Bachelor's project

NTNU
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Comparing Structural Features of Flavonoid Derivatives for Enhancing Inhibition of Multidrug Resistant Cancer Mediating ABC transporters: P-gp and BCRP

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Supervisor: Eirik Johansson Solum
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Abstract

ABC transporters are a family of transmembrane proteins that serve multiple physiological functions by pumping unwanted substances out of different cells (efflux).[1] However, some ABC transporters, including Breast Cancer Resistance Protein (BCRP) and Permeability-glycoprotein (P-gp), can be overexpressed by cancer cells and cause multidrug resistance (MDR).[1] The transporters enable the cancer cells to efflux multiple chemotherapeutics and therefore cause treatment failure.[1] Inhibitors for these MDR mediating ABC transporters have been developed but most of them fail in clinical trials because of toxic effects to healthy cells.[2] In this regard, recent research has investigated the potential of flavonoids as BCRP and P-gp inhibitors. Flavonoids are a class of natural polyphenols with little to no toxic effects.[2] In order to identify and develop BCRP/P-gp inhibiting flavonoids, studies have identified structural features (i.e. functional groups) that give flavonoids the highest inhibitory activity.[3–6] For both BCRP and P-gp inhibiting flavonoids some of these beneficial features include: a flavone double bond, absence of hydrogen bond donating groups, and aromatic groups for establishing flavonoid-protein Pi-interactions.[2] This paper presents and discusses additional inhibition enhancing structural features for both BCRP and P-gp and the possibility of combining these results for the identification or development of a dual BCRP/P-gp inhibitor.

Contents

Abbreviations

ABC	ATP-binding cassette
P-gp	Permeability-glycoprotein
ABCB1	Gene enconding P-gp
BCRP	Breast Cancer Resistance Protein
ABCG2	Gene encoding BCRP
MDR	Multidrug Resistance
NBD	Nucleotide Binding Domain
TMD	Transmembrane Domain
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
SAR	Structure-Activity Relationship
QSAR	Quantitative Structure-Activity Relationship
IC_{50}	Half maximal inhibitory concentration
RI	Relative Inhibition

1 Introduction

Although the ongoing fight against cancer has in recent years seen great success, especially in the development of chemotherapeutic drugs, unforeseen challenges have arisen, such as the appearance of multidrug resistant cancers.[7] The research that is necessary to overcome these challenges requires a profound understanding of complex cellular processes in both healthy and malignant cells. One of the most vital functions of a cell is its ability to control which substances enter and exit it. When unwanted compounds cross the cell's protective membrane into the cytosol (inside of the cell), transport mechanisms are able to pump them out again. This process is called efflux.[8] These mechanisms involve specific transport proteins, a class of which are the ABC transporters.[1] ABC transporters generally contribute to the health of the cell by protecting it against toxic compounds. Unfortunately, some cancer cells have developed the ability to produce a large quantity of certain ABC transporters (overexpression) on their cell membranes.[1] This can provide them with protection against numerous chemotherapeutics, and therefore give them multidrug resistance. An idea for overcoming this problem is to identify substances to be administered in addition to the chemotherapeutic, which can inhibit the ABC transporter causing multidrug resistance.[1] The problem doesn't end here however. Observations have been made that the successful inhibition of only one of such ABC transporters might not overcome multidrug resistance in cancer cells because they compensate by expressing a different type of ABC transporter.[9] The new ABC transporter also achieves resistance yet is not affected by the present inhibitor. This suggests that the best inhibitors are those which simultaneously inhibit several of the possibly multidrug resistance mediating ABC transporters. The identification and development of these inhibitors is the subject of ongoing research. The biggest challenge is that many of the so far identified inhibitors have led to toxicities above a clinically acceptable threshold in healthy cells.[1] It is therefore of great interest to find inhibitors with low toxicity yet high inhibitory activity.

Flavonoids are promising candidates for low toxicity ABC transport inhibitors.[10] They are a class of natural substances, which are abundantly found in plants, fruits and vegetables. They are known for their low toxicities, health benefits, appearance in many traditional Chinese medicines and recently their inhibitory activity against some of these troublesome ABC transporters.[10] The base structure of flavonoids is depicted in figure 1.1.

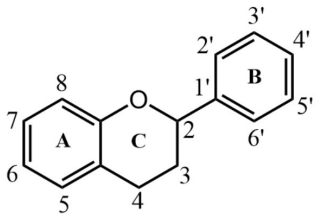

Figure 1.1: The flavonoid skeleton consists of 15 carbons, arranged in two benzene rings (ring A and ring B). The two rings are connected through a heterocyclic ring (ring C) which contains an oxygen. The conventional numbering of the carbon atoms is provided, adapted from Gonçalves et al.[10]

The aim of this paper is to present and discuss recent studies, which have identified flavonoids that are able to inhibit two of the most common ABC transporters responsible for multidrug resistance in cancers. These transporters are BCRP (breast cancer resistance protein) and P-gp (permeation glycoprotein). An in depth discussion of two studies focusing on BCRP inhibition by flavonoids will provide an insight into the methods which are used for the identification of ABC transport inhibitors. Because the inhibitory activity of a flavonoid depends on its functional groups and structure, a comparison and explanation of the beneficial structural features for increasing BCRP and P-gp inhibition will be provided. Furthermore, there will be a brief discussion of the discovery of one dual BCRP/P-gp inhibitor. Clinical considerations such as the possibility of food-drug interactions that may occur with the proposed inhibitors is not within the scope of this work.

2 Theory

ATP-binding cassette (ABC) transporters are a class of transmembrane proteins found in both eukaryotes and prokaryotes.[11,12] They play a vital role in the regulation of cellular concentrations of a wide range of small molecules, including ions, lipids and xenobiotics.[1] In brief, ABC transporters are able to efflux unwanted substances across the lipid bilayer and out of the cytosol.[12] This process requires energy because the translocation occurs against the hereby created concentration gradient. The energy is provided by ATP hydrolysis.[1] 48 human genes coding for ABC transporters have been found. They are further classified into 7 subfamilies (ABC A through G).[1]

2.1 Structure and mechanism of ABC transporters

Most ABC transporters consist of four domains, two cytoplasmic nucleotide-binding domains (NBDs) and two transmembrane domains (TMDs).[13] The TMDs bind substrates and the NBDs efflux the substrate out of the cytosol.[13] TMDs of different ABC transporters tend to have large structural differences, a feature that enables the efflux of many different substrates.[1,12] The NBDs of ABC transporters are less structurally diverse and several motifs are highly conserved throughout the transporter families.[12]

The exact mechanism for substrate translocation of ABC transporters has not yet been determined.[1] The transport cycle involves considerable conformational changes in both the TMDs and NBDs. Therefore protein crystallization only provides a snapshot of a complex choreography. These snapshots lack information about the mechanism.[11] One of the proposed mechanism starts with the substrate binding within the TMD dimer. Subsequently one ATP is hydrolyzed, causing a conformational change in the TMD, such that the substrate is effluxed. The second ATP is then hydrolyzed to reestablish the protein's initial conformation.[1] This mechanism is illustrated in figure 2.1 below.

