To my wife, my parents and my brother.
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LIST OF ABBREVIATIONS

HMG-CoA: 3-Hydroxy-3-methylglutaryl Coenzyme A
CNS: central nervous system
LDL: low density lipoprotein
LDL-C: low density lipoprotein cholesterol
CYP: cytochrome P450
PGP: p-glycoprotein
CK: creatine kinase
NLA: national lipid association
FDA: Food and Drug Administration
apoB: apolipoprotein B
ACAT: acyl-CoA:cholesterol acyltransferase
DS: double strength
RS: regular strength
DHB: dihydroxybergamottin
BG: bergamottin
NAR: naringin
NAG: naringenin
AUC: area under the curve
CMAX: peak plasma concentration
CMIN: trough plasma concentration
TMAX: time of peak plasma concentration
T1/2: half life
CL/F: clearance over bioavailability
Vz/F: volume of distribution over bioavailability
GFJ: grapefruit juice
OTC: over the counter
PHP: PHP hypertext preprocessor
MYSQL: structured query language
RDBMS: relational database management system
GNU GPL: GNU general public licence
CMC: carboxymethyl cellulose
GGT: gamma glutamyltransferase
ALT: alanine aminotransferase
AST: aspartate aminotransferase
CPK: creatine phosphokinase
PVDF: polyvinylidene fluoride
DMSO: dimethyl sulfoxide
SEM: standard error of the mean
SV: simvastatin
I.P.: intraperitoneal
ACN: acetonitrile
H2O: water
ESI: electrospray ionization
SRM: single reaction monitoring
CID: collision induced dissociation
Grapefruit juice (GFJ) has been shown to increase the plasma concentrations of several drugs including HMG-CoA reductase inhibitors. The predominant mechanism for this interaction is the irreversible inhibition of intestinal drug metabolizing enzymes, mainly Cytochrome P450 3A4 (CYP 3A4). CYP 3A4 metabolizes roughly 60% of all drugs available to patients to a greater or lesser extent. Changes in exposure of HMG-CoA reductase inhibitors have been shown to reach increases of up to 16-fold. However, the long term and clinical effects of the interaction remain unclear.

The purpose of the presented work was to investigate the potential clinical relevance of the interaction between GFJ and HMG-CoA reductase inhibitors using GFJ quantities comparable to human consumption (5mL/kg). For this purpose data from animals experiments and a clinical trial have been analyzed.

During the animal experiment male Sprague-Dawley rats were dosed with either 20mg/kg or 80mg/kg simvastatin concomitantly with regular (RS) and double strength (DS) GFJ. The primary outcome parameters were defined as the changes in exposure of simvastatin lactone and simvastatin acid levels. Our findings suggest that grapefruit juice increases the exposure only
minimally when administered with 20mg/kg simvastatin. Exposure increased significantly when double strength GFJ was dosed concomitantly with 80mg/kg simvastatin in laboratory animals. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine phosphokinase (CPK), total cholesterol (tCHOL) and changes in muscle histology were analyzed as secondary parameters. Changes of the primary parameters however did not result in an increase in the incidence of muscle damage, changes in other secondary parameters or decrease in survival rate. Additionally our results suggest that GFJ is capable of lowering cholesterol by 19% and 15% when administered as RS and DS respectively.

The data from the clinical trial suggest that steady state atorvastatin plasma concentrations do not change significantly over 90 days when the drug was coadministered with 10oz of GFJ once daily.

Our results indicate that GFJ consumption in moderation is unlikely to result in clinically relevant interactions. Further research in humans however is needed to confirm this hypothesis.
CHAPTER 1
INTRODUCTION

3-Hydroxy-3-methylglutaryl (HMG) Coenzyme A (CoA) Reductase Inhibitors

Despite the increased effort, coronary heart diseases remain the main cause of death in the western world (Figure 1-1) [1], even though the main risk factors have long been identified. These risk factors include smoking, hypertension, lipid disorders and diabetes mellitus. In 1984 the Lipid Research Clinic Coronary Primary Prevention Trial (LRC-CPPT) concluded that there is a positive relationship between the reduction of plasma cholesterol and decrease of myocardial infarction [2]. Subsequently numerous drugs have been developed to treat lipid disorders such as hypercholesterolaemia. The drugs include bile acid sequestrants, fibrates, selective cholesterol absorption inhibitors, and HMG-CoA reductase inhibitors. One of the most prescribed class of drugs in this category are the HMG-CoA reductase inhibitors or statins [3]. Lipitor® (atorvastatin) and Zocor® (simvastatin) were the number 1 and 7 prescription drugs in the USA in 2006 generating a sales volume of 11.6 billion dollars according to IMS National Sales Perspectives [3] (Figure 1-2).

History of Development and Mechanism of Action

The biosynthesis of cholesterol from acetyl-CoA accounts for 60-70% of the total cholesterol available to the human body. This high amount of endogenous production makes this pathway the perfect target for cholesterol lowering therapy [4]. The first inhibitors of the cholesterol biosynthesis triparanol (MER-29) and AY-9944 acted on a later step of the biosynthesis pathway [5-7]. AY-994 prevented the conversion from 7-dehydrocholesterol to cholesterol whereas MER-29 inhibits the conversion from 24-dehydrocholesterol (desmosterol) to cholesterol [8]. However the inhibition of the later stages of the cholesterol synthesis resulted in the increase of desmosterol in plasma [5] and researchers believe that the accumulation of this
sterol led to ichthyosis [9] and posterior lenticular cataracts [10]. The effects of the compounds resulted in the withdrawal of triparanol from the market in 1962 and reduced the initial enthusiasm for the inhibition of the cholesterol pathway as a therapeutic target.

Later in 1968 Dietschy et al. [11] reported that the endogenous biosynthesis of cholesterol in the liver was nearly completely inhibited when high amounts of cholesterol are added to the diet. Further investigation revealed that this feedback mechanism is due to the changes in activity of the HMG-CoA reductase [12]. These findings encouraged Endo and Kuroda that the concept of inhibition of the HMG-CoA reductase could be a potential target for treatment of hypercholesterolemia in humans [13].

In 1971 Endo and Kuroda began their search for inhibitors of the HMG-CoA reductase isolated from bacteria, assuming that some microorganisms would use this pathway a defense mechanism against other microbes. Both researchers tested close to 6000 bacterial strains and in 1973 isolated the first irreversible inhibitor of the HMG-CoA reductase, citrinin, an antibiotic compound from *Pythium ultimum* [14]. By the end of 1973 another promising structure was isolated from *Penicillium citrium*. Mevastatin (ML-236B) was shown to inhibit the cholesterol synthesis in the early stages of the biosynthesis pathway from both $[^{14}\text{C}]$acetate and $[^{14}\text{C}]$HMG-CoA, but showed no significant effect on the conversion of $[^{3}\text{H}]$mevalonate into successive sterols [13]. Subsequent search for additional inhibitors of the HMG-CoA reductase led to several synthetic and semi-synthetic compounds currently marketed including atorvastatin and simvastatin, which are the number 1 and 7 best selling prescription drugs in the USA in 2005 [15]. All of the chemical moieties work by the same mechanism of action: they inhibit the rate limiting step of the endogenous cholesterol biosynthesis, the conversion from HMG-CoA in
mevalonate by the HMG-CoA reductase (Figure 1-3), resulting ultimately in the reduction of overall produced cholesterol [13, 16].

**Statin Metabolism and Pharmacokinetics**

Simvastatin and lovastatin are administered as the inactive prodrug which must be hydrolyzed *in vivo* to the corresponding β-hydroxy acid form to achieve activity. All other statins are dosed as the active open β- hydroxy acid form [4, 16]. Differences also exist in their physical-chemical properties. Atorvastatin, lovastatin and simvastatin are lipophilic drugs, whereas pravastatin and fluvastatin are hydrophilic drugs (Figure 1-4). Lennernaes and Fager reported in 1997 that simvastatin and lovastatin cross the blood brain barrier in their inactive lactone form [17]. The distribution of statins into the CNS is dependent on lipophilicity as reported by Sirtori et al. [18]. Furthermore Vickers et al. [19] were able to measure brain simvastatin levels both after intravenous and oral administration.

The pharmacokinetic properties of statins show a high degree of variation when administered via the oral route. Therapeutic doses range from 5-80 mg/day (simvastatin), 10-80mg/day (atorvastatin, lovastatin), 10-40mg/day (pravastatin) to 20-40 mg/day (fluvastatin). Mean LDL cholesterol lowering ranges from 21-42% (lovastatin), 22-25% (fluvastatin), 22-34% (pravastatin), 26-47% (simvastatin) up to 43-60% (atorvastatin) [20]

The inactive lactone forms of simvastatin and lovastatin are readily converted *in vivo* into the active open acid form (Figure 1-5). Gender differences exist in the conversion rates of lactone to acid in rats for simvastatin [19]. The same has been shown in healthy volunteers for simvastatin and lovastatin [21]. Both the lactone [22, 23] and the acid [24] form of simvastatin undergo extensive metabolism (Figure 1-6) by CYP 3A resulting in more than 10 metabolites. Furthermore lovastatin and atorvastatin are known CYP3A substrates [4]. The acid form of simvastatin can further undergo beta-oxidation [19, 25]. Lovastatin [26, 27] and pravastatin [28-
are known p-glycoprotein substrates. Due to its extensive first pass metabolism, bioavailability of simvastatin and lovastatin is below 5%. Pravastatins low bioavailability of 18% despite the lack of CYP3A metabolism can be explained by its poor mucosal absorption [4]. Additionally, the acid form can relactonize in vivo (Figure 1-7) [31, 32]. Metabolism of both active and inactive form takes place in the enterocyte as well as in the liver and can lead to a variety of active and inactive metabolites [19]. The small intestine is therefore a potential site for drug-drug or drug-food interactions.

**Adverse Events**

As mentioned before, the mean decrease of LDL cholesterol can be as high as 60%. The large number of statin prescriptions [15], revealed more markedly the potential of this drug class for a rare but severe adverse event. Statins can be associated with diffuse myalgia, myopathy and rhabdomyolysis. Myalgia is defined as muscle complaints without creatine kinase (CK) level elevation. Myopathy is traditionally defined as muscle pain or weakness accompanied by creatine kinase levels 10 times above the upper limit of normal. Myopathy can progress to rhabdomyolysis (CK levels higher than 10 times the normal range) and result in renal failure but rhabdomyolysis does not have to be preceded by myopathy. Determination is complicated since definitions for myopathy and rhabdomyolysis vary. The National Lipid Association’s (NLA) Muscle Safety Expert Panel has been charged to examine the definitions and causative factors of statin myopathy [33]. These severe side effects are estimated to occur in less than 1 per 100,000 prescriptions [20]. Thompson et al. [34] found a total of 3339 cases of rhabdomyolysis when reviewing the FDA database from 1990 to 2002. Interestingly 57% of the cases where assigned to cerivastatin, 18% to simvastatin and 12% to atorvastatin. It is also noteworthy that approximately half of the cases occurred in 51-75 year old patients, with an additional 17% occurring in patients older than 75 years. According to Dresser et al. myalgia and
rhabdomyolysis seems to occur in conditions where the plasma concentrations of both parent
drug and metabolite are elevated [35] and is generally dose related. Conversely cases have been
reported where myopathy occurs without elevated plasma levels of creatine kinase [36].

The Grapefruit (Citrus paradisi)

Botany

The grapefruit tree (7-10m large) belongs to the genus Citrus, Rutaceae. It bears white
flowers and its fruits are a modified berry (Figure 1-8) [37]. Even though much controversy
exists regarding the classification of the genus Citrus, the main focus here should not be the
history of the Citrus genus, but the origin of the grapefruit and appearance in Northern America.
Research suggests that centuries after the first mentioning of the citrus genus in south-east Asian
culture, the oranges, lemons and sweet oranges reached Europe [38]. It is believed that
Columbus brought the first Citrus biotypes to parts of the Caribbean. Furthermore the English-
Dutch speaking territories of the Caribbean seem to have undertaken the cultivation of another
plant of the Citrus genus, the Citrus grandis [39]. Biochemical [40] and genetic analysis [41]
suggests indeed, that the grapefruit originated as a cross between Citrus grandis and Citrus
sinensis. Furthermore, Scoras analysis of the amylase patterns in C. paradisi strengthens the
assumption that it is a cross between C. grandis and C. sinensis [38]. These results seem to
match the historical descriptions from James Macfayden who in 1837 first assigned the name C.
paradisi to the forbidden fruit described prior by Griffith Hughes in 1750 and Patrick Browne in
1789. Even though some contradiction exists between Hughes’ and Browne’s description of the
fruit, the colloquial name ‘Barbados grapefruit’ indicated clearly the origin of this fruit.

Grapefruit Economy

It is unclear as to when the grapefruit was introduced to the northern part of the American
continent, but indicators exist that Citrus fruits were brought to Florida, most likely St.
Augustine, around the year 1565 laying the foundation for the Florida Citrus economy [38].

Other researchers believe that the grapefruit was brought to Florida by Count Odette Phillip, who settled close to Tampa Bay [37]. Florida seems to be one of ideal regions for the Citrus cultivation, since times where temperatures reach the fruit freezing temperature are rare and temperatures in general are in the interval of optimal growth. Citrus trees do not grow when the air temperature reaches lower than 50°F and growth is imperceptible when the air temperature is above 95°F [42]. It is noteworthy, that in case of grapefruit marketing the Duncan grapefruit is predominately sold in the first five months of the harvesting period, however sales are shifted towards the Marsh Seedless grapefruit in later time points [43]. The main percentage of the Florida grapefruit harvest is processed to frozen concentrate or chilled juice. In total, the United States of America produce a quarter of the world’s grapefruit production of which Florida contributes 13.5% of the world grapefruit crop. Despite the increase in personal disposable income however, the per capita grapefruit consumption has decreased by 50% since 1999[44].

Health Benefits

Already in the year 1928 Bertha M. Wood included a pear and grapefruit salad in her Dietetic treatment of Hypertension [45]. Even today consumers of grapefruit and grapefruit juice believe in the beneficial attributes for their health. In fact 14% of grapefruit juice consumers believe that grapefruit juice can prevent heart disease and 21% of the consumers believe that grapefruit juice can lower cholesterol [46]. In 2006 Gorinstein et al. investigated the effect of red grapefruit on serum cholesterol levels and found that the consumption of grapefruit over thirty days can lower serum total cholesterol, LDL-cholesterol and triglyceride levels in hyperlipidemic patients [47]. Intake of 200 mL juice daily for four weeks lowered cholesterol by 9%, low density lipoprotein cholesterol (LDL-C) by 21% and triglycerides by 25% [48]. It could furthermore been shown that a diet supplemented with grapefruit or flavonoids contained in
grapefruit can increase the plasma antioxidant potential [49, 50], effects which have also been attributed to sweetie [48, 51]. Research suggests, that the flavonoid naringenin, which is also contained in grapefruit, inhibits the apolipoprotein-B (apoB) secretion in cell models [52-54], whereas naringin inhibits 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase and acyl-CoA:cholesterol acyltransferase (ACAT) in rats [55]. Furthermore the grapefruit industry cooperated with the American Heart Association and launched the heart healthy campaign certifying grapefruit juice with the “heart check” mark for certain nutritional levels.

**Grapefruit Juice-Drug Interactions**

**Discovery**

The combination of facts about grapefruit related and drug related health benefits can be intriguing for the consumer, who might consume his drug together with grapefruit juice or consume grapefruit juice during the day to achieve maximum beneficial effect, because of the 1989 finding that grapefruit juice can increase the oral bioavailability of certain medications. This was originally discovered by Bailey *et al.* [56] who were investigating the interaction between the calcium channel antagonist felodipine and ethanol. In this study grapefruit juice was used to mask the taste of ethanol. After the discovery of this interaction more than 160 scientific papers have been published concerning grapefruit juice drug interactions.

**Mechanism of Action**

It has been shown that the predominant mechanism for this interaction is the irreversible inhibition of intestinal Cytochrome P450 3A4 (CYP 3A4) enzyme with a marked decrease in presystemic metabolism [57]. This is of great interest to the community of health professionals since roughly 60% of the drugs available to patients are metabolized to a greater or lesser extent by CYP 3A [58, 59]. In addition, another mechanism has been proposed, potentially leading to
increased fractions of drug absorbed by inhibition of the enterocytic efflux transporter P-glycoprotein (Pgp) [60].

The increase in fraction absorbed however is limited to presystemic metabolism in the enterocyte. In most cases, studies have shown that half-life of the drug under investigation does not change and the interaction does not change liver metabolism. This is further confirmed by a study comparing the raise in felodipine concentrations after intravenous and oral dosing concomitantly with grapefruit juice. No significant increase in exposure could be found after intravenous dosing of felodipine with oral consumption of grapefruit juice [61].

