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INTERACTION OF BAICALIN WITH TRANSPORTERS

PhD thesis

Bernadett Kalaposné Kovács

Doctoral School of Pharmaceutical Sciences

Semmelweis University

Supervisor: Dr. Imre Klebovich, D.Sc

Official reviewers:
Dr. Éva Szökő, D.Sc
Dr. Györgyi Horváth, Ph.D

Head of the Final Examination Committee:
Dr. Tamás Török, D.Sc

Members of the Final Examination Committee:
Dr. László Tóthfalusi, Ph.D
Dr. Gábor Halmos, Ph.D

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1. Abbreviations

ABC transporters: ATP-binding cassette transporters

ADME: absorption, distribution, metabolism, excretion

ATP: adenosine triphosphate

AMP: adenosine monophosphate

B: baicalein

BCRP: Breast cancer resistant protein

BCS: Biopharmaceutical Classification System

BG: baicalin

B-GS: bimane-glutathion conjugate

CHO cells: chinese hamster ovary cells

C_{max}: maximal concentration

Cl_{int}: intrinsic clearance

ctrl K: control membrane for K562 cells

ctrl M: control membrane for mammalian cells

defMRP: functionally defective mutant MRP

DHEAS: dehydroepiandrosterone sulfate

DMSO: dimethyl sulfoxide

$E_2$17βG: estradiol-17-β-D-glucuronide

EMA: European Medicines Agency

E3S: estrone-3-O-sulfate

FDA: Food and Drug Administration

GFJ: grapefruit juice

GSH: glutathione

HEK293: human embryonic kidney cells

HEK ctrl: functionally defective control membrane for HEK293 cells

K562: human myelogenous leukemia cells

K_m: substrate concentration at half V_{max}

LC-MS: liquid chromatography–mass spectrometry

LLOQ: lower limit of quantification

M: Michigan Cancer Foundation-7 (Breast cancer cell line) cells

MCF7: Michigan Cancer Foundation-7 (Breast cancer cell line)

MDCK: Madin-Darby canine kidney cells

MDR: multidrug resistance

MRP: multidrug resistance associated protein

NMQ: N-methyl-quinidine

OATP: organic anion-transporting polypeptide

OCT: organic cation transporter

OCTN: carnitine/organic cation transporter

P_{app}: apparent permeability coefficient

QC: quality control

RS: *Radix Scutellariae*

±SD: standard deviation

Sf9: *Spodoptera frugiperda* ovarian cells

SLC: solute carrier transporter

SULT: sulfotransferase

UGT: uridine 5'-diphospho-glucuronosyltransferase

V_{max}: maximal reaction velocity

VT: vesicular transport

2. Introduction

In the last decade, interest in studying the pharmacologic effects of phytomedicines has grown extensively. This is largely a result of the higher tendency among the general population to use complementary and alternative medicine.

Flavonoids, the main bioactive components found in herbs, are a group of polyphenolic compounds, diverse in chemical structure and characteristics, found ubiquitously in plants. Until now, more than 9000 different flavonoids have been studied and described. There has been increasing interest in their research due to growing evidence of their versatile health benefits including anti-inflammatory, antioxidant, ant proliferative and anticancer activity, free radical scavenging capacity, antihypertensive effects, coronary heart disease prevention and anti-human immunodeficiency virus functions. Additionally, flavonoids are safe and associated with low toxicity, making them excellent candidates for chemo preventive agents (Conseil *et al.*, 1998; Nabekura *et al.*, 2005). However, to achieve successful therapeutic efficacy, flavonoids must be absorbed adequately and consistently after oral administration; this behavior depends heavily on the drug delivery system (Tian *et al.*, 2009).

Clinical studies and case reports have identified a number of herb-drug interactions potentiated by the concurrent use of herbal medicines with prescription drugs, raising concerns by health professionals regarding the potential for flavonoids to affect pharmacokinetics and pharmacodynamics of drugs (Bailey *et al.*, 1993; Fasinu *et al.*, 2012; Kantamreddi *et al.*, 2009; Rajnarayana *et al.*, 2004). The clinical consequences of herb-drug interactions varies, from being well-tolerated to moderate or serious adverse reactions, or possibly life-threatening events. Undoubtedly, the early and timely identification of herb-drug interactions is imperative to prevent potentially dangerous clinical outcomes (Chen *et al.*, 2011; Hu *et al.*, 2005). The potential of pharmacokinetic interactions occurring between phytomedicines and conventional drugs is therefore increasingly being recognized (Kennedy *et al.*, 2010; Laki *et al.*, 2013; Mohamed *et al.*, 2011; Nguyen *et al.*, 2015).

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2.1. Baicalin

Radix Scutellariae (RS), officially listed in the *Chinese Pharmacopoeia*, is the dried root of the medicinal plant *Scutellariae baicalensis*, known as *Huang Qin* in Chinese traditional medicine (***Figure 1***) (CP, 2005).

Figure 1: *Scutellaria baicalensis (https://en.wikipedia.org/wiki/scutellaria_baicalensis; 2016. September 26.)*

RS is widely used for the prevention and treatment of various ailments including cardiovascular diseases, hypertension, bacterial infection, inflammation, and cancer (Blach-Olszewska *et al.*, 2008; Gao *et al.*, 2011b; Jung *et al.*, 2012; Tseng *et al.*, 2010; Zhang *et al.*, 2011c). More than 50 flavonoids have been purified and identified from *RS* (Chen *et al.*, 2014). The major components are baicalin (baicalein-7-O-glucuronide, BG), and its aglycone baicalein (5, 6, 7 -trihydroxyfavone, B) (Li-Weber, 2009; Li *et al.*, 2009). Due to their relatively low toxicity and high abundance in *RS*, BG

and B became the most widely researched components in recent years (***Figure 2***) (Li-Weber, 2009).

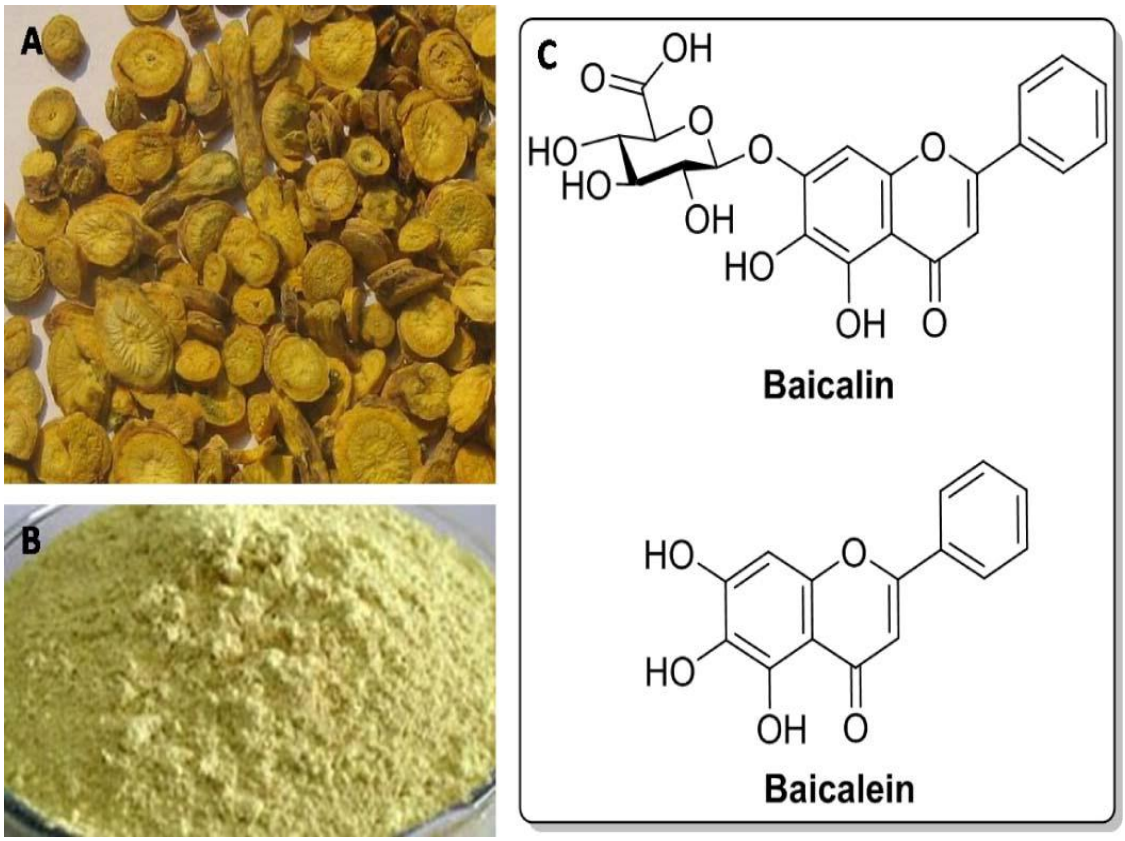

Figure 2: *(A) The roots of Scutellaria baicalensis; (B) the powder of BG; (C) chemical structures of BG and B(Zhang et al., 2011c).*

Numerous in vivo and in vitro studies carried out in the last decade demonstrated that BG and its aglycone B were important medical agents with a variety of pharmacological activities such as chemopreventive, hepatoprotective, anti-aging, antioxidant, anti-fibrotic, anti-allergic, anti-depressant, anti-microbial, anti-inflammatory, antimutagenic, neuroprotective, memory improving, endotoxin, as well as anxiolytic effects (Dou *et al.*, 2007; Gao *et al.*, 2016; Hu *et al.*, 2009; Kim *et al.*, 2012; Kumagai *et al.*, 2007; Oga *et al.*, 2012; Sahebkar, 2012; Shang *et al.*, 2010; Takahashi *et al.*, 2011; Waisundara *et al.*, 2011; Wang *et al.*, 2015; Woo *et al.*, 2005; Xu *et al.*, 2011; Yu *et al.*, 2016b). The clinical applications of BG include the treatment of pneumonia, hepatitis and cardiovascular diseases. BG might serve as a novel approach for the treatment of patients with Parkinson's disease (Xue *et al.*, 2014). BG can exert anti-H1N1 and H5N1

effects (Chu *et al.*, 2015; Sithisarn *et al.*, 2013) and antiviral activity against dengue virus (Moghaddam *et al.*, 2014).

Treatment with BG showed to be a potential therapeutic strategy for acute lung injury (Ding *et al.*, 2016). BG pre-treatment attenuated brain ischemia reperfusion injury by suppressing cellular apoptosis (Zhou *et al.*, 2016). Other in vivo findings demonstrated that BG had significant potential as a novel anti-inflammatory agent for therapy of autoimmune diseases such as multiple sclerosis (Zhang *et al.*, 2015). B exhibited anti-tumor effects in several types of cancers by inducing cancer cell apoptosis and suppressing metastasis. B might also be used in the treatment of pancreatic cancer, bladder cancer, lung cancer, hepatoma, breast cancer and skin carcinoma (Chao *et al.*, 2007; Chen *et al.*, 2000; Chiu *et al.*, 2011; Du *et al.*, 2010; Jiang *et al.*, 2010; Li-Weber, 2009; Mu *et al.*, 2016; Takahashi *et al.*, 2011; Wu *et al.*, 2011b; Yang *et al.*, 2011; Yu *et al.*, 2015). BG exerted anti-aging effects likely through attenuating oxidative stress (Gao *et al.*, 2016). B has also shown to affect xenobiotic and carcinogen metabolism by inhibiting several metabolizing enzymes' activity (Moon *et al.*, 2006).

Moreover, BG extracts are easily accessible over-the-counter herbal remedies, purchasable online and in numerous stores in liquid or bulk powder form. Recommended daily dosage of BG powder is 60-500 mg meaning a 540-4480 μM dose (in 0.25 liters).

Since BG and B have such enormous therapeutic potentials, a better understanding of their pharmacokinetics and bioavailability is necessary to specify clinical effects, developing clinical regimens and elucidating potential drug interactions.

Oral administration is a popular drug delivery route because it is usually convenient for both doctors and patients. To achieve successful therapeutic efficacy, BG must be absorbed adequately and consistently after oral administration (Tian *et al.*, 2009).

Glucuronidation is a significant metabolic pathway that facilitates efficient elimination and detoxification of numerous endogenous substances (e.g., bilirubin and estradiol) and xenobiotics (e.g., SN-38 and indinavir) (Wu *et al.*, 2011a).

In recent decades, numerous studies have accumulated evidence indicating that natural polyphenols are rapidly and extensively metabolized to glucuronides and sulfates after oral ingestion. Based on a previous pharmacokinetic study of *RS*, the glucuronides and/or sulfates of B were the major molecules in the bloodstream after dosing a *RS* decoction to rats (Hou *et al.*, 2011).

Several animal studies showed that BG, instead of B, was the predominant form in the general blood circulation after oral administration of B or BG (Akao *et al.*, 2000; Akao *et al.*, 2013; Cai *et al.*, 2016; Fong *et al.*, 2015; Gao *et al.*, 2011a; Gao *et al.*, 2012; Lai *et al.*, 2003; Taiming *et al.*, 2006; Xing *et al.*, 2005).

Upon oral intake, BG is either directly absorbed from the upper intestinal tract (Akao *et al.*, 2000; Lu *et al.*, 2007; Zhang *et al.*, 2005a) or undergoes hydrolysis by intestinal glucuronidase or intestinal microflora to release its aglycone B, which will then be absorbed via passive diffusion (Abe *et al.*, 1990; Kang *et al.*, 2014; Lu *et al.*, 2007; Noh *et al.*, 2016; Wang *et al.*, 2012; Xing *et al.*, 2014; Zhang *et al.*, 2005a).

Concomitantly upon oral intake of B, B is absorbed via passive diffusion. Absorbed B undergoes extensive first-pass intestinal Phase II metabolism in enterocytes, including glucuronidation (>90%), catalyzed by the enzyme UDP-glucuronosyltransferase (UGT) and less significant sulfation, catalyzed by sulfotransferase (SULT) resulting in its conjugated metabolites, BG and baicalein-7-O-sulfate (Akao *et al.*, 2000; Zhang *et al.*, 2007a).

Although B demonstrates good permeability due to its good lipophilicity, its metabolite BG formed inside the intestinal epithelial cells is too polar to cross the lipid bilayer by passive diffusion (Dai *et al.*, 2008).

Because of low oral bioavailability, various formulations have been developed to improve the gastrointestinal absorption of BG. Solid dispersions, microcapsules, cyclodextrins, emulsions, phospholipid complex, liposomes and nanoparticles have been described (Gabrielska *et al.*, 1997; Li *et al.*, 2011b; Liu *et al.*, 2011; Luo *et al.*, 2010; Wu *et al.*, 2014). BG belongs to Class IV of Biopharmaceutical Classification System (BCS) due to its extremely low hydrophilicity (solubility 0.052 mg/mL in water)

and lipophilicity ($P_{app} = 0.037 \times 10{-}6$ cm/s) (Wu *et al.*, 2014). B is highly permeable ($P_{app} = 1.7 \times 10{-}5$ cm/s) but poorly water soluble, which is classified as a Class II compound according to BCS (Zhang *et al.*, 2007b; Zhang *et al.*, 2014).

Accordingly, after administration of a single ascending dose of B (100-2800 mg) chewable tablets to healthy subjects, the C_{max} values of BG were about ten-fold higher than C_{max} values of B (Li *et al.*, 2014). Another study using rat intestine perfusion model and Caco-2 monolayer model uncovered that B was rapidly converted to BG, before being transported to the mesenteric system (Zhang *et al.*, 2005a). In addition, significant biliary as well as sinusoidal transport of BG from hepatocytes was shown (Akao *et al.*, 2009).

There have been many studies reporting that the glucuronides and sulfates of xenobiotics are the substrates of multidrug resistance–associated proteins (MRPs) or breast cancer resistance protein (BCRP). The involvement of efflux transporters such as BCRP is necessitated by the fact that a glucuronide is too polar to passively diffuse out of cells. Therefore, in addition to UDP-glucuronosyltransferases (UGTs) that catalyze glucuronidation reaction (i.e., glucuronide formation), efflux transporter is another element that enables glucuronide clearance (Xu *et al.*, 2009; Zamek-Gliszczynski *et al.*, 2006). In recent years, numerous studies have shown that BCRP is involved in intestinal and/or biliary excretion of glucuronides of a diverse group of compounds including flavonoids (Xu *et al.*, 2009). Thus, the effective transport of intracellularly formed glucuronides of B from enterocytes likely depends on a carrier-mediated transport.

Owing to the various mechanisms involved in the absorption, reconversion dynamics between BG and B, and metabolism of BG, the attainment of peak concentrations in plasma for BG appeared to be prolonged, suggesting a significant role of the enterohepatic recycling of BG. The extensive enterohepatic recycling distributive phase has been confirmed after both oral and intravenous routes of dosing in rats (Xing *et al.*, 2005). Biliary excretion plays a major role in bringing the glucuronide and sulfate conjugates of B back to the small intestine where it undergoes hydrolytic cleavage through intestinal beta-glucuronidase (Liu *et al.*, 2010).

