Effect of blueberry juice on clearance of buspirone and flurbiprofen in human volunteers

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WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- Many citrus and fruit juices inhibit the activity of human cytochromes P450 (CYPs) *in vitro*.
- *In vitro* studies showing CYP inhibition by nutrients and natural products are not necessarily predictive of clinical drug interactions.
- Only grapefruit juice (GFJ) contains naturally occurring substances that produce irreversible (as opposed to reversible) inhibition of CYP3A *in vitro*, and only GFJ has so far been shown to produce significant drug interactions with CYP3A substrates *in vivo*.

WHAT THIS STUDY ADDS

- Blueberry juice (BBJ) at low concentrations produces inhibition of CYP3A and CYP2C9 activity *in vitro*.
- The mechanism of inhibition of BBJ is reversible as opposed to irreversible.
- In clinical studies with human volunteers, BBJ produces slight impairment of CYP3A activity and no detectable inhibition of CYP2C9 activity.
- There is minimal if any risk of drug interactions involving BBJ and substrate drugs metabolized by CYP3A or CYP2C9.

AIM

The present study evaluated the possibility of drug interactions involving blueberry juice (BBJ) and substrate drugs whose clearance is dependent on cytochromes P4503A (CYP3A) and P4502C9 (CYP2C9).

METHODS

A 50:50 mixture of lowbush and highbush BBJ was evaluated *in vitro* as an inhibitor of CYP3A activity (hydroxylation of triazolam and dealkylation of buspirone) and of CYP2C9 activity (flurbiprofen hydroxylation) using human liver microsomes. In clinical studies, clearance of oral buspirone and oral flurbiprofen was studied in healthy volunteers with and without co-treatment with BBJ.

RESULTS

BBJ inhibited CYP3A and CYP2C9 activity *in vitro*, with 50% inhibitory concentrations (IC₅₀) of less than 2%, but without evidence of mechanism-based (irreversible) inhibition. Grapefruit juice (GFJ) also inhibited CYP3A activity, but inhibitory potency was increased by pre-incubation, consistent with mechanism-based inhibition. In clinical studies, GFJ significantly increased area under the plasma concentration–time curve (AUC) for the CYP3A substrate buspirone. The geometric mean ratio (GMR = AUC with GFJ divided by AUC with water) was 2.12. In contrast, the effect of BBJ (GMR = 1.39) was not significant. In the study of flurbiprofen (CYP2C9 substrate), the positive control inhibitor fluconazole significantly increased flurbiprofen AUC (GMR = 1.71), but BBJ had no significant effect (GMR = 1.03).

CONCLUSION

The increased buspirone AUC associated with BBJ is quantitatively small and could have occurred by chance. BBJ has no effect on flurbiprofen AUC. The studies provide no evidence for concern about clinically important pharmacokinetic drug interactions of BBJ with substrate drugs metabolized by CYP3A or CYP2C9.

Introduction

Interactions of prescription drugs with nutrients and natural products have become an issue of medical and public health concern. Much of the attention has focused on citrus and other fruit beverages, in particular grapefruit juice (GFJ) [1–6]. GFJ contains a number of natural substances, classified as furanocoumarins, which have the property of producing irreversible inhibition (also termed ‘mechanism-based inhibition’) of cytochrome P4503A (CYP3A) enzymes in the mucosal cells of the gastrointestinal tract lining [4–12]. For drugs that ordinarily undergo presystemic extraction (first pass metabolism) due to biotransformation by enteric CYP3A, ingestion of GFJ can cause a decrease in presystemic extraction, increased extent of absorption, and higher systemic plasma concentrations. Although relatively few drugs are significantly affected by this interaction, there are a few cases in which the GFJ interaction effect can be of clinical importance [4–6].

Clinical drug–drug interaction (DDI) studies are needed to confirm or rule out the possibility of a drug interaction between any given pair of medications, or a medication with a dietary component or nutrient [13–23]. Over the last two decades, *in vitro* models have become widely applied to identify or exclude potential clinical DDIs. While the *in vitro* paradigms cannot precisely predict the magnitude of clinical DDIs, the data from *in vitro* models has in many cases proved to be useful in identifying high probability DDIs, thereby allowing resources available for clinical studies to be targeted in a more informed way. Unfortunately, *in vitro* data have been of limited value in identifying clinical DDIs involving fruit and citrus products. The inhibitory effect of GFJ on CYP3A activity is predicted by such models [4–7]. However, many other fruit beverages, such as pomegranate and cranberry juices, are inhibitors of CYP3A (and other CYP enzymes) *in vitro*, but to date none of these products other than GFJ has produced clinically important DDIs in humans [6].

Anthocyanins and other flavonoids present in blueberries and cranberries are reported to have beneficial effects in terms of disease prevention and health maintenance [24–28]. The two principal varieties of blueberry produced commercially in North America are *Vaccinium angustifolium* Aiton and *Vaccinium corymbosum* L., also termed lowbush ‘wild’ blueberries and highbush ‘cultivated’ blueberries, respectively. The two varieties differ in flavonoid content and in methods for extraction, processing, and storage [27, 28].

