DETERMINATION OF MITRAGYNINE AS A SUBSTRATE, INDUCER, OR INHIBITOR OF P-GLYCOPROTEIN DRUG TRANSPORTER, AND PREDICTION OF DRUG-HERB INTERACTION RISKS

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LIST OF ABBREVIATIONS

2D	two-dimensional
ABC	ATP binding cassette
ABCB1	ATP binding cassette subfamily B member 1
ABCG2	ATP binding cassette subfamily G member 2
ADMET	Absorption, distribution, metabolism, excretion and toxicity
ADT	AutoDockTools
ALD	adrenoleukodystrophy
ATP	adenosine triphosphate
BCRP	breast cancer resistance protein
CAM	complementary and alternative medicine
cDNA	complementary deoxyribonucleic acid
CFTR	cystic fibrosis transmembrane conductance regulator
CYP	cytochrome
DDI	drug-drug interaction
ER	efflux ratio
FDA	Food Drug and Administration
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCN20	ATP binding cassette subfamily F
HDL	high-density lipoproteins
HIV	human immunodeficiency virus
HPLC	high performance liquid chromatography
IC_{50}	half maximal inhibitory concentration
K_i	inhibition constant
LLOQ	lower limit of quantification

LSGGQ	ABC signature motif or C motif
MDR1	multi drug resistance 1
Mg^{2+}	magnesium ion
mRNA	messenger ribonucleic acid
MRP	multi-drug resistance associated protein
NBD	nucleotide binding domain
OABP	ATP binding cassette subfamily E member 1
OAT	organic anion transporter
OATP	organic anion-transporting polypeptide
OCT	organic cation transporter
Papp	apparent permeability
PDB	Protein Data Bank
P-gp	P-glycoprotein
QSAR	quantitative structure–activity relationship
RM	Ringgit Malaysia
RNA	ribonucleic acid
RT-qPCR	reverse transcription quantitative polymerase chain reaction
SD	standard deviation
SEM	standard error of the mean
SLC	solute carrier
TAP	transporter associated with antigen processing
TEER	transepithelial electrical resistance
TMD	transmembrane domain
Tyr	tyrosine
US	United States

US$	United States dollar
UV	ultraviolet
v/v	volume/volume
w/v	weight/volume
WHO	World Health Organization

LIST OF SYMBOLS

α	alpha
~	approximately
*	asterisk
β	beta
–	dash
=	equals
>	greater than
-	hyphen
<	less than
≤	less-than or equal to
/	or
±	plus-minus
®	registered trademark
×	times
™	trade mark

LIST OF UNITS

Å	Ångström
°C	degree Celcius
Da	Dalton
g	gram (weight per unit mass)
h	hour
kcal/mol	kilocalorie per mole
µg	microgram
µL	microliter
µm	micrometer
µM	micromolar
mL	milliliter
mm	millimeter
mM	millimolar
min	minute
M	molar
nm	nanometer
nM	nanomolar
Ω	ohm
%	percentage
psi	pounds per square inch
U/mL	unit per milliliter

PENENTUAN MITRAGININA SEBAGAI SUBSTRAT, PENGARUH, ATAU PERENCAT P-GLIKOPROTEIN DAN RAMALAN RISIKO INTERAKSI DRUG-HERBA

ABSTRAK

P-Glikoprotein (P-gp) adalah protein pengangkut yang banyak terdapat di dalam tisu usus dan berfungsi dengan merembes keluar pelbagai substrat dari sel. Perubahan terhadap aktiviti, serta gen dan protein P-gp yang disebabkan oleh ubat-ubatan atau sebatian herba akan menjejaskan bioketersediaan ubat yang dimakan dan berpotensi untuk meyebabkan interaksi di antara ubat dan ubat atau ubat dan herba. *Mitragyna speciosa* Korth atau Ketum digunakan secara tradisional untuk pelbagai penyakit tetapi disebabkan oleh kesan euforia, tumbuhan ini sering disalahgunakan oleh penduduk tempatan. Walaupun Ketum adalah bahan terkawal di negara-negara Asia, termasuk Malaysia, namun penggunaannya tidak dikawal selia dengan ketat di negara lain. Mitragynine adalah satu komponen bioaktif utama dalam ekstrak mentah Ketum dan keselamatan alkaloid ini dalam menyebabkan interaksi ubat dan herba melalui P-gp masih tidak disiasat sepenuhnya. Oleh itu objektif utama kami adalah untuk menentukan sama ada mitragynine adalah substrat P-gp atau mempunyai potensi untuk merencat atau meningkatkan aktiviti pengangkutan serta ekspresi P-gp di dalam sel kultur Caco-2. Satu kaedah penyaringan *in silico*, bagi meramal bentuk pengikatan mitragynine kepada P-gp telah dijalankan dengan menggunakan Autodock 4.2 dan seterusnya disahkan dengan menggunakan kaedah *in vitro* asai pengangkutan dwiarah. Pengoptimuman asai pengangkutan dwiarah telah dijalankan dan semua kompaun dianalisis menggunakan pengesan HPLC/UV dengan teknik elusi isokratik. Kesan mitragynine kepada ungkapan mRNA dan protein P-gp telah

dijalankan menggunakan optimisasi RT-qPCR, analisis Western blot serta immunofluorescence. Mitragynine tidak bertindak sebagai substrat P-gp berdasarkan kedua-dua simulasi dok molekul dan asai pengangkutan dwiarah. Walau bagaimanapun, mitragynine membentuk ikatan hidrogen dan interaksi hidrofobik dengan P-gp dan didapati menghalang aktiviti pengangkutan oleh P-gp dengan penurunan sebanyak 30% dibandingkan dengan kontrol. Mitragynine didapati merencatkan ungkapan mRNA serta protein P-gp. Pada kepekatan tertinggi, iaitu 10 µM, perencatan ungkapan mRNA dan protein adalah sebanyak 35% dan 40% dan adalah selaras dengan penurunan dalam aktiviti pengangkutan digoxin melalui P-gp. Oleh itu, mitragynine adalah merupakan perencat P-gp secara *in vitro*. Rifampicin didapati mengaruh ekspresi protein P-gp setelah dinilai menggunakan kedua-dua analisis Western blot dan imunositokimia. Manakala, quinidine telah didapati merencat P-gp pada tahap transkripsi dan juga aktiviti pengangkutan P-gp.

