The antigenotoxic effects of grapefruit juice on the damage induced by benzo(a)pyrene and evaluation of its interaction with hepatic and intestinal Cytochrome P450 (Cyp)1a1

I. Alvarez-Gonzalez a, R. Mojica a, E. Madrigal-Bujaida a, R. Camacho-Carranza b, D. Escobar-García b, J.J. Espinosa-Aguirre a,b,*

a Laboratorio de Genética, Escuela Nacional de Ciencias Biológicas, I.P.N., Unidad Profesional A López Mateos, Av. Wilfredo Massieu, Lindavista, 0740 México, DF, Mexico
b Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Apartado Postal 70228, Cd. Universitaria, 04510 México, DF, Mexico

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Abstract
We determined the capacity of grapefruit juice (GJ) to inhibit the rate of micronucleated polychromatic erythrocytes (MNPE) in mice treated with benzo(a)pyrene (BaP), an environmental contaminant that is biotransformed by Cyp1a1 and is a strong genotoxic agent. For this study, we administered 4.1, 20.8, and 41.6 l l/g body weight (b.w.) of GJ to BaP-treated mice (340 mg/kg). We found a significant decrease in the frequency of MNPE at 48 and 72 h compared to BaP-only treated animals. In turn, no prevention of the cytotoxic damage induced by BaP was found. We next explored whether GJ’s antigenotoxic mechanism of action was related to an inhibitory effect on the activity of the Cyp1a1 enzyme. A reduction in microsomal hepatic and intestinal ethoxyresorufin-O-deethylase (EROD) activity of 20% and 44%, respectively, was found in mice treated with BaP and GJ compared to BaP-only treated animals. Furthermore, when EROD inhibition was tested in vitro, we found a concentration-dependent EROD inhibition by GJ, which reached 85% of the maximum level. Together, these results suggest that the protective effect of GJ against the genotoxicity of BaP may be related to the inhibition of Cyp1a1 enzyme activity.

1. Introduction
Grapefruit (Citrus paradisi Macfad.) come from a perennial tree of about 6 m in height from the Rutaceae family, which grows mainly in subtropical climates. This tree produces a globular fruit of about 15 cm in diameter, which is consumed as juice, in combination with other fruits and vegetables or in preserves (Morton, 1987). Grapefruit contains carbohydrates, lipids, proteins, vitamins, minerals, and other compounds, such as flavonoids, coumarins, and hydroxicynamic acids. Its consumption has been suggested as beneficial for cardiac diseases, obesity, diabetes, and cancer chemoprevention (Adeneye, 2008; Diaz-Juarez et al., 2009). Investigations using various experimental models have shown the capability of various grapefruit juice (GJ) constituents to inhibit DNA damage induced by xenobiotics. This capability is also found in compounds such as vitamin C, vitamin E, naringin, zinc, and selenium (Alvarez-Gonzalez et al., 2001; Bronzetti et al., 2001; Record et al., 1996). In recent years, the antigenotoxic potential of GJ has also been investigated. A report showed GJ’s protective effect against aflatoxin B1-induced liver DNA damage in rats (Miyata et al., 2004), and another study demonstrated suppression of DNA damage induced by 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) (Miyata et al., 2002) in the colon. Furthermore, a significant reduction in the levels of micronuclei induced by daunorubicin in mice was reported (Alvarez-Gonzalez et al., 2004).

