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Inhibition of in-vitro simvastatin metabolism in rat liver microsomes by bergamottin, a component of grapefruit juice

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Abstract

Grapefruit juice can modify the pharmacokinetic parameters of many drugs, in particular simvastatin, an orally active cholesterol-lowering agent. The exact components in grapefruit juice responsible for drug interactions are not perfectly known. However, it seems that bergamottin, a furocoumarin derivative, is one of the main active components within grapefruit juice. The objective of this paper was to quantify and to characterize in-vitro the inhibitory effect of bergamottin on simvastatin metabolism by using rat and human liver microsomes. In rat liver microsomes, the incubation conditions (± NADPH) of bergamottin were found to influence its inhibiting capacity. In co-incubation with simvastatin, the K_i value (the equilibrium dissociation constant for the enzyme–inhibitor complex) was higher ($K_i = 174 \pm 36\ \mu M$) than in pre-incubation ($K_i = 45 \pm 6\ \mu M$ and $4 \pm 2\ \mu M$, without and with NADPH, respectively). It thus seems that the pre-incubation of bergamottin (in particular with NADPH) increases its inhibiting capacity on simvastatin metabolism. Bergamottin metabolism study in rat liver microsomes showed the formation of two metabolites that were CYP-450 dependent. In contrast, in human liver microsomes, the incubation conditions of bergamottin did not influence its inhibiting capacity of simvastatin metabolism ($K_i = 34 \pm 5\ \mu M$, $K_i = 22 \pm 5\ \mu M$, $K_i = 27 \pm 11\ \mu M$ in co-incubation and pre-incubation without and with NADPH, respectively). In rat and man, bergamottin was found to be a mixed-type inhibitor of simvastatin hepatic metabolism. However, in rat, bergamottin was partially a mechanism-based inhibitor by involvement of either bergamottin alone or one of its metabolites. The results highlight the importance of validating in-vitro models to help verify the suitability of the in-vitro model for predicting the nature and degree of metabolic drug interactions.

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Introduction

3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) inhibitors (statins) represent the main drugs used in the treatment of hypercholesterolaemia. Plasma cholesterol is lowered due to the inhibition of endogenous cholesterol synthesis and the subsequent increased expression of the low-density lipoprotein (LDL) receptors, which results in an upregulated catabolic rate for plasma LDL.

Of the statins, simvastatin is a prodrug used in the treatment of hypercholesterolaemia (Mauro 1993). Following the conversion of this lactone prodrug to its hydroxy acid form, the compound is a potent inhibitor of HMG-Co A reductase, the rate limiting enzyme in cholesterol biosynthesis (Alberts et al 1980). Simvastatin undergoes a very important hepatic first-pass metabolism (Prueksaritanont et al 1997), and this may be responsible for its low bioavailability. Simvastatin is either converted by some cytosolic esterases to the active corresponding hydroxy acid form or biotransformed by oxidation into metabolites by cytochrome P450 (CYP).

Statins are generally well tolerated. However, adverse events are associated with concomitant therapy, leading to myopathy and potentially fatal rhabdomyolysis (Igel et al 2001). In fact, prolonged exposure to a low serum concentration of simvastatin acid considerably increases the risk of skeletal muscle toxicity (Bogman et al 2001).

Oral co-administration of grapefruit juice has been demonstrated to significantly increase the oral bioavailability of several clinically used drugs, including dihydropyridines (Miniscalco et al 1992), ciclosporin (Edwards et al 1999), saquinavir

(Kupferschmidt et al 1998) and simvastatin (Lilja et al 1998). Concomitant administration of simvastatin with high amounts of grapefruit juice (corresponding to three glasses of 200 mL) increased the mean plasma AUC (0–∞) of simvastatin by 13.5 fold compared with water. However, the effect of grapefruit juice was only about one-tenth of the maximum when studies were performed 24 h after stopping the intake of grapefruit juice (Lilja et al 2000).

The exact constituents in grapefruit juice that are responsible for drug metabolism inhibition are not perfectly known. The grapefruit-juice composition varies from variety to variety and from lot to lot and also depends on the preparation method (Ho et al 2000). In all cases, the grapefruit juice contains different components, the majority being flavonoids and also furocoumarin derivatives.

The possible components include a few flavonoids such as naringenin (the aglycone form of naringin), quercetin and kaempferol. Within the grapefruit juice responsible for increasing the bioavailability of some drugs, these substances have been chosen as possible candidates because they have been shown to competitively inhibit CYP3A4 activity in-vitro (Ubeaud et al 1999; Le Goff et al 2002). Indeed, if substances or drugs interfere with the CYP isoenzymes involved in simvastatin metabolism, the bioavailability of simvastatin and the formation of simvastatin acid may be increased. However, in-vivo the oral administration of these flavonoids does not reproduce the grapefruit juice effect (He et al 1998). So it seems that these are not the principal components responsible for the effect of grapefruit juice in-vivo.