The role of hepatic biliary excretion in the modulation of pharmacokinetics of BG has been recently clarified. In that study, the pharmacokinetics of BG were evaluated in wild type rats and Mrp2-deficient rats. Following oral administration of B to Mrp2-deficient rats, the peak concentration and AUC value for BG were five-fold and eight-fold higher than the relative values obtained in normal rats (Akao et al. 2009). When B was dosed into the portal vein of Mrp2-deficient rats, a four-fold reduction in the biliary excretion and a 30-fold elevation in systemic exposure was observed as compared with a similar B dose administration into the portal vein of regular rats. Therefore, this work not only clarified the biliary excretory pathway for BG, but also indicated the propensity of the sinusoidal efflux mechanism (Akao *et al.*, 2009; Srinivas, 2010). The potential biotransformation pathway of BG and B can be seen on ***Figure 3*** (Chen *et al.*, 2014).

The liver is regarded as the most important organ for the disposition of various endogenous and exogenous substances in the body. As for the hepatic disposition of conjugates already existing in the circulation, the hepatic uptake of metabolites is critical due to their difficulty to traverse the basolateral cell membrane. Before hepatic metabolism and biliary excretion, drugs need to enter the hepatocytes first, either through passive diffusion or mediated by transporters. Uptake transporters are membrane proteins that modulate the cellular influx of numerous substances including clinically important agents such as antibiotics, anti-cancer agents, and non-steroidal antiinflammatory drugs.

Because of its poor passive permeability, the hepatic uptake of BG mediated by an uptake transporters could be a key determinant in hepatobiliary excretion of BG.

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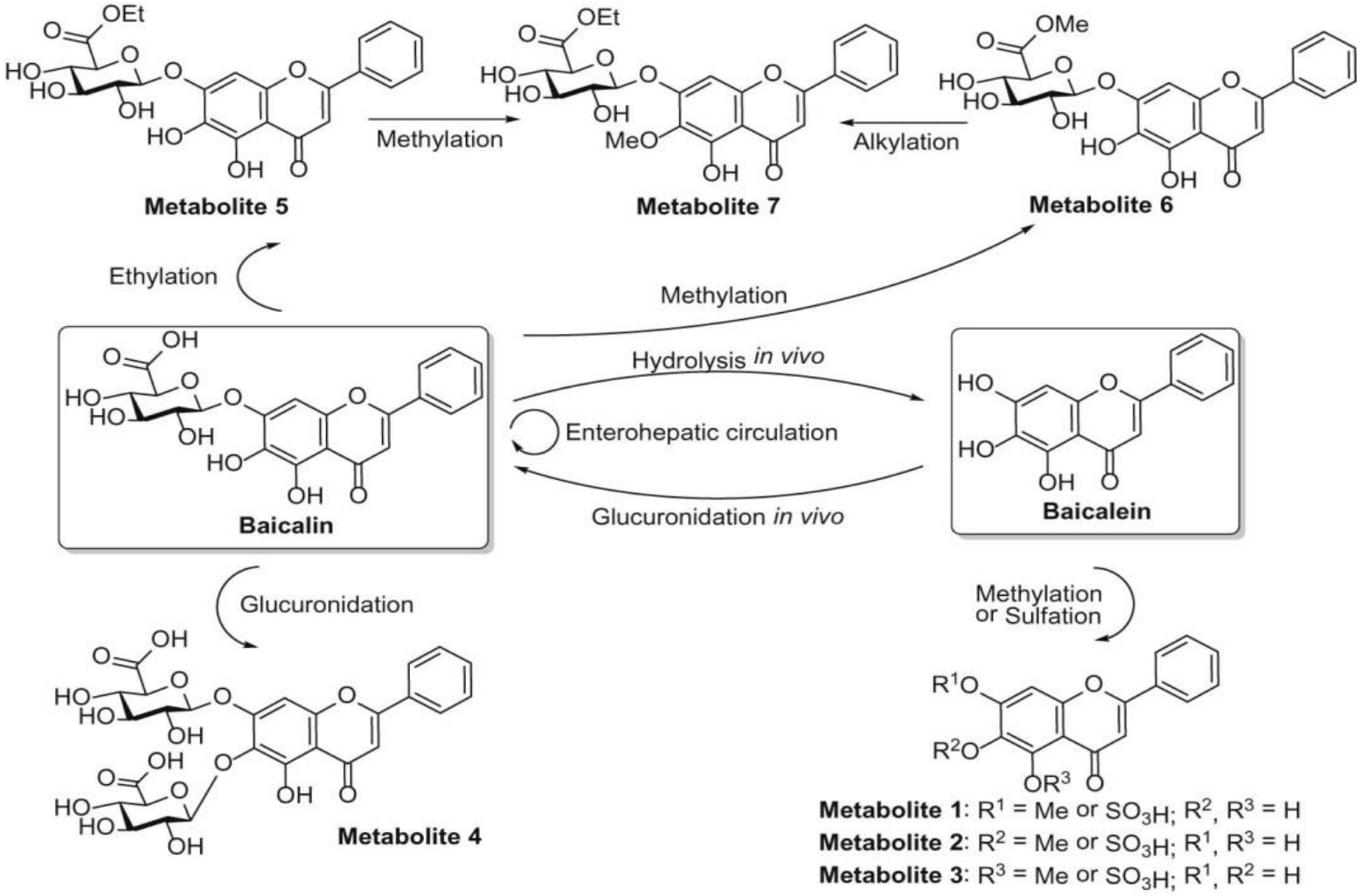

Figure 3: *Biotransformation pathway of BG and B (Chen et al., 2014).*

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2.2. Efflux transporters

Drug transporters are multispecific transmembrane proteins that facilitate the membrane transport of a large number of drugs. Drug transporters have a distinct expression pattern in the human body lining pharmacological barrier tissues, most importantly the small intestinal epithelium **(*Figure 4*)**, the endothelial cells in the blood–brain barrier, the epithelium of the proximal tubule cells in the kidney, and hepatocytes in the liver (Chandra *et al.*, 2004; Feng *et al.*, 2010; Klukovits *et al.*, 2015; Muller *et al.*, 2011; Suzuki *et al.*, 2000; Tamai *et al.*, 2000).

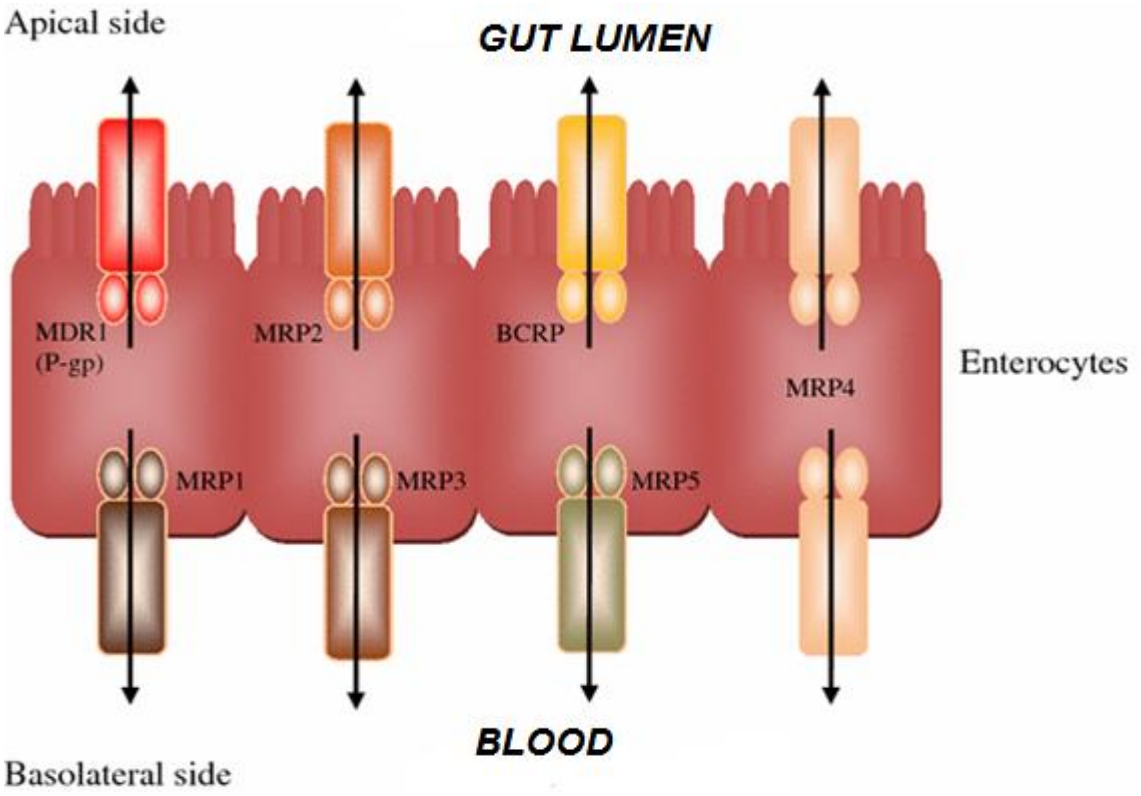

Figure 4: *ABC transporter localisation in gut epithelial cells. P-glycoprotein (Pgp/MDR1), MRP2 and BCRP are localised in the apical membrane, effluxing compounds back in to the gut lumen; whereas MRP1, 3 and 5 are localised in the basolateral membranes pumping substrates in to the blood stream. MRP4 is present in both the apical and basolateral membranes of gut epithelia (Brand et al., 2006).*

The superfamily of human ATP-binding cassette (ABC) proteins comprises 49 members divided into 7 subfamilies (ABCA – ABCG) (Dean *et al.*, 2001; Vasiliou *et al.*, 2009). The first ABC transporter involved in drug trafficking (ABCB1) was described in 1976 (Jani *et al.*, 2014b). ABC transporters transport a wide variety of substrates across plasma- and intracellular membranes, including metabolic products, lipids and sterols, and drugs **(*Figure 4*)** (Brand *et al.*, 2006).

The members of multidrug resistance-associated proteins (MRPs), especially MRP2(ABCC2), and MRP3(ABCC3), possess similar substrate selectivity and prefer to transport organic anion and phase II metabolites, including glutathione, glucuronide, and sulfate conjugates (Borst *et al.*, 2000). It also seems likely that MRP2 and/or BCRP (ABCG2) contribute to the efflux of flavonoid conjugates across the intestinal apical membrane (Sesink *et al.*, 2005).

In a recent study performed on MDCKII-MRP2 and MDCKII-BCRP cell lines, *RS* inhibited transport by the selected transporters. Consistently, the cell study further confirmed that BG inhibited the efflux transport mediated by MRP2 (Yu *et al.*, 2016a). Other study results indicated that, in rat, a large proportion of B absorbed is retained, transformed into BG within the intestinal mucosal cells, and coordinately excreted through MRP2 into the intestinal lumen (Akao *et al.*, 2004; Cao *et al.*, 2008). In other studies, BG was shown to inhibit very efficiently BCRP-, MRP2- and MRP3-mediated vesicular transport and to activate the ATPase activity of BCRP, MRP2 and MRP3 (Gao *et al.*, 2012). Another inhibitory study on MDCKII-MRP2 and MDCKII-BCRP cell lines indicated that BG inhibited both the BCRP- and MRP2-mediated efflux transports (Yu *et al.*, 2016a). Moreover experiments performed on Caco-2 cells showed that BG may be a P-gp inhibitor (Miao *et al.*, 2016). An increase of the sinusoidal transport of BG was seen in Mrp2-deficient rats (Akao *et al.*, 2009).

These findings indicate that MRP3 could likely play a role in the basolateral transport of BG from intestinal cells while MRP2 and BCRP might be the transporters effluxing BG on the apical side of enterocytes and hepatocytes (Akao *et al.*, 2007; Akao *et al.*, 2009; Li *et al.*, 2012; Zhang *et al.*, 2007a). The ATPase assay, however, is not a functional transport assay hence the substrate or inhibitory potential needs to be confirmed by a

functional vesicular transport assay, which is optimal for testing low passive permeability substrates.

On the other hand, by inhibiting the activity of ABC transporters, BG could modulate absorption and disposition of drugs, increasing the risk of therapeutic failure, adverse effects and toxicity (Li *et al.*, 2013; Zamek-Gliszczynski *et al.*, 2006). Impaired transporter activity could result in reduced (intestinal/hepatic) glucuronide clearance and elevated glucuronide accumulation in systemic circulation (Xu *et al.*, 2009). Therefore elucidating the modulation of intestinal and hepatic efflux transport by BG is of crucial importance for the evaluation of flavonoid–drug interactions, since the majority of drug products and food supplements are given orally at large quantities.

Driven mainly by the needs of the industry, in vitro methods that assess transporter interactions have matured into routine tools in the past two decades and are widely applied to predict in vivo and clinical phenomena as well as to characterize interactions on a molecular level (Sjogren *et al.*, 2014).

The first membrane vesicle applications on ABCB1 were demonstrated in 1986 using selected mammalian and transduced Sf9 cells (Doige *et al.*, 1992; Horio *et al.*, 1988; Sarkadi *et al.*, 1992). Since then the methods have been further refined and extended on other ABC transporters and are presently widely used in the study of interactions between drugs and ABC transporters (Stieger *et al.*, 2000). Vesicular transport is very efficient in the characterization of inhibitors, where the studied compound modulates transport rate of a reporter probe (Glavinas *et al.*, 2008).

The vesicular transport assay relies on membrane preparations enriched in inside-out vesicles (Heredi-Szabo *et al.*, 2012). In this orientation, the binding sites of the transporter are facing the solvent; thus concentrations and other conditions affecting the transport process can be precisely defined. Substrate molecules will be pumped into the membrane vesicles in an ATP dependent manner, and typically incubations containing AMP provide the baseline (Doige *et al.*, 1992). Transported and non-transported substrate molecules are separated by filtration through glass fiber or nitrocellulose membranes. Vesicles with trapped molecules will be retained on the filters, and

substrate molecules can be quantified via common analytical methods after elution with a suitable agent, such as methanol.

It has been demonstrated that the vesicular transport assay is a useful tool to investigate the contribution of transporters in the permeability of flavonoids (Cooray *et al.*, 2004; Dreiseitel *et al.*, 2009; Tan *et al.*, 2014; Tan *et al.*, 2013; Valdameri *et al.*, 2012; Zhang *et al.*, 2004). Vesicular transport is also very efficient in the characterization of inhibitors, where the studied compound modulates transport rate of a reporter probe (Glavinas *et al.*, 2004; Heredi-Szabo *et al.*, 2012; Heredi-Szabo *et al.*, 2013; Jani *et al.*, 2014b; Klukovits *et al.*, 2015). In the drug transporter area, the potential for inhibition is commonly assessed via the determination of an in vitro IC_{50} value. Current FDA and EMA guidance for drug transporter interactions is dependent on IC_{50} measurements as these are utilized in determining whether a clinical interaction study is warranted (FDA, 2012). These guidances contain decision trees on whether a clinical drug-drug interaction study is warranted which are based on the IC_{50} value in combination with clinical drug concentrations (Ellens *et al.*, 2013; FDA, 2012).

2.3. Uptake transporters

The organic anion transporting polypeptides (OATPs) are essential solute carrier (SLC) transporters expressed in key human organs / tissues, in particular the intestine, kidney, and liver. OATPs mediate the sodium-independent transport of a diverse range of amphiphilic organic compounds. These include bile acids, steroid conjugates, thyroid hormones, anionic peptides, numerous drugs and other xenobiotic substances (Giacomini *et al.*, 2010). OATP1A2, OATP2B1, OCT1, OCTN1, and OCTN2 transporters may assist in the intestinal absorption of many clinically important and frequently prescribed drugs at the lumen facing apical membrane of enterocytes, whereas OCT1 and OCT2 may mediate the drug uptake from blood at the basolateral membrane of enterocytes. OATP1B1, OATP1B3, OATP2B1, OAT2, OCT1, and OCT3 are responsible for the uptake of drugs into the liver at the basolateral membrane of

hepatocytes, which is the first step of the subsequent biliary excretion and/or drug metabolism (Roth *et al.*, 2012).

It was already shown that BG impacted on the pharmacokinetic performance of rosuvastatin, a substrate of several hepatic SLC transporters, including OATP1B1, OATP1B3, OATP2B1 and OATP1A2 (Fan *et al.*, 2008).

In another inhibitory study performed on CHO-OATP1B1, MDCKII-OATP2B1 and CHO-OATP1B3 transfected cell lines, BG has shown to inhibit transport by OATP2B1 and OATP1B3, uptake transporters expressed in the apical membranes of enterocytes and sinusoidal membranes of hepatocytes. However, BG did not affect transport by OATP1B1 (Zhang *et al.*, 2011b). In addition, another study performed on HEK293 cells exhibited that BG did not inhibit transport by OATP1A2 and OATP1B1, but affected transport by OATP1B3 and OATP2B1 (Xu *et al.*, 2013).