DETERMINATION OF MITRAGYNINE AS A SUBSTRATE, INDUCER, OR INHIBITOR OF P-GLYCOPROTEIN DRUG TRANSPORTER, AND PREDICTION OF DRUG-HERB INTERACTION RISKS

ABSTRACT

P-glycoprotein (P-gp) is a multidrug transporter, mainly expressed in the intestinal tissue as a secretory efflux protein. Changes in the activity, gene and protein expression of P-gp caused by drugs or herbal compounds will affect oral drug bioavailability and may potentially lead to drug-drug or drug-herb interactions. *Mitragyna speciosa* Korth or Ketum is traditionally used for various ailments but due to its euphoric effects, this plant is often misused by the local population. Although Ketum is a controlled substance in most Asian countries, including Malaysia, its use is not strictly regulated in other parts of the world. Mitragynine is a major bioactive component in the crude extract of the plant and the safety of this alkaloid causing adverse drug interaction via P-gp has not been fully investigated. Therefore our main objective is to determine if mitragynine is a substrate of P-gp or has the potential to inhibit or induce the P-gp transport activity and expression in Caco-2 cells. An *in silico* computational method to predict the binding conformation of mitragynine to the substrate binding site as well as the nucleotide binding domain (NBD) of the P-gp was carried out using Autodock 4.2 and further validated using *in vitro* bidirectional transport assay. Optimization of the bidirectional transport assay was carried out and both mitragynine and digoxin were analyzed using HPLC/UV detector with isocratic elution. The effects of mitragynine on mRNA and protein expression of P-gp were carried out using an optimized RT-qPCR, Western blot analysis and immunofluorescence. Mitragynine is unlikely a P-gp substrate based on

both the molecular docking simulation and bidirectional transport assay. However, it appears to form hydrogen bonds and hydrophobic interactions with P-gp and was found to inhibit the P-gp transport activity by 30% reduction when compared with control. Mitragynine was found to inhibit mRNA and protein expression of P-gp. For the highest concentration of 10 μM, inhibition of mRNA and protein were approximately 35% and 40% that of the control respectively and were consistent with the decrease in the transport activity of digoxin via P-gp. Thus, mitragynine is a significant *in vitro* P-gp inhibitor. Rifampicin was only found to significantly induce the protein expression evaluated using both Western blot analysis and immunocytochemistry. Meanwhile, quinidine was found to significantly inhibit the P-gp at the transcriptional level as well as its transport activity.

CHAPTER 1

INTRODUCTION

1.1 Background

Herbal medicine is used by herbalists and indigenous communities to prevent and treat various diseases over years. According to the definition by the World Health Organization (WHO), herbal medicines include herbs, herbal materials, herbal preparations, and finished herbal products that contain parts of plants, other plant materials, or combinations as active ingredients (Nworu et al. 2015). There are more than 35,000 species of plants worldwide which are known and used for medicinal purposes and about 250-500 species of plants that are commonly used in Asian and other countries (Chen et al. 2011). Herbal medicine is part of complementary and alternative medicine (CAM) where it comprises a diverse medical and health care systems, therapies, and products that are not presently considered to be part of conventional medicine (Barnes et al. 2004, Poonthananiwatkul et al. 2015). The usage of complementary and alternative medicine (CAM) has been increased in developing countries including Malaysia, and recently, the usage of CAM has also expanded to developed countries (Aziz and Tey 2009).

The 2012 National Health Interview Survey of the United States reported that, 38.3% of adults and 11.8% of children used herbal medicine and supplements which resulted in total expenditure of $30.2 billion (Black et al. 2015, Clarke et al. 2015). In addition, other studies showed that an approximately 25% of adults in developed countries and more that 80% of the population in most developing

countries including Malaysia are using herbal medicine either for treatment of disease or as a supplement (Chen et al. 2011, Jayaray 2010, Mukherjee et al. 2011). From a survey conducted in Malaysia, about 63.9% Malaysian population are practicing and using herbal medicine and supplements. From 2000 to 2005, the annual sales for traditional medicines in Malaysia has increased from US$ 385 million (RM 1 billion) to US$ 1.29 billion (RM 4.5 billion) (Aziz and Tey 2009).

The number of herbal medicine and/or natural products users are increasing worldwide, partly due to the increasing preference for natural therapies and/or preventive medicine (Silvanathan and Low 2015). Rising concerns about the undesirable side effects of conventional medicine and the spreading belief that natural products are safer are known to be the contributing factors (Calixto 2000, Zhang et al. 2015). Other common reasons for herbal medicine consumption include the desire to improve physical and mental symptoms, and quality of life as well as to help deal with the disease and its unpleasant treatment (Poonthananiwatkul et al. 2015). In Malaysia, the popularity of herbal medicine usage among the elderly was associated with its lower cost, a lower rate of side effects, more effective, more natural and better accessibility compared to conventional Western medicines (Mitha et al. 2013, Silvanathan and Low 2015). In addition, the usage of herbal medicine was also relatively high in middle-aged adults. Both patients and the public have been known to use herbal medicines to improve and maintain health, treatment or prevention of minor ailments as well as chronic diseases (Barnes 2003, Chang et al. 2007, Mehta et al. 2007). Among Malaysian adults, being a woman, Malay, suffering from health problem, earning a high income, and having favourable opinions about herbal medicines were significant positive forecasters of use of herbal medicines (Aziz and Tey 2009).

However, with increasing number of herbal medicine usage, the number of adverse events which occurred after consumption of herbal product as well as herbal product abuse are also increasing. Ingestion of herbal products such as yohimbine (an alkaloid from *Pausinystalia yohimbe*), maca (*Lepidium meyenii*), horny goat weed (*epimedium sp.*), *Mitragyna speciosa* and *Ginkgo biloba* were reported to cause modification of the function of central nervous system which leads to changes in psychological behavioral and addiction (Corazza et al. 2014). Several herbal medicines, such as Ma-Huang (*Ephedra sinica*), kava (*Piper methysticum*), and chaparral leaf (*Larrea divaricate*), have been implicated as hepatotoxins where hepatotoxicity may be the most frequent adverse reaction to these herbal remedies when taken in excessive quantities. Other herbal plants such as *Aspalathus linearis* (red bush tea), *Echinacea angustifolia* and *Valeriana officinalis* may also cause hepatotoxicity as well as proved to have effects on coagulation and platelets function (Reddy et al. 2016, Wang et al. 2015).

In Malaysia, the increasing use of herbal plant either for a herbal supplement, herbal medicine or herbal addiction and abuse by the community is of special concern because these herbal preparations are not strictly regulated by the Drug Control Authority (DCA) of Malaysia (Aziz and Tey 2009). The evaluation of the quality and safety of herbal medicines and supplements by the DCA of Malaysia before approval is only limited to control the content of specified adulterants and contaminants such as heavy metals and microorganisms (Aziz 2004, Bas and Oliu Castillo 2016). Thus, there is lack of information on the standard preparation procedure, general effects and its safety information for the herbal medicine to be considered safe for consumption. In addition, the main concern regarding the use of herbal medicine and supplements is the potential for dangerous adverse effects and

drug-herb interactions, especially when the herbal products were taken simultaneously with conventional medicine (Silvanathan and Low 2015). Hence, it is important to further investigate the safety, quality and therapeutic efficacy of herbal medicine as well as herbal plant derived compound.

1.2 Drug interactions

Drug-drug interaction (DDI) is defined as a pharmacological or clinical response to the administration of two or more drugs that is different from the response they initiate when individually administered (Rodrigues et al. 2015). Drug interactions occur when either pharmacokinetics or pharmacodynamics or both of one drug are altered by the co-administration of another drug. Changes in the pharmacokinetics and pharmacodynamics of one drug will affect the effectiveness of another either by causing treatment failure or toxicity (Chen and Raymond 2006). Drug interactions usually occur when one of the drug or xenobiotics having properties to interact with the mechanism of action, metabolism or transportation of the other drug. The effect may mimic, magnify or oppose the effect of drugs and usually produce adverse effects (Fugh-Berman 2000). These adverse reactions caused by the concomitant use of drugs or other xenobiotics can potentially lead to severe, and perhaps even life-threatening, adverse reactions. The severity of the adverse reaction can range from theoretical to clinically significant, including prolonged morbidity and even death (Chen et al. 2011, Manzi and Shannon 2005).