We are interested in another component, bergamottin, the major furocoumarinic derivative present in grapefruit juice. We have shown that bergamottin qualitatively inhibits simvastatin metabolism (Le Goff-Klein et al 2003). So, in this paper, we proposed to characterize the mechanism of inhibition by bergamottin by calculation of inhibition parameters at different concentrations and experimental conditions.

The aim of this paper was to investigate the in-vitro metabolic interaction between simvastatin and bergamottin to further characterize the mechanism of inhibition. The results of these in-vitro studies should allow the early identification of potential interactions between simvastatin and grapefruit juice for predicting interactions in man.

Materials and Methods

Chemicals

Simvastatin was obtained from Merck Sharp and Dohme (USA). Bergamottin was purchased from Extrasynthese (France). All other chemicals were commercially available and of high analytical grade.

Sample analysis

Simvastatin analysis by liquid chromatography-UV method

Simvastatin was determined by using a reversed-phase LC-UV method according to the modified technique of Carlucci et al (1992). The analytical system consisted of an automatic sample injector (360 model; Kontron) connected with a pump (model 420; Kontron) and a UV detector (model Spectroflow 773; Kratos).

The chromatographic analysis was performed on a Lichrospher 100 RP-18 (150 × 4.6 mm, 5 μm particles) protected by a Supelguard LC-18 precolumn (20 mm × 4.6, 5 μm particles). The mobile phase consisted of sodium dihydrogen phosphate (0.025 M; pH 4.5)–acetonitrile (35:65, v/v) at a total flow rate of 0.8 mL min^{-1}. The column was maintained at room temperature (20°C) and the eluted compounds were recorded on the detector at a constant wavelength of 238 nm. The injected volume was 50 μL. The retention time for simvastatin was 10 min. The quantification limit was 200 ng mL^{-1} and the inter-assay precision was $<2\%$ over the concentration range 1–80 μg mL^{-1}.

Simvastatin was dissolved in acetonitrile and bergamottin in methanol. The final concentration of solvent in the incubation mixture was 0.5%.

Initially, the stability of simvastatin and bergamottin was investigated under experimental conditions and was shown to be stable throughout the whole incubation period in different media.

Bergamottin analysis by liquid chromatography–mass spectrometry (LC-MS-MS)

Bergamottin was analysed on an HPLC Waters 1525 coupled with a triple quadripole mass spectrophotometer (Quattro, micromass) interfaced with an API-ES (atmosphere pressure ionization-electrospray) ionization chamber. The HPLC apparatus was equipped with 4 pumps and an Inertsil ODS-3 250 × 2 mm (particle size 5 μM) analytical column. The injected volume was 100 μL. The column was perfused at a flow rate of 1 mL min^{-1} with a linear elution gradient containing a mixture of 2 solvents as the mobile phase: solvent A, $H_2O + 0.5\%$ ammonium acetate pH 4.5–methanol (95:5, v/v); solvent B, $H_2O + 0.5\%$ ammonium acetate pH 4.5–methanol (10:90, v/v). The separation was obtained using 0–100% linear gradients of A for 40 min followed by elution with 100% B for 17 min. The retention time of bergamottin was 53 min.

The mass spectrophotometry (MS) analysis was performed under the following conditions: capillary voltage, 3 kV; API temperature, 100°C; nebulising gas (N_2) flow rate, 0.4 L min^{-1}; desolvation temperature, 240°C; acquisition mode, Scan 40–700 m/z.

Figure 1 presents an LC-MS-MS chromatogram of bergamottin and its metabolites after incubation in rat liver microsomes.

Liver microsomal fractions

Liver microsomes from male Wistar rats (240–260 g) were prepared according to a technique previously described (Ubeaud et al 1999). Human liver microsomes were provided from In Vitro Technology, Inc. (Baltimore MD). The microsomal protein and the total CYP contents were estimated according to the Bradford (1976) and Omura & Sato

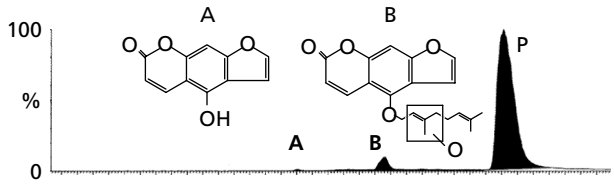

Figure 1 LC-MS-MS chromatogram of bergamottin and its metabolites. The structures of the metabolites of bergamottin (A and B) are identified: A was formed by *o*-deakylation of bergamottin and B was formed by hydroxylation of the double bond in the lateral chain.

