THE EFFECT OF SELECTED METHOXY FLAVONOIDS ON THE IN VITRO EFFLUX TRANSPORT OF RHODAMINE123 USING RAT JEJUNUM

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ABSTRACT

Abstract: Many orally administered drugs must overcome several barriers before reaching their target site. The first major obstacle to cross is the intestinal epithelium. Although lipophilic compounds may readily diffuse across the apical plasma membrane, their subsequent passage across the basolateral membrane and into blood is by no means guaranteed. Efflux proteins located at the apical membrane, which include P-glycoprotein (P-gp, MDR1) and Multidrug Resistance-associated Protein (MRP2), may drive compounds from inside the cell back into the intestinal lumen, preventing their absorption into the blood. Intestinal P-gp is localised to the villus tip enterocytes, i.e. the main site of absorption for orally administered compounds and in close proximity to the lumen. P-gp is therefore ideally positioned to limit the absorption of compounds by driving efflux back into the lumen. Drugs may also be modified by intracellular phase I and phase II metabolizing enzymes. This process may not only render the drug ineffective, but it may also produce metabolites that are themselves substrates for P-gp and/or MRP2. Drugs that reach the blood are then passed to the liver, where they are subjected to further metabolism and biliary excretion, often by a similar system of ATP-binding cassette (ABC) transporters and enzymes to that present in the intestine. Thus a synergistic relationship exists between intestinal drug metabolizing enzymes and apical efflux transporters, a partnership that proves to be a critical determinant of oral bioavailability. **Aim:** The aim of this study was to investigate the effect of selected methoxy flavonoids (3-methoxyflavone, 5-methoxyflavone, 6-methoxyflavone and 7-methoxyflavone) on the mean ratio of Rhodamine123 (Rho 123) transport across rat intestine (jejunum) and to investigate structure activity relationships (SAR) of the selected flavonoids with reference to inhibition of P-gp. **Methods:** 3-Methoxyflavone, 5-methoxyflavone, 6-methoxyflavone and 7-methoxyflavone were evaluated at a concentration of 10 µM and 20 µM as modulators of Rho 123 transport across rat jejunum. The Sweetana-Grass diffusion cells were used to determine the transport of Rho 123. Each modulator was studied 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. Each modulators P_{app} ratio was then compared with that of the control. **Results:** 3-Methoxyflavone decreased the P_{app} ratio from 3.34 (control) to 1.66 (10 μM) and 1.33 (20 μM) and showed statistical significant differences. 7-Methoxyflavone decreased the P_{app} ratio to 1.94 (10 μM) and 1.55 (20 μM) but only showed a statistical significant difference at 10 μM. 5-Methoxyflavone decreased the P_{app} ratio to 2.41 (10 μM) and 1.71 (20 μM) and 6-methoxyflavone decreased the P_{app} to 3.03 (10 μM) and 2.49 (20 μM). Both 5- and 6-methoxyflavone showed no statistical significant differences from the control. The structure activity relationships with reference to P-gp inhibition clearly indicated that the C3 and C7 positioning of the methoxy-group on the A ring played a major role in the inhibition of Rho 123 transport. **Conclusion:** All the selected modulators showed inhibition of Rho 123 transport across the jejunum. This should affect the bioavailability of the substrates of P-gp and other active transporters. In summary, this study describe the inhibitory interaction of selected flavonoids with P-gp. Structure activity relationships were identified describing the inhibitory potency of the flavonoids based on methoxy groups positioning. The inhibitory potency results were 3-methoxyflavone > 7-methoxyflavone > 5-methoxyflavone > 6-methoxyflavone. **Keywords:** P-glycoprotein, Rhodamine 123, Sweetana-Grass diffusion cells, 3-methoxyflavone, 5-methoxyflavone, 6-methoxyflavone and 7-methoxyflavone

UITTREKSEL

Uittreksel: Talle orale geneesmiddels moet biologiese skanse oorkom om die teiken van werking te bereik. Die eerste en belangrikste skans om te oorkom, is die intestinale epiteel. Lipofiliese verbindings diffundeer geredelik oor die apikale plasmamembraan, maar hul deurgang deur die basolaterale membraan na die bloed is nie verseker nie. Effluksproteïene soos P-glikoproteïen (P-gp, MDR1) en multigeneesmiddelresistente geassosieerde proteïen (MRP2) wat in die apikale membraan geleë is, kan verbindings van binne die sel af na die intestinale lumen uitdryf en sodoende absorpsie van die verbindings deur die bloed verhoed. Intestinale P-gp is gelokaliseerd in die villus van die enterosiete wat in die nabyheid van die lumen is en ook die belangrikste plek van absorpsie vir oraal toegediende verbindings is. Geneesmiddels kan ook deur intrasellulêre fase I en fase II metaboliese ensieme gemodifiseer word, wat die afbraak van geneesmiddels na 'n onaktiewe vorm is en wat substrate van P-gp en/of MRP2 is. Verder word geneesmiddels wat die bloedstroom bereik na die lewer vervoer waar dit blootgestel word aan verdere metaboliese prosesse wat ooreenstem met 'n effluksproteïensisteem wat ook in die intestinum voorkom. Daar bestaan dus 'n sinergistiese verhouding tussen intestinale metaboliserende ensieme en die apikale efflukstransporteerders. **Doelwitte:** Die doel van die studie was om geselekteerde metoksiflavone (3-metoksiflavoon, 5-metoksiflavoon, 6-metoksiflavoon en 7-metoksiflavoon) se effek op die gemiddelde verhouding van Rhodamien123 (Rho 123) transport oor rotintestinum (jejenum) te bestudeer en om die struktuuraktiwiteitsverwantskappe (SAV) van die geselekteerde flavone ten opsigte van inhibering van P-gp te verklaar. **Metode:** 3-metoksiflavoon, 5-metoksiflavoon, 6-metoksiflavoon en 7-metoksiflavoon as moduleerders van Rho 123transport ge-evalueer by 'n konsentrasie van 10 µM en 20 µM. Sweetana-Grass diffusieselle is gebruik om die transport van Rho 123 oor die rotintestinum te bepaal. Elke moduleerder is bidireksioneel gemeet met die eerste twee selle as transport in die apikale na basolaterale (AP/BL) rigting en die laaste twee selle as transport in die basolaterale na die apikale (BL/AP) rigting. Die tempo van transport is as die waarneembare permeabiliteitskoëffisiënt (P_{app}) uitgedruk en die mate van aktiewe transport is uitgedruk deur die verhoudings van BL/AP en AP/BL te bepaal. Elke moduleerder se P_{app}-verhouding is dan met die kontrole vergelyk. **Resultate:** 3-Metoksiflavoon het die P_{app}-

CHAPTER 1

INTRODUCTION AND STATEMENT OF THE PROBLEM

Multidrug resistance (MDR) is a kind of resistance of cancer cells to multiple classes of chemotherapeutic drugs that can be structurally unrelated. The development of MDR by tumor cells is a major impediment to the success of cancer chemotherapy. P-glycoprotein (P-gp) and multidrug resistance protein (MRP1) are the most important and widely studied members of the family that belongs to the ATP-binding-cassette superfamily of membrane transorters (ABC transporters). P-gp, the multidrug resistance-associated protein (MRP) and the breast cancer resistance protein (BCRP) are members of the ABC transporters (Cole *et al.*, 1992:1651). These proteins are believed to function as energy-dependent efflux pumps for a variety of structurally diverse chemotherapeutic agents, thereby decreasing the level of intracellular drug accumulation. Consequently, tumor cells can evade the cytotoxic effects of drugs. The over expression of the P-gp confers MDR to cancer cells. This protein acts as an efflux pump for a number of commonly used cytotoxic agents, e.g., doxorubicin, vincristine, vinblastine, paclitaxel, colchicines, actinomycin D and mitomycin C (Endicott & Ling, 1989:137). Besides their role in cancer cell resistance, these proteins also have multiple physiological functions since they are also expressed in many important non-tumoural tissues and are largely present in prokaryotic organisms. A number of drugs have been identified which are able to reverse the effects of P-gp, MRP1 and sister proteins, on multidrug resistance. The first MDR modulators discovered and studied in clinical trials were endowed with definite pharmacological actions, therefore the doses required to overcome MDR were associated with unacceptably high side effects. As a consequence, much attention has been focused on developing more potent and selective modulators with proper potency, selectivity and pharmacokinetics that can be used at lower doses. Several novel MDR reversing agents (also known as chemosensitisers) are undergoing clinical evaluation for the treatment of resistant tumors (Teodori *et al.*, 2002:385).

This study is concerned with the reversing ability and structure activity relationship requirements of selected flavonoids on P-gp.

The aims of this study were to:

- study the effects of selected flavonoids (3-methoxyflavone, 5-methoxyflavone, 6-methoxyflavone and 7-methoxyflavone) on the transport of the P-gp substrate Rho 123 across rat jejunum;

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

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

CHAPTER 2
FACTORS EFFECTING ABSORPTION AND TRANSPORT OF DRUGS

2. Introduction

Oral administration is the most popular route for drug administration since dosing is convenient, non-invasive and most drugs are well absorbed by the gastrointestinal tract (Chan *et al.*, 2004:26). The small intestine is the principal site of absorption for any ingested compound, whether dietary, therapeutic or toxic. The permeability of a drug at the absorption site into the systemic circulation is dependent on the physical and biochemical properties of the cell membranes (Shargel & Yu, 1999:99). Systemic bioavailability may be influenced by a variety of factors which are shown in Table 2.1.

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

Physiological factors	Formulation factors	Pharmacokinetic factors	Physicochemical factors
Membrane transport	Crystal form	Gastro-intestinal metabolism	Lipophiliity
Gastro-intestinal motility	Particle size	Liver metabolism	Solubility
Stomach emptying	Absorption enhancers	Chemical instability	Degree of ionization
Disease state	Dissolution Rate	Absorption	Molecular size and shape
	Dosage form	Distribution	Hydrogen-bonding potential
		Elimination	

Membranes are a major structure in cells that surrounds the entire cell and act as a boundary between the cell and the interstitial fluids. Membranes enclose most of the cell organelles and act as a selective barrier for the passage of molecules. Cell membranes

are semi-permeable allowing water, some selected small molecules, and lipid-soluble molecules to pass through such membranes, whereas highly charged molecules and large molecules, such as proteins and protein-bound drugs, are excluded (Shargel & Yu, 1999:99).

For the absorption of drugs into the cell it must penetrate the cell membrane. This can be achieved either by trans- or paracellular absorption. Transcellular absorption is the process of a drug movement across the cell. Some polar molecules may however not be able to penetrate the cell membrane, but instead, pass through the gaps or tight junctions between the cells. This is a process known as paracellular drug absorption (Shargel & Yu, 1999:99). 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).

To understand the mechanism of absorption, it is important to have knowledge of the morphology of the small intestine, which is the main site for drug absorption.

2.1 Morphology of small intestine

The small intestine is the largest component of the digestive tract and the major site of digestion and absorption. The small intestine is divided into three anatomic divisions namely the duodenum, the jejunum and the ileum (Carr & Toner, 1984:1). In addition to receiving gastric chyme from the stomach, the initial segment of the small intestine, the duodenum, receives bile from the gall bladder and digestive enzymes from the pancreas. The pancreatic enzymes are produced in an inactive form and only become active in the lumen of the duodenum (University of Ottawa, 2005). It provides the environment for digestion and absorption, while its muscular layers propel the dietary residue to the large bowel where water is reabsorbed, leading progressively to the formation of the faeces (Carr & Toner, 1984:1).

2.1.1 Layers of the small intestine

The different layers of the small intestine are illustrated in Figure 2.1.

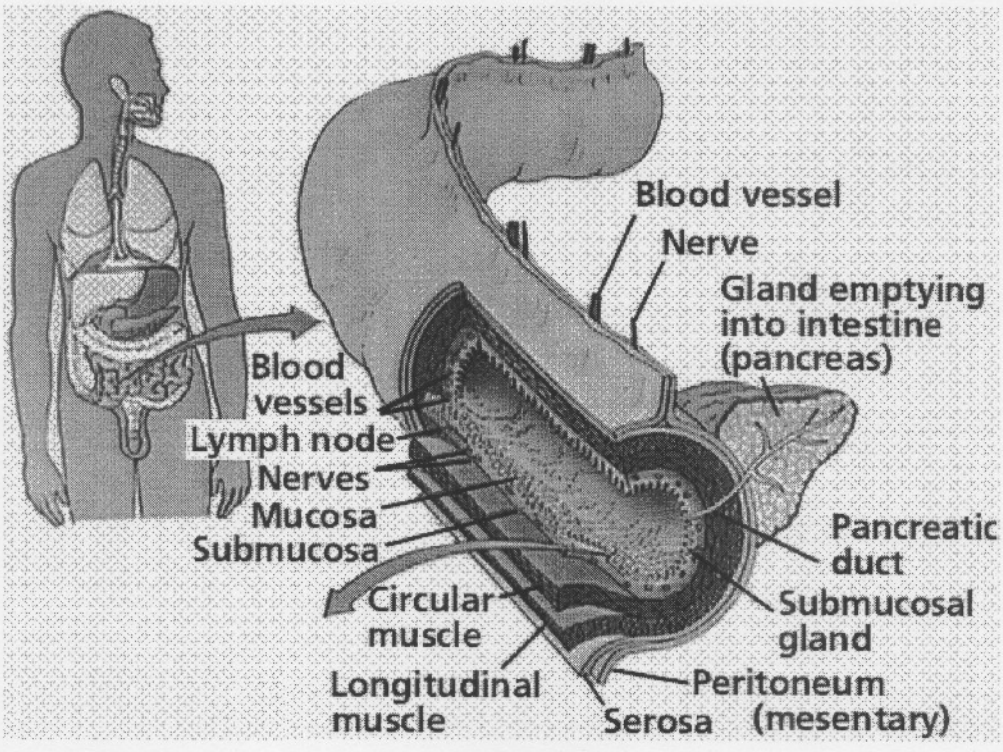


Figure 2.1: Layers of the small intestine (Garcia-Diaz, 2005).

2.1.1.1 Serosa

The serosa is a thin layer of connective tissue. It carries blood vessels and nerves to supply the intestine (Rogers, 1983:205).

2.1.1.2 Submucosa

The submucosa consists of dense connective tissue. Adipose cells may be present. Both the duodenum and the jejunum are characterised by modifications of the

submucosa. The ileum is also modified, but its modifications, Peyer's patches, arise from the lamina propria (University of Ottawa, 2005).

2.1.1.3 Mucosa

The mucosa of the small intestine is highly modified. The histological organisation of the mucosa is illustrated in Figure 2.2. The luminal surface is completely covered by a number of finger- or leaf like projections called villi. The core of a vilus is an extension of the lamina propria, and its surface is covered by a simple columnar epithelium layer.

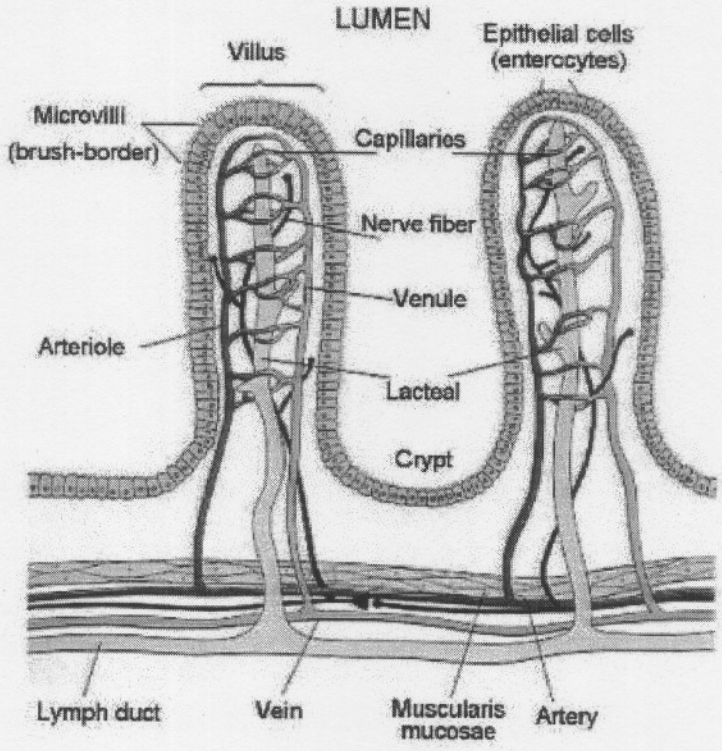


Figure 2.2: Histological organisations of the mucosa of the small intestine (Garcia-Diaz, 2005).

Opening towards the luminal surface at the bases of the villi are simple tubular structures called intestinal glands or crypt of Lieberkühn. The crypts extend downward toward the muscularis mucosae and the simple columnar epithelium lining is continuous with that covering the villi (University of Ottawa, 2005).

The predominant cell type of the epithelium is the enterocyte or absorptive cell. Each enterocyte has about 3000 microvilli at its luminal surface, which appear in the light microscope as the fuzzy striated border on the surface of the villi (University of Ottawa, 2005). The villi and microvilli increase the absorptive surface of the small intestine about 600 times. The epithelium of the small intestine consists of the following cell types:

- **Enterocytes or absorptive cells**. These are tall columnar cells with microvilli and a basal nucleus, specialised for the transport of substances. They are bound to one another and other cell types by junctional complexes. Amino acids and monosaccharides are absorbed by active transport while monoglycerides and fatty acids cross the microvilli membranes passively. Absorbed substances enter either the fenestrated capillaries in the lamina propria just below the epithelium or the lymphatic lacteal. Most lipids and lipoprotein particles follow the latter way. Enterocytes have a lifespan of about 5-6 days (University of Ottawa, 2005).

- **Goblet cells**. Small intestinal goblet cells are polarised, mucous secreting cells that are present throughout the epithelium with increased relative frequency from the proximal jejunum to the distal ileum (Trier & Madara, 1981:949). Goblet cells are found in the crypts, where they mature from stem cell precursors and migrate to the villus surface. They occur singly, their typical shape being determined by the pressure of surrounding enterocytes, balanced by the accumulation of secretion within the cell apex. A goblet cell is a unicellular gland, secreting mucus into the lumen for lubrication and protection (Carr & Toner, 1984:28).

- **Paneth cells**. Paneth cells are found only in the bases of the crypts of Lieberkühn. These cells have an oval basal nucleus and large, refractile acidophilic granules at their apical end. The granules contain the antibacterial enzyme lysozyme, other glycoprotein, an arginine-rich protein and zinc (an essential trace metal for a number of enzymes). Paneth cells also phagocytise some bacteria and protozoa and also may have a role in regulating intestinal flora. Paneth cells have a lifespan of about four weeks and are easy to identify with a light microscope (University of Ottawa, 2005).

- **Entero-endocrine cells**. In the intestine entero-endocrine cells are most often found in the lower part of the crypts but can occur at all levels of the epithelium. Their most abundant products are cholecystokinin (CCK), secretin and gastric inhibitory peptide (GIP). CCK stimulates pancreatic enzyme secretion and gall bladder contraction, secretin stimulates pancreatic and biliary bicarbonate secretion and GIP inhibits gastric acid secretion. These cells are not easily observed without special preparations (University of Ottawa, 2005).

