Evaluation of metabolism and cytochrome P450 mediated interaction liabilities of naringenin

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Abstract

Naringenin is one of the major components of grapefruit juice. It has a broad spectrum of pharmacological activities, and many studies report that grapefruit juice inhibits cytochrome P450 (CYP) 3A4 leading to drug interactions. Naringenin was profiled through various *in vitro* studies like metabolic stability and glucuronidation in rat and human liver microsomes while, CYP inhibition using human liver microsomes. In addition, pharmacokinetic profiling was conducted upon intravenous (*i.v.*) and oral administration in rats. Naringenin undergoes both phase I and phase II metabolism in rat liver microsomes, and in human liver microsomes, it is predominantly metabolized by phase II. Glucuronidation which is addition (conjugation) of glucuronic acid to various functional groups is one of the major metabolic pathways of Naringenin. Naringenin, at 1.0 µM and 10.0 µM, did not elicit any appreciable inhibition of the 5 major CYP isoforms (CYP1A2, CYP3A4, CYP2C9, CYP2C19, and CYP2D6). Oral pharmacokinetic studies at 100, 300,and 1000 mg/kg dose and intravenous pharmacokinetic studies at 1 mg/kg dose were performed in male SD rats. Naringenin exhibited very short half-life (0.27 h) and rapid elimination (Clearance=110.65 mL/min/kg) after *i.v.* administration. There was saturation in C_{max} and exposure beyond 100 mg/kg, and the absolute bioavailability was found to be ≤ 5% at the tested oral doses. This present experiment suggests that naringenin does not substantially inhibit CYP3A4 (or any of the tested five isoforms) isoforms *per se*. Given the minimal involvement of CYP enzymes in the metabolism of naringenin and minimal inhibition of CYP enzymes (IC_{50}> 10 µM), the potential for drug-drug interactions involving CYP substrates and inhibitors is very minimal in humans.

Introduction

Drug Metabolism and Pharmacokinetics (DMPK) is one of the essential components in drug development.[1] Evaluation of drug metabolism and pharmacokinetics from *in vitro* and *in vivo* data gives us an insight into the potential drug candidates. These *in vitro* studies are useful in determining drug stability or its liability to pharmacokinetic drug interactions. It would be futile if the new chemical entities are taken into clinical trials without appreciable evaluation in DMPK, *i.e.*, there would be financial risks as well as time and resources. The objective of these pre-clinical studies is not only to identify the most active leads with the most appropriate safety profiles but also, to select the closest animal species to humans for toxicity studies.[2] In designing appropriate human clinical trials, understanding pharmacokinetics and metabolism characteristics of the selected compounds is a prerequisite. There is a large scientific and public interest in flavonoids due to their presumed association with beneficial effects on health issues.

Grapefruit is an excellent source of many nutrients and phytochemicals that contribute to a healthy diet.[3] Grapefruit is endorsed by the American Heart Association as a "heart-healthy food" because it contains compounds that may reduce the formation of atherosclerotic plaques. It may also inhibit cancer cell proliferation. Grapefruit contains many important nonflavonoid (furanocoumarins) constituents like bergamottin,6,7-dihydroxy bergamottin, bergaptol,6,7-epoxy bergaptol and flavonoids like naringin, naringenin, kaempferol, quercetin, sesquiterpenes (nootkatone), polyamines (*e.g.*, putrescine), and limonoids. Naringin is the most abundant flavonoid in grapefruit juice, present in concentrations of up to 1mM/L.[4] Naringenin is an aglycone, and it is also present in tomato skin (*Solanum lycopersicum*) and oranges (*Citrus sinensis*), *i.e.,* mostly in citrus species. It is weakly acidic in nature. Grapefruit and its products are very much prone to drug interactions because of the presence of many flavonoids and furanocoumarins. The actual cause for the drug interactions is due to the presence of these constituents. Because most of the constituents have the ability to block CYP450 enzymes, these CYP450 enzymes play a major role in the metabolism of most of the xenobiotics, so there is more chance for causing drug interactions.

Naringenin was selected owing to its desirable pharmacological actions, including anti-microbial, anti-ulcer, anti-oxida-

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Contributions: MS and RRS, both participated in all aspects starting from study design, execution, data collection and preparing the manuscript.