Moreover, OATP2B1 was found to be primarily responsible for the hepatic uptake of Scutellarin-6-G, a structural analog of BG (Gao *et al.*, 2012).

Therefore, OATP2B1 and OATP1B3 might be ideal candidates for the role of hepatic uptake of BG ***(Figure 5).***

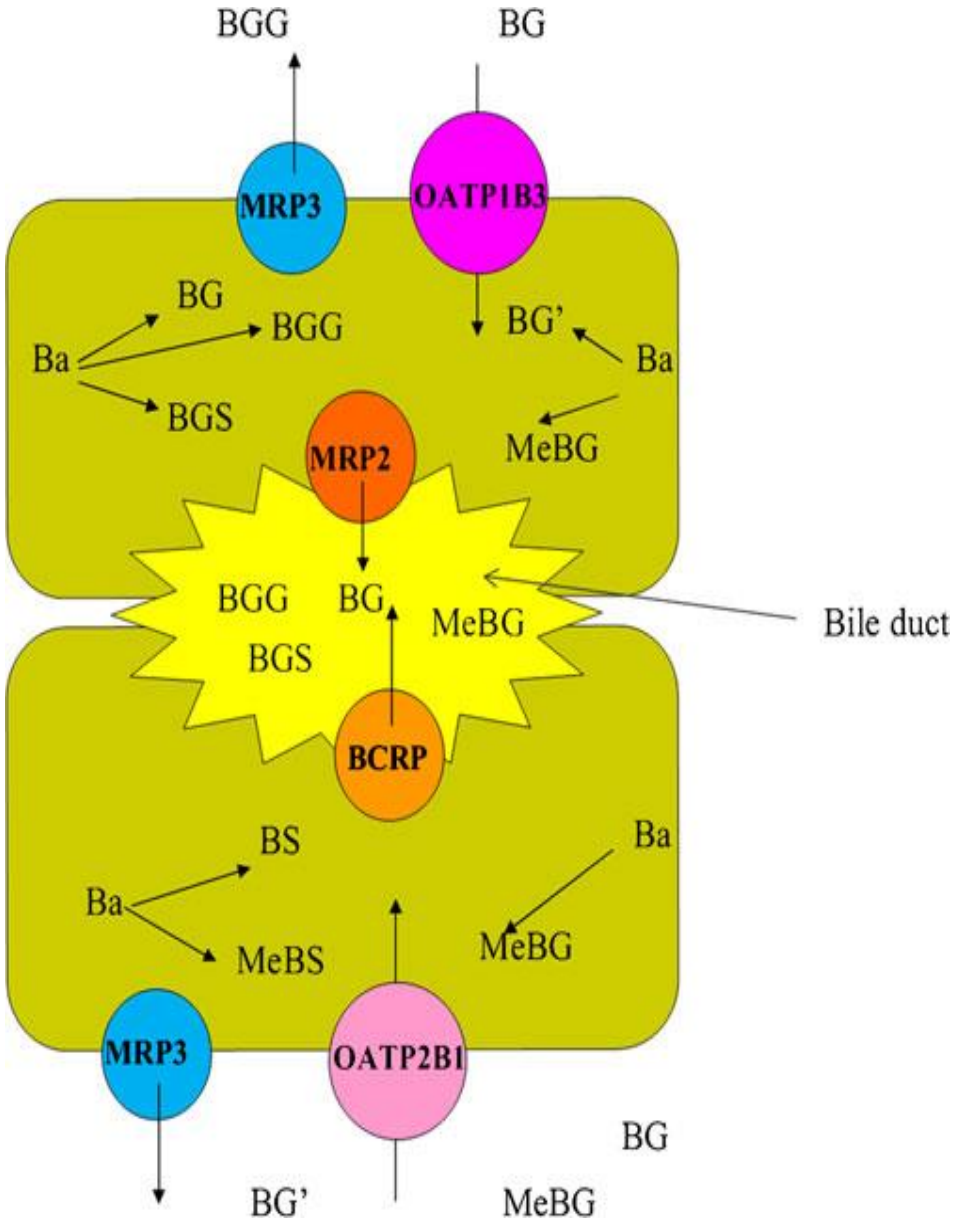

Figure 5*: Proposed diagram of hepatic metabolism and disposition of B (Zhang et al., 2011a). Ba: Baicalein, BGG: baicalein-O-diglucuronide, BGS baicalein-O-glucuronide-O-sulfate, BGGlu:baicalein-O-glucose-O-glucuronoide, BG: baicalein-7-O-glucuronide, MeBG: methyl-O-baicalein-O-glucuronide, BG' : baicalein-6-O-glucuronide, BS: baicalein-O-sulfate, MeBS: methyl-O-baicalein-O-sulfate.*

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3. Objectives

As explained in the introduction, in recent years, a wealth of evidence has been generated from *in vitro* and *in vivo* studies showing that BG could interact extensively with drug transporters and might play critical roles in multidrug resistance reversal and drug disposition. Altered drug disposition due to pharmacokinetic interactions may result in clinically relevant changes in drug ADME properties and therefore drug efficacy or toxicity. Moreover, since BG and B have such enormous therapeutic potentials, a better understanding of their pharmacokinetics and bioavailability is necessary for extrapolating the data from pharmacological assays to clinical effects and developing clinical regimens.

The objectives of this thesis were therefore:

- to investigate the inhibitory effect of BG on ABC transporters
- to identify the transporters responsible for the efflux of BG from enterocytes and hepatocytes
- to identify transporters responsible for the uptake of BG into hepatocytes and enterocytes

The information obtained from these studies will help us better understand and predict the potential in vivo BG-drug interactions mediated by these drug transporters and to elucidate pharmacokinetics of BG.

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4. Materials and methods

4.1. Materials

^{3}H-Estradiol-17-β-D-glucuronide (^{3}H-E$_2$17βG), ^{3}H-Estrone-3-O-sulfate (^{3}H-E3S) and ^{3}H-Dehydroepiandrosterone sulfate (^{3}H-DHEAS) were purchased from Perkin Elmer Inc. (Waltham, MA, USA) and ^{3}H-N-Methyl-quinidine (^{3}H-NMQ) from BRC Radio-Lab Ltd. (Szeged, Hungary).

Membranes isolated from BCRP-, MDR1-, MRP1-, MRP2-, MRP3- and MRP4-overexpressing cells and bimane-glutathion conjugate (B-GS) were provided by Solvo Biotechnology Ltd. (Budaörs, Hungary). HEK293-OATP1B3 and MDCKII-OATP2B1 cells stably overexpressing the human transporters of interest and control cells (HEK293-Mock and wild type MDCKII) were obtained from Solvo Biotechnology Ltd. (Budaörs, Hungary).

All other chemicals were of analyitical grade and were purchased from Sigma–Aldrich Ltd. (Budapest, Hungary).

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4.2. Vesicular transport inhibition studies

The interaction of BG with the transporter can be detected as the modulation of the initial rate of a labeled radioactive substrate transport by the transporter into membrane vesicles purified from Sf9 or MCF7 or HEK293 cells expressing the transporter (***Figure 6***).

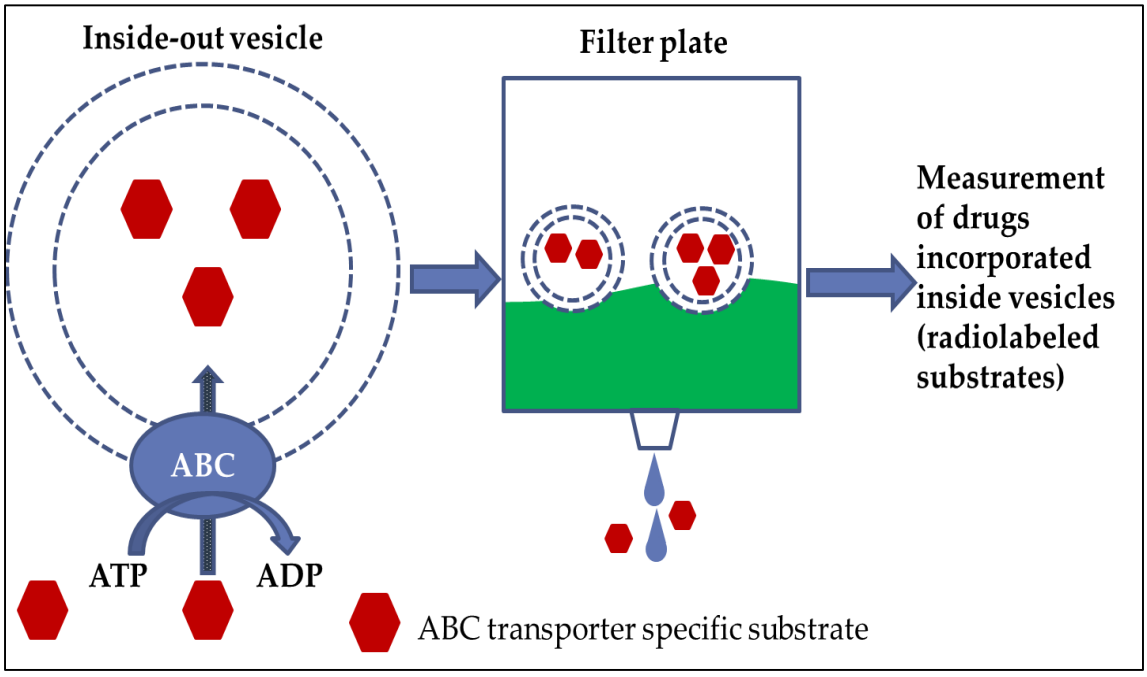

Figure 6: *Vesicular transport inhibition study principles*

Inhibitory effects of BG on transport of NMQ in membrane vesicles from K562 cells overexpressing MDR1, on transport of BGS in membrane vesicles from Sf9 cells overexpressing MRP1, on transport of $E_2$17βG in membrane vesicles from Sf9, then HEK293 cells overexpressing human MRP2, on transport of $E_2$17βG in membrane vesicles from Sf9, then HEK293 cells overexpressing human MRP3, on transport of DHEAS in membrane vesicles from HEK 293 cells overexpressing human MRP4 and on transport of E3S in vesicles from MCF7 cells expressing high levels of BCRP were investigated with rapid filtration techniques as described previously (Bodo *et al.*, 2003; Pal *et al.*, 2007).

The baculovirus insect cell system (Sf9) is easy-to-use and gives high expression of the transduced gene. Obviously, no other mammalian transporters are present in the insect cells. In case of membrane preparations from insect cells, the baseline activity is very high, so most interacting compounds actually inhibit this baseline activity. In case of membranes prepared from mammalian cells (HEK293, MCF7, K562), the baseline activity is lower, and some interacting compounds inhibit, while known transported substrates activate the baseline activity. Experiments were performed in Sf9 and mammalian cell system as well, if available, to compare the systems.

4.2.1. MDR1, MRP2, MRP3, MRP4 and BCRP

Experiments were performed on MDR1-K562, MRP1-Sf9, MRP2-Sf9, MRP2-HEK293, MRP3-Sf9, MRP3-HEK293, MRP4-HEK293 and BCRP-MCF7 membranes.

A 30 mM BG stock solution and a 3-fold serial dilution was prepared in dimethyl sulfoxide (DMSO).

Inside-out membrane vesicles were preincubated. The ^{3}H labeled transporter specific substrate was added to the mixture (***Table 1***). The reaction volume was 75 µl in each well, with 50 µg protein/well.

0.75 µl of the BG dilution series was added to each well. The dilution series was diluted 150-fold when added to the wells. Transport was initiated with the addition of 4 mM ATP or AMP in the appropriate well. Transport was carried out on specific cell lines, under specific buffer solution, incubation temperature and time conditions for each transporter (***Table 1***).

The transport was stopped by the addition of cold wash buffer, after specific incubation time. The samples were transferred to class B glass fiber filters, 1-µm pore size (Millipore, Billerica, MA, USA). Filters were washed with 5 x 200 µL of ice-cold wash buffer and dried. After adding 100 µL of scintillation cocktail to each well, radioactivity retained on the filter was measured by liquid scintillation counting (Perkin Elmer 1450 LSC, Luminescence counter, Microbeta Trilux). Results were obtained in cpm.

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Table 1: *Experiment parameters for inhibition type vesicular transport experiments*

	Transporters					
	MDR1 (P-gp, ABCB1)	**MRP1 (ABCC1)**	**MRP2 (ABCC2)**	**MRP3 (ABCC3)**	**MRP4 (ABCC4)**	**BCRP (ABCG2)**
Cell type	K562	Sf9	Sf9, HEK293	Sf9, HEK293	HEK293	MCF7
Assay buffer	250 mM sucrose, 10 mM Tris–HCl 10 mM $MgCl_2$	7.5 mM MgCl2 40 mM MOPS-Tris (pH 7.0) 70 mM KCl 2 mM GSH	7.5 mM MgCl2 40 mM MOPS-Tris (pH 7.0) 70 mM KCl 2 mM GSH	7.5 mM MgCl2 40 mM MOPS-Tris (pH 7.0) 70 mM KCl 2 mM GSH	250 mM sucrose, 10 mM Tris–HCl 10 mM $MgCl_2$	250 mM sucrose, 10 mM Tris–HCl 10 mM $MgCl_2$
Substrate	NMQ (2 μM)	B-GS (5 μM)	50 μM $E_2$17βG	50 μM $E_2$17βG	0.026 μM DHEAS	1 μM E_3S
Incubation time	3 min	10 min	8 min	10 min	4 min	1 min
Incubation temperature	37°C	37°C	37°C	37°C	37°C	32°C
Cold buffer	10 mM Tris-HCl 250 mM sucrose 10 mM NaCl	40 mM MOPS-Tris (pH 7.0) 70 mM KCl	40 mM MOPS-Tris (pH 7.0) 70 mM KCl	40 mM MOPS-Tris (pH 7.0) 70 mM KCl	10 mM Tris-HCl 250 mM sucrose 10 mM NaCl 200 mg/l BSA	10 mM Tris-HCl 250 mM sucrose 10 mM NaCl

Calculations:

ATP dependent transport (cpm): the mean cpm values measured in the absence of ATP were substracted from the mean cpm values measured in the presence of ATP.

ATP dependent transport (pmol/mg/min): *Total activity (cpm)* was calculated by multiplying the cpms measured in the designated well. The rate of transport in pmol/mg membrane protein/min was calculated using the following formula:

$$\frac{ATP\,dependent\,transport\,(cpm)}{Total\,activity\,(cpm)} * \frac{Substrate\,concentration\,(nM)*Volume\,(ml)}{membrane\,protein\,(mg)*time\,(min)}$$

ATP dependent transport (%): the percent activation or inhibition of the test drug. In this representation the ATP dependent transport determined in the *drug fee control* were taken as 100% and all other values were represented on this relative scale, using the following formula:

$$\frac{ATP\,dependent\,transport\,in\,the\,presence\,of\,\,test\,drug\,(cpm)}{ATP\,dependent\,transport\,in\,drug\,\,free\,control\,(cpm)} * 100$$

All experiments were performed 3 times. All concentrations were tested in duplicates.

BG concentration-relative transport (%) "dose-response curve" was generated for each transporter.

The IC_{50} is defined as the concentration needed to inhibit transport of the reporter substrate by 50%. The IC_{50} parameters were derived from the equation of a one-binding site, dose-response curve fitted onto the relative activity against the concentration of BG, plotted by non-linear regression using GraphPad (San Diego, CA) Prism version 5.

4.2.2. MRP1

Inhibitory effects of BG on transport of B-GS in membrane vesicles from Sf9 cells overexpressing MRP1 were investigated with rapid filtration techniques. The interaction was detected as the modulation of the initial rate of bimane-glutathion conjugate (B-GS) transport of MRP1 into membrane vesicles purified from Sf9 cells expressing the transporter.

Same inhibition vesicular transport experiments as described before were conducted in the presence of BG concentrations in duplicates in MRP1-Sf9 vesicles. The BG stock solution and the dilution series were prepared in dimethyl sulfoxide (DMSO) and were diluted 100-fold when added to the wells

The transport was stopped by the addition of cold wash. The samples were transferred to class B glass fiber filters, 1-µm pore size (Millipore, Billerica, MA). Filters were washed with 5 x 200 µL of ice-cold wash buffer. After adding 100 µl of the detector solution (0.01 M HCl), fluorescence was measured at Ex: 430 nm, Em: 538 nm (BMG Fluostar optima). Data was analyzed following the preparation of a B-GS calibration curve.

Calculations:

ATP dependent transport (fluorescence): we took the average of the duplicates. Fluorescence values measured in the absence of ATP were substracted from the fluorescence values measured in the presence of ATP for control and samples.