Enzymes that belong to the Cytochrome P450 (CYP) superfamily are known for their diverse functions, including their participation in the biosynthesis and regulation of different molecules and in pathways involved in the metabolic disposition of chemicals (Alfin, 2003). With respect to this last function, modulating the action of compounds may lead to at least two scenarios: drug–drug interactions that may cause adverse reactions and the promotion or inhibition of the metabolic activation of carcinogens (Lin, 2006; Nassar et al., 2007). GJ is known to inhibit intestinal CYP3A4 when ingested together with a number of drugs, to modify their pharmacokinetics and to provoke an increase in the oral bioavailability of these drugs compound (Bailey et al., 1991; Ducharme et al., 1995; Kupferschmidt et al., 1995; Benton et al., 1996). However, this inhibition may also have beneficial consequences, as shown by the antimutagenic ability of GJ against aflatoxin B1 in vivo (Miyata et al., 2004). This effect could be attributed to the inhibitory effect of GJ on CYP3A activity.
Overall, the above mentioned data indicate a complex action of GJ over different xenobiots, which is probably related to the effects of specific constituents in the juice. Moreover, it is also known that GJ alters the metabolism of coumarin and caffeine, chemicals that are biotransformed by CYP2A6 and CYP1A2, respectively (Runkel et al., 1997; Maish et al., 1996). This suggests that drugs affected by GJ may not be limited to CYP3A substrates. In this context, it is pertinent to mention that Cyp1a1 is an enzyme involved in the oxidation of polycyclic aromatic hydrocarbons, including benzo(a)pyrene (BaP). BaP is an inducer of Cyp1a1 and is a well-known environmental contaminant with high genotoxic potential (Miller and Ramos, 2001).

To gain a better understanding of the antigenotoxic capacity of GJ, GJ was administered to mice treated with BaP. To explore the mechanism of action involved, we evaluated whether GJ inhibited Cyp1a1 activity in the mouse, the homologous enzyme of CYP1A1 in the rat, and if this was related to a decrease in micronuclei function.

2. Materials and methods

2.1. Chemicals
Acidine orange (AO), BaP, 7-ethoxyresorufin, NADPH, phosphate buffered saline (PBS), tween 20, tris, and corn oil were obtained from Sigma Chemicals (St. Louis, MO, USA). GJ was obtained from fresh Citrus paradisi Macf. (Ruby red) and cultivated in a pesticide-free field in Albeciras, Veracruz, 400 km southwest of Mexico City. A single oral administration of freshly prepared GJ was given to mice before each assay.

2.2. Animals
Male mice, weighing 25 g, were obtained from the National Institute of Hygiene (NIH, Mexico City) and used in this study. The animals were maintained in polypropylene cages at 23 °C in a 12 h dark–light cycle, and they had access to food (Rodent Lab Chow 5001) and water ad libitum. The Committee of Ethics and Biosecurity in the National School of Biological Sciences approved this experimental protocol.

2.3. Determination of micronuclei
Thirty-six animals were obtained and organized into six groups receiving the following oral treatments: group 1 was given 340 mg/kg body weight (b.w.) of BaP; group 2 was given 0.01 ml/g of corn oil; and group 3 was administered GJ (41.6 μl/g b.w.). Groups 4, 5 and 6 were given GJ 4.1, 20.8, and 41.6 μl/g b.w. respectively, and 1 h later received 340 mg/kg b.w. of BaP. The doses of GJ selected in this study showed no genotoxicity or cytotoxicity in earlier studies using the same strain of mice and similar experimental conditions (Alvarez-Gonzalez et al., 2004). The dose of BaP was based on previous reports (Awogi and Sato, 1989) and data obtained in a preliminary experiment. Micronuclei determination was made before the oral administration of the compounds and at 24, 48, and 72 h post-administration. For this purpose, two drops of blood from the tail of each mouse were smeared onto cleaned slides, fixed in methanol for 3 min, and stained for 1 min in AO (made in PBS, pH 6.8). The slides were then rinsed twice in PBS, air dried, and observed under a fluorescent microscope (Axioskop, Carl Zeiss). To analyze the antigenotoxic potential of GJ, we quantified the frequency of MNPE in 1000 polychromatic erythrocytes (PE) per mouse.