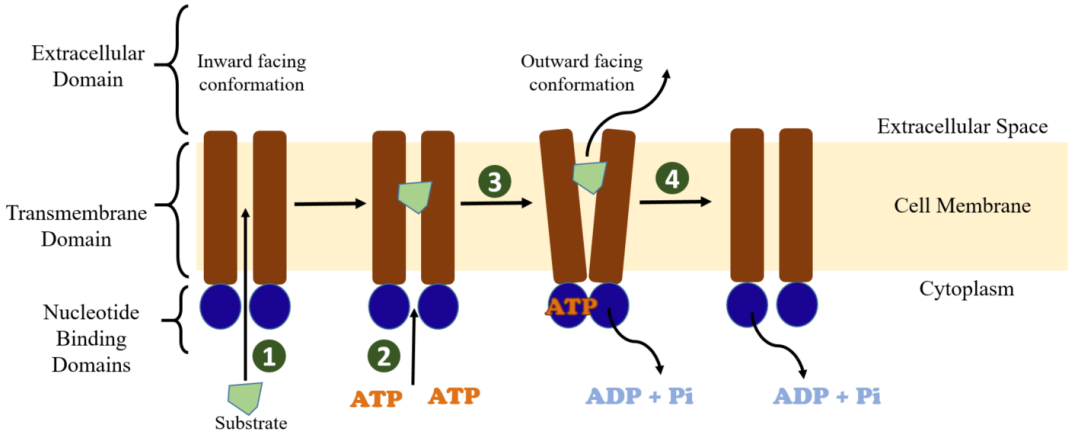

Figure 2.1: The general substrate efflux mechanism for ABC transporters.[1,10] **1)** The substrate (green) binds within the TMD binding site, **2)** two ATPs bind at the NBD binding site, **3)** ATP hydrolysis ($ATP + H_2O \rightarrow ADP + P_i$) causes a conformational change (from inward facing to outward facing), causing substrate efflux, **4)** ATP hydrolysis reestablishes the original conformation and $ADP + P_i$ are released

2.2 ABC transporter mediated multidrug resistance in cancer

ABC transporters are expressed throughout the body, including in the blood brain barrier, the placenta and the stomach lining.[1] Although the proper functioning of ABC transporters is vital for protecting healthy tissue against unwanted substances, the presence of ABC transporters in the wrong cells can be harmful.[1] As is the case in cancer cells that start effluxing

chemotherapeutics causing their intracellular concentrations to drop below therapeutic levels.[14] Consequently they become multidrug resistant (MDR).[15] ABC transporter mediated MDR is seen amongst others in blood, breast, ovarian, and lung cancers.[16] 19 out of the 48 ABC transporters are related to multidrug resistance and are those with low substrate specificity.[1] This low specificity allows the recognition of multiple chemotherapeutics. For example, the MDR mediating ABC transporter, P-gp, has such a flexible drug binding pocket that it can bind different chemicals from 100-4000Da, including many common chemotherapeutics.[17] The expression of ABC transporters, such as P-gp, in cancers often worsens a patient's prognosis significantly because it increases the chance of chemotherapy failure.[16]

Three of the most studied and common MDR inducing ABC transport inhibitors are P-gp (encoded by ABCB1), Multidrug Resistance Associated Protein 1: MRP1 (ABCC1) and Breast Cancer Resistance Protein: BCRP (ABCG2).[2] Due to their normal physiological roles in amongst others the blood-brain barrier, placenta and small intestine, the inhibition of these transporters doesn't come without side effects, often due to increased bioavailability and decreased clearance of their substrates out of the body.[2,5]

Figure 2.2A shows the structures of P-gp and BCRP.[10] The aforementioned transmembrane domains (TMDs) and nucleotide binding domains (NBDs) are visualised. Figure 2.2B illustrates the structural variations between the two transporters, BCRP only having one TMD and one NBD. Numerous experimental data suggests that homodimerization of BCRP is required for substrate efflux.[18]

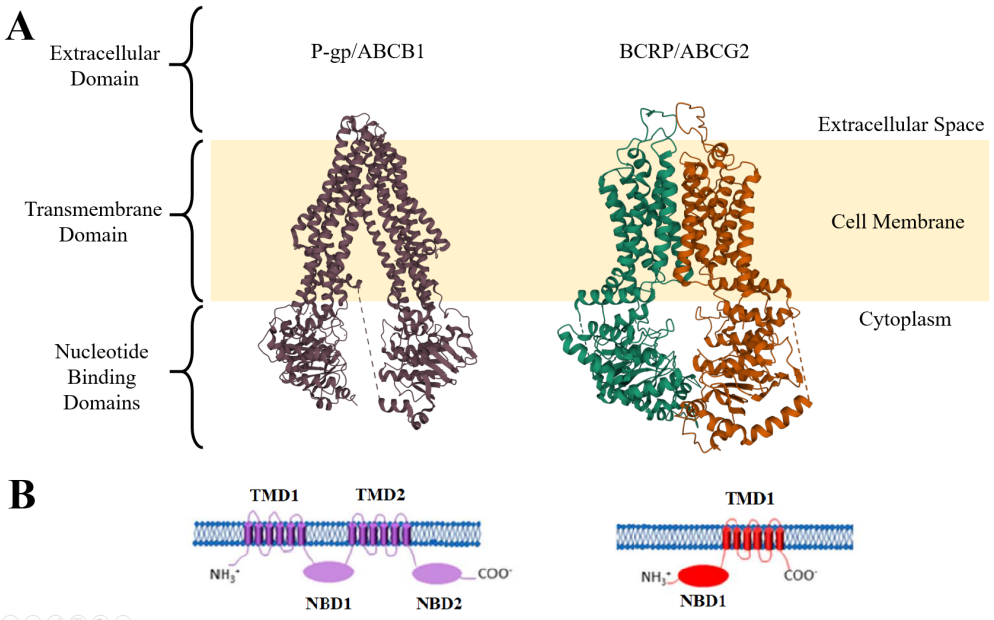

Figure 2.2: **A:** 3D structure of mouse P-gp (PDB-ID:4M1M),[19] and human BCRP (PDB-ID:6VXF)[20] (inspired by Gonçalves et al.[10]) **B:** membrane topology models of P-gp and BCRP. Figures adapted from Gonçalves et al.,[10] licensed under CC BY 4.0.

2.3 Methods for ABC transport inhibitor identification

The identification process of ABC transporter inhibitors usually involves specified in vitro studies. Recently, more studies have started including machine learning approaches, in order to establish quantitative structure-activity relationships (QSAR). Modeling the interactions between the inhibitor and transporter binding site in molecular docking models can also be found in some research.[4,5] A brief explanation of these three methods follows below.

In Vitro Studies

The purpose of in vitro studies for the identification of ABC transport inhibitors is to determine to what extent they inhibit substrate efflux. One of the most common in vitro approaches are accumulation studies.[21] Cell lines that express one of the ABC transporters are exposed to a suitable substrate and the accumulated intracellular concentration of it, with and without the potential inhibitor is quantified. These accumulation measurements are often achieved by means of fluorescence spectroscopy where the intensity of fluorescence emitted by the substrate from within the cell is measured.[21] Cells with strongly inhibited ABC transporters will fluoresce the strongest, because substrate efflux is less effective.[21] The intensity of the emitted light is proportional to the intracellular substrate concentration.[21] Figure 2.3 illustrates the Calcein AM assay, a common fluorescence study used to study P-gp inhibition. Results are conventionally given in IC_{50} values, which is the inhibitor concentration needed to inhibit the efflux activity by 50%. The activity of the transporter is said to be 100% in the absence of an inhibitor. 50% activity can be found graphically by plotting the concentration of the inhibitor against the activity of the transporter, which is inversely proportional to the intracellular substrate concentration. Good inhibitors will exhibit low IC_{50} values because one requires only a small amount to achieve 50% inhibition. Another common value that is calculated is the relative inhibition (RI), usually found through the following equation:

$$RI(\%) = 100 * \frac{C_i - C_{min}}{C_{max} - C_{min}} \tag{2.1}$$

where C_i is the substrate concentration with the test compound, C_{min} is the basal cellular substrate concentration without an inhibitor and C_{max} is the substrate concentration at maximal inhibition, which is usually found by incubating the cells with a significant concentration of a control compound that is known to be a potent inhibitor. If a set of potential inhibitors is compared at a given concentration, those giving the highest RI values indicate the better inhibitors.