- **M-Cells.** These cells overlie the apex of Peyer's patches (Carr & Toner, 1984:37). According to Owen and Jones (as quoted by Trier & Madara, 1981:953) Peyer's patches are aggregates of mucosal lymphoid follicles and may occur at any level of the small intestine, but are the largest and most abundant in the ileum. These cells are able to take up antigens from the gut lumen and pass them to the underlying lymphocytes. It has been proposed that they act as part of the afferent limb of the intestinal immune response (Carr & Toner, 1984:37).

- **Undifferentiated cells**. These stem cells are found only at the base of the crypts. These cells are responsible for the development of all the other cell types. A cell destined to be a goblet cell or enterocyte undergoes about 2 additional divisions after leaving the pool of stem cells and migrates from the crypt to the villus. It will be shed at the tip of the villus (University of Ottawa, 2005).

Within the lamina propria core of each villus is a lymphatic capillary called a lacteal, as well as numerous capillaries. The lacteal is accompanied by smooth muscle fibers arising from the muscularis mucosae. The smooth muscle in the villus allows it to contract intermittently, expelling the contents of the lacteal into the lymphatic network surrounding the muscularis mucosae. The lamina propria is very cellular, with numerous lymphocytes, plasma cells, macrophages and eosinophils. Lymphatic nodules which originate in the lamina propria may extend into the submucosa. The muscularis mucosae may be partially or totally disrupted by the nodules. The ileum is characterised by having large aggregates of lymph nodules, called Peyer's patches, in the submucosa (University of Ottawa, 2005).

For a drug to be absorbed it should pass across the cell membrane. The different mechanisms according to which a drug may be absorbed will therefore be discussed.

2.2 Passage of drugs across cell membranes

The absorption, distribution, biotransformation and excretion of a drug involves its passage across cell membranes (Benet *et al.*, 1996:3). 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, 1984b:53).

There are two principal routes by which compounds may cross the intestinal epithelium, namely paracellular or transcellular (Chan *et al.*, 2004:26). In general, the transport pathways can be divided into the following which is shown in Figure 2.3. (Chan *et al.*, 2004:26).

In Figure 2.3 **(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).

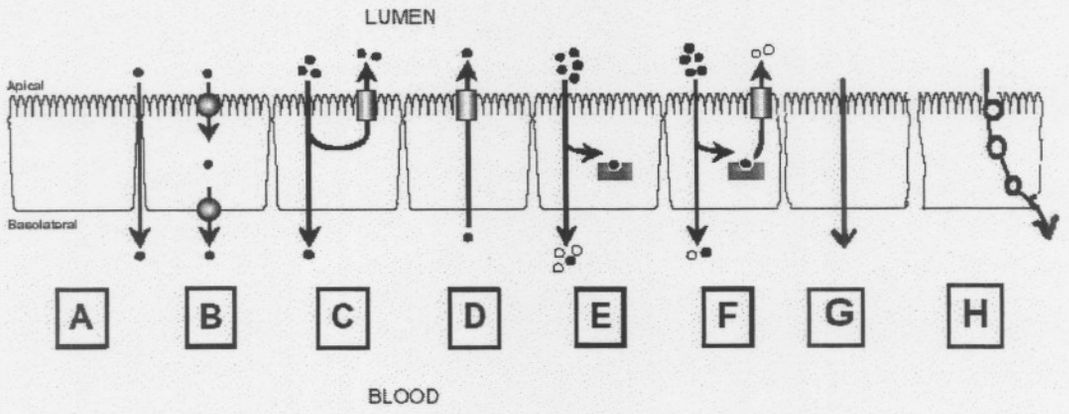


Figure 2.3: Diagram of pathways and mechanisms that mediate transepithelial transport (Chan *et al.*, 2004:26).

A. Paracelllar transport is the main route for transepitheal permeation of hydrophilic compounds: The difference between the electrical potential and hydrostatic pressure on opposite sides of the epithelium forms an electrochemical potential gradient. This gradient is the driving force for the aqueous extra cellular route across the epithelium (Hidalgo, 2001:388).

B. Carrier-mediated transporters (active transport): The first step during transcellular absorption is the crossing over of compounds from the lumen through the apical membrane. Compounds will then be transported across the cytosol to exit the basolateral membrane into the blood (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: P-glycoprotein (P-gp) and Multi Drug Resistance-associated Protein 2 (MRP2) limit the absorption of potentially toxic compounds. Active blood-to-lumen secretion of compounds is facilitated by these transporters. These apical efflux transporters are principally ATP (Adeno-Triphospate)-binding

cassette [ABC] proteins and act as the first line of defense against toxic compounds (Watkins, 1997:165).

E. Intracellular metabolising enzymes: Small intestinal enterocytes provide the first site for Cytochrome P450 3A4 (CYP3A4)-mediated metabolism of orally ingested drugs and xenobiotics. The transcellular route of absorption exposes drugs to intracellular metabolic systems (Watkins, 1992:520).

F. Apical efflux transporters and intracellular metabolising enzymes: The CYP3A4-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.*, 1992:520).

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

2.2.1 Passive Diffusion

Passive diffusion is the major absorption process for most drugs. This process is passive because no external energy is required for transportation of these drugs. The driving force for passive diffusion is due to higher drug concentrations on the mucosal side of the intestine compared to that on the side of the blood. According to Fick's first law of diffusion, passive diffusion is the process by which drug molecules diffuse from a region of high concentration to a region of low drug concentration. The following equation is used to determine the rate of diffusion:

$$\frac{dQ}{dt} = \frac{DAK}{h}(C_{GI} - Cp)$$

Where dQ/dt is the rate of diffusion; D is the diffusion coefficient; K is the lipid water partition coefficient of drug in the biologic membrane that controls drug permeation; A is the surface area of membrane; h is membrane thickness and $C_{GI} - Cp$ is difference

between the concentrations of drug in the gastrointestinal tract and in the plasma (Shargel & Yu, 1999:102)..

According to Fick's law of diffusion, several other factors may influence the rate of passive diffusion of drugs. For example, the degree of lipid solubility of the drug will influence the rate of drug absorption. The partition coefficient (K) represents the lipid-water partitioning of a drug across the hypothetical membrane in the mucosa. Drugs that are more lipid-soluble will have a larger value for K. Therefore drugs that are more lipid-soluble tend to penetrate cell membranes more easily than less lipid-soluble or water-soluble molecules, thus increasing the rate of diffusion (Shargel & Yu, 1999:102).

2.2.2 Active Transport

Active transport is a carrier-mediated transmembrane process that plays an important role in the gastrointestinal absorption and in renal and biliary secretion of many drugs and metabolites. Active transport is characterised by the transport of drug against a concentration gradient – that is, from regions of low drug concentrations to regions of high concentrations. Therefore, this is an energy-consuming system. In addition, active transport is a specialised process that requires 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 (Shargel & Yu, 1999:105).

The carrier molecule is highly selective for the drug molecule. A drug is likely to be actively transported by the same carrier mechanism if its structure resembles a natural substrate that is actively transported. Therefore, drugs of similar structure may compete for sites of absorption on the carrier. Furthermore, because only a fixed number of carriers are available, all the binding sites on the carrier may become saturated if the drug concentration is high. (Shargel & Yu, 1999:105).

2.2.3 Facilitated diffusion

Facilitated diffusion is also a carrier-mediated transport system that differs from active transport in that the drug moves along a concentration gradient (i.e., moves from a region of high-drug concentration to a region of low-drug concentration). Because the system is carrier mediated, it is saturable and structurally selective for drug and show competition kinetics for drugs of similar structure. In terms of drug absorption, facilitated diffusion seems to play a 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 therapeutic 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.

2.3 Cytochrome P450 3A4 (CYP3A4)

CYP3A4 plays an important role in the metabolism of drugs. The principal efflux mechanisms and intracellular metabolising enzymes in intestinal epithelia are given in Figure 2.4. CYP3A4 and P-gp are functionally interactive. For example, in the small intestine, CYP3A4 and P-gp are both localised to mature enterocytes on the villus tip, with P-gp expressed on the apical brush border membranes of villus enterocytes (Thiebaut *et al.*, 1987:7738), and CYP3A4 present in the endoplasmic reticulum just below the brush border membrane (Watkins, 1997:26,161). Expression of CYP3A4 is highest in the proximal intestine. This information has led to the hypothesis that CYP3A4 metabolises substrates inside the cell, with P-gp mediating their efflux, thus maximising the effectiveness of drug excretion in the tissue where they are co-expressed (Chan *et al.*, 2004:35).

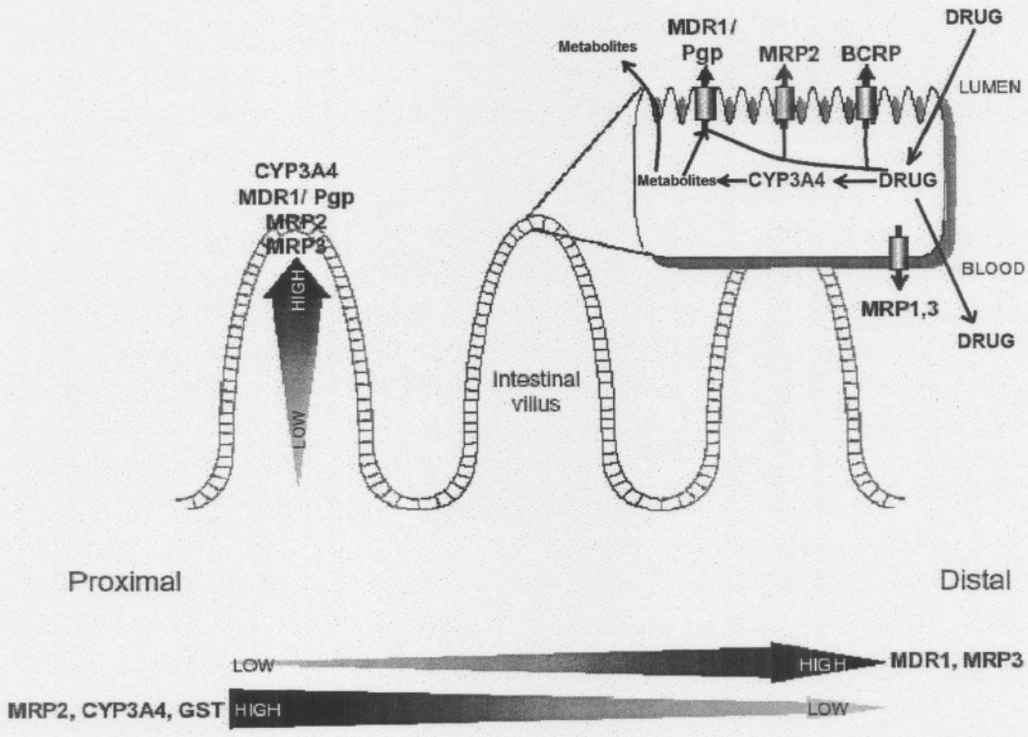


Figure 2.4: Principal efflux mechanisms and intracellular metabolising enzymes in intestinal epithelia (Washington, 2001:131; Chan *et al.*, 2004:33).

The inter-relationship of P-gp and CYP3A4 operates in a complex manner. Firstly, P-gp limits the total drug transport across the membrane to prevent saturation of CYP3A4 in the enterocytes (Washington *et al.*, 2001:131). Secondly, the duration of exposure of the drug to CYP3A4 in the enterocytes is increased by the slow drug absorption caused by P-gp, thus providing greater opportunity for metabolism. In addition the metabolites generated by CYP3A4 are substrates for P-gp. These metabolites are actively transported from the cell to the lumen by P-gp to prevent them from competing with the metabolism of the parent drug.

CYP3A4 and P-gp share a remarkable number of substrates and inhibitors which are shown in Table 2.2. It has been suggested that both P-gp and CYP3A4 are responsible for the poor bioavailability observed for many orally administered drugs (Benet *et al.*, 1996:39,143). It also appears that the physico-chemical properties may not be the only factors determining the bioavailability of an orally administrated drug. Gut metabolism

by CYP3A4 and countertransport by P-gp, or other active transporters, should also be taken into consideration (Wacher *et al.*, 2001:98).

Table 2.2: CYP3A4 substrates that also interact with P-glycoprotein (Wacher *et al.*, 1995:129-134).

P-gp Inhibitors	P-gp Substrates	P-gp Inhibitors and Substrates
Amiodarone	Dexamethasone	Cortisol
Erythromycin	Etopside	Cyclosporine
Felodipine	Rapamycin	Diltiazem
Itraconazole	Taxol	FK-506
Ketoconazole	Vinca alkaloids	Nicardipine
Lidocaine		Verapamil
Nifedipine		
Nitrendipine		
Progesterone		
Quinidine		
RU486		
Tamoxifen		
Terfenadine		
Testosterone		

2.4 ABC Transporters

The ATP(Adenosine-Triphospate)-binding cassette (ABC) transporter super family contains membrane proteins that translocate a wide variety of substrates across extra- and intracellular membranes, including metabolic products, lipids, sterols, and drugs (Dean *et al.*, 2002:1). Although the ABC transporters first characterized were nutrient uptake systems, it is now clear that many ABC transporters are exporters. Many of them participate in elimination of waste products or toxins from the cell. Although, in general, each ABC transporter is relatively specific for its own particular substrate(s), it is

remarkable that there is an ABC transporter for essentially every type of molecule that crosses cellular membranes (Higgins *et al.*, 2001:205).

Transmembrane arrangement of ABC transporters is given in Figure 2.5. The basic unit of an ABC transporter consists of four core domains. The general structure of ABC transporters comprises 12 Transmembrane Regions (TMS), split into two halves, each with a Nucleotide Binding Domain (NBD). This configuration is seen in P-gp and Multi Drug Resistant Transporters [(MDR)-1, (MDR)-3, (MRP)-4, (MRP)-5 and (MRP)-8] (Chant *et al.*, 2004:28). The TMS form the pathway through which a solute crosses the membrane and determine the specificity of the transporter through substrate-binding sites. The NBD's are hydrophilic and peripherally associated with the cytoplasmic face of the membrane (Higgins, 2001:208).

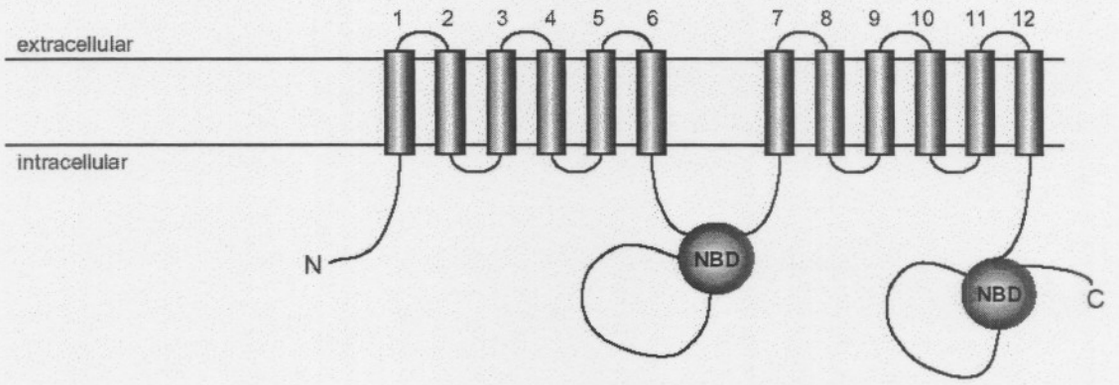


Figure 2.5: Transmembrane arrangement of ABC efflux proteins (Chan *et al.*, 2004:28).

Among the ABC transporters the best studied example is mammalian P-gp (Jones & George, 2000:5299). P-gp and other ABC transporters are proven to play a role in the absorption, distribution, metabolism and elimination which affect the therapeutic efficacy of some compounds (Brinkman *et al.*, 2001:837).

2.4.1 Multidrug resistant transporters (MDR)

The generally accepted basic mechanism of multidrug resistance is that the MDR proteins actively expel the cytotoxic drugs from the cells, maintaining the drug level below a cell-killing threshold. Drug extrusion mediated by these primary active transporters is driven by the energy of ATP hydrolysis. The most intriguing characteristic that distinguishes the MDR proteins from other mammalian transporters is their wide specificity. Unlike other, selective (classical) transporter proteins, multidrug transporters recognise and handle a wide range of substrates (Bodo *et al.*, 2003:134).

P-gp, a prototypical MDR protein, was originally identified as a 170-kDa glycoprotein abundantly expressed in MDR cells, that was later purified, cloned and found to mediate unidirectional ATP-dependent drug efflux. Pgp-MDR1 is a transporter for large hydrophobic, either uncharged or slightly positively charged compounds, while the MRP (multi drug associated proteins) family is mostly responsible for the transportation of hydrophobic anionic conjugates, and also extrudes hydrophobic uncharged drugs (Bodo *et al.*, 2003:134). The putative routes of carrier mediated drug transport are illustrated in Figure 2.6.

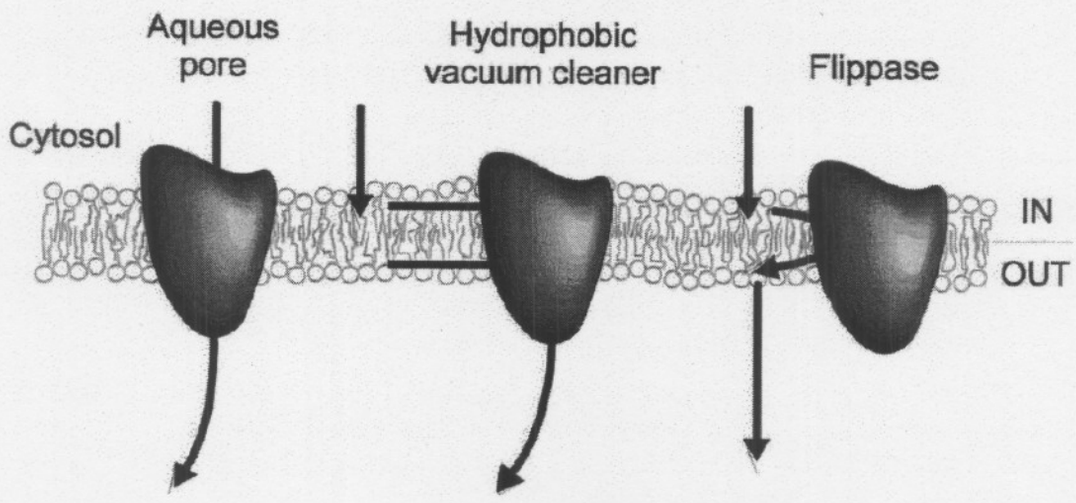


Figure 2.6: Putative routes of carrier mediated drug transport (Bolhuis *et al.*, 1997:70).

Several models have been postulated for the pump function of multidrug transporters to explain their broad specificity for chemically unrelated compounds. Conventional ideas about carrier mediated substrate transport comprise the initial capturing (binding) of substrates from the aqueous phase, followed by translocation across the lipid bilayer, release of the substrate into the aqueous phase at the trans site of the membrane, and re-orientation of the empty binding site(s) (Bolhuis *et al.*, 1997:70). Alternatively, multidrug transporters could recognize the lipophilic drugs by their physical property to intercalate into the lipid bilayer, and transport drugs from the lipid bilayer to the exterior (vacuum cleaner hypothesis), or from the inner leaflet to the outer leaflet of the lipid bilayer (flippase hypothesis).

