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Effect of Grapefruit Juice, Naringin, Naringenin, and Bergamottin on the Intestinal Carrier-Mediated Transport of Talinolol in Rats

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The effect of two varieties of grapefruit juice (white and ruby red) and its selected components (naringin, naringenin, and bergamottin) was investigated on the activity of the P-glycoprotein (P-gp) in male Sprague−Dawley rats. Talinolol, a nonmetabolized P-gp substrate, was used as a marker compound. The white grapefruit juice (GFJ) had a minor effect on talinolol pharmacokinetics, but the ruby red GFJ reduced the C_{max} and the $AUC_{(0-\infty)}$ by 60% and 50% of the control, respectively. However, among the GFJ constituents tested, bergamottin (0.22 mg/kg) was the most potent component augmenting the C_{max} and the $AUC_{(0-\infty)}$ of talinolol by 2.4- and 1.8-fold, respectively, if compared to the control group. The flavonoids naringenin (0.7 mg/kg) and naringin (2.4 and 9.4 mg/kg) had a similar effect increasing the talinolol C_{max} and $AUC_{(0-\infty)}$ by 1.5- to 1.8-fold, respectively. In conclusion, the effect of GFJ on P-gp activity seems to depend on the variety, the concentration of compounds in the juice, and the composition of different ingredients.

KEYWORDS: Grapefruit; juice; talinolol; flavonoids; furanocoumarins; rats; intestinal transport; P-glycoprotein

INTRODUCTION

Grapefruit juice (GFJ) has been demonstrated to inhibit the drug-metabolizing enzyme cytochrome P450 (CYP) 3A4 specifically located in the small intestine (*1*). As a result, the oral bioavailability of a variety of medications is significantly augmented, including some calcium channel blockers (felodipine, nicardipine, nisoldipine) (*2, 3*), 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (lovastatin, simvastatin) (*4, 5*), and benzodiazepines (midazolam, diazepam) (*6, 7*). Additional efforts revealed the involvement of GFJ on the permeability of P-glycoprotein (P-gp) (*8, 9*) and the organic anion transporting polypeptide (OATP) activities (*10, 11*). It is becoming increasingly evident that intestinal transporters play an important role in the oral absorption of compounds, with both influx and efflux transporters (*12, 13*). Oral absorption of compounds can be limited by efflux transporters located in the intestine such as P-gp while influx transporters such as OATP can aid intestinal drug absorption (*14–16*). Additionally, there appears to be an overlap in the substrate specificity between the efflux transporter P-gp and the influx transporter OATP (*14*), which could lead to opposing influences on the net absorption of a shared substrate.

However, the components present in GFJ with potential to interact with drugs and their tendency to preferentially affect the intestinal uptake or efflux remain uncertain. It may depend on the net balance of the influx and efflux transporters (*17*), on the concentrations, and on the kind of drug-interacting compounds present in GFJ (*18*).

Takanaga and co-workers (*19*) suggested that GFJ and the components distinct from flavonoids (naringin and naringenin) significantly inhibited the P-gp efflux of vinblastine in Caco-2 cells. Conversely, from five GFJ constituents (quercetin, naringin, naringenin, bergamottin, and 6′,7′-dihydroxybergamottin) tested, only naringin and 6′,7′-dihydroxybergamottin had a considerable effect reducing the net secretion of the P-gp substrate, saquinavir, across the Caco-2 cells from 25 to 7.6 and 7.1, respectively (*20*). On the other hand, bergamottin (BG) has been shown to inhibit P-gp ($IC_{50} = 40$ μM), whereas the flavonoids quercetin, naringin, naringenin, and kaempferol had a slight or no significant effect (*21*).

Recent investigations reported that the OATP-mediated uptake of fexofenadine in HeLa cells was greatly inhibited by naringin ($IC_{50} = 3.6$ μM) and hesperidin ($IC_{50} = 2.7$ μM) (*17*), whereas in Caco-2 cells both flavonoids had a minor effect on P-gp-mediated transport of talinolol (*22, 23*). Satoh et al. (*24*), demonstrated that naringin, naringenin, and bergamottin at 10 μM reduced the uptake of estrone-3-sulfate into HEK/293 cells by 39, 28, and 60%, respectively. The same group has shown that the OATP-mediated transport of glibenclamide was significantly inhibited by 6′,7′-dihydroxybergamottin and moder-