Despite the extensive consumption of blueberry juice in North America and Europe, no clinical information is available on the possibility of interactions of blueberry juice with prescription medications. The present study evaluated the effect of blueberry juice on the activity of human CYP3A and CYP2C9 *in vitro* and in human volunteer trials. For *in vitro* studies, triazolam and buspirone were

used as the index substrates for CYP3A and flurbiprofen was the index substrate for CYP2C9. For clinical studies, buspirone and flurbiprofen were the probe substrates for CYP3A and CYP2C9, respectively.

Methods

Constituents of blueberry juice and grapefruit juice preparations

To assure representation of the two principal varieties of blueberry juice, the juice used in the *in vitro* and clinical study was a 50:50 mixture of lowbush (100% Pure Wild Blueberry Juice, van Dyk’s Health Juice Products Ltd, Nova Scotia, Canada) and highbush (Just Blueberry, R.W. Knudsen Family Co., Orrville, OH, USA) juices. The positive control inhibitor for the buspirone study was commercially-purchased regular strength shelf stable white GFJ from concentrate, manufactured by Ocean Spray Inc., Lakeville-Middleboro, MA, USA.

The concentrations of 23 different anthocyanin derivatives (Table 1) were determined in the blueberry juice preparation used in the *in vitro* and clinical studies. The high performance liquid chromatographic (HPLC) procedure was based on a previously reported method [29]. A 1000 µg ml⁻¹ solution of cyanidin-3-glucoside chloride was prepared by dissolving 2 mg of the pure powder in 2 ml of 5% formic acid in water. This solution was serially diluted with 5% formic acid in water to yield 13 calibration standards containing concentrations of cyanidin-3-glucoside ranging from 927 to 0.23 µg ml⁻¹.

The analytical column was a 5 µ, 4.6 × 250 mm, Agilent Zorbax SB-C₁₈. The two mobile phases were 5% formic acid in water, and methanol. The flow rate was 1.0 ml min⁻¹. Separation of anthocyanins was achieved using a methanol gradient from 5% to 70% over 84 min. Column effluent was monitored by ultraviolet detection at 520 nm. Identification of individual anthocyanins was done by comparing the retention times with those reported previously [29].

The total phenolic content of blueberry juices was determined using the method of Singleton *et al.* [30]. Juice samples were diluted 1:10 with water prior to analysis. A calibration curve was generated using gallic acid as the reference compound. Gallic acid was dissolved in water, at concentrations ranging from 62.5 to 1000 µg ml⁻¹. A 50 µl aliquot of each sample – calibration standard or blueberry juice – was added to a 2 ml microcentrifuge tube. Water (865 µl) and the Folin-Ciocalteu phenol reagent (75 µl) were added and each tube was vortexed. After 5 min, 225 µl of 20% sodium carbonate in water was added to each tube. After mixing, the volume was brought to 1.5 ml by the addition of 285 µl of water. Tubes were vortexed and allowed to stand at room temperature, in the dark, for 2 h. A 1 ml aliquot of each sample was transferred to a cuvette and the absorbance measured at 765 nm. All samples were analyzed in duplicate.

Table 1Concentrations of individual anthocyanins, total anthocyanins, and total phenolics in blueberry juice preparations used for *in vitro* and clinical studies

Anthocyanin derivative	Concentration ($\mu\text{g ml}^{-1}$)			Flurbiprofen study		
	Buspirone study Mean ($n = 12$)	SD	%CV	Mean ($n = 12$)	SD	%CV
Delphinidin 3-galactoside	44.5	4.0	9.0	42.9	2.7	6.2
Delphinidin 3-glucoside	61.0	2.6	4.2	53.3	4.7	8.7
Cyanidin 3-galactoside	22.7	1.0	4.4	20.9	1.5	7.1
Delphinidin 3-arabinoside	31.8	4.5	14.2	29.7	2.5	8.5
Cyanidin 3-glucoside	35.7	2.2	6.2	30.1	4.1	13.6
Petunidin 3-galactoside	29.5	3.0	10.3	29.5	2.4	8.0
Cyanidin 3-arabinoside	16.8	1.1	6.2	14.2	1.5	10.5
Petunidin 3-glucoside	53.0	2.7	5.1	46.6	3.4	7.3
Peonidin 3-galactoside	9.6	1.0	10.2	9.1	0.6	7.0
Petunidin 3-arabinoside	17.5	2.5	14.5	16.3	1.7	10.4
Peonidin 3-glucoside	25.7	1.5	5.8	23.5	1.6	6.9
Malvidin 3-galactoside	81.6	10.8	13.2	85.5	10.3	12.1
Malvidin 3-glucoside	117.4	8.4	7.1	109.4	7.5	6.8
Malvidin 3-arabinoside	45.8	7.3	16.0	44.8	5.5	12.2
Cyanidin-3-(malonoyl)glc/cyanidin 3-(6"-acetyl)gal	2.9	0.4	15.2	1.7	0.4	22.5
Malvidin + acetyl + hexose/petunidin + pentose	3.9	0.6	14.6	2.5	0.4	14.1
Delphinidin 3-(6"-acetyl)glucoside	20.4	2.4	11.5	15.2	3.6	23.5
Peonidin 3-(6"-acetyl)galactoside	1.7	0.1	7.8	1.1	0.2	16.6
Cyanidin 3-(6"-acetyl)glucoside	15.1	1.5	10.1	11.2	2.6	23.2
Malvidin 3-(6"-acetyl)galactoside	15.3	1.0	6.7	12.0	1.8	14.6
Petunidin 3-(6"-acetyl)glucoside	16.9	2.0	11.8	12.2	3.4	27.7
Peonidin 3-(6"-acetyl)glucoside	10.9	1.5	14.1	8.5	1.8	20.6
Malvidin 3-(6"-acetyl)glucoside	51.1	5.8	11.3	39.5	8.5	21.6
Total anthocyanin	730.9	47.6	6.5	659.8	43.5	6.6
Total phenolic	2013.8	127.0	6.3	2447.5	222.2	9.1

