

Inhibitory Effects of Fruit Juices on Cytochrome P450 2C9 Activity *in Vitro*

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There is limited information on the effect of fruits on human cytochrome P450 (CYP) 2C9 activity. The objective of this study was to determine the effect of fruit juice on CYP2C9-mediated drug metabolism. Nine citrus fruits and eight tropical fruits were chosen. We investigated effects of the fruits on diclofenac 4'-hydroxylation and tolbutamide hydroxylation by human liver microsomes. Among the fruits, pineapple juice showed potent inhibition of CYP2C9 activity. The addition of 25 μ l (5.0% v/v) of pineapple juice resulted in almost complete inhibition. Next we examined the inhibitory effect of bromelain, a cysteine protease in pineapple. Bromelain also strongly inhibited CYP2C9 activity. In addition, E-64, a cysteine protease inhibitor, almost entirely blocked inhibition by pineapple juice and bromelain. Thus we found that pineapple juice was a potent inhibitor of CYP2C9, and that the inhibitory effect might be due to the bromelain contained in pineapple.

Key words: pineapple; cytochrome P450; cytochrome P450 2C9 (CYP2C9); bromelain

It has been reported that grapefruit juice interacts with therapeutic drugs that undergo substantial presystemic metabolism mediated by cytochrome P450 (CYP) 3A4,¹ and that furanocoumarin derivatives identified in grapefruit juice strongly inhibited the catalytic activity of CYP3A.² The mechanism of action probably involved irreversible (mechanism-based) inhibition of CYP3A in the small intestine,³ which resulted in a decrease in the first-pass metabolism of orally administered therapeutic drugs. Furthermore, in recent years, reports have indicated that various kinds of fruits have an inhibitory effect on CYP3A activities *in vitro* and/or *in vivo*.⁴⁻⁷

The inhibitory effect is assumed to be dependent on the kind of fruit, and is attributed to the type of chemical components contained in the fruit.²

Recent reports indicate that some fruit juices inhibit CYP2C9 activities and cause food-drug interactions.^{8,9} When CYP2C9 substrates such as warfarin and phenytoin with low therapeutic margins diminish metabolic capacity because of drug-food interactions, these drugs can lead to toxicity even at normal therapeutic doses, but few reports are available on the inhibition of CYP2C9 activity by fruit juice or extract. Hence it is important to evaluate the effect of fruit juice on CYP2C9 activity.

In the present study, we investigated to determine whether fruit juice would inhibit the CYP2C9-mediated drug metabolism using human liver microsomes. The fruits that we chose for this study were citrus fruits and tropical fruits, because people living in all over the world consume them. Tropical fruits are produced in areas with warm climates, such as Southwest Asia and the southern part of Japan. We used diclofenac and tolbutamide as substrates for CYP2C9, since both drugs are recommended as probe substrates for *in vitro* metabolic studies.¹⁰

Materials and Methods

Chemicals. Tolbutamide, 4-hydroxytolbutamide, sulfaphenazole, E-64, pepstatin A, aprotinin, and bromelain were purchased from Sigma-Aldrich (St. Louis, MO). Diclofenac and 4'-hydroxydiclofenac were from Daiichi Pure Chemicals (Tokyo). Pooled human liver microsomes were from BD Gentest (Woburn, MA). All chemicals and solvents were of the highest grade commercially available. All aqueous solutions were prepared using ultra-pure grade water.

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Abbreviation: CYP, cytochrome P450

Fruit samples. Citrus fruits, hyuga-natsu, unshu mandarin, banpeiyu, hirami lemon, valencia orange, pomelo, grapefruit, lemon, and lime, and tropical fruits, melon, mango, litchi, pineapple, papaya, mangosteen, passion fruit, and kiwi fruit, were obtained from local commercial sources. These species and origin information are shown in Table 1. The fruits were stored at 4 °C until use. Fruit juice was obtained by squeezing the edible portion of the fruit, and the juice was filtered to remove the residues. All samples were treated soon after they were squeezed and filtered.