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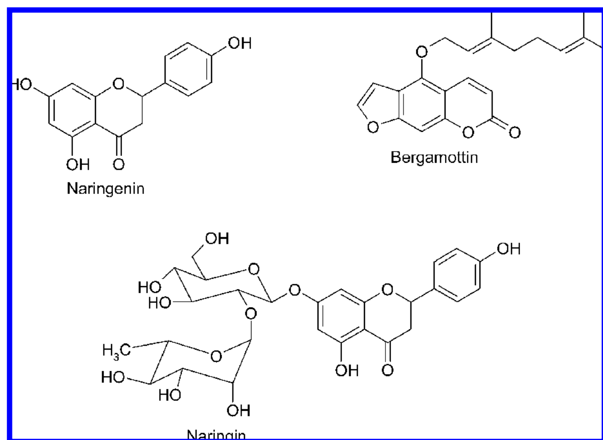

Figure 1. Structures of naringin, naringenin, and bergamottin.

ately inhibited by naringin and naringenin. In contrast, no effect was observed for quercetin and bergamottin.

It was the aim of this study to investigate the effect of two varieties of GFJ (white and ruby red) and its components naringin (NAR), naringenin (NAG), and bergamottin (BG) (structures are shown in **Figure 1**) on the activity of P-glycoprotein in male Sprague−Dawley rats. These compounds together with 6′,7′-dihydroxybergamottin (DHB), 6′,7′-epoxy-bergamottin, and their dimers have been suggested to contribute to the GFJ−drug interaction (*25, 26*). In the present experiment, talinolol, a P-gp substrate not metabolized by CYP3A4 (*27*), was selected as a marker compound. Most of the in vivo experiments already reported in the literature evaluated the interaction between GFJ and its components with the CYP3A4 enzymatic system or the effects of only one juice on the activity of P-gp (*28–31*). To our knowledge, this is the first time that outcomes of an in vivo study comparing the modulatory effects of two different varieties of GFJ and their pure compounds on P-gp activity are presented.

MATERIALS AND METHODS

Chemicals. The following materials were used: (rac)-verapamil hydrochloride and halothane were obtained from Sigma Chemical Company (St. Louis, MO); talinolol (99.9% purity) was gently provided by AWD-Pharma GmbH & Co. KG (Dresden, Germany), naringin (NAR) and naringenin (NAG), both >95% pure, were from Roth GmbH & Co. (Karlsruhe, Germany), and bergamottin (BG) (98% purity) was bought from Indofine Chemical Company, Inc. (Somerville, NJ, USA); triethylammonium phosphate buffer (1M) was obtained from Fluka (Buchs, Switzerland); methanol HPLC grade was purchased from Fisher Scientific (Fair Lawn, NJ). All others reagent were analytical grade.

Animals and Experimental Protocol. All animals were housed and all experiments performed according to the policies and guidelines of the Institutional Animal Care and Use Committee (IACUC) of the University of Florida (Gainesville, FL) (NIH publication #85-23). Male Sprague−Dawley rats weighing 350−400 g were used in this study. The animals were not fasted and had free access to food and water during the experiment except for the first 2 h after drug administration. A dose of talinolol (10 mg/kg) was orally administered in the absence or presence of verapamil HCl (4 mg/kg) (positive control) or naringenin (NAG, 0.7 mg/kg), naringin (NAR, 2.4 and 9.4 mg/kg), and bergamottin (BG, 0.05 and 0.2 mg/kg). Similarly, the effects of the grapefruit juices ruby red (RR-GFJ) (Florida Natural) and white GFJ (W-GFJ) (Minute Maid) on the intestinal carrier-mediated transport of talinolol were evaluated. All compounds and juices were orally administered by gavage in a volume of 5 mL/kg. Except for NAG, which was found in the juices at concentrations <0.56 μM, the amount of NAR (2.4 mg/kg) and BG (0.05 mg/kg) used in this study correspond to those present in the W-GFJ (BG: 31.8 μM; NAR: 811 μM) (*32*). The higher concentrations of NAR (9.4 mg/kg) and BG (0.22 mg/kg) were chosen to mimic the effects of a double-strength frozen concentrate GFJ (Minute Maid), which has been used in clinical studies (*33–35*). Concentrations of NAG and BG found in the RR-GFJ were 469 and 12.2 μM, respectively (*32*). The administered volume of the RR-GFJ was 5 mL/kg; thus, the rats received 1.4 mg/kg of NAR and 0.02 mg/kg of BG. All compounds were dissolved in vehicle (propylene glycol/isotonic saline; 20:80). After drug administration, blood samples (500 μL) were collected from the sublingual vein (*36*) of each rat at 0 min, 45 min, 1, 2, 3, 4, 5, and 6 h. Prior to blood collection, the rats were anaesthetized with halothane, and after the sampling procedures, approximately 1000 μL of isotonic saline were replaced via i.p. injection in order to maintain the blood fluid. The blood samples were centrifuged at 2800*g* for 15 min at room temperature, and the serum separated. The collected samples were stored at −70 °C until analysis.