2.4. Effect of GJ on the activity of Cyp1a1 in vivo

2.4.1. Experimental groups and microsomal preparation
Four groups of six mice were organized as follows: group 1 received corn oil (0.01 ml/g b.w., i.p.); group 2 was treated with 100 mg/kg b.w. of BaP (i.p. (Cyp1a1 inducer)); a third group received a p.o. administration of 41.6 μl/g b.w. of GJ; and a fourth group was administered BaP (100 mg/kg b.w., i.p.). The 4th group was treated with 41.6 μl/g b.w. Twenty-four hours after the last treatment, all mice were cervicaly dislocated, the liver and small intestine dissected, and microsomes obtained according to the procedure described by Maron and Ames (1983). Briefly, organs were placed in a 150 mM solution of KCl at 4 °C. After centrifugation, the supernatant (S9 fraction) was constituted by 7-ethoxyresorufin (25 μM), 1 mg/ml of microsomal protein, and buffer (50 mM tris and 25 mM MgCl2, pH 7.4). The mixture was pre-incubated at 37 °C for 3 min and the reaction began with the addition of 0.2 ml of 50 mM NADPH. The spectrfluorometric readings were recorded every 15 s for 3 min using excitation and emission filters of 530 and 585 nm, respectively. A resorufin solution was used for the calibration curve (5–250 pmol). All measurements were made in triplicate.

2.4.2. Activity of Cyp1a1
The Cyp1a1-associated ethoxyresorufin-O-dealkylation (EROD) activity was measured according to the method described by Burke et al. (1994) with some modifications. The production of resorufin was spectrfluorometrically assessed with the microsomes previously obtained. For measuring EROD activity we used 2 ml of the reaction mixture (at 4 °C) constituted by 7-ethoxyresorufin (25 μM), 1 mg/ml of microsomal protein, and buffer (50 mM tris and 25 mM MgCl2, pH 7.4). The mixture was pre-incubated at 37 °C for 3 min and the reaction began with the addition of 0.2 ml of 50 mM NADPH. The spectrfluorometric readings were recorded every 15 s for 3 min using excitation and emission filters of 530 and 585 nm, respectively. A resorufin solution was used for the calibration curve (5–250 pmol).

3. Results

3.1. Effect of GJ on the frequency of micronuclei
The ability of GJ to inhibit the genotoxicity induced by BaP is shown in Fig. 1. A significant increase in the frequency of micronuclei was observed in response to treatment with BaP. This was determined to be 6.5 and 2.8 times higher than the frequency of MNPE observed in the control group at 48 and 72 h, respectively. On the other hand, we found that administration of GJ was not genotoxic to mice and showed a frequency of micronuclei in the range observed for the control group. Also, a statistically significant decrease in the amount of BaP-induced MNPE was observed with the three doses of GJ given. Protection against the genotoxicity of BaP was observed with a GJ dose of 4.1 mg/kg b.w., reducing by 29% and 57% the amounts of micronuclei after 48 h and 72 h of BaP treatment respectively.

Fig. 2 shows the bone marrow mitotic activity estimated with the PE/NE index. The GJ doses tested in the present study did not modify this index compared to control animals. However, a cytotoxic effect of BaP was evident at 48 h and 72 h after its administration to mice, showing an index decrease of 20% and 27%, respectively. The cytotoxic damage produced by BaP was not ameliorated by any of the three GJ doses tested.

3.2. Effect of GJ on the activity of Cyp1a1 in vivo

Table 1 presents the results of the GJ inhibitory effect on microsomal EROD activity. From the hepatic and intestinal microsomes collected in mice treated with BaP alone, we found EROD activity increases of eight- and twofold, respectively, compared to the level in control microsomes. In contrast, administration of GJ to BaP-treated mice inhibited EROD activity by 20% and 44% in liver and intestinal microsomes, respectively.
3.3. Effect of GJ on the activity of Cyp1a1 in vitro

