THE EFFECT OF SELECTED HYDROXY FLAVONOIDS ON THE IN VITRO EFFLUX TRANSPORT OF RHODAMINE 123 USING RAT JEJUNUM

S. VAN HUYSSTEEN B. Pharm

Dissertation submitted in the partial fulfilment of the requirements for the degree Magister Scientiae in Pharmaceutics at the North West University

Supervisor: Dr. M.M. Malan

Co-Supervisor: Mr. K. Swart

2005

POTCHEFSTROOM

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION AND STATEMENT OF THE PROBLEM

1.1 Introduction

Oral bioavailability requires the absorption of drugs across the intestinal epithelium. This may be mediated by either the paracellular and/or transcellular routes. Passive transcellular absorption requires the appropriate physicochemical properties to allow permeation across the apical and basolateral membrane domains. Compounds demonstrating these properties are more likely to be recognised as substrates for intracellular metabolism, such as by cytochrome P450 isozymes and/or secretory drug efflux systems, including Pgp, such that oral bioavailability will be limited. Pgp, which leads to MDR in tumor cells, is an ATP-dependent secretory drug efflux pump, encoded by the MDR1 gene in humans. One of the working mechanisms of Pgp is to clear the membrane lipid bilayer of lipophilic drugs in the manner of a flippase. In the intestine, as well as at specific other epithelial and endothelial sites, Pgp expression is localised at the apical membrane, consistent with secretory detoxifying and absorption limitation functions. Other secretory efflux systems, such as multidrug-resistance associated protein (a glutathione S-conjugate transporter), fluorochrome efflux systems and the methotrexate efflux systems, together with drug ionic charge and the intestinal pH microclimate, may mediate intestinal secretion of a wide variety of drugs. Direct evidence for Pgp limiting drug absorption comes from studies *in vitro* with human Caco-2 cells and includes non-linear dependence of absorption on substrate concentration, increased absorption upon saturation of secretion and increased absorption upon inhibition of Pgp function, with modulators such as verapamil. Pgp function may be integrated with drug metabolism, with several drugs being common substrates for Pgp and cytochrome P450 (CYP3A4) (Hunter & Hirst, 1997:129).

Other potential problems concerning Pgp involve specificity. If the inhibitor is not specific enough, it blocks multiple ATP-binding cassette proteins (ABC) members, or it binds with such high affinity that it does not dissociate, then normal

physiologic/protective functions of these transporters may be compromised, leading to unwanted effects (Chan *et al.*, 2004:43). Many Pgp inhibitors are compounds which occur in foods such as soy beans and grapefruit juice and inhibition as well as induction of this protein could lead to enhanced or diminished absorption of drugs that are substrates for Pgp (Wagner *et al.*, 2001:S14).

Different compounds have been shown to reverse the Pgp-mediated MDR. MDR cells can be sensitised to anticancer drugs when treated with a Pgp inhibitor, which is known as a chemosensitiser (Choi *et al.*, 2004:672). The search for chemosensitisers which have the advantages of being a non-transportable inhibitor without side effects, has led to a great deal of research in flavonoids derived from plants (Conseil *et al.*, 1998:9831).

During this study a few selected flavonoids (morin, galangin, kaempferol and quercetin) were used to test whether inhibition of active transport can be facilitated in this model.

To assess the absorption potential of chemical entities numerous *in vitro* and *in vivo* model systems have been used. Many laboratories rely on cell culture models of intestinal permeability such as Caco-2 cell monolayers. The successful application of *in vitro* models of intestinal drug absorption depends on the extent to which the model comprises the relevant characteristics of the *in vivo* biological barrier (Hidalgo, 2001:389). For the purposes of this study, an *in vitro* method using intestinal mucosal sheets suitable for mounting in Ussing chambers (Sweetana-Grass diffusion cells) was chosen. Using this method, the rate of transport of selected compounds can be determined in the presence of Pgp inhibitors.

The aims of this study are to:

- study the effects of selected flavonoids (morin, galangin, kaempferol and quercetin) on the transport of the Pgp substrate Rhodamine 123 (Rho 123) across rat jejunum using Sweetana-Grass diffusion cells;

- evaluate the structure activity relationships (SAR) of the selected flavonoids with reference to the inhibition of Pgp, and

- compare the effect of the selected flavonoids at two different concentrations.

1.2 Structure of this dissertation

In this dissertation, the introductory chapter is followed by a review of the relevant literature (Chapter 2). In Chapter 3, flavonoids as modulators will be discussed with reference to the inhibition of Pgp. In Chapter 4 the experimental procedure as well as the validation of this method will be given. In Chapter 5 the results of the study will be reported and discussed. In Chapter 6 final conclusions are drawn and recommendations are made.

CHAPTER 2

DRUG DELIVERY AND ORAL BIOAVAILABILITY

2.1 Introduction

The oral route is the most convenient and widely used means of drug administration. Estimates of effective clinical dose are based on a combination of estimates of oral absorption, bioavailability, clearance and volume of distribution (Pelkonen *et al.*, 2001:622). Bioavailability indicates a measurement of the rate and amount of therapeutically active drug that reaches the systemic circulation and is available at the site of action (Shargel & Yu, 1999:247). Bioavailability relates to more than just absorption. It is determined by the extent of absorption and presystemic metabolism. It is also the result of several competing processes, some of which favor the entry into the systemic circulation and others which impede it. The most important processes are the following:

- Extent of absorption which is determined by passive diffusion, active and facilitated transport, paracellular transport, endocytosis and gut flora metabolism;
- Efflux proteins located at the apical membrane of the small intestine, which include P-glycoprotein (Pgp; MDR1) and MRP2 (multidrug resistance-associated protein), may drive compounds from inside the cell back into the intestinal lumen, preventing their absorption into the blood, thus although lipophilic compounds may readily diffuse across the apical plasma membrane, their subsequent passage across the basolateral membrane and into the blood is by no means guaranteed and
- Presystemic metabolism which describes the rapid metabolism of orally administered drugs prior to reaching the general circulation. It is determined by cytochrome P450 (CYP)3A4, other CYPs and phase-II enzymes in the gut and by contribution of the liver.

(Chan *et al.*, 2004:25; Hunter & Hirst, 1997:132; Pelkonen *et al.*, 2001:622; Shargel & Yu, 1999:378).

Efforts to formulate oral products to optimise their bioavailability are continually being researched. These efforts also include changes in the physico-chemical and dissolution properties of the drugs to be able to formulate such products. It is however widely recognised that intestinal metabolism and active drug efflux must also be addressed in order to maximise oral bioavailability and decrease variability that exist in drug delivery (Wacher *et al.*, 1998:1322).

The principal site of absorption of most nutrients is the small intestine which has a more elaborate system that determines its permeability (Csáky, 1984:51). In order to understand the absorption of drugs as well as the efflux of drugs that may take place, the anatomy of the small intestine as well as the different absorption processes and mechanisms of the efflux of drugs that may take place will be discussed.

2.2 Anatomy of the small intestine

The small intestine represents the principal site of absorption for any ingested compound, whether dietary, therapeutic, or toxic (Chan *et al.*, 2004:26). The three anatomic divisions of the small intestine are the duodenum, jejunum and ileum with a total length of approximately 6 m in humans (Carr & Toner, 1984:1).

The intestinal mucosa is divided into three distinct layers (Figure 2.1). The deepest layer is the muscularis mucosa, a continuous thin sheet of smooth muscle separating the mucosa from the submucosa. Contraction of the muscularis mucosa facilitates emptying of crypt luminal contents by causing luminal compression. The second layer, the lamina propria is the connective tissue inside the villus and surrounding crypt and carries out several immunologic functions as well as providing structural support to the epithelial layer (Trier & Madara, 1981:926). The epithelial layer, which is the third layer, is a continuous sheet of epithelial cells that is only one cell thick. It lines the villi and their surrounding crypts. It consists of a monolayer of heterogenous cells and includes absorptive cells, crypt cells, endocrine cells and goblet cells which secrete mucin. This layer is in direct contact with the luminal content. The epithelium is separated from the underlying lamina propria by a thin, continuous basement membrane. In certain parts of the intestine (Peyer`s patches) the epithelial cell layer contains M (microfold) cells, which

sample antigens from the intestinal lumen to the lymph (Trier & Madara, 1981:928). The anatomy of the villi of the small intestine is shown in Figure 2.1 (Trier & Madara, 1981:926).

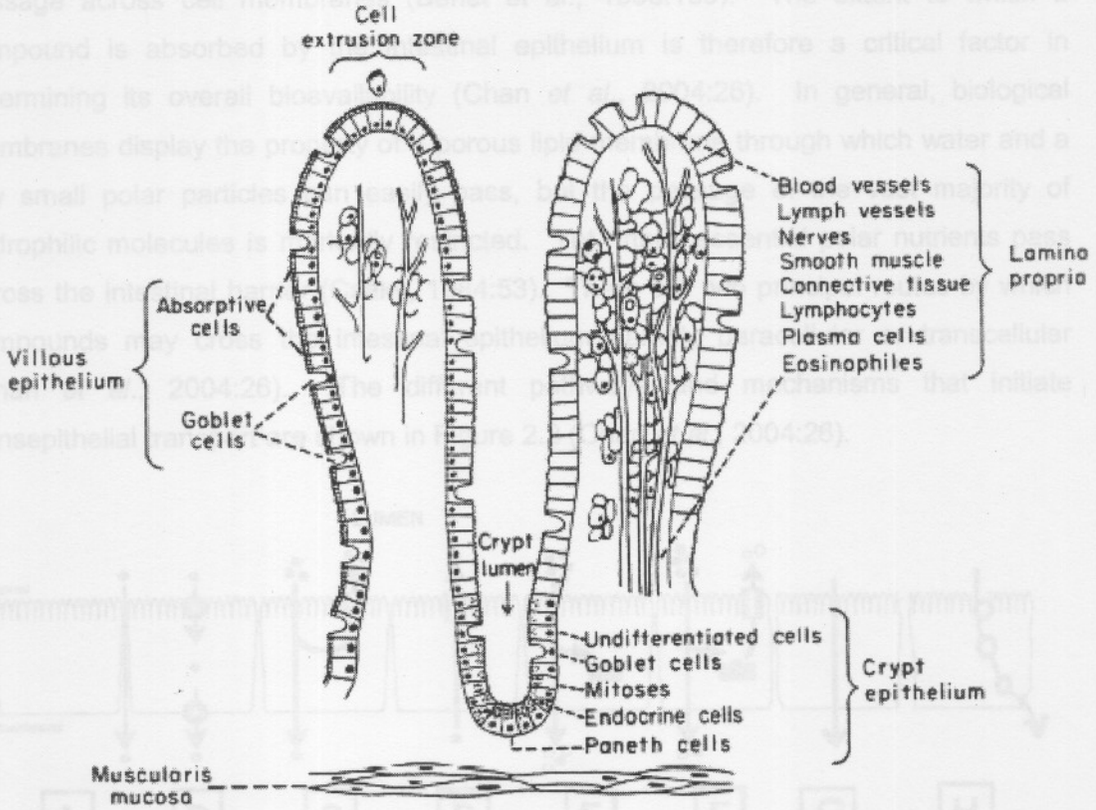


Figure 2.1: Schematic presentation of the intestinal mucosa (Trier & Madara, 1981:926).

The surface facing the lumen has a fuzzy appearance called the brush border or microvilli (Rogers, 1983:201). The microvillous border increases the surface area available for absorption by a factor of 25 (Carr & Toner, 1984:13).

Although the primary function of the villus epithelium is absorption, other functions can also be performed. Known functions of the crypt epithelium include epithelial cell renewal by means of undifferentiated and young goblet cells and exocrine secretion into the crypt lumen by means of goblet, Paneth and undifferentiated cells and endocrine secretion by means of the endocrine epithelial cells (Trier & Madara, 1981:929).

2.3 Passage of drugs across biologic membranes

The absorption, distribution, biotransformation and excretion of a drug involve its passage across cell membranes (Benet *et al.*, 1996:139). The extent to which a compound is absorbed by the intestinal epithelium is therefore a critical factor in determining its overall bioavailability (Chan *et al.*, 2004:26). In general, biological membranes display the property of a porous lipid membrane through which water and a few small polar particles can easily pass, but the passage of the vast majority of hydrophilic molecules is markedly restricted. Yet, many essential polar nutrients pass across the intestinal barrier (Csàky, 1984:53). There are two principal routes by which compounds may cross the intestinal epithelium namely paracellular or transcellular (Chan *et al.*, 2004:26). The different pathways and mechanisms that initiate transepithelial transport are shown in Figure 2.2 (Chan *et al.*, 2004:26).

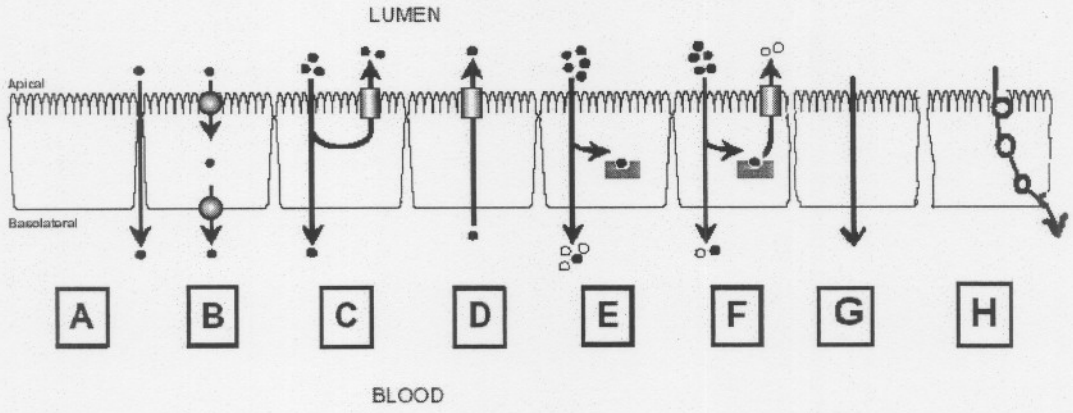


Figure 2.2: Diagram of pathways and mechanisms that mediate transepithelial transport (Chan *et al.*, 2004:26).

In Figure 2.2 **(A)** passive paracellular transport is illustrated on the left side followed by **(B)** carrier-mediated transport, **(C)** efflux transporters, **(D)** apical efflux, **(E)** intracellular metabolising enzymes **(F)** apical efflux and intracellular metabolising enzymes **(G)** passive transcellular diffusion and **(H)** vesicular transport which is illustrated on the right side (Chan *et al.*, 2004:26).

In general, the transport pathways can thus be divided into the following:

A. **Passive paracellular transport (passive diffusion).** This is an aqueous, extracellular route across the epithelium and the driving forces for this route are the electrochemical potential gradients derived from differences in concentration, electrical potential and hydrostatic pressure between the two sides of the epithelium. The transepithelial permeation of hydrophilic compounds occurs mainly through the paracellular route (Hidalgo, 2001:388).

B. **Carrier-mediated transporters (active transport).** Transcellular absorption from the lumen to the blood requires uptake across the apical membrane, followed by transport across the cytosol, then exit across the basolateral membrane and into the blood. Transcellular absorption of hydrophilic drugs may be facilitated via specific carrier-mediated pathways by means of utilising the same route of absorption followed by nutrients and micronutrients. Many orally administered drugs are lipophilic and undergo passive transcellular absorption (Hunter & Hirst, 1997:131).

C. **Efflux transporters.** Drugs that cross the apical membrane may be substrates for apical efflux transporters, which extrude compounds back into the lumen (Evers *et al.*, 1998:1318).

D. **Apical efflux.** These apical efflux transporters are principally ABC proteins such as Pgp and MRP2, and are ideally situated to act as the first line of defence by limiting the absorption of potentially toxic compounds. Compounds that are already present in the blood may undergo active blood-to-lumen secretion facilitated by these transporters (Watkins, 1992:520).

E. **Intracellular metabolising enzymes.** The transcellular route of absorption exposes drugs to intracellular metabolic systems, small intestinal enterocytes (which provides the first site for cytochrome P450 (CYP)-mediated metabolism of orally ingested drugs) and xenobiotics (Watkins, 1992:520).

F. **Apical efflux transporters and intracellular metabolising enzymes.** The CYP-system (phase I metabolism), as well as other intracellular metabolic systems, such as phase II conjugating enzymes, may yield metabolites that are themselves substrates for efflux pumps, thus providing additional possibilities for interactions (Keppler *et al.*, 1999:237).

It is however only passive diffusion, active transport and facilitated diffusion that are of importance in drug transport (Shargel & Yu, 1993:112).

2.3.1 Passive diffusion

The most common method of drug absorption is passive diffusion, the process where molecules diffuse spontaneously from a region of higher concentration to a region of lower concentration. This process is passive because no external energy is needed (Shargel & Yu, 1993:112). A prerequisite for passive diffusion is that the compound should be lipophilic and of low molecular weight. The rate of transport across a membrane by means of passive diffusion is derived from the first law of diffusion by Fick which states that the rate of transport is dependant on the surface area of the diffusion coefficient and the concentration gradient across the membrane (Lund, 1994:220).

According to Fick (as quoted by Csáky, 1984:51) diffusion is the unrestricted flow of substances from one compartment to the other. It is caused by thermal agitation of the molecules. Fick's law quantitatively expresses the rate of diffusion by means of the following equation:

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

Where *dn* is the number of molecules (ions) crossing an area *A* in 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 unit area per unit time where *dc/dx=1*.

Permeation is essentially a two-way process, namely the flow of substances from the lumen into the bloodstream (absorption) and the flow from the bloodstream into the lumen (exorption), which occurs simultaneously. The primary physiologic function of the intestine is absorption. Therefore, the net result of permeation is usually absorption. Nonetheless, exorption cannot be completely neglected (Csáky, 1984:52). Lipids or lipid-soluble substances, including the majority of drugs and other xenobiotics, are transported by passive diffusion (Csáky, 1984:52).

2.3.2 Active transport

Active transport is characterised by the transport of drug against a concentration gradient which is, from regions of low drug concentrations to regions of high drug concentrations. Therefore, this is an energy-consuming system. In addition, active transport is a specialised process requiring a carrier that binds the drug to form a carrier-drug complex that shuttles the drug across the membrane and then dissociates the drug on the other side of the membrane. The carrier molecule may be highly selective for the drug molecule. If the drug structurally resembles a natural substrate that is actively transported, then it is likely to be actively transported by the same carrier mechanism. Therefore, drugs of similar structure may compete for sites of adsorption on the carrier. Furthermore, because only a fixed number of carriers are available, all the binding sites on the carrier may become saturated with an increased drug concentration (Shargel & Yu, 1999:105).

2.3.3 Facilitated diffusion

Facilitated diffusion is also a carrier-mediated transport system, differing from active transport in that the drug moves along a concentration gradient. Therefore, this system does not require an energy input. However, because this system is carrier mediated, it is saturable and structurally selective for the drug and shows competition kinetics for drugs of similar structure. In terms of drug absorption, facilitated diffusion seems to play a very minor role (Shargel & Yu, 1999:106).

Absorption mechanisms such as efflux may limit intestinal drug absorption. Therefore, drug concentrations may not be satisfactory to deliver the desirable therapeutically effects. It is of great importance to study processes like efflux more extensively in order to find ways to circumvent these mechanisms and to improve a drug's absorption and ultimately, its bioavailability.

2.4 Efflux mechanisms

Pgp is the first member of what is now a large and diverse superfamily comprising around fifty human ATP-binding cassette (ABC) proteins that perform many and varied functions (Chan *et al.*, 2004:25). Overexpression of Pgp is found to prevent the accumulation of cytotoxic drugs in resistant cells. MDR (multidrug resistance) is of clinical importance because many human tumors have inherent or acquired Pgp-mediated drug resistance and do not respond to chemotherapy. Pgp acts as an energy-dependent pump to translocate a wide variety of structurally and functionally diverse substrates. These compounds tend to be moderately hydrophobic, planar and natural products which are often substrates for/or metabolites of detoxification enzymes such as cytochromes P450 (CYPs) (Bard, 2000:357). CYP represents a superfamily of microsomal heme-thiolate enzymes that catalyse the biotransformation (oxidation) of numerous endogenous and foreign compounds (McKinnon *et al.*, 1995:259). CYPs identified in the human intestine include CYP1A1, CYP2C8-10, CYP 2D6, CYP2E1 (trace amounts), CYP3A4 and, more recently, CYP3A5 (Wacher *et al.*, 2001:90). The major congener of the CYP3A superfamily is CYP3A4, the predominant form in the liver and intestine. The enzyme is considered to be the most important enzyme in drug metabolism (Wacher *et al.*, 2001:90). Pgp also prevent the cellular accumulation of endogenous metabolites, phospholipids and xenobiotics in exposed animals and cell cultures (Bard, 2000:357).

Drugs may also be modified by biotransformation processes which include phase I and phase II reactions. The process biotransformation or metabolism is the enzymatic conversion of a drug to a metabolite. Phase I, or asynthetic reactions, include oxidation, reduction and hydrolysis. Phase II reactions, or synthetic reactions include conjugations (Shargel & Yu, 1999:367). These processes may not only render the drug ineffective,

but it may also produce metabolites that are themselves substrates for Pgp and/or MRP2 (Chan *et al.*, 2004:25).

Drugs that eventually reach the blood are transported to the liver, where they are subject to further metabolic processes and biliary excretion, often by a system of ABC transporters and enzymes similar to those present in the intestine. Thus a synergistic relationship exists between intestinal drug metabolising enzymes and apical efflux transporters, a partnership that proves to be a critical determinant of oral bioavailability. The effectiveness of this system is optimised through dynamic regulation of transporter and enzyme expression. Tissue has a remarkable capacity to regulate the amounts of protein in order to maintain homeostasis (Chan *et al.*, 2004:25).

Members of the ABC family of transporter proteins and knowledge on the mechanism of action of Pgp as a representative of this fascinating, rapidly growing group of membrane transport proteins, is of great interest and are involved in diverse physiological processes which include antigen presentation, drug efflux from cancer cells, bacterial nutrient uptake and cystic fibrosis. In order to understand the role of these transporters, an integrated approach combining structural, pharmacological and biochemical methods are being studied extensively (Germann, 1996:928; Kerr, 2002:47).

2.4.1 ABC transporters

Over the years, tremendous progress has been made to understanding the pharmacological and toxicological impact of ABC drug efflux transporters. Once thought to be perhaps just of relevance in making cancer cells resistant to anticancer drugs, it is now clear that they can have a pronounced role in the oral bioavailability and hepatobiliary, direct intestinal and most likely renal excretion of an extensive range of drugs and toxins. In addition, they contribute to important pharmacological sanctuary sites such as the brain, cerebrospinal fluid, testis and fetus and they can protect individual cells from drug and toxin penetration (Schinkel & Jonker, 2003:22). Many other xenotoxins, pre-carcinogens and endogenous compounds are also influenced by the ABC transporters (Schinkel & Jonker, 2003:3).

The typical structure of an ABC protein consists of membrane-embedded transmembrane domains (TMD) and ATP binding domains (ABC) (Figure 2.3). Typically, the transmembrane regions anchor the protein to the membrane and form a pore through which the transport of a surprisingly large variety of substrates occurs. The cytoplasmic nucleotide binding domains provide the molecular compartment where the energy of ATP is released (Sigma Aldrich, 2005).

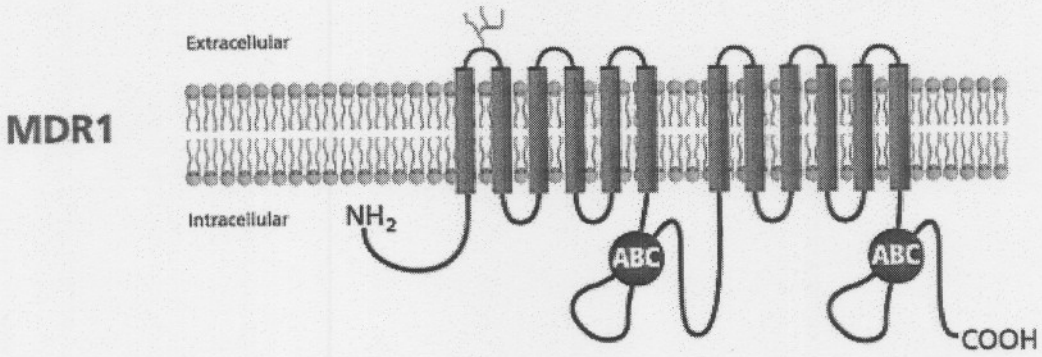


Figure 2.3: Schematic presentation of membrane topology of an ABC transporter (Sigma Aldrich, 2005).

2.4.1.1 P-glycoprotein

With the realisation of the importance of drug efflux transporters in the treatment of diseases, inhibitors of these transporters have been sought to use as adjuncts during treatment with these specific drugs. To date, inhibitors that have best been studied are modulators of Pgp, a transporter that is important in the removal of anticancer agents from cells and over expression of this transporter will result in multidrug resistance (Dantzig *et al*., 2003:133). *In vitro* and *in vivo* studies have demonstrated that Pgp plays a significant role in drug absorption and disposition. Like cytochrome P450 enzymes, inhibition and induction of Pgp has been reported as the cause of drug-drug interactions (Lin, 2003:53).