ATP dependent transport (%): percent activation or inhibition of the test drug. In this representation the ATP dependent transport determined in the *drug free control* was taken as 100% and all other values were represented on this relative scale, using the following formula:

$$\frac{ATP\,dependent\,transport\,in\,the\,presence\,of\,\,test\,drug}{ATP\,dependent\,transport\,in\,drug\,\,\,free\,control} * 100$$

ATP dependent transport (pmol/mg/min): after setting up a calibration curve with the help of the measured fluorescence values and the B-GS concentrations used, we substituted the fluorescence values into the equation of the calibration curve and calculated the amount of B-GS / well (pmol). After that, we divided this value by the amount of protein per well (0.05 mg) and by the time (10 min).

Relative transport values (%): This curve shows the effect of the test drug on B-GS transport by MRP1 in percentages. 100% represent B-GS transport by MRP1 in the absence of test drug ,while 0% is the transport in the absence of ATP (non-specific binding of B-GS).

If the test drug interacts with the B-GS transport, then a dose-dependent decrease in transport is observed. The IC_{50} value for the test drug is the concentration where the B-GS transport is inhibited by 50%. In case of a non-interactor, the transport of the reporter substrate typically does not change.

4.2.3. Follow-up experiments

After the first set of experiments, starting concentration of BG was optimized for each transporter according to the first obtained inhibition curves, if needed (***Table 2***). The inhibition curve for each transporter has to contain at least 2 data points on the inflection and on both plateaus. The same experiments were then performed.

The IC_{50} can be used for ranking a series of compounds based on their inhibition potential. Compounds inhibiting the transport can be either substrates or inhibitors of the transporter protein investigated. In order to determine which is the case, further experiments (named as substrate accumulation assay, direct vesicular assay or feasibility assay) were performed to reveal the mechanism of inhibition.

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***Table 2**: BG concentrations in mixture for follow-up experiments in inhibition type vesicular transport experiments*

	MDR1-K562	**MRP2-Sf9**	**MRP2-HEK293**	**MRP3-Sf9**	**MRP3-HEK293**	**MRP4-HEK293**	**BCRP-MCF7**	**MRP1-Sf9**
BG concentrations (µM)	0.41-300	0.41-300	0.41-300	0.41-300	0.41-300	0.41-300	0.41-300	0.41-300
Follow-up experiments BG concentrations (µM)	0.41-300	3.7-2700	3.7-2700	0.41-300	0.41-300	0.41-300	0.05-33.33	3.7-2700

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4.3. Vesicular transport substrate study

Vesicular transport substrate experiments, also known as feasibility assay, were performed at 2 concentrations and at 2 reaction times, to evaluate whether ATP-dependent accumulation of BG could be detected directly in transporter-overexpressing membrane vesicles, with rapid filtration techniques (Heredi-Szabo *et al.*, 2012). Accumulation of BG in membrane vesicles from K562 cells overexpressing MDR1, in membrane vesicles from Sf9 cells overexpressing MRP1, in membrane vesicles from HEK293 cells overexpressing human MRP2, in membrane vesicles from HEK293 cells overexpressing human MRP3, in membrane vesicles from HEK293 cells overexpressing human MRP4 and in vesicles from MCF7 cells expressing high levels of BCRP were investigated with rapid filtration techniques as described previously (Bodo *et al.*, 2003), (Pal *et al.*, 2007) (***Figure 7***).

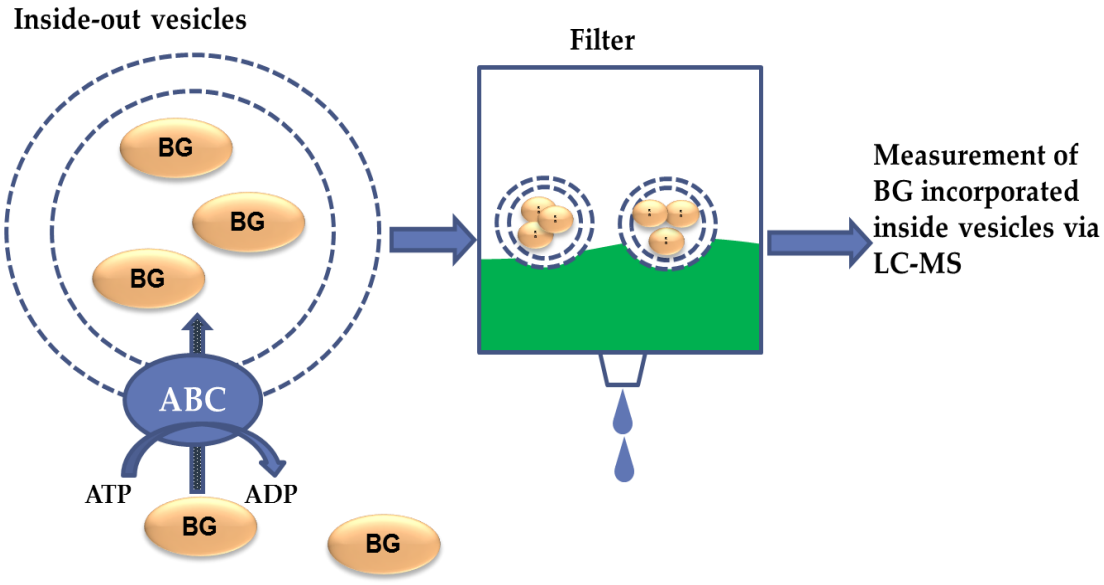

Figure 7: *Vesicular transport experiment principle*

Vesicular transport of BG was also tested on control membranes (control K562 for K562 vesicles, defMRP for Sf9 vesicles, control M for M vesicles, HEK293 control for

HEK293 vesicles) with no, or significantly lower transporter activity, in order to elucidate transporter dependent accumulation inside the vesicles.

1 mM BG stock solution was prepared in dimethyl sulfoxide (DMSO) and were diluted 100-fold when added to the wells. The reaction was performed at one or two BG concentrations (low and high) - adjusted to the previous IC_{50} data for each transporter, and one or two time points. Experiments were repeated 2 more times with criteria were the accumulation rate was maximal.

Inside-out membrane vesicles were preincubated. BG was added to the mixture. The inside-out membrane vesicles were incubated in the presence or absence of 4 mM ATP in the appropriate assay buffer and temperature (***Table 1***). The reaction volume was 75 µl in each well, with 50 µg protein/well.

The transport was stopped by the addition of the appropriate cold wash buffer. The samples were transferred to class B glass fiber filters, 1-µm pore size (Millipore, Billerica, MA, USA). Filters were washed with 5 x 200 µL of ice-cold wash buffer.The vesicles were lysed with 2 x 150 µl methanol and the eluted volumes, containing BG, were collected, the organic solvent dried with a speedvac concentrator (Thermo DNA 120) and the BG on the plates were subjected to bioanalysis.

The amount of transported BG was determined by LC-MS/MS analysis (Magda *et al.*, 2015).

Experiments were performed 3 times, at the concentration and incubation time giving the most adequate result. All concentrations were tested in triplicates. The effectiveness of the membranes was controlled with a specific substrate for each transporter.

Statistical significance was calculated using ANOVA (one-way analysis of variance).

4.4. Uptake transport studies

4.4.1. Cell culture and preparation

OATP2B1-MDCKII, OATP1B3-HEK293, wild type MDCKII and HEK293 mock cells were plated in 24-well tissue culture plates at a density of 4 x 10^5 cells/ well. For HEK293 cells, plates were precoated with poly-D-lysine. Feasibility studies were performed 24 hours after seeding. Before experiments, cell culture medium was removed and the reaction was initiated by adding transport buffer (Henseleit–Krebs buffer: KCl 4.83 mM, KH_2PO_4 0.96 mM, $NaHCO_3$ 23.8 mM, NaCl 142 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 1.53 mM, 4-(2-hydroxyethyl)- 1-piperazineethanesulfonic acid (HEPES) 12.5 mM, D-glucose 5 mM, and pH 7.4).

4.4.2. Incubations

For feasibility screening, experiments were performed by adding BG at two different concentrations (1 μM and 10 μM) and the cells were incubated at 37ºC for 2 or 20 minutes. Experiments were carried out 3 times in triplicates.

For time course, cells were incubated at 37ºC for the indicated periods of time (1-45 minutes) after adding 5 μM BG to the transport medium in transfected and control cells. Experiments were carried out twice in triplicates.

For concentration dependence, experiments were performed by adding different concentrations of BG (1 μM - 100 μM) to the transport medium in transfected and control cells. The cells were incubated at 37ºC for 3 minutes. Experiments were carried out three times in triplicates.

4.4.3. Sample preparation and analysis

The uptake was terminated by the addition of ice-cold transport medium and immediate rinsing of cells twice with ice-cold transport medium. Cells were lysed with methanol-water (2:1) solution, and the plates were centrifuged at 5000 g for 10 min, 4°C.

Supernatants were transferred into a U-bottom plate and vacuum dried. Samples were dissolved in eluent. The amount of accumulated BG was determined by LC-MS/MS analysis (Magda *et al.*, 2015).

4.4.4. Protein quantitation

Bicinchoninic acid kit (Sigma-Aldrich, St Louis, MO, USA) was used to check the total protein concentrations in cells. Positive control experiments with specific substrates of the transporters were performed to control the activity of the cells.

4.4.5. Data analysis

GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA) was used for curve fitting and calculation of kinetic parameters. Data shown in the figures are arithmetic means with standard deviation (± SD). Statistical significance was calculated using ANOVA (one-way analysis of variance).

4.5. Analytics

LC-MS/MS method was developed to quantify BG in membrane vesicles (***Figure 8***). The analytical method was developed in collaboration with the Institute of Organic Chemistry, Research Centre for Natural Sciences, Hungarian Academy of Sciences. The chromatographic separation used was an "inverse gradient elution" on a reversed phase column.

A QTRAP 6500 triple quadruple - linear iontrap mass spectrometer, equipped with a Turbo V Source in electrospray mode (AB Sciex, Redwood City, CA, USA) and a Perkin Elmer Series 200 micro LC system (Waltham, MA, USA) was used for LC-MS/MS analysis of BG.

Chromatographic separation was achieved by an Agilent Zorbax SB-C8 column (250 mm × 4.6 mm, i.d.: 5 μm) (Waldbronn, Germany). Sample was eluted with a gradient of solvent A (0,1% formic acid in water) and solvent B (0,1% formic acid in MeOH). The MS/MS system was operated under positive mode and multiple reaction monitoring mode.

To each well containing the transported BG, 200 μl water in methanol (2:8, v/v) containing 0.1% formic acid was added and kept at room temperature for one hour. The samples were transferred to 200 μl vials before injection into the LC-MS system. The calibration curve was linear (r=0.9987) from 1-1000 nM over the 3 concentration range. The coefficient of variation and relative error of BG for 4 intra- and inter-assay at three quality control (QC) levels was 2.0-10.2 % and -6.1-6.7 %, respectively. The lower limit of quantification (LLOQ) for BG was 1 nM (0.446 ng/ml), without preconcentration of the sample (Magda *et al.*, 2015).

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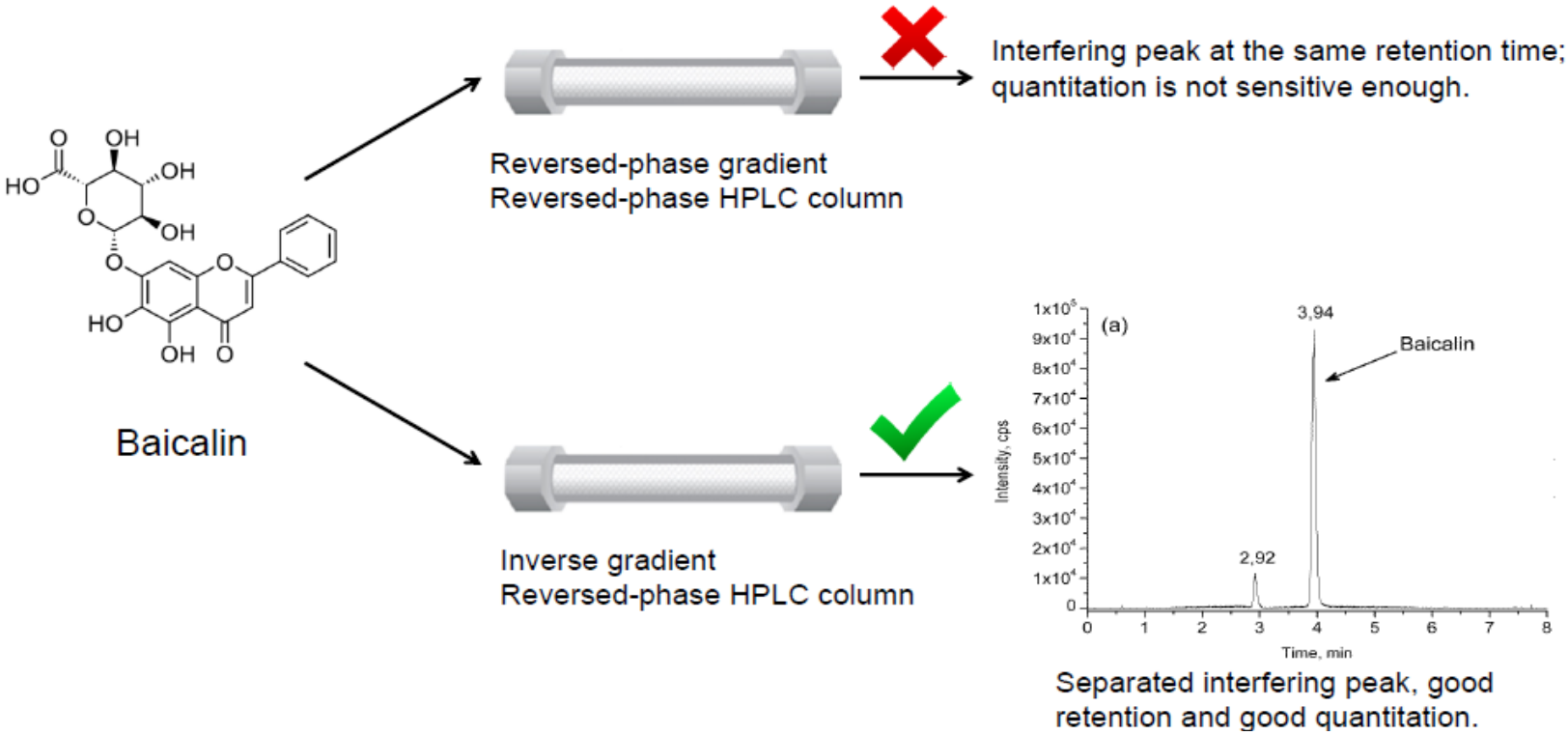

Figure 8: *Graphical abstract for the quantification of BG in membrane vesicles (Magda et al., 2015)*

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5. Results

Phase II metabolism of flavonoids in the intestinal cells and in hepatocytes as well as transport by active transporters greatly affect the disposition and bioavailability of flavonoids (Li *et al.*, 2012). The purpose of the thesis was to provide data on the interaction of BG with efflux and uptake transporters playing a role in intestinal and hepatic transport and reported to interact with BG (Akao *et al.*, 2009; Li *et al.*, 2012; Zhang *et al.*, 2007a).

5.1. Inhibition of efflux transporters by baicalin

Inhibitory vesicular transport assays were performed to determine wether BG is an inhibitor of the selected transporter. These assays are ideal for testing low permeability compounds. Therefore, inhibitory effect of BG on the efflux of transporter specific substrates was studied using membrane vesicles from cells overexpressing the transporter of interest (MDR1, MRP1, MRP2, MRP3, MRP4 and BCRP).

In case of MRP2 and MRP3, the experiments were performed in 2 cell systems, HEK293 and Sf9, to determine whether there is a difference in inhibition potential.

5.1.1. Dose-response curves

BG concentration-relative transport (%) curve was generated for each transporter (***Figures* 9*a-d***). From this curve, the IC_{50} value was calculated using GraphPad Prism 5.

The IC_{50} is defined as the concentration needed to inhibit transport of the reporter substrate by 50%. The IC_{50} parameters were derived from the equation of a one-binding site model, dose-response curve fitted onto the relative activity against the concentration of BG, plotted by log inhibitor-vs response-variable slope using GraphPad (San Diego, CA) Prism 5.

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Concentration-dependent percent inhibition by BG of transport of specific substrates by MDR1, MRP1 (***Figure 9a***), MRP2 (***Figure 9b***), MRP3 (***Figure 9c)***, MRP4 and BCRP (***Figure 9d),*** was observed, with 2 plateaus.