Sample Preparation and HPLC Method. Talinolol was extracted from the samples by using 96-well solid-phase extraction (SPE) disks (Polaris, Varian, Lake Forest, CA). Briefly, the disks were equilibrated with 400 μL of methanol and preconditioned by adding the same volume of 1 mM HCl. All samples were diluted (1:2) with the conditioning solvent and vortexed for 30 s before they were applied to the SPE disks at a final volume of 750 μL. Propranolol (500 ng/mL) was used as an internal standard. The interferents were eluted with 400 μL of methanol/water (20:80). Finally, the analysates were recovered by rinsing the SPE disks twice with 200 μL of methanol/water (90:10). The solvent was evaporated to dryness, and the residue was redissolved in 125 μL of mobile phase before analysis.

A reversed phase high-performance liquid chromatograph (HPLC) method with UV detection was applied for the quantification of talinolol in plasma. Samples (75μL) were injected into a Shimadzu LC2010C HPLC system (Kyoto, Japan) equipped with a 100 μL loop and Class VP 7.2 SP1 chromatographic software. The wavelength was set at 241 nm, the flow rate at 1.00 mL/min, and the temperature at 40 °C. The analytical column was a Lichrospher 60RP-Select B column, 250 × 4.6 mm i.d., 5 μm, preceded by a Lichrospher 60RP-Select B guard column (Merck GaA, Darmstadt, Germany). Talinolol was eluted isocratically using a mobile phase consisted of 0.025 mol/L triethyl-ammonium phosphate buffer (pH 3.0) and acetonitrile (77:23) (**Figure 2**). The range of validation (5−1000 ng/mL) was linear with coefficients of correlation more than 0.998 when using $1/X^2$ as weighing factor, where X was the talinolol concentration (ng/mL). The precisions intra- and interday for talinolol were satisfactory with values between 1.2 and 7.3%. Similarly, the accuracies were between 96.4 and 106.8% of the nominal values. Recovery assessed at three distinct levels of concentration (10, 250, and 500 ng/mL) ranged from 83.7 to 92.2% of the expected values.

Pharmacokinetic Analysis. The pharmacokinetics of talinolol was evaluated by a noncompartmental method with WinNonlin Professional Edition (version 3.1; Pharsight Corp, Mountain View, CA). The maximum talinolol serum concentration (C_{max}) and the time to reach C_{max} (t_{max}) were obtained directly from the concentration−time profile curves. The $AUC_{(0-6)}$, corresponding to the area under the concentration−time profile curve from time 0 to the time of the last measurable concentration (C_{6h}), was determined by the linear trapezoidal method. The terminal elimination rate constant (λz) was determined by log-linear regression of at least the last three points. The terminal half-life ($t_{1/2}$) was calculated as ln(2/λz). The AUC from time 0 to infinity ($AUC_{(0-\infty)}$) was the sum of $AUC_{(0-6)}$ and $C_{6h}/\lambda z$.

Statistical Analysis. Descriptive and comparative statistics were calculated with the use of GraphPad Prism (version 4.0; GraphPad Software Inc., San Diego, CA). Pharmacokinetic data are presented as mean values ($n = 8$) ± standard error of the mean (SEM). Comparison among the groups initially used one-way ANOVA followed by Tukey's multiple comparison tests as posthoc analysis. A probability of less than 0.05 ($p < 0.05$) was considered to be statistically significant. Grubb's test was applied to detect possible outliers ($\alpha = 0.05$), and the Kolmogorov−Smirnov test was used to determine if the obtained data were normally distributed.

