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Flavonoids diosmetin and luteolin inhibit midazolam metabolism by human liver microsomes and recombinant CYP 3A4 and CYP3A5 enzymes

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ABSTRACT

We evaluated the effects of increasing concentrations of the flavonoids salvigenin, diosmetin and luteolin on the in vitro metabolism of midazolam (MDZ), a probe substrate for cytochrome P450 (CYP) 3A enzymes, which is converted into 1′-hydroxy-midazolam (1′-OH-MDZ) and 4-hydroxy-midazolam (4-OH-MDZ) by human liver microsomes. Salvigenin had only a modest effect on MDZ metabolism, whereas diosmetin and luteolin inhibited in a concentration-dependent manner the formation of both 1′-OH-MDZ and 4-OH-MDZ, with apparent K_i values in the 30–50 μmol range. Both diosmetin and luteolin decreased 1′-OH-MDZ formation by human recombinant CYP3A4, but not CYP3A5, whereas they decreased 4-OH-MDZ formation by both recombinant enzymes. To assess whether any relationship exists between the physico-chemical characteristics of flavones and their effects on MDZ metabolism, we tested the effects of three other flavones (flavone, tangeretin, chrysin) on MDZ metabolism by human liver microsomes. Whereas flavones possessing more than two hydroxyl groups (luteolin, diosmetin) inhibited MDZ biotransformation, flavones lacking hydroxyl groups in their A and B rings (flavone, tangeretin) stimulated MDZ metabolism. We also found close relationships between the maximum stimulatory or inhibitory effects of flavones on 1′-OH-MDZ and 4-OH-MDZ formation rates and their log of octanol/water partition coefficients (log *P*) or their total number of hydroxyl groups. The results of the study may be of clinical relevance since they suggest that luteolin and diosmetin may cause pharmacokinetic interactions with co-administered drugs metabolized via CYP3A.

1. Introduction

The human cytochrome P450 (CYP) 3A subfamily is responsible for the metabolism of more than 50% of currently marketed drugs [1]. CYP3A4 is the major isoform, largely expressed in human liver and gastrointestinal tract [2], whereas CYP3A5 is a polymorphic isoform, present in significant amounts in 20–60% of human livers [3]. When present, however, it accounts for at least 50% of total CYP3A [3–5]. CYP3A enzymes metabolize numerous structurally unrelated compounds, this ability being responsible for the large number of documented drug–drug and drug–food interactions.

Among dietary substances potentially affecting drug metabolism, flavonoids, a large class of polyphenolic compounds, play a crucial role, due to their massive presence in fruits and fruit products, vegetables, and plant-derived beverages such as tea and wine. Intake of flavonoids by

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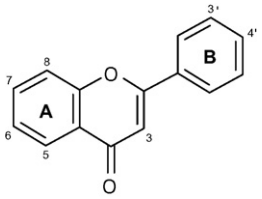
Table 1 – Structures and log *P* values of the flavones used in the study



	Substitutions in flavone basal structure						log *P*
	C5[a]	C6[a]	C7[a]	C8[a]	C3′[b]	C4′[b]	
Flavone	–	–	–	–	–	–	3.6
Tangeretin	OCH_3	OCH_3	OCH_3	OCH_3	–	OCH_3	2.7
Salvigenin	OH	OCH_3	OCH_3	–	–	OCH_3	2.7
Diosmetin	OH		OH	–	OH	OCH_3	1.5
Chrysin	OH		OH	–	–	–	2.8
Luteolin	OH		OH	–	OH	OH	0.7

Log of octanol/water partition coefficients (log *P*) of the flavones used were obtained from the PubChem web site of the US National Institute of Health (http://pubchem.ncbi.nlm.nih.gov/).
[a] A ring.
[b] B ring.

human beings is difficult to evaluate, since consumption of flavonoid-containing foods differs markedly among populations in various countries [6]. Furthermore, in recent years the use of dietary supplements and herbal preparations containing flavonoids has become very popular and is constantly increasing [7], since flavonoids have long been associated with a variety of beneficial properties (see [8,9] for reviews). Due to their antioxidant activity [10], they are presumed to protect tissues against oxidative stress and associated pathologies such as cancer, coronary artery disease and inflammation. They may also prevent degenerative diseases through inhibition of several protein functions ([11–13] and references therein). In particular, although many mechanisms may be responsible for their protective effect against cancer, this beneficial effect has frequently been ascribed to the inhibition of CYP1A1, CYP1A2 and CYP1B1, the CYP isoforms principally involved in the metabolic activation of pro-carcinogens [13–15].

Although flavonoid intake produces beneficial effects, it also constitutes a possible risk factor for pharmacokinetic interactions with co-administered conventional drugs, since some flavonoids behave as stimulators [16–18] and others as inhibitors [13] of CYP3A4, the main CYP isoform involved in drug metabolism. The best-known example is the interaction between grapefruit juice and some cardiovascular drugs [19,20], due to the presence in the former of certain flavonoids that inhibit intestinal CYP3A4 [21–23].

This study focuses on some compounds of the flavone subclass (Table 1), salvigenin (5-hydroxy-6,7,4′-trimethoxyflavone), luteolin (3′,4′,5,7-tetrahydroxyflavone) and diosmetin (3′,5,7-trihydroxy-4′methoxyflavone). Salvigenin is present in several species of *Salvia* [24], an important plant genus cultivated all over the world for culinary purposes and widely used in folk medicine for its many biological activities [24]. Luteolin, also present in *Salvia officinalis* [25], is found abundantly in thyme, celery, green pepper, and many other vegetables (http://www.ars.usda.gov/nutrientdata. USDA Database for the Flavonoid Content of Selected Foods; Release 2.1, January 2007). Diosmin, the 7-rutinoside of diosmetin, to which it is readily converted upon administration [26], is present in high amount (450 mg) in various phlebotonic preparations, e.g., Daflon500® which is widely used in the management of chronic venous insufficiency, venous ulcers and haemorrhoids [27,28], its recommended dose being up to six tablets a day.

In spite of the widespread use of the above-mentioned vegetables and medicinal preparation, and the in vivo observation that diosmin interferes with the disposition of metronidazole [29], a drug partly metabolized by CYP3A4, to the best of our knowledge no data are available in the literature about the in vitro effects of these flavones on drug biotransformations by the CYP3A4/5 enzymes. Therefore, the main aim of this study was to assess their possible in vitro effects on the metabolic activity of CYP3A enzymes by measuring the conversion of midazolam (MDZ), an established probe substrate for this enzyme system [30], to its two metabolites, 1′-hydroxy-midazolam (1′-OH-MDZ) and 4-hydroxy-midazolam (4-OH-MDZ) [31,32]. An additional aim was to compare the effects of salvigenin, luteolin and diosmetin on MDZ metabolism with those caused by other flavones provided with different substitutions in the basic flavone skeleton (Table 1) in order to verify if any relationship exists between the effects of this series of flavones on MDZ metabolism and their structural and physico-chemical characteristics. In particular, we chose the non-substituted compound 2-phenyl-4H-chromen-4-one (flavone), 4′,5,6,7,8-pentamethoxyflavone (tangeretin), and 5,7-dihydroxyflavone (chrysin).

