

✓

**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.

Candidate: **Peter M. O. Owira**

BSc (Clinical Biochem) (NRB), BSc (Med, Hons) (UCT), MSc
(Med), (UCT).


Promoter: **Professor John A. O. Ojewole**

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'.

116125



.....

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.

TABLE OF CONTENTS

Declaration	i
Dedication	ii
Acknowledgements	iii
Table of contents	iv
List of contents	v
List of figures	x
List of tables	xii
List of abbreviations	xiii
Abstract	xv

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1	The Citrus Fruit	1
	1.1.2 Medicinal Properties of grapefruit	2
	1.1.3 Phytochemistry of grapefruit	3
1.2	Grapefruit juice-drug interactions	4
	1.2.1 Cardiovascular drugs	5
	1.2.2 Antimicrobials	6
	1.2.3 Central nervous system drugs	7
	1.2.4 Other drugs	8
1.3	Mechanism of grapefruit juice-drug interaction	8
	1.3.1 Molecular insight	9
	1.3.2 Cytochrome P450 enzymes	10
	1.3.3 Drug transporter proteins	13
	1.3.3.1 P-glycoprotein (P-gp)	16
	1.3.3.2 Organic anion transporter protein (OATP)	20
	1.3.3.3 Organic cation transporter protein (OCT)	23
	1.3.3.3.1 Structure and function of OCT	23
	1.3.3.3.2 Regulation of OCT	25
	1.3.4 Identifying the culprits in grapefruit juice	27
	1.3.4.1 Candidate ingredients for CYP3A4 inhibition	28
	1.3.4.2 Grapefruit ingredients that interact with P-gp	29
1.4	Clinical considerations	29
	1.4.1 Pharmacodynamics of grapefruit juice	30

1.4.1.1	CYP3A4	30
1.4.1.2	P-gp	32
1.4.1.3	OATPs	33
1.4.1.4	OCT	33
1.4.2	Grapefruit juice effects on metabolic syndrome	37
1.5	Aims and Objectives	39

CHAPTER 2

MATERIALS, STUDY DESIGN AND PROCEDURE

2.1	Chemicals, Reagents and accessories	40
2.2	Study design and Procedure	41
	2.2.1 Animal experiments	41
	2.2.1.1 Determination of LD₅₀ for grapefruit juice	41
	2.2.1.2 Grapefruit juice treatment	42
	2.2.1.3 Glucose tolerance test	42
	2.2.1.4 Determination of grapefruit juice effects on glycaemic	
	Index	43
	2.2.1.5 PCN treatment	43
	2.2.1.6 Dexamethasone treatment	44
	2.2.1.7 Metformin treatment	44

CHAPTER 3

METHODS

3.1	Grapefruit juice fingerprinting	45
3.1.1	Determination of flavonoids	45
3.1.2	Determination of furanocoumarins	46
3.1.3	Component identification	46
3.2	Blood glucose measurement	46
3.3	Blood lactate	47
3.4	Metformin assay	47
3.5	Insulin assay	47
3.6	Immunoblot assays	48
3.7	Glycogen assay	49
3.8	Glucokinase activity	49
3.9	Glucose-6-phosphatase activity	50
3.10	Phosphoenolpyruvate carboxykinase (PEPCK) activity	50
3.11	Adenosine-Monophosphate-Activated Protein Kinase (AMPK) activity	51
3.12	Statistical analysis	51

CHAPTER 4

RESULTS

4.1	Acute toxicity study	52
4.2	GFJ chemical constituents	52
4.3	Effect of grapefruit juice on blood glucose and glycaemic Index (GI)	52

4.3.1	Dose-response effects of grapefruit juice	52
4.3.2	Fasting blood glucose	53
4.3.3	PCN-induced hyperglycaemia	53
4.3.4	Glucose tolerance	53
4.4	Effect of grapefruit juice on plasma insulin	54
4.5	Effect of grapefruit juice on hepatic glucose homeostasis	54
4.5.1	Hepatic glycogen content	54
4.5.2	Hepatic glucokinase activity	55
4.5.3	Hepatic glucose-6-phosphatase activity	56
4.5.4	Hepatic phosphoenolpyruvate activity	57
4.5.5	Hepatic adenosine monophosphate-activated protein kinase activity	58
4.6	Effect of grapefruit juice on metformin-induced lactic acidosis	58
4.7	Effect of grapefruit juice on hepatic metformin uptake	59
4.8	Effect of grapefruit juice on hepatic expression of OCT1 protein	59

CHAPTER 5

5.1	DISCUSSION	61
5.2	CONCLUSION AND RECOMMENDATIONS	72
5.3	REFERENCES	74

6.0	APPENDICES	122
6.1	Figures and Tables	122
6.2	Ethics approval	162
6.3	Publications	163
6.4	Reprint permission	164

LIST OF FIGURES

Figure 1	The grapefruit	74
Figure 2	Enzymatic hydrolysis of naringin	75
Figure 3	PXR-mediated regulation of cellular expression of drug transporter proteins and drug metabolising enzymes	76
Figure 4	Transporters involved in hepatic drug disposition	77
Figure 5	Schematic illustration of P-gp structure	78
Figure 6	Proposed mechanism of action of P-gp	79
Figure 7	Schematic representation of rOatp1	80
Figure 8	Proposed secondary structure of OCT1	81
Figure 9	Schematic illustration of transcriptional and post-transcriptional regulation of OCT1 expression	82
Figure 10	Animal weights at the beginning and end treatment period	83
Figure 11	HPLC chromatogram of the grapefruit extract	83
Figure 12	MS analysis of HPLC chromatograms of grapefruit juice extracts	84
Figure 13	Dose-response effects of grapefruit juice on plasma glucose levels	85
Figure 14	Fasting blood glucose levels	86
Figure 15	AUC calculated from random blood glucose sampling versus time curves	87
Figure 16	Fasting blood glucose, dose-response curves of GTT and the calculated AUC	88-89
Figure 17	Dose-response curves of GTT and the calculated AUC	90
Figure 18	Fasting plasma insulin levels	91
Figure 19	Glycogen content of liver tissue	92
Figure 20	Michaeli-Menten kinetics of glucokinase activity	93
Figure 21	Linear-regression analysis of Eadie-Hofstee plots of glucokinase activity	94

Figure 22	Michaeli-Menten kinetics of G6Pase activity	95
Figure 23	Linear-regression analysis of Eadie-Hofstee plots of G6Pase activity	96
Figure 24	Michaeli-Menten kinetics of PEPCK	97
Figure 25	Linear-regression analysis of Eadie-Hofstee plots of PEPCK activity	98
Figure 26	Michaeli-Menten kinetics of AMPK activity	99
Figure 27	Linear-regression analysis of Eadie-Hofstee plots of AMPK activity	100
Figure 28	Blood lactic acid levels	101
Figure 29	Linear regression analysis of metformin concentration in the liver	102
Figure 30	Ponceau S stain of PVD membrane	103
Figure 31	Autoradiograph of OCT1 protein following Western blot analysis	103
Figure 32	Densitometry scans of OCT1 immunoblot assays	104
Figure 33	Schematic illustration of proposed grapefruit juice-mediated regulation of hepatic glucose metabolism and OCT1 protein expression	105

LIST OF TABLES

Table 1	Bioactive compounds of the grapefruit	106
Table 2	Substrates of P-gp	107
Table 3	Animal treatment schedule	108
Table 4	Buffers used in SDS-PAGE	109
Table 5	MS analysis of grapefruit juice HPLC chromatograms	110
Table 6	Glycaemic index calculated from blood glucose dose-response curves	111
Table 7	V_{max} , K_m and linear regression analysis of glucookinase activity	112
Table 8	V_{max} , K_m and linear regression analysis of G6Pase activity	113
Table 9	V_{max} , K_m and linear regression analysis of PEPCK activity	114
Table 10	V_{max} , K_m and linear regression analysis of AMPK activity	115
Table 11	Metformin levels in plasma and liver tissue	116

LIST OF ABBREVIATIONS

ABC	ATP-binding Cassette
ABCBA	Multi-drug resistance protein
AMPK	Adenosine Monophosphate Protein Kinase
ATPIII	Adult Treatment Panel III
AUC	Area Under the Concentration-Time Curve
BCRP	Breast Cancer Resistance Protein
BRU	Biomedical Resource Unit
BSEP	Bile Salt Export Pump
CAR	Constitutive Androsterone Receptor
DR	DNA-response element
EXD	Extracellular Domain
FXR	Farnesoid X Receptor
GFJ	Grapefruit Juice
GI	Glycaemic Index
G6Pase	Glucose-6-phosphate
GR	Glucocorticoid Receptor
GTT	Glucose Tolerance Test
HNF	Hepatocyte Nuclear Factor
ISSR	Inter-Simple Sequence Repeat
LXR	Liver X Receptor
MDR	Multidrug Resistant
MMP	1-methyl-4-phenylpyrinium
MRP	Multidrug Resistant Protein
NBD	Nucleotide Binding Domain
OATP	Organic Anion Transporter Protein
OCT	Organic Cation Transporter
PEPCK	Phosphoenolpyruvate carboxy kinase
PCN	Pregnenolone-16 α -carbonitrile

P-gp	P-glycoprotein
PKA	Protein Kinase A
PKC	Protein Kinase C
PPAR	Peroxisome-Ploriferator-Activated Receptor
PXR	Pregnane X Receptor
RAPD	Random Amplified Polymorphic DNA
RLFP	Restriction Fragment Length Polymorphism
RXR	Retinoid X Receptor
SCAR	Sequence Characterised Amplified Region
SLC	Solute Carrier transpoters
SHP	Small Heterodimer Patner
TMD	Transmembrane Domain
TEA	Tetraethylammonium
UKPDS	United Kingdom Prospective Diabetes Study

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. Subgroups 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 LD₅₀ 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 ($K_m = 2.29 \text{ mM}$; $V_{max} = 14.02 \pm 0.45 \mu \text{mol/min/g}$ liver tissue), compared with the controls ($K_m = 2.068 \text{ mM}$; $V_{max} = 13.4 \pm 0.45 \mu \text{mol/min/g}$ liver tissue). Similarly, GFJ significantly ($p < 0.05$) reduced ($K_m = 2.78 \text{ mM}$; $V_{max} = 10.72 \pm 0.3630 \mu \text{mol/g}$ of liver tissue/ min) dexamethasone-induced PEPC activity ($K_m = 0.738 \text{ mM}$; $V_{max} = 26.78 \pm 0.41 \mu \text{mol/g}$ of liver tissue/ min). GFJ alone ($K_m = 0.02 \text{ mM}$; $V_{max} = 96.44 \pm 2.47 \text{ nmol/ min/}\mu\text{g}$ of liver tissue), or in combination with dexamethasone ($K_m = 0.0321 \text{ mM}$; $V_{max} = 84.71 \pm 3.949 \text{ nmol/ min/}\mu\text{g}$ of liver tissue), significantly ($p < 0.0001$) increased AMPK activity in the 'test', compared to control ($K_m = 0.146 \text{ mM}$; $V_{max} = 7.442 \pm 0.411 \text{ nmol/ min/}\mu\text{g}$ of liver tissue) rats.

Blood lactic acid levels were significantly higher ($p = 0.0079$) in rats that were treated with either metformin alone ($5.38 \pm 2.53 \text{ mmol/L}$), or metformin in combination with GFJ ($8.31 \pm 2.5 \text{ mmol/L}$), than in control ($2.54 \pm 0.7 \text{ mmol/L}$) 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, quinone 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 atherosclerosis [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 for 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 amlodipine, 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 α_1 -adrenergic antagonists, such as doxazosin, terazosin and prazosin, have so far shown no interaction with grapefruit juice [49], but antiarrhythmic 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, artemeter, 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, sertraline, 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 16 α -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 RXR α 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 (eg, 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 *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-Proliferator-Activated Receptor (PPAR α), 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 canalicular 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 canalicular membrane) [145]. Members of the ABC family are predominantly found on the canalicular 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]. MRPs 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 canalicular 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 *mdr1 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 canalicular 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 tempcaprilat [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]. Digoxin 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 fingers 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 (*SLC22A4*), 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 OCT1 and OCT3 in humans are localized within a cluster on chromosome 6.q26-7 [235, 236, 237]. The rat OCT1 (rOct1) 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 OCT1 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 segments 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, prazosin, amantadine, oxaliplatin, imatinib

and verapamil), and model compounds, such as 1-methyl-4-phenylpyridinium (MPP), [225]. However, some cations {such as tetrapethylammonium (TEA), decaynium 22 and disprocynium}, non-charged compounds (corticosterone, deoxycorticosterone and β -estradiol), and anions (probenecid 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, amphetimine, 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 fetuses, 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-4 α (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-16 α -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 rOct1 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 μ M 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-I-1 and GF-I-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 purpureus*, *Pleurotus 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 bergamottin 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 idarubicin 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 bromosulphophthalein 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-4 α is inhibited by bile acid (chenodeoxycholic acid), via component small heterodimer partner (SHP) of the bile acid-inducible transcription repressor [260]. HNF-4 α 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-4 α inclusive [336, 337]. HNF-4 α is one of the nuclear factors that regulate PEPCK and or G6Pase expression [333]. It is, however, intriguing that HNF-4 α 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 of 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, 345].

Therapeutic effects of metformin in diabetic patients, therefore, include decreased fasting and post-prandial glucose, decreased glycosylated haemoglobin (HBA_{1C}), 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.

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 rOct1 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 (HMRSAMSGHLHLVKRR) 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, $25\pm 2^{\circ}\text{C}$, humidity, $55\pm 5\%$), 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 LD₅₀ for grapefruit juice**

The median lethal dose (LD₅₀) 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 LD₅₀ 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) = $AUC_{(GFJ)}/AUC_{(Glucose)} \times 100$; where $AUC_{(GFJ)}$ and $AUC_{(Glucose)}$ 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-16 α -carbonitrile (PCN), {17 mg/kg body weight (15 mg/ml in propylene glycol)} on the 10th 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 Lichrospher 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 $[\text{M} + \text{H}]^+$ ions of the relevant flavonoids and furanocoumarins. The $[\text{M} + \text{H}]^+$ and $[\text{M} = \text{Na}]^+$ 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 μ l 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 μ Bondpack (10 μ m) (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 °C, cooled and then run on a 10% polyacryl amide 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 °C, 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, 2.5mM 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/g 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 dithiothreitol, 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, furanocoumarins 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'-dihydroxybermottin 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 \pm 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).

4.5.2 Glucokinase activity

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_{max} , respectively. Rats treated with dexamethasone (344 μM) and metformin (369 μM) had parallel slopes, suggesting comparable K_m values, while grapefruit juice-treatment significantly ($p < 0.05$) reduced K_m (144 μM), even in the presence of dexamethasone (269 μM), compared to control rats (308 μM), respectively. Metformin alone (369 μM) or with PCN (351 μM) had no significant effect on K_m compared to control rats, respectively. Grapefruit juice treatment (26.65 ± 1.11 $\mu\text{mol/g}$ of liver tissue/min) doubled the V_{max} compared to control (13.56 ± 0.72 $\mu\text{mol/g}$ of liver tissue/min) rats, and decreased V_{max} when co-administered with dexamethasone (16.83 ± 0.36 $\mu\text{mol/g}$ of liver tissue/min), compared to dexamethasone alone (18.86 ± 0.476 $\mu\text{mol/g}$ of liver tissue/min), respectively. Metformin treatment (20.04 ± 0.406 $\mu\text{mol/g}$ of liver tissue/min) significantly ($p < 0.05$) increased V_{max} compared to control rats, but had no significant effect on V_{max} when co-administered with PCN (13.72 ± 0.876 $\mu\text{mol/g}$ of liver tissue/min), compared to PCN alone (11.51 ± 11.51 $\mu\text{mol/g}$ 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_{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).

4.5.3

Glucose-6-phosphatase (G6Pase) activity

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_{max} , respectively. Both dexamethasone- ($K_m = 1.705$ mM; $V_{max} = 23.19 \pm 0.577$ $\mu\text{mol}/\text{min}/\text{g}$ liver tissue) and PCN- ($K_m = 1.299$ mM; $V_{max} = 23.84 \pm 1.102$ $\mu\text{mol}/\text{min}/\text{g}$ liver tissue) treatments significantly ($p < 0.05$) increased glucose-6-phosphatase activity, compared to controls ($K_m = 2.068$ mM; $V_{max} = 13.4 \pm 0.45$ $\mu\text{mol}/\text{min}/\text{g}$ liver tissue). Metformin administered alone ($K_m = 2.32$ mM; $V_{max} = 13.85 \pm 0.34$ $\mu\text{mol}/\text{min}/\text{g}$ liver tissue) or concurrently with PCN ($K_m = 2.26$ mM; $V_{max} = 13.4 \pm 0.80$ $\mu\text{mol}/\text{min}/\text{g}$ liver tissue), significantly ($p < 0.05$) reduced glucose-6-phosphatase activity compared to PCN. Grapefruit juice ($K_m = 2.29$ mM; $V_{max} = 14.02 \pm 0.45$ $\mu\text{mol}/\text{min}/\text{g}$ 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_{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; $V_{max} = 12.44 \pm 0.41$ $\mu\text{mol/g}$ of liver tissue/ min) did not significantly affect the activity of PEPCK compared to controls ($K_m = 1.379$ mM; $V_{max} = 13.73 \pm 0.399$ $\mu\text{mol/g}$ of liver tissue/ min), but significantly ($p < 0.05$) reduced ($K_m = 2.78$ mM; $V_{max} = 10.72 \pm 0.3630$ $\mu\text{mol/g}$ of liver tissue/ min) dexamethasone-induced effects ($K_m = 0.738$ mM; $V_{max} = 26.78 \pm 0.41$ $\mu\text{mol/g}$ of liver tissue/ min) on PEPCK activity. Similarly, metformin alone ($K_m = 1.615$ mM; $V_{max} = 12.1 \pm 0.481$ $\mu\text{mol/g}$ of liver tissue/ min) did not significantly affect PEPCK activity compared to controls, but significantly ($p < 0.05$) opposed ($K_m = 1.398$ mM; $V_{max} = 14.32 \pm 0.749$ $\mu\text{mol/g}$ of liver tissue/ min) PCN-induced ($K_m = 0.7151$ mM; $V_{max} = 27.77 \pm 0.81$ $\mu\text{mol/g}$ 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_{max} , respectively. Grapefruit juice alone ($K_m = 0.02$ mM; $V_{max} = 96.44 \pm 2.47$ nmol/ min/ μ g of liver tissue), or in combination with dexamethasone ($K_m = 0.0321$ mM; $V_{max} = 84.71 \pm 3.949$ nmol/ min/ μ g of liver tissue), significantly ($p < 0.0001$) increased AMPK activity in the 'test' compared to control ($K_m = 0.146$ mM; $V_{max} = 7.442 \pm 0.411$ nmol/ min/ μ g of liver tissue) rats (Figures 26 and 27). Similarly dexamethasone alone ($K_m = 0.0194$ mM; $V_{max} = 89.94 \pm 4.75$ nmol/min/ μ g of liver tissue), or metformin alone ($K_m = 0.0411$ mM; $V_{max} = 79.0 \pm 3.887$ nmol/min/ μ g of liver tissue) significantly ($p < 0.0001$) increased AMPK activity compared to controls. PCN treatment with ($K_m = 0.0217$ mM; $V_{max} = 93.74 \pm 6.55$ nmol/ min/ μ g of liver tissue) or without ($K_m = 0.017$ mM; $V_{max} = 89.75 \pm 10.08$ nmol/min/ μ 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_{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 ($p = 0.0122$; $r^2 = 0.9080$) and grapefruit juice-treated 'test' rats ($p = 0.0005$; $r^2 = 0.9893$) (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 rOct1 protein, in response to different agonists (Figure 31). Densitometry scans expressed as a percentage of the controls showed that dexamethasone ($80.6 \pm 40.28\%$) significantly ($p < 0.0001$) downregulated rOct1 expression, compared to grapefruit juice ($285.6 \pm 33.4\%$), PCN ($260 \pm 25.7\%$), or metformin and PCN ($275 \pm 48.4\%$), respectively (Figure 30). However, grapefruit juice

treatment significantly ($p < 0.05$) reversed dexamethasone-induced down-regulation of rOct1 ($165.4 \pm 26.4\%$) (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 dimers 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 μ l) 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; $V_{max} = 23.84 \pm 1.102$ $\mu\text{g/mol/min/g}$ liver tissue) significantly ($p < 0.05$) in PCN-treated compared to control ($K_m = 2.068$ mM; $V_{max} = 13.4 \pm 0.45$ $\mu\text{mol/min/g}$ 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; $V_{max} = 13.85 \pm 0.34$ $\mu\text{mol/min/g}$ liver tissue) (Table 8). Likewise, PEPCK activity increased in PCN-treated ($K_m = 0.7151$ mM; $V_{max} = 27.77 \pm 0.81$ $\mu\text{mol/g}$ of liver tissue/ min) compared to control ($K_m = 1.379$ mM; $V_{max} = 13.73 \pm 0.399$ $\mu\text{mol/g}$ 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; $V_{max} = 14.32 \pm 0.749$ $\mu\text{mol/g}$ of liver tissue/ min) (Table 9). However, PCN did not significantly affect the activity of glucokinase ($K_m = 422$ μM ; $V_{max} = 11.51 \pm 0.83$ $\mu\text{mol/min/g}$ of liver tissue), compared to the controls ($K_m = 308$ μM ; $V_{max} = 13.56 \pm 0.72$ $\mu\text{mol/min/g}$ 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 ($K_m = 396 \mu\text{M}$; $V_{\text{max}} = 20.4 \pm 0.406 \mu\text{mol}/\text{min}/\mu\text{g}$ of liver tissue) compared to controls (288 ± 14 mg/g of liver tissue), ($K_m = 308 \mu\text{M}$; $V_{\text{max}} = 13.6 \pm 0.72 \mu\text{mol}/\text{min}/\mu\text{g}$ 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 ($K_m = 0.0411 \text{ mM}$; $V_{\text{max}} = 79.0 \pm 3.887 \text{ nmol}/\text{min}/\mu\text{g}$ of liver tissue), or in combination with PCN ($K_m = 0.0217 \text{ mM}$; $V_{\text{max}} = 93.74 \pm 6.55 \text{ nmol}/\text{min}/\mu\text{g}$ of liver tissue), significantly ($p < 0.0001$) increased AMPK activity compared to controls ($K_m = 0.146 \text{ mM}$; $V_{\text{max}} = 7.442 \pm 0.411 \text{ nmol}/\text{min}/\mu\text{g}$ 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 $\{3.48 \pm 0.47$ (oral), 3.68 ± 0.19 (IP) mmol/L $\}$, than in control $\{4.12 \pm 0.34$ (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 ($K_m = 144 \mu\text{M}$; $V_{\max} = 26.65 \pm 1.11 \mu\text{mol/min/g}$ of liver tissue) compared to controls ($K_m = 308 \mu\text{M}$; $V_{\max} = 13.56 \pm 0.72 \mu\text{mol/min/g}$ of liver tissue), but decreased ($K_m = 2.69 \mu\text{M}$; $V_{\max} = 16.83 \pm 0.36 \mu\text{mol/min/g}$ of liver tissue) the enzyme activity when co-administered with

dexamethasone, compared to dexamethasone alone ($K_m = 344 \mu\text{M}$; $V_{\text{max}} = 18.66 \pm 0.46 \mu\text{mol/min/g}$ 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 ($K_m = 2.29 \text{ mM}$; $V_{\text{max}} = 14.02 \pm 0.54 \mu\text{mol/min/g}$ liver tissue) or metformin alone ($K_m = 2.23 \text{ mM}$; $V_{\text{max}} = 13.85 \pm 0.348 \mu\text{mol/min/g}$ liver tissue) significantly ($p < 0.05$) reduced glucose-6-phosphatase activity, compared to dexamethasone ($K_m = 1.705 \text{ mM}$; $V_{\text{max}} = 23.19 \pm 0.58 \mu\text{mol/min/g}$ liver tissue), and PCN ($K_m = 1.299 \text{ mM}$; $V_{\text{max}} = 23.84 \pm 1.10 \mu\text{mol/min/g}$ liver tissue) respectively (Figures 22 and 23). When both grapefruit juice and metformin were co-administered with dexamethasone ($K_m = 1.738 \text{ mM}$; $V_{\text{max}} = 19.26 \pm 0.41 \mu\text{mol/min/g}$ liver tissue) and PCN ($K_m = 2.26 \text{ mM}$; $V_{\text{max}} = 13.34 \pm 0.8 \mu\text{mol/min/g}$ liver tissue), respectively, glucose-6-phosphatase activity was similarly reduced compared to either dexamethasone or PCN alone, respectively (Table 8). Similarly grapefruit juice ($K_m = 1.55 \text{ mM}$; $V_{\text{max}} = 12.44 \pm 0.41 \mu\text{mol/min/g}$ liver tissue) did not have any significant effect on PEPCK activity compared to controls ($K_m = 1.379 \text{ mM}$; $V_{\text{max}} = 13.37 \pm 0.399 \mu\text{mol/min/g}$ liver tissue), but significantly ($p < 0.05$) reduced ($K_m = 2.78 \text{ mM}$; $V_{\text{max}} = 10.72 \pm 0.363 \mu\text{mol/min/g}$ liver tissue) dexamethasone-induced increase in PEPCK activity ($K_m = 0.0738 \text{ mM}$; $V_{\text{max}} = 26.78 \pm 0.41 \mu\text{mol/min/g}$ liver tissue) (Figures 24 and 25). Likewise, metformin alone ($K_m = 1.615 \text{ mM}$; $V_{\text{max}} = 12.1 \pm 0.481 \mu\text{mol/min/g}$ liver tissue) did not significantly affect PEPCK activity compared to controls, but significantly ($p < 0.05$) antagonised ($K_m = 1.398 \text{ mM}$; $V_{\text{max}} = 14.32 \pm 0.749 \mu\text{mol/min/g}$ liver tissue) PCN-induced ($K_m = 0.7151 \text{ mM}$; $V_{\text{max}} = 27.77 \pm 0.81 \mu\text{mol/min/g}$ 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 hesperidine, 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 ($K_m = 0.0298$ mM; $V_{max} = 96.44 \pm 2.478$ nmol/min/g liver tissue) or in combination with dexamethasone ($K_m = 0.0321$ mM; $V_{max} = 84.71 \pm 3.949$ nmol/min/g liver tissue), significantly ($p < 0.0001$) increased AMPK activity in the 'test' compared to 'control' ($K_m = 0.146$ mM; $V_{max} = 7.442 \pm 0.411$ 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 μ g 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 ($80.6\pm 40.28\%$) significantly ($p<0.0001$) down-regulated rOct1 expression, compared to grapefruit juice ($285.6\pm 33.4\%$), PCN ($260\pm 25.7\%$), or metformin and PCN ($275\pm 48.4\%$), respectively (Figure 32). However, grapefruit juice treatment significantly ($p<0.05$) reversed dexamethasone-induced down-regulation of rOct1 ($165.4\pm 26.4\%$). 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

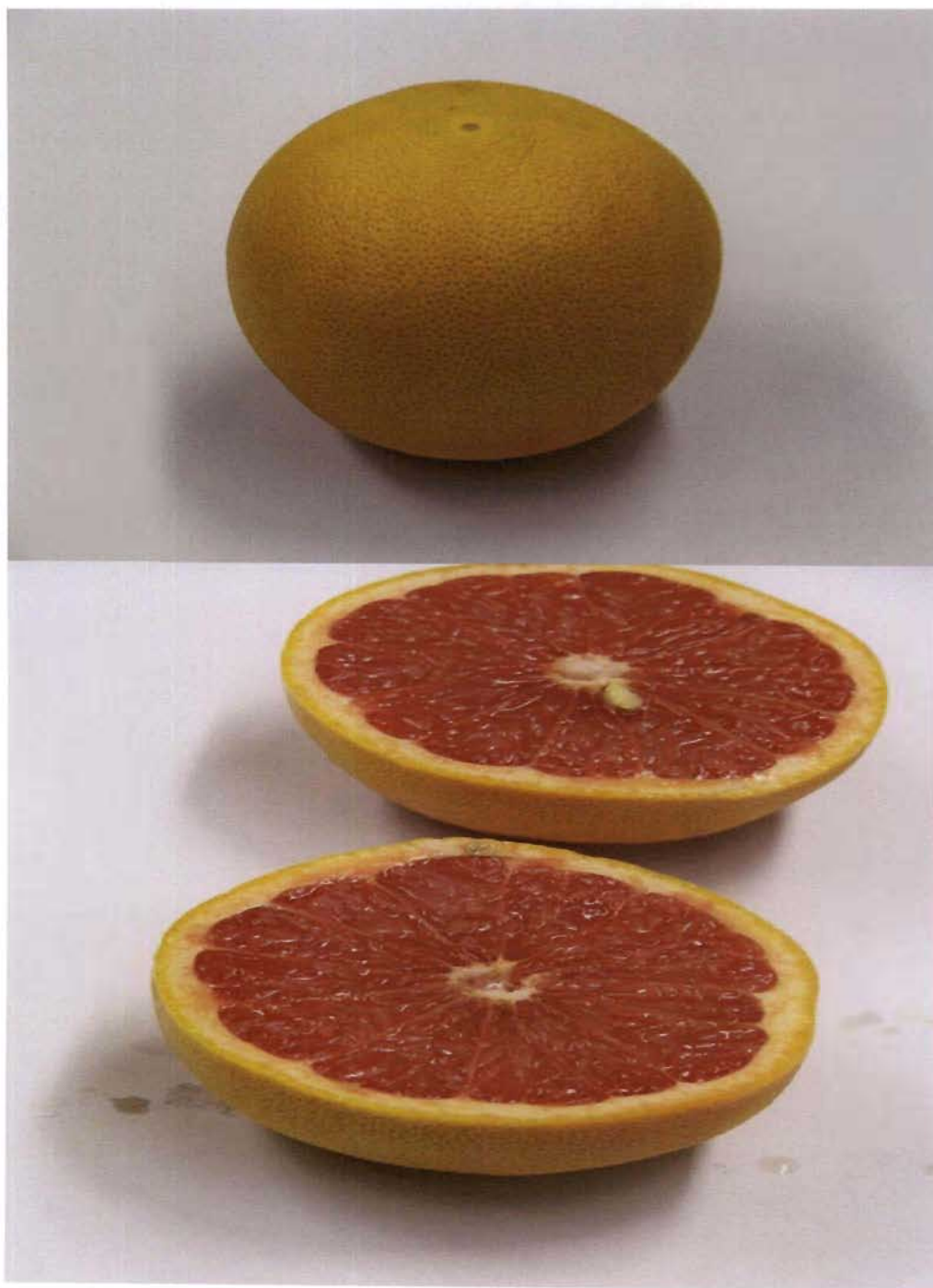


Figure 1. Ruby grapefruit.

