GRAPE FRUIT JUICE IMPROVES GLYCEMIC INDEX AND UP-REGULATES EXPRESSION OF HEPATIC ORGANIC CATION TRANSPORTER PROTEIN (OCT1) IN THE RAT

Submitted in Partial Fulfilment of the Requirements for the Award of the Degree of Doctor of Philosophy (Pharmacology) the Department of Pharmacology, School of Pharmacy and Pharmacology, Faculty of Health Sciences, University of KwaZulu-Natal, Private Bag X54001, Durban 4000, South Africa.

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March 2009
DECLARATION

I hereby certify that the work presented in this thesis is original and that all ideas and opinions expressed in this article are my own creation. This work has not previously been presented in part or in full by me to any other university for any other degree. Referenced materials have duly been acknowledged in the text. There are no 'competing interests'.

PMO Owira
Student Reg No: 206526650

March 2009
DEDICATION

To my mother who taught me how to read and write, and to my students who always think that I know more than I actually do.
ACKNOWLEDGEMENTS

I extend sincere thanks and gratitude to my supervisor, Prof John A. O. Ojewole, for his relentless support and encouragement. Without him, this work would not have been completed.

I would like to extend my special thanks to the following people:
Linda Bester and the technical staff at the Biomedical Research Unit;
Youna Moodley and Kheswa Nokwethemba for helping with in vivo work;
Kogi Moodley and Ms Rudo Mapanga of Physiology Department, UKZN;
Chemical Pathology Laboratory at Prince Albert Luthuli Hospital;
Dr Thavi Govender and the staff at Chemistry Department, UKZN;
The University of KwaZulu-Natal Research Office, for funding part of this study.
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<tr>
<td>ABC</td>
<td>ATP-binding Cassette</td>
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<td>ABCBA</td>
<td>Multi-drug resistance protein</td>
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<td>AMPK</td>
<td>Adenosine Monophosphate Protein Kinase</td>
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<td>ATPIII</td>
<td>Adult Treatment Panel III</td>
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<tr>
<td>AUC</td>
<td>Area Under the Concentration-Time Curve</td>
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<tr>
<td>BCRP</td>
<td>Breast Cancer Resistance Protein</td>
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<tr>
<td>BRU</td>
<td>Biomedical Resource Unit</td>
<td></td>
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<tr>
<td>BSEP</td>
<td>Bile Salt Export Pump</td>
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<tr>
<td>CAR</td>
<td>Constitutive Androsterone Receptor</td>
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<tr>
<td>DR</td>
<td>DNA-response element</td>
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<tr>
<td>EXD</td>
<td>Extracellular Domain</td>
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<tr>
<td>FXR</td>
<td>Farnesoid X Receptor</td>
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<tr>
<td>GFJ</td>
<td>Grapefruit Juice</td>
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<tr>
<td>GI</td>
<td>Glycaemic Index</td>
<td></td>
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<tr>
<td>G6Pase</td>
<td>Glucose-6-phosphate</td>
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<tr>
<td>GR</td>
<td>Glucocorticoid Receptor</td>
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<tr>
<td>GTT</td>
<td>Glucose Tolerance Test</td>
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<tr>
<td>HNF</td>
<td>Hepatocyte Nuclear Factor</td>
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<tr>
<td>ISSR</td>
<td>Inter-Simple Sequence Repeat</td>
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<tr>
<td>LXR</td>
<td>Liver X Receptor</td>
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<tr>
<td>MDR</td>
<td>Multidrug Resistant</td>
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<td>MMP</td>
<td>1-methyl-4-phenylpyriminium</td>
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<tr>
<td>MRP</td>
<td>Multidrug Resistant Protein</td>
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<td>NBD</td>
<td>Nucleotide Binding Domain</td>
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<tr>
<td>OATP</td>
<td>Organic Anion Transporter Protein</td>
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<td>OCT</td>
<td>Organic Cation Transporter</td>
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<td>PEPCK</td>
<td>Phosphoenolpyruvate carboxy kinase</td>
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<tr>
<td>PCN</td>
<td>Pregnenolone-16α-carbonitrile</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>P-gp</td>
<td>P-glycoprotein</td>
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<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
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<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
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<tr>
<td>PPAR</td>
<td>Peroxisome-Ploriferator-Activated Receptor</td>
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<tr>
<td>PXR</td>
<td>Pregnane X Receptor</td>
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<tr>
<td>RAPD</td>
<td>Random Amplified Polymorphic DNA</td>
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<tr>
<td>RLFP</td>
<td>Restriction Fragment Length Polymorphism</td>
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<tr>
<td>RXR</td>
<td>Retinoid X Receptor</td>
<td></td>
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<tr>
<td>SCAR</td>
<td>Sequence Characterised Amplified Region</td>
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<tr>
<td>SLC</td>
<td>Solute Carrier transporters</td>
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<tr>
<td>SHP</td>
<td>Small Heterodimer Patner</td>
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<tr>
<td>TMD</td>
<td>Transmembrane Domain</td>
<td></td>
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<tr>
<td>TEA</td>
<td>Tetraethylammonium</td>
<td></td>
</tr>
<tr>
<td>UKPDS</td>
<td>United Kingdom Prospective Diabetes Study</td>
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ABSTRACT
Recent clinical studies have shown that grapefruit improves insulin resistance, reduces weight gain in humans and is associated with unwanted drug interactions.
However, despite the abundant knowledge on the effects of grapefruit juice (GFJ) on intestinal drug transporter proteins [organic cation transporter protein (OATP), P-glycoprotein (P-gp)] and drug metabolizing enzymes (CYP3A4), the effect of GFJ on hepatic metabolism has not previously been critically examined. Potential effects of GFJ on other hepatic drug transporter proteins, such as organic cation transporter protein (OCT1), could affect the pharmacokinetics and pharmacodynamics of substrates such as metformin. The effects of GFJ on glucose tolerance, OCT1 expression in the liver and metformin-induced lactic acidosis in normal, non-diabetic in rats, are hereby investigated.
Male Wistar rats, were divided into 3 groups (A, B, C) which were further divided into 4 sub-groups designated A1-A4, B1-B4, and C1-C4 of 5 animals each, respectively. Sub-groups A1-A4 were treated with 0, 1.0, 2.0, and 3.0 ml/kg body weight of grapefruit juice (GFJ), respectively, while sub-groups B1 and B3 were each treated with an oral dose of 3.0 ml/kg body weight of GFJ, respectively. Sub-groups B2 and B4 were similarly treated with an equal volume of distilled water. Sub-groups C3 and C4 were given oral doses of 3.0 ml/kg body weight of GFJ, while sub-groups C1 and C2 were treated with an equal volume of distilled water. On the 14th day of treatment, the rats in sub-groups C2 and C4 were treated with 1.0 ml oral dose of 250 mg/kg body weight of metformin in distilled water. Sub-groups B2, B3 and C2, were further treated with subcutaneous injections of pregnenolone-16-alpha-carbonitrile (PCN) [17 mg/kg body weight (15 mg/ml in propylene glycol)] on the 10th day of treatment, while sub-groups A4 and C1 were treated with subcutaneous injections of 1.0 mg/kg body weight/day of dexamethasone for 3 consecutive days prior to end-point.
Glucose tolerance test (GTT) was done on all treatment groups on the 14th day of treatment by oral administration, or intraperitoneal injection (i.p), of 3.0 g/kg body weight of glucose in distilled water, or in normal saline, respectively. Blood glucose concentrations were measured at times 0, 30, 60, and 90 minutes. Area-under-the-curve \{ (AUC), (mmol/L X minutes = AUC units) \} was calculated from blood glucose-time.
curves. Glycaemic index was determined by trapezoid rule using the formula: Glycaemic Index (GI) = AUC_{GFJ}/AUC_{Glucose} \times 100.

No deaths, or adverse effects, were observed in the LD_{50} study. Net weight gain due to natural growth was 76.0±5.7 g in the control rats, compared to 72.8±4.9 g in the GFJ-treated ‘test’ rats. GFJ significantly (p<0.05) lowered plasma glucose and GI, respectively, in a dose-dependent manner. However, there was no significant difference in plasma insulin levels among all treatment groups.

GFJ- (409±25 mg/g of liver tissue) or dexamethasone- and GFJ (439±35 mg/g of liver tissue)-treated animals had significantly (p<0.05) increased hepatic glycogen levels, compared to control (288±14 mg/g of liver) rats, respectively. GFJ-treatment significantly (p<0.05) reduced glucokinase activity (K_m = 144 \ \mu M), even in the presence of dexamethasone (K_m = 269 \ \mu M), compared to control rats (K_m = 308 \ \mu M). However, GFJ significantly (p<0.05) reduced G6Pase activity (K_m = 2.29 mM; V_{max} = 14.02±0.45 \ \mu mol/min/g liver tissue), compared with the controls (K_m = 2.068 mM; V_{max} = 13.4±0.45 \ \mu mol/min/g liver tissue). Similarly, GFJ significantly (p<0.05) reduced (K_m = 2.78 mM; V_{max} = 10.72±0.3630 \ \mu mol/g of liver tissue/min) dexamethasone-induced PEPCK activity (K_m = 0.738 mM; V_{max} = 26.78±0.41 \ \mu mol/g of liver tissue/min). GFJ alone (K_m = 0.02 mM; V_{max} = 96.44±2.47 \ \text{nmol/min/\mu g of liver tissue}), or in combination with dexamethasone (K_m = 0.0321 mM; V_{max} = 84.71±3.949 \ \text{nmol/min/\mu g of liver tissue}), significantly (p<0.0001) increased AMPK activity in the ‘test’, compared to control (K_m = 0.146 mM; V_{max} = 7.442±0.411 \ \text{nmol/min/\mu g of liver tissue}) rats.

Blood lactic acid levels were significantly higher (p=0.0079) in rats that were treated with either metformin alone (5.38 ± 2.53 mmol/L), or metformin in combination with GFJ (8.31 ±2.5 mmol/L), than in control (2.54 ± 0.7 mmol/L) rats, respectively. Linear regression analysis showed significant correlation between liver tissue metformin concentrations, and plasma lactic acid levels in both control (p= 0.0122; r^2 = 0.9080) and GFJ-treated ‘test’ rats (p= 0.0005; r^2 =0.9893).

Densitometry scans expressed as a percentage of the controls showed that GFJ (285.6±33.4%) significantly (p<0.05) up-regulated the expression of OCT1 in the liver, compared to controls.
Although GFJ improves glucose tolerance, and may be beneficial in diabetic patients with metabolic syndrome, caution should be exercised in patients who are on concurrent medication with metformin.
CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 The Citrus fruit

Grapefruit (*Citrus paradise* Macf., family, Rutaceae), is consumed worldwide not only because of its taste and nutritional value, but also because of its accredited medicinal properties [1]. Other members of the genus *Citrus* are oranges, lemons, limes and mandarins (tangerines) [2]. Citrus is an ancient crop with records of cultivation dating back to 2100BC [3], and is thought to have originated from South East Asia (East India, Burma and South West China), [3, 4], but is currently distributed worldwide, courtesy of ancient explorers and travellers [2]. Commercial grapefruit varieties were mainly developed in Florida, but at present, grapefruit is largely produced in USA, Israel, Cuba, Argentina and South Africa [5].

Classification of the *Citrus* fruit is still contentious among plant taxonomists, who nevertheless, believe that the grapefruit originated in the Caribbean Islands (West Indies) in the early 1700s, by hybridization between orange and shaddock [1, 5]. However, recent molecular biology techniques using inter-simple sequence repeat (ISSR), microsatellite probes, random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), and sequence characterised amplified region (SCAR) have shown that the grapefruit is most probably a hybrid between pummelo (*C. grandis*) and sweet orange (*C. sinensis*), followed by introgression back to pummelo [2,6,7]. The original grapefruit was white-fleshed and very seedy, but other mutated fruit varieties have been selected for either being seedless or increasingly red in colour [2]. Such varieties include, Duncan/Walters (seedy white), Marsh (seedless, white), Foster (seedy, pink), Thompson (seedless, pink), Redblush (seedless, red), and Ruby, Ray Ruby, Flame (seedless, very red) (Figure 1) [2]. These pigmented cultivars have now become more popular and are generally preferred to white grapefruit in the market [8].
1.1.2 Medicinal properties of grapefruit

Traditionally, consumption of grapefruit juice was indicated for such diverse ailments as anorexia, microbial infections, cancers, rheumatism, insomnia, dysuria and cardiovascular disorders [1]. Citrus fruits in general, and grapefruit in particular, are considered to be functional foods that promote good health, and have been shown by preliminary medical evidence, to be associated with a reduction in the development of atherosclerotic plaque, inhibition of breast cancer cell proliferation, and suppression of mammary cell tumorigenesis [9, 10, 11]. Although bioactive compounds present in Citrus fruits have been studied for many years, the cellular mechanisms involved in their biological actions have not been completely understood. Many pharmacological activities of citrus fruits appear to be linked to their ability to modify the activity of mammalian enzyme systems such as, kinases, phospholipases, ATPase, lipooxygenases and phosphodiesterases [12]. The antineoplastic properties of grapefruit or grapefruit juice, for example, have partially been attributed to its modulation of the expression and activity of phase II hepatic enzymes (glutathione S-transferase, quinine reductase [13]) and free radical scavenging antioxidant activity [8]. Studies by Gorinstein et al [14] have suggested that diets supplemented with grapefruit improve plasma lipid levels and increase plasma antioxidant activity in experimental animals. Similar studies conducted on human subjects have shown that red grapefruit significantly lowers serum total cholesterol, low density lipoprotein cholesterol and triglycerides in patients with coronary artherosclerosis [15]. Feeding grapefruit juice to senescent male rats has also been shown to improve antioxidant status and protect the animals against osteoporosis [16].

A recent study by Fujioka et al [17] has reported that consumption of whole grapefruit or grapefruit juice is associated with significant weight loss and improved insulin resistance in patients with metabolic syndrome, compared to placebo. Grapefruit has been part of many diets since its incorporation into the ‘Hollywood’ diet of hard-boiled eggs, green vegetables and ‘melba’ toast in 1930, as an anti-obesity ingredient [18]. These observations, therefore, seem to suggest that consumption of grapefruit or grapefruit juice may have beneficial effects in patients with type 2 diabetes mellitus and other
degenerative diseases, hence, scientifically justifying the age-old tradition of diet supplementation with grapefruit.

1.1.3 Phytochemistry of the grapefruit

A wide variety of bioactive compounds in grapefruit juice have been isolated and characterised. Their relative abundance varies according to the variety, geographical location, time of harvesting and the method of processing of the grapefruit [19]. Four types of flavonoids (flavonones, flavones, flavonols and anthocyanins) have been identified in the Citrus fruits [12]. Flavonoids constitute the most abundant bioactive constituents of the grapefruit, besides limonoid aglycones, glucosides, furocoumarins, ascorbic acid, folic acid, glucaric acid, carotenoids, pectin and potassium [20, 21, 22, 23] (Table 1). These compounds are believed to be responsible for many of the accredited medicinal properties of the grapefruit. The flavonoids (naringin and hesperidin) and limonoids (limonin) are responsible for the bitter taste commonly associated with grapefruit [24]. Flavonoids exist in grapefruit as glycosides with naringin being the most abundant but converted to aglycones and sugars by intestinal bacteria upon ingestion [1]. Enzymatic hydrolysis of naringin by naringinase yields naringenin (Figure 2), which has been shown experimentally to have pharmacological properties such as anticancer, anti-inflammatory, antithrombotic, vasodilator as well as antiatherosclerotic activities [25]. Naringin has been shown to reduce intestinal glucose absorption, and to modulate the expression of key hepatic glucose-regulating enzymes, suggesting that it may be beneficial in the treatment of type 2 diabetes [26, 27, 28]. A recent study by Purushotham et al. [29] has, however, shown that it is naringenin, rather than its glucoside naringin, that is responsible the suppression of hepatic glucose production, suggesting that the observed effects of naringin could be as a result of in vivo hydrolysis by naringinase to its active compound, naringenin.

Laboratory animal model studies have shown that limonin and abacunone, both commonly found in grapefruit, reduce the incidence of colonic adenocarcinomas in rats [30], while hesperidine reduced proliferation and induced apoptosis in colonocytes [31]. Vanamala et al [32] have shown that consumption of grapefruit or limonin suppresses the
development of colon cancer in rats through inhibition of the expression and activity of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). Glucaric acid is found in grapefruit in high levels [33], and appears to be beneficial in the prevention of carcinogenesis. Currently, oral supplementation with calcium D-glucarate is used as adjunct therapy against hormone-dependent cancers, such as breast, prostate and colon cancers [34]. The protective effects of grapefruit against degenerative diseases in general, and cancer in particular is, therefore, not in dispute.

1.2 Grapefruit juice-drug interaction

Despite the many health benefits of grapefruit juice as a dietary component, it has, surprisingly, come to light that consumption of grapefruit juice is associated with drug interactions. Drug-drug interactions have regulatory processes monitored through legislation; but food-drug interactions are difficult to regulate or legislate. The accidental observation of pharmacokinetic interaction between ethanol and dihydropyridine calcium channel antagonist (felodipine) when grapefruit juice was used as a flavour to mask ethanol taste in a study by Bailey et al [35] opened the flood gates to many investigations that indeed confirmed grapefruit-drug interactions. Grapefruit juice is frequently taken with medications at breakfast by many patients, and this discovery, therefore, came as a surprise not only to the citrus industry, but also clinicians and patients alike. Many drugs have since been investigated for potential interactions with grapefruit juice, and the results now confirm that grapefruit juice does indeed increase oral bioavailability of many of such therapeutic drugs. Since 1998, grapefruit juice has been known to increase the area under the concentration-time curve (AUC), or maximum plasma concentrations, of 34 out of 40 drugs that it has been reported to interact with after oral ingestion, thus increasing their oral bioavailability [36].
1.2.1 Cardiovascular drugs

Calcium channel blockers, commonly used to treat hypertension, have been extensively studied for possible interactions with grapefruit juice following accidental discovery of interaction of grapefruit juice with a dihydropyridine, felodipine. A study by Lundahl et al [37] showed that grapefruit juice increased oral bioavailability of felodipine by 112% in healthy men without significantly altering intravenous pharmacokinetics, suggesting that grapefruit effects were mediated by gut wall metabolism. Grapefruit-felodipine interaction has subsequently been shown to increase with dosing frequency and the amount of grapefruit ingested, and that a dosing interval of 2-3 days between grapefruit ingestion and felodipine intake was required to overcome this interaction [37, 38]. These observations, therefore, provided initial clues that grapefruit juice-felodipine interactions could not be as a result of direct chemical interaction, but rather, a consequence of some physiological response, and that these effects were partially reversible.

Grapefruit juice increases oral bioavailability albeit to a lesser extent than felodipine, of nondihydropyridines, diltiazem and verapamil, when co-administered [40, 41]. The maximum plasma concentration increased as the time taken to reach peak plasma concentration of nisoldipine reduced when grapefruit juice was taken concomitantly [42]. Other dihydropyridines, such as nimlodipine and nitrendipine, exhibit a lesser but still significant interaction with grapefruit juice than felodipine [43]. Amlodipine and nifedipine are similarly less affected by grapefruit juice than felodipine [44, 45, 46]. These studies, therefore, led to the conclusion that, the extent of interaction of calcium channel blockers with grapefruit juice is inversely proportional to the inherent bioavailability, such that drugs with high oral bioavailability, like nifedipine and amoldipine, are less affected than felodipine, when co-administered with grapefruit juice. Pharmacokinetics studies have subsequently shown that calcium channel blockers with smaller bioavailability or lower plasma free fraction are likely to exhibit a more potent interaction with grapefruit juice and vice versa [47], suggesting that grapefruit juice could be affecting intestinal metabolism of these drugs prior to their pre-systemic circulation.

Unlike calcium blockers, angiotensin converting enzyme (ACE) inhibitors have not shown significant interaction with grapefruit juice. However, grapefruit juice has been
shown to inhibit the bioactivation of angiotensin receptor blocker losartan, to its active metabolite, thus reducing its efficacy [48].

Thiazide diuretics and $\alpha_1$-adrenergic antagonists, such as doxasozin, terasozin and prazosin, have so far shown no interaction with grapefruit juice [49], but antiarrythmic prodrug, amiodarone, is less effective when co-administered with grapefruit juice [36]. Grapefruit juice completely inhibited conversion of amiodarone to its active metabolite, N-desthylamiodarone, resulting in 50% and 84% increases in AUC and maximum plasma concentration of amiodarone, respectively; clinically leading to prolongation of QT intervals (which is a measure of the time between the start of the Q wave and the end of the T wave in the heart's electrical cycle) [50].

Among the cholesterol-lowering agents, grapefruit has been demonstrated to increase serum concentration of simvastatin and its active metabolite, simvastatin acid, and that this interaction subsides within 3-7 days after ingestion of the last dose of grapefruit juice [51, 52, 53]. Similar observations have been made (to a lesser extent though) when other HMG-CoA reductase inhibitors, such as lovastatin and atorvastatin, are taken concomitantly with grapefruit juice [36], but pravastatin’s (not metabolised in the body) bioavailability is not affected by grapefruit juice ingestion, indicating that grapefruit juice effect on HMG-CoA reductase inhibitors is a consequence of intestinal metabolism of such drugs [54]. Other cholesterol-lowering agents like nicotinic acid and common fibric acid derivatives, as well as bile acid sequestrants, have not been reported to interact with grapefruit juice [49].

1.2.2 Antimicrobials

Grapefruit juice has significant interaction with saquinavir, a protease inhibitor used in the treatment of HIV infection. Oral bioavailability of saquinavir increases by a factor of two following ingestion of grapefruit juice, without affecting systemic clearance after intravenous administration [55]. Like calcium channel blockers, saquinavir has inherent low oral bioavailability [56], suggesting that grapefruit inhibits intestinal metabolism of saquinavir. However, grapefruit juice does not appear to significantly affect oral bioavailability of indinavir and amprenavir [57, 58, 59].
Grapefruit juice significantly increases oral bioavailability of the antimalarial drug, artmether, and anthelmintic agent, praziquantel, respectively; without affecting their pharmacokinetics, thus suggesting a role of intestinal metabolism [59, 60, 61, 62]. On the other hand, the pharmacokinetics of quinine, which is known to have a high inherent oral bioavailability, is not affected by grapefruit [36, 63], yet again implicating intestinal metabolism as the site of action of grapefruit. However, grapefruit has been demonstrated to reduce total clearance and increase elimination half-life of quinidine by 19% [64]. Administration of grapefruit juice increases time-to-peak concentration of clarithromycin without affecting other pharmacokinetic parameters [65], suggesting a facilitation of clarithromycin uptake by grapefruit juice.