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Human and animal rights: no humans were involved in this study. The reported experiments on animals were in accordance with the standards set forth in the 8th Edition of Guide for the Care and Use of Laboratory Animals (http:// grants.nih.gov/grants/olaw/Guide-for-thecare-and-useof- laboratory-animals.pdf) published by the National Academy of Sciences.

Availability of data and materials: the European Medicines Agency (EMA) Guideline on the Investigation of Drug Interactions (Adopted in 2012).

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tion, superoxide scavenging, and anti-cancer effects. Nevertheless, a lot of them studied *in vitro* outcomes. Naringenin is currently undergoing phase I clinical trials, which proves the interest of researchers to develop it as a possible clinical candidate in some of the therapeutic areas. Grapefruit juice constituents has broad spectrum of pharmacological activities,[5-25] and many studies report that grapefruit juice inhibits cytochrome P450 (CYP) 3A4 leading to drug interactions.[26-40] On the basis of previous literature reviews supporting the CYP mediated drug-drug interactions of grapefruit juice, naringenin being one of the principal constituents of grapefruit juice, was screened *per se* to investigate its role in causing drug interactions. Hence the present study was chosen in order to predict various parameters of naringenin and evaluating its drug interaction potential. The profiling studies included *in vitro* metabolic stability and *in vitro* CYP inhibition potential to generate evidence about CYP mediated drug interaction liability of naringenin.

Majority of the articles, which reported interactions with naringenin were in preclinical species.[41-44] However, there are no human microsomal studies evaluated for inhibitory potential. Although NCEs (new chemical entities) are thoroughly evaluated prior to animal testing, the same approach is not being followed for natural products. Present study helps researchers understand the importance of *in vitro* tests prior to animal testing for active components derived from natural products especially to evaluate drug interaction potential. This approach helps in identifying if flavonoids like naringenin could cause drug interactions.

Materials and Methods

Chemicals

Naringenin, Uridene diphosphoglucuronic acid (UDPGA), Alamethacin, Saccharolactone, Losartan, Phenacetin, Diltiazem, α-Napthoflavone, Testosterone, Ketoconazole, Diclofenac, Sulphaphenazole, *S*-Mephenytoin, Ticlopidine, Dextromethorphan, Qunidine, and Tolbutamide were purchased from Sigma Chemical Co. Dimethyl sulfoxide (DMSO) and Potassium di-hydrogen phosphate (KH_2PO_4) were purchased from Aldrich. Acetonitrile, methanol and methyl tertiary butyl ether (TBME) were of HPLC grade and were purchased from JT Baker. Ammonium acetate and potassium hydroxide were purchased from Merck. β-nicotinamide adenine dinucleotide phosphate (NADPH) was obtained from Sisco research labs. Rat and human liver microsomal proteins were obtained from Xenotech. 1-Aminobenzotriazole (1-ABT) and formic acid were obtained from Fluka. Other reagents were of analytical grade. Milli-Q plus water (in-house) was used throughout this study.

Instrumentation and LC/MS/MS Conditions

The LC/MS/MSwas equipped with a pump (LC-10AT, Shimadzu, Japan), a diode array detector (SPD-M10AVP, Shimadzu, Japan), an automatic injector (SIL-10A, Shimadzu, Japan) and a Phenomenex C18 column (5 µm, 50 x 3.0 mm, Nacalai tesque, Kyoto, Japan). The column oven temperature and autosampler temperature are maintained at 40 ± 2ºC and 10 ± 2ºC, respectively. The mobile phase consisted of 0.1% formic acid in water: 5 mM ammonium acetate buffer (pH ~ 3.4) and acetonitrile (premix 5: 95). Gradient elution was done at a flow rate of 0.6 mL/min for naringenin assay in serum. Detection was done using positive ionization in MRM (multiple reaction monitoring) mode using API3000 mass spectrometer and protonated molecule was *m/z* 273.4 for naringenin The mass spectrometer operated using electrospray ionization (ESI) with an ion spray voltage of +4500V.

