Effects of pomegranate juice on human cytochrome P450 2C9 (CYP2C9) and tolbutamide pharmacokinetics in rats

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Abbreviations: HPLC, high-performance liquid chromatography; AUC, area under the concentration-time curve; MRT, mean residence time
Abstract

In this study, we investigated whether pomegranate juice could inhibit cytochrome P450 (CYP) 2C9 activity. The ability of pomegranate juice to inhibit the diclofenac 4'-hydroxylase activity of human CYP2C9 was examined using human liver microsomes. Pomegranate juice was shown to be a potent inhibitor of human CYP2C9. The addition of 25 µl (5% v/v) of pomegranate juice resulted in almost complete inhibition of human CYP2C9 activity. In addition, we investigated the effect of pomegranate juice on the pharmacokinetics of tolbutamide (substrate for CYP2C9) in rats. Relative to the control group, the area under the concentration-time curve (AUC) was approximately 1.2-fold greater when pomegranate juice (3 ml) was orally injected 1 h before the oral administration of the tolbutamide (20 mg/kg). The elimination half-life of tolbutamide was not altered by pomegranate juice administration. These results suggest pomegranate juice ingestion inhibits the intestinal metabolism of tolbutamide without inhibiting the hepatic metabolism in rats. Thus, we discovered that pomegranate juice inhibited human CYP2C9 activity, and furthermore, increased tolbutamide bioavailability in rats.
Introduction

It has been reported that some fruit juices affect the oral bioavailability of drugs. Grapefruit, star fruit, and pomelo juices increase the oral bioavailability of cytochrome P450 (CYP) 3A substrates and the mechanism of these interactions is mainly thought to be due to the inhibition of CYP3A in the small intestine (Bailey et al., 1991, 1998; Culm-Merdek et al., 2006; Hidaka et al., 2006; Grenier et al., 2006).

CYP2C9 is one of three human microsomal CYPs in subfamily 2C that contributes extensively to the hepatic metabolism of therapeutic drugs (Miners and Birkett, 1998). When CYP2C9 substrates such as warfarin and phenytoin which have low therapeutic margins exhibit diminished metabolic rates due to drug-drug or drug-food interactions, these drugs could become toxic even at the normal therapeutic doses (Gilbar and Brodribb, 2001; Murphy and Wilbur, 2003; Suvarna et al., 2003). Thus, the inhibition of CYP2C9 is clinically important for the drug therapy. Furthermore, a recent report revealed that CYP2C9 is also expressed at a significant level in the human small intestine (Obach et al., 2001). Therefore, if some fruits can inhibit CYP2C9 activity in the human small intestine, then food-drug interactions may occur, similar to those observed in the case of CYP3A. However, few reports are available regarding the inhibition of CYP2C9
activity by fruit juice or fruit extracts (Greenblatt et al., 2006). Hence, it is important to evaluate the effect of fruit juice on CYP2C9 activity.

We previously reported that a component(s) of pomegranate inhibited human CYP3A activity in vitro. It almost completely inhibited midazolam 1'-hydroxylase activity and carbamazepine 10,11-epoxidation activity in human liver microsomes (Hidaka et al., 2004, 2005). In addition, pomegranate altered the pharmacokinetics of carbamazepine in rats (Hidaka et al., 2005). However, it is still unknown whether pomegranate juice can inhibit CYP2C9 activity.

In the present study, we investigated whether pomegranate juice could inhibit CYP2C9-mediated drug metabolism by using human liver microsomes. We used diclofenac as a substrate for CYP2C9, since diclofenac is a recommended probe substrate for in vitro metabolic studies (Bjornsson et al., 2003). Furthermore, we investigated the effect of pomegranate juice on tolbutamide pharmacokinetics in rats, since tolbutamide is also recommended as a CYP2C9 probe substrate for in vivo studies (Bjornsson et al., 2003).
Materials and Methods

**Chemicals.** Diclofenac and 4’-hydroxydiclofenac were obtained from Daiichi Pure Chemicals (Tokyo, Japan). Tolbutamide was purchased from Sigma-Aldrich (St. Louis, MO). Pooled human liver microsomes were obtained from Daiichi Pure Chemicals (Tokyo, Japan). All chemicals and solvents were of the highest commercially available grade.

**Fruit Samples.** Pomegranate (California, U.S.A.) was obtained from local commercial sources. The fruit was stored at 4°C until use. Pomegranate juice was obtained by squeezing the edible portion of the pomegranate and filtering it to remove the residue. All samples were used within 1 h after they were squeezed and filtered.