2. Materials and methods

2.1. Reagents

Flavone and tangeretin were purchased from Indofine Chemical Co. (Hillsborough, NJ, USA), diosmetin and luteolin

from ChromaDex Inc. (Santa Ana, CA, USA). Chrysin was a kind gift from Prof. G. Zagotto (Department of Pharmaceutical Sciences, University of Padova, Italy). Salvigenin was kindly given by Dr. G. Delogu (Institute of Biomolecular Chemistry of CNR, Unit of Sassari, Sassari, Italy) and Prof. M. Usai (Department of Drug Sciences, University of Sassari, Italy). NADPH was from Sigma–Aldrich Co. (St. Louis, MO, USA). MDZ, 1′-OH-MDZ and 4-OH-MDZ were from Roche S.p.A. (Milan, Italy). Methanol, ethanol, acetone and acetonitrile (all HPLC grade) were from Carlo Erba Reagenti (Milan, Italy). Ultrapure water was obtained by means of a Millipore (Bedford, MA, USA) MilliQ apparatus.

Flavone and tangeretin were dissolved in acetonitrile, luteolin in methanol, and chrysin and diosmetin in acetone. Solutions of salvigenin were prepared in methanol/acetone (1:1). Flavonoid solutions were prepared daily and kept in ice until use. Final solvent concentration in the incubation medium was 1%, since preliminary experiments had shown that higher concentrations strongly modified the metabolic activity of human liver microsomes. MDZ was prepared daily in 50% methanol, final solvent concentration in the assay medium being 0.5%.

Log of octanol/water partition coefficients (log *P*) of the flavones were obtained from the PubChem web site of the US National Institute of Health (http://pubchem.ncbi.nlm.nih.gov/).

2.2. Human liver microsomes and human cDNA-expressed CYP3A4 and CYP3A5 microsomal preparations

Pooled mixed gender human liver microsomes were provided by XenotechLLC (Lenexa, KS, USA) and were stored in aliquots at −80 °C until use.

Recombinant CYP3A4 (rCYP3A4) and CYP3A5 (rCYP3A5), co-expressed with human NADPH-cytochrome P450-reductase and cytochrome b_5 in insect cell microsomes (Supersomes™), were purchased from BD Gentest (Woburn, MA, USA). The expression level of CYP, NADPH-cytochrome P450-reductase, and cytochrome b_5, as well as their specific activities, were provided in the manufacturer's data sheets.

2.3. Evaluation of MDZ metabolism by human liver microsomes

Incubation of human liver microsomes was carried out in linear reaction conditions with respect to incubation time and microsomal protein concentration. Microsomal proteins (final concentration 0.5 mg/ml) were incubated in a mixture (total volume of 0.2 ml) containing 0.2 M KH_2PO_4 (pH 7.4), 0.5 mM NADPH, and increasing concentrations of MDZ (from 0.5 to 200 μM, $n = 8$). Reactions were started by the addition of microsomes, following thermal equilibration at 37 °C of incubation mixtures. They were conducted in a shaking water bath at 37 °C in aerobic conditions and stopped after 5 min by adding 0.2 ml of ice-cold methanol. Denatured proteins were then removed by centrifugation for 10 min at 20,000 × *g*, and an aliquot (0.1 ml) of the supernatant was analyzed by HPLC with UV detection, as described below.

2.4. Evaluation of MDZ metabolism by human cDNA-expressed CYP3A4 and CYP3A5 microsomal preparations

MDZ metabolism by rCYP3A4 and rCYP3A5 was studied in experimental conditions identical to those described for liver microsomes, except that final rCYP3A4 or rCYP3A5 concentration in the incubation medium was 12.5 pmol/ml. Incubation was carried out in linear reaction conditions with respect to incubation time and CYP concentration. Substrate depletion was less than 10% at all MDZ concentrations.

2.5. Evaluation of the effects of flavones on MDZ metabolism by human liver microsomes, rCYP3A4 and rCYP3A5

Human liver microsomes (final protein concentration: 0.5 mg/ml) were incubated in the absence or presence of increasing concentrations (from 1 to 100 μM, $n = 5$) of the various flavones in the above-specified incubation mixture containing 4 or 25 μM MDZ, in order to evaluate 1′-OH-MDZ or 4-OH-MDZ formation, respectively (see below). Human rCYPs (final concentration: 12.5 pmol CYP/ml) were incubated in identical conditions, except that final MDZ concentration was 25 μM. Control samples contained 1% (final concentration) of the flavone solvent. Incubations were carried out as specified above and stopped after 5 min of incubation, by addition of 0.2 ml of ice-cold methanol.

2.6. Characterization of the mechanism of inhibition of 1′-OH-MDZ formation by diosmetin and luteolin in human liver microsomes

In order to characterize the mechanism of inhibition of 1′-OH-MDZ formation by diosmetin and luteolin, 1′-OH-MDZ formation catalyzed by human liver microsomes (final protein concentration: 0.5 mg/ml) was determined in the above-specified incubation mixture containing increasing concentrations of MDZ ranging form 0.5 to 25 μM ($n = 5$) in the absence or presence of two concentrations (30 and 100 μM) of diosmetin or luteolin.

2.7. HPLC analysis

Quantitative evaluation of 1′-OH-MDZ and 4-OH-MDZ formation was carried out essentially as described by Wrighton and Ring [33], using a Hewlett-Packard series 1100 HPLC system equipped with degasser, quaternary pump, auto-sampler, and multiple-wavelength detector (Agilent, formerly Hewlett-Packard GMBH, Germany); chromatographic data were collected and integrated by means of the Hewlett-Packard ChemStation software (Version A.06.03). Chromatographic conditions were as follows: column, Zorbax Rx-C18 (4.6 mm × 250 mm, 5 μm, Agilent Technologies Inc., Palo Alto, CA, USA); mobile phase, 10 mM KH_2PO_4 (pH 7.4)/methanol/acetonitrile (44:35:21, v/v/v); flow rate, 1.0 ml/min; injection volume, 100 μl; column temperature, 30 °C; detection, 220 nm. In the above conditions, retention times of MDZ, 1′-OH-MDZ, and 4-OH-MDZ were 21, 11.4, and 8.5 min, respectively. For analysis of 1′-OH-MDZ formation in the samples containing tangeretin, aliquots (100 μl) of the supernatants were injected