In addition, many medicinal herbs and pharmaceutical drugs are therapeutic at one dose and toxic at another especially for drugs with narrow therapeutic indices such as digoxin and warfarin (Chen et al. 2011). Alteration in the pharmacology of those drugs leads to changes in the concentration of the drugs at the site of action producing side effects. Drug interactions are the most common causes for medication

error in a developed country, with a prevalence of 20-40% which mostly occurred in elderly due to polytherapy (Palleria et al. 2013). Most of the drug interactions are potentiated by the concurrent use of herbals medicine and prescription drugs as identified by case reports and clinical studies worldwide (Chen et al. 2011). An example of DDIs is by co-administration of quinidine, dronedarone or verapamil with edoxaban and it has been found to increase the concentration of the edoxaban and lead to bleeding potential (Mendell et al. 2013). In addition, administration of warfarin together with some known herbs such as cranberry, soya, St John's wort, and danshen had been found to alter the concentration of warfarin and leading to drug-herb interactions (Ge et al. 2014). Generally, the mechanism for drug interactions is divided into two types, namely pharmacodynamics and pharmacokinetics mechanism of drug interactions as shown in Figure 1.1.

1.3 Pharmacodynamic mechanism of drug interactions

Pharmacodynamics is the study of how drugs have effects on the body (Maxwell 2016, Meibohm and Derendorf 1997). Pharmacodynamics interactions are the result of the effects of combined treatment at the site of action which altered pharmacological actions at standard plasma concentration (Kashuba and Bertino 2005, Palleria et al. 2013). Pharmacodynamic interactions are more difficult to identify and measure than pharmacokinetic interactions since they result in a modification of the pharmacological action of a drug without any change in the plasma concentration (Spina et al. 2016). Pharmacodynamic interactions are divided into three subgroups based on the mechanism either by direct effect at receptor function, interference with a biological or physiological control process, or additive or opposed pharmacological effect (Palleria et al. 2013). The effects of

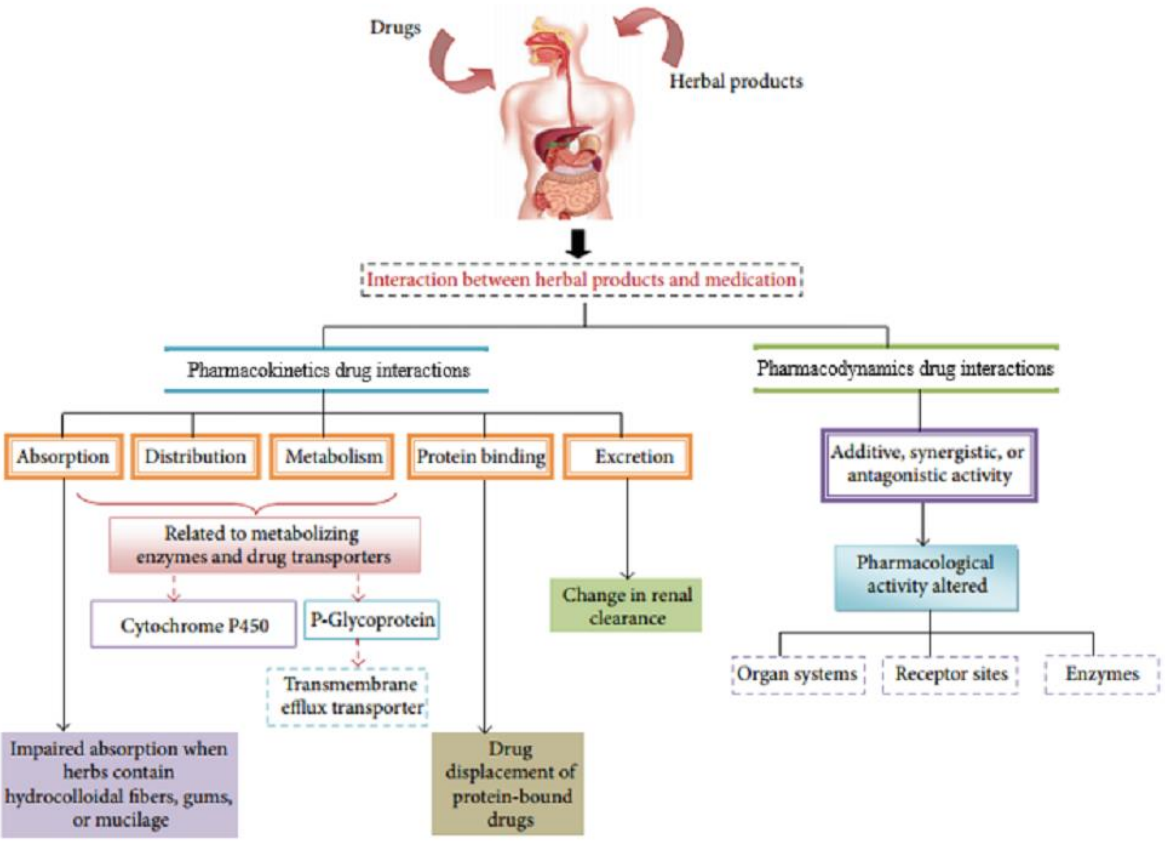

Figure 1.1 Generalized mechanistic insight into drug interactions where it can be divided into two based on the mechanism, pharmacokinetics drug interactions and pharmacodynamics drug interactions. Adapted from Underestimating the Toxicological Challenges Associated with the Use of Herbal Medicinal Products in Developing Countries, page 6 (Neergheen-Bhujun 2013).

pharmacodynamics type of drug interactions can either be synergistic where the combined effects are greater than expected from the sum of individual effects, additive where the effects are equal to the sum of the effects of the individual drugs, or antagonistic where the combined effects are less than additive (Spina and Italiano 2015). The ability of diverse xenobiotics to interact with different receptor sites and alter the physiological environment can also partly explain pharmacodynamic interactions.

1.4 Pharmacokinetic mechanism of drug interactions

Pharmacokinetics interaction is defined as changes in the absorption, distribution, metabolism or excretion of a drug and/or its metabolite(s) after the co-administration of another drug (Spina et al. 2016). These interactions affect the way xenobiotics or drugs are absorbed, distributed, metabolized and excreted, resulting in altered plasma drug concentration (Ge et al. 2014, Spina et al. 2016). Over the past decade, drug metabolism was known as a major contributor in drug interactions. However, other than metabolism, drug absorption, distribution, and excretion are also shown to have important influences on pharmacokinetics, bioavailability, and consequently therapeutic efficacy of drugs (Faber et al. 2003, Meyer et al. 2015, Muller and Fromm 2011). Both *in vitro* and *in vivo* studies have indicated that hepatic or intestinal drug-metabolizing enzymes and drug transporters both equally contribute to the pharmacokinetic interactions (Zhou et al. 2003).

1.4.1 Drug absorption and distribution

Drug absorption refers to the movement of the drug from its site of administration (either via gastrointestinal tract or skin) into the blood circulation. Once a drug has gained access to the blood circulation, it will be distributed to other

tissues for any effects to be produced. Drugs absorption can occur via passive diffusion, facilitated diffusion or active transport by protein transporters into the blood circulation before distribution takes place (Artursson et al. 2012, Chillistone and Hardman 2014). For drugs with oral route of administration, absorption occurs in the lower part of the gastrointestinal tract (jejunum and ileum of the small intestine and large intestine) due to the larger surface area of the intestine (Bergström et al. 2014). The absorption process occurs via the enterocytes lining the intestinal tract. Enterocytes are polarized simple columnar epithelial cells with microvilli on the apical surface of the cells and joined together by tight junctions (Snoeck et al. 2005).