Analytical procedures for human CYP2C9 activity. Assay of tolbutamide methyl hydroxylase activity was performed according to the method of Tang *et al.*,¹¹ with minor modifications. Briefly, the incubation mixtures (final volume, 0.5 ml) consisted of the following: 0.1 M phosphate buffer (pH 7.4), 10 mM MgCl₂, 1 mM EDTA, 1 mM NADP⁺, 10 mM D-glucose 6-phosphate, 10 units/ml D-glucose 6-phosphate dehydrogenase, and 0.2 mg/ml of microsomal protein. The concentration of tolbutamide was 250 μM. The reaction time was predetermined based on linearity between the reaction time *versus* the metabolite formation rate. Based on the results obtained, the reaction time was determined to be 75 min. The reaction mixture was preincubated at 37 °C for 5 min, and the reaction was started by the addition of substrate, and terminated with 2 ml of ice-cold acetonitrile. Midazolam (1 nM) was added as an internal standard. Following centrifugation (3,000 rpm, 10 min), the organic phase was evaporated at 40 °C. The residue was dissolved in 200 μl of HPLC mobile phase, and 100 μl of the resulting mixture was injected into an HPLC.

The HPLC system consisted of an LC-10ADvp pump (Shimadzu, Kyoto, Japan), a Shimadzu L-4200 UV absorbance detector, and a Shimadzu SIL-10ADvp auto injector. The system was equipped with a Cadenza CD-C18 column (3 μm, 4.6 × 250 mm; Intact, Kyoto, Japan) preceded by a precolumn (5 μm, 2 × 5 mm). The mobile phase consisted of acetonitrile and 0.1% of pH 7.4 phosphate buffer (20:80, v/v; solvent A) and acetonitrile (solvent B). The initial mobile phase consisted of 5% of solvent B, which increased linearly to 40% over 8 min. The mobile phase was delivered at a flow rate of 0.7 ml/min at 40 °C. Quantification was performed by determining the UV peak areas monitored at 230 nm.

Assay of diclofenac 4'-hydroxylase activity was performed according to a previously reported method.⁹

Inhibitory effect of fruit and bromelain on CYP2C9 activity. The inhibitory effects of fruits on CYP activity were investigated according to a previously reported method,⁶ with minor modifications. Briefly, an appropriate amount of fruit juice was applied to fresh tubes. The reaction mixture described above (before the addition of substrate) was added to the tubes and resuspended with a mixer. The maximum amount of fruit juice was 25 μl (5.0% v/v), and the pH of the reaction

mixtures was constant under these conditions. After preincubation of the mixture at 37 °C for 5 min, the substrate, diclofenac or tolbutamide, was added. The reaction was performed as described above. The inhibitory effects of a fruit juice on diclofenac 4'-hydroxylation and tolbutamide hydroxylation were expressed in terms of percentage of residual activity as compared with the control in the absence of the fruit juice. Similarly, the inhibitory effect of bromelain was also examined after dissolving bromelain in water. The final concentration of bromelain was 0.5 to 50 μg/ml.

Effect of protease inhibitors on CYP2C9 inhibition by pineapple juice and bromelain. Pineapple juice (2% v/v) and bromelain (50 μg/ml) were incubated with a protease inhibitor at 37 °C for 5 min prior to CYP2C9 inhibition assay. E-64, pepstatin A, and aprotinin, protease inhibitors, were dissolved in appropriate solvents and diluted with phosphate buffered saline at 4 °C. The final concentrations of E-64, pepstatin A, and aprotinin were 100 μM, 100 μM, and 10 μM respectively. After incubation, the effect of these samples on CYP2C9 activity was examined according to the method mentioned above.

Effect of ultrafiltration and heat treatment on CYP2C9 inhibition by pineapple juice and bromelain. Low molecular weight fractions were isolated by ultrafiltration (Ultracent-10; Tosoh, Tokyo) of 1-ml pineapple juice and bromelain (50 μg/ml) in a centrifuge at 2,200 g at 25 °C for 15 min.

Heat treatment was performed according to the method of Uesawa *et al.*¹² One ml each of pineapple juice and bromelain (50 μg/ml) was treated at 95 °C for 60 min. The effect of these samples on CYP2C9 activity was examined according to the method described above.

Enzyme assay. The proteolytic activities of pineapple juice and bromelain were assayed by the procedure of Ahmad *et al.*,¹³ with minor modifications. Briefly, a denatured casein solution (2%) at pH 7.0 was incubated for 15 min at room temperature with pineapple juice or bromelain solution. The reaction was stopped by adding 6% trichloroacetic acid, and the undigested casein was removed by centrifugation or filtration. The amount of peptide remaining in supernatant was determined spectrophotometrically at 275 nm against a blank containing all the reaction mixture except for the activity. Enzyme activity was expressed in terms of ΔA_{275} of the supernatant. The absorbance values obtained correlated with the amounts of product formed.