Table 2 – Comparison of kinetic parameters for 1′-OH-MDZ and 4-OH-MDZ formation by human liver microsomes

	Metabolite	
	1′-OH-MDZ	4-OH-MDZ
Model	Michaelis–Menten with substrate inhibition	Michaelis–Menten
V_{max} (nmol/min/mg protein)	1.73 ± 0.13	0.59 ± 0.03
K_m (μM)	4.04 ± 0.47	28.12 ± 3.38
K_{si} (μM)	365.16 ± 74.92	–
Apparent CL_{int} (μl/mg protein/min)	454.14 ± 65.44	21.65 ± 2.23

The apparent CL_{int} was expressed as μl/mg protein/min and was calculated as V_{max}/K_m. Kinetic parameters were determined by non-linear regression analysis of untransformed data (Prism 3.03 software, GraphPad Inc., San Diego, CA, USA) using one-site hyperbolic Michaelis–Menten model or Michaelis–Menten model with uncompetitive substrate inhibition [34]. Results are the means ± S.E.M. of data from five different experiments performed in duplicate.

in a Symmetry® C8 column (4.6 mm × 250 mm, 5 μm, Waters Corp., Milford, MA, USA); in this case, the retention times of MDZ, 1′-OH-MDZ, and 4-OH-MDZ were 22.0, 14.5, and 13 min, respectively. Quantitative determination of MDZ metabolites was carried out by comparing peak areas with external standard calibration curves obtained daily with authentic 1′-OH-MDZ and 4-OH-MDZ at concentrations ranging from 0.01 to 1 nmol/0.2 ml ($n = 7$). The calibration curves were linear in this concentration range ($r^2 \geq 0.98$), the lowest value of the range representing the limit of quantification of the assay. The inter-assay coefficients of variation (CV) for 1′-OH-MDZ determination ($n = 5$) at 0.01 and 1 nmol/0.2 ml were 5.52 and 3.48%, respectively. For 4-OH-MDZ determination ($n = 5$) at 0.01 and 1 nmol/0.2 ml, the CVs were 9.61 and 3.48%, respectively.

2.8. Data analysis

Initial velocity data for MDZ biotransformation by human liver microsomes or human rCYP3A4 and rCYP3A5, in the absence of flavones, were analyzed by one of the following models: one-site hyperbolic Michaelis–Menten model (Eq. (1)) or Michaelis–Menten kinetics with uncompetitive substrate inhibition [34] (Eq. (2)):

$$v = \frac{V_{max}[S]}{K_m + [S]} \quad (1)$$

$$v = \frac{V_{max}[S]}{K_m + [S] + [S]^2/K_{si}} \quad (2)$$

The F test was used to discriminate between the two models. Kinetic parameters were determined by non-linear regression analysis of untransformed data using GraphPad Prism 3.03 software (GraphPad Inc., San Diego, CA, USA). Estimated parameters were: V_{max}, maximum velocity of uninhibited reaction; K_m, substrate concentration yielding 50% of V_{max}; K_{si}, substrate inhibition constant, and CL_{int}, intrinsic metabolic clearance, calculated as V_{max}/K_m. Maximal inhibition (i_{max}) and apparent inhibitor dissociation constant (K_i^a) values were evaluated by means of the graphical method described by Palatini [35] (see Section 3). Lineweaver–Burk (double-reciprocal) plots were used for graphical presentation of the inhibition of 1′-OH-MDZ formation by diosmetin and luteolin. Their mechanism of inhibition was inferred from their effects on the kinetic parameters (V_{max} and K_m), obtained by non-linear regression analysis using the one-site hyperbolic Michaelis–Menten model.

Statistical analyses were performed with GraphPad Prism 3.03 software. All values are expressed as arithmetic means ± S.E.M. When two or more data points were compared with their control, one-way analysis of variance (ANOVA) was performed, followed by Dunnett's post hoc test. $P < 0.05$ was considered statistically significant. For comparisons of the effects of each flavone on 1′-OH-MDZ and 4-OH-MDZ formation rates, or of the effects of two flavones on a single metabolic pathway, Student's t-test for unpaired data was used. Correlations were examined by linear regression analysis.

3. Results

3.1. Metabolism of MDZ by human liver microsomes

In the substrate concentration range used (0.5–200 μM), initial velocity data for 1′-OH-MDZ formation were best fitted by Michaelis–Menten kinetics with substrate inhibition [34]. For 4-OH-MDZ formation, the one-site hyperbolic Michaelis–Menten kinetics proved to be the best model (Fig. 1). Mean kinetic parameters for MDZ hydroxylation by human liver microsomes are shown in Table 2. Results indicate that V_{max}

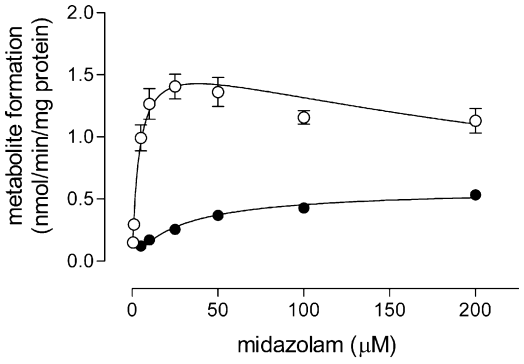


Fig. 1 – Kinetics of 1′-OH-MDZ (○–○) and 4-OH-MDZ (●–●) formation by human liver microsomes. Each point represents the mean ± S.E.M. of five determinations. S.E.M. values are not shown where size of data point is larger than S.E.M. bar.

was greater for 1′-OH-MDZ than 4-OH-MDZ formation, and K_m for 1′-hydroxylation of MDZ was about 7.5-fold lower than that for the 4-hydroxylation pathway. As a consequence, values of MDZ intrinsic metabolic clearance (CL_{int}) through 1′-hydroxylation and 4-hydroxylation represented 95.5 and 4.5% of the total CL_{int} value, respectively.