The aforementioned effects seem to be dependent on individual patient variability, batch, amount, and type of grapefruit juice, as well as dosing schedule [62]. This can be shown when comparing two studies, investigated by the same group of researchers. Lilja et al. [63-65] performed two clinical trials assessing the interaction of simvastatin and grapefruit juice. In the first study, double strength (DS) grapefruit juice (grapefruit juice frozen concentrate diluted with half the regular amount of water) was given three times a day for three days. This resulted in a 13 fold increased plasma levels of simvastatin. Years later a similar study performed with regular strength (RS) grapefruit juice resulted in only a 3.5 fold increase in simvastatin plasma concentrations. The effect of the interaction can last up to 3 days and has been extensively studied after single dose administration of drugs. Available sources for long term effect studies after multiple drug administration however are limited.

**Compounds of Interest**

Furanocoumarins and flavonoids are currently suggested to play an active role in the inhibition of intestinal CYP 3A4 by grapefruit juice. 6’,7’-Dihydroxybergamottin (DHB) and a furanocoumarin dimer have been identified to be inhibitors of this CYP enzyme [57]. In vitro experiments also confirmed the potential of naringin (NAR) and naringenin (NAG) as an
inhibitor of CYP 3A4 in humans (Figure 1-9). Concentrations of 50 µM showed a significant
decrease in simvastatin intrinsic clearance in rat hepatocytes [66]. Bergamottin (BG) has also
been reported to have the same inhibitory activity as naringenin [67]. Furthermore some studies
with furanocoumarin free grapefruit juice have shown that furanocoumarins account for some
but not all of the inhibitory effect [68]. Roughly one third of the inhibition could be attributed to
the concentrations of DHB and BG (Figure 1-10). Further inhibitory potential is attributed to
DHB dimers, epoxides and orthospirioesters. Tassaneeyankul et al. [69] determined the content
of the two tail/tail dimers (Figure 1-11) to range from not detectable to 0.39 ppm for GF-I-1 also
called Paradisin A and 0.08-0.44 ppm for GF-I-4 Paradisin B. Guo et al. [70] quantified the
contents of the BG epoxide and the DHB orthospirioester. Concentrations of the epoxide resulted
to vary from 0.17-0.27 ppm and the orthospirioester ranged from not detectable to 6.31 ppm. The
dimers have great potential for CYP 3A4 inhibition. GF-I-1 and GF-I-4 seem to be potent
inhibitors in human liver microsomes, with K_I of 40.0 µM and 5.56 µM respectively [71]. When
compared to DHB, GF-I-1 seems to be a far more potent inhibitor of CYP 3A4 with IC_{50} values
of 0.075 µM compared to 0.45 µM [72]. Epoxibergamottin showed slightly greater inhibition
potential that DHB with a reported IC_{50} of 0.33 µM [73]. No definitive data about the inhibition
potential of grapefruit spiroesters has been published so far, however in 2000 Bioavailability
Systems, LLC received a patent for the development of CYP 3A4 inhibitors that resemble
orthospirioesters from grapefruit juice to increase the bioavailability of CYP 3A4 substrates [74].

**Influence of Grapefruit Juice on Statin Pharmacokinetics**

The effects of high doses of statins regarding muscle toxicity have been extensively studies
and the risk has been identified [34]. It is particularly worrying that patients and consumers
attribute cholesterol lowering and cardio-protective properties to grapefruit juice consumption
and take their cholesterol lowering drug and grapefruit juice concomitantly at breakfast in order
to maximize the effect. The Food and Drug Administration (FDA) classifies grapefruit juice as a moderate inhibitor of CYP 3A metabolizing enzymes which can lead to a 2 to 5 fold increase in drug concentrations. However the FDA also notes that the effects of grapefruit juice vary widely [75]. Interestingly recent experiments have shown that components of grapefruit juice also seem to exhibit an esterase inhibiting effect. Influence on lovastatin metabolism has already been shown [76, 77] and might also be true for simvastatin.

The effects of grapefruit juice on the pharmacokinetic properties of statins have been shown by a battery of clinical trials. The degree of increase in plasma AUC correlates with the bioavailability of the respective drug. Simvastatin and lovastatin, both exhibiting a bioavailability lower than 5%, show the greatest degree of interaction.

The highest degree of interaction was discovered when healthy subjects were administered double strength grapefruit juice three times a day over a period of three days. The AUC after a single dose of 60 mg simvastatin and simvastatin acid increased 16 fold and 7 fold respectively. Changes in C$_{\text{max}}$ were 9 fold and 7 fold respectively. An assessment of active and total inhibitors in the plasma revealed an increase of 2.4 fold for the former and 3.6 fold for the later [63]. A later study performed with regular strength grapefruit juice and 40mg of simvastatin resulted in smaller increases in AUC and C$_{\text{max}}$. Changes were 3.6 fold and 3.9 fold for simvastatin and 3.3 fold and 4.3 fold for simvastatin acid. Active or total inhibitors were not assessed in this trial [65]. The half life decreased slightly in the later but not in the former clinical trial. Significant changes after a single dose of grapefruit juice were measured up to 3 days. Changes in half-life however could also be attributed to simvastatins own potential to inhibit CYP 3A4 metabolism [78].
The extent of increase in pharmacokinetic parameters of lovastatin when taken with grapefruit juice varied greatly with the type of juice administered. Double strength grapefruit juice resulted in increases of 12 fold and 15 fold in lovastatin \( C_{\text{max}} \) and AUC respectively after an 80mg dose. Changes for lovastatin acid were 4 fold and 5 fold for \( C_{\text{max}} \) and AUC respectively [79]. Regular strength juice and a dose of 40mg led to approximately 2 fold increases in lovastatin \( C_{\text{max}} \) and AUC and to an increase of 1.6 fold in lovastatin acid \( C_{\text{max}} \) and AUC. The AUC of both active and total inhibitors increased around 1.3 fold [80].

Changes in atorvastatin acid, which has a bioavailability of 12% [4] were far less pronounced. Increases in atorvastatin acid plasma exposure (AUC) range from 1.4 fold [81] and 1.8 fold [82] with single strength grapefruit juice to 2.5 fold when given with double strength grapefruit juice [83].

Statistically significant though small increases in AUC were also found with pitavastatin [82].

As expected, grapefruit juice had no effect on pravastatin, since pravastatin is not a substrate for CYP 3A [81, 83].

Company information and trials with other CYP 3A inhibitors suggest that fluvastatin [84] and rosuvastatin[85, 86] are not substrates for this metabolizing enzyme. It is therefore unlikely that they might interact with grapefruit juice.

Since only the enterocytic metabolism is affected by the grapefruit juice-drug interaction, metabolic ratios might be shifted and might lead to different concentrations of active and inactive metabolites. This has been shown by assessing the amount of active inhibitors in human samples after grapefruit juice and statin administration. Levels of active inhibitors were far less elevated as one would expect from the increase in simvastatin levels [63].
Hypothesis and Objectives

Grapefruit juice has been shown to increase exposure of some drugs. The effect however varies greatly. Furthermore, the potential for clinically significant drug interaction is most likely limited to low bioavailability drugs or drugs given at the higher end of the therapeutical range. We hypothesize, that most of the investigated drugs do not interact in a clinically relevant manner with grapefruit juice (GFJ) and that the degree of the interaction is dependent on the batch and strength of the grapefruit juice. Regarding the interaction with the cholesterol lowering drug we hypothesize, that the interaction of grapefruit juice with simvastatin is not clinically relevant and in low doses does not lead to greater muscle damage than simvastatin alone. Furthermore we expect that grapefruit juice can lower cholesterol as it has been shown with pomelo juice.

To test these hypotheses, the following specific aims were proposed:

Specific Aim 1

Create an online database summarizing all published clinical trials to assess the overall interaction potential. A comprehensive, dynamic, and expandable database was created covering prescription and OTC drugs with tailored information for patients and health care professionals.

Specific Aim 2

Assess the magnitude to which GFJ changes the simvastatin plasma levels in rats and atorvastatin levels in humans after long term use. The magnitude of the interaction was assessed in a time and dose dependent manner. The study was performed over a 4-week period using two different strengths of grapefruit juice and two different doses of simvastatin.

Specific Aim 3

Assess the potential risk of myopathy development in rats due to the grapefruit juice-simvastatin interaction. A mid-term study over four weeks was performed, using behavioral,
physiological, biochemical, and histological markers to assess the potential development of myopathy after the alteration of the metabolic profile.
Figure 1-1. Causes of death in the year 2004 as reported by the US Census Bureau [1].

Figure 1-2. Top 10 selling prescription drugs in 2006 (total dollars in billions) [3]
Figure 1-3. Mevalonate pathway in mammalian cells [16]
Figure 1-4. Structures of HGM-CoA reductase inhibitors compared to mevalonic acid [4]
Figure 1-5. Structure of simvastatin and simvastatin acid [23]
Figure 1-6. Metabolism pathway of simvastatin SV=simvastatin, SVA=simvastatin acid, I=6'-OH-SV, II=3’-OH-SV, III=3’’-OH-SV, IV=6’-exomethylene SV, V=6’-CH2OH-SV, VI=6’-COOH-SV, VII=1’’,2’’,6’’,7’’,8’’,8a’’-hexahydroxy-2’’,6’’-dimethyl-8’’-(2’’,2’’-dimethyl-1-oxobutoxy)-1’’naphthalene-pentanoic acid [23]
Figure 1-7. Proposed mechanism of the relactonization of simvastatin acid into simvastatin [32]
Figure 1-8. *Citrus paradisi* Macfaden (grapefruit)
Figure 1-9. Compounds in grapefruit juice shown to interact with CYP 3A4, NAR=naringin, NAG=naringenin

Figure 1-10. Compounds in grapefruit juice shown to interact with CYP 3A4, BG=bergamottin, DHB=6',7'-dihydroxybergamottin
Figure 1-11. Structures of DHB Tail/Tail dimers identified in grapefruit juice
Bergamottin-6',7'-epoxide (GF-I-5)

GF-I-6

Figure 1-12 Structure of BG epoxide and DHB orthospiroester
CHAPTER 2
ASSESSMENT OF THE OVERALL INTERACTION POTENTIAL OF GRAPEFRUIT JUICE

**Background**

So far there have only been two case reports involving statins and potentially severe consequences of grapefruit consumption [87]. As of now, there is only one case report involving a woman that developed severe muscle damage after starting to consume one grapefruit a day [88]. Her simvastatin dose was on the higher end of the therapeutic spectrum. Another case report involved atorvastatin [87]. This patient reported severe fatigue and muscle pain after exercising and consumption of grapefruit juice with his cholesterol lowering medication. Case reports like the above mentioned can only provide limited information regarding the clinical relevance of this interaction. However the mass media impact of those case reports can have a high consumer impact. Regularly public mass media companies report about grapefruit juice drug interactions. However the public awareness intentions of multiple major television stations also publicize common misconceptions and misunderstandings. To clarify the interaction potential, a MSNBC reporter said that grapefruit juice can double your drug concentration by as much as three times [89].

In contrast to the mass media publication of the grapefruit juice drug interactions there has been a large quantity of scientific publications that focus on pre clinical, clinical or mechanistic investigation of this interaction. It is challenging however, to find this information especially for consumers of grapefruit juice who are not also healthcare professionals, since for them scientific database are largely unavailable or the information presented on the internet is not comprehensive and does not show the overall picture or does not include all drugs.
Specific Aim

Create an online database summarizing all published clinical trials to assess the overall interaction potential. A comprehensive, dynamic, and expandable database was created covering prescription and OTC drugs with tailored information for patients and health care professionals.

Material and Methods

To create a comprehensive database of all relevant publications regarding the grapefruit juice drug interactions, database searches in PubMed, Web of Sci, Physicians’ Desk Reference, Clinical Pharmacology database, WebSpires and Google were performed. The search terms included ‘grapefruit’, ‘grapefruit juice’, a combination of either with ‘drug interaction’ or a combination of a drug know to interact or ‘citrus paradisi’. Furthermore searches in review articles, books (Drug Interaction Facts 2004 [90]) and other web databases focusing on grapefruit drug interactions were performed. All papers found were categorized into in vitro and in vivo experiments, where in vivo experiments were subcategorized in clinical trials and animal experiments. In cases where no human in vivo data was available, animal in vivo data was considered. Each compound was assigned an interaction level according to Bjornson et al.: weak or no interaction < 2.0-fold, moderate interaction > 2.0- to 4.9-fold, strong interaction > 5-fold increase in plasma AUC after grapefruit juice administration [91]. When different studies resulted in a categorization of two categories, the higher category was chosen for safety reasons.

The database was then programmed in MySQL (MySQL AB, Sweden). The programming for the user frontend was performed in PHP4.

The one page summaries were written in MS Word and converted to PDF using Adobe Acrobat Professional. Webalizer 2.01 was used to record the websites access statistics.
Database Design

Programming Language

MySQL is a free, open source Relational Database Management System (RDBMS), which means that the data is organized and stored in a set of related tables. MySQL is licensed via the GNU GPL (General Public License). In order for a MySQL server to understand a request sent by a user, the command needs to be communicated using the Structured Query Language (SQL). In order to send the commands to the MySQL server we use the PHP scripting language. PHP stands for PHP:Hyper Text Preprocessor. PHP however does not understand SQL commands; it just establishes the connection to the MySQL server and sends the SQL message over that connection [92]. Through the combination of MySQL and PHP scripting language it is possible to create truly dynamic websites. This means that not every user will see the same website and website are tailored towards the users need. This enables us to create differentiated websites for health care professionals or for grapefruit juice consumers out of the same database.

The principle of a dynamic website is illustrated in figure 2-1. The visitor of the website requests a certain dataset. The MySQL server receiving that request via PHP then accesses the database to gather the requested data. The data is then merged with a previously determined website template and sent to the user computer. The advantages of this principle are clearly visible. Dynamic web programming enables us to update the database without changing the overall website template, or to change the website without having to reprogram the dataset.

Database Layout

As mentioned before, MySQL is a Relational Database Management System, meaning that the data is stored and organized in sets of related tables. A graphical illustration of the Table layout can be found in figure 2-2. The database layout consists of three different tables. Table 1 contains the information about the drug categories. Each category is assigned a unique category
identification (ID) number. The drug category table contains the information fields category name (c_name) and category general information (c_info).

Table 2 is the table that lists the information for a specific drug in each category. Again, each drug is assigned a drug identifier (ID). Table 2 contains the information fields drug name (d_name), drug brand name (d_product), drug pharmacokinetic information (d_pkinfo), drug pharmacodynamic information (d_pdinfo), a differentiated information text for consumers (d_textconsumer) or health care professionals (d_textprofessional) and an identifier for the classification in different drug interaction categories (d_interactionlevel). In order to link this table to the previously defined drug category, a category reference field (categoryID) is also contained in table 2.

To deliver the most comprehensive information on grapefruit juice drug interactions, one page summaries of the respective literature were created and published together with the respective pubmed link and fulltext for Center members. Table 3 of the database stores the relevant information of the studies, the study title (s_title) the hyperlink to the study summary (s_summary), pubmed abstract (s_pubmed) and fulltext pdf (s_fulltext). The studies are crosslinked to the respective drug in table 2 using a drug identification reference field (drugID).

One disadvantage of the RDBMS system is that the table layout has to be implemented before the data is input into the database. Once the dataset is created, changes of the database structure are difficult and may result in manually reentering the data.

**Results**

The review of online databases and current literature resulted in a dataset of 74 drugs. A total of 163 scientific publications were reviewed and summarized in simple one page summaries, consisting of a reference section, a material and methods sections, a results section and a conclusions section (Figure 2-3). The database is currently online under
www.druginteractioncenter.org. Out of the 74 drugs analyzed, 51 (69%) drugs showed a weak or no interaction with grapefruit juice, 14 drugs (19%) showed a moderate interaction with grapefruit juice and 6 drugs (8%) showed a strong interaction with grapefruit juice. 3 drugs (4%) have not been studied in humans (Figure 2-4). The necessity of a comprehensive online database can be reflected in the great number of hits that the website received. A total of roughly 9 million hits were registered on the website. A total of more than 1.5 million pages were submitted to the users. Results of the access parameters can be found in Figure 2-5. Rough estimation of returning visitors shows a relatively stable number of users accessing the website multiple times (Figure 2-6).

Interaction Potential by Drug Category

Antiallergics

Several clinical trials have been performed assessing the interaction potential of grapefruit juice with antiallergics. Studied drugs include terfenadine [93-96], desloratadine [97] and fexofenadine [97]. Exposure for desloratadine was not altered when the drug was consumed concomitantly with grapefruit juice, whereas the AUC of fexofenadine was decreased. Three performed studies showed an increase in terfenadine exposure when administered simultaneously with grapefruit juice[94-96]. The reported effects seem improbable to be of high clinical relevance.

Antibiotics

Three antibiotics where studied in the published literature: clarithromycin [98], erythromycin [99] and telithromycin [100]. Overall the clinical relevance of the interaction with grapefruit juice appears to be low.
**Anticoagulants**

The data resulting from 4 published clinical trials is inconsistent. Merkel et al. [101] found the percentage of 7-hydroxycoumarin, a metabolite of coumarin excreted in the urine, to be decreased, controversially in a second study the appearance of the metabolite in urine was delayed, but the recovery remained unchanged[102]. The delay in appearance of the metabolite has been confirmed in a third study [103]. No interaction with warfarin has been reported [104]. Currently, no clinically relevant interactions have been confirmed for this drug class. More extensive clinical studies are necessary to assess the overall effect of GFJ on this drug class.