2.4.2 P-glycoprotein (P-gp)

P-glycoprotein is an energy dependant drug efflux pump and belongs to the family of ATP binding cassette transport proteins. It is highly expressed in different types of tumor cells and on the apical surfaces of several epithelial cells (e.g., intestine) and endothelial cells (e.g., human brain capillary blood vessels that represent the blood-brain-barrier). P-gp is not only limited to humans but is also expressed in normal rat intestinal epithelium (Hsing *et al.*, 1992:879). P-gp transports different classes of substrates, like chemotherapeutic drugs, steroid hormones, HIV protease inhibitors and fluorescent dyes. Although these substrates differ considerably in chemical structures, the general feature of P-gp substrates is their relatively hydrophobic nature (van der Sandt *et al.*, 2000:207).

The importance of P-gp's ability to cause efflux transport and influence oral bioavailability of certain drugs is continuously being recognized (Wacher *et al.*, 2001:90).

2.4.2.1 Structure of P-gp

P-gp is composed of two blocks each containing six trans-membrane regions (TMS) and a site for binding ATP on each half. Analysis of the nucleotide and the deduced amino acid sequence of human P-gp 170 genes have provided strong evidence for the structure and function of the protein, P-gp 170. The gene products, which are approximately 1280 amino acids long and contain 2 homologous halves is joined by a linker region. Each half has six membrane-spanning domains that form three transmembrane loops and an intracellular nucleotide-binding site for ATP-binding and hydrolysis (Van der Heyden *et al.*, 1995:223).

A hypothetical 2-D model of human P-glycoprotein, based on hydropathy analysis of the amino acid sequence and its functional domains is given in Figure 2.7. In this diagram, each circle represents an amino acid residue, with blue solid circles showing the positions of mutations that alter the substrate specificity of P-gp (for clarity, not all mutations are shown). The NBD's sites are circled, with Walker A, B and the signature or C region indicated as A, B, and C, respectively (Ambudkar *et al.*, 2003:7476).

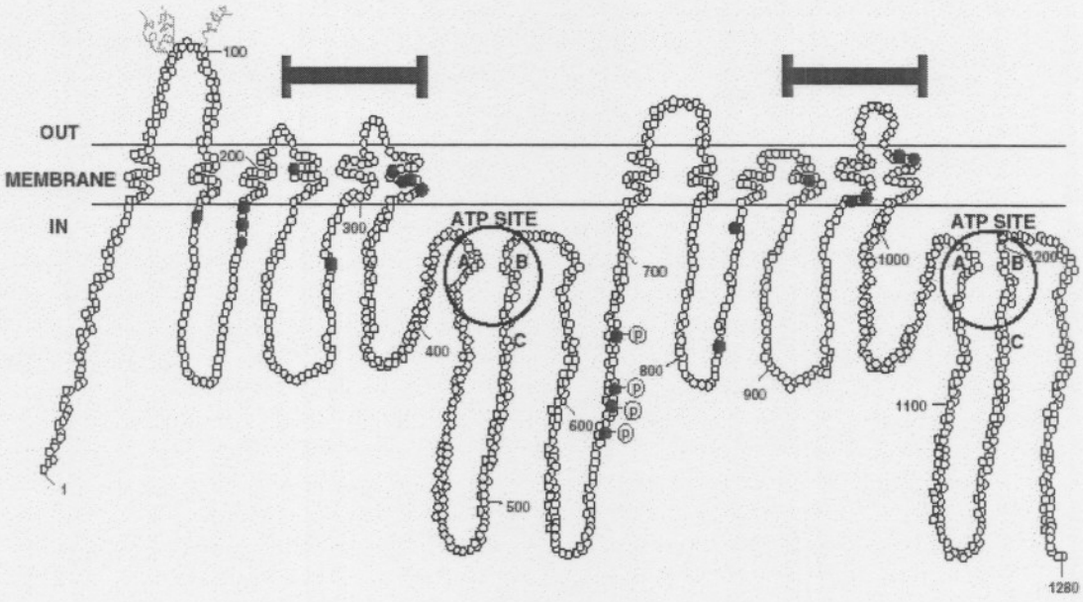


Figure 2.7: A hypothetical 2-D model of human P-glycoprotein, based on hydropathy analysis of the amino acid sequence and its functional domains (Ambudkar *et al.*, 2003:7476).

2.4.2.2 Mechanism of action

There is a big difference between conventional transporter proteins and P-gp. P-gp extracts its substrates directly from the membrane and will rather translocate a multitude of diverse compounds than exhibiting specificity for one substrate (Seelig & Landwojtowicz, 2000:31). Initial efflux mechanism of drugs by P-gp hypothesized that P-gp forms a hydrophilic pathway (pore). Substrates are shielded from the hydrophobic lipid phase because drugs are transported from the cytosol to the extracellular media through the middle of a pore (Johnstone *et al.,* 2000:1). Figure 2.8 illustrates possible mechanisms of action for drug efflux by P-gp.

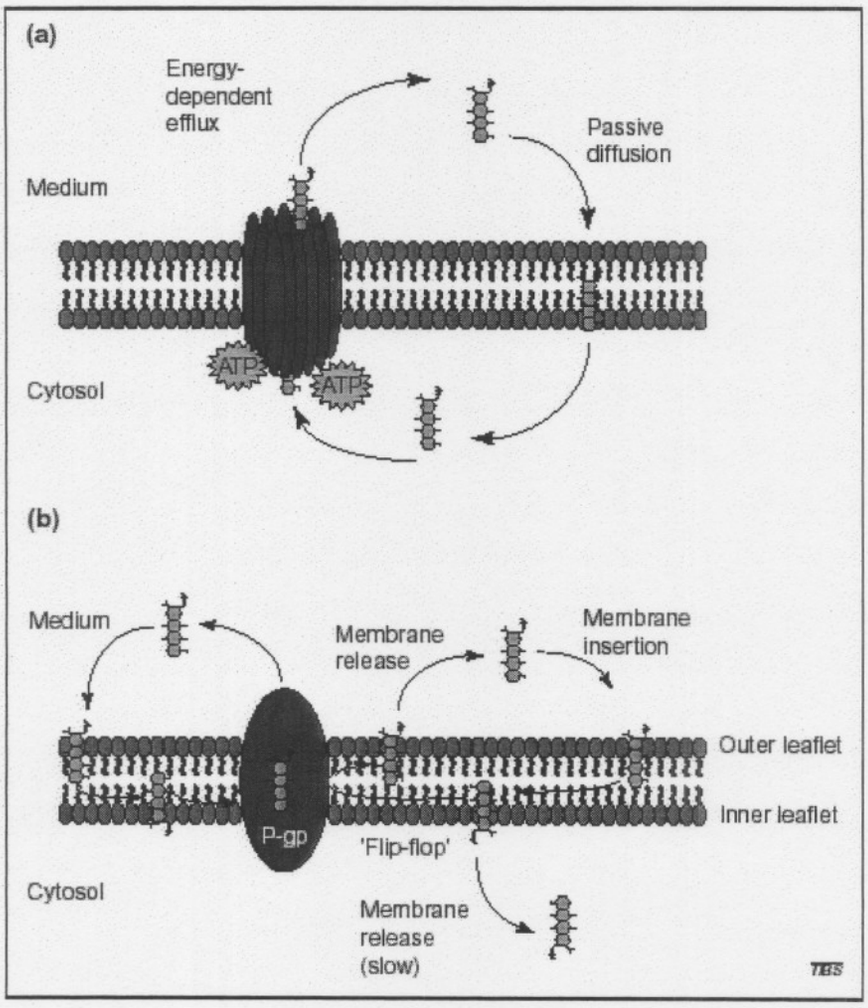


Figure 2.8: Two-model mechanism of action (Johnstone *et al.*, 2000:2).

Model (a) is the 'pump' model for drug transport. The three-dimensional structure of P-gp consists of a single drug-pore (shown in red). Chemotherapeutic drugs (green) diffuse through the lipid membrane and are transported in an ATP-dependent manner out of the cell by P-gp.

Model (b) is the 'flippase' model for drug transport. Before interacting with P-gp, a drug will interact with lipids of the membrane. The drug will immediately after interacting with P-gp be transported from the inner leaflet directly into the extracellular medium. In this model P-gp acts as a lipid translocase (Borges-Walmsley & Walmsley, 2001:77). Drugs are intercalated into the inner leaflet of the lipid bilayer. These drugs are 'flipped' into the outer leaflet and released into the extracellular space. Movement of drugs from the inner to the outer leaflet is a relatively quick process in comparison with the movement of drugs from the inner leaflet to the cytosol, which is a relatively slower process (Johnstone *et al.*, 2000:2).

There is still uncertainty regarding the mechanism of P-gp and the linking utilisation of ATP-derived energy to the mechanism (Borges-Walmsley & Walmsley, 2001:76). A detailed three-dimensional structure of the protein and additional biochemical studies must be conducted to attain further insight into the mechanism of drug transport (Borst & Schinkel, 1997:221).

2.4.2.3 Distribution of P-gp

Monoclonal antibody MRK16 was used to localise P-gp in normal human tissues. Most tissues examined revealed very little P-gp. However, P-gp was found in the:

- Liver in hepatocytes on the biliary canalicular form and on the apical (luminal or central canal) surface of epithelial cells in small biliary ductules;
- Pancreas on the apical surface of the epithelial cells of small ductules, but not in the larger pancreatic ducts;
- Kidney on the apical surface of epithelial cells of the proximal tubules;

- Colon and jejunum in high concentrations on the apical surfaces of superficial solemner epithelial cells, and in the
- Adrenal gland in high levels on the surface of cells in both the medulla and the cortex (Thiebaut *et al*,. 1987:7736)

In the small intestines of rats and mice, regional variation in P-gp expression has been demonstrated, with moderate P-gp expression found in the duodenum and jejunum and maximal expression in the ileum (Yumoto, 1999:153).

P-gp was also found in the sub-apical surface of the epithelium of the choroid plexus of the brain (which forms the blood-cerebrospinal fluid (CSF) barrier) as well as the luminal surface of the endothelium of blood capillaries of the brain (the blood-brain barrier) (Delph, 2000).

In mice, MDR mRNA expression levels increase dramatically during pregnancy and are expressed at extremely high levels in the gravid compared with the non-gravid uterus. This expression is specifically localized to the luminal surface of the secretory epithelial cells of the endometrium. Murine placental P-gp is present in the fetus-derived epithelial cells that make up the exchange border between the fetal and maternal blood circulation (Delph, 2000). P-gp is also expressed in the testis and ovaries of mice and in the steroid-producing endometrial glands of the pregnant uterus (Schinkel, 2003:17). P-gp expression was detected in the capillary endothelial cells of the 7th and 8th peripheral nerves of the guinea pig by immunohistochemical staining and Western blot analysis. Levels of immunoreactivity were similar to those in the brain. Somewhat lower levels of immunoreactivity were also found in the sciatic nerve (Delph, 2000).

P-gp has been found in normal bone marrow in hematopoietic stem cells and in peripheral blood mononuclear cells (PBMCs), mature macrophages, natural killer (NK) cells, antigen-presenting dendritic cells (DCs) and T- and B-lymphocytes (Gottesman *et al.*, 1993:395; Johnstone *et al.*, 2000:5).

2.4.2.4 Function of P-gp

The differential expression of P-gp in normal tissues and its conservation among species suggest that the protein may have distinct physiological roles associated with specialised cell functions.

Protection from drugs and toxins:

Expression of P-gp on the luminal surfaces of the epithelial cells of the small and large intestine, biliary ductules and proximal tubules of the kidney suggest a role in decreasing the absorption from the gut and/or the excretion of endogenous and exogenous hydrophobic amphipathic toxins. P-gp plays a role in the intestinal excretion and, hence, in the reduced bioavailability after oral ingestion of several drugs (Schinkel, 2003:18).

Expression in the capillary endothelial cells of the brain, nerves, testis and placenta suggest a barrier function to keep toxins out of the nervous system, gonads and fetus. (Gottesman 1996:615; Schinkel 2003:21). It has been noted that P-gp can be detected in human placental trophoblasts from the first trimester of pregnancy to full term, making it very likely that placental P-gp protects the developing embryo and fetus from toxic insult in humans as well (Delph, 2000).

P-gp has been found in hematopoietic stem cells and probably contributes significantly to the removal of drugs and toxins from the bone marrow. Pleuripotent hematopoietic stem cells from P-gp KO mice demonstrated markedly decreased rates of rhodamine 123 efflux compared with WT cells and confirming the substantial contribution of P-gp to the extrusion of drugs from the bone marrow (Schinkel 2003:18).

Steroid metabolism:

The presence of P-gp in the adrenal and in steroid-producing cells of the endometrium suggest it may also have a role in the handling of steroids, possibly providing a protective function for the plasma membranes of steroid-producing cells. Furthermore, it has been found that P-gp expressing epithelial monolayers of cells are able to transport steroids and that some lymphoid cells expressing P-gp are resistant to the cytotoxic effects of steroids (Gottesman, 1996:615).

Cholesterol metabolism:

P-gp appears to have a role in cholesterol metabolism. Cholesterol esterification is one of the mechanisms that cells use to control the amount of free cholesterol, which is toxic to cells. Under conditions of excess cholesterol, cholesterol is transported from the plasma membrane, where 90% of it resides, to the endoplasmic reticulum (ER), where it is esterified by acyl-CoA:cholesterol acyl transferase (ACAT). The rate of cholesterol esterification is limited by the availability of cholesterol substrate in the ER, rather than by ACAT.

Within a given cell type, greater expression of P-gp was correlated with increased esterification of plasma membrane cholesterol. P-gp functions to increase esterification of cholesterol derived from plasma membrane by facilitating the movement of cholesterol from the plasma membrane to the ER. The exact mechanism by which P-gp does this in not known, but expression of P-gp does not seem to confer any change in cholesterol content of plasma membrane as examined with cholesterol oxidase (Delph, 2000).

Immune system:

Immune responses in a peripheral organ like skin are initiated when antigen-presenting cells, especially dendritic cells (DCs), capture antigens locally. The DCs then migrate via lymphatic vessels to draining lymph nodes where they select T lymphocytes that bear receptors for the presented antigen. *In vitro* models have demonstrated that P-gp facilitates this migration of DCs and that in the presence of P-gp antagonists, DCs are retained in the epidermis (Delph, 2000).

There is also evidence that P-gp may be involved in the transport of some cytokines (CKs), particularly interleukin-1 (IL-1), IL-2, IL-4 and interferon-gamma (IFN-y) out of activated normal lymphocytes. However, P-gp does not seem to transport IL-6. The biological importance of P-gp to CK secretion during an immune response is still to be clarified (Johnstone *et al.*, 2000:4).

Chloride channel:

P-gp does not seem to have intrinsic channel activity, but may regulate an endogenous chloride channel which is yet to be identified. The physiological importance of this indirect modulation of chloride channels remains controversial (Gottesman, 1998:55).

Cytochromes:

Many of the same drugs that are transported by P-gp are metabolized 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. Human variation in CYP3A expression is believed to influence drug response for up to one-third of all drugs (Gottesman, 1998:55).

2.4.2.5 P-gp Substrate interaction

It has been shown that the apparent cut-off for penetration into the brain at a molecular mass of 700 Da is another consequence of P-gp action. The presence of P-gp in the blood-brain barrier may explain many (if not all) exceptions to the rule that the penetration of drugs into the brain tends to increase with increasing hydrophobicity. Large drugs are merely good P-gp substrates (Borst & Schinkel, 1996:989).

The passive permeability of substrates to the apical membrane, its affinity for the active transport site and the maximal capacity of P-gp contained in the apical membrane are factors that determine the permeability of a substrate for P-gp through a membrane. This is illustrated in Figure 2.9.

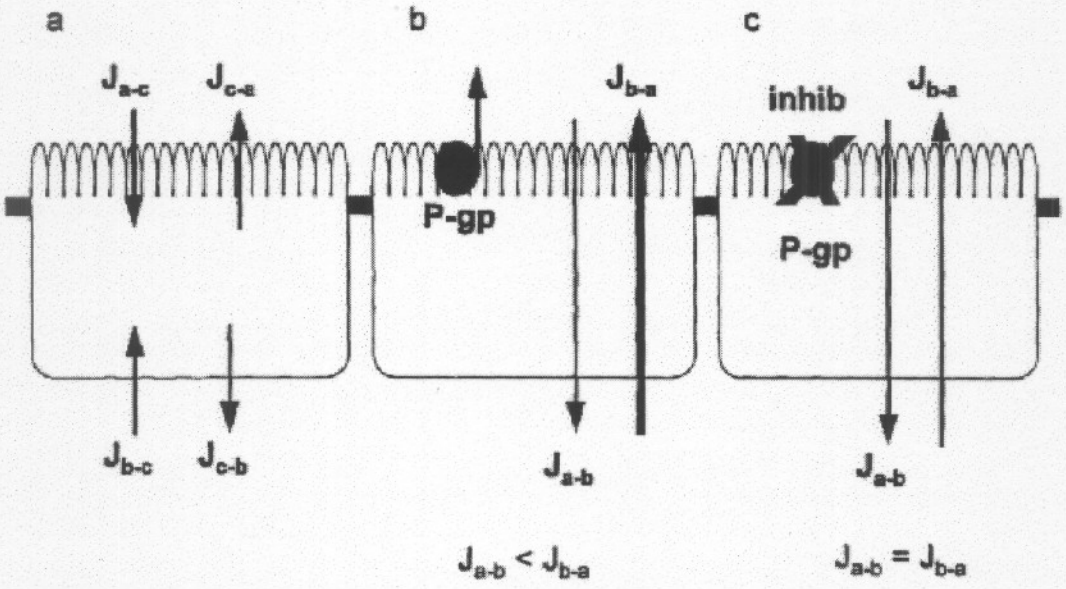


Figure 2.9: Model of p-gp substrate interaction (Hunter & Hirst, 1997:141).

(a) Shows transepithelial permeabilities for a drug, with J_{a-b} (shown in (b)) the sum of J_{a-c} and J_{c-b} and J_{b-a} (shown in (b)) the sum J_{b-c} and J_{c-a}. (b) shows that in the presence of P-gp, J_{c-a} is increased as the drug is actively pumped out across the apical membrane, illustrated by increased secretory flux, J_{b-a}. This active removal of drug results in reduced J_{a-b}, i.e., reduced absorption. (c) Shows inhibition of P-gp activity, resulting in a decrease in J_{b-a} as efflux (J_{c-a}) is reduced, while absorption is no longer limited by secretion, such that J_{a-b} increases (Hunter & Hirst, 1997:142).

2.4.2.6 The ATPase activity of P-glycoprotein

Although we know that both ATP sites are needed for this activity to occur efficiently, and that the drugs themselves stimulate the ATPase activity, very little is known about how the energy of ATP is harnessed in this transporter (Hunter & Hirst, 1997:134).

Hrycyna *et al* (1998:16634) have demonstrated that both ATP sites of human P-gp are capable of hydrolyzing ATP, but that this hydrolysis does not take place simultaneously. Thus, it has been proposed that hydrolysis takes place by an alternating site mechanism.