The most common mechanisms of interaction occurring during absorption are an alteration in the active and passive intestinal transport, alteration in the intestinal cytochrome P450 isozyme activity, alteration in the intestinal P-glycoprotein (P-gp) activity, and alteration of gastric pH, gastric emptying, intestinal motility, and intestinal blood flow (Kashuba and Bertino 2005). In addition, absorption across the intestinal membrane is also affected by other transporters on both luminal and basolateral membrane. These transporters can be either influx or efflux type of transporter (Chillistone and Hardman 2014). Other than P-glycoprotein, other drug transporters such as organic anion transporter (OAT), organic cation transporter (OCT) and solute carrier (SLC) transporter which are also abundantly expressed in the drug's absorption site are responsible for maintaining balanced absorption in the intestine (Tsuji 2002).

1.4.2 Drug metabolism

Drug metabolism and drug excretion represent the detoxification processes that protect the human body from xenobiotics and their toxic metabolites (Leslie et al. 2005). Generally, drug metabolism or biotransformation is a chemical alteration

of the drug in the body (Peng and Zhong 2015). Liver is the primary site for drug metabolism and this process can be divided into two phases, which are phase I metabolism and phase II metabolism (Konstandi et al. 2014, Nowak et al. 2014). Hepatic detoxification is generally initiated by the uptake of xenobiotics into liver hepatocytes, by uptake transporter followed by phase I metabolism such as reduction, oxidation, and hydrolysis. Subsequently, phase II metabolism by conjugation processes such as glutathione conjugation, glucuronidation, and sulfation are performed before proceeding with excretions of xenobiotics and/or their metabolites to the bile or through renal excretion (Song et al. 2013).

In drug interactions, potential mechanisms involving metabolism are the genetic polymorphism, inhibition, and induction of enzyme activity (Kashuba and Bertino 2005). Nonrandom genetic modification generates polymorphisms which occurred in at least 1% of a population and give rise to distinct subgroups that differ in their ability to metabolized xenobiotics (Daly et al. 1998). For the inhibition of enzyme activity, there are several mechanisms of inhibition exist such as reversible and irreversible inhibition where reversible is the most common type of enzyme inhibition. Reversible inhibition do not permanently disable the enzyme activity and it occurs when weak bonds were quickly formed between compounds and CYP P450 isozyme (Kashuba and Bertino 2005). This reversible inhibition can occur both competitively and noncompetitively. In addition, there is also reversible inhibition due to the oxidation of inhibitor which forms a slowly reversible reactions (Thummel and Wilkinson 1998). The formation of CYP-mediated reactive metabolite caused irreversible inhibition and this metabolite can covalently and irreversibly bind to the catalytic site residue which permanently inactivate the enzyme (Ho et al. 2015). For

induction of enzyme activity, it generally happens with an increase in P450 synthesis either by mRNA stabilization or receptor-mediated transcriptional activation.

1.4.3 Drug elimination

Kidneys play an important role in drug excretion. Liver and kidney transporters such as solute carrier (SLC) transporter family and ATP-binding cassette (ABC) transporter family are known to play an important role in excretion and elimination of drugs and other foreign substances from the body (Le Vee et al. 2015). Generally, elimination can occur via tubular secretion, glomerular filtration, or a combination of both pathways. Recently, the role of these transporters in the excretion of drugs have drawn major attention because of their involvement in drug interactions (Moss et al. 2014). SLC transporter family can be subdivided into cationic transporters, anionic transporters, and other transporters. These SLC transporters family are expressed in both apical and basolateral membrane of the proximal tubule cells and control the entry of xenobiotics into the epithelial cells (Morrissey et al. 2013). Meanwhile, ATP-binding cassette (ABC) transporter help to eliminate and excrete xenobiotics and endogenous compounds across the proximal tubule cells in kidney, the apical membrane of hepatocytes, capillary endothelial cells at the blood-brain barrier as well as the brush border membrane of enterocytes (Giacomini et al. 2010, van Montfoort et al. 2003).

There are five potential mechanisms of drug interactions affecting excretion at the site of renal elimination (Bonate et al. 1998). The three most common mechanisms are glomerular filtration, tubular secretion, and tubular reabsorption. Changes in renal blood flow, cardiac output, and extend of protein binding will affect rates of glomerular filtration which disturb the normal excretion process (Kashuba and Bertino 2005). However, the most common renal drug interactions occur at the

transport site of tubular secretion. Many organic anions and cationic drugs and metabolites compete with each other for secretion as they are sharing the same proximal tubular active transport system. In addition, inhibition of renal P-gp which has been identified in the apical membrane of the proximal tubule leads to an increase in plasma drug concentrations and potentially contribute to significant drug interactions. In tubular reabsorption, changes in the urinary pH can alter the reabsorption process but these interactions are not known to be clinically significant.

1.5 Membrane transporter

As discussed earlier, transporters and drug metabolizing enzymes are two major components, playing an important role in pharmacokinetics drug interactions (Wu et al. 2016). While many researches have been focused on the role of drug metabolizing enzymes in drug interactions, less emphasis has been placed on the importance of transporter in drug interactions. Transporters mostly involve in the absorption, distribution and elimination of xenobiotics either into or outside of the cells, whereas drug metabolizing enzymes are mostly involved in the metabolism process (Scherrmann 2009). The major physiological functions of transporters are to facilitate the transfer of nutrients or endogenous substrate across the cell membrane, such as endogenous metabolites and signaling molecules. There are two main transporter superfamilies, namely SLC and ABC transporters and both are involved in transporter-mediated drug interactions (Giacomini et al. 2010). SLC transporters comprise of facilitated and ion-coupled transporters, including organic cation transporters (OCTs), organic anion transporters (OATs) and organic anion-transporting polypeptides (OATPs), where most of the SLC transporters mediate uptake of substrates into the cells (Koepsell 2013, Wessler et al. 2013). On the other

hand, ABC transporters, including P-glycoprotein (P-gp or MDR1), breast cancer resistance protein (BCRP or ABCG2), and multi-drug resistance associated proteins (MRPs), are considered to be responsible for efflux of xenobiotics where they rely on ATP to actively pump their substrate across cell membranes (Leslie et al. 2005). These specific uptake and efflux transporters are extensively expressed in the apical and luminal membrane of epithelia of many organs as shown in Figure 1.2 (Giacomini et al. 2010, Sai 2005). Their expression in cells of all of these major barriers such as intestine, blood-brain barrier as well as in metabolic organs such as liver (hepatocytes) and kidney (kidneys proximal tubules) also explains their influence on the pharmacology properties of drugs and drugs candidates (Estudante et al. 2013, Montanari and Ecker 2015). Among all the protein transporters, ABC transporters especially P-gp has drawn major attention for its great influence on drug resistance and drug interactions problem (Hennessy and Spiers 2007, Li et al. 2014).

