THE EFFFECT OF DIFFERENT MODULATORS ON THE TRANSPORT OF RHODAMINE 123 ACROSS RAT JEJUNUM USING THE SWEETANA-GRASS DIFFUSION METHOD

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GLOSSARY

The definition or meanings of the various symbols, abbreviations and terminology used in this dissertation are as follows:

ABC-transporters	: ATP-binding casette transporters
ABS	: ATP-binding sites
ANOVA	: Analysis of variance
AP-BL	: Apical to basolateral
BL-AP	: Basolateral to apical
BBMV`	: Brush border membrane vesicles
BLMV	: Basolateral membrane vesicles
CYP3A	: Cytochrome-P450 family 3, subfamily A
CYP3A4	: Cytochrome-P450 family 3, subfamily A, member 4, the predominant form in adult liver and intestine
DBSs	: Drug-binding sites
GJ	: Grapefruit juice
HCT-15	: Human colon tissue
KR	: Krebs-Ringer bicarbonate buffer
MBEC4	: Mouse brain endothelial cells
MDR	: Multidrug resistance
MDR1	: Multidrug resistance gene product class 1
MDR2	: Multidrug resistance gene product class 2
MDR3	: Multidrug resistance gene product class 3
MRP	: Multidrug resistance-associated protein
MSD	: Membrane-spanning domains
NBDs	: Nucleotide binding domains
NBS	: Nucleotide binding site

P_{app}	: Average apparent permeability coefficient
Pgp	: P-glycoprotein
PKC	: Protein kinase C
Rho123	: Rhodamine 123
TMD	: Transmembrane domain

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ABSTRACT

Background: P-glycoprotein (Pgp), which leads to multidrug resistance in tumour cells, is an ATP-dependent secretory drug efflux pump. In the intestine, as well as at specific other epithelial and endothelial sites, P-glycoprotein expression is localised to the apical membrane, consistent with secretory detoxifying and absorption limitation functions. The primary function of Pgp is to clear the membrane lipid bilayer of lipophilic drugs. Results from *in vitro* studies with human Caco-2 cells provide direct evidence for Pgp limiting drug absorption. Limitation has non-linear dependence of absorption on substrate (eg. vinblastine) concentration, increased absorption upon saturation of secretion and increased absorption upon inhibition of Pgp function, with modulators such as verapamil. **Aim:** The aim of this study was to investigate the effect of a known Pgp inhibitor (verapamil) and grapefruit juice components (naringenin, quercetin and bergamottin) on the transport of Rhodamine 123 across rat jejunum and to compare these results with those obtained in similar studies done in Caco-2 cells and in rat intestine (monodirectional). **Methods:** Verapamil, naringenin (442 µM, 662 µM and 884 µM), quercetin (73 µM, 183 µM and 292 µM) and bergamottin (12 µM, 30 µM and 48 µM) were evaluated as modulators of rhodamine 123 transport across rat jejunum using Sweetana-Grass diffusion cells. This study was done bidirectionally, with three cells measuring transport in the apical to basolateral direction (AP / BL) and three cells measuring transport in the basolateral to apical direction (BL / AP). The rate of transport was expressed as the apparent permeability coefficient (P_{app}) and the extent of active transport was expressed by calculating the ratio of BL/AP to AP/BL. **Results:** The BL-AP/AP-BL ratio calculated for Rhodamine 123 with no modulators added was 2.31. The known modulator verapamil decreased the BL-AP/AP-BL ratio to 1.52. This was statistically significant and inhibition of active transport was clearly demonstrated. All modulators inhibited active transport. Only naringenin 884 µM, quercetin 183 µM and bergamottin 30 µM did not show a statistically significant decrease in the BL-AP/AP-BL ratio. **Conclusion:** All three components of grapefruit juice showed inhibition of active transport and should have an effect on the bioavailability of the substrates of Pgp and other active transporters. The results obtained in this study are similar to the results

found in Caco-2 cells, which suggests that Sweetana-Grass diffusion method can be used for diffusion studies.

Keywords: P-glycoprotein, naringenin, quercetin, bergamottin, Rhodamine 123, Sweetana-Grass diffusion cells

UITTREKSEL

Agtergrond: P-glikoproteïen (Pgp) is 'n ATP-afhanklike sekretoriese geneesmiddel effluks pomp wat lei tot weerstand van verskeie geneesmiddels in tumor selle. In die intestinum, asook in ander epiteel en endoteel areas, is Pgp gelokaliseerd tot die apikale membraan. Hierdie lokalisasie is in ooreenstemming met sy sekretoriese detoksifikasie en absorpsie beperkende funksies. Die primêre funksie van Pgp is om lipofiele geneesmiddels uit die selmembraan te verwyder. *In vitro* studies met menslike Caco-2 selle het bewys dat Pgp geneesmiddelabsorpsie beperk. Absorpsie van die substraat (bv. vinblastien) toon nie-liniêre afhanklikheid met 'n verandering in konsentrasie. Absorpsie neem toe met versadiging van sekresie en tydens inhibisie van Pgp funksie met moduleerders soos verapamil. **Doel:** Die doel van hierdie studie was om ondersoek in te stel na die effek van 'n bekende Pgp inhibeerder (verapamil) en pomelosap komponente (naringenien, kwersetien en bergamottien) op die transport van Rhodamien 123 oor rot jejenum en om hierdie resultate te vergelyk met resultate verkry in Caco-2 selle en rot intestinum (monodireksioneel). **Metode:** Verapamil, naringenien (442 µM, 662 µM en 884 µM), kwersetien (73 µM, 183 µM en 292 µM) en bergamottien (12 µM, 30 µM en 48 µM) was geëvalueer as moduleerders van Rhodamien 123 transport oor rot jejenum met Sweetana-Grass diffusie kamers. Hierdie studie was bidireksioneel uitgevoer, met drie kamers wat transport in die apikaal-basolaterale rigting bepaal het (AP-BL), en drie kamers wat transport in die basolateraal-apikale rigting bepaal het (BL-AP). Die tempo van transport was uitgedruk as die waarneembare permeabiliteits koëffisiënt (P_{app}) en die mate van aktiewe transport was uitgedruk deur die verhouding BL-AP/AP-BL te bereken. **Resultate:** Die BL-AP/AP-BL verhouding vir Rhodamien 123 sonder die toevoeging van enige moduleerders was 2.31. Die bekende moduleerder verapamil het die BL-AP/AP-BL verhouding verminder na 1.52. Hierdie verlaging van die verhouding was statisties betekenisvol en inhibisie van aktiewe transport was duidelik sigbaar. Al die moduleerders het aktiewe transport geïnhibeer. Net naringenien 884 µM, kwersetien 183 µM en bergamottien 30 µM het nie 'n statisties betekenisvolle verlaging in die BL-AP/AP-BL verhouding teweeg gebring nie. **Gevolgtrekking:** Al drie komponente van pomelosap het aktiewe transport geïnhibeer en behoort 'n effek te hê op die biobeskikbaarheid van die substrate van Pgp en ander aktiewe transporters. Die resultate in hierdie studie stem ooreen met die resultate wat verkry is tydens 'n soortgelyke studie waar Caco-2 selle gebruik is. Die Sweetana-Grass diffusie metode word dus aanbeveel vir uitvoering van diffusie studies.

Sleutelwoorde: P-glikoproteïen, naringenien, kwersetien, bergamottien, Rhodamien 123, Sweetana-Grass diffusie metode

CHAPTER 1

INTRODUCTION AND STATEMENT OF PROBLEM

1 Introduction

Poor oral bioavailability is generally thought to be due to physico-chemical processes, such as poor solubility in gastrointestinal fluids, lack of permeability through the intestinal membranes, or alternatively due to marked first-pass metabolism in the liver. For many drugs however, poor oral bioavailability could be due to the coordinated action of intestinal enzymes and efflux transporters (Benet *et al.*, 1996:139; Wacher *et al.*, 1996:99).

Drug absorption can be decreased by efflux transporters in the intestine. P-glycoprotein (Pgp) is a plasma membrane-bound drug efflux protein primarily found in drug-eliminating organs. In the small intestine, Pgp has been localized in the apical membrane of the intestinal epithelial cells (Thiebault *et al.*, 1987:7735), consistant with its role in effluxing compounds back into the intestinal lumen. Pgp is the product of the multidrug resistance gene (MDR1) in humans and was first characterised as the ATP-dependent transporter responsible for efflux of chemotherapeutic agents from resistant cancer cells (Gottesman & Pastan, 1993:385). Substrates for Pgp cover a broad range of structures with diverse therapeutic indications. There are no clear structural features defining Pgp substrates, however the molecules tend to be large and amphipathic, containing one or more aromatic rings (Wang *et al.*, 2003:205). Wacher *et al.* (1998:1322) noted that most substrates of Cytochrome P450 3A4 (CYP3A4) are also substrates of Pgp, demonstrating the mutually broad selectivity of these proteins.

Pgp modulators can be roughly divided into three categories, namely:

- high-affinity substrates of the pump;
- efficient inhibitors of ATP hydrolysis coupled Pgp transport, and
- partial substrates / inhibitors according to the interaction between modulators and Pgp (Wang *et al.*, 2003:205).

Although most modulators share some common chemical features, such as aromatic rings structures, a tertiary or secondary amino group (protonated under physiological pH) and high lipophilicity, certain compounds may be without some of these features. Neutral molecules such as progesterone and flavonoids, for example, still posses resistance reversal activity (Wang *et al.*, 2003:205). The modulators can be grouped into seven classes according to their structural features namely:

- calcium or sodium channel blockers;
- calmodulin antagonists;
- Protein kinase C inhibitors;
- flavonoid and steroidal compounds;
- indole alkaloids and polycyclic compounds;
- cyclic peptides and macrolide compounds, and
- miscellaneous compounds (Wang *et al.*, 2003:206).

During this study one calcium channel blocker (verapamil) will be used to test if inhibition of active transport can be facilitated in this model. Subsequently several compounds in the category of the flavonoid and steroidal compounds will be evaluated as potential modulators.

Several *in vitro* and *in vivo* methods may be used to screen possible modulators of Pgp activity (Smith, 1996:13). These include *in vitro* transport studies using cultured cell membranes, as well as intact human or animal intestine and *in vivo* testing in humans and animals. There are several considerations to be taken into account when deciding which of these methods should be used, including time, cost and Pgp expression (Smith, 1996:13).

In a study done with Caco-2 cell membranes (Janse van Vuuren, 2000:1) the effects of the individual components of grapefruit juice were investigated. Various problems were encountered using this technique which includes the high costs to grow and sustain these cultures as well as time consuming procedures. It has also been reported that expression and activity of Pgp in Caco-2 cells varied with culturing conditions (Anderle *et al.*, 1998:760). In order to address these problems an *in vitro* method using Sweetana-Grass diffusion cells was chosen because the rate of transport of selected compounds could be determined in the presence of Pgp inhibitors or enhancers using excised biological membranes. Rat jejunum segments were used to perform this study.

The aims of this study are to:

- determine the validity of the model by testing the effect of a known modulator (verapamil) on the transport of the Pgp substrate rhodamine 123 across rat intestine using Sweetana-Grass diffusion cells;

- study the effects of grapefruit juice components (naringenin, quercetin and bergamottin) on the transport of the Pgp substrate rhodamine 123 across rat intestine, and

- compare these results to those found in a previous study done with Caco-2 cells (Janse van Vuuren, 2000:1)

CHAPTER 2

FACTORS AFFECTING INTESTINAL DRUG ABSORPTION AND TRANSPORT

2 Introduction

For systemic absorption, a drug must pass from the absorption site through or around one or more layers of cells to gain access into the general circulation. The permeability of a drug at the absorption site into the systemic circulation is intimately related to the molecular structure of the drug and to the physical and biochemical properties of the cell membranes. For absorption into the cell, a drug must traverse the epithelial cell membrane to reach the circulation. This can be done either by trans- or paracellular absorption. *Transcellular absorption* is the process of a drug movement across the cell. Some polar molecules may not however be able to traverse the cell membrane, but instead, go through gaps or "*tight junctions*" between cells, a process known as *paracellular drug absorption.*

Membranes are a major structure in cells, surrounding the entire cell (plasma membrane) and act as the boundary between the cell and the intestinal fluid. Cell membranes are semi-permeable membranes and act as selective barriers for the passage of molecules. Water, some selected small molecules and lipid-soluble molecules pass through such membranes, whereas highly charged molecules and large molecules, such as proteins and protein-bound drugs, do not (Shargel & Yu, 1999:99).

The oral route of administration is preferred for many classes of drugs and the main reasons therefore are the ease of administration and patient compliance. The bioavailability of a medicine is defined as the rate at which the drug becomes available to the body and the extent to which the dose is ultimately absorbed after administration (Lund, 1994:244). Systemic bioavailability is influenced by a variety of factors which are shown in Table 2.1

Table 2.1: Factors influencing bioavailability (Van der Waterbeemd, 2000:32)

Physiological factors	**Pharmacokinetic factors**
Membrane transport	Gastro-intestinal and liver metabolism
Gastro-intestinal motility	(first pass effect)
Stomach emptying	Chemical instability
Disease state	Absorption
Formulation factors	Distribution and elimination
Crystal form (polymorphism)	**Physicochemical factors**
Particle size	Lipophilicity
Absorption enhancers	Solubility
Dissolution rate	Degree of ionization (pKa)
Dosage form (solution, capsule, tablet or	Molecular size and shape
other)	Hydrogen-bonding potential

The most common detrimental influences on drug absorption include poor solubility, poor permeation, intestinal and liver metabolism, and P-glycoprotein (Pgp)-mediated efflux (van der Waterbeemd, 2000:31).

2.1 Passage of substances across biologic membranes

The passage of drugs across biological membranes can take place by means of different mechanisms which include simple diffusion, specific transport and persorption.

2.1.1 Simple Diffusion

Simple diffusion is caused by the thermal agitation of the solvent and solute particles. Fick's law quantitatively expresses the rate of diffusion:

$$\frac{dn}{dt} = DA\frac{dc}{dx}$$

where *dn* is the number of molecules (ions) crossing an area *A* in the time *dt* in proportion to the concentration difference *dc* over a distance of *dx*. *D* is the diffusion coefficient and is expressed by the amount of substance diffusing across a unit area per unit time where *dc*/*dx*=1 (Fick, 1855:59). Lipids or lipid-soluble substances, including the majority of drugs and other xenobiotics, are transported by simple diffusion (Csàky, 1984:53).

2.1.2 Specific Transport Mechanisms

There is ample evidence that highly polar substrates pass across the lipid intestinal membranes, even against a higher concentration or electrochemical gradient. In these cases the involvement of a specific transport mechanism is assumed and these mechanisms include carrier-mediation and pinocytosis (Csàky, 1984:53).

2.1.2.1 Carrier-mediation

Various carrier-mediated systems (transporters) are present at the intestinal brush border and basolateral membrane for the absorption of specific ions and nutrients essential for the body. Because of the structural similarity of these carriers to natural substrates they also aid in the absorption of many drugs. A transmembrane protein, P-glycoprotein (Pgp), has been identified in the intestine and appears to reduce apparent intestinal epithelial cell permeability from the lumen to the blood for various lipophilic drugs. There is however also other transporters present in the intestines (Thiebaut *et al.*, 1987:7735; Tsuji *et al.*, 1996:963).

2.1.2.1.1 Facilitated Diffusion

Facilitated diffusion is also a carrier mediated system and differs from active transport in that the drug moves along a concentration gradient. As its basic function the carrier simply facilitates the permeation of the polar substrate across the lipid membrane. Consequently, as long as the carrier sites are not saturated, the kinetics of the transport is the same as that for simple diffusion where the net transport ceases when equilibrium is reached on both sides of the barrier (Csàky, 1984:54).

2.1.2.1.2 Exchange Diffusion (Countertransport)

In the case of exchange diffusion the carrier may combine with the substrate on one side of the membrane and deliver it to the other side where it may combine with another substrate of similar structure, facilitating its transport in the opposite direction. This process is called exchange diffusion or countertransport (Csàky, 1984:54). In terms of active transport and absorption of drugs exchange diffusion plays a minor role.

2.1.2.1.3 Active transport

Active transport is a carrier-mediated process and is defined as an energy-requiring process whereby a substance permeates across a membrane barrier from a lower to a higher concentration (or electrochemical) gradient, yet the substance is neither bound on either side of the membrane nor produced or consumed during the transport. Thus transport take place against a concentration gradient and the process requires the expenditure of metabolic energy (Csàky, 1984:55).

2.1.2.2 Pinocytosis

Pinocytosis is a process where the cell membrane produces a deep infolding which is eventually detached as an intracellular vesicle (Lewis, 1931:17). Subsequently, the membrane of this vesicle is dissolved and its content is emptied into the cytosol. Alternately the pinocytotic vesicle attaches itself to the opposite membrane of the cell, fuses with it, and "releases" its content into the opposite extracellular space (Csàky, 1984:55).

2.1.3 Persorption

This is a special permeation across the intestinal wall in which the cell membranes are not involved. The intestinal epithelium turns over rapidly. New cells are continuously produced in the crypts of Lieberkühn. These cells migrate toward the tip of the villi where they are sloughed of, leaving temporarily a hiatus in the cell layer through which drugs are absorbed (Csàky, 1984:56).

2.2 ABC Transporters

A typical ATP-binding casette (ABC) transporter protein consists of four units namely two membrane-spanning domains (MSD), each with six transmembrane (TM) segments and two nucleotide-binding domains (NBDs), which bind and hydrolyze ATP. These four modular units can be expressed as separate polypeptides, or they may be fused together in one of several alternative arrangements, with the number of genes varying from one to four (Sharom, 1997:161). A schematic representation of an ABC transporter is shown in Figure 2.1.

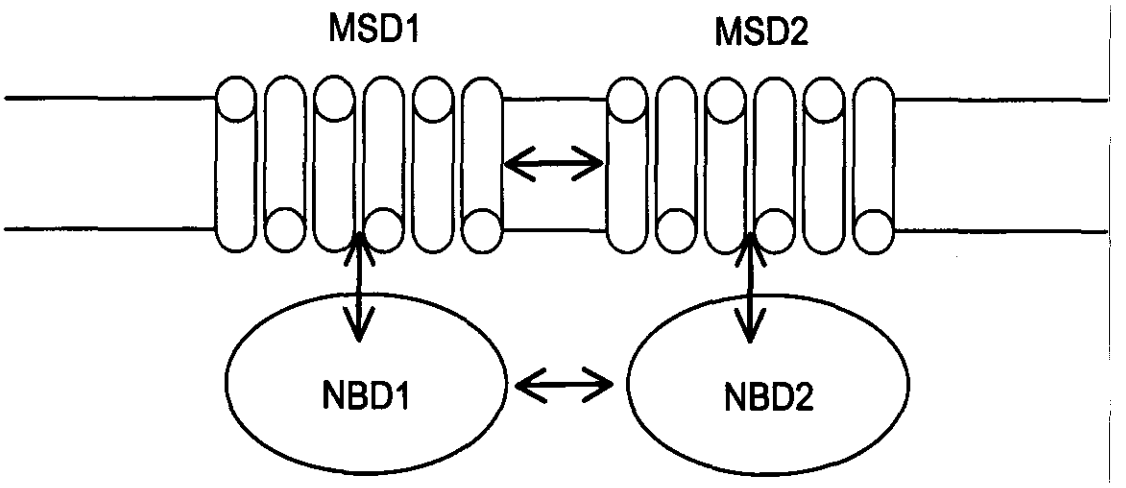


Figure 2.1: Schematic representation of ABC transporter membrane topology (Kerr, 2002:2)

Pgp is one of the most thoroughly studied proteins among the ABC family, and a significant amount of information has been acquired regarding the structure and function of ABC-transporters, based on analysis of Pgp (Brinkmann *et al.*, 2001:835). Absorption, distribution, metabolism and elimination are major factors that affect the therapeutic efficacy of compounds. Pgp and other ABC transporters are proven to play a role in these processes, by providing a barrier for the entry of compounds into the body, as well as controlling their rate of transfer between different tissues and compartments (Brinkmann *et al.*, 2001:837). Although identified as drug transporters these ABC-transporters frequently transport a number of substrates including dyes, ionophoric peptides, lipids and steroids (Walmsley *et al.*, 2001:71).