2.4.1.2 Structure of Pgp

Pgp is a transmembrane protein which is 1280 amino acids long and consists of two homologous halves of 610 amino acids joined by a flexible linker region of 60 amino acids. Each half has an N-terminal hydrophobic domain containing six transmembrane domains followed by a hydrophilic domain containing a nucleotide binding site. The nucleotide binding sites can bind ATP and its analogues and both sites are essential since inactivation of either site inhibits substrate-stimulated ATPase activity. However, the two sites are likely to be functionally independent and cleavage probably only occurs at one site at a time (Loo & Clarke, 1999:24759). The membrane topology of the human multidrug resistance protein (MDR1) is illustrated in Figure 2.4. The ATP-binding sites are circled (Sarkadi *et al*., 1996:216).

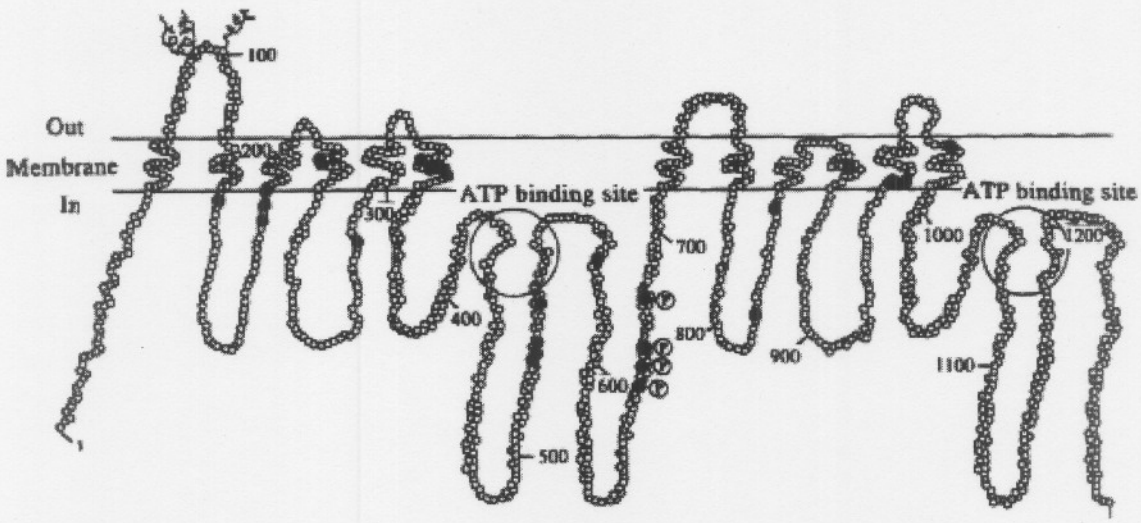


Figure 2.4: Schematic model of the human multidrug resistance gene product, Pgp and its functional domains (Sarkadi *et al*., 1996:216).

The nucleotide binding domain must interact with the membrane spanning domains in order to transmit conformational changes resulting from the hydrolysis of ATP. In eukaryotic systems fusion of the domains into one polypeptide chain assures their physical proximity (Schneider & Hunke, 1998:12).

2.4.1.3 Location and function of Pgp

Pgp expression is detected in three distinct subsets of normal human tissue namely those involved in secretion and absorption or those that have a barrier function. It is also present in some tumors derived from these tissues which include a subset of columnar epithelial cells, endothelial cells of capillary beds in specific anatomic locations and placental trophoblasts. The trophoblast is a thin layer of ectoderm that constitutes the wall of a mammalian blastula and is important for the nutrition and implantation of an embryo (van der Heyden *et al*., 1995:223).

It is suggested that Pgp helps to maintain the integrity of the blood-brain and blood-testis barrier in addition to preventing accumulation of xenobiotics in multiple organs (Bard, 2000:368). Studies by Borst and Schinkel (1996:985) support the positive expectation that clinical chemosensitiser treatment in cancer patients to block functional Pgp, may not have major effects on human physiology, beyond altered drug metabolism. The location and functions of Pgp is represented in Table 2.1 (Delph, 2000).

Table 2.1: Location and function of Pgp (Delph, 2000).

Location and function of Pgp		
Organ/tissue	**Site**	**Function**
Gut • Colon • Jejunum	Apical (luminal) surfaces of superficial columnar epithelial cells	Intestinal excretion and reduced absorption of drugs and toxins
Liver and biliary system	Hepatocytes on biliary canalicular cells of small biliary ductules	Hepatobiliary excretion of drugs and toxins
	Apical surfaces of epithelial cells of small biliary ductules	
	Hepatocytes	Regulation of cytochrome expression
Pancreas	Apical surfaces of epithelial cells of small ductules	Unknown
Kidney	Apical surfaces of epithelial cells of proximal tubules	Urinary excretion of drugs and toxins
Brain	Luminal surfaces of endothelial cells of cerebral capillaries	Contributes to the blood-brain barrier, keeping drugs and toxins out of the brain
	Choroid plexus	Unknown
Peripheral nerves	Endothelial cells of nerve capillaries	Contributes to the blood-nerve barrier, keeping drugs and toxins out of the nerves
Uterus	Fetally-devided epithelial cells of placenta	Contributes to the materno-fetal barrier, keeping drugs and toxins out of the fetus
	Steroid-producing cells of endometrial glands	Unknown – possible role in steroid production
Testis and ovary	Capillary endothelial cells	Contributes to the blood-testis/ovary barrier, keeping

		drugs and toxins out of the gonads
Immune system	Skin dendritic cells	Migration of dendritic cells to lymph nodes
	Activated lymphocytes	Transport of some cytokines (especially interleukins-1,2,4 and interferon-y) out of cell
	Natural killer and CD8+ cytotoxic T cells	Reduced cytolytic activity
Bone marrow	Hematopoietic stem cells	May remove drugs and toxins from the bone marrow
Adrenal gland	Medulla and cortex	Unknown – possible role in steroid production
Large arteries	Endothelium	Unknown – possible role in accumulation of intracellular cholesterol ester in atherosclerotic lesions

2.4.1.4 Mechanism of efflux

For many years, the model for drug resistance conferred by Pgp has been a relatively simple one. According to that model, cytotoxic drugs are actively transported out of cells that express Pgp against a concentration gradient thereby reducing intracellular drug accumulation and inhibit drug-mediated cell death. Three models have been described by Bard (2000:361-362) to explain Pgp-mediated transport and include the following:

- Hydrophobic vacuum cleaner model (Higgins & Gottesman,1992:18);
- Flippase (Higgins & Gottesman, 1992:18) and
- Extrusion from the inner leaflet (Eytan & Kuchel, 1999:217).

In the hydrophobic vacuum cleaner model, drugs were transported from the plasma membrane to the extracellular medium (Higgins & Gottesman, 1992:18). This model was not satisfactory because Pgp has been shown to specifically bind to substrates with different specifities (Liu & Sharom, 1996:11873).

The second model proposed that Pgp flips drugs from the inner to the outer leaflet of the plasma membrane against an intramembrane concentration gradient and is shown in Figure 2.5 (Higgins & Gottesman, 1992:19; Borst & Schinkel, 1997:220).

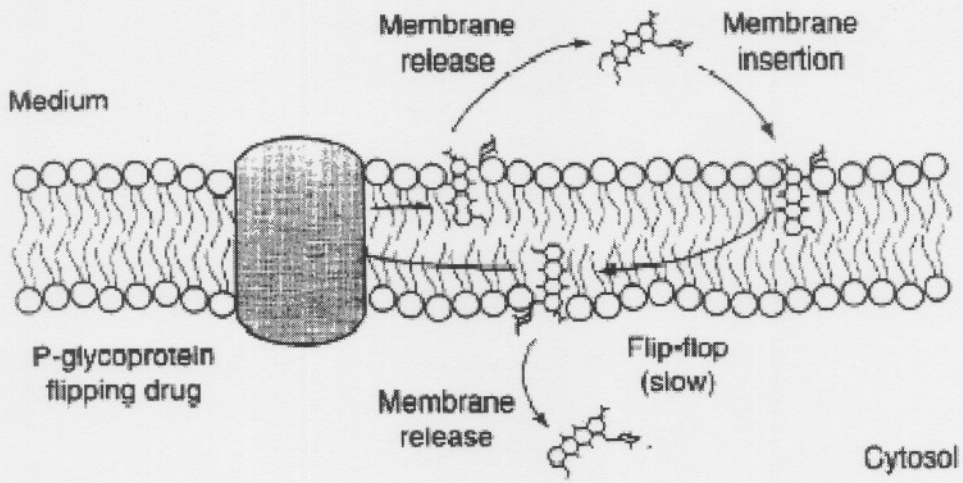


Figure 2.5: Flippase model for Pgp action (Borst & Schinkel, 1997:220).

This model is based on the analogy between amphipathic drugs and the normal phospholipids constituents of membranes. The term flippase was originally used to describe membrane enzymes that translocate phospholipids. Although the lateral mobility of phospholipids is high, movement between the leaflets is low because the polar head group of the phospholipids has difficulty passing through the hydrophobic interior of the membrane. Enzymes that can speed up this flipping reaction have been described and these enzymes are called flippases or phospholipids translocators (Borst & Schinkel, 1997:220).

A third model has been proposed by Eytan and Kuchel (1999:218), which combines elements of the two previous models and is shown in Figure 2.6.

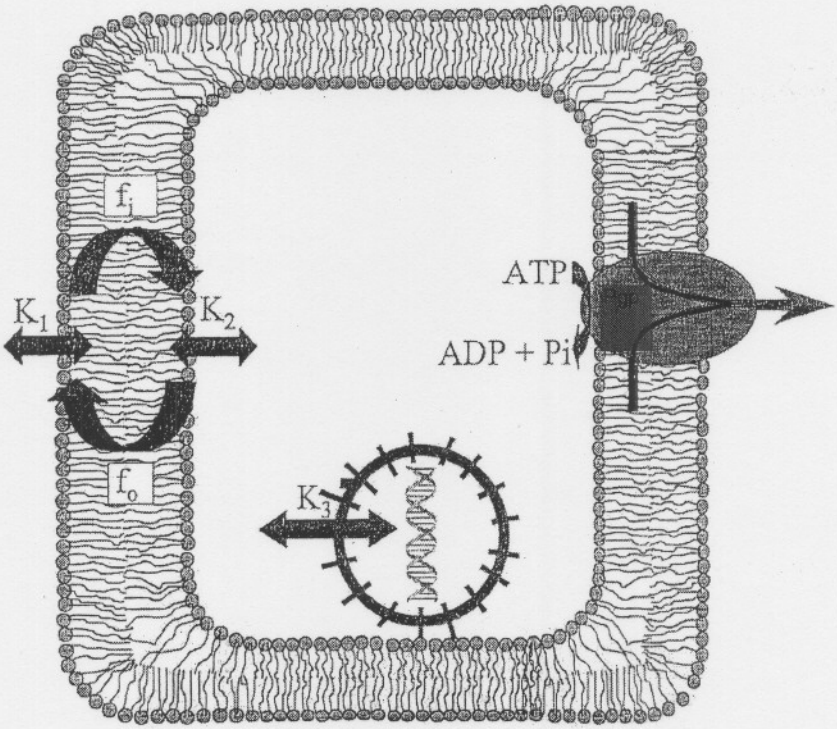


Figure 2.6: Schematic diagram describing movement of MDR-type drugs in Pgp-overexpressing cells (Eytan & Kuchel, 1999:180).

In this model, MDR drugs added to the extracellular medium, bind to and are in practical equilibration (K_1) with the drug pool in the outer leaflet of the plasma membrane. The drugs flip-flop across the plasma membrane (f_1 and f_0). The drug pool in the inner leaflet of the membrane is in "effective" equilibrium with the drug pool in the cytoplasm (K_2). Drugs in the cytoplasm are largely bound and form equilibrium (K_3) with intracellular molecular sinks represented here, for simplicity, as DNA. Pgp seems to extract its substrates from the inner leaflet of the plasma membrane and flip those outwards (Eytan & Kuchel, 1999:180).

2.4.1.5 The ATPase activity of P-glycoprotein

It has been previously stated that ABC-transporters have two NBDs. Both of these domains were found to be able to hydrolyse ATP, but substrate-stimulated ATPase

activity requires interaction between the two halves of the molecule (Borges-Walmsley & Walmsley, 2001:76; Schneider & Hunke, 1998:11; Sharom *et al.,* 1999:329). Both domains are sensitive to the same set of inhibitors and seem to be functionally identical (Schneider & Hunke, 1998:15). Inactivation by chemical modification of either of the NBDs causes a loss of activity, suggesting Cooper-activity between the sites. In addition to the site for the nucleotide, the NBDs of Pgp have a site for ligands, such as flavonoids, which modulate drug transport, probably by inhibiting ATPase activity (Borges Walmsley & Walmsley, 2001:76).

It has also been suggested that mammalian sites function by an alternating-site mechanism. This implies that one site hydrolyses ATP and inactivates the other site for that period. After hydrolysation, the site becomes inactive and the previously inhibited site becomes able to hydrolyse ATP (Borges-Walmsley & Walmsley, 2001:76; Schneider & Hunke, 1998:7). This alternating-site drug transport process and its relationship to the hydrolysis of ATP is shown in Figure 2.7 (Senior *et al*., 1995:288).

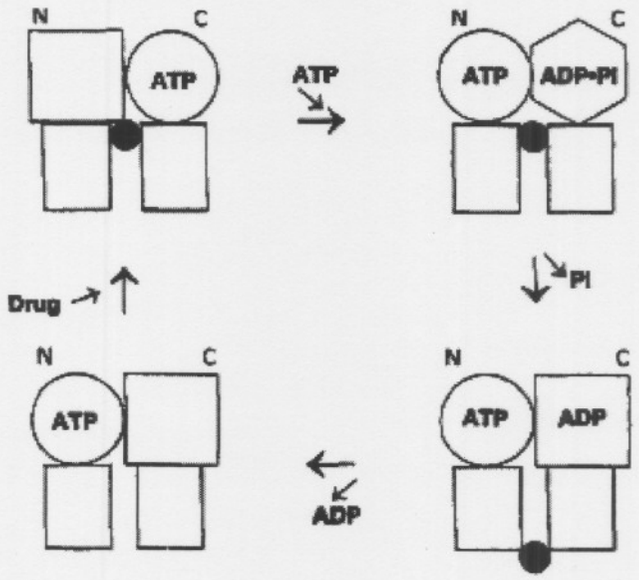


Figure 2.7: Postulated alternating catalytic sites cycle of ATP hydrolysis by P-glycoprotein (Senior *et al*., 1995:288).

In Figure 2.7 the rectangles represent the two Pgp trans-membrane domains (TMD). The circles, squares and the hexagon, represent different conformations of the N- and

C-catalytic sites (NBS1 and NBS2, respectively). The mechanism can be explained in the following four steps:

- The N-catalytic site is empty where the C-catalytic site has bound ATP and the drug is bound at the inside-facing transport site (top left).
- In the illustration on the top right side it is suggested The ATP binding at the N-site allows ATP hydrolysis at the C-site, inducing a conformation at the C-site which prohibits hydrolysis at the N-site (top right). The conformation at the C-site immediately after bound-cleavage is a high chemical potential state with bound ADP.P_i (shown as a *hexagon).*
- Relaxation of the C-site conformation occurs and coupled to the drug movement from the inside-facing, higher-affinity to the outside-facing, lower-affinity, and P_i is released (bottom right).
- The drug and ADP dissociate (bottom left). The drug binds at the inner side and the N- and C-sites have now reversed their relationship (top left). In the next cycle, ATP hydrolysis will occur in the N-site (Senior *et al.*, 1995:288).

2.4.1.6 Multidrug resistance-associated protein – MRP

Several of the substrates and inhibitors of MRP are anionic. In contrast Pgp has typically been associated with cationic substrates. MRP is inhibited by anionic compounds, such as probenecid and indomethacin (Aungst, 1999:110). Since the discovery of MRP in 1992, understanding of its biological properties has progressed rapidly. It is now firmly established that MRP can confer a multidrug resistance phenotype that *in vitro* is similar in many respects to that conferred by Pgp. Insufficient data is available to assess whether these two proteins play a similar role *in vivo* in drug resistant malignancies, but the normal physiological functions of these two proteins clearly differ (Loe *et al.*, 1996:950).

Human MRP is a 190 kDa protein and its primary structure shares approximately 15% amino acid identity with Pgp (Quan *et al.*, 2000:197). The expression of MRP1 in the body is widespread, with high levels in the intestine, brain, kidney, lung, testis and lower expression in the liver (Cherrington *et al.*, 2002:97). MRP1 is localised to the basolateral membranes of polarised epithelial cells including those of the intestinal crypt, renal distal

and collecting tubules (Peng *et al.*, 1999:757) and liver (Mayer *et al.*, 1995:138). The basolateral localisation of MRP1 in normal tissues suggests that it protects cells by extruding its substrates into blood. Indeed, confocal microscopic studies show that MRP1-positive staining on the basolateral membranes of rat hepatocytes is markedly increased following bile duct ligation (Pei *et al.*, 2002:59), indicating an adaptive response to maintain cellular detoxification when elimination into bile is restricted. The increased levels of toxins present in the blood presumably undergo subsequent elimination by the intestine and kidneys. The barrier formed by MRP1 against the cellular accumulation of toxins is yet more effective when coupled with apical Pgp expression (Tada *et al.*, 2002:634).

2.5 Synergism between Pgp and Cyp3A4

Many of the same drugs that are transported by Pgp are metabolised by some of the cytochromes (CYPs), especially CYP450 3A which constitutes 30% and 70% of the total CYP450s in most human livers and intestines, respectively. CYP3A substrates include HIV protease inhibitors, the antibiotic erythromycin, the lipid-lowering agent lovastatin; the calcium channel blocker nifedipine; rifampicin; and the sedative midazolam. CYP3A can be induced by many agents, including glucorticoids, barbiturates and rifampicin. Human variation in CYP3A expression is believed to influence drug response for up to one-third of all drugs (Delph, 2000). There are several mechanisms according to which the functions of Pgp and CYP3A4 could be complimentary and are shown in Figure 2.8 (Watkins, 1997:166).

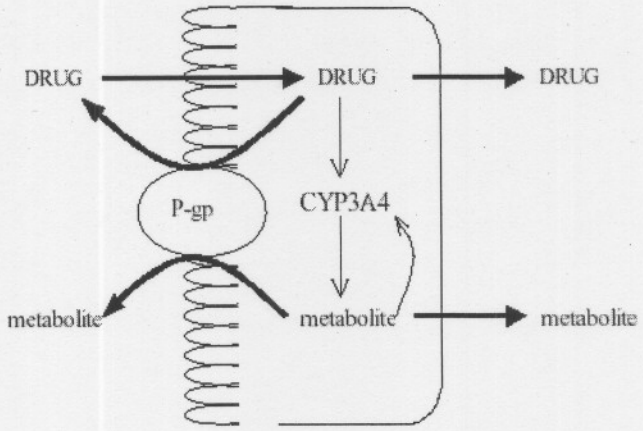


Figure 2.8: Potential functional metabolism between enterocyte Pgp and CYP3A4 (Watkins, 1997:166).

Firstly, Pgp may prevent parent drug diffusion across the apical brush border membrane and therefore prevent absorption. Secondly, Pgp may function to prolong the duration of absorption. This might increase the duration of exposure of drugs to CYP3A4 and, hence, the extent of metabolism by enterocyte CYP3A4. Finally, Pgp may preferentially remove primary drug metabolites that are themselves substrates for CYP3A4. This would prevent or limit product inhibition by these metabolites and facilitate primary metabolism catalysed by CYP3A4 (Watkins, 1997:161).

Tissue are equipped with two lines of defense namely phase I (e.g. hydroxylation via cytochromes P450) and phase II (e.g. conjugation with glutathione) detoxification enzymes which metabolise xenobiotics (as well as endogenous substrates) to more hydrophilic compounds, which can be more easily excreted from the body. An examination of the structure-activity relationships and molecular characteristics for xenobiotic transport substrates and inhibitory ligands of Pgp indicate that Pgp may function in the elimination of hydroxylated metabolites of xenobiotics after modification by phase I enzymes (Bain *et al*, 1997:812). Many phase II conjugates are also transported by MRP. A schematic presentation of the roles of ABC transporters and phase I and II enzymes in xenobiotic resistance are shown in Figure 2.9 (Bard, 2000:370).

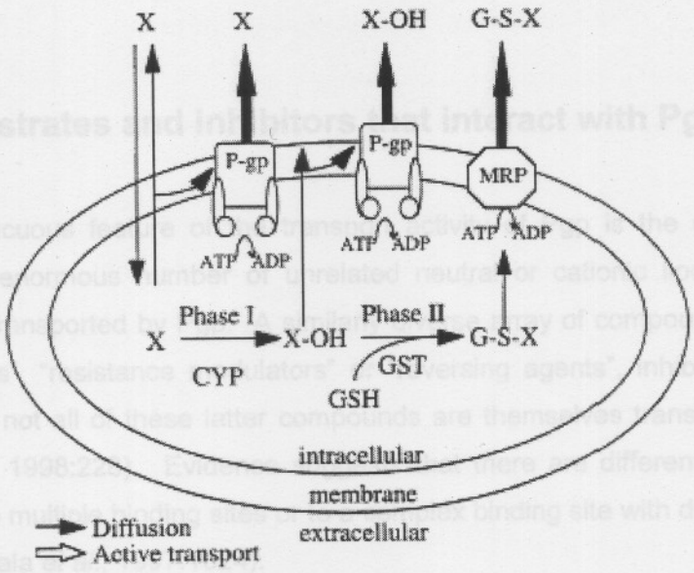


Figure 2.9: A schematic presentation of the roles of ABC transporters and phase I and II enzymes in xenobiotic resistance (Bard, 2000:370).

This is a speculative model of xenobiotic resistance provided by transmembrane active transporters, Pgp and MRP, and phase I and II detoxification enzymes, cytochromes P450 (CYP) and glutathione-*S*-transferase **(GST)** which gives an indication of the different pathways by which each exerts its action. A moderately hydrophobic natural product **(X)** diffuses in and out of the cell and at low concentrations little accumulates in the cell due to the active efflux of parent compound by Pgp. At high concentrations, X accumulates and is metabolised by one or more of the CYP enzymes to form a hydroxylated metabolite **(X-OH)**. The hydroxylated metabolite can either be removed by Pgp mediated transport or may further be modified by conjugation to glutathione **(GSH)** catalysed by **GST**. The glutathione conjugate **(G-S-X)** is then expelled from the cell by MRP active transport as illustrated in Figure 2.9 (Bard, 2000:370).

2.6 Drug substrates and inhibitors that interact with Pgp

The most conspicuous feature of the transport activity of Pgp is the diversity of its substrates. An enormous number of unrelated neutral or cationic lipophilic organic compounds are transported by Pgp. A similarly diverse array of compounds, known as "chemosensitisers", "resistance modulators" or "reversing agents", inhibit transport by Pgp. Many, but not all of these latter compounds are themselves transported by Pgp (Shapiro & Ling, 1998:228). Evidence suggests that there are different categories of substrates due to multiple binding sites or to a complex binding site with different regions of interaction (Scala *et al.*, 1997:1024).

Studies done by Scala *et al.* (1997:1024) defined compounds as Pgp substrates if cytotoxicity was increased more than four times by the addition of cyclosporin, and as Pgp antagonists if inhibition of efflux increased Rhodamine 123 (Rho 123) accumulation more than four times. Their studies also imply that antagonists bind to Pgp but fail to be transported, therefore, prevent the transport of other agents. Pgp substrates are transported but do not block the transport of other substrates. Pgp substrates and inhibitors are shown in Tables 2.2 and 2.3 respectively (Delph, 2000).

Table 2.2: Pgp substrates (Delph, 2000).

Pgp substrates	
Chemicals transported by Pgp include	
Cancer drugs • Doxoubicin • Daunorubicin • Vinblastine • Vincristine • Actinomycin D • Paclitaxel • Teniposide • Etoposide **Immunosuppressive drugs** • Cyclosporin A • FK506 **Lipid-lowering agent** • Lovastatin **Antihistamine** • Terfenadine **Steroids** • Aldosterone • Hydrocortisone • Cortisol • Corticosterone • Dexamethasone **Dopamine antagonist** • Domperidone	**HIV protease inhibitors** • Amprenavir • Indinavir • Nelfinavir • Ritonavir • Sequinavir **Cardiac drugs** • Digoxin • Quinidine **Antiemetic** • Ondansetron **Anti-diarrhoeal agent** • Loperamide **Anti-gout agent** • Colchicine **Antibiotic** • Erythromycin **Anti-helminthic agent** • Ivermectin

Table 2.3: Pgp inhibitors (Delph, 2000).