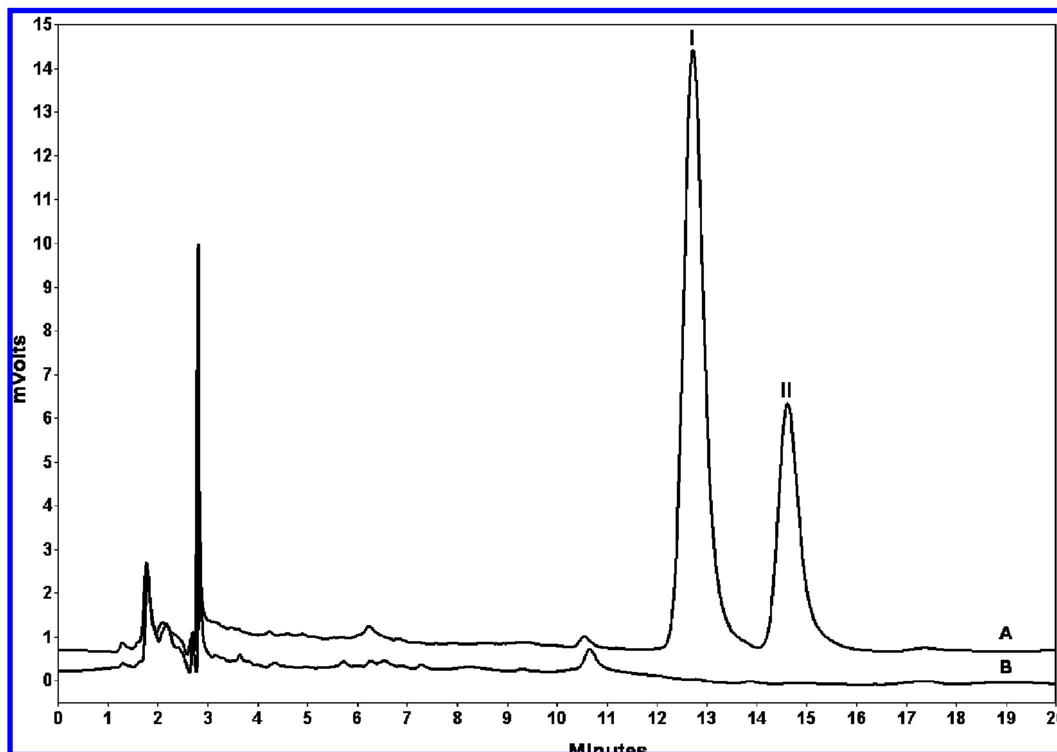

Figure 2. Comparison of chromatograms corresponding to the talinolol (I) and the internal standard propranolol hydrochloride (II) after extraction from rat serum (**A**) with that obtained after extraction of a blank sample (**B**).

Table 1. Pharmacokinetics of Talinolol in Rats after a Single Oral Dose (10 mg/kg) in the Absence (Control), White GFJ (W-GFJ), and Ruby Red GFJ (RR-GFJ) (5 mL/kg Each)[a]

treatment	AUC(0−6) (ng · h/mL)	AUC(0−∞) (ng · h/mL)	C_{max} (ng/mL)	t_{max} (h)	$t_{1/2}$ (h)
control	538 ± 36	558 ± 36	294 ± 29	1.9 ± 0.2	1.0 ± 0.1
W-GFJ	684 ± 102	698 ± 102	337 ± 56	2.1 ± 0.2	0.7 ± 0.07
RR-GFJ	249 ± 43[b]	259 ± 43[b]	121 ± 21[b]	2.2 ± 0.2	0.9 ± 0.2

[a] Data are expressed as mean (n = 8) ± SEM. [b] $p < 0.05$ vs control.

Table 2. Pharmacokinetics of Talinolol in Rats after a Single Oral Dose (10mg/kg) in the Absence (Control) or Presence of Verapamil, Naringenin (NAG), Naringin (NAR), and Bergamottin (BG)[a]

treatment	AUC(0−6) (ng · h/mL)	AUC(0−∞) (ng · h/mL)	C_{max} (ng/mL)	t_{max} (h)	$t_{1/2}$ (h)
control	501 ± 57	512 ± 55	274 ± 35	2.0 ± 0.1	0.8 ± 0.1
verapamil (4 mg/kg)	827 ± 133	856 ± 132	385 ± 84	2.7 ± 0.2[d]	1.0 ± 0.2
NAG (0.7 mg/kg)	801 ± 78	812 ± 77	493 ± 66	1.7 ± 0.2	0.8 ± 0.1
NAR (2.4 mg/kg)	906 ± 72[b]	937 ± 70[b]	482 ± 59	2.1 ± 0.1	1.2 ± 0.3
NAR (9.4 mg/kg)	771 ± 81	797 ± 83	430 ± 64	2.0 ± 0.1	1.0 ± 0.1
BG (0.05 mg/kg)	403 ± 42	429 ± 45	222 ± 30	2.0 ± 0.1	1.6 ± 0.2
BG (0.22 mg/kg)	866 ± 108[b]	896 ± 102[b]	650 ± 91[c]	1.8 ± 0.2	1.3 ± 0.3