Evaluation of metabolic stability and glucuronidation of naringenin in liver microsomes

The metabolic stability of naringenin in human and rat liver microsomes was evaluated at 1 µM. A 5 mM stock of naringenin was prepared in DMSO from which a working stock of 0.1 mM in DMSO was prepared. A 5 µL aliquot of test solution from the working stock and 25 µL of 20 mg/mL microsomal protein were spiked into 445 µL of KH_2PO_4 buffer (pH 7.4) to get a final concentration of 1.0 µM naringenin and 1.0 mg/mL microsomal protein, respectively. Reaction was initiated by the addition of 25 µL of 48 mM of NADPH (final concentration of 2.4 mM) solution in water. The reaction mixture was incubated at 37°C for 60 min and reaction was terminated by the addition of 3 mL of TBME. For the 0 hr samples, reaction was terminated with 3 mL of TBME prior to the addition of NADPH. All the incubations were carried out in triplicate. As an internal standard, 25 µL of 20 µg/mL of losartan in methanol was spiked in both 0 hr and 1 hr samples. The samples were processed by vortex mixing for 5 min and centrifuging at 3000 rpm for 5 min. The clear supernatant was separated and dried under nitrogen. The residue was reconstituted by adding 500 µL of acetonitrile: 2 mM ammonium acetate buffer [pH 3.4 (90:10 v/v)]. The samples were injected into LC/MS/MS system. Diltiazem (highly metabolized) and tolbutamide (highly stable) were used as positive controls to ensure the system suitability. The data was compared with the historical values determined in house during method validation. The assay was considered acceptable if the experimental values match with the historical values.

The glucuronidation potential of naringenin was evaluated based on the structural liability of naringenin (presence of –OH groups). The active site of the Uridene Diphospho Glucuronosyltransferase (UGT) faces the lumen of the Endoplasmic Reticulum (ER). Non polar substrates can diffuse through the ER membrane and be conjugated in the ER lumen. However, UDPGA must be transported into the ER to facilitate the reaction. Since the experiment was done with liver microsomes, a pore forming agent alamethacin was used which facilitates the transport of UDPGA into the ER. For glucuronide quantitation, an aliquot of 25 µL of 20 mg/mL liver microsomal protein was spiked into 302.5 µL of KH_2PO_4 buffer to get a final concentration of 1 mg/mL protein. To this 12.5 µL of 1 mg/mL alamethacin solution in ethanol was added to get a final alamethacin concentration of 0.025 mg/mL. The above preparation was pre incubated at 2-8ºC for 10 min. After pre incubation, 40µL of 50 mM $MgCl_2$ in water (final concentration 4 mM), 25 µL of 48 mM of NADPH (final concentration of 2.4 mM), 50 µL of 20 mM UDPGA solution in water (final concentration 2 mM), 40 µL of 100 mM saccharolactone in water (final concentration of 8 mM) were added and the preparation was incubated at 37ºC for 10 min. A 5 mM stock of naringenin was prepared in DMSO from which a working stock of 0.1 mM in DMSO was prepared. A 5 µL aliquot of test solution from the working stock was added to get a final concentration of 1.0 µM naringenin. After addition of naringenin, 0 hr samples were immediately terminated with 500 µL of acetonitrile: methanol (80:20 v/v). The test samples were terminated similarly after incubation of 60 min. As an internal standard (IS), 5 µL of 2.0 µg/mL of losartan in methanol was spiked in both 0 hr and 60 min test samples. The samples were processed by vortex mixing for 5 min and centrifuging at 3000 rpm for 5 min. The clear supernatant was transferred into labelled vials and the samples were analyzed by LC/MS/MS system.

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Evaluation of role of CYP enzymes in the metabolism of naringenin *in vitro* and *in vivo*

The function of CYP450 *in vitro* in the phase I metabolism of naringenin in rat and human liver microsomes was evaluated at 1 µM. A 60 mM working stock of 1-ABT in DMSO was prepared. A 2.5 µL aliquot of 1-ABT from the working stock, 25 µL of 20 mg/mL microsomal protein and 25 µL of 48 mM NADPH solution were spiked into 445 µL of KH_2PO_4 buffer (pH 7.4) and this mixture was pre incubated for 30 min at 37°C. The final concentration of 1-ABT and NADPH in the incubation mixture was 300 µM and 2.4 mM, respectively and that of microsomal protein was 1 mg/mL. For the control samples, all the above constituents were spiked except 1-ABT, instead of 1-ABT solution 2.5 µL of DMSO (vehicle) was spiked and the reaction mixture was incubated for 30 min at 37°C. After 30 min of pre incubation, 2.5 µL of 0.2 mM naringenin was spiked into the reaction mixture to both 1-ABT and control samples. The 1-ABT samples were allowed to incubate for 60 min at 37°C, whereas the control samples were immediately terminated with 3 mL of TBME. After 60 min, the reaction was terminated by the addition of 3 mL of TBME. All the reactions were carried out in triplicate. As an internal standard, 25 µL of 20.0 µg/mL of losartan in methanol was spiked in control and 1-ABT samples. The samples were processed by vortex mixing for 5 min and centrifuging at 3000 rpm for 5 min. The clear supernatant was separated and dried under nitrogen. The residue was reconstituted by adding 500 µL of acetonitrile: 2 mM ammonium acetate buffer [pH 3.4 (90:10 v/v)]. The samples were injected into LC/MS/MS system.