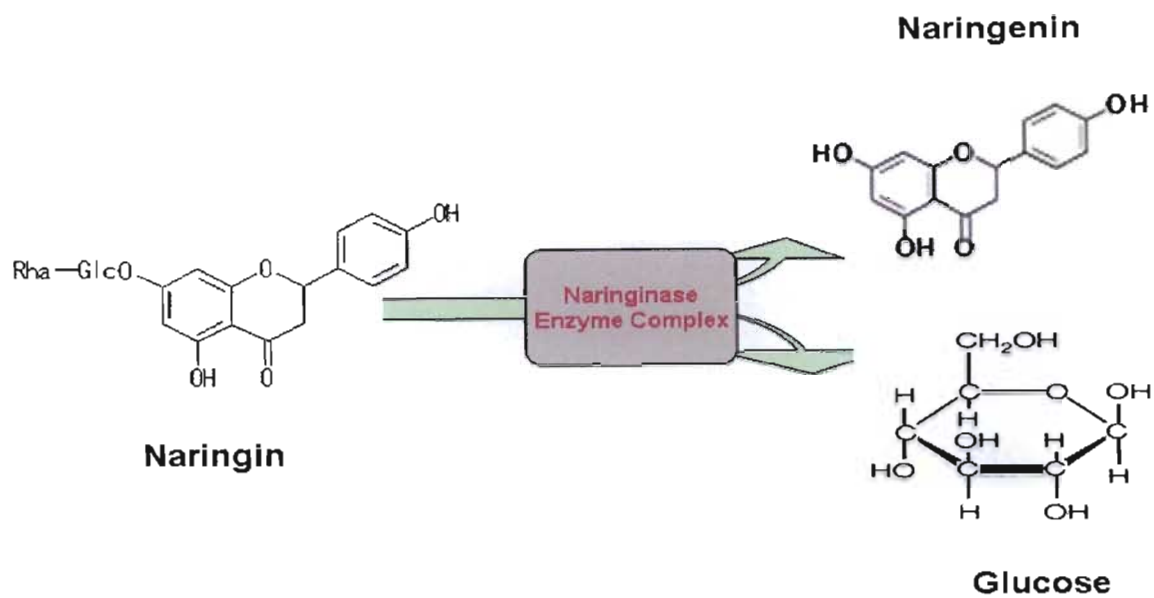


Figure 2.

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].

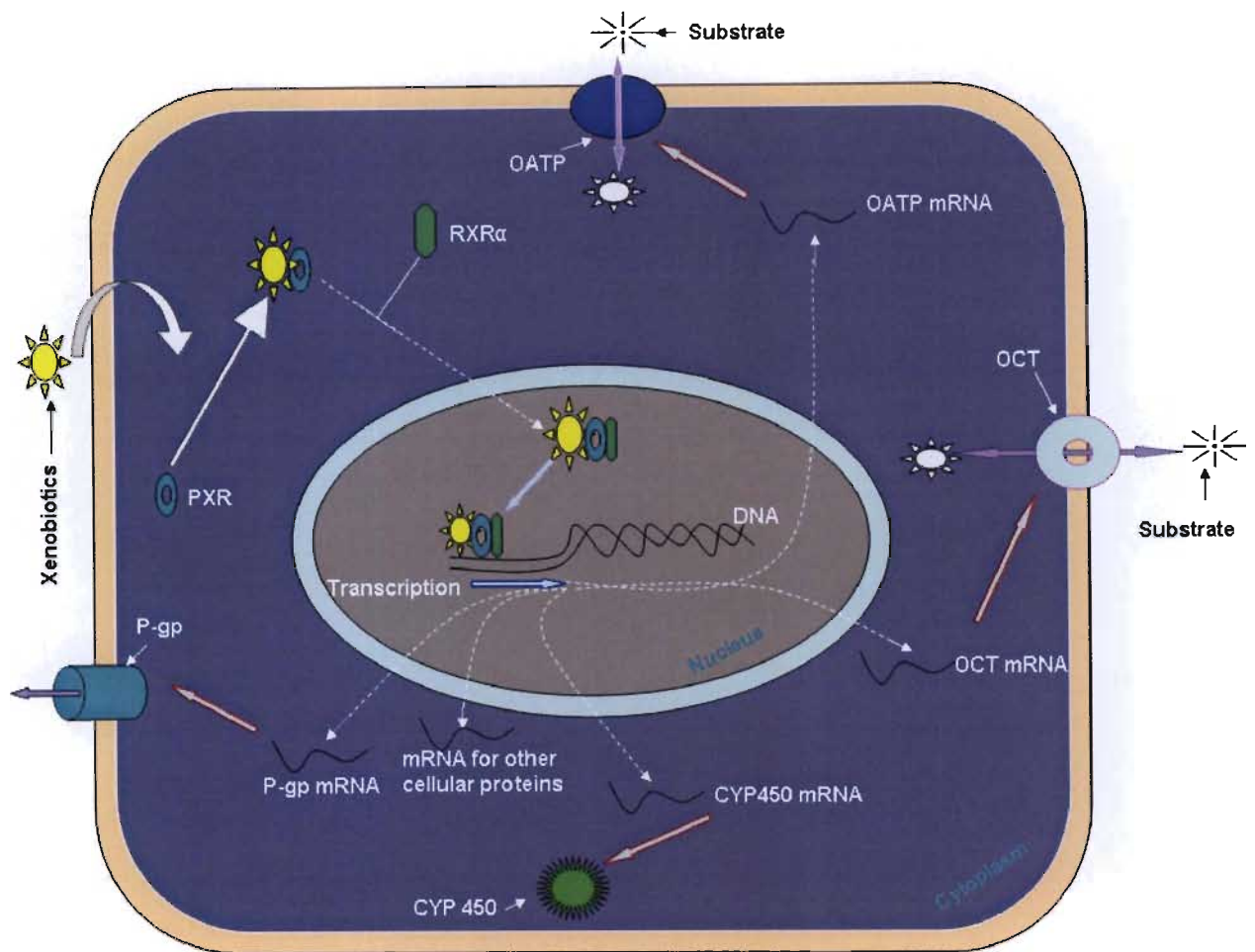


Figure 3.

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.

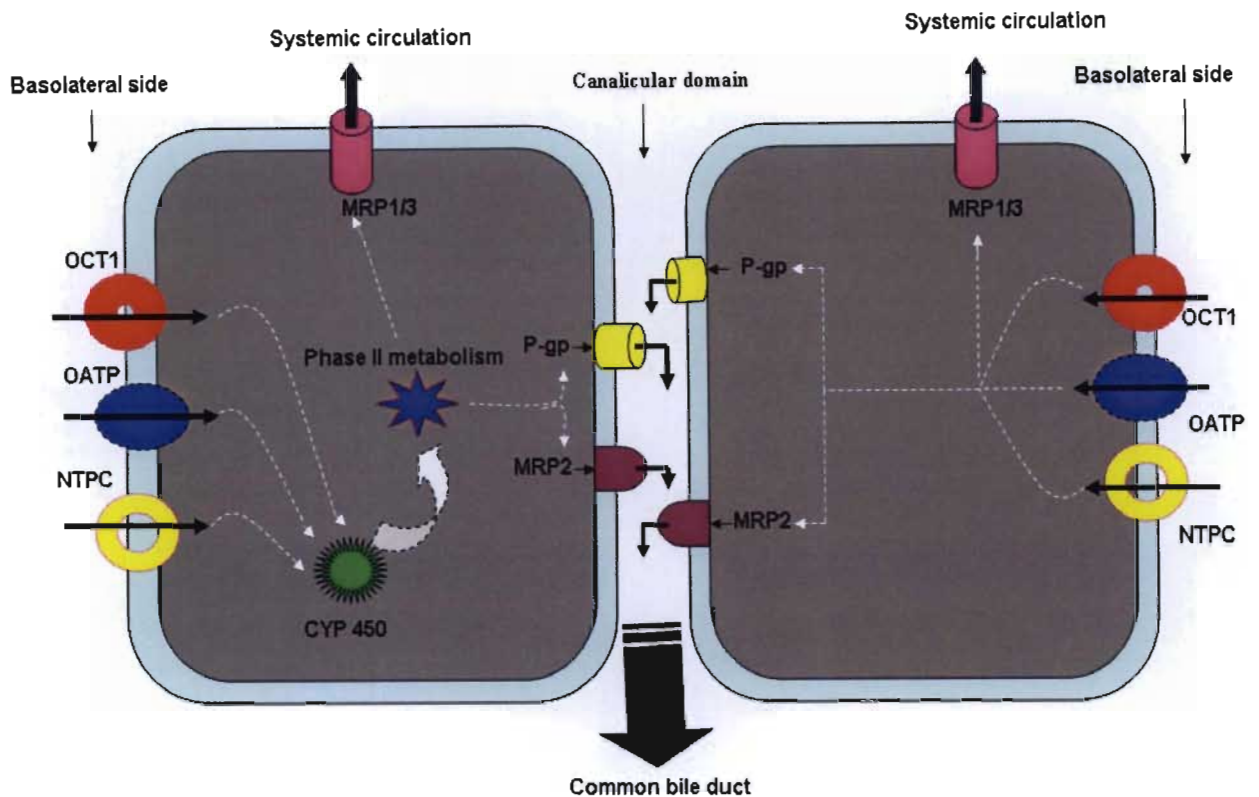


Figure 4.

Transporters 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 transporters on the basolateral membrane.

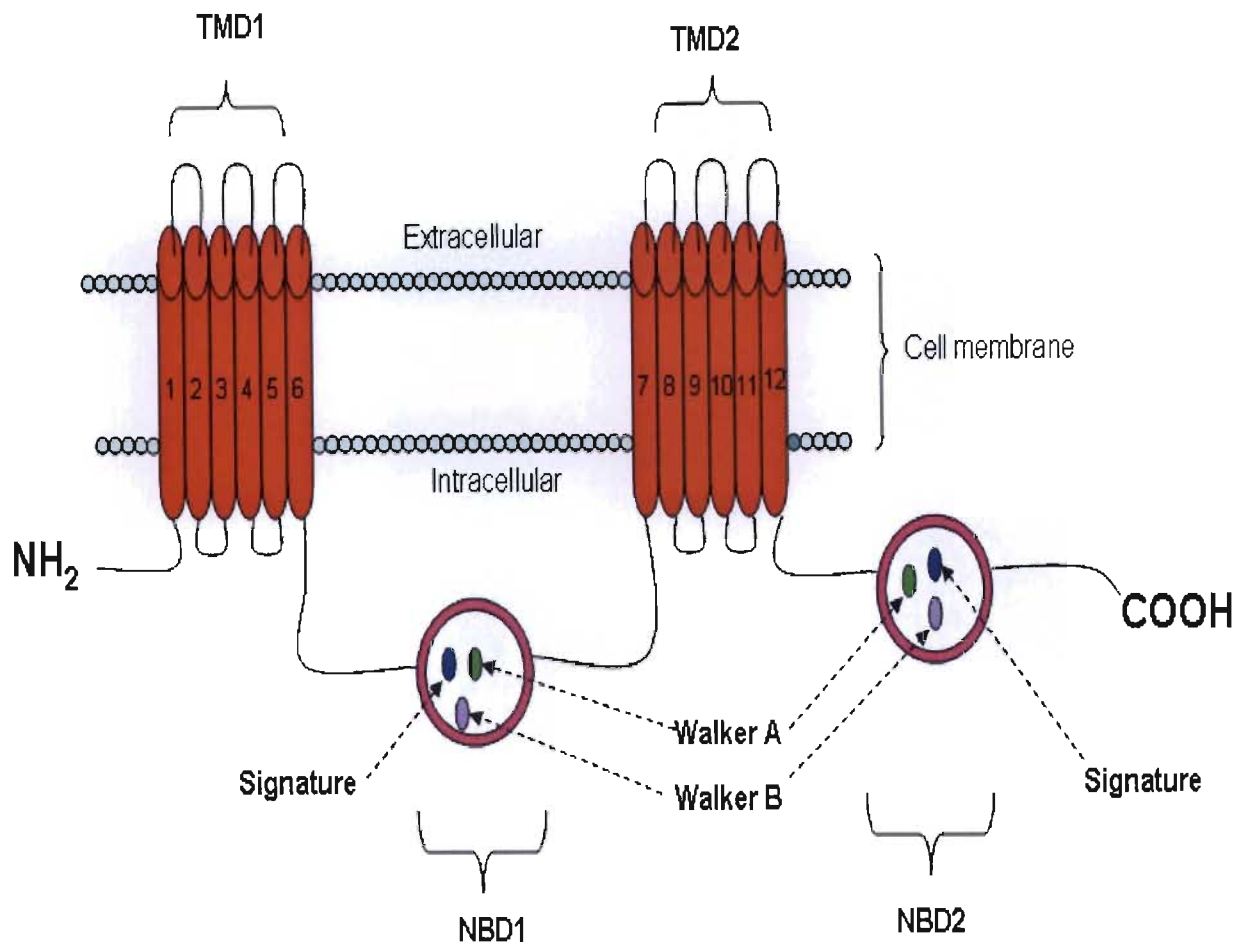


Figure 5.

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].

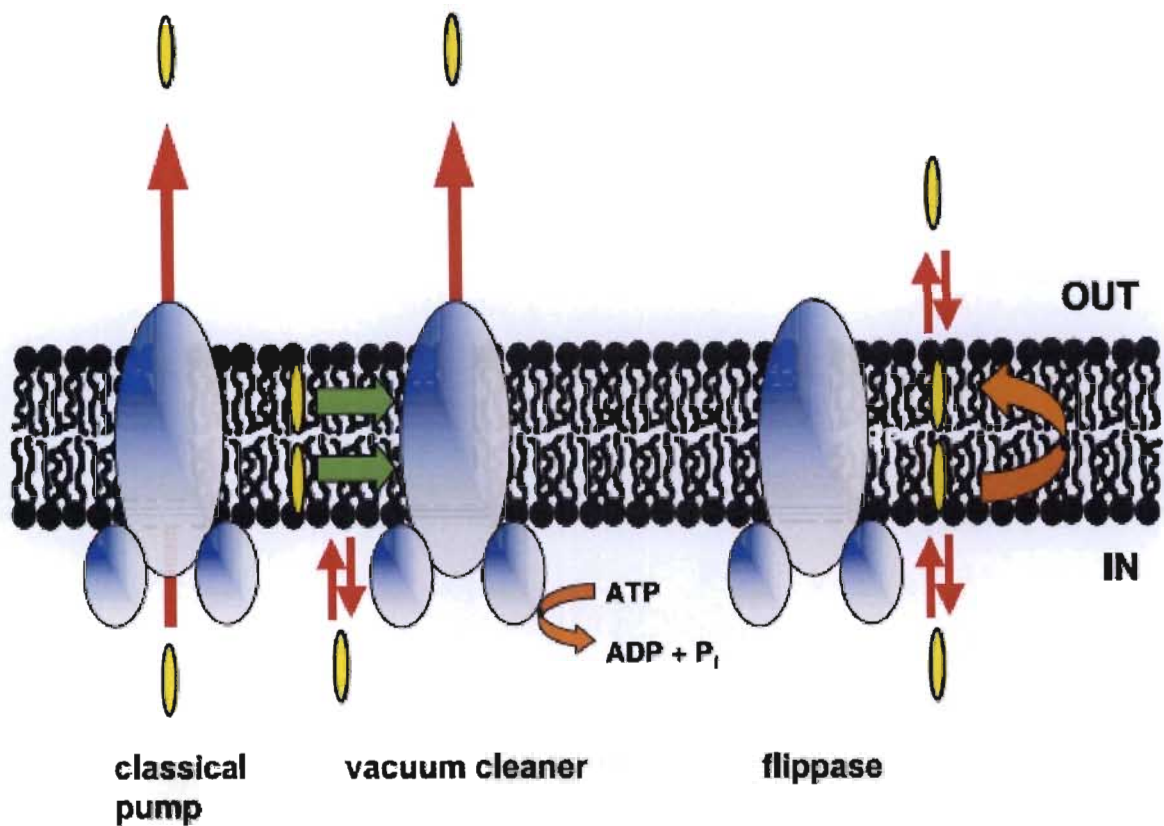


Figure 6.

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 “vacuum 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].

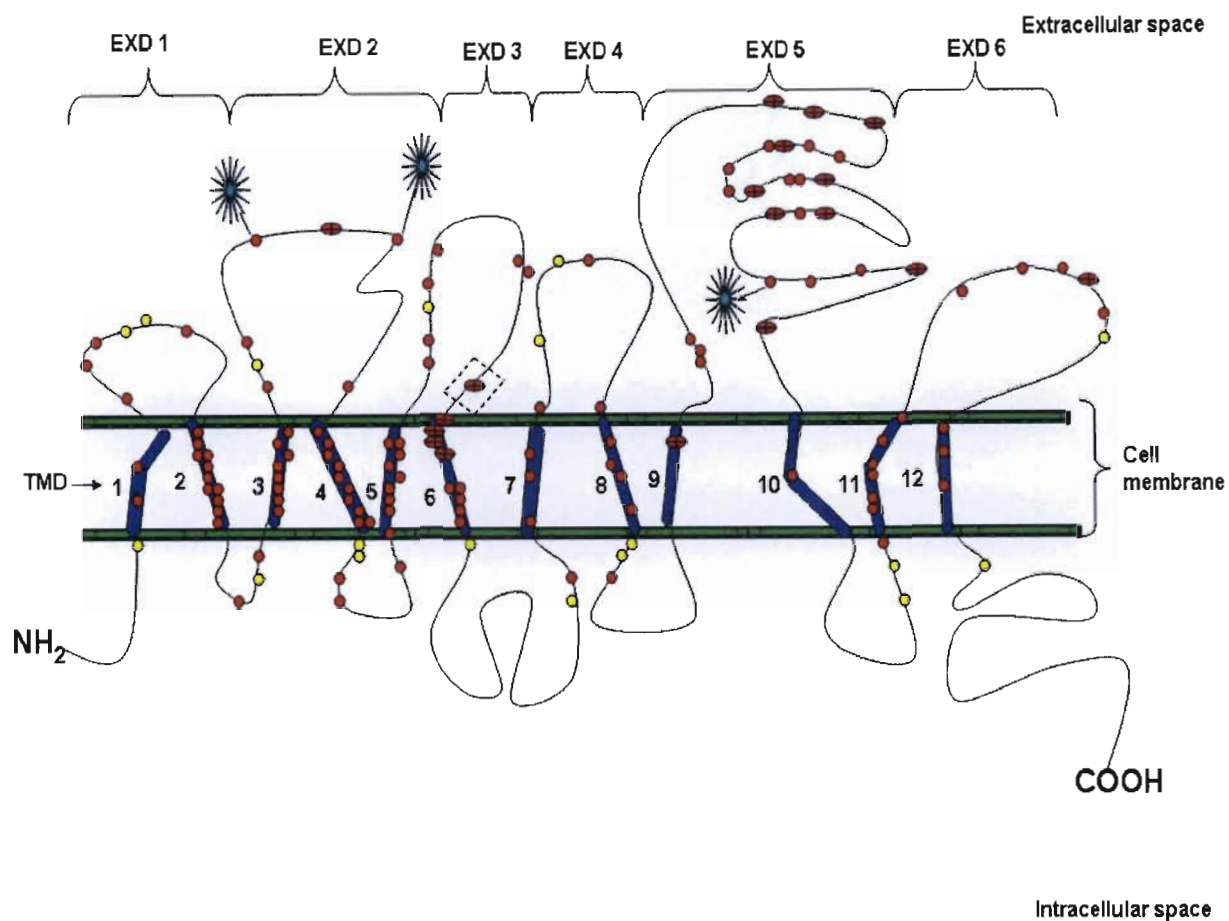


Figure 7.

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 cysteine (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.

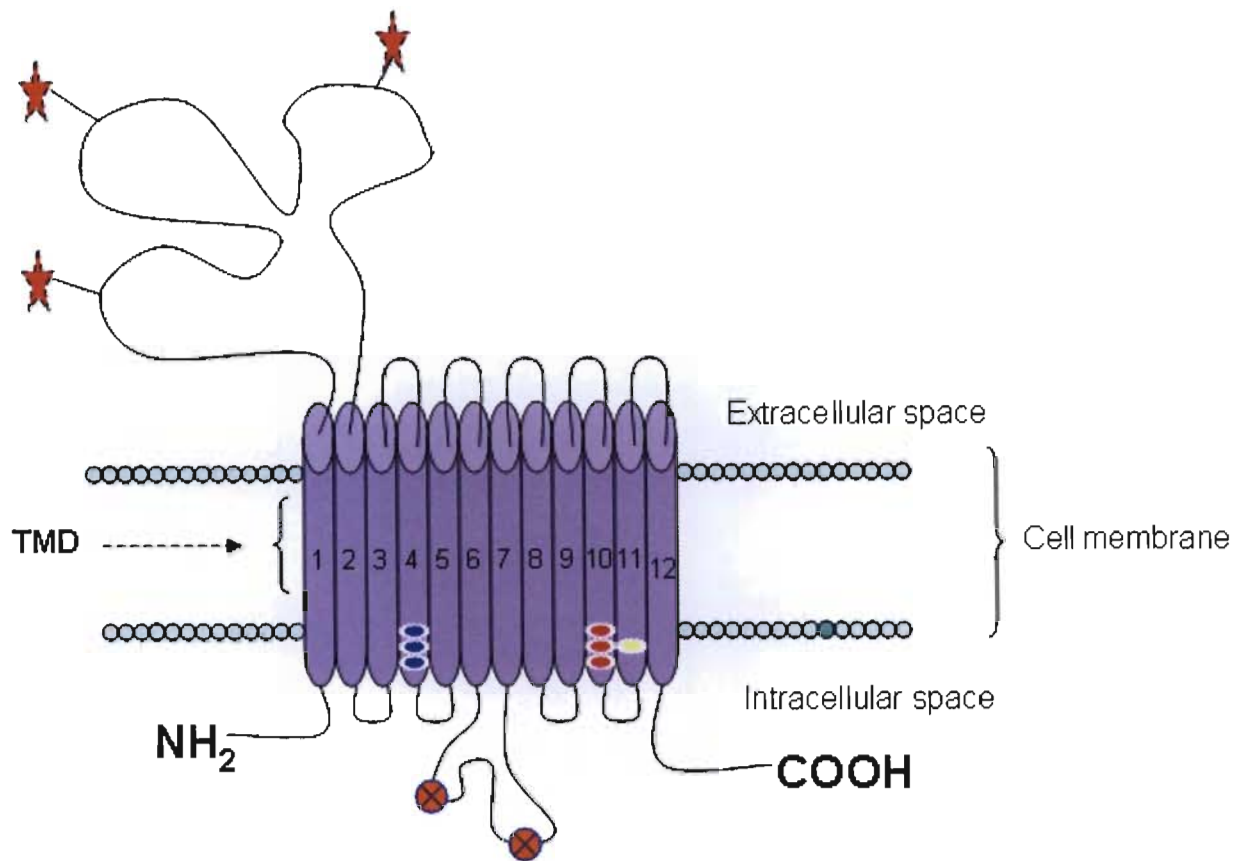


Figure 8.

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].