Pgp inhibitors	
Chemicals that block Pgp-mediated transport include:	
Cyclopropyldibenzosuberane • LY335979 **Immunosuppressant** • Cyclosporin A • Valspodar (PSC833) **HIV protease inhibitors** • Ritonavir • Sequinavir • Nelfinavir • Indinavir **Calcium channel blocker** • Verapamil **Progesterone antagonist** • Mefipristone (RU486)	**Anti-estrogen cancer agent** • Tamoxifen **Antiarrhythmic agent** • Quinidine **Antifungal agent** • Ketoconazole **Sedative** • Midazolam **Acridonecarboxamide derivative** • GG918 (GF120918) **Peptide chemosensitisers** • Reversin 121 • Reversin 205

It has also been known for some time that flavonoids present in the diet are capable of interfering with drug metabolism *in vitro*, and it was thought that a flavonoid present in grapefruit juice might be responsible for the interaction with CYP3A4 substrates (Evans, 2000:133). One of the most abundant flavonoids in grapefruit juice is naringen. The compound is converted *in vivo* to the aglycone, naringenin (Ho *et al.*, 2000:379). Naringenin, like many other flavonoids, is a potent inhibitor of CYP3A4 (Fuhr, 1998:265). It was found by Mitsunaga *et al.* (2000:199) that naringenin increased the uptake of vincristine into MBEC4 cells, indicating inhibition of Pgp activity.

Non-cytotoxic compounds such as fluorescent Rho 123 dye and the calcium channel blocker verapamil, a cardiovascular medication, have been used respectively as a model substrate as well as a model competitive inhibitor (Neyfakh, 1998:168). The competitive inhibition of Rho 123 efflux in a model vesicle or cellular system by the addition of verapamil is considered evidence of Pgp-activity. Furthermore, the ability of a chemical to inhibit Rho 123 transport in an assay system is considered evidence of a potential Pgp-substrate or inhibitor (Bard 2000:363).

Verapamil which can competitively inhibit the transport of Pgp substrates functions as a chemosensitiser or modulator of multidrug resistance. MDR chemosensitisers tend to be more lipophilic than other substrates and once extruded by Pgp, diffuse back into the cell before being transported again (Sharom, 1997:162). Drugs referred to as substrates are found to diffuse relatively slowly across membranes during experimental studies (in the order of minutes to hours) compared to chemosensitisers which diffuse bilayers at a speed unable to measure (Eytan *et al.*, 1996:12901). Clinicians have co-administered verapamil with chemotherapeutic drugs in the hope to eradicate MDR tumors in cancer patients. Unfortunately, the chemosensitiser dose required to inhibit Pgp provokes cardiovascular side effects (Lehnert *et al.*, 1998:1155).

2.7 Substrate binding of Pgp

Pgp has wide substrate specificity and is an intriguing problem and much work has been directed toward characterising the binding site(s). Recently Shapiro and Ling (1998:227) presented evidence that Pgp contains at least two distinct substrate binding and transport sites and that these sites interact in a positively cooperative fashion. They propose that one site marked **R** (for the substrate Rho 123 and anthracyclenes) binds one set of compounds and the other site **H** (for the substrate Hoechst 33342 and colchicine) binds a second set as shown in Figure 2.10 (Shapiro & Ling, 1998:227).

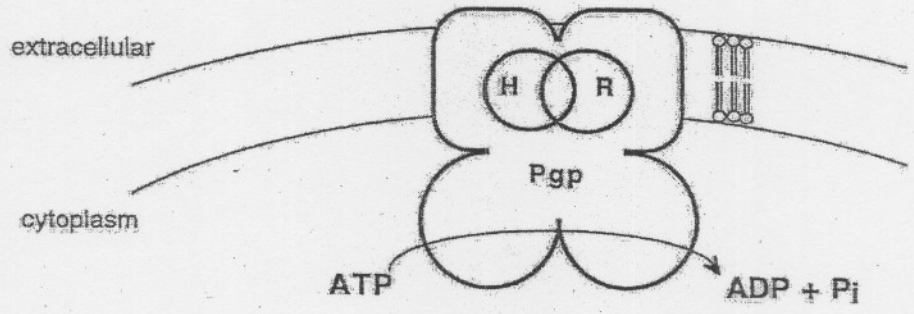


Figure 2.10: Two transport-competent drug binding sites of Pgp with distinct but overlapping specificities (Shapiro & Ling, 1998:227).

Each **R** substrate stimulates the Pgp-mediated transport of an **H** substrate and *vice versa*. Two **R** substrates would compete for the **R** site and inhibit binding and transport of the other. Competitive inhibition would also be observed between two **H** substrates or between substrates which can bind to both **R** and **H** sites. A third drug-binding site (for the substrates prazosin and progesterone) on the Pgp exerts a positive allosteric effect on drug transport by the **H** and **R** sites but is not thought to be capable of drug transport itself (Shapiro *et al.*, 1999:848). The three-site model described by Shapiro and Ling is consistent with previous observations of both competitive and non-competitive interactions between Pgp substrates (Bard, 2000:362).

2.8 Structure-activity relationships of Pgp substrates

Little information is available regarding the structure-activity relationships of Pgp substrates. It is, however, evident that the substrates are of large molecular weight, amphipathic and contain at least one aromatic ring (Wacher *et al.,* 1998:1323). Other important structure-activity relationship properties that influence the complexation interaction (between a drug and a drug-binding site on Pgp) include large molecular surface, high polarisability and hydrogen binding properties (Österberg & Norinder, 2000:295). The only physical similarities substrates share are that they are moderately hydrophobic, cationic or neutral but never anionic and that they are natural products (Gottesman & Pastan, 1988:55).

2.9 Conclusion

According to Steyn (1999:579) the properties of Pgp that has to be taken into account in attempting to test for possible modulators or inhibitors of this protein are summarised as follows:

- Pgp is an intrinsic membrane protein with a polypeptide sequence that suggests it consists of two similar halves. Each half has six sectors that cross the membrane as well as an ATP-binding cytoplasmic region;
- Pgp is a transport ATPase and can transport its substrates out of the cell in which it is present. The substrates are pumped from the membrane by itself. Substrates can be pumped from the outer bilayer of the membrane, i.e., before it crosses the membrane and enters the cell, as well as from the inner half bilayer;
- Pgp displays a very broad specificity to its substrates. Most are weak bases, but non-protonable molecules can also be good substrates, as well as molecules bearing a permanent positive charge. Although all the substrates should be lipophilic it is not clear whether extremely lipophilic molecules can be substrates;
- Transport out of the cell can be blocked by reversers or modulators of Pgp. The latter consist of a very wide range of molecular types with the same general characteristics as the substrates themselves;
- Pgp is an ATPase of moderate ATP-splitting capacity. The rate of hydrolysis of ATP is enhanced up to 5- to 10-fold by many substrates and modulators of Pgp's function, but reduced by others;
- Transport of substrates by Pgp is not influenced by the membrane potential, is independent of the pH gradient and is not associated with any co- or countertransport of protons, cations or anions. Thus Pgp seems to be a primary ATPase;
- No evidence has been found for an intermediate phosphorylated state of Pgp when it hydrolyses ATP, nor for a tightly bound ATP molecule and
- The two ATP-binding sites in Pgp appear to co-operate in the transport of substrate but can independently hydrolyse ATP. The two halves of Pgp thus seem to interact during substrate transport. Consistent with this is the evidence

> that pairs of substrate molecules and of some modulators and inhibitors, co-operate in the action of Pgp.
>
> (Stein, 1997:579)

At present, available therapy cure a little more than half cancer patients. Surgery is considered the most efficient treatment, possibly because it is used to cure very small tumours, which are rarely accompanied by metastasis. Chemotherapy appears to be less efficient, possibly because it is used when metastases are already present, the tumour is large, spreading and moreover, anticancer drug resistance is frequent. A successful solution to this resistance would lead to a great increase in therapeutic success. As novel anti-cancer drugs often offer only a small improvement over older agents, a change in the target of chemotherapy must be considered. Instead of looking for a new cytotoxic drug, it may be an option to look for methods of circumventing drug resistance. In order to address this problem most efficiently, it is essential to establish the dominant mechanism of resistance to chemotherapy in human tumours. This is often considered to be the so-called MDR mechanism (Desoize & Jardillier, 1999:194).

Nevertheless, although the first drug able to reverse MDR was discovered more than 20 years ago, a potent, specific and safe drug, able to reverse multidrug resistance and to assist in the chemotherapy of cancer is still lacking. However, several drug candidates presently under development look very promising and some of them, such as valspodar, biricodar and LY 335979 are in a good position to gain approval in the near future (Teodori *et al.*, 2002:405).

Present data are consistent with the hypothesis that certain flavonoids affect MRP-mediated transport of anticancer drugs by a direct interaction with MRP (Hooijberg *et al.*, 1997:344). The benefits of flavonoids as chemopreventive dietary or dietary supplemental agents are still only potential. Much has been learned about possible mechanisms of action of these agents, but whether they can reach their multiple intended sites of action, particularly in humans, is largely unknown (Walle, 2004:829).

In the following study, with reference to the inhibition of Pgp. Certain flavonoids will be extensively studied to provide an inspiration for a rational drug and/or chemopreventive

agent design of future pharmaceuticals. The potential inhibition of selected flavonoids will be evaluated using an *in vitro* transport model with Rho 123 as a Pgp substrate.

CHAPTER 3

THE POTENTIAL STRUCTURE ACTIVITY RELATIONSHIP OF FLAVONOIDS WITH REFERENCE TO THE INHIBITION OF P-GLYCOPROTEIN

3.1 Introduction

Diverse compounds have been shown to reverse the Pgp-mediated MDR, including calcium channel antagonists such as verapamil (Tsuruo *et al.*, 1981:1967) and immunosuppressants such as cyclosporin A (Twentyman *et al.*, 1991:24). MDR cells can be sensitised to anticancer drugs when treated with a Pgp inhibitor, which is known as a chemosensitiser. The search for chemosensitisers which have the advantages of being a non-transportable inhibitor without side effects, has led to a great deal of research in flavonoids derived from plants (Conseil *et al.*, 1998:9831).

The diverse nature of the compounds which interact with Pgp has lead to the realisation that there is a greater possibility for drug-drug interaction than was initially thought. Many of the Pgp inhibitors are compounds which occur in foods such as soya beans and grapefruit juice, and inhibition as well as induction of this protein could lead to enhanced or diminished absorption of drugs that are substrates for Pgp (Wagner *et al.*, 2001:S14).

3.1.1 Flavonoids

Flavonoids represent a group of phytochemicals exhibiting a wide range of biological activities arising mainly from their antioxidant properties and ability to modulate several enzymes or cell receptors. Flavonoids have been recognised to exert anti-bacterial, anti-viral, anti-inflammatory, anti-angionic, analgesic, anti-allergic, hepato-protective, cytostatic, apototic, estrogenic and anti-estrogenic properties. However, not all flavonoids and their actions are necessarily beneficial. Some flavonoids have mutagenic

and/or pro-oxidant effects and can also interfere with essential biochemical pathways. Cytochromes P450, mono-oxygenases, metabolising xenobiotics (e.g. drugs and carcinogens) and endogenous substrates (e.g. steroids) also play a prominent role among the proteins that interact with flavonoids. Flavonoids in the human diet may reduce the risk of various types of cancers, especially hormone-dependent breast and prostate cancer, as well as preventing menopausal symptoms. For these reasons the structure-function relationship of flavonoids is extensively studied to provide an inspiration for a rational drug and/or chemopreventive agent design of future pharmaceuticals (Hodek, 2002:1).

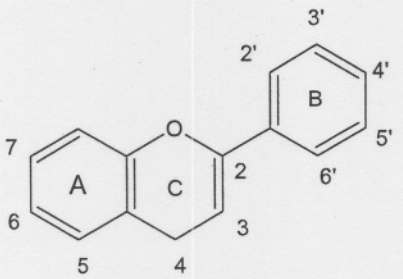
3.1.2 Basic flavonoid structure

The basic flavonoid structure is the flavan nucleus, which consists of 15 carbon atoms arranged in three rings (C_6-C_3-C_6), which are labeled **A**, **B**, and **C** (Figure 3.1a). The various classes of flavonoids differ in the level of oxidation and pattern of substitution of the **C** ring, while individual compounds within a class differ in the pattern of substitution of the **A** and **B** rings (Pietta, 2000:1035-1036).

The benzene ring **A** is condensed with a six-member ring **(C)**, which in the 2-position carries a phenyl pyran, which yields flavanols (catechins) and anthocyanidins, or pyrone which yields flavonols, flavones and flavonones. The term *4-oxo-flavonoids* is often used to describe flavonoids, such as flavanols (catechins), flavanones, flavonols and flavones, which carry a carbonyl group on **C-4** of ring **C** (Figure 3.1b) (Aherne & O`Brien, 2002:75).

The chemical nature of the flavonoids depends on structural class, degree of hydroxylation, other substitutions and conjugations and degree of polymerisation (Harborne, 1986:15).

(a)



(b)

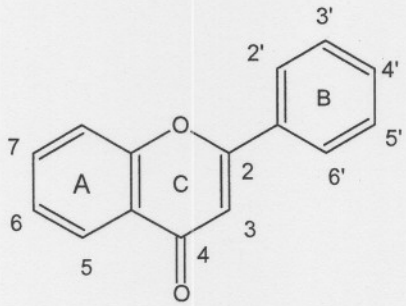


Figure 3.1: (a) Flavan nucleus and (b) 4-oxo-flavonoid nucleus (Aherne & O`Brien, 2002:75).

The structures of the basic flavonoids are given in Figure 3.2 (Aherne & O'Brien, 2002:75).

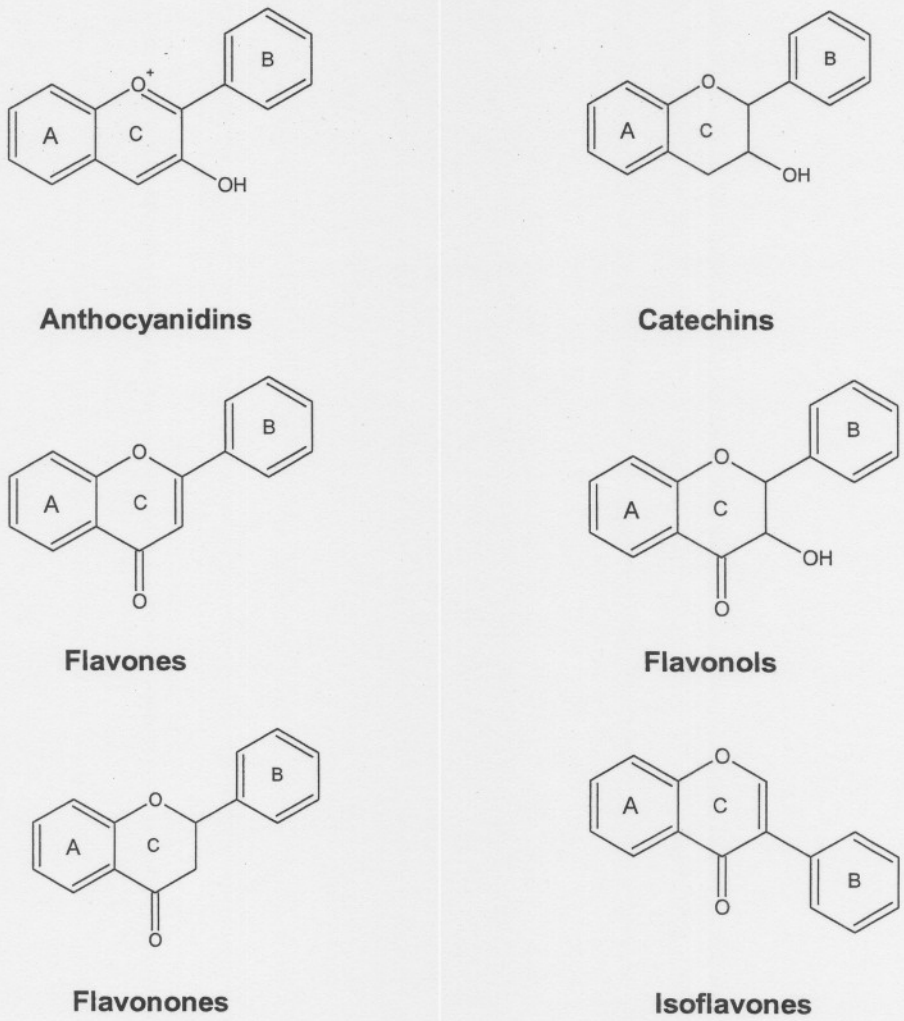


Figure 3.2: Structures of the basic flavonoids (Aherne & O'Brien, 2002:76).

3.2 Flavonoids and MDR

It has previously been demonstrated that flavonoids are efficient Pgp inhibitors, by binding to cytosolic sites which partly overlap the ATP-binding site and the modulators interacting region (Conseil *et al.*, 1998:9831). It has been reported that quercetin affected the expression of MDR1 in the human hepatocarcinoma cell line HepG2. The increase of Pgp synthesis (the gene product) and MDR1 mRNA accumulation in these

cells caused by exposure to arsenite were inhibited by quercetin (Kioka *et al.*, 1992:308). This appeared to be the first report to describe the inhibition of MDR1 expression by any chemical. Not only did certain flavonoids inhibit the expression of the multidrug resistance gene but, in addition, could act as potent stimulators of the Pgp-mediated efflux of the carcinogen 7,12-dimethylbenz[a]anthracene, resulting in a decreased intracellular burden of this polycyclic compound. The active flavonoids were kaempferol, quercetin and galangin (Phang *et al.*, 1993:5979).

In a study conducted by Chieli and co-workers (1995:1748), it has been shown that flavonols are able to modulate liver Pgp activity *in vitro* and that the interference with the transporter is related not only to the flavonol concentration and to the Pgp amount, but, more interestingly, to the kind of substrate used. In particular, the resulting effect may also be of opposite sign. Thus the overall pattern would be consistent with the possibility that the flavonols are substrates of Pgp. In fact, this hypothesis could explain the dose-dependent flavonol-depressed Rho 123 efflux from hepatocytes as a competitive inhibition on the same transporter.

It has also been shown that (iso) flavonoids genistein, kaempferol and flavopiridol stimulate the membrane ATPase activity of the MRP-overexpressing cell line CLC4/ADR. This data is consistent with the hypothesis that certain (iso)flavonoids affect MRP-mediated transport of anticancer drugs by a direct interaction with MRP (Hooijberg, 1997:344).

However, contradictory effects were reported with different multidrug resitant cell lines. Quercetin, kaempferol and galangin were found to increase adriamycin efflux from HCT-15 colon cells whereas quercetin and a methoxylated derivative of quercetin inhibited Rho 123 efflux and reverted MDR in MCF-7 breast cells (Conseil *et al.*, 1998:9831).

3.3 Structure-activity relationship between Pgp and flavonoids

To date, no elements of molecular recognition have been found to interact with Pgp. However, the analysis of three-dimensional structures of a much larger (n>100) and chemically more heterogeneous set of compounds than used in previous investigations revealed specific recognition patterns for Pgp. They consist of hydrogen bond acceptor

(or electron donor) groups (e.g. carbonyl, ether, hydroxyl, or tertiary amino groups) with a specific spatial separation. Recognition patterns formed by two electron donor groups with a spatial separation of 2.5±0.3 Å are called type I and recognition elements formed either by two electron donor groups with a spatial separation of 4.6±0.6 Å, or three electron donor groups with a spatial separation of the two outer groups of 4.6±0.6 Å are called type II patterns. For binding with Pgp at least one of the type I or, one of the type II pattern are required. Binding was shown to increase with the strength and the number of electron donor groups involved in type I and type II patterns. For transport at least two of the type I patterns and one of the type II patterns are required. The same substrate recognition principle was also observed for MRP. Type II patterns are also responsible for Pgp induction and thus the development of drug resistance (Seelig & Landwojtowicz, 2000:32).

Studies of structure-activity relationships have concluded that flavones are more efficient than isoflavones, while flavonols and chalcones are even more active (Boumendjel, 2001:75). Hydroxyl groups at positions 3 and 5 are essential for high-affinity binding to Pgp as in quercetin, galangin, kaempferol and morin shown in Figure 3.4. In a recent study conducted with chalcones, Boumendjel and co-workers (2001:76) showed that the presence of a hydrophobic substituent on the B-ring, considerably enhanced the binding affinity.

In a study conducted by Conseil *et al.*, (1998:9831) the binding of flavonols to the purified C-terminal nucleotide-binding domain (NBD2) of Pgp was directly measured by the quenching of protein intrinsic fluorescence due to a single tryptophan residue. Flavones (like quercetin or apigenin) bound more strongly than flavanones (naringenin), isoflavones (genistein), or glycosylated derivatives (rutin). Kaempferide, a 4' –methoxy 3,5,7-trihydroxy flavone, was even more reactive and induced a complete quenching of H_6 -NBD2 intrinsic fluorescence. Kaempferide binding was partly prevented by preincubation with ATP, or partly displaced upon ATP addition. Interestingly, kaempferide was also able to partly prevent the binding of the antiprogestin RU 486 to a hydrophobic region similar to that recently found, close to the ATP site, in the N-terminal cytosolic domain. Conversely, RU 486 partly prevented kaempferide binding, the effect being additive to the partial prevention by ATP. Furthermore, MANT-ATP binding, that occurred at the ATP site and extended to the vicinal steroid interacting hydrophobic

region, was completely prevented or displaced by kaempferide. Flavonoids behave as bifunctional modulators able to bind partially to the ATP-binding site and partially to a vicinal hydrophobic steroid-binding site. Despite several studies on the structural features of flavonoids that lead to inhibition of MDR transport, nothing is known about the orientation of the flavonoid molecules within the binding pocket. It has been indicated that the flavonoid, luteolin and it's 7-0-glucoside bind to the purified NBD2 fragment (Conseil *et al*, 1998:9831). This ability qualifies them as suitable ligands for investigating the alignment of both flavonoids inside the ATP binding pocket (Nissler *et al*., 2004:1045). The assumed binding of luteolin (1) and luteolin 7-0-glucoside (2) in the binding pocket of the NBD2 is presented in Figure 3.3 (Nissler *et al*., 2004:1048).

The flavonoid molecule is bound to the protein by its polar groups on C-5, C-4, and C-3' (solid lines in Figure 3.3). The latter requires a conformation of the side chain phenyl ring as drawn. This molecular attachment causes the protein to transmit the magnetisation mainly to the protons H-6, H-3, and H-2' (dashed lines in Figure. 3.3) and this holds for both the luteolin 1 and its glucoside derivative 2. Since the side-chain phenyl ring is attached by a single bond, it is more flexible and thus the protons H-6' and H-5' also obtain some magnetisation transfer. It could also be that the protein pocket reaches around the side-chain phenyl ring. These structural requirements predict that modification of the polar group at C-5 should result in less pronounced binding and/or functional activity of the flavonid derivative. Indeed, by using quenching of intrinsic tryptophan fluorescence (see above) it was found that by introducing a methoxy group at C-5 of luteolin increases the EC_{50} value from 22.9 to 33.4 M, and substitution with hexyl groups at C-5 and C-3' further enhanced it to 46.5 M (Nissler *et al*., 2004:1048).

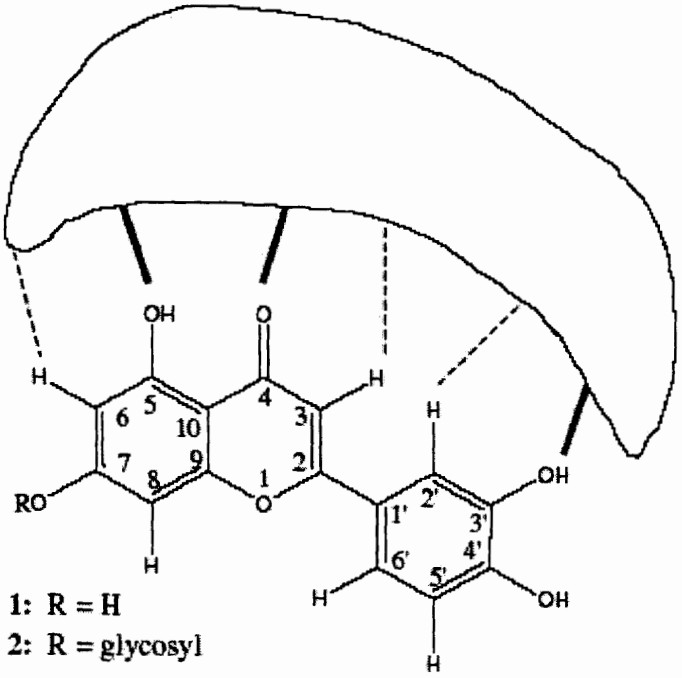
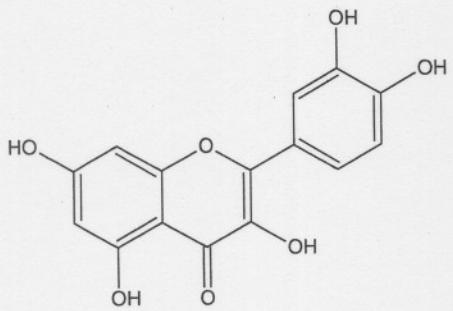
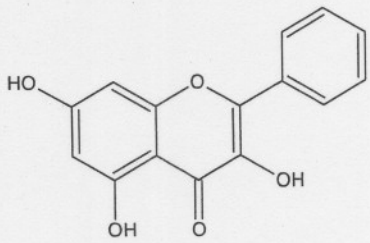


Figure 3.3: Assumed binding of luteolin (1) and luteolin-7-O-glucoside (2) in the binding pocket of the NBD2 (Nissler *et al.*, 2004:1048).