(1964) methods, respectively. The rat total microsomal CYP content and the protein rate were 2070 pmol mL^{-1} and 22.3 mg mL^{-1}, respectively. The human total microsomal CYP content and the protein rate were 281 pmol mL^{-1} and 20 mg mL^{-1}, respectively.

Co-incubation of bergamottin and simvastatin

Human and rat liver microsomes (0.5 mg protein/mL) were co-incubated at 37°C for 15 min with various concentrations of simvastatin (10–250 μM) and bergamottin (25–100 μM) in a final volume of 0.5 mL of 0.1 M phosphate buffer (pH 7.4) containing 3 nmol of NADPH in a shaking water bath. The reaction was stopped with one volume of cold ethyl acetate. The supernatant was vortexed and centrifuged. The organic phase was collected and the pellet was washed three times with 500 μL of ethyl acetate. All organic phases were evaporated and the pellet was re-suspended with 500 μL of acetonitrile and was injected into the HPLC column.

Control experiments, realized in the same conditions in the presence of heat-inactivated liver microsomes, were carried out in parallel to check the stability of simvastatin under the incubation conditions.

Pre-incubation of bergamottin and incubation with simvastatin

Human and rat liver microsomes (0.5 mg protein/mL) were pre-incubated with bergamottin (25–100 μM) in the presence or absence of NADPH (3 nM) in a final volume of 0.5 mL of 0.1 M phosphate buffer (pH 7.4) at 37°C in a shaking water bath. At several pre-incubated times, simvastatin was added for a 15-min period at 37°C. The reaction was stopped with one volume of cold ethyl acetate. Each sample was extracted as previously described.

Calculation

In kinetic studies, the Michaelis–Menten parameters (K_m and V_{max}) were determined by using non-linear regression analysis with Enzymekinetics software (Condorcet 1996). V_{max} represented maximum velocity expressed in nmol min^{-1} (mg protein)$^{-1}$ and K_m represented the Michaelis–Menten affinity constant expressed in μM.

Apparent K_i values were calculated by two methods. The first method used $K_{m\ app}$ based on the linear regression plot of $K_{m\ app}/V_{max}$ ratio as a function of inhibitor concentrations. The equation describing this relationship is (Bourrie et al 1996; Kakkar et al 1999):

$$K_{m\ app}/V_{max\ app} = (K_m[I]/K_i) + (K_m/V_{max}) \quad (1)$$

with $K_{m\ app}$ and $V_{max\ app}$ representing the values of K_m and V_{max} in the presence of different concentrations of inhibitor.

The apparent K_i value (the equilibrium dissociation constant for the enzyme–inhibitor complex) was determined from the x-intercept of a plot of apparent K_m/V_{max} (obtained from the slope of the Lineweaver–Burk plots) versus inhibitor concentration. K_i was obtained by the x-intercept of the linear plot.

The second method used was the Voet & Voet (1990) equation defined according to the inhibition type as follows: $K_i = K_m[I]/(K_{m\ app} - K_m)$ for competitive inhibition; $K_i = V_{max\ app} \times K_m[I]/(V_{max} - V_{max\ app})$ for non-competitive inhibition; $K_i = V_{max\ app} \times K_m[I]/(K_{m\ app}V_{max} - K_mV_{max\ app})$ for mixed inhibition; $K_i = [I]/(([S]/([S] + K_m) - 1)$ for uncompetitive inhibition; K_m, $K_{m\ app}$, V_{max}, $V_{max\ app}$ were obtained in the absence and in the presence of inhibitor. [S] and [I] were substrate and inhibitor concentrations respectively.

Lineweaver–Burk plots of the enzyme kinetics data were generated to determine the mode of inhibition (Chang et al 2001).

Statistics

All data were expressed as mean ± standard deviation of n experiments. Statistical evaluation of the K_i values was performed with analysis of variance; P values less than 0.05 were considered to be statistically significant.

Results

Calculation of V_{max} and K_m

In kinetic microsomal fraction studies (human and rat liver microsomes), the Michaelis–Menten kinetic parameters V_{max} and K_m were determined by incubating simvastatin in the presence of microsomal fractions.

Preliminary experiments were performed in the presence of 0.2–1.0 mg protein for different times (5–20 min) to give activities that are linear with respect to time and protein concentrations. The optimal conditions giving activities that are linear with respect to time and protein concentrations were 0.5 mg of protein for 15 min.

In rat liver microsomal fractions, a range of simvastatin concentrations (10–1000 μM) was incubated for 15 min in the presence of 0.5 mg mL^{-1} microsomal proteins.

In human microsomal fractions, a range of simvastatin concentrations (10, 25, 50, 100, 150 and 300 μM) was incubated for 10 min with 0.5 mg mL^{-1} microsomal proteins.