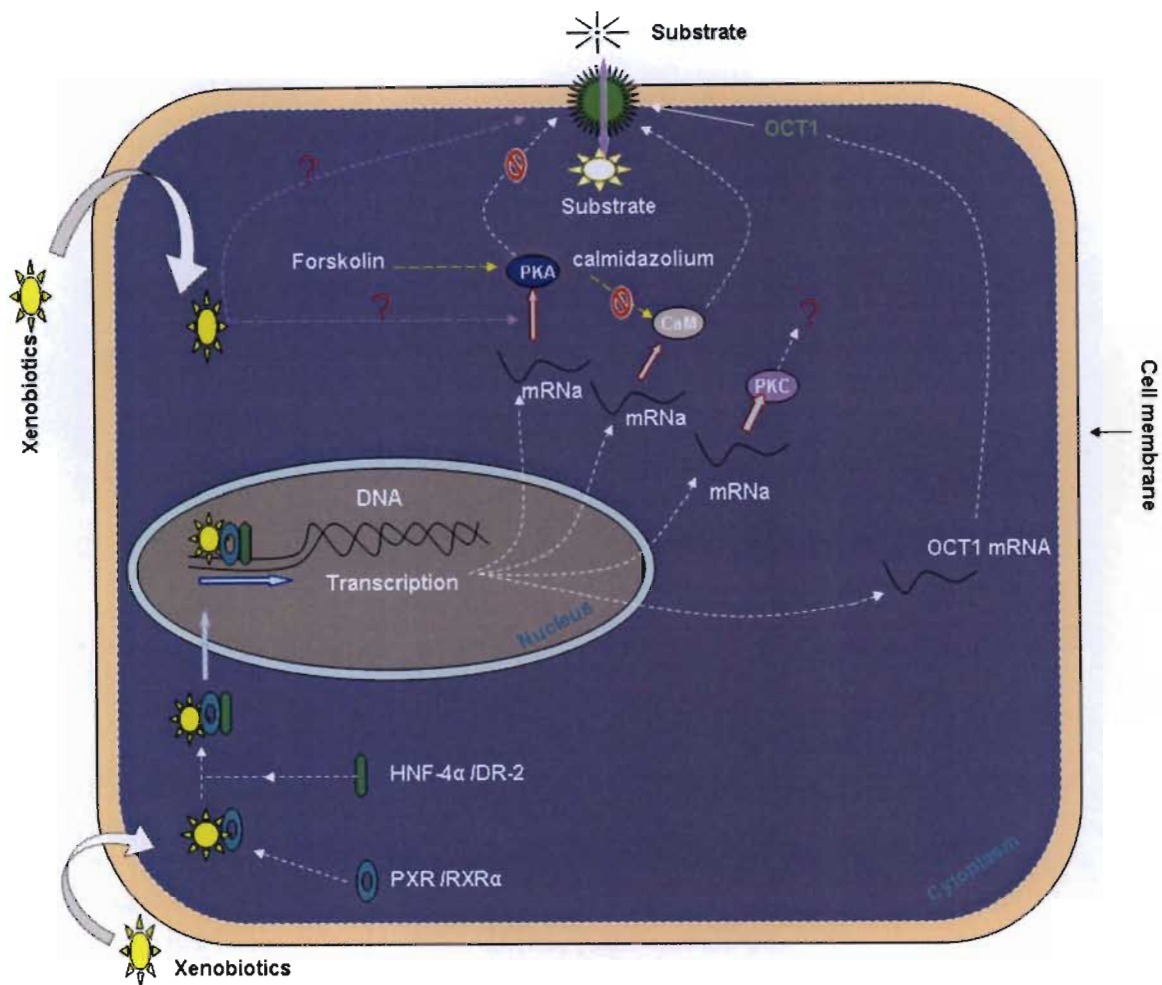


Figure 9.

Schematic illustration of the transcriptional and post-transcriptional regulation of OCT1. In response to endogenous or exogenous agonists, HNF-4 α binds DR-2 in the promoter region, and activates transcription of OCT1. Other ligands may alternatively activate PXR which dimerizes with RXR α , 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].

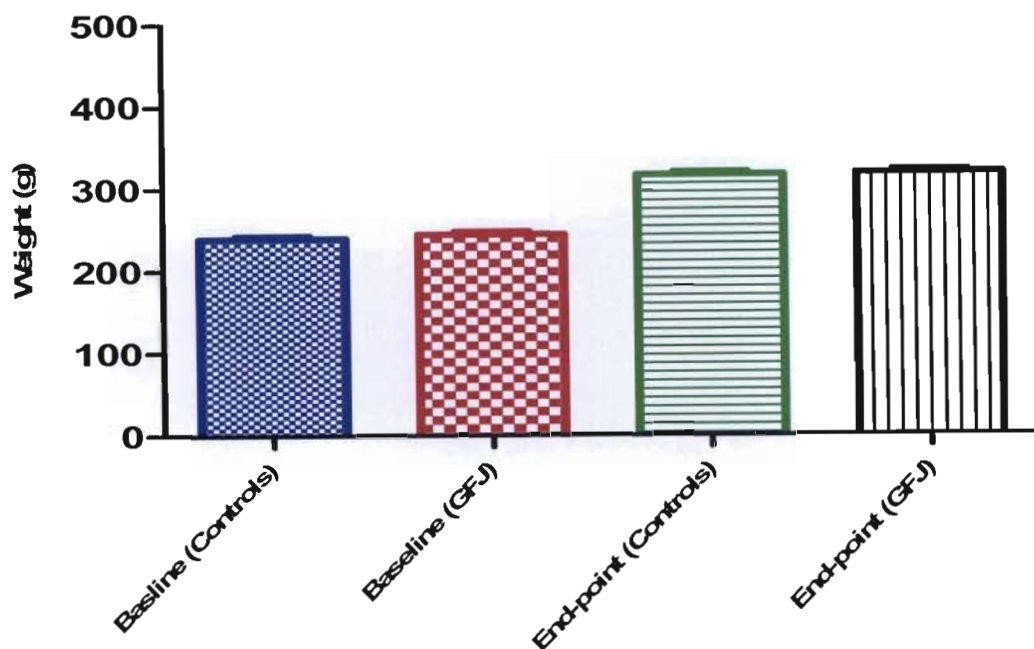


Figure 10.

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.

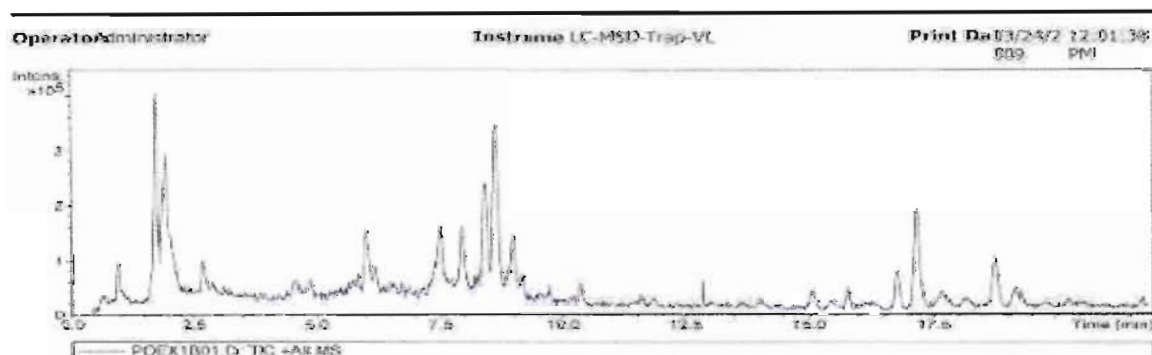


Figure 11.

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.

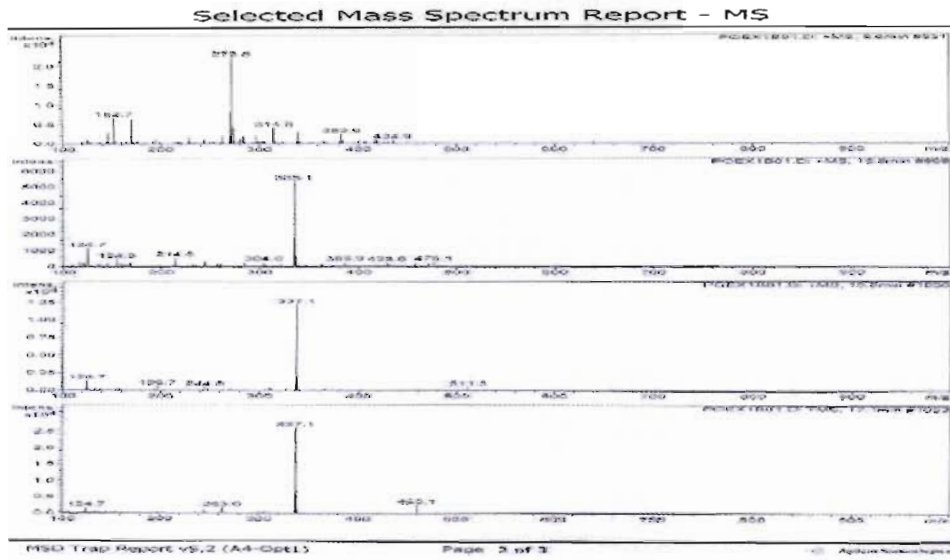
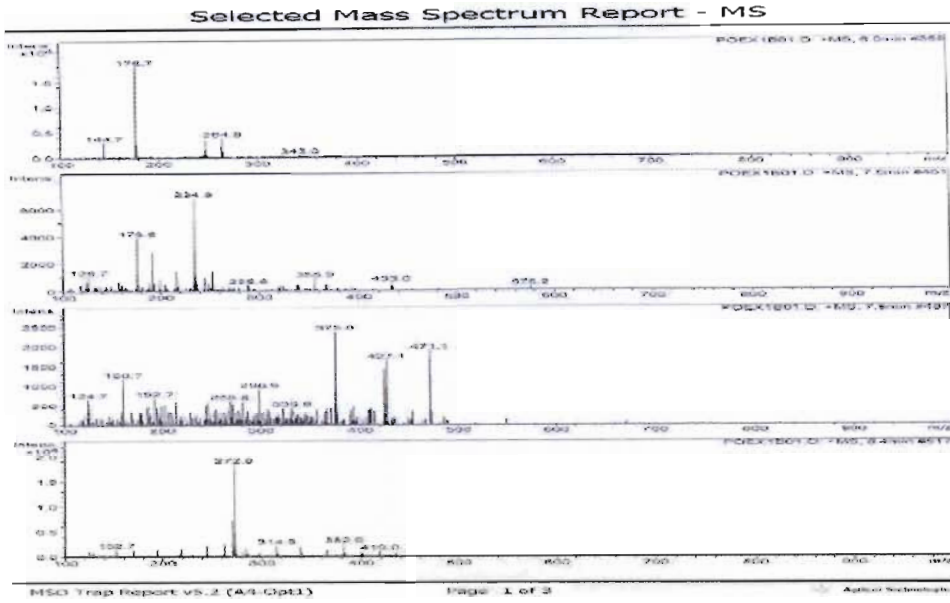


Figure 12.
Mass spectrometry analysis of HPLC chromatograms of the grapefruit juice extract.

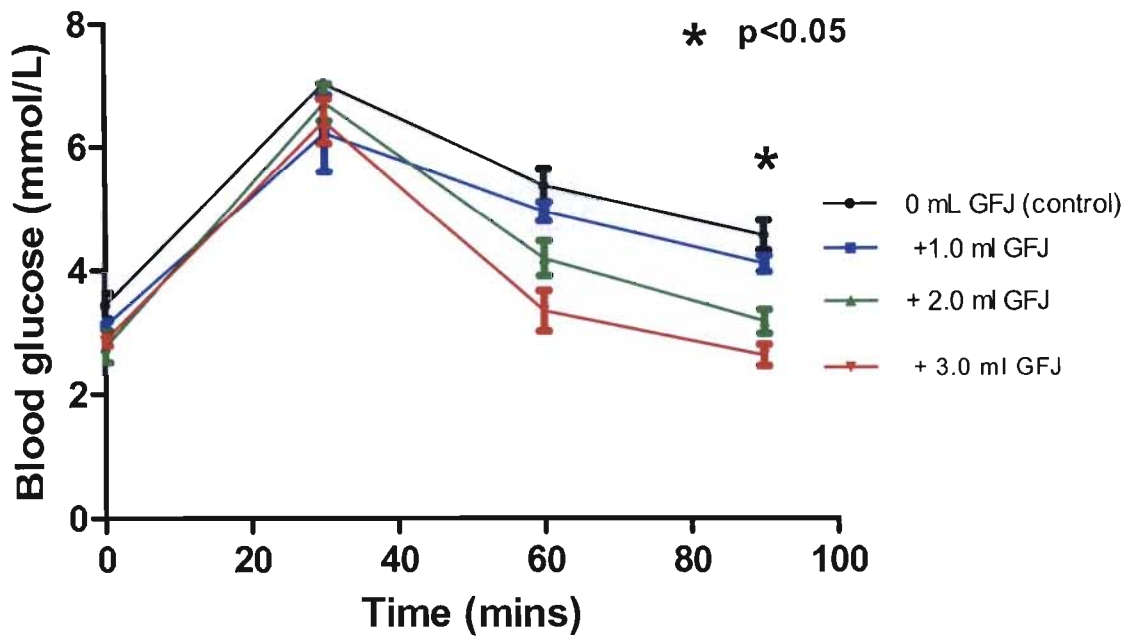


Figure 13.

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.

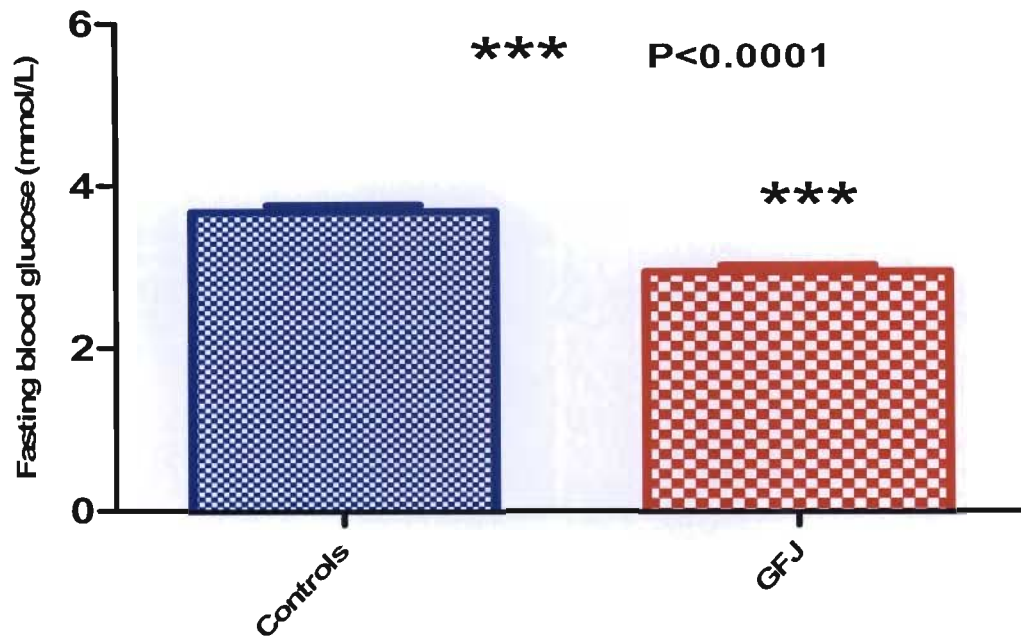


Figure 14.

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.

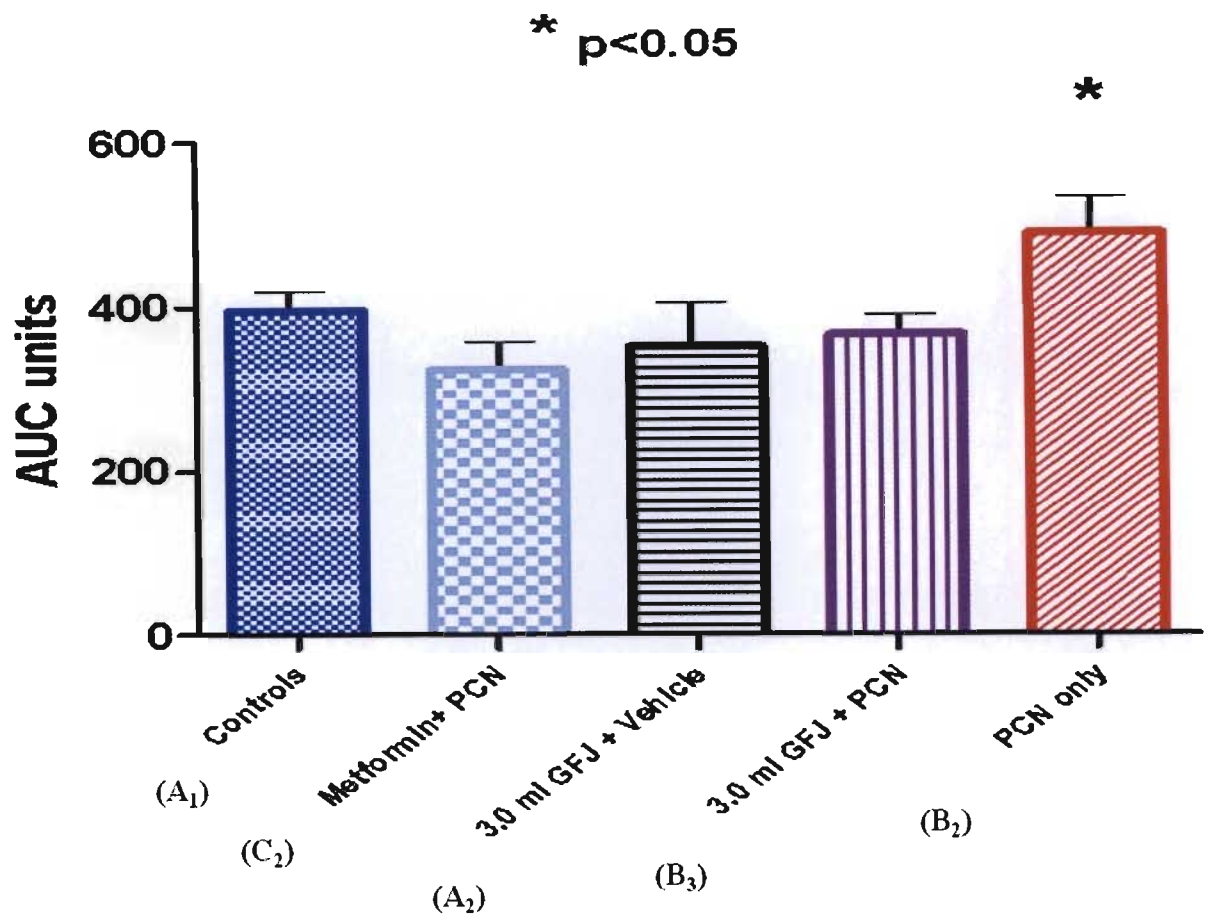
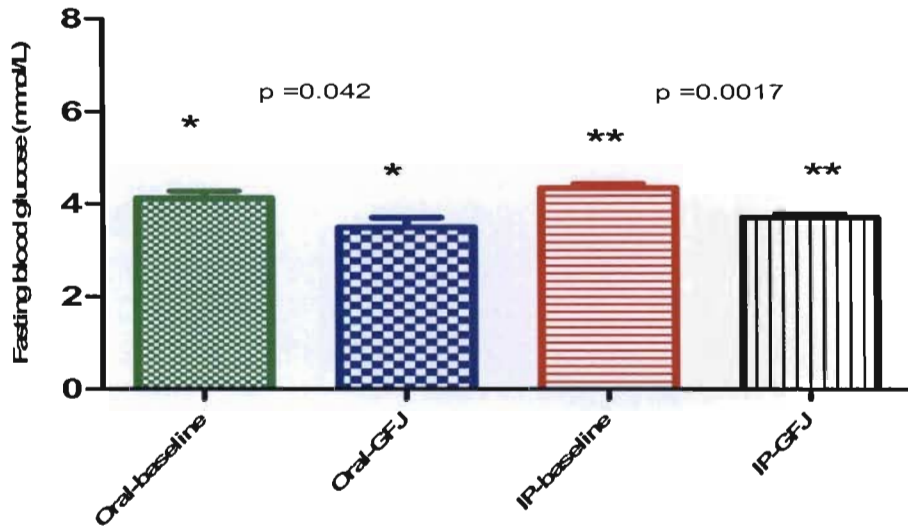


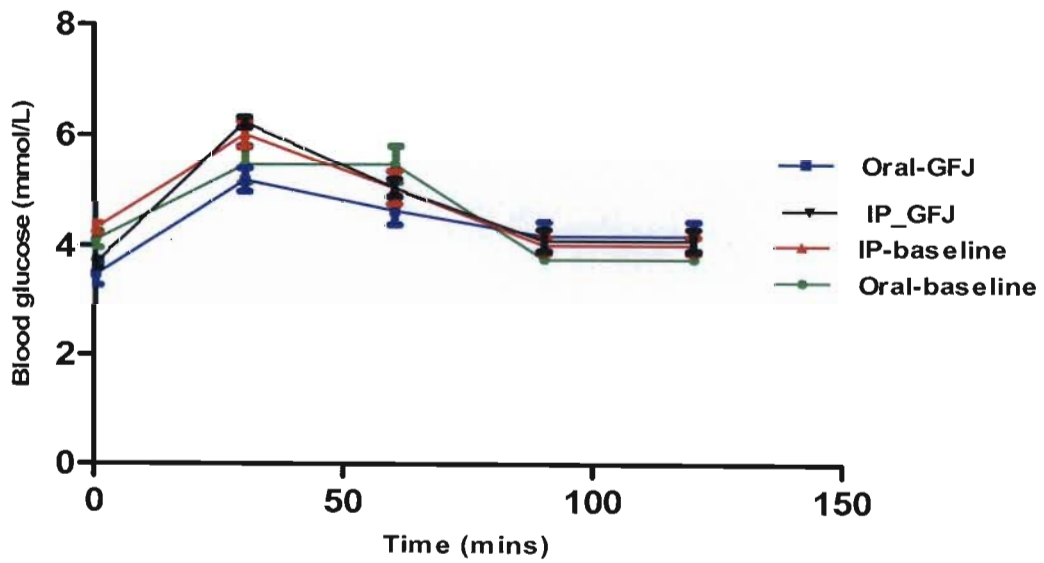
Figure 15.

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)



(B)



(C)

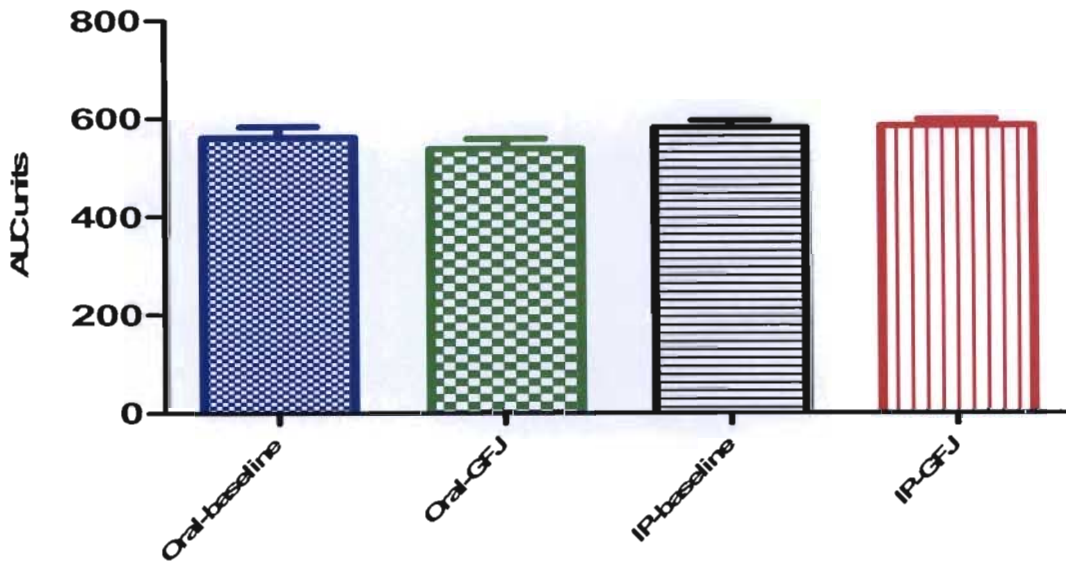


Figure 16.

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.

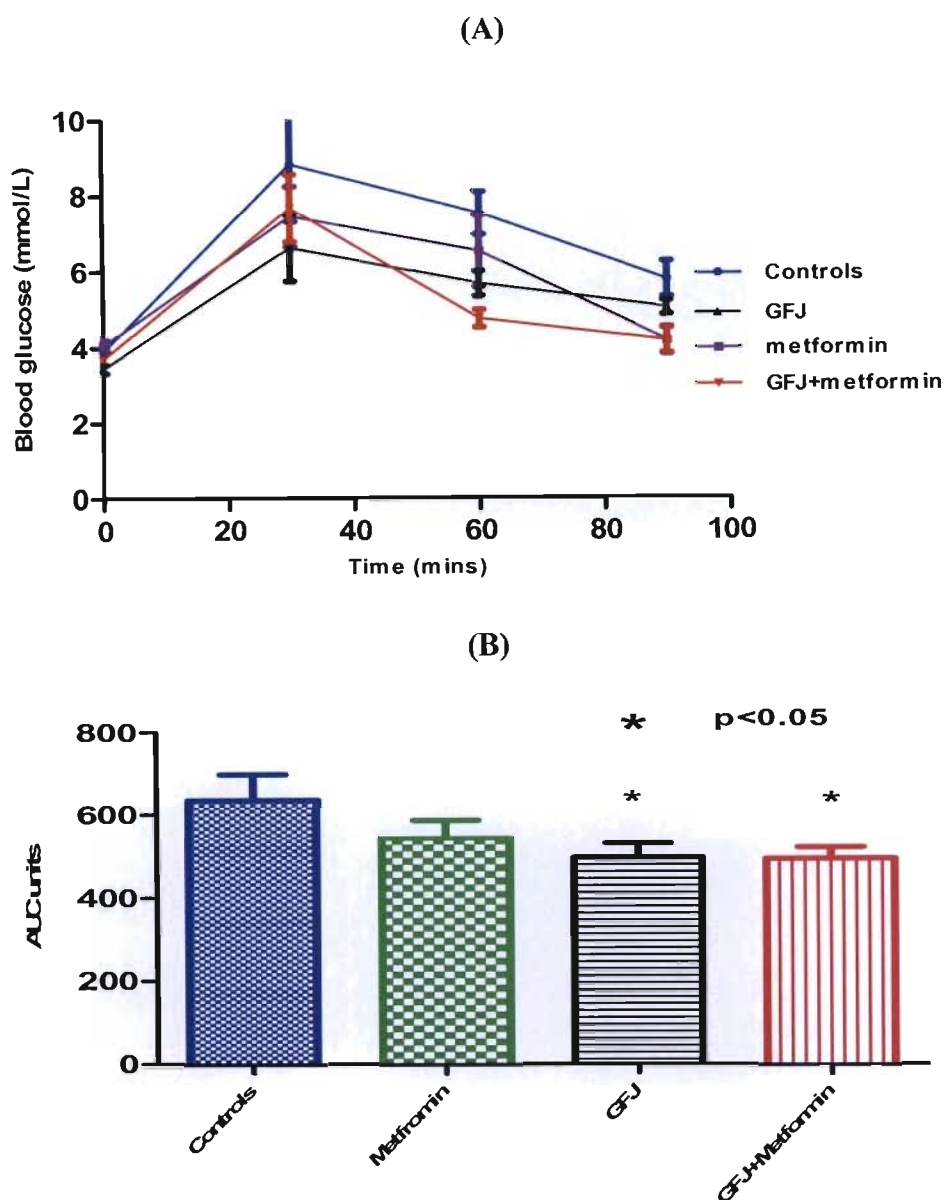


Figure 17.