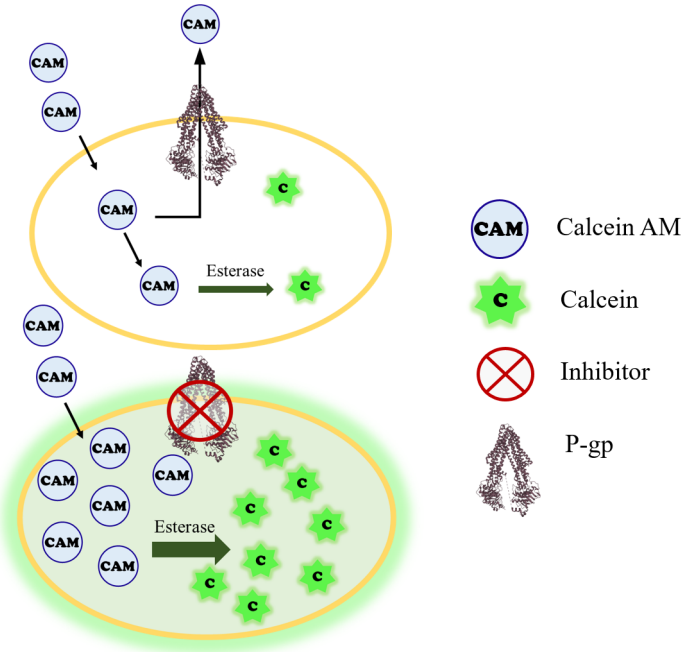

Figure 2.3: Calcein AM-assay, adapted from E.J.Solum.[22] Calcein AM is converted by esterases within the cell to fluorescing calcein. **Top:** Calcein AM is a substrate of P-gp and therefore effluxed out of the cell, meaning only a few fluorescing calcein molecules are produced. **Bottom:** An inhibitor strongly reduces the calcein AM efflux, allowing its accumulation, and therefore increased production of calcein, causing an increase in fluorescence intensity.[23] The figure shows mouse P-gp (PDB-ID:4M1M).[19]

Quantitative Structure-Activity Relationship (QSAR)

The structure-activity relationship (SAR) refers to the assumption that molecules with similar structures have similar activities.[24] To find SARs, the different chemical features of a molecule must be identified. Examples of such chemical features are lipophilic areas and hydrogen bond donating regions. Quantitative SAR (QSAR) is an approach in which the degree to which each chemical feature influences the chemical activity is quantified, for example, the effect of a lipophilic area within the structure of an inhibitor, on the efflux activity of a transporter.[24] 3D-QSAR are QSAR models that take into consideration another variable, which is the placement of these chemical feature in 3D space.[24] 3D-QSAR thereby takes into account that chemical features in different regions of the compound can have different effects.[24]

There are several different approaches to achieve 3D-QSAR models. Amongst these, the alignment-based technique, CoMSIA, is relevant in the studies discussed later. The method allows for the calculation of the field strengths for five different features of a compound throughout 3D space (steric, electrostatic, hydrophobic, hydrogen bond donor, and hydrogen bond acceptor).[24] Contours can be found for regions of the compound where these features have equal field strength. The contours can then be related to the biological activity of the assessed compound. If the procedure is repeated for each compound, and the compounds are aligned according to molecular similarity, one can create a so-called pharmacophore that identifies the optimal regions for each of the five properties, such that the highest biological activity is achieved.[24] The requirement for such alignment strategies is that the compounds all have the same binding site.[24,25]

Molecular Docking Models

Molecular docking techniques are computational methods that give insight into the probable positioning of a substrate within a protein binding site.[26] This can provide a further understanding of SARs and possible binding mechanisms. Access to the structure of the protein, for example by X-ray crystallography, is required.[26] In a modeling program, the favorable and unfavorable stereochemical and electrostatic interactions between the ligand and protein are taken into account. A "best fit" of the substrate is computed by iteration towards the binding conformation with the smallest binding energy.[26] Furthermore, one can identify protein residues that are involved in binding (hydrogen bond, van der Waals, Pi-Pi, and Pi-alkyl interactions).[5] Weaknesses of molecular docking models must be considered, such that the validity of the results can be weighted appropriately. A source of error emerges from inaccuracies in predicting the bonding energetics, which is calculated based on a set of selected properties.[26] The summation of these parameters in a scoring function provides an estimation of bonding energy. Inaccurate estimations can cause systematic errors, especially if some unexpected yet significant parameters have been neglected.[26] Furthermore, it is difficult to compute the flexibility of the protein. As discussed previously, the ABC transporters undergo significant conformations changes, and these are difficult to model. Therefore, the docking models may not be accurate throughout the protein's conformational cycle.[11,26] Keeping in mind these possible errors, molecular docking is still a valuable tool, especially when used in tandem with other methods.

2.4 Challenges in ABC transport inhibitor identification

Although many inhibitors of MDR mediating ABC transporters have been found, they have shown minimal clinical success for cancer treatment.[2] Many of the identified inhibitors are non-specific for inhibition of the target ABC transporters, meaning that they have other physiological impacts that can lead to side effects and even major toxicity.[27] Furthermore, because the MDR-related ABC transporters have a large substrate range, inhibitors will increase the cellular concentration of chemotherapeutics and other possibly harmful substrates, in both cancererous and healthy cells.[1] For example, BCRP is highly expressed within a healthy stomach lining. Amongst others, it is responsible for decreasing the absorption of a range of substances, including some common medications.[28] If BCRP is inhibited throughout the body, then the

absorption of these medications through the stomach lining will increase and thus the concentration of them may exceed their therapeutic window.

These are only some of the challenges that must be considered when evaluating possible inhibitors. In the early stages of drug development, it is beneficial to use these considerations as guidelines. However, we are only starting to understand ABC transporters and it is of value to find good inhibitors, regardless of later clinical concerns. Hereby one gains more understanding about where and how the inhibitors bind, and which chemical structures are involved in increased inhibitory activity. Further development of inhibitors into clinically approved drugs is not within the scope of preliminary research and this paper.

A problem worth considering in the early stages of research is the possibility of failure if the inhibitor only inhibits one type of MDR mediating ABC transporter. Studies have shown that the cells can adapt by expressing a different type of ABC transporter that is not affected by the inhibitor. This makes the inhibitor ineffective.[9,14] This phenomenon is possible due to the significant substrate overlap of the various MDR mediating transporters, demonstrated for P-gp and BCRP in figure 2.4(left). Therefore, there is value in finding inhibitors that can inhibit more than one MDR mediating ABC transporter, as demonstrated by the overlapping region of inhibitors in figure 2.4(right).[14]

Due to the toxic side effects that have been caused by many developed ABC transport inhibitors, increasing attention has been given to potential inhibitors found in nature. Many of these have high bioactivities and low cytotoxicity. This can make them ideal candidates for new medicines.[10] Amongst these natural products are flavonoids, which will be discussed in the next section.

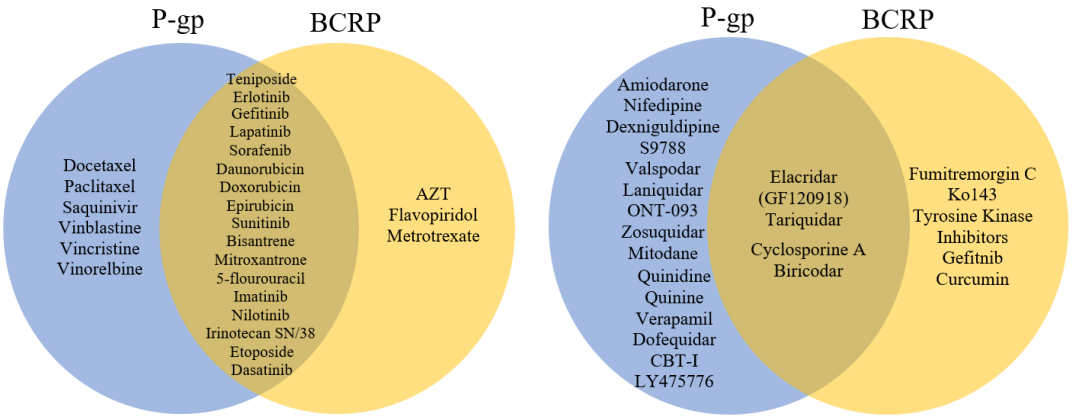

Figure 2.4: **Left:** substrates and substrate overlap of P-gp and BCRP, **Right:** examples of known inhibitors and inhibitor overlap for P-gp and BCRP[14 29]

2.5 Flavonoids

Flavonoids are naturally occurring polyphenols present within a large variety of plants.[2] Several subclasses of flavonoids exist. These include chalcones, flavanones, flavanonols, flavones, flavonols, anthocyanidins, and isoflavones.[2,10] As depicted in figure 2.5, the different subclasses of flavonoids are mainly classified based on where ring B attaches to ring C, to which degree ring C is unsaturated, and the presence/absence of hydroxyl groups on ring C (naming of rings for flavonoids found in figure 1.1).