**Antimalaria drugs**

Grapefruit juice has been reported to increase the exposure of artemether [105, 106] and halofantrine [107]. The increased exposure of artemether did not result in bradicardia or changes in QTc interval. No changes in pharmacokinetic parameters were registered during simultaneous administration of quinidine [108, 109] or quinine [110]. Though no changes in pharmacokinetic parameters were observed, the QTc interval in the grapefruit juice quinine group was prolonged at the 1 hour timepoint after drug administration. According to the aforementioned drug classification the interactions with quinidine and quinine would be considered weak and unlikely to be clinically relevant, the interaction with halofantrine however might result in a change in clinical effect.

**Antiparasitic drugs**

Moderate and weak interaction were reported for albendazole [111] and praziquantel respectively [112]. A clinical significant interaction with praziquantel seems unlikely; however grapefruit juice should only be consumed with albendazole after a careful risk benefit assessment.
Sedative-hypnotics

Concurrent administration of grapefruit juice and alprazolam [113] did not result in changes in pharmacokinetic parameters during a single dose experiment. Midazolam [114-117], triazolam [117-121] and quazepam [120], when administered concomitantly with grapefruit juice exhibit minor exposure increases. In a recent clinical trial with triazolam, the acute and extended exposure to GFJ produced a significant inhibition of enteric, but not hepatic, CYP3A4 and also caused significant pharmacodynamic effects [121]. Apparently clinically relevant interactions have also been observed for buspirone [122] and diazepam [123]. More clinical trials are necessary to definitively assess the extent of the interaction for this drug class.

Calcium channel blockers

Amlodipine [124, 125], diltiazem [126, 127], nimodipine [128], nifedipine [129-131], pranidipine [132] and verapamil [133-135] were shown to interact weakly with GFJ. However, only for amLodipine, diltiazem, nimodipine and verapamil no changes in pharmacodynamic parameters heart rate and blood pressure were reported. No pharmacodynamic alterations were reported for nifedipine and GFJ. Felodipine [61, 124, 133-146], nicardipine [147], nisoldipine [148, 149], nitrendipine[150] and manidipine [151] moderately interacted with GFJ. Changes in pharmacodynamic parameters were reported after GFJ administered with felodipine. Overall, felodipine, nicardipine, nisoldipine and nitrendipine containing products should only be consumed with GFJ after a cautious risk and benefit assessment.

HIV protease inhibitors

Amprenavir [152] and indinavir [153, 154] pharmacokinetic parameters did not change when the drugs were administered concomitantly with GFJ. The AUC of saquinavir was increased by GFJ [155, 156]. However, overall the reported interactions can be considered weak and are unlikely to be clinically relevant.
HMG-CoA reductase inhibitors

Increased exposures were reported for atorvastatin [81-83], lovastatin [79, 80] and simvastatin [10, 63-65], but not for pravastatin [81, 83]. Small but statistically significant changes were found for pitavastatin [82]. Atorvastatin exhibited a moderate interaction with GFJ regarding the overall exposure. Lovastatin and simvastatin exhibited a strong interaction. Pravastatin or pitavastatin could be chosen as an alternative drug if clinically recommendable and patients want to ensure a lack of interaction.

Hormones

GFJ did not affect 17-beta estradiol [157]or prednisone [158] pharmacokinetics. AUCs were weakly elevated for ethinylestradiol [159]and methylprednisolone [160]. A decreased morning cortisol plasma concentrations was found after methylprednisolone administration with GFJ. The absorption of levothyroxine was mildly decreased after repeated consumption of grapefruit juice, however here the authors conclude a low clinical relevance [161]. Anecdotal reports that grapefruit juice may cause contraceptives to lose their effect could not be confirmed by scientific studies.

Immunosuppressants

Eleven out of a total of thirteen studies reported increased cyclosporine exposure up to 2-fold [162-172]. Conversely, two studies reported no GFJ-induced change in AUC. Overall, the clinical significance of interactions is assumed to be low [158, 173].

Antitumor drugs

Few studies have been performed with human subjects for this drug class. The mean AUC of etoposide was increased when administered concomitantly with GFJ [174]. However, the report does not state the statistical significance of the reported data. In order to develop recommendations for this drug class further research will have to be conducted.
Over the counter drugs

Contradicting results were reported when caffeine was administered with GFJ. Whereas no changes in AUC, blood pressure, and heart rate were reported by Maish et al. [175], a second study found increased AUC and half life, without an assessment of pharmacodynamic parameters [176]. Overall, no clinically significant interactions of an over-the-counter drug with GFJ have been reported.

Beta receptor blocker

AUC and Cmax of celiprolol decreased by 95%, when administered concomitantly with GFJ [177], however heart rate and blood pressure remained unchanged. Conclusively, observed interactions to be clinically relevant. The plasma concentrations of acebutolol [178] were slightly decreased by grapefruit juice. Further human studies will have to be conducted in order to derive reliable conclusions. Currently, clinically significant interactions appear to be unlikely.

Antiarrhythmics

Coadministration of amiodarone [179] with regular strength GFJ resulted in a significant but weak increase in AUC and Cmax, however a decrease in the alteration on the PR and QTc interval was observed. These data imply that not only pharmacokinetic data should be considered when determining the extent of the significance of a given GFJ-drug interaction. Overall, the changes in pharmacodynamic parameters should be taken into account when prescribing this drug to patients who wish to consume GFJ.

Other drugs

GFJ has shown to increase the exposure of carbamazepine [180], cisapride [181-184], fluvoxamine [185], losartan [186], methadone [187], scopolamine [188], sertraline [189] and repaglinide[190]. However, only the interaction of GFJ and carbamazepine and cisapride appear to be clinically relevant. No alteration in exposure was observed for clozapine [191, 192],
theophylline [193] and haloperidol [194], omeprazole [69] and phenytoin [195]. Contradicting results were reported for itraconazole [196-198] digoxin [199, 200], and sildenafil [201, 202].

**Conclusions**

The results of the creation of this database support the importance of a comprehensive online database. Additionally a dynamic, updateable MySQL database combined with PHP scripting seems to be the ideal tool for patient and health care professional information and education. The amount of visitors of the online database strengthens the need for up to date scientific information. The work performed demonstrates that the current conception that most of the drugs interact with grapefruit juice cannot hold. On the other side however is it also unsafe and irresponsible to say that patients can consume grapefruit juice to lower their drug dose and reduce drug costs. Strong indicators are present that suggest a need for the measurement of certain furanocoumarins and flavonoids in the juice prior to conducting a study and report those results in the literature to achieve comparable results.
Figure 2-1. Principle of a dynamic website.
Figure 2-2. MySQL table layout for the drug interaction website
**Cyclosporine**

*In Vivo Study*

<table>
<thead>
<tr>
<th>Drug</th>
<th>Article</th>
</tr>
</thead>
</table>

*Title:* 7-Dihydroxybergamottin in grapefruit juice and Seville orange juice: Effects on cyclosporine disposition enterocyte CYP3A4, and P-glycoprotein


**Methods**

*Subjects:* five men and two women; age range – 23 to 41 years

*Study design:* randomized crossover study

*Dose:* 7.5 mg/kg cyclosporine

*Food parameters:* Subjects received a single oral dose of cyclosporine with 8 ounces of water, grapefruit juice, and Seville orange juice. Each treatment was separated by a 1-week washout period. After an overnight fast, subjects ingested water or juice and 3 ¼, 7 ¼, and 11 ½ hours after the cyclosporine dose.

**Results**

AUC and C<sub>max</sub> were increased by 55% and 35%, respectively, with grapefruit juice compared with water. Seville orange juice had no influence on cyclosporine disposition but reduced enterocyte concentrations of CYP3A4 by an average of 40%.

6. 7-Dihydroxybergamottin did not inhibit P-glycoprotein at concentrations up to 50 μmol/L.

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>Gender</th>
<th>Water</th>
<th>Grapefruit juice</th>
<th>Seville orange juice</th>
<th>Water</th>
<th>Grapefruit juice</th>
<th>Seville orange juice</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Male</td>
<td>6,561.4</td>
<td>13,190.0</td>
<td>8,419.0</td>
<td>1,114.0</td>
<td>1,666.0</td>
<td>1,197.0</td>
</tr>
<tr>
<td>2</td>
<td>Female</td>
<td>3,220.5</td>
<td>5,415.0</td>
<td>5,157.0</td>
<td>875.0</td>
<td>916.0</td>
<td>367.0</td>
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<tr>
<td>3</td>
<td>Male</td>
<td>7,866.0</td>
<td>13,586.5</td>
<td>7,875.0</td>
<td>689.0</td>
<td>1,481.0</td>
<td>1,043.0</td>
</tr>
<tr>
<td>4</td>
<td>Male</td>
<td>8,022.2</td>
<td>6,395.5</td>
<td>5,969.1</td>
<td>1,275.0</td>
<td>1,185.0</td>
<td>1,129.0</td>
</tr>
<tr>
<td>5</td>
<td>Female</td>
<td>8,311.0</td>
<td>10,289.5</td>
<td>7,302.5</td>
<td>1,096.0</td>
<td>1,300.0</td>
<td>402.0</td>
</tr>
<tr>
<td>6</td>
<td>Male</td>
<td>5,035.5</td>
<td>12,434.0</td>
<td>5,640.0</td>
<td>875.0</td>
<td>1,468.0</td>
<td>965.0</td>
</tr>
<tr>
<td>7</td>
<td>Male</td>
<td>11,800.5</td>
<td>14,336.5</td>
<td>7,616.0</td>
<td>1,688.0</td>
<td>2,289.0</td>
<td>1,407.0</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>6,973.9</td>
<td>10,805.0*</td>
<td>6,981.5</td>
<td>1,087.4</td>
<td>1,471.8*</td>
<td>938.6</td>
</tr>
<tr>
<td>SD</td>
<td></td>
<td>2,732.5</td>
<td>5,392.1</td>
<td>1,911.2</td>
<td>328.0</td>
<td>435.5</td>
<td>384.7</td>
</tr>
</tbody>
</table>

**Conclusions**

6. 7-Dihydroxybergamottin is not responsible for the effects of grapefruit juice on cyclosporine. Because the interaction did not occur with Seville orange juice despite reduced enterocyte concentrations of CYP3A4, inhibition of P-glycoprotein activity by other compounds in grapefruit juice may be responsible. Reduced enterocyte CYP3A4 by 6. 7-dihydroxybergamottin could be more important for other drugs whose bioavailability is less dependent on P-glycoprotein.

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**Figure 1.** Blood concentration - time profile for cyclosporine in subject 1 after ingestion of cyclosporine with water, grapefruit juice, or Seville orange juice.

**Figure 2.** Microfluorometric evaluation of p-glycoprotein inhibition by 6. 7-dihydroxybergamottin (DHB), LLC-PK1 (open squares) and L-MDR1 cells were incubated in the absence (solid circles) or presence of 5 μmol/L (triangle), 10 μmol/L (open circles), and 50 μmol/L (solid squares) 6. 7-dihydroxybergamottin for 15 minutes.

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**Figure 2-3.** Sample of the one page summaries uploaded to the database
Figure 2-4. Number of interaction drugs in each category: 1=no to weak interaction, 2=moderate interaction, 3=strong interaction, N/A=not tested in humans.
Figure 2-5. Website access statistics per month: A=number of hits, B=number of submitted pages, C=number of files, D=number of visits

Figure 2-6. Website access statistics hits-files as rough estimate of returning visitors per month.
CHAPTER 3
EFFECT OF LONG TERM INGESTION OF GRAPEFRUIT JUICE ON THE PHARMACOKINETICS AND TOXICOLOGY OF SIMVASTATIN

Background

Besides cholesterol lowering drugs, it seems that consumers also attribute healthy and cholesterol lowering qualities to grapefruit juice. 14% of grapefruit juice consumers believe that grapefruit juice can prevent heart disease and 21% of consumers are convinced that grapefruit juice lowers cholesterol [46]. Furthermore the grapefruit industry cooperated with the American Heart Association and launched the heart healthy campaign certifying grapefruit juice with the “heart check” mark for certain nutritional levels. This combination of facts can be intriguing for the consumer, who might consume his drug together with grapefruit juice or consume grapefruit juice during the day to achieve maximum beneficial effect, because of the 1989 finding that grapefruit juice can increase the oral bioavailability of certain medications [56]. As mentioned in the previous chapter, grapefruit juice has been shown to alter the disposition of simvastatin in healthy subjects. Lilja et al. [63] demonstrated in 1998 as well as 2004 [65] that grapefruit juice can increase the AUC of simvastatin by as much as 13 fold. Lower increases have been found in changes in the main active metabolite simvastatin acid, as well as the influence on active and total inhibitors of the HMG-CoA reductase. Lilja et al. [63] conclude that large amount of grapefruit juice should be avoided, or the dose of simvastatin should be adjusted in order to prevent the increased risk of muscle toxicity associated with increased serum concentration of simvastatin. It remains unclear however if the increase in simvastatin serum concentrations leads in fact to a higher incidence of muscle toxicity.

In experiments performed by Smith et al. [203, 204] this research group showed that doses of 100 mg/kg x day\(^{-1}\) and 150 mg/kg x day\(^{-1}\) do not lead to any detectable muscle damage after 2-4 weeks in Sprague Dawley rats. However a dose of 180 mg/kg x day\(^{-1}\) has been shown to
induce muscle damage ranging from very slight to moderate in 4 out of 15 rats during a subjective evaluation. In contrast to the findings by Smith et al. recently performed experiments by Westwood et al. [205] indicate that muscle damage might be induced in female Wistar Hannover rats with a maximum tolerated dose of 80 mg/kg x day\(^{-1}\). The rats in the dose finding experiment exhibited detectable muscle damage in this dosing group after 10 days ranging from mild to severe depending on the muscle under investigation. A dose of 60 mg/kg x day\(^{-1}\) however showed no muscle necrosis after 43 days. It is widely believed that the occurrence of myopathy is correlated to the inhibition of the HMG-CoA reductase. Experiments have shown that a dietary substitution of mevalonate, the subsequent reaction product of the HMG-CoA reductase can reduce the severity of muscle damage [205]. On the other hand experiments conducted with 3 week old Wistar rats have shown that muscle damage can also occur at a dose of 100 mg/kg x day\(^{-1}\) in seven out of nine rats ranging from moderate to severe without showing significantly different levels of cholesterol compared to the untreated control [206]. It has also been shown that administration of HMG-CoA reductase inhibitors in rats might not lead to a cholesterol lowering effect in this species. The rat can upregulate the cholesterol synthesis overcoming the effect of the hypercholesterolemic drug, to a point where the cholesterol synthesis rate and cholesterol levels seems normal even in the presence of a competitive inhibitor [207, 208].

Many researchers therefore assume that the high simvastatin exposure after concomitant grapefruit juice consumptions exhibit the same muscle damaging potential as the administration of a regular increased dose. That this fact is not necessarily accurate might be demonstrated by the fact that simvastatin is a prodrug that needs to be metabolized in order to be activated and that the prodrug itself has a low bioavailability [4]. As mentioned before, grapefruit juice only
interacts with the CYP 3A4 in the gut wall and seems to have no influence on liver metabolism (Figure 3-1). Simvastatin however is already metabolized to an extent of almost 95% when reaching systemic circulation to metabolites which account for the gross part of the activity. High doses of simvastatin (as they are reached by increase in simvastatin dosing) thus results in an increased amount of metabolites. High doses of simvastatin that are achieved as a result of an enterocyte sided inhibition of the drug metabolizing enzyme CYP 3A4 however lead to predominantly increased concentrations of the prodrug lactone form of simvastatin which is inactive. It is therefore crucial to investigate the relationship between the frequency of muscle damage after concomitant grapefruit juice simvastatin administration over a increased period of time.

**Specific Aim**

The objective of this study was to assess the magnitude by which GFJ changes the simvastatin plasma levels after long term use in rats. The magnitude of the interaction was assessed in a time and dose dependent manner. The study was performed in rats over a 4-week period using two different strengths of grapefruit juice and two different doses of simvastatin.

Further to assess the potential risk of myopathy development due to the grapefruit juice-simvastatin interaction. A mid-term study in rats over four weeks was performed, using behavioral, physiological, biochemical, and histological markers to assess the potential development of myopathy after the alteration of the metabolic profile.

**Material and Methods**

**Chemicals**

Floridas Natural grapefruit juice Ruby Red was purchased at Publix supermarkets (Gainesville, FL, USA) at a total amount of 2 Gal. Lot: 98 and lot 2A6 and frozen in 250mL aliquots on 06/21/06.
Minute Maid Premium 100% pure frozen concentrate grapefruit juice (with Calcium added) was purchased on 06/23/06 at Publix supermarkets (Gainesville, FL, USA) in amount of 4 cans. Lot AD3 1712 and frozen in 125 mL aliquots after dilution with half the regular amount of water. 1 can was diluted with 532.5 mL regular tap water.