Data analysis. Data from the experiments are expressed as mean ± S.D. The concentration of pineapple juice or bromelain required for 50% inhibition of CYP2C9 activity (IC₅₀) was calculated by linear regression analysis of the log inhibitor concentration *versus* percentage residual activity plots.

Table 1. Effect of Components of Citrus and Tropical Fruits on the CYP2C9 Activity of Human Liver Microsomes

Data are presented as mean \pm S.D. of triplicate assays. The amount of fruit juice used in assays was 25 μ l (5.0% v/v). The control activities of diclofenac 4'-hydroxylation and tolbutamide hydroxylation by human liver microsomes determined in the absence of fruit juice were 972 and 254 pmol/min/mg protein respectively.

Samples (25 μ l)	Species	Origin	Residual activity (%)	
			Diclofenac 4'-hydroxylation	Tolbutamide hydroxylation
Banpeiyu	<i>Citrus grandis</i> OSBECK	Kumamoto, Japan	54.1 \pm 1.4	45.3 \pm 2.6
Grapefruit	<i>Citrus paradisi</i>	California	31.5 \pm 1.0	49.6 \pm 2.2
Hirami lemon	<i>Citrus depressa</i>	Okinawa, Japan	54.1 \pm 7.6	75.2 \pm 3.8
Hyuga-natsu	<i>Citrus tamurana</i>	Miyazaki, Japan	98.4 \pm 4.9	78.2 \pm 3.9
Lemon	<i>Citrus limon</i>	California	102.5 \pm 2.9	97.8 \pm 3.6
Lime	<i>Citrus aurantifolia</i>	Veracruz, Mexico	42.8 \pm 1.2	62.7 \pm 5.6
Pomelo	<i>Citrus grandis</i>	California	57.2 \pm 3.5	65.3 \pm 3.6
Unshu mandarin	<i>Citrus reticulata</i>	Miyazaki, Japan	81.6 \pm 2.2	72.5 \pm 5.2
Valencia orange	<i>Citrus sinensis</i>	California	97.4 \pm 4.5	93.0 \pm 8.0
Kiwi fruit	<i>Actinidia chinensis</i>	Bay of Plenty, New Zealand	91.5 \pm 3.0	95.8 \pm 1.7
Litchi	<i>Litchi chinensis</i>	Kuangtung, China	74.3 \pm 7.4	65.1 \pm 6.3
Mango	<i>Mangifera indica</i>	Miyazaki, Japan	89.2 \pm 2.9	81.7 \pm 4.7
Mangosteen	<i>Garcinia mangostana</i>	Chaing Mai, Thailand	44.0 \pm 1.1	71.0 \pm 4.0
Melon	<i>Cucumis melo</i>	Miyazaki, Japan	65.2 \pm 3.5	79.6 \pm 2.1
Papaya	<i>Carica papaya</i>	Hawaii	19.4 \pm 2.3	37.7 \pm 2.2
Passion fruit	<i>Punica granatum</i>	Okinawa, Japan	113.6 \pm 8.8	97.0 \pm 7.2
Pineapple	<i>Ananas comosus</i>	Mindanao, Philippine	0.4 \pm 0.1	1.4 \pm 0.3
Pineapple	<i>Ananas comosus</i>	Okinawa, Japan	0.3 \pm 0.1	0.8 \pm 0.2
Sulfaphenazole	(10 μ M)		38.1 \pm 1.1	15.9 \pm 2.0
	(100 μ M)		1.7 \pm 0.7	1.9 \pm 0.3

Results

Inhibition of microsomal human CYP2C9 activity by fruit juice

To evaluate an inhibitory effect of fruit juice on CYP2C9 activity, we examined diclofenac 4'-hydroxylase and tolbutamide hydroxylase activity with and without fruit juices using human liver microsomes. Since we have reported that citrus fruits and tropical fruits inhibited CYP3A,^{6,7,14} we chose these fruits for this study. The results are summarized in Table 1. Among the various fruit juices evaluated, pineapple juice showed the strongest inhibition. The addition of 25 μ l (5.0% v/v) of pineapple juice resulted in almost complete inhibition of human CYP2C9 activity, and the inhibition potency was more than a match for that of sulfaphenazole, a potent inhibitor of CYP2C9.¹⁵ In addition, when we evaluated CYP2C9 activity for pineapples from two different sources, reproducibility of the inhibitory effect was observed. On the other hand, citrus fruits and other tropical fruits had weak or negligible CYP2C9 inhibitory capacity. Hence we conducted further studies of the inhibitory characteristics of pineapple juice. After that we used pineapples grown in Okinawa.