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).

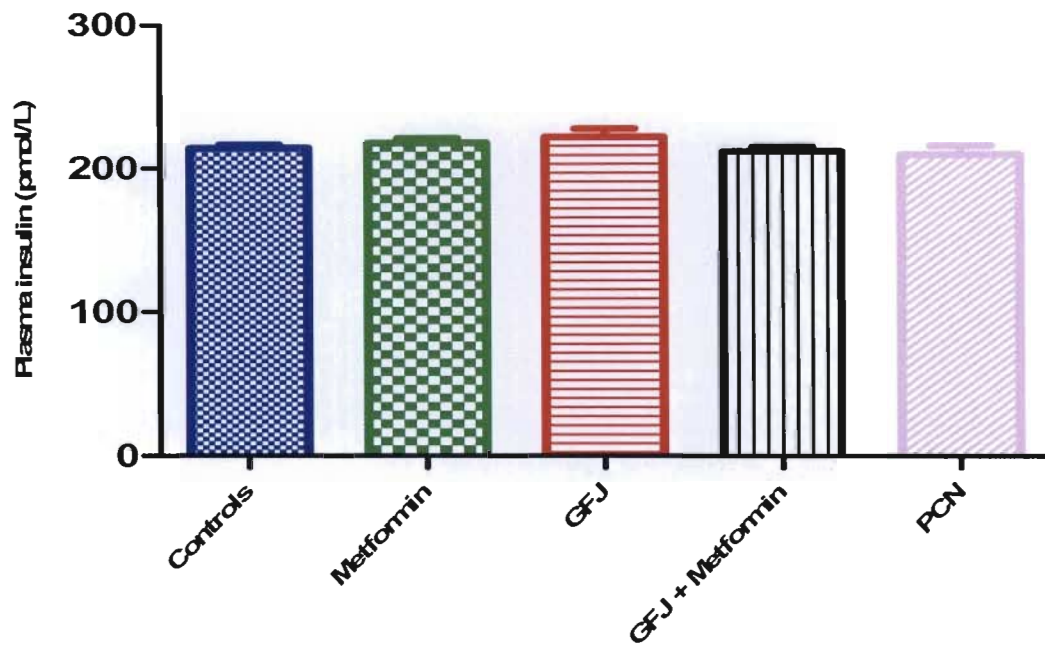


Figure 18.

Fasting plasma insulin levels in rats before intraperitoneal injection of 3.0 g/kg body weight of glucose in saline.

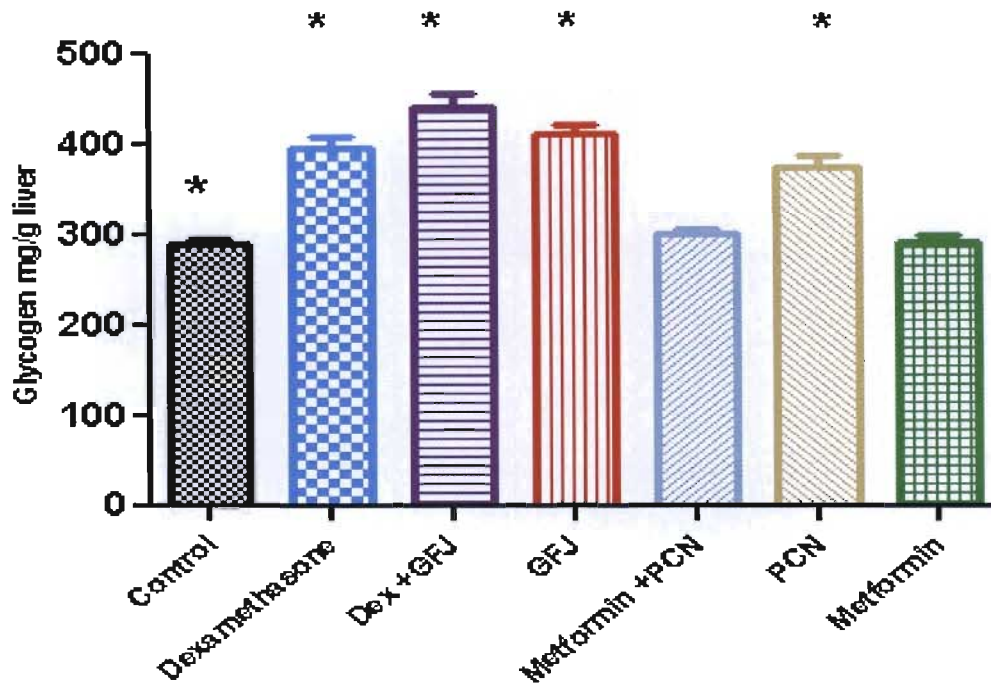


Figure 19.

Glycogen content of liver tissue after treatment with indicated mediators of glucose homeostasis. (* $p < 0.05$).

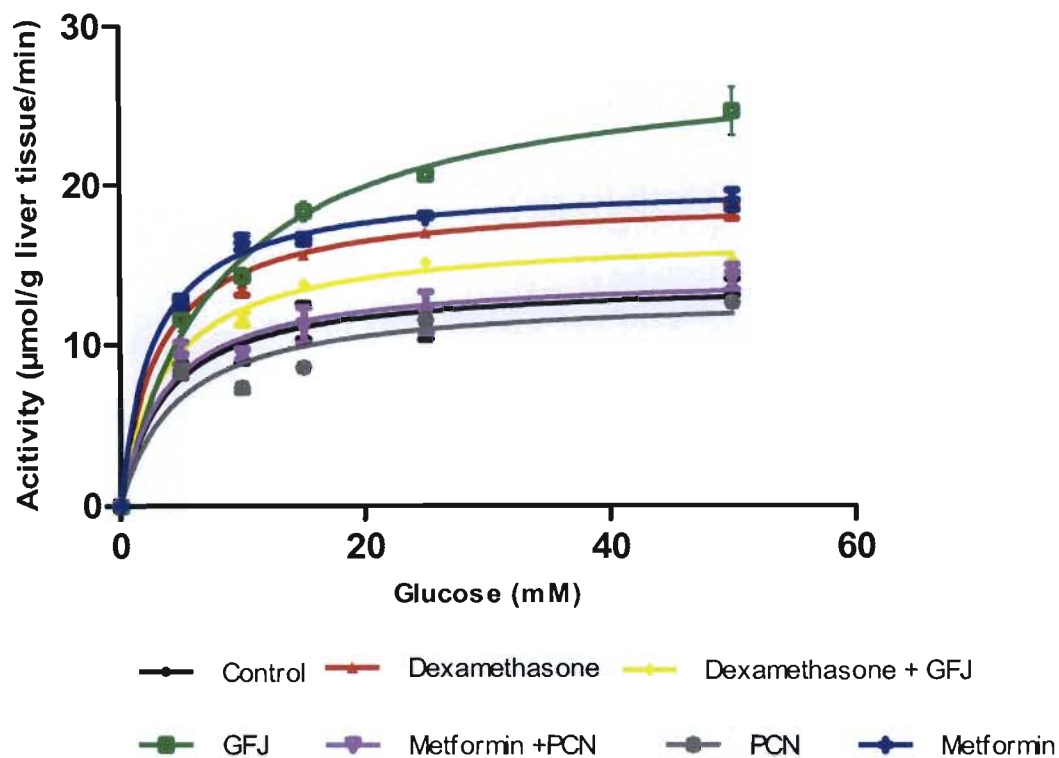


Figure 20.

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.

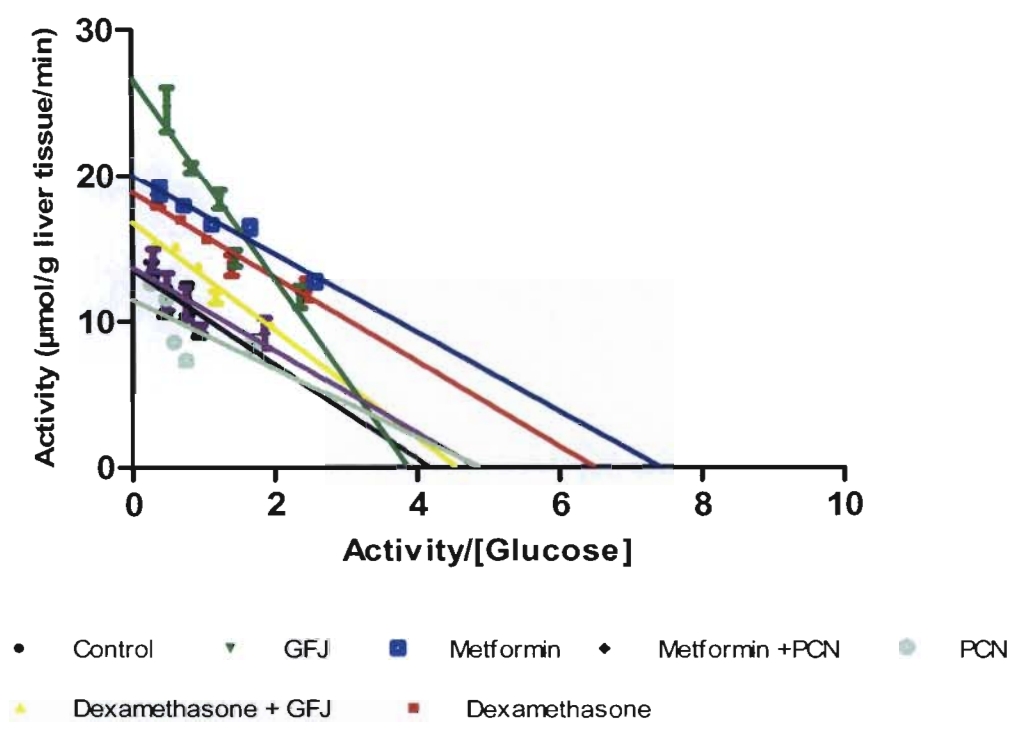


Figure 21.

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

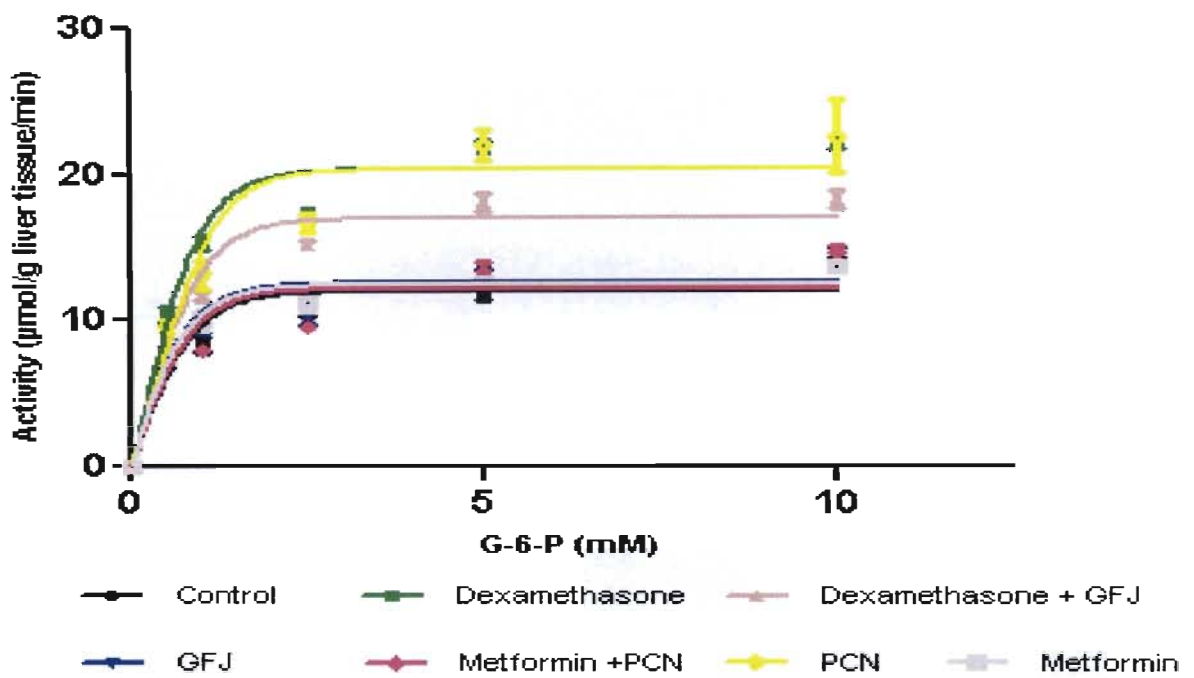


Figure 22.

Michaelis-Menten kinetics of glucose-6-phosphatase activity in the rat liver samples previously treated with different agonists *in vivo*. Enzyme activity was plotted against substrate concentrations.

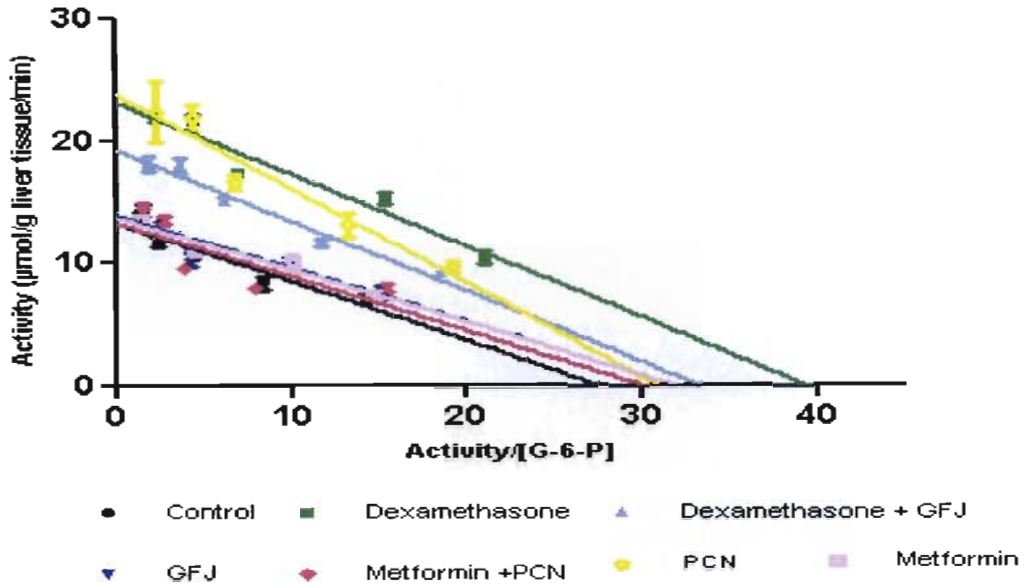


Figure 23.

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. $K_m = 1/\text{slope}$.

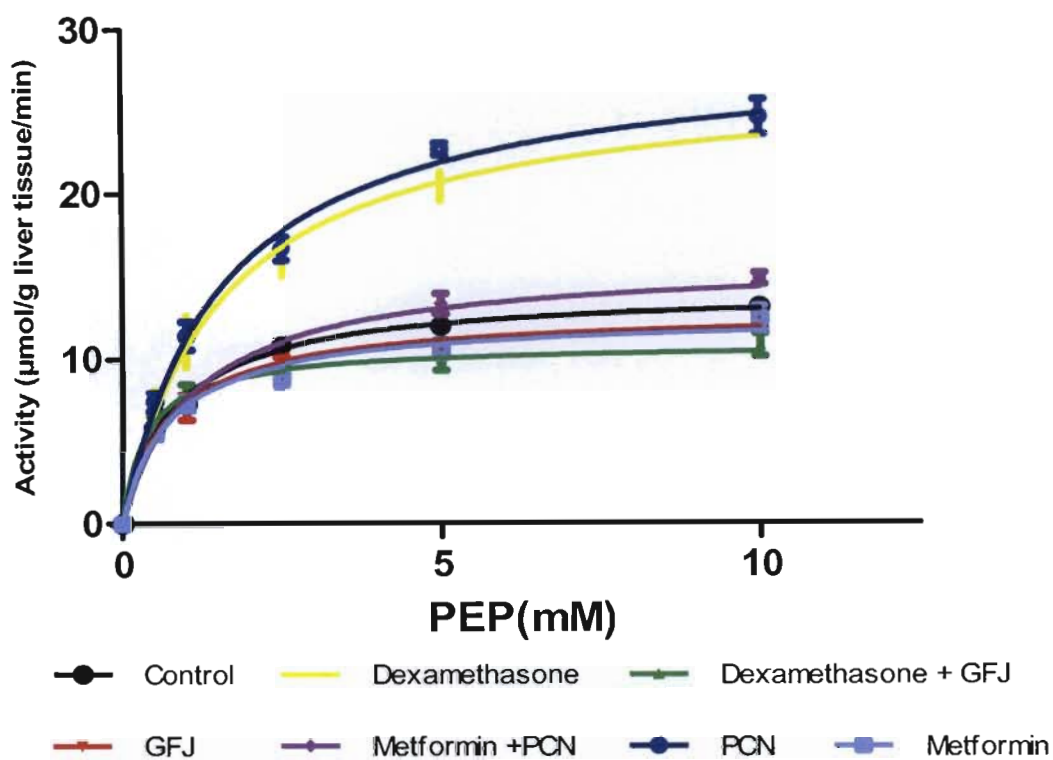


Figure 24.

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.

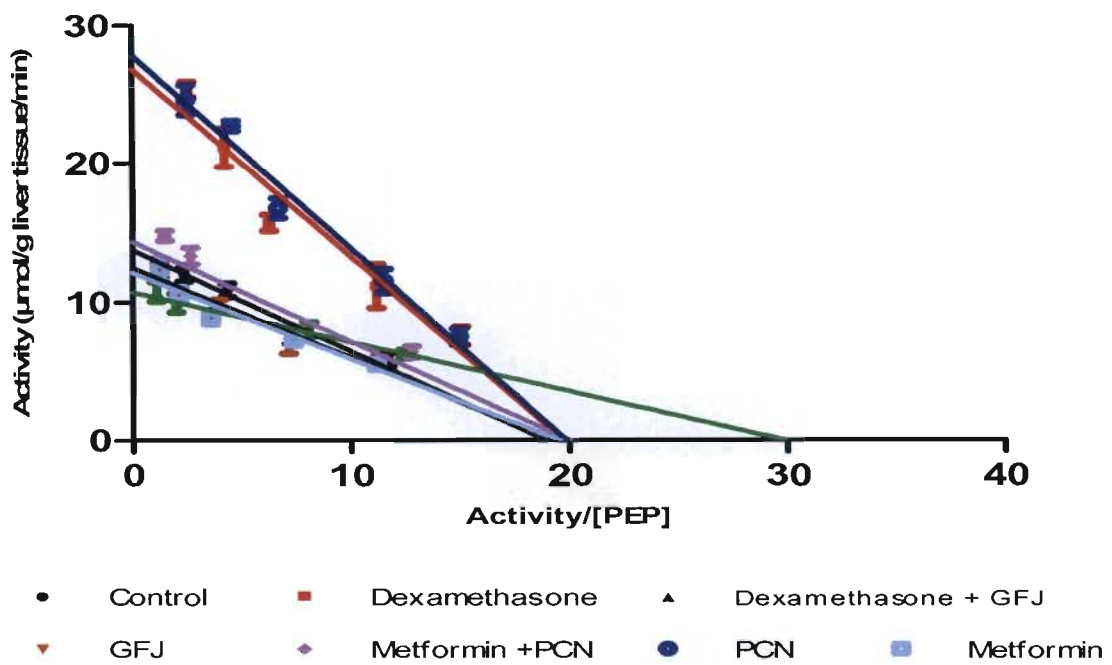


Figure 25.

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. $K_m = 1/\text{slope}$.

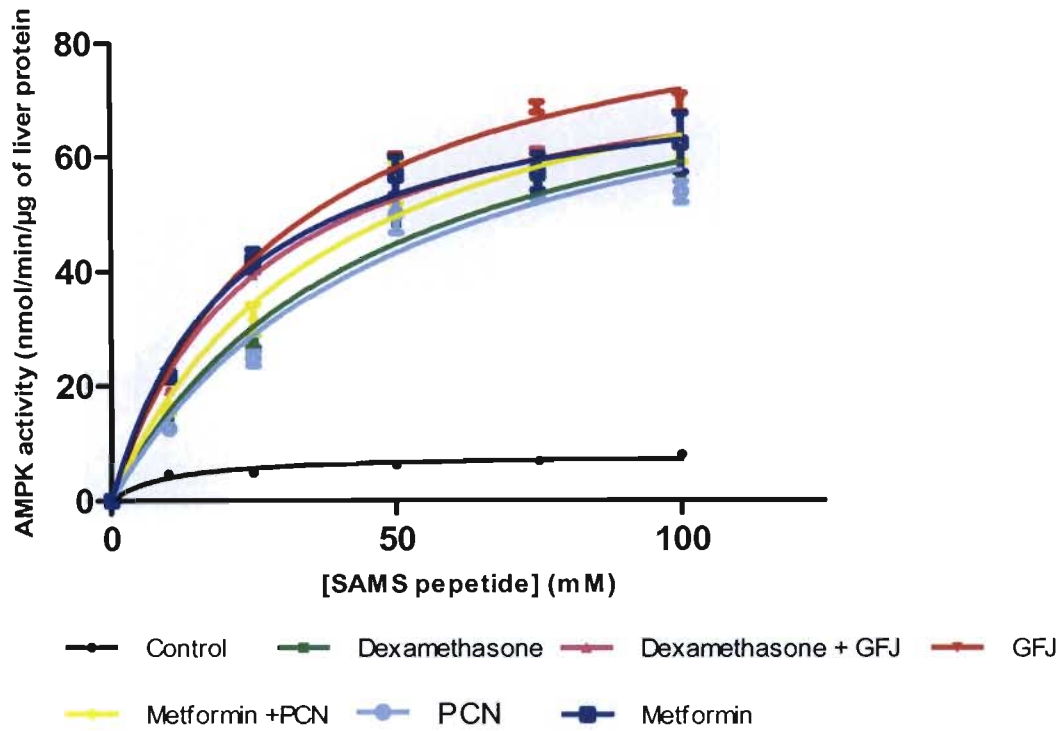


Figure 26.

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.

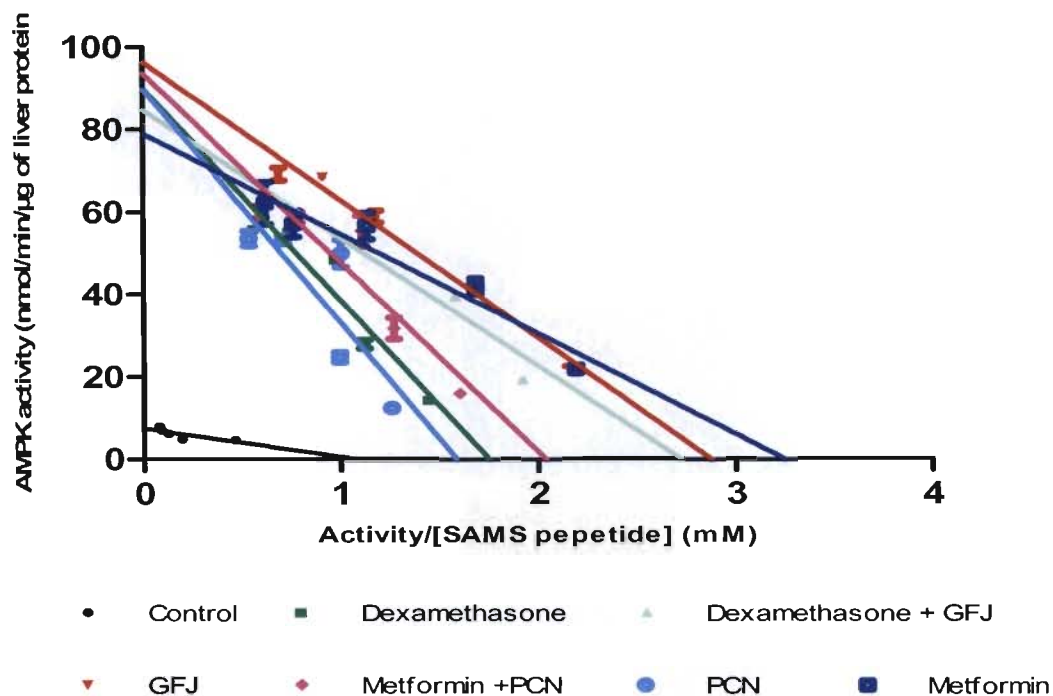


Figure 27.

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. $K_m = 1/\text{slope}$.



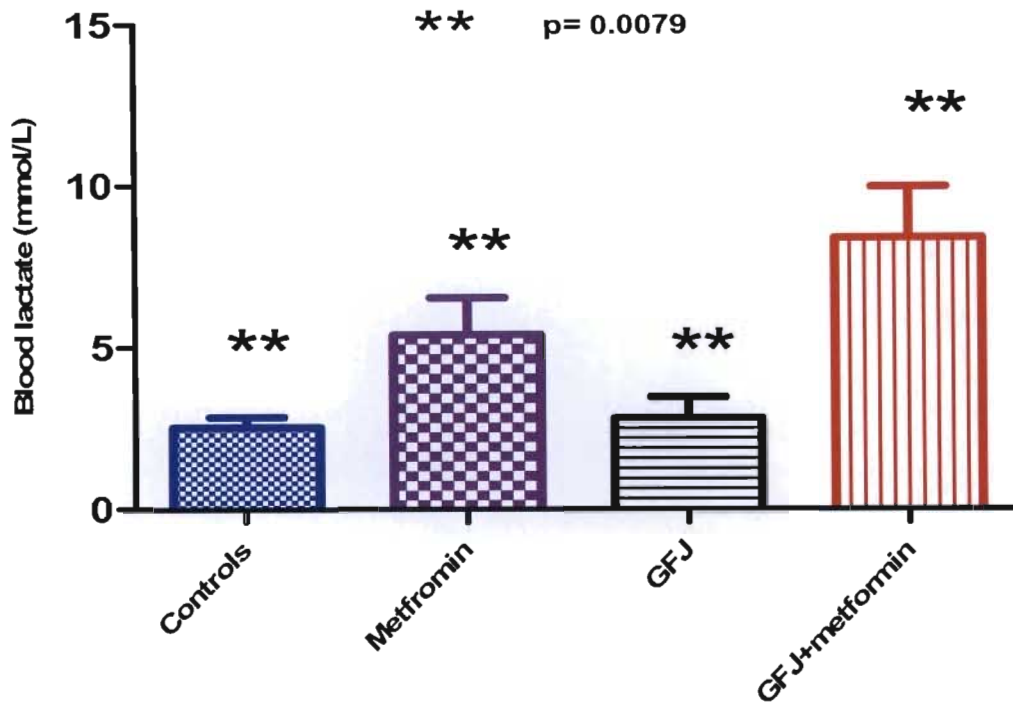


Figure 28.

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.