The K_m and V_{max} values are given in Table 1.

Table 1 Michaelis–Menten parameters for the metabolism of simvastatin in rat and human liver microsomes

	Rat liver microsomal fractions	Human liver microsomal fractions
K_m (μM)	449 ± 25	23 ± 3
V_{max} (nmol min^{-1} (mg protein)$^{-1}$)	82 ± 33	7 ± 2

Data are presented as means ± standard deviation (n = 3 experiments).

Simvastatin metabolism inhibition by bergamottin: K_i values of bergamottin determination

Bergamottin co-incubation with rat liver microsomes

To determine estimated K_i values of bergamottin, simvastatin was incubated at 10, 25 and 100 μM in the presence of increasing bergamottin concentrations (10, 25, 50 μM) for 15 min with 0.5 mg mL^{-1} microsomal proteins.

Lineweaver–Burk analysis was carried out to determine the nature of inhibition and values of inhibition constants (K_i). With increasing bergamottin concentrations, there was a concentration-dependent increase in the apparent K_m values as well as a decrease in the V_{max}. This is consistent with a mixed-type inhibition between simvastatin and bergamottin (Figure 2A). The apparent K_i value was computed ($K_{m\ app}/V_{max}$ as a function of inhibitor concentration) at ≈ 188.5 μM (Figure 2B).

The second method, using Voet and Voet formula, gave $K_i = 174 \pm 36$ μM, which was not significantly different from the graphically determined value ($P < 0.05$) (Table 2).

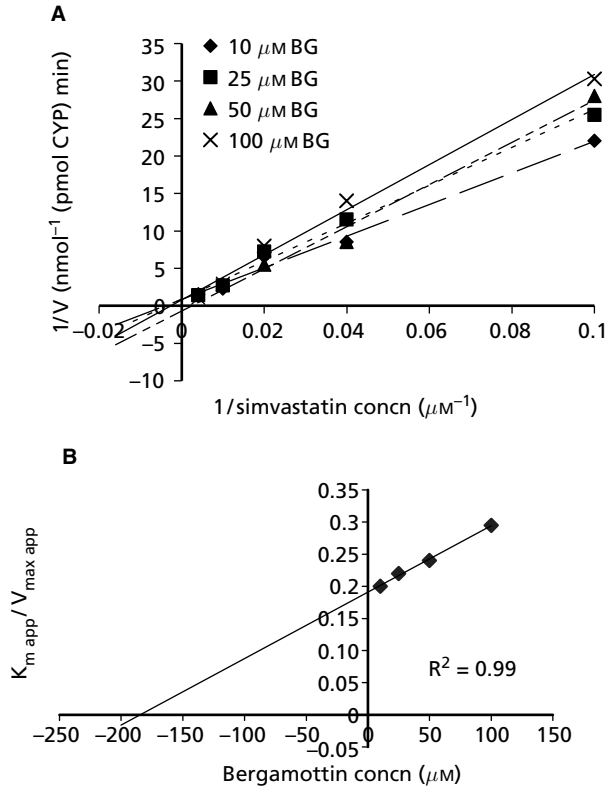

Figure 2 Lineweaver–Burk plot showing the inhibitory effect of bergamottin (BG) on the metabolism of simvastatin by rat liver microsomes (A). The secondary plot (B) illustrates the inhibitory effect of bergamottin on simvastatin metabolism by plotting K_m/V_{max} ratio as a function of bergamottin concentration. Each data point represents an average of three separate experiments. The standard deviation is not represented to not overload the graphic representation. The units of reaction velocity (V) are nmol min^{-1} (mg protein)$^{-1}$.

Bergamottin co-incubation with human liver microsomes

The representative Lineweaver–Burk and the corresponding secondary plots for the inhibitory effect of bergamottin on simvastatin metabolism by human liver microsomal samples obtained from pooled different livers are shown in Figure 3. With increasing bergamottin concentrations (25, 50, 75 and 100 μM), there was a concentration-dependent increase in the apparent K_m values as well as a decrease in the V_{max} values. This is consistent with a mixed-type inhibition.

The apparent K_i value was computed at ≈43 μM by plotting K_m/V_{max} as a function of inhibitor concentrations

Table 2 Calculation of inhibition constant (K_i) values

	K_i values (μM)					
	Co-inbubation		Pre-incubation – NADPH		Pre-incubation + NADPH	
	(K_m/V_{max})	Voet & Voet (μM)	(K_m/V_{max})	Voet & Voet	(K_m/V_{max})	Voet & Voet
Rat liver microsomes	188	174 ± 36	34	45 ± 6	3	4 ± 2
Human liver microsomes	43	34 ± 5	18	22 ± 5	33	27 ± 11

Data are presented as means ± standard deviation (n = 3 experiments). The K_i values are significantly different between species for one incubation type ($P < 0.01$). In rat liver microsomes, the K_i values are significantly different as a function of incubation conditions ($P < 0.01$) but in human liver microsomes, the difference between K_i values is not significant ($P < 0.05$).