When comparing the two cell systems in case of MRP2 and MRP3, the depicted curves showed similar inhibitory tendency (***Figures 9b and 9c*****).**

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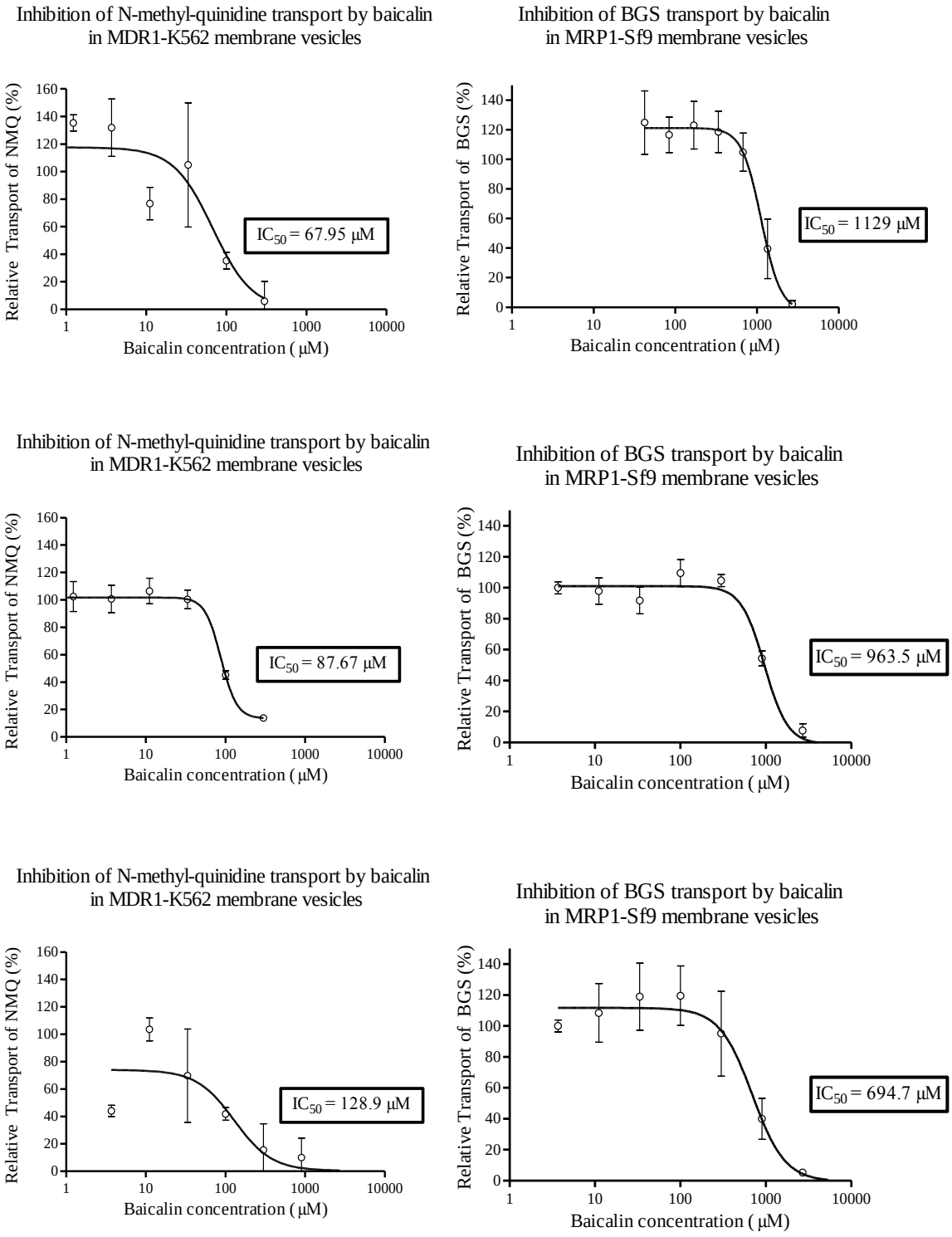

Figure 9 a: *Dose-response curves: inhibition of transporter-specific substrate transport by BG in membrane vesicles.*

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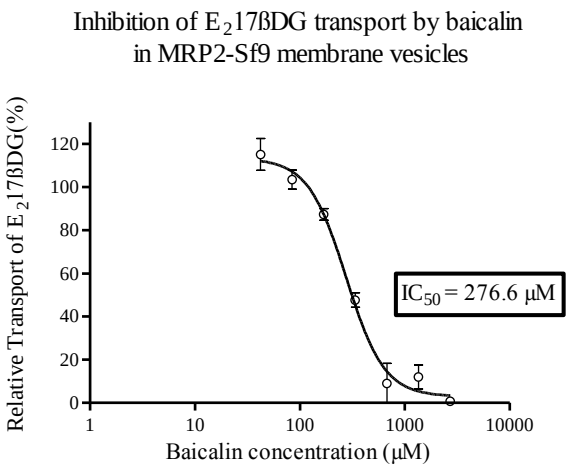

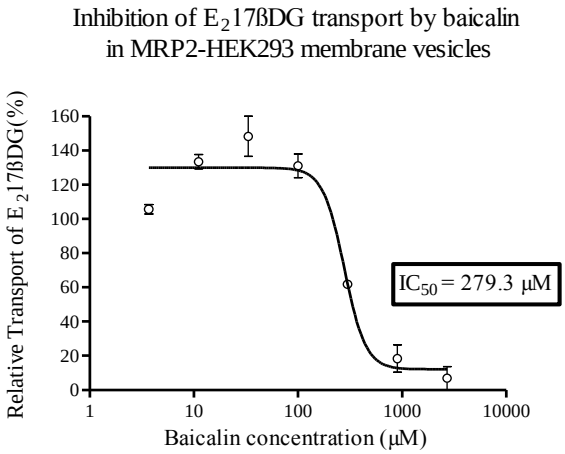

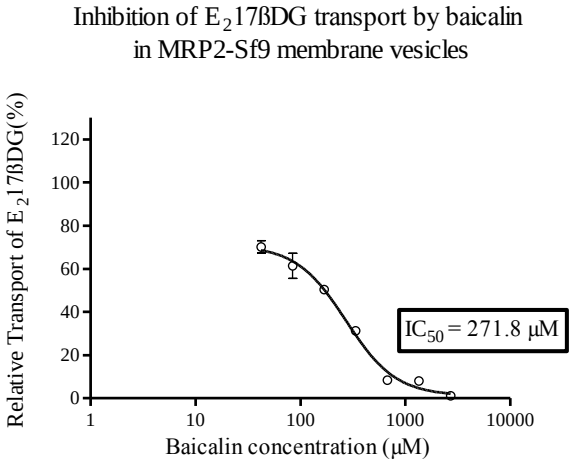

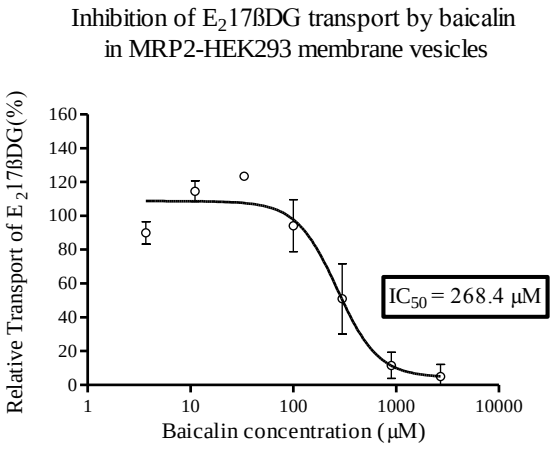

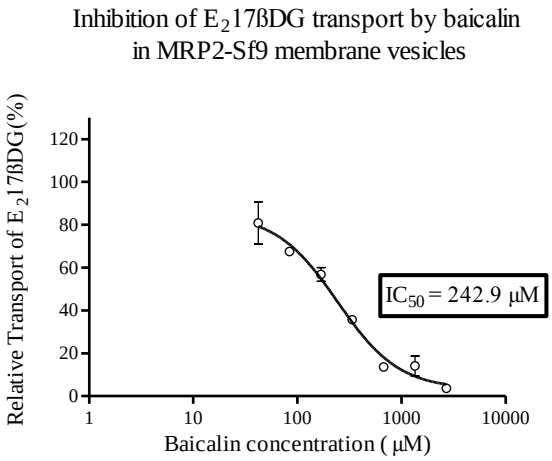

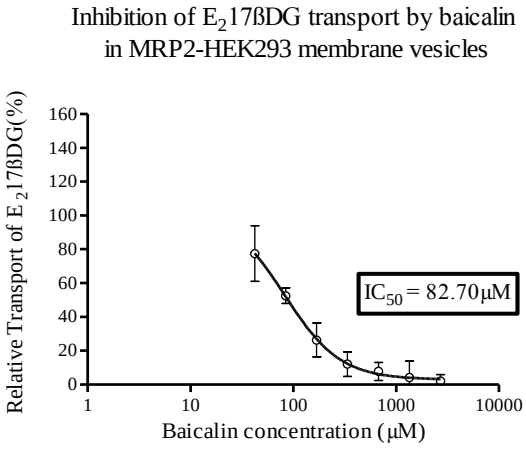

Figure 9 b: *Dose-response curves: inhibition of transporter-specific substrate transport by BG in membrane vesicles.*

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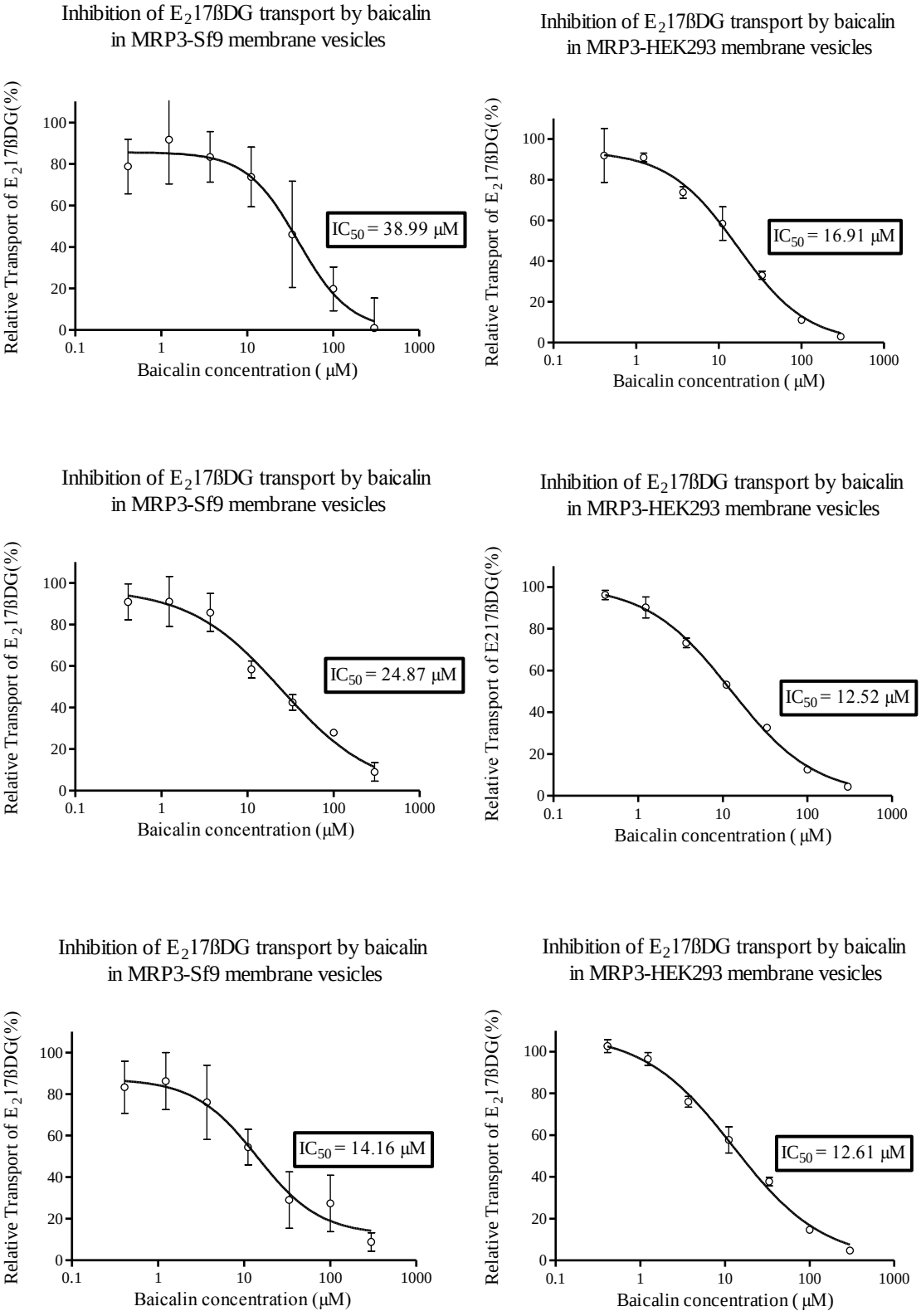

Figure 9 c: *Dose-response curves: inhibition of transporter-specific substrate transport by BG in membrane vesicles.*

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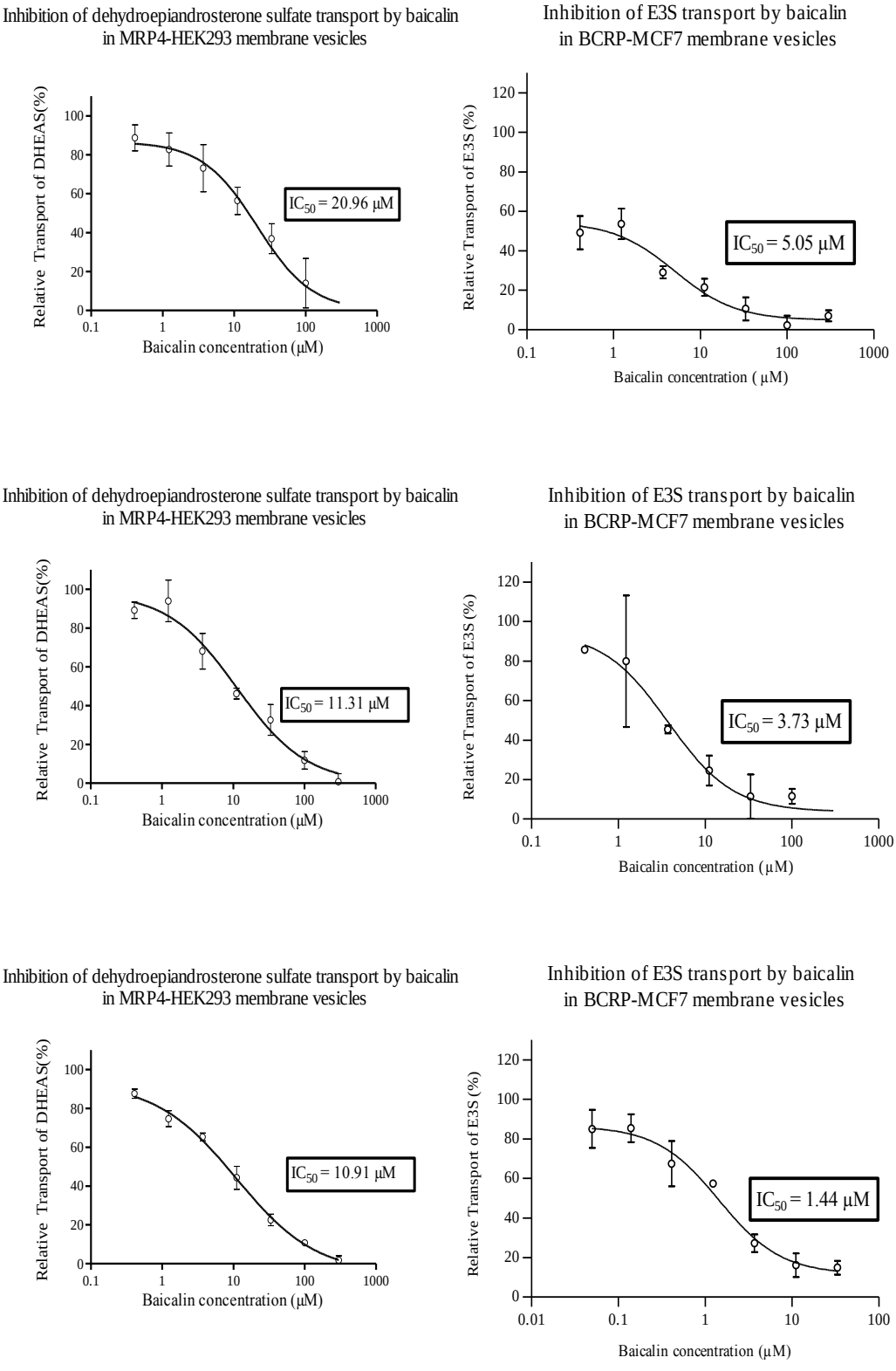

Figure 9 d: *Dose-response curves: inhibition of transporter-specific substrate transport by BG in membrane vesicles.*

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5.1.2. IC_{50} values

IC_{50} measures the effectiveness of BG in inhibiting the specific substrate transport.