1.2.3 Central nervous system drugs

Benzodiazepines, being the most commonly-used sedatives-hypnotics, have been investigated for potential interactions with grapefruit juice. A single glass of regular strength grapefruit juice was shown by Ozdemir et al [66] to increase the AUC of diazepam by more than 3-fold, while a similar amount of grapefruit juice increased the AUC and peak plasma concentration of triazolam and midazolam by 50%, without affecting their half-lives [67, 68]. Chronic consumption of grapefruit juice also increases the AUC of triazolam by as much as 150% [69]. No pharmacokinetic effects have, however, been observed with aprazolam even after repeated ingestions of grapefruit juice [70]. A 40% increase in AUC has been reported with anticonvulsant carbamazepine after oral ingestion of grapefruit juice [71], without affecting pharmacokinetics of phenytoin [72]. Increased bioavailability has also been shown when grapefruit is ingested concurrently with buspirone, which has inherent low bioavailability [73], and serotonin selective reuptake inhibitor, sertralin, has been reported [74]. Antipsychotics, such as clozapine and haloperidol, however, remain unaffected by grapefruit juice consumption [75, 76].
Antihistamines and serotonin analogues most affected by grapefruit juice include cisapride [77], racemic nitrendipine which also inhibits metabolism of nitrendipine in humans [78, 79].

1.2.4 Other drugs

Sildenafil citrate, a phosphodiesterase inhibitor, commonly used for the treatment of erectile dysfunction, has been shown to interact with grapefruit juice. Ingestion of 250 ml grapefruit juice 1.0 hour before, or concomitantly with grapefruit juice, increases the oral bioavailability of sildenafil by 23% [80]. Ingestion of similar amount of grapefruit juice also increases oral bioavailability of cisapride, a prokinetic agent, by 50% [81, 82]. An increase in cyclosporine (an immunosuppressant) AUC of more than 60% has been observed after grapefruit ingestion [83, 84]. In paediatric transplant patients, grapefruit juice altered oral cyclosporine AUC, peak plasma concentration, as well as elimination parameters following oral [85], but not intravenous, administration [83]. Similar observations have been made in healthy volunteers [86]. Grapefruit juice has recently been shown to affect the formation and/or elimination of cyclosporine metabolites M1 and M9 [85, 86, 87, 88], suggesting inhibition of intestinal metabolism of cyclosporine by grapefruit juice. Concurrent administration of grapefruit juice in postmenopausal women has been shown to increase oral bioavailability of estradiol [89]. Similarly, Dasgupta et al, [90] reported that grapefruit juice increases plasma concentration of paracetamol by increasing elimination half-life in mice, thus suggesting for the first time that there could be hepatic involvement in grapefruit juice-drug interactions.

1.3 Mechanism of grapefruit juice-drug interactions

Grapefruit juice-drug interactions have been investigated using regular or double strength (reconstituted with less water) juice, with a single glass, or repeated ingestions [91], and the results consistently show that ingestion of single glass (250 ml) of grapefruit juice is enough to produce maximum effect [79, 92], even though repeated dosing produces
higher intensity interactions [69]. These studies, therefore, confirm that grapefruit juice-drug interaction is not dependent on concomitant administration of both. Lovastatin bioavailability is doubled even if taken 12 hours after grapefruit ingestion [93], and felodipine-grapefruit juice interaction persists at 30% of its maximum effect 24 hours after grapefruit juice ingestion [94]. Studies by Takanga et al, [95] have reported that grapefruit juice impact can last up to 3 days. These observations have led investigators to believe that grapefruit juice-drug interactions are mediated by enzyme inhibition, since it became apparent that an interval of 24 hours between ingestions of grapefruit juice is required to overcome the interaction [53]. This theory was further re-enforced by the consistent reports that drugs with inherently low bioavailability are most affected by grapefruit juice, suggesting that these drugs undergo extensive intestinal metabolism by enzymes that may be inhibited by grapefruit juice or its constituent chemicals.

1.3.1 Molecular insight

In the human body, organs concerned with drug disposition, such as the liver, the intestines and the kidney, actively express drug metabolising enzymes and transporter proteins in response to local or systemic exposure to xenobiotics. The response to xenobiotics exposure may be in the form of induction, constitutive or repressed expression of these enzymes and proteins [96]. Such responses are mediated and regulated by nuclear receptors, such as Pregnane X Receptor (PXR), Constitutive Androstane Receptor (CAR) and many others.

PXR is a member of the nuclear receptor superfamily of proteins which constitute a large group of transcription factors that regulate expression of target genes of endobiotics as well as xenobiotics. Ligand binding activates these receptors to induce target gene expression by binding to specific response elements within the promoter regions of the target genes [97, 98]. These proteins, therefore, mediate signal transduction cascade, which leads to the synthesis of drug metabolizing enzymes and transporters of endobiotics and xenobiotics. Other members of this superfamily include Retinoid X Receptors (RXR) [99], Peroxisome-Ploriferator-Activated Receptors (PPAR) [100], Farnesoid X Receptors (FXR) [101], Liver X Receptors (LXR) [102] and Constitutive
Androstane Receptors (CAR), [103]. Expression of PXR, also referred to as steroid and xenobiotic receptor (SXR) in humans [104], predominantly occurs in the liver and to a lesser extent, in the intestines [105], and is activated by many structurally-diverse compounds, such as pregnenolone 16α-carbonitrile (PCN), rifampicin and dexamethasone [105, 106]. PXR is known to be promiscuous with regard to ligand specificity, and is a powerful regulator of phase I and phase II enzymes of hepatic metabolism as well as several cellular transporters [107, 108]. In the liver and intestines, PXR regulates the expression of CYP3A and 2B genes [107], and many other proteins involved in drug disposition. Molecular studies have shown that, following ligand (xenobiotics as well as endobiotics) binding, PXR translocates to the nucleus by facilitated nucleus diffusion, dimerizes with RXRα and then binds the 5'-regulatory regions of target genes including CYP3A family [109], and causes transcription of relevant genes (Figure 3). In the absence of an activating ligand, or in the presence of an antagonist, corepressors are thought to bind PXR and deacetylate histones, preventing transcription of target genes [110]. PXR up-regulation of CYP3A4 in human tissues is particularly significant, taking into account the grapefruit juice-drug interactions. The potential of grapefruit juice to modulate the activity of this enzyme via PXR can not be ruled out. The combined activity of cytochrome P450 enzymes and drug transporters is responsible for the drug interactions involving many therapeutic agents. However, factors controlling regulation of the expression of drug metabolising enzymes and transporter proteins in different tissues in the human body are still not well understood.

1.3.2 Cytochrome P450 enzymes

Drug metabolising systems are primarily located in the liver and small intestines, and to a lesser extent in other organs such as the lungs and adrenal glands [111]. Oxidative drug metabolism occurs mainly to terminate its action and render it less lipophilic for enhanced renal clearance. Cytochrome P450 is a large multi-gene family of heme-containing enzymes located in the endoplasmic reticulum of cells [1], and constitutes major drug metabolising enzymes in humans, comprising many isoforms which, to a large extent, are drug specific. These enzymes have been named by a code-like
nomenclature with Arabic numerals for the families, followed by a letter defining the sub-family and the last number for the enzyme within the sub-family (e.g., CYP 1A2, CYP2C9, CYP3A4, etc). About half of the therapeutic drugs used in humans are metabolised by CYP 3A4, which appears to be more promiscuous with respect to drug specificity [112]. Cytochrome P450 enzymes are inducible by, amongst others, increased substrate concentrations, such that exposure to a drug or dietary substances triggers increased expression/activity of the relevant isoform. This leads to increased drug metabolism, and hence, elimination from the body. It, therefore, follows that inhibition of these enzymes conversely leads to accumulation of the substrate drug in plasma, and vice versa.

Cytochrome P450 may be inhibited or induced by many diverse drugs, and xenobiotics, such as food components, leading to food-drug interactions [113]. Unlike drug-drug interactions, which have been well studied, food-drug interactions have only recently been noticed [113, 114].

Grapefruit juice-drug interactions are known to be mediated by inhibition of intestinal, but not liver, CYP 3A4 [115], since grapefruit juice does not affect the pharmacokinetics of the same drugs when administered intravenously [37, 83]. Grapefruit juice inhibition of CYP3A4 leads to reduced first-pass metabolism and increased intestinal absorption, resulting in elevated maximum plasma concentration. Hence, drugs which are substrates of CYP3A4 are the most affected. The half-life and distribution of the substrate drug is not affected by grapefruit juice inhibition of CYP3A4 [116]. Grapefruit juice inhibition of drug metabolism appears to be partially reversible as the normal CYP3A4 activity is restored only after 24 hours post administration of grapefruit juice [94, 117]. The duration of the inhibition is dose-dependent, and may persist in reduced magnitude for up to 3 days [94, 118]. Currently, laboratory evidence indicates that grapefruit juice accelerates the degradation of cytochrome P450 enzymes, and also reduces translation of these enzymes from mRNA without affecting transcription of mRNA from cellular DNA [1, 36, 94, 119, 120]. This, therefore, suggests that grapefruit juice inhibition of CYP 3A4 is mechanism-based, rather than competitive. It has been noted that grapefruit juice reduces the levels of CYP3A4 in the cells by as much as 47% within 4 hours after
ingestion of grapefruit juice, with a resulting increase in bioavailability detectable at 30%, 24 hours later [1, 36, 94, 119]. This reduction in CYP3A4 is not accompanied by an increase in cellular mRNA content, suggesting that there could be no feedback regulatory mechanism on intestinal expression of CYP3A4 in this context [1]. However, restoration of intestinal activity of CYP3A4 has been noted to require de novo synthesis or enterocyte replacement [49], which may, therefore, account for the prolonged duration of grapefruit juice effects, and also explain the apparent partial reversibility of grapefruit juice effects observed by Greenblatt et al [121].

Experimentally, it has been demonstrated that grapefruit juice, or its component(s), metabolically activate CYP3A4, which in turn gets irreversibly inactivated by covalent binding in a mechanism-based interaction [36, 122], suggesting that certain ingredients in grapefruit act as suicide substrates of CYP3A4. In vitro experiments using cell-free enzyme assay systems and human carcinoma cell (Caco-2 cells) have recently indicated that grapefruit juice-CYP3A4 interaction is mechanism-based [119, 123, 124]. Some researchers have, however, suggested that some chemicals in grapefruit juice exhibited competitive (reversible) inhibition of CYP3A4 in vitro [125, 126]. It is not yet known for certain which one of these two different mechanisms precisely contributes to grapefruit juice-drug interactions, but available evidence so far favours mechanism-based interaction [124, 127]. Clinical studies have, however, shown that grapefruit juice inhibits intestinal, but not hepatic, CYP3A4 [37, 68, 83], which may explain the fact that grapefruit juice reduces oral bioavailability of many drugs without affecting their pharmacokinetics. What is puzzling, however, is why would this interaction occur only with intestinal and not hepatic CYP3A4? Does the intestine express a different isoform of the enzyme from the liver, or is it because the intestine is exposed to high concentrations of grapefruit chemical compounds which do not effectively reach systemic circulation to influence hepatic metabolism? These questions remain unanswered to date. However, there is evidence to suggest that grapefruit juice inhibition of CYP3A4 could affect hepatic CYP3A4 upon long-term exposure, since the current studies have only looked at acute effects [128]. A study by Lilja et al [69] suggested that grapefruit juice-triazolam interaction is probably as a result of inhibition of hepatic CYP3A4 after prolonged exposure.
Recent studies have, however, shown that inhibition of cytochrome P450 enzymes by grapefruit juice is not limited to CYP3A4 only [129]. CYP 3A4 and CYP2C9 (constituting 80% and 15%, respectively) are the predominant intestinal cytochrome P450 enzymes, while others such as CYP2D6, 2C19 and 2J2 are expressed to a lesser extent [130, 131]. Grapefruit extracts have recently been shown to inhibit the activities of CYP2D6 and 2C9 \textit{in vitro} [130]. Grapefruit juice chemical constituents, such as the furanocoumarins, have been reported to inhibit other isoforms, such as CYP2C19, 2E1, 1A1,1A2 and 1B1 [120, 132, 133, 134, 135].

Evidence from molecular studies indicate that cytochrome P450 gene expression in response to xenobiotic activation is mediated by nuclear hormone receptors, such as PXR, Constitutive Androsterone Receptor (CAR) and Peroxisome-Ploriferator-Activated Receptor (PPARα), which bind to characteristic DNA response elements in the 5'-regulatory regions of the genes [136] (Figure 3). Dietary chemicals are known to directly or indirectly modulate steroid hormone receptors that regulate gene expression of cytochrome P450 enzymes, but no studies have been reported on grapefruit juice chemical constituents to that effect.

1.3.3 **Drug transporter proteins**

The intestinal barrier, being the gateway to systemic circulation, is endowed with gatekeepers that limit the absorption of many xenobiotics which may be derived from dietary factors, intestinal microbiota or therapeutic medicines. These proteins work in concert with cytochrome P450 enzymes in the enterocytes to limit oral bioavailability of many drugs [137]. Such proteins include ATP-binding Cassette drug transporters (ABC transporters) and Solute Carrier (SLC) transporters [138, 139]. Different families of ABC transporters (also referred to as drug efflux pumps) have been described in the intestinal tissue, including ABCBA \{multidrug resistance protein (MDR1)\}, ABCC1-6 \{Multidrug Resistance-associated Protein (MRP1-6)\} as well as ABCG2, \{Breast Cancer Resistance Protein (BCRP)\} [138]. These transporters extrude drugs, xenobiotics and metabolites from the intestines by active transport in an ATP-dependent manner, hence, the name ABC. This leads to reduced absorption of drugs or
xenobiotics into systemic circulation, thus protecting the body against acute and chronic toxicity of toxins [138]. ABC1 substrates include anticancer agents, calcium channel blockers, antiviral, immune suppressive agents and dietary components [138] (Table 2). Consequently, the occurrence of these proteins in the brush-border of intestinal epithelia, and their broad substrate specificity, has been associated with multidrug-resistance, especially in cancer chemotherapy.

Different families of SLC drug transporting proteins, such as Organic Anion Transporting Polypeptides {OATP (Oatp in rat)}, Organic Cation Transporters{OCT, (Oct in rat)}, Organic Anion Transporters (OAT), have similarly been described in intestinal tissues, but unlike ABC drug transporters, these proteins improve absorption of many substrate drugs and xenobiotics [138, 140].

However, drug transporting proteins, are not only expressed in the intestines, but also in the liver, kidney, placenta, testis, brain and other tissues concerned with drug disposition [141]. SLC family members show wide tissue distribution, but in the liver, they mainly mediate hepatic uptake of substrates, and may also support bidirectional transport, depending on relative concentrations of their substrates across the sinusoidal membrane [142]. Hepatic uptake of organic anions, cations, prostaglandins and other xenobiotics is facilitated by dedicated transport proteins in the basolateral (sinusoidal) membrane of hepatocytes [142] (Figure 4). These proteins extract drugs, metabolites and other xenobiotics into hepatocytes for oxidative enzyme metabolism. Efflux pumps on the canalicular domain of hepatocytes, working in concert with these proteins, extrude metabolised drugs and other compounds from the cell interior, thus maintaining a concentration gradient across the cell [142] (Figure 4). Expression of these proteins is subsequently regulated by their substrate xenobiotics/endobiotics, which may lead to drug interactions. Many of the hepatic carrier proteins belong to the SLC family, but unlike ABC family of proteins, they are mainly found in the basolateral membranes, where it has been suggested that they may act both as influx and efflux pumps, depending on substrate concentration gradient across cell membrane [142] (Figure 4). An inward proton gradient at the brush-border maintained by Na⁺/H⁺ exchanger has been proposed to be the main facilitator of SLC-mediated transport in the intestines [143]. SLC substrates are mainly low molecular weight compounds, which may be metabolised in
phase I hepatic metabolism by CYP450 enzymes, then followed by phase II conjugation/hydroxylation reactions [142]. The products of phase II reaction may exit the cell via efflux pumps, such as MRP1/3 (into systemic circulation), or P-glycoprotein (P-gP) and MRP2 located on the canilicular membrane (Figure 4). Alternatively, SLC substrates may be taken out of the cell by the same efflux pumps without being metabolised (Figure 4).

Efflux pump substrates are usually relatively high molecular weight compounds, which are either secreted via the bile canaliculi, courtesy of canalicular membrane embedded efflux pumps, or urine, after extrusion into systemic circulation by MRP1/3 or other pumps on the basolateral membrane [144] (Figure 4). Therefore, it can be argued that substrate specificity for hepatic transporter proteins of drugs and their metabolites determines whether a drug will be excreted in urine or in bile. For example, paracetamol which is biotransformed in phase II hepatic metabolism to a glucuronic acid conjugate, is excreted in urine because glucuronide has a higher affinity for MRP3 (located on basolateral membrane) than MRP2 (located on the canilicular membrane) [145]. Members of the ABC family are predominantly found on the canilicular membrane of hepatocytes where they function as efflux pumps that transfer drugs and their metabolites from hepatocyte to bile, uphill against a 100-1000-fold concentration gradient [142]. MRP3 are expressed in many tissues and organs, especially those that protect delicate organs like the blood-brain-barrier, placental barrier, liver, lungs, kidney and intestine [142]. MDRs equally have a wide tissue distribution, while some, such as MDR3 and MDR2, are exclusively expressed in the liver [142]. Expression of drug transporters in the liver has been shown to exhibit adaptive response as shown by the observation that ligation of bile duct in rats decreases expression of MRP2 in the liver, but increases its expression in the kidney, and at the same time upregulates MRP3 expression in the kidney [146, 147]. Thus, by down-regulating the expression of hepatic canilicular membrane-bound MRP2, and up-regulating the expression of hepatic basolateral membrane-bound MRP3 as well as kidney MRP2, the adaptive response in this case enhanced drug efflux when the normal exit route (bile duct) was blocked. A recent clinical study by Chen HL et al. [148] reported that in children with biliary atresia, bile salt export pump (BSEP), MDR3, NTCP, OATP, MRP2 and FXR are all down-regulated
at early stages, while at late-stage of the disease, MDR1 and MDR3 are up-regulated, while PXR is down-regulated in response to cholestasis. These observations suggest that the expression of hepatic transport proteins is adjustable to disease states, such that at late-stage cholestasis, the overall response is to decrease bile acid uptake, but not canalicular export, or biliary pressure.

1.3.3.1 P-glycoproteins (P-gp)

P-glycoprotein (also known as P-gp, ABC1 or MDR1), a product of the *mdrl* gene, is the most studied member of ABC family of proteins [149, 150]. The 170 kDa protein was first discovered in the plasma membrane of mammalian cells that had been selected for resistance to drugs [151]. The human P-gp has 4 domains fused into a single polypeptide in an H2N-(MD-NBD-MD-NBD)-COOH configuration [149]. Two transmembrane domains (TMD) with 6 segments each, form the ligand binding sites, and two cytosolic nucleotide binding domains (NBD) bind and hydrolyze ATP to facilitate substrate translocation [150] (Figure 5). TMDs are believed to form the pathway through which the substrates cross the membrane, while NB domains couple the energy associated with ATP binding and hydrolysis to ligand transport [152]. Mutagenesis studies have shown that drugs bind TMD4, TMD5 and TMD6 in the N-terminal half, and TMD9, TMD10, TMD11 and TMD12 in the C-terminal half [153] (Figure 5). The NB domains are highly conserved, including the Walker A and B motif, that are found in other ATPases, and the characteristic C (signature) motif that is unique to the ABC family of proteins [149]. The C motif is thought to be involved in the transduction of ATP hydrolysis energy to the conformational changes in the TMD responsible for the translocation of substrates.
The precise mechanism by which P-gp mediates transport of ligands across cell membranes is still not fully understood, but it is believed that the transport cycle is initiated by substrate binding in the transmembrane domain, which increases ATP affinity for the protein [150]. After binding and hydrolysis of ATP in one of the NB domains, the protein undergoes conformational change, which facilitates the release of the drug/ligand into the extracellular space, and then returns to its native state after hydrolysis of a second ATP molecule [150]. However, the mechanism that drives the ligand from low to high affinity site is still contentious. One model proposes the formation of a closed NB domain dimer, which provokes conformational changes that are transmitted to the drug binding site followed by two-step sequential hydrolysis of ATP molecules that resets the P-gp protein to its native state [158]. The other models propose either one or two hydrolysis reactions, which facilitate the efflux of the drug, and the resetting of the protein for another cycle [159, 160]. Despite the recent advances in understanding the precise mechanism by which P-gp affects ligand efflux, it is still not known precisely how the pump mechanically operates, given that its ligands are largely hydrophobic. Classical membrane pumps, such as Na⁺K⁺-ATPase, transport polar or charged substrates across cell membranes by moving the ligands through a polar channel within the protein molecule, thus avoiding contact with the hydrophobic lipid bilayer of the membrane [149]. But given the hydrophobic nature of its ligands, it has been suggested that P-gp operates in part like “a hydrophobic vacuum cleaner”, binding non-polar molecules that partition into the membrane and expelling them into the extracellular space [161]. However, other schools of thought have it that P-gp operates like a drug “flippase”, moving substrates from the cytoplasmic membrane leaflet to the extracellular leaflets where they can partition into the aqueous phase [162, 163, 164] (Figure 6).

P-gp substrates include natural products, common therapeutic drugs, steroids, fluorescent dyes, linear and cyclic peptides and ionophores [149]. Physiologically, it is known to play a central role in drug disposition in many organisms [165]. P-gp is expressed in the apical
surface of many epithelial cells such as gastro-intestinal tract, hepatocyte canalicul
cellular membrane, and endothelial cells that line the brain capillaries, where it forms a major
component of blood-brain-barrier [149, 166]. In the epithelial cells lining the gut wall, P-
gp is a major factor limiting drug bioavailability [167, 168, 169, 170].