2.4.2.6.1 Alternating two-site transport model

The alternating two-site transport model is shown in Figure 2.10. The six-helix membrane domain is shown as a cylinder, the drug as the small circle with the wedge missing and the nucleotide-binding sites (NBS) as oval shapes. The transporter operates like a two-cylinder engine, with transport catalyzed by each half of the transporter alternately. Each six-helix membrane domain of the transporter can expose a high-affinity binding site intracellularly (upwards) and a low-affinity binding site extracellular (downwards), and ATP hydrolysis is used to drive the transporter between these alternate, mutually exclusive, conformations. In the absence of ATP, one membrane domain exposes the high-affinity site intracellularly, whilst the other domain exposes the low-affinity site extracellular. Drugs can bind to both the high and low-affinity sites in the absence of ATP. During drug efflux, ATP binds to the NBS of one-half of the transporter, triggering interaction of the drug with the intracellular facing high-affinity binding site (Steps 1-2). ATP is hydrolyzed, occluding the drug binding site (Steps 2-3), and release of product phosphate leads to exposure of the low affinity extracellular facing site on the same domain, from which the drug can be released (Steps 3-4). As a result of the bound ADP, and co-operative interactions between the two NBSs, ATP is more likely to bind to the unliganded NBS. This probably induces a further conformational change (Steps 4-5), so that the membrane domain associated with the liganded NBS exposes a high affinity intracellular facing drug binding site. The drug can bind to this site and is occluded as the ATP is hydrolyzed (Steps 5-6). As product phosphate is released, the low affinity site is exposed extracellular and the drug is released (Steps 7-1). The release of the drug and ADP allows the transporter to return to its original conformation, a process that might be accelerated by the binding of more ATP to the alternate NBS (Borges-Walmsley & Borges, 2001:77).

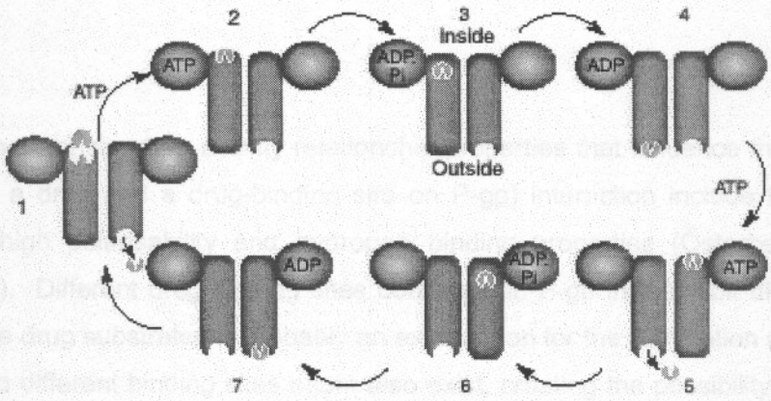


Figure 2.10: Alternating two site transport model (Borges-Walmsley & Borges, 2001:77).

2.4.2.7 Compounds interacting with P-gp

Although it is evident the substrates for P-gp are of large molecular weight, amphipathic and contain at least one aromatic ring, there is little information available regarding the structure-activity relationships of P-gp (Wacher *et al.,* 1998:1323). Substrates, inhibitors, substrates and inhibitors and enhancers are the four classes of compounds that interact with P-gp. Excluded from this list are a large number of hydrophobic natural products (derived from plants, or micro-organisms), semi-synthetic analogs of such products and synthetic organic compounds. Examples of compounds that interact with P-gp are given in Table 2.3. They are amphipathic compounds that are preferentially soluble in lipid (Gottesman & Pastan, 1993:386; Wacher *et al.*, 1995:131).

Translocation of drugs is an ATPase-dependent function. The translocation of drugs can therefore be influenced by their ATPase profile. The function of P-gp can be modulated in a bimodal fashion by some drugs. Some modulators can stimulate or inhibit ATPase activity in a concentration-dependent fashion. In this process the bimodal profile of a drug is complicated even more because of the additional modulators. Stimulation of ATPase function is seen at low drug concentrations whilst inhibition is demonstrated at higher concentrations. Some modulators in contrast display only inhibition regardless of concentration (Sharom *et al.,* 1999:329).

Other important structure-activity relationship properties that influence the complexation (between a drug and a drug-binding site on P-gp) interaction include large molecular surface, high polarisability and hydrogen binding properties (Österberg & Norinder 2000:295). Different drug binding sites contained in P-gp that exhibit different affinities toward the drug substrates is probably an explanation for the stimulation and inhibition of P-gp. Two different binding sites might also exist, creating the possibility for a substrate to interact with one or both of them. This might also lead to a positive coöperative interaction between different drug binding sites. The effect of P-gp activity induction might therefore become saturated at high concentrations of substrates and inhibition of P-gp, than seemed evident (Sharom *et al.*, 1999:336).

Table 2.3: Compounds that interact with P-gp (Wacher *et al.*, 1995:131).

Substrates	Inhibitors	Substrates/Inhibitor	Enhancers
Chemotherapeutic Agents	**Calcium-channel blockers**	**Calcium-channel blockers**	**Flavonoids**
Etoposide	Felodipine	Diltiazem	Kaempferol
Doxorubicin	Nitrendipine	Nicardipine	Quercetin
Paclitaxel	Nifedipine	Verapamil	
Vinblastine	**Antiarrhythmics**	**Hormones**	
Vincristine	Amiodarone	Hydrocortisone	
Vindesine	Lidocaine	**Immunosuppressants**	
Hormones	Quinidine	Cyclosporine	
Dexamethasone	**Antifungals**		
17-β Estradiol glucoronide	Itraconazole		
	Ketoconazole		
	Hormones		
	Progesterone		
	Testosterone		
	Other		
	Erythromycin		
	Tamoxifen		
	Terfenadine		

2.4.2.7.1 Rhodamine 123

Rhodamine 123, a lipophilic cation, constitutes a typical P-gp substrate and is subject to P-gp extrusion through the plasma membrane (Hirsch-Ernst *et al.*, 2001:48). Studies in a P-gp knockout mice model showed an increase in the uptake of Rho 123 into the brain in mdr1a (–/–) mice, which lack P-gp at the level of the blood-brain barrier, while in mdr1a (+/+) mice the uptake was a factor 3-4 times lower. However, although Rho 123 is often used to study the functionality of P-gp it seems not to be a specific P-gp substrate. Thus, other transporters may play a role in the efflux of Rho 123 across the intestinal membrane (van der Sandt *et al.*, 2000:208).

2.4.3 Multidrug Resistance-associated Protein, MRP

MRP (Multidrug resistance-associated protein) is another membrane transporter associated with drug efflux from drug resistant tumour cells. To date, multidrug resistance in model systems is known to be conferred by two different integral membrane proteins, the 170 kDa P-gp and the 190-kDa multidrug resistance associated protein (MRP) (Cole *et al.*, 1992:1650). These proteins belong to the ATP-binding casette proteins (ABC) or traffic ATPase (Higgins *et al.*, 1992:20).

MRP is expressed in most tissues and cell types and may be overexpressed in tumor cells. It probably has a six + six membrane spanning topology similar to P-gp. However, it also possesses an additional *N*-terminal amino acid sequence that has no counterparts in P-gp (Quan *et al.*, 2000:197). Substrate specificity of MRP is overlapping but distinct from that of P-gp. Some examples of compounds known to interact with MRP in drug resistant cells are given in Table 2.4.

Table 2.4: Compounds interacting with MRP in MDR cells (Aungst, 1999:110).

Calcein	Leukotrine C4
Duanorubicin	Probenecid
Doxorubicin	Rhodamine 123
Estradiol-17β-glucoronide	Vincristine
Indomethacin	Dinitrophenyl glutathione

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 P-gp. 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.*, 1996:953).

2.5 Conclusion

Chemotherapy is the treatment of choice in many malignant diseases. A major form of resistance against a variety of the anti-neoplastic agents currently used involves the function of a group of membrane proteins that extrude cytotoxic molecules, thus keeping intracellular drug concentration below a cell-killing threshold. The medical significance of ABC proteins exceeds their role in cancer therapy resistance; the transport function of several members was found to hinder the effective therapy of many other widespread diseases (e.g. malaria, AIDS), and inherited diseases were also linked to mutations in these genes. The transport activity of ABC proteins has an important effect in general pharmacology, that is, in modulating the absorption, distribution and excretion of numerous pharmacological compounds (Sigma Aldrich, 2005).

Prevention of clinical MDR should significantly improve the therapeutic response in a large number of cancer patients. One way to prevent MDR may be to develop anticancer agents that do not interact with any of the multi-drug transporters. However, as cytotoxic drugs must penetrate the cell membrane, and the MDR proteins have an extremely wide recognition pattern, this seems to be a remote possibility. There are several suggested methods to prevent the expression or function of multi-drug transporters, but pharmacological modulation seems to be the first choice at present (Sigma Aldrich, 2005).

In an attempt to find naturally occurring dietary compounds which may stimulate the P-gp-mediated efflux of carcinogens, it has been found that certain flavonols are potent stimulators of the P-gp-mediated efflux of 7,12-dimethylbenz(a)-anthracene. The increased efflux decreased the cellular burden of 7,12-dimethylbenz(a)anthracene. Since these flavonol compounds are widely distributed in fruits and vegetables, their inhibitory effect on P-gp may be a mechanism relevant to carcinogenesis and the observed lowered cancer risk in humans with higher dietary intake of fruits and vegetables (Phang *et al.*, 1993:245977).

CHAPTER 3

SELECTED FLAVONOIDS AND THEIR POTENTIAL STRUCTURE ACTIVITY RELATIONSHIPS

3.1 Introduction

Different compounds have been shown to reverse the P-gp-mediated MDR, which also include calcium channel antagonists such as verapamil and immunosuppressants such as cyclosporine A. MDR cells can be sensitised to anticancer drugs when treated with a P-gp inhibitor, which is known as a chemosensitiser. Because the classical first-generation chemosensitisers have side effects at the doses required for clinical effectiveness, there has been a great deal of interest in the development of second-generation chemosensitisers with a higher selectivity and potency. The search for chemosensitisers which have the advantages of being non-transportable inhibitors without side-effects initiated research in flavonoids derived from plants (Choi *et al.*, 2004:672).

3.1.1 Flavonoids

Flavonoids are a class of secondary plant phenolics with significant antioxidant and chelating properties. In the human diet, flavonoids are concentrated in fruits, vegetables, wines, teas and cocoa. Flavonoids occur in food primarily as glycosides and polymers that are degraded to variable extents in the digestive tract. Flavonoids in the human diet may reduce the risk of various types of cancer, especially hormone-dependent breast and prostate cancer. Flavonoids have the ability to inhibit lipid peroxidation, chelate redox-active metals and attenuate other processes involving reactive oxygen species and these characteristics are responsible for their cardio-protective effects. The structure of the flavonoids is based on the flavan nucleus. The number, position and types of substitution may influence the flavonoid activity (Heim *et*

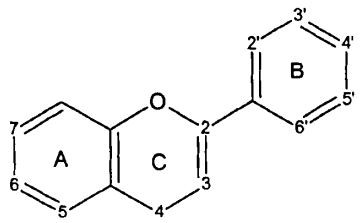
al., 2002:572). The structure-activity relationship of flavonoids is extensively studied to provide an inspiration for a rational drug and/or chemopreventive agent design of future pharmaceuticals (Hodek *et al.*, 2002:1).

3.1.2 The basic flavonoid structure

The chemical nature of the flavonoids depends on structural class, degree of hydroxylation, other substitutions and conjugations and degree of polymerisation. The structure of the flavonoids is based on the flavan nucleus (Figure 3.1a), which consists of three phenolic rings referred to as the A, B end C rings. The benzene ring A is condensed with a six-member ring (C), which carries a phenyl benzene ring (B) as a substituent in the 2-position. Ring C may be a heterocyclic pyran which yields flavanols (catechins) and anthocyanidins, or pyrone, which yields flavonols, flavones and flavanones. 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 basic structure of the flavonoid nucleus allows for a multitude of substitution patterns in the A, B and C rings, resulting in various subgroups. The flavonoids are divided into classes according to their oxidation level on the C-ring, which include anthocyanidins, flavanols (catechins), flavonones, flavonols, flavanones and isoflavonoids which are shown in Figure 3.2. Flavones and flavonols have been identified in almost all plants and the ones found most frequently being those with B-ring hydroxylation in the C-3' and C-4' positions (Aherne & O`Brien, 2002:75).

3.1a



3.1b

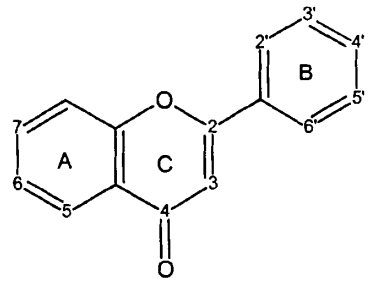
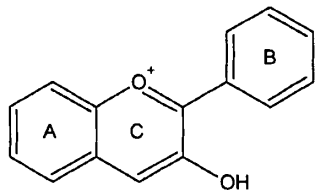


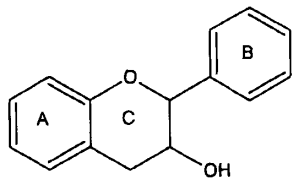
Figure 3.1: (a) Flavan nucleus and (b) 4-oxo-flavonoid nucleus (Aherne & O`Brien, 2002:75).

3.2 Modulation by flavonoids of MDR

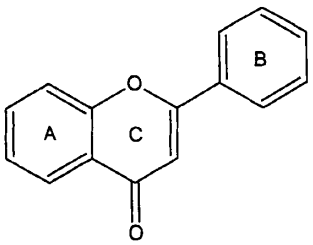
It has been reported by Phang and co-workers (1993:5979) that the active flavonoids, kaempferol, quercetin and galangin have the unique property of stimulating drug transport by P-gp *in vivo*. Since flavonoids are abundant in food, it is important to understand their effects on the function of P-gp, because of the implications for cancer chemotherapy (Shapiro & Ling, 1997:587).



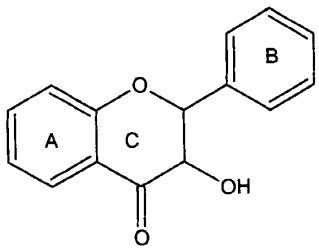
Anthocyanidins



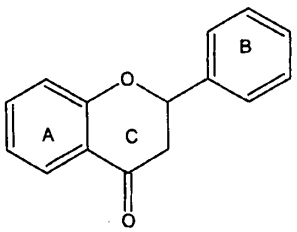
Catechins



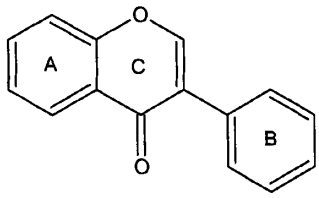
Flavones



Flavonols



Flavonones



Isoflavones

Figure 3.2: Classes of flavonoids according to oxidation level (Aherne & O'Brien, 2002:76).

Among others, several members of one group of natural compounds, the so-called (iso) flavonoids, were found to be able to modulate P-gp- and MRP-mediated drug transport in tumor cell lines. These compounds are important constituents of our daily food, since they are metabolic products of plant species, such as vegetables, red wine and tea (Hooijberg, 1997:344).

It has also been shown that certain (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 flavonoids affect MRP-mediated transport of anticancer drugs by a direct interaction with MRP (Hooijberg, 1997:347).

The inhibitory activity of the flavonoids is dependent on the transport model used, the model substrate, the inhibitor and the concentration of the inhibitor used. It has been shown that quercetin (10mg/kg) increases the bioavailability of moxidectin in sheep (Dupuy *et al.*, 2003:377), that quercetin (10μM) increases the activity of P-gp in mouse brain capillary endothelial (MBEC4) cells and that quercetin (50μM) decreases the activity of P-gp in MBEC4 cells. It has also been shown that chrysin (10-50μM) increased the uptake of vincristine into MBEC4 cells (Mitsunaga *et al.*, 200:199). It was found that quercetin and morin (20μM) had no effect on the uptake of vincristine into K562 (human myelogenous leukemia) and K562/ADM (adriamycin-resistant human myelogenous leukemia) cells (Ikegawa *et al.*, 2002:92).

3.3 Structure-activity relationship between P-gp and flavonoids

Flavonoid compounds were shown to interact with recombinant NBD2 in a bifunctional manner, by partly overlapping both sites. However, differences in binding affinities were observed between the various classes of flavonoidic compounds. Flavones and flavonols display better affinities than corresponding isoflavones and flavanones (Conseil *et al.*, 1998:9831). On the other hand, the introduction of a hydrophobic isoprenoid or alkhoxy substituents on chalcones further improve binding affinity. Silybin (3-hydroxy flavanone) exhibited good binding affinity towards NBD2 according to Maitrejean and co-workers (2000:157) due to extensive modification of ring B.

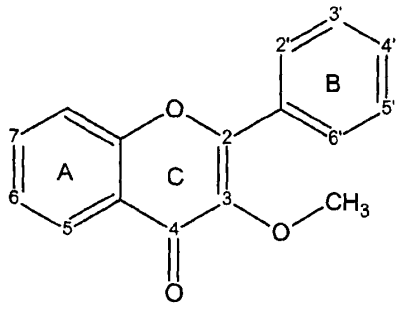
Glycosylation, as well as, the presence of several hydroxyl groups and/or addition of methoxy-groups results in drastic decrease in their inhibitory activities. Based on the observation that catechins had no effect on the CYP3A4 enzyme activity, the oxo-group (position C4) in the C ring is also an essential factor for the enzyme inhibitions (Hodek *et al.*, 2002:11).

Studies of structure-activity relationships have concluded that flavones are more efficient than isoflavones, while flavonols and chalcones are even more active. The relative activity of flavonoids with respect to P-gp inhibition, are as follows: flavonols > flavones > isoflavones. Studies showed that hydroxyl groups at positions 3 and 5 are essential for

high-affinity binding to P-gp. In a study conducted with chalcones, it has been shown that the prescence of a hydrophobic substituent on the B-ring considerably enhanced the binding affinity (Boumendjel, 2001:76).

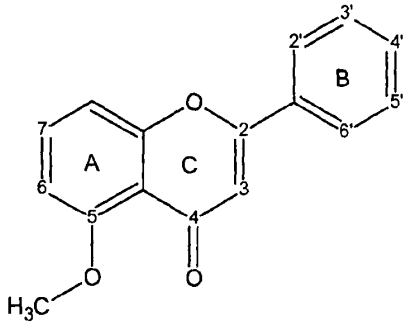
The flavonoids that will be investigated in this study for their structure-activity relationship are given in Figure 3.3.

(a)



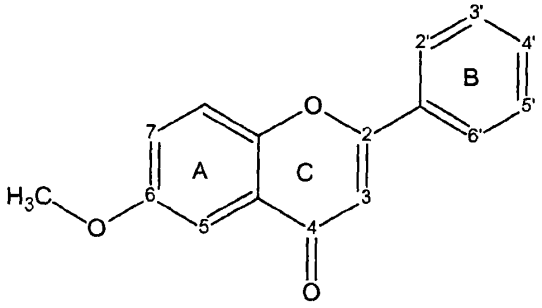
3-methoxyflavone

(b)



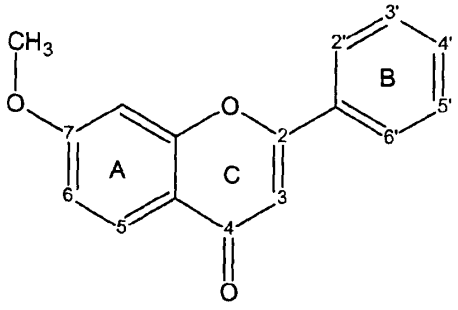
5-methoxyflavone

(c)



6-methoxyflavone

(d)



7-methoxyflavone

Figure 3.3: Flavonoids investigated for their structure-activity relationship.