The results obtained in this experiment are shown in Fig. 3. We determined a sevenfold enhancement of EROD activity in the microsomes of BaP-treated mice in comparison to the level in untreated microsomes. This result confirms the significant inducing capacity of BaP on Cyp1a1. However, a clear concentration–response inhibitory curve was obtained when GJ was added (from 0.5% to 5%) to the reaction mixture of ethoxyresorufin and hepatic microsomes. Under the experimental conditions used, we found that the highest concentration of GJ returned EROD activity to values corresponding to the untreated mice microsomes. The possibility of an unspecific quench of resorufin fluorescence by GJ was addressed, and the results indicated that GJ does not interfere with the fluorescence of resorufin at concentrations of up to 5% (Fig. 4).

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**Table 1**

<table>
<thead>
<tr>
<th>EROD activitya</th>
<th>Microsomes</th>
<th>Control (100 mg/kg)</th>
<th>BaP (100 mg/kg)</th>
<th>GJ (41.6 mL/kg)</th>
<th>BP (100 mg/kg) + GJ (41.6 mL/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatic</td>
<td>37.1 ± 4.6</td>
<td>303.8 ± 14.84</td>
<td>339.7 ± 5.36</td>
<td>241.94 ± 22.2</td>
<td></td>
</tr>
<tr>
<td>Intestinal</td>
<td>3.7 ± 0.54</td>
<td>7.3 ± 1.167</td>
<td>3.3 ± 0.03</td>
<td>4.06 ± 0.44</td>
<td></td>
</tr>
</tbody>
</table>

a pmoles resorufin/mg protein/min.

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Fig. 1. Inhibitory effect of grapefruit juice (GJ) on the frequency of micronucleated polychromatic erythrocytes (MNPEs) induced by benzo[a]pyrene (BaP) in mice. Three doses of GJ were administered to each of three groups of animals (6 mice/group) to test its antigenotoxicity. BaP (340 mg/kg, b.w., p.o.) was administered 1 h after GJ administration, and blood samples were collected 24 h, 48 h and 72 h later. Each bar represents the mean rate (± standard deviation) of MNPEs in 1000 polychromatic erythrocytes (PEs) per mouse. *Significantly different from the control group (p < 0.05). †Significantly different from the BaP group (p < 0.05). ‡Significantly different from the BaP group (p < 0.001). RM ANOVA and Student–Newman–Keuls tests (α = 0.05). GJ exposure reduces the genotoxic activity of BaP in mice erythrocytes.

Fig. 2. Failure of grapefruit juice (GJ) to protect from the cytotoxicity induced by benzo[a]pyrene (BaP) in mouse erythrocytes. Three doses of GJ were administered to each of three groups of animals (6 mice/group). BaP (340 mg/kg, b.w., p.o.) was administered 1 h after GJ administration, and blood samples were collected 24 h, 48 h and 72 h later. Each bar represents the proportion of PEs with respect to the number of normochromatic erythrocytes (NEs) (± standard deviation) in 1000 erythrocytes per mouse (PE/NE index). *Significantly different from the positive group (p < 0.001). †Significantly different from the control group (p < 0.05). ‡Significantly different from the control group (p < 0.001). RM ANOVA and Student–Newman–Keuls tests (α = 0.05). GJ does not protect from the cytotoxic effect of BaP in mouse erythrocytes.

Fig. 3. Inhibitory effect of grapefruit juice on ethoxyresorufin activity of hepatic microsomes obtained from benzo[a]pyrene (BaP)–treated mice. A group of four mice were treated intraperitoneally for 3 days with benzo[a]pyrene (BaP) at a dose of 50 mg/kg b.w. Twenty-four hours after the last treatment; hepatic microsomes were obtained and used for the in vitro inhibition experiments as described in the Section 2. Grapefruit juice (GJ) was added at final concentrations of 0.5%, 2.5%, 3.25%, and 5% into the reaction mixture. *Significantly different from the control group (p < 0.001). †Significantly different from the induced group (p < 0.01). ‡Significantly different from the induced group (p < 0.001). ANOVA and Tukey–Kramer tests (α = 0.05). GJ inhibits ethoxyresorufin activity in mice hepatic microsomes.