Figure 1 shows the effect of pineapple juice on the diclofenac 4'-hydroxylase activity of human CYP2C9. The degree of inhibition depended on the amount of pineapple juice added to the reaction mixture. The IC₅₀ value was 0.08% v/v. Next we examined to determine whether the components of pineapple juice would inhibit CYP2C9 reversibly or irreversibly. The effect of the

preincubation period on the inhibition of diclofenac 4'-hydroxylase activity by pineapple juice was tested. The results are shown in Fig. 1. The inhibition potency of pineapple juice was altered by lengthening of the preincubation period. The mean residual CYP2C9 activities observed with 0.25 μ l (0.05% v/v) of pineapple juice at preincubation periods of 0, 5, 15, and 30 min were 79.4%, 72.4%, 64.9%, and 55.1% respectively. These data suggest that pineapple juice causes irreversible inhibition of human CYP2C9 activity. Thus, we discovered that pineapple juice potently inhibited the CYP2C9-mediated metabolism of diclofenac and tolbutamide *in vitro*.

Bromelain inhibits CYP2C9 activity

Bromelain is a mixture of cysteine proteases obtained from pineapple stems, and is also present in pineapple fruits.¹⁶ The mixture of cysteine proteases is different as between pineapple stems and fruits, but proteolytic activity is observed in both mixtures.¹⁷ Hence we concluded that bromelain is a candidate for CYP2C9 inhibitor, and we examined the effect of bromelain on diclofenac 4'-hydroxylase activity. The addition of bromelain resulted in almost complete inhibition of CYP2C9 activity at a final concentration of 50 μ g/ml. The inhibition depended on the concentration of bromelain (data not shown), and the IC₅₀ value was calculated to be 1.2 μ g/ml. The proteolytic activities of pineapple juice and bromelain were 0.138 Δ A₂₇₅/min/ml and 0.043 Δ A₂₇₅/min/mg respectively. These data suggest that bromelain is a potent inhibitor of CYP2C9 and is related to the inhibitory effect of pineapple juice.

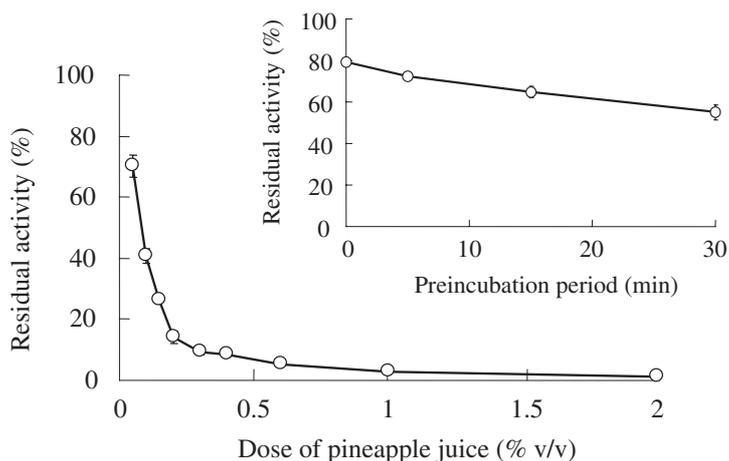


Fig. 1. Inhibition of Human CYP2C9 by Pineapple Juice.

The amounts of fruit juice added to the incubation mixture were 0.05, 0.1, 0.15, 0.2, 0.3, 0.4, 0.6, 1, and 2% v/v. The control activity of diclofenac 4'-hydroxylation by human liver microsomes determined in the absence of fruit juice was 1,019 pmol/min/mg. Each point and each bar represents the mean and S.D. of three independent assays. The inset is the determination of effect of the preincubation period on inhibition. Pineapple juice (0.05% v/v) was added to the reaction mixture and incubated for the indicated periods before the start of the reaction by the addition of a substrate. The control activity of diclofenac 4'-hydroxylation by human liver microsomes determined in the absence of fruit juice was 983 pmol/min/mg. Each point and each bar represents the mean and S.D. of three independent assays.