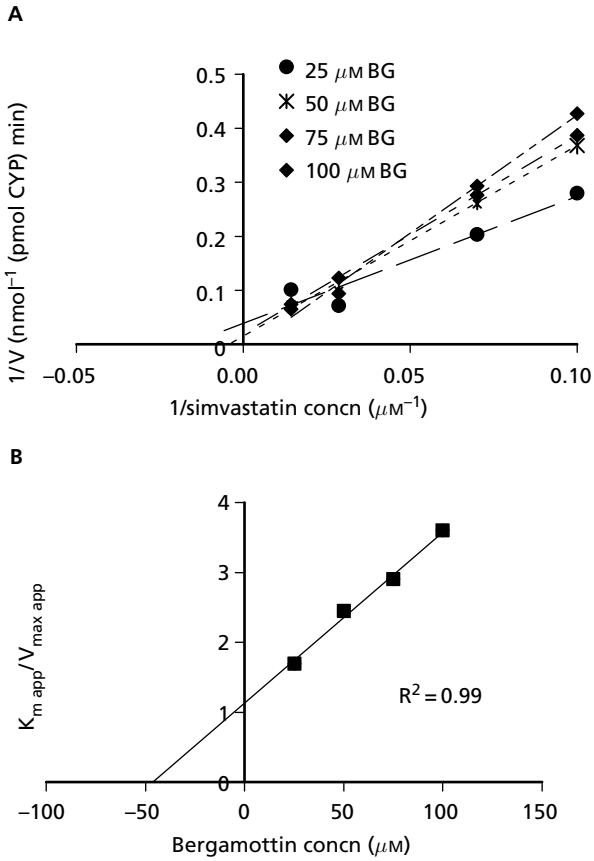

Figure 3 Linewaver–Burk plot showing the inhibitory effect of bergamottin (BG) on the metabolism of simvastatin by human liver microsomes (A). The secondary plot (B) illustrates the inhibitory effect of bergamottin on simvastatin metabolism by plotting K_m/V_{max} ratio as a function of bergamottin concentration. Each data point represents an average of three separate experiments. The standard deviations are not represented to not overload the graphic representations. The units of reaction velocity (V) are nmol min^{-1} (mg protein)$^{-1}$.

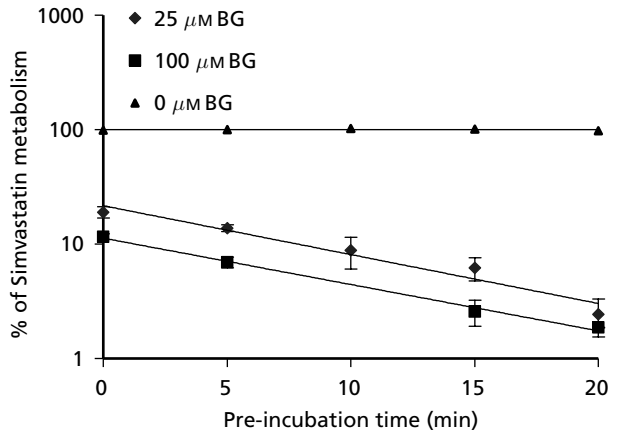

Figure 4 Inhibition of simvastatin metabolism in rat liver microsomes as a function of time and bergamottin (BG) concentration. Each data point represents an average of three separate experiments; bars represent standard deviation.

and $\approx 34 \pm 5\,\mu$M by the formula for the mixed-type inhibition.

These values were not significantly different ($P < 0.05$) (Table 2).

Bergamottin pre-incubation with rat liver microsomes

Determination of pre-incubation time. Different concentrations of bergamottin (25–100 μM) were pre-incubated with NADPH (0.3 mM) and microsomal protein (0.5 mg mL^{-1}). After different pre-incubation times (0, 5, 10, 15, 20 min), 50 μM of simvastatin was added to the mixture for 15 min at 37°C. The reaction was stopped by addition of one volume of cold ethyl acetate.

The inhibition of CYP activity was dependent on the time of pre-incubation and bergamottin concentration (Figure 4). Therefore, subsequent experiments were performed with a 15-min pre-incubation time, to ensure maximum CYP inhibition (98%).

Pre-incubation in the presence of NADPH. Bergamottin at different concentrations (0–100 μM) was pre-incubated with microsomal protein (0.5 mg mL^{-1}). After 15 min pre-incubation, NADPH (0.3 mM) and simvastatin at different concentrations (25–250 μM) was added to the mixture.