3.2. *Effects of flavones on MDZ hydroxylation by human liver microsomes*

We tested the effects of the flavones on MDZ metabolism by human liver microsomes at substrate concentrations corresponding to K_m values, i.e., 4 μM for 1′-OH-MDZ formation and 25 μM for 4-OH-MDZ formation. Because of the solubility limits of flavones in our experimental conditions, we tested the effects of five concentrations ranging from 1 to a maximum of 100 μM. Fig. 2 shows the effects of increasing concentrations of the flavones on 1′-OH-MDZ and 4-OH-MDZ formation. The data indicate that diosmetin and luteolin decreased MDZ metabolism in a concentration-dependent manner. However, whereas luteolin decreased to a similar extent the formation of both 1′-OH-MDZ and 4-OH-MDZ, diosmetin proved to be significantly more active as an inhibitor of 1′-hydroxylation. By contrast, salvigenin did not

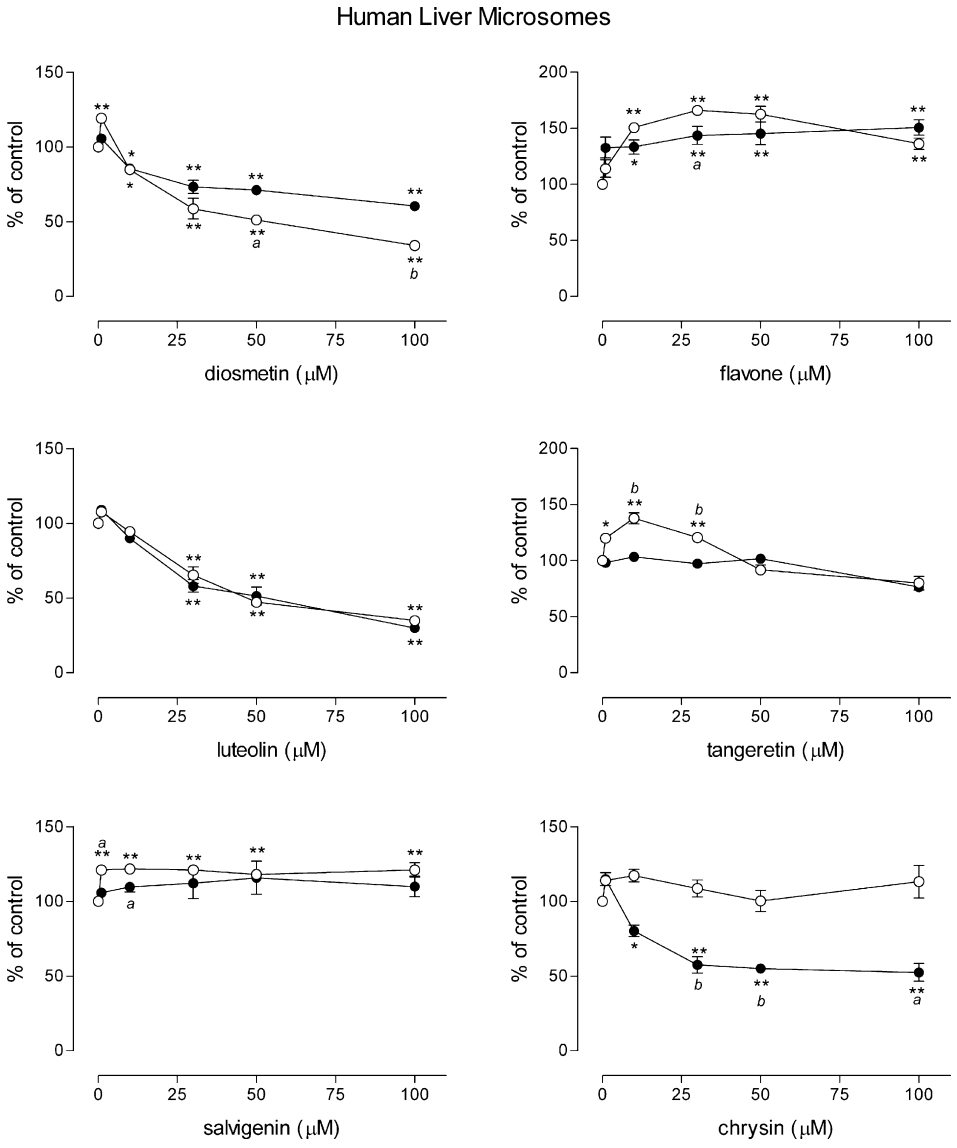


Fig. 2 – Effect of increasing concentrations of various flavones on 1′-OH-MDZ (○–○) and 4-OH-MDZ (●–●) formation by human liver microsomes. 1′-OH-MDZ and 4-OH-MDZ formations were evaluated at 4 and 25 μM, respectively. In these conditions, the mean values of 1′-OH-MDZ and 4-OH-MDZ formation rates, determined in absence of flavones, were 0.76 ± 0.01 and 0.25 ± 0.02 nmol/mg protein/min, respectively. Results are means ± S.E.M. of at least three experiments performed in duplicate. S.E.M. values are not shown where size of data point is larger than S.E.M. bar. Statistical analyses of the data were performed by means of the Prism 3.03 software, using ANOVA followed by Dunnett's post hoc test to evaluate the effect of increasing concentrations of each flavone compound vs. its control value (*$P < 0.05$ and **$P < 0.01$). Student's t-test for unpaired data was used to compare the effects of each flavone concentration on 1′-OH-MDZ and 4-OH-MDZ formation rates ($^{a}P < 0.05$, $^{b}P < 0.01$).

significantly modify 4-OH-MDZ formation and had a modest stimulatory effect on 1′-OH-MDZ formation (~20% stimulation vs. control, $P < 0.01$). The latter effect did not appear concentration-dependent since very similar stimulations were also observed at lower salvigenin concentrations (18 and 21% at 0.1 and 0.5 μM, respectively; data not shown).

The effects of other flavones on MDZ metabolism were also tested, in an attempt to verify if any relationship exists for this series of compounds between their activity on MDZ metabolism and their structural and physico-chemical characteristics. Chrysin proved to be an effective inhibitor of 4-OH-MDZ formation, but did not significantly affect 1′-hydroxylation of MDZ. Almost opposite effects were observed with tangeretin and flavone. Tangeretin did not significantly modify MDZ 4-hydroxylation, since it caused only 24% inhibition of 4-OH-MDZ formation at 100 μM. By contrast, it exerted a biphasic effect on 1′-OH-MDZ formation, which was increased significantly ($P < 0.01$) up to 50 μM and was then slightly inhibited. Flavone significantly increased ($P < 0.01$) the formation rates of both 1′-OH-MDZ and 4-OH-MDZ in a concentration-dependent manner. However, whereas it showed a peak stimulatory effect (66%) on 1′-OH-MDZ formation at 30 μM, it reached its maximum effect (50% stimulation) on 4-OH-MDZ formation at 100 μM.