ATP-binding casette transporters are responsible for the uptake and efflux of a multitude of substances across both eukaryotic and prokaryotic membranes. Members of this family of proteins are involved in diverse physiological processes including antigen presentation, drug efflux from cancer cells, bacterial nutrient uptake and cystic fibrosis.

In contrast to prokaryotic drug transporters, many eukaryotic drug transporters belong to the ABC superfamily of membrane transporters, which hydrolyse ATP to drive drug efflux. This family includes the multidrug resistance (MDR), Pgp a 1280-amino-acid protein that confers resistance to anti-cancer drugs (Gottesman *et al.*, 1996:610).

2.2.1 Multidrug resistant transporters

Certain membrane proteins, collectively known as multidrug transporters, play an important role in different processes when they act as cellular antitoxins. Multidrug transporters provide an innate, but in many respects also an adaptive defence against toxic products of various infectious agents, harmful components of our metabolism as well as clinically applied therapeutic compounds. The key defence system of the individual cells against water-soluble harmful agents is the lipid bilayer of the plasma membrane, which provide an effective barrier against such compounds. However, hydrophobic toxic materials easily penetrate the core structure of the cell membrane, thus they have to deal with either intracellularly, or at the place of their entry which is the cell membrane itself. The major intracellular protective systems against hydrophobic agents are those that make these compounds water-soluble either by oxidation (e.g. the P450 enzymes) or by conjugation to glutathione or other hydrophilic small molecules. Thereafter the oxidized and / or conjugated, thus partially detoxified molecules are exported from the cells by special transport systems. The other possibility for the cellular defence is to eliminate the hydrophobic toxins before they actually enter the cytoplasm, thus removing them from the hydrophobic environment of the plasma membrane (Sarkadi *et al.*, 1996:215).

The multidrug transporters are key components for both of these defense mechanisms where some of them will most probably act in the manner described as "hydrophobic vacuum-cleaners" in the cell membrane (Higgins, 1992:67; Gottesman *et al.*, 1993:385; Croop, 1993:1). However, some of the recently discovered homologues for example the multidrug resistance-associated protein (MRP) also seem to contribute to the export of already water-soluble toxic products or conjugates (Cole *et al.*, 1992:1650; Zaman *et al.*, 1994:8822; Jedlitschky *et al.*, 1994:4833; Zaman *et al.*, 1995:7690).

Thus the multidrug transporters act as cellular antitoxic mechanisms and are adapted to recognize a variety of potential substrates, but do not remove essential hydrophobic or conjugated elements from the living cell. A detailed discussion of the exact mechanism of multidrug transporter, Pgp will be given in paragraph 2.2.1.1.3.

2.2.1.1 P-glycoprotein

Pgp is an energy dependant efflux pump associated with multidrug resistance in tumor cells and is also expressed in a variety of normal human tissues which include the liver, brain, kidney and the gastrointestinal tract (Thiebault *et al.*, 1987:7735). Pgp is not limited to humans but is also expressed in normal rat intestinal epithelium (Hsing *et al.*, 1992:879). In the small intestine, Pgp is located on the apical membrane of the mature intestinal cells and acts as a pump that transports drugs back into the lumen as they are absorbed across the intestinal mucosa (Hebert *et al.*, 1997:201).

2.2.1.1.1 Function and distribution of Pgp

The differential expression of Pgp in normal tissues and its conservation among species suggest that the protein may have distinct physiological roles associated with specialized cell functions. The tissue distribution of Pgp which is mainly in the epithelia of excretory organs, and the ability of Pgp to transport a wide range of lipophilic substrates, confirm the hypothesis that Pgp serves a detoxification function in the body (Gatmaitan *et al.*, 1993:77), although in tissues like the adrenal gland the protein is likely involved in the transport of specific endogenous cellular products (Ueda & Okamura *et al.*, 1992:24248; Wolf *et al.*, 1992:141).

The possible physiological functions of Pgp in mammals include protection against exogenous toxins ingested with food, excretions of metabolites or toxins, transport of steroid hormones, extrusion of (poly-) peptides (cytokines) not exported from the cell via the classical signal/cleavage pathway, ion transport and cell volume regulation, lymphocyte cytotoxicity, transport of prenylcysteine methyl esters and intracellular vesicular transport of cholesterol (Borst & Schinkel, 1996:986).

Since Pgp has a role in clinical drug resistance, many investigators have focused on strategies to inhibit the action of this protein. It is now well documented that many drugs, including verapamil and cyclosporin A (Miller *et al.*, 1991:17; List *et al.*, 1993:1652) are able to reverse multidrug resistance. This suggests a common role as a protective mechanism. The mechanism by which such a wide range of compounds are transported is unknown, but it appears that the drug is effluxed by flipping the drug from the inner to the outer leaflet of the bilayer membrane as illustrated in Figure 2.3 (Schinkel *et al.*, 1999:179).

2.2.1.1.2 Structure of pgp

Pgp is composed of two blocks each containing six transmembrane domains (TMD) that form three transmembrane loops and an intracellular nucleotide-binding site (NBS) for ATP-binding and hydrolysis on each block. Pgp is approximately 1280 amino acids long and consists of 2 homologous halves joined by a linker region (Van der Heyden *et al.*, 1995:223). A schematic model of the human multidrug resistance gene product Pgp is shown in Figure 2.2.

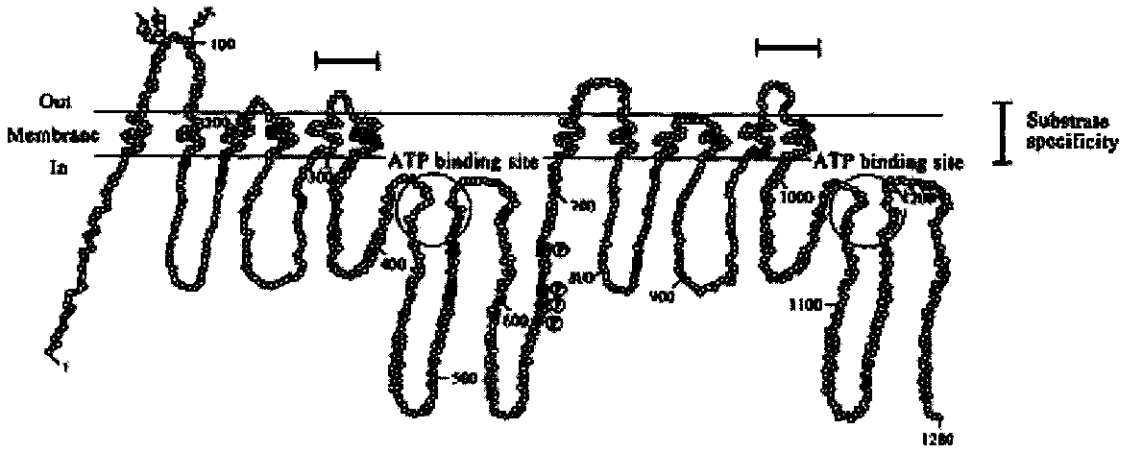


Figure 2.2: Schematic model of the human multidrug resistance gene product Pgp and its functional domains (Germann, 1996:929)

Studies on purified Pgps have confirmed that both ATP-binding sites (ABS) are capable of hydrolyzing ATP as the energy source for drug-translocation but not simultaneously, and that ATP hydrolysis and drug binding are intimately coupled together. This means that there exists a direct interaction between drug binding sites and the ATP binding site, probably involving conversion of the energy of hydrolysis of the pyrophosphate bond to the energy used to change the conformation of the peptide of the efflux pump (Urbatsch *et al.*, 1995:269576; Ramachandra *et al.*, 1998:5010).

Early photoaffinity labelling studies with Pgp substrate analogues have determined that there are two major drug interaction sites, TMDs 5,6 and 11,12 (Horizontal bars Figure 2.2), as well as the extracellular loop connecting them together (see Fig. 2.2) (Greenberger, 1993:5010; Morris *et al.*, 1994:329). But studies indicated that at least four distinct drug-binding sites (DBSs) exist on Pgp and they can be classified as transport and modulating sites, which can switch between high- and low-affinity states for substrates / inhibitors. Because of the mobility of Pgp subunits, these binding sites may be situated in distinct regions of Pgp or serve as parts of a large binding pocket with discrete domains for specificity. Thus, the presence of multiple DBSs and NBSs on Pgp and the

interaction among them may account for 'feeding' on a diverse range of structurally and functionally unrelated modulators and substrates and their behaviour as a drug efflux pump (Martin, *et al.*, 2000:624).

2.2.1.1.3 Mechanism of action

For many years, the model for drug resistance conferred by MDR1 Pgp has been a relatively simple one. In such a model cytotoxic drugs were actively transported out of cells that express Pgp against a concentration gradient, thereby reducing intracellular drug accumulation and inhibiting drug-mediated cell death (Persidis, 1999:94). The initial mechanistic models used to define efflux of drugs by Pgp hypothesized that Pgp formed a hydrophilic pathway, and drugs were transported from the cytosol to the extracellular media through the middle of the pore (Gottesman & Pastan, 1993:385), thereby shielding the substrate from the hydrophobic lipid phase as illustrated in Figure 2.3a. The current model proposes that Pgp intercepts the drug as it moves through the lipid membrane and flips the drug from the inner leaflet to the outer leaflet and into the extracellular media (Higgins & Gottesman, 1997:18) as illustrated in Figure 2.3b. A schematic model of the possible mechanism of action for the drug efflux by Pgp is shown in Figure 2.3.

This "flippase" function has also been described for related ABC molecules encoded by the human MDR3 (MDR2) and MRP genes (van Helvoort *et al.*, 1996:507; Kamp & Haest, 1998:91), indicating a possible conservation of function between this family of proteins. There is evidence for at least two allosterically coupled drug-binding sites, but the exact number of acceptor sites is still uncertain (Ferry, *et al.*, 1992:440; Martin *et al.*, 1997:765).

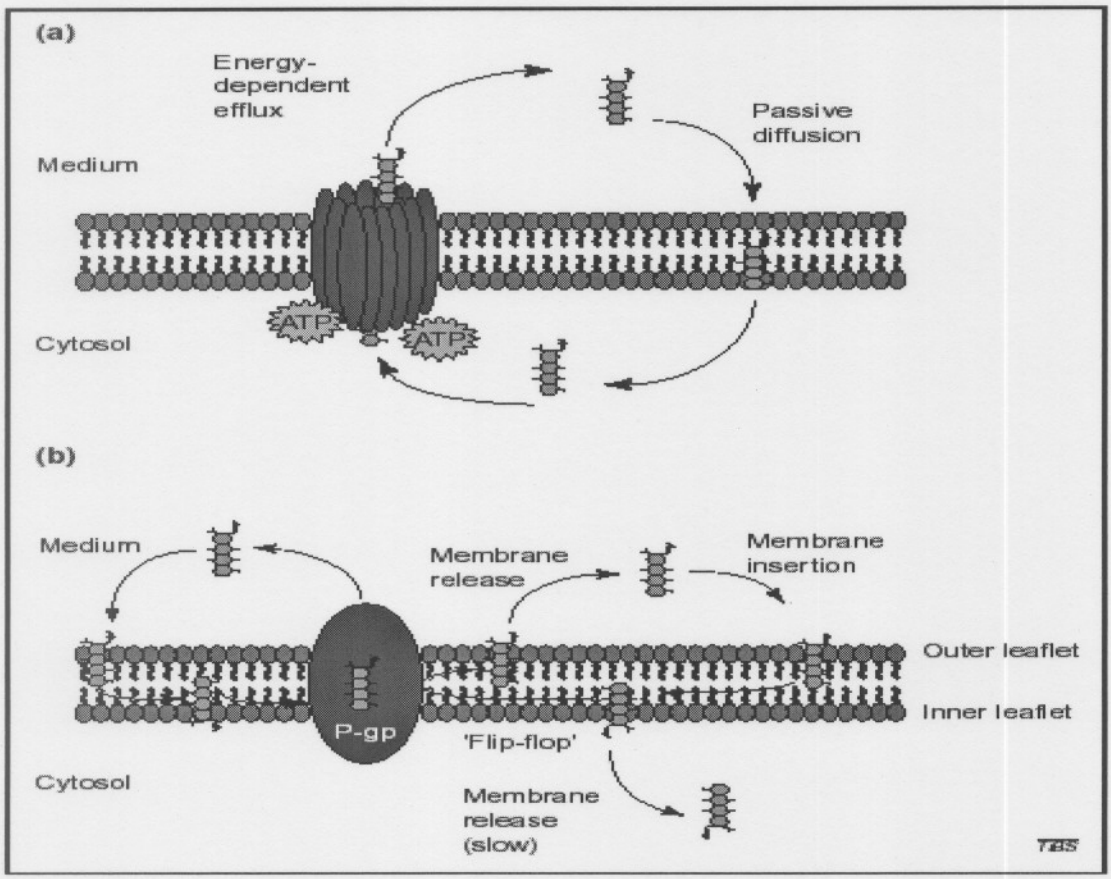


Figure 2.3: Possible mechanisms of action for drug efflux by P-glycoprotein (Pgp). (a) The "pump" model for drug transport. (b) The "flippase" model for drug transport (Persidis, 1999:94)

2.2.1.1.4 Compounds that interact with Pgp

Intestinal and tumor Pgp appear to have similar substrates and inhibitors. Initially it was observed that typical Pgp substrates are lipophilic and have a cationic functional group. A recent structural analysis of Pgp substrates suggested that the common structural components are two or three electron groups separated by a fixed space (Taburet *et al.,* 1996:385). Some examples revealing the structural diversity of Pgp substrates in the MDR spectrum are listed in Table 2.2.

Table 2.2: Pgp substrates included in the multidrug resistance spectrum (Sharom, 1997:162; Ambudkar *et al.*, 1999:368; Fertè, 2000:279)

Anthracyclines	**Cytotoxic agents**	**Steroids**
Doxorubicin	Colchicine	Aldosterone
Daunorubicin	Emetine	Dexamethasone
Epirubicin	Actinomycin D	**Miscellaneous**
***Vinca* alkaloids**	Puromycin	Rhodamine 123
Vinblastine	Mitoxantrone	Hoechst 33342
Vincristine	Ethidium bromide	Triton X-100
Epipodophyllotoxins	**Linear and cyclic peptides**	Prenyl-Cys methyl esters
Etoposide	NAc-Leu-Leu-norLeu-al	Calcein acetoxymethylester
Teniposide	NAc-Leu-Leu-Met-al	^{99m}Tc-SESTAMIBI
Taxanes	Leupeptin	JC-1
Paclitaxel (Taxol)	Pepstatin A	TPP^+
Docetaxel	Gramicidin D	**HIV protease inhibitors**
	Nonactin	Ritonavir
	Yeast *a*-factor	Indinavir
	Valinomycin	Saquinavir

A number of compounds known to interact with MDR cell Pgp have also been shown to be transported in the secretory direction by the intestinal epithelium. The identification of secretory transport has been primarily based on *in vitro* studies where transport in the secretory (basolateral-to-apical) direction exceeded that in the absorptive direction (apical-to-basolateral). Often, the involvement of Pgp in secretory transport was proposed because secretory transport was inhibited by Pgp antibodies or by inhibitors of Pgp, such as verapamil. Inhibition of secretory transport results in an increase in the net absorptive permeation (Ford & Hait, 1990:155).

Some examples of compounds that are recognised as inhibitors for intestinal secretion by Pgp are listed in Table 2.3.

Table 2.3: Chemosensitizing compounds which reverse multidrug resistance (Sharom, 1997:162; Fertè, 2000:279).

Calcium channel blockers	**Steroids**	**Cyclic peptides**
Verapamil	Progesterone	Cyclosporin A
Nifedipine	Tamoxifen	SDZ PSC 833
Azidopine	Cortisol	Valinomycin
Dexniguldipine	**Detergents and amphiphiles**	**Miscellaneous**
Nicardipine	Cremophor EL	Quinidine
Calmodulin antagonists	Solutol HS-15	Chloroquine
Trifluoperazine	Tween 80	Reserpine
Chlorpromazine		Amiodarone
Trans-flupenthixol		Terfenadine
		Dipyridamole
		FK 506

The possible mechanisms of action of chemosensitizers are illustrated in Figure 2.4.

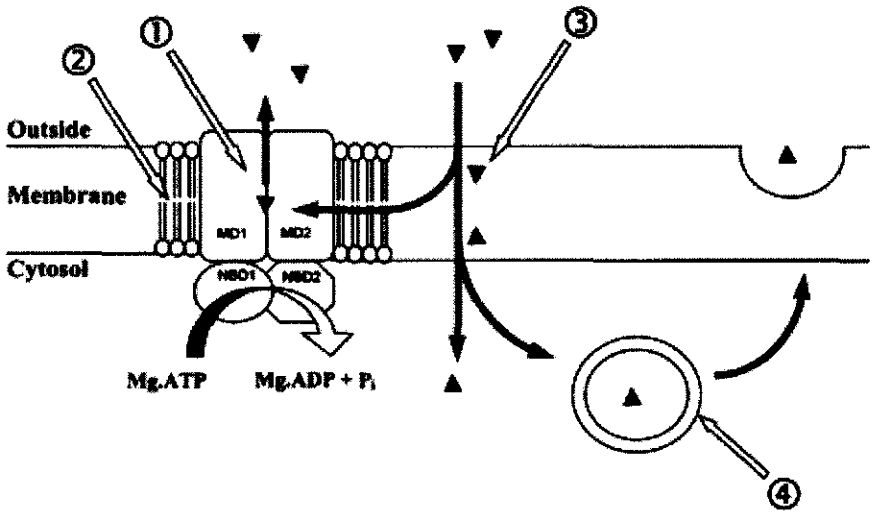


Figure 2. 4: Possible mechanisms of action of chemosensitizers (Fertè, 2000:285)

The cellular accumulation of a transport substrate in MDR cells depends on the permeation properties through the lipid bilayer as well as on the handling by Pgp. A chemosensitizer may interfere at different levels. It can impair the drug transport by Pgp through a direct interaction with the protein (1). Alternatively, chemosensitizers are able to alter membrane properties in different ways. This can result either in Pgp inhibition through perturbation of its membrane environment

(2), or in a modification of drug-membrane interactions (3). Interference with vesicular membranes can also lead to a redistribution of drug entrapped in endosomes (4) (Fertè, 2000:285).