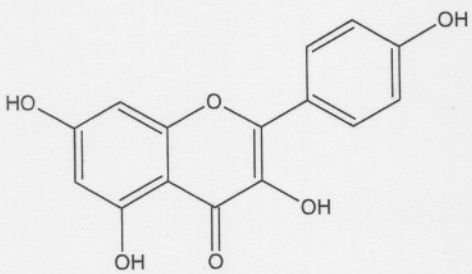
The structures of the flavones that will be investigated in this study are given in Figure 3.4.



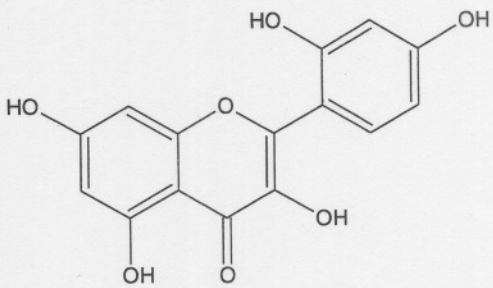
Quercetin (3,5,7,3',4'-pentahydroxyflavone)



Galangin (3,5,7-trihydroxyflavonone)



Kaempferol (3, 4´, 5, 7-tetrahydroxyflavone)



Morin (2´, 3, 4´, 5, 7-pentahydroxyflavone)

Figure 3.4: Flavones that will be evaluated during this study.

3.4 Rhodamine 123

Rho 123 is the model compound that will be used in this study. Rho 123 is a fluorescent lipophilic cation and is classified as a typical Pgp substrate and subject to Pgp-dependent extrusion through the plasma membrane. Rho 123 shows selectivity for mitochondrial compartments. Due to its fluorescence, dye levels can easily be measured in cell extracts and accumulation can be observed in intact cells. Thus, Rho 123 accumulation in (or efflux from) cells is often used as a measure of Pgp-dependent

transport activity. Initially used to study mitochondria, Rho 123 has been introduced in the study of multidrug resistance by Neyfakh (1988:168), who showed that this dye was a definite substrate for Pgp. Since then, various investigators have used the dye as a probe for the investigation of Pgp functional activity (Hirsch-Ernst *et al.*, 2001:48; Marques-Santos *et al.*, 1999:103).

3.5 Conclusion

The effectiveness of Pgp modifiers in chemosensitising multidrug resistance cancer cells has stimulated a serious effort to define a common pharmacophore necessary to circumvent Pgp mediated transport (Seelig & Landwojtowicz, 2000:31). Many successful anticancer drugs are themselves either natural products or have been developed from naturally occurring lead compounds. Great interest is currently being given to flavonoids. Flavonoids are one of the major classes of natural products with widespread distribution in fruits, vegetables, spices, tea and soy-based foodstuff (Pouget *et al.*, 2001:3095).

Flavonoids constitute a class of bifunctional modulators which are able to partly overlap the ATP binding site and a vicinal hydrophobic region interacting with steroids, within a cytosolic domain of Pgp. This important finding opens exciting perspectives for studying the molecular mechanism of interaction with flavonoid modulators through structural and functional approaches and for further design of a new generation of bifunctional and specific inhibitors of Pgp activity (Conseil *et al.*, 1998:1836).

The aim of this study is to determine the potential inhibition of Pgp by selected flavonoids using an *in vitro* transport model and to explore the potential structure-activity relationship and requirements of the selected flavonoids in order to identify an optimal candidate to circumvent MDR.

CHAPTER 4

EXPERIMENTAL PROCEDURE

4.1 Introduction

The ability of an orally administered compound to permeate the intestinal mucosa may be limited by the physical and/or the biochemical components of the intestinal mucosal barrier. Thus, *in vitro* intestinal permeability models should not only predict intestinal drug absorption potential but also provide some understanding of the absorption mechanism(s). This can be accomplished to the extent in which the permeability model incorporates the functionality of the physical and biochemical barrier components (Hidalgo, 2001:389).

4.1.1 *In vitro* models of intestinal drug absorption

The successful application of *in vitro* models for intestinal drug absorption depends on the extent to which the model comprises the relevant characteristics of the *in vivo* biological barrier. Despite the obvious difficulties associated with trying to reproduce all the characteristics of the intestinal mucosa *in vitro*, various systems have been developed which mimic, to varying degrees, the relevant barrier properties of the intestinal mucosa. These systems include excised tissue (e.g. rat and rabbit), cell cultures (e.g. Caco-2, HT-29, T84 and MDCK), physicochemical methods (e.g. Log D/P, immobilised artificial membrane and parallel artificial membrane permeation assay), mucosal cell membrane vesicles, isolated mucosal cells and computational (*in silico*) methods (Hidalgo, 2001:389).

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

4.2.1 Method validation

Whether the methods used to predict the behavior of drugs *in vivo* in humans are *in vitro*, *ex vivo* or in experimental animals, some validation is necessary. Validation is a process by which the reliability and relevance of a procedure are established for a particular purpose. With the respect to the importance of *in vitro* methods in the assessment of chemicals, there is an urgent need to perform this exercise (Pelkonen *et al.*, 2001:627).

For the validation of the HPLC method, the following parameters for Rho 123 were used:

- linearity
- precision (intra-day and inter-day)
- sensitivity
- selectivity
- system repeatability

4.2.1.1 Linearity

The linearity for Rho 123 was determined by performing linear regression analysis on the plot of the peak AUC versus concentration. Six standard solutions were prepared as described in Appendix B, to obtain concentrations ranging from 34.38 to 68.75µg/ml. The regression value (r^2) was greater than 0.9940 and the Y-intercept was 31.129.

4.1.2 *In vitro* measurement of gastrointestinal tissue permeability using a diffusion cell

A diffusion cell (Sweetana-Grass diffusion chambers), derived from the Ussing chamber, was developed for the measurement of tissue permeability. This cell incorporates the attributes of using a single material and laminar flow across the tissue surface. In addition, the design allows the cell to be manufactured in a wide range of sizes to allow optimisation of surface area to volume for a variety of tissue. The apparatus is applicable for the evaluation of transport of compounds through mucosal/epithelial barriers such as the gastrointestinal tissue. Active transport, permeability enhancers, enzymatic degradation and absorption in various tissue sections can be explored (Grass & Sweetana, 1988:373).

4.2 HPLC Analysis and validation

A high pressure liquid chromatographic method was validated to analyse the samples, 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	
	Conditions:	Injection volume:	200 μL
		Flow rate:	1.5 mL/min
	Mobile phase:	32% Acetonitrile: 68% K_2HPO_4 0.1M (pH = 3.2)	
	Excitation wavelength:	510 nm	
	Emission wavelength	546 nm	

4.2.1.2 Precision

Intra-day precision:

Precision (repeatability) was determined by performing HPLC analyses (n=5) of five different concentrations ranging from 34.38 to 68.75 ug/ml of Rho 123 on the same day. Data for the intra-day precision is given in Table 4.1.

Table 4.1: Data obtained for intra-day precision

Rho 123 concentrations (µg/ml)	Mean recovered (%)	Standard deviation (%)	RSD (%)
34.38	106.46	12.16	7.93
39.29	106.57	7.51	4.27
44.20	107.45	6.55	3.27
54.02	100.55	9.75	4.25
68.75	97.05	8.11	2.87

Inter-day precision:

The inter-day precision was determined by performing HPLC analyses (n=3) on a low, medium and a high concentration (34.38 µg/ml, 44.20µg/ml and 68.75 µg/ml) of Rho 123 on three consecutive days. The inter-day precision complied with pharmaceutical standards and is presented in Table 4.2.

Table 4.2: Data obtained for inter-day precision

Rho 123 concentration (µg/ml)	Mean recovered (%)	Standard deviation (%)	RSD (%)
34.38	104.09	5.56	3.71
44.20	106.14	5.79	2.23
68.75	97.61	8.11	2.77

4.2.1.3 Sensitivity

The sensitivity of an analytical method can be measured by determining the limit of quantification and limit of detection. The limit of quantification is defined as the lowest concentration of an analyte in a sample that can be quantitatively determined with acceptable precision and accuracy (RSD < 15%). The limit of detection is defined as the lowest concentration of an analyte in a sample that can be detected, but not necessarily quantified as an exact value. The limit of quantification for Rho 123 studied was 7.79 µg/ml and the limit of detection was 23.43 µg/ml.

4.2.1.4 Selectivity

Selectivity is the ability of an analytical method to determine the analyte selectively and accurately in the presence of other compounds that may interfere. A test for selectivity is imperative when the drug is unstable and degradation products are formed which may interfere with the analysis or when any other compound in the sample may cause interference. The Krebs-ringer bicarbonate buffer (KR) (pH 7.4) and the mobile phase were separately analysed by HPLC. This method was selective since there were no interfering peaks with the same retention time as Rho 123.

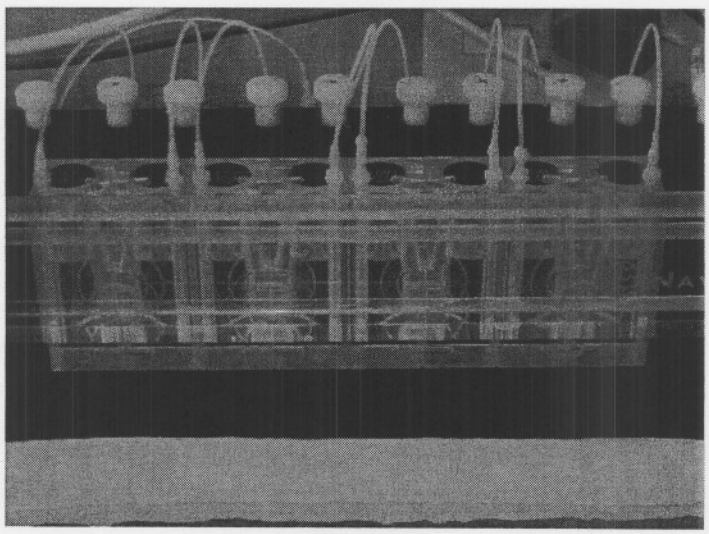
4.2.1.5 System repeatability

In order to evaluate the repeatability of the peak area and the retention time, a sample of Rho 123 with a known concentration (44.2 µg/ml) was injected six times. The variation in the response (RSD) of the detection system when six determinations of Rho 123 were made on the same day, and under the same conditions, was found to be 3.00% for the peak area and 1.20% for retention time. The system repeatability for Rho 123 was within acceptable limits.

4.3 Instrument used for *in vitro* transport studies

To prepare intestinal mucosal sheets suitable for mounting in diffusion chambers a longitudinal cut of an intestinal segment of the jejunum is made to produce a long mucosal sheet. This sheet is cut as needed to produce mucosal strips of adequate size to fit in the opening of the diffusion chamber. Although mucosal sheets are used with or without the underlying muscle layer, the removal of this muscle layer, a process known as stripping, is advantageous for two reasons. Firstly, it removes an artificial permeability barrier, and secondly, stripped tissues can be oxygenated more efficiently (Ungell, 1998:360). In a trial study conducted with Rho 123 transport across isolated jejunum segments mounted in Sweetana-Grass diffusion cells, it was found that the serosa had to be removed in order for the Rho 123 to be transported (Hattingh, 2002:48).

The rate of Rho 123 transport under the influence of selected flavonoids was determined using a vertical diffusion chamber system, comprising four Sweetana-Grass diffusion chambers, one heating block and one gas manifold (Corning Costar Corporation, Cambridge, USA) (Slide 1).



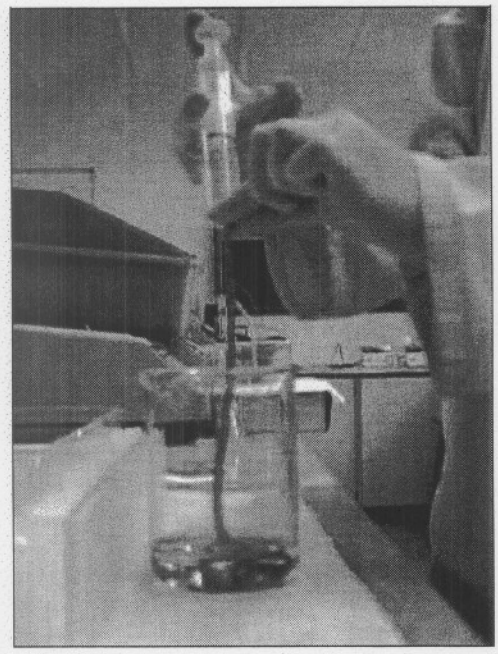
Slide 1

4.3.1 Materials

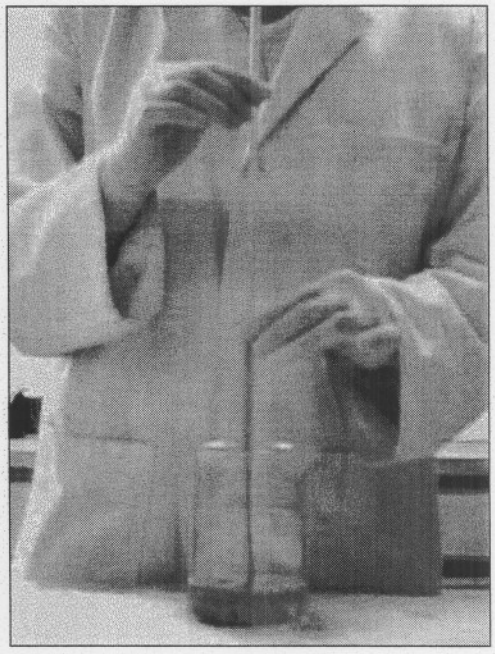
KR, Rho 123, galangin, kaempferol, morin and quercetin (Sigma Chemical Company Ltd., St. Louis, Missouri, USA) were obtained from Sigma-Aldrich (Pty) Ltd, Johannesburg and absolute ethanol, acetonitrile for HPLC, K_2HPO_4 were obtained from Merck (Pty) Ltd, Germiston. Certificates of analysis for Rho 123, galangin, kaempferol, morin, quercetin and Krebs-ringer bicarbonate buffer are presented in Appendix C.

4.3.2 Tissue preparation

The use of unfasted adult male Sprague-Dawley rats (350-370g) obtained from the Animal Research Centre (North-West University) were approved by the Ethical Committee of the North-West University under protocol number 03D03 (Appendix C). The rats were anaesthetised by inhalation of halothane. An abdominal incision was made and starting 10 cm from the stomach a 20-30 cm strip of the jejunum was excised, 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) and then pulled onto a glass rod (Slide 3).

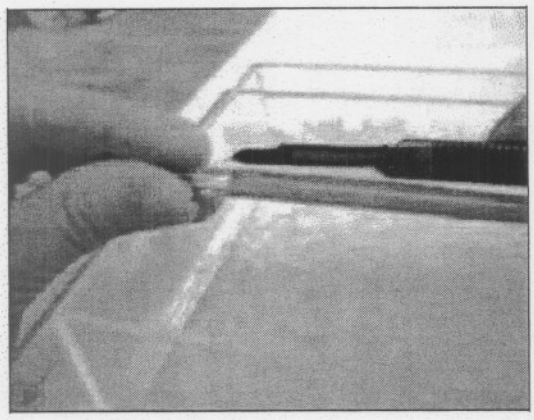


Slide 2

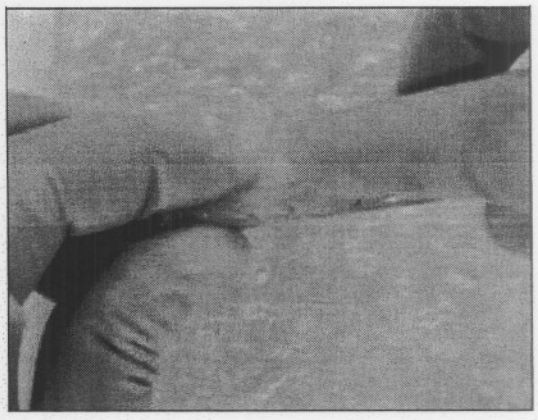


Slide 3

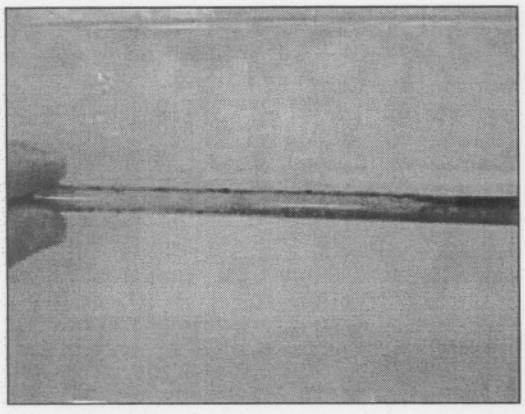
One hand was used to lift and hold the one end of the rod. The jejunum was then gently scoured long the mesenteric border with the back of a scalpel (Slide 4). Using the index finger along the length of the jejunum, the serosa and muscle layer were then gently pushed back and peeled off (Slide 5). The jejunum was kept moist with cold KR at all times which was kept in an ice bath. The jejunum was cut above and along the mesenteric border with a scalpel blade (Slide 6). The jejunum was then washed off the glass rod with a buffer-filled syringe onto a strip of filter paper (Slide 7).



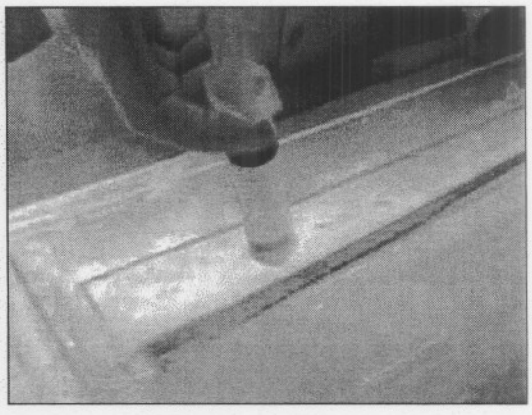
Slide 4



Slide 5

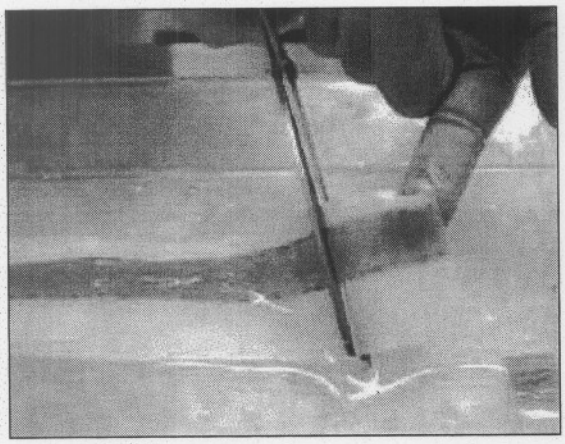


Slide 6

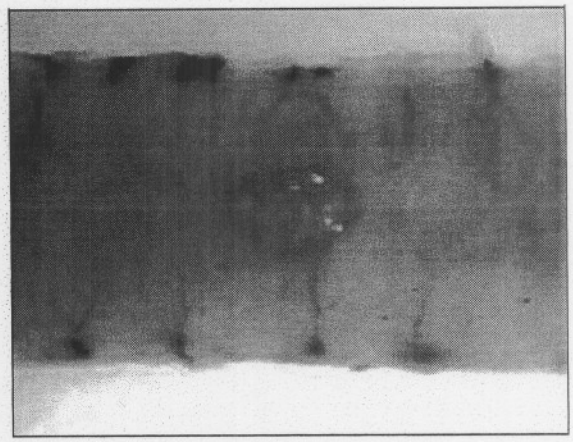


Slide 7

The jejunum and filter paper were then cut simultaneously into lengths approximately 3 cm (Slide 8). The segments were kept moist with ice cold KR and were kept on ice during the procedure. Segments containing Peyer`s patches were identified visually and avoided (Slide 9), as these lymph like tissues would cause greater variation in the rates of transport because of altered morphology and thickness of the epithelial layer.

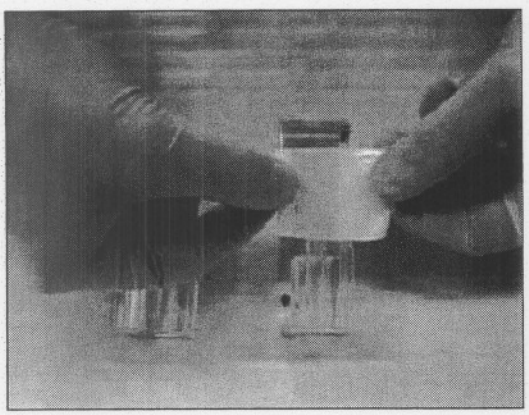


Slide 8

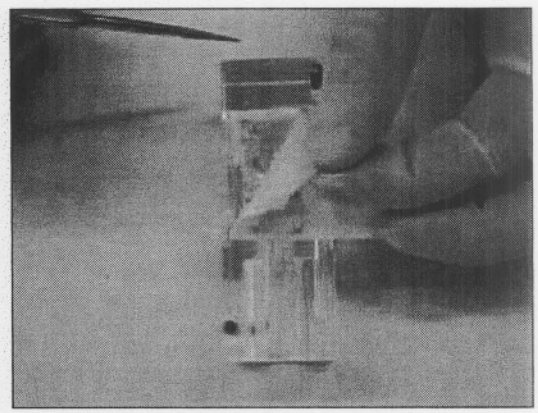


Slide 9

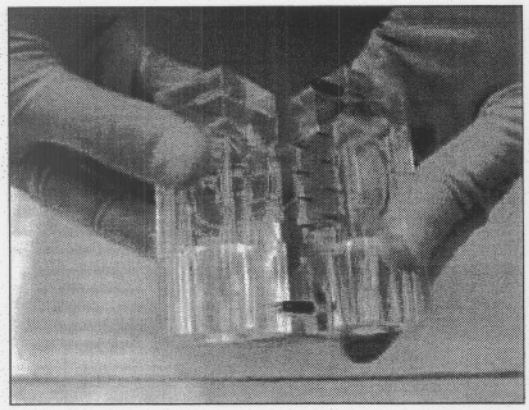
The segments were then carefully mounted onto the half cells (preheated to 37 °C) with the side containing the basolateral side of the jejunum face-down onto the pins (Slide 10 & 11). The filter paper was then removed and the matching half-cells were then carefully clamped together (Slide 12 & 13). Thereafter the assembled chamber was placed in the heating block (37 °C) and 5 ml KR preheated to 37 °C was added. 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.



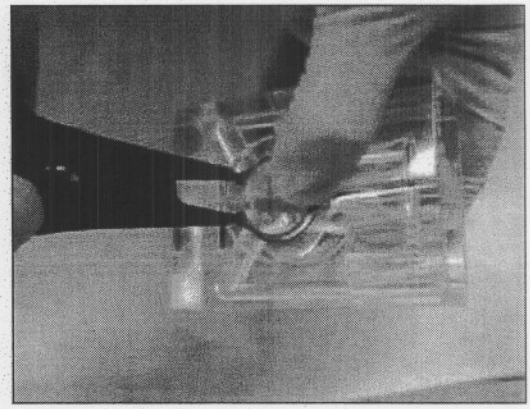
Slide 10



Slide 11



Slide 12



Slide 13

After all four cells were mounted with jejunum segments and filled with KR, the cells were left for 15 min to reach a state of equilibrium before a transport study was started by adding the various compounds under investigation to the donor cell. The temperature of the cells was maintained at 37 °C throughout the experiment.

4.3.3 Procedures used during transport studies

The transport of Rho 123 was determined 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. Galangin, kaempferol, morin and quercetin were screened as potential Pgp modulators. Each experiment was done in quadruplet for each modulator. Galangin, kaempferol, morin and quercetin were dissolved in 700 µl absolute ethanol as these flavonoids are poorly soluble in water. These solutions were then made up to volume with KR containing Rho 123. The final ethanol concentration in the various sample solutions was kept ≤1%, a concentration proven to not alter cell viability or permeability (Soldner *et al.*, 1999: 479).

The effects of these compounds on the transport of Rho 123 were assessed by the concomitant addition of the individual compounds with Rho 123 in the first two chambers. Sufficient Rho 123, to give a final Rho 123 concentration of 10.1 µM in the donor cell, was added to the apical side and 2 ml KR was added to the basolateral side. For the last two chambers, Rho 123 (sufficient to give 10.1 µM) was added to the

basolateral side and the 2 ml KR was added to the apical side. The total volume in each half chamber 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 of the chambers at 30, 60, 90, and 120 min after the addition of Rho 123, 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.

4.3.4 Apparent permeability coefficient

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 Rho 123 (100%) and *A* is the area of exposed tissue (1.78 cm^2). An example of the calculations performed is presented in Appendix B.

4.4 Statistical analysis

Statsoft® Statistica for Windows (Statsoft Inc., Tulsa, Oklahoma, USA) was used to perform the statistical analyses on the data obtained. For the comparison of all the values with the control a non-parametric one-tailed Kruskal-Wallis test was done (Steyn *et al.*, 1998:604). This test was also used to test for statistical differences between different modulators at the same concentration. Statistically, this was the preferred test to perform the statistical analysis on the data obtained because each experiment was done in quadruplet and normality could not be assumed. Effect sizes were performed to determine whether a practical significance exist between each modulator at two different concentrations.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 Transport studies

The effect of different flavonoid modulators on the transport of Rho 123 was investigated using the Sweetana-Grass diffusion method comprising four cells. Each experiment was done in quadruplet. The transport of Rho 123 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 Rho 123 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 individual values for the cumulative transport of Rho 123 in the absence and in the presence of selected flavonoids are presented in Appendix A. Statistical analyses were used to compare the results of the experiments with the control.