Table 2Summary of experimental conditions for *in vitro* studies using human liver microsomal preparations

CYP isoform	Substrate (concentration)	Product(s)	Positive control inhibitors
CYP3A	Triazolam (250 μM)	α -OH- and 4-OH-triazolam	GFJ
	Buspirone (25 μM)	1-PP*	
CYP2C9	Flurbiprofen (5 μM)	4'-OH-flurbiprofen	Fluconazole

*1-pyrimidinyl piperazine.

The furanocoumarin content in the GFJ samples ingested by each of the 12 clinical study participants was determined by HPLC as described previously [31].

In vitro inhibition of CYP3A and CYP2C9 by blueberry juice and grapefruit juice preparations

The *in vitro* metabolic model using human liver microsomal preparations has been described previously [6, 7, 12–15, 32, 33].

Fixed concentrations of index substrates were used for inhibition studies (Table 2). The rates of transformation of the index substrates to their respective metabolites were determined in the control condition (without inhibitor) and with varying concentrations of BBJ. Also evaluated

were positive control index inhibitors: GFJ for CYP3A, and fluconazole for CYP2C9. Reactions were started by the addition of microsomal protein, 0.1–0.2 mg ml⁻¹ for triazolam and flurbiprofen, and 0.125–0.25 mg ml⁻¹ for buspirone. Each assay was allowed to proceed for 20 min at 37°C. Studies were done both without and with pre-incubation of inhibitors with microsomal protein and necessary cofactors prior to addition of index substrate. Increased inhibitory potency attributable to pre-incubation is consistent with mechanism-based (irreversible) inhibition [7, 12, 14, 32]. The pre-incubation duration was 20 min.

Buspirone incubates were analyzed using a 150 $\times$ 4.6 mm Agilent Zorbax RX-C₈ column. Mobile phase A was 0.01% trifluoroacetic acid in water. Mobile phase B was

acetonitrile. The initial conditions were 92% A and 8% B. After 8 min, the percentage of B was increased linearly to 40% over 22 min. From 30 to 35 min, the percentage of B was increased linearly to 90%. The mobile phase was returned to its initial conditions over the next 5 min, and the column was re-equilibrated for an additional 5 min with the initial mobile phase conditions. The total run time was 45 min and the flow rate was held constant at 1.0 ml min⁻¹. Column effluent was monitored with ultraviolet detection at a wavelength of 248 nm. Under these conditions, 1-pyrimidinylpiperazine (1-PP), 2-acetamidophenol (internal standard) and buspirone eluted at 4.2, 12.9 and 25.1 min, respectively.

Reaction velocities with co-addition of inhibitor were expressed as a percentage ratio vs. the corresponding velocity with no inhibitor present. The relation of reaction velocity to inhibitor concentration was analyzed by non-linear regression (using SAS PROC NLIN) to determine the inhibitor concentration reducing velocity to 50% of inhibitor-free control (IC₅₀) [32]. IC₅₀ values for juice preparations were expressed as the volume of juice (after appropriate dilution) in the total incubation volume (%). Lower IC₅₀ values indicate greater inhibitory potency [7, 12].

Clinical study: subjects

The clinical study protocol and consent form were reviewed and approved by the Institutional Review Board serving Tufts University School of Medicine and Tufts Medical Center.

Study participants were healthy male and female volunteers aged 19 to 54 years. They were active ambulatory adults with no history of significant medical disease and taking no prescription medications.

Subjects were initially recruited through advertising or word of mouth. After a telephone screening interview, potential candidates were invited for on-site screening and evaluation. After signing informed consent, subjects underwent medical history screening, physical examination, blood haematology and chemistry testing, and screening for hepatitis (B and C) and HIV. Potentially child-bearing female subjects underwent a pregnancy test.

Candidates were accepted as study participants if the screening procedure indicated that they were healthy active adults without significant medical disease or any condition that would make participation unsafe or not appropriate.