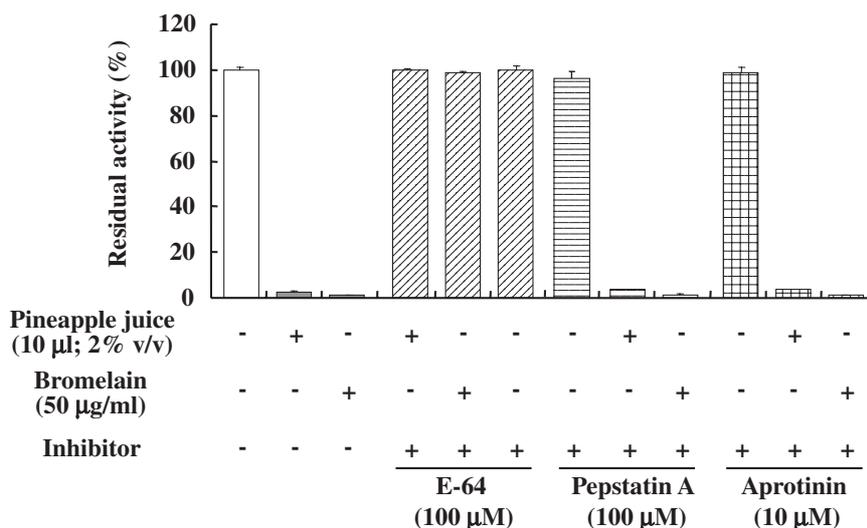


Fig. 2. Effect of Protease Inhibitor on the Inhibition of Diclofenac 4'-Hydroxylation by Pineapple Juice and Bromelain.

Pineapple juice (2% v/v) and bromelain (50 µg/ml) were incubated with a protease inhibitor or phosphate-buffered saline at 37°C for 5 min prior to the CYP2C9 inhibition assay. After incubation, the samples were added to the reaction mixture and assays were performed. The proteolytic activities of pineapple juice and bromelain were 0.138 ΔA_{275} /min/ml and 0.043 ΔA_{275} /min/mg respectively. Each point and each bar represents the mean and S.D. of three independent assays.

To determine whether bromelain makes a contribution to the inhibitory effect of pineapple juice on CYP2C9 activity, we used a specific cysteine protease inhibitor, E-64, to inactivate its protease activity.¹⁸⁾ Figure 2 shows the effect of the protease inhibitor on the CYP2C9 inhibition of pineapple juice and bromelain. Treatment with E-64 diminished the inhibitory effect of pineapple juice as well as bromelain did, and it almost recovered to the control level. In addition, we examined the effect of other protease inhibitors on the inhibitory effect of pineapple juice and bromelain. Serine protease

inhibitor, aprotinin, and aspartic protease inhibitor, pepstatin A, did not affect the inhibitory effect of pineapple juice or bromelain.

In addition, we investigated whether other components of pineapple juice would inhibit CYP2C9 activity using ultrafiltration with a centrifugal filter and heat treatment. We confirmed in a preliminary way that the proteolytic activity of pineapple juice was abolished by these treatments. A low molecular fraction of pineapple juice separated by ultrafiltration did not inhibit CYP2C9 activity (Table 2). Heat treatment inactivates proteins

Table 2. Effects of Ultrafiltration and Heat Treatment on CYP2C9 Inhibitory Activity of Pineapple Juice and Bromelain

The inhibitory effects of pineapple juice and bromelain on diclofenac 4'-hydroxylation by human liver microsomes are shown. Data are presented as mean \pm S.D. of triplicate assays. Control activity determined in the absence of samples was 1,002 pmol/min/mg protein.