3.4 Conclusion

Different ways have been undertaken to restore chemosensitivity of the MDR tumor cells to cytotoxic drugs. Besides attempts to discover anticancer drugs (which would not be transported substrates for P-gp) through maintaining a sufficient activity towards their molecular target, another pharmacological approach consists of trying to inhibit P-gp pump functioning sufficiently in order to restore cell sensitivity to anticancer drugs. It has been demonstrated by Boumendjel and co-workers (2001:75) that flavonoids are efficient P-gp inhibitors. Flavonoids bind to cytosolic sites which partly overlap the ATP-binding site and the modulators interacting region (Boumendjel *et al.*, 2001:75).

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

CHAPTER 4

EXPERIMENTAL PROCEDURE

4.1 Introduction

In a trial study conducted with Rho123 transports across isolated jejunum segments mounted in Sweetana-Grass diffusion cells, it was found that the serosa had to be removed in order for the Rho123 to be transported. One of the problems that are faced with this model system is that the presence of the serosa and muscle layers may prevent or decrease the permeability of lipophilic drugs. Another problem is tissue viability, especially in cases where it is necessary to apply stripping of some of the layers in order to facilitate permeability of drugs (Hattingh, 2002:48).

P-gp is expressed in the rat intestine and reduces apparent intestinal epithelial penetration of many drugs from the lumen to the blood. The contribution of other efflux transporters limiting the intestinal permeability of drugs cannot be ruled out (Terao *et al.*, 1996:1088).

The effect of different methoxy flavonoids on the transport of Rho123 across rat intestine was investigated using a vertical diffusion cell system (Figure 4.1), comprising four Sweetana-Grass diffusion cells, one heating block and one gas manifold (Corning Costar Corporation, Cambridge, USA).

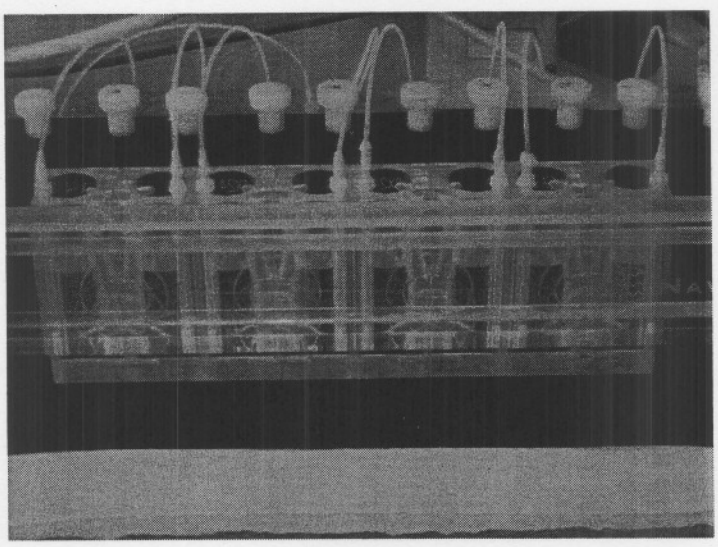


Figure 4.1

4.2.1 Materials

Krebs-Ringer bicarbonate buffer, Rho123, 3-methoxyflavone, 5-methoxyflavone, 6-methoxyflavone and 7-methoxyflavone (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 Rho123, 3-methoxyflavone, 5-methoxyflavone, 6-methoxyflavone and 7-methoxyflavone and Krebs-ringer bicarbonate buffer are presented in Appendix C.

4.2.2 Tissue preparation

3-Methoxyflavone, 5-methoxyflavone, 6-methoxyflavone and 7-methoxyflavone were dissolved in 700 µl absolute ethanol as they are poorly soluble in water. These solutions were then made up to volume with Krebs-Ringer bicarbonate buffer (KR) containing Rho123. The final ethanol concentration in the various sample solutions was kept ≤1%, a concentration proven not to alter cell viability or permeability (Soldner *et al.*, 1999: 479).

The use of unfasted adult male Sprague-Dawley rats (350-370 g) from the Animal Research Centre (North-West University) was approved by the Ethical Committee of the North-West University under protocol number 03D03. 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 (Figure 4.2) after which it was pulled onto a glass rod (Figure 4.3).

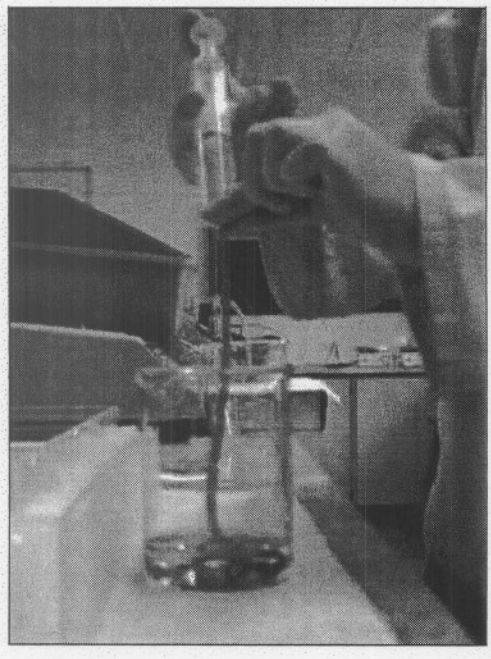


Figure 4.2

Figure 4.3

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 (Figure 4.4). Using the index finger along the length of the jejunum, the serosa and muscle layer were then gently pushed back and peeled off (Figure 4.5). 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 (Figure 4.6). The jejunum was then washed off the glass rod with a buffer-filled syringe onto a strip of filter paper (Figure 4.7).

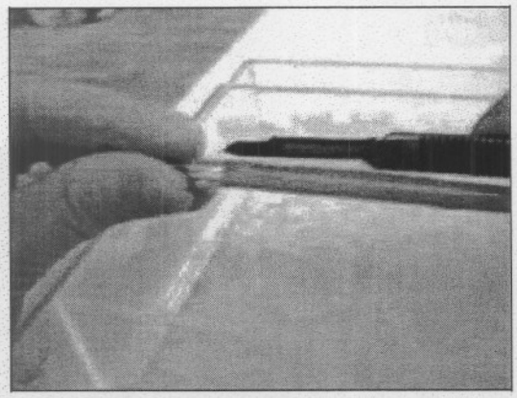


Figure 4.4

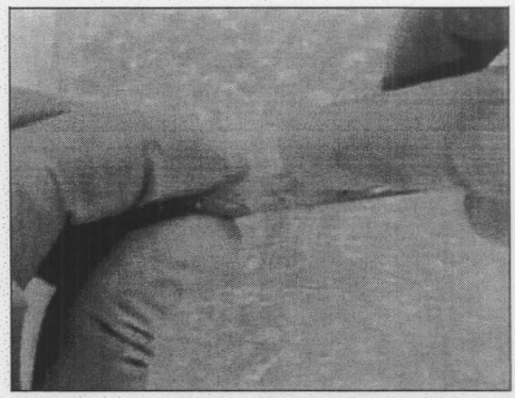


Figure 4.5

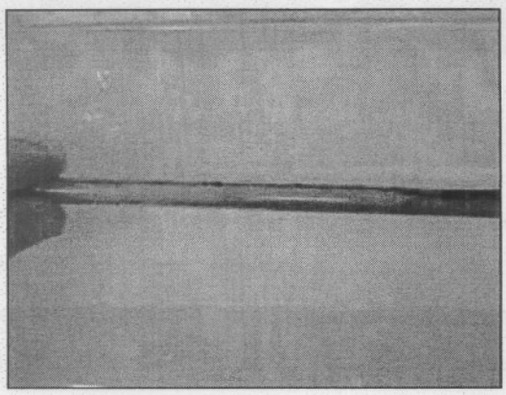


Figure 4.6

Figure 4.7

The jejunum and filter paper were then cut simultaneously into lengths approximately 3 cm long (Figure 4.8). The segments were kept moist with ice cold KR and were kept on ice throughout the procedure. Segments containing Peyer`s patches were identified visually and avoided (Figure 4.9), as these lymph like tissues would cause greater variation in the rates of transport because of the altered morphology and thickness of the epithelial layer.

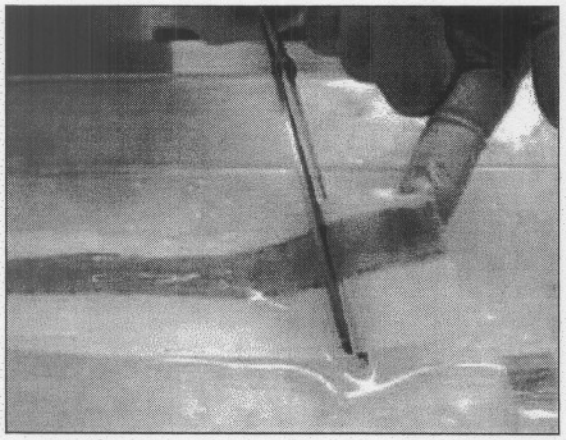


Figure 4.8

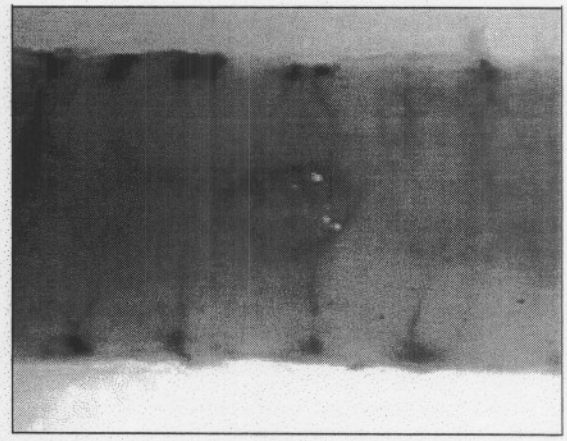


Figure 4.9

The segments were then carefully mounted onto the half-cells (preheated to 37 °C) with the serosal side face-down onto the pins (Figure 4.10 & 4.11). The filter paper was removed and the matching half-cells were carefully clamped together (Figure 4.12 & 4.13). 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.

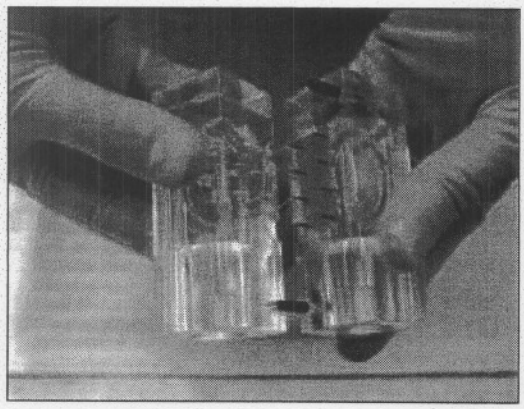


Figure 4.10

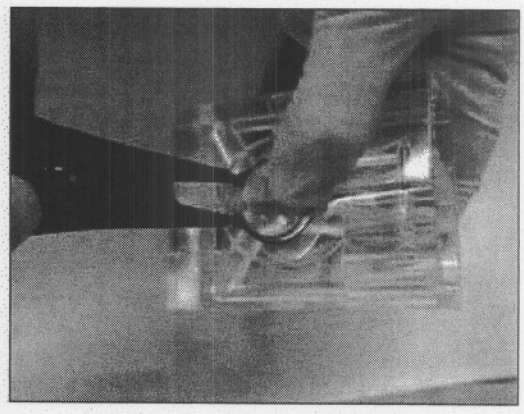


Figure 4.11

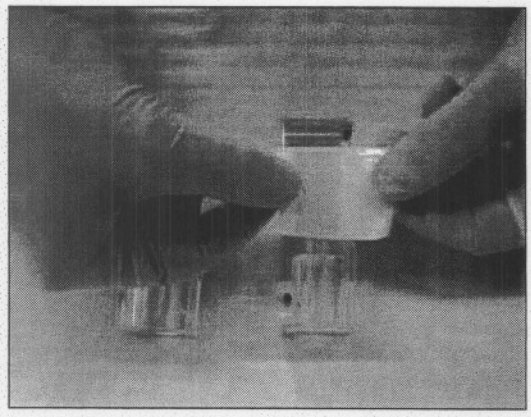


Figure 4.12

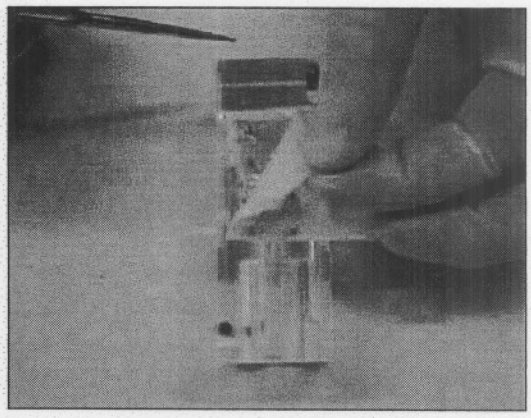


Figure 4.13

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 experimental procedures.

3-Methoxyflavone, 5-methoxyflavone, 6-methoxyflavone and 7-methoxyflavone were screened as potential P-gp modulators. The effects of these compounds on the transport of Rho123 were assessed by the concomitant addition of the individual compounds with Rho123 to the donor cell. Sufficient Rho123, to give a final Rho123 concentration of 10.1 µM in the donor cell, was added to the donor compartment and 2 ml KR was added to the receiver cell. In the first two cells, the serosal side was the donor compartment (AP/BL) and in the third and fourth cells (BL/AP), the basolateral side was the donor compartment. In this study a total of four experiments were done for each modulator.

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 30, 60, 90, and 120 min after the addition of Rho123, and replaced with an equivalent amount of fresh KR. It was previously shown (Hattingh, 2002:83) that the maximum period to perform transport studies was approximately 120 min before structural damage to the epithelium occurred. The aliquots were analysed by HPLC.

4.2.3 Average apparent permeability used during transport studies

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 Rho123 (100%) and A is the area of exposed tissue (1.78 cm^2). An example of the calculations performed is presented in Appendix B.

4.2.4 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. 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 as each experiment was done in quadruplet. Effect sizes were performed to determine whether a practical significance exist between each modulator at two different concentrations (Steyn *et al.*, 1998:604).

4.2.5 HPLC Analysis

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
	Auto sampler:	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	

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.

4.3 Validation of the HPLC method

For the purposes of this study the following performance parameters were validated:

- Selectivity
- Recovery
- Repeatability
- Linearity
- Sensitivity
- Accuracy and precision

4.3.1 Linearity

The linearity for Rho123 was determined by performing linear regression analysis on the plot of the peak AUC versus concentration. Six standard solutions were prepared as described in 4.2.2, to obtain concentrations ranging from 34.4 to 68.8 µg/ml. The regression value (r^2) was greater than 0.9929 and the Y-intercept was 28.2.

4.3.2 Precision

Intra-day precision

Precision (repeatability) was determined by performing HPLC analyses (n=5) on five different concentrations ranging from 34.4 to 68.4µg/ml of Rho123 on the same day. The intra-day precision complied with pharmaceutical standards (see Table 4.1).

Table 4.1: Data obtained for intra-day precision.

Rhodamine 123 concentrations (µg/ml)	Mean % recovered	Standard deviation	%RSD
34.38	104.32	2.86	1.90
39.29	105.49	5.38	3.09
44.20	104.47	6.28	3.23
54.02	100.84	5.64	2.45
68.75	95.58	11.41	4.10

Inter-day precision

The inter-day precision was determined by performing HPLC analyses (n=3) on a low, medium and a high concentration (34.4 µg/ml, 44.2 µg/ml and 68.8 µg/ml) of Rho123 on three consecutive days. The intra-day precision complied with pharmaceutical standards (See Table 4.2)

Table 4.2: Data obtained for inter-day precision.

Rhodamine 123 concentrations (µg/ml)	Mean % recovered	Standard deviation	%RSD
34.38	103.42	4.79	3.22
44.20	104.29	6.91	3.26
68.75	95.19	10.71	3.86

4.3.3 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 on the other hand, 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 Rho123 studied was 5.5 µg/ml and the limit of detection was 16.7 µg/ml.

4.3.4 Selectivity

Selectivity is the ability to detect and analyse a specific compound in the presence of other compounds. The components of the 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 Rho123 (± 5.2 min).

4.3.5 System repeatability

In order to evaluate the repeatability of the peak area and the retention time, samples of Rho123 with a known concentration (44.2µg/ml) was injected six times. The variation in the response (RSD) of the detection system when six determinations of Rho123 were made on the same day, and under the same conditions, was found to be 3.0% for the peak area and 1.3% for retention time.

CHAPTER 5
RESULTS AND DISCUSSION

5.1 Transport studies

The potential modulating effect of selected flavonoids was investigated on the transport of Rho 123 across rat jejunum. The Sweetana-Grass diffusion method comprising out of four chambers was used. 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 AB-BL direction was calculated. The mean rate of transport (P_{app}) observed in the control experiments were compared to the mean rate of transport observed in the experiments where the modulators were added. The individual values for the cumulative transport of Rho 123 in the presence of selected flavonoids are presented in Appendix A. Each experiment was done in quadruplet.

5.1.1 Statistical analysis

A statistical analysis was performed to compare the results obtained in the experiments with the control. As each experiment was done in quadruplet, it was recommended that a one-tailed non-parametric Kruskal-Wallis test was done to test for statistical significant differences between the ratio's obtained after the addition of modulators at both concentrations (10 μM and 20 μM) and the control. This test was also used to test for statistically differences between different modulators at the same concentration. Tables 5.1 and 5.2 present the p-values at concentrations of 10 μM and 20 μM respectively to indicate which mean ratio's showed statistical significant difference from the control. $p<0.05$ was taken as statistically significant.

Table 5.1: p-Values (1-tailed) obtained by the Kruskal-Wallis test comparing the P_{app} ratio's in the presence of modulators with the P_{app} ratio obtained in the control at 10 µM.

	Statistical analyses (p-Values) at 10 µM				
	Control	3M	5M	6M	7M
Control		0.00211*	0.2791465	0.5	0.024864*
3-Methoxyflavone	0.00211*		0.5	0.024864*	0.5
5-Methoxyflavone	0.2791465	0.5		0.5	0.5
6-Methoxyflavone	0.5	0.024864*	0.5		0.182349
7-Methoxyflavone	0.02486*	0.5	0.5	0.182349	

*Values representing statistically significant difference

Table 5.2: p-Values (1-tailed) obtained by the Kruskal-Wallis test comparing the P_{app} ratio's in the presence of modulators with the P_{app} ratio obtained in the control at 20 µM.

	Statistical evaluation (p-Values) at 20 µM				
	Control	3M	5M	6M	7M
Control		0.009431*	0.084137	0.5	0.06037
3-Methoxyflavone	0.009431*		0.5	0.157219	0.5
5-Methoxyflavone	0.084137	0.5		0.5	0.5
6-Methoxyflavone	0.5	0.157219	0.5		0.5
7-Methoxyflavone	0.060369	0.5	0.5	0.5	

*Value representing statistically significant difference

One of the aims of this study was to determine whether a practical significant difference exists between two concentrations of each modulator. Effect sizes were determined in order to evaluate practical significant differences. 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, $\bar{x}F_{10} - \bar{x}F_{20}$ the difference between the means of the two concentrations at 10 µM and 20 µM and S_{max} the ratio at the maximum standard deviation (Steyn, 1999). The results of the effect sizes between the two concentrations for each modulator are given in Table 5.3.