Simvastatin (SV), 99.4% pure, was a kind gift from Merck & Co., Inc. (West Point PA., USA).

Naringin (NAR) and naringenin (NAG), both > 95% pure, were from Roth GmbH & Co. (Karlsruhe, Germany), bergamottin (BG) (> 98% purity) was obtained from Indofine Chemical Company, Inc. (Somerville, NJ, USA), 6’,7’-dihydroxybergamottin (DHB) (> 97% purity) was purchased from Sigma-Aldrich (Saint Louis, MO.). Sodium heparin was purchased from Sigma-Aldrich (Saint Louis, MO).

**Stock and Work Solutions**

Solutions of Simvastatin were made by suspending SV in a 0.8% Carboxymethylcellulose (CMC) solution in water. Suspensions for simvastatin were prepared daily by the scheme in table 3-1. Solutions for NAR, NAG and BG, DHB were prepared according to table 3-2 and 3-3.

**Material and Methods**

**Laboratory animals**

Male Sprague-Dawley weighting 230-270g rats were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN). The animals were housed in conventional housing, two animals per cage. The experiment was approved by the University of Florida Institutional Animal Care and Use Committee (IACUC).

**Open field test**

This method is usually used to evaluate possible sedative or stimulating activities of animals [209]. The test is also used as an indicator for locomotor activity. The open field consists
of a round grey plastic arena measuring 70 cm in diameter surrounded by a grey plastic wall of 34 cm height and is evenly lighted with three 40 W light bulbs. The floor of the arena is divided into several concentric units by black painted lines, dividing the arena into 19 equally sized fields. Each rat will be placed in the center of the arena and recorded for 5 min after the 28 day experiment. The parameters evaluated in this test include number of field crossings and total distance covered by the animal. We assume that drug effects on the muscle would be present if both number of field crossings and distance are significantly lower in the treatment groups compared to control.

Open field tests were digitally recorded using a high-resolution video camera WV-CP244 (Panasonic, Secaucus, NJ, U.S.A). The analysis of the videos was performed using TopScan (Figure 3-2), Top View Animal Behavior Analyzing System (version 1.00, Clever Sys Inc. Preston, VA, U.S.A) by a blinded person.

The open field test arena was thoroughly cleaned with a soap solution after each animal, to avoid influencing factors such as odor left of the previous animal.

**Grip strength test**

This test is used to assess muscular strength in rodents that can be influenced not only by sedative drugs and muscle relaxants but also by toxic compounds. Reliable assessment of gripping ability requires that animals are used to handling. Accordingly, the first 5 sessions of training were limited to handling each animal for 5 min. During the training sessions, animals were held with both hands and placed on the grid of the grip strength meter (Linton grip strength meter for the pilot study, GSM, PANLAB grip strength meter for the final study, distributed by Stoelting & Co, IL, USA figure 3-3). When the animals are placed on the grid, the animals readily hold on to the grid as a natural reflex, and the animals are then gently pulled away from the device. The GSM then measures the maximal force before the animal releases the grip. GSM
testing (3 trials/animal/session) was carried out once per week for the duration of the entire treatment. The area under the grip strength curve was compared.

**Histological experiment**

Muscle biopsies of the gastronemius and the extensor digitorum long muscle were taken on the final day of the experiment. The muscle biopsies were immediately preserved in 10% neutral buffered formalin. After completion of both experiments, the samples were processed to wax blocks and the 5µm thick sections were stained with Haematoxylin & Eosin stain. Transversal and longitudinal sections of the muscle were evaluated. The occurrence of muscle damage was analyzed objectively by a blinded analyst under light microscopy, evaluating eight randomly chosen sections in the muscle. Total muscle cells and damaged muscle cells were counted. The observed sections were equally distributed between transversal and longitudinal sections. The following parameters were used in the assessment of muscular damage:

Variability in size and shape of muscle fibers, muscle fiber striation or loss of striation as well as size and localization of nuclei.

The assessment of muscle damage was performed by assigning the observed damaged muscle cells into four categories: No damage to Minimal – less than 10% damaged fibers, Mild – less than 20% damaged fibers, Moderate – less than 50% damaged fibers, Severe – More than 50% damaged fibers following a similar classification by Westwood et al. [205]

**Clinical chemistry**

Plasma chemistry parameters were evaluated using the Vitalab Selectra II Autoanalyzer (Vital Scientific NV, Spankeren, The Netherlands). Reagent, Control and Calibrator Kits for total cholesterol, gamma-glutamyltransferase (GGT), alanine amino transferase (ALT), aspartate amino transferase (AST) and Creatine kinase (CPK) were purchased from Clinical Data,
Smithfield USA. The Vitalab Selectra II was a kind gift from Steigerwald Arneimittelwerk GmbH (Darmstadt, Germany).

**Grapefruit Juice Analysis**

**HPLC System**

Samples were analyzed by a Shimadzu VP series HPLC system (Kyoto, Japan) equipped with a SPD-M10A VP diode array detector, a LC-10ATvp solvent delivery unit, a SIL-10AF autosampler, a CTO-10A VP column oven, a SCL-10A VP system controller, a DGU-14A on-line degasser, a FCV-10A VP low-pressure gradient unit, and Class VP 7.2 SP1 chromatographic software. Additionally, the peak purity software (Class VP 7.2 SP1 chromatographic software, Shimadzu) was applied to the diode array data to test for impurities in all of the chromatographic peaks of interest.

**Determination of Flavonoids (Naringin and Naringenin)**

The GFJ and the homogenate of tissues (200 µL) were centrifuged at 5000 rpm for 5 min and were mixed with cold methanol (1 mL for DS juices 600 microL for RS juices), vortexed for 1 min, and centrifuged at 2500 g for 15 min, as previously described [210]. After filtration through a 0.45 µm PVDF membrane filter (Millipore Corp., Bedford, MA), the supernatant (25 mL) was injected and analyzed at 285 nm. The flow rate and the temperature were set to 0.5 mL/min and 35 °C, respectively. Mobile phases A and B consisted of water (pH 2.4) (adjusted with orthophosphoric acid) and water (pH 2.4) (adjusted with orthophosphoric acid)/methanol (40:60), respectively. The 250 × 4.6 mm i.d., 5 µm, Lichrospher RP-18 column and Lichrospher 100 RP-18 guard column (Merck KGaA, Darmstadt, Germany) were initially equilibrated during 30 min with solvent A. After sample injection, an initial isocratic run for 5 min was followed by a linear gradient from 100% of A at 5 min to 100% of B at 55 min. This condition was
maintained until 70 min and then returned to 100% of A, which was kept constant during 5 min before proceeding to the next injection.

**Determination of Furanocoumarins (Bergamottin and 6',7'-Dihydroxybergamottin)**

GFJ and the homogenate of tissues (3 mL) were mixed with ethyl acetate (2 mL). The extraction was performed by shaking the mixtures four times over 30 min. The mixture was centrifuged at 3200g for 20 min; the organic phase was collected and evaporated under vacuum. The residue was reconstituted with 600 µL of a DMSO/methanol solution (1:3 v/v) as described previously [62].

The reconstituted residues were filtered through a 0.45 µm PVDF membrane filter (Millipore Corp.). Volumes of 25 µL of each sample were injected and analyzed at 310 nm. The flow rate and the temperature were set to 1 mL/min and 35 ºC, respectively. Solvents A and B consisted of water and methanol, respectively. The column and guard column (as used for flavonoids) were initially equilibrated with mobile phase consisting of a mixture of solvents A and B (45:55), respectively. Twenty minutes after injection, solvent B was increased linearly from 55 to 100% in 20 min. This condition was maintained for 5 min, after which the system returned to the original mobile phase and was equilibrated for a further 5 min before the next injection.

**Animal Experiment Design**

The animal experiment was separated in two parts:

Part 1: a pilot study to assess the detectability of muscle damage in male Sprague Dawley rats after two different doses of Simvastatin

Part 2: a major (final) study to assess the influence of two different concentrations of grapefruit juice on two different doses of simvastatin.
**Pilot Study**

A 4 week animal experiment using two doses of simvastatin was performed. Non-fasted male Sprague-Dawley rats weighing 214-245g were used. Simvastatin was given in doses of 53 mg/kgxday-1 (SV53), 200 mg/kgxday-1 (SV200) both in 0.8% CMC. Water was given as control also with 0.8% CMC. The aim of this study was to assess whether it is possible to detect myopathy after different doses of simvastatin in rats. A total of 24 male Sprague Dawley rats were used. 8 rats were randomly assigned to one of the following three groups and dosed daily (Table 3-1). On each day, we measured the forelimb grip performance using a Linton grip strength meter. Furthermore, the daily body weight was recorded. On the last day the animals were sacrificed and the organs (liver, spleen, kidneys, adrenal glands, heart and testes) were weighted. Additional muscle biopsies from the hindlimb were taken. Two muscles were sampled, the gastronemius and the extensor digitorum long. These muscles have been shown to be susceptible for simvastatin muscle damage in the literature [203, 205]. As potential parameters, the extremity temperature was measured using a non contact infrared thermometer and an open field test (to evaluate potential locomotor impairment) was performed on the last day prior to scarification.

**Final Study**

Non-fasted male Sprague-Dawley rats weighing 239-346 g were used. The animals were handled daily a couple of days before the study starts in order to reduce stress and adapt them to the researcher. Body weight was checked daily as well. The male Sprague-Dawley rats had free access to food and water during the experiment except for the first two hours after drug administration. The animals were divided in the groups shown in table 3-3. On the first day of the experiment the rats were dosed with their respective simvastatin dose (SVlowdose = 20 mg/kgxday-1 (SV20) Simvastatin, SVhighdose = 80 mg/kgxday-1 (SV80) Simvastatin) and their
grapefruit juice permutations (simvastatin and regular strength grapefruit juice; SV20RS and SV80RS, and simvastatin and double strength grapefruit juice; SV20DS and SV80DS). The grapefruit juice lot # 98 was used for regular strength dosing and lot # AD3 was used for double strength dosing. To establish a pharmacokinetic baseline profile, blood samples (500 µl) were taken from the sublingual vein at 0, 1, 2, 4, 6, 8, 12 hours after drug administration (three blood collections per day; e.g. time points 1, 4, 8; then two weeks washout, then the remaining time points 0, 2, 6, 12 in the same animals). After the acute phase two blood samples (1000 µl) was taken each week for 4 weeks right before dosing the drug (trough level) and 2 hours after dosing (peak level) figure 3-4. After each blood sample, approximately 1000 µl of isotonic saline were replaced by i.p. injection in order to maintain the blood fluid. The blood samples were analyzed for Simvastatin, Simvastatin acid. Liver enzymes were also measured to observe the influence of simvastatin on its target organ (the liver) and total cholesterol was measured as an effect parameter.

Muscle strength parameters were measured before blood sampling of each of the acute study days and the chronic study phase.

The animals were fed grapefruit juice (GFJ) (5 mL/kg) at different concentrations (double and regular strength), or water (5mL/kg) and Simvastatin through an oral feeding needle daily. At the end of the study the rats were sacrificed, blood was collected (for determination of simvastatin plasma levels) and muscle samples were taken to assess differences in muscle histology and organs were weight to assess organ changes.

**Statistical Analysis**

Pilot study: One-way ANOVA with Dunnett’s post test was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. Differences were considered statistically significant at P<0.05.
Final study: Samples were analysed using SAS version 9 (Cary, NC, USA). All samples were analyzed using a Two Way ANOVA with a Bonferroni multiple comparison post hoc test to compare treatments to each other. Differences were considered statistically significant at P<0.05, As set a priori, the following comparisons were evaluated:

- All parameters except cholesterol:
  - Water control, SV20, SV20RS and SV20DS
  - Water control, SV80, SV80RS and SV80DS
- Cholesterol:
  - Water control, RS, DS, SV20 and SV80
  - Water control, RS, SV20 SV80 SV20RS, and SV80RS
  - Water control, DS, SV20, SV80, SV20DS and SV80DS

Outliers were detected with Grubbs’ test using Graphpad QuickCalcs, GraphPad Software, San Diego California USA, www.graphpad.com.

**Results**

**Juice Analysis**

No naringenin could be found in either of the juices analyzed. Contents of naringin were 1475 ± 42.3 µM and 684 ± 12.0 µM for both batches of Floridas Natural Ruby Red, lot 98 and 2A6 respectively. The Minute Maid frozen concentrate contained 1476 ± 34.3 µM naringin. Concentrations of bergamottin were 31.64 ± 1.1 µM (batch 98) and 19.13 ±0.1 µM (batch 2A6); the dihydroxybergamottin content was 6.53 ± 0.3 µM and 4.9 ± 1.3 µM respectively. The juice concentrate AD3 contained 47.77 ± 1.9 µM bergamottin and 39.2 ± 1.6 µM dihydroxybergamottin. Values are given as mean (n=3) ± SD. Figure 3-5

**Survival Rate**

One animal died at the beginning of the Pilot study in the control group due to complications during blood sampling. All animals survived in the low dose 53 mg/kgxday⁻¹ and four rats died in the 200 mg/kgxday⁻¹ before the end of the study. The study was prematurely aborted due to the strong signs of toxicity in the 200 mg/kgxday⁻¹ group.
The grapefruit juice final study was completed as planned. All animals survived during this study (Figure 3-6).

**Body Weight**

During the pilot study, the control group and the 53 mg/kgxday-1 group show an increase in body weight over 10 days; the 200 mg/kgxday-1 group shows a significant decrease in body weight (Figure 3-7). During the final study, all groups showed an increase in body weight over the period of 28 days. There was no significant difference in weight gain comparing the a priori selected groups (Figure 3-8).

**Behavioral Experiments**

**Open field test**

The question to be answered was whether simvastatin in combination with grapefruit juice decreases the locomotor activity as a sign of muscle damage. After the completion of the pilot study, the 53 mg/kgxday-1 group exhibited a significantly higher number of line crossing than the control (P<0.05). The number of line crossings was decreased in the 200 mg/kgxday-1 group. These differences however were not statistically significant. Figure 3-9

No differences in the number of line crossings could be found in the SV 20 group. Within the SV80 group, the number of line crossing of the SV80DS was increased compared to the SV80 control and the water control (both P<0.01). Figure 3-10

The total distance travelled by the rat resulted in the following differences. In the pilot study the animals in the 53 mg/kgxday-1 group travel more distance in the recorded 5 minutes than the control group. The 200 mg/kgxday-1 had a much lower travelling distance in millimeters than the control group, however the difference was not statistically significant (Figure 3-11).
In the final study, the distance covered by the animals in the SV80DS group was significantly higher than its respective control SV80 and the water control (P<0.01 and P<0.05 respectively). No differences were observed in the SV20 group (Figure 3-12).

**Grip strength test**

Comparisons of the grip strength results were made as area under the grip strength curve. No significant differences were found in the pilot study (Figure 3-13, 3-14) or in the final study (Figure 3-15,3-16).

**Extremity temperature**

The measurements of the paw temperature in the pilot study resulted in significant differences when comparing the 200 mg/kgxday-1 group to control (Figure 3-17).

In the final study, differences were observed in when comparing the SV20ERS group with the water control. No differences were observed in the SV80 juice combinations with the SV80 control (Figure 3-18).

**Histological Experiment**

During the pilot study, all animals in the control group and the 53 mg/kgxday-1 group exhibited no or only minimal muscle damage; out of the four surviving animals in the 200 mg/kgxday-1 group three showed no to minimal sign of muscle damage. One rat in this group exhibited clear signs of muscle damage and was classified as moderate muscle damage with 32% of damaged muscle fibers.

The incidence of muscle damage in the final study was minor. Throughout the groups no damage to minimal muscle fiber damage was prevalent. Light microscopical analysis at different magnifications revealed one animal with mild muscle damage in the regular strength grapefruit juice control group, furthermore 1 one rat in the SV20DS group exhibited mildly damaged muscle fibers. (Figure 3-19,3-20,3-21)
Clinical Chemistry

Values below 10 mg/dl for Cholesterol were considered measuring errors and were not considered.

No significant differences in the cholesterol levels in between the groups during the pilot study were found after 10 days of treatment. Although two rats in the 200 mg/kgxday-1 had slightly elevated cholesterol levels (Figure 3-22).

During the final study, we found that the cholesterol levels of RS and DS juice are significantly lower than the water control (P<0.05 for both) and that the cholesterol levels in each was not different from either the SV20 or SV 80 group. The levels for SV20 and SV80 however were not different from the water control. Furthermore SV80RS cholesterol levels were significantly lower than SV20RS or the water control. Additionally cholesterol levels after SV20DS treatment were significantly higher when compared to a regular dose of DS GFJ (Figure 3-23).

ALT values measured in the pilot study were elevated in two rats out of the four surviving rats in the 200 mg/kgxday-1 group. One Way analysis of variance resulted in a significant difference between this group and the control (P<0.05) (Figure 3-24). In the final study no elevated ALT values were identified. The SV80RS group however showed significantly lower ALT values than the water control group, the SV80DS group or the SV80 group. (P<0.01) group (Figure 3-25).