The role of CYP enzymes in the metabolism of naringenin was evaluated *in vivo* in male SD rats, weighing 180 to 220 g. They were housed in a temperature (22±1°C) and relative humidity (55±10%) in a controlled room. They were allowed free access to food and water in a controlled 12-hr light/dark cycle. For this study, all animals were fasted overnight (12 hours) before dosing and continued until 4 h after administration of test sample, there after rat chew diet was provided *ad libitum*. Drinking water was deprived of cages before dosing and continued until 2 h post dose, thereafter it was provided *ad libitum*. The vehicle [2.5 µL/mL tween 80 + 0.5% (w/v) methyl cellulose suspension] and 1-ABT suspension (100 mg/kg) were administered to control group (Group I) and 1-ABT group (Group II), respectively. Two hours post administration, naringenin suspension (50 mg/kg) was administered to both the groups by oral gavage. The blood samples (~ 200 µL at each collection time-point) were collected according to the sampling schedule from orbital sinus into microfuge tubes containing disodium EDTA as anticoagulant. Blood samples were centrifuged within 10 min at 13000 rpm for 3 min at 4°C. The separated plasma samples were analyzed for naringenin concentrations using LC/MS/MS. The plasma concentration *vs.* time data was subjected to non-compartmental analysis for estimation of pharmacokinetic parameters using WinNonlin® software (Pharsight version 5.2).

CYP inhibition of naringenin

CYP inhibition potential of naringenin on CYP1A2, CYP3A4, CYP2C9, CYP2C19 and CYP2D6 isoforms was evaluated in pooled human liver microsomes at 1 µM and 10 µM. The test compound was allowed to incubate with the recommended standard substrates of the specific isoforms in human liver microsomes. After termination of the incubation; the samples were processed and analyzed for the standard metabolites using LC/MS/MS system. A 5 mM stock of naringenin was prepared in DMSO from which a working stock of 0.4 mM and 4 mM in DMSO was prepared. A 2.5 µL aliquot of test item from the working stocks and 25 µL of 10 mg/mL microsomal protein were spiked into 920 µL of KH_2PO_4 Buffer (pH 7.4) to get a final concentration of 1.0 µM and 10.0 µM of test item and 0.25 mg/mL protein, respectively. For the control samples, 2.5 µL of DMSO was spiked instead of naringenin and rest of the constituents remained same. A 2.5 µL of respective substrates (as shown in Table 1) were spiked in both control and test samples. The reaction mixture was allowed to pre incubate for 10 min at 37°C. Reaction was initiated by the addition of 50 µL of 48 mM of NADPH (final concentration of 2.4 mM) solution in water. The reaction mixture was incubated at 37°C for 15 min (except for CYP2C19 for which the incubation is carried out for 30 min) and reaction was terminated by the addition of 3.0 mL of TBME. All the incubations were carried out in triplicate. As an internal standard, 25 µL of respective standards were spiked in both control and test samples. The samples were processed with the same procedure that was followed for glucuronidation of naringenin. In each experiment, a positive control (known inhibitor) was used at a concentration close to its IC_{50} in order to assess the assay suitability. The assay was considered valid if the suitability criteria were met in accordance with the corresponding validation data.

Pharmacokinetic profiling of naringenin upon i.v. administration in rats

The intravenous pharmacokinetic profile of naringenin was evaluated in male SD rats at 1 mg/kg dose. The intravenous formulation (2 mg naringenin + 10% Pharmasolve + 10% Ethanol + 80% PEG 200: water (3:2 pre mix)) was freshly prepared on the day of administration. Rats were placed in the anesthesia chamber and were anesthetized by isoflurane for about 1 min. The anesthetized animals were placed in the rat restrainer and the intravenous solution was administered through the lateral tail vein. The blood samples were collected and analyzed using Liquid Chromato-graphy/Mass Spectrometry (LC/MS/MS).