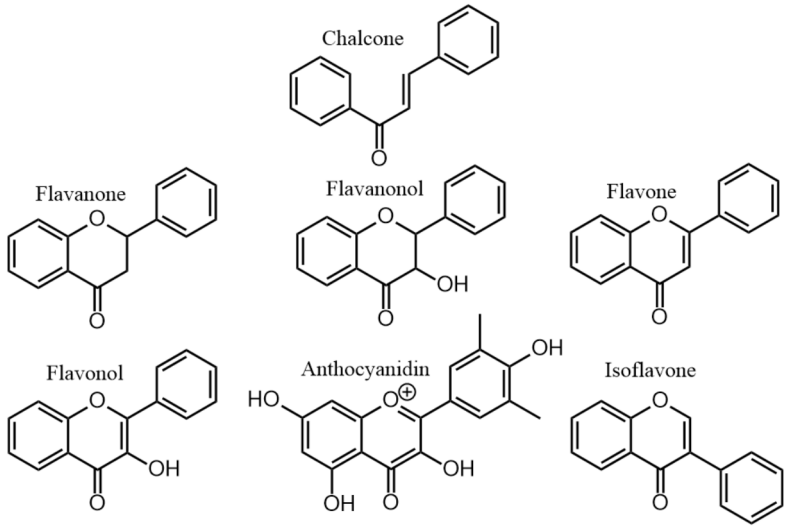

Figure 2.5: Major Flavonoid Subclasses[10]

2.5.1 Medical significance of flavonoids

In nature, flavonoids serve plants as protection against stresses from the environment. These protective functions have been shown to be beneficial in humans as well. They include antioxidative functions, reduction of procarcinogen activation, and detoxification of carcinogens.[3] Due to the prevalence of flavonoids in nature, humans already ingest significant amounts of flavonoids, with an estimated 200mg-1g per day.[5] They can also be found in traditional Chinese medicines and increasingly in health products and drugs.[3 5] Flavonoids have been incorporated into treatments for cancer, Alzheimer's disease, and atherosclerosis.[2] As the discussion below shall reveal, various flavonoids have been shown to contribute to the inhibition of P-gp and BCRP.[2]

2.5.2 Flavonoids as MDR mediating ABC transport inhibitors - Downside

Because of frequent exposure and absorption of flavonoids from nutrition, health products, and medicines, and their potential to alter the pharmacokinetics of various drugs by inhibiting drug transporters, possibilities for food-drug and drug-drug interactions exist.[5] For instance, a study demonstrated that flavonoids present in fruit juice interfered with BCRP and thus reduced the efflux of dasartinib into BCRP expressing cells.[30] Such food-drug interactions can become dangerous if they change the pharmacokinetics of drugs to a degree that causes unforeseen toxicities. Although most flavonoids are not directly cytotoxic, it should be noted that they could cause indirect toxicities due to their potency to interfere with the pharmacokinetics of endogenous and exogenous substances.

3 Results and Discussion

Certain flavonoids have revealed their potential for increasing the inhibition of BCRP and P-gp.[2] The aim is to identify the potentiating structural features of flavonoids such that flavonoids with the highest possible inhibitory activity can be found or synthesized. Some general features have at present been found to increase BCRP and P-gp inhibition, including a hydroxyl group at carbon 5, a methoxyl group at carbon 3, and a double bond between carbon 2 and 3 (for numbering of the flavonoid skeleton, see figure 1.1).[2] Moreover, the substitution of a methoxy group at carbon 3 with a hydroxy group has shown to decrease the inhibitory activity against BCRP and P-gp.[2] In the following sections, studies, which have further identified such features, have been reviewed and compared. There are limited studies on dual BCRP/P-gp inhibitors.

The discussion focuses on studies looking at BCRP inhibition by flavonoids. This will provide insight into the applied methods for identifying and evaluating ABC transport inhibitors. A discussion about the identification of a dual BCRP/P-gp inhibitor will follow.

3.1 BCRP inhibiting Flavonoids

In 2011, Pick et al.[3] studied the inhibition activity of 31 flavonoids on BCRP. They included a range of flavonoid subgroups. The study consisted of an uptake study, in which the previously identifies BCRP substrate, Hoechst 33342, was used as a fluorescent marker. Two BCRP expressing cell lines, MCF-7 MX and MDCK BCRP were tested. IC_{50} values for all 21 flavonoids in addition to the standard BCRP inhibitors Ko143 and tariquidar (for control) were given. The inhibitory activity of the 31 flavonoids against P-gp in P-gp overexpressing A2780adr cells was also found.[3] A calcein AM assay was used for this purpose. Testing for the inhibition of both transporters allows the potential identification of dual BCRP/P-gp inhibitors. Furthermore, a 3D QSAR study was conducted to identify the structural features which increased BCRP inhibition.

Amongst the 31 flavonoids in the study by Pick et al., were four flavonoid glycosides (two of which are depicted in figure 3.1), with substitution of galactose, glucose, and/or rhamnose at various positions on the flavonoid skeleton.(*3*) None of these showed BCRP or P-gp inhibition. No explanation is proposed for this observation, but it may be reasonable to suggest that the steric bulk of the saccharides and the addition of their hydrogen bond donating hydroxyl groups are responsible for decreasing the inhibitory potential.

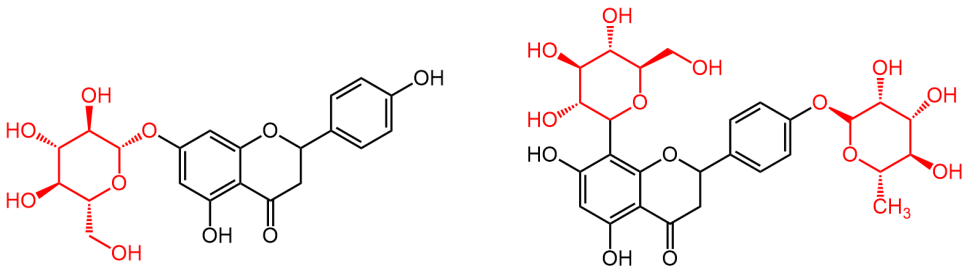

Figure 3.1: Flavonoid glycosides with no BCRP inhibitory activity, saccharide substituents are shown in red, demonstrating the steric bulk and abundance of hydrogen bond donating hydroxyl groups, **Left:** Apigetrin, **Right:** Vitexin-4'-rhamnoside[3]

Furthermore, it was systematically observed that flavones showed greater BCRP inhibitory activity than their analog flavanones, with IC_{50} values for example changing from 19±8 μM to 3.4±1.8 μM when exchanging 6-methoxyflavanon with its flavone analog 6-methoxyflavone. In figure 2.5 one sees that the difference between flavanones and flavones is the presence of a double bond between carbon 2 and 3 in the latter. The benefit of this double bond is in agreement with the results of prior studies, as mentioned above.[2] The reason for this observation is not discussed by Pick et al..[3] The double bond could be beneficial due to its introduction of a planar arrangement of the substituent at carbon 3 and the linker to ring B. Also, the double bond increases the electron density in ring C, which may be beneficial for binding interactions within the binding site of the transporter. However, these are only speculations and need further investigation for verification.