P-glycoprotein exhibits broad substrate specificity consistent with its role in transporting
compounds absorbed into the gut wall back into the lumen [172]. Many therapeutic
agents are substrates of P-gp (Table 2). It, therefore, follows that any agent that
modulates the activity of P-gp will equally affect the pharmacokinetics of these drugs.
This implies that modulators which can block drug efflux activity of P-gp would have an
impact on the disposition of these drugs. These modulators have been shown to bind to P-
gp at the substrate-binding site, and compete with the transport of substrates in a complex
fashion [149]. P-gp modulators are known to have similar molecular features to the
normal transport substrates [175]. Such modulators include calcium channel blocker,
verapamil, and cyclosporine A, but many others yet to be identified may be found in
natural products.

Consumption of grapefruit juice has been reported to modify the activity of enterocyte P-
gp. However, there is no conclusive evidence whether grapefruit juice components inhibit
or activate P-gp. Evidence supporting grapefruit juice activation of P-gp is scanty, save
for one study by Sodner et al [176]. The inhibitory effect of P-gp by grapefruit in the
intestinal wall is supported by the observation that grapefruit effects on bioavailability are
not apparent when drugs which are substrates P-gp are administered intravenously [55,
68, 83, 177]. Grapefruit juice has recently been shown in vitro to inhibit P-gp-mediated
transport of talinolol [178, 179, 180] in Caco-2 cells. Clinical studies have suggested
that 200 ml of grapefruit juice significantly increases oral bioavailability of
dextromethorphan, probably by inhibition of P-gp, in human volunteers [181]. A more
dramatic observation has been made on grapefruit juice interaction with cyclosporin in
kidney transplant patients, where grapefruit juice increased oral bioavailability of cyclosporin, a known P-gp substrate [168], rather than reducing it. However, Seville (sour) orange, a citrus fruit like grapefruit with similar chemical compounds, but which is known to reduce enterocyte CYP3A4 concentration, did not affect the pharmacokinetics of cyclosporin in the same patients [182]. This observation strongly suggests that grapefruit juice increased cyclosporine bioavailability in these patients by P-gp inhibition.

Despite the fact that some drugs are known to be specific substrates of P-gp, grapefruit juice does not dramatically affect their oral bioavailability. Digoxin, a well characterised substrate of P-gp with negligible metabolism in humans [183, 184], exhibited only a modest increase in oral bioavailability when co-administered with grapefruit juice, contrary to expectations [185]. It has been argued that, it is the inherent high oral bioavailability (70 – 80%) [186] of digoxin, rather than lack of grapefruit juice effect on P-gp, that contributed to the apparent reduced absorption [187]. A more confusing observation came from studies with fexofenadine, a known substrate of P-gp with an absolute oral bioavailability estimated at 33% in humans [187], and eliminated from the body unchanged, mainly in the gastrointestinal tract via the biliary tract [186], but exhibited a reduced, rather than increased, oral bioavailability when co-administered with grapefruit juice [187]. This led to the suggestion that there may be some other drug transporter proteins, or metabolic enzymes, which could be involved in transporting or metabolising these drugs concurrently, since there is no conclusive evidence at present to suggest that grapefruit juice induces or activates P-gp [187]. Surprisingly, these observations were similarly made with orange and apple juices, lending further credibility to the suspicion that other proteins are involved in the transport of fexofenadine [188].

Given the fact that both P-gp and CYP3A4 have broad overlapping substrate specificity (Table 2), co-localization and functional similarity (reduce bioavailability of drugs), one would expect that there exists some kind of co-regulation of the functions of these proteins. Both proteins are highly expressed in the liver and the intestines. In the intestine, P-gp effluxes drugs absorbed into the enterocyte, while CYP3A4 metabolises them, and in the hepatocyte, CYP3A4 and oxidative enzymes metabolise intracellular drugs, while P-gp effluxes such drugs and their metabolites into the canalicular space.
(Figure 4) for biliary excretion. The pertinent question to ask is: is the expression of P-gp and CYP3A4 co-ordinately regulated in both the liver and the intestines, given the apparent synergistic nature of their functions in both organs? Several studies seem to suggest otherwise [168]. However, the fact that both proteins share common regulatory transcription factors (PXR and RXR-α) in both the intestines and the liver, (Figure 3) seems to support co-regulation hypothesis taking into account the aforementioned adaptive response of the proteins to disease in the liver.

Despite the paucity of knowledge and conflicting data generated by many researchers, the synergistic interplay between P-gp and CYP3A4 has been demonstrated in human subjects, using quinidine as a common substrate [190]. In this study, it was shown that dose-adjusted plasma quinidine concentrations negatively correlated with intestinal P-gp and CYP3A4 protein content, suggesting that oral bioavailability of quinidine was limited by both proteins, concurrently. Similar findings have been reported in perfused rat liver, where quinidine increased the metabolism of digoxin (metabolised by CYP3A4 in the rat) by inhibiting P-gp, which pumps digoxin out of the hepatocyte into canilicular space [191]. Even though there is a clear case for co-regulation of P-gp and CYP3A4 in the liver and intestines, it has to be taken into account that many other proteins are involved in drug disposition in these organs, further complicating the matter and making the search for evidence rather elusive. Understanding the mechanism by which grapefruit juice fits into this scientific puzzle is indeed a challenging task.

1.3.3.2 Organic anion transporter protein (OATP)

Since the isolation of Oatp1 from the rat brain in 1994, 11 human OATPs have been identified with their genes (SLC2A1), classified by the Human Gene Nomenclature Committee, within the gene superfamily (2A1) of the solute carriers [140, 192]. Hydropathy analysis has predicted that OATPs contain 12 transmembrane domains (TMDs), which include a large extracellular domain between TMs 9 and 10, corresponding to extracellular loop 5, and containing many conserved cysteine residues that resemble zinc fingers of the DNA binding proteins [193] (Figure 7). Extracellular loops 2 and 5 contain N-glycosylation sites [194], while the “OATP superfamily
signature” is found at the border between extracellular loops 3 and TMD 6 [140] (Figure 7). At the boundary of TM domains on cytoplasmic side, conserved amino acid sequences are common.

Functionally, the transport mode of OATPs has been suggested to involve sodium independent bile salt and organic ion transport systems, which uses anion exchange as well as taurocholate/HCO₃⁻ exchange mechanisms [195, 196, 197, 198, 199, 200]. Details of these mechanisms are at present unknown, but it has been suggested that physiologic glutathione efflux from hepatocytes creates a driving force that facilitates substrate uptake by Oatp1 in experimental conditions [201, 202].

OATPs have a broad substrate specificity, but in general common substrates are mainly anionic amphipathic molecules with relatively high molecular weights (>450), and normally bound to proteins [140]. Common features of the substrates tend to include steroid nucleus (e.g. bile acids, steroid hormones and their conjugates), and small linear and cyclic peptides [140]. It is, therefore, not surprising that OATPs are predominantly expressed on the basolateral side of the hepatocytes, since, their substrates, amongst others, constitute bile constituents, which are extracted from blood capillaries. In the liver, OATP-B, OATP-C and OATP8 are expressed on the sinusoidal (basolateral membrane) where they effect uptake and elimination of xenobiotics [140]. On the other hand, hydrophilic compounds with low protein binding capacity are excreted in urine, and are mainly substrates of OCTs and OATPs, which are predominantly found on basolateral membranes of the hepatocytes [203, 204] (Figure 4). Drugs which are substrates of OATPs include opioid receptor antagonists, enkephalin and deltorphin [205], enalapril and temcaprilat [206, 207], and fexofenadine [188], methotrexate [208], repaglinide [209] and levothyroxine [210]. OATPs have also been reported to transport digoxin across cell membranes in the rat liver, the brain and the kidney [196, 211, 212, 213, 214]. In the rat, hepatic uptake of digoxin has been demonstrated to be mediated by Oatp2, but inhibited by amiodarone [214]. Dogixin which is known to have a narrow therapeutic window [215], is not metabolised in the body [216], and is a substrate of P-gp. Therefore, it is rational at this point to suggest that P-gp and OATP co-regulated
digoxin's pharmacokinetics in the body, such that OATP facilitates cellular uptake of digoxin while P-gp concurrently enhances its cellular exit.

HMG-CoA reductase inhibitor, pravastatin, is a substrate of OATP [217]. Pravastatin uptake by hepatocytes was demonstrated to be inhibited by OATP substrates, such as bile acid and bromosulfophthalein, in a competitive manner [218, 119]. Subsequently, Oatp2 has been confirmed to mediate pravastatin uptake in the rat liver [220]. Potential inhibitors of OATPs may include natural products found in foods, herbs, and other sources. The surprise finding that grapefruit juice reduces, rather than increases, fexofenadine bioavailability contrary to expectations, has led researchers to point accusing figures at OATPs as the “other protein” which antagonises P-gp-mediated fexofenadine uptake in the enterocytes. Both P-gp and OATP are located on the apical membrane of the enterocytes, where the former acts as an efflux, while the latter as an influx facilitator [208]. However, on the hepatocytes membranes, these roles appear to be reversed, such that OATPs located on the sinusoidal membrane facilitate the influx, while P-gp located on the canalicular membrane, facilitates efflux of their substrates. This, therefore, suggests that, in the intestines, the actions of these proteins oppose each other, while in the liver, their combined activity facilitates drug metabolism and elimination.

Recent evidence has confirmed that indeed human OATP1A2 co-localizes with P-gp in immunohistochemical staining [221] solely mediates fexofenadine uptake in enterocytes, and is inhibited by grapefruit juice [222]. Glaeser et al. [222] have showed that co-administration of grapefruit juice with fexofenadine reduces the AUC of fexofenadine by 50% when compared to grapefruit juice given 2.0 hours before (38%), without affecting the absorption rate, and that this inhibition is abolished when grapefruit juice is administered 4 hours before fexofenadine. This shows that grapefruit juice-OATP interaction is more complex and difficult to predict than previously thought, and needs to be further investigated as there may be other players yet unidentified.
1.3.3.3 Organic cation transporter protein (OCT1), the missing link?

Grapefruit juice interaction with drug metabolising enzymes and transport proteins presented here may involve other transporter proteins not yet identified or investigated, given the complexity of the interactions so far described. One such drug transporter proteins could be organic cation transporter protein 1 (OCT1).

1.3.3.3.1 Structure and functions of OCT

The OCTs belong to the family of polyspecific organic cation transporter family of proteins designated SLC22, which contains 3 subtypes referred to as OCT1 (SLC22A1), OCT2 (SLC22A2), OCT3 (SLC22A3), and the cation and carnitine transporter OCTN1 (SLC2A4), etc [224]. OCT1 was cloned in 1994, and subsequently 16, additional human family members from different species were identified [224, 225]. The members of this family have a predictable membrane topology comprising 12 α-helix transmembrane domains (TMDs) and an intracellular N-terminus, with a large glycosylated extracellular loop between TMDs 1 and 2, a large intracellular loop with phosphorylation sites between TMDs 6 and 7, as well as an intracellular C-terminus (Figure 8) [224, 226, 227, 228, 229]. Tertiary structure model of TMDs and site-directed mutagenesis of amino acids have revealed that substrate-binding cleft has conserved amino acids located on the 4th TMD (tryptophan 218, tyrosine 222 and threonine 226), 10th TMD (alanine 443, leucine 447 and glutamine 448), and 11th TMD (aspartate 475), respectively (Figure 8) [224, 230, 231]. The leucine 447 residue on the 10th TMD is believed to play a role in substrate specificity in the rabbit OCT2 [233]. The glycosylation sites may be involved in maintaining protein stability, intracellular routing or protection from extracellular proteases [234]. Available evidence from these studies so far suggest that OCTs contain a single substrate binding region accessible both from inside and outside the cell membrane, but flips direction of substrate transport either inwardly or outwardly during the transport cycle [223]. This change in orientation may be accompanied by conformational changes within the protein molecule that alters the affinity for substrates or inhibitors [223].
The genes coding for OCTI and OCT3 in humans are localized within a cluster on chromosome 6.q26-7 [235, 236, 237]. The rat OCTI (rOctI) encodes 556-amino acid protein, its homologue (67% identity) rOCT2 encodes 593-amino acid protein, while rOCT3 (with 48% homologous identity), encodes 551-amino acid protein with a predicted molecular mass of 61 kDa [234, 238].

Using molecular biology techniques, such as Northern and Western Blots, PCR and immunohistochemistry, researchers have shown that OCTI is expressed in epithelial cells and some neurons, and that in humans, it is mainly expressed in the liver, whereas in rodents, it is strongly expressed in the liver, the kidney and the small intestine, [224, 225, 240, 241, 242, 243]. These techniques have further shown that in human and rat livers, OCT1 is located on the sinusoidal membrane of hepatocytes [225, 234] (Figure 4), while in the mouse small intestine, it is located on the basolateral membranes of enterocytes [246], and in rat kidney, it is found in the basolateral membranes of the epithelial cells in the S1 and S2 segements of the proximal tubules [240, 241, 244]. OCT2 has a more restricted pattern of expression than OCT1, with the kidney being the major organ of expression, where it is found in the basolateral membrane of the proximal tubules, like OCT1 [227, 241, 244]. Unlike OCT1 and OCT2, OCT3 has a broad range of expression and in humans, the strongest expression occurring in the skeletal muscle, liver, placenta and heart, where it is localized largely in the basolateral membrane [243, 245, 246].

Functionally, the OCTs (OCT1-3) have similar basic characteristics in various species. OCT1 and OCT2 share 70% sequence homology (68-69% in humans, rats and mice; and 71% for rabbits) [247]. The substrates include a variety of small organic cations with different molecular structures, with relative molecular mass of less than 500, and the smallest diameter of less than 4 Å [248]. Unlike P-gp, OCTs execute bidirectional transport of organic cations and weak bases, as well as non-charged compounds [249, 250]. At physiological pH, 40% of all drugs are cations, most of which are cleared from the body into hepatocytes by active transport [251]. Models of data obtained from cell-based assay systems have identified 47 novel inhibitors, and confirmed 15 previously known inhibitors of OCT1 [251]. Physiologically known substrates of OCT include endogenous compounds (dopamine, noradrenaline), xenobiotics, therapeutic drugs (metformin, procainamide, atropine, cisplatin, prazocin, amantadine, oxaliplatin, imatinib
and verapamil), and model compounds, such as 1-methyl-4-phenylpyridinium (MPP), [225]. However, some cations (such as tetrapethylammonium (TEA), decynium 22 and disprocynium), non-charged compounds (corticosterone, deoxycorticosterone and β-estradiol), and anions (probencid and α-ketoglutarate) are not substrates, but do inhibit OCTs [225]. However, there are differences in substrate/inhibitor specificity of the OCTs among animal species, despite the overlap in substrate affinities, such that a substrate of one may be an inhibitor of the other, and vice versa [225]. It has been shown for the rat and human OCT transporters, that organic cations are transported in an electrogenic manner, suggesting that OCTs operate independently of Na⁺ and membrane proton gradients [227, 238, 249, 250, 253].

Despite close structural and functional similarities, OCTs show remarkable differences in substrate affinity, which can be used to distinguish between them. OCT1 has higher affinity for verapamil, prazosin, atropine, phencyclidine, desipramine, phenoxybenzamine, and quinine, than OCT2, which has higher affinity for cisplatin, amphetamine, methylenedioxymetamphetamine, amantadine, cimetidine, and diphenhydramine, than OCT1 in decreasing order of intensity, respectively [225].

Substrate affinities and tissue distribution of OCTs suggest a physiological role in the distribution of cationic drugs in the liver, kidney, heart and brain, and biliary and renal excretion of their substrates [254]. Studies with genetic knockout mice have shown that Oct1 knockout mice (Oct1- (Slc22a1 gene knockout)), Oct2- (Slc22a2 gene knock out) and Oct3- (Slc22a3 gene knockout) are phenotypically normal, but Oct1- knockout mice showed dramatically reduced hepatic uptake of metformin and TEA [254, 255, 256, 257, 258]. It has further been shown that a combined double knockout in Oct1/2, led to reduced renal excretion and increased plasma level of TEA, which means that a combined deficiency of Oct1 and Oct2 better reflects the effects of Oct2 deficiency in renal function, since Oct1 is also expressed in the kidney [254, 256]. Similarly, Oct3-deficient mice showed reduced uptake of MPP in their hearts and foetuses, compared to the wild type [254, 258]. These observations, therefore, emphasize the role of OCTs in hepatic and renal uptake, distribution and elimination of drugs and other substrates.
1.3.3.3.2 Regulation of OCTs

Regulation of OCTs involves many factors acting at transcription and post-transcription level. Transcriptional control has recently been reported to involve two adjacent putative DNA-response elements (DR-2) for hepatocyte nuclear factor-4α (HNF-α) [259]. mRNA expression of OCT1 was increased by overexpression of HNF-α, and targeted disruption of HNF-α dramatically decreased OCT1 mRNA in the liver, kidney and duodenum [260, 261]. A peroxisome proliferator agonist receptor (PPAR) response element has been identified in the promoter of mouse Oct1, and it has been shown to be transactivated by PPAR-γ agonists, such as clofibrate and ciglitazone [262]. Further to that, it has been shown that the mRNA of rat Oct1 and Oct2 was increased by treatment with pregnenolone-16α-carbonitrile (PCN) which is a known ligand of PXR (Figures 3 and 9) in the primary cultured cells, rat liver and kidney; and also that biliary expression and tissue to plasma ratio of metformin, MPP and TEA (which are known substrates of OCT1) increased following subcutaneous administration of PCN [263]. However, exposure to dexamethasone, a ligand of glucocorticoid receptor (GR), simultaneously decreased rOctl mRNA level and MPP uptake, respectively, which was reversed by exposure to a glucocorticoid receptor ligand antagonist, RU486 [264], suggesting that dexamethasone down-regulates the expression of rOct1. It is thought that GR-mediated down-regulation of rOct1 expression may be as a result of decreased PXR expression (> 10 μM dexamethasone suppresses PXR expression) or by direct GR effects on rOct1 expression [264].

Post-transcriptional regulation of OCT has been proposed to be controlled by its phosphorylation status, implying that inhibitors of protein kinase A (PKA), Serc-like p56, and calmodulin, can modulate the activity of OCTs [265, 266, 267, 268]. OCT1 and OCT2 seem to share common regulatory mechanisms involving PKA and calmodulin, but not PKC which down-regulates OCT2, but not OCT1. It is rational to speculate that these regulatory mechanisms involve phosphorylation sites located within the loop, forming intracellular domain between TMD 6 and 7, and strategically located within the substrate binding pocket (Figure 8). Thus, it would appear that PKA phosphorylation of this site deactivates it, and vice versa, while calmodulin stimulates it (Figure 9).
However, these observations are made following in vitro studies, and the data obtained are still controversial, considering an earlier observation by Mehrens et al., [269] that rOCT1 is stimulated by PKC, PKA and endogenous tyrosine kinase activation, and that PKC phosphorylates rOCT1 and leads to a conformational change at the substrate binding site. Some of these post-transcriptional regulatory pathways could be influenced by many ligands, some of which may be components of natural products.

In spite of the strategic role that OCT1 plays in drug disposition in the intestines, the liver and the kidney, not much is known about drug-interactions mediated by OCT1. Grapefruit juice effects on drugs disposition in the intestines have been investigated in detail, but no studies have been done to date, to explore the possibility of such interactions taking place in the liver, and to interrogate the involvement of OCT1 in this regard, given the strategic role it plays in drug disposition in the liver.

1.3.4 Identifying the culprits in grapefruit juice

Grapefruit juice is known to contain hundreds of chemical ingredients, some of which have been identified as having biological/medicinal properties (Table 1) [271]. Even though many studies have been conducted on pharmacological actions of grapefruit juice ingredients, definite conclusions on the actual chemical constituents have not been made. Indeed, only a handful of such actions have been attributed to specific chemical constituents of the grapefruit juice.

1.3.4.1 Candidate ingredients for CYP3A4 inhibition

Flavonoids and furanocoumarins commonly found in grapefruit juice and Seville (sour) orange, but not in orange juice, have been proposed as the main inhibitors of CYP3A4 [36, 19]. Although naringin is the predominant chemical ingredient in grapefruit juice, recent clinical studies have shown that neither naringin, nor its aglycone, naringenin, is responsible for the intestinal inhibition of CYP3A4 by grapefruit juice (Figure 1) [127, 272]. In vitro studies have suggested that naringenin does not inhibit CYP3A4 by
mechanism-based inhibition, but by competitive antagonism, which is insignificant clinically [273]. Co-administration of naringin with felodipine and its primary oxidative metabolite, dihydrofelodipine, in vivo produced minimal effects on drug bioavailability, suggesting that naringin is not a major inhibitor of CYP3A4 [272].

However, furanocoumarins have been shown to inhibit CYP3A4 by mechanism-based inhibition, albeit in in vitro, since clinical studies can not be done because furanocoumarins have not been approved for human use [36, 274]. Although bergamottin is the major furanocoumarin in fresh grapefruit juice [275, 276], available evidence suggests that 6',7'-dihydroxybergamottin, and paradisin A, are the major CYP3A4 inhibitors of CYP3A4 in vivo [217, 278, 279].

Other furanocoumarins that have been reported to inhibit CYP3A4 include bergapten, also found in grapefruit juices, and shown to inhibit CYP3A4 in vitro by mechanism-based inhibition, but with a lesser potency than 6',7'-dihydroxybergamottin [280]. However, bergapten is likely to be a minor ingredient of grapefruit juice. Epoxybergamottin, also present in grapefruit juice in minor quantities, has been reported to inhibit CYP3A4 in vitro by mechanism-based inhibition [127, 274]. However, it is chemically unstable, and may be rapidly converted to 6',7'-dihydroxybergamottin (in the gastrointestinal tract), which may be responsible for the observed inhibitory effects [36]. The list of furanocoumarins capable of CYP3A4 inhibiting is still growing, with the recent isolation of bergatol and geranylcoumarin from grapefruit, both of which appear to be potent inhibitors of CYP3A4 in vitro [281]. Furanocoumarin dimers, GF-1-1 and GF-1-4, occurring in minute quantities in grapefruit juice, have been isolated and shown to be potent inhibitors of CYP3A4 in vitro [282, 283, 284].

Despite spirited attempts to attribute CYP3A4 inhibition to certain ingredients of grapefruit juice, it may be the combined effect of furanocoumarins that produces these inhibitory effects.

1.3.4.2 Grapefruit ingredients that interact with P-gp

Unlike CYP3A4, P-gp interaction with grapefruit juice has not been finger-pointed to individual chemical ingredients in grapefruit juice. There is inconclusive evidence on the
effects of flavonoids, (naringin and naringenin), despite suggestions that they do inhibit P-gp [36], and furanocoumarins (6’,7’-dihydroxybergamottin), which are found in both grapefruit juice and Seville orange, and exert inhibitory effects on P-gp in vitro, only in grapefruit juice [36, 274]. Other furanocoumarins, such as psoralens (dermatological photosensitizing agents) in citrus fruits have been shown to inhibit P-gp, thus enhancing bioavailability of saquinavir [55].