Clinical study procedures

The two index substrates for the clinical study were buspirone and flurbiprofen. Buspirone is a non-benzodiazepine anxiolytic having bioavailability in the range of 3%, mainly due to presystemic extraction by enteric CYP3A [34–37]. It is classified as a 'sensitive' substrate for DDI studies involving CYP3A [38–40]. In a previous study, buspirone AUC was substantially increased by co-administration of GFJ [41]. Flurbiprofen is a non-

steroidal anti-inflammatory agent metabolized almost exclusively by CYP2C9 [33, 42]. Clearance of flurbiprofen is used to profile CYP2C9 activity [42–46]. Fluconazole, the positive control, is an azole antifungal agent known to be an inhibitor of CYP2C9 [45, 47].

For the buspirone study, the three co-treatment trials (in random sequence) were 1) water (300 ml), 2) grapefruit juice (300 ml) and 3) blueberry juice (300 ml).

For the flurbiprofen study, the three trials (in random sequence) were 1) water (300 ml), 2) fluconazole (200 mg) with 300 ml water and 3) blueberry juice (300 ml).

The three trials in each study were separated by at least 1 week. Within each trial, the co-treatments were administered twice: once on the afternoon (between 16.00h and 18.00 h) of the day before the index substrate, and again 30 min prior to the index substrate. The index substrate itself was given with 300 ml water at 08.00 h (buspirone hydrochloride 10 mg or flurbiprofen 100 mg). Eleven venous blood samples were drawn during 12 h after index substrate administration.

Using total area under the plasma concentration curve (AUC) for the index substrate as the principal outcome measure, a change in AUC of 35% (Trial 3 vs. Trial 1) was considered to be clinically important. Based on prior DDI studies, the relative standard deviation of the mean difference in AUC was estimated to be 40% of the mean difference itself. Under these conditions, a sample size of $n = 12$ allowed power of 0.80 with $\alpha = 0.05$.

Analysis of plasma samples

Venous blood samples were centrifuged, and the plasma separated and stored at –18°C until the time of assay. All samples from a given subject's set of three trials were extracted and analyzed on the same day using one set of calibration standards.

Plasma concentrations of buspirone were determined by liquid chromatography-mass spectroscopy (LC/MS). Stock solutions of buspirone and the internal standard, d₈-buspirone, were prepared in methanol and further diluted as appropriate. Stock solutions were stored at –20°C until use. For the calibration samples, 2.5 ng of d₈-buspirone were added to a series of tubes containing buspirone in quantities ranging from 0.0125 ng to 10 ng. The solvent was evaporated to dryness under mild vacuum. Drug-free control serum (bovine serum, 0.5 ml) was added to the calibration samples. Study sample plasma (0.5 ml) was added to tubes containing only the internal standard. Calibration standards and study samples were alkalized by adding 50 µl of 0.1 mol l⁻¹ sodium carbonate buffer. Buspirone and d₈-buspirone were extracted with 1.5 ml of methyl tert-butyl ether. Samples were vortexed for 5 min and the phases were separated by centrifugation. Samples were placed at –80°C for 30 min to freeze the aqueous layer. The organic layer was removed and evaporated to dryness under mild vacuum. The residue was reconstituted in 90 µl of 0.1% formic acid in water.

HPLC-MS/MS analysis was conducted using a 150×2.00 mm, 4 μ , Synergi Fusion RP-80A column (Phenomenex). Solvent A was 0.1% formic acid in water. Solvent B was acetonitrile. The flow rate was $0.300 \text{ ml min}^{-1}$. From 0 to 2 min, the percentages of A and B were 75% and 25%, respectively. At 2 min, the concentration of solvent B was increased linearly over 4 min to 90%. At 6 min, the mobile phases were returned to their initial conditions and the column re-equilibrated for 5 min prior to the next injection. Buspirone and d_8 -buspirone coeluted at approximately 3.5 min.

Multiple reaction monitoring was conducted using m/z transitions of $386 \rightarrow 122$ for buspirone and $394 \rightarrow 122$ for d_8 -buspirone. The mass spectrometer was an Applied Biosystems API3000 equipped with a turbo electrospray ionization source operated in positive ion mode. The relevant gas values were: nebulizer gas, 10 psi; curtain gas, 12 psi; and collision gas, 7 psi. The ion spray voltage was 5000 V and the source temperature was 400°C . The parameters for buspirone and d_8 -buspirone were: declustering potential, 26.0 V; focusing potential, 370.0 V; entrance potential, 10.5 V; collision energy, 43.0 V; and collision cell exit potential, 2.0 V.

At 0.1, 1 and 20 ng ml^{-1} , the percent recovery of buspirone exceeded 95%. Recovery of d_8 -buspirone was essentially 100% at 5 ng ml^{-1} . The intra- and inter-day precision was $\leq 17\%$ at all concentrations on the calibration curve. Quality control (QC) samples – 0.2 and 2 ng ml^{-1} – were analyzed with each analytical run. The mean $\pm$ SD ($n = 10$) measured QC concentrations were 0.23 ± 0.03 and $2.1 \pm 0.07 \text{ ng ml}^{-1}$. The mean slope of the standard curve was 0.3674 with a coefficient of variation of 2%.