After fitting the dose-response curves for the ATP dependent transport (%) against the concentration of BG, IC_{50} values were derived using GraphPad Prism 5. ***Table 3*** and ***Figure 10*** show the summary of the experiments.

As shown in ***Table 3***, IC_{50} values were in different range for each transporter.

BG inhibited transport of the transporter-specific substrate by MDR1-K562, MRP1-Sf9, MRP2-Sf9, MRP2-HEK293, MRP3-Sf9, MRP3-HEK293, MRP4-HEK293 and BCRP – MCF7 with average IC_{50} values of 94.84 ± 31.10 μM, 929.07 ± 219.88 μM, 263.77 ±18.23 μM, 210.13 ±110.49 μM, 26.01 ± 12.45 μM, 14.01 ± 2.51 μM, 14.39 ± 5.69 μM and 3.41 ± 1.83 μM respectively.

Overall, inhibition potential of each transporter by BG differed regarding IC_{50} range**,** with all inhibition curves depicting 2 plateaus (***Figure 10*****)**.

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Table 3: *Summary of IC_{50} values after fitting dose-response curve in inhibition type vesicular transport experiments.*

Transporter	IC_{50} (µM)	Average IC_{50}(µM)	± SD
MDR1-K562	67.95	94.84	31.10
	87.67		
	128.9		
MRP1-Sf9	1129	929.07	219.88
	963.5		
	694.7		
MRP2-Sf9	276.6	263.77	18.23
	271.8		
	242.9		
MRP2-HEK293	82.7	210.13	110.49
	279.3		
	268.4		
MRP3-Sf9	38.99	26.01	12.45
	24.87		
	14.16		
MRP3-HEK293	16.91	14.01	2.51
	12.52		
	12.61		
MRP4-HEK293	20.96	14.39	5.69
	11.31		
	10.91		
BCRP-MCF7	3.728	3.41	1.83
	5.053		
	1.439		

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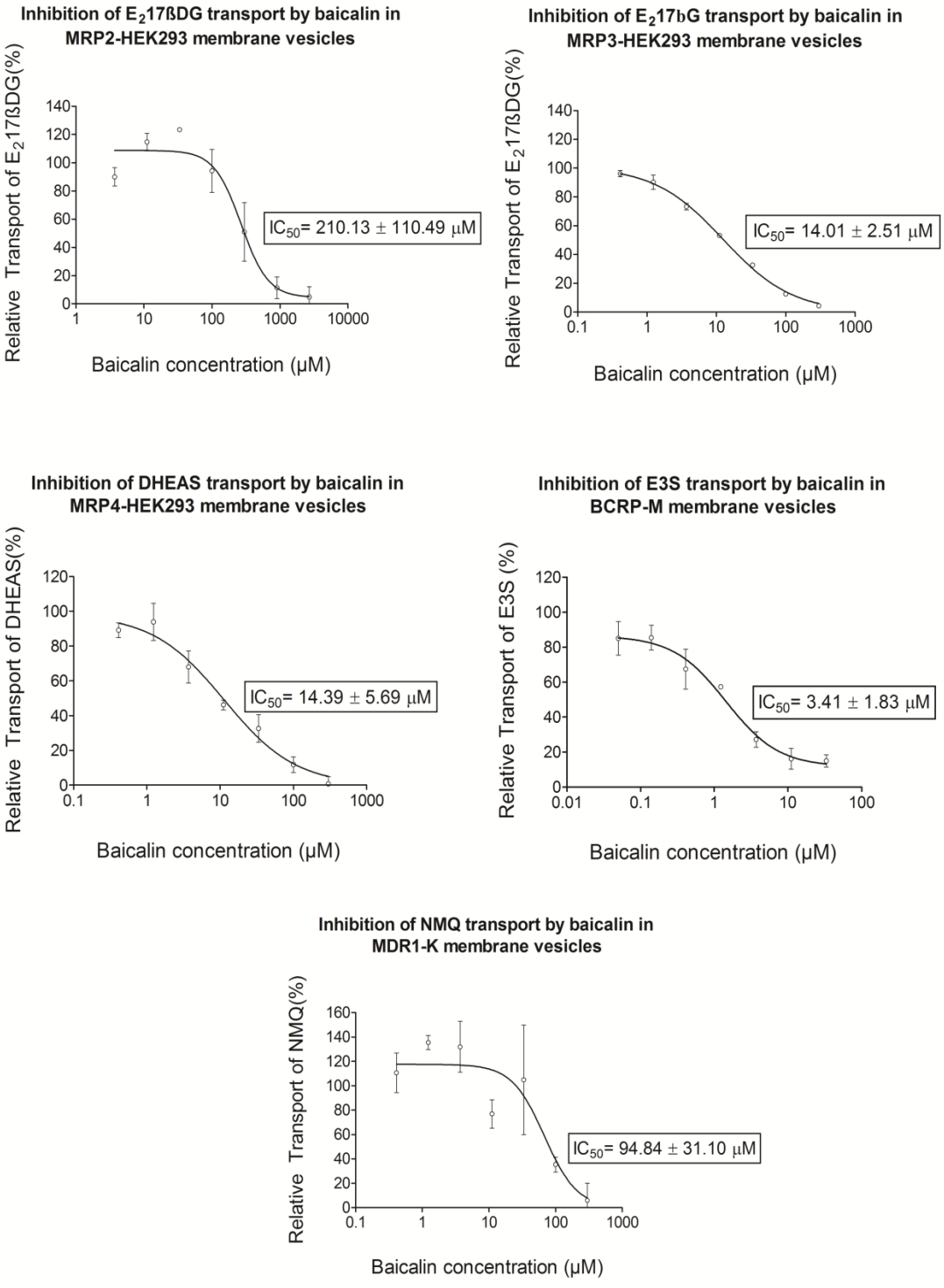

Figure 10: *Summary of experiments: inhibition of intestinal and hepatic ABC transporters by BG in vesicular transport assays. IC_{50} data represent the average of three experiments (± SD) (Kalapos-Kovacs et al., 2015).*

5.2. Efflux of baicalin by selected transporters

Direct transport studies were carried out to determine whether BG is a substrate of these transporters or BG is just an inhibitor.

The accumulation of BG was measured directly in transporter-overexpressing membrane vesicles, with rapid filtration techniques, in the presence of ATP or AMP. Starting concentrations of BG were adjusted based on IC_{50} data. The amount of transported BG was determined by LC-MS/MS analysis. ATP dependent transport was calculated by subtracting accumulation in the presence of AMP from accumulation measured in the present of ATP (***Tables 4 to 9***).

Transport studies were also performed on control membranes (K562 control, HEK293 control, defMRP and M control) to determine the transporter specific transport rate.

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5.2.1. MDR1

No ATP-dependent transport of BG was observed in MDR1-transfected vesicles and control vesicles (***Table 4***).

Table 4*: Accumulation of BG in MDR1-K562 and ctrl K (control) vesicles.*

	Accumulation (pmol/mg protein)				
	ATP		**AMP**		***ATP dependent***
Transporter (Starting BG concentration, reaction time)	*Average*	*± SD*	*Average*	*± SD*	
MDR1-K562 (100 µM, 2 min)	642.67	25.72	1165.33	118.75	***-522.67***
MDR1-K562 (100 µM, 2 min)	445.33	124.92	484.00	21.17	***-38.67***
MDR1-K562 (100 µM, 2 min)	334.00	138.69	470.27	136.76	***-136.27***
Ctrl K (100 µM, 2 min)	275.47	16.40	317.20	78.48	***-41.73***
MDR1-K562 (100 µM, 20 min)	397.47	28.20	405.73	61.51	***-8.27***
MDR1-K562 (100 µM, 20 min)	380.27	110.46	460.00	34.64	***-79.73***
MDR1-K562 (100 µM, 20 min)	322.80	45.38	343.87	54.54	***-21.07***
Ctrl K (100 µM, 20 min)	288.93	29.71	261.07	28.80	***27.87***
Ctrl K (100 µM, 20 min)	349.60	38.20	361.87	84.98	***-12.27***
Ctrl K (100 µM, 20 min)	299.87	15.14	332.93	31.77	***-33.07***

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5.2.2. MRP1

No ATP-dependent transport of BG was observed in MRP1-transfected vesicles and control vesicles (***Table 5***).

Table 5*: Accumulation of BG in MRP1-Sf9 and defMRP (control) vesicles.*

	Accumulation (pmol/mg protein)				
	ATP		**AMP**		***ATP dependent***
Transporter (Starting BG concentration, reaction time)	*Average*	*± SD*	*Average*	*± SD*	
MRP1-Sf9 (100 µM, 20 min)	103.60	19.77	195.87	112.19	***-92.27***
MRP1-Sf9 (100 µM, 20 min)	167.87	13.32	341.07	29.83	***-173.20***
MRP1-Sf9 (100 µM, 20 min)	35.56	4.89	30.64	6.13	***4.92***
MRP1-Sf9 (100 µM, 20 min)	34.81	4.70	34.40	8.09	***0.41***
defMRP (100 µM, 20 min)	4.52	2.34	3.05	0.61	***1.47***

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5.2.3. MRP2

ATP-dependent transport of BG by MRP2 was observed reaching 1533.07 pmol/mg protein. No ATP-dependent transport was detected for control cells (***Table 6***).

Table 6*: Accumulation of BG in MRP2-HEK293 and HEK ctrl (control) vesicles.*

	Accumulation (pmol/mg protein)				
	ATP		**AMP**		***ATP dependent***
Transporter (Starting BG concentration, reaction time)	*Average*	*± SD*	*Average*	*± SD*	
MRP2-HEK293 (100 µM, 20 min)	961.33	80.13	80.53	22.29	***880.80***
MRP2-HEK293 (100 µM, 20 min)	1797.33	224.33	264.27	19.67	***1533.07***
MRP2-HEK293 (100 µM, 20 min)	1552.00	65.48	115.60	30.18	***1436.40***
HEK293 Ctrl (100 µM, 20 min)	78.27	11.49	158.27	50.17	***-80.00***
HEK293 Ctrl (100 µM, 20 min)	178.93	27.74	224.80	11.05	***-45.87***
HEK293 Ctrl (100 µM, 20 min)	115.60	23.80	132.27	20.72	***-16.67***

5.2.4. MRP3

ATP-dependent accumulation of BG by MRP3 was observed reaching 2033.07 pmol/mg protein. No ATP-dependent transport was detected for control cells (***Table 7***).

Table 7*: Accumulation of BG in MRP3-HEK293 and HEK293 ctrl (control) vesicles.*

	Accumulation (pmol/mg protein)				
	ATP		**AMP**		***ATP dependent***
Transporter (Starting BG concentration reaction time)	*Average*	*± SD*	*Average*	*± SD*	
MRP3-HEK293 (10 µM, 20 min)	631.73	379.30	52.71	11.98	***579.03***
MRP3-HEK293 (10 µM, 20 min)	1424.00	204.39	82.80	18.71	***1341.20***
MRP3-HEK293 (10 µM, 20 min)	2138.67	626.10	105.60	11.39	***2033.07***
HEK293 Ctrl (10 µM, 20 min)	24.03	16.61	64.53	19.00	***-40.51***
HEK293 Ctrl (10 µM, 20 min)	33.08	7.75	64.53	19.00	***-31.45***
HEK293 Ctrl (10 µM, 20 min)	75.44	55.98	98.00	13.36	***-22.56***

5.2.5. MRP4

ATP-dependent transport of BG by MRP4 was observed reaching 120.67 pmol/mg protein. Low ATP-dependent transport was detected for control cells (***Table 8***).

***Table 8**: Accumulation of BG in MRP4-HEK293 and HEK ctrl vesicles.*

	Accumulation (pmol/mg protein)				
	ATP		**AMP**		***ATP dependent***
Transporter (Starting BG concentration, time)	*Average*	*± SD*	*Average*	*± SD*	
MRP4-HEK293 (0.1 µM, 2 min)	8.63	5.41	2.59	0.46	***6.03***
HEK293 Ctrl (0.1 µM, 2 min)	2.67	1.26	1.61	0.08	***1.06***
MRP4-HEK293 (0.1 µM, 20 min)	2.94	0.14	10.56	8.03	***-7.62***
HEK293 Ctrl (0.1 µM, 20 min)	1.53	0.00	2.91	2.36	***-1.39***
MRP4-HEK293 (1 µM, 2 min)	20.41	7.37	21.36	7.87	***-0.95***
HEK293 Ctrl (1 µM, 2 min)	3.38	2.79	1.87	0.17	***1.51***
MRP4-HEK293 (1 µM, 20 min)	29.27	8.70	17.64	1.94	***11.63***
HEK293 Ctrl (1 µM, 20 min)	1.69	0.26	5.15	3.53	***-3.47***
HEK293 ctrl (10 µM, 2 min)	36.45	2.23	43.07	5.28	***-6.61***
MRP4-HEK293 (10 µM, 2 min)	80.53	16.86	195.07	10.74	***-114.53***
MRP4-HEK293 (10 µM, 2 min)	67.47	3.61	74.53	14.80	***-7.07***
MRP4-HEK293 (10 µM, 2 min)	184.67	33.98	308.80	12.20	***-124.13***
MRP4-HEK293 (10 µM, 2 min)	128.67	43.23	42.80	35.68	***85.87***
MRP4-HEK293 (10 µM, 20 min)	101.11	60.80	135.73	14.32	***-34.63***
MRP4-HEK293 (10 µM, 20 min)	157.73	29.95	85.33	13.86	***72.40***
MRP4-HEK293 (10 µM, 20 min)	347.07	18.07	226.40	13.38	***120.67***
MRP4-HEK293 (10 µM, 20 min)	353.33	12.96	240.80	16.92	***112.53***
HEK293 ctrl (10 µM, 20 min)	24.03	16.61	64.53	19.00	***-40.51***
HEK293 ctrl (10 µM, 20 min)	162.80	22.20	101.87	17.08	***60.93***
HEK293 ctrl (10 µM, 20 min)	151.07	16.49	120	9.71	***31.07***

5.2.6. BCRP

ATP-dependent transport of BG by BCRP was observed reaching 2615.36 pmol/mg protein. Low ATP-dependent transport was detected for control cells (***Table 9***).

Table 9: *Accumulation of BG in BCRP-MCF7 and ctrl M (control) vesicles.*

	Accumulation (pmol/mg protein)				
	ATP		**AMP**		***ATP dependent***
Transporter (Starting BG concentration reaction time)	*Average*	*± SD*	*Average*	*± SD*	
BCRP-MCF7 (0.1 µM, 2 min)	179.87	9.10	5.29	0.37	***174.57***
BCRP-MCF7 (0.1 µM, 2 min)	889.33	354.39	33.15	7.32	***856.19***
BCRP-MCF7 (0.1 µM, 2 min)	60.80	25.75	7.71	1.55	***53.09***
Ctrl M (0.1 µM, 2 min)	8.56	0.29	7.87	1.64	***0.69***
BCRP-MCF7 (0.1 µM, 20 min)	474.00	181.92	8.51	1.22	***465.49***
BCRP-MCF7 (0.1 µM, 20 min)	2325.33	79.03	21.69	2.38	***2303.64***
BCRP-MCF7 (0.1 µM, 20 min)	70.27	26.09	1.82	0.47	***68.45***
CtrlM (0.1 µM, 20 min)	13.55	1.54	5.11	1.33	***8.43***
BCRP-MCF7 (1 µM, 2 min)	720.00	96.99	27.57	8.45	***692.43***
BCRP-MCF7 (1 µM, 2 min)	73.73	5.72	32.99	52.50	***40.74***
BCRP-MCF7 (1 µM, 2 min)	163.87	38.41	5.47	2.17	***158.39***
Ctrl M (1 µM, 2 min)	0.00	0.00	0.36	0.33	***-0.36***
BCRP-MCF7 (1 µM, 20 min)	471.47	134.06	3.82	1.08	***467.65***
BCRP-MCF7 (1 µM, 20 min)	400.80	24.95	4.27	2.42	***396.53***
BCRP-MCF7 (1 µM, 20 min)	474.67	66.01	5.85	1.17	***468.81***
Ctrl M (1 µM, 20 min)	2.43	0.56	0.00	0.00	***2.43***
Ctrl M (1 µM, 20 min)	0.00	3.52	5.43	0.48	***-5.43***
Ctrl M (1 µM, 20 min)	26.07	3.52	5.43	0.48	***20.64***
Ctrl M (1 µM, 20 min)	21.23	1.90	0.00	0.00	***21.23***
BCRP-MCF7 (10 µM, 2 min)	598.67	78.42	50.40	3.60	***548.27***
Ctrl M (10 µM, 2 min)	13.20	0.88	24.67	2.85	***-11.47***
BCRP-MCF7 (10 µM, 20 min)	2661.33	140.87	45.97	13.89	***2615.36***
Ctrl M(10 µM, 20 min)	25.87	2.14	17.87	5.46	***8.00***

5.2.7. Summary of efflux transport experiments

Figure 11 and ***Table 10*** summarize the ATP dependent accumulation of BG after 20 minutes reaction time, on transporter specific and control membrane. Statistical significance was calculated using ANOVA. ATP dependent accumulation of BG was observed with potentially different transport rates, as of 64.17 ± 17.60, 92.69 ± 8.20, 5.09 ± 1.29 and 22.22 ± 2.07 pmol/mg protein/min for MRP2, MRP3, MRP4 and BCRP respectively, comparable to low, or even no transport in the case of control cells. Transport in transfected cells compared to control cells was significant for BCRP, MRP2 and MRP3. No significant ATP dependent transport was observed in the case of MDR1.