1.4 Clinical considerations

Positive aspects of grapefruit-induced drug interactions are related to the potential reduction in costs incurred on reduced treatment regimens of different ailments. Grapefruit juice contains a number of health-promoting compounds, which may be exploited for therapeutic use. Traditionally, grapefruit-drug interactions have been viewed in terms of enhancement of unwanted adverse effects. But recently attempts have been made to limit such effects by either modifying the chemistry of the chemical constituents of grapefruit juice, or eliminating them altogether. Various laboratories have synthesized furanocoumarin dimers, which are believed to be as potent as the natural forms but selective in their inhibition of CYP3A4 [285, 286]. It is believed that such dimers may be therapeutically exploited to customize grapefruit juice-drug interactions to specific patients’ needs. A furanocoumarin-free grapefruit juice created by using food grade solvents and absorption resins, failed to inhibit CYP3A4 activity, and did not increase felodipine’s bioavailability in healthy human volunteers, thus confirming that furanocoumarins are the actual ingredients in grapefruit juice that enhance felodipine bioavailability [287]. A recent study by Mying et al. [288] has suggested that autoclaved edible fungi (Morchella esculenta, Monascus pupureus, Pleuratus sapidus and Agaricus bisporus) bind bergamottin and 6’,7’-dihydroxybergamottin, and can, therefore, be used to remove furanocoumarins from grapefruit juice, without affecting its food quality. Previous studies have suggested that heat treatment or UV radiation inactivates bergamotting and 6’,7’-dihydroxybergamottin in grapefruit juice, and therefore, eliminates pharmacokinetic interaction of grapefruit juice with drugs [289, 290]. Clinical benefits of such interventions are yet to be seen.
doses, may increase the risk of rhabdomyolysis [52, 54, 294]. With the current trend towards a more aggressive lipid lowering therapy with the statins, the risk of rhabdomyolysis is even greater in patients taking grapefruit juice concomitantly [295].

The danger of grapefruit juice-induced drug reaction is particularly grave in drugs with narrow therapeutic index, such as terfenadine and cyclosporine. Ingestion of grapefruit juice has been reported to increase oral bioavailability of terfenadine, and prolong the QT interval in the electrocardiogram, and precipitate ventricular arrhythmia of Torsade-des-pointes [296]. A case report has been made of a 29-year-old man who had been taking terfenadine twice daily for more than a year, but collapsed and died on the day he took two glasses of grapefruit juice [297, 298]. His post-mortem revealed terfenadine concentration of 35 ng/ml, which was, therefore, suggested to be the cause of death. Fortunately, terfenadine has been withdrawn from the market globally, due to cardiac arrhythmias caused by its interaction with other drugs, when taken concurrently [1].

Grapefruit juice was noted to increase plasma concentration of cyclosporine in renal transplant patients and normal adult volunteers. Such interactions are potentially fatal, considering known adverse effects of cyclosporine [299, 300, 301]. However, serious calcineurin toxicity has not been reported on these patients, prompting researchers and clinicians to believe that regular and moderate grapefruit consumption may allow maintenance of cyclosporine plasma levels within a therapeutic range, at a reduced dosage [302, 303].

Geriatric patients and those with liver cirrhosis are particularly at risk. Liver cirrhosis patients are more dependent on intestinal CYP3A4 for drug metabolism than those with normal liver function [1]. The elderly are particularly vulnerable to grapefruit juice-induced drug interactions, since they are often on multiple medications, and they experience diminished drug disposition capacity [39, 112, 304].

Another crucial factor to consider in grapefruit juice-drug interaction is genetic polymorphism of the CYP3A4 enzyme. It would be expected that patients who express intestinal CYP3A4 would extensively metabolise substrate drugs, and hence, experience a greater impact of grapefruit juice-drug interactions, and vice versa. However, no large scale genotyping data is available to make conclusive evidence in this regard.
1.4.1.2 P-gp

P-gp is the main culprit in multidrug resistance in cancer chemotherapy. Neoplastic cells resistant to treatment are known to express P-gp which is responsible for the efflux of a large number of drugs [179, 305]. Pharmacological agents which are inhibitors of P-gp are linked to adverse effects when taking drugs which are also P-gp substrates (Table 2). Calcium channel blockers, such as nifedipine, flunarizine, verapamil, etc, are known to inhibit P-gp activity, causing increased cellular concentration of anthracyclines used in cancer chemotherapy such as doxorubicin, daunorubicin, and idiarubicin in cardiomyocytes, hence potentiating their cardiotoxicities [306]. Quinidine, verapamil and itraconazole are known to increase digoxin blood concentrations and change the pharmacodynamics of digoxin in the body by competitive inhibition of P-gp transporting functions [307]. The full extent of pharmacodynamic interactions of grapefruit juice interactions with P-gp substrates is not fully understood and known.

1.4.1.3 OATPs

The clinical impact of OATP modulation on pharmacodynamics has not been fully established, but it has been reported that single nucleotide polymorphism of the OATP gene affects pharmacokinetics of pravastatin [308]. It may, therefore, be speculated that OATPs modulators have the potential to enhance or impair cholesterol-lowering effects of pravastatin, since patients who express high OATPs in their hepatocytes would experience increased hepatic uptake of pravastatin, and vice versa. Considering drug interactions, this may, therefore, have serious implications on the pharmacodynamics of therapeutic agents, which are OATP substrates. Such potential dangers have been demonstrated by the realisation that rifamycin SV and rifampicin used in the treatment of tuberculosis cause hyperbilirubinaemia and reduce elimination of bromosulphthalein in the liver, due to OATP inhibition [140]. In vitro studies have since confirmed that rifamycin is a potent inhibitor of rat Oatp1 and Oatp2, as well as human OATPs, while
its structural analogue, rifampacin, mainly inhibits rat Oatp2 and human OATP8 [309, 310]. This, therefore, strongly suggests that OATPs play an important role in the enterohepatic circulation of drugs and endobiotics, such that their inhibition would cause an increase in plasma concentrations of these substrates, which potentially could lead to adverse drug reactions or treatment failure.

1.4.1.4 OCT1

Considering the central role that OCT1 plays in hepatic uptake and disposition of many therapeutic agents, it would be expected that OCT1 modulators influence pharmacodynamic effects of such drugs. It has been shown that activation of human OCT1 (hOCT1) by HNF-4α is inhibited by bile acid (chenodeoxycholic acid), via component small heterodimer partner (SHP) of the bile acid-inducible transcription repressor [260]. HNF-4α is an activator of OCT1 expression in the hepatocytes (Figure 9). Hepatic expression of OCT1 is decreased during cholestasis [267]. OCT1 expression decreases in the liver, but not kidney, after bile duct ligation in the rat, with concomitant decreased hepatic accumulation of intravenously injected TEA [267]. This suggests that during cholestasis, hepatic bile acid influx pumps are switched off. It has also been reported that nephrotoxicity induced by platinum-based antineoplastic agents, such as cisplatin and oxaliplatin, is affected by the activity of OCTs, which determines the concentration of these drugs in the kidney [311].

That PPAR-α and −γ response element occurs in the promoter region of mouse Oct1 [312], suggests that PPAR agonists can regulate the expression of OCT1 in hepatocytes, and currently, they are used in the treatment of diabetes mellitus [312].

Interaction of drugs with hepatic transporters may lead to treatment failures or adverse reactions when substrate drugs are co-administered, as recently demonstrated in a study by Backmakov et al [313], which showed that OCT1-mediated uptake of metformin (1, 1, dimethylbiguanide) is inhibited by repaglinide and rosiglitazone in vitro. Studies with Oct1 gene knock-out mice previously showed that accumulation of metformin in the liver is reduced, compared to the wild-type [314], confirming that rOct1 is the major
transporter of metformin into hepatocytes. However, the same study showed that kidney metformin levels are comparable in both Oct1 knock-out mice and wild type, further supporting the hypothesis that Oct1 is the major determinant of metformin disposition, both in the liver and in the kidney.

Recently, Shu et al, [315] showed that OCT1 polymorphisms affect metformin actions mediated by adenosine monophosphate-activated protein kinase (AMPK) such as phosphorylation and gluconeogenesis, such that glucose-lowering effects are completely abolished in Oct1-deficient mice. The study also showed that the effect of metformin on glucose tolerance tests were significantly lower in human subjects carrying reduced function polymorphisms of OCT1. A similar study further showed that individuals carrying reduced function OCT1 allele had increased metformin bioavailability [316]. These reports, therefore, confirm that OCT1 is important for therapeutic actions of metformin, and that genetic variations in OCT1 gene may contribute to the variations in therapeutic response to metformin. It is, therefore, likely that any grapefruit juice effect on hepatic OCT1 would most probably affect metformin pharmacodynamics.

Excessive accumulation of metformin and other biguanides in hepatocytes has been associated with lactic acidosis commonly seen in diabetic patients taking these medications, and OCT1 has been implicated [317]. Wang et al, [317] have showed that blood lactate levels increased significantly in wild-type mice, compared to Oct1 knock-out mice, and also in isolated rat hepatocytes with concomitant reduction in oxygen consumption in response to metformin and other biguanides. Metformin has been shown in in vitro studies, to reduce oxygen consumption and glucose production in isolated rat hepatocytes in a dose-depend manner, by inhibition of mitochondrial respiratory complex 1 [318, 319]. It would be expected that this inhibition causes oxidative stress, which may lead to conversion of pyruvate to lactate in the cytosol, and hence, increased lactate levels.

Lactic acidosis is a life-threatening condition characterized by low arterial pH (<7.35), and elevated lactate levels (5.0 mEq/L in humans), and electrolyte disturbances with an increased anion gap [317, 320]. It has been shown that among the therapeutically-used biguanides, the affinity of OCT1 decreases in the order of phenformin>buformin>metformin, which is also reflected in their ability to cause lactic acidosis [317]. The
incidence of lactic acidosis has been reported by epidemiological surveys to be significantly less in diabetic patients taking metformin, than in those taking phenformin [320], and indeed, phenformin was subsequently withdrawn from the market when 50% of the patients who were taking it died due to lactic acidosis [322]. Metformin is poorly metabolised, does not accumulate in the liver, and is eliminated unchanged in urine [321, 323, 324]. However, there is a growing body of evidence that metformin increases glucose utilisation in the small intestine through anaerobic metabolism, and that this leads to production of lactic acid, which can be found in hepatic portal vein [325, 326, 327]. A recent report by Bailey et al. [327] has shown that metformin accumulates and enhances glucose utilisation in human intestinal mucosa, which leads to significant production of lactic acid. However, lactate produced from the intestines is likely to be rapidly removed by hepatic metabolism in the liver.

At cellular level, metformin activates AMPK by phosphorylation, which suppresses glucagon-stimulated glucose production, and causes increased glucose uptake by hepatocytes and skeletal muscles [328, 329]. The AMPK has previously been referred to as “a master sensor, integrator and regulator” of cell and body energy homeostasis [328, 329]. Activation of AMPK, a well known serine/threonine kinase, is regulated by AMP/ATP ratio, and upstream kinases, and affects many pathways that increase cellular ATP content [330] in response to such physiological stimuli as exercise, muscle contraction and hormones (adiponectin and leptin), as well as physiological stresses, such as glucose deprivation, hypoxia, oxidative stress and osmotic shock [331, 332]. Once activated, AMPK inhibits gluconeogenesis and lipogenesis, while promoting both fatty acid oxidation and lipolysis [332]. The precise mechanism by which metformin up regulates AMPK expression is not known to date, but it has been shown that metformin inhibits the expression of two key hepatic gluconeogenic enzymes, namely phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase), through AMPK-mediated transcriptional regulation [334, 335]. A recent study by Kim et al. [333] has suggested that metformin inhibition of gluconeogenesis (through down-regulation of PEPCK and G6Pase expression) is mediated by AMPK-dependent regulation of small heterodimer partner (SHP), a typical orphan nuclear receptor (which lacks DNA-binding domain) that represses the transcriptional activity of a number of
nuclear receptors, HNF-4α inclusive [336, 337]. HNF-4α is one of the nuclear factors that regulate PEPCK and or G6Pase expression [333]. It is, however, intriguing that HNF-4α activation of hOCT1 expression is inhibited by chenodeoxycholic acid via SHP [260]. This would, therefore, suggest that hepatic uptake of metformin via OCT1 is regulated by SH. Taking into account the fact that metformin is neither metabolised nor stored in the liver, OCT1 expression and subsequent metformin uptake and physiological effects in hepatocytes may be co-regulated to some extent, could SHP be the focal point? A recent study by Kodama et al [338] reported that treatment with PCN, a PXR activator, repressed cAMP-dependent induction of G6Pase gene in primary hepatocytes in wild-type mice, which, therefore, suggests that PXR-mediated up-regulation of OCT1 expression could parallel suppression of gluconeogenesis in hepatocytes. This implies that any xenobiotic ligand that affects OCT1 expression via PXR could have an influence on regulation of blood sugar. Does grapefruit juice have a role to play in this? No studies reported in the current biomedical literature have been done to determine the effects of grapefruit juice consumption on hepatic expression of OCT1 on metformin pharmacodynamics.

1.4.2 **Grapefruit juice effects on metabolic syndrome**

Metabolic syndrome, commonly characterised by abdominal obesity, hypertriglyceridemia, low high density lipoprotein, high blood pressure and elevated fasting blood sugar, as defined by Adult Treatment Panel III (ATPIII) [339], is a growing public health problem [340]. Measures taken to manage metabolic syndrome have previously included weight reduction, either through exercise, diet or both. Grapefruit juice has traditionally been incorporated into many diets, including the famous “Hollywood diet” of hard-boiled eggs, green vegetables, and melba toast [18]. Despite the fact that good health promoting benefits associated with grapefruit juice consumption have been known for many years, not many studies have been carried out to confirm this. The study by Fujioka et al, [17] was the first and only one to date that has been published to confirm that consumption of grapefruit is associated with weight loss and improved insulin resistance in non-diabetic patients, with or without metabolic syndrome. In this
study, it was suggested that grapefruit could be improving insulin resistance by some as yet unknown mechanisms, possibly involving liver enzymes that regulate glucose homeostasis.

Metformin is one the most commonly-used oral antihyperglycaemic agents for the management of type 2 diabetes. A United Kingdom Prospective Diabetes Study (UKPDS) published in 1998 showed that patients treated with metformin have a reduction of 36% in all-cause mortality, 42% reduction in diabetes-related mortality, and 32% reduction in diabetes-related end-points [341]. Unlike insulin and sulphonylureas, metformin administration leads to weight loss in patients with type 2 diabetes mellitus, and does not cause hypoglycaemia [342]. Metformin has a much shorter half-life (1.5–5 h), and is less lipophilic (compared to other biguanides); it is not metabolised by liver enzymes, and is eliminated (90%) unchanged by glomerulus’s filtration and tubular secretion [323, 324, 343]. Metformin is absorbed primarily in the upper part of the intestine, and has negligible plasma binding capacity [344].

Therapeutic effects of metformin in diabetic patients, therefore, include decreased fasting and post-prandial glucose, decreased glycosylated haemoglobin (HBA1C), weight loss, decreased low-density lipoprotein (LDL), and increased high-density lipoprotein (HDL) [344].

Like other biguanides, metformin-associated lactic acidosis is common in patients with renal dysfunction, congestive heart failure, and other conditions that cause tissue hypoxia (such as hepatic disease, sepsis, chronic pulmonary disease), which lead to metabolic acidosis, and in whom metformin is contraindicated [317, 346, 347, 348]. These co-morbidities mask the true incidence of metformin-induced lactic acidosis in population studies, but a mortality rate of 50% has been reported in patients with lactic acidosis taking metformin [349]. Despite claims that metformin-associated lactic acidosis does not occur in patients without these co-morbidities, a recent study by Bruijstens et al [343], reported the occurrence of lactic acidosis in patients treated with metformin in the absence of renal impairment. This suggests that lactic acidosis may occur with or without these co-morbidities in patients on metformin therapy.

The metformin-like effects of grapefruit juice in human subjects, such as weight reduction, improved glycaemic index and insulin resistance as reported by Fujioka et al
[17], are indeed, intriguing. The as yet unanswered questions in this regard are: does grapefruit juice lower blood glucose like metformin? What are the potential implications of concurrent consumption of grapefruit juice with metformin - which, unlike other medications, is not a substrate of drug metabolising enzymes? If grapefruit juice has metformin-like effects on glucose homeostasis, does its consumption affect metformin pharmacodynamics? If that is the case, are such effects mediated by modulation of hepatic expression of OCT1 and other cellular proteins critical to glucose homeostasis? Does dexamethasone, a known down-regulator of OCT1 expression in hepatocytes antagonise these effects? GFJ effects on glucose homeostasis and OCT1 expression could impact negatively on the therapeutic benefits of metformin in the treatment and management of type 2 diabetes. The present study is therefore, aimed at determining the potential effects of grapefruit juice on glucose tolerance and hepatic expression of OCT1 in normal, non-diabetic rats in vivo, using metformin as a reference drug.
Aims and Objectives

The objectives of this study were to:

(i) determine the effects of chronic ingestion of grapefruit juice on glucose tolerance in normal, non-diabetic rats in vivo, using metformin as the 'reference drug';

(ii) determine the effect of grapefruit juice on the activity of key enzymes (glucokinase, glucose6-phosphatase, phosphoenolpyruvate carboxylase and adenosine monophosphate-activated protein kinase) regulating hepatic glucose homeostasis;

(ii) determine the effects of grapefruit juice on the expression of hepatic OCT1, using PCN and dexamethasone as positive and negative controls, respectively in normal non-diabetic rats in vivo; and

(iii) establish if grapefruit juice-mediated modulation of hepatic expression of OCT1 affects metformin pharmacodynamics with respect to glucose homeostasis and induction of lactic acidosis in normal, non-diabetic rats in vivo.
CHAPTER 2

MATERIALS, STUDY DESIGN AND PROCEDURE

2.1 CHEMICALS REAGENTS AND ACCESSORIES

Unless otherwise stated, all chemicals and reagents used were purchased from Sigma-Aldrich™ (SA).

Animal treatment: glucose, phosphate buffer, normal saline, dexamethasone, metformin, portable glucometer and glucose test strips (Ascensia Elite™, Bayer Schering Pharma, Germany) were purchased from a local pharmacy. Halothane and other accessories were provided by Biomedical Resource Unit (BRU) of the University of KwaZulu-Natal, Durban, South Africa. Vacutainer tubes, insulin kit ((DRG Diagnostics, Marburg, Germany)

Immunoblot assays: Tris HCl, SDS, B-mercaptoethanol, Bromophenol blue, Bis-acryl amide, ammonium persulphate, TEMED, Trizma base, glycine, methanol, Tween 20, KCl, NaCl, rabbit 'anti-rat rOctI antibody, HRP-conjugated goat anti-rabbit antibody (BioRad, Hercules, CA, USA), rat anti-rabbit Na/K-ATPase antibody (Alpha Diagnostics, Texas, USA), PVDF membrane filter (Millipore Corp, Bedford, MA), Teflon glass homogeniser (Thomas Scientific, Philadelphia, PA), protease inhibitor (Complete™ protease inhibitor cocktail, Roche Diagnostics, Mannheim, Germany).

HPLC: acetonitrile, methanol, ethyl acetate, DMSO, orthophosphoric acid.

Enzyme kinetic studies: KOH, anthrone reagent, H₂SO₄, HEPES, KCl, dithiothreitol EDTA, MgCl₂, ATP, albumin, NAD, glucose-6-phosphate dehydrogenase (from Leuconostic mesenteroides), IDP, NADH, phosphoenolpyruvate, Na₂CO₃, malate dehydrogenase, SAMS (HMRSAMSGLHLVKRR) peptide and AMP.

Grapefruit juice (GFJ), commercially processed from Ruby grapefruit (with the following declared nutritive contents per 100 ml: energy, 190 kJ; protein, 0.6 g; carbohydrate, 10.0 g; total fat, 0.0 g; total dietary fibre, 0.4 g; sodium 0.0 g), was purchased from a local
Woolworths Groceries Stores in Durban, South Africa. No preservatives or any other food additives were used in this preparation (as per manufacturer’s declaration).

2.2

**STUDY DESIGN AND PROCEDURE**

2.2.1 **Animal experiments**

Male Wistar (*Rattus norvegicus*) rats (200-300 g body weight), housed 5 rats per cage in Biomedical Resource Unit (BRU) with free access to commercial chow and drinking tap water, maintained on 12-hour dark/light cycle in an air-controlled room (temperature, 25±2°C, humidity, 55±5%), were handled with humane care according to the guidelines of the Animal Ethics Committee of the University of KwaZulu-Natal - which approved the study see (Appendix 1).

2.2.1.1 **Determination of LD$_{50}$ for grapefruit juice**

The median lethal dose (LD$_{50}$) of grapefruit juice was determined according to a modified method of Lorke [350]. The rats previously fasted for 12 hours were randomly divided into two groups of 8 animals each. Stepwise, escalated doses of grapefruit juice (0, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 ml/kg body weight, diluted with distilled water to make a final volume of 1.0 ml) were administered via gastric gavage twice daily to each of the rats (per test group, respectively). Control group rats received 1.0 ml of distilled water instead of grapefruit juice. The animals in both ‘test’ and ‘control’ groups were allowed free access to food and drinking tap water and observed for 4 days, for signs of acute toxicity. These observations were recorded in a score sheet, and log-dose response plots were constructed, from which LD$_{50}$ was determined.
2.2.1.2 Grapefruit juice treatment

Group A animals were divided into 4 sub-groups (designated A₁-A₄, respectively) of 5 animals each, and treated with 0, 1.0, 2.0, and 3.0 ml/kg body weight of grapefruit juice, respectively, for 14 consecutive days. The pure grapefruit juice was diluted with a corresponding volume of distilled water, depending on the relevant weight, to make a total final volume of 1.0 ml, which was administered twice daily (Table 3). The weights of the rats were recorded daily.