5.1.1 Transport of Rho 123

An example of the cumulative transport of Rho 123 with no modulators added, is presented in Figure 5.1 and the P_{app} values in Table 5.1. The ratio's obtained served as the control values to which the ratio's obtained after addition of the modulators, could be compared.

Table 5.1: Individual and mean P_{app} values of Rho 123 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	1.67	5.60	3.35	3.29±0.220*
2	1.15	3.63	3.15	
3	0.67	2.40	3.57	
4	3.59	11.05	3.07	

*Each value represents the mean ± standard deviation

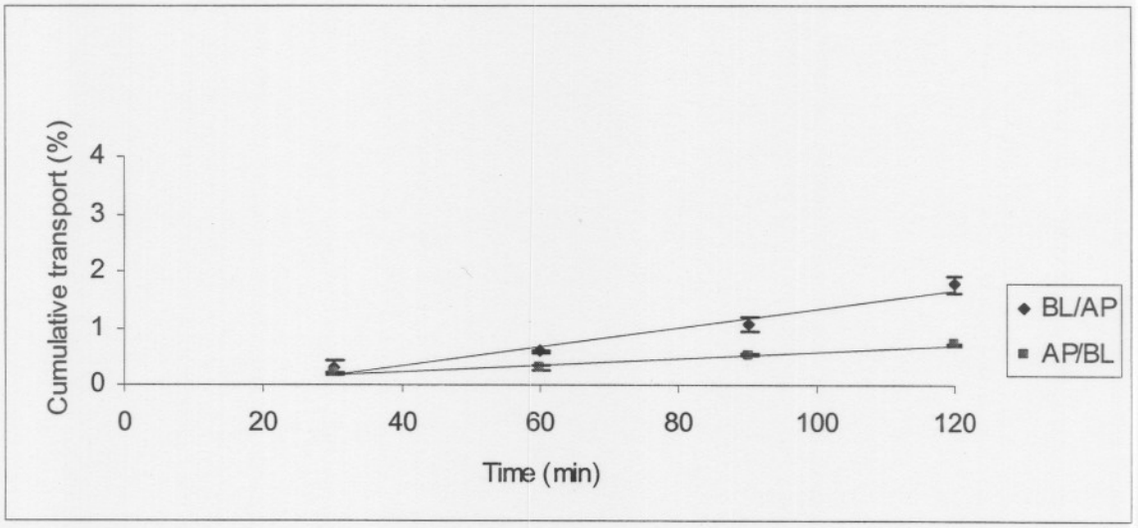


Figure 5.1: Example of the cumulative transport of Rho 123 with no modulators added.

Conclusion

According to Neyfakh (1998:168), Rho 123 is classified as a typical Pgp substrate and is subject to Pgp-dependent extrusion through the plasma membrane. Thus, Rho 123 accumulation in (or efflux from) cells is used as a measure of MDR1-dependent transport activity. The results obtained during these experiments also indicated that Rho 123 is a substrate for active transporters. The mean ratio calculated was 3.29. The mean ratio's observed in the absence of a modulator were compared to the mean ratio's obtained after the addition of the modulators. Table 5.2 presents the p-values obtained

by the statistical analysis (Kruskal-Wallis test) to show which mean ratio's showed a statistical significant difference from the control. $p<0.05$ was taken as statistical significant.

Table 5.2: Multiple comparisons p-values (1-tailed) obtained by the non-parametric Kruskal-Wallis test comparing the P_{app} ratio's in the presence of modulators with the P_{app} ratio obtained in the control experiment.

	Statistical evaluation (p-values) at 10 µM				
	Control	Morin	Galangin	Kaempferol	Quercetin
Control		0.006252*	0.050887	0.242980	0.500000
Morin	0.006252*		0.500000	0.500000	0.098850
Galangin	0.050890	0.500000		0.500000	0.471320
Kaempferol	0.242980	0.500000	0.500000		0.500000
Quercetin	0.500000	0.098850	0.471322	0.500000	

	Statistical evaluation (p-values) at 20 µM				
	Control	Morin	Galangin	Kaempferol	Quercetin
Control		0.024864*	0.042755*	0.2108245	0.182349
Morin	0.024864*		0.500000	0.500000	0.500000
Galangin	0.042755*	0.500000		0.500000	0.500000
Kaempferol	0.210825	0.500000	0.500000		0.500000
Quercetin	0.182349	0.500000	0.500000	0.500000	

* Value representing a statistical significant difference

In order to determine whether a practical significance exists between the two concentrations of each modulator, effect sizes were determined. Tables for the descriptive statistics of each modulator are given in Table 5.3.

Table 5.3 Descriptive statistics of modulators at concentrations 10 µM and 20 µM.

10 µM	Mean	Standard deviation
	ratio	ratio
Control	3.245	0.277
Morin	1.880	0.091
Galangin	2.184	0.375
Kaempferol	2.514	0.554
Quercetin	2.976	0.200

20 µM	Mean	Standard deviation
	ratio	ratio
Control	3.245	0.277
Morin	1.490	0.611
Galangin	1.604	0.544
Kaempferol	1.816	0.557
Quercetin	1.952	0.272

Results of the effect size were determined by the following equation:

$$d = \left| \frac{\bar{x}F_{10} - \bar{x}F_{20}}{S_{\max}} \right|$$

Where *d* is the effect size, $\bar{x}F_{10} - \bar{x}F_{20}$ is the difference between the means of the two concentrations at 10 µM and 20 µM and *Smax* is the ratio at the maximum standard deviation (Steyn, 1999:28). The results of the effect sizes between the two concentrations of each modulator are given in Table 5.4.

Table 5.4: Effect size results.

Modulator	Effect size (d)
Morin	0.64
Galangin	1.06*
Kaempferol	1.30*
Quercetin	3.80*

*Value representing practical significance

Guidelines for the interpretation of the effect sizes are the following:

(a) Small effect: $d \leq 0.2$

(b) Medium effect: $0.2 \leq d \leq 0.8$

(c) Large effect: $d \geq 0.8$

Data where $d \geq 0.8$ is considered as practically significant as it is the result of a difference having a large effect (Cohen, 1988).

5.1.2 Transport of Rho 123 in the presence of selected flavonoids

The modulating effect of four selected flavonoids on the transport of Rho 123 was investigated. During screening for flavonoid chemosensitisers in a study done by Choi and co-workers (2004:672), it was found that a certain flavonoid 5,7,3',4',5'-pentamethoxyflavone (PMF) was equipotent to verapamil *in vitro* with respect to the chemosensitising effect. However, the effect of PMF on the level of Rho 123 accumulation was lower than that of the Pgp substrate, verapamil. In this study, morin, galangin, kaempferol and quercetin (all flavonoids) were investigated as modulators. The effect of these components on the transport of Rho 123 across rat jejunum was evaluated using two concentrations (10 µM and 20 µM). The concentrations were chosen according to findings by Scambia and co-workers (1994:459). They have demonstrated that concentrations of quercetin ranging between 1 and 10 µM had a dose-dependent effect on reducing Pgp in MCF-7 adriamycin-resistant human breast cancer cells. Van Zanden and co-workers (2005:699) found that quercetin and

kaempferol were potent MRP inhibitors at concentrations 25 μM and 19.4 μM respectively. They also found that the highest flavonoid concentration tested was 50 μM, because some flavonoids are either cytotoxic or poorly soluble at concentrations above 50 μM.

Structure activity relationships (SAR) of the selected flavonoids were also investigated in this study with reference to inhibition of Pgp. The effect of the different hydroxyl group positioning on the B-ring was investigated. SAR studies for the inhibition of Pgp ATP-ase activity by flavonoids showed that the presence of a 5-hydroxyl group, the 3-hydroxyl group and the C2-C3 double bond are required for high potency binding to the NBD of Pgp (Boumendjel *et al.*, 2002:512). In a study by van Zanden and co-workers (2005:704), multiple regression analysis was used for the identification and quantification of the effects of different structural characteristics regarding potent inhibition of MRP1 and MRP2 activity. For MRP1, an optimal multiple parameter QSAR model was obtained which revealed that three structural characteristics are of importance for MRP1 inhibition:

- The total number of methoxylated moieties;
- The total number of hydroxyl groups and
- The dihedral angle between the B- and C-ring which quantifies the planarity of the flavonoid modulators.

The measured lipophilicity and calculated dihedral angle between the B- and C-ring for the flavonoids tested are given in Table 5.5. The lipophilicity of the flavonoids was calculated using the capacity factor (K') calculated by:

$$K' = \frac{t_r - t_o}{t_o}$$

in which K' is the capacity factor, t_r is the retention time (min) and t_0 is the retention time of unretained substances (min) (van Zanden *et al.*, 2004:1613).

Table 5.5: Measured lipophilicity (K') and calculated dihedral angle between the B- and C-ring for the following flavonoids tested (van Zanden *et al.*, 2004:705).

	Lipophilicity (K')	Dihedral angle (degrees)
Morin	2.8	19.3
Galangin	18.3	14.5
Kaempferol	8.8	14.4
Quercetin	4.4	14.7

As seen from the results in Table 5.5, galangin is the most lipophilic compound. It is also interesting to take note that galangin is the only flavonoid without hydroxyl groups at the B-ring. Therefore it can be expected that galangin has a higher net transfer across the brush border and therefore probably will have a profound effect on inhibiting Rho 123 transport. Morin is shown to have the lowest lipophilicity. It may be due to the presence of the two hydroxyl groups at the B-ring. Therefore it can be expected for morin to have a less inhibitory effect on the transport of Rho 123. Kaempferol has only one hydroxyl group at the B-ring and also has a have relative high lipophilicity. Therefore kaempferol also may have a relative effect on the inhibition of Rho 123 efflux. There exists a definite correlation between the amount of hydroxyl groups at the B-ring and the lipophilicity of the flavonoids. According to the dihedral angles given in Table 5.5, there does not seem to be major differences between the angles of the compounds. Morin however is shown to have the largest dihedral angle (19.3°).

5.1.2.1 Transport of Rho 123 in the presence of morin

The effect of morin on the transport of Rho 123 across rat jejunum was investigated in this study using two concentrations. An example of the cumulative transport of Rho 123 in the presence of morin (10 µM and 20 µM) is presented in Figures 5.2 and 5.3 respectively and the P_{app} values in Table 5.6.

Table 5.6: Individual and mean P_{app} values of Rho 123 transported (AP-BL and BL-AP) in the presence of morin (10 µM and 20 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
Morin (10 µM)				
1	2.24	4.25	1.90	1.88±0.091*
2	4.49	8.98	2.00	
3	4.52	8.21	1.82	
4	1.59	2.86	1.80	
Morin (20 µM)				
1	6.65	12.07	1.82	1.49±0.611*
2	5.15	11.20	2.18	
3	9.62	8.24	0.86	
4	22.65	25.20	1.11	

*Each value represents the mean ± standard deviation

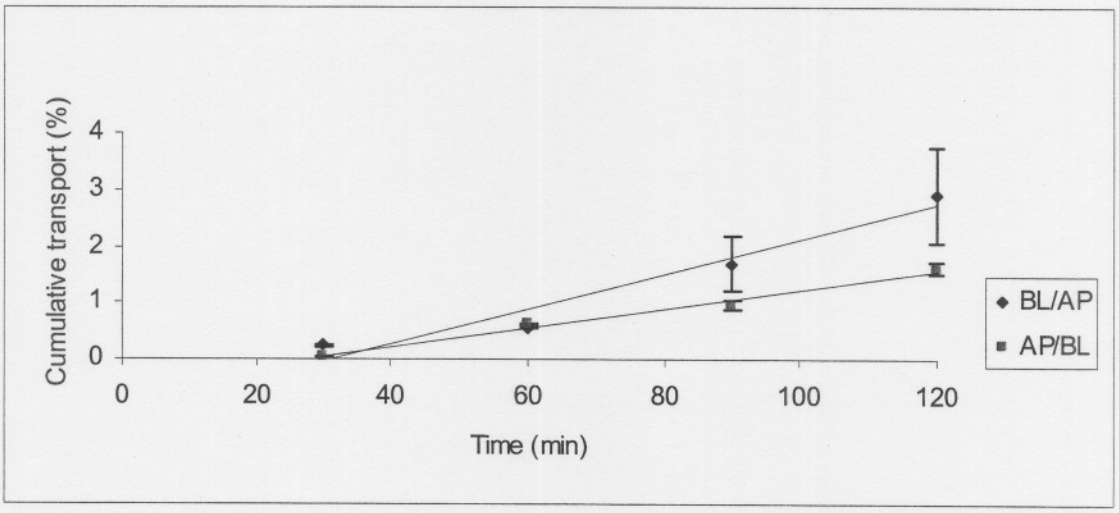


Figure 5.2: Example of cumulative transport of Rho 123 in the presence of morin (10 µM).

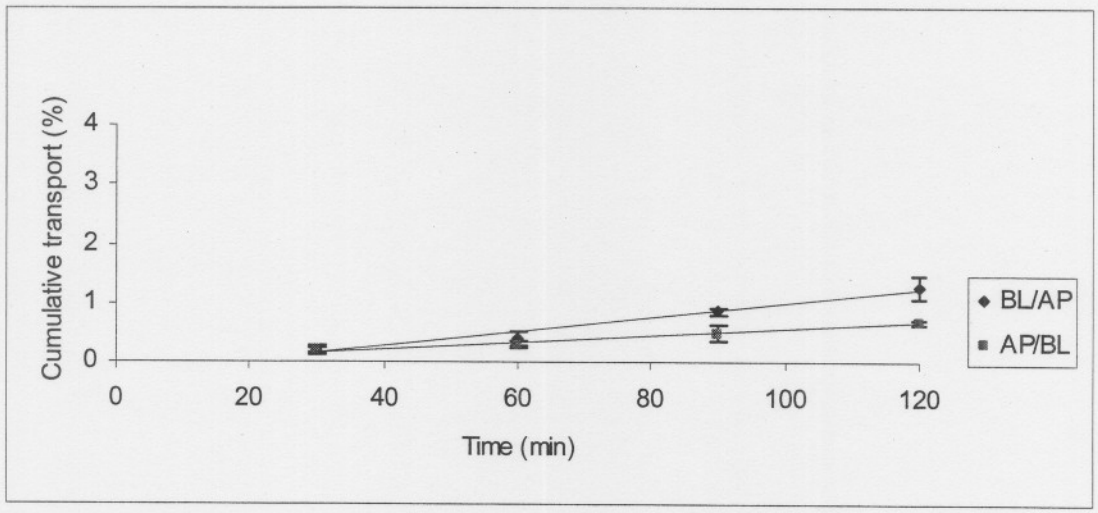


Figure 5.3: Example of cumulative transport of Rho 123 in the presence of morin (20 µM).

Conclusion

At concentrations of 10 µM and 20 µM the mean ratio's calculated were 1.88 and 1.49 respectively, indicating that the transport of Rho 123 in the basolateral to apical direction was decreased. The p-values at the two concentrations were 0.006252 and 0.024864 respectively. Both p-values were < 0.05 and therefore indicated that there was a statistically significant difference between the mean ratio of morin and the mean ratio of the control. Morin however, did not show a practically significant effect between the two different concentrations according to the effect size. It can be concluded that morin at concentrations of 10 µM and 20 µM inhibited the ration of transport of Rho 123. It acts therefore as an inhibitor of the transporter proteins responsible for the efflux of Rho 123. The chemical structure of morin is given in Figure 5.4.

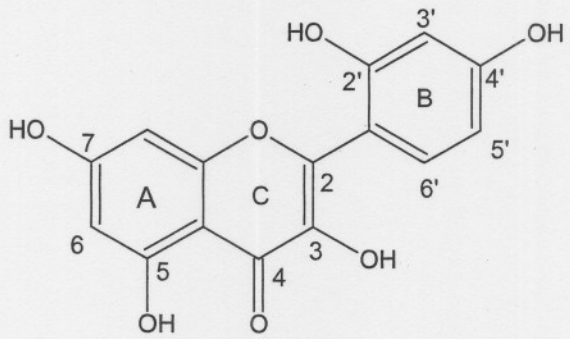


Figure 5.4: Chemical structure of morin (2',3,4',5,7-pentahydroxyflavone).

Morin is the only flavonoid investigated in this study possessing a hydroxyl group at the C2' atom. In a study done by Nguyen and co-workers (2003:250) it was found that the most potent inhibitors on MRP1-mediated transport in Panc-1 cells were morin > kaempferol > quercetin > genistein. Therefore, it can be assumed for the purposes of this study that the hydroxyl groups at the C2' atom plays a definite role in the inhibition of Rho 123 efflux. Contradictory results were found regarding the lipophilicity. In Table 5.5 morin had the lowest lipophilicity, but regardless of that, still showed inhibition of Rho 123 transport more effectively than the other flavonoids. Morin also has the largest dihedral angle which probably played a definite role in the Rho 123 transport inhibition and this may be due to the hydroxyl group on the C2' atom of the B-ring.

5.1.2.2 Transport of Rho 123 in the presence of galangin

The effect of galangin on the transport of Rho 123 across rat jejunum was investigated using two concentrations. An example of the cumulative transport of Rho 123 in the presence of galangin (10 μM and 20 μM) is presented in Figures 5.5 and 5.6 respectively and the P_{app} values in Table 5.7.

Table 5.7: Individual mean P_{app} values of Rho 123 transported (AP-BL and BL-AP) in the presence of galangin (10 µM and 20 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
Galangin (10 µM)				
1	4.20	11.38	2.71	2.18±0.375*
2	16.76	36.58	2.18	
3	19.36	36.21	1.87	
4	13.25	26.11	1.97	
Galangin (20 µM)				
1	22.54	23.58	1.05	1.60±0.544*
2	9.91	21.27	2.15	
3	4.33	8.60	1.99	
4	17.76	21.93	1.23	

*Each value represents the mean ± standard deviation

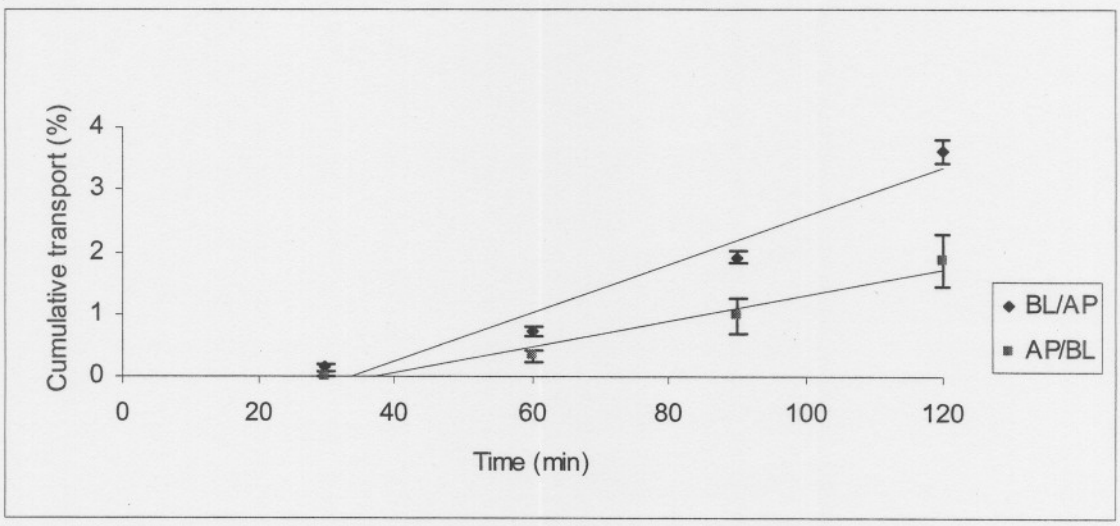


Figure 5.5: Example of the cumulative transport of Rho 123 in the presence of galangin (10 µM).

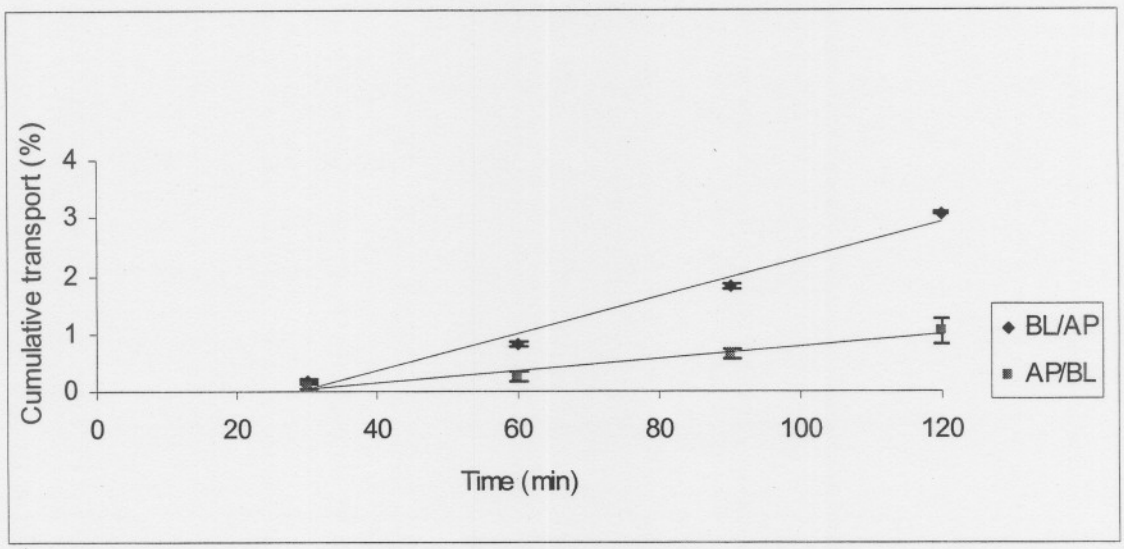


Figure 5.6: Example of the cumulative transport of Rho 123 in the presence of galangin (20 µM).

Conclusion

At a concentration of 10 µM the mean ratio calculated was 2.18 indicating a decrease in transport of Rho 123 but this was not statistical significant. At the concentration of 20 µM the mean ratio calculated was 1.60. The p-value at the 20 µM concentration was 0.042755 which is <0.05 and therefore indicated that there was a statistical significant difference between the mean ratio of galangin and the mean ratio of the control. It can be concluded that galangin at concentration 20 µM inhibited the rate of transport of Rho 123 in the basolateral to apical direction. It acts therefore as an inhibitor of the transporter proteins responsible for the efflux of Rho 123. This corresponds to the findings of Conseil and co-workers (1998:3835) who reported that galangin was able to modulate drug efflux in MDR cancer cells. Galangin did have a practically significant difference at the two different concentrations according to the effect size. The chemical structure of galangin is given in Figure 5.7.

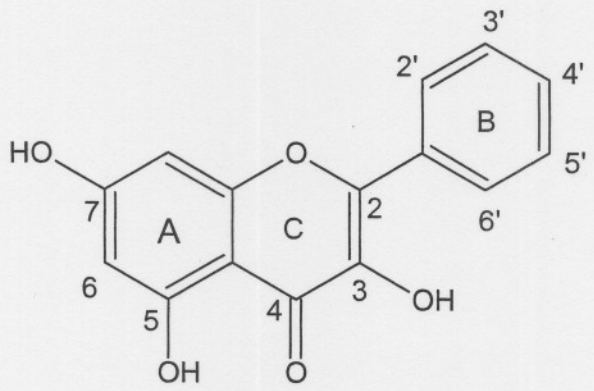


Figure 5.7: Chemical structure of galangin (3,5,7-trihydroxyflavonone).

Galangin is the only flavonoid used in this study possessing no hydroxyl groups at the B-ring. Van Zanden and co-workers (2004:1607) investigated the structural requirements for inhibition of glutathione -S-transferase P1-1 (GSTP1-1) and MRP1 and MRP2 activity by structurally related flavonoids. They found that especially galangin was able to inhibit almost all cellular GSTP1-1 activity upon exposure of the cells to a concentration of 25 µM. They found that the absence of B-ring hydroxyl groups in galangin might result in relatively higher GSTP1-1 inhibition potency. The results obtained in this study may be correlated to the above findings where galangin with no B-ring hydroxyl groups, successfully inhibited the transport of Rho 123 at a concentration of 20 µM. Although galangin was shown to be the most lipophilic compound in Table 5.5, its inhibition of Rho 123 transport was smaller than that of morin. The dihedral angle of galangin does not significantly differ from that of kaempferol and quercetin.

5.1.2.3 Transport of Rho 123 in the presence of kaempferol

The effect of kaempferol on the transport of Rho 123 across rat jejunum was investigated using two concentrations. An example of the cumulative transport of Rho 123 in the presence of kaempferol (10 µM and 20 µM) is presented in Figures 5.8 and 5.9 respectively and the P_{app} values in Table 5.8.