[a] Data are expressed as mean (n = 8) ± SEM. [b] $p < 0.05$ vs control. [c] $p < 0.01$ vs control. [d] $p < 0.001$ vs control.

RESULTS

The pharmacokinetic parameters of talinolol are presented in **Tables 1** and **2**. When coadministered with talinolol, verapamil (VER) (4 mg/kg), a known inhibitor of P-gp, promoted a slight but not significant increase on the C_{max} and the $AUC_{(0-\infty)}$ of the drug by 1.4- and 1.7-fold, correspondingly. Additionally, the absorption (t_{max}) was delayed from 2 to 2.7 h when compared to the control group. The elimination half-life ($t_{1/2}$) remained practically unchanged among all treatments. The W-GFJ provided a slight increase on the C_{max} and the $AUC_{(0-\infty)}$ of talinolol but also without statistical significance when compared to the control. Interestingly, the RR-GFJ significantly decreased the C_{max} and the $AUC_{(0-\infty)}$ of talinolol by 41% and 46%, respectively.

Among the GFJ components, the furanocoumarin BG (0.22 mg/kg) significantly modified the talinolol disposition, augmenting the C_{max} and the $AUC_{(0-\infty)}$ by 2.4- and 1.8-fold, respectively, but no effect was observed at the lower dose of BG. Compared to the control group, the flavonoids NAG (0.7 mg/kg) and NAR (2.4 and 9.4 mg/kg) increased the talinolol C_{max} and $AUC_{(0-\infty)}$ by 1.5- to 1.8-fold, respectively. However, the effect of the flavonoids as well as BG in a concentration of 0.22 mg/kg on talinolol bioavailablity was in the same range as that of the positive verapamil.

DISCUSSION

In the present study, we investigated the potential effects of two varieties of grapefruit juice and its selected ingredients on the intestinal carrier-mediated transport of talinolol in Sprague−Dawley rats. To our knowledge, this is the first preclinical study involving the administration of pure GFJ components in rats in order to evaluate their effects on the intestinal transport of drugs. Although the effect of GFJ on the pharmacokinetics of several drugs has been studied intensively, there is still a lack of in vivo studies investigating the interaction of the active ingredients of GFJ with drugs. Goosen et al. (*37*) were the first to give bergamottin as a pure compound to humans. The authors could show that bergamottin enhanced the oral bioavailability of felodipine by 30−40% compared to water, confirming the contribution of this compound for the inhibition of CYP3A4 after ingestion of GFJ (*37*). A more recent clinical investigation demonstrated that coadministration of fexofenadine with GFJ, or an aqueous solution containing naringin concentrations of 1234 and 1210 μM (∼215 mg), respectively, lowered the drug AUC to 55 and 75% of that with water (*17*). If GFJ inhibited

only P-gp, then the bioavailability of fexofenadine should have increased rather than decreased. Because fexofenadine is also a substrate for OATP (*38*), the hypothesis put forth was that the decrease in fexofendaine oral bioavailability by GFJ is a result of greater inhibition of OATP over P-gp (*39*). Thus, on the basis of these results, it was suggested that naringin is the major and selective OATP inhibitor present in GFJ.

In the present study, the terminal half-life of talinolol after oral administration in the absence or presence of GFJ, naringin, naringenin, or bergamottin was not affected (**Tables 1** and **2**), indicating that any effect on the talinolol pharmacokinetics parameters can be attributable to processes that occur in the gut rather than to a modification of its systemic clearance (*29, 40, 41*). The lack of a significant influence of verapamil on talinolol disposition in rats (**Table 2**) may be dose-related, since this drug is also subjected to presystemic metabolism by intestinal CYP3A4 (*42*). Although VER at the same dose used in this study has been described not to affect the t_{max} of talinolol in rats (*40*), our results showed a prolongation, probably by delaying the gastric emptying time as previously described (*43*).