Group B animals were similarly divided into 4 sub-groups of 5 animals each (designated as B₁-B₄, respectively). Sub-groups B₁ and B₃ were each treated with an oral dose of 3.0 ml/kg body weight of grapefruit juice (diluted as in group A), respectively, for 14 consecutive days. Sub-groups B₂ and B₄ were similarly treated with an equal volume of distilled water (Table 3). All the sub-groups were subsequently treated as in group A.

Group C animals were also divided into 4 sub-groups of 5 rats each (designated C₁-C₄). Sub-groups C₃ and C₄ were given oral dose of 3.0 ml/kg body weight of grapefruit juice for 14 consecutive days, while sub-groups C₁ and C₂ were treated with an equal volume of distilled water. All the sub-groups were subsequently treated as in group A, but on the 14th day, the rats in sub-groups C₂ and C₄ were treated with 1.0 ml oral dose of 250 mg/kg body weight of metformin in distilled water (Table 3).

2.2.1.3 Glucose tolerance test

All animals in groups A-C were starved overnight on the 13th day of treatment. Fasting blood glucose concentrations were determined after tail pricking and analysed by a portable glucometer, followed by oral administration or intraperitoneal injection (i.p) of 3 g/kg body weight of glucose in distilled water, or in normal saline, respectively. Glucose solutions for oral and intraperitoneal administrations were prepared by dissolving 18.75 g of glucose in 50 ml of distilled water or normal saline, respectively, from which aliquot amounts were withdrawn to obtain a dose of 3.0 g/kg body weight for each rat. Subsequently, blood glucose levels of the animals were monitored at times 0, 30, 60, and
90 minutes in all treatment groups. Area-under-the-curve (AUC) was calculated from blood glucose-time curves, and the units (mmol/L X minutes) presented as AUC units.

2.2.1.4 Determination of grapefruit juice effects on glycemic index

A group of rats was treated as in subgroups A₁ (controls) and A₄ (test groups), respectively. The animals were maintained under similar conditions with unrestricted access to normal chow and water for 14 days. Random blood (non-fasted) samples were drawn at time 0 minute followed by oral administration of 3.0 g/kg body weight of glucose in distilled water. Blood glucose level was then monitored at 30-minute intervals for 90 minutes, the concentrations plotted against time, and the area-under-the-curve (AUC) calculated using trapezoid rule according to the FAO/WHO guidelines [351]. Thus Glycemic Index (GI) = \( \frac{\text{AUC}_{(GFJ)}}{\text{AUC}_{(Glucose)}} \times 100; \) where \( \text{AUC}_{(GFJ)} \) and \( \text{AUC}_{(Glucose)} \) represented the AUC calculated from blood glucose-time plots in rats that were treated with GFJ and controls respectively.

2.2.1.5 PCN treatment

A separate group of rats was treated as in subgroups B₂, B₃ and C₂, respectively with subcutaneous injections of pregnenolone-16alpha-carbonitrile (PCN), \( \{17 \text{ mg/kg body weight (15 mg/ml in propylene glycol)}\} \) on the 10\(^{th}\) day of treatment. Random blood sugar levels were determined in non-fasted rats for 90 minutes at 30 minute intervals. At the end of the treatment period, all the animals were euthanized by halothane overdose. Blood samples were collected via cardiac puncture. The livers were excised, snap frozen in liquid nitrogen, homogenised in phosphate buffered-saline, and stored at -180 °C for further analysis.
2.2.1.6 **Dexamethasone treatment**

Rats in subgroups A4 and C1 were treated with subcutaneous injections of 1.0 mg/kg body weight/day of dexamethasone for 3 consecutive days starting on 10th day of treatment. At the end of the treatment period, the rats were euthanized by halothane overdose. Blood samples were collected via cardiac puncture. The livers were excised, snap frozen in liquid nitrogen, homogenised in phosphate-buffered saline, and stored at -180 °C for further analysis.

2.2.1.7 **Metformin treatment**

Metformin (500 mg tablets) purchased from a local pharmacy were crushed and dissolved (62.5 mg/ml) in distilled water, and the volume (ml) of metformin solution administered orally was calculated by dividing the weight of rat (g) by 250 g to obtain a volume corresponding to the dose of 250 mg/kg body weight. This means that a rat weighing 250 g would get (250/250) 1.0 ml solution of metformin and another rat weighing 300 g would get (300/250) 1.2 mls of metformin solution since no more than 1.0 ml metformin solution could be administered via gastric gavage.
CHAPTER 3

METHODS

3.1 Grapefruit juice fingerprinting

Five aliquots of 500 ml each of GFJ were made from freshly opened containers and the pH adjusted to 5 (to avoid degradation of furanocoumarins) and concentrated at 40°C using rotary evaporator.

3.1.1 Determination of flavonoids

Each residue was redissolved in 1.0 ml of methanol vortexed for 1 minute and centrifuged at 2500 g for 15 minutes as per the modified methods of De Castro et al., [19] and Ho et al., [352]. The mixture was filtered through 0.45 μm PVDF membrane filter (Millipore Corp, Bedford, MA), and the supernatant analysed by high-pressure liquid chromatography (HPLC) equipped with photodiode array (PDA) detector and single quadrupole mass spectrophotometer (LC-MSD), and analysed at 285 nm. Separation was accomplished with a multistep linear water/acetonitrile/0.05% formic acid gradient at a flow rate of 0.75 ml/min. The temperature was set at 35 °C. Column used was Lichrospher RP-18, 250 x 4.6 internal diameter, 5 μm and Lichropher 100 RP-18, guard column (Merck KGaA, Darmstadt, Germany). MS parameters used were: ionisation mode, ES+; capillary voltage, 30 kV; extractor voltage 5V, source temperature, 100°C; desolvation temperature 225°C; desolvation N₂ flow 465 L/h, cone N₂ flow, 70 L/h; scan range m/z 150 and 100.
3.1.2 Determination of furanocoumarins

Each of the 5 aliquots was mixed with an equal volume of ethyl acetate. Extraction was performed by shaking the mixture 4 times for over 30 mins. The mixture was then centrifuged at 3200 g for 20 minutes, and, the organic phase collected and evaporated under vacuum. The residue was reconstituted with 600 µl of a DMSO/methanol solution (1:3 v/v) as previously described by De Castro et al [19]. The reconstituted residues were then filtered through 0.45 µm PVDF membrane filter (Millipore Corp, Bedford, MA). The extracts were analysed according to the modified method of Manthey et al, [353]. For analysis in the HPLC-LC-MSD, 25 µl of each sample was injected and analysed at 310 nm under similar conditions as for the flavonoids.

3.1.3 Component identification

The chromatographic peaks were characterized by a combination of UV (PDA) and ESI-MS analysis. The UV traces of the HPLC chromatograms were compared with TICs corresponding to [M + H]+ ions of the relevant flavonoids and furanocoumarins. The [M + H]+ and [M = Na]+ peaks (if present), obtained at 20 V cone volts, were used to determine the molecular mass of most compounds. Fragmentation data at higher cone volts provided additional structural information.

3.2 Blood glucose measurement

Droplets of blood obtained by tail prick and mounted on glucose strips were analysed by portable glucometer (Ascensia Elite TM, Bayer Schering Pharma, Germany) using glucose oxidase method.
3.3 **Blood lactate**

Whole blood was collected in vacutainer tubes containing sodium fluoride (NaF) and potassium oxalate (KO), and lactic acid was analysed by automated spectrophotometric method using Chem Profile 20 analyser. The L(+)-lactic acid is selectively oxidized in the presence of lactate dehydrogenase (LDH) and diphosphopyridine nucleotide to form a light absorbing species and a reduced form of nicotine amide dinucleotide (NADH). The amount of light absorbing species formed is proportional to the amount of LDH in plasma.

3.4 **Metformin assay**

Blood samples collected in vacutainer tubes were centrifuged and plasma samples deproteinized with 4 times their volume of acetonitrile before HPLC analysis. Liver samples were homogenized with 4 times their volume of phosphate buffered-saline, and deproteinized with acetonitrile, and then evaporated to dryness. Thereafter, the pellets were re-dissolved in 100 μl of double distilled water before HPLC analysis as per the modified method previously described by Wang et al., [314]. The HPLC column used was a 300 x 3.9 mm internal diameter, C18 μBondpack (10 μm) (Waters, Milford, MA). The mobile phase consisted of 0.01 M phosphate buffer, pH 6.5, and acetonitrile in the ratio of 30:70. The UV detection wavelength was 236 nm, and flow rate was adjusted to 1.0 ml/min, with a metformin peak retention time of 16 minutes.

3.5 **Insulin assay**

Plasma insulin levels were analysed by Ultrasensitive Rat Insulin Enzyme-Linked Immunoassay kit (DRG Diagnostics, Marburg, Germany) as per manufacturer’s manual. The assay is based on direct sandwich in which two monoclonal antibodies which were directed against separate antigenic determinants on the insulin molecule react with peroxidase-conjugated anti-insulin antibodies bound to microtitre wells upon incubation. Unbound enzyme labelled antibodies were removed by washing, while the bound
conjugates were detected by a reaction with 3,3',5,5'-tetramethylbenzidine. The reaction was stopped by adding 0.5 M sulphuric acid, which gave a coloured product that was read spectrophotometrically at 450 nm.

3.6 Immunoblot assays

Crude hepatocyte membrane was prepared as per the method previously described by Denk et al [267]. Frozen livers were homogenised in ice-cold Tris-sucrose buffer (100 mmol/L Tris, 250 mmol/L sucrose, pH 7.6) using motor driven Teflon glass homogeniser (Thomas Scientific, Philadelphia, PA) at 3000 rpm in the presence of protease inhibitor (Complete™ protease inhibitor cocktail, Roche Diagnostics, Mannheim, Germany). The homogenate was then ultracentrifuged at 100,000 g for 1.0 hr at 4°C, to obtain a membrane-enriched microsomal pellet from post nuclear supernatant. The pellet was then resuspended in HEPES-sucrose buffer (10 mmol/L HEPES, 300 mmol/L sucrose, pH 7.5, protease inhibitors).

Total protein content was determined by Bradford method [354], and 50 µg protein from the membrane fractions were diluted 1:4 with sample loading buffer (Table 4). The fractions were then heated at 95 ºC, cooled and then run on a 10% polyacrylamide gel (Table 4) with a molecular weight marker (Precision Plus Western Standard, BioRad) for 1.0 hr at 200V at room temperature, using PowerPac Universal Power Supply (BioRad (Hercules, CA)). The proteins were then transferred to Immun-Blot PVDF membrane (BioRad) in transfer buffer (Table 4) at 4 ºC, using 40 mA supplied by PowerPac HC (BioRad), and then stained with Ponceau S (Sigma-Aldrich) to ensure equal loading and complete transfer. The stain was then washed off with Tris-buffered saline containing 0.1% Tween (TBS-T) (Table 4), and the blots blocked with TBS-T containing 5% dried milk for 2.0 hrs at room temperature. The blots were then incubated overnight at 4°C with rabbit anti-rat primary antibody (Alpha Diagnostics, Texas, USA), diluted 1:5000 in TBS-T for 1.0 hr at 4°C. After incubation, the membrane was washed 3 times with TBS-T and goat anti-rabbit-horseradish peroxidase secondary antibody conjugate (BioRad), applied in a dilution of 1:5000 in TBS-T. Immunoreactive bands were exposed to autoradiography films which were developed and quantified, using ChemiDoc Image.
Analyser Software (BioRad). To correct for variations in protein enrichment in the basolateral membrane, the blots were re-probed by washing twice for 10 min with TBS-T, followed by incubation in digestion strip buffer (containing 62.5 mM Tris pH 6.8, 0.1 M β-mercaptoethanol and 2.0% SDS). The membranes were then washed with TBS-T blocked and incubated with 1:000 dilution of rat anti-rabbit Na/K-ATPase antibody (Alpha Diagnostics, Texas, USA). Immunoreactive bands were semiquantitated as previously.

3.7 **Glycogen assay**

Hepatic glycogen content was measured by the modified method of Seifter *et al* [355]. The liver tissues were homogenised in 5 volumes of an ice-cold 4.0 M KOH solution and dissolved in a boiling water bath (100°C) for 30 minutes. The glycogen was then precipitated with ethanol, pelleted, washed, and resolubilized in distilled water. The concentration of glycogen in the liver tissues was then assayed by treatment with anthrone reagent [2 g/L anthrone in 95% (v/v) H₂SO₄], and the absorbance measured at 620 nm [27]. Glycogen content was expressed as mg/g liver protein.

3.8 **Glucokinase activity**

Tissues from the liver samples which had previously been snap-frozen were sampled from different parts of the liver and aliquoted into 100 mg per tube. Homogenates were prepared in buffer containing 50 mM HEPES, 100 mM KCl, 2.5mM dithiothreitol, 1 mM EDTA and 5 mM MgCl₂. Homogenates were centrifuged at 100,000 g for 1.0 hr at 4°C, to sediment the microsomal fraction (which was kept for glucose-6-phosphate assay). The postmicrosomal supernatant was used for the spectrophotometric measurement as per previously described methods of Davidson and Arion [356], and Barzila and Rosetti [357]. Total protein content was determined by Bradford method [354]. The formation of glucose-6-phosphate from glucose was coupled to oxidation by glucose-6-phosphate dehydrogenase and NAD⁺ in a continuous reaction mix containing 50 mM HEPES, 100
mM KCl, 2.5 mM dithiothreitol, 7.5 mM MgCl₂, 5 mM ATP, 10 mg/ml albumin, glucose (0, 5, 10, 15, 25, 50 mM respectively), 0.5 mM NAD⁺, 4 units of glucose-6-phosphate dehydrogenase (from *Leuconostic mesenteroides*), and the equivalent of 1 mg of liver wet-weight. The reaction was initiated by addition of ATP, and the rate of NAD⁺ reduction recorded at 340 nm for 30 minutes at 37 °C, using Beckman DU-70 spectrophotometer equipped with temperature controller. The enzyme activity was expressed as µmol/g liver protein/min.

3.9 Glucose-6-phosphatase activity

Liver content of glucose-6-phosphatase content was measured spectrophotometrically as per the modified method of Lange et al, [358]. Total protein content was determined by Bradford method [354]. The microsomal fraction obtained from glucokinase assay and certified to contain no glucokinase activity was incubated with 0, 0.5, 1.0, 2.5, 5, and 10 mM glucose-6-phosphate as previously described [356]. The reaction was carried out at 37°C, and stopped after 30 minutes with a solution containing acid molybdate, with 2/9 volumes of 10% SDS, and 1/9 volume of 10% ascorbic acid. The reaction mixture was then incubated at 45 °C for 20 minutes, and the absorbance read at 820 nm, using Beckman DU-70 spectrophotometer equipped with temperature controller. The assay was based on the hydrolysis of glucose-6-phosphate by tissue microsomal fraction containing glucose-6-phosphatase. A standard curve was constructed using different concentrations of free phosphate, and the enzyme activity was expressed as µmol/min/µg of liver protein.

3.10 Phosphoenolpyruvate carboxykinase (PEPCK) activity

PEPCK activity was determined as per modified methods of Bentle and Lardy [359] and Stiffin et al, [360]. Cytosolic fraction was obtained from homogenised liver tissues by centrifugation at 100,000 g for 1.0 hr at 4°C, and the activity of enzymes measured in a final reaction volume of 1.0 ml, at pH 7.0, containing 50 mM sodium HEPES/KOH.
buffer, 10.0 mM IDP, 1.0 mM MgCl₂, 1.0 mM dithothreitol, 0.25 mM NADH, 2.0 mM phosphoenolpyruvate, 50 mM Na₂CO₃ and 10 U of malate dehydrogenase (1 unit defined as 1 μmol of malate produced/min/mg of liver protein). Total protein content was determined by Bradford method [354]. All assay components were pre-incubated for 3 minutes. The enzyme activity was measured at 25°C and 340 nm, using Beckman DU-70 spectrophotometer equipped with temperature controller, and expressed as μmol of OAA formed/min/g of liver protein.

3.11 Adenosine-Monophosphate-Activated Protein Kinase (AMPK) activity

Microsomal fraction of homogenised liver tissue was obtained by centrifugation of the homogenate suspended in final buffer concentration of 50 mM Tris-HCl, pH 7.5, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM EDTA, 10% glycerol, 1 mM dithiothreitol with 1 % Triton X-100 at 4000 g for 15 minutes and the resulting supernatant removed. The pellet was then adjusted to 10% with polyethylene glycol and kept on ice for 10 minutes and then centrifuged for 7000g for 15 minutes. The pellet was finally resuspended in 400 μl of sample preparation buffer and aliquoted into 5 μl portions, which were then assayed for AMPK activity by measuring phosphorylation of a synthetic peptide substrate, SAMS (His-Met-Arg-Seri-Ala-Met-Ser-Gly-Leu-His-Leu-Val-Lys-Arg--Arg) in the presence of saturating concentrations of 5′-AMP (200 μM), according to the methods of Davies et al, [361], Foretz et al, [362] and Sullivan et al, [363]. Total protein content was determined by Bradford method [354]. The enzyme activity, representing cpm incorporated into SAMS peptide/min/μg of liver protein, was expressed as nmol/min/μg of liver protein.

3.12 Statistical analysis

Data obtained were presented as mean±SD. Statistical analysis was done by One-way ANOVA or non-parametric Mann Whitney, Student’s t-test, where applicable, using Graphpad Prism®, V5.0 (Graphpad Prism® Software, Inc. San Diego CA). A probability level of 0.05 was used for the rejection of null hypothesis.
CHAPTER 4

RESULTS

4.1 Acute toxicity study

No deaths or adverse effects were observed in the rats exposed to graded doses of grapefruit juice. There was no significant difference in the baseline and end-point animal weights between control and grapefruit juice-treated test rats, respectively (Figure 10). Net weight gain due to natural growth was 76.0±5.7 g in the control rats, compared to 72.8±4.9 g in the grapefruit juice-treated test rats.

4.2 Chemical constituents of grapefruit juice

The HPLC chromatograms were dominated by peaks attributable to flavonoids, furancoumarins and other related compounds. The flavonoids (naringin, hesperidin, neohesperidin, etc) were extracted into DMSO/methanol and eluted in the early phase of the chromatogram (retention times, <7.5 mins) (Figure 11). In contrast, the furanocoumarins, which were extracted in ethyl acetate, eluted much later (retention times >7.5 mins) than the polar flavonoids with distinctive UV spectra (absorption wavelength 310 nm) easily detectable by PDA analysis (Figures 12) (Table 5). Mass Spectrometry fragmentations showed consistency with 6',7'-dihydroxyermottin structures, which were identified by fragment-ions m/z 317 (Figure 12) (Table 5). Other ionisation fragments were, however, unidentifiable.

4.3 Effects of grapefruit Juice on blood glucose and glycemic Index (GI)

4.3.1 Dose-response effects of grapefruit juice

Glucose-response curves showed that grapefruit juice lowered plasma glucose in a dose-dependent manner. Plasma glucose levels were significantly (p<0.05) reduced in rats
treated with 3.0 ml/kg body weight of grapefruit juice at 90 minutes after glucose challenge (Figure 13). GI was calculated from the mean±SD of AUC as explained in the methods (Table 6). Grapefruit juice significantly (p<0.05) reduced GI in a dose-dependent manner. In all subsequent experiments, 3.0 ml/kg body weight of grapefruit juice was used as a standard dose.

4.3.2 **Fasting blood glucose**

Fasting blood glucose levels were significantly lowered (p<0.0001) in grapefruit juice-treated 'test' rats (2.9±0.4 mmol/L), compared to the control (3.7±0.39 mmol/L) rats in all treatment groups (Figure 14).

4.3.3 **PCN-induced hyperglycemia**

Treatment of the rats with 15 mg/ml of PCN in propylene glycol resulted in a significant (p<0.05) rise in blood glucose levels compared to control- or grapefruit juice-treated rats, respectively (Figure 15). However, PCN-induced hyperglycemia was reversed by both metformin and grapefruit juice. Co-administration of Grapefruit juice with polyethylene glycol (PCN vehicle) reduced blood sugar level insignificantly compared to controls.

4.3.4 **Glucose tolerance**

In order to determine whether grapefruit juice affects intestinal absorption of glucose, GTT responses after oral and intraperitoneal administrations of glucose were compared. Fasting blood glucose levels were significantly reduced (p<0.05) in grapefruit juice-treated {3.48±0.47 (oral), 3.68±0.19 (IP) mmol/L} than in control 4.12±0.34 (oral), 4.32±0.21 (IP) mmol/L} rats (Figure 16A), but the AUC calculated from GTT after the animals were challenged with 3.0 g/kg body weight of glucose either orally or intraperitoneally, showed no significant difference in both the control and grapefruit-
treated test animals, respectively (Figures 16B and C). All subsequent experiments were, therefore, performed with intraperitoneal injection of glucose.

When rats were challenged with intraperitoneal injection of 3.0 g/kg body weight of glucose, grapefruit juice alone (495±77 AUC units), or in combination with metformin (491±61 AUC units), significantly (p<0.05) lowered blood glucose levels compared to control (636±59 AUC units) animals, but insignificantly (p>0.05) compared to metformin (543±96 AUC units) (Figures 17A and 17B). Metformin alone lowered blood glucose levels compared to control animals, but the difference was not statistically significant.

4.4 Effects of Grapefruit juice on plasma insulin

There was no significant difference in fasting (0 hr) or 1.5-hour plasma insulin levels among all treatment groups (Figure 18), suggesting that neither metformin nor grapefruit juice significantly influenced insulin response in the rats after glucose challenge. Similarly, PCN did not affect insulin response.