Fig. 4. Lack of interference by grapefruit juice on resorufin fluorescence. A calibration curve was prepared with different concentrations of resorufin (5–500 Pmo/ml) in pH 7.4 buffer (50 mM tris and 25 mM MgCl₂) with or without grapefruit juice (2.5%, 3.5%, and 5.0%). Spectrofluorometric readings were recorded using excitation and emission filters of 530 and 585 nm, respectively. There are no significant differences between the calibration curve and those obtained with different concentrations of grapefruit juice.
4. Discussion

The antigenotoxic and/or cancer chemopreventive effects of various plant juices, such as pomegranate, orange, lime, grape, apple, and tomato, have been reported (Faria et al., 2007; Franke et al., 2005; Patil et al., 2009; Shi and Jiang, 2002; Iriti and Faoro, 2009; Ferreira et al., 2007). These effects of vegetable and fruit juices have raised interest in determining specific chemical(s) responsible for such responses. Also of interest is the analysis of a possible synergism among the constituents of juices that could induce a stronger protection than the one observed by the specific compounds tested alone. Another point of interest is related to the identification of the mechanism of action involved, which has been suggested to be more than one considering the complex composition of juices, the variety of mutagens and the type of genetic lesions produced. In the case of GJ, previous experiments performed in prokaryotic and eukaryotic models have revealed its potential to reduce the genotoxic damage induced by different types of mutagens, such as 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), daunorubicin, and aflatoxin B1 (AFB1) (Miyata et al., 2002, 2004). These experiments have suggested at least two mechanisms of action to explain such effects: one is the well-identified antioxidant potential of GJ and, in the case of the mutagen AFB1, an inhibition of CYP3A4 activity, which is the isosform responsible for the metabolic activation of the mycotoxin.

In the present study, we determined a clear capacity of BaP to induce micronuclei in mice (Fig. 1), a result which agrees with previous reports made in the same model system (Vanparys et al., 1992). Earlier studies demonstrated no hematological or related health disturbances in the animal by the daily extraction of about 1–2 drops of blood (CSCMT, 1992). However, the proportion of young to mature erythrocytes has been a useful indicator for erythropoietic toxicity. In this study we found no change in this index in control mice, confirming that blood extraction was harmless, contrary to the significant decrease of polychromatic erythrocytes induced by BaP. This compound is a widely-distributed environmental contaminant that may produce ecological disturbances as well as significant human health damage (Miller and Ramos, 2001). The chemical is biotransformed by Cyp1a1 to oxides and one of these (7,8 BaP-oxide) is converted into 7,8 dihydrodiol-BaP-epoxide. This epoxide can also be metabolized by the same Cyp1a1 to 7,8 dihydrodiol-9,10-BaP-epoxide, which is recognized as the metabolite that induces mutagenesis and carcinogenesis. Also, 7,8 BaP-oxide may give rise to quinone and semiquinone structures that are involved in the production of free radicals (Parkinson, 1996). Our study showed the capacity of GJ to reduce the frequency of micronuclei induced by BaP (Fig. 1), such effect was observed with the three tested doses of GJ (4.1, 20.8, and 10 glasses of GJ by a human weighing 60 kg. In a related experiment, we also observed that oral administration of the higher dose of GJ inhibited the increase of hepatic and intestinal Cyp1a1 activity resulting from BaP exposure (Table 1). Furthermore, additional in vitro experiments proved that GJ is capable to inhibit hepatic EROD activity (Fig. 3). Although these data were obtained using independent experiments designed to answer specific questions regarding: (i) antigenotoxic potential of GJ; (ii) capacity of GJ to prevent Cyp1a1 induction, and (iii) in vitro inhibition of Cyp1a1-associated EROD activity by GJ, we may hypothesize that the above-mentioned results are related. Thus, the reduction in micronuclei observed in the BaP-GJ treated groups could be related to the Cyp1a1 inhibition properties of GJ.