Treatment	Residual activity (%)		
	Pineapple juice (0.1% v/v)	Pineapple juice (2% v/v)	Bromelain (50 μ g/ml)
Intact sample	40.8 \pm 2.5	0.5 \pm 0.1	0.4 \pm 0.1
Ultrafiltration	94.3 \pm 1.5	95.9 \pm 2.0	92.6 \pm 2.9
Heat (95 °C, 60 min)	96.9 \pm 4.9	95.5 \pm 1.3	94.3 \pm 1.0

and some chemicals. Heated pineapple juice also did not inhibit CYP2C9 activity. These data suggest that bromelain is a principal ingredient of pineapple juice that inhibits CYP2C9 activity, and that other components in pineapple juice are negligible in CYP2C9 inhibition by the juice.

Discussion

In this study, we evaluated the effect of various citrus fruits and tropical fruits on the CYP2C9 activity of human liver microsomes *in vitro*. Among the fruits evaluated, pineapple showed the strongest inhibition of CYP2C9 activity (Table 1). Pineapple is commonly grown in the southern part of Japan as well as southern China, Taiwan, the Philippines, and Hawaii, and it is consumed in all over the world. It sometimes is taken concomitantly with various types of drugs, but few data are available as to whether pineapple influences drug pharmacokinetics. Hence it is important to assess the interaction between pineapple and CYP-mediated drugs. Therefore, we further investigated the inhibitory characteristics of pineapple juice on CYP2C9 activity *in vitro*, and discovered that inhibition occurs in a dose-dependent and mechanism-based manner. It has been reported that cranberry juice, grape juice, and green tea all cause significant inhibition of CYP2C9 *in vitro*, but there is no evidence of inhibitory activity *in vivo*.¹⁹⁾ However, since pineapple juice showed very strong inhibitory activity, we think there is a possibility of food-drug interactions caused by pineapple juice *in vivo*.

Next we attempted to elucidate the mechanism of CYP2C9 inhibition. Since pineapple contains bromelain, known to be a cysteine protease, we examined the effect of bromelain on CYP2C9 activity. Bromelain strongly inhibited CYP2C9 activity. Furthermore, the inhibitory activities of pineapple juice and bromelain were almost entirely blocked by E-64, but were not blocked by other protease inhibitors (Fig. 2). In addition, the inhibitory effect of pineapple juice decreased with ultrafiltration and heat treatment (Table 2). Ultrafiltration can remove the high molecular weight components of pineapple juice, and this result indicates that the low molecular weight components of pineapple juice did not inhibit

CYP2C9 activity. Heat treatment under this condition would inactivate almost all proteins and some chemicals. This result indicates that the heat-tolerant components of pineapple juice are not involved in the CYP2C9 inhibitory activity. Taken together, these data suggest that the inhibitory effect of pineapple juice depends on the proteolytic activity of bromelain, and that other components are negligible in the inhibitory effect. The mechanism of inhibition is perhaps that bromelain degrades CYP2C9 protein and thus decreases CYP2C9 activity. Accordingly, we concluded that bromelain was the principal component in pineapple juice that inhibited CYP2C9 activity. Papaya and kiwi fruit include cysteine protease, papain, and actinidin respectively. In this study, papaya showed weak inhibition of CYP2C9, and kiwi fruit did not inhibit CYP2C9 activity. We concluded that bromelain has an especially strong ability to inhibit CYP2C9 activity, and that the pineapple-drug interaction should be especially noted.

Furthermore, bromelain itself is taken as a nutritional supplement, and recently its medical contribution has been identified.²⁰⁾ It is absorbed from the intestine, and the plasma concentration reaches as much as 5 ng/ml after oral administration (3 g/d), with partial proteolytic activity.²¹⁾ Further, Bock *et al.* investigated the absorption mechanism of proteolytic enzymes by the Caco-2 monolayer method. They found that bromelain absorption can occur by self-enhanced paracellular transport,²²⁾ but the obtained IC₅₀ value was 1.2 μ g/ml, and not sufficient to inhibit hepatic CYP2C9. Generally, early after ingestion, the concentrations of orally ingested chemicals are much greater in the intestine than in the plasma. Hence there is a possibility that bromelain makes enteric CYP2C9 inactive even if the uptake of bromelain into systemic circulation is not sufficient to inhibit hepatic CYP2C9. CYP2C9 is responsible for the metabolic clearance of numerous drugs, such as warfarin and phenytoin, within a narrow range of therapeutic plasma concentrations. Hence patients receiving CYP2C9-metabolized drugs should suffer a disadvantage even from small changes in plasma concentrations caused by food-drug interactions. Therefore, further investigation in humans is necessary in order to develop our findings.

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