The oral dose proportionality and absolute bioavailability of naringenin were evaluated at 100, 300 and 1000 mg/kg doses in male SD rats. The freshly prepared naringenin suspension in 2.5 µL/mL tween 80 + 0.5% (w/v) methyl cellulose suspension (q.s.) as vehicle was administered to the animals by oral gavage.

Table 1. Positive control substrates and inhibitors used for reaction phenotyping.

CYP isoenzyme	Positive control	Positive control concentration (µM)	Inhibitor	Positive control values (IC_{90})
CYP1A2	Phenacetin	125	α-Naphthoflavone	86.54
CYP2D6	Dextromethorphan	8	Quinidine	94.18
CYP3A4	Testosterone	75	Ketoconazole	91.10
CYP2C9	Diclofenac	25	Sulphaphenazole	92.86
CYP2C19	S-Mephenytoin	125	Ticlopidine	93.18

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Results and Discussion

Evaluation of metabolic stability and glucuronidation of naringenin in liver microsomes

To evaluate the metabolic stability data of naringenin in human and rat liver microsomes at 1 µM, the peak area ratios of test sample to internal standard in all samples were analyzed by LC/MS/MS method (using Analyst software version 1.4.1). Based on the comparison of area ratios of test sample to internal standard in the 60 min test samples in relation to the 0 min sample, the percent remaining of the parent was estimated using following equation:

% remaining = [(Mean peak area ratio of analyte/IS in 60 min sample)/ (Mean peak area ratio of analyte/IS in 0 min sample)] x 100

It was observed that naringenin was stable in human liver microsomes with 88.72% remaining, indicating very minimal phase I mediated metabolism in human. But naringenin showed low metabolic stability in rat liver microsomes with 16.2% remaining, indicating high phase I mediated metabolism in rats (Table 2). Naringenin showed 100% metabolism through glucuronidation in both rat and human liver microsomes, indicating glucuronidation as the major metabolic pathway of naringenin.

Evaluation of role of CYP enzymes in the metabolism of naringenin *in vitro* and *in vivo*

Role of CYP enzymes in the metabolism of naringenin in vitro

Substantial improvement in the percentage stability of naringenin was observed in rat liver microsomes from ~ 16% to ~ 68% in the presence of 1-ABT. This shows the significant role of CYP450 in the phase I metabolism of naringenin in rat. However, in human liver microsomes there was a marginal improvement in the stability with ~ 94% remaining in the presence of 1-ABT (Table 2).

The analyte to IS peak area ratios (p.a.r.) at 0 mins to 60 mins were used to calculate the percent metabolism observed in the presence and absence of inhibitor using the below mentioned formula:

Percent metabolism = 100 – [(Area ratio at 60 mins) / (Area ratio at 0 mins)] x 100

Area ratio = Analyte area / IS area

Role of CYP enzymes in the metabolism of naringenin in vivo

The following pharmacokinetic parameters were estimated from *in vivo* data: C_{max}(maximum observed concentration), AUC $_{(0-t)}$ (area under curve at time 0-t), AUC $_{(0-\infty)}$(area under curve at 0-inifinity), T_{max}(time to reach maximum concentration) and $T_{1/2}$ (elimination half-life). The AUC (area under curve) was estimated using linear trapezoidal rule. The median T_{max} observed was 0.5 hr in the control group and 6 h in the 1-ABT group showing a delayed absorption in the 1-ABT pretreated animals. The mean C_{max} observed was 110 ng/mL and 111 ng/mL in the control group and 1-ABT pretreatment group, respectively. The mean AUC_{0-t} observed was 251 ng.h/mL and 499 ng h/mL in the control group and 1-ABT pretreatment group, respectively. Though there was no change in the C_{max} between the groups, the exposure increased ~ 2-fold in the 1-ABT group which shows the possible role of CYP450 in the metabolism of naringenin *in vivo* as shown in Figure 1.