2.2.1.1.4.1 Verapamil

Verapamil is a calcium-channel blocker and is classified as a class IV anti-arrhythmic agent (Reynolds *et al.*, 1989:89). The chemical structure of verapamil is shown in Figure 2.5.

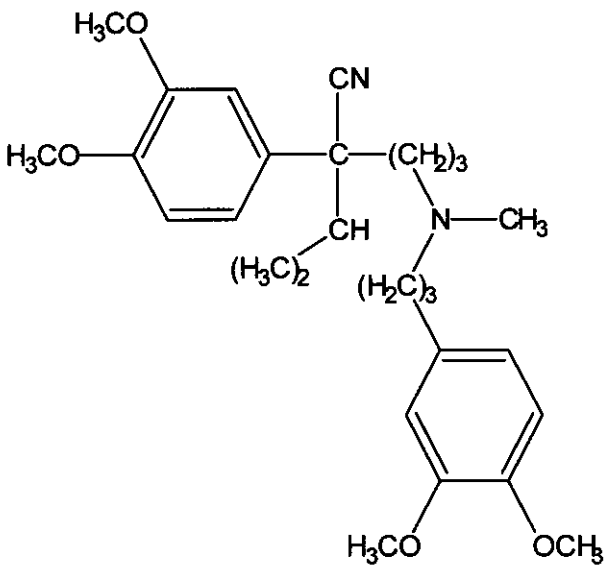


Figure 2.5: Structure of verapamil (Özkan *et al.*, 2000:376)

Verapamil is an effective MDR reversing agent (Ford & Hait, 1990:155). In humans, however, verapamil plasma concentrations necessary to reverse MDR may cause unacceptable cardiovascular side effects due to verapamil calcium antagonist activity (Salmon *et al.*, 1991:44; Miller *et al.*, 1991:17). *In vivo* and *in vitro* studies have shown that there is no correlation between the reversal potency and calcium antagonist activity (Plumb *et al.*, 1990:787; Mickisch *et al.*, 1991:447). R-verapamil is, in fact 10-fold less potent as a calcium antagonist than the S-isomer (Echizen *et al.*, 1985:210), but displays a similar reversal potency (Häubermann *et al.*, 1991:53).

2.2.1.1.4.2 Rhodamine 123 (Rho123)

Rhodamine 123 (Rho123), a fluorescent dye that is accumulated by mitochondria, is a Pgp substrate and a well-established tool to study Pgp transport activity. Inhibitors of Pgp-dependent transport such as verapamil or cyclosporin A have been found to decrease Rho123 efflux from Pgp-expressing cells (Hirsch-Ernst *et al.*, 2001:47). The chemical structure of Rho123 is shown in Figure 2.6.

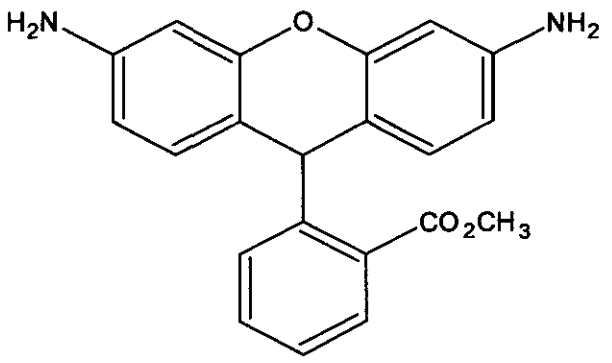


Figure 2.6: Structure of Rhodamine 123 (Eytan *et al.*, 1997:105)

Rho123, a lipophilic cation, constitutes a typical Pgp substrate, and is subject to Pgp-dependent extrusion through the plasma membrane. Due to its fluorescence, dye levels can easily be measured in cell extracts and accumulation can be observed in intact cells. Thus, Rho123 accumulation in (or efflux from) cells is often used as a measure of Pgp-dependent transport activity (Neyfakh, 1988:168; Chieli *et al.*, 1993:235; Wigler, 1996:279; Zubercova & Babusikova, 1998:53). High intracellular steady-state accumulation of the dye is interpreted in terms of low Pgp activity and *vice versa*. Accordingly, Pgp inhibitors would be expected to lead to enhanced accumulation of the dye in Pgp-expressing cells, as they interfere with dye extrusion. On the other hand, it has to be taken into account that Rho123, like many other lipophilic cations with aromatic groups, is not uniformly distributed throughout the cytosol but is accumulated in the mitochondria (Johnson *et al.*, 1980:990), driven by the mitochondrial internally negative membrane potential (Emaus *et al.*, 1986:436).

2.2.1.2 Multidrug resistance-associated protein, MRP

Multidrug resistance (MDR) is defined as the ability of cells, exposed to a single drug, to develop resistance to a broad range of structurally and functionally unrelated drugs due to enhanced outward transport (efflux) of these drugs mediated by a membrane glycoprotein (Hunter & Hirst, 1997:132).

MDR is thus a condition encountered in cancer patients where tumors become resistant to a variety of cytotoxic chemotherapeutic agents (Riordan & Ling, 1985:51). Resistance to multiple drugs is frequently encountered during treatment of various types of cancer by chemotherapy. Resistance may develop during drug treatment for example during the treatment of lung cancer, or the resistance may be an inherent feature of the particular tumor type (Cole *et al.*, 1996:169).

To date, MDR in model systems is known to be conferred by two different integral membrane proteins namely the 170 kDa P-glycoprotein (Pgp) (Riordan *et al.*, 1985:817; Gros *et al.*, 1986:728; Ueda *et al.*, 1987:3004; Lincke *et al.*, 1990:1779) and the 190-kDa multidrug resistance associated protein (MRP) (Cole *et al.*, 1992:1650; Cole *et al.*, 1993:879; Grant *et al.*, 1994:357; Zaman *et al.*, 1994:8822; Kruh *et al.*, 1994: 1649). Multidrug resistance associated protein (MRP) is another membrane transporter associated with drug efflux from tumour cells. MRP and Pgp proteins belong to the ATP-binding casette proteins (ABC) (Higgins *et al.*, 1992:67) or traffic ATPase (Ames *et al.*, 1992:1).

The cDNA encoding ATP-binding casette (ABC) multidrug resistance protein MRP1 was originally cloned from the drug-selected lung cancer cell line resistant to multiple natural product chemotherapeutic agents. MRP1 is the founder of a branch of the ABC superfamily whose members (from species as diverse as plants and yeast to mammals) share several distinguishing structural features that may contribute to functional and mechanistic similarities among this subgroup of transport proteins. In addition to its role in resistance to natural product drugs, MRP1 (and related proteins) functions as a primary active transporter of structurally diverse organic compounds, many of which are formed by the biotransformation of various endo- and xenobiotics by Phase II conjugating enzymes, such as the glutathione *S*-transferases. MRP1 is involved in a number of glutathione-related cellular processes. Glutathione also appears to play a key role in MRP1-mediated drug resistance (Hipfner *et al.*, 1999:359).

Compounds that interact with Pgp in MDR cells and which are also secreted by intestinal epithelial cells are listed in Table 2.4.

Table 2.4: Representative compounds interacting with Pgp in MDR cells and also secreted by intestinal epithelial cells

Compound	Model	Inhibited by	Reference
Duanomycin Rhodamine 123	Rat jejunum and ileum in vitro	Verapamil Diltiazem	Hsing *et al.*, 1992:879
Vinblastine	Caco-2 cells	Verapamil	Hunter *et al.*, 1993:14991
Vinblastine	HCT-8 cells	Verapamil Cyclosporin	Zacherl *et al.*, 1994:125
Cyclosporin A	Caco-2 cells	Chlorpromazine Progesterone	Augustijns *et al.*, 1993:360
Methylprogesterone	Rat ileum in situ	Verapamil Quinidine	Saitoh *et al.*, 1998:73
Verapamil	Rat intestine in vivo	Chlorpromazine Pgp antibody	Saitoh *et al.*, 1995:1304
Etoposide	Rat intestine in vivo	Quinidine Pgp antibody	Leu *et al.*, 1995:1304
Quinidine	Rat intestine in vivo	Verapamil Vinblastine	Emi *et al.*, 1998:295
Digoxin	Caco-2 cells	Verapamil Vinblastine	Cavet *et al.*, 1996:1389

2.3 Factors that may influence the bioavailability of drugs

Various factors may influence the transport of drugs across biological membranes and thus also the bioavailability of the drugs. One of these factors include cytochrome P450 3A4.

2.3.1 Cytochrome P450 3A4 (CYP3A4)

In the past, it was always assumed that the liver, rather than the intestines was the main guardian of the systemic circulation and that metabolism of xenobiotic compounds by the gut was not significant. Although the importance of hepatic metabolism cannot be over emphasized, overwhelming evidence exist that the intestinal metabolism by CYP3A4 is a major determinant of systemic bioavailability of orally administered drugs (Wacher *et al.*, 1998:1322). It appears that Pgp and CYP3A4 are functionally integrated as illustrated in Fig.2.7

The inter-relationship between Pgp and CYP3A4 operates in a complex manner as illustrated in Figure 2.7. Firstly, Pgp limits the total drug transport across the membrane resulting in drug concentrations that do not saturate CYP3A4 in the enterocytes (Washington *et al.*, 2001:131). Secondly, the decreased rates of drug absorption by Pgp increase the duration of exposure of the drug to the CYP3A4 in the enterocyte, thus providing greater opportunity for metabolism. In addition the metabolites generated by CYP3A4 are substrates for Pgp. These metabolites are actively transported out of the cell by Pgp and therefore it does not compete with the metabolism of the parent drug (Washington, 2001:131).

Both complexes are localised in the tips of the villi and not present in the crypts of Lieberkühn and CYP3A4 and Pgp genes appear to be close to each other on the same chromosome. An overlap between the substrates for both systems also exist (Watkins, 1997:161).

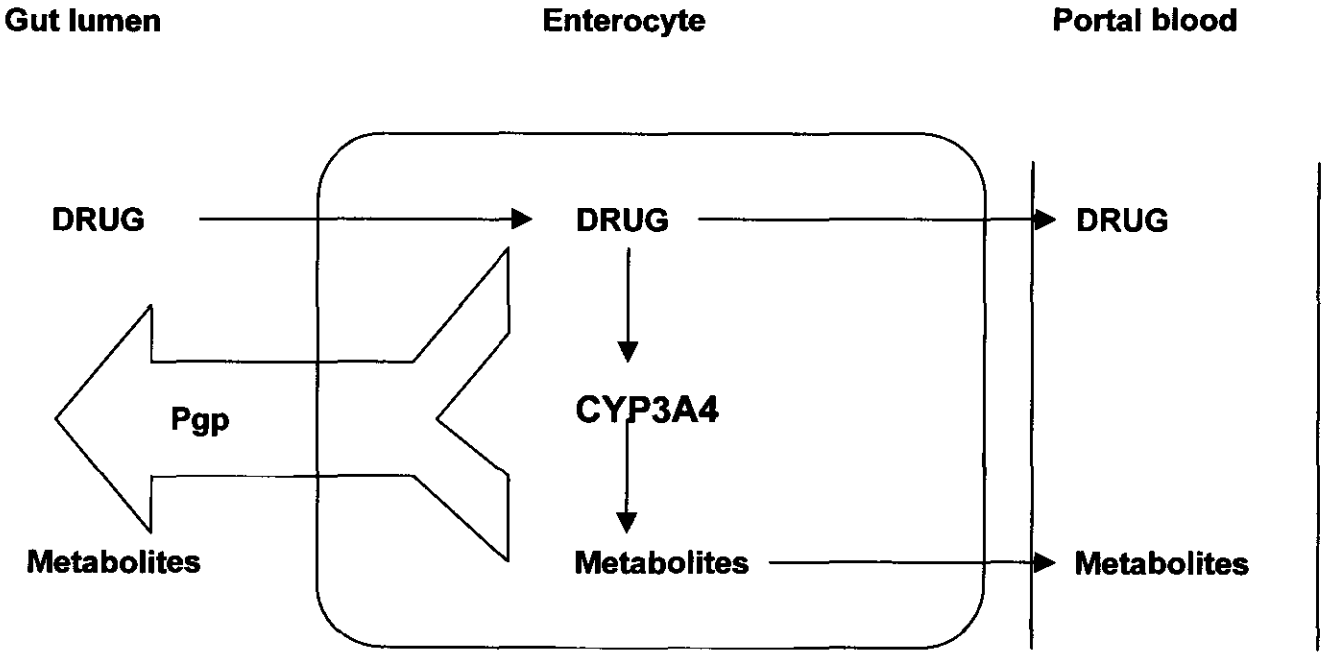


Figure 2.7: Possible mechanism of interaction between Pgp and cytochrome P450 3A4 (CYP3A4) (Washington, 2001:131)

Since the substrate specificity of CYP3A4 and Pgp overlap each other, these two proteins act synergistically in reducing the bioavailability of their substrates after oral administration. After being taken up by enterocytes, some of the substrate drug molecules are metabolized. Drug molecules which escaped metabolic conversion are eliminated from the cells into the lumen via Pgp. The drug molecules in the lumen may repeat the same cycle, resulting in repeated exposure to metabolic enzymes further reducing the intestinal bioavailability (Watkins *et al.*, 1997:161; Wacher *et al.*, 1998:1322; Benet *et al.*, 1999:25).

2.4 Grapefruit-drug interactions

The opportunity for a food-drug interaction is an everyday occurrence. This interaction may be of particular importance when the total amount of drug absorbed is altered (Bailey *et al.*, 1994:91). Grapefruit juice (GJ) for example can markedly increase the oral bioavailability of a number of medications when taken orally in conjunction with the drugs. Grapefruit juice has been shown to increase the bioavailability of various orally administered CYP3A4 substrates which include cyclosporine (Ducharme *et al.*, 1995:485; Min *et al.*, 1996:123; Ioannides-Demos *et al.*, 1997:49; Ku *et al.*, 1998:959), felodipine (Edgar *et al.*, 1992:313; Bailey *et al.*, 1993:637; Lundahl *et al.*, 1995:61; Lown *et al.*, 1997:2545; Lundahl *et al.*, 1997:139), midazolam (Kupferschmidt *et al.*, 1995:20), terfenadine (Benton *et al.*, 1996:383; Lundahl *et al.*, 1998:75), verapamil (Fuhr *et al.*, 1994:134) and many other therapeutic agents (Bailey *et al.*, 1993:589; Ameer *et al.*, 1997:103; Kane *et al.*, 2000:933). However, it has little effect on intravenously administered drugs (Ducharme *et al.*, 1995:485, Lown *et al.*, 1997:2545).

Identification of the active ingredient(s) in grapefruit juice would permit evaluation of this type of interaction with other foods. The apparently non-toxic active ingredients present in grapefruit juice might also be used commercially to be able to administer drugs orally that are currently only active when administered intravenously (Bailey *et al.*, 1994:91). In addition, because hepatic CYP3A4 activity does not appear to be altered by grapefruit juice, a major mechanism for systemic drug inactivation is not jeopardized. However, the persistence of hepatic CYP3A4 activity means that it would not likely be possible to produce complete oral drug bioavailability (Bailey *et al.*, 1989:357).

The grapefruit juice components that will be studied to determine their influence on the transport of Pgp substrate Rho123 across rat intestines include the furanocoumarin, bergamottin and the flavonoids, naringin and naringenin.

2.4.1 Bergamottin

Several furanocoumarins in grapefruit juice are effective *in vitro* CYP3A4 inhibitors and are suggested to be clinically active constituents (Edwards *et al.*, 1996:1287; Fukuda *et al.*, 1997:391; He *et al.*, 1998:252; Guo *et al.*, 2000:766). The most abundant furanocoumarins are bergamottin and 6', 7'-dihidroxybergamottin (Guo *et al.*, 2000:766). Bergamottin is the furanocoumarin found in the highest concentration in the ethyl acetate extract of grapefruit juice (He *et al.*, 1998:252). The structure of Bergamottin is shown in Figure 2.8.

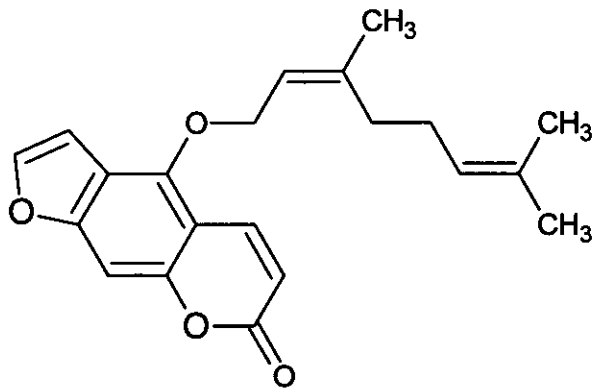


Figure 2.8: Structure of bergamottin (Guo *et al.*, 2000:767)

However, bergamottin was found to be a more potent competitive and mechanism-based inhibitor of CYP3A4 activity than its 6', 7'-dihidroxy derivative *in vitro* (Bailey *et al.*, 2000:468). It is even more important that bergamottin was concentrated in the fraction of grapefruit juice (particulate portion) that produced the most pronounced drug interaction in humans (Bailey *et al.*, 1998:248) when taken orally with felodipine. Consequently, bergamottin may be the primary inhibitor of clinical CYP3A4 activity in grapefruit juice.

2.4.2 Naringin

Naringin is the most abundant flavonoid in grapefruit juice attaining relatively high concentrations (1mM). This flavonoid is absent from orange juice (Blychert *et al.*, 1991:15). Naringin inhibited *in vitro* felodipine and nifedipine metabolism but was much less potent than its aglycone, naringenin to which it is converted *in vivo* (Deslypere *et al*,. 1991:342; Miniscalco *et al.*, 1992:1196). The structure of naringin is given in Figure 2.9.

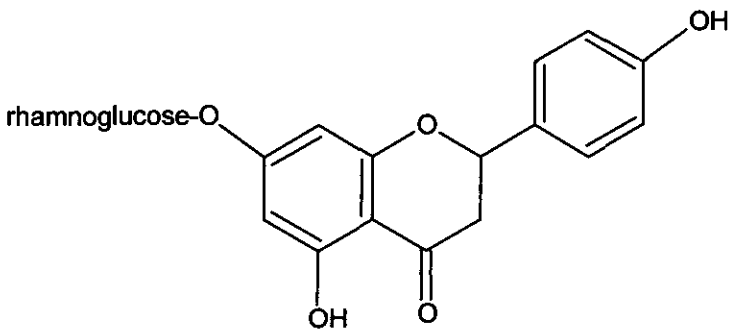


Figure 2.9: Structure of Naringin (Bailey *et al.*, 1993:637)

2.4.3 Naringenin

Naringenin is not normally present in grapefruit juice, but is produced *in vivo* through hydrolysis of naringin and narirutin (Takanaga *et al.*, 1998:1064). Fuhr and Kummert (1995:370) also found that naringin is partly metabolized to naringenin, indicating that enteral bacteria played an important role in this metabolic pathway. Bailey *et al.* (1998:250) did not detect naringenin in extracts from supernatant or particulate fractions of grapefruit juice, but Ameer *et al.* (1996:35) found a concentration of 241,1mg/l naringenin in grapefruit juice.