The lower limit of quantification for the assay was 0.025 ng ml^{-1} . Measured concentrations less than this were assigned a value of zero for purposes of calculating mean concentrations at corresponding times.

Plasma concentrations of flurbiprofen and 4'-OH-flurbiprofen were determined by HPLC with fluorescence detection as described previously [41]. QC concentrations for 4'-OH-flurbiprofen were 0.08 and $0.8 \mu\text{g ml}^{-1}$. For flurbiprofen, the concentrations analyzed were 0.75 and $7.5 \mu\text{g ml}^{-1}$. The mean $\pm$ SD ($n = 12$) measured QC concentrations for 4'-OH-flurbiprofen were 0.09 ± 0.03 and $0.84 \pm 0.15 \mu\text{g ml}^{-1}$. The analogous values for flurbiprofen were 0.78 ± 0.12 and $7.64 \pm 1.24 \mu\text{g ml}^{-1}$.

For Trial 2 of the flurbiprofen study, plasma concentrations of fluconazole were determined by HPLC with ultraviolet detection [48]. QC samples were prepared at 0.75 and $7.5 \mu\text{g ml}^{-1}$. Across three analytical runs, the mean measured QC concentrations were 0.75 and $7.73 \mu\text{g ml}^{-1}$, with coefficients of variation less than 2%.

Pharmacokinetic and statistical analysis

The terminal log-linear phase of each plasma concentration curve (buspirone or flurbiprofen) was identified visually, and the slope (β) determined by linear regression

Table 3

Furanocoumarin content of grapefruit juice preparations ($n = 12$) used in the buspirone clinical study

Compound	Concentration ($\mu\text{g ml}^{-1}$)		%CV
	Mean	SD	
6',7'-dihydroxybergamottin	1.5	0.4	23.5
Bergamottin	7.3	0.7	9.5
Bergaptol	20.0	2.9	14.5
Paradisins C	0.59	0.17	28.3
Isoimperatorin	0	–	–
Cnidicin	0	–	–
Auraptene	2.9	0.3	9.9

analysis. The slope was used to calculate the elimination half-life ($t_{1/2}$). Area under the plasma concentration curve from time zero until the last non-zero concentration was calculated by the linear trapezoidal method, and extrapolated to infinity by addition of the final concentration divided by β . This yielded the total area under the curve from time zero to infinity (AUC). Apparent oral clearance (CL/F) was calculated as the administered dose divided by AUC. Observed maximum plasma concentration (C_{max}) and time of maximum concentration (t_{max}) were used as indicators of the rate of absorption.

For each pharmacokinetic variable for each trial, values were arrayed in tabular form, with calculation of arithmetic mean, SD, median, geometric mean, and 90% confidence interval (CI). The principal statistical procedure was analysis of variance (ANOVA) for repeated measures (Trial 1 vs. 2 vs. 3), with subsequent comparison of Trials 2 and 3 each vs. Trial 1 using Dunnett's test. Also calculated were ratios (Trial 2 divided by Trial 1, and Trial 3 divided by Trial 1) of individual values for C_{max} and AUC. The arithmetic mean, SD, geometric mean and 90% CIs were determined for these ratios. SAS GLM was used for all pharmacokinetic and statistical analyses.

Results

Anthocyanin and phenolic content of blueberry juice preparations

The anthocyanins present in highest concentrations were malvidin 3-glucoside and malvidin 3-galactoside (Table 1). Total anthocyanin content averaged just under $700 \mu\text{g ml}^{-1}$, and total phenolic content just under $2100 \mu\text{g ml}^{-1}$. There was relatively low variation (less than 20%) in content among the 12 blueberry juice aliquots ingested by subjects within each of the two clinical studies, or between the mean values for the two studies.

Furanocoumarin content in grapefruit juice preparation

Table 3 shows mean concentrations of various furanocoumarin derivatives measured in the GFJ preparations given

to the 12 participants in the buspirone clinical study. Bergaptol and bergamottin were present in the highest concentrations, though these two compounds have relatively low inhibitory potency [6, 12]. The compounds with the highest inhibitory potency, 6',7'-dihydroxybergamottin (DHB) and Paradisin C, were present, but in lower concentrations.

In vitro inhibition of CYP3A and CYP2C9 by blueberry juice preparations

BBJ inhibited *in vitro* biotransformation of the CYP3A substrates triazolam and buspirone (Figure 1). Pre-incubation did not cause a meaningful leftward shift in the inhibition curve. GFJ also inhibited biotransformation of triazolam and buspirone. However, the inhibition curves were shifted significantly leftward by pre-incubation, consistent with mechanism-based inhibition (Figure 2).