1.6 ABC transporter superfamily

The ATP-binding cassette (ABC) transporters form a large superfamily of membrane proteins which are responsible for various functions, including the active transportation of ions and peptides, excretion of harmful compounds, and cell signaling (Leslie et al. 2005). There are more than 100 membrane transporters/channels in the ABC transporter superfamily and they are universally expressed in all living organisms ranging from prokaryotes to mammals (Hennessy and Spiers 2007, Montanari and Ecker 2015). In humans, 49 ABC transporters have

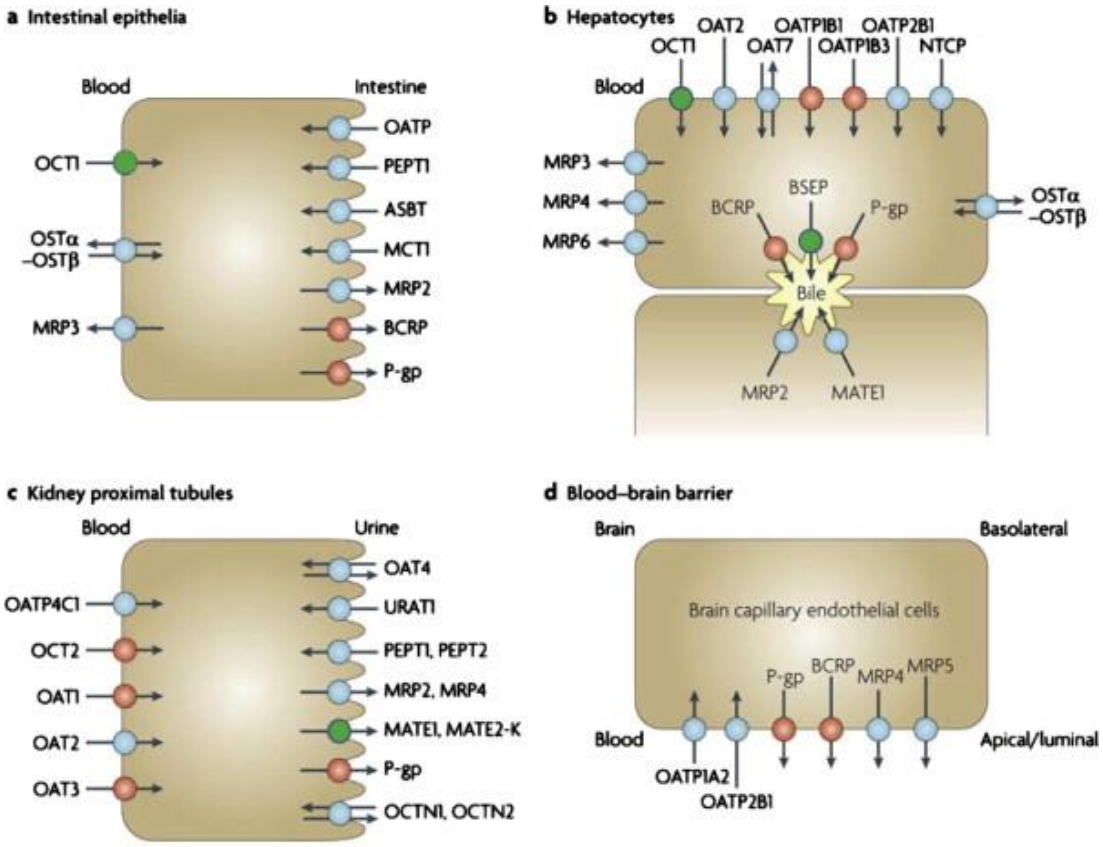

Figure 1.2 Various distribution of protein transporter in the membrane of cells in the a) intestine, b) liver, c) kidney, and d) brain are shown. Arrows represent the direction of substrate transport for each transporter. Orange circles: key drug transporters mentioned in both US FDA draft guidance and EMA guideline on DDIs (Giacomini et al. 2010, Maeda and Sugiyama 2013).

been identified, and organized into 7 subfamilies: ABCA (12 members; previously ABC1), ABCB (11 members; previously MDR/TAP), ABCC (13 members; previously MRP/CFTR), ABCD (4 members; previously ALD), ABCE (1 member; previously OABP), ABCF (3 members; previously GCN20) and ABCG (5 members; previously White) (Table 1.1) (Dean and Allikmets 2001, Dean et al. 2001, Vasiliou et al. 2009). These ABC transporters consist of several core domains (transmembrane domains and intracellular nucleotide binding domains) depending on different subfamilies. The nucleotide binding domains (NBDs) are usually well conserved across subfamilies while transmembrane domains (TMDs) are less conserved and possibly account for substrate specificity of the different transporters. In addition, these TMDs form the translocation chamber across the transporter which explained the ability for their substrate to be transported (Tarling et al. 2013).

Generally, the TMD of ABC transporters consists of few membrane spanning segments (α-helices) separates by hydrophilic loops and intracellular NBD (Kast et al. 1995, Loo and Clarke 1995). In each NBDs, two sequence motifs known as Walker A and Walker B which located 100-200 amino acids apart, are conserved among all ABC transporter superfamily members, as well as numerous other ATP-binding proteins (Walker et al. 1982). The lysine residue in the Walker A motif is involved in the H-bonding with the ATP while the aspartic acid residue in the Walker B motif interacts with Mg^{2+} (Hung et al. 1998, Sharom et al. 1999). In addition, another highly conserved amino acid sequence, ABC signature motif or C motif (ALSGGQ) which located between the Walker A and B motifs, as well as the D,H, Q, and A loops were known to have implication in the recognition, binding, and hydrolysis of ATP. Several of these motifs shown to interact with the adenine ring of ATP and appear to form part of the ATP-binding site (Loo et al. 2002).

Table 1.1 Human ABC transporter genes, and their functions (Dean et al. 2001).

Gene	Chromosome location	Exons	AA	Accession number	Function
ABCA1	9q31.1	36	2261	NM005502	Cholesterol efflux onto HDL
ABCA2	9q34	27	2436	NM001606	Drug resistance
ABCA3	16p13.3	26	1704	NM001089	Multidrug resistance
ABCA4	1p22	38	2273	NM000350	N-retinylidene-phosphatidylethanolamine (PE) efflux
ABCA5	17q24.3	31	1642	NM018672	Urinary diagnostic marker for prostatic intraepithelial neoplasia (PIN)
ABCA6	17q24.3	35	1617	NM080284	Multidrug resistance
ABCA7	19p13.3	31	2146	NM019112	Cholesterol efflux
ABCA8	17q24	31	1581	NM007168	Transports certain lipophilic drugs
ABCA9	17q24.2	31	1624	NM080283	Might play a role in monocyte differentiation and macrophage lipid homeostasis
ABCA10	17q24	27	1543	NM080282	Cholesterol-responsive gene
ABCA12	2q34	37	2595	NM173076	Has implications for prenatal diagnosis
ABCA13	7p12.3	36	5058	NM152701	Inherited disorder affecting the pancreas
ABCB1	7q21.1	20	1280	NM000927	Multidrug resistance
ABCB2	6p21.3	11	808	NM000593	Peptide transport
ABCB3	6p21.3	11	703	NM000544	Peptide transport
ABCB4	7q21.1	25	1279	NM000443	Phosphatidylcholine (PC) transport
ABCB5	7p15.3	17	812	NM178559	Melanogenesis
ABCB6	2q36	19	842	NM005689	Iron transport
ABCB7	Xq12-q13	14	753	NM004299	Fe/S cluster transport
ABCB8	7q36	15	718	NM007188	Intracellular peptide trafficking across Membranes
ABCB9	12q24	12	766	NM019625	Located in lysosomes
ABCB10	1q42.13	13	738	NM012089	Export of peptides derived from proteolysis of inner-membrane proteins
ABCB1	2q24	26	1321	NM003742	Bile salt transport
ABCC1	16p13.1	31	1531	NM004996	Drug resistance
ABCC2	10q24	26	1545	NM000392	Organic anion efflux