Although naringenin is not normally present in grapefruit juice (Kuhnau, 1976:117), oral administration of grapefruit juice resulted in renal excretion of naringenin conjugates demonstrating *in vivo* formation of this potentially active species (Fuhr & Kummert, 1995:365). The structure of naringenin is shown in Figure 2.10.

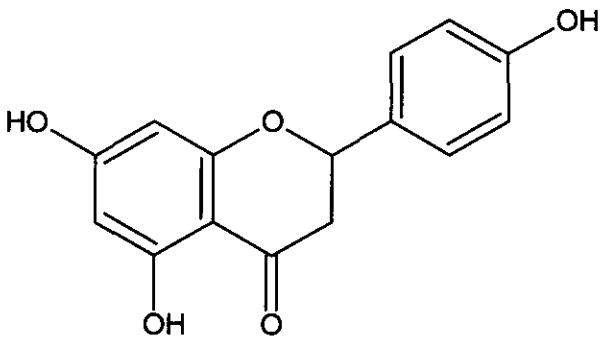


Figure 2.10: Structure of naringenin (Bailey *et al.*, 1993:637)

Naringenin, like many other flavonoids, is a potent inhibitor of CYP3A4 (Fuhr, 1998:265). Naringenin also inhibits Pgp action. It was found by Mitsunaga *et al.* (2000:199) that naringenin

increased the uptake of vincristine into MBEC4 cells and this observation indicates that there was Pgp inhibition.

2.4.4 Quercetin

Quercetin is widely distributed mainly as glycosides in components of the daily diet such as onions, apples, berries, tea and red wine (Hertog *et al.*, 1992:1591; Hertog *et al.*, 1995:381) as well as in herbal remedies and dietary supplements available worldwide such as *Sophora japonica* and *Ginkgo biloba* (Watson *et al.*, 1999:203; Hibatallah *et al.*, 1999:1435). Evidence showed that orally administered quercetin glycosides were significantly broken down to absorbable quercetin by enterobacteria (Kuhnau, 1976:117; Bokkenheuser *et al.*, 1987:953). Quercetin is however non-toxic and displays a variety of biological actions. Quercetin exhibit antioxidation activity (Takahama, 1985:1443; Frankel *et al.*, 1993:454), antiviral activity (Vrijsen *et al.*, 1988:1749; Ohnishi *et al.*, 1993:327), antiulcer activity (de la Alarcon *et al.*, 1994:56), antiallergic activity (Murray, 1998:10) as well as anticancer activity (Davis *et al.*, 2000:196). The structure of quercetin is shown in Figure 2.11.

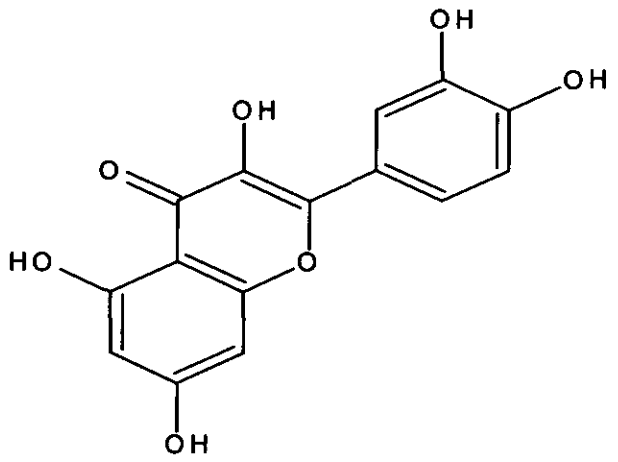


Figure 2.11: Structure of Quercetin (Hsiu *et al.*, 2002:228)

Regarding its modulation on Pgp, quercetin was initially described as an inducer in multidrug-resistant breast cancer cells and HCT-15 colon cells (Phang *et al.*, 1993:5977; Critchfiels *et al.*, 1994:1437), but new studies showed quercetin to be an inhibitor of Hoechst 33342 transport by Pgp (Shapiro *et al.*, 1997:587). *In vitro* studies done by Guengerich *et al.* (1990:2275) and Miniscalco *et al.* (1992:1195) indicated that quercetin was a potent inhibitor of CYP3A4.

2.5 Evaluation of intestinal permeability and metabolism *in vitro*

In vitro methods can be employed to determine whether molecules have the required permeability and stability characteristics to traverse the gastrointestinal wall and enter the portal circulation. Compared to *in vivo* absorption studies, evaluation of intestinal permeability *in vitro* requires less of the compound and is relatively easier to perform. In the case of segmental absorption studies, complicated surgery and maintenance of surgically prepared animals are avoided. It is also more rapid to perform and has the potential to reduce the amount of animals used since a number of variables can be examined in each experiment. *In vitro* experiments also provide insight into the mechanisms (e.g., carrier-mediated vs. passive), routes (e.g., transcellular vs. paracellular) and segmental differences (e.g., small vs. large intestine) involved in transepithelial transport. It is also analytically easier to determine because compounds are analyzed in an aqueous buffer solution as opposed to whole blood or plasma samples. In addition to their utility in defining intestinal permeability of compounds, *in vitro* methods can also be employed to study the metabolism of molecules during transport across the intestinal epithelium and to aid in formulation design and provide information to medicinal chemists regarding the molecular features which impede or enhance the absorption of compounds, thereby allowing a rational approach to design orally active molecules (Bondinell *et al.*, 1994:897; Samanen *et al.*, 1996:115).

Evaluation of intestinal permeability can be performed by means of cultured intestinal epithelial cell models as well as intestinal tissue model which both have different advantages and disadvantages.

2.5.1 Cultured intestinal epithelial cell models

In vitro systems such as brush border membrane vesicles (BBMV), basolateral membrane vesicles (BLMV), perfused intestinal loops, stripped intestinal mucosa and isolated enterocytes have been used to study mucosal drug absorption. Isolated enterocytes should permit the determination of transmembrane transport in the presence of cellular metabolism. However, upon isolation enterocytes lose their polarity and show limited viability (Hartmann *et al.*, 1982:G147). Attempts to develop an *in vitro* system derived from cultured intestinal epithelial cells have found this goal to be extremely difficult. Although intestinal enterocytes have been cultured in suspension, they undergo dedifferentiation when cultured as monolayers (Raul *et al.*, 1978:163).

The human colon adenocarcinoma cell lines Caco-2, HT-29 and T84 have been widely used to study various intestinal transport processes (Madara *et al.*, 1988:G416; Audus *et al.*, 1990:435). Because of their high transepithelial electrical resistance (350-1600Ω·cm^2), T84 cells have been mainly used to study tight-junction regulation (Madara *et al.*, 1988:G416). HT-29 and Caco-2 cells however have had greater application to study drug transport and metabolism. Caco-2 cell monolayers do not produce a mucus layer and consequently the role of the mucus layer in drug absorption had to be examined using mucus-secreting HT-29 clones (Lesuffleur *et al.*, 1993:771). Despite their lack of mucus production, Caco-2 cells are commonly used during drug transport and metabolism studies. These cells undergo spontaneous enterocytic differentiation in culture (Pinto *et al.*, 1983:323) and have been evaluated as a transport model system of the small intestinal epithelium (Hidalgo *et al.*, 1989:736; Dix *et al.*, 1990:1272).

Janse van Vuuren (2000) studied the effects of grapefruit juice and its components, alone and in combination, on the transport of cyclosporine by using Caco-2 cell monolayers. The results of this study will be compared to those observed by Janse van Vuuren (2000:37) and to determine if any similarity exists between the results obtained in both studies.

Cultured intestinal epithelial cell models have however several advantages and disadvantages that should be considered during the choice of the model that will be used during specific transport studies.

2.5.1.1 Advantages of cultured intestinal epithelial cell models

The advantages of Caco-2 cells for drug transport and metabolism studies are that these cells:

- can be used to determine both cellular uptake and transepithelial transport;
- permit the determination of drug transport in the presence of cellular metabolic reactions which may be important in active drug transport;
- contain many drug-metabolizing enzymes absent from membrane preparations;
- express cell polarity (a feature absent in BBMV, BLMV and isolated enterocytes), making it possible to determine directionality of uptake/transport;
- remain viable for long periods, and
- are from human origin.

2.5.1.2 Disadvantages of Cultured intestinal epithelial cell models

The disadvantages of Caco-2 cells are that they:

- lack a mucus layer, which may play an important role during drug absorption;
- lack cellular heterogeneity found in the intestinal mucosa for example goblet cell, Paneth cells, and undifferentiated crypt cells and, and
- lack some drug-metabolizing enzymes found in the small intestine, such as CYP4503A4.

The recent isolation of a Caco-2 clone that expresses CYP3A will help overcome above limitations. Another disadvantage of the Caco-2 cells is that their barrier properties resemble more closely that of colonic epithelium than those of intestinal epithelium (Hidalgo *et al.*, 1989:736).

2.5.2 Intestinal tissue models

The *in vitro* techniques resulted from the pioneering work of Ussing (1949:127) and co-workers, who published a series of papers describing the measurement of ion fluxes employing radioisotopes in "short-circuited" frog skin during the late 1940s and early 1950s (Ussing, 1949:127; Ussing, 1950:43; Ussing & Zerahn, 1951:110; Koefoed-Johnson *et al.*, 1952:150; Koefoed-Johnson *et al.*, 1953:38; Koefoed-Johnson & Ussing, 1953:60). These techniques have subsequently been applied not only to frog skin but also to a variety of epithelia including intestine (Schultz & Zalusky, 1964:567; Field *et al.*, 1971:1388).

Over the years, the design of the Ussing setup has been modified to incorporate the water-jacketed reservoirs and tissue cell into one piece (Grass & Sweetana, 1988:372; Sutton *et al.*, 1992:316), to accommodate different tissue surface areas (White, 1982:343), and to allow for alternative experimental procedures such as determination of the cell membrane potential or intracellular ion activities or for uptake studies (Rose & Schultz, 1971:639; Nellans *et al.*, 1974:1131; Frizzel *et al.*, 1979:27). However, the majority of transport/metabolism studies described in the literature have been conducted with a setup essentially identical to that described by Ussing and Zerahn (1951:110).

Techniques for preparation of intestinal tissue for use in Ussing chambers vary with the animal used as well as the segment used during the studies. Studies have been conducted with both "unstripped" and "stripped" tissue. For stripped tissue, the intestine is prepared by opening it along the mesenteric border, removing the circular and longitudinal muscle layers, after which these muscle-deficient tissues are placed in the Ussing chambers. For studies designed to determine the mechanisms and rates of drug transport and metabolism, stripped tissue are preferred because they resemble the *in vivo* situation more closely. Drug absorption into the intestinal vasculature for example does not involve permeation through the intestinal smooth muscle (Smith, 1996:17).

The *in vitro* Ussing technique does not provide information on the potential for hepatic first-pass effects or instability in any compartment other than the intestinal epithelial cells. However, the *in vitro* Ussing technique does provide a method for comparing intestinal epithelial permeability of molecules as well as monitoring intestinal viability and integrity (Smith, 1996:29).

2.5.2.1 Advantages of intestinal tissue model

The advantages of intestinal tissue models are that they:

- monitor viability and integrity of the system;
- determine the mechanisms involved in transepithelial transport;
- compare segmental differences in transport;
- evaluate sites and types of metabolism and/or degradation;
- identify interaction of molecules with apical recycling mechanisms;
- determine the effects of potential enhancers on the barrier properties and viability of the epithelium, and
- identify structural features of molecules that allow them to interact with a transporter (Smith, 1996:29).

7.2.1.2 Disadvantages of intestinal tissue models

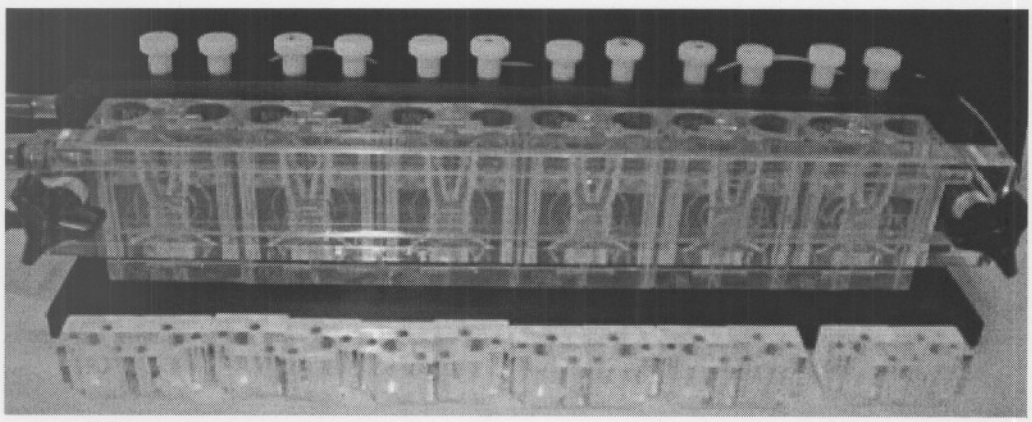
The major disadvantage of the Ussing technique in particular and the use of *in vitro* techniques used to predict human bioavailability is in general the limited database that is currently available. With the molecules that have been studied and reported, an acceptable similarity exists in general between the *in vitro* permeability of molecules and their bioavailability in humans.

CHAPTER 3

EXPERIMENTAL PROCEDURE

3 Introduction

The effects of different modulators on the transport of Rhodamine 123 across rat intestine was investigated using a vertical diffusion cell system, comprising six Sweetana-Grass diffusion cells, one heating block and one gas manifold (Corning Costar Corporation, Cambridge, USA) (Slide 1).



Slide 1

Although the Sweetana-Grass apparatus consists of six cells only four cells were used to perform the transport studies.

The Sweetana-Grass diffusion cells were derived from the Ussing chamber and have several advantages when compared to the classical Ussing chamber apparatus (Sutton *et al.*, 1992:316). The Sweetana-Grass diffusion cells were developed for the measurement of tissue permeability. This cell incorporates the attributes of using a single material and laminar flow across the tissue surface. The design of the cells allows the cell to be manufactured in a wide range of sizes to allow optimization of surface area to volume for a variety of tissues. The apparatus is also applicable for the evaluation of transport of compounds through mucosal/epithelial barriers for example gastrointestinal tissue. Active transport, permeability enhancers, enzymatic degradation and absorption in various tissue sections can also be determined.

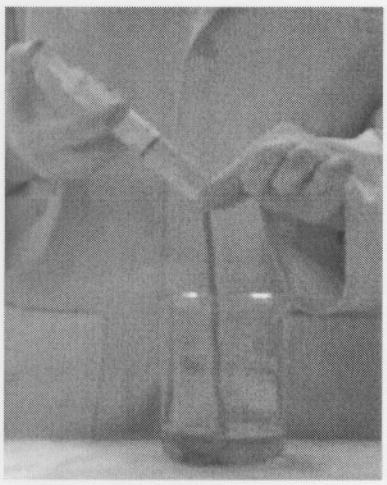
3.1 Materials

Krebs-Ringer bicarbonate buffer, Rhodamine 123, naringenin, quercetin, verapamil, (Sigma Chemical Company Ltd., St. Louis, Missouri, USA) was obtained from Sigma-Aldrich (Pty) Ltd, Johannesburg. Bergamottin (Indofine Chemical Company Inc., Somerville, New Jersey, USA) and absolute ethanol, acetonitrile for HPLC, were obtained from Merck (Pty) Ltd, Germiston.

3.2 Sweetana-Grass diffusion method

3.2.1 Tissue preparation

It was found to be necessary to remove the serosal muscle layer, as it reduced and in some cases completely blocked the transport of drugs. As this layer is not between the lumen and the blood vessels it is not normally an impediment to absorption and may be removed.

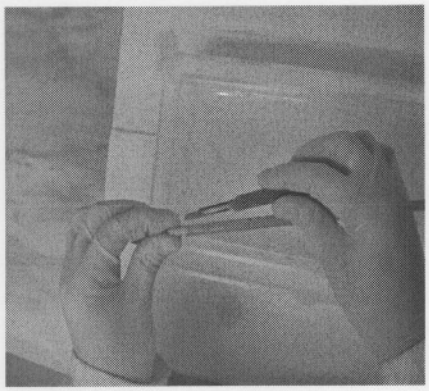


Slide 2

Slide 3

Adult Sprague-Dawley rats (350-370 g) from the animal research centre (North West University) were used. The ethical committee of the North West University approved the project under protocol number 03D03. Rats were anaesthetised by inhalation of halothane. An abdominal incision was made and a single jejunum segment of approximately 30 cm was removed beginning 5 cm from the stomach. The jejunum segment was rinsed with ice cold Krebs-Ringer bicarbonate buffer (KR) through which 95% O_2 / 5% CO_2 had been bubbled for 10 minutes (Slide 2) after which it was pulled over a glass rod (Slide 3) and placed into a long Perspex container filled with ice cold KR. The container was kept on ice.

The excised tissue was then gently scoured along the mesenteric border with the back of a scalpel (Slide 4). The serosal muscle layer was removed by gentle rubbing along the mesenteric border with the forefinger (Slide 5). Throughout the procedure, the tissue was immersed in ice cold KR which was kept in an ice bath. The excised strip was then cut along the mesenteric border (Slide 6) and washed off the glass rod with KR onto a strip of filter paper (Slide 7).