Table 5.3: Effect size results.

Effect size between the same modulators at 10 µM and 20 µM	
Modulator	**Effect size (d)**
3-Methoxyflavone	0.74
5-Methoxyflavone	1.03*
6-Methoxyflavone	0.87*
7-Methoxyflavone	0.10

*Value representing practically significant effect

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

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

(b) Medium effect: $0.2 < d < 0.8$

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

Data $d \geq 0.8$ is considered as practically significant as it is the result of a difference having a large effect. Variable concentrations play a major role in the inhibitory effects of modulators with a large effect (Cohen, 1998: 225).

5.1.2 Transport of Rhodamine123

Rho 123 is a fluorescent dye which exhibits a positive charge at physiological pH. Since Rho 123 is relatively lipophilic, its uptake into cells through the plasma membrane has been assumed to be due to passive diffusion (Eytan *et al.*, 1996:23). Rho 123 is classified as a substrate of MDR1-type P-gp (Neyfakh, 1998:168). Following cellular Rho 123 accumulation or retention in the presence or absence of P-gp modulators, valuable information on P-gp activity as well as efficiency of these modulators in reversing P-gp activity can be obtained (Choi *et al.*, 2000:124).

The cumulative transport of Rho123 without modulators is presented in Figure 5.1 and the P_{app} values in Table 5.4. The results and ratio's obtained served as the control values to which the ratio's obtained after addition of the modulators, could be compared.

Table 5.4: Individual and mean P_{app} values of Rho123 transported (AP-BL and BL-AP) with no modulators added.

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
Control				
1	1.44	5.30	3.68	3.382±0.230
2	0.75	2.50	3.33	
3	1.08	3.70	3.43	
4	1.36	4.25	3.13	

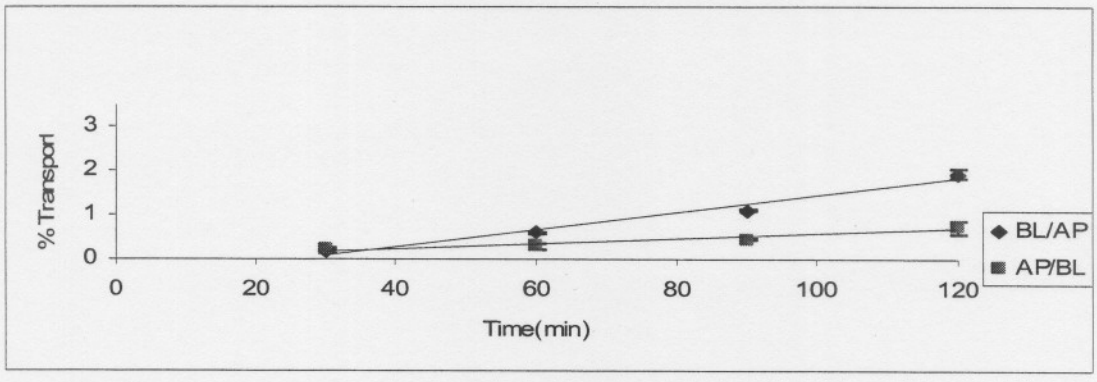


Figure 5.1: Cumulative transport of Rho123 with no modulators added.

Discussion

The mean ratio calculated was 3.382 which indicated that active transport in the BL-AP direction occurred. The results also indicated that Rho 123 is a substrate for active transporters.

5.1.3 Transport of Rho123 in the presence of selected flavonoids

Many herbal constituents, in particular flavonoids, were reported to modulate P-gp by directly interacting with the ATP-binding site, the steroid-binding site or the substrate-binding site. The inhibition of P-gp by flavonoids may provide a novel approach for reversing MDR in tumor cells, whereas the stimulation of P-gp expression or activity has implications for chemo-preventive enhancement. Certain natural flavonoids (e.g.,

kaempferol, quercetin and galangin) are potent stimulators of the P-gp-mediated efflux of 7,12-dimethylbenza-anthracene (a carcinogen). The modulation of P-gp activity and expression by these herb constituents may result in altered absorption and bioavailability of drugs that are P-gp substrates (Zhang & Cowby, 2004:57). In this study, the modulating effect of the following methoxy flavonoids was investigated:

- 3-methoxyflavone
- 5-methoxyflavone
- 6-methoxyflavone
- 7-methoxyflavone

The effect of these components at two concentrations (10 µM & 20 µM) was used to determine the transport of Rho 123 across rat jejunum. One of the purposes of this study was to evaluate the structure activity relationships (SAR) of the selected methoxy flavonoids with reference to the inhibition of P-gp. The chemical structures of the selected flavonoids are given in Figure 5.2.

Van Zanden and co-workers (2005:703) have found that several of the methoxylated flavonoids are among the best MRP1 inhibitors. For inhibition of MRP1, the total number of methoxylated moieties was found to be of major importance. They also found that the methoxylated flavonoids 5,7,3',4'-tetramethoxyflavone, diosmetin, chrysoeriol, amarixetin and isorhamnetin are among the best MRP1 inhibitors, except for kaempferide and acacetin which are less potent inhibitors than the other methoxylated flavonoids. The methoxylation pattern of the above-mentioned methoxy flavonoids is given in Table 5.5.

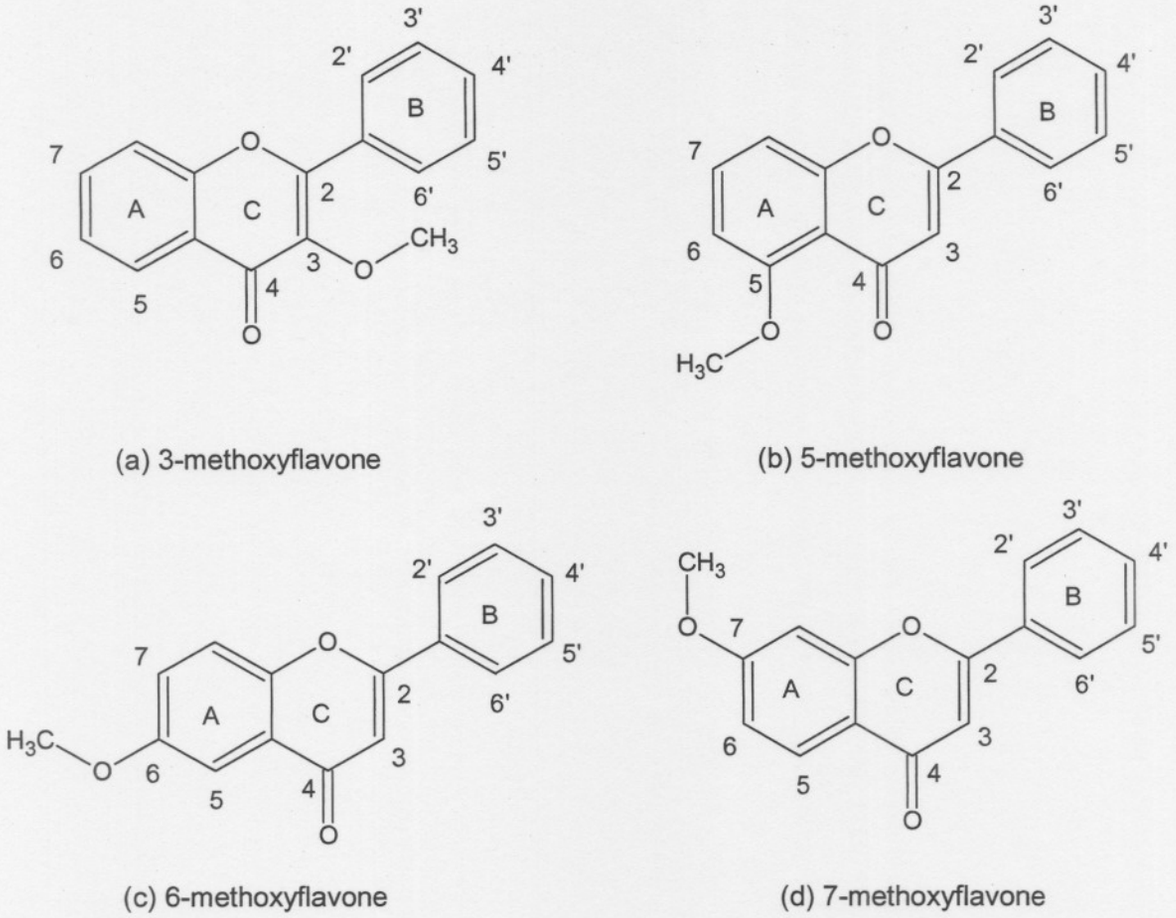


Figure 5.2: Chemical structures of the selected flavonoids used in this study.

Table 5.5: Methoxylation pattern of certain flavonoids (van Zanden, 2005:701).

Flavonoids	Methoxylation pattern	Hydroxylation pattern
Acacetin	4'	5,7
Kaempferide	4'	3,5,7
5,7,3',4'-Tetramethoxyflavone	5,7,3',4'	-
Diosmetin	4'	5,7,3'
Chrysoeriol	3'	5,7,4'
Tamarixetin	4'	3,5,7,3'
Isorhamnetin	3'	3,5,7,4'

Van Zanden and co-workers (2005:705) also measured the lipophilicity (K') and calculated the dihedral angle between the B- and C-ring. The tested methoxylated flavonoids are given in Table 5.6.

Table 5.6: Measured lipophilicity (K') and calculated dihedral angle between the B- and C-ring for selected flavonoids tested (Van Zanden, 2005:705).

Flavonoid	Lipophilicity (K')	Dihedral angle (degrees)
Acacetin	28	6.7
Kaempferide	32.4	14
5,7,3',4'-Tetramethoxyflavone	20.4	9.1
Diosmetin	12.1	7.1
Chrysoeriol 3	11.1	2.3
Tamarixetin	7.2	11.9
Isorhamnetin 3	10.4	13.9

Choi and co-workers (2004:677) found that when the number of hydroxyl groups on a ring is increased, it results in a smaller chemosensitising effect. This suggests that methoxylated substitution and its numbers on the site of a ring are more important than the hydroxylation of one of the counterparts. This indicates that the hydrophobicity of both the A/C and B rings plays an important role in the binding to the flavonoid-binding pocket of P-gp. The flavonoid they used, was sinensetin (5,6,7,3',4'-pentamethoxyflavone). They have also shown that the lipophilicity of the flavonoid was shown to influence MDR-modulating activity.

As seen from the results above, there does not seem to be a correlation between the specific methoxylated atom, the lipophilicity and the dihedral angle (Choi *et al.*, 2004:677). It can therefore be assumed that these factors will not influence the activity of the flavonoids used in this study.

5.1.4 Transport of Rho 123 in the presence of 3-methoxyflavone

The effect of 3-methoxyflavone on the transport of Rho 123 across rat jejunum was investigated in this study using two concentrations. Cumulative transport of Rho 123 in

the presence of 3-methoxyflavone (10 µM and 20 µM) is presented in Figures 5.3 and 5.4 respectively and the P_{app} values in Table 5.7.

Table 5.7: Individual and mean P_{app} values of Rho 123 transported (AP-BL and BL-AP) in the presence of 3-methoxyflavone (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
3-Methoxyflavone (10 µM)				
1	9.04	15.18	1.68	1.66±0.088
2	8.52	14.84	1.74	
3	8.33	14.06	1.69	
4	9.99	15.33	1.54	
3-Methoxyflavone (20 µM)				
1	31.07	32.82	1.06	1.33±0.447
2	46.07	56.90	1.24	
3	14.23	28.30	1.99	
4	22.54	23.58	1.05	

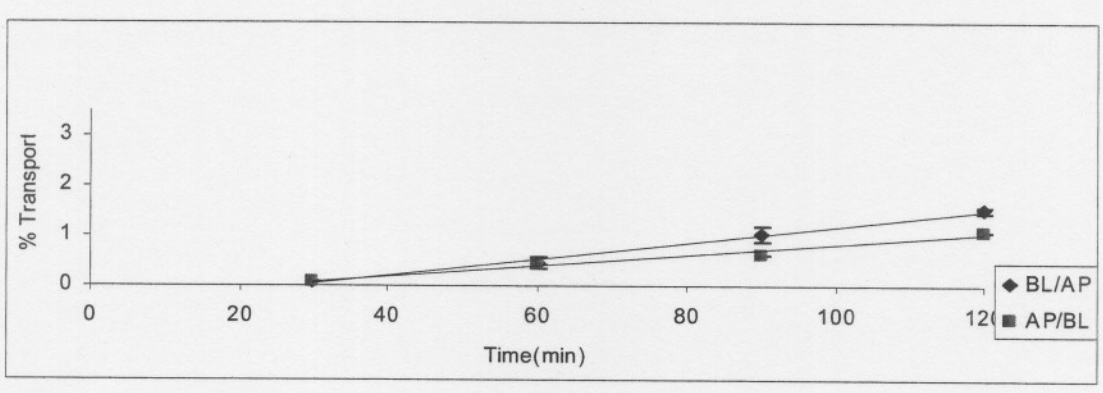


Figure 5.3: Cumulative transport of Rho 123 in the presence of 3-methoxyflavone (10 µM).

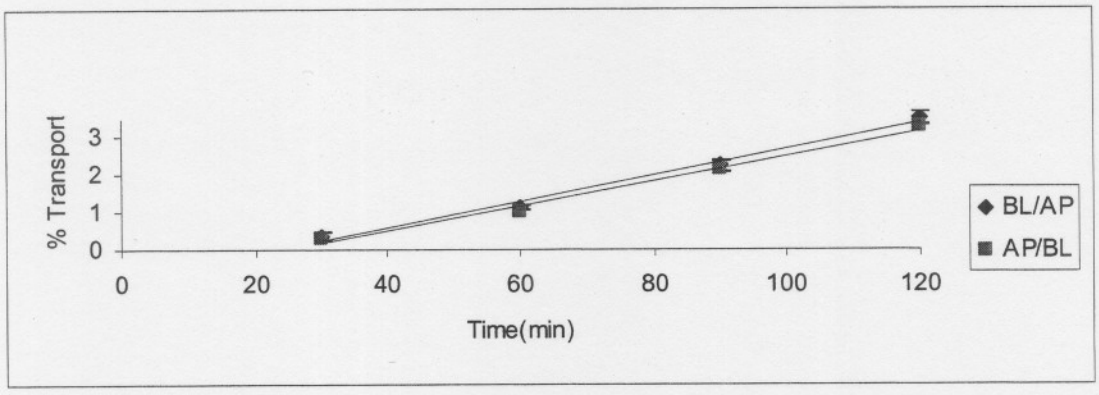


Figure 5.4: Cumulative transport of Rho123 in the presence of 3-methoxyflavone (20 µM).

Discussion

The results indicated that 3-methoxyflavone was able to decrease the mean ratio of transport (P_{app}) of Rho 123. The mean ratio calculated were 1.66 at a concentration of 10 µM and 1.33 at a concentration of 20 µM. The p-values at a concentration of 10 µM and 20 µM were both <0.05 which indicated a statistically significant difference in comparison with the control. Therefore it can be concluded that 3-methoxyflavone is an inhibitor of transporter proteins responsible for the efflux of Rho 123. There was also a statistically significant difference between 3-methoxyflavone and 6-methoxyflavone at a 10 µM concentration which indicated that a methoxylation pattern 6 on the A-ring has less inhibitory activity. There was also a medium effect size between the two concentrations of 3-methoxyflavone, which indicated that concentration played a minor role in this modulator's inhibitory properties.

5.1.5 Transport of Rho 123 in the presence of 5-methoxyflavone

The effect of 5-methoxyflavone on the transport of Rho 123 across rat jejunum was investigated in this study using two concentrations. Cumulative transport of Rho 123 in the presence of 5-methoxyflavone (10 µM and 20 µM) is presented in Figures 5.5 and 5.6 respectively and the P_{app} values in Table 5.8.

Table 5.8: Individual and mean P_{app} values of Rho 123 transported (AP-BL and BL-AP) in the presence of 5-methoxyflavone (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
5-Methoxyflavone (10 µM)				
1	7.76	19.78	2.55	2.41±0.261
2	8.85	21.48	2.43	
3	7.98	20.98	2.63	
4	9.83	20.04	2.04	
5-Methoxyflavone (20 µM)				
1	7.65	19.69	2.57	1.71±0.678
2	6.76	7.96	1.18	
3	10.89	21.16	1.94	
4	40.31	46.95	1.16	

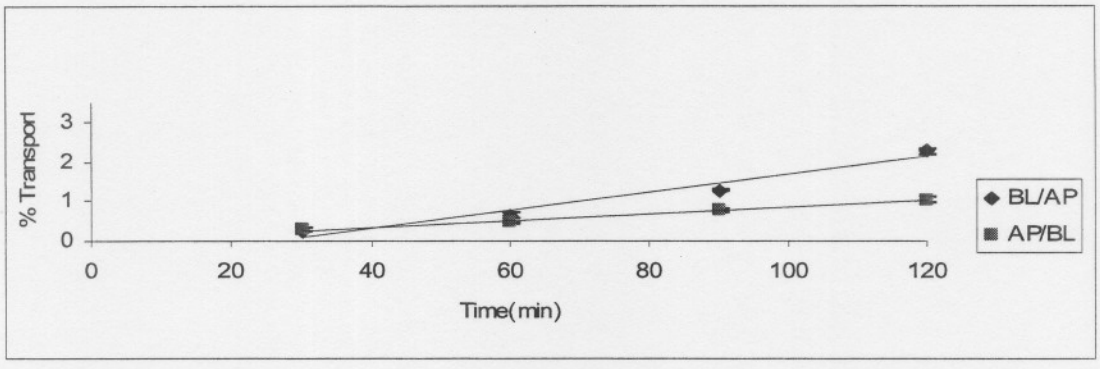


Figure 5.5: Cumulative transport of Rho123 in the presence of 5-methoxyflavone (10 µM).

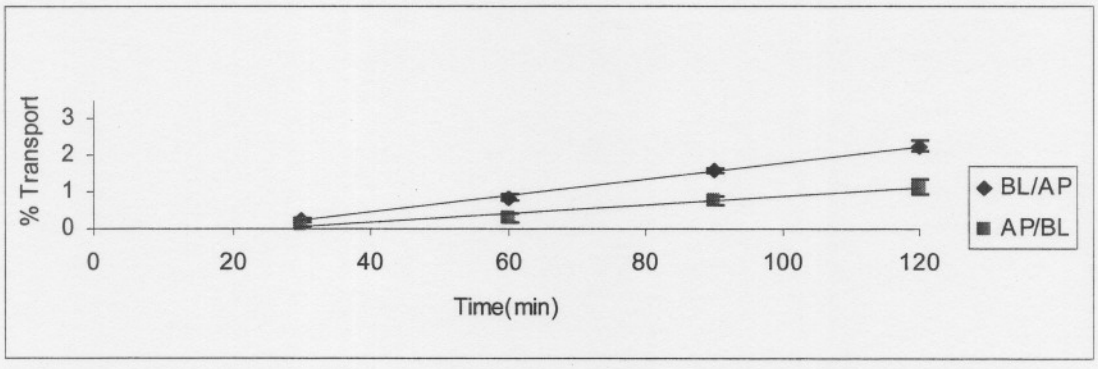


Figure 5.6: Cumulative transport of Rho123 in the presence of 5-methoxyflavone (20 µM).