As Fig. 2 also shows, neither 1′-OH-MDZ nor 4-OH-MDZ formation was totally inhibited by any of the flavones up to the highest (100 μM) concentration that could be tested. In certain cases, the effect appeared to plateau at rather low levels of inhibition, thereby preventing determination of IC_{50} values. Therefore, in order to compare the effects of the various inhibitors, their maximal inhibition (i_{max}) and apparent inhibitor dissociation constant (K_i^a) values were determined for both 1′-OH-MDZ and 4-OH-MDZ formations, by using the plot previously described by Palatini [35]. In this plot, if the experimental concentrations of the inhibitor are marked on a negative horizontal axis and the corresponding fractional inhibition (i) values are marked on a vertical axis, the straight lines drawn through each pair of points intersect at a common point whose ordinal and abscissal coordinates represent i_{max} and K_i^a, respectively. This plot allows a straightforward determination of maximal inhibition even when saturating inhibitor concentrations cannot be tested. Fig. 3 shows representative plots obtained from two single experiments performed with luteolin on 1′-OH-MDZ and 4-OH-MDZ formation. They clearly show that luteolin was a partial inhibitor of both 1′-OH-MDZ and 4-OH-MDZ formation. Table 3 lists the mean values of i_{max} and K_i^a obtained for each flavone. The results clearly indicate that diosmetin and luteolin, as well as chrysin, were partial inhibitors, since their fractional i_{max} values were lower than unity. i_{max} values were consistent with the results obtained from the concentration–response curves, since maximum inhibition by diosmetin and luteolin

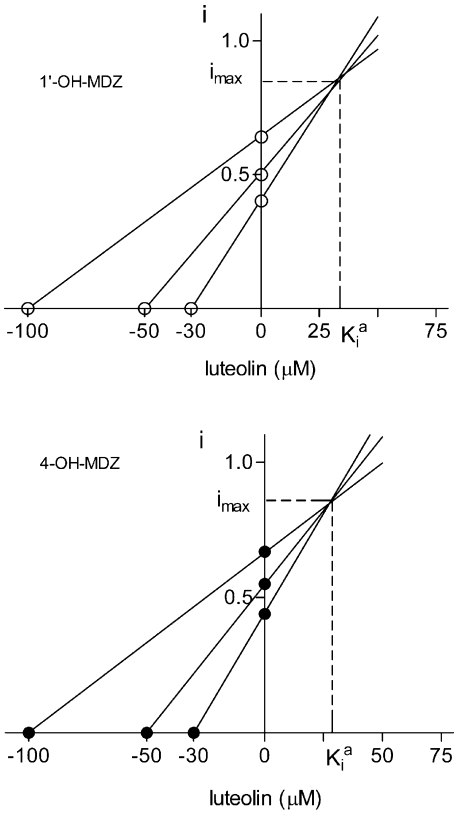


Fig. 3 – Determination of maximal inhibition (i_{max}) and apparent dissociation constant (K_i^a) values of luteolin on 1′-OH-MDZ and 4-OH-MDZ formation by means of the plot described by Palatini [35]. 1′-OH-MDZ and 4-OH-MDZ formation was evaluated at 4 and 25 μM, respectively. The results of two representative experiments are reported.

Table 3 – Maximal inhibition (i_{max}) and apparent inhibitor dissociation constant (K_i^a) values of diosmetin, luteolin and chrysin on 1′-OH-MDZ and 4-OH-MDZ formation by human liver microsomes

Flavone	1′-OH-MDZ formation		4-OH-MDZ formation	
	i_{max}	K_i^a (μM)	i_{max}	K_i^a (μM)
Diosmetin	0.89 ± 0.06**	44.5 ± 6.4	0.53 ± 0.05	37.0 ± 8.1
Luteolin	0.92 ± 0.04	41.2 ± 3.0	0.83 ± 0.02[a]	34.2 ± 2.7
Chrysin	–		0.62 ± 0.05	32.3 ± 5.9

i_{max} and K_i^a values were determined graphically as reported in Section 2 by means of the plot described by Palatini [35]. Results are means ± S.E.M. of data obtained from at least three different experiments performed in duplicate. Statistical significance was calculated by Student's *t*-test for unpaired data.

** $P < 0.01$ vs. i_{max} value on 4-OH-MDZ formation.

[a] $P < 0.01$ vs. diosmetin i_{max} value on 4-OH-MDZ formation.

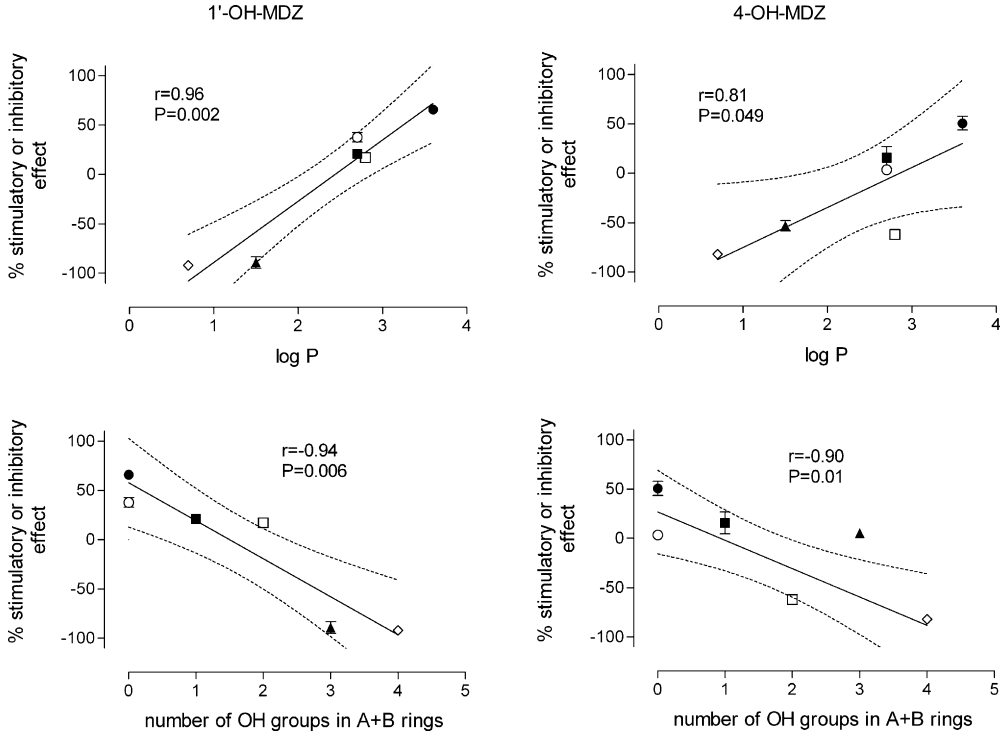


Fig. 4 – Correlation analysis between physico-chemicals characteristics of flavones and their effects on MDZ metabolite formation in human liver microsomes: ●, flavone; ○, tangeretin; ■, salvigenin; □, chrysin; ▲, diosmetin; ◇, luteolin. Peak stimulatory effects or maximum inhibitory effects (i_{max}) of the various flavones are reported on the ordinal axis (expressed as percent of control activity). The log *P* values were obtained from http://pubchem.ncbi.nlm.nih.gov. Data are means ± S.E.M. from at least three experiments in duplicate. S.E.M. values are not shown where size of data point is larger than S.E.M. bar. Values of the correlation coefficient (*r*) were determined by linear regression analysis.

on 1′-OH-MDZ formation were similar, whereas luteolin inhibited 4-OH-MDZ formation to a significantly greater extent than diosmetin. Diosmetin kinetic parameters were very similar to those of chrysin as an inhibitor of 4-hydroxylation of MDZ. Moreover, no significant differences were observed between the K_i^a values calculated for each flavone on both 1′OH-MDZ and 4-OH-MDZ formation, all values ranging from 32 to 44 μM.