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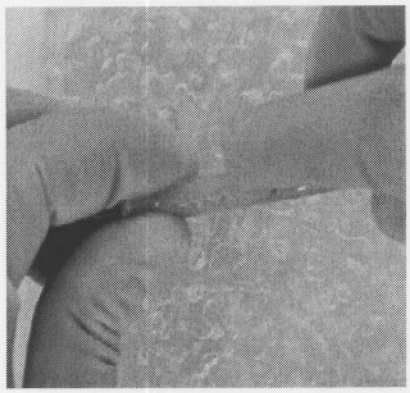

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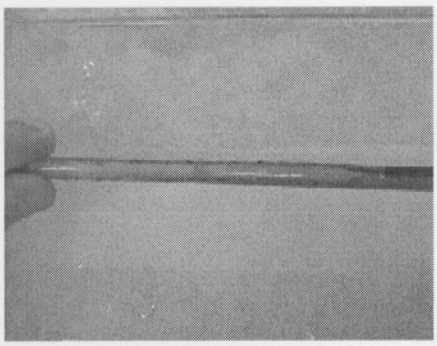

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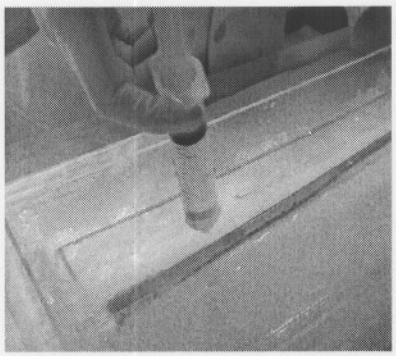
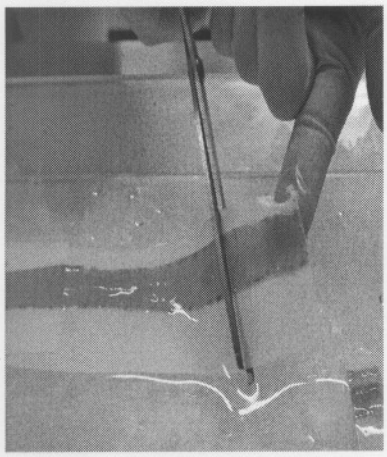


Slide 7

The strip was then cut into lengths approximately 3 cm long (Slide 8). The segments were kept on ice and moist with ice cold KR (Slide 9). Care was taken to avoid segments containing Payer's patches (Slide 10), as this lymph like tissues could probably cause greater variation in the rates of transport because of altered morphology and thickness of the epithelial layer.



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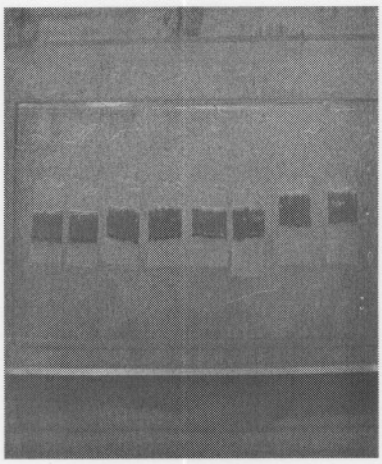

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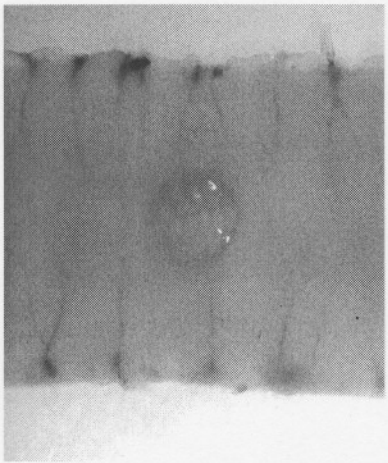
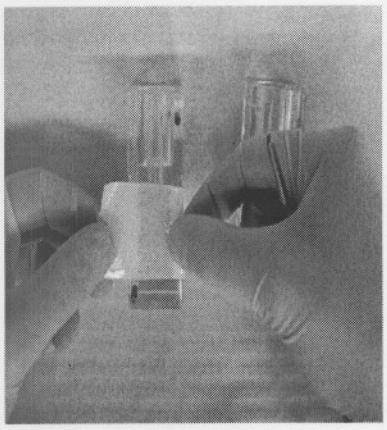


Slide 10

3.2.2 Mounting of tissue

The segments were then carefully mounted onto the half cells containing pins (Slide's 11 & 12). The half cells were preheated to 37 °C. The matching half-cells were then carefully clamped together without damaging the jejunal membrane (Slide's 13 & 14). The assembled cell was then placed in the heating block (37 °C) and 5 ml KR preheated to 37 °C was added (Slide 15). Circulation of the buffer was maintained by a gas-lift using 95% O_2 / 5% CO_2 at a flow rate of 15-20 ml/min.



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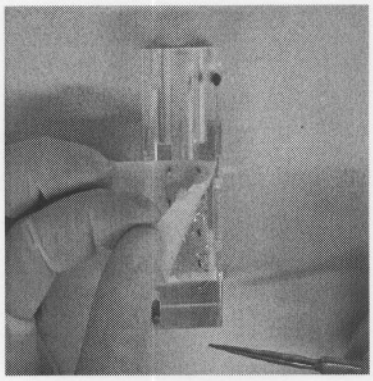

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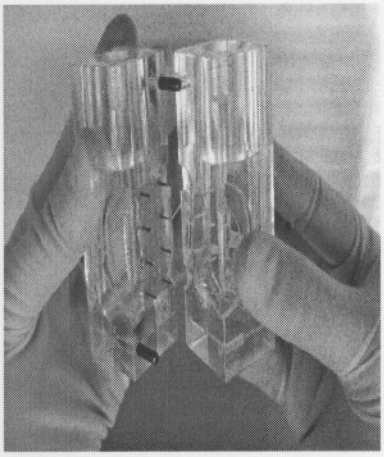

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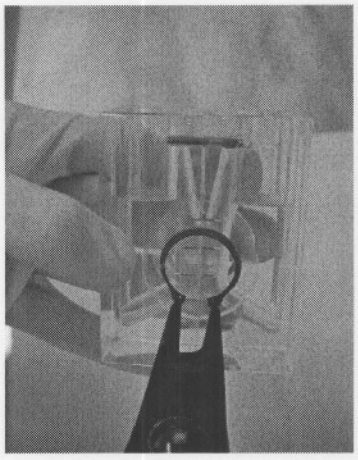
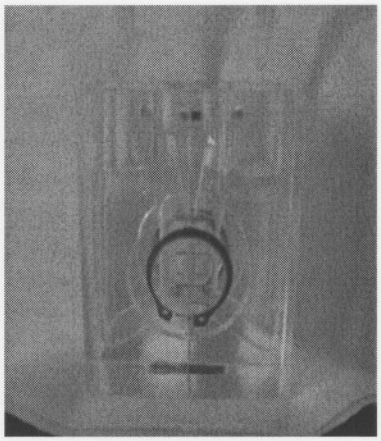


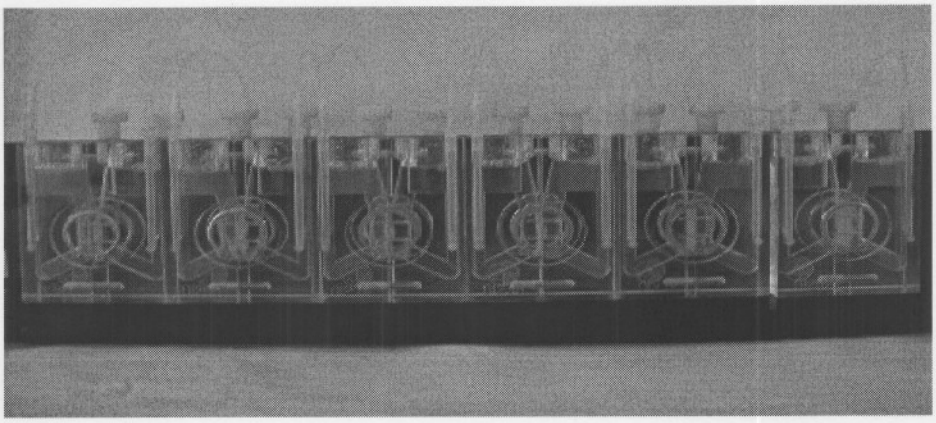
Slide 14



Slide 15

The cells were assembled and placed in the heating block (Slide 16). The entire procedure from removal of the intestine from the rat until all the cells were mounted took approximately 25 minutes. The tissue was acclimatised for 15 minutes before a transport study could be performed. The

various compounds under investigation were added to the receiver and donor cells respectively and cells were kept in the heating block at 37 °C for the duration of the experimental procedures.



Slide 16

3.2.3 HPLC Analysis

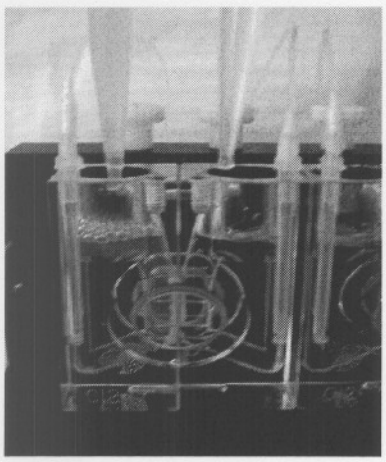
The samples were analysed by a previously validated (Hattingh, 2002: 65) high performance liquid chromatography (HPLC) method, using the apparatus and conditions given below:

Apparatus:	Pump:	Spectra Physics SP 8810
	Autosampler:	Spectra Physics AS 3000
	Detector:	Spectra Physics FL 2000
	Integrator:	Computerised integration system, with Chromquest chromatographic database for Windows® NT as software
	Column:	Luna 5μ C_{18}(2) reverse phase, 250 x 4mm
Conditions:	Injection volume:	200 μL
	Flow rate:	1.5 mL/min
	Mobile phase:	32% Acetonitrile: 68% KH_2PO_4 0.01M (pH=3)
	Excitation wavelength:	510 nm
	Emission wavelength	546 nm

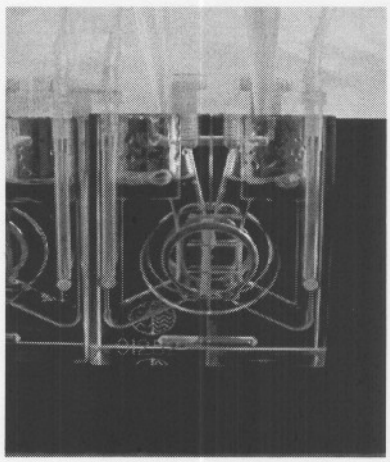
The mobile phase was mixed using HPLC grade reagents and Milli Q50 water for HPLC. The mobile fase was filtered through a MN 85/90 glass fibre filter (Macheney-Nagel, Germany) prior to use.

3.2.4 Analytical procedures used during transport studies

Bergamottin, quercetin, naringenin, verapamil were screened as potential Pgp modulators. The effects of these compounds on the transport of Rho123 were assessed by the concommitant addition of the individual compounds with Rho123 in the 2 ml solution. 2 ml Rho123 and 2 ml KR were added to the apical (AP) and basolateral (BL) sides respectively in two cells (Slide 17) and to the basolateral and apical sides respectively in the other two cells (Slide 18).
Sufficient Rho123, to give a final Rho123 concentration of 10.1 μM in the cell was in the 2 ml solution.



Slide 17



Slide 18

The total volume in each half cell after the final additions was 7 ml. The total exposed tissue surface area was 1.78 cm^2. 250 μl Aliquots were taken from the receiver cell at 30, 60, 90, and 120 min after the addition of Rho123, and replaced with an equivalent amount of fresh KR. It was previously shown (Hattingh, 2002:83) that the maximum period to perform transport studies was approximately 120 min before structural damage to the epithelium occurred. The aliquots were analysed by HPLC.

Bergamottin, quercetin, naringenin, verapamil and were dissolved in absolute ethanol. These solutions were then made up to volume with KR containing Rho123. The final ethanol concentration in the various solutions was kept ≤1%, a concentration proven not to alter cell viability or permeability (Soldner *et al.*, 1999: 479). In order to compare the results found using the Caco-2 cell layer technique (Janse van Vuuren, 2002:41) with the Sweetana-Grass technique, concentrations similar to those used by Janse van Vuuren (2002:41) were chosen. Concentrations of the various compounds investigated in the transport studies are given in Table 3.1.

Table 3.1: Concentrations (μM) of the various compounds investigated in the transport studies

Compound	Concentration (μM)
Bergamottin	12, 30, 48
Quercetin	73, 183, 292
Naringenin	442, 662, 884
Verapamil	900

The average apparent permeability coefficient (P_{app}) was calculated according to the following equation:

$$P_{app} = \frac{dQ/dt}{60 \times A \times C_0}$$

where dQ/dT is the transport rate, C_0 is the initial concentration of rhodamine (100%) and A is the area of exposed tissue (1.78cm^2). An example of the calculations performed is presented in Appendix C.

3.2.5 Statistical analysis

Statsoft® Statistica for Windows (Statsoft Inc., Tulsa, Oklahoma, USA) was used to perform the statistical analyses on the data obtained. The Dunnett test (Dunnett, 1964:482) was done to calculate the comparison between the mean ratios of each experiment with the mean ratio of the control. For the comparison between the different concentrations of each component, the Tukey Honest Significant Difference test was used (Steyn *et al.*, 1997:736).

CHAPTER 4

RESULTS AND DISCUSSION

4 Introduction

The effect of different modulators on the transport of Rho123 was investigated by means of the Sweetana-Grass diffusion method consisting of six cells, however only four cells were used. Experiments were done in triplicate. The transport of Rho123 was calculated in the apical to basolateral (AP-BL) direction for the first two cells and basolateral to apical (BL-AP) direction for the last two cells. The net transport of Rho123 was determined and the ratio of the P_{app} value in the BL-AP to the P_{app} value in the AP-BL direction was calculated. The results obtained from this study were compared to the results from a previous studies done with Caco-2 cells (Janse van Vuuren, 2000:76) and monodirectional transport of Rho123 across rat jejunum using the Sweetana-Grass diffusion method (Swart, 2002:35). Statistical analyses were used to compare the results of the experiments with the control. An analysis of variance (ANOVA) was done to test for statistical differences between the ratios from the same modulators at different concentrations.

4.1 Transport of Rhodamine 123

The cumulative transport of Rho123 where no modulators were present is presented in Figure 4.1 and the P_{app} values in Table 4.1. The results and ratio's obtained served as the control values to which the ratio's obtained after addition of the modulators, could be compared.

Table 4.1: Individual and mean P_{app} values of Rho123 transported (AP-BL and BL-AP) with no modulators present

Experiment no.	P_{app} x 10^{-7}cm/s AP-BL	P_{app} x 10^{-7}cm/s BL-AP	Ratio BL-AP/AP-BL	Mean Ratio BL-AP/AP-BL
1	3.05	7.81	2.56	2.31±0.258*
2	13.89	32.19	2.32	
3	20.77	42.45	2.04	

*Each value represents the mean ± standard deviation

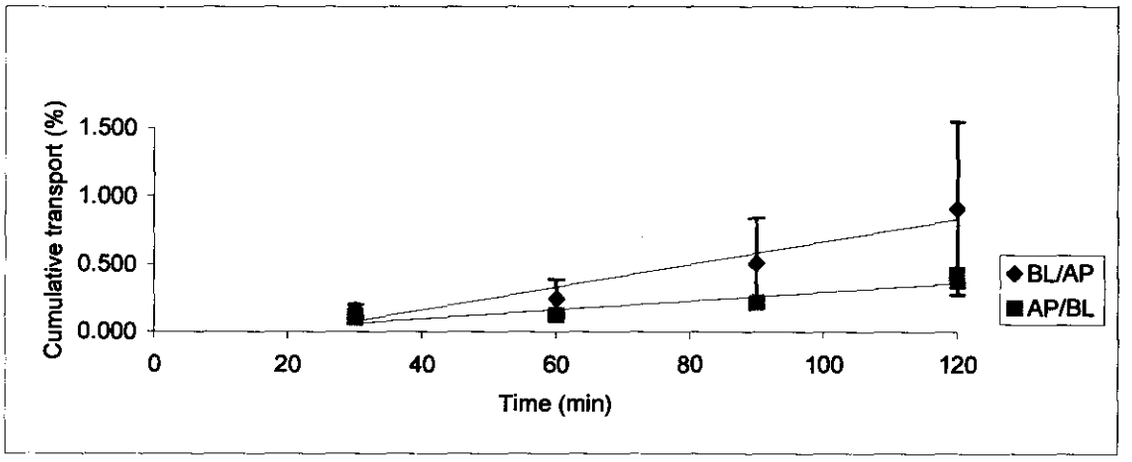


Figure 4.1: Cumulative transport of Rhodamine 123 (N=2) with no modulators added

According to Sharom (1997:162), Ambudkar *et al.* (1999:368) and Fertè (2000:279) Rho123 is a substrate for Pgp and multidrug resistance-associated protein (Hsing *et al.*, 1992:879). The results obtained during this experiment also indicated that Rho123 is a substrate for active transporters. The mean ratio calculated was 2.31 which indicated that active transport in the BL-AP direction occurred.

4.1.1 Transport of rhodamine 123 in the presence of Verapamil

According to the literature verapamil, a calcium channel blocker is an inhibitor of Pgp (Gottesman & Pastan, 1993:387; Sharom, 1997:162; Fertè, 2000:279). The cumulative transport of Rho123 in the presence of verapamil (900 µM) is shown in Figure 4.2 and the P_{app} values in Table 4.2.

Table 4.2: Individual and mean P_{app} values of Rho123 transported (AP-BL and BL-AP) in the presence of verapamil (900 µM)

Experiment no.	P_{app} x 10^{-7}cm/s AP-BL	P_{app} x 10^{-7}cm/s BL-AP	Ratio BL-AP/AP-BL	Mean Ratio BL-AP/AP-BL
	Verapamil (900 µM)			
1	4.45	6.91	1.55	1.52±0.279*
2	3.95	4.84	1.23	
3	1.65	2.94	1.78	

*Each value represents the mean ± standard deviation

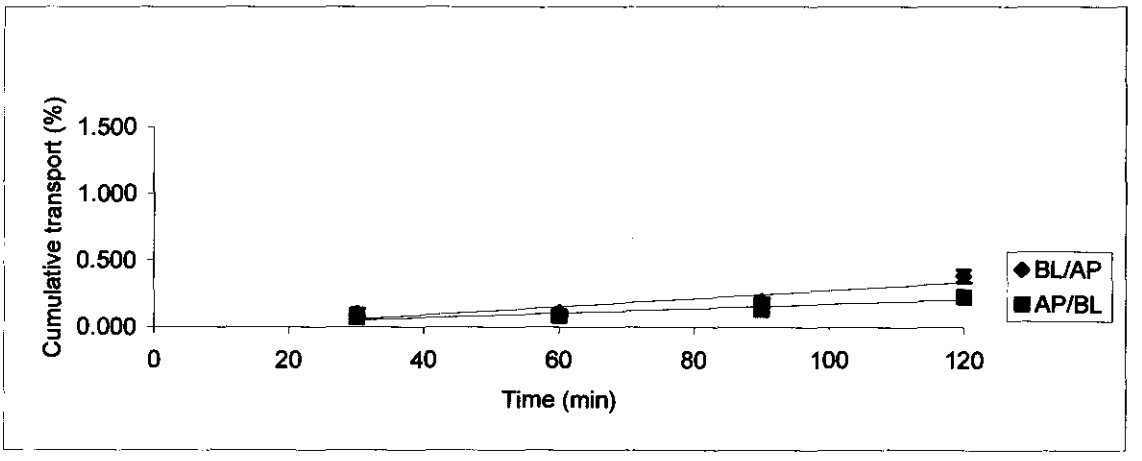


Figure 4.2: Cumulative transport of Rhodamine 123 (N=2) in the presence of verapamil (900 µM)

The mean ratio calculated was 1.52. The ratio observed showed that the rate of transport of Rho 123 in the basolateral to apical direction was inhibited by verapamil. The specific concentration (900µM) of verapamil was chosen as a concentration that had a statistically significant influence on Rho123 transport. The ratio of BL-AP to AP-BL transport of Rho123 was decreased by verapamil. The p-value was 0.023. This indicated that there was a statistically significant difference between mean ratio of verapamil and the mean ratio of the control.