Although this relationship could have been assessed more precisely if we had used the same route of administration of BaP in the two assays, we privileged protocols which have proved to efficiently produce the expected results, that is, induction of micronuclei and CYP activity.

However, the relationship between GJ inhibition of Cyp1a1 and genotoxic damage is supported by results reported elsewhere (Tadashi et al., 2008; Girennavar et al., 2006) indicating that specific molecules contained in GJ such as bergamottin, naringin, paradisin, and dihydroxybergamottin, inhibit CYP activity, and those indicating that CYP3A inhibition by GJ is related to its protective action against AFB1 genotoxicity (Miyata et al., 2004). Nevertheless, we cannot rule out additional explanations concerning GJ action including the stimulation of DNA repair, in the light of the higher protection exerted by GJ at 72 h after BaP exposure observed (Fig. 1). It is pertinent to indicate that the absence of a linear dose–response in the micronuclei assay suggest the need of further research to determine the factors involved. In the present experiment, it is possible that Cyp1a1 could be saturated by GJ (or some of its components) at low doses avoiding the formation of the active genotoxic metabolite, 7,8 BaP-oxide; this would explain a similar amount of MN even at higher doses of GJ. In this study we tested a single dose of GJ to inhibit EROD activity in vivo; therefore, it is possible that by increasing the number of GJ doses, more precise information can be obtained regarding the relationship between the antigenotoxic and enzyme activity results.

In vitro assays are thoroughly applied to study the involved mechanisms of action; in our case, such study was made by using multiple concentrations of GJ and results obtained strongly support our hypothesis, however, an in vivo confirmation seems pertinent in the light of the fact that whole animal assays incorporate dynamic physiological processes such as uptake and systemic distribution by the circulatory system, phases I and II metabolic process, and elimination/excretory activities (Recio et al., 2010).

Although this is the first report showing an inhibitory effect of GJ on EROD activity, this type of inhibition has been shown to be a reliable predictor of antimutagenesis against agents metabolized by Cyp1a1. For example, by quantifying EROD activity, Feng et al. (2003) explained the antimutagenic effect of alkyl gallates on 2-aminoanthracene using the Salmonella typhimurium test. They also explained the suppressive effect of the damage induced by 3-methylcholanthrene in human hepatoma (Hep G2) cells with this quantification. Other antigenotoxic effects related to the inhibition of the Cyp1a1 subfamily have also been reported. These include the following: the effect of a Phyllanthus orbicularis aqueous extract on genotoxic damage induced by 4-aminobiphenyl, and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (Ferrer et al., 2004), the effect of amino acids derived from allyl isothiocyanate on the mutagenicity of 2-amino-3-methylimidazo[4,5-f]quinoline (Takahashi et al., 2005), and interestingly, in the protective activity of an extract of the mushroom Pleurotus cornucopiae on damage elicited by BaP, showed by using the Ames and comet assays (El Bohi et al., 2005).

The final biological implications resulting from the modulation of CYP depend on the pharmacological effects of the involved molecules metabolized by it. Although our results suggest that GJ could protect from the genotoxic activity of promutagens belonging to the family of aromatic hydrocarbon molecules, we cannot disregard a possible retardation in the elimination of other substances metabolized by the same CYP leading to unspecified toxic effects.

In summary, our study established the capacity of GJ to reduce the amount of micronuclei induced by BaP in mice and provided data suggesting that this result could be related to an inhibitory effect of GJ on the activity of the Cyp1a1 enzyme. Our report adds new information on the antigenotoxic potential of GJ as well as on the mechanism of action involved.
Conflict of interest

The authors declare that there are no conflicts of interest.

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