116125

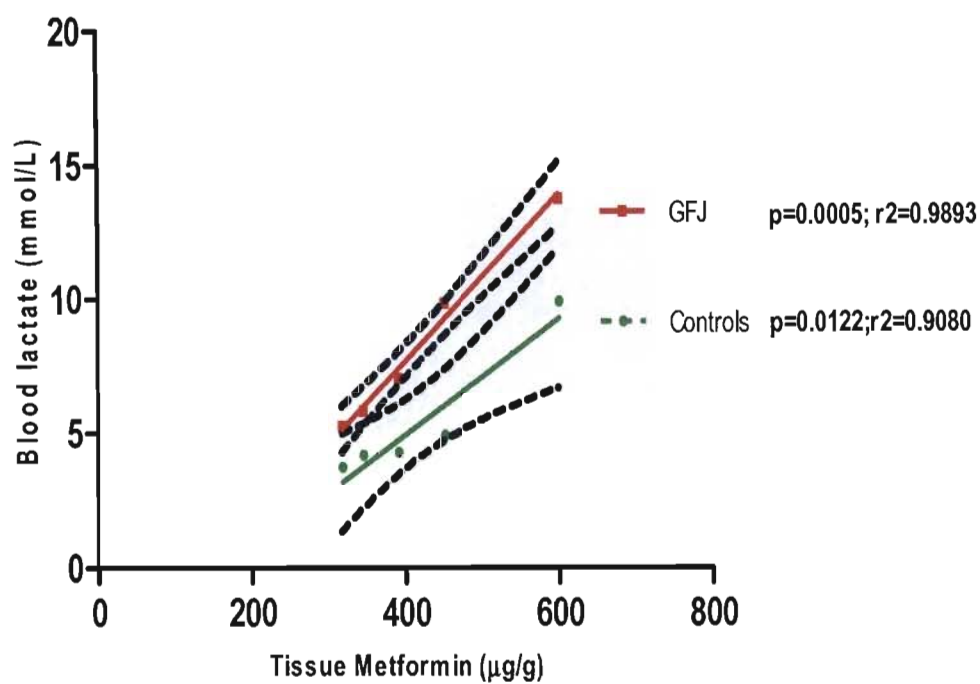


Figure 29.

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.

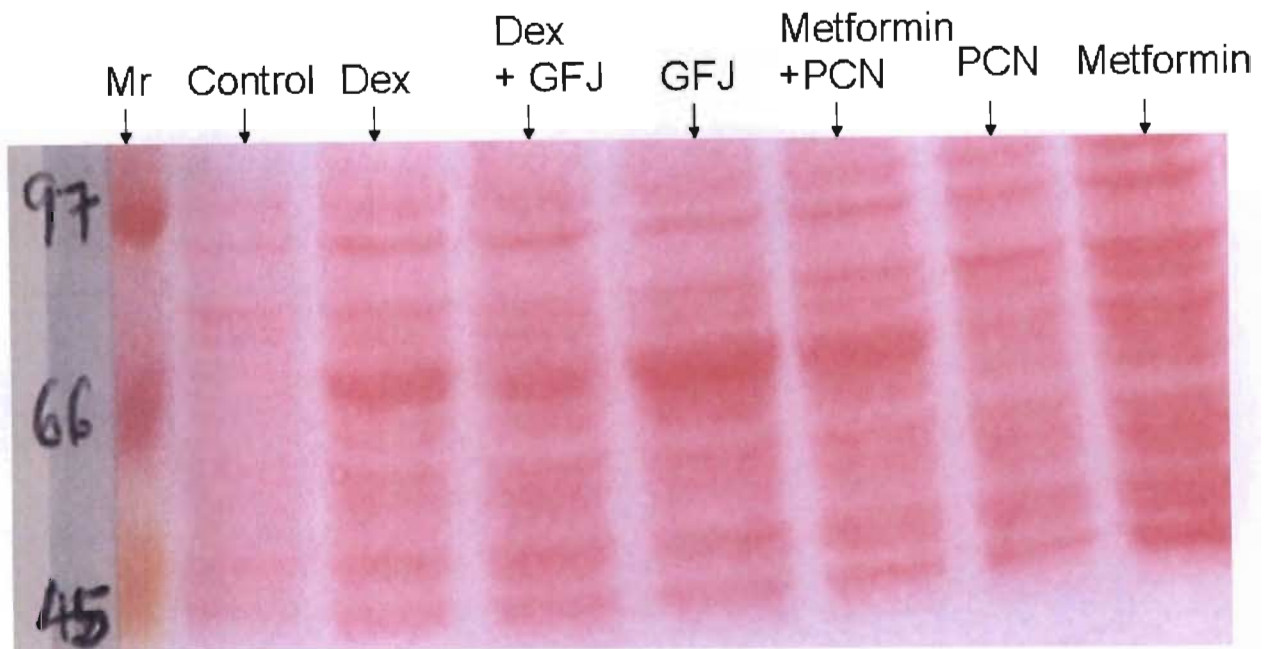


Figure 30.

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.

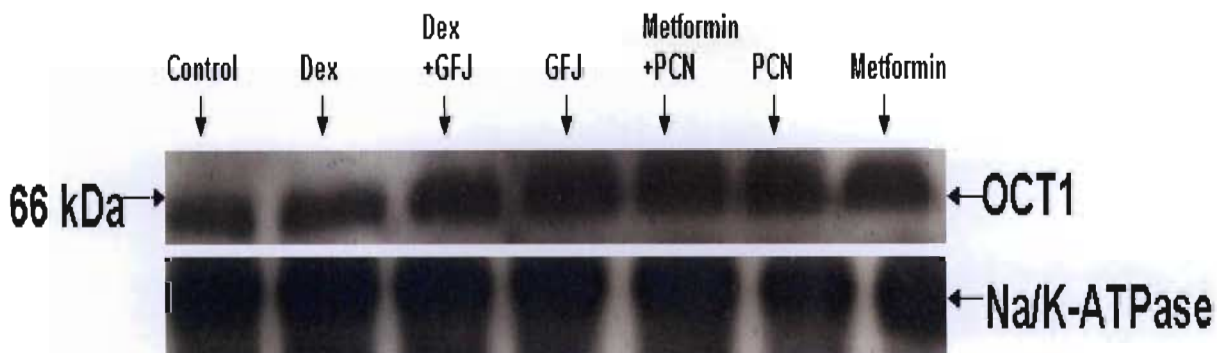


Figure 31.

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.

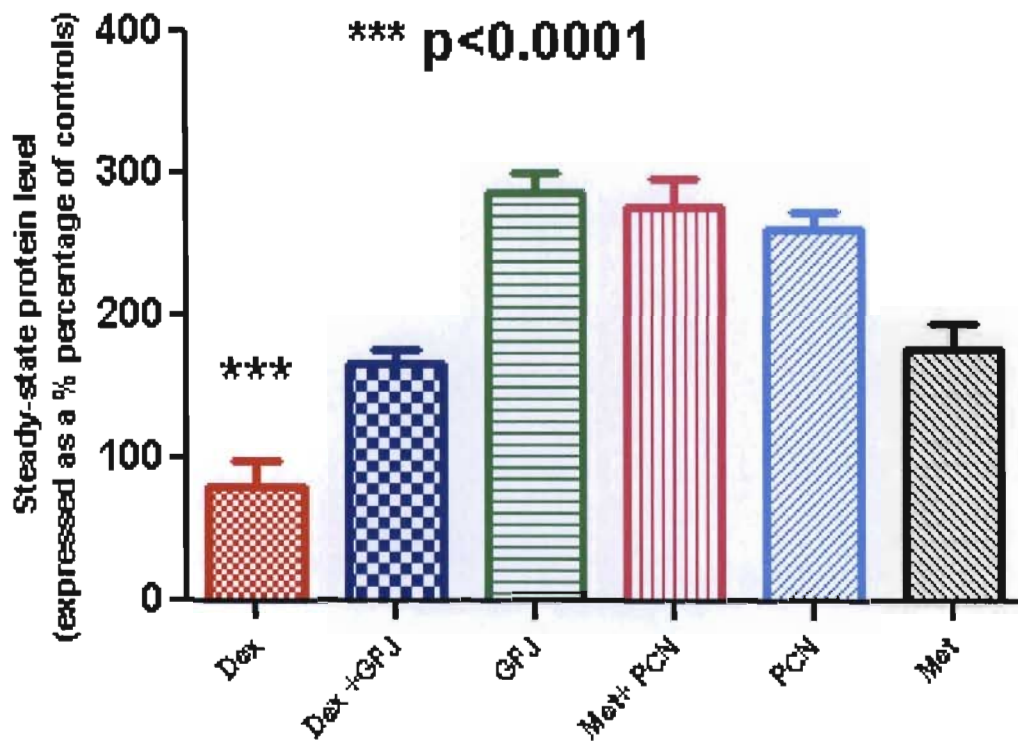


Figure 32.

Densitometry scans of rOct1 expression in the rat liver. Steady-state protein level was expressed as a percentage of the control.

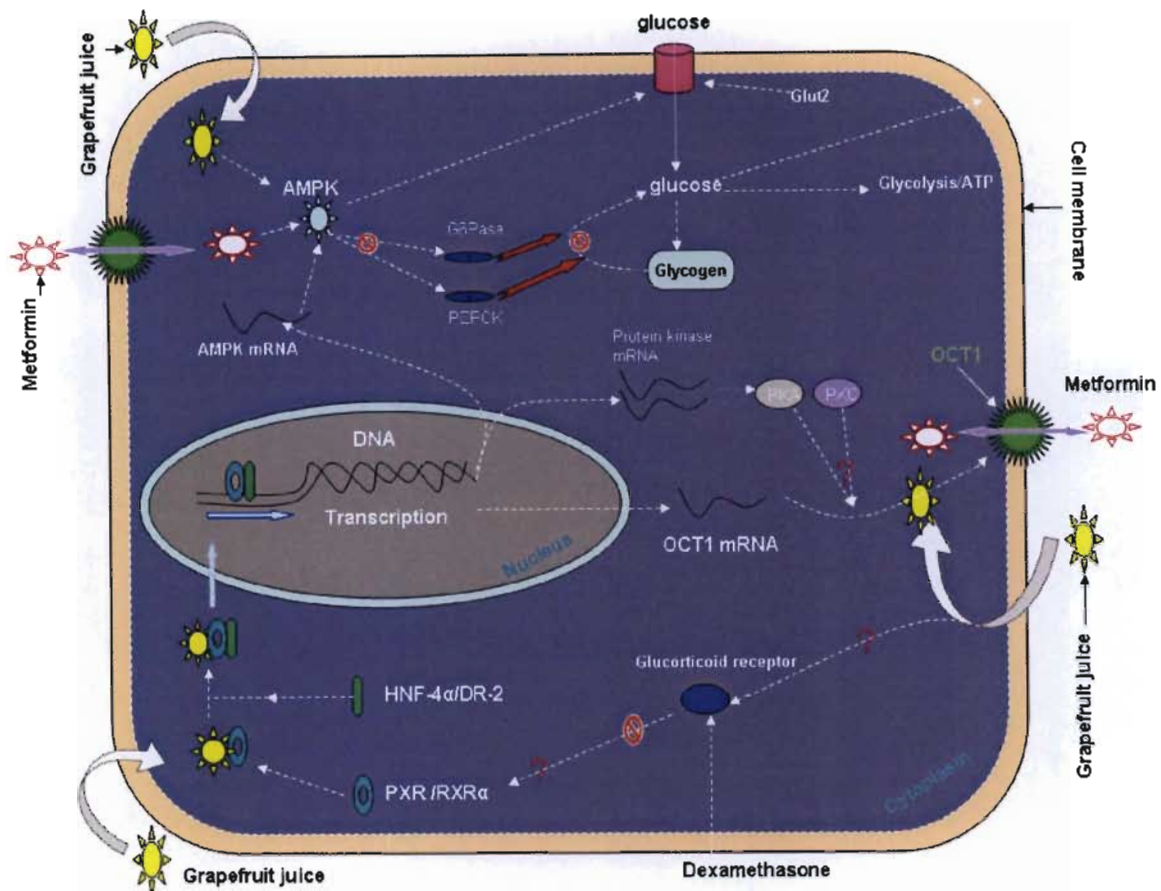


Figure 33

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.

Compounds	Bioactive constituents	Pharmacological Effects	Potential Clinical Applications	References
Flavonoids (flavanones)	Naringin/ Naringenin Hesperidin	Antioxidant Antineoplastic Anti-microbial Anti-thrombotic Vasodilator Antiinflammatory Antiatherosclerotic Hypoglycemic Antineoplastic Antiinflammatory	Cancer /diabetes Cancer Infection Cardiovascular/ degenerative disorders, Diabetes, Cancer, Degenerative disorders, Cancer Antihistamine	1, 15, 22, 26, 27, 29, 31
Limonoid	Limonin Nomilin Abacunone	Antineoplastic	Cancer	22, 30, 32
Furanocoumarins	Bergamottin Dihydrobergamottin Epoxybergamottin Paradisins A Gergamotin	Antioxidant, CYP 3A4 inhibitors	Cancer	1, 12, 20, 22, 35, 36, 40
Carotenoids	β -carotene Lycopene			1, 12,
Ascorbic acid		Antioxidant	Cancer/Diabetes	1, 12
Folic acid				
Potassium				1,
Glucaric acid	Glucaric acid	Antineoplastic, Antihypercholesterolemia	Diabetes	23, 33, 34
Highly soluble fibre	Pectin			1, 12

Table 1.

Some of the bioactive compounds so far identified in the grapefruit and their pharmacological actions.

Anticancer Agents	Beta blockers	Calcium channel blockers	Other cardiovascular drugs	HIV protease inhibitors	Corticosteroids
Docetaxel* Etoposide* Paclitaxel* Vinblastin* Teniposide* Vincristine*	Bunitrol Carvedilol* Celiprolol Talinolol	Diltiazem* Milbefradil* Verapamil*	Digitoxin Digoxin Quinidine*	Amprenavir* Indinavir* Nelfinavir* Saquinavir* Ritonavir*	Dexamethasone* Methylprednisolone*
Immuno-Suppressants	Antiemetics	Statins	Antihistamines	Antibiotics	Others
Cyclosporine* Sirolimus* Tacrolimus*	Ondansetron*	Atovastatin* Lovastatin*	Fexofenadine Terfenadine*	Erythromycin* Levofloxacin Clarithromycin	Amytriptyline* Itraconazole* Losartan Morphine* Phenytoin Rifampin*

Table 2.

Selected substrates of P-gp. The substrates marked with (*) are also substrates of CYP3A4 [172, 173, 174].

		Treatment				
Group	Sub-group	Distilled H ₂ O (ml/kg body weight)	Grapefruit juice (ml/kg body weight)	Metformin (mg/kg body weight)	Glucose (g/kg body weight)	n
A	A ₁	-	0.0	-	3.0	5
	A ₂	-	1.0	-	3.0	5
	A ₃	-	2.0	-	3.0	5
	A ₄	-	3.0	-	3.0 (DEX)	5
B	B ₁	-	3.0	-	3.0 i.p	5
	B ₂	3.0	-	-	3.0 (PCN)	5
	B ₃	-	3.0	-	3.0 (PCN)	5
	B ₄	3.0	-	-	3.0 i.p	5
C	C ₁	3.0	-	-	3.0 (DEX)	5
	C ₂	3.0	-	250	3.0 (PCN)	5
	C ₃	-	3.0	-	3.0	5
	C ₄	-	3.0	250	3.0	5

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).

Ingredients	Loading Buffer	Loading Buffer	Running Buffer	Running Buffer	Transfer Buffer	Transfer Buffer	TBS_T
	Quantity	Conc.	Separating gel (0.375 M Tris pH 8.8)	Stacking gel (4%), (0.125 M pH 6.8)	Quantity	Conc. pH 8.3	Quantity pH 7.4
0.5 M Tris HCl	1.0	62.5 M					
Glycerol	1.6	20%					
10% SDS	1.6	20%	0.5 ml	100 µl			
B-mercaptoethanol	0.4	5%					
0.5% Bromophenol blue (w/v)	0.4	5%					
30% Bis-acryl amide			16.65 ml	1.3 ml			
1.5 M Tris HCl pH 8.8			12.5 ml	-			
0.5 M Tris HCl pH 6.8			-	2.5			
10% ammonium persulphate			250 µl	50 µl			
TEMED			25 µl	50 µl			
Trizma base					60.06 g	25 mM	3.0 g
Glycine					28.8 g	192 mM	
Methanol					400 ml	20% (v/v)	
Tween 20							900 µl
KCl							0.2 g
NaCl							8.0 g
Distilled water	3.0 ml	-	20.1 ml	6.1 ml	1600 ml		900 ml

Table 4.

Buffers used in SDS-PAGE analysis of OCT1 proteins in the liver tissue. The quantities added and effective concentrations are as indicated.

Category	Compound	MS Fragment ions (<i>m/z</i>)
Flavonoids	Unidentified	160.7
	Unidentified	176.7
	Unidentified	176.8
	Unidentified	234.9
	Unidentified	272.8
Furanocoumarins	7-geranyloxy coumarin	298.9
	Unidentified	335.1
	6',7'-dihydroxybermottin	337.1
	Unidentified	339.1
	Unidentified	375.0
	Unidentified	427.1
	Unidentified	471.1

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).

	0 ml GFJ	1.0 ml GFJ	2.0 ml GFJ	3.0 ml GFJ
AUC	491.1±28	442.8±39	416.1±35	375.3±41
GI	-	0.902	0.847	0.764
%	-	90.2	84.7	76.4
p-value	-	<0.05	<0.05	<0.05

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 minutos) indicated is for plasma glucose concentrations.

	Control	Dexamethasone	Dexamethasone +GFJ	GFJ	Metformin +PCN	PCN	Metformin
V_{max} ($\mu\text{mol/g}$ of liver tissue/min)	13.56 \pm 0.72 ^{*Φ}	18.66 \pm 0.476 ^{*Θ}	16.83 \pm 0.36 ^{*Θ}	26.65 \pm 1.11 [*]	13.72 \pm 0.87	11.51 \pm 0.83	20.04 \pm 0.406 ^{Φ}
K_m (μM)	308 [*]	344	269 [*]	144 [*]	351	422	369
r^2	0.5914	0.846	0.9107	0.8535	0.4569	0.3342	0.8835
V_{max}/K_m	44	54.2	62.6	184	39.1	27.3	54.2

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; Φ = metformin or dexamethasone with/without GFJ vs controls;

	Control	Dexamethasone	Dexamethasone +GFJ	GFJ	Metformin +PCN	PCN	Metformin
V_{max} ($\mu\text{mol/g}$ of liver tissue/min)	$13.4 \pm 0.45^{*\Phi\Theta}$	$23.19 \pm 0.58^{\Theta}$	$19.26 \pm 0.41^{*\Theta}$	14.02 ± 0.54	$13.4 \pm 0.80^{\partial}$	$23.84 \pm 1.10^{\Phi\partial}$	13.85 ± 0.348
K_m (mM)	$2.06^{\#}$	$1.705^{\#}$	1.738^{Φ}	2.292^{Φ}	2.26	$1.299^{\#}$	2.326
r^2	0.8412	0.9219	0.9400	0.7800	0.6050	0.8219	0.8805
V_{max}/K_m	6.5	13.6	11.08	6.11	5.9	18.35	5.96

Table 8.

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

	Control	Dexamethasone	Dexamethasone +GFJ	GFJ	Metformin +PCN	PCN	Metformin
V_{\max} ($\mu\text{mol/g}$ of liver tissue/min)	13.73 \pm 0.399*#	26.78 \pm 1.17* ^{∂}	10.72 \pm 0.363 ^{∂}	12.44 \pm 0.413	14.32 \pm 0.749 ^{∂}	27.77 \pm 0.815 ^{#Φ}	12.1 \pm 0.481
K_m (mM)	1.379*#	0.783*	2.784	1.552	1.398	0.7151 [#]	1.615
r^2	0.8141	0.9162	0.782	0.8817	0.7716	0.9506	0.8337
V_{\max}/K_m	9.9	34.2	3.82	8.0	10.2	38.7	7.49

Table 9.

V_{\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; ∂ = dexamethasone vs dexamethasone with GFJ; Φ = PCN vs PCN with metformin

	Control	Dexamethasone	Dexamethasone +GFJ	GFJ	Metformin +PCN	PCN	Metformin
V_{max} (nmol/g of liver tissue/min)	7.44±0.4115 ^Φ	89.94±4.757 ^Φ	84.71±3.949 ^Φ	96.44±2.478 ^Φ	93.74±6.55 ^Φ	89.75±10.08 ^Φ	79.0±3.887 ^Φ
K_m (mM)	0.146*	0.01948*	0.03217*	0.02987*	0.0217*	0.1770	0.04116*
r^2	0.5479 [#]	0.9017 [#]	0.8898 [#]	0.9663 [#]	0.8283 [#]	0.6782	0.8557 [#]
V_{max}/K_m	51	4619	270.7	3228	4319	507	1919

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, Φ = controls vs all treatment groups; * = controls vs all treatment groups except PCN; # = controls vs all treatment groups except PCN.

	Metformin	Metformin	Metformin
	Plasma ($\mu\text{g/ml}$)	Liver ($\mu\text{g/g}$)	Plasma: liver ratio (%)
Controls	95 \pm 8.1	280 \pm 15	33.9 \pm 5.4
PCN	107 \pm 23	402 \pm 58*	26.6 \pm 4.0*
Grapefruit Juice	108 \pm 20	397 \pm 66*	27.2 \pm 3.5*
p-value	>0.05	<0.05	<0.05

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.

REFERENCES

1. Kiani J and Imam SZ. Medicinal importance of grapefruit juice and its interaction with various drugs. *Nutrition Journal*, 2007;6:33
2. Moore GA. Oranges and lemons: clues to the taxonomy of citrus from molecular markers. *Trends in Genetics*, 2001;17(9):536-540
3. Webber HJ. History and development of the citrus industry. In *The Citrus industry*. (1967), (Vol 1), (Reuther *et al.*, eds), pp.1-39, University of California Press; Scora RW. Biochemistry, taxonomy and evolution of modern cultivated Citrus.(1988). In *The Proceedings of the International Society of Citriculture* (Goren R and Mendel K., eds), pp. 227-289, International Society of Citriculture
4. Takana T. Species problem in Citrus. (1954). Japanese Society for the Promotion of Science; Webber HJ. History and development of the citrus industry. In *The Citrus industry*. (1967), (Vol 1), (Reuther *et al.*, eds), pp.1-39, University of California Press; Scora RW. Biochemistry, taxonomy and evolution of modern cultivated Citrus.(1988). In *The Proceedings of the International Society of Citriculture* (Goren R and Mendel K., eds), pp. 227-289, International Society of Citriculture
5. Hodgson RW. Horticultural varieties of Citrus. In *the Citrus Industry*; Rether W, Batchelor LD, Webber HJ, Eds; *University of California Press: Berkeley, CA, 1967;Vol 1, pp431-531*

6. E. Nicolosi E, Deng ZN, Gentile A, Malfa SJ, Continella G, Tribulato E. Citrus phylogeny and genetic origin of important species as investigated by molecular markers. *TAG Theoretical and Applied Genetics*, 2000;100:1155-1166
7. Gmitter FG, Jr. Origin, evolution and breeding of the grapefruit. *Plant Breeding Reviews*, 1995;13:345-363
8. Jayapaskasha GK, Girenavar B, Patil BS. Radical scavenging activities of Rio Red grapefruits and sour orange fruit extracts in different in vitro model systems. *Bioresource Technology*, 2008;99:4484-4494
9. So SV, Guthrie N, Chambers AF, Moussa M, Carol KK. Inhibition of human cancer cell proliferation and delay of mammary cell tumorigenesis by flavonoids and citrus juices. *Nutrition and Cancer*, 1996; 26:167-18
10. Guthrie N, Carol KK. Inhibition of mammary cancer by citrus flavonoids. *Advanced Experimental Medical Biology*, 1998; 439:227-236
11. Kim EH, Hakinnson SE, Elliasen AH and Willet WC. A prospective study of grapefruit and grapefruit juice intake and breast cancer risk. *British Journal of Cancer*, 2008;98:240-241
12. Benavente-Garcia O and Castillo J. Updates on uses and properties of Citrus flavonoids: new findings in anticancer, cardiovascular and anti-inflammatory activity. *Journal of Agricultural Food Chemistry*, 2008;56:6185-6205
13. Breiholt VM, Nielsen SE, Knuthsen P, Lauridsen ST, Daneshvar B, Sorensen A. Effects of commonly consumed fruit juices and carbohydrates on redox status and anticancer biomarkers in female rats. *Nutrition and Cancer*, 2003;45(1):46-52
14. Gorinstein S, Leontowicz H, Leontowicz M, Drzewiecki J, Jastrzebski Z, Tapia MS, Katrich E, Trakhtenberg S. Red Star Ruby (Sunrise) and blood quantities of

- Jaffa grapefruits and their influence on plasma lipid levels and plasma antioxidant activity in rats fed with cholesterol-containing and cholesterol-free diets. *Life Science*, 2005;77:2384-2397
15. Gorinstein S, Caspi A, Libman I, Lerner HT, Huang D, Leontowicz H, Leontowicz M, Tashma Z, Katrich E, Feng S, Trakhtenberg S. Red grapefruit positively influences serum triglycerides level in patients suffering from coronary atherosclerosis: studies in vitro and in humans. *J. Agric. Food Chem*, 2006;54:1887-1892
 16. Deyhim F, Garica K, Lopez E, Gonzalez J, Ino S, Garcia M, Patil BS. Citrus juice modulates bone strength in male senescent rat model of osteoporosis. *Nutrition*, 2006;22:559-563
 17. Fujioka K, Greenway F, Sheard J, Ying Y. The effects of grapefruit juice on weight and insulin resistance: relationship to metabolic syndrome. *Journal of Medicinal Food*, 2006;9(1)49-54
 18. Florida Department of citrus: *History of dieting*, Florida, Department of Citrus, Lakeland FL, 2003.
 19. De Castro WV, Mertens-Talcott S, Rubner A, Butterweck V, Derendorf H. Variation of flavonoids and furanocoumarins in grapefruit juices: a potential source of variability in grapefruit juice-drug interaction studies. *Journal of Agricultural Food Chemistry*, 2006;54:249-255
 20. Girenavar B, Jayaprakasha GK, Patil BS. Influence of pre- and post-harvest factors and processing on the levels of furocoumarins in grapefruits. *Food Chemistry*, 2008;111:387-392
 21. Gorski C, Huang SM, Pinto A, Hamman MA, Janna K, Hilligoss JK, Narjis A, Zaheer NA, Desai M, Miller M, Hall SD. The effect Echinacea (Echinacea