4.5 Effect of grapefruit juice on hepatic glucose homeostasis

4.5.1 Hepatic glycogen content

Grapefruit juice- (409±25 mg/g of liver tissue), dexamethasone- (395±27 mg/g of liver tissue) or dexamethasone and grapefruit juice- (439±35 mg/g of liver tissue) as well PCN- (372±27 mg/g of liver tissue) treated animals had significantly (p<0.05) increased glycogen levels compared to control (288±14 mg/g of liver) rats, respectively. Metformin (290±15 mg/g of liver tissue) did not change glycogen levels compared to controls, but when co-administered with PCN, significantly (p= 0.0079) reduced PCN-induced glycogen storage (Figure 19).
Hepatic glucokinase activity was measured *in vitro* in liver samples which had previously been snap-frozen. The activity of glucokinase obeyed Michaelis-Menten kinetics when plotted against substrate concentration (Figure 20). The data was then transformed into Eadie-Hofstee plots [356], which were used to calculate $K_m$ and $V_{\text{max}}$, respectively. Rats treated with dexamethasone (344 $\mu$M) and metformin (369 $\mu$M) had parallel slopes, suggesting comparable $K_m$ values, while grapefruit juice-treatment significantly ($p<0.05$) reduced $K_m$ (144 $\mu$M), even in the presence of dexamethasone (269 $\mu$M), compared to control rats (308 $\mu$M), respectively. Metformin alone (369 $\mu$M) or with PCN (351 $\mu$M) had no significant effect on $K_m$ compared to control rats, respectively. Grapefruit juice treatment (26.65±1.11 $\mu$mol/g of liver tissue/min) doubled the $V_{\text{max}}$ compared to control (13.56±0.72 $\mu$mol/g of liver tissue/min) rats, and decreased $V_{\text{max}}$ when co-administered with dexamethasone (16.83±0.36 $\mu$mol/g of liver tissue/min), compared to dexamethasone alone (18.86±0.476 $\mu$mol/g of liver tissue/min), respectively. Metformin treatment (20.04±0.406 $\mu$mol/g of liver tissue/min) significantly ($p<0.05$) increased $V_{\text{max}}$ compared to control rats, but had no significant effect on $V_{\text{max}}$ when co-administered with PCN (13.72±0.876 $\mu$mol/g of liver tissue/min), compared to PCN alone (11.51±1.51 $\mu$mol/g of liver tissue/min), (Table 7). Linear regression analysis showed significant correlation between substrate concentration and glucokinase activity in grapefruit juice- ($r^2 = 0.8535$), grapefruit juice with dexamethasone- ($r^2 = 0.9137$), dexamethasone- ($r^2 = 0.8460$) and metformin- ($r^2 = 0.8835$) treatments respectively (Figure 21) (Table 7). Calculations of $V_{\text{max}}/K_m$ ratio suggested that glucokinase activity is increased in the order of decreasing magnitude following treatment with grapefruit juice, grapefruit juice with dexamethasone, dexamethasone and metformin compared to controls, respectively (Table 7). PCN treatment with or without metformin did not increase glucokinase activity compared to controls (Table 7).
Hepatic glucose-6-phosphatase activity was measured in microsomal fractions of the liver tissues (as prepared in the glucokinase assay). The activity of glucose-6-phosphatase similarly obeyed Michaelis-Menten kinetics when plotted against substrate concentration (Figure 22). The data was then transformed into Eadie-Hofstee plots [356], which were used to calculate $K_m$ and $V_{\text{max}}$, respectively. Both dexamethasone- ($K_m = 1.705 \, \text{mM}; \, V_{\text{max}} = 23.19 \pm 0.577 \, \mu\text{mol/min/g liver tissue}$) and PCN- ($K_m = 1.299 \, \text{mM}; \, V_{\text{max}} = 23.84 \pm 1.102 \, \mu\text{mol/min/g liver tissue}$) treatments significantly ($p<0.05$) increased glucose-6-phosphatase activity, compared to controls ($K_m = 2.068 \, \text{mM}; \, V_{\text{max}} = 13.4 \pm 0.45 \, \mu\text{mol/min/g liver tissue}$). Metformin administered alone ($K_m = 2.32 \, \text{mM}; \, V_{\text{max}} = 13.85 \pm 0.34 \, \mu\text{mol/min/g liver tissue}$) or concurrently with PCN ($K_m = 2.26 \, \text{mM}; \, V_{\text{max}} = 13.4 \pm 0.80 \, \mu\text{mol/min/g liver tissue}$), significantly ($p<0.05$) reduced glucose-6-phosphatase activity compared to PCN. Grapefruit juice ($K_m = 2.29 \, \text{mM}; \, V_{\text{max}} = 14.02 \pm 0.45 \, \mu\text{mol/min/g liver tissue}$) exhibited similar effects to metformin on the activity of glucose-6-phosphatase, but slightly decreased dexamethasone-induced activity (Figure 22), (Table 8). Linear regression analysis showed a strong correlation coefficient between substrate concentration and enzyme activity in dexamethasone-treated with ($r^2 = 0.9219$) or without ($r^2 = 0.9400$), grapefruit juice, and in metformin-treated animals compared to control ($r^2 = 0.8412$) rats, respectively (Figure 25). The correlation coefficient was poor in grapefruit juice- ($r^2 = 0.7800$) and metformin (with PCN)-treated rats ($r^2 = 0.6050$), respectively, suggesting a down-regulation/repression of enzyme activity. Calculations of $V_{\text{max}}/K_m$ ratio suggested that glucose-6-phosphatase activity increased following treatments with PCN, dexamethasone, and grapefruit juice with dexamethasone (in the order of decreasing magnitude) but not grapefruit juice, metformin or metformin with PCN, compared to controls, respectively (Table 8).
4.5.4 **Phosphoenolpyruvate carboxykinase (PEPCK)**

The activity of PEPCK similarly obeyed Michaelis-Menten kinetics when plotted against substrate concentration (Figure 24). The data was then transformed into Eadie-Hofstee plots [356], which were used to calculate $K_m$ and $V_{max}$, respectively. Grapefruit juice ($K_m = 1.55$ mM; $V_{max} = 12.44 \pm 0.41$ μmol/g of liver tissue/ min) did not significantly affect the activity of PEPCK compared to controls ($K_m = 1.379$ mM; $V_{max} = 13.73 \pm 0.399$ μmol/g of liver tissue/ min), but significantly ($p<0.05$) reduced ($K_m = 2.78$ mM; $V_{max} = 10.72 \pm 0.3630$ μmol/g of liver tissue/ min) dexamethasone-induced effects ($K_m = 0.738$ mM; $V_{max} = 26.78 \pm 0.41$ μmol/g of liver tissue/ min) on PEPCK activity. Similarly, metformin alone ($K_m = 1.615$ mM; $V_{max} = 12.1 \pm 0.481$ μmol/g of liver tissue/ min) did not significantly affect PEPCK activity compared to controls, but significantly ($p<0.05$) opposed ($K_m = 1.398$ mM; $V_{max} = 14.32 \pm 0.749$ μmol/g of liver tissue/ min) PCN-induced ($K_m = 0.7151$ mM; $V_{max} = 27.77 \pm 0.81$ μmol/g of liver tissue/ min) effects on PEPCK activity when administered concurrently with PCN. Dexamethasone and PCN treatments significantly increased PEPCK activity compared to controls (Table 9). Linear regression analysis showed significant correlation between PEPCK activity and substrate concentrations in dexamethasone- ($r^2 = 0.9162$) and PCN- ($r^2 = 0.9506$) treated animals compared to control groups ($r^2 = 0.8141$), respectively, but this correlation was reduced in the presence of grapefruit juice ($r^2 = 0.782$) and metformin ($r^2 = 0.7716$) respectively (Figure 25) (Table 9). Grapefruit juice alone ($r^2 = 0.8817$), or metformin alone ($r^2 = 0.8337$), showed relatively poor correlation between the enzyme activity and substrate concentration, suggesting a repression of PEPCK activity by both. Calculations of $V_{max}/K_m$ ratio suggested that PEPCK activity increased in the order of decreasing magnitude following treatments with PCN, dexamethasone, but not grapefruit juice or metformin, compared to controls, respectively. Grapefruit juice and metformin appeared to decrease dexamethasone- and PCN- induced increases in PEPCK activity, compared to controls, respectively (Table 9).
4.5.5  Hepatic Adenosine Monophosphate Activated Protein kinase (AMPK)

Hepatic AMPK activity similarly obeyed Michaelis-Menten kinetics when plotted against substrate concentration (Figure 26). The data was then transformed into Eadie-Hofstee plots [356], which were used to calculate \( K_m \) and \( V_{\text{max}} \), respectively. Grapefruit juice alone (\( K_m = 0.02 \) mM; \( V_{\text{max}} = 96.44\pm2.47 \) nmol/ min/\( \mu \)g of liver tissue), or in combination with dexamethasone (\( K_m = 0.0321 \) mM; \( V_{\text{max}} = 84.71\pm3.949 \) nmol/ min/\( \mu \)g of liver tissue), significantly (\( p<0.0001 \)) increased AMPK activity in the 'test' compared to control (\( K_m = 0.146 \) mM; \( V_{\text{max}} = 7.442\pm0.411 \) nmol/ min/\( \mu \)g of liver tissue) rats (Figures 26 and 27). Similarly dexamethasone alone (\( K_m = 0.0194 \) mM; \( V_{\text{max}} = 89.94\pm4.75 \) nmol/min/\( \mu \)g of liver tissue), or metformin alone (\( K_m = 0.0411 \) mM; \( V_{\text{max}} = 79.0\pm3.887 \) nmol/min/\( \mu \)g of liver tissue) significantly (\( p<0.0001 \)) increased AMPK activity compared to controls. PCN treatment with (\( K_m = 0.0217 \) mM; \( V_{\text{max}} = 93.74\pm6.55 \) nmol/min/\( \mu \)g of liver tissue) or without (\( K_m = 0.017 \) mM; \( V_{\text{max}} = 89.75\pm10.08 \) nmol/min/\( \mu \)g of liver tissue) metformin, significantly (\( p<0.05 \)) increased AMPK activity compared to controls. Linear regression analysis of Eadie-Hofstee plots showed significant (\( p<0.05 \)) positive correlation between substrate concentration and the activity of AMPK in grapefruit juice (\( r^2 = 0.966 \)), dexamethasone with (\( r^2 = 0.8898 \)) or without (\( r^2 = 0.9017 \)) grapefruit juice, PCN with metformin (\( r^2 = 0.8283 \)), and metformin alone (\( r^2 = 0.8557 \)) (Figure 27). Poor correlation coefficient was observed between substrate concentration and the activity of AMPK in rats treated with PCN alone (\( r^2 = 0.6782 \)). Calculations of \( V_{\text{max}}/K_m \) ratio suggested that AMPK activity increased in the order of decreasing magnitude following treatments with dexamethasone, PCN with metformin, grapefruit juice, metformin, PCN and dexamethasone with grapefruit juice, compared to controls, respectively (Table 10).

4.6  Effect of grapefruit juice on metformin-induced lactic acidosis

Blood lactic acid levels were significantly higher (\( p=0.0079 \)) in rats that were treated with either metformin alone (5.38 ± 2.53 mmol/L), or metformin in combination with grapefruit juice (8.31 ±2.5 mmol/L), than in control (2.54 ± 0.7 mmol/L) rats.
respectively. However, lactate levels after treatment with grapefruit juice alone (2.81 ± 1.4 mmol/L) were similar to those of control rats, but significantly lower than in the combination of grapefruit juice and metformin, respectively (Figure 28).

4.7 Effect of grapefruit juice on hepatic metformin uptake

Grapefruit juice- and PCN- treated rats had significantly (p<0.05) higher metformin levels in their liver tissues than the control rats (metformin only). Plasma metformin levels were lower in control rats than in the treated groups, but the difference was not statistically significant (Table 11). However, the plasma: liver ratio of metformin was significantly (p<0.05) reduced in the grapefruit juice- and PCN-treated groups, compared to controls. Linear regression analysis showed significant correlation between liver tissue metformin concentrations and plasma lactic acid levels in both control (p=0.0122; r² = 0.9080) and grapefruit juice-treated ‘test’ rats (p=0.0005; r² = 0.9893) (Figure 29). The correlation coefficient was, however, stronger in the latter group. PCN treatment showed similar correlation co-efficient between blood lactate levels and liver metformin as with grapefruit juice treatment (data not shown on graph for clarity). Surprisingly, no correlation was observed between plasma lactate and metformin levels in both groups.

4.8 Effect of grapefruit juice on hepatic expression of OCT1 protein

Hepatocyte membrane fractions were separated by SDS-PAGE and transferred to PVD membranes. Ponceau S stains of the membrane before probing indicated that the protein bands were clearly separated and transferred (Figure 30). Similar staining of the gel confirmed that no proteins remained on it. Immunoblot assay showed the expression of a distinct band of 66 kDa protein, corresponding to rOctl protein, in response to different agonists (Figure 31). Densitometry scans expressed as a percentage of the controls showed that dexamethasone (80.6±40.28%) significantly (p<0.0001) downregulated rOctl expression, compared to grapefruit juice (285.6±33.4%), PCN (260±25.7%), or metformin and PCN (275±48.4%), respectively (Figure 30). However, grapefruit juice
treatment significantly (p<0.05) reversed dexamethasone-induced down-regulation of rOct1 (165.4±26.4%) (Figure 32). Metformin alone did not up-regulate rOct1 expression compared to grapefruit juice or PCN.
CHAPTER 5

DISCUSSION

A commercial preparation of grapefruit juice was used in this study in order to guard against variations in the chemical constituents/active ingredients due to harvesting time - which may influence ripening of the fruits [20]. The method of processing has previously been reported to influence the relative abundance of bioactive constituent compounds [19]. Batch processed commercial preparations would, therefore, be expected to minimize such variations. No signs of toxicity, or significant difference in weight gain were noted between grapefruit juice-treated (76.0±5.7 g) and control (72.8±4.9 g) rats, respectively, suggesting that grapefruit juice did not influence the natural growth of the animals (Figure 10).

HPLC and LC-MSD analysis showed that the grapefruit extract used in this study contained flavonoids and furanocoumarins (Figures 11, 12 and 13). Mass spectrometry fragmentations showed consistency with 6',7'-dihydroxybergamottin as well as 7-geranyloxycoumarin structures which were identified by fragment ions m/z 317 and 298.9, respectively (Figures 12 and 13). Even though other ionisation fragments were not identifiable, their fragmentation patterns were consistent with the presence of flavonoids (naringin, hesperidin, neohesperidin etc) and furanocoumarins (bergamottin, 6',7'-epoxybergamottin etc) respectively [353] (Table 5). Some of the fragment ions could have been part of dimmers of these chemical entities making direct identification of molar masses difficult.

Grapefruit juice lowered blood glucose levels in a dose-dependent manner (Figure 13) after the rats were challenged with 3.0 g/kg body weight of glucose, which corresponded to concomitant significant reduction in GI index (Table 6). Glycemic index is defined as “blood glucose response to a test food consumed by an individual under standard conditions, and expressed as a percentage of the AUC following consumption of reference food consumed by the same person on a different day” [351, 364]. By this definition, the test food in this study was the normal chow, and the reference food was glucose, which is normally assigned a GI of 100. Other carbohydrates were not used as
they would have interfered with glucose homeostasis. In this study, a maximum of 3.0 ml/kg body weight of glucose was administered to rats which weighed 316.4±16.3 g at end-point (Figure 10). The grapefruit juice used had a declared carbohydrate content of 0.6 g/100 ml. This implies that at a mean weight of 316 g, each rat was dosed with a maximum of 0.948 ml (948 µl) of grapefruit juice which contained an equivalent 6 mg of carbohydrate. Since 24 hrs had elapsed between the last grapefruit juice dosing and the time the test was done, it was highly unlikely that the negligible carbohydrate content of grapefruit juice could have influenced glycemic index calculation. Higher doses of grapefruit juice were not possible to administer since only a maximum of 1.0 ml was permissible orally by the weight of the rats.

Grapefruit juice significantly (p<0.05) lowered fasting blood glucose levels (Figure 14) without any significant effect on fasting or 1.5-hour post-glucose insulin level (Figure 18) in grapefruit juice-treated test (2.9±0.4 mmol/L), compared to control (3.7±0.39 mmol/L) rats. This observation, therefore, suggests that grapefruit juice or its constituent chemicals, could be mediating hepatic glucose homeostasis, rather than pancreatic insulin release. The study by Fujioka et al, [17] reported that grapefruit juice improved insulin resistance in patients with metabolic syndrome, with or without diabetes, but in this study, normal non-diabetic rats were used. In any case, the phenomenon of weight reduction associated with grapefruit, suggests that grapefruit effects on glucose control is more complicated than is currently understood, and may involve control of glucose and lipid metabolism in other organs, such as the intestines, liver, skeletal muscle and even adipose tissue. The current study, therefore, supports the hypothesis that grapefruit juice consumption promotes efficient glucose utilisation, and may, therefore, be beneficial in the management of metabolic syndrome.

PCN administration significantly elevated blood sugar, compared to control or grapefruit juice-treated rats, respectively (Figure 15). This elevation of blood sugar was, however, reversed by concomitant administration of metformin or grapefruit juice respectively. PCN is a known hepatic microsomal enzyme inducer [338], but its effects on blood glucose control have hardly been investigated. A study by Szabo et al, [365] reported that PCN-induced marked changes in blood glucose in rats; and another study by Kourounakis et al, [356] reported PCN induced depletion of hepatic glycogen. However,
in the present study, when liver glycogen content was measured in rats previously treated with PCN, significant (p<0.05) increase (372±27 mg/g of liver tissue) in hepatic glycogen content was observed compared to control rats (288±14 mg/g of liver tissue) (Figure 19). A recent study by Kodama et al, [338], reported that PCN activates PXR, which mediates down-regulation of glucagon stimulated gluconeogenic and glycogenolytic rate limiting step enzymes (glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK1). However, in this study, glucose-6-phosphatase activity increased (K_m = 1.299 mM; V_max = 23.84±1.102 µmol/min/g liver tissue) significantly (p<0.05) in PCN-treated compared to control (K_m = 2.068 mM; V_max = 13.4±0.45 µmol/min/g liver tissue) rats (Figures 22 and 23). PCN-induced increased activity of glucose-6-phosphatase was, however, abolished in the presence of metformin (K_m = 2.32 mM; V_max = 13.85±0.34 µmol/min/g liver tissue) (Table 8). Likewise, PEPCK activity increased in PCN-treated (K_m = 0.7151 mM; V_max = 27.77±0.81 µmol/g of liver tissue/ min) compared to control (K_m = 1.379 mM; V_max = 13.73±0.399 µmol/g of liver tissue/ min) rats (Figures 24 and 25). PCN-induced increased activity of PEPCK was significantly (p<0.05) reversed by metformin treatment (K_m = 1.398 mM; V_max = 14.32±0.749 µmol/g of liver tissue/ min) (Table 9). However, PCN did not significantly affect the activity of glucokinase (K_m = 422 µM; V_max = 11.51±0.83 µmol/min/g of liver tissue), compared to the controls (K_m = 308 µM; V_max = 13.56±0.72 µmol/min/g of liver tissue), despite the fact that it increased hepatic glycogen content (Table 7). Glucokinase is a regulatory enzyme controlling the first step in hepatic glycolytic pathway, and the calculated V_max/K_m ratios suggest that PCN (27.3) repressed glucokinase activity, compared to the controls (44.0) (Table 7). It is, therefore, possible that in this in vivo model, unlike the study of Kodama et al [338], which was done in vitro, PCN causes hyperglycemia by up-regulation of key gluconeogenic enzymes (G-6-Pase, and PEPCK), and concomitantly repressing glucokinase activity. This could be through modulation of key enzymes controlling the upstream regulators of G-6-Pase, PEPCK as well as glucokinase expression, such as AMPK. That metformin opposed PCN-mediated effects on both G-6-Pase and PEPCK suggests that there could be a common regulatory pathway that controls the activities of these enzymes.
Metformin is known to lower blood sugar by, amongst other mechanisms, up-regulation of AMPK, which inhibits the expression of PEPCK1 and G6Pase, respectively [334, 335]. Metformin did not affect hepatic glycogen content (290±15 mg/g of liver tissue) (Figure 19) but marginally increased glucokinase activity ($K_m = 396 \mu M; V_{\text{max}} = 20.4\pm0.406 \mu \text{mol/min/\mu g of liver tissue}$) compared to controls (288±14 mg/g of liver tissue), ($K_m = 308 \mu M; V_{\text{max}} = 13.6\pm0.72 \mu \text{mol/min/\mu g of liver tissue}$) respectively, (Figures 21 and 22), (Table 7), despite opposing PCN-induced activation of both G6Pase and PEPCK (Tables 8 and 9). However, metformin alone ($K_m = 0.0411 \text{mM}; V_{\text{max}} = 79.0\pm3.887 \text{nmol/min/\mu g of liver tissue}$), or in combination with PCN ($K_m = 0.0217 \text{mM}; V_{\text{max}} = 93.74\pm6.55 \text{nmol/min/\mu g of liver tissue}$), significantly ($p<0.0001$) increased AMPK activity compared to controls ($K_m = 0.146 \text{mM}; V_{\text{max}} = 7.442\pm0.411 \text{nmol/min/\mu g of liver tissue}$) (Figures 26 and 27) (Table 10). It is important to point out at this stage that, PCN is known to activate OCT1, which transports metformin into hepatocytes [314]. It is, therefore, logical to speculate that PCN enhanced hepatic uptake of metformin, which inhibited gluconeogenic/glycogenolytic pathways through upregulation of AMPK, which may, therefore, suggest that PCN-induced hyperglycemia may be mediated by up-regulation of G6Pase and PEPCK, which is abolished in the presence of metformin. However, there may be many other nuclear receptors regulating synthesis of hepatic gluconeogenic/glycogenolytic enzymes, which are PCN responsive. Such factors may up-regulate enzymes like glycogen synthase, or down-regulate glycogen phosphorylase, which may account for the observed increase in hepatic glycogen storage in the present study. These observations, however, need to be verified by hepatic gene expression of these enzymes to confirm whether they occur at gene transcription level, or at post-translation processing of the relevant proteins, or both. However, preliminary data available so far seem to suggest the latter [331, 332, 333, 334, 336].

Grapefruit juice improved glucose tolerance and exerted metformin-like effect on blood sugar levels (Figure 16). Fasting blood glucose levels were significantly ($p<0.05$) lower in grapefruit juice treated {3.48±0.47 (oral), 3.68±0.19 (IP) mmol/L}, than in control {4.12±0.34 (oral), 4.32±0.21 (IP) mmol/L} rats, respectively, prior to administration of 3.0 g/kg body weight of glucose (Figure 16). However, blood glucose-time response
curves constructed showed no significant difference between oral and intraperitoneal administration of glucose (Figure 16B and C). This suggests that grapefruit juice administration did not significantly affect oral absorption of glucose, taking into account the known effect of grapefruit juice on intestinal glucose metabolism, and that potential hypoglycaemic effect of grapefruit juice could be due to an influence on hepatic metabolism of glucose. Glucose tolerance test was done with metformin as a 'reference drug'. Grapefruit juice alone (495±77 AUC units), or in combination with metformin (491±61 AUC units), significantly (p<0.05) lowered blood glucose levels in the 'test' compared to 'control' (636±59 AUC units) animals, but insignificantly (p>0.05) compared to metformin (543±96 AUC units) (Figures 17A and B). Metformin alone (at a dose of 250 mg/kg body weight) lowered blood glucose levels compared to controls, but less than grapefruit juice- or grapefruit juice in combination with metformin-treated rats, respectively, but the difference was not statistically significant. Perhaps more profound differences could have been observed with an increased dose of metformin, since we previously noted a dose-dependent reduction in blood glucose levels with grapefruit juice alone (Figure 13). These observations, therefore, suggest that grapefruit juice could be exhibiting metformin-like effects on hepatic regulation of blood glucose. Metformin is a widely-used antidiabetic agent that lowers blood glucose levels by inhibiting hepatic gluconeogenesis, enhancing hepatic and skeletal muscle glucose uptake, and reducing intestinal glucose absorption [344]. It is unlikely that metformin alone, or in combination with grapefruit juice, could have interfered with glucose absorption in this study, since glucose was administered intraperitoneally.