AST values measured in the pilot study were elevated in two rats, the same rats that had elevated ALT values, out of the four surviving rats in the 200 mg/kgxday-1 group. One Way analysis of variance resulted in a significant difference between this group and the control (P<0.05) (Figure3-26).
After the final study, AST values of all SV80 and AV20 groups were significantly lower than the water control (P<0.001), however SV20DS showed higher AST values than SV20 or SV20RS. (Figure 3-27).

CPK values for the pilot study could not be evaluated. The plasma samples for this study were taken after decapitation and additional studies have shown that decapitation dramatically increases CPK values (Figure 3-28). Consequently the CPK results for the final study also resulted in higher values for the Control, RS and DS group since these values were also taken after decapitation. Peak values for the other groups were taken before sacrificing and are therefore comparable. CPK values in the SV20DS group were significantly higher than in the SV20 group (P<0.001) or the SV20RS group (P<0.001). No differences were observed within the SV80 group (Figure 3-29).

**Organ weights**

After removal of the organs during the pilot study, no significant differences could be found in the absolute organ weight of the liver, adrenal gland and testes, the absolute spleen, kidney and heart weight was significantly lower in the 200 mg/kgxday-1 group compared to control. The organ index was higher in the 200 mg/kgxday-1. All other organ indices were not significantly different. During the final study, no difference could be observed in absolute spleen, kidney, testis, and adrenal gland weight. Absolute liver weight was significantly lower in all SV20 and all SV80 groups. Furthermore absolute heart weight was decreased in the SV20Rs group compared to the water control (Figure 3-30). Furthermore no differences were observed in organ indices of heart, testis, spleen, liver, and adrenal gland weight. Organ indices were increased in SV20RS and SV20Ds compared to water and in SV80, SV80RS and SV80Ds compared to water (Figure 3-31)
Conclusions

The results of our study to evaluate the risk of muscle damage after chronic coadministration of grapefruit juice with simvastatin demonstrate that no increased muscle damage can be observed when simvastatin is taken with grapefruit juice than taken alone. We were not able to observe increased organ toxicity or a decreased survival rate. This study also indicates that grapefruit juice can lower cholesterol in laboratory animals; however this result will need to be confirmed humans.
Figure 3-1. Schematics of first pass metabolism

Figure 3-2. Open field arena and TopScan software interface.
Figure 3-3. PANLAB grip strength meter

Figure 3-4. Sampling schedule during the final study

- Once daily administration of grapefruit juice (5 ml/kg) and simvastatin
Figure 3-5. Concentration of 6',7'-dihydroxybergamottin (DHB), bergamottin (BG) and Naringin (NAR) in three different juices (mean ± SD) n=3

Figure 3-6. Survival rate during both studies A=pilot study, B=final study
Figure 3-7. Slope of the linear regression performed of the body weight of rats in the pilot study from day 0 to day 10 (mean ± SEM), Control n=7, SV53 n=8, SV200 n=4. (*=P<0.05)

Figure 3-8. Slope of the linear regression performed of the body weight of rats in the final study from day 0 until day 28 shown as mean ± SEM (n=13).
Figure 3-9. Number of line crossings of the open field test in the pilot study (mean ± SEM), Control n=7, SV53 n=8, SV200 n=4. (*=P<0.05)

Figure 3-10. Number of line crossings of the open field test in the final study shown as mean ± SEM (n=13). (**=P<0.01)
Figure 3-11. Distance traveled in the open field test of the pilot study (mean ± SEM), control n=7, SV53 n=8, SV200 n=4. (*=P<0.05).

Figure 3-12. Distance traveled in the open field test of the final study shown as mean ± SEM (**=P<0.01).
Figure 3-13. Grip strength development during the pilot study mean ± SEM, control n=7, SV53 n=8, SV200 n=4.

Figure 3-14. Comparison of the grip strength AUC mean ± SEM, control n=7, SV53 n=8, SV200 n=4
Figure 3-15. Grip strength development during the final study n=13, data shown as mean ± SEM.

Figure 3-16. Comparison of the grip strength AUC during the final study, n=13 data shown as mean ± SEM.
Figure 3-17. Comparison of the extremity temperature in the pilot study, mean ± SEM, control n=7, SV53 n=8, SV200 n=4 (** P<0.01).

Figure 3-18. Comparison of the extremity temperature in the final study mean ± SEM, n=13
Figure 3-19. Muscle damage (arrows) observed during the pilot:

A) control group
B) SV53
C) SV200
Figure 3-20. Histological changes in the rat muscle during the final study A) Water B) SV20 C) SV80 D) RS juice E) DS juice
Figure 3-21 Histological changes in the rat muscle during the final study A) SV20RS B) SV20DS C) SV80RS D) SV80DS
Figure 3-22. Cholesterol levels after 12 day dosing during the pilot study mean ± SEM, Control n=7, SV53 n=8, SV200 n=4.

Figure 3-23. Cholesterol levels after 28 day dosing during the final study mean ± SEM, n=13 (*=P<0.05).
Figure 3-24. Alanine amino transferase (ALT) levels after 12 days during the pilot study, mean ± SEM, Control n=7, SV53 n=8, SV200 n=4 (*=P<0.05).

Figure 3-25. Alanine amino transferase (ALT) levels after 28 days during the final study, mean ± SEM, n=13 (**=P<0.01).
Figure 3-26. Aspartate amino transferase (AST) levels after 12 days during the pilot study mean ± SEM, Control n=7, SV53 n=8, SV200 n=4 (*=P<0.05).

Figure 3-27. Aspartate amino transferase (AST) levels after 28 days during the final study, mean ± SEM, n=13 (**=P<0.01).
Figure 3-28. Creatine kinase (CPK) levels before and after decapitation n=9.

Figure 3-29. Creatine kinase (CPK) levels after 28 days during the final study, mean ± SEM, n=13 (**=P<0.01).
Figure 3-30. Absolute organ weights after 28 days during the final study, mean ± SEM, n=13 A) liver B) liver c) Spleen D) heart E) testis F) adrenal glands G) kidney
Figure 3-31. Organ indices after 28 days during the final study, mean ± SEM, n=13 A) liver B) spleen C) heart D) testis E) adrenal glands F) kidney G) kidney
Table 3-1. Preparation procedure for simvastatin suspensions with respective dosing volume.

<table>
<thead>
<tr>
<th>Study</th>
<th>Dose</th>
<th>Amount simvastatin</th>
<th>Amount CMC</th>
<th>Vehicle (30mL)</th>
<th>Dosing volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pilot</td>
<td>SV 53</td>
<td>160mg</td>
<td>240mg</td>
<td>Water</td>
<td>0.1mL/10g</td>
</tr>
<tr>
<td></td>
<td>SV200</td>
<td>600mg</td>
<td>240mg</td>
<td>Water</td>
<td>0.1mL/10g</td>
</tr>
<tr>
<td>Final</td>
<td>SV20</td>
<td>120mg</td>
<td>240mg</td>
<td>Water</td>
<td>0.05mL/10g</td>
</tr>
<tr>
<td></td>
<td>SV20RS</td>
<td>120mg</td>
<td>240mg</td>
<td>RS</td>
<td>0.05mL/10g</td>
</tr>
<tr>
<td></td>
<td>SV20DS</td>
<td>120mg</td>
<td>240mg</td>
<td>DS</td>
<td>0.05mL/10g</td>
</tr>
<tr>
<td></td>
<td>SV80</td>
<td>480mg</td>
<td>240mg</td>
<td>Water</td>
<td>0.05mL/10g</td>
</tr>
<tr>
<td></td>
<td>SV80RS</td>
<td>480mg</td>
<td>240mg</td>
<td>RS</td>
<td>0.05mL/10g</td>
</tr>
<tr>
<td></td>
<td>SV80DS</td>
<td>480mg</td>
<td>240mg</td>
<td>DS</td>
<td>0.05mL/10g</td>
</tr>
</tbody>
</table>

Table 3-2. Naringin (NAR) / naringenin (NAG) standard curve

<table>
<thead>
<tr>
<th>Concentration</th>
<th>NAR(µM)</th>
<th>NAG(µM)</th>
<th>Add both and fill to 5mL with 50:50 methanol:water</th>
<th>Take</th>
<th>Fill to 10mL</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>450</td>
<td>0.058g</td>
<td>49.6</td>
<td>Take 0.45mL</td>
<td></td>
<td>10mL</td>
<td>50:50 methanol:water</td>
</tr>
<tr>
<td>350</td>
<td></td>
<td>38.6</td>
<td>Take 0.350mL</td>
<td></td>
<td>10mL</td>
<td>50:50 methanol:water</td>
</tr>
<tr>
<td>250</td>
<td></td>
<td>27.5</td>
<td>Take 0.250mL</td>
<td></td>
<td>10mL</td>
<td>50:50 methanol:water</td>
</tr>
<tr>
<td>150</td>
<td></td>
<td>16.5</td>
<td>Take 0.150mL</td>
<td></td>
<td>10mL</td>
<td>50:50 methanol:water</td>
</tr>
<tr>
<td>50</td>
<td></td>
<td>5.5</td>
<td>Take 0.050mL</td>
<td></td>
<td>10mL</td>
<td>50:50 methanol:water</td>
</tr>
</tbody>
</table>
Table 3-3. Bergamottin and dihydroxybergamottin standard curve

<table>
<thead>
<tr>
<th>Concentration</th>
<th>BG (µM)</th>
<th>DHB (µM)</th>
<th>Take</th>
<th>Fill to</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>125</td>
<td>125</td>
<td>50</td>
<td>1mL</td>
<td>2mL</td>
<td>50:50 methanol:water</td>
</tr>
<tr>
<td>62.5</td>
<td>62.5</td>
<td>25</td>
<td>0.5mL</td>
<td>2mL</td>
<td>50:50 methanol:water</td>
</tr>
<tr>
<td>25</td>
<td>25</td>
<td>10</td>
<td>0.2mL</td>
<td>2mL</td>
<td>50:50 methanol:water</td>
</tr>
<tr>
<td>6.25</td>
<td>6.25</td>
<td>2.5</td>
<td>0.05mL</td>
<td>2mL</td>
<td>50:50 methanol:water</td>
</tr>
<tr>
<td>2.5</td>
<td>2.5</td>
<td>1</td>
<td>0.01mL</td>
<td>2mL</td>
<td>50:50 methanol:water</td>
</tr>
</tbody>
</table>

BG (0.0169 g) Dissolve in 1mL DMSO (50,000 µM)

DHB (0.0186 g) Dissolve in 1mL DMSO (50,000 µM)

Add both and fill to 5mL with 50:50 methanol:water (=BG 250µM DHB 100µM)
Table 3-4. Dosing scheme of the final study

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>N per group</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Water control</td>
<td>13</td>
</tr>
<tr>
<td>2</td>
<td>Grapefruit juice (regular strength) control (RS)</td>
<td>13</td>
</tr>
<tr>
<td>3</td>
<td>Grapefruit juice (double strength) control (DS)</td>
<td>13</td>
</tr>
<tr>
<td>4</td>
<td>Simvastatin (low dose) control (SV20)</td>
<td>13</td>
</tr>
<tr>
<td>5</td>
<td>Simvastatin (high dose) control (SV80)</td>
<td>13</td>
</tr>
<tr>
<td>6</td>
<td>SV\textsubscript{low dose} + GFJ\textsubscript{RS} (SV20RS)</td>
<td>13</td>
</tr>
<tr>
<td>7</td>
<td>SV\textsubscript{low dose} + GFJ\textsubscript{DS} (SV20DS)</td>
<td>13</td>
</tr>
<tr>
<td>8</td>
<td>SV\textsubscript{high dose} + GFJ\textsubscript{RS} (SV80RS)</td>
<td>13</td>
</tr>
<tr>
<td>9</td>
<td>SV\textsubscript{high dose} + GFJ\textsubscript{DS} (SV80DS)</td>
<td>13</td>
</tr>
</tbody>
</table>

SV\textsubscript{low dose} = 20 mg/kg Simvastatin, SV\textsubscript{high dose} = 80 mg/kg Simvastatin, GFJ\textsubscript{RS} = Grapefruit juice concentrate dilute with the same volume of water, GFJ\textsubscript{DS} = Grapefruit juice concentrate dilute with half the volume of water.
### Table 3-5. Muscle damage incidence and severity.

<table>
<thead>
<tr>
<th>Study</th>
<th>Dose (mg/kg x day⁻¹)</th>
<th>Incidence</th>
<th>Severity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pilot</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SV53</td>
<td>0/7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SV200¹</td>
<td>1/4</td>
<td>**</td>
<td></td>
</tr>
<tr>
<td>Final</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0/12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RS</td>
<td>1/13</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>DS</td>
<td>0/13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SV20</td>
<td>0/11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SV20RS</td>
<td>0/10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SV20DS</td>
<td>1/12</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>SV80</td>
<td>0/11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SV80RS</td>
<td>0/12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SV80DS</td>
<td>0/13</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹ four animals died in this group due to toxicity. * = mild muscle damage, ** = moderate muscle damage, *** = severe muscle damage
CHAPTER 4
DETERMINATION OF ACUTE AND CHRONIC EFFECT OF GRAPEFRUIT JUICE ON SIMVASTATIN PLASMA CONCENTRATIONS IN RATS

Specific Aim

Assess the magnitude to which GFJ changes the simvastatin (SV) plasma levels after long term use in the rat model and assess the possibility of adaptive processes.

Material and Methods

Chemicals

Simvastatin (SV), 99.4% pure, was a kind gift from Merck Sharp Dome (MSD, West Point PA., USA. Simvastatin hydroxy acid (98% pure) for calibration and quality control standards was purchased from Toronto Research chemicals, North York, ON, Canada. D6-simvastatin and D6-simvastatin hydroxy acid as internal standards were also purchased from Toronto Research Chemicals. Isotopic purity for both compounds was >99%. Methanol and Acetonitrile (Optima Grade) and Formic and acetic acid were purchased from Fisher Scientific. Methylamine was purchased from Sigma-Aldrich (Saint Louis, MO). Solid Phase Extraction columns (Versaplate C8 100mg) were purchased from Varian Inc. Heparinized, mixed gender pooled rat plasma from Sprague DAwley rats was purchased from Biocemed (Winchester, VA).

Stock and Work Solutions

Stock solutions for Simvastatin acid were prepared by dissolving 1000µg simvastatin hydroxy acid in 1 mL ACN/H2O 60:40. Stock solutions for simvastatin lactone were prepared by dissolving 1000µg simvastatin lactone in 1 mL ACN. 100µg and 0.5 µg stock solutions were prepared as follows:

Simvastatin acid:

100 µg/mL: 0.2 mL (1000 µg/mL SV-acid Stock)/2mL 60:40 ACN:H2O
0.5 µg/mL: 0.010 mL (100 µg/mL SV-acid Stock)/2mL 60:40 ACN:H2O
Simvastatin lactone:

100 µg/mL: 0.2 mL (1000 µg/mL SV-acid Stock)/2mL ACN
0.5 µg/mL: 0.010 mL (100 µg/mL SV-acid Stock)/2mL ACN

**Calibration Quality Control Standards**

Calibration and quality control standards were prepared according to the spiking scheme in table 4-1.

**Analytical System and Chromatographic Conditions**

The LC/MS/MS system consisted of a Thermo Finnigan Surveyor HPLC autosampler, Thermo Finnigan Surveyor MS quaternary pump and a Thermo Finnigan TSQ Quantum Discovery triple quadrupole mass spectrometer. The TSQ Quantum mass spectrometer was equipped with an electrospray (ESI) ion source and operated in the negative mode from 0 to 2.9 minutes and in positive mode from 2.9 to 4.5 minutes. The ESI source spray was set orthogonal to the ion transfer capillary tube. The mass spectrometer was calibrated with a solution of polytyrosine-1,3,6 according to the manufacturer. The MS/MS conditions were optimized by infusing simvastatin and simvastatin hydroxy acid in the mobile phase.

For quantification, the TSQ Quantum was operated in single reaction monitoring mode (SRM). The acquisition parameters were: spray voltage 3kV for simvastatin hydroxy acid and 5 kV for simvastatin lactone, source CID 5 V, and heated capillary temperature at 325oC. Nitrogen was used as the sheath and auxiliary gas and set to 45 and 15 (arbitrary units), respectively.

The argon collision gas pressure was set to 1.5 mTorr. The collision energy was 23 eV for Simvastatin hydroxy acid and simvastatin hydroxy acid-D6 (internal standard) and. 19 eV for Simvastatin lactone and simvastatin lactone-D6 (internal standard).
The selected reaction monitoring scheme followed transitions of the [M-H]- precursor to selected product ions with the following values: m/z 435.3 → 319.1 for simvastatin acid and 441.3 → 319.1 for simvastatin acid-D6 and [M+CH₃NH₃]+ precursor to selected product ions m/z 450.3 → 285.1 for simvastatin lactone and 456.3 → 285.1 for simvastatin lactone-D6.