Table 1.1 Continued

Gene	Chromosome location	Exons	AA	Accession number	Function
ABCC3	17q22	19	1527	NM003786	Drug resistance
ABCC4	13q32	19	1325	NM005845	Nucleoside transport
ABCC5	3q27	25	1437	NM005688	Nucleoside transport
ABCC6	16p13.1	28	1503	NM001171	Expressed primarily in liver and kidney
ABCC7	7q31.2	23	1480	NM000492	Chloride ion channel (same as CFTR gene in cystic fibrosis)
ABCC8	11p15.1	30	1581	NM000352	Sulfonylurea receptor
ABCC9	12p12.1	32	1549	NM005691	Encodes the regulatory SUR2A subunit of the cardiac Kþ(ATP) channel
ABCC10	6p21.1	19	1464	NM033450	Multidrug resistance
ABCC11	16q12.1	25	1382	NM033151	Drug resistance in breast cancer
ABCC12	16q12.1	25	1359	NM033226	Multidrug resistance
ABCC13	21q11.2	6	325	NM00387	Encodes a polypeptide of unknown Function
ABCD1	Xq28	9	745	NM000033	Very-long-chain fatty acid (VLCFA) Transport
ABCD2	12q11-q12	10	740	NM005164	Major modifier locus for clinical diversity in X-linked ALD (X-ALD)
ABCD3	1p22-p21	16	659	NM002858	Involved in import of fatty acids and/or fatty acyl-coenzyme As into the peroxisome
ABCD4	14q24	19	606	NM005050	May modify the ALD phenotype
ABCE1	4q31	14	599	NM002940	Oligoadenylate-binding protein
ABCF1	6p21.33	19	845	NM001025 091	Susceptibility to autoimmune pancreatitis
ABCF2	7q36	14	634	NM005692	Tumour suppression at metastatic sites and in endocrine pathway for breast cancer/ drug resistance

Table 1.1 Continued

Gene	Chromosome location	Exons	AA	Accession number	Function
ABCF3	3q27.1	21	709	NM018358	Also present in promastigotes (one of five forms in the life cycle of trypanosomes)
ABCG1	21q22.3	13	678	NM004915	Cholesterol transport
ABCG2	4q22	16	655	NM004827	Toxicant efflux, drug resistance
ABCG4	11q23.3	15	646	NM022169	Found in macrophage, eye, brain and spleen
ABCG5	2p21	11	651	NM022436	Sterol transport
ABCG8	2p21	10	673	NM022437	Sterol transport

1.7 P-glycoprotein (P-gp)

1.7.1 Background and structure of P-gp

P-glycoprotein (P-gp) is one of the drug transporters that play an important role in the pharmacokinetics of drugs or xenobiotics. It is a 170 kDa (~170-180 kDa) polypeptide consisting of approximately 1280 amino acids (Chen et al. 1986, Miyata et al. 2016). P-gp is a multidrug transporter and one of the members of the ABC (ATP-binding cassette) superfamily encoded by the MDR1 gene in human which is now known as ABCB1 (Hennessy and Spiers 2007). Since P-gp is one of ABC transporter, the basic units of P-gp consists of two units of TMDs and two units of NBDs as depicted in Figure 1.3. In addition, it appears that P-gp is generated by a gene duplication event, fusing two related half molecules (van Veen et al. 2000). Each half molecules consists of one unit of TMD and one unit of NBD (Miyata et al. 2016). Generally, the two homologous halfs of the protein were connected by a central sequence known as the "linker" region. The two half share 43% sequence identity and 78% similarity (Grandjean-Forestier et al. 2009).

The TMD unit was made up of six transmembrane segments where each segment is connected by extracellular or cytosolic loops as shown in Figure 1.3b. The last cytosolic loop was then, followed by a large cytosolic domain containing an ATP-binding site or NBD where the first half is NBD1 and the second half is NBD2 (Dean et al. 2001, Silva et al. 2015). Furthermore, the secondary structure of P-gp shows that it consists of approximately 32-43% α-helix, 16-26% β-sheet, 15-29% β-turn and 13-26% unordered structure (Dong et al. 1998, Sonveaux et al. 1996).

In P-gp, two TMDs units constitute the drug transport pore while, the NBDs bind and hydrolyze ATP to provide energy for the movement of its substrates across membranes (Wessler et al. 2013). For efficient ATP hydrolysis, there is evidence that

the two NBDs have to interact by forming a sandwich dimer so that the LSGGQ motif of one NBD comes into contact with the loop of the other NBD to form the nucleotide-binding pocket (Leslie et al. 2005). The linker region also plays a role in P-gp function by creating flexible secondary structure. This flexibility is sufficient for the coordinate functioning of both halfs of P-gp, which are likely required for the proper interaction of the two ATP-binding sites (Grandjean-Forestier et al. 2009).

In normal cells expressing P-gp, this protein is synthesized in the endoplasmic reticulum as a core glycosylated intermediate with a molecular weight of 150 kDa before subsequently modified in the Golgi apparatus prior to export to the surface of the cell (Grandjean-Forestier et al. 2009). P-gp protects the cells from xenobiotics and toxins by pumping them out from the cells. The efflux of the drug helps in preventing it from reaching the systemic circulation (Vaalburg et al. 2005). P-gp is mainly expressed at the apical (luminal) side of the epithelial cells of the intestine (enterocytes), in the brain capillary endothelial cell of the blood brain barriers, renal proximal tubular cells, hepatocytes, blood-testis barrier in testis as well as in placental fetal-maternal barrier (Figure 1.4).

Generally, P-gp works in two steps where the first is a catalytic cycle of ATP hydrolysis, while second is the movement of the substrate from the cytoplasmic side to the extracellular side of the membrane (Grandjean-Forestier et al. 2009). In normal state, the P-gp pore opens towards the cytoplasm of the cells with both NBDs and TMDs open inward as shown in Figure 1.5. The substrate from the cytoplasm will bind to the substrate binding-site located within the cytoplasmic membrane leaflet (Higgins and Gottesman 1992). Then, low-affinity binding of ATP to both