In the presence of increasing bergamottin concentrations, a concentration-dependent inhibition of simvastatin metabolism was observed. This inhibition was characterized by a concentration-dependent increase in K_m value as well as a decrease in V_{max} value. This is consistent with a mixed-type inhibition (Lineweaver–Burk plots are shown in Figure 5A).

The K_i value was computed at $K_i = 3\,\mu$M by plotting $K_{m\ app}/V_{max}$ as a function of inhibitor concentration and $4 \pm 2\,\mu$M by the Voet and Voet formula. These values were not significantly different ($P < 0.05$) (Table 2).

Pre-incubation in the absence of NADPH. Bergamottin at different concentrations (0–100 μM) was pre-incubated with rat liver microsomal protein (0.5 mg mL^{-1}). After 15 min pre-incubation, simvastatin at different concentrations (25–250 μM) was added to the mixture.

Lineweaver–Burk plots are reported in Figure 5B and represent a mixed-type inhibition between simvastatin and bergamottin.

The K_i value was computed at $K_i = 34\,\mu$M by plotting $K_{m\ app}/V_{max}$ as a function of inhibitor concentration and $45 \pm 6\,\mu$M by the Voet and Voet formula. These values were not significantly different ($P < 0.05$) (Table 2).

Bergamottin pre-incubation with human liver microsomes

The same experiment was realized in human liver microsomes to compare the type of inhibition by bergamottin in these two models.

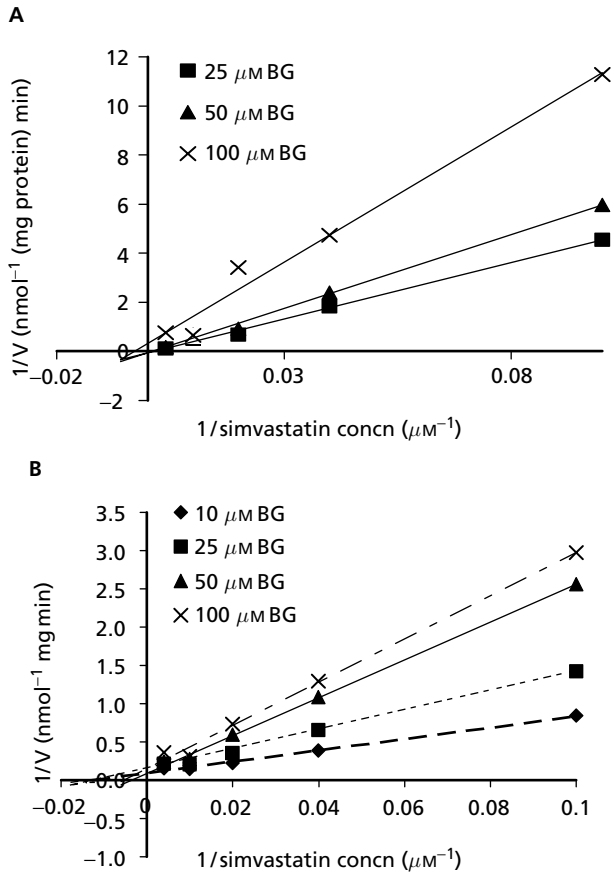

Figure 5 Lineweaver–Burk plots showing the inhibition of simvastatin metabolism by bergamottin (BG) in rat liver microsomes after pre-incubation of bergamottin with NADPH (A) or without NADPH (B). Each data point represents an average of three separate experiments. The standard deviation is not represented to not overload the graphic representations. The units of reaction velocity (V) are nmol min^{-1} (mg protein)$^{-1}$.