**Analytical Procedures for Human CYP2C9 Activity.** Assay of diclofenac 4’-hydroxylase activity was performed according to the method of Tang et al. (Tang et al., 2000) with minor modifications. Briefly, the incubation mixtures (final volume of 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, 1 unit/ml D-glucose 6-phosphate dehydrogenase, and 0.1 mg/ml of microsomal protein. The concentration of diclofenac was 30 µM. The reaction time was determined to be 60 min since the rate of production of
the diclofenac metabolite remained constant for up to 60 min under these conditions. The reaction mixture was applied to a fresh 10 ml tube and preincubated at 37°C for 5 min. The reaction was initiated with the addition of substrate, and terminated with 2 ml of ice-cold acetonitrile. Midazolam (1 nM) was added as an internal standard. Following centrifugation (3000 rpm, 10 min), the organic phase was evaporated at 40°C. The residue was dissolved in 200 µl of the HPLC mobile phase, and 100 µl of the resultant mixture was injected into an HPLC.

**Inhibitory Effect of Fruit on CYP2C9 Activity.** The inhibitory effect of pomegranate on CYP activity was evaluated according to our previously reported method (Hidaka et al., 2004) with minor modifications. Briefly, appropriate amounts of pomegranate juice (1, 2.5, 5, 7.5, 10, 15, 20, and 25 µl) were applied to fresh tubes. The reaction mixture described above (before the addition of substrate) was added to the tubes and mixed vigorously for 5 s. The maximum amount of fruit juice was 25 µl (5.0% v/v: the volume of the juice to the total incubation volume), and the pH of the reaction mixture was constant at 7.4 under these conditions. After preincubation at 37°C for 5 min, the substrate (diclofenac) was added. The reaction was performed as described above. The inhibitory effect of pomegranate juice on diclofenac 4’-hydroxylation was expressed as a percentage of the residual activity compared with the control in the absence of
pomegranate juice. IC₅₀ values for the inhibition of the CYP activities were determined by a non-linear least square regression (MULTI, Yamaoka et al., 1981) using the following equation:

\[
\text{Residual Activity (\%) = } 100 \left( 1 - \frac{I^{\gamma}}{I^{\gamma} + IC_{50}^{\gamma}} \right)
\]

where I is the initial concentration of inhibitor in the microsomal incubation, \( \gamma \) is an exponent, and IC₅₀ is the inhibitor concentration that inhibits enzyme activity by 50%. A similar experiment with boiled pomegranate juice (pomegranate juice that was incubated for 30 min in a boiling water bath) was conducted.

**Effect of Preincubation of Pomegranate Juice on CYP2C9 Activity.** As an index of irreversible inhibition, pomegranate juice was preincubated at 37°C for 0, 5, 10, 20, 30 min in the reaction mixture, according to the method described above.

**Animals.** Male Wistar rats (Kyudo Co., Ltd., Kumamoto, Japan) weighing 280 to 310 g and maintained at the Department of Bio-resources, Division of Biotechnology, Frontier Science Research Center, University of Miyazaki, were used in this study. The Committee for the Ethics on Animal Experiments in University of Miyazaki approved the experimental protocol. The animal experiments were performed in accordance with The Guidelines for Animal Experiments in University of Miyazaki.

**Effects of Fruits Juices on the Pharmacokinetics of Tolbutamide in Rats.** The
rats had an indwelling cannula implanted in the left carotid artery under light ether anesthesia. After an overnight fast, three milliliters of pomegranate juice or 5% glucose in phosphoric acid (pH 3) was orally administered by gastric intubation. Since the pH of the fruit juices was approximately 3, we administered 5% glucose in phosphoric acid to the control rats. Tolbutamide was dissolved in 0.02 M sodium hydroxide (3 mg/ml) and administered orally at a dose of 20 mg/kg to rats 1 h after the injection of fruit juice or 5% glucose in phosphoric acid. Blood samples were drawn periodically through the cannula introduced into the carotid artery at 0, 15, and 30 min, and 1, 2, 4, 6, 8, and 12 h after tolbutamide administration. Blood samples were immediately centrifuged at 13000 g for 5 min and the serum was separated. The collected serum samples were stored at -20°C until HPLC analysis.