A close relationship was found between the effects of flavones on 1′-OH-MDZ and 4-OH-MDZ formation rates and their log *P* (reported in Table 1) (Fig. 4). Consistent with this observation, significant inverse correlations were found between peak stimulatory or maximum inhibitory effects of the flavones on 1′-OH-MDZ or 4-OH-MDZ formation and total number of hydroxyl groups present in A and B rings.

3.3. *Mechanism of inhibition by diosmetin and luteolin of 1′-OH-MDZ formation by human liver microsomes*

Since in human liver microsomes MDZ is almost totally converted to 1′-OH-MDZ, only the mechanisms of the inhibitory effects caused by diosmetin and luteolin on 1′-OH-MDZ formation were characterized. In order to determine the type of inhibition caused by the two flavones on 1′-OH-MDZ formation, the effects of two concentrations (30 and 100 μM) of diosmetin and luteolin on the kinetic parameters of 1′-OH-MDZ formation were evaluated. As 1′-OH-MDZ formation by human liver microsomes follows Michaelis–Menten kinetics with substrate inhibition (Fig. 1), in these experiments MDZ concentrations were increased only up to 25 μM, in order to avoid inhibitory substrate concentrations [36]. The results shown in Fig. 5 and the data of Table 4 indicate that both diosmetin and luteolin increased in a concentration-dependent manner K_m and decreased V_{max}. This indicates that their inhibition was of the mixed competitive–noncompetitive type. Table 4 also shows that both flavones markedly reduced CL_{int} values.

3.4. *Effects of diosmetin and luteolin on MDZ hydroxylations catalyzed by rCYP3A4 and rCYP3A5*

As both CYP3A4 and CYP3A5 isoforms can be present in human liver, although in different proportions, and they show overlapping substrate specificity [3,37], we evaluated the effects of diosmetin and luteolin on MDZ metabolism catalyzed by microsomal preparations containing cDNA-expressed human CYP3A4 or CYP3A5. For this purpose, we preliminarily characterized the kinetics of MDZ hydroxylation by these two isoforms. Fig. 6 shows that 1′-OH-MDZ formation by rCYP3A4 could be fitted to Michaelis–Menten kinetics with substrate inhibition, whereas 1′-OH-MDZ formation by rCYP3A5 followed simple Michaelis–Menten kinetics. By contrast, formation of 4-OH-MDZ by both recombinant enzymes could be described by the classic Michaelis–Menten

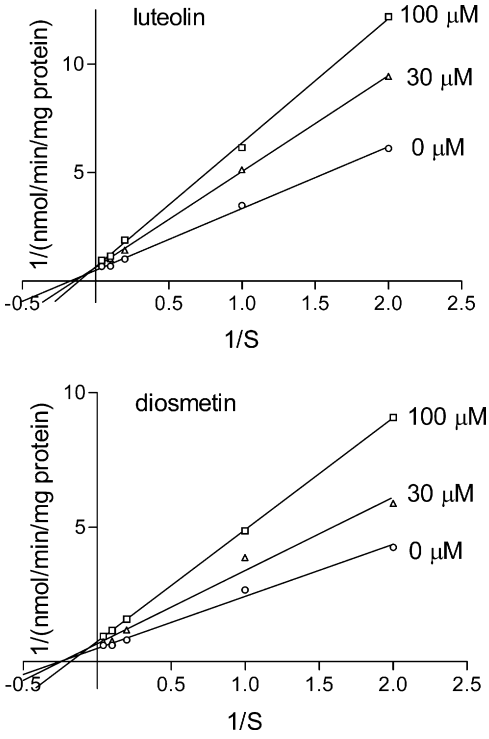


Fig. 5 – Lineweaver–Burk plots for the effects of luteolin and diosmetin on the kinetics of 1′-OH-MDZ formation by human liver microsomes. 1′-OH-MDZ formation was evaluated at five MDZ concentrations (0.5–25 μM) in the absence or presence of 30 and 100 μM luteolin or diosmetin. Data from a typical experiment for each flavone are reported.

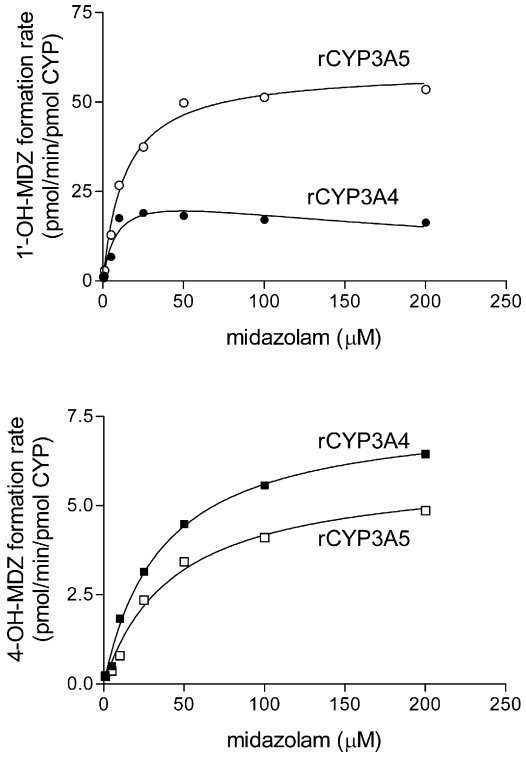


Fig. 6 – Kinetics for 1′-OH-MDZ (upper panel) and 4-OH-MDZ formation (lower panel) catalyzed by human rCYP3A4 and rCYP3A5. Each point represents the mean of two separate determinations performed in duplicate.

model. Kinetic parameters are listed in Table 5. As observed above with human liver microsomes, 1′-OH-MDZ was the major metabolite produced by rCYP3A4 and rCYP3A5, accounting for 93.6 and 96.9% of total CL_{int}, respectively. The V_{max} of the 1′-hydroxylation reaction was approximately twice as high for rCYP3A5 than for rCYP3A4, whereas K_m and CL_{int} values were not significantly different. The kinetic parameters for 4-OH-MDZ formation were quite similar for rCYP3A4 and rCYP3A5.