The flavonoids that showed the most extensive inhibitory activity were the flavone derivatives Penduletin, Ayanin, and Retusin. Their IC_{50} values in MDCK BCRP cells were 1.2±0.6 μM, 0.46±0.04 μM, and 0.39±0.14 μM respectively (± 1 standard deviation). For comparison, the IC_{50} value of the known to be potent BCRP inhibitor Ko143 was found to be only slightly lower at 0.21±0.15 μM in the same cell line. The results, therefore, reveal a group of flavonoids that are very potent BCRP inhibitors. They all belong to the flavone subgroups and have varying

substitutions with methoxy groups. Retusin, which is depicted in figure 3.2 has the strongest inhibitory activity. There are methoxy groups at carbon 3, 7, 3' and 4' (see figure 1.1) and a hydroxy group at carbon 5, indicating additional substituents that can increase inhibitory activity. If instead of all the methoxy groups are hydroxy groups, such as in quercetin, the IC_{50} increases (6.9±1.1 μM in MDCK BCRP cells), demonstrating the importance of methoxylation.

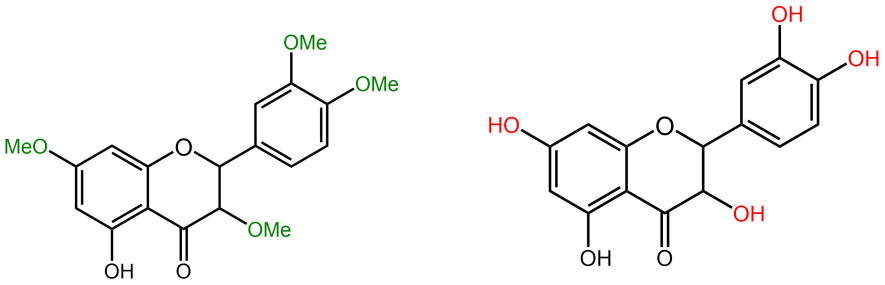

Figure 3.2: Left: Retusin (IC_{50}=0.39±0.14μM), **Right**: Quercetin (IC_{50}=6.9±1.1μM). Demonstration of the positive effect of methoxy groups (green) at carbon 3, 7, 3' and 4' of flavones for inhibition of BCRP compared to hydroxy groups (red) (in Hoechst 33342 assay in MDCK BCRP cells)

Substituting methoxy groups with hydroxy groups could be the reason for increased BCRP inhibition. First, the hydrophobic character of the substituents: hydroxyl groups are more polar than methoxy groups. This generally implies that the fewer hydroxy groups, the less water-soluble the flavonoid becomes. Prior studies have also found that lipophilic compounds generally tend to have higher inhibitory activity towards BCRP and P-gp.[31] Referring to figure 2.2, the binding sites of the inhibitors and substrates of ABC transporters are either within the TMDs or the NBDs, amongst which the TMDs are within the cell membrane thus within a hydrophobic environment.(*31*) Lipophilic compounds are, therefore, a lot more likely to access a binding site within the TMD. This indicates that a/the binding site for inhibition of BCRP may be found within the TMD and that one may find good BCRP inhibitors when looking at lipophilic flavonoids.

Another effect of substituting hydroxy groups with methoxy groups is the decrease in the amount of hydrogen bond donating groups. Hydroxy groups can partake in hydrogen bonds by donating their proton to a hydrogen bond acceptor. Numerous amino acids can be hydrogen bond acceptors and these bonds are favorable in many ligand-protein interactions. However, having hydrogen bond donors in parts of the binding site where there shouldn't be, can cause the compound to improperly bind due to interactions with other amino acids in the vicinity. Methoxy groups are still able to partake in hydrogen bonding, however only through the oxygen atom, being a proton accepting species. This suggests that the binding site in BCRP (for flavonoids) has few hydrogen bond accepting groups. Hydrogen bond donating amino acids may be present but these are still able to interact with the methoxy groups.

The results of the study by Pick et al., for flavonoid inhibition of P-gp, demonstrate that very few of the 31 tested flavonoids also exhibited P-gp inhibition.[3] Those that showed the most P-gp inhibition were biflavonoids. However, none of the biflavonoids showed inhibitory activity against BCRP, which means that no dual P-gp/BCRP inhibitors were identified in this study.

Pick et al.'s 3D QSAR model for the inhibition of BCRP was demonstrated in a contour plot showing the BCRP inhibitor ayanin (figure 3.3). The yellow contour indicates that a hydrogen bond donor such as a hydroxy group would decrease inhibitory activity around the substituent at carbon 3. A red contour is located around the same area, indicating that a partial negative charge such as that of an oxygen atom is favorable here. The red contour in combination with the yellow contour thus explains the benefit of a methoxy group, which is not a hydrogen bond donor, yet it does have a partial negative charge due to its oxygen atom. A smaller red contour is seen above carbon 2 and 3 of ring C. This could explain the increase in inhibition

due to the double bond between carbon 2 and 3, since this increases the electron density and thus the partial negative charge around this area. A blue contour around the hydrogen of ayanin's hydroxy group explains the observed positive effect of a partial positive charge from a hydroxy group at carbon 5. According to the conclusions of Pick et al., the second blue contour is located around an oxygen atom of hydroxy groups at carbon 2', and therefore, is more indicative of the negative effect that negative charge would have in this area. Finally, the green contours explain why hydrogen bond acceptors such as methoxy groups around carbon 6 and 7 increase inhibition.

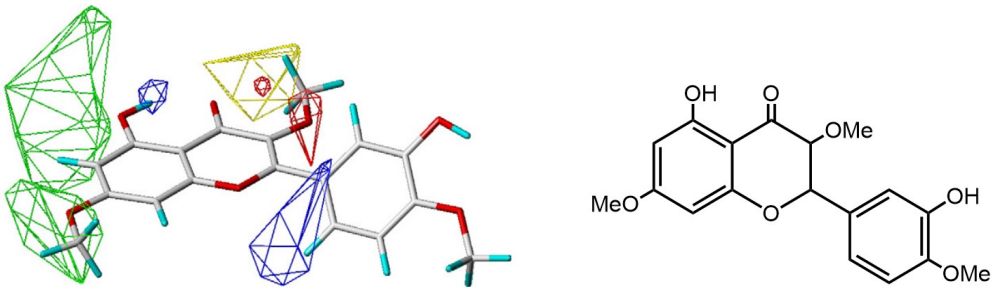

Figure 3.3: **Left:** From the 3D-QSAR study by Pick et al. (2011):[3] Contour Plot (CoMSIA) for BCRP inhibiting flavonoid in MDCK BCRP cells, shown for Ayanin (identified inhibitor). Red shows areas in which negative charge enhances inhibition, blue indicates areas in which positive charge is favored, green shows areas where hydrogen bond acceptors have a positive impact, and yellow highlights areas where hydrogen bond acceptors decrease inhibitory activity. Figure reproduced with pending permission from Pick et al.(3).
Right: Structure of Ayanin provided for clarity

Pick et al. have influenced further investigations of flavonoids' inhibitory activity towards BCRP and P-gp. A more recent study by Fan et al. (2019) tested 99 flavonoids and their BCRP inhibitory activity in BCRP-MDCKII cells..[5] An uptake study was conducted using the known BCRP substrate mitoxantrone with the determination of uptake inhibition via HPLC/MS. Inhibitors were identified as those having an inhibition ratio above 50%. A concentration-dependent uptake study of the flavonoids was also conducted, in which the inhibition ratio was found as a function of flavonoid concentration. This allowed the determination of IC_{50} values. Furthermore, a computational molecular docking model (CDOCKER) was created, which allowed insight into the inhibitor-protein interactions and thus further understanding of inhibition enhancing structural features. Finally, a pharmacophore for flavonoids as BCRP inhibitors was suggested (figure 3.6).