Table 5.8: Individual mean P_{app} values of Rho 123 transported (AP-BL and BL-AP) in the presence of kaempferol (10 µM and 20 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
Kaempferol (10 µM)				
1	14.37	40.83	2.84	2.51±0.533*
2	7.14	17.10	2.39	
3	2.74	4.90	1.79	
4	13.90	42.14	3.03	
Kaempferol (20 µM)				
1	5.28	9.43	1.79	1.82±0.577*
2	11.20	24.40	2.18	
3	5.15	5.35	1.04	
4	3.82	8.63	2.26	

*Each value represents the mean ± standard deviation

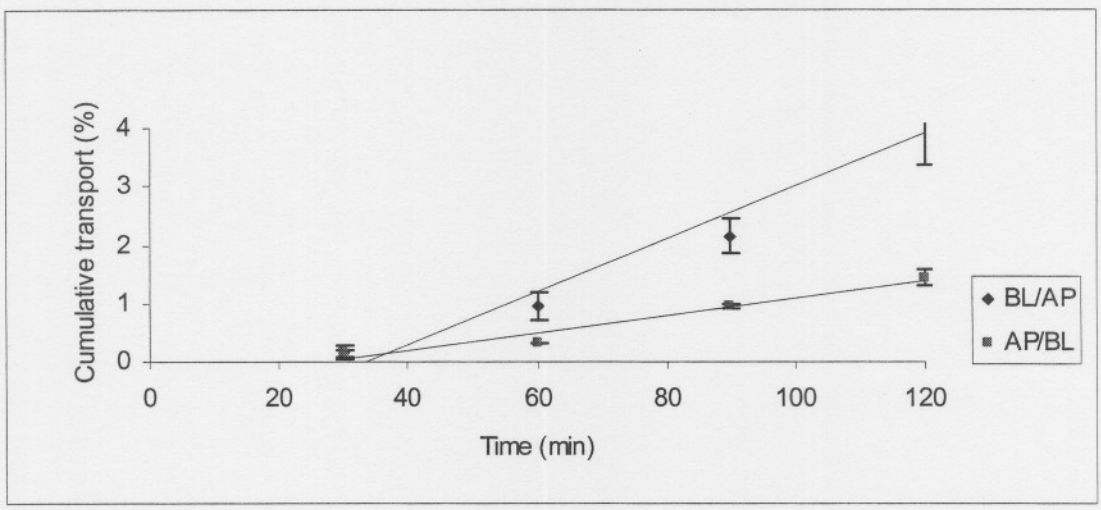


Figure 5.8: Example of the cumulative transport of Rho 123 in the presence of kaempferol (10 µM).

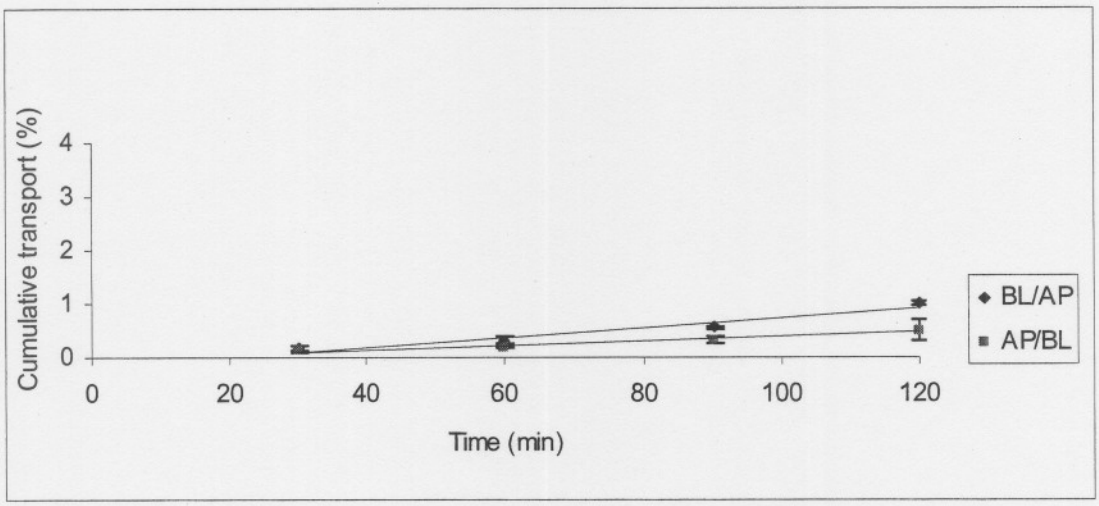


Figure 5.9: Example of the cumulative transport of Rho 123 in the presence of kaempferol (20 µM).

Conclusion

At concentrations 10 µM and 20 µM the mean ratio's calculated were 2.51 and 1.82 respectively but this was not statistical significant. According to the effect size, kaempferol seemed to have a practical significant difference between the two different concentrations.

In a study done by Phang and co-workers (1993:5979) it was found that kaempferol, together with galangin and quercetin was one of the most active compounds on the activity of Pgp. Hooijberg and co-workers (1997:347) found that kaempferol also had a stimulating effect on the ATPase activity on the MRP overexpressing cell line GLC4/ADR. The chemical structure of kaempferol is given in Figure 5.10.

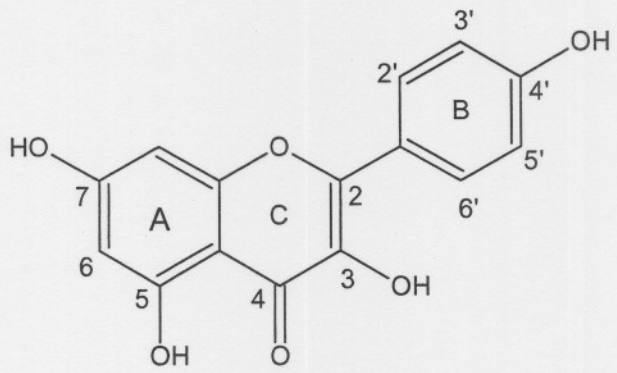


Figure 5.10: Chemical structure of kaempferol (3, 4´, 5, 7-tetrahydroxyflavone).

Kaempferol has four hydroxyl groups. The hydroxyl group at C4' on ring B is of interest. In a study done by Conseil and co-workers (1998:9831) it was found that the addition of a hydroxyl group at C4' of ring B may have a negative effect on the inhibition of modulators (kaempferol compared to galangin). Results obtained in this study also indicated that, although kaempferol was able to inhibit Rho 123 efflux at both concentrations, it had a slight lesser inhibitory effect compared to galangin.

5.1.2.4 Transport of Rho 123 in the presence of quercetin

The effect of quercetin on the transport of Rho 123 across rat jejunum was investigated using two concentrations. An example of the cumulative transport of Rho 123 in the presence of quercetin (10 µM and 20 µM) is presented in Figures 5.11 and 5.12 respectively and the P_{app} values in Table 5.9.

Table 5.9: Individual mean P_{app} values of Rho 123 transported (AP-BL and BL-AP) in the presence of quercetin (10 µM and 20 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 (10 µM)				
1	15.45	50.53	3.27	2.98±0.200*
2	5.90	16.70	2.83	
3	14.97	43.36	2.90	
4	15.15	44.06	2.91	
Quercetin (20 µM)				
1	3.74	8.14	2.17	1.95±0.335*
2	3.49	5.45	1.56	
3	8.80	18.56	2.11	
4	8.65	16.93	1.96	

*Each value represents the mean ± standard deviation

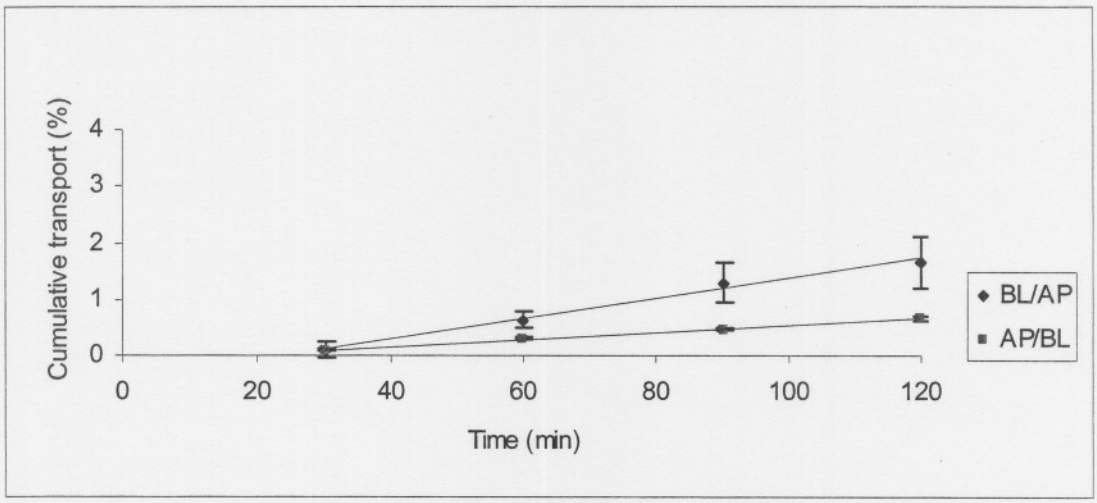


Figure 5.11: An example of the cumulative transport of Rho 123 in the presence of quercetin (10 µM).

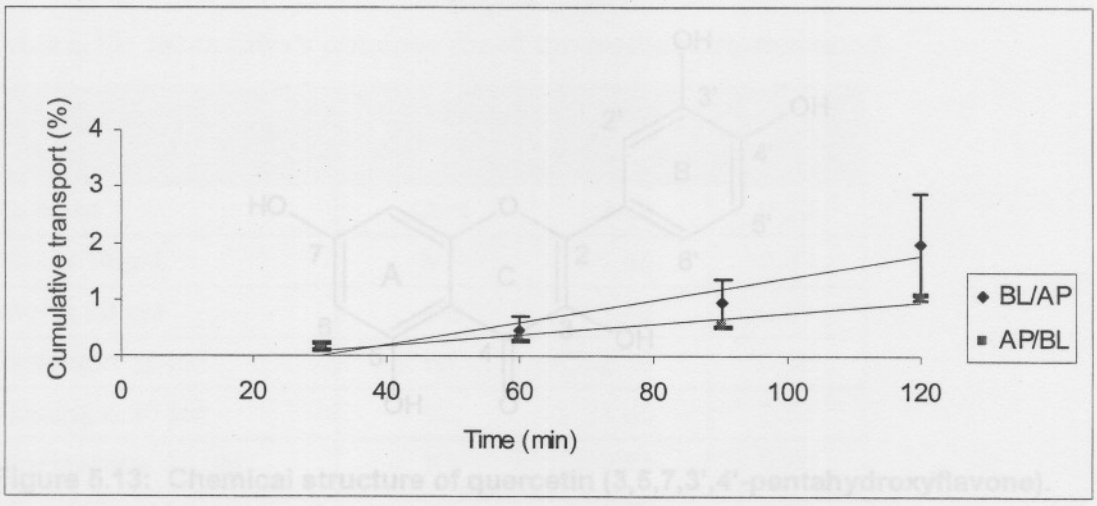


Figure 5.12: An example of the cumulative transport of Rho 123 in the presence of quercetin (20 µM).

Conclusion

At concentrations 10 µM and 20 µM the mean ratio's calculated were 2.98 and 1.95 respectively indicating a decrease in transport of Rho 123 but this was not statistical significant. The results however, indicated that quercetin is an inhibitor of Pgp. These results correlate with those found by van Zanden and co-workers (2005:703), who demonstrated that quercetin was able to inhibit more than 50% of MRP1 activity at 25 µM. There was also a practical significant difference between the two different concentrations of quercetin according to the effect size. The chemical structure of quercetin is given in Figure 5.13.

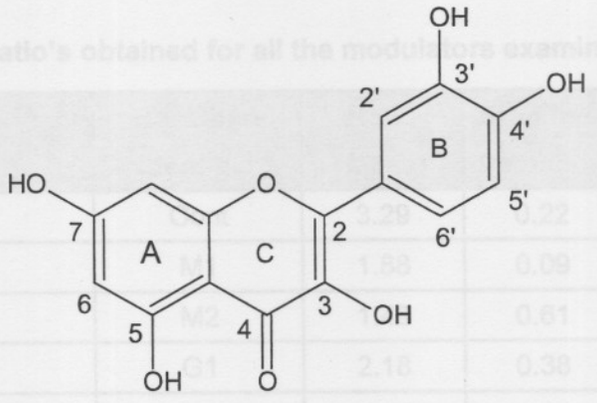


Figure 5.13: Chemical structure of quercetin (3,5,7,3',4'-pentahydroxyflavone).

Quercetin, shown to be the most important MRP1 and MRP2 inhibitor, contains five hydroxyl groups including a B-ring 3',4'-catechol moiety. The presence of a 3',4'-dihydroxy moiety on the B-ring results in strong inhibition as is shown by comparison of quercetin to morin and kaempferol (van Zanden *et al.*, 2005:1615). However, this study indicated that morin showed better Rho 123 efflux inhibition. This may be due to the low concentrations used. Regarding the lipophilicity and dihedral angle, quercetin also did not seem to deviate specifically from the other flavonoids tested. Quercetin was the weakest inhibitor of Rho 123.

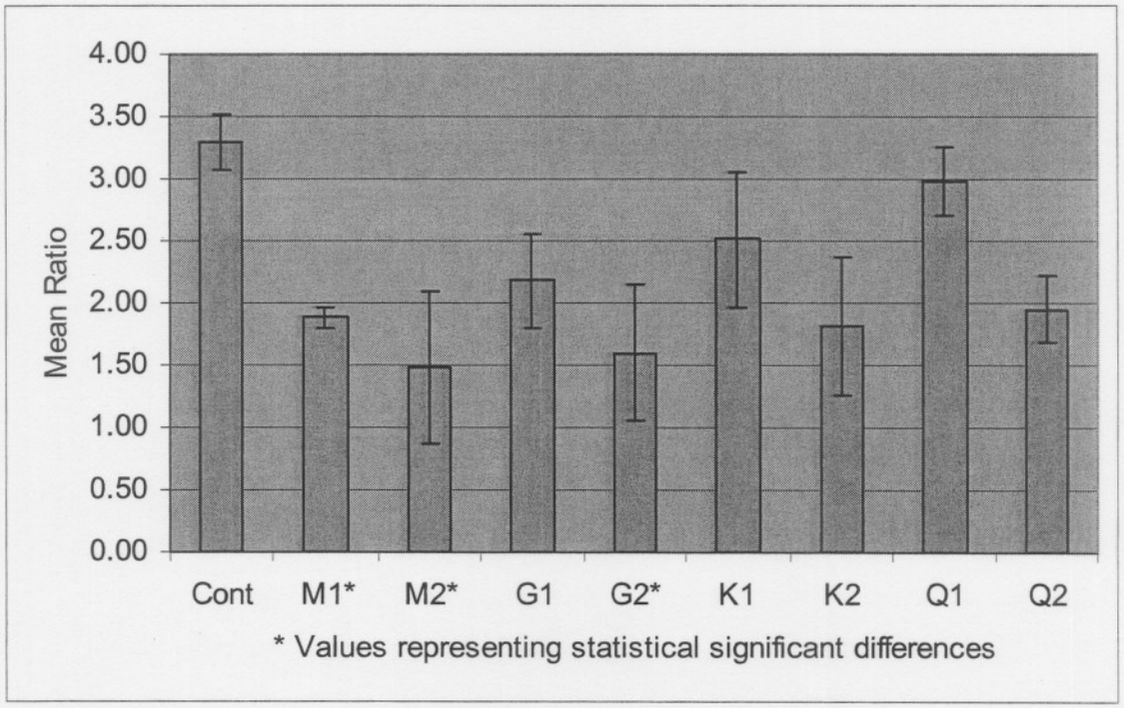
5.2 Summary

Figure 5.14 gives a comparative overview of results obtained in the different studies (as summarised in Table 5.10). As can be seen from this chart, the most pronounced inhibitory effect was due to morin at 20 µM (M2). As previously stated, it was only morin (10 µM and 20 µM) and galangin (20 µM) that showed statistical significant results on the transport of Rho 123, although the other flavonoid concentrations did show inhibition of Rho 123.

Table 5.10: Mean ratio's obtained for all the modulators examined.

Modulator	Bar Chart Identifier	Ratio (Mean)	Standard Deviation
Control	Cont	3.29	0.22
Morin 10 µM	M1	1.88	0.09
Morin 20 µM	M2	1.49	0.61
Galangin 10 µM	G1	2.18	0.38
Galangin 20 µM	G2	1.60	0.54
Kaempferol 10 µM	K1	2.51	0.55
Kaempferol 20 µM	K2	1.82	0.56
Quercetin 10 µM	Q1	2.98	0.27
Quercetin 20 µM	Q2	1.95	0.27

Figure 5.14: Bar chart showing the relative mean ratio's of the various flavonoids



compared to the mean ratio of the control.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 Introduction

Because of the deleterious effect of Pgp on chemotherapeutic efficacy, compounds that modify its function are of potential clinical value. Numerous modulators or chemosensitisers are known that inhibit the ability of Pgp, maintaining subtoxic intracellular drug concentrations (Gottesman & Pastan, 1993:385). Examples include calcium channel blockers like verapamil and trifluoperazine, detergents like Triton X-100 and immunosuppressants like cyclosporine A (Shapiro & Ling, 1997:587). In contrast to chemosensitisers, three flavonoids, kaempferol, galangin and quercetin have been found to stimulate efflux of two Pgp substrates, 7,12-dimethylbenz[a]anthracene and Adriamycin, from multidrug-resistant cells (Phang *et al.*, 1993:5977). This remarkable observation could be of importance for chemotherapy because flavonoids are very common constituents of substances and an increase in the activity of Pgp could potentially reduce the efficacy of chemotherapy (Gottesman & Pastan, 1993:385).

6.2 Conclusion

The final conclusions of this study are as follows:

- Of all the modulators used, morin was the strongest modulator of Rho 123 transport. At a concentration of 10 µM it decreased the mean P_{app} ratio from 3.29 in the control to 1.88 and at a concentration of 20 µM it decreased the mean P_{app} ratio from 3.29 to 1.49. Both concentrations caused a statistical significant decrease of the transport ratio of Rho 123. Structure activity relationhship (SAR) with reference to inhibition of Pgp indicated that the hydroxyl groups at the C2' atom at the B-ring probably played an important role in the inhibition Rho 123 transport.

- Galangin decreased the mean P_{app} ratio from 3.29 in the control to 2.18 at a concentration of 10 µM, indicating decreased efflux activity. This decrease was, however, not statistically significant probably due to the low concentration used. At a concentration of 20 µM it decreased the mean P_{app} ratio from 3.29 to 1.60, also indicating decreased efflux activity and showed a significant difference according to the Kruskal-Wallis test. This decrease in mean P_{app} ratio showed reduced efflux of Rho 123.

- Kaempferol decreased the mean P_{app} ratio from 3.29 in the control to 2.51 at concentration 10 µM and from 3.29 to 1.82 at a concentration of 20 µM indicating inhibition of active transport of Rho 123. No statistical significant differences were observed. SAR with reference to inhibition of Pgp may be due to the hydroxyl group present at C4' at the B-ring.

- Quercetin decreased the mean P_{app} ratio from 3.29 in the control to 2.98 at concentration 10 µM and from 3.29 to 1.95 at a concentration of 20 µM. The decrease at both concentrations however, were not statistically significant. SAR with reference to inhibition of Pgp may be due to the 3',4'-dihydroxy moiety on the B-ring.

- Regarding the effect of the two different concentrations used, effect sizes showed that galangin, kaempferol and quercetin showed practical significant differences. Morin however did not show any practical significant differences at the two different concentrations.

- The lipophilicity and dihedral angle did not seem to have a pronounced effect on the efflux of Rho 123 as the flavonoids tested did not deviate from each other.

- The order of the inhibitory potency on the transport of Rho 123 was as follow: morin > galangin > kaempferol > quercetin.

- In summary, this study described the inhibitory interaction of selected flavonoids with Pgp. Structure activity relationships were identified describing the inhibitory potency of the flavonoids based on hydroxyl groups positioning. Moreover, this

study proved that the Sweetana-Grass diffusion model seems to be efficient and sensitive enough to be used to determine differences in rates of transport caused by various modulators.

6.3 Recommendations

In continuation of this study the following should be investigated:

- An extensive study on structure activity relationships of other selected flavonoid inhibitory activities on Pgp will provide a valuable inspiration for a rational drug and/or chemopreventive agent design of future pharmaceuticals in the prevention and/or treatment of several life-threatening diseases such as cancer.

- Further *in vivo* studies should be pursued by comparing concentrations of *in vitro* studies and whether it will have therapeutic plasma concentrations as a result *in vivo*.

- The potential therapeutic application of flavonoids in cancer therapy, either alone or in combination with other conventional cytotoxic drugs.

- As the potency of Pgp inhibition is largely dependent on the hydrophobic substituent on the B-ring at C4' (Conseil *et al.*, 1998:9836), further studies on different substitutions at this position should be pursued as well as their therapeutic applications.

BIBLIOGRAPHY

AHERNE, S.A. & O'BRIEN, N.M. 2002. Dietary flavonols: chemistry, food content and metabolism. *Nutrition*, 18:75-81.

BAIN, L.J., MCLACHLAN, J.B. & LEBLANC, G.A. 1997. Structure-activity relationships for xenobiotic transport substrates and inhibitory ligands for P-glycoprotein. *Environmental health perspectives*, 105:812-818.

BARD, S.M. 2000. Multixenobiotic resistance as a cellular defense mechanism in aquatic organisms. *Aquatic toxicology*, 48:357-389.

BENET, L.Z., WU, C-Y., HEBERT, M.F. & WACHER, V.J. 1996. Intestinal drug metabolism and antitransport processes: a potential paradigm shift in oral drug delivery. *Journal of controlled release*, 39:139–143.

BORGES-WALMSLEY, M.I. & WALMSLEY, A.R. 2001. The structure and function of drug pumps. *Trends in microbiology*, 9:71-79.

BORST, P. & SCHINKEL, A.H. 1997. Genetic dissection of the function of mammalian p-glycoproteins. *Trends in genetics*, 13:217–222.

BOUMENDJEL, A., BOIS, F., BENEY, C., MARIOTTE, A.M., CONSEIL, G. & DI PIETRO, A. 2001. B-ring substituted 5,7-dihydroxyflavonols with high-affinity binding to P-glycoprotein responsible for cell multidrug resistance. *Bioorganic & medicinal chemistry letters*, 11:75-77.

BOUMENDJEL, A., DI PIETRO, A., DUMONTET, C. & BARRON, D. 2002. Recent advances in the discovery of flavonoids and analogs with high-affiniy binding to P-glycoprotein responsible for cancer cell multidrug resistance. *Medicinal research reviews*, 22:512-529.

CARR, K.E. & TONER, P.G. 1984. Morphology of the intestinal mucosa. (***In*** Csáky, T.Z., ***ed***. Pharmacology of intestinal permeation. Vol.1. Berlin : Springer-Verlag. p.1-50.)

CHAN, L.M.S., LOWES, S. & HIRST, B.H. 2004. The ABCs of drug transport in intestine and liver: efflux proteins limiting drug absorption and bioavailability. *European journal of pharmaceutical sciences*, 21:25-51.

CHERRINGTON, N.J., HARTLEY, D.P., LI, N., JOHNSON, D.R. & KLAASSEN, C.D. 2002. Organ distribution of multidrug resistance proteins 1,2 and 3 (Mrp1,2, and 3) mRNA and hepatic induction of Mrp3 by constitutive androstane receptor activators in rats. *Journal of pharmacology and experimental therapeutics*, 300:97-104.

CHIELI, E., ROMITI, N., CERVELLI, F. & TONGIANI, R. 1995. Effects of flavonols on P-glycoprotein activity in cultured rat hepatocytes. *Life sciences*, 57:1741-1751.

CHOI, C-H., KIM, J-H. & KIM, S-H. 2004. Reversal of P-glycoprotein-mediated MDR by 5,7,3',4',5'-pentamethoxyflavone and SAR. *Biochemical and biophysical research communications,* 320:672-679.

COHEN, J. 1998. Statistical power analysis for the behavioural sciences. 2nd ed. Hillsdale, NJ : Erlbaum. 225 p.

CONSEIL, G., BAUBICHON-CORTAY, H., DAYAN, G., JAULT, J.M., BARRON, D. & DE PIETRO, A. 1998. Flavonoids: a class of modulators with bifunctional interactions at vicinal ATP-and steroid-binding sites on mouse P-glycoprotein. *Proceedings of the National Academy of Sciences of the USA,* 95:9831-9836.

CSÁKY, T.Z. 1984. Intestinal permeation and permeability: an overview. (***In*** Csáky, T.Z., ***ed***. Pharmacology of intestinal permeation. Vol.1. Berlin : Springer-Verlag. p.51-60.)

DANTZIG, A.H., DE ALWIS, D.P. & BURGESS, M. 2003. Considerations in the design and development of transport inhibitors as adjuncts to drug therapy. *Advanced drug delivery reviews*, 55:133-150.

DELPH, Y. 2000. P-glycoprotein and HIV. [Web:] http://www.aidsinfonyc.org/tag/science/pgp.html [Date of access: 20 April 2006].

DESOIZE, B. & JARDILLIER, J. 2000. Multicellular resistance: a paradigm for clinical resistance? *Critical reviews in oncology/hematology*, 36:193-207.