On the basis of these results, we evaluated the effects of diosmetin and luteolin on the metabolism of MDZ catalyzed by human rCYP3A4 and rCYP3A5, using 25 μM midazolam as substrate, in order to evaluate the formation of both metabolites simultaneously. The results of Fig. 7 indicate that, from 30 μM upwards, diosmetin inhibited the rCYP3A4-catalyzed formation of both 1′-OH-MDZ and 4-OH-MDZ by about 45 and 42%, respectively. In these experimental conditions, luteolin poorly affected 1′-OH-MDZ formation (about 18% inhibition at 100 μM), whereas it decreased the formation of 4-OH-MDZ by 34%.

Table 4 – Effect of diosmetin and luteolin on the kinetic parameters of 1′-OH-MDZ formation by human liver microsomes

	V_{max} (nmol/min/mg protein)	K_m (μM)	CL_{int} (μl/min/mg protein)
Control	2.15 ± 0.02	3.90 ± 0.14	553 ± 24
Diosmetin (30 μM)	1.67 ± 0.16*	4.31 ± 0.18	387 ± 32**
Diosmetin (100 μM)	1.50 ± 0.12*	5.26 ± 0.11**	284 ± 22**
Control	1.85 ± 0.03	4.14 ± 0.05	447 ± 2
Luteolin (30 μM)	1.38 ± 0.07*	4.86 ± 0.08**	283 ± 34**
Luteolin (100 μM)	1.19 ± 0.14**	6.83 ± 0.13**	174 ± 17**

Human liver microsomes were incubated, as described under Section 2, with five increasing concentrations (0.5–25 μM) of MDZ in the absence or presence of luteolin or diosmetin. The kinetic parameters were determined for each experiment by non-linear regression analysis of untransformed data (Prism 3.03 software, GraphPad Inc., San Diego, CA, USA) using one-site hyperbolic Michaelis–Menten model. The results represent the means ± S.E.M. of the parameters calculated from three experiments.

* $P < 0.05$.

** $P < 0.01$ vs. the respective control values.

Table 5 – Comparison of the kinetic parameters for 1′-OH-MDZ and 4-OH-MDZ formation by human rCYP3A4 and rCYP3A5

	Metabolite	
	1′-OH-MDZ	4-OH-MDZ
rCYP3A4		
Model	Michaelis–Menten with substrate inhibition	Michaelis–Menten
V_{max} (pmol/pmol CYP/min)	26.31 ± 5.34	7.63 ± 0.27
K_m (μM)	8.52 ± 4.24	36.33 ± 3.80
K_{si} (μM)	287 ± 205	–
Apparent CL_{int} (μl/pmol CYP/min)	3.09	0.21
rCYP3A5		
Model	Michaelis–Menten	Michaelis–Menten
V_{max} (pmol/pmol CYP/min)	58.98 ± 1.78	5.99 ± 0.33
K_m (μM)	13.60 ± 1.66	43.44 ± 6.65
Apparent CL_{int} (μl/pmol CYP/min)	4.33	0.14

The apparent CL_{int} was expressed as μl/pmol CYP/min and was calculated as the V_{max}/K_m ratio. Kinetic parameters were determined by non-linear regression analysis of untransformed data (Prism 3.03 software, GraphPad Inc., San Diego, CA, USA) using one-site hyperbolic Michaelis–Menten model or Michaelis–Menten model with uncompetitive substrate inhibition [34]. Results are means ± S.E. from two determinations, where S.E. represents the standard error of the best-fit value.

Surprisingly, when we determined the metabolism of MDZ by rCYP3A5, both diosmetin and luteolin lost their inhibitory effect on 1′-OH-MDZ formation, whereas they still inhibited, although to a limited extent, 4-OH-MDZ production.

4. Discussion

The results of this study confirm previous indications [37–39] that 1′-hydroxylation is the major metabolic pathway of MDZ catalyzed by human liver microsomes, accounting for about

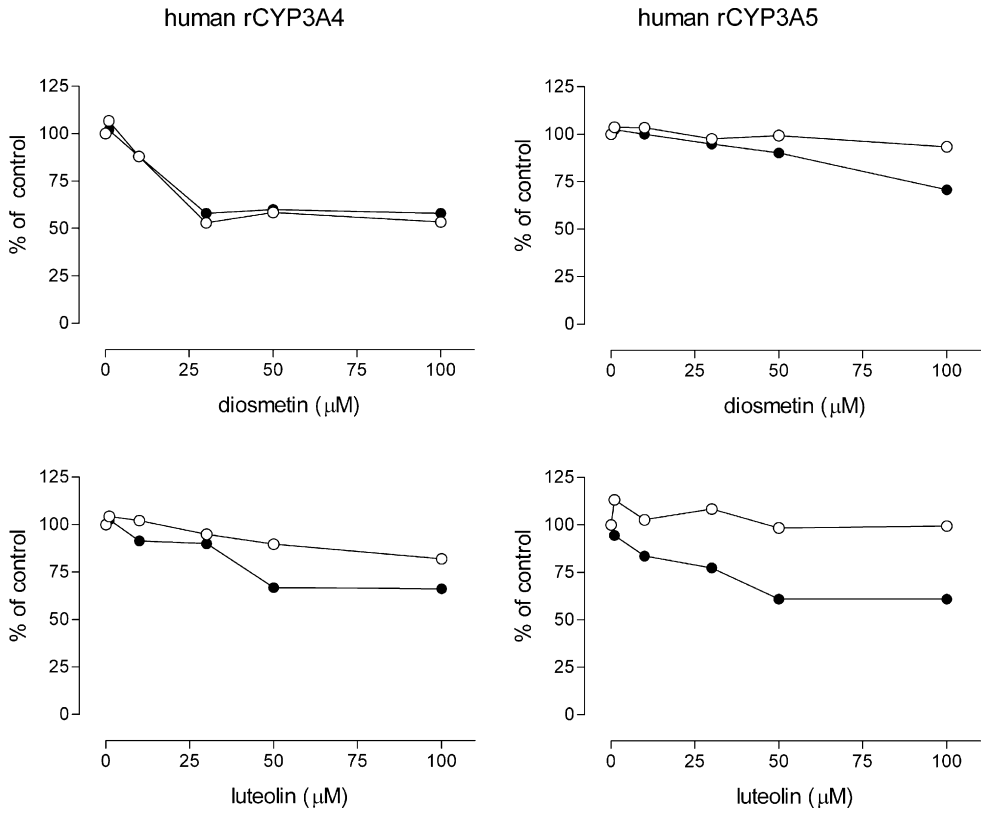


Fig. 7 – Effect of increasing concentrations of diosmetin and luteolin on 1′-OH-MDZ (○–○) and 4-OH-MDZ (●–●) formation by human rCYP3A4 and rCYP3A5. Metabolite formations were evaluated at 25 μM MDZ. Mean control values (in the absence of flavones) for 1′-OH-MDZ and 4-OH-MDZ formation by rCYP3A4 were 19.64 ± 0.47 and 3.48 ± 0.14 pmol/pmol CYP/min ($n = 4$), respectively. Mean control values for 1′-OH-MDZ and 4-OH-MDZ formation by rCYP3A5 were 40.15 ± 0.78 and 2.86 ± 0.30 pmol/pmol CYP/min, respectively. Mean data from two experiments are reported.