This corresponds with the findings of Janse van Vuuren (2000:56) who found that verapamil (500 µM) decreased the basolateral/apical transport of cyclosporine across Caco-2 cells (Table 4.7) and Swart (2002:40) who found that the basolateral/apical transport of Rho123 in the presence of verapamil (200 µM) was smaller than the control in monodirectional studies but that the decrease was not statistically significant. Summers *et al.* (2004:1631) found that verapamil may offer hope for pharmacoresistant patients due to its potential Pgp inhibitory effects during *in vivo* studies. Ito

et al. (1993:399) also found that verapamil inhibited Pgp, thereby increasing serum concentrations of digoxin by renal tubular cells.

4.1.2 Transport of Rhodamine 123 in the presence of Grapefruit juice components

The modulating effect of three of the components of grapefruit juice on the transport of Rho123 was investigated. Grapefruit juice has been shown to increase the bioavailability of various orally administered CYP3A4 substrates, including cyclosporine (Ducharme *et al.*, 1995:48) felodipine (Lundahl *et al.*, 1997:139), midazolam (Kupferschmidt *et al.*, 1995:20), terfenadine (Benton *et al.*, 1996:383; Lundahl *et al.*, 1998:75), verapamil (Fuhr *et al.*, 1994:134) and many other therapeutic agents (Garvan *et al.*, 2000:933). In this study naringenin, quercetin and bergamottin (all grapefruit juice components) were investigated as modulators. The effect of these components on the transport of Rho123 across rat intestine was evaluated.

4.1.2.1 Transport of Rhodamine 123 in the presence of Naringenin

Naringenin is not normally present in grapefruit juice, but is produced *in vivo* through hydrolysis of naringin and narirutin (Takanaga, *et al.*, 1998:1064). Fuhr and Kummert (1995:370) also found that naringin is partly metabolized to naringenin, indicating that enteral bacteria played an important role in this metabolic pathway. Bailey *et al.* (1998:250) did not detect naringenin in extracts from supernatant or particulate fractions of grapefruit juice, but Ameer *et al.* (1996:35) detected naringenin (241,1mg/l) in grapefruit juice.

The effect of naringenin on the transport of Rho123 across rat intestine was investigated using three concentrations. Cumulative transport of Rho123 in the presence of naringenin (442 µM, 662 µM and 884 µM) is presented in Figures 4.3, 4.4 and 4.5 respectively and the P_{app} values in Table 4.3.

Table 4.3: Individual and mean P_{app} values of Rho123 transported (AP-BL and BL-AP) in the presence of naringenin (442 µM, 662 µM and 884 µM)

Experiment no.	P_{app} x 10^{-7}cm/s AP-BL	P_{app} x 10^{-7}cm/s BL-AP	Ratio BL-AP/AP-BL	Mean Ratio BL-AP/AP-BL
	Naringenin (442 µM)			
1	12.47	15.03	1.21	1.25±0.148*
2	45.10	51.25	1.14	
3	5.47	7.76	1.42	
	Naringenin (662 µM)			
1	15.37	14.54	0.95	1.19±0.433*
2	17.62	16.43	0.93	
3	2.97	5.01	1.69	
	Naringenin (884 µM)			
1	6.48	11.04	1.71	1.65±0.261*
2	8.60	11.78	1.37	
3	0.77	1.45	1.88	

*Each value represents the mean ± standard deviation

One-way analysis of variance (ANOVA), F (2; 6) =2.05, p=0.210. $p<0.05$ is statistically significant

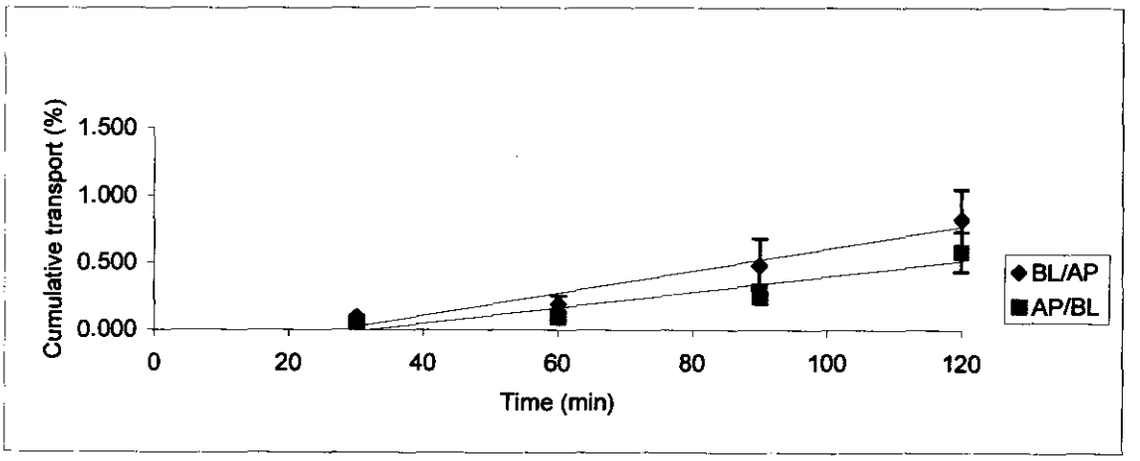


Figure 4.3: Cumulative transport of Rhodamine 123 (N=2) in the presence of naringenin (442 µM)

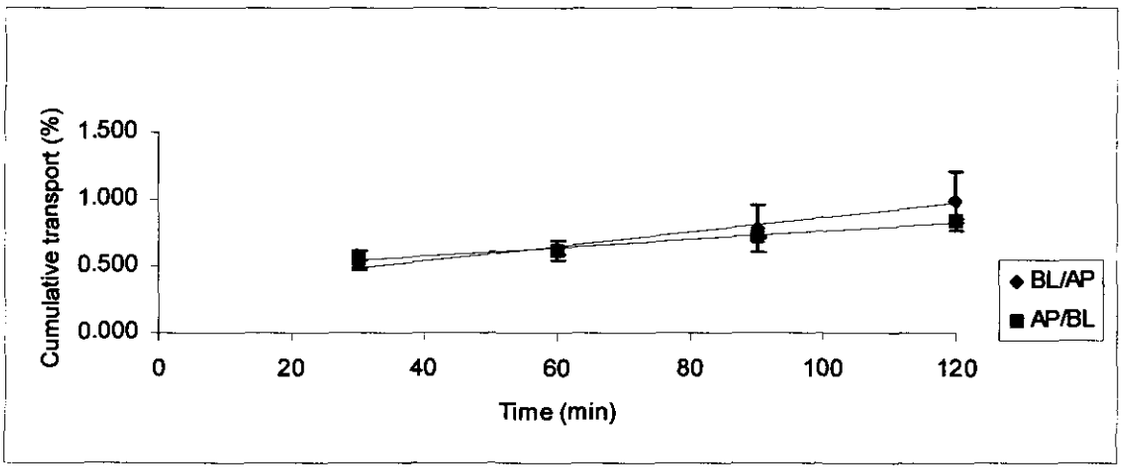


Figure 4.4: Cumulative transport of Rhodamine 123 (N=2) in the presence of naringenin (662 µM)

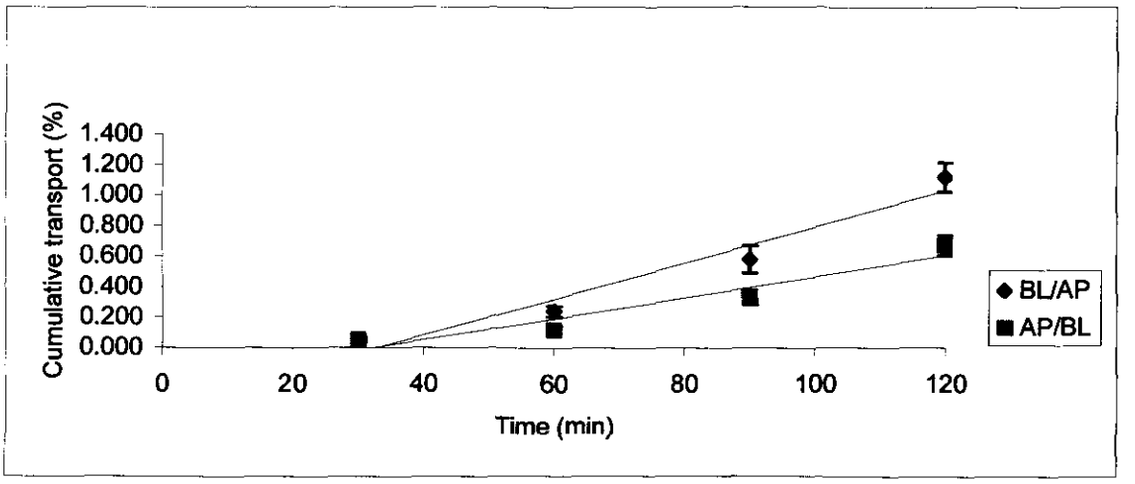


Figure 4.5: Cumulative transport of Rhodamine 123 (N=2) in the presence of naringenin (884 µM)

At the concentrations of 442 µM and 662 µM the mean ratios calculated were 1.25 and 1.19 respectively, indicating that the transport of Rho123 in the basolateral to apical direction was decreased. At the concentration of 884 µM the mean ratio was 1.65 indicating a decrease in transport but this was not statistically significant.

The results are similar to the results found by Janse van Vuuren (2000, 69) in Caco-2 cells which showed that the BL-AP/AP-BL ratio of cyclosporine transport was decreased by naringenin 442 µM, 885 µM and 1770 µM (Table 4.7).

No statistically significant differences existed between the ratios obtained at the three concentrations (Tukey test), indicating that the inhibitory effects of naringenin were not

concentration dependant. The results indicated that naringenin is an inhibitor of Pgp. This is similar to results found by Mitsunaga *et al.*, (2000:199) who found that naringenin increased the uptake of vincristine (substrate of Pgp) into MBEC4 cells indicating inhibition of Pgp activity.

These results differ from those obtained by Swart (2002:47), who did monodirectional transport studies and found that the inhibitory effects of naringenin were concentration dependent. This might be due to the fact that the serosa was not fully removed from the specific segment or that membrane damage occurred during the mounting and stripping thereof.

4.1.2.2 Transport of Rhodamine 123 in the presence of Quercetin

The effect of quercetin on the transport of Rho123 across rat intestine was investigated using three concentrations. The cumulative transport of Rho123 in the presence of quercetin (73 µM, 183 µM and 292 µM) is presented in Figures 4.6, 4.7 and 4.8 respectively and the P_{app} values in Table 4.6.

Table 4.4: Individual and mean P_{app} values of Rho123 transported (AP-BL and BL-AP) in the presence of quercetin (73 µM, 183 µM and 292 µM)

Experiment no.	P_{app} x 10^{-7}cm/s AP-BL	P_{app} x 10^{-7}cm/s BL-AP	Ratio BL-AP/AP-BL	Mean Ratio BL-AP/AP-BL
	Quercetin (73 µM)			
1	15.23	18.82	1.24	1.27±0.080*
2	12.62	15.20	1.20	
3	3.34	4.53	1.36	
	Quercetin (183 µM)			
1	4.20	6.71	1.60	1.82±0.508*
2	8.16	11.95	1.47	
3	0.86	2.07	2.40	
	Quercetin (292 µM)			
1	32.79	38.35	1.17	1.47±0.353*
2	4.95	9.20	1.39	
3	37.65	52.15	1.86	

*Each value represents the mean ± standard deviation

One-way analysis of variance (ANOVA), F (2; 6) =1.8414, p=0.238. p,0.05 is significant

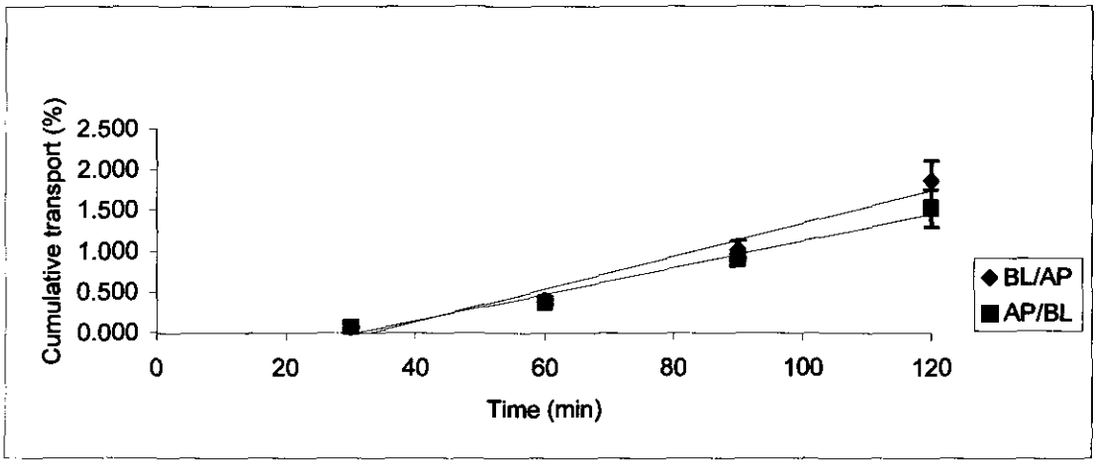


Figure 4.6: Cumulative transport of Rhodamine 123 (N=2) in the presence of quercetin (73 µM)

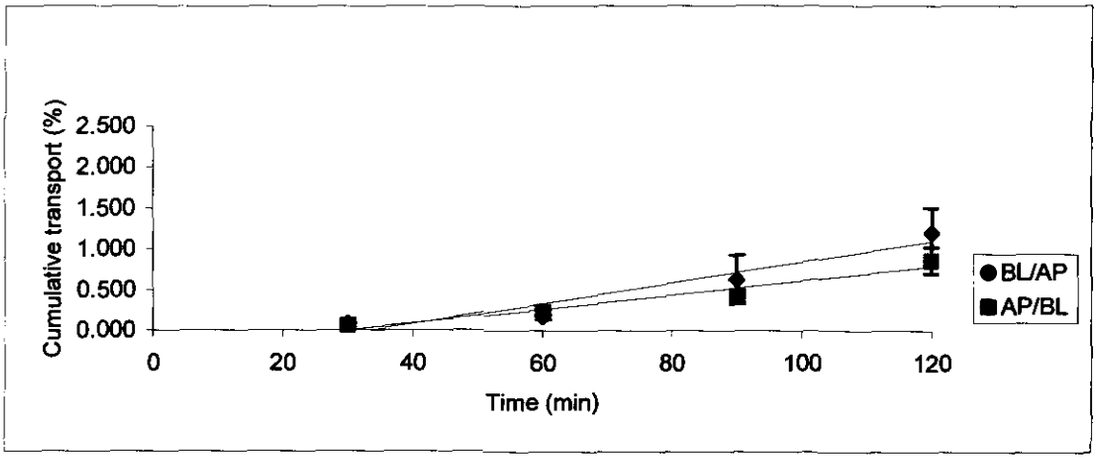


Figure 4.7: Cumulative transport of Rhodamine 123 (N=2) in the presence of quercetin (183 µM)

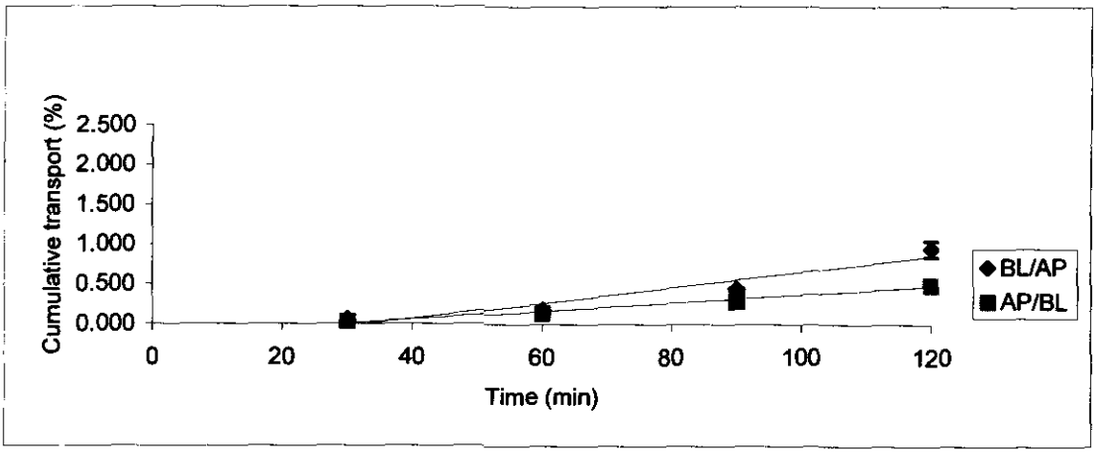


Figure 4.8: Cumulative transport of Rhodamine 123 (N=2) in the presence of quercetin (292 µM)

At the concentrations 73 µM and 293 µM the mean ratio observed were 1.27 and 1.47 respectively and this indicated a statistically significant decrease in the transport of Rho123 in the basolateral to apical direction. At the concentration 183 µM the mean ratio calculated was 1.823 indicating a decrease in transport, but this was not statistically significant.

No statically significant difference was observed between the different concentrations (Tukey test) when they were compared to each other. The transport of Rho123 in the basolateral to apical direction was decreased by the lowest and highest concentration. The transport proteins in the intestine were thus inhibited by quercetin at concentrations of 73 µM and 292 µM.

It was found by Mitsunaga *et al.* (2000:193) that quercetin 50 µM increased the uptake of vincristine (substrate of Pgp) into MBEC4 cells. This shows that there was inhibition of Pgp activity. These results were different from Janse van Vuuren (Table 4.7) who found that quercetin concentrations 73 µM, 146 µM and 293 µM increased the BL-AP/AP-BL ratio indicating increased active transport. This difference between the rat intestine and Caco-2 cells may be due to the more complex nature of the rat jejunum.

Swart (2002:50) found that there was no statistically significant difference in the degree of inhibition between concentrations 73 µM and 292 µM in monodirectional studies.

4.1.2.3 Transport of Rhodamine 123 in the presence of Bergamottin

The effect of bergamottin on the transport of Rho123 across rat intestine was also investigated using three concentrations. The cumulative transport of Rho123 in the presence of bergamottin (12 µM, 30 µM and 48 µM) is presented in Figures 4.9, 4.10 and 4.11 respectively and the P_{app} values in Table 4.4.