EVANS, A.M. 2000. Influence of dietary components on the gastrointestinal metabolism and transport of drugs. *Therapeutic drug monitoring*, 22:131–136.

EVERS, R., KOOL, M., VAN DEEMTER, L., JANSSEN, H., CALAFAT, J., OOMEN, L.C.J.M., PAULUSMA, C.C., OUDE ELFERINK, R.P.J., BAAS, F., SCHINKEL, A.H. & BORST, P. 1998. Drug export activity of the human canalicular multispecific organic anion transporter in polarized kidney MDCK cells expressing cMOAT (MRP2) cDNA. *Journal of clinical investigations*, 101:1310-1319.

EYTAN, G.D. & KUCHEL, P.W. 1999. Mechanism of action of p-glycoprotein in relation to passive membrane permeation. *International review of cytology*, 190:175-250.

EYTAN, G.D., REGEV, R., OREN, G. & ASSARAF, Y.G. 1996. The role of passive transbilayer drug movement in multidrug resistance and its modulation. *Journal of biological chemistry*, 271:12897-12902.

FUHR, U. 1998. Drug interactions with grapefruit juice: extent, probable mechanism and clinical relevance. *Drug safety*, 18:251–272.

GERMANN, U.A. 1996. P-glycoprotein–a mediator of multidrug resistance in tumour cells. *European journal of cancer*, 32A:927–944.

GOTTESMAN, M.M., & PASTAN, I. 1988. Resistance to multiple chemotherapeutic agents in human cancer cells. *Trends in pharmacological sciences,* 9:54-58.

GOTTESMAN, M.M. & PASTAN, I. 1993. Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annual review of biochemistry*, 62:385–427.

GRASS, G.M. & SWEETANA, S.A. 1988. In vivo measurement of gastrointestinal tissue permeability using a new diffusion cell. *Pharmaceutical Research*, 5:373-376.

HATTINGH, O.N. 2002. Validation of the Sweetana-grass diffusion cell technique as a model for studying drug transport. Potchefstroom: PU for CHE. (Dissertation – M.Sc.) 118 p.

HIDALGO, I.J. 2001. Assessing the absorption of new pharmaceuticals. *Current topics in medicinal chemistry*, 1:385-401.

HIGGINS, C.F. & GOTTESMAN, M.M. 1992. Is the multidrug transporter a flippase? *Trends in biochemical sciences*, 17:18-21.

HIRSCH-ERNST, K.I., ZIEMANN, C., RUSTENBECK, I. & KAHL, G.F. 2001. Inhibitors of mdr1-dependent transport activity delay accumulation of the mdr1 substrate rhodamine 123 in primary rat hepatocyte cultures. *Toxicology*, 167:47–57.

HO, P.C., SAVILLE, D.J., COVILLE, P.F. & WANWIMOLRUK, S. 2000. Content of CYP3A4 inhibitors, naringin, naringenin and bergapten in grapefruit and grapefruit juice products. *Pharmaceutica acta Helvetiae*, 74:379–385.

HODEK, P., TREFIL, P. & STIBOROVÁ, M. 2002. Flavonoids- potent and versatile biologically active compounds interacting with cytochromes P450. *Chemico-biological interactions*, 139:1-21.

HOOIJBERG, J.H., BROXTERMAN, H.J., HEIJN, M., FLES, D.L.A., LANKELMA, J. & PINEDO, H.M. 1997. Modulation by (iso)flavonoids of the ATPase activity of the multidrug resistance protein. *FEBS letters*, 413:344-348.

HUNTER, J. & HIRST, B.H. 1997. Intestinal secretion of drugs: the role of p-glycoprotein and related drug efflux systems in limiting oral drug absorption. *Advanced drug delivery reviews*, 25:129–157.

KEPPLER, D., CUI, Y., KÖNIG, J., LEIER, I. & NIES, A. 1999. Export pumps for anionic conjugates encoded by MRP genes. *Advances in enzyme regulations*, 39:237-246.

KERR, I.D. 2002. Structure and association of ATP-binding casette transporter nucleotide-binding domains. *Biochimica et biophysica acta*, 1561:47–64.

KIOKA, N., HOSOKAWA, N., KOMANO, T., HIRAYOSHI, K., NAGATA, K. & UEDA, D. 1992. Quercetin, a bioflavonoid, inhibits the increase of human multidrug resistance gene (MDR1) expression caused by arsenite. *FEBS letters,* 301:307-309.

LEHNERT, M., MROSS, K., SCHUELLER, J., THUERLIMANN, B., KROEGER, N. & KUPPER, H. 1998. Phase II trial of dexverapamil and epirubicin in patients with non-responsive metastatic breast cancer. *British journal of cancer*, 77:1155-1163.

LIN, J.H. 2003. Drug-drug interaction mediated by inhibition and induction of p-glycoprotein. *Advanced drug delivery reviews*, 55:53-81.

LIU, R. & SHAROM, F.J. 1996. Site-directed fluorescence labeling of P-glycoprotein on cysteine residues in the nucleotide binding domains. *Biochemistry*, 35:11865-11873.

LOE, D.W., DEELEY, R.G. & COLE, S.P.C. 1996. Biology of the multidrug resistance-associated protein, MRP. *European journal of cancer*, 32A:945–957.

LOO, T.W. & CLARKE, D.M. 1999. The transmembrane domains of the human multidrug resistance P-glycoprotein are sufficient to mediate drug binding and trafficking to the cell surface. *Journal of biological chemistry*, 274:24759-24765.

LUND, W., ***ed.*** 1994. The pharmaceutical codex. 12th ed. London: The Pharmaceutical Press. 1117 p.

MARQUES-SANTOS, L.F., HARAB, R.C., DE PAULA, E.F. & RUMJANEK, V.M. 1999. The in vivo effect of the administration of resistance-modulating agents on rhodamine 123 distribution in mice thymus and lymph nodes. *Cancer letters*, 137:99-106.

MAYER, R., KARTENBECK, J., BüCHLER, M., JEDLITSCHKY, G., LEIER, I. & KEPPLER, D. 1995. Expression of the MRP gene-encoded conjugate export pump in liver and its selective absence from the canalicular membrane in transport-deficient mutant hepatocytes. *Journal of cell biology*, 131:137-150.

McKINNON, R.A., BURGESS, W.M., HALL, P.M., ROBERTS-THOMSON, S.J., GONZALEZ, F.J. & McMANUS, M.E. 1995. Characterisation of CYP3A gene subfamily expression in human gastrointestinal tissues. *Gut*, 36:259-267.

NEYFAKH, A.A. 1988. Use of fluorescent dyes as molecular probes for the study of multidrug resistance. *Experimental cell research*, 174:168-176.

NGUYEN, H., ZHANG, S. & MORRIS, M.E. 2003. Effect of flavonoids on MRP1-mediated transport in Panc-1 cells. *Journal of pharmaceutical sciences*, 92:250-257.

NISSLER, L., GEBHARDT, R. & BERGER, S. 2004. Flavonoid binding to a multi-drug-resistance transporter protein: an STD-NMRstudy. *Analytical & bioanalytical chemistry*, 379:1045-1049.

ÖSTERBERG, T. & NORINDER, U. 2000. Theoretical calculation and prediction of P-glycoprotein interacting drugs using MolSurf parametrization and PLS statistics. *European journal of pharmaceutical sciences*, 10:295-303.

PELKONEN, O., BOOBIS, A.R. & GUNDERT-REMY, U. 2001. In vitro prediction of gastrointestinal absorption and bioavailability: an experts' meeting report. *European journal of clinical pharmacology*, 57:621-629.

PEI, Q.L., KOBAYASHI, Y., TANAKA, Y., TAGUCHI, Y., HIGUCHI, K., KAITO, M., MA, N., SEMBA, R., KAMISAKO, T. & ADACHI, Y. 2002. Increased expression of multidrug resistance-associated protein 1 (mrp1) in hepatocyte basolateral membrane and renal tubular epithelia after bile duct ligation in rats. *Hepatology research*, 22:58-64.

PENG, K.C., CLUZEAUD, F., BENS, M., VAN HUYEN, J.P., WIOLAND, M.A., LACAVE, R. & VANDEWALLE, A. 1999. Tissue and cell distribution of the multidrug resistance-associated protein (MRP) in mouse intestine and kidney. *Journal of histochemistry and cytochemistry*, 47:757-768.

PHANG, J.M., POORE, C.M., LOPACZYNSKA, J. & YEH, G.C. 1993. Flavonol-stimulated efflux of 7,12-dimethylbenz(a)anthracene in multidrug-resistant breast cancer cells. *Cancer research*, 53:5977-5981.

PIETTA, P. 2000. Flavonoids as antioxidants. *Journal of natural products*, 63:1035-1042.

POUGET, C., LAUTHIER, F., SIMON, A., FAGNERE, C., BASLY, J., DELAGE, C. & CHULIA, A. 2001. Flavonoids: structural requirements for antiproliferative activity on breast cancer cells. *Bioorganic & medicinal chemistry letters*, 11:3095-3097.

QUAN, Z., OZAWA, T., SATO, M. & UMEZAWA, Y. 2000. Screening method for substrates of multidrug resistance-associated protein. *Analytica chimica acta*, 423:197–203.

ROGERS, A.W. 1983. Cells and tissues: an introduction to histology and cell biology. London: Academic Press. 242 p.

SARKADI, B., MÜLLER, M. & HOLLÓ, Z. 1996. The multidrug transporters-proteins of an ancient immune system. *Immunology letters*, 54:215-219.

SCALA, S., AKHMED, N., RAO, U.S., PAULL, K., LAN, L.B., DICKSTEIN, B., LEE, J.S., ELGEMEIE, G.H., STEIN, W.D. & BATES, S.E. 1997. P-glycoprotein substrates and antagonists cluster in two distinct groups. *Molecular pharmacology*, 51:1024-1033.

SCAMBIA, G., RANELLETTI, F.O., PANICI, P.B., DE VINCENZO, R., BONANNO, G., FERRANDINA, G., PIANTELLI, M., BUSSA, S., RUMI, C., CIANFRIGLIA, M. & MANCUSO, S. 1994. Quercetin potentiates the effect of adriamycin in a multidrug-resistant MCF-7 human breast-cancer cell line: p-glycoprotein as a possible target. *Cancer chemotherapy and pharmacology*, 34:459-464.

SCHINKEL, A.H. & JONKER, J.W. 2003. Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview. *Advanced drug delivery reviews*, 55:3-29.

SCHNEIDER, E. & HUNKE, S. 1998. ATP-binding-cassette (ABC) transport systems: functional and structural aspects of the ATP-hydrolyzing subunits/domains. *FEMS microbiology reviews*, 22:1-20.

SEELIG, A. & LANDWOJTOWICZ, E. 2000. Structure-activity relationship of P-glycoprotein substrates and modifiers. *European journal of pharmaceutical sciences*, 12:31-40.

SENIOR, A.E., AL-SHAWI, M.K. & URBATSCH, I.L. 1995. The catalytic cycle of p-glycoprotein. *FEBS letters*, 377:285-289.

SHAPIRO, A.B. & LING, V. 1997. Effect of quercetin on Hoechst 33342 transport by purified and reconstituted P-glycoprotein. *Biochemical pharmacology*, 53:587-596.

SHAPIRO, A.B. & LING, V. 1998. The mechanism of ATP-dependent multidrug transport by P-glycoprotein. *Acta Physiologica Scandinavica,* 163:227-234.

SHAPIRO, A.B., FOX, K., LAM, P. & LING, V. 1999. Stimulation of P-glycoprotein-mediated drug transport by prazosin and progesterone. *European journal of biochemistry*, 259:841-850.

SHARGEL, L. & YU, A.B.C. 1993. Applied biopharmaceuticals and pharmacokinetics. 3rd ed. Norwalk, Conn. : Appleton & Lange. 625 p.

SHARGEL, L. & YU, A.B.C. 1999. Applied biopharmaceutics and pharmacokinetics. 4th ed. Norwalk, Conn. : Appleton & Lange. 766p.

SHAROM, F.J. 1997. The P-glycoprotein efflux pump: how does it transport drugs? *Journal of membrane biology*, 160:161-175.

SHAROM, F.J., LIU, R., ROMISCKI, Y. & LU, P. 1999. Insights into the structure and substrate interactions of the P-glycoprotein multidrug transporter from spectroscopic studies. *Biochimica et biophysica acta*, 1461:327-345.

SIGMA ALDRICH. 2005. Multidrug resistance in cancer – role of ABC transporter proteins. [Web:] http://www.sigmaaldrich.com/Area_of_Interest/ Biochemicals/Enzyme_ Explorer/Cell_Signaling_Enzymes/Xenobiotics/Drug_metabolism_Tech_Review.html [Date of access: 20 April 2006].

SOLDNER, A., CHRISTIANS, U., SUSANTO, M., WACHER, V.J., SILVERMAN, J.A. & BENET, L.Z. 1999. Grapefruit juice activates p-glycoprotein-mediated drug transport. *Pharmaceutical research*, 16:478-485.

STEIN, W.D. 1997. Kinetics of multidrug transporter (P-glycoprotein) and its reversal. *Physiological reviews*, 77:545-590.

STEIN, A.G.W., SMIT, C.F. & DU TOIT, S.H.C. 1998. Moderne statistiek vir die praktyk, J.L. van Schaik: Pretoria. 604p.

STEYN, H.S. 1999. Praktiese beduidendheid: die gebruik van effekgroottes. Potchefstroom: PU vir CHO. Publikasiebeheerkomitee. 28p. (Wetenskaplike bydraes van die PU vir CHO. Reeks B, Natuurwetenskappe; nr. 117.)

TADA, Y., WADA, M., MIGITA, T., NAGAYAMA, J., HINOSHITA, E., MOCHIDA, Y., MAEHARA, Y., TSUNEYOSHI, M., KUWANO, M. & NAITO, S. 2002. Increased expression of multidrug resistance-associated proteins in bladder cancer during clinical course and drug resistance to doxorubicin. *International journal of cancer*, 98:630-635.

TEODORI, E., DEI, S., SCAPECCHI, S. & GUALTIERI, F. 2002. The medicinal chemistry of multidrug resistance (MDR) reversing drugs. *Il Farmaco*, 57:385-415.

TRIER, J.S. & MADARA, J.L. 1981. Functional morphology of the mucosa of the small intestine. (***In*** Johnson, L.R., Christensen, J., Jacobson, E.D., Grossman, M.E. & Schultz, S.G., ***eds***. Physiology of the gastrointestinal tract. Vol.2. New York : Raven Press. p.923-961.)

TSURUO, T., IIDA, H., TSUKAGOSHI, S. & SAKURAI, Y. 1981. Enhancement of vinblastine-induced cytotoxicity by lysolecithin and phosphatidylinositol. *Cancer letters*, 13:133-137.

TWENTYMAN, P.R. & WRIGHT, K.A. 1991. Chemosensitisation of a drug-sensitive parental cell line by low-dose cyclosporine A. *Cancer chemotherapy and pharmacology*, 29:24-28.

UNGELL, A.L., NYLANDER, S., BERGSTRAND, S., SJOBERG, A. & LENNERNAS, H. 1998. Membrane transport of drugs in different regions of the intestinal tract of the rat. *Journal of pharmaceutical sciences*, 87:360-366.

VAN DER HEYDEN, S., GHEUENS, E., DE BRUIJN, E., VAN OOSTEROM, A. & MAES, R. 1995. P-glycoprotein: clinical significance and methods of analysis. *Critical reviews in clinical laboratory sciences*, 32:221-264.

VAN ZANDEN, J.J., GERAETS, L., WORTELBOER, H.M., VAN BLADEREN, P.J., RIETJENS, I.M.C.M. & CNUBBEN, N.H.P. 2004. Structural requirements for the flavonoid-mediated modulation of glutathione S-transferase P1-1 and GS-X pump activity in MCF7 breast cancer cells. *Biochemical pharmacology*, 67:1607-1617.

VAN ZANDEN, J.J., WORTELBOER, H.M., BIJLSMA, S., PUNT, A., USTA, M., VAN BLADEREN, P.J., RIETJENS, I.M.C.M. & CNUBBEN, N.H.P. 2005. Quantitative structure activity relationship studies on the flavonoid mediated inhibition of multidrug resistant proteins 1 and 2. *Biochemical pharmacology*, 69:699-708.

WACHER, V.J., SILVERMAN, J.A., ZHANG, Y. & BENET, L.Z. 1998. Role of P-glycoprotein and cytochrome P450 3A in limiting oral absorption of peptides and peptidomimetics. *Journal of pharmaceutical sciences*, 87:1322-1330.

WACHER, V.J., SALPHATI, L. & BENET, L.Z. 2001. Active secretion and enterocytic drug metabolism barriers to drug absorption. *Advanced drug delivery reviews*, 46:89-102.

WAGNER, D., SPAHN-LANGGUTH, H., HANAFY, A., KOGGEL, A. & LANGGUTH, P. 2001. Intestinal drug efflux: formulation and food effects. *Advanced drug delivery reviews*, 50:S13–S31.

WALLE, T. 2004. Absorption and metabolism of flavonoids. *Free radical biology & medicine*, 36:829-837.

WATKINS, P.B. 1992. Drug metabolism by cytochromes P450 in the liver and small bowel. *Gastroenterology clinics of North America*, 21:511-526.

WATKINS, P.B. 1997. The barrier function of CYP 3A4 and P-glycoprotein in the small bowel. *Advanced drug delivery reviews*, 27:161-170.

APPENDIX A

Table A.1: Cumulative transport amounts of Rho 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	0.03	0.06	0.07	0.02	0.045 ± 0.021	0.045 ± 0.035
	60	0.04	0.08	0.15	0.13	0.060 ± 0.028	0.140 ± 0.014
	90	0.11	0.10	0.32	0.33	0.105 ± 0.007	0.325 ± 0.007
	120	0.18	0.24	0.62	0.53	0.210 ± 0.042	0.575 ± 0.064
Control 2	30	0.02	0.02	0.08	0.06	0.020 ± 0.000	0.070 ± 0.014
	60	0.04	0.07	0.13	0.16	0.055 ± 0.021	0.145 ± 0.021
	90	0.07	0.09	0.23	0.23	0.080 ± 0.014	0.230 ± 0.000
	120	0.09	0.17	0.40	0.46	0.130 ± 0.057	0.430 ± 0.042
Control 3	30	0.09	0.08	0.08	0.08	0.085 ± 0.007	0.080 ± 0.000
	60	0.09	0.09	0.10	0.12	0.090 ± 0.000	0.110 ± 0.014
	90	0.11	0.10	0.23	0.20	0.105 ± 0.007	0.215 ± 0.021
	120	0.15	0.15	0.33	0.27	0.150 ± 0.000	0.300 ± 0.042
Control 4	30	0.05	0.07	0.07	0.07	0.060 ± 0.014	0.070 ± 0.000
	60	0.08	0.10	0.13	0.13	0.090 ± 0.014	0.130 ± 0.000
	90	0.17	0.24	0.48	0.55	0.205 ± 0.049	0.515 ± 0.049
	120	0.32	0.49	1.05	1.07	0.405 ± 0.120	1.060 ± 0.014
Morin 1 (10 µM)	30	0.09	0.11	0.09	0.04	0.100 ± 0.014	0.065 ± 0.035
	60	0.14	0.11	0.23	0.17	0.125 ± 0.021	0.200 ± 0.042
	90	0.16	0.19	0.26	0.27	0.175 ± 0.021	0.265 ± 0.007
	120	0.33	0.32	0.56	0.44	0.325 ± 0.007	0.500 ± 0.085
Morin 2 (10 µM)	30	0.04	0.04	0.13	0.12	0.040 ± 0.000	0.125 ± 0.007
	60	0.10	0.09	0.23	0.26	0.095 ± 0.007	0.245 ± 0.021
	90	0.20	0.14	0.57	0.53	0.170 ± 0.042	0.550 ± 0.028
	120	0.57	0.42	0.97	1.00	0.495 ± 0.106	0.985 ± 0.021
Morin 3 (10 µM)	30	0.04	0.04	0.10	0.11	0.040 ± 0.000	0.105 ± 0.007
	60	0.04	0.04	0.20	0.23	0.040 ± 0.000	0.215 ± 0.021
	90	0.14	0.15	0.51	0.49	0.145 ± 0.007	0.500 ± 0.014
	120	0.49	0.49	0.84	0.93	0.490 ± 0.000	0.885 ± 0.064
Morin 4 (10 µM)	30	0.04	0.04	0.24	0.23	0.040 ± 0.000	0.235 ± 0.007
	60	0.60	0.64	0.54	0.55	0.620 ± 0.028	0.545 ± 0.007
	90	0.92	1.02	2.03	1.37	0.970 ± 0.071	1.700 ± 0.467
	120	1.53	1.71	3.50	2.30	1.620 ± 0.127	2.900 ± 0.849
Morin 1 (20 µM)	30	0.11	0.25	0.37	0.14	0.180 ± 0.099	0.255 ± 0.163
	60	0.45	0.53	0.66	0.62	0.490 ± 0.057	0.640 ± 0.028
	120	0.76	0.96	1.52	1.34	0.860 ± 0.141	1.430 ± 0.127
Morin 2 (20 µM)	30	0.23	0.15	0.17	0.26	0.190 ± 0.057	0.215 ± 0.064
	60	0.33	0.25	0.31	0.47	0.290 ± 0.057	0.390 ± 0.113
	90	0.39	0.59	0.80	0.91	0.490 ± 0.141	0.855 ± 0.078

	120	0.64	0.71	1.12	1.40	0.675 ± 0.049	1.260 ± 0.198
Morin 3	30	0.03	0.04	0.11	0.10	0.035 ± 0.007	0.105 ± 0.007
(20 µM)	60	0.11	0.15	0.22	0.12	0.130 ± 0.028	0.170 ± 0.071
	90	0.28	0.50	0.46	0.37	0.390 ± 0.156	0.415 ± 0.064
	120	0.81	1.14	0.89	0.91	0.975 ± 0.233	0.900 ± 0.014
Morin 4	30	0.20	0.20	0.08	0.08	0.200 ± 0.000	0.080 ± 0.000
(20 µM)	60	0.62	0.63	0.44	0.29	0.625 ± 0.007	0.365 ± 0.106
	90	1.25	1.28	1.24	0.92	1.265 ± 0.021	1.080 ± 0.226
	120	2.25	2.56	2.57	2.50	2.405 ± 0.219	2.535 ± 0.049
Galangin 1	30	0.03	0.10	0.00	0.00	0.065 ± 0.049	0.000 ± 0.000
(10 µM)	60	0.12	0.19	0.24	0.26	0.155 ± 0.049	0.250 ± 0.014
	90	0.20	0.32	0.60	0.66	0.260 ± 0.085	0.630 ± 0.042
	120	0.51	0.45	1.13	1.06	0.480 ± 0.042	1.095 ± 0.049
Galangin 2	30	0.20	0.06	0.23	0.13	0.130 ± 0.099	0.180 ± 0.071
(10 µM)	60	0.25	0.39	0.94	1.04	0.320 ± 0.099	0.990 ± 0.071
	90	0.68	0.67	1.77	2.33	0.675 ± 0.007	2.050 ± 0.396
	120	2.03	1.57	3.44	4.03	1.800 ± 0.325	3.735 ± 0.417
Galangin 3	30	0.01	0.01	0.10	0.19	0.010 ± 0.000	0.145 ± 0.064
(10 µM)	60	0.26	0.39	0.68	0.77	0.325 ± 0.092	0.725 ± 0.064
	90	0.78	1.18	1.84	1.98	0.980 ± 0.283	1.910 ± 0.099
	120	1.56	2.17	3.49	3.74	1.865 ± 0.431	3.615 ± 0.177
Galangin 4	30	0.05	0.03	0.11	0.13	0.040 ± 0.014	0.120 ± 0.014
(10 µM)	60	0.45	0.30	0.46	0.68	0.375 ± 0.106	0.570 ± 0.156
	90	0.91	0.67	1.23	1.73	0.790 ± 0.170	1.480 ± 0.354
	120	1.57	1.07	2.13	3.08	1.320 ± 0.354	2.605 ± 0.672
Galangin 1	30	0.20	0.23	0.12	0.12	0.215 ± 0.021	0.120 ± 0.000
(20 µM)	60	0.70	0.64	0.53	0.45	0.670 ± 0.042	0.490 ± 0.057
	90	1.41	1.22	0.99	1.15	1.315 ± 0.134	1.070 ± 0.113
	120	2.64	2.17	2.44	2.45	2.405 ± 0.332	2.445 ± 0.007
Galangin 2	30	0.18	0.18	0.15	0.14	0.180 ± 0.000	0.145 ± 0.007
(20 µM)	60	0.24	0.32	0.29	0.35	0.280 ± 0.057	0.320 ± 0.042
	90	0.46	0.81	1.07	1.01	0.635 ± 0.247	1.040 ± 0.042
	120	0.92	1.32	2.45	1.89	1.120 ± 0.283	2.170 ± 0.396
Galangin 3	30	0.06	0.06	0.21	0.16	0.060 ± 0.000	0.185 ± 0.035
(20 µM)	60	0.20	0.10	0.43	0.53	0.150 ± 0.071	0.480 ± 0.071
	90	0.37	0.16	0.81	0.75	0.265 ± 0.148	0.780 ± 0.042
	120	0.69	0.28	1.07	0.95	0.485 ± 0.290	1.010 ± 0.085
Galangin 4	30	0.30	0.27	0.38	0.34	0.285 ± 0.021	0.360 ± 0.028
(20 µM)	60	0.83	0.94	1.12	0.85	0.885 ± 0.078	0.985 ± 0.191
	90	1.36	1.61	1.75	1.69	1.485 ± 0.177	1.720 ± 0.042
	120	1.85	2.11	2.46	2.45	1.980 ± 0.184	2.455 ± 0.007
Kaempferol 1	30	0.06	0.36	0.17	0.14	0.210 ± 0.212	0.155 ± 0.021
(10 µM)	60	0.25	0.79	1.53	1.21	0.520 ± 0.382	1.370 ± 0.226
	90	0.71	1.25	2.89	2.49	0.980 ± 0.382	2.690 ± 0.283