CYP inhibition of naringenin

CYP inhibition of naringenin for 5 major CYP isoforms (CYP1A2, CYP3A4, CYP2C9, CYP2C19 and CYP2D6) was evaluated in pooled human liver microsomes under standardized conditions. The peak area ratio of the metabolite to Internal Standard (IS) in control and test samples was obtained to calculate the percent inhibition using following equation:

% Inhibition = 100 –[(peak area ratio of metabolite/IS in test sample)/ (peak area ratio of metabolite/IS in control sample)] x 100

Naringenin, at 1.0 µM and 10.0 µM, did not elicit any appreciable inhibition (Table 3) of the 5 major CYP isoforms (CYP1A2, CYP3A4, CYP2C9, CYP2C19 and CYP2D6). Grape fruit juice was known to be a potent inhibitor of CYP3A4 isoform and also many drug interactions with standard CYP3A4 substrates were reported. The present experiment concludes that naringenin, one of the principal constituents of grape fruit juice do not substantially inhibit CYP3A4 (or any of the tested five isoforms) isoforms *per se.*

From the previous experiment, it was proven that the *in vitro* metabolic stability of naringenin was improved in 1-ABT preincubated microsomal preparations indicating the involvement of CYP450 enzymes in the metabolism of naringenin. The exposure observed in the 1-ABT pretreatment group was ~2-fold higher compared to the control group, which concludes the role of CYP450 mediated metabolism of naringenin. This was in correlation with the *in vitro* data, where the percent stability increased from 16% to 68% in 1-ABT incubated microso-

Table 2. Percentage stability of naringenin in rat and human liver microsomes.

Liver microsomes	% Stable	
	Absence of 1-ABT	In presence of 1-ABT
Rat	16.2	68.4
Human	88.7	94.0

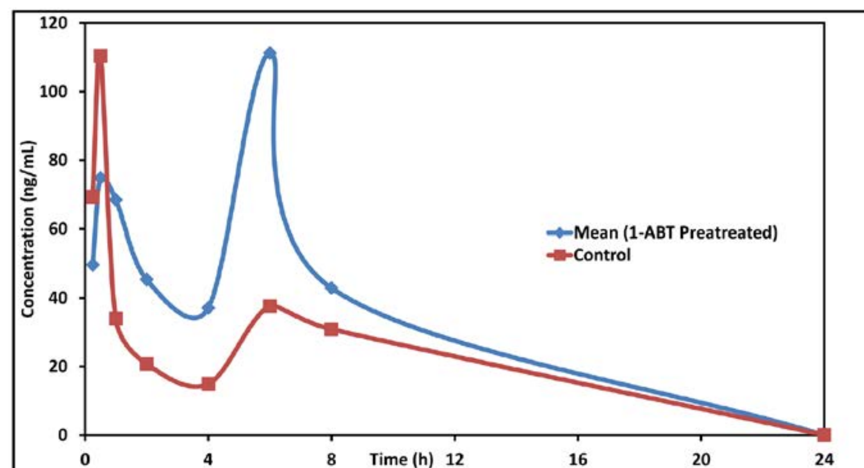

Figure 1. Mean plasma concentration profiles of naringenin *versus* time.

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mal preparations. The incubation data of positive controls confirmed the integrity of the test system and showed approximately 90% inhibition of the metabolite formation in comparison to the control at the IC_{90} concentrations of the inhibitor.

Prediction of drug interaction potential of naringenin

As per the FDA recommendation, I/Ki ratio serves as a standard tool to predict the possibility of any possible clinically relevant Drug-Drug Interactions (DDI). Naringenin did not show any appreciable inhibition of the five major CYP isoforms tested at 10 µM. So the IC_{50} will be greater than 10 µM. The therapeutic concentration of naringenin given in clinics be: 1 µM, then I/Ki will be less than 0.1; hence the possibility of DDI will be remote. Between 1 and 10 µM, then I/Ki ratio will be between 0.1 and 1.0; hence the possibility of DDI will be possible, Greater than 10 µM, then I/Ki ratio will be >1.0; hence the possibility of DDI will be likely.

Pharmacokinetic profiling of naringenin up on i.v. administration in rats

Analyte concentrations were determined by evaluating the peak area ratio of drug to IS against a standard curve. The standard curve was prepared by plotting the relationship between peak area ratio of naringenin to IS against the concentration of standard calibration samples by using linear regression y = ax + b and putting the $1/x^2$ as aweighting factor. The measured peak area ratio of the QC and unknown samples were converted into concentrations using the following equation:

Analyte concentration =
[p.a.r.(analyte / IS)/b]– a

Where a: intercept of the corresponding standard curve, b: slope of the corresponding standard curve.