Fan et al.(2019) identified 11 inhibitors, the strongest were amentoflavone (IC_{50} = 4 ± 1 μM, RI = 80.99%), kaempferide (IC_{50} = 5±1 μM, RI = 59.54%) and hypericin (IC_{50} = 7±1 μM, RI = 81.22%) (figures 3.4, 3.6 and 3.5 respectively). The IC_{50} values are higher, and thus the inhibitory activities are weaker than for penduletin, ayanin, and retusin from Pick et al.'s study.[3] The values are not completely comparable due to the different methods and cell lines. Most notable are the molecular docking models which demonstrated additional electronic factors for BCRP inhibition. The models for amentoflavone, hypericin, and kaempferide are displayed in the same figures. The docking diagram for myricetin, which is a non-inhibiting kaempferide analog, is provided for comparison. Dark green indicates hydrogen bond interactions, light green: van Der Waals type, light pink: Pi-Alkyl, and dark pink: Pi-Pi interactions. Pi-Pi stacking interactions with Phe439 and Pi-alkyl interactions with Val546, were found in all docking models of the 11 inhibiting flavonoids and the known inhibitor Ko143. All of these have Pi-Pi interactions with Ph439 at ring B for instance. This might explain the positive effect of methoxy and hydroxy groups at this aromatic ring since both substituents are electron-donating groups, which increases the electron density in the ring and increases the strength of the Pi-Pi interactions. The docking models also demonstrated that an increase in

hydrogen bonding interactions, such as are present with myricetin (figure 3.7), systematically decreased the inhibitory activity of flavonoid analogs. This agrees with the findings of Pick et al., as discussed above. A speculative explanation for this effect could be that hydrogen bond accepting amino acids near the BCRP binding site cause unfavorable interactions with the compound, and decrease its affinity for the correct binding site. The fact that methoxy groups can donate electron density into the Pi system of the flavonoid rings, yet are unable to act as unfavourable hydrogen bond donors, explains why they have shown to have such a good impact on BCRP inhibition.

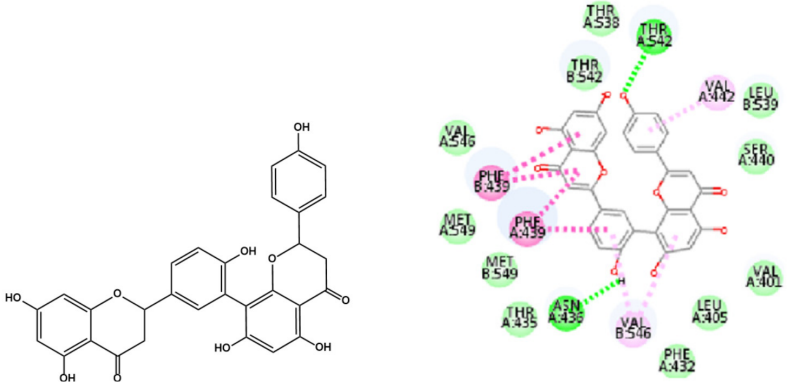

Figure 3.4: **Left:**BCRP inhibitor, Amentoflavone (IC_{50} = 4±1 μM, RI = 80.99, in BCRP-MDCKII cells).[5] **Right**: Molecular docking model (CDOCKER) for amentoflavone and human BCRP, dark green: hydrogen bond interactions, light green: van Der Waals type interactions, light pink: Pi-Alkyl interactions, dark pink: Pi-Pi interactions. Figure reproduced with pending permission from Fan et al.[5]

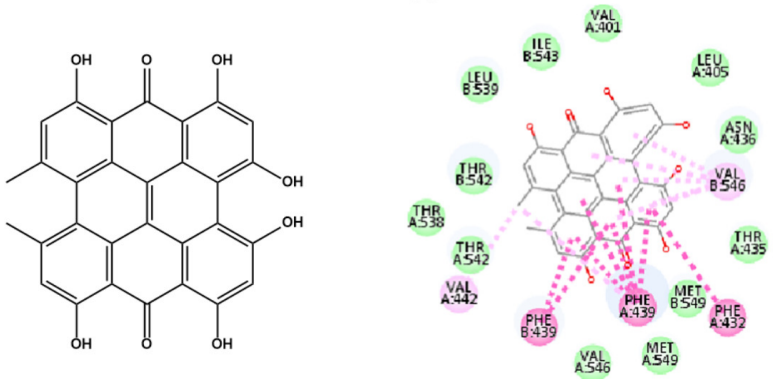

Figure 3.5: **Left:**BCRP inhibitor, hypericin (IC_{50} = 7±1 μM, RI = 81.22, in BCRP-MDCKII cells).[5] **Right**: Molecular docking model (CDOCKER) for hypericin and human BCRP, dark green: hydrogen bond interactions, light green: van Der Waals type interactions, light pink: Pi-Alkyl interactions, dark pink: Pi-Pi interactions. Figure reproduced with pending permission from Fan et al.[5]

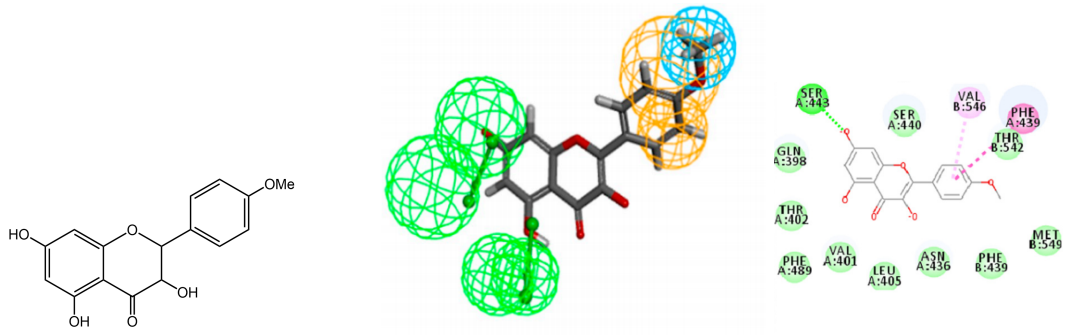

Figure 3.6: **Left:**BCRP inhibitor, kaempferide (IC_{50} = 5±1 μM, RI = 59.54, in BCRP-MDCKII cells).[5] **Middle:** pharmacophore for BCRP inhibitors, shown with kaempferide, green: hydrogen bond acceptors, blue: hydrophobic groups, orange: aromatic ring. **Right**: Molecular docking model (CDOCKER) for kaempferide and human BCRP, dark green: hydrogen bond interactions, light green: van Der Waals type interactions, light pink: Pi-Alkyl interactions, dark pink: Pi-Pi interactions. Figure reproduced with pending permission from Fan et al.[5]

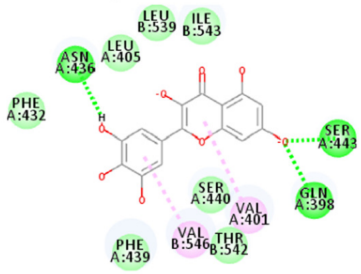

Figure 3.7: Molecular docking model (CDOCKER) for myricetin and human BCRP, dark green: hydrogen bond interactions, light green: van Der Waals type interactions, light pink: Pi-Alkyl interactions, dark pink: Pi-Pi interactions. Figure reproduced with pending permission from Fan et al.[5]

Fan et al.'s pharmacophore (see figure 3.6), supports the findings of Pick et al. Seen in green, in figure 3.6, are areas in which hydrogen bond acceptors such as hydroxy groups and methoxy groups are preferable (carbon 5 and 7). This is similar to Pick et al.'s findings. Ring B is marked with orange, indicating that an aromatic ring is preferable, probably because it allows Pi-Pi interactions with Phe439. The blue marking around the methoxy group at carbon 4' indicates that a hydrophobic group enhances inhibition. This may suggest that the binding site of BCRP contains some hydrophobic pocket into which ring B and its substituents fit. In this case, the hydrophilic hydroxy groups would weaken binding, whilst the more hydrophobic methoxy groups would increase binding.(*5*) This observation is also in agreeance with the findings of Pick et al. A summary of beneficial structural features for BCRP inhibition, found by the two studies is depicted in figure 4.1.