Table 4.5: Individual and mean P_{app} values of Rho123 transported (AP-BL and BL-AP) in the presence of bergamottin (12 µM, 30 µM and 48 µM)

Experiment no.	P_{app} x 10^{-7}cm/s AP-BL	P_{app} x 10^{-7}cm/s BL-AP	Ratio BL-AP/AP-BL	Mean Ratio BL-AP/AP-BL
	Bergamottin (12 µM)			
1	4.57	5.36	1.17	1.17±0.044*
2	1.80	2.02	1.12	
3	1.87	2.25	1.21	
	Bergamottin (30 µM)			
1	3.79	5.98	1.58	2.10±0.531*
2	8.42	22.21	2.64	
3	18.27	38.10	2.09	
	Bergamottin (48 µM)			
1	19.72	26.18	1.328	1.25±0.214*
2	4.02	5.71	1.42	
3	2.54	2.56	1.01	

*Each value represents the mean ± standard deviation

One-way analysis of variance (ANOVA), F (2; 6) =7.2507, p=0.251. p<0.05 is significant

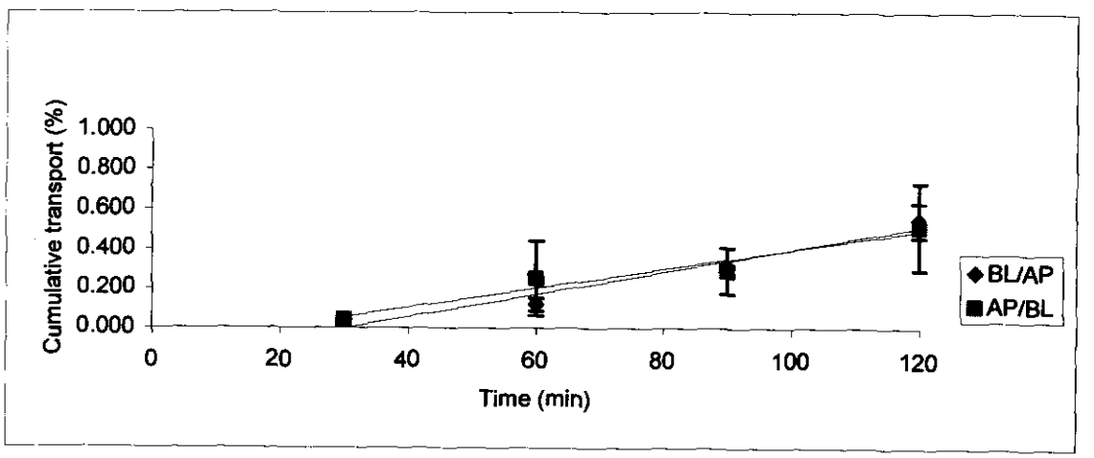


Figure 4.9: Cumulative transport of Rhodamine 123 (N=2) in the presence of bergamottin (12 µM)

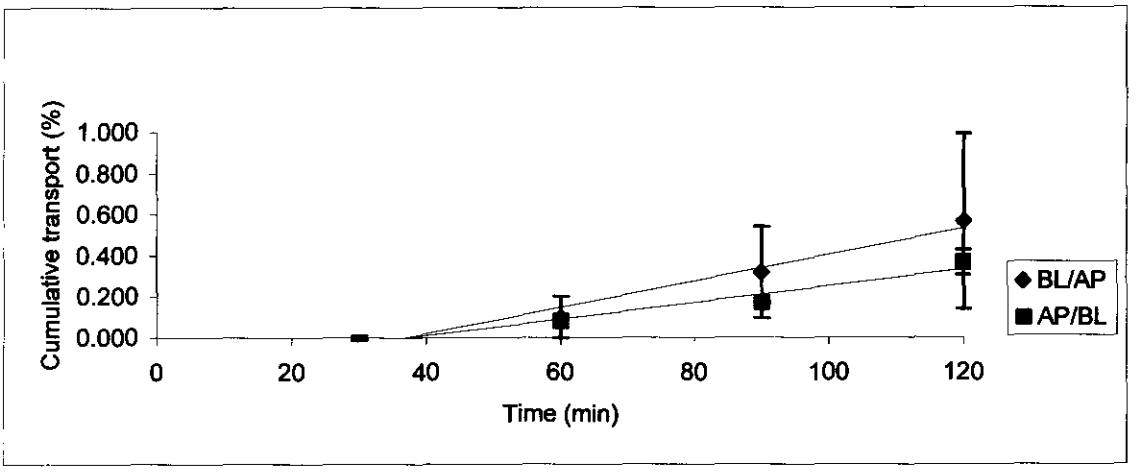


Figure 4.10: Cumulative transport of Rhodamine 123 (N=2) in the presence of bergamottin (30 µM)

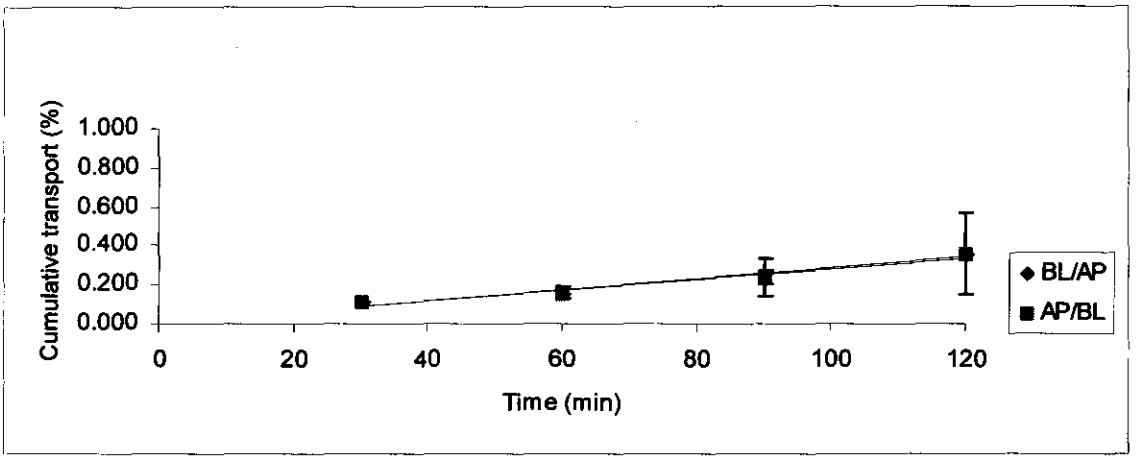


Figure 4.11: Cumulative transport of Rhodamine 123 (N=2) in the presence of bergamottin (48 µM)

At the concentrations (12 µM and 48 µM) the mean ratios observed were 1.17 and 1.25 respectively indicating that the transport of Rho123 in the basolateral to apical direction was decreased statistically significantly. The concentration 30 µM showed no statistically significant difference compared to the control. Tukey test showed that when the three concentrations were compared, bergamottin 30 µM differed statistically significantly from the other two concentrations.

The results differ from Swart (2002:54) that found bergamottin 48 µM was statistically significantly from the control, and indicating that the rate of Rho123 increased in the BL-AP direction and active transport occurred.

In contrast with the results obtained by Janse van Vuuren (2000:68) bergamottin 6 µM produced the highest AP-BL transport of cyclosporine. Bergamottin 12 µM and 24 µM caused statistically

significant increases in the BL-AP/AP-BL ratio indicating active transport. This difference between the rat intestine and Caco-2 cells may be due to the more complex nature of the rat jejunum.

4.2 Summary

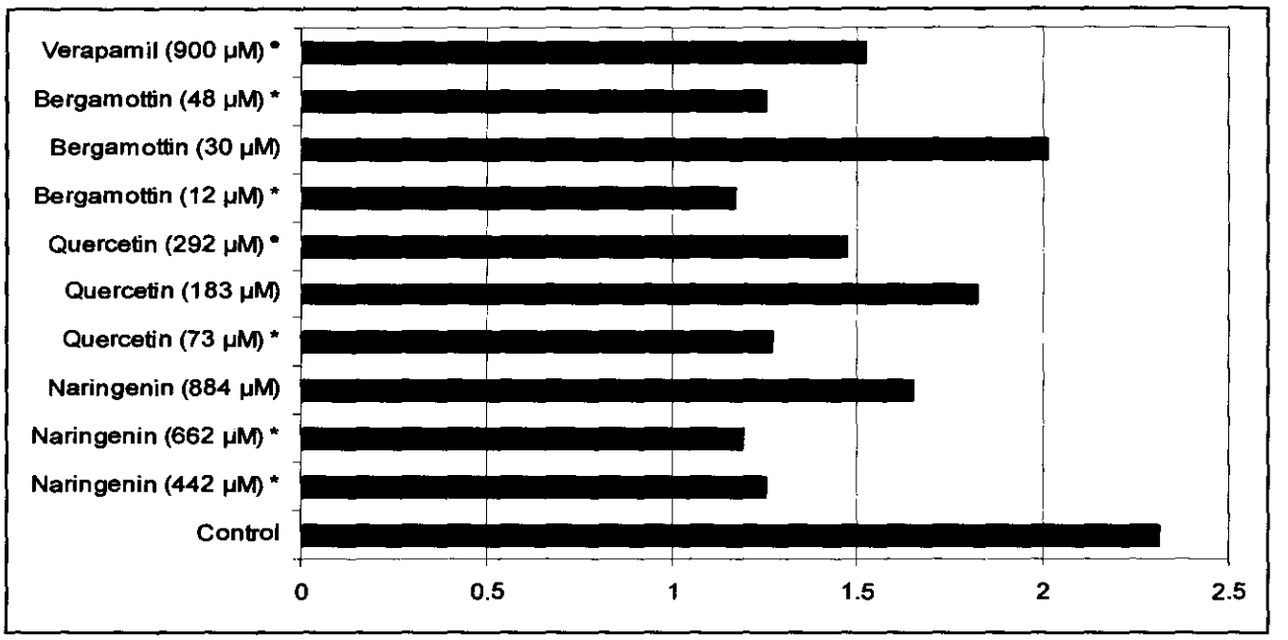
Figure 4.12 gives a comparative overview of results obtained in the different experiments. The mean ratio of the control and each modulator at a specific concentration is given in Table 4.6 and the mean ratio of each modulator examined by Janse van Vuuren (2000) is given in Table 4.7.

Table 4.6: Mean ratio of the different modulators

Modulator	Mean ratio	Standard deviation
Control	2.31	0.26
Naringenin (442 µM)	1.25	0.15
Naringenin (662 µM)	1.19	0.43
Naringenin (884 µM)	1.65	0.26
Quercetin (73 µM)	1.27	0.08
Quercetin (183 µM)	1.82	0.51
Quercetin (292 µM)	1.47	0.35
Bergamottin (12 µM)	1.17	0.04
Bergamottin (30 µM)	2.01	0.53
Bergamottin (48 µM)	1.25	0.21
Verapamil (900 µM)	1.52	0.28

Table 4.7: Mean ratio of each modulator examined by Janse van Vuuren (2000) in Caco-2 cell monolayers

Modulator	Mean ratio BL-AP/AP-BL
Control (Cyclosporine 1.1 µM)	3.52
Verapamil (500 µM)	3.01
Naringenin (442 µM)	1.37
Naringenin (885 µM)	1.39
Naringenin (1770 µM)	1.06
Quercetin (73 µM)	4.24
Quercetin (146 µM)	4.19
Quercetin (293 µM)	4.07
Bergamottin (6 µM)	2.11
Bergamottin (12 µM)	4.73
Bergamottin (24 µM)	4.16



*Value representing a statistically significant difference

Figure 4.12: Comparison between the mean ratio of each modulator at a specific concentration and the mean ratio of the control

From Figure 4.12 it can be observed that all the modulators decreased the transport of Rho123when compared to the control. Only three modulators at a specific concentration did not differ statistically significantly from the control namely naringenin 884 µM, quercetin 183 µM and bergamottin 30 µM.

Naringenin

At the concentrations of 442 µM and 662 µM the mean ratios observed were 1.25 and 1.19 respectively (Table 4.6.), indicating that the transport of Rho123 in the basolateral to apical direction was decreased. This can be observed from the Figure 4.12. At the concentration of 884 µM the mean ratio was 1.65 indicating a decrease in transport but this was not statistically significant. This corresponds to Janse van Vuuren (Figure 4.7) who also found that naringenin 442 µM and 884 µM decreased the ratio BL-AP/AP-BL.

Quercetin

At the concentrations 73 µM and 293 µM the mean ratio observed were 1.27 and 1.47 respectively (Table 4.6) and this indicated a statistically significant decrease in the transport of Rho123 in the basolateral to apical direction. At the concentration 183 µM the mean ratio calculated was 1.823 indicating a decrease in transport, but this was not statistically significant. It was found by Mitsunaga *et al.* (2000:193) that quercetin 50 µM increased the uptake of vincristine (substrate of Pgp) into MBEC4 cells. This showed that there was inhibition of Pgp activity

These results differ from Janse van Vuuren (Table 4.7) who found that quercetin increased active transport, thus inducing Pgp. This difference between the rat intestine and Caco-2 cells may be due to the more complex nature of the rat jejunum.

Bergamottin

At the concentrations (12 µM and 48 µM) the mean ratios observed were 1.17 and 1.25 respectively (Table 4.6) indicating that the transport of Rho123 in the basolateral to apical direction was decreased and was statistically significantly. The concentration 30 µM showed no statistically significant difference compared to the control. The results differ from Swart (2002:54) who found bergamottin 48 µM was statistically significant from the control and indicating that the rate of Rho123 increased in the BL / AP direction and active transport occurred.

These results differ from Janse van Vuuren (Table 4.7) who found that 12 µM and 24 µM bergamottin increased the ratio BL-AP/AP-BL, indicating active transport. This difference between the rat intestine and Caco-2 cells may be due to the more complex nature of the rat jejunum.

Table 4.8: Dunnett test: Statistical evaluation (p-values) obtained from the ratio between P_{app} (N=2) in the presence of modulators and the P_{app} ratio obtained from the control

Modulator	Dunnett test p-values
Verapamil (900μM)	0.023024*
Naringenin (442μM)	0.002358*
Naringenin (662μM)	0.001332*
Naringenin (884μM)	0.064555
Quercetin (73μM)	0.002623*
Quercetin (183μM)	0.197708
Quercetin (292μM)	0.015478*
Bergamottin (12μM)	0.001093*
Bergamottin (30μM)	0.642085
Bergamottin (48μM)	0.002365*

*Value representing a statistically significant difference

The mean ratio observed in the absence of a modulator was compared to the mean ratios after the addition of modulators at selected concentrations. Table 4.8 presents the p-values obtained after statistical analyses to determine which mean ratio's showed a statistically significant difference from the control. $p<0.05$ was taken as statistically significant.

CHAPTER 5

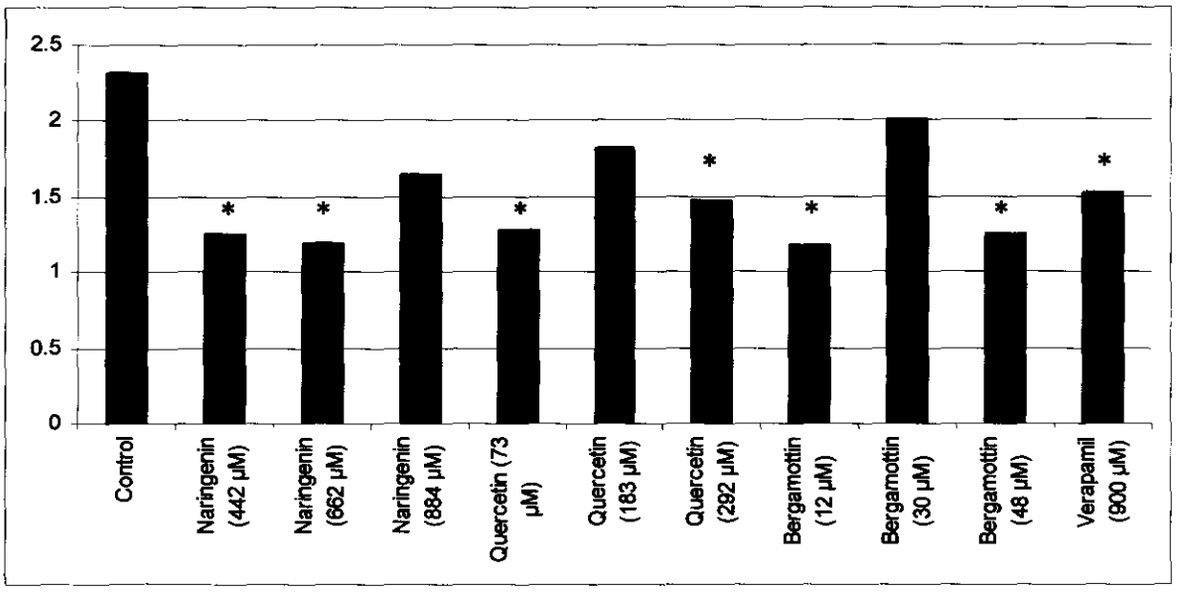
CONCLUSION AND RECOMMENDATIONS

5 Introduction

Intestinal secretion of drugs is an important mechanism of clearance which affects oral bioavailability of a wide variety of drugs. In extreme situations, these secretory systems may compromise oral bioavailability. No single efflux system explains the diversity of drug secretion displayed by the intestine, while interactions with intestinal drug metabolism systems add an extra dimension to the equation describing oral bioavailability. First isolated as one feature associated with a phenotype of cross-resistance to antitumoral agents, P-glycoprotein is receiving great attention as a possible physiological determinant of the disposition of drugs. Pgp is expressed in the intestinal mucosa and presents a barrier to oral bioavailability (Hunter & Hirst, 1997:134). The aim of this study was to investigate the effect of a known Pgp inhibitor (verapamil) and grapefruit juice components namely naringenin, quercetin and bergamottin on the transport of rhodamine 123 across rat jejunum and to compare these results with those obtained in similar studies done previously in Caco-2 cells and monodirectionally in rat intestine.

5.1 Conclusion

The mean ratio of the control compared to the mean ratios of the modulators at specific concentrations is presented in Figure 5.1. All the modulators showed inhibition and only three concentrations namely naringenin 884 µM, quercetin 183 µM and bergamottin 30 µM did not differ statistically significantly from the control.



*M

odulators which produced statistically significant inhibition of active efflux

Figure 5.1: Comparison between the mean ratio of the control and the mean ratio of the different modulators at specific concentrations

The final conclusions are as follows:

- Verapamil 900 µM decreased the BL-AP/AP-BL ratio. The ratios observed showed that the rate of transport of Rho 123 in the basolateral to apical direction was inhibited by verapamil. This corresponds with the literature that verapamil is a inhibitor of active transporters.

- Naringenin 442 µM and 662 µM decreased the BL-AP/AP-BL ratio statistically significant and showed inhibition of active transport. This inhibition was statistically significant. Naringenin 884 µM showed inhibition, but was not statistically significant. Inhibitory effects of naringenin were not concentration dependent.

- Quercetin 73 µM and 292 µM decreased the BL-AP/AP-BL ratio statistically significant. The ratios observed showed that the rate of transport of Rho 123 in the basolateral to apical direction was inhibited. Inhibition of active transporters occurred. Quercetin 183 µM showed inhibition, but this was not statistically significant. It was found that the inhibitory effects of quercetin were not concentration dependent.