- purpurea root) on cytochrome P450 activity in vivo. *Clinical Pharmacology and Therapeutics*, 2004;75(1):89-100
22. Yu J, Wang L, Walzem RL, Miller EG, Pike LM, Patil BS. Antioxidant activity of citrus limonoids, flavonoids and coumarins. *Journal of Agricultural and Food Chemistry*, 2005;53(6):2009-2014
 23. Dwivedi C, Heck WJ, Downie AA, Larroya S, Webb TE. Effect of calcium glucarate on beta-glucuronidase activity and glucarate content of certain vegetables and fruits. *Biochem Med Metab Biol*, 1990;43(2):83-92
 24. Ribeiro IA and Ribeiro MHL. Naringin and naringenin determination and control in grapefruit juice by a validated HPLC method. *Food Control*, 2008;19:432-438
 25. Chen Y, Shen S and Lin H. Rutin at C7 attenuates the apoptosis-inducing activity of flavonoids. *Biochemical Pharmacology*, 2003;66:1139-1150
 26. Jung, Lee MK, Park YB, Kang MA, Choi MS. Effects of citrus flavonoids on lipid metabolism and glucose-regulating enzymes mRNA levels in type-2 diabetic mice. *International Journal of Biochemistry & Cell Biology*, 2006;38:1134-1145
 27. Jung UJ, Lee MK, Jeong KS, Choi MS. Hypoglycemic effects of hesperidin and naringin are partly mediated by hepatic glucose-regulating enzymes in C57BL/KsJ-db/db mice. *J. Nutr*, 2004;134:2499-2503
 28. Li JM, Chun Tao Che CT, CBS, Leung PS, Cheng CHK. Inhibition of intestinal and renal Na⁺-glucose cotransporter by naringenin. *International Journal of Biochemistry & Cell Biology*, 2006;38:985-995

29. Purushotham A, Tian M, Belury MA. The citrus fruit flavonoid naringenin suppresses hepatic glucose production from Fao hepatoma cells. *Mol Nutr Food Res*, 2008;53(2):300-307
30. Tanaka T, Maeda M, Kohno H, Murakami M, Kagami S, Miyake M, Wada K. Inhibition of azoxymethane-induced colon carcinogenesis in male F344 rats by citrus limonoids abacunone and limonin. *Carcinogenesis*, 2001;22:193-198
31. Tanaka T, Makita H, Kawabata K, Mori H, Kakumoto M, Satoh K, Hara A, Sumida T, Tanaka T, Ogawa H. Chemoprevention of azoxymethane-induced rat colon carcinogenesis by the naturally occurring flavonoids, diosmin and hesperidin. *Carcinogenesis*, 1997;18:957-965
32. Vanamala J, Leonardi T, Patil BS, Taddeo SS, Murphy ME, Pike LM, Chapkin RS, Lupton JR, Turner ND. Suppression of colon carcinogenesis by bioactive compounds in grapefruit. *Carcinogenesis*, 2006;27(6):1257-1265
33. Perez JL, Jayaprakasha GK, Yoo KS, Patil BS. Development of a method for the quantification of D-glucaric acid in different varieties of grapefruits by high performance liquid chromatography and mass spectra. *Journal of Chromatography A*, 2008;1190:394-397
34. Walaszek Z, Szemraj J, Narog M, Adams AK, Kilgore J, Sherman U, Hanausek M. Metabolism, uptake, and excretion of a D-glucaric acid salt and its potential use in cancer prevention. *Cancer Detect Prev*, 1997;21:178-190
35. Bailey DG, Spence JD, Edgar B, Bayliff CD, Arnold JM. Ethanol enhances the hemodynamic effects of felodipine. *Clin Invest Med*, 1989 Dec;12(6):357-62
36. Saito, Mitsuo S, Mutsuko HK, Mariko M, Tsutomu U, Ryuichi H. Undesirable effects of citrus juice on pharmacokinetics of drugs: focus on recent studies. *Drug safety*, 2005;28:677-694

37. Lundahl J, Regårdh CG, Edgar B, Johnsson G. Effects of grapefruit juice ingestion-pharmacokinetics and haemodynamics of intravenously administered felodipine in healthy men. *Eur J Clin Pharmacol*, 1997;52(2):139-145
38. Takanaga H, Ayako Ohnishi A, Matsuo H, Murakami H, Sata H, Kuroda K, A Urae A, Higuchi S, Sawada Y. Pharmacokinetic analysis of felodipine-grapefruit interaction based on an irreversible enzyme inhibition model. *Brit J Clin Pharmacol*, 2000;49:49-58
39. Dresser GK, Bailey DG, Carruthers SG. Grapefruit juice-felodipine interaction in the elderly. *Clin Pharmacol Ther*, 2000;68:28-34
40. Christensen H, Asberg A, Holmboe AB, berg KJ. Co-administration of grapefruit juice increases systemic exposure of diltiazem in healthy volunteers. *Eur J Clin Pharmacol*, 2002;58:515-520
41. Zaidenstein R, Dishi V, Gips M, Soback S, Cohen N, Weissgarten J, Blatt A, Golik A. The effect of grapefruit juice on pharmacokinetics of orally administered verapamil. *Eur J Clin Pharm*, 1998;45:337-340
42. Bailey DG, Arnold JMO, Strong HA, Munoz C , Spence JD. Effect of grapefruit juice and naringin on nisoldipine pharmacokinetics. *Clin Pharm Ther*, 1993;54:589-594
43. Fuhr U, Maier-Brüggemann A, Blume H, Mück W, Unger S, Kuhlmann J, Huschka C, Zaigler M, Rietbrock S, Staib AH. Fuhr *et al.* Grapefruit juice increases oral nimlodypine bioavailability. *Int J Clin Pharmacol Ther*, 1998;36:126-132

44. Rashid TJ, Martin U, Clarke H, Waller DG, Renwick AG, George CF. Factors that affecting the absolute bioavailability of nifedipine. *Brit J Clin Pharmacol*, 1995;40:51-58
45. Josefsson M, Zackrisson AL, Ahlner J. Effect of grapefruit juice on the pharmacokinetics of amlodipine in healthy volunteers. *Eur J Clin Pharmacol*, 1996;51:189-193
46. Vincent J, Harris SI, Foulds G, Dogolo LC, Willavize S, Friedman HL. Lack of effect of grapefruit juice on the pharmacokinetics and hemodynamics of amlodipine. *Br J Clin Pharmacol*, 2000;50:455-463
47. Ohnishi A, Ohtani H, Sawada Y. Major determinant factors of the extent of interaction between grapefruit juice and calcium channel antagonists. *Brit J Clin Pharmacol*, 2006;62(2):196-199
48. Zaidenstein R, Soback S, Gips M, Avni B, Dishi V, Weissgarten Y, Golik A, Scapa E. Effect of grapefruit juice on the pharmacokinetics of losartan and its active metabolite E3174 in healthy volunteers. *Ther Drug Monit*, 2001;23(4):369-373.
49. Bailey DG, Dresser GK. Interaction between grapefruit juice and cardiovascular drugs. *Am J Cardiovasc Drugs*, 2004;4:281-297
50. Libersa CC, Brique SA, Motte KB, Caron JF, Guédon-Moreau LM, Luc Humbert L, Vincent A, Devos P, Lhermitte MA. Dramatic amiodarone metabolism induced by grapefruit juice. *Br J Clin Pharmacol*, 2000;49:373-37
51. Le Goff-Klein N, Koffel JC, Jung L, Ubeaud G. *In vitro* inhibition of simvastatin metabolism, a HMG-CoA reductase inhibitor in human and rat liver by bergamottin, a component of grapefruit juice. *Eur J Pharm Sci*, 2003;18:31-35

52. Lilja JJ, Kivisto KT, Neuvonen PJ. Grapefruit juice-simvastatin interaction: effect on serum concentrations of simvastatin, simvastatin acid, and HMG-Co A reductase inhibitors. *Clin Pharmacol Ther*, 1998;64:477-483
53. Lilja JJ, Kivisto KT, Neuvonen PJ. Duration of effects of grapefruit juice on the pharmacokinetics of the CYP3A4 substrate, simvastatin. *Clin Pharmacol Ther*, 2000;68:384-90
54. Lilja JJ, Kivisto KT, Neuvonen PJ. Grapefruit juice increases serum concentrations of atorvastatin and has no effect on pravastatin. *Clin Pharmacol Ther*, 1999;66(2):118-27
55. Kupferschmidt HH, Fattinger KE, Ha HR, Krahenbuhl S. Grapefruit juice enhances the bioavailability of the HIV protease inhibitor saquinavir in man. *Br J Clin Pharmacol*, 1998;45:355-359
56. Fitzsimmons MR, Collins JM. Selective biotransformation of the human immunodeficiency virus protease inhibitors saquinavir by human small-intestinal cytochrome P450: potential contribution of to high first pass metabolism. *Drug Metab Dispos*, 1997;25:256-266
57. Shelton MJ, Wynn HE, Hewitt RG, DiFrancisco R. Effects of grapefruit juice on pharmacokinetic exposure to indinavir in HIV positive subjects. *J Clin Pharmacol*, 2001;41:435-42
58. Penzak SR, Acosta EP, Turner M, Edwards MD, Hon YY, Desai HD, Jann MW. Effects of Seville orange juice and grapefruit juice on indinavir pharmacokinetics. *J Clin Pharmacol*, 2002;42:1165-70

59. Demarles D, Gillotin C, Bonaventure-Paci S, Vincent I, Fosse S, Taburet AM. Single dose pharmacokinetics of amprenavir co-administered with grapefruit juice. *Antimicrob Agents Chemother*, 2002;46:1589-90
60. Van Agtmael MA, Gupta V, van der Graaf CA, van Boxtel CJ. The effect of grapefruit juice on the time-dependent decline of artemether plasma levels in healthy subjects. *Clin Pharmacol Ther*, 1999;66:408-414
61. Van Agtmael MA, Gupta V, van der Wosten TH, Rutten JP, van Boxtel CJ. Grapefruit juice increases the bioavailability of artemether. *Eur J Clin Pharmacol*, 1999;55:405-410
62. Castro N, Jung H, Medina R, González-Esquivel D, Lopez M, Sotelo J. Interaction between grapefruit juice and praziquantel in humans. *Antimicrob Agents Chemother*, 2002;46:1614-1616
63. Ho PC, Chalcroft SC, Coville PF, Wanwimolruk S. Grapefruit juice has no effect on quinine pharmacokinetics. *Eur J Clin Pharmacol*, 1999;55:393-398
64. Damkier P, Hansten LL, Brosen K. Effect of diclofenac, disulfiram, itraconazole, grapefruit juice and erythromycin on pharmacokinetics of quinidine. *Br J Clin Pharmacol*, 1999;48:829-838
65. Cheng KL, Nafziger AN, Peloquin CA, Amsden GW. Effect of grapefruit juice on clarithromycin pharmacokinetics. *Antimicrob Agents Chemother*, 1998;42:927-929
66. Ozdemir M, Aktan Y, Boydag BS, Cingi MI, Musul A. Interaction between grapefruit juice and diazepam. *Eur J Drug Metab Pharmacokin*, 1998;23:55-59

67. Hukkinen SK, Vahre A, Olkkola KT, Neuvonen PJ. Plasma concentrations of triazolam are increased by concomitant ingestion of grapefruit juice. *Clin Pharm Ther*, 1995;58:127-131
68. Kupferschmidt HHT, Ha HR, Ziegler WH, Meier PJ, Krähenbühl S. Interaction between grapefruit juice and midazolam in humans. *Clin Pharm Ther*, 1995;58:20-28
69. Lilja JJ, Kivisto KT, Backman JT, Neuvonen J. Effect of grapefruit dose on grapefruit-juice-triazolam interaction: repeated consumption prolongs triazolam half-life. *Eur J Clin Pharmacol*, 2000;56:411-415
70. Yasui N, Kondo T, Furukori H, Kaneko S, Ohkubo T, Uno T, Osanai T, Sugawara K, Otani K. Effects of repeated ingestion of grapefruit juice on the single and multiple oral-dose pharmacokinetics and pharmacodynamics of aprazolam. *Psychopharmacology (Ber)*, 2000;150:185-190
71. Garg SK, Kumar N, Bhargava BK, Prabhaka SK. Effect of grapefruit juice on carbamazepine bioavailability in patients with epilepsy. *Clin Pharmacol Ther*, 1998;64:286-288
72. Kumar N, Garg SK, Prabhakar S. Lack of pharmacokinetic interaction between grapefruit juice and phenytoin in healthy male volunteers and epileptic patients. *Methods Find Exp Clin Pharmacol*, 1999;21:629-633
73. Lilja JJ, Kivistö KT, Backman JT, Lamberg TS, Neuvonen PJ. Grapefruit juice substantially increases plasma concentration of buspirone. *Clin Pharmacol Ther*, 1998;64:655-660

74. Lee AJ, Chan WK, Harralson AF, Buffum J, Bui BCC. The effects of grapefruit juice on sertraline metabolism: an in vitro and in vivo study. *Clin Ther*, 1999;21(11):1890-1899
75. Lane HY, Jann MW, Chang YC, Chiu CC, Huang MC, Lee SH, Chang WH. Lane HY, Jann MW, Chang YC, Chiu CC, Huang MC, Lee SH, Chang WH. Repeated ingestion of grapefruit juice does not alter clozapine's steady-state plasma levels, effectiveness and tolerability. *J Clin Psychiatry*, 2001;62:812-817
76. Yasui N, Kondo T, Suzuki A, Otani K, Mihara M. Lack of significant pharmacokinetic interaction between haloperidol and grapefruit juice. *Int Clin Psychopharmacol*, 1999;14:113-118
77. Kvisto KT, Lilja JJ, Backman JT, Neuvonen PJ. Repeated consumption of grapefruit juice considerably increases plasma concentration of cisapride. *Clin Pharmacol Ther*, 1999;66:448-453
78. Soons PA, Vogels BAPM, Roosemalen MCM, Schemaker HC, Uchida E, Edgar B, Lundahl J, Cohen AF, Breimer DD. Grapefruit juice and cimetidine inhibit stereoselective metabolism of nitrendipine in humans. *Clin Pharmacol Ther*, 1991;50:394-403
79. Rau SE, Bend JR, Arnold JMO, Tran LT, Spence JD, Bailey DG. Grapefruit juice-terfenadine single-dose interaction: magnitude, mechanism and relevance. *Clin Pharmacol Ther*, 1997;61:401-409.
80. Hyland R, Roe EG, Jones BC, Smith DA. Identification of the cytochrome P450 enzymes involved in the N-demethylation of sildenafil. *Br J Clin Pharmacol*, 2001;51:239-248

81. Gross AS, Goh YD, Addison RS, Shenfield GM. Influence of grapefruit juice on cisapride pharmacokinetics. *Clin Pharmacol Ther*, 1999;65:395-401
82. Offman EM, Freeman DJ, Dresser GK, Munoz C, Bend JR, Bailey DG. Red wine-cisapride interaction: comparison with grapefruit juice. *Clin Pharmacol Ther*, 2000;70:17-23
83. Duchame MP, Warbasse LH, Edwards DJ. Disposition of intravenous and oral cyclosporine after administration with grapefruit juice. *Clin Pharm Ther*, 1995;57:485-491
84. Ionannides-Demos LL, Christophis N, Ryan P, Angelis P, Liolios L, Maclean AJ. Dosing implications of a clinical interaction between grapefruit juice and cyclosporine and metabolite concentrations in patients with autoimmune diseases. *J Rheumatol*, 1997;24:49-54
85. Brunner LJ, Pai KS, Munar MY, Lande MB, Olyaei AJ, Mowry JA. Effect of grapefruit juice on cyclosporine A pharmacokinetics in paediatric renal transplant patients. *Paediatr Transplant*, 2001;4:313-321
86. Yee GC, Stanley DL, Pessa LJ, Dalla Costa T, Beltz SE, Ruiz J, Lowenthal DT. Effect of grapefruit juice on blood cyclosporine concentrations. *Lancet*, 1995;345:955-956
87. Ku YM, Min DI, Flanigan M. Effect of grapefruit juice on the pharmacokinetics of microemulsion cyclosporine and its metabolite in healthy volunteers: does the formulation difference matter? *J Clin Pharmacol*, 1998;38:959-965
88. Hermann M, Asberg A, Reubsæet JL, Sather S, Berg KJ, Christensen H. Intake of grapefruit juice alters the metabolic pattern of cyclosporine A in renal transplant recipients. *Int J Clin Pharmacol Ther*, 2002;40(10):451-456

89. Weber A, Jäger R, Börner A, Klinger G, Vollanath R, Matthey K, Balogh A. Can grapefruit juice influence ethinylestradiol bioavailability? *Contraception*, 1996;53(1):41-47
90. Dasgupta A, Reyes MA, Risin SA, Actor JK. Interaction of white and pink grapefruit juice with acetaminophen (paracetamol) in vivo in mice. *Journal of Medicinal Food*, 2008;4(11):795-798
91. Dahan A, Altman H. Food-drug interaction: grapefruit juice augments drug bioavailability-mechanism, extent and relevance. *Eu J Clin Nutr*, 2004;58:1-9
92. Kane GC, Lipsky JJ. Drug-grapefruit juice interactions. *Mayo Clin Proc*, 2000;75:933-942
93. Rogers JD, Zhao J, Liu L, Amin RD, Gagliano KD, Porras AG, Blum RA, Wilson MF, Stepanavage M, Vega JM. Grapefruit juice has minimal effects on plasma concentration lovastatin-derived 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors. *Clin Pharm Ther*, 1999;66:358-366
94. Lundahl J, Regardh CG, Edgar B, Johnson G. Relationship between time of intake and of grapefruit juice and its effect on pharmacokinetics and pharmacodynamics of felodipine in healthy subjects. *Eu J Clin Pharmacol*, 1995;49:61-67
95. Takanga, Ohnishi A, Murakami H *et al.* Relationship between time after intake of grapefruit juice and the effect of pharmacokinetics and pharmacodynamics of nisoldipine in healthy subjects. *Clin Pharm Ther*, 2000;67:201-214
96. Dixit SG, Tirona RG, Kim RB. Beyond CAR and PXR. *Current Drug Metab*, 2005;6:385-397

97. Mangelsdorf DJ and Evans RM. The RXR heterodimers and orphan receptors. *Cell*, 1995;83(6):841-50
98. Shulman AI, Larson C, Mangelsdorf DJ. Structural determinants of allosteric ligand activation in RXR heterodimers. *Cell*, 2004;116(3):417-29
99. Mangelsdorf DJ, Borgmeyer U, Heyman RA, Zhou JY, Ong ES, Oro AE, Kakizuka A, Evans RM. Characterization of three RXR genes that mediate the action of 9-cis retinoic acid. *Genes Development*, 1992;6(3):329-44
100. Kliewer SA, Forman BM, Blumberg B, Ong ES, Mangelsdorf DJ, Borgmeyer U, Umesono U, Evans RM. Differential expression and activation of a family of murine peroxisome proliferator-activated receptors. *Proc Natl Acad Sci USA*, 1994; 91(15):7355-9
101. Makishima M, Okamoto AY, Repa JJ, Tu H, Learned RM, Luk A, Hull MV, Lustig KD, Mangelsdorf DJ, Shan B. Identification of a nuclear receptor for bile acids. *Science*, 1999;284(5418):1362-5
102. Willy P J , Umesono K, Ong ES, Evans RM, Heyman RA, Mangelsdorf DJ. LXR, a nuclear receptor that defines a distinct retinoid response pathway. *Genes Dev*, 1995; 9(9):1033-45
103. Forman BM, Tzameli I, Choi HS, Chen J, Simha D, Seol W, Evans RM, Moore DD. Androstane metabolites bind to and deactivate the nuclear receptor CAR-beta. *Nature*, 1998;395(6702):612-5
104. Lehmann JM, McKee DD, Watson MA, Willson TM, Moore JT, Kliewer SA. The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. *J Clin Invest*, 1998;102:1016-1023

105. Moore JT, Kliewer SA. Use of the nuclear receptor PXR to predict drug interactions. *Toxicology*, 2000;153(1-3):1-10
106. Moore LB, Derek J. Parks DJ, Jones SA, Bledsoe RK, Consler TG, Stimmel JB, Goodwin B, Liddle C, Blanchard SG, Willson TM, Collins JL, Kliewer SA. Orphan nuclear receptors constitutive androstane receptor and pregnane X receptor share xenobiotic and steroid ligands. *J Biol Chem*, 2000;275(20):15122-7
107. Mandlekar S, Jin-Liern H, Kong AT. Modulation of metabolic enzymes by dietary phytochemicals : a review of mechanisms underlying beneficial versus unfavourable effects. *Current Drug Metabolism*, 2006;7:671-675
108. Kretschmer XC, Baldwin WS. CAR and PXR: xenosensors of endocrine disrupters? *Chem Biol Interact*, 2005;155(3):111-28
109. Stanley LA, Horsburgh BC, Ross J, Scheer N, Wolf CR. PXR and CAR: nuclear receptors which play a pivotal role in drug disposition and chemical toxicity. *Drug Metabolism Reviews*, 2006;38:515-597
110. Carnahan VE, Redinbo MR. Structure and function of the human nuclear xenobiotic receptor PXR. *Current Drug Metabolism*, 2005;6(4):357-367
111. Wilkinson GW. Pharmacokinetics: the dynamics of drug absorption, distribution and elimination. In: Hardman JG, Limbird LE, eds. Goodman and Gilman's: *The pharmacological basis of Therapeutics*, 10th ed. New York: MacGraw Hill, 2001:3-29
112. Bressier R, Bahl JJ. Principles of drug therapy for the elderly patient. *Mayo Clin Proc*, 2003;78(12):1564-77

113. Murray M. Altered CYP expression and function in response to dietary factors: potential roles in disease pathogenesis. *Current Drug Metabolism*, 2006;7:67-81
114. Tomilson B, Chow MSS. Stereoselective interaction of manidipine and grapefruit juice: a new twist on an old tale. *Bri J Clin Pharmacol*, 2006;61(5):529-532
115. Dresser GK, Spencer JD, Bailey DG. Pharmacokinetic-pharmacodynamic consequences and clinical relevance of cytochrome P450 inhibition. *Clin Pharmacokinet*, 2000;38(1):41-57
116. Blesser R. Grapefruit juice and prescription drug interactions: exploring the mechanism of this interaction and potential toxicity for certain drugs. *Geriatrics*, 2006;61(11):12-18
117. Greenblatt DJ, von Moltke LL, Harmatz JS, Chen G, Weemhoff JL, Jen C, Kelley CJ, LeDuc BW, Zinny MA. Time course of recovery of cytochrome P450 3A function after single dose of grapefruit juice. *Clin Pharmacol Ther*, 2003;74(2):121-129
118. Kane GC, Lipsky JJ. Drug-grapefruit juice interactions. *Mayo Clin Proc*, 2000;75:933-942
119. Schmiedlin-Ren P, Edwards DJ, Fitzsimmons ME, He K, Lown KS, Woster PM, Rahman A, Thummel KE, Fisher JM, Hollenberg PF, Watkins PB. Mechanism of enhanced oral bioavailability of CYP3A4 substrates by grapefruit juice constituents: decreased enterocyte CYP3A4 concentration and mechanism based activation by furanocoumarins. *Drug Metab Dispos*, 1997;25:1228-1233

120. Lown KS, Bailey DG, Fontana RJ, Janardan SK, Adair CH, Fortlage LA, Brown MB, Guo W, Watkins PB. Grapefruit increases felodipine oral bioavailability in humans by decreasing intestinal CYP3A protein expression. *J Clin Invest*, 1997;99:2545-53
121. Greenblatt DJ, von Moltke LL, Harmatz JS, Chen G, Weemhoff JL, Jen C, Kelley CJ, LeDuc BW, Zinny MA. Time course of recovery of cytochrome P450 3A function after single dose of grapefruit juice. *Clin Pharmacol Ther*, 2003;74(2):121-129
122. Silverman RB. Mechanism-based enzyme inactivation; chemistry and enzymology, Vol. 2. Boca Raton (FL): CRC Press, 1988:224; Silverman RB. *Mechanism-based enzyme inactivation; chemistry and enzymology, Vol. 2. Boca Raton (FL): CRC Press, 1988:228*
123. Chan WK, Nguyen LT, Miller VP, Harris RZ. Mechanism-based inactivation of human cytochrome P450 3A4 by grapefruit juice and red wine. *Life Sci*, 1998;62(10):PL135-42
124. Bailey DG, Dresser GK, Bend JR. Bergamottin, lime juice and red wine as inhibitors of cytochrome P450 activity: comparison with grapefruit juice. *Clin Pharmacol Ther*, 2003;73:529-37
125. Bailey DG, Malcolm J, Arnold O, Spence JD. Grapefruit juice-drug interactions. *Br J Clin Pharmacol*, 1998;46:101-110
126. Fuhr U. Drug interactions with grapefruit juice: extent, probable mechanism and clinical relevance. *Drug Saf*, 1998;18:251-72

127. Bailey DG, Dresser GK, Kreeft JH, Munoz C, Freeman DJ, Bend JR. Grapefruit juice-felodipine interaction: effect of unprocessed fruit and probable active ingredients. *Clin Pharmacol Ther*, 2000;28:482-486
128. Mohri K, Uesawa Y, Sagawa K. Long-term grapefruit juice ingestion on nifedipine pharmacokinetics: induction of rat hepatic P-450 by grapefruit juice. *Drug Metab Dispos*, 2000;56:411-415
129. Lin HL, Kent UM, Hollenberg PF. The grapefruit juice effect is not limited to cytochrome P450 (P450) 3A4: evidence for bergamottin-dependent inactivation, heme destruction, and covalent binding to protein in P450s 2B6 and 3A5. *J Pharmacol Exp Ther*, 2005;313:154-164
130. Fujita T, Kawase A, Niwa T, Tomohiro N, Masuda M. Comparative evaluation of 12 immature citrus fruit extracts for the inhibition of cytochrome P450 isoform activities. *Biol Pharm Bull*, 2008;31(5):925-930
131. Paine MF, Hart HL, Ludington SS, Haining RL, Rettie AE, Zeldin DC. The human intestinal cytochrome P450 "pie". *Drug Metab Dispos*, 2006;34:880-886
132. Miniscalco A, Lundahl J, Regardh CG, Edgar B, Eriksson UG. Inhibition of dihydropyridine metabolism in rat and human liver microsomes by flavonoids found in grapefruit juice. *Pharmacol Exp Ther*, 1992;261(3):1195-1199
133. Tassaneeyakul W, Guo LQ, Fukuda K, Ohta T, Yamazoe Y. Inhibition selectivity of grapefruit juice components on human cytochromes P450. *Arch Biochem Biophys*, 2000;387(2):356-363

134. Mays DC, Hilliard JB, Wong DD, Chambers MA, Park SS, Gelboin HV, Gerber N. Bioactivation of 8-methoxypsoralen and irreversible inactivation of cytochrome P-450 in mouse liver microsomes: modification by monoclonal antibodies, inhibition of drug metabolism and distribution of covalent adducts. *J Pharmacol Exp Ther*, 1990;254:720-731
135. Murray GI, Taylor MC, McFadyen MCE, McKay JA, Greenlee WF, Burke MD, Melvin WT. Tumor-specific expression of cytochrome P450 CYP1B1. *Cancer Res*, 1997;57:3026-3031
136. Kliewer SA. The nuclear pregnane X receptor regulates xenobiotic detoxification. *J Nutr*, 2003;133(7 suppl):2444S-2447S
137. Suzuki H, Sugiyama Y. Role of metabolic enzymes and efflux transporters in the absorption of drugs from the small intestine. *Eur J Pharm Sci*, 2000;12:3-12
138. Oostendorp RL, Beijnen JH, Schellens JH. The biological and clinical role of drug transporters at the intestinal barrier. *Cancer Treat Rev*. 2009 Apr;35(2):137-47
139. Ofer M, Wolfram S, Koggel A, Spahn-Langguth H, Langguth P. Modulation of drug transport by selected flavonoids: involvement of P-gp and OCT? *Eur J Pharm Sci*, 2005;25:263-271
140. Hagenbuch B, Meier PJ. The superfamily of organic anion transporting polypeptides. *Biochim Biophys Acta*, 2003;1609(1):1-18
141. Tamai I, Nezu J, Uchino H, Sai Y, Oku A, Shimane M, Tsuji A. Molecular identification and characterization of novel members of the human organic anion transporter (OATP) family. *Biochem Biophys Res Comm*, 2000;273(1):251-260