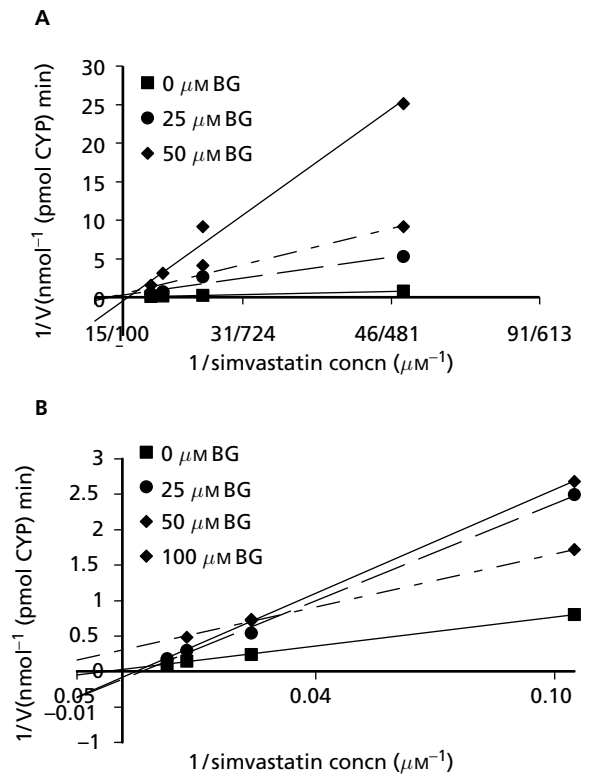

Figure 6 Lineweaver–Burk plots showing the inhibition of simvastatin metabolism by bergamottin (BG) in human liver microsomes after pre-incubation of bergamottin with NADPH (A) or without NADPH (B). Each data point represents an average of three separate experiments. The standard deviation is not represented to not overload the graphic representations. The units of reaction velocity (V) are nmol min^{-1} (mg protein)$^{-1}$.

Incubation in the presence of NADPH. Bergamottin at different concentrations (0–100 μM) was pre-incubated with human liver microsomal protein (0.5 mg mL^{-1}). After 15 min pre-incubation, simvastatin at different concentrations (10–100 μM) and NADPH were added to the mixture.

The Lineweaver–Burk analysis was carried and represented a mixed-type inhibition between simvastatin and bergamottin (Figure 6A).

The K_i value was computed at ≈33 μM by plotting $K_{m\ app}/V_{max}$ as a function of inhibitor concentration and ≈27 ± 11 μM by the Voet and Voet formula. These values were not significantly different ($P < 0.05$) (Table 2).

Incubation in the absence of NADPH. Bergamottin at different concentrations (0–100 μM) was pre-incubated with human liver microsomal protein (0.5 mg mL^{-1}). After 15 min pre-incubation, simvastatin at different concentrations (10–100 μM) was added to the mixture. The Lineweaver–Burk analysis was carried out and represented a mixed-type inhibition between simvastatin and bergamottin (Figure 6B).

The K_i value was computed at ≈18 μM by plotting $K_{m\ app}/V_{max}$ as a function of inhibitor concentration and ≈22 ± 5 μM by the Voet and Voet formula. These values were not significantly different ($P < 0.05$) (Table 2).

Metabolism of bergamottin

Bergamottin at different concentrations (50, 100, 200 μM) was incubated with rat liver microsomes in phosphate buffer in the presence of NADPH (0.3 mM) for 15 min. Each sample was analysed by LC and by LC-MS-MS.

The chromatogram showed that bergamottin was metabolized into two metabolites (A and B). The major metabolite was metabolite B. The bergamottin metabolites' structures were identified by mass spectrometry.

Metabolite A was formed by an *o*-dealkylation (cutting up to ether on the lateral chain). As for metabolite B, it could be formed by a hydroxylation of the double bond in the lateral chain (Figure 1).

Discussion

In-vivo studies have shown that grapefruit juice can modify the pharmacokinetic parameters of many drugs, notably simvastatin. Lilja et al (1998, 2000) showed that grapefruit juice increased the $AUC_{(0-\infty)}$ of simvastatin by 13.5 fold, but only by 1.5 fold if simvastatin was administered 1 day after the last dose of grapefruit juice. Meanwhile, recent studies have shown that grapefruit juice could modify pharmacokinetic parameters of dextropropoxyphene for three days after the last dose of grapefruit. So, it seems that grapefruit juice components can have a lasting effect on the inhibition of drug metabolism (Di Marco et al 2002).

In this paper, the metabolic interaction between simvastatin and bergamottin was studied in the liver because the bioavailability of simvastatin was low in spite of a good intestinal absorption (60–85%) (Igel et al 2001), which could be explained by an important hepatic first-pass metabolism. The results showed that bergamottin inhibited hepatic CYP-mediated metabolism of simvastatin.

The apparent K_i values were determined by two different methods: the secondary plots of Lineweaver–Burk ($K_{m\ app}/V_m$ as a function of inhibitor concentration) and the Voet and Voet formula. These two methods gave similar results, thus validating the methodology.

The type of inhibition was similar, as a mixed-type inhibition in rat and human liver microsomal fractions in our experimental conditions. However, the apparent K_i values were different as a function of incubation conditions. In human liver microsomes, K_i values for inhibition were not modified by the type of incubation (co-incubation, or pre-incubation with or without NADPH) ($K_i = 31 \pm 5\,\mu M$, $K_i = 27 \pm 11\,\mu M$, $K_i = 22 \pm 5\,\mu M$ for a co-incubation, for a pre-incubation without NADPH and with NADPH, respectively) (Figures 3 and 6). In our experimental conditions, bergamottin pre-incubation did not modify the capacity of bergamottin to inhibit simvastatin hepatic metabolism. In rat liver microsomal fractions, simvastatin metabolism was strongly inhibited by bergamottin pre-incubation in comparison with bergamottin co-incubation (Figures 2 and 5) ($K_i = 45 \pm 6\,\mu M$ and $K_i = 174 \pm 36\,\mu M$ for pre-incubation without NADPH and co-incubation respectively). The difference between the K_i values was significant (analysis of variance test, $P < 0.01$). Simvastatin metabolism inhibition is otherwise more potent when NADPH co-factor is present during the pre-incubation time than in the absence of NADPH co-factor (Table 1).