BBJ inhibited *in vitro* biotransformation of flurbiprofen to 4'-OH-flurbiprofen (Figure 3). Pre-incubation did not increase inhibitory potency. Fluconazole inhibited flurbiprofen biotransformation with an aggregate IC_{50} of 20.3 μ M. This was reduced to 14.3 μ M with pre-incubation.

Clinical studies

Two different groups of 12 healthy volunteers completed each of the two clinical studies. For the buspirone study, there were nine men and three women (nine Caucasian, two Asian, one Hispanic). For the flurbiprofen study, there were nine men and three women (eight Caucasian, two Asian, one African-American, one Hispanic). All clinical trials were completed without adverse reactions.

Figure 4 shows mean plasma buspirone concentrations in each of the three treatment conditions. Total AUC and CL/F differed significantly among the three treatment conditions (Table 4). Individual comparisons using Dunnett's test indicated that co-administration of GFJ significantly increased buspirone AUC and significantly reduced buspirone clearance. However, BBJ had no significant effect on any of these variables.

Analysis of ratios indicated that the arithmetic mean ratios for buspirone C_{max} and AUC (value with BBJ divided by control value with water) did not differ significantly from 1.0 (Table 5). However, the upper boundary of the 90% CI for the geometric mean AUC ratio exceeded the default boundary of 1.25, as specified in guidelines from the American Food and Drug Administration. For GFJ, arithmetic mean ratios for C_{max} and AUC both were significantly different from 1.0. For total AUC, the geometric mean ratio was 2.12, and the entire 90% CI exceeded the default boundary value of 1.25.

Figure 5 shows mean plasma flurbiprofen concentrations in each of the three treatment conditions. Plasma concentrations of the principal metabolite, 4'-OH-flurbiprofen, are also shown. Plasma concentrations of fluconazole during the fluconazole-flurbiprofen trial are shown in Figure 6.

$t_{1/2}$, total AUC, and CL/F for flurbiprofen differed significantly among the three treatment conditions (Table 6). Individual comparisons using Dunnett's test indicated that co-administration of fluconazole significantly increased flurbiprofen AUC, prolonged $t_{1/2}$ and reduced clearance. Reduced clearance of flurbiprofen was mirrored by

Figure 1

IC_{50} determination for the blueberry juice preparation vs. CYP3A substrates. A) Formation of α -OH-triazolam from triazolam. B) Formation of 1-PP from buspirone. Each point is the mean ($\pm$ SEM, $n = 4$) reaction velocity expressed as a percentage ratio vs. the inhibitor free control. IC_{50} values for the aggregate data points were determined by nonlinear regression. Similar results were seen for 4-OH-triazolam (data not shown). A) ●, without pre-incubation $IC_{50} = 1.41\%$; ○, with pre-incubation $IC_{50} = 1.16\%$; B) ●, without pre-incubation $IC_{50} = 1.43\%$; ○, with pre-incubation $IC_{50} = 1.48\%$

Figure 2

Inhibition of formation of α -OH-triazolam from triazolam (A) and formation of 1-PP from buspirone (B) by a grapefruit juice sample used in the clinical trials. *In vitro* incubation studies were done as in Figure 1. A) ●, without pre-incubation $IC_{50} = 1.51\%$; ○, with pre-incubation $IC_{50} = 0.78\%$; B) ●, without pre-incubation $IC_{50} = 1.31\%$; ○, with pre-incubation $IC_{50} = 1.00\%$

Figure 3

Inhibition of 4'-OH-flurbiprofen formation from flurbiprofen by the mixture of blueberry juices. Studies were performed as in Figures 1 and 2. ●, without pre-incubation $IC_{50} = 1.57\%$; ○, with pre-incubation $IC_{50} = 1.57\%$

reduced formation of 4'-OH-flurbiprofen (Figure 5, Table 6). BBJ had no significant effect on any of the kinetic variables for flurbiprofen or 4'-OH-flurbiprofen.

The arithmetic mean ratios for flurbiprofen C_{max} and AUC (value with BBJ divided by control value with water) did not differ significantly from 1.0 (Table 5). The 90% CI for the geometric mean ratios for C_{max} and AUC fell entirely within the 0.8, 1.25 default limits. For fluconazole, the arithmetic mean ratio for AUC was significantly larger than 1.0

Figure 4

Mean ($\pm$ SEM) plasma buspirone concentrations in each of the three treatment conditions. ●, with water; ■, with grapefruit juice; ▽, with blueberry juice

and the entire CI for the geometric mean AUC ratio exceeded the 1.25 boundary.