The concentrations were reported in nanogram per milliliter plasma.

The plasma concentration vs. time profile obtained after a single dose intravenous administration of naringenin at 1 mg/kg dose is shown in Figure 2. Levels of naringenin were observed up to 1.0 hours post dose in plasma. The mean C_0 and exposure (AUC_{0-t}) were 628.10 ng/mL, and 145.25 ng hr/mL, respectively. The mean terminal half life was 0.27 hr, and naringenin was distributed with a mean volume of distribution of 2553.87 mL/kg and was eliminated very rapidly with a mean clearance of 110.65 mL/min/kg after intravenous administration. The mean plasma concentration profile of naringenin when administered orally at 100, 300 and 1000 mg/kg of dose (Figure 3), showed a quick absorption with median T_{max} ranging from 0.5 to 2 h across the doses. The animals showed a mean C_{max} of 180.53, 134.33 and 295.19 ng/mL and a mean AUC_{0-t} of 739.73, 622.42 and 1463.08 ng.hr/mL at 100, 300 and 1000 mg/kg, respectively. The mean apparent elimination half-life was 4.09, 2.94 and 2.99 hr at 100, 300 and 1000 mg/kg doses, respectively. Dose proportionality in mean C_{max} and mean AUC_{0-t} across the doses was evaluated by dose normalizing the mean C_{max} and mean AUC_{0-t}. The dose normalized mean C_{max} was 1.08, 0.4 and 0.3 ng/mL/mg/kg at 100, 300 and 1000 mg/kg dose levels, respectively and the dose normalized mean AUC_{0-t} was 7.4, 2.1 and 1.5 ng.hr/mL/mg/kg at 100, 300 and 1000 mg/kg dose levels, respectively indicating saturation in C_{max} and exposure beyond 100 mg/kg dose (Figure 3). The mean elimination half life of naringenin was found to be

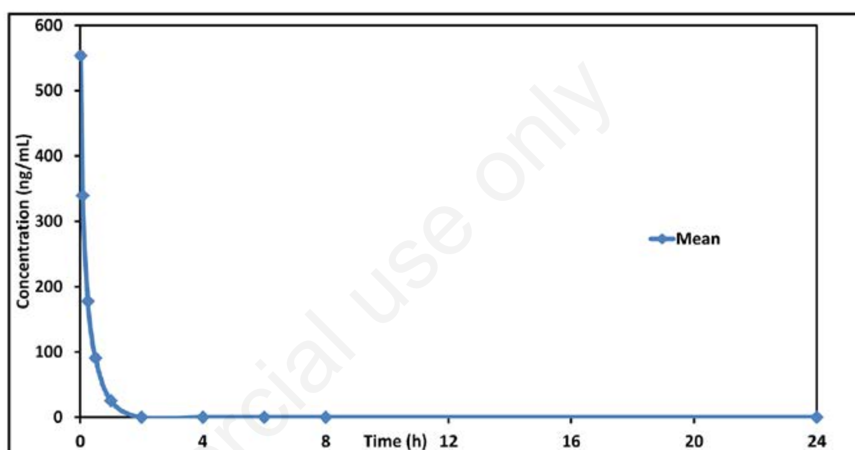

Figure 2. Mean plasma concentration profiles of naringenin *versus* time upon i.v. administration.

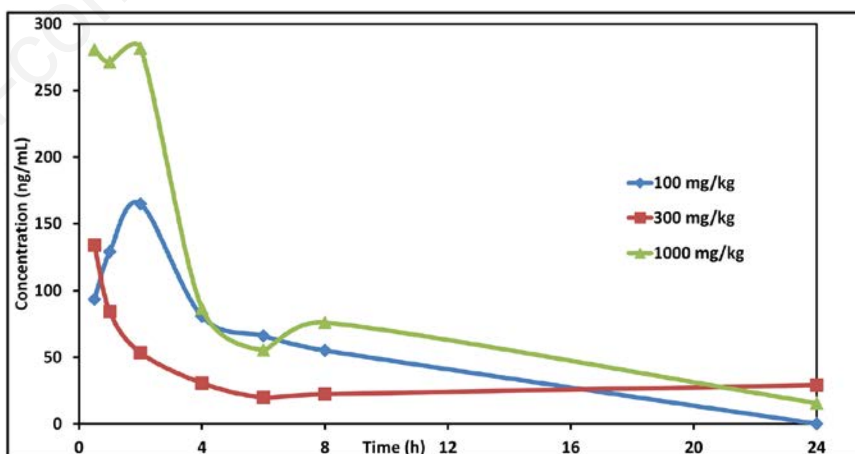

Figure 3. Mean plasma concentration profiles of naringenin *versus* time upon oral administration.