**Assay of Tolbutamide or Glucose Concentration in Serum.** For the determination of tolbutamide in serum, 50 µl of serum and 100 µl acetonitrile were mixed vigorously for 10 s, and then centrifuged at 13000 g for 5 min. Fifty microliters of supernatant and 20 µl of 0.02 M sodium hydroxide were mixed and 20 µl of the mixture was injected onto the HPLC column. The serum concentration of glucose was determined by the glucose oxidase method using a Glucose CII-test Wako (Wako Pure Chemical Industries, Osaka, Japan).
**HPLC conditions.** The HPLC system consisted of an LC-10ADvp pump (Shimadzu, Kyoto, Japan), a Shimadzu L-4200 UV absorbance detector, a Shimadzu SIL-10ADvp auto injector, and a Shimadzu SCL-10A vp system controller. 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 for diclofenac metabolite consisted of acetonitrile and 0.1% of phosphoric acid (50:50, v/v). The mobile phase for tolbutamide consisted of acetonitrile (20:80) in 0.1% of pH 7.4 phosphate buffer (solvent A) and acetonitrile (solvent B). The initial 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 274 nm for the diclofenac metabolite, and 230 nm for tolbutamide).

**Pharmacokinetic Analysis.** The peak plasma concentration ($C_{\text{max}}$) and the time required to reach $C_{\text{max}}$ ($T_{\text{max}}$) of tolbutamide were obtained from the actual data recorded after oral administration. The elimination half-life ($t_{1/2}$) was calculated by dividing the natural logarithm of 2 by $k_{\text{el}}$, which is the apparent elimination rate constant that is obtained from the elimination phase slope. The area under the concentration-time curve (AUC) and mean residence time (MRT) were estimated by moment analysis (Yamaoka et al., 1978).

**Statistical Analysis.** Pharmacokinetic parameters were expressed as the geometric
mean and 90% confidence intervals. All other results were expressed as the arithmetic mean ± S.D. Differences in the sample means between two groups were evaluated by the F-test for equality of variances followed by a Student’s t-test or Welch’s t-test. Differences were considered significant at $p < 0.05$. 
Results

Inhibitory Effect of Pomegranate Juice on CYP2C9 Activity in Vitro. To examine the effects of pomegranate juice on CYP2C9 activity, we investigated diclofenac 4’-hydroxylase activity with or without pomegranate juice by using human liver microsomes. As shown in Fig. 1, the inhibitory effect of pomegranate juice on CYP2C9 activity depended on the amount of pomegranate juice added to the reaction mixture. The addition of 25 µL (5% v/v) of pomegranate juice resulted in almost complete inhibition of human CYP2C9 activity. The mean IC₅₀ value determined for pomegranate juice was 4.84 µL (0.97% v/v). The inhibition potency of pomegranate juice for CYP2C9 was found to be similar to that for CYP3A (Hidaka et al., 2005). The mean IC₅₀ value determined for boiled pomegranate juice was 5.03 µL (1.01% v/v). There was no difference in the inhibition potency for CYP2C9 between pomegranate juice and boiled pomegranate juice. These results suggest that the inhibitory effect of pomegranate would not be due to a proteolytic activity contained in the juice.

To evaluate whether pomegranate juice inhibits CYP2C9 enzyme in a mechanism-based manner, we next investigated the effect of the length of the preincubation period on the inhibition of diclofenac 4’-hydroxylase activity by
pomegranate juice. The inhibition potency of pomegranate juice was altered by elongation of the preincubation period (Fig. 2). The mean residual CYP2C9 activities observed with pomegranate juice (1 µl) at the preincubation times of 0, 5, 10, 20, and 30 min were 95.0%, 89.8%, 84.6%, 82.4% and 79.7%, respectively. The activities observed with pomegranate juice (5 µl) were 65.9%, 55.1%, 49.8%, 42.8%, and 38.3%, respectively. These results suggest that pomegranate juice contains a mechanism-based inhibitor(s).

**Effects of Pomegranate Juice on the Pharmacokinetics of Tolbutamide in Rats.**

Because pomegranate juice strongly inhibited CYP2C9 activity *in vitro*, we wondered whether the coadministration of pomegranate juice with tolbutamide might alter the pharmacokinetics of tolbutamide. Therefore, we investigated the effect of pomegranate juice on tolbutamide pharmacokinetics in rats. Results are shown in Fig. 3 and Table 1. In a preliminary study, we investigated whether 5% glucose in phosphoric acid by itself could alter tolbutamide pharmacokinetics. There was no significant difference in serum tolbutamide concentrations between 5% glucose in phosphoric acid -treated rats and water-treated rats (data not shown). Pomegranate juice significantly increased the AUC of tolbutamide by 22%. The $t_{1/2}$, $T_{\text{max}}$, and MRT of tolbutamide in pomegranate-treated rats were not significantly different from those of their controls. These results suggest that
pomegranate juice increases tolbutamide bioavailability in rats.