The instrument was operated in normal resolution with peak width (FWHM) set to 0.7 Th at Q1 and to 0.7 Th at Q3. The scan time was 300 ms for each transition. SRM data were acquired and processed using ThermoFinnigan XCalibur® software version 1.4, service release 1 (Thermo Electron Corporation, San Jose, CA, USA).

Separation of the analytes was achieved using a Phenomenex (Phenomenex, Torrance, CA) Luna PhenylHexyl, 100 × 2.0 mm, 3µ analytical column. The mobile phases used for the analysis were ACN (mobile phase A) and 1mM methylammonium acetate in deionized water (mobile phase B). The mobile phases were degassed and filtered through a 0.22 Nylon 66 membrane before use. The analyses were performed under isocratic conditions using 70% mobile phase A and 30% mobile phase B. The flow rate was 0.2 mL/min.

Experiment Design

Non-fasted male Sprague-Dawley rats weighing 239-346 g were used. The animals were handled daily a couple of days before the study started in order to reduce stress and adapt them to the researcher. Body weight was checked daily as well. The male Sprague-Dawley rats had free access to food and water during the experiment except for the first two hours after drug administration. The animals were divided in the groups shown in table 3-3. On the first day of the experiment the rats were dosed with their respective simvastatin dose (SV_lowdose = 20 mg/kgxday⁻¹ (SV20) Simvastatin, SV_highdose = 80 mg/kgxday⁻¹ (SV80) Simvastatin) and their grapefruit juice permutations (simvastatin and regular strength grapefruit juice; SV20RS and SV80RS, and simvastatin and double strength grapefruit juice; SV20DS and SV80DS). To
establish a pharmacokinetic baseline profile, blood samples (500 µl) were taken from the sublingual vein at 0, 1, 2, 4, 6, 8, 12 hours after drug administration (three blood collections per day; e.g. time points 1, 4, 8; then two weeks washout, then the remaining time points 0, 2, 6, 12 in the same animals). After the acute phase two blood samples 1000 µl was taken each week for 4 weeks right before dosing the drug (trough level) and 2 hours after dosing (peak level) figure 3-4. After each blood sample, approximately 1000 µl of isotonic saline were replaced by i.p. injection in order to maintain the blood fluid. The blood samples were analyzed for Simvastatin, Simvastatin acid.

The animals were fed grapefruit juice (GFJ) (5mL/kg) at different concentrations (double and regular strength), or water (5mL/kg) and Simvastatin through an oral feeding needle daily. At the end of the study the rats were sacrificed, blood was collected for determination of simvastatin plasma levels.

**Sample Preparation and Extraction**

Samples were collected into heparinized tubes and stored on ice until further processing. Samples were centrifuged for 15 minutes at room temperature at 2800 rpm. The plasma was collected, aliquoted and stored at -80°C until analysis. The plasma was thawed at room temperature and 100 microL were transferred into a 2 mL deep well plate. 400 microL of DDwater and 10 µL of internal standard were added to each well. The plate was sealed and vortexed for 1 min. The C8 columns were preconditioned with 2x 1mL Optima grade methanol and equilibrated 2x1mL DDwater. The previously prepared samples were then loaded onto the columns and low vacuum <7in Hg was applied. The SPE cartridges were then washed with 1x1mL DDwater, 1x1mL 3.5% formic acid and 1x1mL DDwater. Low vacuum (<7in Hg) was applied for each step. After the last washing step, high vacuum (20 in Hg) was applied for 3 min to dry the cartridges. The elution was then performed using 1x1mL of optima grade methanol.
and 1x1mL of 2% formic acid in methanol. The columns were dried for 1 min. after each elution step.

The samples were then dried in a centrivap overnight at 37°C and reconstituted in 200μL 70:30 Acetonitrile:Methylammoniumacetate buffer (1 mM pH4.5). 20 µL of the reconstitute was injected onto the HPLC column.

**Pharmacokinetic Analysis**

Pharmacokinetic analysis was performed using WinNonlin 5.2 Professional Build 200701231637 (Pharsight Corp., Mountain View, CA). The Noncompartmental analysis algorithm was used and the parameters AUC<sub>last</sub>, AUC<sub>inf</sub>, T<sub>1/2</sub>, T<sub>max</sub>, C<sub>max</sub>, V<sub>z</sub>/F and CL/F were evaluated.

**Statistical Analysis**

Final study: Samples were analyzed using SAS version 9 (Cary, NC, USA). All samples were analyzed using a Two Way ANOVA with a Bonferroni multiple comparison post hoc test to compare treatments to each other. Differences were considered statistically significant at P<0.05. As set a priori, the following comparisons were evaluated, SV20 compared with SV20Rs and SV20DS. We also compared SV80 with SV80RS and SV80DS.

**Results**

The inter- and intraday variability during validation was less than 20%. The calibration range was linear from 0.5ng/mL to 750 ng/mL.

Grapefruit juice did not have a significant effect on the pharmacokinetic parameter of either Simvastatin lactone or its hydroxy acid form when administered as 20 mg/kg over 28 days. There were no significant differences in either regular strength or double strength grapefruit juice group. RS and DS GFJ significantly increased the exposure of a 80 mg/kg simvastatin lactone during the first administration by 1.3 fold. The main active metabolite exposure increase by 1.3
fold. During the chronic treatment with RS GFJ the $C_{\text{max}}$ of SV on day 7 and day 28 increased by around 1.8 fold and 1.7 fold for the hydroxy acid.

$C_{\text{max}}$ of SV increased maximally by 2-fold on day 28 and 2.1 fold on day 14 for SVA.

**Acute Treatment**

**Simvastatin lactone**

**SV20:**

The Area Under the Curve from 0 to 12 hours ($AUC_{0-12}$) of Simvastatin lactone in the 20 mg/kg group increased from 127.7±10.1 (h*ng/mL) when give with water to 228.3±27.1 (h*ng/mL) and 187.2±15.9 (h*ng/mL) when given with RS GFJ and DS GFJ respectively. These changes however were not statistically significant. The AUC from 0 to infinity ($AUC_{\text{inf}}$) increased from 137.1±9.9 (h*ng/mL) when dosed with water to 241.8±25.4 (h*ng/mL) and 365.0±164.4 (h*ng/mL) when given with RS GFJ and DS GFJ respectively. Similar to $AUC_{0-12}$ these changes were not statistically significant. (Figure 4-1)

$C_{\text{max}}$, $T_{\text{max}}$ and half life remained unchanged. The volume of distribution ($Vz/F$) decreased in the SV20DS group compared to the water control from 690.7±107.8 (L/kg) to 360.3±439.4 (L/kg) (P<0.05). The clearance of simvastatin decreased from 156.7±13.4 (L/h/kg) when the drug was dosed with water to 95.3±10.4 (L/h/kg) and 99.6±12.3 (L/h/kg) when dosed with RS and DS GFJ respectively (Table 4-2).

**SV80:**

The Area Under the Curve from 0 to 12 hours ($AUC_{0-12}$) of simvastatin lactone in the 80 mg/kg group increased significantly from 651.4±41.6 (h*ng/mL) when administrated with water to 869.6±39.5 (h*ng/mL) and 889.5±536.6 (h*ng/mL) when dosed with RS (P<0.001) and DS (P<0.001) GFJ respectively. The AUC from 0 to infinity ($AUC_{\text{inf}}$) increased from 807.5±78.3
in the control group to 963.0±41.0 (h*ng/mL) and 1104.5±99.4 (h*ng/mL) when dosed with RS (P<0.001) and DS (P<0.001) GFJ respectively.

The $C_{\text{max}}$ in the SV80RS group increased from 226.2±18.0 (ng/mL) to 312.2±32.6 (ng/mL) when comparing to the control group (P<0.05). The increase in the SV80DS group was not statistically significant. In addition the $T_{\text{max}}$ increased from 0.8±0.1 (h) to 1.2±0.1 (h) comparing the control group to the RS (P<0.001) GFJ group. The increase to 1.0±0.0 (h) in the DS GFJ group was not statistically significant. The half life, volume of distribution and Clearance ($V_{z}/F$ and $CL/F$) remained unchanged (Table 4-3).

**Simvastatin hydroxy acid**

**SV20:**

The Area Under the Curve from 0 to 12 hours ($AUC_{0-12}$) of simvastatin lactone in the 20 mg/kg group increased from 92.7±6.9 (h*ng/mL) when coadministered with water to 117.4±10.2 (h*ng/mL) and 135.5±9.2 (h*ng/mL) when administered with RS and DS GFJ. However these changes were not statistically significant. The AUC from 0 to infinity ($AUC_{\text{inf}}$) increased from 99.8±6.8 (h*ng/mL) in the control group to 128.5±9.4 (h*ng/mL) and 142.6±11.5 (h*ng/mL) when dosed with RS (P<0.001) and DS (P<0.001) GFJ respectively. Similar to $AUC_{0-12}$ these changes were not statistically significant. (Figure 4-3)

$C_{\text{max}}$, $T_{\text{max}}$, $T_{1/2}$ remained unchanged. The $V_{z}/F$ decreased from 871.3±111.9 (L/kg) in the water control group to 425.1±46.4 (L/kg) when administered concomitantly with DS GFJ (<0.05). The $CL/F$ decreased from 212.1±14.8 (L/kg) in the water control group to 148.6±11.8 (L/kg) when administered concomitantly with DS GFJ (<0.05) (Table 4-4).

**SV80:**

The Area Under the Curve from 0 to 12 hours ($AUC_{0-12}$) of simvastatin lactone in the 80 mg/kg group increased from 546.9±37.5 (h*ng/mL) when coadministered with water to
659.3±34.9 (h*ng/mL) and 716.3±41.5 (h*ng/mL) when administered with RS and DS GFJ. The increase with DS GFJ was statistically significant (P<0.001), however the increase in plasma concentration when the drug was administered with RS GFJ did not reach significance. The AUC from 0 to infinity (AUC_{inf}) increased from 648.6±52.7 (h*ng/mL) in the control group to 735.8±33.7 (h*ng/mL) and 857.1±68.1 (h*ng/mL) when dosed with RS (P<0.001) and DS (P<0.001) GFJ respectively. Of these changes only the increase with coadministration of DS GFJ showed to be statistically significant (P<0.01) (Figure 4-4)

\[ C_{\text{max}}, T_{\text{max}}, T_{1/2}, V_z/F \text{ and } C_l/F \text{ remained unchanged. (Table 4-5)} \]

**Chronic Treatment**

**Simvastatin lactone**

**SV20:**

The \( C_{\text{min}} \) as well as the \( C_{\text{max}} \) during the 28 days of study remained unchanged when comparing the two juice groups with the control group (Figure 4-5)

**SV80:**

There was no difference in the trough concentration of simvastatin lactone in the RS GFJ group. The peak concentrations in the RS GFJ group were increased only on day 7 and 28 (168.5±19.5 ng/mL compared to 96.8±8.8 ng/mL and 111.4±16.3 ng/mL compared to 64.0±9.5 ng/mL respectively). We did find a significant increase in the trough concentration of Simvastatin lactone in the DS GFJ group on day 7 (137.8±31.2 ng/mL compared to 13.8±3.3 ng/mL). Furthermore the peak concentration was increase on day 14 (186.0±29.0 ng/mL to 109.4±13.9 ng/mL), and 28 (130.4±13.3 ng/mL to 64.0±9.5 ng/mL), but did not reach statistical significance on day 7 and day 21 (Figure 4-6, Table 4-6).
Simvastatin hydroxy acid

SV20:

The $C_{\text{min}}$ as well as the $C_{\text{max}}$ during the 28 days of study remained unchanged when comparing the two juice groups with the control group (Figure 4-7).

SV80:

There was no difference in the trough concentration of simvastatin lactone in the RS GFJ group. The peak concentrations in the RS GFJ group were increased only on day 7 and 28 (111.0±12.5 ng/mL compared to 68.6±6.8 ng/mL and 98.5±16.8 ng/mL compared to 57.4±7.8 ng/mL respectively). We found a significant increase in the trough concentration of Simvastatin lactone in the DS GFJ group on day 7 (41.0±10.0 ng/mL compared to 9.1±2.0 ng/mL). Furthermore the peak concentration was increase on day 14 (140.9±21.6 ng/mL to 65.9±8.2 ng/mL), 21 (151.1±25.8 ng/mL to 90.9±13.1 ng/mL) and 28 (96.6±9.6 ng/mL to 57.4±7.8 ng/mL), but did not reach statistical significance on day 7 (Figure 4-8, Table4-7).

Conclusions

As hypothesized, Grapefruit juice in either concentration (DS or RS) administered daily did not seem to have a relevant effect on the pharmacokinetics on a low acute or chronic dose of simvastatin. When administered with a high dose of simvastatin however, concentrations were elevated up to the last day of the study. Concentrations of the main active metabolite were also elevated up to the end of the study. It seems that the interaction persist over the full range of the study.
Figure 4-1. Plasma concentrations of simvastatin lactone in rat plasma after a 20 mg/kg oral dose. Data is shown as mean±SEM (n=13)

Figure 4-2. Plasma concentrations of simvastatin lactone in rat plasma after a 80 mg/kg oral dose. Data is shown as mean±SEM (n=13)
Figure 4-3. Plasma concentrations of simvastatin hydroxy acid in rat plasma after a 20 mg/kg oral dose. Data is shown as mean±SEM (n=13)

Figure 4-4. Plasma concentrations of simvastatin hydroxy acid in rat plasma after a 80 mg/kg oral dose. Data is shown as mean±SEM (n=13)
Figure 4-5. Plasma concentrations of simvastatin lactone in rat plasma after a 20 mg/kg oral dose from day 7 to day 28. Data is shown as mean±SEM (n=13)

Figure 4-6. Plasma concentrations of simvastatin lactone in rat plasma after a 80 mg/kg oral dose from day 7 to day 28. Data is shown as mean±SEM (n=13)
Figure 4-7. Plasma concentrations of simvastatin hydroxy acid in rat plasma after a 20 mg/kg oral dose from day 7 to day 28. Data is shown as mean ± SEM (n=13)

Figure 4-8. Plasma concentrations of simvastatin hydroxy acid in rat plasma after a 80 mg/kg oral dose from day 7 to day 28. Data is shown as mean ± SEM (n=13)
| Standard Concentration (ng/mL) | Total Plasma Amount (mL) | Stock Solution Stock Solution Stock Spiking Amount (µg/mL) (µg/mL) Solution Amount (µL) |
|-------------------------------|------------------------|-----------------------------------------------|-----------------------------------------------|
| 0                             | 10                     | 0                                             | 0                                             |
| 0.5                           | 10                     | 0.5                                           | 0.5                                           |
| 1                             | 10                     | 0.5                                           | 0.5                                           | 20 |
| 10                            | 10                     | 0.5                                           | 0.5                                           | 200 |
| 100                           | 10                     | 100                                           | 100                                           | 10 |
| 250                           | 10                     | 100                                           | 100                                           | 25 |
| 500                           | 10                     | 100                                           | 100                                           | 50 |
| 750                           | 10                     | 100                                           | 100                                           | 75 |
| 2.0 LC                        | 10                     | 0.5                                           | 0.5                                           | 40 |
| 150.0 MC                      | 10                     | 100                                           | 100                                           | 15 |
| 400.0 HC                      | 10                     | 100                                           | 100                                           | 40 |
| 2.0 LC                        | 5                      | 0.5                                           | 0.5                                           | 20 |
| 150.0 MC                      | 5                      | 100                                           | 100                                           | 7.5 |
| 400.0 HC                      | 5                      | 100                                           | 100                                           | 20 |
Table 4-2. Pharmacokinetic parameters of simvastatin lactone after a 20 mg/kg oral dose in rats. Data is shown as mean ± SEM (n=13) (*=p<0.05, **=P<0.01, ***=P<0.001)

<table>
<thead>
<tr>
<th></th>
<th>AUC&lt;sub&gt;last&lt;/sub&gt;</th>
<th>AUC&lt;sub&gt;inf&lt;/sub&gt;</th>
<th>C&lt;sub&gt;max&lt;/sub&gt;</th>
<th>T&lt;sub&gt;max&lt;/sub&gt;</th>
<th>T&lt;sub&gt;1/2&lt;/sub&gt;</th>
<th>Vz/F</th>
<th>Cl/F</th>
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<tr>
<td></td>
<td>(h*ng/mL)</td>
<td>(h*ng/mL)</td>
<td>(ng/mL)</td>
<td>(h)</td>
<td>(h)</td>
<td>(L/kg)</td>
<td>(L/h/kg)</td>
</tr>
<tr>
<td>0 GFJ</td>
<td>127.7±10.1</td>
<td>137.1±9.9</td>
<td>61.0±8.8</td>
<td>0.9±0.1</td>
<td>2.9±0.2</td>
<td>690.7±107.8</td>
<td>156.8±13.5</td>
</tr>
<tr>
<td>RS GFJ</td>
<td>228.3±27.1</td>
<td>241.8±25.4</td>
<td>90.8±15.2</td>
<td>1.1±0.2</td>
<td>2.7±0.4</td>
<td>429.6±98.4</td>
<td>95.4±9.4***</td>
</tr>
<tr>
<td>DS GFJ</td>
<td>187.2±15.9</td>
<td>365.0±164.4</td>
<td>77.1±13.4</td>
<td>1.6±0.5</td>
<td>5.3±3.1</td>
<td>360.3±43.9*</td>
<td>99.6±12.4**</td>
</tr>
</tbody>
</table>