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Table 3. Cyp inhibition potential of naringenin in human liver microsomes.

% Inhibition	Conc.	CYP1A2	CYP3A4	CYP2C9	CYP2C19	CYP2D6
Human	1 µM	21.88	-10.14	-7.92	0.58	-15.39
	10 µM	35.91	14.82	9.43	27.45	-19.91

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0.27 hr and mean clearance was 110.65 mL/min/kg which indicate a very short half life and rapid elimination of naringenin.

Naringenin showed both phase I and phase II metabolism (glucuronidation) in rat liver microsomes, whereas in humans, metabolism was predominantly by phase II and especially by glucuronidation pathway. The percent stability of naringenin in rat liver microsomes was 16%, which substantially increased to 68% in 1-ABT preincubated microsomes, indicating the involvement of CYP450 in the phase I mediated metabolism of naringenin. This was further concluded by a 2-fold increase in exposure in 1-ABT pretreated rats compared to control animals. However, in human liver microsomes, very minimal phase I mediated metabolism was seen with ~ 90% stability post 60 min incubation at 37ºC; hence very minimal CYP involvement in the metabolism of naringenin was expected in humans. Naringenin showed 100% metabolism in both rat and human liver microsomal incubations done in the presence of alamethacin and UDPGA, indicating glucuronidation is the major metabolic pathway. CYP inhibition of naringenin for 5 major CYP isoforms (CYP1A2, CYP3A4, CYP2C9, CYP2C19, and CYP2D6) was evaluated in pooled human liver microsomes under standardized conditions. Naringenin, at 1.0 µM and 10.0 µM, did not elicit any appreciable inhibition of the 5 major CYP isoforms (CYP1A2, CYP3A4, CYP2C9, CYP2C19, and CYP2D6). Considering the minimal involvement of CYP enzymes in the metabolism of naringenin and minimal inhibition of CYP enzymes by naringenin (IC50 > 10 µM), the potential for drug-drug interactions involving CYP substrates and inhibitors is very minimal in humans. Oral PK studies at 100, 300, and 1000 mg/kg dose, and *I.V.* PK study at 1 mg/kg dose was performed in male SD Rats. The absolute bioavailability of naringenin was found to be low with 5%, 1.4%, and 1.0% bioavailability at 100, 300, and 1000 mg/kg doses, respectively. There was saturation in C_{max} and exposure beyond 100 mg/kg dose.

Conclusions

Naringenin, a major constituent of grapefruit juice has majorly been investigated in preclinical studies to characterize its drug interaction potential; however, the translation of preclinical drug interaction potential to clinical is not known[43] until it is proven. In the current investigation, we tried to profile the CYP mediated drug interaction potential of naringenin using human liver microsomes in addition to profiling in pre-clinical species (in rat using both *in vitro* and *in vivo*). Findings from human liver microsomal studies can be better correlated with human *in vivo* experience. Naringenin undergoes both phase I and phase II metabolism in rat liver microsomes, and in human liver microsomes, it is predominantly metabolized by glucuronidation. This confirms that the role of CYP enzymes in the metabolism of naringenin in human is minimal. Naringenin, at 1.0 µM and 10.0 µM, did not elicit any appreciable inhibition of the 5 major CYP isoforms (CYP1A2, CYP3A4, CYP2C9, CYP2C19, and CYP2D6). Results from our study suggests that naringenin does not substantially inhibit CYP3A4 (or any of the tested five isoforms) isoforms *per se*. Given the minimal involvement of CYP enzymes in the metabolism of naringenin and minimal inhibition of CYP enzymes (IC_{50}> 10 µM), the potential for drug-drug interactions involving CYP substrates and inhibitors is very minimal in humans.

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