	120	1.07	2.10	4.14	4.01	1.585 ± 0.728	4.075 ± 0.092
Kaempferol 2 (10 µM)	30	0.15	0.21	0.25	0.16	0.180 ± 0.042	0.205 ± 0.064
	60	0.34	0.52	0.55	0.47	0.430 ± 0.127	0.510 ± 0.057
	90	0.46	0.71	1.11	1.41	0.585 ± 0.177	1.260 ± 0.212
	120	0.70	1.08	1.78	1.78	0.890 ± 0.269	1.780 ± 0.000
Kaempferol 3 (10 µM)	30	0.23	0.14	0.23	0.27	0.185 ± 0.064	0.250 ± 0.028
	60	0.29	0.25	0.38	0.43	0.270 ± 0.028	0.405 ± 0.035
	90	0.35	0.36	0.52	0.62	0.355 ± 0.007	0.570 ± 0.071
	120	0.42	0.47	0.65	0.79	0.445 ± 0.035	0.720 ± 0.099
Kaempferol 4 (10 µM)	30	0.08	0.25	0.20	0.10	0.165 ± 0.120	0.150 ± 0.071
	60	0.32	0.31	1.12	0.79	0.315 ± 0.007	0.955 ± 0.233
	90	0.98	0.91	2.36	1.94	0.945 ± 0.049	2.150 ± 0.297
	120	0.53	1.35	4.87	3.63	0.940 ± 0.580	4.250 ± 0.877
Kaempferol 1 (20 µM)	30	0.07	0.07	0.19	0.15	0.070 ± 0.000	0.170 ± 0.028
	60	0.16	0.16	0.28	0.32	0.160 ± 0.000	0.300 ± 0.028
	90	0.25	0.18	0.59	0.62	0.215 ± 0.049	0.605 ± 0.021
	120	0.62	0.60	1.22	0.93	0.610 ± 0.014	1.075 ± 0.205
Kaempferol 2 (20 µM)	30	-0.20	-0.11	-0.20	-0.09	- 0.155 ± 0.064	- 0.145 ± 0.078
	60	0.02	0.01	0.18	0.38	0.015 ± 0.007	0.280 ± 0.141
	90	0.20	0.28	0.78	0.85	0.240 ± 0.057	0.815 ± 0.049
	120	0.96	0.96	2.25	2.32	0.960 ± 0.000	2.285 ± 0.049
Kaempferol 3 (20 µM)	30	0.12	0.18	0.17	0.11	0.150 ± 0.042	0.140 ± 0.042
	60	0.27	0.24	0.30	0.25	0.255 ± 0.021	0.275 ± 0.035
	90	0.38	0.43	0.45	0.44	0.405 ± 0.035	0.445 ± 0.007
	120	0.60	0.70	0.60	0.71	0.650 ± 0.071	0.655 ± 0.078
Kaempferol 4 (20 µM)	30	0.12	0.14	0.20	0.11	0.130 ± 0.014	0.155 ± 0.064
	60	0.18	0.21	0.35	0.26	0.195 ± 0.021	0.305 ± 0.064
	90	0.28	0.36	0.57	0.53	0.320 ± 0.057	0.550 ± 0.028
	120	0.36	0.63	1.01	0.98	0.495 ± 0.191	0.995 ± 0.021
Quercetin 1 (10 µM)	30	0.05	0.08	0.51	0.46	0.065 ± 0.021	0.485 ± 0.035
	60	0.24	0.37	1.55	1.65	0.305 ± 0.092	1.600 ± 0.071
	90	0.70	1.04	3.14	3.60	0.870 ± 0.240	3.370 ± 0.325
	120	1.16	1.89	5.04	5.55	1.525 ± 0.516	5.295 ± 0.361
Quercetin 2 (10 µM)	30	0.08	0.11	-0.01	0.21	0.095 ± 0.021	0.100 ± 0.156
	60	0.33	0.28	0.54	0.73	0.305 ± 0.035	0.635 ± 0.134
	90	0.50	0.45	1.05	1.54	0.475 ± 0.035	1.295 ± 0.346
	120	0.70	0.64	1.34	1.99	0.670 ± 0.042	1.665 ± 0.460
Quercetin 3 (10 µM)	30	0.05	0.07	0.40	0.42	0.060 ± 0.014	0.410 ± 0.014
	60	0.26	0.33	1.34	1.51	0.295 ± 0.049	1.425 ± 0.120
	90	0.67	0.97	2.91	3.45	0.820 ± 0.212	3.180 ± 0.382

	120	1.16	1.80	3.99	4.92	1.480 ± 0.453	4.455 ± 0.658
Quercetin 4 (10 µM)	30	0.06	0.08	0.37	0.46	0.070 ± 0.014	0.415 ± 0.064
	60	0.24	0.37	1.19	1.61	0.305 ± 0.092	1.400 ± 0.297
	90	0.69	1.03	2.30	3.48	0.860 ± 0.240	2.890 ± 0.834
	120	1.14	1.86	3.75	5.48	1.500 ± 0.509	4.615 ± 1.223
Quercetin 1 (20 µM)	30	0.09	0.07	0.16	0.09	0.080 ± 0.014	0.125 ± 0.049
	60	0.13	0.16	0.40	0.46	0.145 ± 0.021	0.430 ± 0.042
	90	0.28	0.29	0.57	0.60	0.285 ± 0.007	0.585 ± 0.021
	120	0.45	0.41	0.91	0.97	0.430 ± 0.028	0.940 ± 0.042
Quercetin 2 (20 µM)	30	0.18	0.20	0.09	0.09	0.190 ± 0.014	0.090 ± 0.000
	60	0.20	0.21	0.09	0.18	0.205 ± 0.007	0.135 ± 0.064
	90	0.35	0.27	0.29	0.21	0.310 ± 0.057	0.250 ± 0.057
	120	0.54	0.51	0.69	0.58	0.525 ± 0.021	0.635 ± 0.078
Quercetin 3 (20 µM)	30	0.14	0.23	0.20	0.10	0.185 ± 0.064	0.150 ± 0.071
	60	0.28	0.29	0.63	0.31	0.285 ± 0.007	0.470 ± 0.226
	90	0.53	0.52	1.24	0.63	0.525 ± 0.007	0.935 ± 0.431
	120	1.08	1.01	2.62	1.34	1.045 ± 0.049	1.980 ± 0.905
Quercetin 4 (20 µM)	30	0.04	0.07	0.17	0.12	0.055 ± 0.021	0.145 ± 0.035
	60	0.32	0.48	0.52	0.16	0.400 ± 0.113	0.340 ± 0.255
	90	0.56	0.77	0.75	0.87	0.665 ± 0.148	0.810 ± 0.085
	120	0.78	1.00	1.76	1.83	0.890 ± 0.156	1.795 ± 0.049

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} ($x10^{-7}$ cm/s)
3928.57	Cell 1							
	0	38	38	9.79	0.25	0.01830	1.61	1.71
	30	93	94	21.56	0.55			
	90	197	200	43.71	1.11			
	120	319	326	69.98	1.78			
	Cell 2							
	30	21	21	6.23	0.16	0.01174	1.81	
	60	79	79	18.35	0.47			
	90	257	257	55.51	1.41			
	120	325	325	69.77	1.78			

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			
4.58	11	10	16	12	4.7855	-8.8333	0.9992
9.17	36	48	24	36			
13.75	55	60	57	57			
18.33	72	81	82	78			

a) Reference concentration =Concentration of Rho123 in 7mL
=275µg x 1000 /20ml x 2ml/7ml
=3928.57ng/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: 38/28 + 56 = 57.35
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): 4.7855

y-intercept (c): -8.8333 ng/mL

Thus $x = (y-c)/m$

$x = (38 - (-8.8333 \text{ ng/mL})/4.7855$

$x = 9.786$ ng/mL

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

Example: $9.786/3928.57 \times 100 = 0.249$

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

f) Calculated by the equation on page 54 given in the experimental procedure chapter

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

$$P_{app} = \frac{0.0183}{60 \times 1.78 \times 100}$$

$$= 1.71 \times 10^{-7} \text{ cm/s}$$

APPENDIX C

SIGMA-ALDRICH

Certificate of Analysis

Product Name	Rhodamine 123,
Product Number	R8004
Product Brand	SIGMA
CAS Number	62669-70-9
Molecular Formula	$C_{21}H_{16}N_2O_3 \cdot HCl$
Molecular Weight	380.82

TEST	SPECIFICATION	LOT 013K3744 RESULTS
APPEARANCE	RED TO BROWN POWDER	RED-BROWN POWDER
SOLUBILITY	CLEAR TO SLIGHTLY HAZY YELLOW-ORANGE TO RED SOLUTION AT 1MG/ML IN ETHANOL	CLEAR YELLOW-ORANGE
IR SPECTRUM	CONFORMS TO STRUCTURE	CONFORMS
WATER BY KARL FISCHER	NMT 10%	7.2%
CARBON *	62.2 TO 70.2%	65.0%
NITROGEN *	6.9 TO 7.8%	7.1%
PURITY BY HPLC		96.7%
SHELF LIFE SOP QC-12-006	4 YEARS	DECEMBER 2003
QC ACCEPTANCE DATE		JANUARY 2003

Lori Schulz, Manager
Analytical Services
St. Louis, Missouri USA

SIGMA-ALDRICH

Certificate of Analysis

Product Name	Krebs-Ringer Bicarbonate Buffer, powder cell culture tested
Product Number	K4002
Product Brand	SIGMA
CAS Number	
Molecular Formula	
Molecular Weight	

TEST	SPECIFICATION	LOT 034K8309 RESULTS
APPEARANCE	WHITE TO OFF-WHITE POWDER	PASS
SOLUBILITY	CLEAR SOLUTION AT 9.5 G/L IN WATER	PASS
WATER BY KARL FISCHER	NMT 2.0%	0.8%
PH TEST	6.1 - 6.7	6.4
PH TEST WITH NAHCO3	7.0 - 7.6	7.4
OSMOLALITY	235 - 260 MOSM/KG H2O	237 MOSM/KG H2O
OSMOLALITY WITH NAHCO3	253 - 280 MOSM/KG H2O	265 MOSM/KG H2O
GLUCOSE	17.0 - 20.8%	18.6%
ENDOTOXIN ASSAY	NMT 1.0 EU/ML AT 1X	<0.05 EU/ML
KEY ELEMENTS BY ICP	CONSISTENT WITH FORMULATION	PASS
CELL CULTURE TEST	PASS	PASS
CELL LINE		M2E6
EXPIRATION DATE	24 MONTHS	MARCH 2006
QC ACCEPTANCE DATE		APRIL 2004

Lori Schulz, Manager
Analytical Services
St. Louis, Missouri USA

SIGMA-ALDRICH

Certificate of Analysis

Product Name	Morin, powder
Product Number	M4008
Product Brand	SIGMA
CAS Number	480-16-0
Molecular Formula	$C_{15}H_{10}O_7$
Molecular Weight	302.24

TEST	**LOT 062K2504 RESULTS**
APPEARANCE	BROWN POWDER
SOLUBILITY	RED SOLUTION AT 0.5 G PLUS 10 ML OF METHANOL
WATER BY KARL FISCHER	7.1%
UV-VIS SPECTRUM	E1% = 417 AT LAMBDA MAX 360 NM IN METHANOL
PURITY BY HPLC	88.9%
QC ACCEPTANCE DATE	JUNE 2002
PRODUCT CROSS REFERENCE INFORMATION	REPLACEMENT FOR ALDRICH #M87630

Lori Schulz

Lori Schulz, Manager
Analytical Services
St. Louis, Missouri USA

SIGMA-ALDRICH

Certificate of Analysis

Product Name	Galangin,
Product Number	28,220-0
Product Brand	ALDRICH
CAS Number	548-83-4
Molecular Formula	$C_{15}H_{10}O_5$
Molecular Weight	270.24

TEST	**LOT S08982 RESULTS**
QC Acceptance date	23-NOV-2001
APPEARANCE - COLOUR	YELLOW
APPEARANCE - STATE	POWDER
HPLC - PURITY	96.10%
FT-IR SPECTROSCOPY - FTIR SPECTRUM	CONFORMS TO STRUCTURE
MELTING POINT	220.5-223.6 DEG C

Claudia Mayer, Manager
Quality Control
Steinheim Germany

SIGMA-ALDRICH

Certificate of Analysis

Product Name	Kaempferol, BioChemika ≥96% (HPLC)
Product Number	60010
Product Brand	FLUKA
CAS Number	520-18-3
Molecular Formula	$C_{15}H_{10}O_6$
Molecular Weight	286.24

TEST	**LOT 437695/1 RESULTS**
ASSAY (HPLC)	97.8 % REL
APPEARANCE	LIGHT YELLOW POWDER
WATER	6.1 %
INFRARED SPECTRUM	CORRESPONDS
DATE OF QC-RELEASE	02/MAY/02

Fluka guarantees the 'Sales-Specification' values only, non-specified tests may be included as additional information. The current 'Sales-Specifications' sheet is available on request. For further inquiries, please contact our Technical Service. Fluka warrants, that its products conform to the information contained in this and other Fluka publications. Purchaser must determine the suitability of the product for its particular use. See reverse side of invoice for additional terms and conditions of sale. The values given on the 'Certificate of Analysis' are the results determined at the time of analysis.

Dr. Gert van Look, Manager
Quality Control
Buchs Switzerland

SIGMA-ALDRICH

Certificate of Analysis

Product Name	Quercetin dihydrate, ≥98% (HPLC) powder
Product Number	Q0125
Product Brand	SIGMA
CAS Number	6151-25-3
Molecular Formula	$C_{15}H_{10}O_7 \cdot 2H_2O$
Molecular Weight	338.27

TEST	SPECIFICATION	LOT 039H0598 RESULTS
APPEARANCE	YELLOW TO YELLOW WITH A GREEN TO BROWN CAST POWDER	YELLOW POWDER
SOLUBILITY	DARK RED SOLUTION AT 200 MG PLUS 4 ML OF 1 M SODIUM HYDROXIDE	CONFORMS
PURITY BY HPLC	NOT LESS THAN 98%	99.7%
QC ACCEPTANCE DATE		APRIL 1999

Lori Schulz, Manager
Analytical Services
St. Louis, Missouri USA

ACKNOWLEDGEMENTS

My gratitude to the following people:

Unto my heavenly Father; Your everlasting love and mercy makes everything in my life possible and I praise You with all my heart and soul.

My dear parents Stefan and Estelle van Huyssteen. Thank you for all your unconditional love, advice and support. I will always look up to you. You are everything that I am and I love you dearly.

My sisters Andri and Esté van Huyssteen. You were always a beam of sunshine to come home to after work. Thank you for all your love and support.

To my future husband Stanley Dodd. Without you, my days would have been dark and difficult. Thank you for all your help, patience and love. I treasure it deeply.

My dearest grandpartents Pine and Rensa Pienaar and Lettie van Huyssteen. Thank you for all your love and support.

Mr. Kobus Swart, you were always someone to rely on and thank you for helping me with every aspect of this study.

Dr. Maides Malan, I treasure all your support, leadership and help deeply within my heart. Thank you for taking over the project and in helping me to get onto my feet again.

Prof. D.G. Müller, you will always be in my thoughts and I am privileged to know someone like you.

Prof. A.W. Kotze, thank you for the use of the diffusion cells.

Mrs. Antoinette Fick and Mr. Cor Bester. Thank you for always providing me with the rats for the experiments.

Mrs. J.W. Breytenbach, thank you for your help with the statistical analysis of the data.

To everyone at Kosmos, Secunda and President Pharmacies, thank you for all your caring and support.

To all my friends and the friendly faces at the Department of Pharmaceutics, thank your for your friendship and support.

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Background: Multidrug resistance (MDR) is resistance of cancer cells to multiple classes of chemotherapeutic drugs that can be structurally unrelated. MDR involves altered membrane transport that results in a lower cell concentration of cytotoxic drugs which plays an important role during cancer treatment. P-glycoprotein (Pgp) is localised at the apical surface of epithelial cell in the intestine and it functions as a biological barrier by extruding toxic substances and xenobiotics out of cells (Lin, 2003:54). The ATP-binding-cassette superfamily is a rapidly growing group of membrane transport proteins and are involved in diverse physiological processes which include antigen presentation, drug efflux from cancer cells, bacterial nutrient uptake and cystic fibrosis (Germann, 1996:928; Kerr, 2002:47). A number of drugs have been identified which are able to reverse the effects of Pgp, multidrug resistance protein (MRP1) and their associated proteins on multidrug resistance. The first MDR modulators discovered and studied during clinical trials were associated with definite pharmacological actions, but the doses required to overcome MDR were associated with the occurrence of unacceptable side effects. As a consequence, more attention has been given to the development of modulators with proper potency, selectivity and pharmacokinetic characteristics that it can be used at a lower dose. Several novel MDR reversing agents (also known as chemosensitisers) are currently undergoing clinical evaluation for the treatment of resistant tumors (Teodori *et al.*, 2002:385). **Aim:** The aim of this study was to investigate the effect of selected flavonoids (morin, galangin, kaempferol and quercetin) at two different concentrations (10 µM and 20 µM) on the transport of a known Pgp substrate, Rhodamine 123 (Rho 123) across rat intestine (jejunum) and to investigate structure activity relationships (SAR) of the selected flavonoids with reference to the inhibition of Pgp. **Methods:** Morin, galangin, kaempferol and quercetin were evaluated as potential modulators of Rho 123 transport, each at a concentration of 10 µM and 20 µM across rat jejunum using Sweetana-Grass diffusion cells. This study was done bidirectionally, with two cells measuring transport in the apical to basolateral direction (AP-BL) and two 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 to AP-BL ratio calculated for Rho 123 with no modulators added was 3.29. Morin decreased the BL-AP to AP-BL ratio to 1.88 at a concentration of 10 µM and to 1.49 at a concentration of 20 µM. Galangin decreased the BL-AP to AP-BL ratio to 1.60 at a concentration of 20 µM. These two flavonoids showed statistically significant results and inhibition of active transport were clearly demonstrated. However, the other flavonoids inhibited active transport of Rho 123 but according to statistical analysis, the results were not significantly different. The two different concentrations (10 µM and 20 µM) indicated that galangin, kaempferol and quercetin showed practically significant differences according to the effect sizes. Morin, however, did not show any practically significant differences at the different concentrations. Regarding the SAR, it was shown by Boumendjel and co-workers (2002:512) that the presence of a 5-hydroxyl group and a 3-hydroxyl group as well as the C2-C3 double bond are required for high potency binding to the nucleotide binding domain (NBD) of Pgp. All the flavonoids tested had the above-mentioned characteristics. **Conclusion:** All the selected flavonoids showed inhibition of active transport of Rho 123 and should have an effect on the bioavailability of the substrates of Pgp and other active transporters. This study described the inhibitory interaction of selected flavonoids on Pgp activity. Practical significant differences between the same modulator at different concentrations were also observed. Structure activity relationships were identified describing the inhibitory potency of the flavonoids based on hydroxyl group positioning.

Keywords: P-glycoprotein, Rhodamine 123, morin, galangin, kaempferol, quercetin, structure activity relationship, Sweetana-Grass diffusion cells

UITTREKSEL

Agtergrond: Multi-geneesmiddel resistensie (MGR) is die weerstandigheid van kankerselle teen verskeie klasse struktureel onverwante chemoterapeutiese geneesmiddels. MGR behels die veranderde membraan transport wat lei tot 'n laer sellulêre konsentrasie sitotoksiese geneesmiddels en speel dus 'n belangrike rol tydens kankerbehandeling. P-glikoproteïen (Pgp) is gelokaliseerd in die apikale oppervlakte van die epiteelselle in die dunderm en funksioneer as 'n biologiese skans deur toksiese bestandele en xenobiotika uit selle te verwyder (Lin, 2003:54). Pgp is 'n groep transport proteïene wat vinnig uitbrei en is betrokke in diverse fisiologiese prosesse wat insluit antigeen uitdrukking, geneesmiddel effluks vanuit kankerselle, bakteriële nutriënt opname en sistiese fibrose (Germann, 1996:928; Kerr, 2002:47). 'n Aantal geneesmiddels is reeds geïdentifiseer wat die vermoë het om die effekte van Pgp, multigeneesmiddel resistente proteïen (MRP1) en hul verwante proteïene tydens MGR om te keer. Die eerste MGR moduleerders wat tydens kliniese toetse ontdek en bestudeer is het definitiewe farmakologiese eienskappe besit, in so 'n mate dat die dosisse wat nodig was om MGR om te keer, geassosieer was met 'n onaanvaarbare hoë voorkoms van newe-effekte. Dit het daartoe aanleiding gegee dat meer aandag geskenk was aan die ontwikkeling van moduleerders, met 'n aanvaarbare sterkte, selektiwiteit en farmakokinetiese eienskappe, wat gebruik kan word by laer dosisse om MGR te verhoed. Verskeie bestaande MGR moduleerders (ook bekend as chemosensitiseerders), word tans klinies geëvalueer vir die behandeling van resistente tumore (Teodori *et al.*, 2002:385). **Doel:** Die doel van die studie was om die effek van geselekteerde flavenoïede (morien, galagnien, kaempferol en kwersetien) op die transport van 'n bekende Pgp substraat, Rhodamien 123 (Rho 123) oor rot jejenum te ondersoek en om die struktuuraktiwiteitsverwantskappe (SAR) van die geselekteerde flavenoïede vas te stel met verwysing na die inhibisie van Pgp. **Metode:** Morien, galagnien, kaempferol en kwersetien was bestudeer as potensiële moduleerders van Rho 123 transport, elk by 'n konsentrasie van 10 µM en 20 µM oor rot jejenum deur middel van Sweetana-Grass diffusie kamers. Hierdie studie was bidireksioneel uitgevoer, met twee selle wat die transport in die apikaal na basolaterale (AP-BL) rigting en twee selle wat die transport in die basolateraal na apikale (BL-AP) rigting gemeet het.

Die tempo van transport was uitgedruk as die waarneembare permeabiliteits koëffisiënt (P_{app}) en die mate van die aktiewe transport was uitgedruk deur die verhouding van BL-AP tot AP-BL te bereken. **Resultate:** Die BL-AP tot AP-BL verhouding wat bereken was vir Rho 123 met geen moduleerders teenwoordig nie, was 3.29. Morien het die BL-AP tot AP-BL verhouding verminder na 1.88 by 'n konsentrasie van 10 µM en na 1.49 verminder by 'n konsentrasie van 20 µM. Galagnien het die BL-AP tot AP-BL verhouding na 1.60 verminder by 'n konsentrasie van 20 µM. Hierdie twee flavenoïede se verlaging in die verhouding was statisties betekenisvol en inhibisie van aktiewe transport was aangetoon. Alhoewel die ander flavenoïede aktiewe transport van Rho 123 geïnhibeer het, was dit volgens die statistiese analise nie statisties betekenisvol nie. Die effek tussen die verskillende konsentrasies (10 µM en 20 µM) het aangetoon dat galagnien, kaempferol en kwersetien prakties betekenisvolle verskille volgens die berekende effekgroottes getoon het. Morien het egter geen prakties betekenisvolle verskille by die verskillende konsentrasies getoon nie. Wat SAR betref, was dit voorheen aangedui deur Boumendjel en mede-werkers (2002:512) dat die aanwesigheid van 'n 5-hidroksielgroep en 'n 3-hidroksielgroep sowel as 'n C2-C3 dubbelbinding nodig is vir sterk binding van die flavenoïed aan die nukleotied-bindings area (NBD) van Pgp. Al die flavenoïede wat ondersoek was het hierdie karakteristieke getoon. **Gevolgtrekking:** Die geselekteerde flavenoïede het almal inhibisie van aktiewe transport van Rho 123 getoon en behoort 'n effek te hê op die biobeskikbaarheid van Pgp substrate en ander aktiewe transporteerders. Hierdie studie beskryf die inhibisie van geselekteerde flavenoïede op Pgp aktiwiteit. Praktiese betekenisvolle verskille tussen dieselfde moduleerder by verskillende konsentrasies is ook waargeneem. SAR is ook geïdentifiseer wat die sterkte van inhibisie van flavenoïede aandui, gebasseer op die posisie van die hidroksielgroepe.

Sleutelwoorde: P-glikoproteïn, Rhodamien 123, morien, galagnien, kaempferol, kwersetien, struktuuraktiwiteitsverwantskappe, Sweetana-Grass diffusie kamers