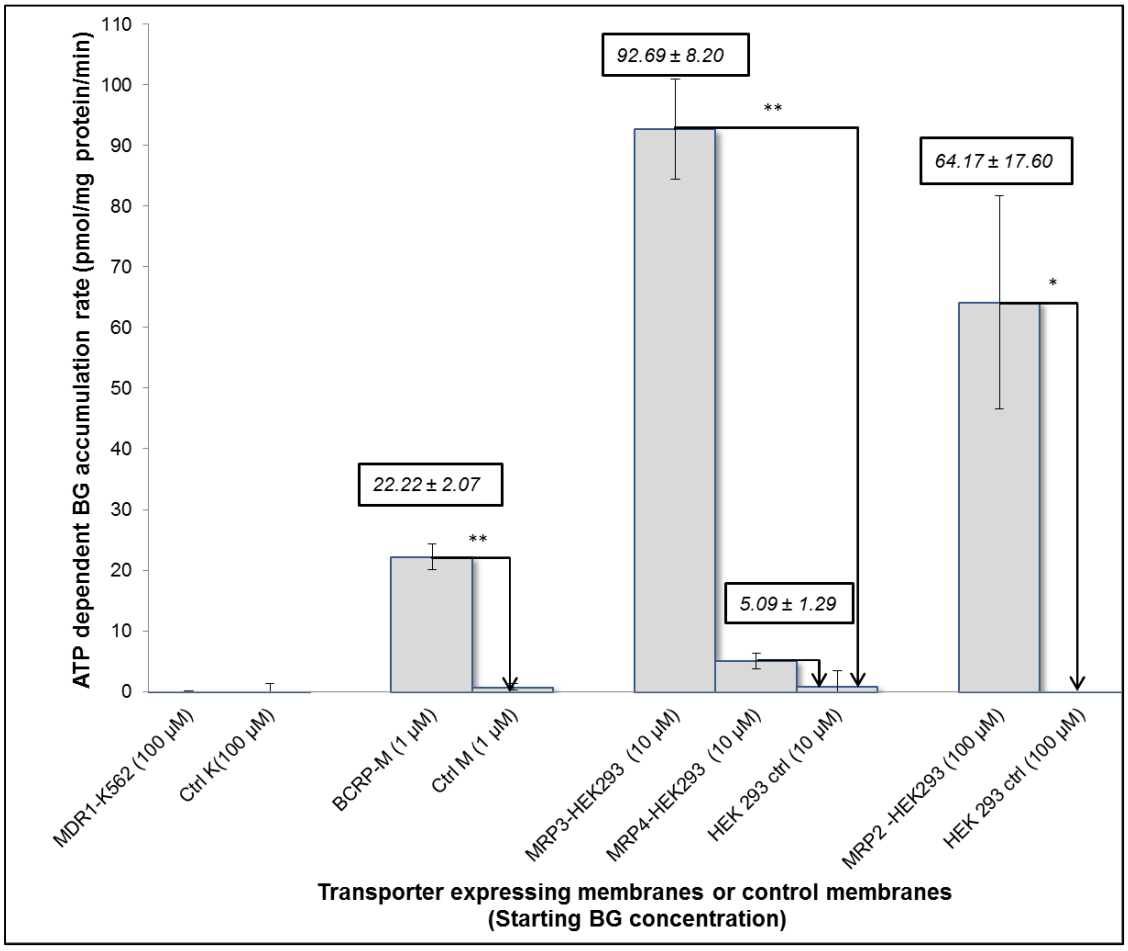

Figure 11: *ATP-dependent transport of BG by selected transporters in vesicular transport assay in transporter overexpressing and non-transfected or non-selected control membranes. *p<0.01, **p<0.001 (Kalapos-Kovacs et al., 2015).*

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***Table 10**: Summary of vesicular transport experiments of BG.*

Transporter (BG) concentration, reaction time: 20 min	**BG accumulation (pmol/mg protein)**				**ATP dependent BG accumulation rate (pmol/mg protein/min)**		
	ATP		**AMP**		**Rate**	***Average***	**± *SD***
	Average	**± *SD***	***Average***	**± *SD***			
MDR1-K562 (100 µM)	380.27	110.46	460.00	34.64	-3.99	-1.82	1.91
	397.47	28.20	405.73	61.51	-0.41		
	322.80	45.38	343.87	54.54	-1.05		
Ctrl K (100 µM)	288.93	29.71	261.07	28.80	1.39	-0.29	1.55
	349.60	38.20	361.87	84.98	-0.61		
	299.87	15.14	332.93	31.77	-1.65		
BCRP-MCF7 (1 µM)	474.67	66.01	5.85	1.17	23.44	22.22	2.07
	471.47	134.06	3.82	1.08	23.38		
	400.80	24.95	4.27	2.42	19.83		
Ctrl M (1 µM)	26.07	3.52	5.43	0.48	1.03	0.74	0.53
	21.23	1.90	0.00	0.00	1.06		
	2.43	0.56	0.00	0.00	0.12		
MRP3-HEK293 (10 µM)	2138.67	626.10	105.60	11.39	101.65	92.69	8.20
	1938.67	54.31	121.73	20.01	90.85		
	1813.33	306.79	101.87	8.10	85.57		
MRP4-HEK293 (10 µM)	347.07	18.07	226.40	13.38	6.03	5.09	1.29
	353.33	12.96	240.80	16.92	5.63		
	157.73	29.95	85.33	13.86	3.62		
HEK 293 Ctrl (10 µM)	151.07	16.49	120.00	9.71	1.55	0.86	2.61
	162.80	22.20	101.87	17.08	3.05		
	24.03	16.61	64.53	19.00	-2.03		
MRP2 - HEK293 (100 µM)	961.33	80.13	80.53	22.29	44.04	64.17	17.60
	1797.33	224.33	264.27	19.67	76.65		
	1552.00	65.48	115.60	30.18	71.82		
HEK 293 Ctrl (100 µM)	178.93	27.74	224.80	11.05	-2.29	-2.38	1.58
	115.60	23.80	132.27	20.72	-0.83		
	78.27	11.49	158.27	50.17	-4.00		

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5.3. Uptake of baicalin by transporters

Inhibitory effect of BG on uptake by OATP2B1 and OATP1B3 has already been shown in previous studies. Transport studies were carried out at 2 concentrations (1μM and 10 μM) and 2 time points (2 and 20 minutes), using MDCKII-OATP2B1 and HEK293-OATP1B3 cells to determine whether BG is a substrate of these uptake transporters, or just an inhibitor.

Transporter specific accumulation was determined by subtracting accumulation in control cells from accumulation in transfected cells.

5.3.1. Feasibility results

Feasibility experiments, performed at 2 BG concentrations and 2 incubation times, showed no transporter specific accumulation of BG in HEK293-OATP1B3 cells and HEK293 control cells (***Table 11a*****).**

Meanwhile, transporter specific uptake of BG was observed in MDCKII-OATP2B1 cells, reaching 376.90 ± 30.07 pmol/mg protein/min (***Table 11b*****).**

Further experiments were carried out for OATP2B1 to specify transport of BG.

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Table 11a: *Uptake transporter - mediated accumulation of BG at 2 concentrations 2 time points.*

Transporter	BG concentration, reaction time	Accumulation (pmol/mg protein)				Fold	Accumulation rate (pmol/mg protein/min)				Specific transport rate (pmol/mg protein/min)	
		Transfected cells		Control cells			Transfected cells		Control cells			
		Average	*± SD*	*Average*	*± SD*		*Average*	*± SD*	*Average*	*± SD*	*Average*	*± SD*
HEK293FT-OATP1B3 and HEK293FT mock	**1 µM, 20 min**	17.03	6.54	8.02	0.15	*2.12*	0.85	0.33	0.40	0.01	0.45	0.33
	10 µM, 20 min	66.50	7.55	46.80	7.40	*1.42*	3.33	0.38	2.34	0.37	0.99	0.53
	1 µM, 2 min	32.43	5.09	32.12	4.15	*1.01*	16.22	2.54	16.06	2.08	0.16	3.28
	10 µM, 2 min	60.17	9.44	51.55	8.79	*1.17*	30.08	4.72	25.78	4.39	4.31	6.45
	1 µM, 20 min	90.17	2.36	74.67	6.53	*1.21*	4.51	0.12	3.73	0.33	0.78	0.35
	10 µM, 20 min	352.83	19.53	203.67	22.19	*1.73*	17.64	0.98	10.18	1.11	7.46	1.48
	1 µM, 2 min	78.33	4.01	73.00	9.01	*1.07*	39.17	2.01	36.50	4.51	2.67	4.93
	10 µM, 2 min	229.33	3.25	177.00	10.97	*1.3*	114.67	1.63	88.50	5.48	26.17	5.72
	1 µM, 20 min	71.67	12.29	149.00	15.16	0.48	3.58	0.61	7.45	0.76	-3.87	0.98
	10 µM, 20 min	337.17	25.85	161.00	16.80	2.09	16.86	1.29	8.05	0.84	8.81	1.54
	1 µM, 2 min	62.50	14.29	57.17	9.17	1.09	31.25	7.15	28.58	4.58	2.67	8.49
	10 µM, 2 min	236.50	17.26	149.00	15.16	1.59	118.25	8.63	74.50	7.58	43.75	11.48

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Table 11b: *Uptake transporter - mediated accumulation of BG at 2 concentrations 2 time points*

Transporter	BG concentration, reaction time	Accumulation (pmol/mg protein)				Fold	Accumulation rate (pmol/mg protein/min)				Specific transport rate (pmol/mg protein/min)	
		Transfected cells		Control cells			Transfected cells		Control cells			
		Average	*± SD*	*Average*	*± SD*		*Average*	*± SD*	*Average*	*± SD*	*Average*	*± SD*
MDCKII-OATP2B1 and MDCKII wild type	**1 µM, 20 min**	98.50	4.77	38.57	12.51	*2.55*	4.93	0.24	1.93	0.63	3.00	0.67
	10 µM, 20 min	511.00	29.72	41.78	8.38	*12.23*	25.55	1.49	2.09	0.42	23.46	1.54
	1 µM, 2 min	94.00	24.66	59.00	6.54	*1.59*	47.00	12.33	29.50	3.27	17.50	12.76
	10 µM, 2 min	261.83	52.25	70.50	6.38	*3.71*	130.92	26.13	35.25	3.19	95.67	26.32
	1 µM, 20 min	1411.67	75.06	66.33	7.59	21.28	70.58	3.75	3.32	0.38	67.27	3.77
	10 µM, 20 min	7883.33	596.52	345.33	76.87	22.83	394.17	29.83	17.27	3.84	376.90	30.07
	1 µM, 2 min	911.67	103.96	84.00	6.26	10.85	455.83	51.98	42.00	3.13	41.38	5.21
	10 µM, 2 min	3098.33	930.17	305.50	0.00	10.14	1549.17	465.08	152.75	0.00	139.64	46.51
	1 µM, 20 min	1548.33	426.92	284.00	46.81	5.45	77.42	21.35	14.20	2.34	63.22	21.47
	10 µM, 20 min	7933.33	1039.63	393.00	167.54	20.19	396.67	51.98	19.65	8.38	377.02	52.65
	1 µM, 2 min	666.67	98.66	135.17	54.40	4.93	333.33	49.33	67.58	27.2	26.58	5.63
	10 µM, 2 min	2401.67	228.82	284.00	46.81	8.46	1200.83	114.41	142.00	23.4	105.88	11.68

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5.3.2. Time dependence of OATP2B1-mediated BG uptake

Time dependence of uptake of BG by OATP2B1 was studied in transfected and control cells at different incubation times, after adding 5 μM BG, to determine optimal incubation time. Time dependent transport of BG by OATP2B1 showed linear kinetics until 10 minutes, with depicted saturation plateau. Optimal incubation time was determined at 3 minutes (***Figure 12***).

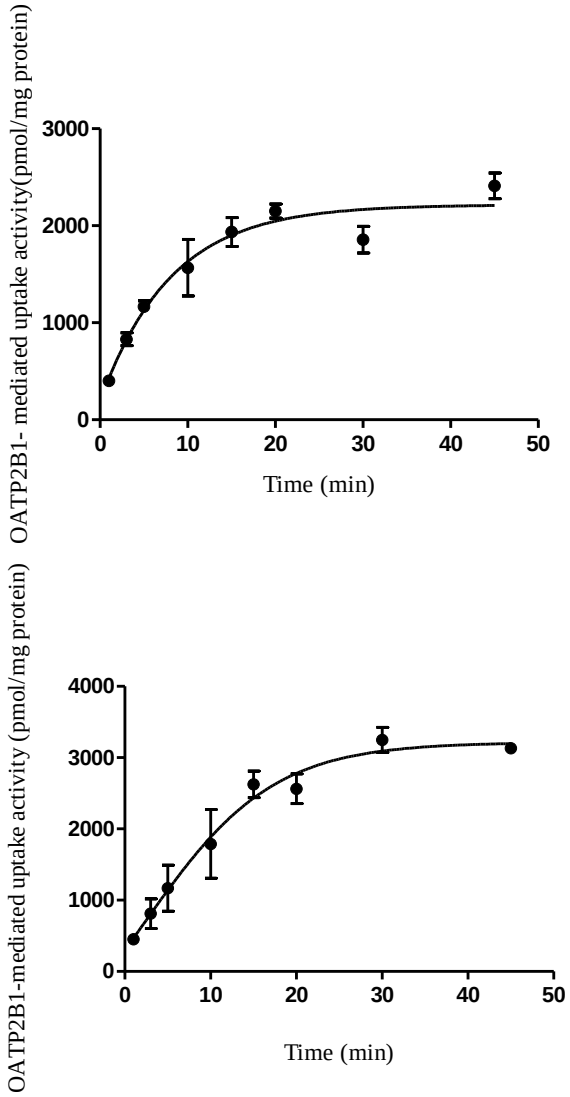

Figure 12: Time dependence of OATP2B1 –mediated transport of 5 μM BG.

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5.3.3. Concentration dependence of OATP2B1-mediated BG uptake

Concentration dependence of transport by OATP2B1 was carried out at the optimal incubation time of 3 minutes by adding different concentrations of BG, to transfected and control cells. Concentration-dependent transport of BG by OATP2B1 could be observed with V_{max} reaching 2052 pmol/mg protein/min and K_m values in the range of 10 μM or below (***Figure 13***).