Discussion

In vitro inhibition studies demonstrated that BBJ inhibited CYP3A and CYP2C9 metabolic activity, based on probe substrates commonly applied for this purpose. IC_{50} values

Table 4

Buspirone–blueberry interaction study (CYP3A): summary of pharmacokinetic variables ($n = 12$)

Kinetic variables for buspirone	Mean ($\pm$ SEM) value			Value of F from ANOVA	Dunnett's test	
	With water	With BBJ	With GFJ		BBJ vs. water	GFJ vs. water
$C_{\max}$ (ng ml ⁻¹)	1.14 ($\pm$ 0.24)	1.10 ($\pm$ 0.17)	1.53 ($\pm$ 0.20)	2.02 (NS)	NS	NS
$t_{\max}$ (h)*	0.75 (0.5–1.5)	1.0 (0.5–2.0)	1.5 (0.5–3.0)	3.85 ($P < 0.05$)	NS	$P < 0.05$
$t_{1/2}$ (h)	2.9 ($\pm$ 0.3)	2.9 ($\pm$ 0.1)	2.7 ($\pm$ 0.1)	0.54 (NS)	NS	NS
Total AUC (ng ml ⁻¹ h)	3.11 ($\pm$ 0.52)	4.06 ($\pm$ 0.59)	6.15 ($\pm$ 0.92)	8.74 ($P < 0.002$)	NS	$P < 0.05$
CL/F (l min ⁻¹)	78 ($\pm$ 20)	50 ($\pm$ 9)	33 ($\pm$ 7)	3.97 ($P < 0.05$)	NS	$P < 0.05$
Geometric Mean (90% CI)						
$C_{\max}$ (ng ml ⁻¹)	0.83 (0.5–1.3)	0.92 (0.6–1.3)	1.41 (1.1–1.8)			
Total AUC (ng ml ⁻¹ h)	2.55 (1.8–3.7)	3.54 (2.6–4.8)	5.39 (4.0–7.2)			

*Median and range. BBJ, blueberry juice; GFJ, grapefruit juice.

Table 5

Analysis of ratios for interaction studies

	$C_{\max}$ ratio BBJ / Water	GFJ / Water	Total AUC ratio BBJ / Water	GFJ / Water
Blueberry–buspirone (CYP3A)				
Arithmetic				
Mean	1.81	2.19*	1.89	2.47*
SEM	$\pm$ 0.60	$\pm$ 0.49	$\pm$ 0.45	$\pm$ 0.47
Range	0.22–7.21	0.56–5.93	0.39–5.59	1.18–6.65
Geometric				
Mean	1.1	1.68	1.39	2.12
90% CI	0.64, 1.87	1.14, 2.49	0.90, 2.14	1.59, 2.82
	$C_{\max}$ ratio BBJ/water	Fluconazole/water	Total AUC ratio BBJ/water	Fluconazole/water
Blueberry–flurbiprofen (CYP2C9)				
Arithmetic				
Mean	1.03	1.18*	1.05	1.78*
SEM	0.10	0.07	0.06	0.15
Range	0.64–1.84	0.80–1.53	0.78–1.46	0.88–2.84
Geometric				
Mean	0.98	1.15	1.03	1.73
90% CI	0.82, 1.16	1.02, 1.30	0.94, 1.14	1.46, 2.00

*Significantly different from 1.0. Bold value indicates a 90% CI boundary exceeding 1.25. BBJ, blueberry juice; GFJ, grapefruit juice.

for BBJ were lower than 2% for inhibition of both isoforms. However, pre-incubation of the inhibitory juice sample with metabolic enzyme (in human liver microsomes) prior to addition of substrate did not substantially enhance inhibitory activity of BBJ. We have previously hypothesized that mechanism-based (irreversible) inhibition is a requisite for clinically significant pharmacokinetic interactions involving citrus or fruit beverages and drug substrates of enteric CYP3A [4–7, 12]. Though many citrus and fruit beverages inhibit CYP3A activity *in vitro*, only the furanocou-

marins in GFJ produce mechanism-based inhibition, and only GFJ produces clinically important interactions with drugs metabolized by enteric CYP3A [4–7, 49]. In the case of CYP2C9, a number of fruit beverages and herbal products inhibit *in vitro*, but inhibit minimally if at all *in vivo* [4, 45, 46].

The commercially available GFJ used as a positive control inhibitor in the present study contained the furanocoumarin derivatives 6,7'-DHB and Paradisin C, considered to be two of the mechanism-based CYP3A inhibi-

Figure 5

Mean ($\pm$ SE) plasma flurbiprofen concentrations (A) and 4'-OH-flurbiprofen concentrations (B) in each of the three treatment conditions. ●, with water; ▽, with blueberry juice; ◆, with fluconazole

Figure 6

Mean ($\pm$ SEM, $n = 12$) plasma fluconazole concentrations during the flurbiprofen-fluconazole trial

tors of principal importance in terms of drug interactions with CYP3A substrates [4–7, 9–12]. However, the concentrations of these two components was relatively low compared to values reported for many grapefruit juice entities from different regions [12, 31]. In any case, the GFJ used in this study clearly produced *in vitro* inhibition of CYP3A that was enhanced by pre-incubation.