Table 4-3. Pharmacokinetic parameters of simvastatin lactone after a 80 mg/kg oral dose in rats. Data is shown as mean ±SEM (n=13) (*=p<0.05, **=P<0.01, ***=P<0.001)

<table>
<thead>
<tr>
<th></th>
<th>AUC&lt;sub&gt;last&lt;/sub&gt;</th>
<th>AUC&lt;sub&gt;inf&lt;/sub&gt;</th>
<th>C&lt;sub&gt;max&lt;/sub&gt;</th>
<th>T&lt;sub&gt;max&lt;/sub&gt;</th>
<th>T&lt;sub&gt;1/2&lt;/sub&gt;</th>
<th>Vz/F</th>
<th>Cl/F</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(h*ng/mL)</td>
<td>(h*ng/mL)</td>
<td>(ng/mL)</td>
<td>(h)</td>
<td>(h)</td>
<td>(L/kg)</td>
<td>(L/h/kg)</td>
</tr>
<tr>
<td>0 GFJ</td>
<td>651.4±41.6</td>
<td>807.5±78.3</td>
<td>226.2±18.0</td>
<td>0.8±0.1</td>
<td>3.6±0.6</td>
<td>504.7±40.4</td>
<td>111.4±11.6</td>
</tr>
<tr>
<td>RS GFJ</td>
<td>869.6±39.5***</td>
<td>963.0±41.0</td>
<td>312.2±32.6*</td>
<td>1.2±0.1**</td>
<td>3.4±0.3</td>
<td>417.2±37.5</td>
<td>85.0±3.9</td>
</tr>
<tr>
<td>DS GFJ</td>
<td>889.5±53.6***</td>
<td>1104.5±99.4</td>
<td>291.7±23.0</td>
<td>1.0±0.0</td>
<td>3.8±0.5</td>
<td>405.2±34.6</td>
<td>79.1±6.4</td>
</tr>
</tbody>
</table>
Table 4-4. Pharmacokinetic parameters of simvastatin hydroxy acid after a 20 mg/kg oral dose in rats. Data is shown as mean±SEM (n=13) (*=p<0.05)

<table>
<thead>
<tr>
<th></th>
<th>AUC(_{last}) (h*ng/mL)</th>
<th>AUC(_{inf}) (h*ng/mL)</th>
<th>C(_{max}) (ng/mL)</th>
<th>T(_{max}) (h)</th>
<th>T(_{1/2}) (h)</th>
<th>V(_z/F) (L/kg)</th>
<th>Cl/F (L/h/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 GFJ</td>
<td>92.7±6.9</td>
<td>99.8±6.8</td>
<td>37.9±4.6</td>
<td>1.1±0.3</td>
<td>2.8±0.2</td>
<td>871.3±111.9</td>
<td>212.1±14.8</td>
</tr>
<tr>
<td>RS GFJ</td>
<td>117.4±10.2</td>
<td>128.5±9.4</td>
<td>41.9±7.7</td>
<td>1.2±0.4</td>
<td>3.2±0.6</td>
<td>812.0±16.8</td>
<td>166.4±12.7</td>
</tr>
<tr>
<td>DS GFJ</td>
<td>135.5±9.2</td>
<td>142.6±11.5</td>
<td>41.9±7.7</td>
<td>2.2±2.6</td>
<td>2.0±0.1</td>
<td>425.1±46.4*</td>
<td>148.6±11.8*</td>
</tr>
</tbody>
</table>

Table 4-5. Pharmacokinetic parameters of simvastatin hydroxy acid after a 80 mg/kg oral dose in rats. Data is shown as mean±SEM (n=13) (**=P<0.01, ***=P<0.001)

<table>
<thead>
<tr>
<th></th>
<th>AUC(_{last}) (h*ng/mL)</th>
<th>AUC(_{inf}) (h*ng/mL)</th>
<th>C(_{max}) (ng/mL)</th>
<th>T(_{max}) (h)</th>
<th>T(_{1/2}) (h)</th>
<th>V(_z/F) (L/kg)</th>
<th>Cl/F (L/h/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 GFJ</td>
<td>546.9±37.5</td>
<td>648.6±52.7</td>
<td>199.2±16.8</td>
<td>0.9±0.3</td>
<td>3.3±0.4</td>
<td>594.6±51.8</td>
<td>135.8±13.4</td>
</tr>
<tr>
<td>RS GFJ</td>
<td>659.3±34.9</td>
<td>735.8±33.7</td>
<td>234.6±22.3</td>
<td>1.0±0.0</td>
<td>3.4±0.3</td>
<td>549.3±52.7</td>
<td>111.9±5.9</td>
</tr>
<tr>
<td>DS GFJ</td>
<td>716.3±41.5***</td>
<td>857.1±68.1**</td>
<td>244.6±17.0</td>
<td>1.0±0.0</td>
<td>4.1±0.7</td>
<td>534.8±63.0</td>
<td>100.6±8.0</td>
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</table>
Table 4-6. Trough($C_{\text{min}}$) and peak($C_{\text{max}}$) concentrations of simvastatin lactone after a 20 mg/kg and 80 mg/kg oral dose in rats. Data is shown as mean±SEM (n=13) (*=p<0.05, **=P<0.01, ***=P<0.001)

<table>
<thead>
<tr>
<th></th>
<th>C$_{\text{min}}$7 (ng/mL)</th>
<th>C$_{\text{max}}$7 (ng/mL)</th>
<th>C$_{\text{min}}$14 (ng/mL)</th>
<th>C$_{\text{max}}$14 (ng/mL)</th>
<th>C$_{\text{min}}$21 (ng/mL)</th>
<th>C$_{\text{max}}$21 (ng/mL)</th>
<th>C$_{\text{min}}$28 (ng/mL)</th>
<th>C$_{\text{max}}$28 (ng/mL)</th>
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<tbody>
<tr>
<td><strong>20 mg/kg</strong></td>
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<td></td>
</tr>
<tr>
<td>0 GFJ</td>
<td>1.9±0.6</td>
<td>24.1±2.4</td>
<td>1.7±0.4</td>
<td>26.2±2.2</td>
<td>0.5±0.1</td>
<td>18.6±1.7</td>
<td>5.3±1.7</td>
<td>15.4±1.0</td>
</tr>
<tr>
<td>RS GFJ</td>
<td>5.0±3.0</td>
<td>18.0±3.2</td>
<td>3.0±1.3</td>
<td>26.5±3.4</td>
<td>1.5±0.2</td>
<td>21.0±2.7</td>
<td>0.2±0.1</td>
<td>17.7±2.9</td>
</tr>
<tr>
<td>DS GFJ</td>
<td>2.1±0.7</td>
<td>26.1±2.0</td>
<td>2.2±0.7</td>
<td>18.5±1.4</td>
<td>1.3±0.2</td>
<td>24.6±3.5</td>
<td>5.1±1.4</td>
<td>24.8±3.3</td>
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<tr>
<td><strong>80 mg/kg</strong></td>
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</tr>
<tr>
<td>0 GFJ</td>
<td>13.8±3.3</td>
<td>96.8±8.8</td>
<td>16.8±7.3</td>
<td>109.4±13.9</td>
<td>16.6±4.9</td>
<td>121.0±15.2</td>
<td>8.1±2.1</td>
<td>64.0±9.5</td>
</tr>
<tr>
<td>RS GFJ</td>
<td>49.8±11.9</td>
<td>168.5±19.5***</td>
<td>4.8±0.9</td>
<td>109.9±11.0</td>
<td>18.5±6.4</td>
<td>111.0±16.0</td>
<td>60.5±36.0</td>
<td>111.4±16.3*</td>
</tr>
<tr>
<td>DS GFJ</td>
<td>137.8±31.2***</td>
<td>155.9±24.6</td>
<td>14.7±3.2</td>
<td>186.0±29.0**</td>
<td>9.9±2.6</td>
<td>189.7±32.0</td>
<td>4.1±1.0</td>
<td>130.4±13.3***</td>
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Table 4-7. Trough($C_{\text{min}}$) and peak($C_{\text{max}}$) concentrations of simvastatin hydroxy acid after a 20 mg/kg and 80 mg/kg oral dose in rats. Data is shown as mean±SEM (n=13) (*=p<0.05, **=P<0.01, ***=P<0.001)

<table>
<thead>
<tr>
<th></th>
<th>$C_{\text{min}}$7 (ng/mL)</th>
<th>$C_{\text{max}}$7 (ng/mL)</th>
<th>$C_{\text{min}}$14 (ng/mL)</th>
<th>$C_{\text{max}}$14 (ng/mL)</th>
<th>$C_{\text{min}}$21 (ng/mL)</th>
<th>$C_{\text{max}}$21 (ng/mL)</th>
<th>$C_{\text{min}}$28 (ng/mL)</th>
<th>$C_{\text{max}}$28 (ng/mL)</th>
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<td></td>
</tr>
<tr>
<td></td>
<td>0 GFJ</td>
<td>0.9±0.3</td>
<td>20.4±1.8</td>
<td>1.7±0.4</td>
<td>16.3±1.3</td>
<td>0.4±0.1</td>
<td>13.2±0.8</td>
<td>3.5±1.0</td>
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<tr>
<td></td>
<td>RS GFJ</td>
<td>2.6±1.6</td>
<td>13.9±2.2</td>
<td>3.0±1.3</td>
<td>15.8±1.8</td>
<td>1.0±0.2</td>
<td>17.8±2.0</td>
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<tr>
<td></td>
<td>DS GFJ</td>
<td>1.4±0.5</td>
<td>19.3±1.3</td>
<td>2.2±0.7</td>
<td>18.4±2.1</td>
<td>1.0±0.2</td>
<td>18.7±2.5</td>
<td>3.4±0.9</td>
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<tr>
<td>80 mg/kg</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 GFJ</td>
<td>9.1±2.0</td>
<td>68.6±6.8</td>
<td>16.8±7.3</td>
<td>65.9±8.2</td>
<td>12.6±3.8</td>
<td>90.9±13.1</td>
<td>6.3±1.8</td>
</tr>
<tr>
<td></td>
<td>RS GFJ</td>
<td>8.7±1.4</td>
<td>111.0±12.5**</td>
<td>4.8±0.9</td>
<td>89.4±8.5</td>
<td>12.2±4.2</td>
<td>78.1±11.7</td>
<td>39.2±23.2</td>
</tr>
<tr>
<td></td>
<td>DS GFJ</td>
<td>41.0±10.0***</td>
<td>103.2±13.0</td>
<td>14.7±3.2</td>
<td>140.9±21.6***</td>
<td>7.3±2.0</td>
<td>151.1±25.8*</td>
<td>3.1±0.8</td>
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CHAPTER 5
DETERMINATION OF CHANGES IN STEADY STATE CONCENTRATIONS OF ATORVASTATIN AFTER LONG TERM GRAPEFRUIT JUICE CONSUMPTION

Background

Current research demonstrates the increases in plasma concentration of certain statins when taken concomitantly with grapefruit juice. Even though a number of clinical trials have been published about this interaction, it remains unclear whether this interaction is clinically relevant and results in a higher incidence in muscle damage than a regular high dose of statins. In 2004 a case report about a 59 year old man from the north of the country was published in the scientific literature and soon afterwards was picked up by the mass media [87]. This patient suffered from hypercholesterolaemia and his initial dose of 10 mg/day atorvastatin was steadily increased to 60 mg/day, which is on the higher and of the therapeutic dosing range. Additionally to changes in his diet and exercise, he also started to consume grapefruit juice that he squeezed himself. This patient was diagnosed with rhabdomyolysis [87]. A similar case was reported with another patient that was diagnosed with rhabdomyolysis after having tolerated simvastatin well for 2 years and after tolerating the increase in dose from 40-80mg for 6 month. She stared consuming grapefruit juice 4 days before the appearance of the first symptoms [88]. These case reports demonstrate the necessity to evaluate the long term effects of grapefruit on the exposure of atorvastatin.

Specific Aim

Assess the magnitude by which GFJ changes the steady state atorvastatin plasma levels after long term concomitant use. The magnitude of the interaction was assessed in a time dependent manner over a period of 90 days.
Material and Methods

Chemicals

Atorvastatin (compound # 0134298-0038A batch # 62) and D₅-Atorvastain (compound # 0134298-0038A batch # 53) reference standard were provided as a kind gift by Pfizer, USA. Human Plasma was provided by Civitan Regional Blood System.

Grapefruit juice was provided by the Florida Department of Citrus. The juice was 100% Florida juice as found on the domestic market meeting USDA grade A standards. Grapefruit juice was from one homogeneous lot, packaged in a shelf stable form.

Stock and Work Solutions

Atorvastatin:

Stock solution 1 (1mg/mL) was prepared by dissolving 0.0250 g of Atorvastatin in methanol up to a volume of 25 mL in a graduated flask.

Stock solution 2 (100 μg/mL) was prepared by transferring a 0.1 mL aliquote of Stock solution 1 and adding a volume of 0.9 mL 50/50 water/acetonitrile in a micro centrifuge tube.

Stock solution 3 (10 μg/mL) was prepared by transferring a 0.1 mL aliquot of standard solution 2 and adding a volume of 0.9 mL plasma in a micro centrifuge tube.

Stock solution 4 (1 μg/mL) was prepared by transferring a 0.2 mL aliquot of standard solution 3 and adding a volume of 1.8 mL plasma in a micro centrifuge tube.

Stock solution 5 (100 ng/mL) was prepared by transferring a 0.2 mL aliquot of standard solution 4 and adding a volume of 1.8 mL plasma in a micro centrifuge tube. Next, these solutions were used to spike the calibration and quality control plasma samples. Where calibration curve samples were made in concentrations from 25-0.25 ng/mL and quality control samples were made in concentrations 18-0.5 ng/mL.
D₅-Atorvastatin:

0.010 g was dissolved in 10 mL methanol to obtain a stock solution of 1 mg/mL.

**Calibration Standards**

Atorvastatin calibration standards were prepared in human plasma from standard solutions 3, 4 and 5, according to the following pipetting scheme (Tbl. 5-1). Calibration Curve total volume: 40 mL –a set of 40 was prepared. 1mL was transferred from each tube to obtain 40 complete calibration sets with a total volume of 1mL/standard sample.

**Atorvastatin Quality Control Standards**

Atorvastatin quality control standards were prepared in human plasma from standard solutions 2, 3 and 4, according to the following pipetting scheme. (Tbl. 5-2) Quality controls total volume: 1mL a set of 2 was prepared. 1mL was transferred from each tube to obtain 40 complete calibration sets with a total volume of 1mL/standard sample.

**Analytical System and Mobile Phase**

The HPLC system consists of a LDC CM 4000 pump, Perkin Elmer ISS-200 autoinjector, an adequate precolumn, a YMC J’sphere H80 S-4 2.0 x 15 mm column, part #JH085041502WT followed by a Quattro LC-Z MS/MS system. The mobile phase consisted of acetonitrile : 0.1 % formic acid (70:30). The mobile phase was filtered and degassed prior to use. The flow rate was 0.2 mL/min, injection volume was 40µL.

**Analytical Method**

The daughter ions of Atorvastatin (parent ion 559 m/z) 440 m/z and D₅-Atorvastatin (parent ion m/z 564) daughter ion m/z 445 were detected after electrospray ionization MS/MS analysis. The capillary voltage was set to 2.80 kVolt, the cone voltage to 35 Volt, the extractor voltage and RF Lens voltage to 10 Volt and 0.20 Volt respectively. The source block was heated
to 140 °C and the desolvation temperature was 350 °C. The setting for the collision energy was 25.0 eV.

**Experiment Design**

The clinical trial was performed at the Watson Clinic in Lakeland, Fl under the supervision of Patrick Reddy. Samples were kindly provided for analysis to the Department of Pharmaceutics, University of Florida, Gainesville, FL.

Patients were on atorvastatin for a 90 day run in phase. Patients with steady state levels of atorvastatin were eligible for this study. Plasma was taken before commencement of the study. After the 0 time point was taken 10 ounces of grapefruit juice daily were added to the diet and monthly blood samples were taken. Blood sampling time points were 30, 60 and 90 days after enrollment.