3.2 Dual BCRP/P-gp inibiting Flavonoids

As discussed in section 2.4, it would be highly valuable to find an inhibitor that is capable of being multispecific towards two or more MDR mediating ABC transporters. Studies have identified numerous flavonoids that specifically inhibit BCRP and P-gp, but amongst these, there are only a few dual BCRP/P-gp inhibitors.

Despite the transporters being very similar, in structure,[10] the flavonoids which have been studied so far, could be too specific towards the differences between each inhibitor. It suggests

that the inhibiting effect, which the flavonoids have on one of the transporters, is not achieved in the other.

To demonstrate the differences in favorable structural features in BCRP and P-gp inhibitors one can compare pharmacophores. Above, a suggested pharmacophore for BCRP inhibitors was discussed (Fan et al.[5]). Bai et al. (2019) conducted a study to test the P-gp inhibiting activity of 75 flavonoids, five of which were found to be quite potent (RI>50%): tangeretin, sinensetin, isosinensetin, sciadopitysin and oroxylin A. A pharmacophore was created, just like in Fan et al.'s study, and is displayed below with the structure of sinensetin (figure 3.8).

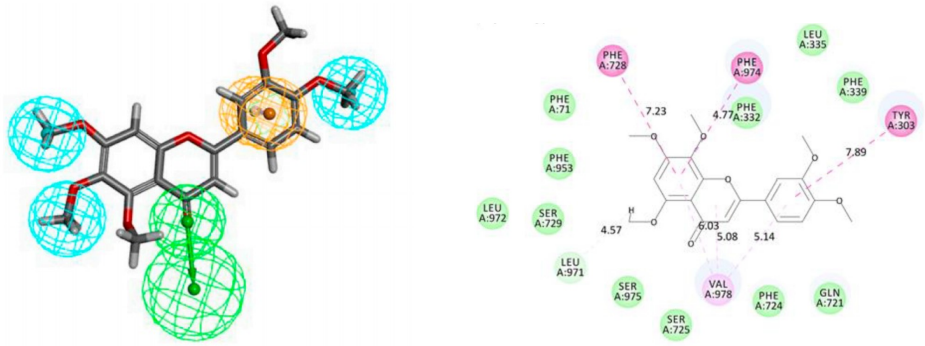

Figure 3.8: **Left:** pharmacophore for P-gp inhibitors, shown with P-gp inhibitor sinensetin, green: hydrogen bond acceptors, blue: hydrophobic groups, orange: aromatic ring.[4]
Right: Molecular docking model (CDOCKER) for sinensetin and mouse P-gp, dark green: hydrogen bond interactions, light green: van Der Waals type interactions, light pink: Pi-Alkyl interactions, dark pink: Pi-Pi interactions. Figure reproduced with pending permission from Bai et al.[4]

Compared to Fan et al.'s pharmacophore for BCRP inhibitors (figure 3.6) there are some similarities but also differences. The presence of an aromatic ring at ring B seems to increase inhibition in both transporters, in addition to the hydrophobic groups at carbon 4'. The presence of a hydrogen bond acceptor at carbon 4 seems to increase P-gp inhibition, yet no such preference is seen for inhibition of BCRP. Hydrophobic groups at carbon 6 and 7 increase P-gp inhibition, whilst for BCRP, hydrogen bond acceptors in this area enhance inhibition.

Bai et al. also conducted molecular docking studies and the model for the potent inhibitor sinensetin is displayed in figure 3.8. They found that effective P-gp binding was due to interaction with Phe974, Phe728, Val978, and Phe332 residues, as can be seen in the figure. As in the BCRP inhibitors, inhibition seems to be dependent on Pi interactions (dark and light pink) with these amino acids. In addition, the same negative effect of hydrogen bonding was found for inhibition of P-gp as for inhibition of BCRP. A summary of the inhibitor inducing structural features found by Bai et al. is provided in figure 4.2.

The question remains, whether it is possible to find a combined P-gp/BCRP inhibitor pharmacophore, yet for this purpose more dual P-gp/BCRP inhibiting flavonoids should be identified and compared. In a study by Yuan et al. (2012) one dual P-gp/BCRP inhibitor was found (figure 3.9). Several features such as the flavone double bond, methoxy groups at carbon 7, 3', and 4' are beneficial for BCRP inhibition as discussed above.[5] Methoxy groups at carbon 4 and 7 are also part of the P-gp pharmacophore.[4] The compound also has an aromatic ring B, with two methoxy substituents having an electron-donating effect, that can strengthen Pi interactions in the binding site of both P-gp and BCRP. Notable is the large 2-((4-Methoxybenzoyl)oxy)ethyl substituent at carbon 3 with an ethyleneoxycarbonyl linker. Yuan et al. compared several analogs of this compound and found that this substituent enhanced dual BCRP/P-gp inhibition, making the compound equally potent against both transporters. Compared to retusin, which was found to be the best BCRP inhibitor also in this study (19.3 fold increase in substrate

concentration with 1.0 μmol retusin in MCF7-MX100 cells), the dual BCRP/P-gp inhibitor is only somewhat less inhibiting towards BCRP (1.0 μmol causing 7.3 fold increase in substrate concentration in MCF7-MX100 cells). It was the most effective P-gp inhibitor (1.0 μmol causing 11.3 fold increase in substrate concentration in LCC6MDR cells), with a ca. 3.4 fold higher activity than verapamil (a potent inhibitor of P-gp used as control). Evidence of dual P-gp/BCRP inhibitors, with high inhibitory activity against both transporters, therefore exists. It also demonstrates that substitution such as that at carbon 3 in the dual inhibitor may overcome the differences in the binding sites of the two transporters. For further investigation, it may be of value to further investigate analogs of this compound. This may allow the construction of a dual BCRP/P-gp inhibitor pharmacophore, and provide a deeper understanding of the binding mechanisms in the transporters.

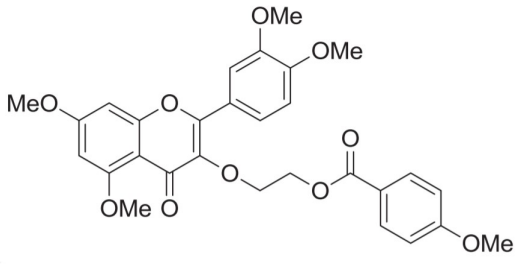

Figure 3.9: Dual BCRP/P-gp inhibitor found by Yuan et al.[6]

Overall the discussed studies have provided further insight into flavonoid's inhibitory activity towards BCRP and P-gp. One must keep in mind that there are always uncertainties, especially when studies use modeling techniques such as QSAR and molecular docking. Nevertheless, the results of the models give good indications of which structural features are beneficial, and the findings of the models agree with in vitro studies. As mentioned throughout the discussion, different studies come to similar conclusions, which strengthens the results. The next step is to further validate the results, possibly by conducting studies in which derivatives of the best inhibitors are synthesized, and tested in different cell lines.