142. Fabe KN, Müller M, Jansen PLM. Drug transport proteins in the liver. *Advanced Drug Delivery Reviews*, 2003;55:107-124
143. Adibi SA. The oligopeptide transporter (Pept-1) in human intestine: biology and function. *Gastroenterology*, 1997;113:332-40
144. Hirom PC, Millburn P, Smith RL. Bile and urine as complementary pathways for the excretion of foreign organic compounds. *Xenobiotica*, 1976;6(1):55-64
145. Xiong H, Turner KC, Ward ES, Jansen PLM, Brouwer KLRM. Altered hepatobiliary disposition of acetaminophen glucuronide in isolated perfused livers from multidrug resistance-associated protein 2-deficient TR(-) rats. *J Pharmacol Exp Ther*, 2000;295(2):512-518
146. Soroka CJ, Lee JM, Azzaroli F, Boyer JL. Cellular localization and up-regulation of multidrug resistance-associated protein 3 in hepatocytes and cholangiocytes during obstructive cholestasis in rat liver. *Hepatology*, 2001;33(4):783-791
147. Donner MG, Keppler D. Up-regulation of basolateral multidrug resistance protein 3 (Mrp3) in cholestatic rat liver. *Hepatology*, 2001;34(2):351-359
148. Chen HL, Liu YJ, Chen HL, Wu SH, Ni YH, Ho MC, Lai HS, Hsu WM, Hsu HY, Tseng HC, Jeng YM, Chang MH. Expression of hepatocyte transporter and nuclear receptors in children with early and late-stage biliary atresia. *Pediatric Research*, 2008;63(6):667-673
149. Sharom FJ. Shedding light on drug transport: structure and function of the P-glycoprotein multidrug transporter (ABC1). *Biochem Cell Biol*, 2006;84:979-992

150. Becker JP, Depret G, Van Bambeke F, Tulkens PM, Prévost M. Molecular models of human P-glycoprotein in two different catalytic states. *BMC Struct Biol.* 2009 Jan 22;9:3 (ahead of print)
151. Gottesman MM and Ling V. The molecular basis of multidrug resistance in cancer: the early years of P-glycoprotein research. *FEBS lett*, 2006;580:998-1009
152. Ambudkar SV, Dey S, Hrycyna CA, Ramachandra M, Pastan I, Gottesman MM. Biochemical, cellular and pharmacological aspects of the multi-drug transporter. *Ann Rev Pharmacol Toxicol*, 1999,39(5-6):361-398
153. Loo TW, Clarke DM. Recent progress in understanding the mechanism of P-glycoprotein-mediated drug efflux. *J Membr Biol*, 2005;206:173-185
154. Couture L, Nash JA, Turgeon J. The ATP-binding cassette transporters and their implication in drug disposition: a special look at the heart. *Pharmacol Rev*, 2006;58:244-258
155. Hyde SC, Emsley P, Hartshorn MJ, Mimmack MM, Gileadi U, Pearce SR, Gallacher MP, Gill DR, Hubbard RE, Higgins CF. Structural model of ATP binding proteins associated with cystic fibrosis, multidrug resistance, and bacterial transport. *Nature (London)*, 1990;346:362-365
156. Takada Y, Yamada K, Taguchi Y, Kino K, Matsuo M, Tucker SJ, Komano T, Amachi T, Ueda K. Non-equivalent cooperation between the two nucleotide binding folds of P-glycoprotein. *Biochim Biophys Acta*, 1998;1373:131-136
157. Urbatsch IL, Beaudet L, Carrier I, Gros P. Mutations in either nucleotide-binding site of P-glycoprotein (Mdr3) prevent vanadate trapping of nucleotide at both sites. *Biochemistry*, 1998;37:4592-4602

158. Higgins CF, Linton KJ. The ATP switch model for ABC transporters in clinical practice. *Nat Struct Mol Biol*, 2004;11(10):918-926
159. Sauna ZE, Ambudkar SV. Evidence for a requirement for ATP hydrolysis at two distinct steps during a single turnover of the catalytic cycle of human P-glycoprotein. *Proc Natl Acad Sci, USA*, 2000;97(6):2515-2520
160. Senior A, Al-Shawi MK, Urbatsch IL. The catalytic cycle of P-glycoprotein. *FEBS Lett*, 1995;377(3):285-289
161. Higgins CF, Gottesman MM. Is the multidrug transporter a flippase? *Trends Biochem Sci*, 1992;17:18-21
162. Sharom FJ. The P-glycoprotein efflux pump: how does it transport drugs? *J Membr Biol*, 1997;160:161-175
163. Shapiro AB, Ling V. Extraction of Hoechst 33342 from the cytoplasmic leaflet of the plasma membrane by P-glycoprotein. *Eur J Biochem*, 1997;250:130-137
164. Lugo MR, Sharom FJ. Interaction of LDS-751 with P-glycoprotein and mapping of the location of the R drug binding site. *Biochemistry*, 2005;44:643-655
165. Fromm MF. Importance of P-glycoprotein for drug disposition in humans. *Eur J Clin Invest*, 2003;33:6-9
166. Silverman JA. Multidrug resistance transporters. *Pharm Biotechnol*, 1999;12:353-386.
167. Lin JH, Yamazaki M. Role of P-glycoprotein in pharmacokinetics: clinical implications. *Clin Pharmacokinetic*, 2003;42:59-58

168. Lown KS, Mayo RR, Leichtman AB, Hsiao H, Turgeon DM, Schmiedlin-Ren P, Brown MB, Guo W, Rossi SJ, Benet LZ, Watkins PB. Role of intestinal P-glycoprotein (mdr1) in interpatient variation in oral bioavailability of cyclosporine. *Clin Pharmacol Ther*, 1997;62:248-260
169. Alex Sparreboom A, van Asperen J, Mayer U, Schinkel AH, Smit JW, Meijer DKF, Borst P, Nooijen WJ, Beijnen JH, van Tellingen O. Limited oral bioavailability and active epithelial excretion paclitaxel (Taxol) caused by P-glycoprotein in the intestine. *Proc Natl Acad Sci USA*, 1997;94:2031-2035
170. Kim RB, Fromm MF, Wandel C, Leake B, Wood AJ, Roden DM, Wilkinson GR. The drug transporter P-glycoprotein limits oral bioavailability and brain entry of HIV-1 protease inhibitors. *J Clin Invest*, 1998;101:289-294
171. John A, Köpke K, Gerloff T, Mai I, Rietbrock S, Meisel C, Hoffmeyer S, Kerb R, Fromm MF, Brinkmann U, Eichelbaum M, Brockmöller J, Cascorbi I, Roots I. Modulation of steady state kinetics of digoxin by halophytes of the P-glycoprotein MDR1 gene. *Clin Pharmacol Ther*, 2002;72:584-594
172. Kivisto KT Niemi M, Fromm MF. Functional interaction of intestinal CYP3A4 and P-glycoprotein. *Fundamental and Clinical Pharmacology*, 2004;18:621-629
173. Fromm MF. The influence of MDR1 polymorphisms on P-glycoprotein expression and function in humans. *Ad Drug Deliv Rev*, 2002;54:1295-1310
174. Marzolini C, Paus E, Buclin, Kim RB. Polymorphisms in human MDR1 (P-glycoprotein): recent advances and clinical relevance. *Clin Pharmacol Ther*, 2004;75:13-33

175. Wiese M, Pajeva IK. Structure-activity relationships of multidrug reversers. *Curr Med Chem*, 2001;8(6):685-713
176. Soldner A, Christians U, Susanto M, Wachter VJ, Silverman JAS, Benet LZ. Grapefruit juice activates P-glycoprotein-mediated drug transport. *Pharm Res*, 1999;16:478-85
177. Rashid TJ, Martin U, Clarke H, Waller DG, Renwick AG, George CF. Factors affecting the absolute bioavailability of nifedipine. *Br J Clin Pharmacol*, 1995;40:51-58
178. de Castro WV, Mertens-Talcott S, Derendorf H, Butterweck V. Grapefruit juice-drug interactions: grapefruit juice and its components inhibit P-glycoprotein (ABC1) mediated transport of talinolol in Caco-2 cells. *Journal of Pharmaceutical Sciences*, 2007;96:2808-2817
179. Takanga H, Ohnishi A, Matsuo H, Sawada Y. Inhibition of vinblastin efflux mediated by P-gp by grapefruit juice in Caco-2 cells. *Clin Pharmacol Ther, Biol Pharm Bull*, 1998;21:1062-6
180. Xu J, Go ML, Lim LY. Modulation of digoxin transport across Caco-2 monolayers by citrus fruit juices: lime, lemon, grapefruit, and pummelo. *Pharm Res*, 2003;20:169-176
181. Di Marco MP. The effect of grapefruit juice and Seville orange on the pharmacokinetics of dexomethorphan: the role of gut CYP3A4 and P-glycoprotein. *Life Sci*, 2002;71:1149-1160
182. Edwards DJ, Fitzsimmons ME, Schuetz EG, Yasuda K, Ducharme MP, Warbasse LH, Woster PM, Schuetz JD, Watkins P. 6', 7'-dihydroxybergamottin in grapefruit

- juice and Seville orange juice: effects on cyclosporine disposition, enterocyte CYP3A4 and P-glycoprotein. *Clin Pharmacol Ther*, 1999;65:237-44
183. Mayer U, Wagenaar E, Beijnen JH, Smit JW, Meijer DK, van Asperen J, Borst P, Schinkel AH. Substantial excretion of digoxin via the intestinal mucosa and prevention of long-term digoxin accumulation in the brain by the mdr 1a P-glycoprotein. *Br J Pharmacol*, 1996;119(5):1038-1044
184. Cavet ME, West M, Simmons NL. Transport and epithelial secretion of the cardiac glycoside, digoxin, by human intestinal epithelial (Caco-2) cells. *Br J Pharmacol*, 1996;118(6):1389-96
185. Becquemont L, Verstuyft C, Kerb R, Brinkmann U, Lebot M, Patrice Jaillon P, Funck-Brentano C. Effect of grapefruit juice on digoxin pharmacokinetics in humans. *Clin Pharmacol Ther*, 2001;70:311-6
186. Mooradian AD. Digitalis: An update of clinical pharmacokinetics, therapeutic monitoring techniques and treatment recommendation. *Clin Pharmacokinet*, 1988;15:165-79
187. Dresser GK, Bailey DG. The effects of fruit juices on drug disposition: a new model for drug interactions. *Eur J Clin Invest*, 2003;33:(suppl 2):10-16
188. Cvetkovic M, Leake B, Fromm MF, Wilkinson GR, Kim RB. OATP and P-glycoprotein transporters mediate cellular uptake and excretion of fexofenadine. *Drug Metab Dispos*, 1999;27:866-71
189. Lown KS, Kolars JC, Thummel KE, Barnett JL, Kunze KL, Wrighton SA, Watkins PB. Interpatient heterogeneity in expression of CYP3A4 and CYP3A5 in small bowel. Lack of prediction by erythromycin breath test. *Drug Metab Dispos*, 1994;22:947-955

190. Drescher S, Glaeser H, Mürdter T, Hitzl M, Eichelbaum M, Fromm MF. P-glycoprotein-mediated intestinal and biliary digoxin transport in humans. *Clin Pharm Ther*, 2003;73:223-231
191. Lau YY, Wu CY, Okochi H, Benet LZ. Ex situ inhibition of hepatic uptake and efflux significantly changes metabolism: hepatic enzyme-transporter interplay. *J Pharmacol Exp Ther*, 2004;308:1040
192. Abe T, Suzuki T, Unno M, Tokui T, Ito S. Thyroid hormone transporters: recent advances. *Trends Endocrinol Metab*, 2002;13:215-220
193. Hakes DJ, Berezney R. Molecular cloning of matrix F/G: A DNA binding protein of the nuclear matrix that contains putative zinc finger motifs. *Proc Natl Acad Sci USA*, 1991;88:6186-6190
194. Meier-Abt F, Mokrab Y, Mizuguchi K. Organic anion transporting polypeptides of the OATP/SLCO superfamily: identification of new members in non-mammalian species, comparative modeling and a potential transport mode. *J Membr Biol*, 2005 Dec;208(3):213-27
195. Jacquemin E, Hagenbuch B, Stieger B, Wolkoff AW, Meier PJ. Expression cloning of a rat liver Na(+)-independent organic anion transporter. *Proc Natl Acad Sci USA*, 1994;91:133-137;
196. Noé B, Hagenbuch B, Stieger B, Meier PJ. Isolation of a multispecific organic anion and cardiac glycoside transporter from rat brain. *Proc Natl Acad Sci USA*, 1997;94:10346-10350

197. Walters HC, Craddock AL, Fusegawa H, Willingham Mc, Dawson PA. Expression, transport properties, and chromosomal location of organic anion transporter subtype 3. *Am J Physiol Gastrointest Liver Physiol*. 2000 Dec;279(6):G1188-200
198. Kullak-Ublick GA, Hagenbuch B, Stieger B, Scheingart CD, Hofmann AF, Wolkoff AW, Meier PJ. Molecular and functional characterization of an organic anion transporting polypeptide cloned from human liver. *Gastroenterology*, 1995;109:1274-1282
199. Shi X, Bai S, Ford AC, Burk RD, Jacquemin E, Hagenbuch B, Meier PJ, Wolkoff AW. Stable Inducible Expression of a Functional Rat Liver Organic Anion Transport Protein in HeLa Cells. *J Biol Chem*, 1995;270:25591-25595
200. Saltin LM, Amin V, Wolkoff AW. Organic anion transporting polypeptide mediates organic anion/HCO₃⁻ exchange. *J Biol Chem*, 1997;272:26340-26345
201. Li L, Lee TK, Meier PJ, Ballatori N. Identification of glutathione as a driving force and leukotriene C₄ as a substrate for oatp1, the hepatic sinusoidal organic solute transporter. *J Biol Chem*, 1998;273(26):16184-16191
202. Gao B, Stieger B, Noé B, Fritschy JM, Meier PJ. Localization of the organic anion transporting polypeptide 2 (Oatp2) in capillary endothelium and choroid plexus epithelium of rat brain. *J Histochem Cytochem*, 1999;47:1255-1264
203. Burckhardt G, Wolff NA. Structure of renal organic anion and cation transporters. *Am J Physiol Renal Physiol*, 2000;278:F853-F866
204. Koepsell H. Organic cation transporters in intestine, kidney, liver, and brain. *Annu Rev Physiol*, 1998;60:243-266

205. Gao B, Hagenbuch B, Kullak-Ublick GA, Benke D, Aguzzi A, Meier PJ. Organic anion-transporting polypeptides mediate transport of opioid peptides across blood-brain barrier. *J Pharmacol Exp Ther*, 2000;294:73-79
206. Ishizuka H, Konno K, Naganuma H, Nishimura K, Kouzuki H, Suzuki H, Stieger B, Meier PJ, Sugiyama Y. Transport of temocaprilat into rat hepatocytes: role of organic anion transporting polypeptide. *J Pharmacol Exp Ther*, 1998;287:37-42
207. Pang KS, Wang PJ, Chung AY, Wolkoff AW. The modified dipeptide, enalapril, an angiotensin-converting enzyme inhibitor, is transported by the rat liver organic anion transport protein. *Hepatology*, 1998;28:1341-1346
208. Mikkaichi T, Suzuki T, Tanemoto M, Ito S, Abe T. The organic anion transporter (OATP) family. *Drug Metab Pharmacokinet*, 2004;19:171-179
209. Niemi M, Backman JT, Kajosaari LI, Leathart JB, Neuvonen M, Daly AK, Eichelbaum M, Kivistö KT, Neuvonen PJ. Polymorphic organic anion transporting polypeptide 1B1 is a major determinant of repaglinide pharmacokinetics. *Clin Pharmacol Ther*, 2005;77:468-478
210. Lilja JJ, Laitinen K, Neuvonen PJ. Effects of grapefruit juice on the absorption of levothyroxine. *Br J Clin Pharmacol*, 2005;60:337-341
211. Kullak-Ublick GA, Ismair MG, Stieger B, Landmann L, Huber R, Pizzagalli F, Fattinger K, Meier PJ, Hagenbuch B. Organic anion transporting polypeptide (OATP-B) and its functional comparison with three other OATPs of human liver. *Gastroenterology*, 2001;120:525-533
212. Cattori V, van Montfoort JE, Stieger B, Landmann L, Meijer DK, Winterhalter KH, Meier PJ, Hagenbuch B. Localization of organic anion transporting polypeptide 4

- (OATP4) in rat liver and comparison of its substrate specificity with Oatp1, Oatp2 and Oatp3. *Pflügers Archiv European Journal of Physiology*, 2001;443:188-195
213. Mikkaichi T, Suzuki T, Onogawa T, Tanemoto M, Mizutamari H, Okada M, Chaki T, Masuda S, Tokui T, Eto N, Abe M, Satoh F, Unno M, Hishinuma T, Inui K, Ito S, Goto J, Abe T. Isolation and characterisation of a digoxin transporter and its rat homologue expressed in the kidney. *Proc Natl Acad Sci USA*, 2004;101:3569-3574
214. Kodawara T, Masuda S, Wakasugi H, Uwai Y, Futami T, Saito H, Abe T, Inu K. Organic anion transporter Oatp2-mediated interaction between digoxin and amiodarone in the rat liver. *Pharm Res*, 2002;19:738-743
215. [No authors listed]. The digitalis investigation group: The effect of digoxin on mortality and morbidity in patients with heart failure. The digitalis investigation group. *N Eng J Med*, 1997;336:525-533
216. Tanigawara Y, Okamura N, Hirai M, Yasuhara M, Ueda K, Kioka N, Komano T, Hori R. Transport of digoxin by human P-glycoprotein expressed in a porcine kidney epithelial cell line (LLC-PKI). *J Pharmacol Exp Ther*, 1992;263:840-845
217. Hsiang B, Zhu Y, Wang Z, Wu Y, Sasseville V, Yang WP, Kirchgessner TG. A novel human hepatic organic anion transporting polypeptide (OATP2). Identification of a liver-specific human organic anion transporting polypeptide and identification of rat and human hydroxymethylglutaryl-CoA reductase inhibitor transporters. *J Biol Chem*, 1999;274:37161-68
218. Komai T, Shigehara E, Tokui T, Koga T, Ishigami M, Kuroiwa C, Horiuchi S. Carrier-mediated uptake of pravastatin by rat hepatocytes in primary cultures. *Biochem Pharmacol*, 1992;43:667-670

219. Yamazaki M, Suzuki H, Hanano M, Tokui T, Komai T, Sugiyama Y. Na(+)-independent multispecific anion transporter mediates active transport pravastatin into rat liver. *Am J Physiol*, 1993;264:G36-G44
220. Tokui T, Nakai D, Nakagomi R, Yawo H, Abe T, Sugiyama Y. Pravastatin, and HMG-CoA reductase inhibitor, is transported by rat organic anion transporting polypeptide, Oatp2. *Pharm Res*, 1999;16:904-908
221. Kirby BJ and Unadkat JD. Grapefruit juice, a glass full of drug interaction? *Clin Pharm Ther*, 2007;81(5):631-633
222. Glaeser H, Bailey DG, Dresser GK, Gregor JC, Schwarz UI, McGrath JS, Jolicoeur E, Lee W, Leake BF, Tirona RG, Kim RB. Intestinal drug transporter expression and the impact of grapefruit juice in humans. *Clin Pharmacol Ther*, 2007;81:362-370
223. Koepsell H, Lips K, Volk C. Polyspecific organic cation transporters: structure, function, physiological roles, and pharmaceutical implications. *Pharmaceutical Research*, 2007;24:1227-1251
224. Gründemann D, Gorboulev V, Gambaryan S, Veyhl M, Koepsell H. Drug excretion mediated by a new prototype of polyspecific transporter. *Nature*, 1994;372:549-552
225. Koepsell H, Schmitt BM, Gorboulev V. Organic cation transporters. *Rev Physiol Biochem Pharmacol*, 2003;150:36-90
226. Zhang L, Dresser MJ, Gray AT, Yost SC, Terashita S, Giacomini KM. Cloning and functional expression of a human liver organic cation transporter. *Mol Pharmacol*, 1997 Jun;51(6):913-21

227. Gorboulev V, Ulzheimer JC, Akhoundova A, Ulzheimer-Teuber I, Karbach U, Quester S, Baumann C, Lang F, Busch AE, Koepsell H. Cloning and characterisation of two human polyspecific organic cation transporters. *DNA Cell Biol*, 1997;16:871-881
228. Terashita S, Dresser MJ, Zhang L, Gray AT, Yost SC, Giacomini KM. Molecular cloning and functional expression of a rabbit renal organic cation transporter. *Biochim Biophys Acta*, 1998;1369:1-6
229. Green RM, Lo K, Sterritt C, Beier DR. Cloning and functional expression of a mouse liver organic cation transporter. *Hepatology*, 1999;29:1556-62
230. Popp C, Gorboulev V, Müller TD, Gorbunov D, Shatskaya N, Koepsell H. Amino acids critical for substrate affinity of rat organic cation transporter 1 line the substrate binding region in model derived from the tertiary structure of lactose permease. *Mol Pharmacol*, 2005;67(5):1600-1611
231. Gorboulev V, Shatskaya N, Volk C, Koepsell H. Subtype-specific affinity for corticosterone of rat organic cation transporters rOCT1 and rOCT2 depends on three amino acids within the substrate binding region. *Mol Pharmacol*, 2005;67(5):1612-1619
232. Gorboulev V, Volk C, Arndt P, Akhoundova A, Koepsell H. Selectivity of the polypeptide cation transporter rOCT1 is changed by mutation of aspartate 475 to glutamate. *Mol Pharmacol*, 1999;56(6):1254-1261
233. Zhang X, Shirahatti NV, Mahadevan D, Wright SH. A conserved glutamate residue in transmembrane helix 10 influences substrate specificity of rabbit OCT2 (SLC22A2). *J Biol Chem*, 2005;280(41):34813-34822

234. Meyer-Wentrup F, Karbach U, Gorboulev V, Arndt P, Koepsell H. Membrane localization of the electrogenic cation transporter rOCT in rat liver. *Biochem Biophys Res Commun*, 1998;248:673-678
235. Gründemann D, Schechinger B, Rappold GA, Schömig E. Molecular identification of the corticosterone-sensitive extraneuronal catecholamine transporter. *Nat Neurosci*, 1998;1:349-351
236. Koehler MR, Wissinger B, Gorboulev V, Koepsell H, Schmid M. The two human organic cation transporter genes SLC22A1 and SLC22A2 are located on chromosome 6q26. *Cytogenet Cell Genet*, 1997;79:198-200
237. Gründeman D, Schömig E. Gene structure of the human non-neuronal monoamine transporters EMT and OCT2. *Human Genet*, 2000;106:627-635
238. Kekuda R, Prasad PD, Wu X, Wang H, Fei YJ, Leibach FH, Ganapathy V. Cloning, and functional characterisation of a potential-sensitive polyspecific organic cation transporter (OCT3) most abundantly expressed in the placenta. *J Biol Chem*, 1998;273:15971-15979
239. Choi Min-Koo, Song Im-sook. Organic cation transporters and their pharmacokinetic and pharmacodynamic consequences. *Drug Metab Pharmacokinet*, 2008;23(4)-243-253
240. Chen JJ, Li Z, Pan H, Murphy DL, Tamir H, Koepsell H, Gershon MD. Maintenance of serotonin in the intestinal mucosa and ganglia of mice that lack the high-affinity serotonin transporter: abnormal intestinal motility and expression of cation transporters. *J Neurosci*, 2001;21:6348-6361

241. Karbach U, Kricke J, Meyer-Wentrup F, Gorboulev V, Volk C, Loffing-Cueni D, Kaissling B, Bachmann S, Koepsell H. Localization of organic cation transporter OCT1 and OCT2 in rat kidney. *Am J Physiol Renal Physiol*, 2000; 279:F679-F687
242. Alnouti Y, Petrick JS, Klaassen CD. Tissue distribution and ontogeny of organic cation transporters in mice. *Drug Metab Dispos*, 2006; 34:477-482
243. Müller J, Lips KS, Metzner L, Neubert RH, Koepsell H, Brandsch M. Drug specificity and intestinal membrane localization of human organic cation transporters (OCT1). *Biochem Pharmacol*, 2005; 70:1851-1860
244. Sugawara-Yokoo M, Urakami Y, Koyama H, Fujikura K, Masuda S, Saito H, Naruse T, Inui K, Takata K. . Differential localization of organic cation transporters rOCT1 and rOCT2 in the basolateral membrane of rat kidney proximal tubules. *Histochem Cell Biol*, 2000; 114:175-180
245. Seithel A, Karlsson J, Hilgendorf C, Björquist A, Ungell AL. Variability in mRNA expression of ABC- and SLC-transporters in human intestinal cells: comparison between human segments and Caco-2 cells. *Eur J Pharm Sci*, 2006; 28:291-299
246. Haag C, Berkels R, Gründemann D, Lazar A, Taubert D, Schömig E. The localization of the extraneuronal monoamine transporter in normal human astrocytes. *J Neurochem*; 2004; 88:291-29
247. Zhang X, Evans KK, Wringht SH. Molecular cloning of rabbit organic cation transporter rbOCT2 and functional comparisons with rbOCT1. *Am J Physiol Renal Physiol*, 2002; 283(1):F124-F133
248. Schmidt BM, Koepsell H. Alkali cation binding and permeation in the rat organic cation transporter rOCT2. *J Biol Chem*, 2005; 280:24481-24490

249. Busch AE, Quester S, Ulzheimer JC, Waldegger S, Gorboulev V, Arndt P, Lang F, Koepsell H. Electrogenic properties and substrate specificity of the polypeptide rat cation transporter rOCT. *J Biol Chem*, 1996;271:32599-32604
250. Keller T, Elfeber M, Gorboulev V, Reiländer H, Koepsell H. Purification and functional reconstitution of the rat organic cation transporter OCT1. *Biochemistry*, 2005;44:12253-12263
251. Ahlin G, Karlsson J, Pedersen JM, Gustavsson L, Larsson R, Matsson P, Norinder U, Bergström CA, Artursson P. Structural requirements for drug inhibition of liver specific human organic cation transport protein 1. *J Med Chem*, 2008;51:5932-5942
252. Busch AE, Karbach U, Miska D, Gorboulev V, Akhoundova A, Volk C, Arndt P, Ulzheimer JC, Sonders MS, Baumann C, Waldegger S, Lang F, Koepsell H. Human neurons express the polyspecific cation transporter hOCT2, which translocates monoamine neurotransmitters, amantadine, and memantine. *Mol Pharmacol*, 1998;54:342-352
253. Dresser MJ, Gray AT, Giacomini KM. Kinetic and selectivity differences between rodent, rabbit, and human organic cation transporters (OCT1). *J Pharmacol Exp Ther*, 2000;292:1146-1152
254. Jonker JW, Schinkel AH. Pharmacological and physiological functions of the polyspecific organic cation transporters: OCT1, 2 and 3 (SLC22A1-3). *J Pharmacol Exp Ther*, 2004;308(1):2-9
255. Jonker JW, Wagenaar E, Mol CA, Buitelaar M, Koepsell H, Smit JW, Schinkel AH. Reduced hepatic uptake and intestinal excretion of organic cations in mice with a targeted disruption of the organic cation transporter 1 (Oct1 [Slc22a1]) gene. *Mol Cell Biol*, 2001;21(16):5471-5477

