcytotoxic spice concentrations, the stronger P-gp-inhibitory action of curcumin was unlikely to be mediated by cell death or cell damage. Curcumin has, however, been reported to inhibit P-gp expression in human KB-V1 cells (Anuchapreeda et al., 2002); whether this was the basis for its action on P-gp function in the L-MDR1 and Caco-2 cells remains to be determined.

The majority of the eight spice components caused either an inhibition or enhancement of CYP3A4 activity in the HLM system. MDZ is a common probe to estimate CYP3A4 activity (Galetin et al., 2005), with modulation of MDZ 1′-hydroxylation generally viewed as playing a more important role than changes in MDZ 4-hydroxylation because 1′-OH-MDZ is the major metabolite (Dundee et al., 1984). Nevertheless, both MDZ 1′-hydroxylation and 4-hydroxylation activities of the HLM CYP3A4 were quantified in this study to provide a comprehensive overview of the effects of the spices on CYP3A4 activity. CYP3A4-mediated transformation of MDZ to 1′-OH-MDZ was inhibited by curcumin at 45 and 60 μM, AMD at 1 and 4 mM, 1,8-cineole at 1 and 4 mM, and β-caryophyllene at 1 and 4 mM. A comparison of IC_{50} values suggests that these spices were weaker CYP3A4 inhibitors compared with the control, ketoconazole. 6-Gingerol at 100 μM, *d*-limonene at 1 and 4 mM, myrcene at 4 mM, and citral at 60 μM stimulated the CYP3A4-mediated 1′-hydroxylation of MDZ, but none was as potent as the control stimulator, α-naphthoflavone. CYP3A4-mediated formation of 4-OH-MDZ was inhibited by curcumin at 30, 45, and 60 μM; by 6-gingerol at 60, 100, and 500 μM; by AMD at 1 and 4 mM; by *d*-limonene at 4 mM; by 1,8-cineole at 0.5, 1, and 4 mM; and by citral at 1 mM. Again, none of the spices was more potent than ketoconazole as inhibitor. Two of the spices, myrcene at 1 and 4 mM and β-caryophyllene at 60 μM, enhanced the formation of 4-OH-MDZ, again at lower potency than testosterone. The collective data suggest, therefore, that the spices were relatively weak, concentration-dependent modulators of CYP3A4 function.

An interesting phenomenon emerged in that, although some spices (curcumin, AMD, 1,8-cineole, and myrcene) had similar effects on MDZ 1′-hydroxylation and 4-hydroxylation, other spices showed opposing effects on these two metabolic pathways. For example, β-caryophyllene inhibited MDZ 1′-hydroxylation but enhanced MDZ 4-hydroxylation, whereas 6-gingerol, *d*-limonene, and citral enhanced MDZ 1′-hydroxylation but inhibited MDZ 4-hydroxylation. CYP3A4 is a complex heme-containing enzyme that exhibits non-Michaelis-Menten kinetics as well as both homotrophic and heterotrophic cooperativity toward several substrates (Hutzler and Tracy, 2002). It has

been shown that MDZ oxidation by CYP3A4 could be altered in favor of either the 1′-OH-MDZ or the 4-OH-MDZ, e.g., α-naphthoflavone promoted the formation of 1′-OH-MDZ but not 4-OH-MDZ, whereas the reverse was true of testosterone (Cameron et al., 2005). The underlying mechanism for the differential effects is not yet known. Another study showed that α-naphthoflavone increased and decreased, respectively, the carboxylic acid and ω-3-hydroxylated metabolites of losartan in cDNA-expressed CYP3A4 microsomes (Shou et al., 2001). One hypothesis proposes the existence of two or more possible MDZ-binding sites in the CYP3A4 enzyme, with at least one favoring the formation of the 1′-hydroxy metabolite and another favoring the 4-hydroxy metabolite. Effectors such as testosterone and α-naphthoflavone and possibly the spices that showed differential effects on 1′-OH-MDZ and 4-OH-MDZ formation could bind at sites that effectively block access to at least one but not all of the possible MDZ-binding sites. Kinetic models (Shou et al., 2001) and theoretical molecular models (Torimoto et al., 2003) have been developed to account for the allosteric interaction within the CYP3A4 active site. These models have attempted to differentiate between two substrates simultaneously bound to the active site, substrates competing for the active site, and changes activated by binding of an effector at a site other than the active site. However, there is to date no firm consensus on the mechanism for the differential effects of CYP3A4 modulators.

There is poor correlation of CYP3A4 and P-gp activities among the spices. Whereas curcumin and 6-gingerol were modulators of both the P-gp and CYP3A4, the other spice components affected only CYP3A4-mediated metabolism of MDZ. Moreover, although 6-gingerol inhibited P-gp function, it promoted CYP3A4-mediated MDZ 1′-hydroxylation and inhibited CYP3A4-mediated MDZ 4-hydroxylation. Of the spices that modulated both CYP3A4-mediated MDZ 1′-hydroxylation and 4-hydroxylation, only AMD appeared to be an irreversible (mechanism-based) inhibitor.

In conclusion, this study has shown spices to be capable of modulating the activities of human intestinal P-gp and CYP3A4. The data may suggest that the spice-modulating effects were modest compared with established controls. Assuming that the 6-gingerol content in ginger is approximately 0.3% (Wang et al., 2002) and the volume of gastrointestinal fluid in the human is approximately 8 liters (Lawson, 2003), typical daily consumption of 8 to 10 g of fresh ginger root in the Indian diet (Longwood Herbal Task Force, http://www.longwoodherbal.org/ginger/ginger.pdf) could result in a 6-gingerol concentration only as high as 13 μM in the human GI tract. In the case of curcumin, the daily turmeric intake in India of approximately 0.6 g could result in a GI curcumin concentration of 10 μM, on the basis that turmeric contains 3 to 5% of curcumin (HighBeam Encyclopedia, http://www.encyclopedia.com/doc/1G1–133803078.html). At these concentrations, 6-gingerol and curcumin are unlikely to cause significant inhibition of P-gp or CYP3A function when administered as a single dose. However, P-gp expression in human KB-V1 cells has been observed to be attenuated by exposure to curcumin at 1 to 10 μM in a concentration-dependent manner (Anuchapreeda et al., 2002). Studies in our laboratory involving the rat model have further demonstrated that chronic curcumin administration could modulate P-gp and CYP3A expression, resulting in changes in the pharmacokinetic profiles of celiprolol, a P-gp substrate, and midazolam, a CYP3A substrate (Zhang et al., 2007). In this case, curcumin was administered intragastrically at a dose of 60 mg/kg/day, equivalent to 4 μM on the basis of a rat GI fluid volume of 7.8 ml (McConnell et al., 2008) for 4 consecutive days. Thus, regular consumption of curcumin could still potentially attenuate P-gp and CYP3A activity through modification of the tissue expression of these two proteins. Until proven otherwise in clinical experiments, it may be prudent therefore to advise against the coadministration of potent drugs that are P-gp and/or CYP3A4 substrates with curcumin.

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