4 Conclusion

The expression of MDR mediating ABC transporters such as BCRP and P-gp in cancers can significantly worsen the chances for a successful chemotherapeutic treatment.[16] This is due to the tansporters' ability to recognize and efflux many of the commonly given chemotherapeutics out of the cancer cells.[1] Research has investigated potential inhibitors for these MDR mediating transporters. Unfortunately, most inhibitors that have made it into clinical trials had too many other toxic effects. Therefore, increasing attention has been given to natural products such as flavonoids, which have low cytotoxicity yet promising inhibitory activities against BCRP and P-gp. To identify flavonoids with good inhibitory activity against BCRP and P-gp, studies have applied a range of methods including accumulation studies, QSAR modeling, and molecular docking models. Such methods have allowed the determination of beneficial structural features on the flavonoid skeleton. Pick et al. and Fan et al. identified such structural features for BCRP inhibition, as is summarised in the following figure.[3,5]

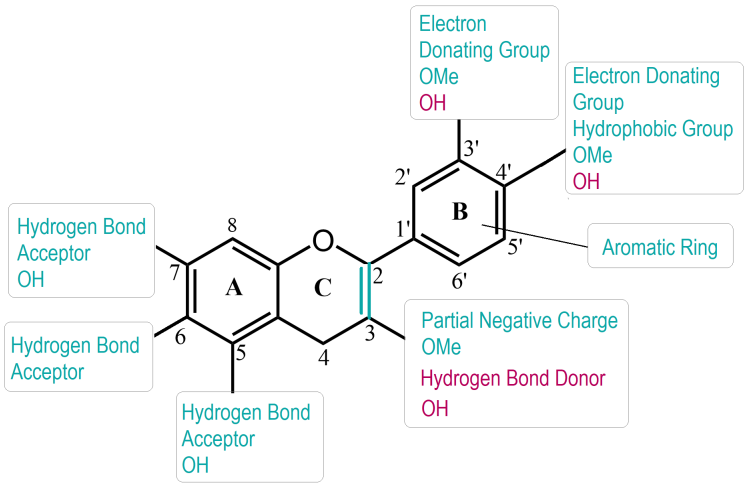

Figure 4.1: Summary of flavonoid structural features and functional groups which enhance/decrease inhibition of BCRP.[2,3,5] **blue:** enhancing features, **pink:** features decreasing inhibition

Studies such as that by Bai et al have identified similar structural features for the inhibition of P-gp, as is summarised in the figure below.[4]

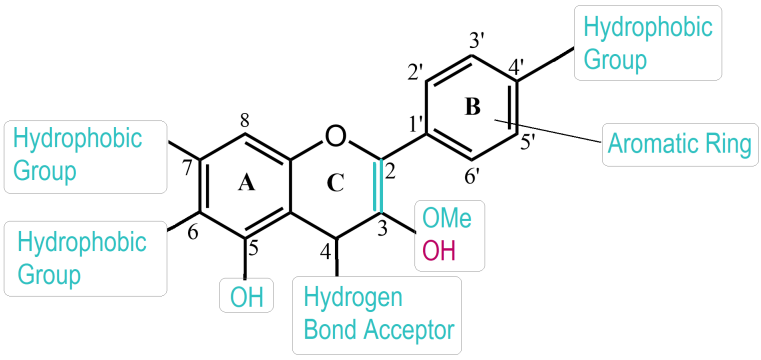

Figure 4.2: Summary of flavonoid structural features and functional groups which enhance/decrease inhibition of P-gp.[2,4] **blue:** enhancing features, **pink:** features decreasing inhibition

Observations have been made that the inhibition of one MDR mediating ABC transporter can cause the expression of another ABC transporter by the cancer cell, which therefore remains multidrug resistant.[9] Therefore inhibitors are often tested for their ability to inhibit more than one transporter, such as for dual BCRP/P-gp inhibitors. Only a couple of dual BCRP/P-gp inhibitors have been identified so far, due to the structural differences of the substrate-binding sites and possibly efflux mechanisms of the two transporters. Yuan et al. found one such dual inhibitor, as seen in figure 3.9. The inhibitor displays beneficial structural features of both the BCRP and P-gp pharmacophore, and with further investigation, it may be possible to establish a pharmacophore for dual BCRP/P-gp transporters.

More research has to be conducted to further verify and improve the pharmacophores for BCRP and P-gp. To improve the comparability between studies it may be beneficial to perform similar studies within the same cell lines, to exclude the possibility of confounding factors within certain cell lines, that may cause an error in the observed inhibitory activities. Further research is also required to fully determine whether there are good dual BCRP/P-gp inhibiting flavonoids.

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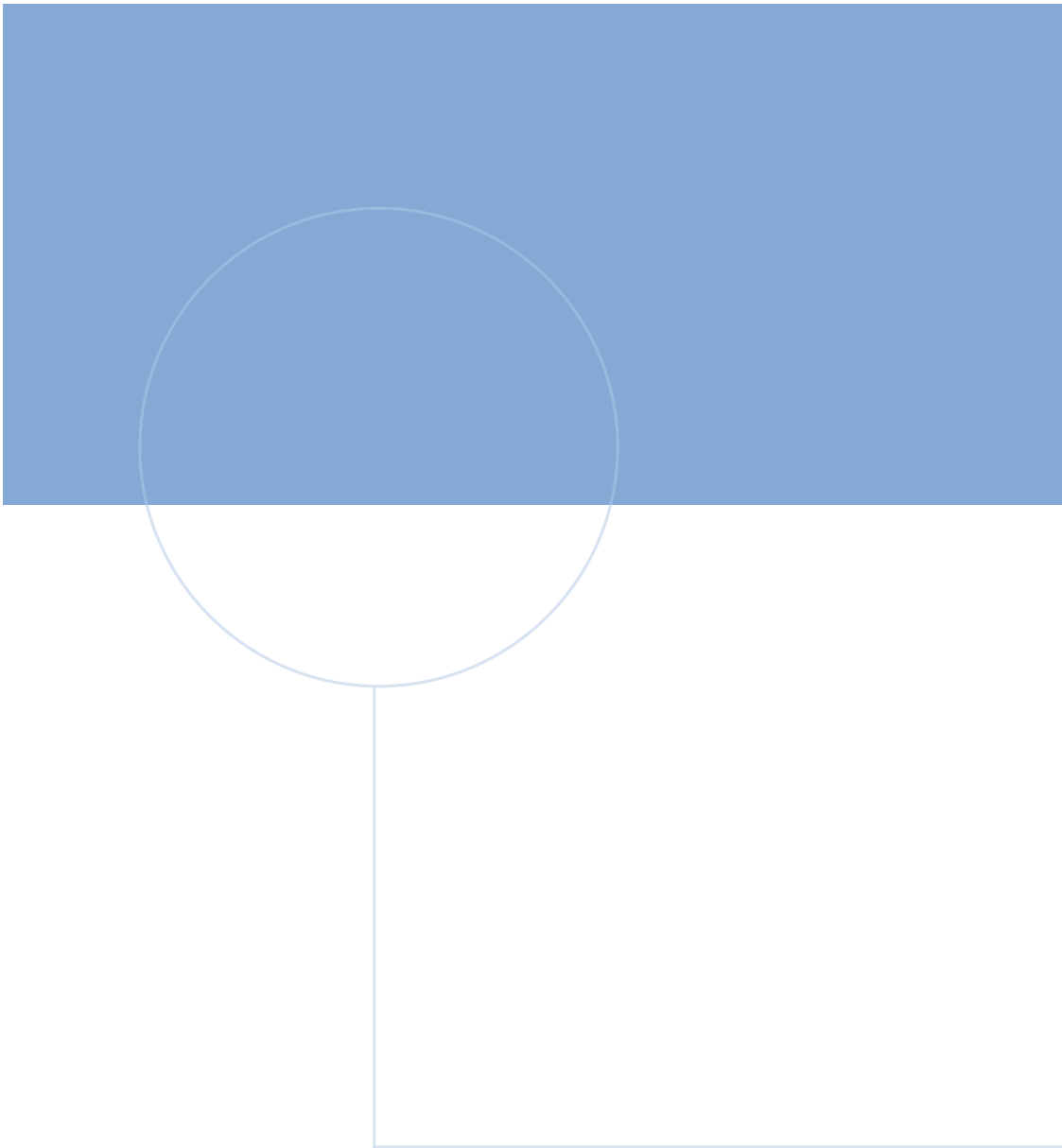

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