Discussion

The results indicated that 5-methoxyflavone was able to decrease the mean ratio of transport (P_{app}) of Rho123. The mean ratio calculated were 2.41 at a concentration of 10 µM and 1.71 at a concentration of 20 µM. Although the results indicated that 5-methoxyflavone inhibited the mean ratio of transport (P_{app}) of Rho123, no statistically significant difference existed, indicating that 5-methoxyflavone was not an active inhibitor of the transport proteins. The p-values for 10µM and 20µM were >0.05. There was also a large effect size between the concentrations of 5-methoxyflavones which indicated that concentration played a major role in this modulator's inhibitory properties.

5.1.6 Transport of Rho 123 in the presence of 6-methoxyflavone

The effect of 6-methoxyflavone on the transport of Rho 123 across rat jejunum was investigated in this study using two concentrations. Cumulative transport of Rho 123 in the presence of 6-methoxyflavone (10 µM and 20 µM) is presented in Figures 5.7 and 5.8 respectively and the P_{app} values in Table 5.9.

Table 5.9: Individual and mean P_{app} values of Rho 123 transported (AP-BL and BL-AP) in the presence of 6-methoxyflavone (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
6-Methoxyflavone (10 µM)				
1	6.62	18.08	2.73	3.03±0.307
2	6.21	17.42	2.81	
3	5.55	18.42	3.32	
4	5.47	17.91	3.27	
6-Methoxyflavone (20 µM)				
1	11.56	24.80	2.15	2.49±0.620
2	21.55	55.76	2.59	
3	5.44	18.11	3.33	
4	11.84	22.79	1.92	

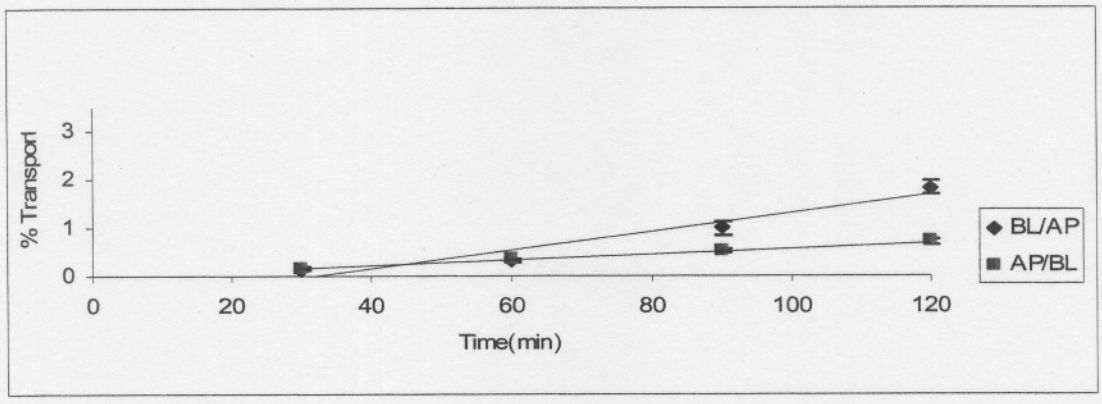


Figure 5.7: Cumulative transport of Rho 123 in the presence of 6-methoxyflavone (10 µM).

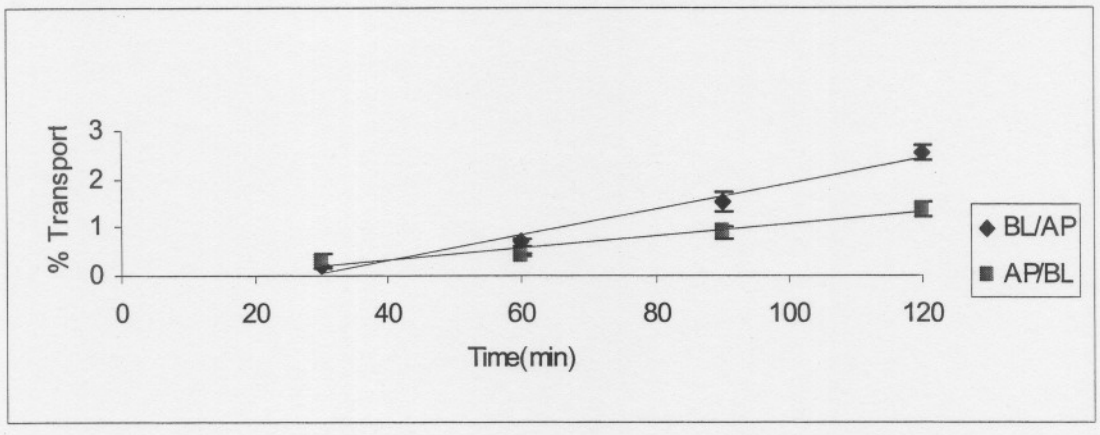


Figure 5.8: Cumulative transport of Rho 123 in the presence of 6-methoxyflavone (20 µM).

Discussion

The results indicated that 6-methoxyflavone was able to decrease the mean ratio of transport (P_{app}) of Rho 123. The mean ratio calculated were 3.03 at a concentration of 10 µM and 2.49 at a concentration of 20 µM. In comparison with the control no inhibition of the transport of Rho 123 was observed. There was also a large effect size between the two concentrations of 6-methoxyflavone which indicated that concentration played a major role in this modulator's inhibitory properties.

5.1.7 Transport of Rho 123 in the presence of 7-methoxyflavone

The effect of 7-methoxyflavone on the transport of Rho 123 across rat jejunum was investigated in this study using two concentrations. Cumulative transport of Rho 123 in the presence of 7-methoxyflavone (10 µM and 20 µM) is presented in Figures 5.9 and 5.10 respectively and the P_{app} values in Table 5.10.

Table 5.10: Individual and mean P_{app} values of Rho123 transported (AP-BL and BL-AP) in the presence of 7-methoxyflavone (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's BL-AP/ AP-BL	Mean Ratio's BL-AP/ AP-BL
7-Methoxyflavone (10 µM)				
1	5.23	8.30	1.59	1.94±0.394
2	6.41	15.97	2.49	
3	8.50	16.35	1.92	
4	6.52	11.43	1.75	
7-Methoxyflavone (20 µM)				
1	19.98	32.60	1.63	1.54±0.186
2	20.86	35.83	1.72	
3	22.73	29.28	1.29	
4	5.81	8.89	1.53	

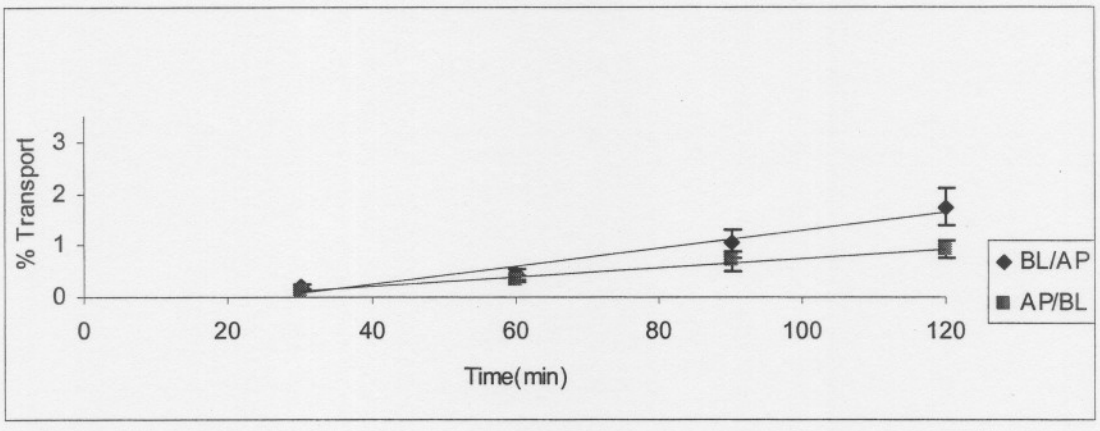


Figure 5.9: Cumulative transport of Rho 123 in the presence of 7-methoxyflavone (10 µM).

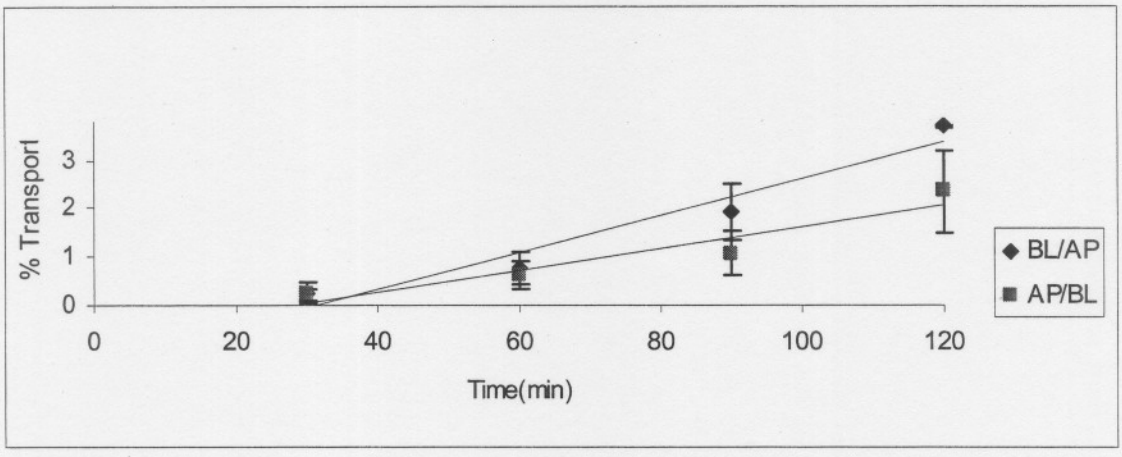


Figure 5.10: Cumulative transport of Rho 123 in the presence of 7-methoxyflavone (20 µM).

Discussion

The results indicated that 7-methoxyflavone was able to decrease the mean ratios of transport (P_{app}) of Rho 123. The mean ratio's calculated were 1.94 at a concentration of 10 µM and 1.55 at a concentration of 20 µM. The Kruskall-Wallis test makes use of ranking orders of data and not of averages. Therefore averages cannot be used to predict statistical significant differences. 7-Methoxyflavone clearly inhibited the mean rate transport of Rho 123 at both concentrations, but was only statistical significant at a concentration of 10 µM. Therefore it can be concluded that 7-methoxyflavone is an inhibitor of transporter proteins responsible for the efflux of Rho 123. There was a small effect size between the two concentrations of 7-methoxyflavone. This indicated that concentration played no role in 7-methoxyflavones inhibitory properties.

5.2 Summary

The mean ratio's of all the modulators investigated during this study are presented in Table 5.11 and the mean ratio's for all the methoxyflavones compared to the control are shown in Figure 5.10. After comparing the mean rate of transport (P_{app}) of the control to that of the methoxyflavones it was obvious that the best inhibitors were 3- and 7-

methoxyflavone followed by 5-methoxyflavone and 6-methoxyflavone. All of the methoxyflavones tested inhibited the mean ratios of transport (P_{app}) of Rho 123 at both concentrations, but only 3-methoxyflavone (10 μM and 20 μM) and 7-methoxyflavone (10 μM) were statistically significant. 6-Methoxyflavone showed insignificant inhibitory characteristics.

Table 5.11: Mean ratio's obtained for all the modulators examined.

Modulator	Mean Ratio's	Standard Deviation
Control	3.382	0.23
3-Methoxyflavone (10μM) *	1.661	0.09
3-Methoxyflavone (20μM) *	1.331	0.45
5-Methoxyflavone (10μM)	2.411	0.26
5-Methoxyflavone (20μM)	1.715	0.68
6-Methoxyflavone (10μM)	3.033	0.31
6-Methoxyflavone (20μM)	2.496	0.62
7-Methoxyflavone (10μM) *	1.939	0.39
7-Methoxyflavone (20μM)	1.546	0.19

*Value representing practical significant differences

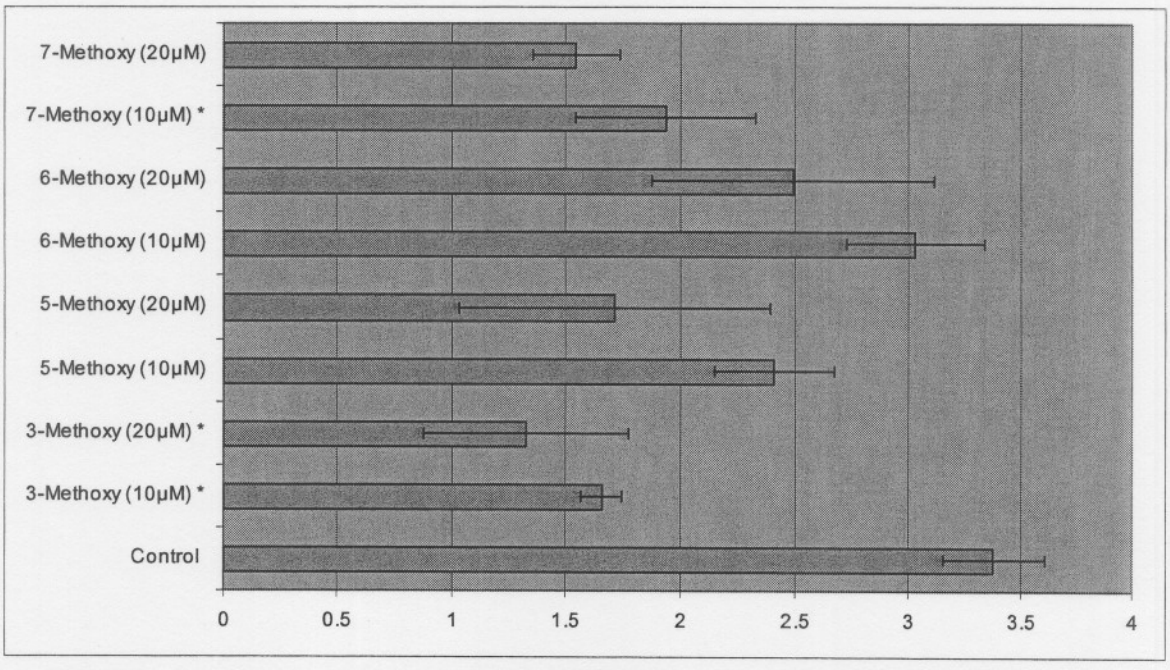


Figure 5.11: Bar chart showing the mean ratio's of the various modulators compared to the mean ratio's of the control.

CHAPTER 6
CONCLUSION AND RECOMMENDATION

Active drug efflux transporters of (ABC)-containing family of proteins have a major impact on the pharmacological behavior of most of the drugs used currently. Pharmacological properties affected by ABC transporters include the oral bioavailability, hepatobiliary, direct intestinal and urinary excretion of drugs and drug-metabolites and conjugates. Moreover, the penetration of drugs into a range of important pharmacological sanctuaries, such as brain, testis, fetus, and the penetration into specific cell- and tissue compartments can be limited extensively by ABC transporters. These interactions with ABC transporters determine to a large extent the clinical usefulness, side effects and toxicity risk of drugs. Many other xenotoxins, carcinogens and endogenous compounds are also influenced by the ABC transporters, with corresponding consequences for the well-being of the individual. (Schinkel & Jonker, 2002:1)

- In this study it is evident that 3-methoxyflavone was the best inhibitor of Rho 123 mediated transport. Both concentrations of 3-methoxyflavone showed statistically significant differences when compared to the control. It decreased the P_{app} ratio from 3.34 (control) to 1.66 (10 µM) and 1.33 (20 µM). The structure activity relationships with reference to P-gp inhibition indicated that the positioning of the methoxy-group on the A ring at position C3, played an important role in the inhibition of Rho 123 transport.

- 7-Methoxyflavone was able to decrease the mean ratio of transport of Rho 123 to 1.94 at a concentration of 10 µM and 1.55 at a concentration of 20 µM. 7-Methoxyflavone inhibited the mean ratio of transport of Rho 123 for both concentrations, but only showed a statistically significant difference at a concentration of 10 µM. The structure activity relationships with reference to P-gp inhibition clearly indicated that the positioning of the methoxy-group on the A ring at position C7, played an important role in the inhibition of Rho 123 transport.

- 5-Methoxyflavone inhibited the mean ratio of transport of Rho 123. The mean ratio's calculated were 2.41 at a concentration of 10 µM and 1.71 at a concentration of 20 µM but there were no statistical significant differences. The structure activity relationships with reference to P-gp inhibition indicated that the positioning of the methoxy-group on the A ring at position C5, may have a role in the inhibition of Rho 123 transport.

- The mean ratio of 6-methoxyflavone at a concentration of 10 µM was 3.03 and 2.49 at a concentration of 20 µM. Almost no sign of inhibition of P-gp were observed and no statistically significant difference when compared to the control existed. The structure activity relationships with reference to P-gp inhibition indicated that the positioning of the methoxy-group on the A ring at position C6, had little effect on the inhibition of Rho 123 transport.

- Different flavonoids with different positioning of the methoxy groups were tested and their inhibitory potencies were compared. There potencies were measured and 3-methoxyflavone showed the highest inhibitory potency followed by 7-methoxyflavone, 5-methoxyflavone and 6-methoxyflavone.

The following recommendations should be considered in further studies:

- The tested methoxyflavones should be tested to determine any signs of synergism between them. Methoxyflavones should also be tested with other inhibitory modulators to evaluate their combined effect on P-gp mediated transport.

- A larger variety of concentrations should be tested to determine if any correlation exist between the selected concentration, the absorption of methoxyflavones and the P-gp binding activity. Studies on higher concentrations should also be conducted.

- Choi and co-workers (2004:677) have found that increasing the number of hydroxyl groups on either ring A/C or B resulted in smaller chemosensitising

effect. The methoxyflavones studied were substituted only on the 3-methoxy ring. Further studies should be performed with single substituted methoxyflavones on the B or C ring. We could obtain a more clear result on the structure activity relationship between different types of methoxyflavones.

- Studies should be performed to measure the difference between the *in vitro* concentration and the *in vivo* therapeutic plasma concentrations.

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

- During this study only methoxyflavones were investigated, and further studies should be conducted with polymethoxylated flavones to determine the effect it might have on the inhibition of P-gp transport.