In regards to the flavonoids, no statistical difference was observed between the low- and high-dose of NAR. The maximum effect was reached when the glycoside was given at 2.4 mg/kg, which significantly increased the $AUC_{(0-\infty)}$ from 512 to 937 ng·h/mL (**Table 2**). Interestingly, the higher dose of NAR (9.4 mg/kg) increased talinolol bioavailability to a lesser extent than the lower concentration if compared to the control group, although the difference between both NAR concentrations was not statistically different. A plausible explanation for this effect could be that, with both concentrations of NAR, a saturation of the P-gp efflux transporter is reached. Further experiments in a concentration below 2.4 mg/kg are therefore necessary to investigate the dose dependent effects of NAR on talinolol bioavailability. Similar results were obtained after ingestion of the NAG (0.7 mg/kg), indicating that this flavonoid aglycone, although not present in the juice (*32*), may contribute to the overall inhibitory effect of GFJ, as a result of the metabolism of the glycoside NAR by intestinal microflora (*44*). This suggests that NAR and NAG at the tested concentrations were able to preferentially inhibit the carrier-mediated efflux of talinolol, probably because of the inhibition of P-gp. Although in vitro studies previously demonstrated that NAR reduced P-gp-mediated transport of talinolol (*19, 20*), in one clinical trial, NAR has been shown to reduce the uptake of fexofenadine (*17*). Interspecies differences in substrate specificity and expression of uptake and efflux transporters may have accounted for this controversy (*45*). Additionally, one clinical study demonstrated that coadministration of the P-gp inhibitor PSC833 did not affect morphine pharmacokinetics or cause adverse events in healthy volunteers (*46*), while in mdr1 knockout mice, the analgesic effect of morphine was augmented (*47*).

Among the components of GFJ tested, the furanocoumarin BG (0.22 mg/kg) provided the greatest effect on the talinolol disposition, suggesting an inhibition of its P-gp-mediated efflux rather than the uptake process. Accordingly, in vitro studies have reported this compound as a potent inhibitor of P-gp activity ($IC_{50} = 40\ \mu M$) compared with NAR or NAG (*21*).

When compared to the control group, W-GFJ did not alter the talinolol disposition (**Table 1**). Interestingly, the juice was less active than the single compounds. Until now, it is unknown if other compounds present in the juice and yet not tested are counteracting the effects of NAR, NAG, and BG, but this needs to be evaluated in future studies involving systematic administration of two or more GFJ components simultaneously.

Our results differ from previous studies investigating the acute effect of white grapefruit juice (Tropicana) on the disposition of talinolol in rats, which reported an increased in the C_{max} and the AUC by 2- and 1.4- fold, respectively (*29*). One reason for these differing results could be that the authors used a 50% diluted juice with a pH adjusted to 7, whereas in our study, no pH adjustments of the juice have been performed. Furthermore, the amounts of the potentially interacting compounds in the juice were not estimated in the previous study.

Conversely, RR-GFJ acted preferentially on the uptake process, reducing the pharmacokinetic parameters of talinolol. The concentrations of NAR, BG, and 6′,7′-dihydroxybergamottin (DHB) were 1.7-, 2.5-, and 10-fold lower than those found in W-GFJ (*32*). It can be speculated that other compounds than those tested present in RR-GFJ may be responsible for the observed effect. Since the concentrations of NAR and BG in the RR-GFJ were lower than those in the W-GFJ, it is also possible that the dosage of the juice plays an important role.

It was previously shown that GFJ reduced the bioavailability of talinolol in humans by half (*31*). However, the authors did not mention the variety of juice given to the subjects. It has been shown that the variability of GFJ depends on the origin, fruit variety, maturity, quality of raw material, manufacturing procedures, and storage conditions (*48–51*). In addition, the expression and the interspecies variability of the intestinal transporters can contribute to these distinct and unpredictable effects (*17*).

In conclusion, we demonstrated that outcomes obtained during this GFJ−talinolol interaction study seem to be dependent on the variety of the juice and the concentration of the GFJ component ingested. It further showed that GFJ and single GFJ components can selectively modulate the activity of intestinal efflux and influx transporters. However, the observed overall result may not be a simply additive effect of all components present in the juice. In fact, synergism or antagonism cannot be excluded. Therefore, further investigations using combinations of two or more GFJ constituents in different concentrations may consist of an important tool to better understand such interactions.

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