The clinical pharmacokinetic study of BBJ effects on CYP3A activity utilized buspirone as the index substrate. Buspirone is considered to be a highly sensitive CYP3A substrate, since its oral bioavailability is estimated to be less than 5% [36–39]. In a previous drug interaction study of buspirone with GFJ [41], buspirone AUC was increased

by a factor of approximately nine-fold. The GFJ administration scheme in that study was 200 ml of double-strength juice three times daily for 2 days prior to buspirone. In contrast, we administered 300 ml of low furanocoumarin juice twice prior to buspirone. Nonetheless, this GFJ preparation significantly increased buspirone AUC by a factor in the range of 2.0 to 2.5. In contrast, co-administration of BBJ caused a non-significant change in buspirone AUC. The geometric mean ratio of buspirone AUC (value with blueberry juice divided by water control) was 1.39, with a 90% CI of 0.90, 2.14. The 'no interaction' default criteria specified by the American Food and Drug Administration require that the 90% CI for the geometric mean AUC ratio falls entirely within the 0.80, 1.25 boundaries. Therefore the findings with buspirone do not qualify as 'no interaction.' Still, the geometric mean ratio of 1.39 itself falls in the range of 1.25 to 2.0, thereby classified as a 'weak' inhibitory effect of BBJ that could have occurred by chance. It is also possible that the effect of BBJ was a non-specific 'food effect,' as reported in the product label for buspirone. The approved label describes a study in which the buspirone AUC increased by 84% (that is, by a factor of 1.84) when administered to healthy volunteers with food as compared with without food. However, the study results have not been published in the peer-reviewed literature. A study of gepirone, structurally and pharmacokinetically similar to buspirone, showed that co-administration with food increased AUC by a factor of 1.48 [50].

Flurbiprofen was the index substrate used to profile CYP2C9 activity *in vivo* [44–46]. The positive control inhibitor, fluconazole, significantly inhibited clearance of flurbiprofen and formation of 4'-OH-flurbiprofen both *in vitro* and *in vivo*. BBJ inhibited biotransformation of flurbiprofen *in vitro*, but in the clinical study had no effect on

Table 6

Flurbiprofen-blueberry interaction study (CYP2C9): summary of pharmacokinetic variables (n = 12)

Kinetic variables	Arithmetic mean (± SEM) values			Value of F from ANOVA	Dunnett's test	
	With water	With BBJ	With fluconazole		BBJ vs. water	Fluconazole vs. water
Flurbiprofen						
C _{max} (µg ml ⁻¹)	11.3 (± 0.8)	11.5 (± 1.3)	13.1 (± 1.0)	1.66 (N.S.)	NS	NS
t _{max} (h)*	1.25 (0.5–3)	1.0 (0.5–4)	2.0 (1–6)	3.23 (P = 0.06)	NS	P < 0.05
t _{1/2} (h)	4.2 (± 0.6)	4.4 (± 0.8)	5.9 (± 0.3)	6.8 (P < 0.005)	NS	P < 0.05
Total AUC (µg ml ⁻¹ h)	69.5 (± 11.3)	72.8 (± 12.4)	112.7 (± 10.3)	19.5 (P < 0.001)	NS	P < 0.05
CL/F (ml min ⁻¹)	28.7 (± 3.1)	28.1 (± 3.3)	16.4 (± 1.7)	30.3 (P < 0.001)	NS	P < 0.05
4'-OH-flurbiprofen						
C _{max} (µg ml ⁻¹)	0.6 (± 0.1)	0.6 (± 0.1)	0.3 (± 0.03)	11.3 (P < 0.001)	NS	P < 0.05
AUC (0–12h) (µg ml ⁻¹ h)	3.4 (± 0.5)	3.4 (± 0.6)	2.0 (± 0.2)	13.8 (P < 0.001)	NS	P < 0.05
Geometric mean (90% CI)						
Flurbiprofen						
C _{max} (µg ml ⁻¹)	11.0 (9.7, 12.6)		10.9 (9.2, 12.9)	12.7 (11.7, 14.5)		
Total AUC (µg ml ⁻¹ h)	62.7 (49.9, 78.8)		64.9 (50.9, 82.7)	107.1 (89.7, 127.9)		
4'-OH-flurbiprofen						
AUC (0–12h) (µg ml ⁻¹ h)	3.0 (2.1, 4.1)		2.8 (2.0, 4.1)	1.7 (1.2, 2.4)		

*Median and range. BBJ, blueberry juice.

flurbiprofen pharmacokinetics. The geometric mean AUC ratio (BBJ value divided by water control value) was 1.03, with a 90% CI of 0.94, 1.14 falling entirely within the 0.80, 1.25 'no interaction' boundaries in American Food and Drug Administration guidance documents.

In summary, the clinical pharmacokinetic studies provided no evidence for concern about clinically important pharmacokinetic drug interactions of BBJ with substrate drugs metabolized by CYP3A or CYP2C9.

Competing Interests

All authors have completed the Unified Competing Interest form at http://www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare the work was supported by grants from the United States Highbush Blueberry Council and the Wild Blueberry Association of North America, DJG has served as a consultant to the Florida Department of Citrus in the previous 3 years, PFC is an employee of the Florida Department of Citrus and no other relationships or activities that could appear to have influenced the submitted work.

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