So, in our experimental conditions, the inhibition of simvastatin metabolism by bergamottin was dependent upon time of pre-incubation and bergamottin concentrations, required the presence of NADPH ($K_i = 4 \pm 2\,\mu M$ and $K_i = 45 \pm 6\,\mu M$ with and without NADPH, respectively) and was shown to exhibit saturation kinetics (Chang et al 2001).

These three points showed that, in rat liver microsomal fractions, bergamottin can inhibit simvastatin metabolism by a mechanism-based inhibition. These results agree with those described in the literature (Koenigs & Trager 1998; Ho et al 2001). NADPH is partially necessary for the effect of bergamottin on simvastatin metabolism because when NADPH was present during pre-incubation, the inhibition of simvastatin metabolism by bergamottin was more potent than when NADPH was absent. It seems that simvastatin metabolism inhibition by bergamottin is irreversible and requires metabolism of bergamottin. Therefore, the necessity of NADPH in the inactivation of CYP by bergamottin indicates that it is probably not the bergamottin, but its metabolites, that are responsible for the inactivation of the CYP.

So, bergamottin seems to be on the one hand, a mixed-type inhibitor and on the other hand, a mechanism-based inhibitor. However, as shown in Figure 5B, the mechanism-based inhibition of bergamottin is not a major factor because when NADPH is present during pre-incubation time, the inhibitory potency of bergamottin increases by only about 20%.

It is thought that the mechanism-based inhibition of CYPs can occur via three different pathways: firstly, the inactivator binding to the apoprotein; secondly, the inactivator binding to the haem; and, thirdly, the inactivator-induced haem fragmentation in products that bind the apoprotein. The CYP active site is large and contains multiple binding domains. Ohta et al (2002) reported that the presence of the 5-geranyloxyfurocoumarin moiety is essential for the CYP3A4 inhibition. Koenigs & Trager (1998) suggested that the mechanism of inactivation was an initial oxidation of the furane ring to generate a fura-epoxide derivative, which then reacts either with water to form a dihydrodiol or with a nucleophilic amino acid at the active site of CYP.

Mechanism-based inactivation accounts for approximately 33% of the spectrally determined CYP lost, based on covalently bound enzyme. The factor(s) responsible for the remaining 67% loss is (are) unknown, but may include unstable adducts, inactive, but spectrally detectable enzyme, or the liberation of reactive oxygen species through uncoupling.

To explain the different inhibition mechanisms of bergamottin in co-incubation and in pre-incubation with simvastatin, we studied bergamottin metabolism in rat liver microsomes in the presence of NADPH. Our results showed that bergamottin was metabolized into two metabolites (A and B) (Figure 1). These two metabolites could be involved in the inhibition of simvastatin metabolism during pre-incubation and could explain that bergamottin was a mechanism-based inhibitor. These metabolites (A and B) are CYP-dependent since they are formed in rat liver microsomes in the presence of NADPH.

Conclusion

Compared with other grapefruit juice components, such as naringenin, bergamottin has the same capacity to inhibit the metabolism of simvastatin in man. In the rat, bergamottin is a more potent inhibitor of simvastatin metabolism, acting partially by a mechanism-based inhibition when it is pre-incubated (in particular in the presence of NADPH). Moreover, interspecies differences in the inhibition mechanism in rat and man highlight the

importance of validating in-vitro models to extrapolate the type and the degree of metabolic drug interactions in-vivo. These interspecies differences could be explained by the different liver CYP isoenzymes involved in the metabolism of simvastatin or the different affinity of bergamottin (or one of its metabolites) for these isoenzymes in rat and man.

Then, bergamottin and naringenin have the potential to interfere with simvastatin metabolism. Our in-vitro results are relevant to the in-vivo situation since the K_i values found (K_i about $\approx 30\,\mu$M) were inferior to the estimated plasma concentrations of naringenin (108–603 mM) and bergamottin (3–11 mM) after ingestion of 250 mL of grapefruit juice containing naringin (420–2345 μM) and bergamottin (12 and 44 μM) (Miniscalco et al 1992; Guo et al 2000; Ho et al 2000; Ross et al 2000).

So, all these results should be taken into account to adjust doses to avoid adverse effects when simvastatin is co-administered with grapefruit juice.

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