Grapefruit juice (409±25 mg/g of liver) significantly (p<0.05) increased hepatic glycogen content compared to controls (288±14 mg/g of liver) (Figure 19). Metformin alone (290±15 mg/g of liver) did not significantly change hepatic glycogen content compared to controls but when co-administered with PCN, significantly (p = 0.0079) reversed PCN-induced glycogen storage in the liver (Figure 18). Grapefruit juice significantly (p<0.05) increased hepatic glucokinase activity (K_m = 144 μM; V_max = 26.65±1.11 μmol/min/g of liver tissue) compared to controls (K_m = 308 μM; V_max = 13.56±0.72 μmol/min/g of liver tissue), but decreased (K_m = 2.69 μM; V_max = 16.83±0.36 μmol/min/g of liver tissue) the enzyme activity when co-administered with
dexamethasone, compared to dexamethasone alone ($K_m = 344 \mu M; V_{max} = 18.66\pm0.46 \mu mol/min/g$ of liver tissue) (Figures 20 and 21). Metformin, on the other hand, had only a modest increase in glucokinase activity (Table 7). It is not possible to determine whether increased glucokinase activity in response to grapefruit juice treatment corresponded to increased hepatic glycogen content since linear regression analysis of glucokinase activity and hepatic glycogen content, showed no positive correlation. It is possible that increased glycolytic flux in this respect translated into glucose expenditure in energy generation (ATP synthesis), or other cellular anabolic reactions such as lipogenesis.

However, grapefruit juice alone ($K_m = 2.29 \text{ mM}; V_{max} = 14.02\pm0.54 \mu mol/min/g$ liver tissue) or metformin alone ($K_m = 2.23 \text{ mM}; V_{max} = 13.85\pm0.348 \mu mol/min/g$ liver tissue) significantly (p<0.05) reduced glucose-6-phosphatase activity, compared to dexamethasone ($K_m = 1.705 \text{ mM}; V_{max} = 23.19\pm0.58 \mu mol/min/g$ liver tissue), and PCN ($K_m = 1.299 \text{ mM}; V_{max} = 23.84\pm1.10 \mu mol/min/g$ liver tissue) respectively (Figures 22 and 23). When both grapefruit juice and metformin were co-administered with dexamethasone ($K_m = 1.738 \text{ mM}; V_{max} = 19.26\pm0.41 \mu mol/min/g$ liver tissue) and PCN ($K_m = 2.26 \text{ mM}; V_{max} = 13.34\pm0.8 \mu mol/min/g$ liver tissue), respectively, glucose-6-phosphatase activity was similarly reduced compared to either dexamethasone or PCN alone, respectively (Table 8). Similarly grapefruit juice ($K_m = 1.55 \text{ mM}; V_{max} = 12.44\pm0.41 \mu mol/min/g$ liver tissue) did not have any significant effect on PEPCK activity compared to controls ($K_m = 1.379 \text{ mM}; V_{max} = 13.37\pm0.399 \mu mol/min/g$ liver tissue), but significantly (p<0.05) reduced ($K_m = 2.78 \text{ mM}; V_{max} = 10.72\pm0.363 \mu mol/min/g$ liver tissue) dexamethasone-induced increase in PEPCK activity ($K_m = 0.0738 \text{ mM}; V_{max} = 26.78\pm0.41 \mu mol/min/g$ liver tissue) (Figures 24 and 25). Likewise, metformin alone ($K_m = 1.615 \text{ mM}; V_{max} = 12.1\pm0.481 \mu mol/min/g$ liver tissue) did not significantly affect PEPCK activity compared to controls, but significantly (p<0.05) antagonised ($K_m = 1.398 \text{ mM}; V_{max} = 14.32\pm0.749 \mu mol/min/g$ liver tissue) PCN-induced ($K_m = 0.7151 \text{ mM}; V_{max} = 27.77\pm0.81 \mu mol/min/g$ liver tissue) increase in PEPCK activity when both metformin and PCN were administered concurrently (Figures 24 and 25), (Table 9). A recent study by Purushotham et al, [29] reported that naringenin, a naringin aglycone commonly found in grapefruit juice (Figure 2) (Table 1), suppressed
hepatic glucose production from hepatoma (Fao) cells, and that like metformin, decreased cellular ATP levels without decreasing cellular toxicity. Previously, Jung et al., [27] reported that hesperidine and naringin reduced blood glucose in diabetic mice by elevating hepatic glucokinase and glycogen concentration, by suppressing the activities of glucose-6-phosphatase and PEPCK, respectively. These studies, like the present one, confirm that grapefruit juice or its constituent chemicals, have metformin-like regulatory properties on hepatic metabolism of glucose by suppressing or activating the key gluconeogenic or glycolytic enzymes reducing blood glucose levels. However, the earlier studies, unlike the present one, were either done on diabetic mice, or used purified grapefruit juice ingredients in vitro. It has to be pointed out that similar attempts at determining constituent chemicals of grapefruit that are responsible for the inhibition of intestinal CYP3A4 gave inconclusive evidence, owing to the diversity of the composition of such chemicals in the grapefruit juice.

It is not clear from these studies [27, 29] whether naringenin, or hespiridin, regulates the activities of these enzymes via direct chemical interaction or regulation of gene transcription/translation of the corresponding proteins. However, the current study confirms that, like metformin, grapefruit juice alone (Km = 0.0298 mM; Vmax = 96.44±2.478 nmol/min/g liver tissue) or in combination with dexamethasone (Km = 0.0321 mM; Vmax = 84.71±3.949 nmol/min/g liver tissue), significantly (p<0.0001) increased AMPK activity in the ‘test’ compared to ‘control’ (Km = 0.146 mM; Vmax = 7.442±0.411 nmol/min/g liver tissue) preparations (Figures 26 and 27) (Table 10). This suggests that down-regulation of glucose-6-phosphatase and PEPCK activities observed with grapefruit juice could be as a result of increased AMPK activity, which, therefore down-regulated the expression of glucose-6-phosphatase and PEPCK respectively.

The present study therefore provides the first evidence that anti-obesity effects of grapefruit juice consumption may be mediated by AMPK which may have downregulated key gluconeogenic enzymes. AMPK-mediated effects of grapefruit juice may be short-term (via phosphorylation of these enzymes) or long-term (via regulation of gene expression of these enzymes) (Figure 33). In the present study, the rats were treated with grapefruit juice for 14 days which was long enough to induce chronic effects such as gene expression of AMPK and its downstream targets. AMPK activation in the liver
leads to metabolic consequences (such as inhibition of gluconeogenesis) that are beneficial to diabetic patients. Grapefruit juice induction of increased AMPK activity may as well establish grapefruit juice or its chemical constituents as one of the AMPK modulators which are currently being vigorously pursued as potential anti-diabetic and anti-obesity agents.

Metformin treatment caused a significant (p=0.0079) increase in blood lactic acid levels, compared to control rats (5.38±2.53 versus 2.54±0.7 mmol/L, respectively) (Figure 28). There was no significant difference in blood lactate levels in grapefruit juice-treated rats (2.8±1.4 mmol/L) and control animals. However, when metformin was administered to rats which had been previously treated with grapefruit juice, there was a significant increase (p<0.05) in lactate levels (8.3±2.5 mmol/L), compared to the control, grapefruit juice- or metformin alone-treated rats, respectively (Figure 28). Metformin-associated lactic acidosis is commonly seen in patients with renal dysfunction, congestive heart failure, and acute or chronic metabolic acidosis [317, 346, 347, 348]. Intestinal lactate production in the absence of conditions associated with tissue hypoxia has previously been described [327]. In the experimental animal model used in this study, lactate could not have been produced from the intestines since glucose was administered intraperitoneally. Experimental animal studies have also shown that metformin does not cause a net increase in lactate production in other tissues such as muscle, fat, brain, and skin [325, 326], hence, the increase in lactate levels observed in the present study could only have been of hepatic origin. In any case, lactate produced from the intestines is likely to be rapidly removed from circulation by hepatic metabolism.

Under normal circumstances, hepatic clearance of lactate by the liver maintains lactate levels below 5 mmol/L. Renal impairment causes metformin-associated lactic acidosis, due to reduced clearance, causing metformin to build up in the body. Metformin is not metabolised in the body, but solely excreted by the kidneys. However, other conditions such as metformin overdose, may also lead to lactic acidosis in the absence of renal failure [349]. Such conditions may thus be described as leading to metformin-induced lactic acidosis. In the present study, normal, non-diabetic rats were used, and, therefore, the increased lactate production can only be attributed to the dose of metformin administered, and its accumulation in hepatocytes. Clinically, lactic acidosis has
previously been reported in patients without chronic renal failure, who took metformin
[343], contrary to the popular belief that metformin, unlike other biguanides, does not
cause lactic acidosis. The present study, therefore, provides direct *in vivo* evidence that
metformin can cause lactic acidosis without the commonly-associated co-morbidities.
The results presented here surprisingly show an enhanced lactate production when rats
that had previously been exposed to grapefruit juice were treated with metformin,
compared to the rats that were given metformin alone (Figure 28). Rats that were treated
with grapefruit juice alone had similar lactate levels to the controls, suggesting that
grapefruit did not induce tissue hypoxia, or cause metabolic disturbances that may have
led to increased lactate levels. It may, therefore, be concluded that grapefruit juice, just
like PCN, facilitated metformin uptake by hepatic tissue because, even though plasma
metformin levels in grapefruit juice-treated and control rats were comparable, metformin
concentrations in the liver tissue was significantly higher in grapefruit juice- and PCN-
treated ‘test’ than in the ‘control’ rats (Table 11). Plasma: liver metformin ratio was
significantly (*p*<0.05) elevated in PCN- and grapefruit juice-treated rats, respectively.
Linear regression analysis showed stronger correlation between metformin concentration
in the liver, and plasma lactate levels in grapefruit juice- and PCN-treated ‘test’ animals
than in the control rats (Figure 29). No such correlation was apparent between plasma
lactate and metformin concentrations in both groups. These results are in tandem with
those of Wang *et al.*, [314], who showed that lactic acidosis is mainly caused by intra-
hepatic metformin, which inhibits lactate metabolism in the liver. In view of this, it is,
therefore, not surprising that Stades *et al.*, [367] found no positive correlation between
plasma metformin concentration and lactic acid. Metformin is a known specific substrate
of organic cation transporter protein (OCT1), which is mainly expressed in the liver, and
to a lesser extent, in the kidney [225, 241, 242, 243]. Wang *et al.*, [314] have clearly
demonstrated that OCT1 mediation of hepatic uptake of metformin is linked to the
development of lactic acidosis in rats. This, therefore, suggests that grapefruit juice could
be up-regulating or stimulating the activity of OCT1, which enhances liver uptake of
metformin.
The expression of rOct1 in hepatocytes in response to grapefruit juice, a known inducer
PCN and down-regulator, dexamethasone, were further investigated. rOct1 is a known
membrane protein, and the 50 μg protein loaded on the SDS-PAGE gel was deemed to be microsomal membrane protein-rich. The Ponceau S staining (Figure 30) confirmed equal loading and successful transfer of the proteins from the gel to the membrane. Immunoreactive bands detected by immunoblot assay, identified a prominent 66kDa protein corresponding to rOct1, to be expressed in rat liver microsomal protein fraction (Figure 31). When the membrane was stripped and re-probed by rat anti-rabbit Na/K-ATPase monoclonal antibody, prominent bands were identified in all the membrane fractions separated by SDS-PAGE, suggesting equal enrichment with membrane fractions. Na/K-ATPase is a membrane protein commonly found in mammalian cells. Densitometry scans of the immunoreactive bands showing steady-state protein levels expressed as a percentage of the controls, showed that dexamethasone (80.6±40.28%) significantly (p<0.0001) down-regulated rOct1 expression, compared to grapefruit juice (285.6±33.4%), PCN 260±25.7%), or metformin and PCN (275±48.4%), respectively (Figure 32). However, grapefruit juice treatment significantly (p<0.05) reversed dexamethasone-induced down-regulation of rOct1 (165.4±26.4%). Metformin alone did not up-regulate rOct1 expression, compared to grapefruit juice or PCN. Maeda et al, [263] previously presented evidence confirming PCN up-regulation, as well as dexamethasone [264] down-regulation of the expression of rOct1.

The present study, therefore, shows for the first time, that despite the well known metabolic effects of grapefruit juice on intestinal metabolism, leading to drug interactions, the liver is another organ where grapefruit juice equally exerts its effects. Although the mechanism by which grapefruit juice up-regulated rOct1 expression could not be established in this study, it is possible that grapefruit juice or its components, just like PCN, are ligands of PXR which gets activated upon exposure, and then binds to the relevant promoter regions, to initiate the transcription of rOct1 mRNA, as well as other post-transcription regulator proteins, such as PKA, PKC, etc, (Figure 33). Grapefruit juice-mediated reversal of dexamethasone-suppressed expression of OCT1 may be as a result of glucocorticoid receptor antagonism, which, therefore, allowed for enhanced expression of PXR. Glucocorticoid receptor has previously been suggested to repress PXR expression, leading to reduced OCT1 expression [264]. Alternatively, grapefruit juice could be exerting a direct antagonistic effect on glucocorticoid receptor, leading to
increased expression of OCT1 expression (Figure 33). It may also be possible that regulation of OCT1 activity occurs at post-transcriptional levels, whereby grapefruit juice either up-regulates translation of the rOCT1 protein, thus increasing the protein content, or increasing the activity of the protein. A functional in vitro study involving physiologically-known substrates of OCT1, such as 1-methyl-4-phenylpyridinium (MPP) or tetrapentylammonium (TEA), using cultured hepatocytes, could have shed more light on this, but such an approach was technically beyond the scope of this study.

PXR activation by grapefruit juice or its constituent chemicals, may also suppress the transcription of genes coding for gluconeogenic/glycogenolytic enzymes, leading to increased hepatic glucose uptake, and reduced gluconeogenesis (Figure 33). This may explain the apparent hypoglycaemic effect of grapefruit juice. Metformin is not metabolised by liver enzymes, and is eliminated in the kidneys unchanged [323, 324, 343], a process mediated by both OCT1 and OCT2. The data presented here clearly demonstrate that metformin does accumulate in the liver tissue, contrary to earlier suggestions [367]. If that is the case, then what happens if hepatocytes are exposed to increased concentrations of metformin? OCT1 is mainly found on the sinusoidal membrane of the hepatocytes [225, 234], and is known to effect bi-directional transport of substrates [249, 250]. It may be possible that OCT1-mediated transport of metformin across hepatocyte membrane is concentration gradient-dependent, such that at high plasma concentrations, metformin is largely transported into the cells, and vice versa. Increased accumulation of metformin in hepatocytes then leads to oxidative stress, due to diminished mitochondrial oxidative phosphorylation. This, therefore, causes conversion of pyruvate to lactate in the cytosol, which leads to lactic acidosis. Metformin has been shown in vitro to reduce oxygen consumption in cultured hepatocytes by inhibition of mitochondrial respiratory complex I [318, 319]. Grapefruit juice, in this case, may be guilty by association, since it facilitates metformin uptake of metformin by hepatocytes.
However, grapefruit juice associated increase in lactate levels in the presence of metformin is a clear case for concern. It appears that grapefruit juice can exacerbate lactic acidosis in the presence of metformin by facilitating its accumulation in hepatocytes. Subject to confirmation of these findings in humans, the present results suggest that despite its beneficial effect in the control of glycaemic index, grapefruit juice should be contra-indicated in diabetic patients who are on concurrent medication with metformin.

5.2 CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE STUDIES

Grapefruit juice has metformin-like effects on hepatic metabolism of glucose: it improves glucose tolerance, does not influence plasma insulin, and is hypoglycaemic. Grapefruit juice ameliorates PCN-induced hyperglycemia, upregulates hepatic glucokinase activity, but down-regulates glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) activities, respectively, by up-regulating hepatic glucose sensor, adenosine monophosphate protein kinase (AMPK). Grapefruit juice also up-regulates hepatic expression of rOct1, which, therefore, facilitates metformin accumulation in hepatocytes, leading to lactic acidosis. This suggests that although grapefruit juice improves glucose tolerance, and may be beneficial in diabetic patients with metabolic syndrome, caution should be exercised in patients who are on concurrent medication with metformin. Further studies in human subjects are suggested.

Recommendations for future studies:

i) determination of chemical ingredients of grapefruit juice responsible for glucose plasma glucose-lowering effect, through modulation of the activities of key hepatic glycolytic, gluconeogenic and glycogenolytic enzymes;

ii) elucidation of the effects of grapefruit up-regulation of adenosine monophosphate protein kinase (AMPK) at molecular level since this enzyme has become a putative pharmacological target in the treatment of metabolic disorders;
iii) determination of the mechanism of grapefruit juice up-regulation of rOCt1 at molecular level, using natural substrates and/or inhibitors of rOct1;

iv) clinical trials to determine the extent of grapefruit juice-induced effects on lactic acidosis in diabetic patients taking metformin.
6.0  APPENDICES

6.1  FIGURES

Figure 1. Ruby grapefruit.
Enzymatic hydrolysis of naringin. Naringinase is an enzymatic complex with α- rhamnosidase activity (hydrolyses naringin to prunin and rhamnose) and β-glucosidase activity which in turn, hydrolyses prunin to naringenine and glucose, respectively. Rha-Glc = Rhamnoglucose [24].
Figure 3.
PXR-mediated regulation of cellular expression of drug-metabolising enzymes, transporter proteins and other cellular proteins. Xenobiotics or endobiotic ligands bind intracellular PXR which gets activated and forms heterodimer with RXR. The complex then diffuses into the nucleus and binds relevant promoter regions of the target genes to initiate transcription of the mRNAs, which in turn, get translated to the relevant proteins. Transporter proteins are membrane bound, while the enzymes remain in the cytosol. P-gp extrudes its substrates from the cytoplasm, while OCT and OATPs may affect bi-directional flow of the substrates across the cell membrane, depending on the concentration gradient.
Transproters involved in hepatic drug disposition. Drugs, bile salts and metabolites are taken up in the hepatocytes by SLC proteins, such as OCT1, OATP and NTPC. Once inside the cell, they may undergo oxidative metabolism by CYP450 enzyme, followed by phase II hepatic metabolism to render them less hydrophobic. Products of phase II reactions may be extruded from the cell by efflux pumps, such as MRP1/MRP3 into systemic circulation, or P-glycoproteins (P-gp) and MRP2 into the bile canaliculi. Alternatively, some substrates of SLC proteins may be taken out of the cell via efflux pumps without metabolism. The efflux pumps help to maintain concentration gradients, which facilitate the uptake of substrates by SLC transpoters on the basolateral membrane.
Schematic illustration of the structure of P-gp. The protein consists of 12 hydrophobic transmembrane segments, split into 2 distinct transmembrane domains, TMD1 and TMD2, on the N- and C-terminus of the peptide, respectively. Each domain has a nucleotide binding domain, NBD1 and NBD2, respectively, on the cytoplasmic side of the membrane. Each NBD has the characteristic signature motif and Walker A and B, respectively, crucial to the hydrolysis of ATP and conformational changes of the proteins [154, 155, 156, 157]. Adapted and modified with permission from [149].
Figure 6.
Proposed mechanism of action of P-gp. The classical pump, such as Na⁺K⁺-ATPase, transports substrates across hydrophobic membranes through hydrophilic channel of the transmembrane region of the protein. But the “vaccum cleaner” model of P-gp proposes that the drugs first partition into the lipid bilayer, and then interact with P-gp within the membrane, before being extruded into the aqueous phase of the extracellular side. In the ‘flippase’ model, drugs partition into the membrane, interact with the drug binding pocket in P-gp within the cytoplasmic leaflet, and are then translocated to the outer membrane leaflet. Reproduced with permission from [149].
Figure 7.
Schematic representation of the proposed structure of rat Oatp1. There are 12 transmembrane (TMD) and 6 extracellular domains (EXD). Conserved amino acid residues are colour coded red, yellow (charged) and cystein (red asterisks). Potential N-glycosylation sites are flagged with stars, while the region containing the OATP superfamily signature is demarcated with dotted lines at the border of EXD3 and TMD6.
Figure 8.
A proposed secondary structure of OCT1. The 12 transmembrane domains (TMD) are flanked by intracellular N- and C-terminus. TMDs 1 and 2 are connected by a large extracellular loop, which is believed to contain 3 N-linked glycosylation sites (flagged with red stars), while TMDs 6 and 7 are interconnected by a large intracellular loop containing potential phosphorylation sites (marked with red crossed circles). TMD4 and TMD10 contain 3 amino acid residues each (marked with blue and red circles, respectively to indicate that they are conserved), while TMD11 has 1 (marked with yellow circle). These amino acids surround a large cleft believed to be the substrate binding pocket [223, 239].
Figure 9.
Schematic illustration of the transcriptional and post-transcriptional regulation of OCT1. In response to endogenous or exogenous agonists, HNF-4α binds DR-2 in the promoter region, and activates transcription of OCT1. Other ligands may alternatively activate PXR which dimerizes with RXRa, and then binds DNA to initiate transcription of OCT1. Such ligands may concurrently trigger transcription of regulatory proteins, such as PKA, PKC, and calmodulin (CaM). In post-transcriptional regulation, these regulatory proteins may phosphorylate membrane-bound OCT1 to either deactivate or activate it. Forskolin may activate PKA, which phosphorylates OCT1, and hence activating it, while antagonism of CaM by calmidazolium may increase substrate affinity of hOCT1. PKC phosphorylation of OCT1 is still not well understood. Some xenobiotics may directly interact with OCT1 on the plasma membrane, either as substrates or modulators [225, 265, 270].
Figure 10.
Animal weights at the beginning of the experiments (baseline), and at the end of the two-week treatment period (end-point), for control and grapefruit juice-treated (GFJ) test rats, respectively.