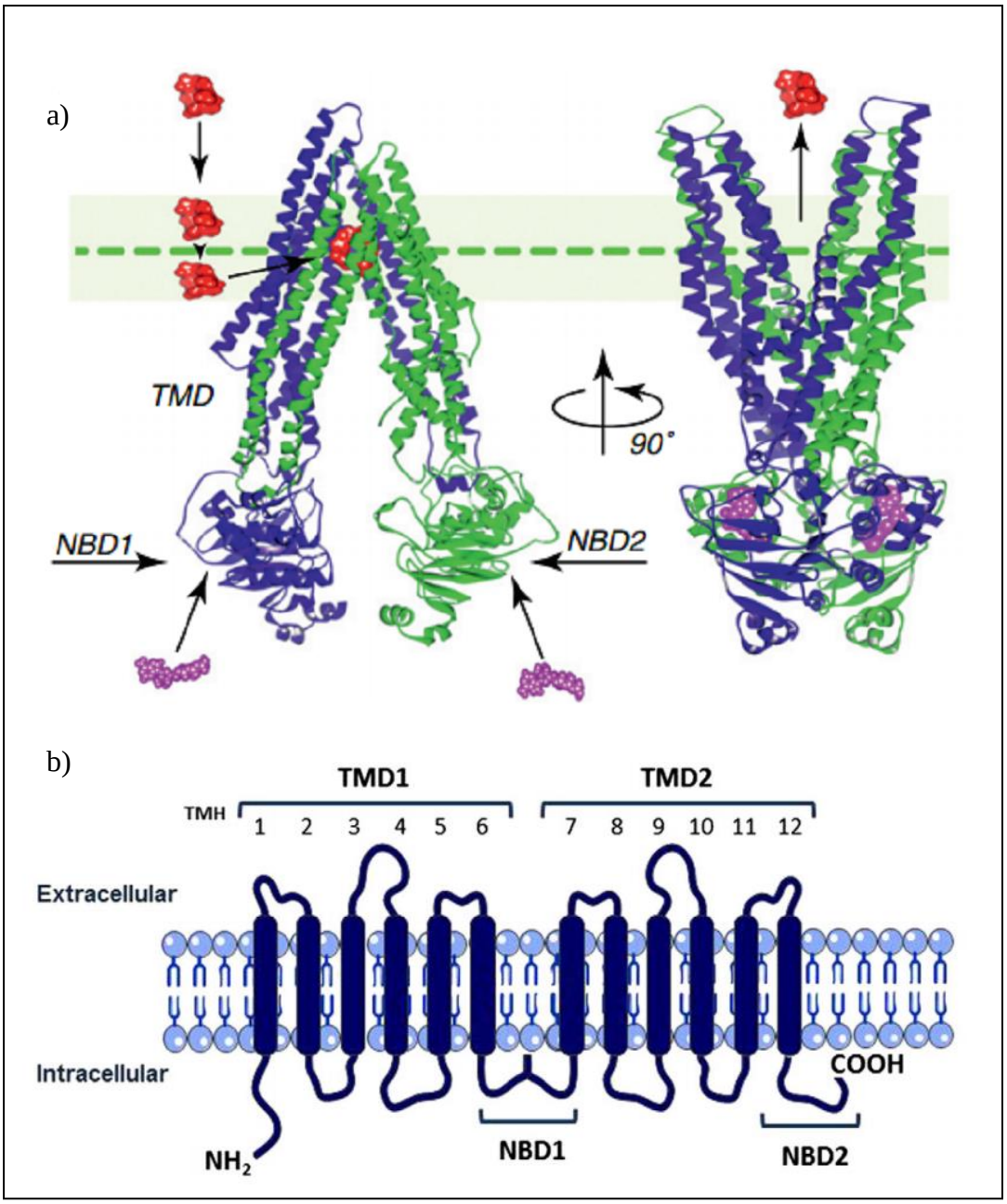

Figure 1.3 Basic units of P-gp which consist of two related half molecules (each molecules consists of one unit of TMD and one unit of NBD). a) Transport cycle for substrate efflux pumped by P-glycoprotein (substrates are colored red and ATP is magenta) b) Topological model of P-gp, showing the two homologous halfs, each with one transmembrane domain (TMD), containing six highly hydrophobic transmembrane α-helices, and one nucleotide binding domain (NBD) located on the cytoplasmic side of the membrane (Chen et al. 2012).

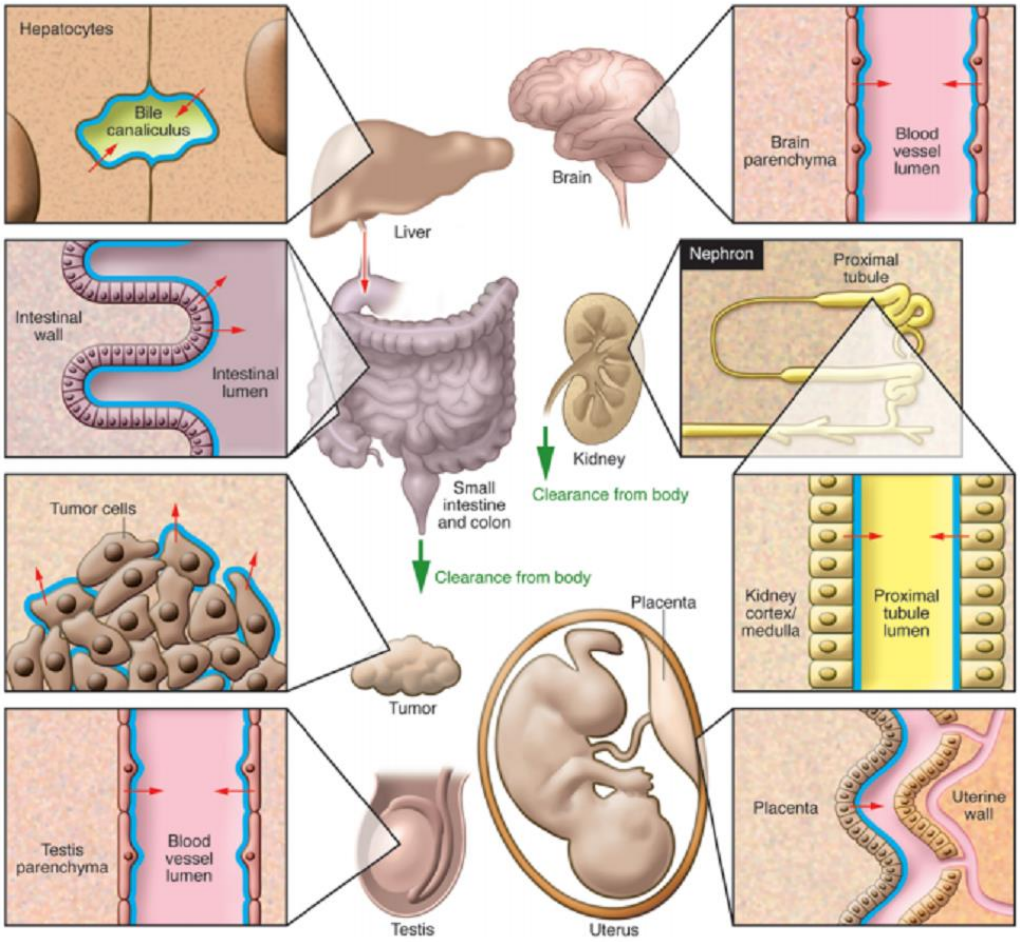

Figure 1.4 Localization of P-gp in various organs of human (Borst and Schinkel 2013).

NBDs will induce the formation of a putative nucleotide sandwich dimer which drive the transportation process (Hennessy and Spiers 2007). At this stage, both the TMDs and NBDs are closed inward and catalysis of ATP into ADP and phosphate occurs. Subsequently, the TMDs open outward allowing the substrate to be transported to the extracellular space (Wu et al. 2016). Finally, P-gp transforms back to its normal conformation releasing the ADP and phosphate.