**Sample Preparation and Extraction**

Plasma samples were thawed at 37°C. They were then vortexed and processed. 1.0 mL plasma, 50µL internal standard and 0.5 mL 1.0 N NaOH were added in that order to a 16 x 125 mm screw cap culture flask and vortexed for 60 sec. 5 mL diethyl ether was added to each tube. The tubes were then capped and shaken horizontally for 15 min. The solutions were then clarified by centrifugation for 10 min at 2600 X g. The bottom aqueous layer was frozen in an isopropanol/dry ice bath for 10 min and the top organic layer was discarded. The aqueous layer was then thawed at 37°C for 5 min. 2.0 mL 1.0 N H₃PO₄ was added to each sample tube and the tubes were vortexed for 60 s. After adding 10 mL of diethyl ether each sample vial was capped, shaken and centrifuged as before. The bottom aqueous layer was frozen and the ether layer was decanted into a clean tissue culture flask. The ether layer was evaporated in a water bath at 37°C. The residue was reconstituted in 0.2 mL ammonium acetate (20 mM,
pH4):acetonitrile:isopropanol (60:40:1), vortexed for 60 s and centrifuged at 2600 x g for 10 min. The supernatants were placed in 0.2 mL injection vials.

**Statistical Analysis**

Repeated measure one way ANOVA with Dunnett’s multiple comparison post hoc test was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. Differences were considered statistically significant at P<0.05.

**Results**

The atorvastatin calibration was linear in the range form 0.25-25 ng/mL. The coefficient of determination of the calibration curve was 0.998228 (Figure 5-1). Interday variation was < 15%.

Subjects that had no sample for the initial pre grapefruit juice time point were not considered in the analysis. After elimination of these subjects, a total of 121 datasets was analyzed. After the statistical analysis, no significant differences could be observed.

Concentrations at timepoint 30 days were not significantly different from concentrations at timepoint 0. Concentrations after 60 days were not different from the beginning. Additionally, atorvastatin concentrations after 90 days were not different from their initial values (Figure 5-2).

**Conclusions**

This study could demonstrate that the chronic concomitant administration of grapefruit juice in a realistic amount of 10 ounces per day does not lead to significantly higher plasma concentrations of the active form of atorvastatin. Simultaneous chronic administration to existing steady state concentrations did not lead to their augmentation either after 30, 60 or 90 days.
Figure 5-1. Calibration curve for atorvastatin acid 0.25ng/ml to 25 ng/ml

Figure 5-2. Concentrations of atorvastatin from day 1 to day 90. Data shown as mean ± SEM (n=121)
Table 5-1. Atorvastatin calibration standards for plasma samples

<table>
<thead>
<tr>
<th>Nr.</th>
<th>Concentration (ng/mL)</th>
<th>Volume to add (µL)</th>
<th>Standard Solution concentration (µg/mL)</th>
<th>Plasma Volume (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>0</td>
<td>----</td>
<td>----</td>
<td>40</td>
</tr>
<tr>
<td>1</td>
<td>25</td>
<td>0.1</td>
<td>10 (µg/mL)</td>
<td>39.9</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>0.08</td>
<td>10 (µg/mL)</td>
<td>39.92</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>0.04</td>
<td>10 (µg/mL)</td>
<td>39.96</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>0.2</td>
<td>1 (µg/mL)</td>
<td>39.8</td>
</tr>
<tr>
<td>5</td>
<td>2.5</td>
<td>0.1</td>
<td>1 (µg/mL)</td>
<td>39.9</td>
</tr>
<tr>
<td>6</td>
<td>1.0</td>
<td>0.4</td>
<td>100 (ng/mL)</td>
<td>39.6</td>
</tr>
<tr>
<td>7</td>
<td>0.5</td>
<td>0.2</td>
<td>100 (ng/mL)</td>
<td>39.8</td>
</tr>
<tr>
<td>8</td>
<td>0.25</td>
<td>0.1</td>
<td>100 (ng/mL)</td>
<td>39.9</td>
</tr>
</tbody>
</table>

Table 5-2. Atorvastatin quality control standards for plasma samples

<table>
<thead>
<tr>
<th>Nr.</th>
<th>Concentration (ng/mL)</th>
<th>Volume to add (µL)</th>
<th>Standard Solution concentration (µg/mL)</th>
<th>Plasma Volume (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>18</td>
<td>0.144</td>
<td>10 (µg/mL)</td>
<td>79.856</td>
</tr>
<tr>
<td>II</td>
<td>5</td>
<td>0.4</td>
<td>1 (µg/mL)</td>
<td>79.6</td>
</tr>
<tr>
<td>III</td>
<td>0.5</td>
<td>0.4</td>
<td>100 (ng/mL)</td>
<td>79.6</td>
</tr>
</tbody>
</table>
Discussion

Grapefruit juice has been shown to interact with several orally administered drugs. This is especially concerning, since most patients who have an existing condition might also want to benefit from the presumed beneficial properties of grapefruit juice. In most cases the expected clinical effect will be minor and of little clinical relevance. Conceptually, drugs which shows a high degree of interaction with grapefruit juice can be substituted by a drug not interacting, if the patient wants to continue his grapefruit juice consumption. This should however be limited to therapeutic equivalence and considered only after a careful risk benefit analysis. From the online database access statistics we can also deduct that there is a great interest in the general population and among health care professionals for comprehensive scientific information. The great number of almost 9 million hits shows the enormous interest in scientific information. This number is however to be considered carefully. A hit is generated whenever a user sends a request to the website. This is also true for sites that don’t exist and return an Error 404- file not found error page. Files might be a better estimator, since this is a count for actual hits resulting in a file being sent back. Site is the number of IP addresses sending the request. Care should be taken in the interpretation of this number; many users can appear to be coming from the same server IP. Visits occur when a remote site makes a request to the server. As long as the same site keeps making the request within a certain time period, it will be considered the same visit. A good estimator for actual pages being requested and not the single items that make up the page is the page access statistic. A relatively constant estimate of returning visitors since June 2006 strengthens our assumption of a great need for a comprehensive information source about grapefruit juice interactions. This estimate can be obtained by subtracting hits from files.
We can further conclude that the degree of interaction and the potential for adverse events of toxic effects depends widely on the type, brand and batch of grapefruit juice used. Different concentrations of grapefruit juice most likely lead to a different degree of effect [63, 65]. De Castro et al. reported in 2006 about the high variation of flavonoid and furanocoumarin content in different brands, batches, and dilutions of grapefruit juice [62]. Many of the publications about grapefruit juice interactions do not measure the amount of potential interaction compounds in their juice. No study has been performed so far where the same juice was given in different dilutions to establish a crude dose relationship curve. Mixing of different varieties of fruits, from different origin and harvest times during the juice production make it almost impossible to achieve a reproducible effect for the patient who just purchases “grapefruit juice”. The Horticultural Science Department of the University of Florida mentions a total of 12 different varieties of grapefruit [37]. Furthermore the interaction seems to be limited to drug with a low bioavailability. In case of a drug with a 95% bioavailability, inhibitions of intestinal metabolism will less likely result in significant changes of plasma concentration than a drug that has a bioavailability of 5%. In this case increase in 5% bioavailability can easily result in a 2 fold increase in plasma concentration. Additionally patients taking medications at the higher end of the therapeutical range might be more prone to adverse events or toxicological manifestations.

Researchers have developed methods such as heat treatment or UV treatment of grapefruit juice to eliminate drug interactions by removing interacting compounds. The removal of bergamottin and dihydroxybergamottin however does not seems to account for the total interaction and other components might be involved. Heat treatment had another detrimental effect: taste, flavor and nutrient content were compromised [68]. Furthermore the physiological safety of UV irradiated grapefruit juice has not been investigated [211]. Paine et al. used
absorption resin to remove furamocoumarins. The consumption of the furanocoumarin free juice resulted in lower felodipine exposure that regular grapefruit juice [212].

Some important questions and puzzles remain for the health care professionals. Adjustment of drug doses is common and mostly linked to clinical effect. How should physicians respond, if a patient had this medication adjusted while he was taking grapefruit juice? It might be common practice to recommend the cessation of grapefruit juice consumption. The fact that his enzyme levels return to normal and that this might result in a lower drug exposure, possibly linked to less therapeutic effect has to be considered.

Therefore we studied the influence of chronic grapefruit juice ingestion on the probability of myopathy development. The main concern about this kind of interaction study with food remains; the reproducibility. Our analysis if the juice shows once more, as it has been shown before by De Castro et al.[62] that the interacting compounds in grapefruit juice vary greatly. Dihydroxybergamottin was clearly more than two fold higher in the double strength juice as in the regular strength juice. Naringin concentrations were even equal in one of the regular strength juices compared to the double strength juice. In retrospect it seems better to make both dilution of juice out of the concentrate in order to have a real two fold difference in concentration of interacting compounds.

We could not repeat the results of Westwood et al. [205] who could show muscle damage in rats at a dose of 80 mg/kgxday-1. It is noteworthy however, that the previous researchers used female rats for their experiments. It has been demonstrated early on in the simvastatin development that female animals have a higher exposure when administered simvastatin than male animals [19]. This could be the reason why muscle damage in our study was only perceptible at a 200 mg/kgxday-1 dose of simvastatin. However the 80 mg/kgxday-1 group
remained unaffected. The survival rate of 50% made it difficult obtain a more suitable picture of 
the muscle damage, since the myopathy development in the dead animals could not be assessed.
It shows however the toxicity of this high dose of simvastatin. A toxicity that could not be 
observed in the SV80RS or SV80DS groups. Assuming the increase in exposure that has been 
observed in humans [63-65] is comparable in rats, that these two groups would have been 
exposed to plasma concentration comparable to a 240 mg/kgxday-1 to almost 1.3 g/kgxday-1 
dose, which would definitively lead to manifestation of toxicity [204, 205] that has not been 
oberved in our study. The 200 mg/kgxday-1 included two animals with high AST and ALT 
values at the end of the study, which could be a sign of muscle or liver toxicity, though no 
changes in absolute or relative liver weight could be demonstrated. Measured GGT values were 
in the negative range and were hence not considered. Due to the choice of scarification, the CPK 
values in the pilot study were not usable. Statistic evaluation and a further study of the influence 
of decapitation on the CPK levels deemed those unusable. Comparison of the remaining groups 
however showed increased CPK levels in two rats in the SV20DS group but none in the SV80DS 
group. We were not capable to lower the cholesterol levels in any of the two studies. Even a dose 
of 200 mg/kgxday-1 did not lead to a reduction in cholesterol. Interestingly the cholesterol levels 
after grapefruit juice administration were significantly lower than in the water control. The fact 
that it might not be possible to lower cholesterol in rats however has been shown before. As 
mentioned earlier, It seems that rats can upregulate the HMG-CoA reductase and cholesterol 
levels appear to be normal even when competitive inhibitors are present [207, 208]. The study 
design did not allow us to answer the question of why RS DS grapefruit juice might lower the 
cholesterol levels. Further research need to be conducted to investigate the exact mechanism of 
this effect. We could however still show muscle damage without lowering the cholesterol levels
as has been reported before [206]. The mechanism for this remains unclear, since other experiments suggest a direct relationship between HMG-CoA reductase inhibition and muscle damage [205]. Assessment of grip strength seems not to be the method of choice to detect and evaluate muscle damage. This test is usually performed to assess neuromuscular damage and shows great variability. Several factors such as operator and pulling angle can further influence the outcome [213]. The most important parameter however is the histological analysis of the muscle biopsies. Moderate muscle damage could be observed on the 200 mg/kgxday-1 group, but only two cases of mild muscle damage could be associated to the RS and SV20Ds group. It is interesting that no significant increase in muscle damage could be observed in the SV80 or SV80 groups combined with grapefruit juice.

As an additional possible indicator for muscle damage a test for locomotor activity was performed. The rational was that muscle necrosis would decrease the locomotor activity. Mobility of the rats in the 200 mg/kgxday-1 group was decreased, though it did not reach statistical significance due to the small number of remaining in this group. It might however prove feasible to use this test as an indicator for myopathy in laboratory animals. Interestingly the 53 mg/kgxday-1 group during the pilot study had increase locomotor activity compared to the water control. Similar observations were made during the final study. The SV80DS group showed a significant increase in locomotor activity compared to the SV80 control. This can be explained with the penetration of simvastatin through the blood brain barrier [17, 19]. Once in the brain, simvastatin seems to exhibit an effect on the neurotransmitter dopamine and the regulation of dopamine receptors. It has been reported that simvastatin can upregulate dopamine (D1 and D2) receptors [214] in the rat prefrontal cortex and can increase the dopamine content in
the stratum by 110% [215]. It has further been shown that changes in the dopaminergic system can lead to changes in behavior and locomotor activity [215].

The results of the muscle biopsies are even more exciting when looked upon in combination with the resulting plasma concentrations after coadministration with regular and double strength grapefruit juice. Regular strength GFJ did not alter the exposure of the low dose of simvastatin and only minimally altered the exposure of the high dose of simvastatin. This is even more interesting considering that we chose a dosing scheme that would reflect a real life situation rather than an artificial overexposure to large quantities of grapefruit juice. The animals were dosed with 5mL/kg juice, which would be equivalent to a regular 8oz glass of RS GFJ or two 8oz glasses when we dosed with DS GFJ. Previous studies have showed that predosing TID with DS[63] or RS[65] GFJ can significantly alter the plasma concentrations in humans. Clear difference however can be seen in the extent to which these plasma concentrations are altered. The fact that large quantities of grapefruit juice can alter the pharmacokinetics of simvastatin has also been recognized by the regulatory authorities and is reflected in the labeling of the drug. We have shown that even the increase in the maximum concentrations over 28 days which reached a maximum of 2-fold in the 80 mg/kg group dosed with DS GFJ did not result in increased occurrence of muscle damage or decreased survival. Assuming dose proportionality plasma concentration would reach into the range of high doses given in the pilot study which showed clear sign of myopathy and a decreased survival rate. The lack of a significant increase in the 20 mg/kg group brings up the question whether the rat model is right to investigate GFJ drug interactions. Researching the presently published literature we encounter previous experiments were a clear interaction has been demonstrated. Mangano et al. [216] showed a significant 31% increase in Cyclosporin-A exposure after coadministration with GFJ in male Sprague-Dawley...
rats. The dose of GFJ however was 10mL/kg and hence double the dose we chose for the previously mentioned reasons. The possibility of inhibiting intestinal CYP 3A4 was also demonstrated by Grundy et al. who report an increase in nifedipine bioavailability after coadministration of 6mL/kg GFJ concentrate. RS GFJ however had no significant effect on bioavailability in their study [217]. Our results seem to fit in this overall picture. Realistic doses of GFJ seem to be able to increase the exposure of simvastatin in the rats but seem not to lead to greater muscle damage. There is however one caveat: these results should be seen in the context of an experiment performed in laboratory animals and might not reflect interspecies differences in metabolism. Further clinical trials are needed to definitively assess the possibility of myopathy after coadministration of simvastatin and GFJ.

It was of great interest to further investigate the effect of chronic administration of grapefruit juice on other statins in humans. Atorvastatin has a bioavailability of 12% and is administered as the active hydroxyacid form [4]. Even though it is metabolized by CYP 3A4 it seems not to be as strongly affected by the grapefruit juice drug interaction as simvastatin [63]. Increases in atorvastatin acid plasma exposure (AUC) range from 1.4 fold [81] and 1.8 fold [82] with single strength grapefruit juice to 2.5 fold when given with double strength grapefruit juice [83]. All the study performed so far however are composed of either multiple administration of grapefruit juice and a single dose administration of the drug or multiple drug administration and single dosing of grapefruit juice. This is the first study were a chronic effect of simultaneous administration of drug and grapefruit juice has been investigated. Our results indicate that there is no significant difference between plasma concentration with or without grapefruit juice consumption. Since atorvastatin is administered in its active form, it seems unlikely that this interaction is clinically relevant.
The results from the in-vivo animal experiments and the clinical trial indicate the necessity to conduct a clinical interaction trial to confirm the lack of myopathy development after increased plasma levels of simvastatin in humans.

**Conclusions**

The results of our investigations suggest that chronic simultaneous administration of grapefruit juice and simvastatin does not lead to an increased incidence of myopathy in laboratory animals. The coadministration with grapefruit juice does not result in the same toxicity as a high dose of simvastatin would. We can further conclude that consumption of grapefruit juice might be capable to reduce cholesterol levels in humans as we have shown in animal experiments. It remains to be investigated whether this is due to the same mechanism as exhibited by HMG-CoA reductase inhibitors. We can further conclude that chronic administration of grapefruit juice and atorvastatin does not result in increased plasma concentration of the active hydroxy acid form. Our research about published clinical interaction trials suggest that most of the investigated drugs show no or only a minor interaction with grapefruit juice. The great number of accesses to the online database indicates the need for comprehensive scientific information.


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BIOGRAPHICAL SKETCH

Immo Zdrojewski was born on February 13th, 1976, in Herten, Germany. He received his license to practice as pharmacist in Germany in January 2003 from the Westfaelische Wilhelms Universitaet in Muenster, Germany. After his graduation he worked as pharmacist for Pharbil GmbH in Waltrop, Germany, performing work in process validation and risk analysis of generic bulk production and primary and secondary packing of liquid and semi-solid dosage forms. He joined the doctoral program under the supervision of Dr. Hartmut Derendorf and Dr. Veronika Butterweck at the Department of Pharmaceutics of the University of Florida in August 2003.

Immo Zdrojewski received his PhD in pharmaceutical sciences in May 2008 with his work about Interactions between grapefruit juice and HMG-CoA reductase inhibitors.