Figure 11.
A representative chromatogram of the grapefruit juice extract. The polar flavonoids eluted earlier (retention times <7.5), while the furanocoumarins eluted at retention times >7.5 minutes.
Figure 12.
Mass spectrometry analysis of HPLC chromatograms of the grapefruit juice extract.
Dose-response effects of grapefruit juice on plasma glucose levels of grapefruit-treated rats challenged with 3.0 g/kg body weight of glucose orally.
Figure 14.
Fasting blood glucose levels in grapefruit juice-treated (GFJ) ‘test’ rats compared to control animals. Blood sugar was tested in rats fasted overnight for 12 hours.
Figure 15.
AUC calculated from random blood glucose sampling (at 30-minute intervals) of non-fasted but food restricted rats for a period of 90 mins. Some of the rats were previously (24 hrs before) treated with subcutaneous injection of PCN or polyethylene glycol (17 mg/kg body weight (15 mg/ml in propylene glycol)). PCN- and polyethylene glycol-treated rats were previously treated with oral GFJ for 14 days, while non-GFJ-treated rats were either given oral metformin (with PCN) or distilled water (controls), respectively.
(A)

(Fasting blood glucose (mmol/L))

- Oral-baseline
- Oral-GFJ
- IP-baseline
- IP-GFJ

\[ P = 0.042 \]

\[ P = 0.0017 \]

(B)

(Blood glucose (mmol/L))

- Oral-GFJ
- IP-GFJ
- IP-baseline
- Oral-baseline

Time (mins)
Figure 16.
Fasting blood glucose was measured in all treatment groups (A), followed by either oral or intraperitoneal administration of 3.0 g/kg body weight of glucose in distilled water or normal saline, respectively. Blood sugar levels were then monitored at 30-minute intervals for 90 minutes, and GTT curves constructed (B). The AUC were then calculated from blood glucose-time curves in control and grapefruit juice-treated test rats, respectively (C). The baseline values represent calculations from the animals that were treated with distilled water (oral) or normal saline (IP) only.
Figure 17.
GTT response (A) when rats were challenged with intraperitoneal injection (i.p.) of 3.0 g/kg body weight of glucose, after treatment with either 3.0 ml/kg body weight of grapefruit juice, 250 mg/kg body weight of metformin or both, respectively. The area-under-the-curves (AUC) were calculated from corresponding plasma glucose-time curves (B).
Figure 18.
Fasting plasma insulin levels in rats before intraperitoneal injection of 3.0 g/kg body weight of glucose in saline.
Figure 19.
Glycogen content of liver tissue after treatment with indicated mediators of glucose homeostasis. (* p<0.05).
Figure 20.
Michaelis-Menten kinetics of glucokinase activity in the rat liver samples previously treated with different agonists in vivo. Enzyme activity was plotted against substrate concentrations.
Figure 21.
Linear regression analysis of Eadie-Hofstee plots used to calculate $K_m$ and $V_{\text{max}}$ of glucokinase. The Y-intercepts correspond to $V_{\text{max}}$, while the X-intercepts correspond to $V/K_m$ values of different treatment groups, respectively. $K_m = 1/\text{slope}$. 
Figure 22.
Michaelis-Menten kinetics of glucose-6-phosphatase activity in the rat liver samples previously treated with different agonists \textit{in vivo}. Enzyme activity was plotted against substrate concentrations.
Figure 23.
Linear regression analysis of Eadie-Hofstee plots used to calculate $K_m$ and $V_{max}$ of glucose-6-phosphatase. The Y-intercepts correspond to $V_{max}$, while the X-intercepts correspond to $V/K_m$ values of different treatment groups, respectively. $K_m = 1/slope$. 
Figure 24.
Michaelis-Menten kinetics of PEPCK activity in the rat liver samples previously treated with different agonists in vivo. Enzyme activity was plotted against substrate concentrations.
Figure 25.

Linear regression analysis of Eadie-Hofstee plots used to calculate $K_m$ and $V_{max}$ of PEPCK. The Y-intercepts correspond to $V_{max}$ while the X-intercepts correspond to $V/K_m$ values of different treatment groups respectively. $K_m = 1/slope$. 

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Michaelis-Menten kinetics of AMPK activity in the rat liver samples previously treated with different agonists in vivo. Enzyme activity was plotted against substrate concentrations.
Figure 27.
Linear regression of Eadie-Hofstee plots used to calculate $K_m$ and $V_{max}$ of AMPK. The Y-intercepts correspond to $V_{max}$, while X-intercepts correspond to $V/K_m$ values of different treatment groups, respectively. $K_m = 1/$slope.
Figure 28.
Blood lactate levels in rats that were treated with 250 mg/kg body weight of metformin, 3.0 ml/kg body weight of grapefruit juice (GFJ), or combination of both, respectively. Blood samples were collected 1.5 hours after the rats were challenged with intraperitoneal injections of 3.0 g/kg body weight of glucose in saline.
Figure 29.
Linear regression analysis of metformin concentrations in liver tissue and plasma lactic acid levels in grapefruit-treated ‘test’ and ‘control’ rats, respectively, 1.5 hr after an oral dose of 250 mg/kg body weight of metformin.
Figure 30.

Ponceau S staining of membrane protein-rich microsomal fraction of the liver homogenate on PVD membrane after SDS-PAGE. 50 µg protein from the membrane fractions were diluted 1:4 with sample loading buffer, and loaded into each well. Molecular weights in kiloDaltons (kDa) are indicated by the marker on the left panel.

Figure 31.

Immunoreactive bands after exposure to autoradiography showing the expression of rOct1 in the rat liver after treatment with different agonists. Protein content of rOct1 was normalised by stripping the membrane with the strip buffer and re-probing with rat anti-rabbit Na/K-ATPase antibody as a basolateral membrane marker, in order to correct for equal enrichment with membrane preparations.
Figure 32.
Densitometry scans of rOct1 expression in the rat liver. Steady-state protein level was expressed as a percentage of the control.
Proposed grapefruit juice-mediated regulation of hepatic glucose metabolism and OCT1 protein expression. Grapefruit juice or its constituent chemicals, may activate the expression of PXR, which in turn up-regulates the expression of rOct1, or alternatively, deactivates glucocorticoid receptor, which may allow for the expression of rOct1. In response to grapefruit juice induction, PXR may also up-regulate the expression of protein kinases (PKA, PKC), which may modulate the activity of rOct1 either through direct phosphorylation, or modulation of translation of its mRNA. In response to grapefruit juice activation, PXR may also up-regulate AMPK (which is activated by metformin) to suppress the expression of gluconeogenic/glycogenolytic enzymes (G6Pase and PEPCK). AMPK may also activate Glut2 transporter protein to facilitate cellular uptake of glucose. Metformin may be transported into, and outside the cell, by OCT1 depending on the relative concentration across the cell membrane.
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<th>Compounds</th>
<th>Bioactive constituents</th>
<th>Pharmacological Effects</th>
<th>Potential Clinical Applications</th>
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Table 1.
Some of the bioactive compounds so far identified in the grapefruit and their pharmacological actions.
### Table 2.
Selected substrates of P-gp. The substrates marked with (*) are also substrates of CYP3A4 [172, 173, 174].

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<th>Other cardiovascular drugs</th>
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*Table 2.*

Selected substrates of P-gp. The substrates marked with (*) are also substrates of CYP3A4 [172, 173, 174].
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<th>Glucose (g/kg body weight)</th>
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<td>-</td>
<td>3.0</td>
<td>-</td>
<td>3.0 (DEX)</td>
<td>5</td>
</tr>
<tr>
<td>B</td>
<td>B₁</td>
<td>-</td>
<td>3.0</td>
<td>-</td>
<td>3.0 i.p</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>B₂</td>
<td>3.0</td>
<td>-</td>
<td>-</td>
<td>3.0 (PCN)</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>B₃</td>
<td>-</td>
<td>3.0</td>
<td>-</td>
<td>3.0 (PCN)</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>B₄</td>
<td>3.0</td>
<td>-</td>
<td>-</td>
<td>3.0 i.p</td>
<td>5</td>
</tr>
<tr>
<td>C</td>
<td>C₁</td>
<td>3.0</td>
<td>-</td>
<td>-</td>
<td>3.0 (DEX)</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>C₂</td>
<td>3.0</td>
<td>-</td>
<td>250</td>
<td>3.0 (PCN)</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>C₃</td>
<td>-</td>
<td>3.0</td>
<td>-</td>
<td>3.0</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>C₄</td>
<td>-</td>
<td>3.0</td>
<td>250</td>
<td>3.0</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 3.
Animal treatment schedule as explained in the experimental procedure. i.p= intraperitoneal injection. PCN = pregnenolone-16alpha-carbonitrile (17 mg/kg body weight), DEX = dexamethasone (1.0 mg/kg body weight).
<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Loading Buffer</th>
<th>Loading Buffer</th>
<th>Running Buffer</th>
<th>Running Buffer</th>
<th>Transfer Buffer</th>
<th>Transfer Buffer</th>
<th>TBS_T</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Quantity</td>
<td>Conc.</td>
<td>Separating gel (0.375 M Tris pH 8.8)</td>
<td>Stacking gel (4%), (0.125 M pH 6.8)</td>
<td>Quantity</td>
<td>Conc. pH 8.3</td>
<td>Quantity pH 7.4</td>
</tr>
<tr>
<td>0.5 M Tris HCl</td>
<td>1.0</td>
<td>62.5 M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td>1.6</td>
<td>20%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% SDS</td>
<td>1.6</td>
<td>20%</td>
<td>0.5 ml</td>
<td>100 µl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-mercaptoethanol</td>
<td>0.4</td>
<td>5%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5% Bromophenol blue (w/v)</td>
<td>0.4</td>
<td>5%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30% Bis-acryl amide</td>
<td></td>
<td></td>
<td>16.65 ml</td>
<td>1.3 ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5 M Tris HCl pH 8.8</td>
<td></td>
<td></td>
<td>12.5 ml</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 M Tris HCl pH 6.8</td>
<td></td>
<td></td>
<td></td>
<td>2.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% ammonium persulphate</td>
<td></td>
<td></td>
<td>250 µl</td>
<td>50 µl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEMED</td>
<td></td>
<td></td>
<td>25 µl</td>
<td>50 µl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trizma base</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>60.06 g</td>
<td>25 mM</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Glycine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>28.8 g</td>
<td>192 mM</td>
<td></td>
</tr>
<tr>
<td>Methanol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>400 ml</td>
<td>20% (v/v)</td>
<td></td>
</tr>
<tr>
<td>Tween 20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>900 µl</td>
</tr>
<tr>
<td>KCl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.2 g</td>
</tr>
<tr>
<td>NaCl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>3.0 ml</td>
<td></td>
<td>20.1 ml</td>
<td>6.1 ml</td>
<td>1600 ml</td>
<td></td>
<td>900 ml</td>
</tr>
</tbody>
</table>

Table 4.
Buffers used in SDS-PAGE analysis of OCT1 proteins in the liver tissue. The quantities added and effective concentrations are as indicated.
<table>
<thead>
<tr>
<th>Category</th>
<th>Compound</th>
<th>MS Fragment ions (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavonoids</td>
<td>Unidentified</td>
<td>160.7</td>
</tr>
<tr>
<td></td>
<td>Unidentified</td>
<td>176.7</td>
</tr>
<tr>
<td></td>
<td>Unidentified</td>
<td>176.8</td>
</tr>
<tr>
<td></td>
<td>Unidentified</td>
<td>234.9</td>
</tr>
<tr>
<td></td>
<td>Unidentified</td>
<td>272.8</td>
</tr>
<tr>
<td>Furanocoumarins</td>
<td>7-geranyloxycoumarin</td>
<td>298.9</td>
</tr>
<tr>
<td></td>
<td>Unidentified</td>
<td>335.1</td>
</tr>
<tr>
<td></td>
<td>6',7'-dihydroxyermottin</td>
<td>337.1</td>
</tr>
<tr>
<td></td>
<td>Unidentified</td>
<td>339.1</td>
</tr>
<tr>
<td></td>
<td>Unidentified</td>
<td>375.0</td>
</tr>
<tr>
<td></td>
<td>Unidentified</td>
<td>427.1</td>
</tr>
<tr>
<td></td>
<td>Unidentified</td>
<td>471.1</td>
</tr>
</tbody>
</table>

Table 5.
Prominent peaks of grapefruit juice extracts separated by HPLC chromatography and analysed by mass spectrometry. Unidentified peaks could represent ionisation fragments of the constituent chemicals or dimers of the same (19).
<table>
<thead>
<tr>
<th></th>
<th>0 ml GFJ</th>
<th>1.0 ml GFJ</th>
<th>2.0 ml GFJ</th>
<th>3.0 ml GFJ</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC</td>
<td>491.1±28</td>
<td>442.8±39</td>
<td>416.1±35</td>
<td>375.3±41</td>
</tr>
<tr>
<td>GI</td>
<td>-</td>
<td>0.902</td>
<td>0.847</td>
<td>0.764</td>
</tr>
<tr>
<td>%</td>
<td>-</td>
<td>90.2</td>
<td>84.7</td>
<td>76.4</td>
</tr>
<tr>
<td>p-value</td>
<td>-</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Table 6.

GI determined by AUC calculated from blood glucose-response to intraperitoneal injection of 3.0 g/kg body weight of glucose (in normal saline) following treatment with graded doses of grapefruit juice. The AUC (mmol/l X minutes) indicated is for plasma glucose concentrations.
<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Dexamethasone</th>
<th>Dexamethasone +GFJ</th>
<th>GFJ</th>
<th>Metformin +PCN</th>
<th>PCN</th>
<th>Metformin</th>
</tr>
</thead>
<tbody>
<tr>
<td>V_{max} (μmol/g of liver tissue/min)</td>
<td>13.56±0.72 $^*$</td>
<td>18.66±0.476 $^\Theta$</td>
<td>16.83±0.36 $^\Theta$</td>
<td>26.65±1.11 $^*$</td>
<td>13.72±0.87</td>
<td>11.51±0.83</td>
<td>20.04±0.406 $^\Phi$</td>
</tr>
<tr>
<td>K_m (μM)</td>
<td>308 $^*$</td>
<td>344</td>
<td>269 $^*$</td>
<td>144 $^*$</td>
<td>351</td>
<td>422</td>
<td>369</td>
</tr>
<tr>
<td>r^2</td>
<td>0.5914</td>
<td>0.846</td>
<td>0.9107</td>
<td>0.8535</td>
<td>0.4569</td>
<td>0.3342</td>
<td>0.8835</td>
</tr>
<tr>
<td>V_{max}/K_m</td>
<td>44</td>
<td>54.2</td>
<td>62.6</td>
<td>184</td>
<td>39.1</td>
<td>27.3</td>
<td>54.2</td>
</tr>
</tbody>
</table>

Table 7.

V_{max}, K_m and linear regression analysis of glucokinase activity determined by Eadie-Hofstee plots. Statistically significant differences are marked by relevant symbols: *<p<0.05; in, $^*$ = GFJ with/without dexamethasone vs controls; $^\Phi$ = metformin or dexamethasone with/without GFJ vs controls;
Table 8.

$V_{\text{max}}$, $K_m$ and linear regression analysis of G6Pase activity determined by Eadie-Hofstee plots. Statistically significant differences are marked by relevant symbols: $p<0.05$; * = dexamethasone with GFJ vs controls; $\Phi$ = PCN vs controls; $\Theta$ = dexamethasone with/without GFJ vs controls; $\delta$ = PCN vs PCN with metformin; $\#$ = Dexamethasone or PCN vs controls; $\phi$ = dexamethasone vs dexamethasone with GFJ

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Dexamethasone</th>
<th>Dexamethasone +GFJ</th>
<th>GFJ</th>
<th>Metformin +PCN</th>
<th>PCN</th>
<th>Metformin</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{max}}$ (µmol/g of liver tissue/min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>13.4±0.45</td>
<td>23.19±0.58</td>
<td>19.26±0.41</td>
<td>14.02±0.54</td>
<td>13.4±0.80</td>
<td>23.84±1.10</td>
<td>13.85±0.348</td>
</tr>
<tr>
<td>$K_m$ (mM)</td>
<td>2.06</td>
<td>1.705</td>
<td>1.738&lt;sup&gt;$\Phi$&lt;/sup&gt;</td>
<td>2.292&lt;sup&gt;$\Phi$&lt;/sup&gt;</td>
<td>2.26</td>
<td>1.299&lt;sup&gt;$\Phi$&lt;/sup&gt;</td>
<td>2.326</td>
</tr>
<tr>
<td>$r^2$</td>
<td>0.8412</td>
<td>0.9219</td>
<td>0.9400</td>
<td>0.7800</td>
<td>0.6050</td>
<td>0.8219</td>
<td>0.8805</td>
</tr>
<tr>
<td>$V_{\text{max}}/K_m$</td>
<td>6.5</td>
<td>13.6</td>
<td>11.08</td>
<td>6.11</td>
<td>5.9</td>
<td>18.35</td>
<td>5.96</td>
</tr>
</tbody>
</table>
Table 9.

$V_{\text{max}}$, $K_m$ and linear regression analysis of PEPCK activity determined by Eadie-Hofstee. Statistically significant differences are marked by relevant symbols: $p<0.05$; in, * = dexamethasone vs controls; # = PCN vs controls; $\dagger$ = dexamethasone vs dexamethasone with GFJ; $\Phi$ = PCN vs PCN with metformin.
Table 10.

$V_{max}$, $K_m$ and linear regression analysis of AMPK activity determined by Eadie-Hofstee. Statistically significant differences are marked by relevant symbols: $p<0.05$; in, $\Phi$ = controls vs all treatment groups; * = controls vs all treatment groups except PCN; # = controls vs all treatment groups except PCN.
<table>
<thead>
<tr>
<th></th>
<th>Metformin Plasma (μg/ml)</th>
<th>Metformin Liver (μg/g)</th>
<th>Metformin Plasma: Liver ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Controls</strong></td>
<td>95±8.1</td>
<td>280±15</td>
<td>33.9±5.4</td>
</tr>
<tr>
<td><strong>PCN</strong></td>
<td>107±23</td>
<td>402±58*</td>
<td>26.6±4.0*</td>
</tr>
<tr>
<td><strong>Grapefruit Juice</strong></td>
<td>108±20</td>
<td>397±66*</td>
<td>27.2±3.5*</td>
</tr>
<tr>
<td><strong>p-value</strong></td>
<td>&gt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Table 11.
Metformin levels in plasma and liver tissue 1.5 hrs after oral treatment with 250 mg/kg body weight of metformin in control (metformin only), grapefruit juice- and PCN-treated animals, respectively. Plasma and liver metformin levels were analysed by HPLC as explained in the methods. (*) = significant difference compared to controls.
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Ethics approval

UNIVERSITY OF KWAZULU-NATAL

RESEARCH OFFICE
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E-Mail: mooderv@ukzn.ac.za
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9 May 2008

Reference: 036/08/Animal

Mr P. Owira
School of Pharmacy and Pharmacology
University of KwaZulu-Natal
WESTVILLE CAMPUS

Dear Mr Owira

Ethical Approval of Research Project using Animals

I have pleasure in informing you that on recommendation of the review panel, the Animal Ethics Sub-committee of the University Ethics Committee has granted ethical approval for 2008 on the following project:

"Potential modulation of the expression organic cation transporter protein (OCT1), P-Glycoprotein, and cytochrome P450 (CYP3A4) in the rat liver by grapefruit juice in vivo".

Yours sincerely

[Signature]

Professor Theresa HT Coetzee
Chairperson: Animal Ethics Sub-committee

Cc Registrar
Research Office
Head of School
6.5 Publications

6.5.1 Submitted manuscript

---

Peter Owire - RE: Manuscript

From: <christine todd@thomsonreuters.com>
To: <Owirep@jukun.ac.za>
Date: 1/19/2009 4:56 PM
Subject: RE: Manuscript

Dear Dr. Owire,

This is to acknowledge receipt of your manuscript submitted to Methods and Findings entitled:

**GRAPEFRUIT JUICE IMPROVES GLYCAEMIC CONTROL BUT EXACERBATES METFORMIN-INDUCED LACTIC ACIDOSIS IN NON-DIABETIC RATS**

Once approved by our editor in chief, your article will be sent out for peer review.

Thank you for your interest in our journal.

Yours sincerely

Christine Todd

Editorial Coordinator
Methods & Findings in Experimental and Clinical Pharmacology
Thomson Reuters

---

6.5.2 Manuscripts in preparation

a) Grapefruit juice increases hepatic glucose uptake and suppresses gluconeogenesis by upregulation of adenosine monophosphate protein kinase (AMPK) activity in normal non-diabetic rats

b) Grapefruit juice enhances hepatic uptake of metformin by upregulating the expression of organic cation transporter protein in the rat liver.
Peter Owira - Re: authorization

From: <jacques.turgeon.chum@ssss.gouv.qc.ca>
To: "Peter Owira" <Owirep@ukzn.ac.za>
Date: 2/6/2009 4:07 PM
Subject: Re: authorization

No problem with me. You also have to obtain permission from the editor.

Jacques Turgeon

Dear Dr. Turgeon,
I am writing to seek to permission to use figure 1A in your paper entitled "The ATP-binding cassette transporters and their implication in drug disposition: a special look at the heart" published in Pharmacology Reviews, 2006;58(2):244-258. Thanks

Kind Regards,
Peter Owira
Department of Pharmacology
University of KwaZulu-Natal
South Africa

Please find our Email Disclaimer here-->: http://www.ukzn.ac.za/disclaimer
Hello Peter

Yes, I give you permission to use figures 2 and 3 in my paper entitled "Shedding Light on drug transport: structure and function of the P-glycoprotein multidrug transporter (ABC1)" published in *Biochem Cell Biol*, 2006;84:979-992 in your PhD thesis. You may also need to obtain permission from the publisher of the journal, since they own the copyright. Please acknowledge the source of the figure.

Best wishes.

Frances Sharom

Dr. Frances J. Sharom
Professor and Canada Research Chair in Membrane Protein Biology
Department of Molecular and Cellular Biology
University of Guelph
Guelph ON Canada N1G 2W1
Phone: (519) 824-4130 Ext. 32287
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