**Effects of Pomegranate Juice on Tolbutamide Efficacy in Rats.** In a preliminary study, three milliliters of pomegranate juice or 5% glucose in phosphoric acid (pH 3) was orally administered by gastric intubation to rats. There was no significant difference in serum glucose level-time profiles between pomegranate-treated rats and their controls (data not shown).

Time-dependent changes in the serum glucose concentrations after oral administration of tolbutamide in rats are shown in Fig. 4. The reduction of serum glucose levels in pomegranate-treated rats tended to be low compared with that of the control rats, but there was no statistical significance between the two groups.
Discussion

Recently, there have been many reports regarding food-drug interactions by the inhibition of drug metabolism mediated by the CYP3A subfamily. However, few reports are available on the inhibition of CYP2C9 activity by fruit juices or the extracts. In this study, we discovered that pomegranate juice inhibited human CYP2C9 activity \textit{in vitro}. This result suggests that pomegranate juice might affect the pharmacokinetics of substrates for CYP2C9 in humans.

Pomegranate is a rich source of several chemicals such as pectin, tannins, flavonoids, and anthocyanins (Gil et al., 2000; Aviram et al., 2002; Noda et al., 2002). If pomegranate also includes an unidentified protease, the effect observed \textit{in vitro} with human microsomes may be due to the proteolytic activity of the juice. Therefore, we used boiled pomegranate juice to determine if the inhibition of CYP2C9 activity \textit{in vitro} was due to the proteolytic activity within the juice or by specific inhibition of the CYP2C9 by a component of the juice. In this study, there was no difference in the mean IC$_{50}$ value between pomegranate juice (4.84 µl) and boiled pomegranate juice (5.03 µl). This result suggests that the inhibitory effect of pomegranate is not due to proteolytic activity of the juice because the protein components of the juice would have been inactivated by boiling.
Tolbutamide, an oral hypoglycemic drug, is mainly metabolized by CYP2C9 (Miners and Birkett, 1998). It has been reported that the coadministration of sulfaphenazole (a CYP2C9 inhibitor, Venkatakrishnan et al., 2001) increased the serum concentration of tolbutamide and induced severe hypoglycemia (Christensen et al., 1963). If pomegranate juice could inhibit the CYP2C9 that is expressed in the small intestine and/or the liver, it could increase the serum concentration of tolbutamide and potentially induce hypoglycemia. Therefore, we further investigated whether pomegranate juice could affect the pharmacokinetics of tolbutamide in rats. The results showed that the coadministration of pomegranate juice significantly increased the AUC of tolbutamide by 22%. Furthermore, the t1/2, which reflects the elimination of tolbutamide, was not altered by pomegranate juice. These results suggest that the increased serum concentrations of tolbutamide in pomegranate juice-treated rats would be caused by an increase in tolbutamide bioavailability.

P-glycoprotein plays an important role in the pharmacokinetics of substrate drugs, i.e., in their absorption, distribution, and elimination, with resulting low oral bioavailability of these drugs. It is well known that there is an overlap between the inhibitors for CYP3A and P-glycoprotein (Kim et al., 1999). Because pomegranate juice is an inhibitor of CYP3A (Hidaka et al., 2005), the juice might be also an inhibitor of
P-glycoprotein in the intestine and enhance the absorption of drugs. However, Nishimura et al. reported that basolateral-to-apical transport of tolbutamide across the Caco-2 cell monolayers was not operated by the carrier-mediated transport system including P-gp efflux (Nishimura et al., 2004). Therefore, the inhibition of P-glycoprotein by pomegranate juice likely would not be associated with the increased bioavailability of tolbutamide.

In this study, serum glucose levels after tolbutamide administration in pomegranate-treated rats were lower than the values in their controls, but these differences were not statistically significant. This result suggests that the 22% increase in AUC of tolbutamide by pomegranate juice ingestion would not affect the efficacy of tolbutamide very much. Therefore, even if pomegranate juice can increase the tolbutamide concentration in humans, this interaction may not be of clinical significance.