256. Jonker JW, Wagenaar E, Van Eijl S, Schinkel AH. Deficiency in the organic transporters 1 and 2 (Oct1/Oct2 [Slc22a1/Slc22a2]) in mice abolishes renal secretion of organic cations. *Mol Cell Biol*, 2003;23(21):7902-7909
257. Wang DS, Jonker JW, Kato Y, Kusuhara H, Schinkel AH, Sugiyama Y. Involvement of organic cation transporter 1 in hepatic and intestinal distribution of metformin. *J Pharmacol Exp Ther*, 2002;302):510-515
258. Zwart R, Verhaagh S, Buitelaar M, Popp-Snijders C, Barlow DP. Impaired activity of the extraneuronal monoamine transporter system known as uptake-2 in rOct3/Slc22a3-deficient mice. *Mol Cell Biol*, 2001;21(13):4188-4196
259. Choi MK, Song IS. Organic cation transporters and their pharmacokinetic and pharmacodynamic consequences. *Drug Metab Pharmacokinet*, 2008;23(4):243-253
260. Saborowski M, Kullak-Ublick GA, Eloranta JJ. The human organic cation transporter-1 gene is transactivated by hepatocyte nuclear factor-4alpha. *J Pharmacol Exp Ther*, 2006;317(2):778-785
261. Maher JM, Slitt AL, Callaghan TN, Cheng X, Cheung C, Gonzalez FJ, Klaassen CD. Alterations in transporter expression in liver, kidney and duodenum after targeted disruption of the transcription factor HNF1 alpha. *Biochem Pharmacol*, 2006;72(4):512-522
262. Nie W, Sweetser S, Rinella M, Green RM. Transcriptional regulation of murine Slc22a1 (Oct1) by peroxisome proliferator agonist receptor-alpha and -gamma. *Am J Physiol Gastrointest. Liver Physiol*, 2004;288(2):G207-G212
263. Maeda T, Oyabu M, Yotsumoto T, Higashi R, Nagata K, Yamazoe Y, Tamai I. Effect of pregnane X receptor ligand on pharmacokinetics of substrates of organic cation transporter Oct1 in rats. *Drug Metab Dispos*, 2007;35(9):1580-1586

264. Maeda T, Yotsumoto T, Oyabu M, Tamai I. Effect of glucocorticoid receptor ligand dexamethasone on the expression of organic cation transporter protein in rat liver. *Drug Metab Pharmacokinet*, 2008;23(1):67-72
265. Cetinkaya I, Ciarimboli G, Yalçinkaya G, Mehrens T, Velic A, Hirsch JR, Gorboulev V, Koepsell H, Schlatter E. Regulation of human organic cation transporter hOCT by PKA, PI3K, and calmodulin-dependent kinases. *Am J Physiol Renal Physiol*, 2003;284(2):F293-F302
266. Ciarimboli G, Koepsell H, Iordanova M, Gorboulev V, Dürner B, Lang D, Edemir B, Schröter R, Van Le T, Schlatter E. Individual PKC-phosphorylation sites in organic cation transporter 1 determine substrate selectivity and transport regulation. *J Am Soc Nephrol*, 2005;16(6):1562-1570
267. Denk GU, Soroka CJ, Mennone A, Koepsell H, Beuers U, Boyer JL. Down-regulation of the organic cation transporter 1 of rat liver in obstructive cholestasis. *Hepatology*, 2004;39(5):1382-1386;
268. Biermann J, Lang D, Gorboulev V, Koepsell H, Sindic A, Schröter R, Zvirbliene A, Pavenstädt H, Schlatter E, Ciarimboli G. Characterisation of regulatory mechanisms and states of human organic cation transporter 2. *Am J Physiol Cell Physiol*, 2006;290(6):C1521-C1531
269. Mehrens T, Lelleck S, Cetinkaya I, Knollmann M, Hohage H, Gorboulev V, Bokník P, Koepsell H, Schlatter E. The affinity of the organic cation transporter rOct1 is increased by protein kinase C-dependent phosphorylation. *J Am Soc Nephrol*, 2000;11:1216-1224

270. Ciarimboli G, Struwe K, Arndt P, Gorboulev V, Koepsell H, Schlatter E, Hirsch JR. Regulation of the human organic cation transporter hOCT1. *J Cell Physiol*, 2004;201:420-428
271. Ranganna S, Govindarajan VS, : Ramana KVR. Citrus fruits: varieties, chemistry, technology and quantity evaluation: Part II: chemistry, techonology and evaluation. *A Chemistry Crit Rev Food Sci Nutr*, 1983;18:313-386
272. Bailey DG, Arnold JM, Munoz C, Spence JD. Grapefruit juice: felodipine interaction. Mechanism, predictability, and effect of naringin. *Clin Pharmacol Ther*, 1993;53:637-642
273. Bailey *et al.* Grapefruit juice-drug interaction issues. In Boullata J, Armeni V, editors. Handbook of drug-nutrient interactions. *Totawa (NJ): The Humana Press*, 2004:175-194
274. Guo LQ, Fukuda K, Ohta T, Yamazoe Y. Role of furanocoumarin derivatives in grapefruit juice-mediated inhibition of human CYP3A4 activity. *Drug Metab Dispos*, 2000;28:766-771
275. He K, Iyer KR, Hayes RN, Sinz MW, Woolf TF, Hollenberg PF. Inactivation of cytochrome P450 3A4 by bergamottin, a component of grapefruit juice. *Chem Res Toxicol*, 1998;11(4):252-259
276. Bailey DG, Dresser GK, Kreeft JH, Munoz C, Freeman DJ, Bend JR. Grapefruit-felodipine interaction: effect of unprocessed fruit and probable active ingredients. *Clin Pharmacol Ther*, 2000;68(5):468-477
277. Ohta T, Nagahashi M, Hosoi S, Tsukamoto S. Dihydroxybergamottin caproate as a potent and stable CYP3A4 inhibitor. *Bioorg med Chem*, 2002;10:969-973

278. Ohta T, Maruyama T, Nagahashi M, Miyamoto Y, Hosoi S, Kiuchi F, Yamazoe Y, Tsukamoto S. Paradisin C: a new CYP3A4 inhibitor from grapefruit juice. *Tetrahedron*, 2002;58:6631-6635
279. Kakar SM, Paine MF, Stewart PW, Watkins PB. 6',7'-dihydroxybergamottin contributes to the grapefruit effect. *Clin Pharmacol Ther*, 2004;75:569-579
280. Malhotra S, Bailey DG, Paine MF, Watkins PB. Seville orange juice-felodipine interaction: comparison with dilute grapefruit juice and involvement of furanocoumarins. *Clin Pharmacol Ther*, 2001;69:14-23
281. Girennavar *et al.* Radical scavenging and cytochrome P450 3A4 inhibitory activity of bergamotol and geranylcoumarin from grapefruit. *Bioorganic and Medicinal Chemistry*, 2007;15:3684-3691
282. Fukuda *et al.* Specific CYP3A4 inhibitors in grapefruit juice: furocoumarin dimers as components of drug interaction. *Pharmacogenetics*, 1997;7:391-396
283. Tassaneeyakul *et al.* Inhibition selectivity of grapefruit juice components on human cytochromes P450. *Arch Biochem Biophys*, 2000;387(2):356-63
284. Guo LQ, Fukuda K, Ohta T, Yamazoe Y. Guo *et al.* Role of furanocoumarin derivatives on grapefruit juice-mediated inhibition of human CYP3A activity. *Drug Metab Dispos*, 2000;28:766-771
285. Row E, Brown SA, Stachulski AV, Lennard MS. Row *et al.* Development of novel furanocoumarin dimers as potent and selective inhibitors of CYP3A4. *Drug Metab Dispos*, 2006;34(2):324-330;

286. Oda K, Yamaguchi Y, Yoshimura T, Wada K, Nishizono N. Synthetic models related to the furanocoumarin, coumarin, and benzofuran dimers as potent inhibitors of CYP3A4. *Chem Pharm Bull*, 2007;59(9):1419-1421
287. Paine MF, Widmer WW, Hart HL, Pusek SN, Beavers KL, Criss AB, Brown SS, Thomas BF, Watkins PB. A furanocoumarin-free grapefruit juice establishes furanocoumarins as the selective mediators of the grapefruit juice-felodipine interaction. *Am J Clin Nutr*, 2006;83:1097-1105
288. Myung K, Narciso JA, Manthey JA. Removal of furanocoumarins in grapefruit juice by edible fungi. *J Agric Food Chem*, 20008;56:12064-12068
289. Uesawa Y, Mohri K. The use of heat treatment to eliminate drug interactions due to grapefruit juice. *Biol Pharm Bull*, 2006;29(110):2274-2278
290. Uesawa Y, Mohri K. UV-irradiated grapefruit juice loses pharmacokinetic interaction with nifedipine in rats. *Biol Pharm Bull*, 2006;29(6):1286-1289
291. Greenblatt DJ, Patki KC, von Moltke LL, Shader RI. Drug interactions with grapefruit juice: an update. *J Clin Psychopharmacology*, 2001;21:357-359
292. Andersen V, Pedersen N, Larsen NE, Sonne J, Larsen S. Intestinal first pass metabolism of midazolam in liver cirrhosis-effect of grapefruit juice. *Br J Clin Pharmacol*, 2002;54:120-124
293. Goho C. Oral midazolam-grapefruit juice interaction. *Paed Dent*, 2001;23:365-366
294. Kantola T, Kivisto KT, Neuvonen PJ. Grapefruit greatly increases serum concentrations of lovastatin and lovastatin acid. *Clin Pharmacol Ther*, 1998;63:397-402

295. Grundy SM, Cleeman JI, Merz CN, Brewer HB Jr, Clark LT, Hunninghake DB, Pasternak RC, Smith SC Jr, Stone NJ; National Heart, Lung, and Blood Institute; American College of Cardiology Foundation; American Heart Association. Implications of recent clinical trials for National Cholesterol Education Programme Adult Treatment Panel II guidelines. *Circulation*, 2004;110:227-239
296. Honig PK, Wortham DC, Lazarev A, Cantilena LR. Grapefruit juice alters the systemic bioavailability of and cardiac repolarization of terfenadine in poor metabolizers of terfenadine. *J Clin Pharmacol*, 1996;36:345-351
297. Dahan A and Altman H. Food-drug interaction: grapefruit juice augments drug bioavailability- mechanism, extent and relevance. *Eur J Clin Nutr*, 2004;58:1-9
298. Spence JD. Drug interactions with grapefruit juice: whose responsibility is it to warn the public? *Clin Pharmacol Ther*, 1997;61:395-400
299. Ku YM, Min DI, Flanigan M. Effect of grapefruit juice on the pharmacokinetics of microemulsion cyclosporine and its metabolite in healthy volunteers: does the formulation difference matter? *J Clin Pharmacol*, 1998;38:959-965
300. Brunner LJ, Pai KS, Munar MY, Lande MB, Olyaei AJ, Mowry JA. Effect of grapefruit juice on cyclosporine A pharmacokinetics in paediatric renal transplant patients. *Paediatr Transplant*, 2000;4:313-321
301. Hermann M, Asberg A, Reubsaet JL, Sather S, Berg KJ, Christensen H. Intake of grapefruit juice alters the metabolic pattern of cyclosporine A in renal transplant recipients. *Int J Clin Pharmacol Ther*, 2002;40:451-456
302. Edwards DJ, Fitzsimmons ME, Schuetz EG, Yasuda K, Ducharme MP, Warbasse LH, Woster PM, Schuetz JD, Watkins P. 6',7'-dihydrobergamottin in grapefruit

- juice and Seville orange juice: effects on cyclosporine disposition, enterocyte CYP4A4, and P-glycoprotein. *Clin Pharmacol Ther*, 1999;65:237-244
303. Nowack R. Cytochrome P450 enzyme, and transport protein mediated herb-drug interactions in renal transplant patients: Grapefruit juice, St John's Wort- and beyond! *Nephrology*, 2008;13:337-347
304. Clotz U. Effect of age on pharmacokinetics and pharmacodynamics in man. *Int J Clin Pharmacol Ther*, 1998;36(11):581-585
305. Gottesman MM Fojo T, Bates SE. Multidrug resistance in cancer: role of ATP-dependent transporters. *Nat Rev Cancer*, 2002;2:48-58
306. Santostasi G, Kutty RK, Krishna G. Increased toxicity of anthracycline antibiotics induced by calcium entry blockers in cultured cardiomyocytes. *Toxicol App Pharmacol*, 1991;108:140-149
307. Xu J, Go ML, Lim LY. Modulation of digoxin transport across Caco-2 monolayers by citrus fruit juices: lime, lemon, grapefruit, and pumelo. *Pharm Res*, 2003;20:169-176.
308. Nishizato Y, Ieiri I, Suzuki H, Kimura M, Kawabata K, Hirota T, Takane H, Irie S, Kusuhara H, Urasaki Y, Urae A, Higuchi S, Otsubo K, Sugiyama Y. Polymorphisms of OATP-C (SLC21A6) and OATP3 (LC22A8) genes: consequences of pravastatin pharmacokinetics. *Clin Pharmacol Ther*, 2003;73:554-565
309. Vavricka SR, Van Montfort J, Ha HR Meier PJ. Interactions of rifamycin SV and rifampicin with organic anion uptake systems of human liver. *Hepatology*, 2002;36:164-172

310. Fattinger K, Cattori V, Hagenbuch B, Meier PJ, Stieger B. Rifamycin SV and rifampicin exhibit differential inhibition of the hepatic rat organic anion transporting polypeptides, Oatp1 and Oatp2. *Hepatology*, 2000;32:82-86
311. Yonezawa A, Masuda S, Yokoo S, Katsura T, Inui K. Cisplatin and oxaliplatin but not carboplatin and nedaplatin are substrates for human organic cation transporters (SLC22A1-3) and multidrug and toxin extrusion family. *J Pharmacol Exp Ther*, 2006;319:879-886
312. Nie W, Sweetser S, Rinella M, Green RM. Transcriptional regulation of murine Slc22A1 (Oct1) by peroxisome proliferator agonist receptor α and γ . *Am J Physiol Gastrointest Liver Physiol*, 2005;288:G207-G212
313. Bachmakov I, Glaeser H, Fromm MF, König J. Interaction of oral antidiabetic drugs with hepatic uptake transporters. *Diabetes*, 2008;57:1463-1468
314. Wang DS, Jonker JW, Kato Y, Kusuhara H, Schinkel AH, Sugiyama Y. Involvement of organic cation transporter 1 in hepatic and intestinal distribution of metformin. *Journal Pharmacology and Experimental Therapeutics*, 2002;302:510-515
315. Shu Y, Sheardown SA, Brown C, Owen RP, Zhang S, Castro RA, Ianculescu AG, Yue L, Lo JC, Burchard EG, Brett CM, Giacomini KM. Effect of genetic variation in the organic cation transporter 1 (OCT1) on metformin action. *J Clin Invest*, 2007;117:1422-1431
316. Shu Y, Brown C, Castro RA, Shi RJ, Lin ET, Owen RP, Sheardown SA, Yue L, Burchard EG, Brett CM, Giacomini KM. Effect of genetic variation in the organic cation transporter 1, OCT1, on metformin pharmacokinetics. *Clin Pharmacol Ther*, 2008;83(2):273-280

317. Wang DS, Kusunohara H, Kato Y, Jonker JW, Schinkel AH, Sugiyama Y. Involvement of organic cation transporter 1 in the lactic acidosis caused by metformin. *Molecular Pharmacology*, 2003; 63:844-848
318. El-Mir MY, Nogueira V, Fontaine E, Avéret N, Rigoulet M, Leverve X. Dimethylbiguanide inhibits cell respiration via an indirect effect target on respiratory chain complex I. *J Biol Chem*, 2000;275:223-228
319. Owen MR, Doran E, Halestrap AP. Evidence that metformin exerts its antidiabetic effects through inhibition of complex I of the mitochondrial complex I of the mitochondrial respiratory chain. *Biochem J*, 2000;348:607-614
320. Salpeter SR, Greyber E, Pasternak GA, Salpeter EE. Risk of fatal and nonfatal lactic acidosis with metformin use in type 2 diabetes mellitus. *Arch Inter Med*, 2003;163:2594-2602
321. Shenoy C. Metformin-associated lactic acidosis precipitated by acute renal failure. *MJ Med Sci*, 2000;331(1):55-57
322. Brown JB, Pedula K, Barzilay J, Herson MK, Latare P. Lactic acidosis rates in type 2 diabetes. *Diabetes Care*, 21:1659-1663;1998
323. Bailey CJ, Turner RC. Metformin. *N Engl J Med*, 1996;334:574-579
324. Schäfer G. Some new aspects of the interaction of hypoglycaemic-producing biguanides with biological membrane. *Biochem Pharmacol*, 1976;25:2075-2076
325. Bailey CJ, Wilcock C, Day C. Effect of metformin on glucose metabolism in the splanchnic bed. *Br J Pharmacol*, 1992;105:1009-1013

326. Bailey CJ, Mynett KJ, Page T. Importance of intestine as a site of metformin-induced glucose utilisation. *Br J Pharmacol*, 1994; 112:671-675
327. Bailey CJ, Wilcock C, Scapelo JHB. Metformin and the intestine. *Diabetologia*, 2008;51:1552-1553
328. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doebber T, Fujii N, Musi N, Hirshman MF, Goodyear LJ, Moller DE. Role of AMPK-activated protein kinase in metformin action. *J Clin Invest*, 2001;108:1167-1174
329. Abbud W, Habinowski S, Zhang JZ, Kendrew J, Elkairi FS, Kemp BE, Witters LA, Ismail-Beigi F. Stimulation of AMPK-activated protein kinase (AMPK) is associated with Glut1-mediated glucose transport. *Arch Biochem Biophys*, 2000;380:347-352
330. Hardie DG. AMP-activated protein kinase as a drug target. *Annu Rev Pharmacol Toxicol*, 2007;47:185-210
331. Carling D. The AMP-activated protein kinase—a unifying system for energy control. *Trends Biochem Sci*, 2004;29:18-24
332. Kemp BE, Stapleton D, Campbell DJ, Chen ZP, Murthy S, Walter M, Gupta A, Adams JJ, Katsis F, van Denderen B, Jennings IG, Iseli T, Mitchell BJ, Witters LA. AMP-activated protein kinase, super metabolic regulator. *Biochem Soc Trans*, 2000;31:162-168
333. Kim YD, Park KG, Lee YS, Park YY, Kim DK, Nedumaran B, Jang WG, Cho WJ, Ha J, Lee IK, Lee CH, Choi HS. Metformin inhibits hepatic gluconeogenesis through AMP-activated protein kinase-dependent regulation of the orphan nuclear receptor SHP. *Diabetes*, 2008;57:306-314

334. Lochhead PA, Salt IP, Walker KS, Hardie DG, Sutherland C. 5-aminoimidazole-4-carboxamide riboside mimics the effects of insulin on the expression of 2 key gluconeogenic genes PEPCK and glucose-6-phosphatase. *Diabetes*, 2000;49:896-903
335. Cool B, Zinker B, Chiou W, Kifle L, Cao N, Perham M, Dickinson R, Adler A, Gagne G, Iyengar R, Zhao G, Marsh K, Kym P, Jung P, Camp HS, Frevert E. Identification and characterisation of a small molecule AMPK activator that treats key components of type2 diabetes and metabolic syndrome. *Cell Metab*, 2006;3:403-416
336. Seol W, Choi SH Moore DD. An orphan nuclear hormone receptor that lacks DNA binding domain and heterodimerises with other receptors. *Science*, 1996;272:1336-1339
337. Yamagata K, Daitoku H, Shimamoto Y, Matsuzaki H, Hirota K, Ishida J, Fukamizu A. Bile acids regulate gluconeogenic gene expression via small heterodimer partner-mediated repression of hepatocyte nuclear factor 4 and Foxo1. *J Biol Chem*, 2004;279:23158-23165
338. Kodama S, Moore R, Yamamoto Y, Negishi M. Human nuclear pregnane X receptor cross-talk with CREB to repress cAMP activation of the glucose-6-phosphatase gene. *Biochem J*, 2007;407:373-381
339. Third report of the National Cholesterol Education Program (NCEP) expert panel on detection, evaluation and treatment of high blood cholesterol in adults final report. *Circulation*, 2002;106:3143-3142
340. Ford ES, Wayne GH, Dietz WH. Prevalence of metabolic syndrome among US adults. *JAMA*, 2002;287:356-359

341. UK Prospective Diabetes Study (UKPDS) Group: Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). *Lancet*, 1998;352(9131):854-865
342. Douek IF, Allen SE, Ewings P, Gale EA, Bingley PJ. Continuing metformin when starting insulin in patients with Type 2 diabetes: a double-blind randomized placebo-controlled trial. *Diabetic Medicine*, 2005;22:634-640
343. Bruijstens LA, van Luin M, Buscher-Jungerhans PM, and Bosch FH. Reality of severe metformin-induced lactic acidosis in the absence of chronic renal impairment. *Netherlands Journal of Medicine*, 2008 66(5):185-190
344. Howlett, HCS and Bailey, C.J. A risk-benefit assessment of metformin in Type 2 diabetes mellitus. *Drug Safety*, 1999;20(6):489-503
345. Scheen AJ. Clinical pharmacokinetics of metformin. *Clinical Pharmacokinetics*, 1996; 30(5):35-37
346. Calabrese AT, Coley KC, DaPos SV, Swanson D, Rao RH. Evaluation of prescribing practices: risk of lactic acidosis with metformin therapy. *Archives of Internal Medicine*, 2002;162:434-437
347. Riesenman PJ, Braithwaite SS and Cairns BA. Metformin-associated lactic acidosis in a burn patient. *Journal of Burn Care Research*, 2007; 28:342-347
348. Dawson D and Conlon C. Case study: metformin-associated lactic acidosis: could orlistat be relevant? *Diabetes Care*, 2003;26(8):2471-2472

349. Lalau, JD, Race, JM. Lactic acidosis in metformin-treated patients: prognostic value of arterial lactate levels and plasma metformin concentrations. *Drug Safety*, 1999;20(4):377-384
350. Lorke D. A new approach to practical acute toxicity testing. *Archives of Toxicology*, 1983;54:275-287
351. FAO. Carbohydrates in human nutrition. *FAO Food Nutr Pap*, 1998;66:1-40
352. Ho CP, Saville JD, Wanwimolruk S. Improved high performance liquid chromatographic method for the analysis of naringin in grapefruit juice without extraction. *Pharm Pharmacol Commun*, 1998;4:473-476
353. Manthey JA, Bulsig BS. Distribution of furanocoumarins in grapefruit juice fractions. *J Agric Food Chem*, 2005;53:5158-5163
354. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal Biochem*, 1976;72:248-254
355. Seifter Dayton S *et al.* The estimation of glycogen with anthrone reagent. *Arch Biochem Biophys* 1950;50:191-200
356. Davidson AL and Arion WJ. Factors underlying significant underestimations of glucokinase activity in crude liver extracts: Physiological implications of higher cellular activity. *Arch Biochem Biophys*, 1987;253:156-167
357. Barzila N, Roseseti L. Role of glucokinase and glucose-6-phosphate in the acute and chronic regulation of hepatic glucose fluxes by insulin. *J Biol Chem*, 1993;268:25019-250-25

358. Lange AJ, Arion WJ, Burchell A, Burchell B. Aluminum ions are required for stabilization and inhibition of hepatic microsomal glucose-6-phosphatase by sodium fluoride. *J Biol Chem*, 1986;261:101-107
359. Bentle LA and Lardy HA. Interaction of anions and divalent metal ions with phosphoenolpyruvate carboxykinase. *J Biol Chem*, 1976;251:2916-2921
360. Stiffin RM, Sullivan SM, Carlson GM, Holyoak T. Differential inhibition of cytosolic PEPCK by substrate analogues. Kinetic and structural characterization of inhibitor recognition. *Biochemistry*, 2008;47(7):2099-2109
361. Davies SP, Carling D and Hardie DG. Tissues distribution of the AMP-activated protein kinase, studied using a specific and sensitive peptide assay. *Eur J Biochem*, 1989;186:123-128
362. Foretz M, Carling D, Guichard C, Ferré P, Foufelle. AMP-activated protein kinase inhibits the glucose-activated expression of fatty acid synthase gene in rat hepatocytes. *J Biol Chem*, 1998;273:14767-14771
363. Sullivan JE, Carey F, Carling D, Beri RK. Characterisation of 5'-AMP-activated protein kinase in human liver using specific peptide substrates and the effects of 5'-AMP analogues on enzyme activity. *Biochem Biophys Res Comm*, 1994;200:1551-1556
364. Venn B, Green TJ. Glycemic index and glycemic load: measurement issues and their effect on diet-disease relationships. *European Journal of Clinical Nutrition*, 2007;61(suppl 1):S122-S131
365. Szabo S, Komblos S, Ignjatovic Z. Effect of pregnenolone-16alpha-carbonitrile (PCN) on drug response in man. *J Pharm Pharmacol*. 1975 Feb;27(2):113-8

366. Kourounakis P, Szabo S, Selye H. Effect of pregnenolone-16alpha-carbonitrile on the rat liver. *Arzneimittelforschung*. 1976;26(1):74-5
367. Stades AM, Heikens JT, Erkelens DW, Holleman F, Hoekstra JB. Metformin and lactic acidosis: cause or coincidence? A review of case reports. *Journal of Internal Medicine*. 2004;255(2):179-87.



UNIVERSITY OF
KWAZULU-NATAL

RESEARCH OFFICE
HOWARD COLLEGE CAMPUS
E-Mail: moodleyv@ukzn.ac.za
Tel.: 27-31-260 2273 Fax: 27-31-260 2384

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

A handwritten signature in cursive script, appearing to read 'Th Coetzer'.

Professor Theresa HT Coetzer
Chairperson: Animal Ethics Sub-committee

Cc Registrar
Research Office
Head of School

6.5 Publications

6.5.1 Submitted manuscript

Peter Owira - RE: Manuscript

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

Dear Dr. Owira,

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.

6.6 Reprint permission

Peter Owira - Re: authorization

From: <jacques.turgeon.chum@sss.gouv.qc.ca>
To: "Peter Owira" <Owirap@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

De : "Peter Owira" [Owirap@ukzn.ac.za]
Envoyé : 02/06/2009 08:39 AM ZÉ2
À : <jacques.turgeon@umontreal.ca>
Objet : authorization

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/dfisclaimer>

Peter Owira - Re: Authorization

From: Frances J Sharom <fsharom@uoguelph.ca>
To: Peter Owira <Owirap@ukzn.ac.za>
Date: 2/6/2009 4:48 PM
Subject: Re: Authorization

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) 824-4120 Ext 52247
Fax: (519) 827-1802
E-mail: fsharom@uoguelph.ca