- Bergamottin 12 µM and 48 µM decreased the BL-AP/AP-BL ratio statistically significant. The ratios observed showed a decrease in the transport of Rho123 in the basolateral to apical direction and inhibition of active transporters occurred. Bergamottin 30 µM showed inhibition, but was this not statistically significant compared to the control. It was found that the inhibitory effects of bergamottin were not concentration dependent.

- One of the main objectives in this study is to differentiate between the inhibition and activation of active transport using certain compounds. Naringenin showed inhibition in both studies although the concentrations were not exactly the same. Quercetin showed activation in Caco-2 cells, but in the literature quercetin was found to be a inhibitor. Bergamottin showed inhibition at lower concentrations in Caco-2 cells, but activation at higher concentrations, whereas in this study inhibition occurred at high concentrations. The results obtained in this study are similar to those obtained in Caco-2 cells. The fact that some of the results showed no statistically significant differences may be due to the model used, as the rat intestine is more complex than the Caco-2 cell membrane. There is also greater variation in rats than in Caco-2 cells and this might be the reason for the variation in the results obtained in this study. This is a disadvantage of the method used.

- According to the results obtained during this study the Sweetana-Grass diffusion model has proved to be the preferred method to perform diffusion studies, because this model is faster, cheaper and less labour intensive compared to the Caco-2 cells. This method is also efficient and sensitive enough to determine differences in rates of transport caused by various modulators. Sweetana-Grass diffusion method is widely used in transport studies. Although the tissue is only viable for a short time, this is sufficient for the purposes of this study.

5.2 Recommendations

- Combinations of the above grapefruit juice components should be tested to determine if any synergistic effects may exist.

- The amount of Pgp in rat intestine should be determined and correlated with the degree of inhibition or activation of transport by the modulators.

- Other flavonoids such as apigenin, genistein and galangin should be evaluated for their effect on Pgp mediated transport.

- Compounds found in other foods and beverages (such as isoflavonoids found in soy beans) should be evaluated for their effect on Pgp mediated transport.

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APPENDIX A

Table A.1: Cumulative transport amounts of Rhodamine 123 (10.1 µM) in the AP-BL and BL-AP direction with and without (control) modulators

Component	Time	Cumulative transport of rhodamine 123				Mean ± Standard deviation	
		AP-BL		BL-AP		AP-BL	BL-AP
		Chamber 1	Chamber 2	Chamber 1	Chamber 2		
Control 1	30	0.1	0.1	0.13	0.19	0.102 ± 0.001	0.159 ± 0.041
	60	0.13	0.11	0.14	0.34	0.122 ± 0.012	0.242 ± 0.140
	90	0.2	0.23	0.27	0.74	0.214 ± 0.017	0.504 ± 0.333
	120	0.35	0.45	0.46	1.36	0.398 ± 0.072	0.907 ± 0.635
Control 2	30	0.06	0.07	0.16	0.25	0.062 ± 0.005	0.208 ± 0.063
	60	0.3	0.35	0.76	1.11	0.323 ± 0.034	0.936 ± 0.244
	90	0.67	0.73	1.7	2.34	0.702 ± 0.043	2.024 ± 0.453
	120	1.28	1.56	2.88	3.69	1.420 ± 0.205	3.283 ± 0.575
Control 3	30	0.06	0.08	0.16	0.33	0.067 ± 0.012	0.243 ± 0.120
	60	0.39	0.43	0.85	1.4	0.407 ± 0.028	1.124 ± 0.384
	90	0.89	1.14	2.08	3.16	1.015 ± 0.174	2.622 ± 0.761
	120	2.09	2.08	3.58	4.97	2.083 ± 0.008	4.278 ± 0.981
Verapamil 1 (900 µM)	30	0.04	0.06	0.05	0.09	0.051 ± 0.009	0.072 ± 0.025
	60	0.11	0.1	0.32	0.28	0.106 ± 0.008	0.299 ± 0.028
	90	0.27	0.25	0.57	0.66	0.263 ± 0.015	0.614 ± 0.065
	120	0.53	0.42	0.7		0.474 ± 0.077	0.705
Verapamil 2 (900 µM)	30	0.43	0.39	0.4	0.14	0.410 ± 0.028	0.268 ± 0.181
	60	0.55	0.52	0.55	0.28	0.536 ± 0.016	0.414 ± 0.192
	90	0.69	0.67	0.65	0.37	0.682 ± 0.017	0.511 ± 0.199
	120	0.81	0.75	0.89	0.62	0.784 ± 0.043	0.753 ± 0.188
Verapamil 3 (900 µM)	30	0.08	0.06	0.13	0.08	0.069 ± 0.015	0.101 ± 0.037
	60	0.09	0.08	0.11	0.11	0.084 ± 0.004	0.110 ± 0.003
	90	0.14	0.12	0.18	0.21	0.134 ± 0.014	0.198 ± 0.021
	120	0.22	0.24	0.35	0.42	0.229 ± 0.011	0.386 ± 0.047
Naringenin 1 (442 µM)	30	0.01	0.01	0.08	0.17	0.006 ± 0.000	0.122 ± 0.060
	60	0.29	0.19	0.32	0.33	0.240 ± 0.071	0.324 ± 0.007
	90	0.72	0.52	1.02	0.67	0.624 ± 0.142	0.846 ± 0.247
	120	1.42	1	1.88	1.23	1.209 ± 0.299	1.554 ± 0.456
Naringenin 2 (442 µM)	30	0.11	0.11	0.06	0.14	0.109 ± 0.005	0.100 ± 0.062
	60	0.95	0.99	0.83	1.09	0.973 ± 0.028	0.959 ± 0.190
	90	2.45	2.55	2.61	3.06	2.504 ± 0.071	2.837 ± 0.316
	120	4.48	4.35	4.61	5.28	4.416 ± 0.086	4.948 ± 0.474
Naringenin 3 (442 µM)	30	0.05	0.07	0.1	0.09	0.057 ± 0.012	0.092 ± 0.009
	60	0.07	0.12	0.15	0.24	0.095 ± 0.029	0.192 ± 0.062
	90	0.22	0.32	0.33	0.62	0.266 ± 0.070	0.477 ± 0.203
	120	0.48	0.69	0.67	0.98	0.584 ± 0.149	0.825 ± 0.220
Naringenin 1 (662 µM)	30	0.07	0.11	0.14	0.04	0.091 ± 0.027	0.092 ± 0.070
	60	0.44	0.51	0.64	0.38	0.475 ± 0.046	0.511 ± 0.186
	90	0.91	0.93	1.52	0.9	0.917 ± 0.013	1.211 ± 0.435
	120	1.62	1.55	1.49	1.33	1.585 ± 0.043	1.412 ± 0.110
Naringenin 2 (662 µM)	30	0.03	0.05	-0.03	0.02	0.039 ± 0.009	-0.009 ± 0.035
	60	0.25	0.41	0.19	0.32	0.328 ± 0.114	0.255 ± 0.093
	90	0.83	1	0.66	0.93	0.913 ± 0.123	0.794 ± 0.187
	120	1.53	1.92	1.35	1.78	1.726 ± 0.272	1.566 ± 0.306
Naringenin 3 (662 µM)	30	0.51	0.6	0.48	0.53	0.555 ± 0.068	0.508 ± 0.035
	60	0.6	0.64	0.56	0.67	0.617 ± 0.028	0.615 ± 0.072
	90	0.71	0.71	0.65	0.9	0.714 ± 0.001	0.778 ± 0.179
	120	0.83	0.85	0.83	1.14	0.839 ± 0.014	0.989 ± 0.218

Naringenin 1 (884 µM)	30	0.05	0.05	0.04	0.06	0.051 ± 0.002	0.051 ± 0.017
	60	0.1	0.14	0.27	0.22	0.119 ± 0.023	0.241 ± 0.034
	90	0.3	0.37	0.64	0.52	0.333 ± 0.048	0.582 ± 0.089
	120	0.62	0.72	1.18	1.05	0.671 ± 0.068	1.116 ± 0.096
Naringenin 2 (884 µM)	30	0	0.06	0.06	0	0.029 ± 0.038	0.030 ± 0.037
	60	0.07	0.2	0.24	0.08	0.134 ± 0.087	0.158 ± 0.116
	90	0.34	0.47	0.59	0.42	0.402 ± 0.089	0.507 ± 0.119
	120	0.79	0.93	1.32	1.03	0.858 ± 0.094	1.172 ± 0.203
Naringenin 3 (884 µM)	30	0.06	0.06	0.06	0.06	0.058 ± 0.000	0.058 ± 0.000
	60	0.06	0.08	0.11	0.09	0.069 ± 0.015	0.099 ± 0.014
	90	0.08	0.08	0.15	0.1	0.082 ± 0.004	0.129 ± 0.036
	120	0.14	0.14	0.24	0.16	0.136 ± 0.000	0.203 ± 0.058
Quercetin 1 (73 µM)	30	0.08	0.07	0.07	0.06	0.079 ± 0.007	0.064 ± 0.007
	60	0.39	0.36	0.41	0.41	0.376 ± 0.022	0.406 ± 0.000
	90	0.88	0.92	0.94	1.1	0.901 ± 0.026	1.022 ± 0.113
	120	1.37	1.69	1.69	2.04	1.530 ± 0.228	1.869 ± 0.246
Quercetin 2 (73 µM)	30	0.15	0.12	0.1	0.16	0.134 ± 0.025	0.131 ± 0.046
	60	0.35	0.26	0.27	0.43	0.306 ± 0.062	0.350 ± 0.110
	90	0.83	0.6	0.65	1.07	0.717 ± 0.159	0.860 ± 0.292
	120	1.56	1.13	1.29	1.88	1.345 ± 0.306	1.583 ± 0.419
Quercetin 3 (73 µM)	30	0.23	0.22	0.23	0.26	0.228 ± 0.005	0.242 ± 0.022
	60	0.29	0.24	0.34	0.29	0.269 ± 0.036	0.314 ± 0.038
	90	0.45	0.31	0.52	0.41	0.381 ± 0.095	0.461 ± 0.079
	120	0.63	0.47	0.75	0.61	0.548 ± 0.115	0.677 ± 0.096
Quercetin 1 (183 µM)	30	0	0.01	-0.01	0	0.004 ± 0.009	-0.003 ± 0.004
	60	0.05	0.06	0.05	0.09	0.058 ± 0.007	0.072 ± 0.025
	90	0.22	0.18	0.2	0.36	0.198 ± 0.025	0.282 ± 0.110
	120	0.43	0.39	0.44	0.85	0.406 ± 0.029	0.644 ± 0.285
Quercetin 2 (183 µM)	30	0.02	0.02	0.02	0.02	0.015 ± 0.000	0.015 ± 0.000
	60	0.08	0.06	0.08	0.06	0.071 ± 0.019	0.069 ± 0.014
	90	0.06	0.06	0.13	0.16	0.058 ± 0.003	0.148 ± 0.024
	120	0.12	0.1	0.21	0.21	0.112 ± 0.013	0.210 ± 0.000
Quercetin 3 (183 µM)	30	0.04	0.07	0.08	0.09	0.058 ± 0.021	0.082 ± 0.004
	60	0.2	0.25	0.19	0.14	0.227 ± 0.033	0.167 ± 0.032
	90	0.44	0.41	0.84	0.41	0.425 ± 0.016	0.627 ± 0.303
	120	0.75	0.98	1.42	0.99	0.863 ± 0.164	1.205 ± 0.306
Quercetin 1 (292 µM)	30	0.43	0.07	0.62	0.4	0.248 ± 0.257	0.509 ± 0.150
	60	1.64	0.83	1.54	1.69	1.235 ± 0.572	1.615 ± 0.101
	90	2.72	2.49	2.9	3.81	2.605 ± 0.161	3.353 ± 0.640
	120	3.46	4.16	4.45	6.55	3.813 ± 0.495	5.500 ± 1.483
Quercetin 2 (292 µM)	30	0.1	-0.05	-0.02	0	0.021 ± 0.107	-0.008 ± 0.013
	60	0.94	0.85	0.51	0.76	0.894 ± 0.066	0.632 ± 0.175
	90	1.89	1.92	1.61	1.66	1.905 ± 0.021	1.636 ± 0.039
	120	3.47	2.9	3.29	4.22	3.187 ± 0.406	3.753 ± 0.655
Quercetin 3 (292 µM)	30	0.01	0.04	0.09	0.03	0.028 ± 0.021	0.059 ± 0.048
	60	0.14	0.13	0.18	0.19	0.136 ± 0.009	0.184 ± 0.008
	90	0.24	0.4	0.46	0.44	0.319 ± 0.116	0.450 ± 0.012
	120	0.44	0.55	1.03	0.88	0.495 ± 0.079	0.953 ± 0.103
Bergamottin 1 (12 µM)	30	0.04	0.04	0.04	0.04	0.041 ± 0.000	0.041 ± 0.000
	60	0.12	0.39	0.1	0.14	0.251 ± 0.190	0.119 ± 0.032
	90	0.21	0.38	0.31	0.32	0.291 ± 0.119	0.315 ± 0.008
	120	0.36	0.67	0.49	0.61	0.515 ± 0.221	0.548 ± 0.086
Bergamottin 2 (12 µM)	30	0	0	0	0	-0.003 ± 0.000	-0.003 ± 0.000
	60	0	0	0.09	0	-0.003 ± 0.000	0.045 ± 0.068
	90	0.05	0.07	0.16	0.09	0.061 ± 0.013	0.122 ± 0.050
	120	0.2	0.13	0.24	0.13	0.168 ± 0.049	0.187 ± 0.079

Bergamottin 3 (12 µM)	30	0.01	0.04	0.05	0.03	0.023 ± 0.018	0.037 ± 0.015
	60	0.05	0.04	0.07	0.09	0.042 ± 0.005	0.081 ± 0.017
	90	0.12	0.11	0.12	0.13	0.114 ± 0.008	0.129 ± 0.007
	120	0.19	0.21	0.29	0.24	0.199 ± 0.018	0.262 ± 0.035
Bergamottin 1 (30 µM)	30	0.14	0.14	0.23	0.32	0.138 ± 0.000	0.277 ± 0.064
	60	0.39	0.6	1.16	1.18	0.495 ± 0.150	1.170 ± 0.015
	90	0.95	1.33	2.49	2.38	1.141 ± 0.272	2.438 ± 0.080
	120	1.67	2.08	3.93	3.91	1.874 ± 0.296	3.923 ± 0.013
Bergamottin 2 (30 µM)	30	-0.01	-0.01	0.01	-0.01	-0.010 ± 0.000	-0.002 ± 0.011
	60	0.06	0.1	0.03	0.17	0.080 ± 0.030	0.099 ± 0.102
	90	0.17	0.17	0.16	0.47	0.170 ± 0.005	0.317 ± 0.221
	120	0.32	0.41	0.26	0.87	0.365 ± 0.061	0.564 ± 0.425
Bergamottin 3 (30 µM)	30	-0.05	0	0.05	-0.03	-0.023 ± 0.038	0.008 ± 0.059
	60	0.19	0.13	0.82	0.27	0.158 ± 0.041	0.548 ± 0.391
	90	0.42	0.38	1.49	0.87	0.396 ± 0.027	1.177 ± 0.441
	120	0.87	0.73	2.73	1.61	0.797 ± 0.100	2.170 ± 0.791
Bergamottin 1 (48 µM)	30	0.02	0.03	0.02	0.07	0.023 ± 0.006	0.043 ± 0.036
	60	0.29	0.43	0.59	0.57	0.362 ± 0.099	0.577 ± 0.011
	90	1.22	1.11	1.56	1.42	1.166 ± 0.076	1.491 ± 0.097
	120	1.91	1.81	2.45	2.62	1.861 ± 0.070	2.535 ± 0.122
Bergamottin 2 (48 µM)	30	0.1	0.09	0.13	0.11	0.069 ± 0.008	0.120 ± 0.021
	60	0.16	0.16	0.22	0.19	0.157 ± 0.002	0.206 ± 0.027
	90	0.32	0.24	0.41	0.3	0.276 ± 0.058	0.354 ± 0.082
	120	0.56	0.41	0.72	0.64	0.486 ± 0.101	0.681 ± 0.058
Bergamottin 3 (48 µM)	30	0.11	0.11	0.11	0.11	0.111 ± 0.000	0.111 ± 0.000
	60	0.15	0.15	0.18	0.14	0.151 ± 0.004	0.162 ± 0.027
	90	0.21	0.26	0.31	0.17	0.239 ± 0.036	0.238 ± 0.101
	120	0.35	0.35	0.5	0.21	0.352 ± 0.003	0.359 ± 0.205

APPENDIX B

Table B.1: Example of the values and calculations done to determine the apparent permeability coefficient (P_{app})

Reference concentration [a] (ng/mL)	Time (min)	Peak Area (mAU)	Transport corrected for dilution[b] (mAU)	Concentration [c] (ng/mL)	Relative transport[d] (%)	Slope of relative transport against time[e]	P_{app}[f] ($x10^{-7}$ cm/s)	Mean P_{app} (10^{-7} cm/s)
3914	Cell 1							
	30	35	35	3.98	0.10	0.0027	2.52	3.053
	60	56	57	5.11	0.13			
	90	110	112	7.89	0.20			
	120	220	224	13.57	0.35			
	Cell 2							
	30	36	36	4.03	0.10	0.0038	3.59	
	60	44	44	4.43	0.11			
	90	129	131	8.83	0.23			
	120	298	303	17.57	0.45			

Table B.2: Values used to obtain standard curve

Concentration (ng/mL)	Peak Area (mAU)				Slope (m)	Y-intercept (c)	Correlation coefficient (r2)
	1	2	3	Mean			
4.57	38	45	79	54	19.693	-43.333	0.9955
9.13	111	134	134	126			
13.70	210	222	242	225			
18.27	307	326	330	321			

a) Concentration of Rho123 in 7 mL

10.278 µM = 10.278 µmol/1000mL x 380.83 µg/

= 3914 µg/1000mL

= 3914 ng/mL

b) It is the transport corrected for dilution at one time interval divided by 28.

This value is then added to the value at the next time interval.

Example: 35/28 + 56 = 57.25

The value of 28 is obtained by dividing the volume of the cell (7000 µL) by the volume of the replaced buffer (250 µL) (7000 µL/250 µL = 28)

c) By using the standard curve generated for each experiment, the peak area is converted to a concentration by using the standard equation for a straight line (y = mx + c).

Slope (m): 19.693

y-intercept (c): -43.333 ng/mL

Thus x = (y-c)/m

x = (35 - (-43.333 ng/mL))/19.693

x =3.978 ng/mL

d) Value calculated by dividing the concentration at each time by the 100% concentration and expressing it as a percentage.

Example: 3.978/3914.171 x 100 = 0.10

e) Slope of line obtained by plotting relative transport against time.

f) Calculated by the equation on page 39 given in the experimental procedure chapter

$$P_{app} = \frac{dQ/dt}{60 \times A \times C_0}$$

$$P_{app} = \frac{0.0027}{60 \times 1.78 \times 100_0}$$ (dQ/dt = Slope (Obtained on (e))

$= 2.52 \times 10^{-7}$ cm/s