On the other hand, in vitro data on metabolic inhibition does not necessarily translate into drug interactions in vivo. Although grape juice, tea, cranberry juice, and a number of natural compounds present in Ginkgo biloba impaired CYP2C9 activity in vitro, these beverages and compounds did not altered CYP2C9-mediated clearance of flurbiprofen in humans (Greenblatt et al., 2006a, 2006b; von Moltke et al., 2004). Furthermore, Eagling et al. (1998) reported that caution must be exercised when
extrapolating the effects of inhibitors from rats to humans. For example, sulfaphenazole selectively inhibited tolbutamide hydroxylation in human liver microsomes but failed to inhibit this reaction in rat liver microsomes (Eagling et al., 1998). In addition, the effects of fruit juice on the pharmacokinetics of drugs in rats are not necessarily consistent with those in humans (Spahn-Langguth and Langguth, 2001; Schwarz et al., 2005). Therefore, further investigations in humans are necessary to elaborate our findings.

In conclusion, we showed that pomegranate juice inhibited human CYP2C9 activity. Furthermore, pomegranate juice increased tolbutamide bioavailability in rats.
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Footnotes

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Legends for figures

FIG 1. Inhibition of human CYP2C9 by pomegranate juice. The amount of pomegranate juice added to the incubation mixture was 1, 2.5, 5, 7.5, 10, 15, 20, and 25 µl (0.2, 0.5, 1, 1.5, 2, 3, 4, and 5%, v/v), respectively. The control activity of diclofenac 4'-hydroxylation by human liver microsomes determined in the absence of pomegranate juice was 1.03 pmol/min/mg protein. Each point and bar represents the mean and S.D. of three independent assays.

FIG 2. The effect of preincubation times on the inhibition of diclofenac 4'-hydroxylase activity by pomegranate juice. The amount of pomegranate juice added to the incubation mixture was 1 or 5 µl (0.2 or 1%, v/v). The concentration of diclofenac was 30 µM. Pomegranate juice was added to the reaction mixture and incubated for the indicated period 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 pomegranate juice was 0.983 pmol/min/mg protein. ●, 1 µl; ○, 5 µl. Each point and bar represents the mean and S.D. of three independent assays.
FIG 3. Serum concentration-time profiles of tolbutamide after oral administration in rats.

Tolbutamide was administered orally at a dose of 20 mg/kg to rats 1 h after the injection of fruit juice or 5% glucose in phosphoric acid (pH 3) (3 ml p.o., each). ●, control; ○, pomegranate juice. Each point and bar represents the mean and S.D. of six (control) or five (pomegranate juice) rats.

FIG 4. Serum glucose level-time profiles after oral administration in rats. Tolbutamide was administered orally at a dose of 20 mg/kg to rats 1 h after the injection of fruit juice or 5% glucose in phosphoric acid (pH 3) (3 ml p.o., each). ●, control; ○, pomegranate juice. Each point and bar represents the mean and S.D. of six (control) or five (pomegranate juice) rats.
TABLE 1

Pharmacokinetic parameters of tolbutamide after an oral administration in rats

Tolbutamide was administered orally at a dose of 20 mg/kg to rats 1 h after the injection of pomegranate juice or 5% glucose in phosphoric acid (pH 3) (3 ml p.o., each).

Pharmacokinetic parameters were expressed as the geometric mean and 90% confidence intervals (Body weight was expressed as the arithmetic mean ± S.D.).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Pomegranate-J</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of animals</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>300 ± 18</td>
<td>292 ± 8</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (µg/ml)</td>
<td>91.1 (74.6-111.2)</td>
<td>106.6 (87.8-116.8)*</td>
</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt; (h)</td>
<td>0.89 (0.46-1.74)</td>
<td>0.87 (0.37-1.30)</td>
</tr>
<tr>
<td>AUC (µg/ml·h)</td>
<td>641.7 (600.3-685.9)</td>
<td>781.7 (708.6-818.6)*</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>7.01 (6.10-8.06)</td>
<td>7.39 (6.80-7.69)</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2&lt;/sub&gt; (h)</td>
<td>4.93 (4.26-5.71)</td>
<td>5.17 (4.79-5.36)</td>
</tr>
</tbody>
</table>

*, p < 0.05 versus control values.
Fig. 1

Residual Activity (%) vs. Dose (µl)

- Dose range: 0 to 25 µl
- Residual Activity range: 0% to 100%
- The graph shows a decrease in residual activity with increasing dose.
Fig. 2

![Graph showing residual activity (%) against pre-incubation time (min)](image-url)

- Residual activity (%)
- Pre-incubation time (min)
Fig. 3

Tolbutamide Concentration (µg/ml) vs. Time (h)

- Solid line with filled circles represents Group A
- Open circles represent Group B

Error bars indicate standard deviation.
Fig. 4

Reduction of Serum Glucose Level (mg/dl)

Time (h)

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