1.7.2 Substrates of P-gp

Due to its extreme broad substrate specificity (poly-specificity or promiscuity) in P-gp, it can actively transport a plethora of substrates compounds with varying size and structure out of the cells (Montanari and Ecker 2015, Pan et al. 2016). P-gp is unique in its ability to recognize and transport its substrates that differ considerably in chemical structure and pharmacological action, including many clinically important agents. In earlier studies, P-gp is known to plays a role in multiple drug resistance especially in cancer therapy (Januchowski et al. 2016). Many of these anti-cancer drugs that are important in managing the cancer patients such as doxorubicin, and daunorubicin were known to be a P-gp substrate (Al-Saraf et al. 2016). Other than these two anthracyclines, P-gp can also transport other anti-cancer drugs such as vinblastine, vincristine, actinomycin D, paclitaxel, teniposide, and etoposide (Hennessy and Spiers 2007).

Other therapeutic drugs such as HIV protease inhibitors (maraviroc), gastrointestinal agents (loperamide, ondansetron) as well as rheumatologic or immunosuppressant agents (cyclosporine, tacrolimus) were found to be transported

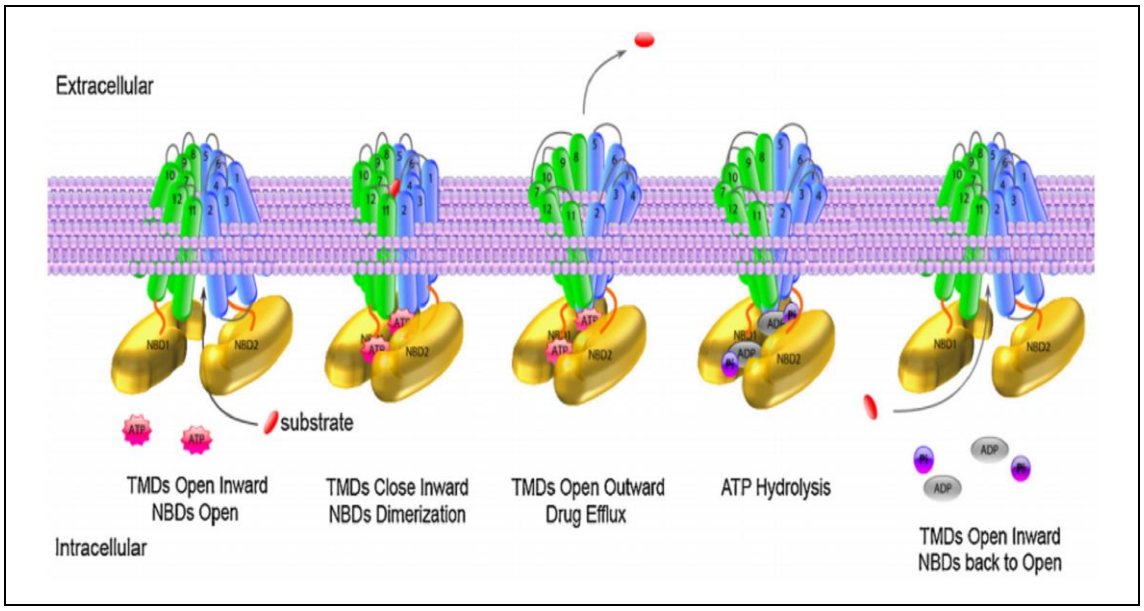

Figure 1.5 Mechanism for transportation of substrate via P-gp (Wu et al. 2016)

by P-gp (Alam et al. 2016, Mendes et al. 2009, Wagner et al. 2001). In addition, antimicrobial agents such as erythromycin, as well as anti-helminthic agent such as ivermectin, were also known to be a P-gp substrates (Griffin et al. 2005, Takano et al. 1998). Neurologic agents which are used in treating pain in post-herpetic neuralgia as well as for local analgesia such as lidocaine are also transported by P-gp (Funao et al. 2003, Wessler et al. 2013). Interestingly, there is rising concern on the roles of P-glycoprotein in cardiovascular as these cardiac agents are found to interact with P-gp (Wessler et al. 2013). For example, antiarrhythmic agents such as digoxin and verapamil which are indicated for congestive cardiac failure and cardiac arrhythmia are known as P-gp substrates (Hansen et al. 1997, Römermann et al. 2013, Tuncok et al. 1997). P-gp can also transports anticoagulant agents such as dabigatran, edoxaban and warfarin as well as antiplatelet agents such as clopidogrel and ticagrelor (Chen et al. 2016, Mendell et al. 2011, Wessler et al. 2013). In addition, statins such as lovastatin and atorvastatin have been found to be transported by P-gp and co-administration of any P-gp inhibitor will affects its bioavailability (Goard et al. 2010). P-gp can also transport talinolol, an antihypertensive agents used for treatment of hypertension and to manage cardiac arrhythmias (Eyal et al. 2009, Nguyen et al. 2014). Other than these therapeutic drugs, plant crude extract as well as its natural compound such as berberine which is an alkaloid extracted from *Berberis vulgaris* is also proved to be transported by P-gp (Gozalpour et al. 2014).

1.7.3 Inhibitor and inducer of P-gp

Some compounds and xenobiotics are found to interact with P-gp by causing inhibition or induction. For example, antimicrobial agents such as ketoconazole and erythromycin produce inhibitory effects on P-gp transport activity (Stappaerts et al. 2013). Tariquidar, an anti-cancer agent which is known as P-gp substrate is also

found to act as P-gp inhibitor (Loo and Clarke 2014). Oral amiodarone and quinidine inhibit intestinal P-gp membrane efflux causing increased plasma concentration of P-gp substrate (Fromm et al. 1999, Robinson et al. 1989). In addition, dronedarone, another antiarrhythmic agents, displays an even greater inhibition on the P-gp transport activity compared to amiodarone (Vallakati et al. 2013). Other than antiarrhythmic agents, calcium-channel blockers such as nicardipine, verapamil, nifedipine and diltiazem inhibit the P-gp mediated transportation of P-gp substrate (Cavet et al. 1996, Takara et al. 2002). Preliminary evidence suggests that warfarin, an oral anticoagulant may inhibit P-gp activity in liver cells. Other than these therapeutic drugs, other xenobiotics such as propiconazole which commonly used on fruits and vegetables as an agricultural fungicide was shown to inhibit the P-glycoprotein transport activity (Mazur et al. 2015). P-gp was also found to be inhibited by spinosad, an oral flea insecticide and was suggested as the underlying cause of the drug interactions with ivermectin (Schrickx 2014). For instance, sinapine which is a small molecular alkaloid extracted from the seeds of cruciferous vegetable (traditional Chinese medicine) plays an important role in downregulation of P-gp expression in tumors (Guo et al. 2014). Another alkaloid compound from gum resin of *Commiphora mukul* (guggulsterone) also showed inhibition potential of cyclooxygenase-2 by downregulating the P-gp expression and was suggested to be used to reverse the imatinib-resistance problem (Xu et al. 2014).

On the contrary, some of these conventional drugs as well as other xenobiotics also act to induce the P-gp activity and might also induce its expression. Rifampicin which is used for treatment of several types of bacterial infections especially tuberculosis and leprosy, upregulate as well as inducing P-gp transport activity. Digoxin which is also known as P-gp substrate, also act to induce both the