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APPENDIX A

Table A.1: Cumulative transport amounts of Rho123 (10.1 µM) in the AP/BL and the 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	Chamber2	Chamber1	Chamber2		
Control 1	30	0.09	0.08	0.08	0.08	0.09 ± 0.007	0.08 ± 0.000
	60	0.09	0.09	0.10	0.12	0.09 ± 0.000	0.11 ± 0.014
	90	0.11	0.10	0.23	0.20	0.11 ± 0.007	0.22 ± 0.021
	120	0.15	0.15	0.33	0.27	0.15 ± 0.000	0.30 ± 0.042
Control 2	30	0.02	0.02	0.08	0.06	0.02 ± 0.000	0.07 ± 0.014
	60	0.04	0.07	0.13	0.16	0.06 ± 0.021	0.15 ± 0.021
	90	0.07	0.09	0.23	0.23	0.08 ± 0.014	0.23 ± 0.000
	120	0.09	0.17	0.40	0.46	0.13 ± 0.057	0.43 ± 0.042
Control 3	30	0.05	0.07	0.07	0.07	0.06 ± 0.014	0.07 ± 0.000
	60	0.08	0.10	0.13	0.13	0.09 ± 0.014	0.13 ± 0.000
	90	0.17	0.24	0.48	0.55	0.21 ± 0.049	0.52 ± 0.049
	120	0.32	0.49	1.05	1.07	0.41 ± 0.120	1.06 ± 0.014
Control 4	30	0.03	0.06	0.07	0.02	0.05 ± 0.021	0.05 ± 0.035
	60	0.04	0.08	0.15	0.13	0.06 ± 0.028	0.14 ± 0.014
	90	0.11	0.10	0.32	0.33	0.11 ± 0.007	0.33 ± 0.007
	120	0.18	0.24	0.62	0.53	0.21 ± 0.042	0.58 ± 0.064
3-Methoxy 1 (10 µM)	30	0.09	0.06	0.11	0.13	0.08 ± 0.022	0.12 ± 0.015
	60	0.34	0.37	0.41	0.45	0.36 ± 0.024	0.43 ± 0.031
	90	0.53	0.57	0.88	0.96	0.55 ± 0.026	0.92 ± 0.056
	120	0.88	0.98	1.51	1.58	0.93 ± 0.070	1.54 ± 0.049
3-Methoxy 2 (10 µM)	30	0.10	0.07	0.08	0.09	0.08 ± 0.021	0.08 ± 0.004
	60	0.45	0.37	0.54	0.39	0.41 ± 0.052	0.46 ± 0.109
	90	0.67	0.64	1.13	0.92	0.65 ± 0.022	1.03 ± 0.148
	120	0.96	0.97	1.56	1.48	0.97 ± 0.008	1.52 ± 0.059
3-Methoxy 3 (10 µM)	30	0.08	0.08	0.08	0.08	0.08 ± 0.000	0.08 ± 0.004
	60	0.43	0.43	0.54	0.38	0.43 ± 0.000	0.46 ± 0.110
	90	0.62	0.60	1.14	0.93	0.61 ± 0.011	1.04 ± 0.150
	120	1.08	1.08	1.57	1.49	1.08 ± 0.000	1.53 ± 0.059
3-Methoxy 4 (10 µM)	30	0.07	0.06	0.09	0.07	0.06 ± 0.004	0.08 ± 0.016
	60	0.38	0.33	0.54	0.33	0.36 ± 0.034	0.44 ± 0.148
	90	0.60	0.56	1.01	0.77	0.58 ± 0.030	0.89 ± 0.168
	120	0.84	0.92	1.45	1.41	0.88 ± 0.056	1.43 ± 0.026

3-Methoxy 1 (20 µM)	30	0.20	0.23	0.12	0.12	0.22 ± 0.021	0.12 ± 0.000
	60	0.70	0.64	0.53	0.45	0.67 ± 0.042	0.49 ± 0.057
	90	1.41	1.22	0.99	1.15	1.32 ± 0.134	1.07 ± 0.113
	120	2.64	2.17	2.44	2.45	2.41 ± 0.332	2.45 ± 0.007
3-Methoxy 2 (20 µM)	30	0.17	0.26	0.32	0.44	0.22 ± 0.064	0.38 ± 0.085
	60	0.40	0.51	0.98	1.27	0.46 ± 0.078	1.13 ± 0.205
	90	0.80	1.13	11.93	2.20	0.97 ± 0.233	7.07 ± 6.880
	120	1.36	1.78	3.04	3.12	1.57 ± 0.297	3.08 ± 0.057
3-Methoxy 3 (20 µM)	30	0.17	0.44	0.39	0.39	0.31 ± 0.191	0.39 ± 0.000
	60	1.26	1.75	1.55	1.23	1.51 ± 0.346	1.39 ± 0.226
	90	2.53	3.83	3.72	2.72	3.18 ± 0.919	3.22 ± 0.707
	120	4.53	4.80	6.42	5.29	4.67 ± 0.191	5.86 ± 0.799
3-Methoxy 4 (20 µM)	30	0.28	0.35	0.45	0.28	0.32 ± 0.049	0.37 ± 0.120
	60	1.18	0.83	1.18	1.10	1.01 ± 0.247	1.14 ± 0.057
	90	2.52	1.70	2.12	2.33	2.11 ± 0.580	2.23 ± 0.148
	120	3.95	2.57	3.39	3.64	3.26 ± 0.976	3.52 ± 0.177
5-Methoxy 1 (10 µM)	30	0.43	0.47	0.26	0.19	0.45 ± 0.027	0.22 ± 0.049
	60	0.59	0.61	0.73	0.57	0.60 ± 0.015	0.65 ± 0.116
	90	0.97	0.91	1.36	1.03	0.94 ± 0.045	1.20 ± 0.233
	120	1.33	1.44	2.40	1.95	1.38 ± 0.079	2.18 ± 0.317
5-Methoxy 2 (10 µM)	30	0.33	0.25	0.26	0.24	0.29 ± 0.055	0.25 ± 0.011
	60	0.48	0.45	0.69	0.61	0.46 ± 0.022	0.65 ± 0.057
	90	0.79	0.74	1.31	1.26	0.77 ± 0.037	1.29 ± 0.039
	120	1.09	0.99	2.33	2.23	1.04 ± 0.075	2.28 ± 0.071
5-Methoxy 3 (10 µM)	30	0.43	0.41	0.28	0.20	0.42 ± 0.011	0.24 ± 0.059
	60	0.58	0.55	0.79	0.58	0.56 ± 0.022	0.68 ± 0.145
	90	0.89	0.80	1.43	1.15	0.84 ± 0.065	1.29 ± 0.203
	120	1.33	1.21	2.65	2.02	1.27 ± 0.086	2.34 ± 0.443
5-Methoxy 4 (10 µM)	30	0.33	0.25	0.26	0.22	0.29 ± 0.055	0.24 ± 0.029
	60	0.48	0.45	0.67	0.58	0.46 ± 0.022	0.63 ± 0.068
	90	0.79	0.74	1.28	1.15	0.77 ± 0.037	1.22 ± 0.087
	120	1.05	0.99	2.23	2.09	1.02 ± 0.042	2.16 ± 0.099
5-Methoxy 1 (20 µM)	30	0.48	0.38	0.49	0.33	0.43 ± 0.071	0.41 ± 0.113
	60	1.47	1.35	1.72	1.37	1.41 ± 0.085	1.55 ± 0.247
	90	2.80	2.31	2.90	2.61	2.56 ± 0.346	2.76 ± 0.205
	120	4.78	3.94	5.15	4.90	4.36 ± 0.594	5.03 ± 0.177
5-Methoxy 2 (20 µM)	30	0.16	0.10	0.27	0.25	0.13 ± 0.042	0.26 ± 0.014
	60	0.39	0.23	0.94	0.78	0.31 ± 0.113	0.86 ± 0.113
	90	0.85	0.67	1.65	1.58	0.76 ± 0.127	1.62 ± 0.049

	120	1.29	0.99	2.17	2.38	1.14 ± 0.212	2.28 ± 0.148
5-Methoxy 3 (20 µM)	30	0.12	0.07	0.12	0.07	0.10 ± 0.035	0.10 ± 0.035
	60	0.35	0.29	0.28	0.30	0.32 ± 0.042	0.29 ± 0.014
	90	0.50	0.53	0.66	0.54	0.52 ± 0.021	0.60 ± 0.085
	120	0.70	0.81	0.84	0.84	0.76 ± 0.078	0.84 ± 0.000
5-Methoxy 4 (20 µM)	30	0.18	0.17	0.14	0.18	0.18 ± 0.007	0.16 ± 0.028
	60	0.42	0.37	0.65	0.38	0.40 ± 0.035	0.52 ± 0.191
	90	0.71	0.72	1.26	0.88	0.72 ± 0.007	1.07 ± 0.269
	120	0.88	0.90	2.40	1.76	0.89 ± 0.014	2.08 ± 0.453
6-Methoxy 1 (10 µM)	30	0.19	0.17	0.13	0.14	0.18 ± 0.013	0.13 ± 0.013
	60	0.37	0.33	0.34	0.30	0.35 ± 0.029	0.32 ± 0.032
	90	0.56	0.49	0.90	1.10	0.53 ± 0.044	1.00 ± 0.142
	120	0.75	0.66	1.72	1.92	0.71 ± 0.060	1.82 ± 0.146
6-Methoxy 2 (10 µM)	30	0.19	0.17	0.13	0.16	0.18 ± 0.013	0.14 ± 0.020
	60	0.37	0.33	0.36	0.32	0.35 ± 0.030	0.34 ± 0.026
	90	0.56	0.50	0.80	1.04	0.53 ± 0.045	0.92 ± 0.175
	120	0.76	0.67	1.94	1.89	0.71 ± 0.061	1.92 ± 0.033
6-Methoxy 3 (10 µM)	30	0.17	0.08	0.13	0.12	0.12 ± 0.062	0.13 ± 0.007
	60	0.36	0.26	0.33	0.37	0.31 ± 0.069	0.35 ± 0.027
	90	0.65	0.45	0.76	0.92	0.55 ± 0.146	0.84 ± 0.119
	120	0.79	0.63	1.91	1.74	0.71 ± 0.110	1.82 ± 0.121
6-Methoxy 4 (10 µM)	30	0.16	0.10	0.13	0.12	0.13 ± 0.042	0.13 ± 0.007
	60	0.34	0.30	0.33	0.37	0.32 ± 0.033	0.35 ± 0.027
	90	0.58	0.52	0.97	1.14	0.55 ± 0.047	1.06 ± 0.122
	120	0.74	0.79	1.90	1.74	0.76 ± 0.031	1.82 ± 0.120
6-Methoxy 1 (20 µM)	30	0.24	0.16	0.22	0.14	0.20 ± 0.057	0.18 ± 0.057
	60	0.35	0.25	0.59	0.60	0.30 ± 0.071	0.60 ± 0.007
	90	0.44	0.46	1.09	1.14	0.45 ± 0.014	1.12 ± 0.035
	120	0.62	0.84	2.02	1.86	0.73 ± 0.156	1.94 ± 0.113
6-Methoxy 2 (20 µM)	30	0.16	0.15	0.28	0.35	0.16 ± 0.007	0.32 ± 0.049
	60	0.55	0.51	1.26	1.45	0.53 ± 0.028	1.36 ± 0.134
	90	1.23	1.08	3.32	3.29	1.16 ± 0.106	3.31 ± 0.021
	120	2.38	2.12	6.04	5.19	2.25 ± 0.184	5.62 ± 0.601
6-Methoxy 3 (20 µM)	30	0.42	0.20	0.18	0.19	0.31 ± 0.156	0.19 ± 0.007
	60	0.42	0.44	0.64	0.74	0.43 ± 0.014	0.69 ± 0.071
	90	0.79	0.99	1.39	1.69	0.89 ± 0.141	1.54 ± 0.212
	120	1.28	1.50	2.64	2.45	1.39 ± 0.156	2.55 ± 0.134
6-Methoxy 4 (20 µM)	30	0.16	0.12	0.17	0.24	0.14 ± 0.028	0.21 ± 0.049
	60	0.69	0.40	0.59	0.62	0.55 ± 0.205	0.61 ± 0.021

	90	1.00	0.61	1.34	1.33	0.81 ± 0.276	1.34 ± 0.007
	120	1.73	0.90	2.53	2.25	1.32 ± 0.587	2.39 ± 0.198
7-Methoxy 1	30	0.11	0.14	0.17	0.11	0.12 ± 0.024	0.14 ± 0.045
(10 µM)	60	0.29	0.22	0.33	0.24	0.25 ± 0.053	0.28 ± 0.064
	90	0.51	0.41	0.55	0.65	0.46 ± 0.067	0.60 ± 0.075
	120	0.60	0.63	0.86	0.98	0.62 ± 0.016	0.92 ± 0.080
7-Methoxy 2	30	0.14	0.15	0.25	0.15	0.15 ± 0.006	0.20 ± 0.067
(10 µM)	60	0.34	0.32	0.45	0.34	0.33 ± 0.014	0.40 ± 0.077
	90	0.55	0.48	0.85	0.79	0.51 ± 0.054	0.82 ± 0.044
	120	0.75	0.82	1.40	1.16	0.79 ± 0.052	1.28 ± 0.176
7-Methoxy 3	30	0.13	0.15	0.16	0.22	0.14 ± 0.017	0.19 ± 0.044
(10 µM)	60	0.31	0.40	0.38	0.51	0.35 ± 0.066	0.45 ± 0.089
	90	0.56	0.85	0.84	1.23	0.70 ± 0.202	1.04 ± 0.277
	120	0.80	1.06	1.49	1.99	0.93 ± 0.183	1.74 ± 0.348
7-Methoxy 4	30	0.13	0.15	0.23	0.24	0.14 ± 0.017	0.23 ± 0.010
(10 µM)	60	0.31	0.40	0.42	0.56	0.35 ± 0.066	0.49 ± 0.101
	90	0.45	0.55	0.89	1.20	0.50 ± 0.077	1.04 ± 0.217
	120	0.71	0.83	1.54	1.97	0.77 ± 0.084	1.75 ± 0.306
7-Methoxy 1	30	0.33	0.35	0.16	0.18	0.34 ± 0.014	0.17 ± 0.014
(20 µM)	60	0.36	0.43	0.39	0.37	0.40 ± 0.049	0.38 ± 0.014
	90	0.63	0.74	0.78	0.72	0.69 ± 0.078	0.75 ± 0.042
	120	0.73	0.99	0.73	1.28	0.86 ± 0.184	1.01 ± 0.389
7-Methoxy 2	30	0.39	0.15	0.49	0.49	0.27 ± 0.170	0.49 ± 0.000
(20 µM)	60	0.69	0.76	1.26	0.90	0.73 ± 0.049	1.08 ± 0.255
	90	1.38	0.80	1.88	1.70	1.09 ± 0.410	1.79 ± 0.127
	120	2.37	2.45	3.45	3.30	2.41 ± 0.057	3.38 ± 0.106
7-Methoxy 3	30	0.11	0.40	0.32	0.15	0.26 ± 0.205	0.24 ± 0.120
(20 µM)	60	0.42	0.83	1.02	0.55	0.63 ± 0.290	0.79 ± 0.332
	90	0.75	1.41	2.34	1.53	1.08 ± 0.467	1.94 ± 0.573
	120	1.75	2.92	3.71	3.65	2.34 ± 0.827	3.68 ± 0.042
7-Methoxy 4	30	0.13	0.39	0.18	0.14	0.26 ± 0.184	0.16 ± 0.028
(20 µM)	60	0.52	0.79	0.73	0.51	0.66 ± 0.191	0.62 ± 0.156
	90	0.84	1.29	1.94	1.33	1.07 ± 0.318	1.64 ± 0.431
	120	1.86	2.65	3.30	3.31	2.26 ± 0.559	3.31 ± 0.007

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	23	23	6.78	0.17	0.0132	1.24	1.42
	30	69	70	15.90	0.40			
	90	148	150	31.61	0.80			
	120	251	262	53.39	1.36			
	Cell 2							
	30	41	41	10.29	0.16	0.0172	1.61	
	60	92	92	20.22	0.47			
	90	213	216	44.43	1.41			
	120	339	347	69.81	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	10	16	9	12	5.1345	-11.833	0.9958
9.17	32	37	41	37			
13.75	51	59	58	56			
18.33	79	79	93	84			

a) Reference concentration =Concentration of Rho123 in 7mL
=275ug 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: 23/28 + 69 = 69.82

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): 5.1345

y-intercept (c): -11.833 ng/mL

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

$x = (23 - (-11.833\ ng/mL)/5.1345$

$x = 6.78\ ng/mL$

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

Example: 6.78/3928.57 x 100 = 0.173

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

f) Calculated by the equation on page 46 given in the experimental procedure chapter

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

$$P_{app} = \frac{0.00132}{60 \times 1.78 \times 100}$$

$$= 1.24 \times 10^{-7}\ cm/s$$

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	3-Methoxyflavone, 99%
Product Number	41,972-9
Product Brand	ALDRICH
CAS Number	7245-02-5
Molecular Formula	$C_{16}H_{12}O_3$
Molecular Weight	252.26

TEST	**LOT 09203CG RESULTS**
APPEARANCE	WHITE POWDER
MELTING POINT	114-115 DEGREES CELSIUS
INFRARED SPECTRUM	CONFORMS TO STRUCTURE.
PROTON, CARBON, APT	CONFORMS TO STRUCTURE.
UV-VISIBLE SPECTRUM	0.01G/L, MEOH E299=16,500 E246=17,700
ELEMENTAL ANALYSIS	CARBON 77.00% HYDROGEN 4.96%
GAS LIQUID CHROMATOGRAPHY	99.8%

Ronnie J. Martin, Supervisor
Quality Control
Milwaukee, Wisconsin USA

SIGMA-ALDRICH

Certificate of Analysis

Product Name	5-Methoxyflavone,
Product Number	M8422
Product Brand	SIGMA
CAS Number	42079-78-7
Molecular Formula	$C_{16}H_{12}O_3$
Molecular Weight	252.26

TEST	LOT 066H0708 RESULTS
APPEARANCE	WHITE POWDER WITH A LIGHT YELLOW CAST
SOLUBILITY	CLEAR VERY FAINT YELLOW SOLUTION AT 200 MG PLUS 4 ML OF CHLOROFORM
PROTON AND CARBON-13 NMR SPECTRA	CONSISTENT WITH STRUCTURE
ELEMENTAL ANALYSIS	75.68% CARBON
PURITY BY THIN LAYER CHROMATOGRAPHY	>99%
QC ACCEPTANCE DATE	AUGUST 1996

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

SIGMA-ALDRICH

Certificate of Analysis

Product Name	6-Methoxyflavone, 99%
Product Number	41,973-7
Product Brand	ALDRICH
CAS Number	26964-24-9
Molecular Formula	$C_{16}H_{12}O_3$
Molecular Weight	252.26

TEST	LOT 01602BG RESULTS
APPEARANCE	WHITE CHUNKY POWDER
MELTING POINT	164-165 DEGREES CELSIUS
INFRARED SPECTRUM	CONFORMS TO STRUCTURE.
PROTON, CARBON, APT	CONFORMS TO STRUCTURE.
ELEMENTAL ANALYSIS	CARBON 75.74%
HIGH PRESSURE LIQUID CHROMATOGRAPHY	99.9%

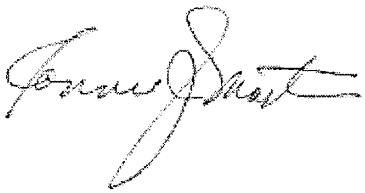
Ronnie J. Martin, Supervisor
Quality Control
Milwaukee, Wisconsin USA

SIGMA-ALDRICH

Certificate of Analysis

Product Name	7-Methoxyflavone, 99%
Product Number	41,974-5
Product Brand	ALDRICH
CAS Number	22395-22-8
Molecular Formula	$C_{16}H_{12}O_3$
Molecular Weight	252.26

TEST	LOT 09303CG RESULTS
APPEARANCE	OFF-WHITE POWDER
MELTING POINT	110-111 DEGREES CELSIUS
INFRARED SPECTRUM	CONFORMS TO STRUCTURE.
PROTON, CARBON, APT	CONFORMS TO STRUCTURE.
UV-VISIBLE SPECTRUM	0.01G/L, MEOH E306=24,100 E251=19,100
ELEMENTAL ANALYSIS	CARBON 75.77%
GAS LIQUID CHROMATOGRAPHY	99.9%



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