95% of total MDZ CL_{int}. We consistently found that 1′-hydroxylation has a sevenfold lower K_m value and a two- to threefold higher V_{max} value than the 4-OH-MDZ formation reaction. Conflicting results have been reported regarding the kinetics of both 1′-hydroxylation and 4-hydroxylation reactions by human liver microsomes [32,37–40]. Our results, obtained in rigorously controlled kinetic conditions, clearly show that 4-OH-MDZ production by human liver microsomes follows classical Michaelis–Menten kinetics, whereas 1′-OH-MDZ formation is best described by Michaelis–Menten kinetics with substrate inhibition. We also found that the Michaelis–Menten kinetics with substrate inhibition observed for 1′-OH-MDZ formation by human liver microsomes is also typical of the 1′-hydroxylation catalyzed by rCYP3A4, whereas 1′-OH-MDZ formation via rCYP3A5 follows classic Michaelis–Menten kinetics, as previously observed by Emoto and Iwasaki [41] and Williams et al. [42]. These results indicate that, with our microsomal preparations, 1′-OH-MDZ formation was mainly catalyzed by the CYP3A4 isoform.

Our novel findings regarding the effects of flavones may be summarized as follows: (1) diosmetin and luteolin inhibit in a concentration-dependent manner the formation of both 1′-OH-MDZ and 4-OH-MDZ by human liver microsomes, whereas salvigenin barely affects MDZ biotransformation; (2) diosmetin and luteolin are partial inhibitors, i.e., they do not completely inhibit the metabolic reactions, and act as mixed competitive–noncompetitive inhibitors of 1′-hydroxylation of MDZ; (3) these flavones decrease 1′-OH-MDZ formation catalyzed by human rCYP3A4, but not by rCYP3A5, whereas they inhibit to similar extents 4-OH-MDZ formation by the recombinant enzymes.

The observation that diosmetin and luteolin inhibit the 1′-hydroxylation of MDZ catalyzed by rCYP3A4 but have no such effect on that catalyzed by rCYP3A5, suggests that rCYP3A4 and rCYP3A5 have different affinities for the two flavones. In spite of the high homology (84% of identity in amino acid sequence) and substantial overlap of substrate specificities [40,43], CYP3A4 and CYP3A5 proteins have been shown to possess different enzymatic properties, including susceptibility to inhibitors. For example, fluconazole and ketoconazole, which are potent inhibitors of CYP3A-mediated activities, are more effective inhibitors of MDZ oxidation by rCYP3A4 than by rCYP3A5 [40,44]. Unlike 1′-hydroxylation, 4-hydroxylation catalyzed by rCYP3A5 is inhibited by diosmetin and luteolin, suggesting that 1′-OH-MDZ and 4-OH-MDZ formations result from the binding of MDZ to two separate sites of the CYP3A5 protein, as previously demonstrated for MDZ oxidation catalyzed by CYP3A4 [45].

Correlation analyses including salvigenin, luteolin and diosmetin, as well as chrysin, tangeretin and flavone, led to a further important finding of our study, i.e., a relationship exists between the structural and physico-chemical characteristics of this series of variously substituted flavones and their effects on MDZ metabolism. Our results indicate that both the qualitative (inhibition or stimulation) and quantitative effects of the flavones are significantly correlated with their hydrophobicity, expressed as log *P*. Consistently, they are inversely related to the presence and number of hydroxyl groups in the A and B rings of the flavone skeleton. The non-substituted flavone and the pentamethoxy-substituted tangeretin, lacking free hydroxyl groups in their A and B rings, stimulate, rather than inhibit, the metabolism of MDZ. This is in accordance with the previous observation of Backman et al. [18] on the effect of tangeretin on 1′-OH-MDZ formation by human liver microsomes. Salvigenin, which has one free hydroxyl group, affects MDZ biotransformation very poorly, whereas the polyhydroxylated flavones chrysin, luteolin and diosmetin, which possess two to three free hydroxyl groups, inhibit the metabolism of the drug. Our results, stressing the importance of the hydroxyl groups in the A and B rings for the inhibitory effect of flavonoids on drug metabolism, confirm results previously obtained by Ho et al. [23] with a different series of flavonoids on the 4-hydroxylation of quinine, which is also catalyzed by CYP3A4.

In conclusion, by demonstrating that luteolin and diosmetin inhibit the formation of the major MDZ metabolite (1′-OH-MDZ) – particularly that catalyzed by CYP3A4, which is the CYP3A isoform expressed to the greatest extent in human liver and gastrointestinal tract [2] – the present study suggests that pharmacokinetic interactions may take place between diosmetin or luteolin and co-administered drugs susceptible to metabolism by CYP3A4. Since hepatic and intestinal CYP3A4 cDNAs are identical, the proteins expressed in these two tissues are most probably the same [46]. Therefore, the observations made here with human hepatic microsomes also appear directly applicable to CYP3A4 in the intestine, where the highest concentrations of flavones are presumably reached. Although flavonoids are generally present as glycosides in herbal or medicinal (e.g., Daflon500®) products, they are generally cleaved into free flavonoids by gut microflora and during passage across the intestine wall [11,26,47]. Thus, the aglycones are likely to be the active moieties interfering with enterocyte and/or liver drug metabolizing enzymes. For example, diosmin, which is known to be hydrolyzed to its aglycone diosmetin in the gastrointestinal tract [26], was shown by Rajnarayana et al. [29] to modify significantly the pharmacokinetics of metronidazole (metabolized by CYP3A4 and CYP2C9) in healthy volunteers, with an approximately 25% increase in drug plasma concentrations and a decrease in urinary excretion of its metabolites. However, as flavonoids are susceptible to quite complex biotransformations, dominated by phase II metabolic reactions [11,48,49], the possibility cannot be excluded that flavone metabolites may also contribute to the observed in vivo effects on drug metabolizing enzymes [29], since both phase I and phase II biotransformations have been shown to give rise to equal or more potent inhibitors of some enzymes [48] and drug transporters [50]. Further studies with flavonoid metabolites are needed to clarify this question.

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