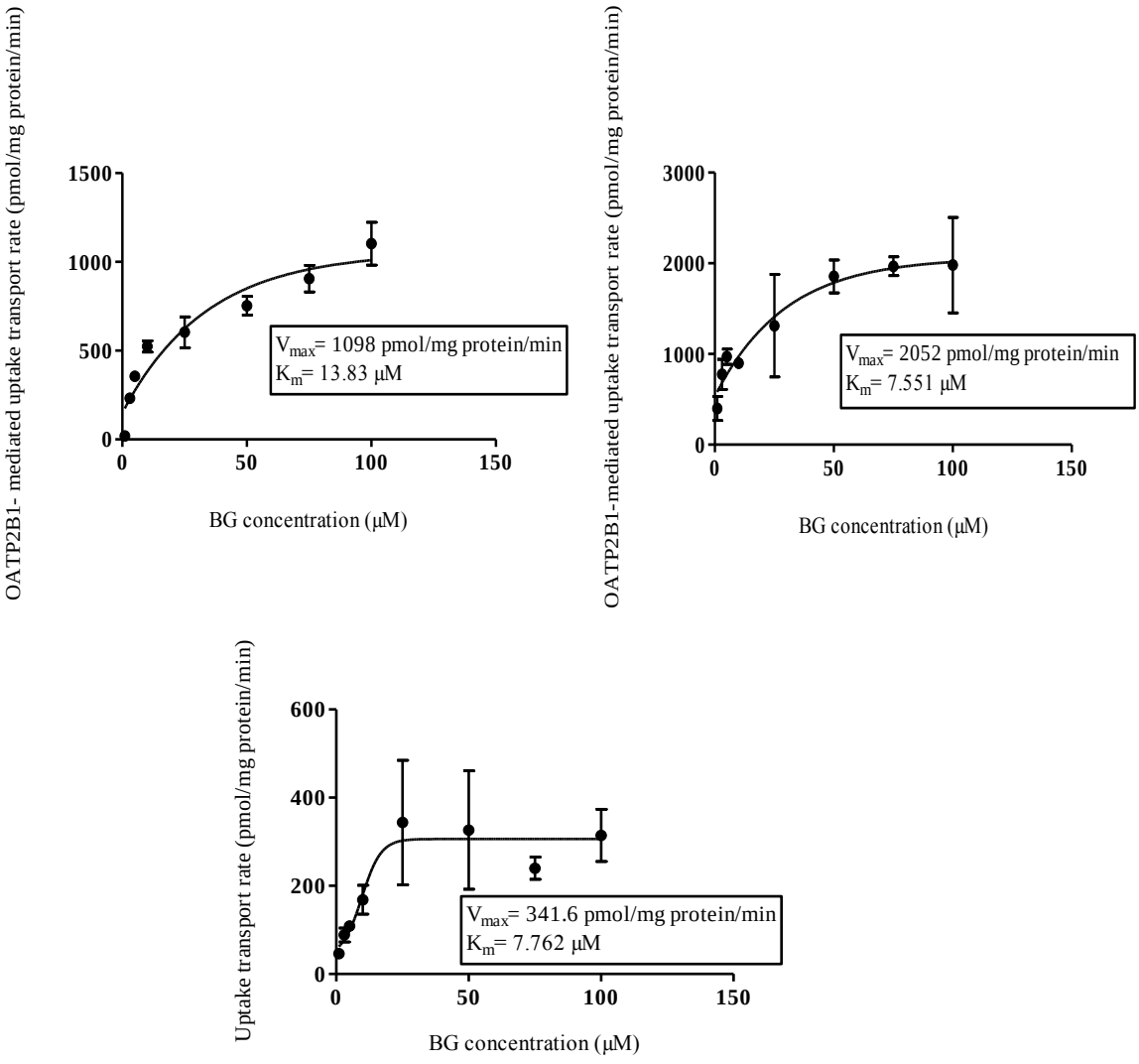

Figure 13: *Concentration dependence of OATP2B1 – mediated BG transport, at 3 minutes incubation time.*

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6. Discussion

Chinese medicines have successfully been used for centuries to treat a wide variety of human ailments and are gaining attraction throughout the world. Chinese herbs and extracts are now receiving more and more courtesies as therapeutic agents, for the treatment of many diseases in human beings (Gong *et al.*, 2002; Kim, 2005). Modern pharmacology research has confirmed that the extracts or monomeric compounds of the genus *Scutellaria* possess antitumor, hepatoprotective, antioxidant, anti-inflammatory, antibacterial and antiviral effects (Gaire *et al.*, 2014). The herb has also been used in the treatment of digestive system cancers, hepatoma, lung cancer and breast cancer. In Canada, the skullcap herb is generally sold as a tea in health food stores, but can also be found as a tonic in combination with other herbs (Awad *et al.*, 2003) .

Radix Scutellariae, the dried root of *Scutellariae baicalensis,* has been used more extensively in Chinese and Japanese medicine and is officially listed in the *Chinese Pharmacopoeia* with broad therapeutic effects (Li *et al.*, 2011a). In China, *RS* has been used to clear away the heat-evil and expel superficial evils, eliminate stasis and activate blood circulation, induce diuresis and reduce edema (Brekhman *et al.*, 1981). Due to its broad application and relatively high intake in our daily diet, it is not uncommon that *RS* may be consumed with other synthetic drugs. As a result, the herb–drug interactions between RS and other synthetic drugs should be paid attention to.

B, BG, wogonin, wogonoside, oroxylin A and oroxylin A-7-O-glucuronide are the main bioactive components found in *RS*.

Studies on content determination of *RS* demonstrated that BG existed in the most abundant amount compared to the other five bioactive flavones (Li *et al.*, 2009). BG and B have also been attracting growing interest from pharmaceutical, cosmetic, and food industries due to their excellent biological action (de Oliveira *et al.*, 2015). Extensive in vivo research carried out in the last decade demonstrated that BG and its aglycone B were important medical agents with a variety of pharmacological activities including anti-cancer, hepatoprotective, antioxidant, anti- inflammatory, anti-RSV, antimutagenic, neuroprotective, memory improving, as well as anxiolytic effects (Chen *et al.*, 2014;

Noh *et al.*, 2016; Sahebkar, 2012; Shang *et al.*, 2010). B might also serve as a novel approach for the treatment of patients with Parkinson's Disease (Xue *et al.*, 2014).

In particular, these two flavonoids have shown anti-inflammatory effects and improvement of mitochondrial dysfunction, while a combination strategy with BG or B as chemotherapeutic adjuvants has been revealed to lead to favourable anticancer activity targeting assorted cancer lines and relevant signalling pathways (Chen *et al.*, 2014; Mu *et al.*, 2009).

As explained in the introduction, in recent years, a wealth of evidence has been generated from *in vitro* and *in vivo* studies showing that BG could interact extensively with efflux drug transporters and might play critical roles in multidrug resistance reversal and drug disposition. Altered drug disposition due to pharmacokinetic interactions may result in clinically relevant changes in drug ADME properties and therefore drug efficacy or toxicity. Moreover, since BG and B have such enormous therapeutic potentials, a better understanding of their pharmacokinetics and bioavailability is necessary to elucidate clinical effects.

Phase II metabolism of flavonoids in the intestinal cells and in hepatocytes as well as transport by ABC transporters greatly affect the disposition and bioavailability of flavonoids (Liu *et al.*, 2007).

After oral administration of B or BG, BG is either directly absorbed from the upper intestinal tract (Lu *et al.*, 2007; Zhang *et al.*, 2005a) or undergoes hydrolysis by intestinal glucuronidase or intestinal microflora to release its aglycone B. B will then be absorbed via passive diffusion (Lu *et al.*, 2007; Zhang *et al.*, 2005a). Absorbed B undergoes extensive first-pass intestinal Phase II metabolism, including glucuronidation (>90%), catalyzed by the enzyme UDP-glucuronosulftransferase (UGT) and less significant sulfation, catalyzed by sulfatransferase (SULT) resulting in its conjugated metabolites, BG and baicalein 7-O-sulfate (Akao *et al.*, 2000; Zhang *et al.*, 2007a).

Although B demonstrates good permeability due to its good lipophilicity, its metabolite BG formed inside the intestinal epithelial cells is too polar to cross the lipid bilayer by passive diffusion (Dai *et al.*, 2008).

However, several animal studies showed that BG, instead of B, was the predominant form in the general blood circulation after oral administration of B or BG (Akao *et al.*, 2000; Lai *et al.*, 2003). Another study using rat intestine perfusion model and Caco-2 monolayer model uncovered that B was rapidly converted to BG, before being transported to the mesenteric system (Zhang *et al.*, 2005a).

In addition, significant biliary as well as sinusoidal transport of BG from hepatocytes was shown. An increase of the sinusoidal transport was seen in Mrp2-deficient rats (Akao *et al.*, 2009).

The objectives of this thesis were to investigate the inhibitory effect of BG on selected efflux transporters, to identify the transporters responsible for the efflux of BG from enterocytes and hepatocytes and to identify transporters responsible for the uptake of BG into hepatocytes and to determine pharmacokinetic values.

These interactions were expected to affect therapeutic outcomes which may be either beneficial or detrimental to the patient (Fekete *et al.*, 2015; Giacomini *et al.*, 2010).

6.1. Inhibition of efflux transporters by baicalin

BG was tested for its potential to inhibit vesicular transport by these transporters. Various cells such as Sf9 and HEK293 are commonly used as host cells to prepare vesicles that are transfected with ABC transporters for mechanistic studies (Sahi, 2005). Since membrane vesicles do not contain metabolic enzymes, the model presents a significant advantage over other models (cell based or in vivo) when dealing with metabolically labile compounds. Assay methods with membrane vesicles have been greatly approved and studies were performed in a high-throughput setting by using 96 well plates.

In the vesicular transport inhibition study series, a BG concentration-relative (%) curve was generated (***Figures 9a -d***) and IC_{50} values were calculated using GraphPad Prism 5.

In the case of MRP2 and MRP3, experiments were performed in 2 systems: the insect (Sf9) and mammalian cell system (HEK293) (***Figures 9b and 9c***).

BG inhibited transport of the reporter substrate in both MRP2-Sf9 and MRP2-HEK293 cells with IC_{50} values in the same concentration range (263.77 ±18.23 μM and 210.13± 110.49 μM respectively). The same tendency was observed when comparing IC_{50} results between MRP3-Sf9 and MRP3-HEK293 (26.01±12.45 μM and 14.01±2.51 μM) (***Figures 9b and 9c***). Biochemical environment of the transporter may be different in human and non-human cells, e.g. cholesterol content of membranes, which may cause difference in the function of the transporters. However, these results suggest no relevant difference while using either systems for vesicular transport studies.

In the indirect vesicular transport studies, concentration-dependent inhibition of BCRP-, MDR1-, MRP1-, MRP2-, MRP3- and MRP4- mediated transport by BG was observed (***Figures 9 and 10***, ***Table 3***).

Inhibition by BG of transport by MRP1 was observed at clinically not relevant concentrations (IC_{50} values of 929.07 ±219.88 μM), since presence of BG in the plasma in this range is unlikely.

Transport by BCRP was inhibited by BG with an IC_{50} of 1.75±1.85 μM.

Inhibition of MRP3 and MRP4 was also potent (IC_{50} values of 14.01±2.50 μM and 14.39±5.69 μM respectively).

Inhibition of MDR1 (IC_{50} = 78.21±9.88 μM) and MRP2 (IC_{50} = 306.40±56.64 μM) was less potent.

In the drug transporter area, the potential for inhibition is commonly assessed via the determination of an in vitro IC_{50} value. Regulatory guidance on the investigation of drug-drug interactions (DDIs) contain decision trees/recommendations on whether a clinical DDI study is warranted which are based on the IC_{50} value in combination with clinical drug concentration. In the 2012 Food and Drug Administration draft guidance on drug-drug interactions (DDIs), a new molecular entity that inhibits MDR1 may need a clinical DDI study with an MDR1 substrate when concentration of inhibitor based on

highest approved dose dissolved in 250 ml divided by IC_{50} ([Dose $_{max}$/IC_{50}) is ≥10. (Ellens *et al.*, 2013; FDA, 2012). Recommended daily dosage of BG is 60-500 mg, meaning a dosage concentration of 540-4480 μM if dissolved in a 0.25 liter.

Table 12: *Summary of average IC_{50} values and potential for inhibition of baicalin based on IC_{50} values. Dose$_{max}$ (4480 μM BG) was determined as the highest approved dose dissolved in 250 ml(Ellens et al., 2013).*

Transporter	Average IC_{50} (μM)	± SD	BG [Dose $_{max}$] / IC_{50}
MDR1-K562	94.84	31.10	47.24
MRP1-Sf9	929.07	219.88	4.82
MRP2-Sf9	263.77	18.23	16.98
MRP2-HEK293	210.13	110.49	21.32
MRP3-Sf9	26.01	12.45	172.24
MRP3-HEK293	14.01	2.51	319.77
MRP4-HEK293	14.39	5.69	311.32
BCRP-MCF7	3.41	1.83	1313.78

In a recent clinical study, total plasma concentrations of BG reached 6.77 μM (Li *et al.*, 2014). Thus, considering the IC_{50} values of 3.41 μM for BCRP and the 14.01 μM and 14.39 μM values measured for MRP3 and MRP4, respectively, and the current suggestion of ([Dose $_{max}$]/IC_{50}) ≥10 for interactions of clinical significance, BG likely modulates pharmacokinetics of co-administered drugs (Ellens *et al.*, 2013; FDA, 2012) (***Table 12***).

Multidrug resistance in cancer has been a major obstacle to successful cancer chemotherapy (Choi *et al.*, 2014). An important mechanism for MDR is the enhanced cellular efflux of anticancer agents due to over-expression of ABC transporter proteins (Teodori *et al.*, 2006). So far, 48 ABC transporters have been identified in humans and these proteins could confer resistance to a broad spectrum of chemotherapeutic agents by exporting drugs out of cancer cells using energy from ATP hydrolysis (Crowley *et al.*, 2010). Twenty-five years ago, it was discovered, essentially by accident, that grapefruit juice can significantly increase felodipine blood concentrations (Bailey *et al.*, 1989). Since then, drug interactions with beverages have become an important research area and have received extensive investigations.

An attractive strategy to overcome ABC transporter-mediated drug efflux is to develop inhibitors to sensitize cancer cells to chemotherapeutic drugs. By co-administering efflux pump inhibitors, such as BG, although it is to note that such reversal agents might actually increase the side effects of therapy by blocking physiological drug efflux from normal cells (Gillet *et al.*, 2011; Nobili *et al.*, 2006). Some polyphenols can overcome cancer chemotherapeutic resistance by modulating cancer cells with multiple drug resistance overexpression phenotype. In solid tumours and hematological malignances, polyphenols, exert an important role in apoptosis induction, cell growth inhibition, cell cycle arrest, oxidative stress, and in cell migration and differentiation. The combination of flavonoids and chemotherapy seems to be an interesting approach for cancer treatment (Anthwal *et al.*, 2016). Several naturally occurring flavonoids as well as few synthetic analogs have been reported to be good inhibitors of ABC transporters (Barrington *et al.*, 2015; Pick *et al.*, 2011; Yuan *et al.*, 2012; Zhang *et al.*, 2005b).

RS, containing an appreciable amount of B and BG, has been recently recognized as a new source of anti-cancer drug. It is therefore highly possible that B could be co-administrated with other drugs used in the treatment of cancer. In such cases, there might be potential competition between BG and the anti-cancer drug, such as methotrexate (a BCRP, MRP2, MRP3 and MRP4 substrate) on MRPs. All of those interactions may lead to the alteration of the clinical pharmacokinetic profiles of drugs coadministered with BG and should be taken into consideration during therapy. In addition, BG may act as a BCRP reversal agent in chemotherapy as well as in

rheumatoid arthritis, diseases where BCRP-mediated multidrug resistance (MDR) has been described (Jani *et al.*, 2014a; Kis *et al.*, 2009)

By inhibiting MDR1, MRP2, MRP3, MRP4 and BCRP, BG might be used as an MDR reversal agent during drug therapy. However, BG is also an important medical agent with a variety of pharmacological activities such as chemopreventive, hepatoprotective, anti-aging, antioxidant, anti-fibrotic, anti-allergic, anti-depressant, anti-microbial, anti-inflammatory, antimutagenic, neuroprotective, memory improving, endotoxin, as well as anxiolytic effects (Dou *et al.*, 2007; Gao *et al.*, 2016; Hu *et al.*, 2009; Kim *et al.*, 2012; Kumagai *et al.*, 2007; Oga *et al.*, 2012; Sahebkar, 2012; Shang *et al.*, 2010; Takahashi *et al.*, 2011; Waisundara *et al.*, 2011; Wang *et al.*, 2015; Woo *et al.*, 2005; Xu *et al.*, 2011; Yu *et al.*, 2016b). To achieve successful therapeutic efficacy, BG must be absorbed adequately and consistently. The next objective of this thesis was to investigate the transport mechanism of BG.

6.2. Several ABC transporters efflux baicalin

On this purpose, transport studies were carried out in membrane vesicles to determine if BG was a substrate of the respective transporter. Concentrations of BG were adjusted to previous IC_{50} data **(*Table 3*).** Experiments were also performed on control membranes with no, or significantly lower transporter activity to determine transporter specific accumulation.

BCRP, the most potent interactor and MRP4 were tested at 0.1 μM, 1 μM and 10 μM of BG. MDR1, MRP1 and MRP2 were tested at 100 μM, whereas MRP3 mediated transport was tested at 10 μM.

MDR1 and MRP1 did not transport BG at a significant level (***Tables 4 and 5***). However, accumulation of BG by MRP2, MRP3, MRP4 and BCRP was observed (***Tables 6 to 10***).

These results clearly identify MRP3 and MRP4 as the transporters implicated in basolateral efflux of BG, and MRP2 and BCRP as the transporters involved in the apical

efflux of BG (***Figure 11***, ***Table 10***). It is of note, that MRP4 transport data are just below statistical significance.

These findings correlate well with previously published ATPase and Caco-2 transport data (Zhang *et al.*, 2007a) (***Table 13***). In that study, BG significantly activated the BCRP, MRP2 and MRP3 ATPases and did not activate the MDR1 ATPase. MRP4 ATPase activity is low, so the ATPase assay is not an established assay for MRP4. Therefore, we did not test effect of BG on MRP4 ATPase. Moreover, in the dose-dependent Caco-2 transport study, a significant basolateral and apical transport of BG was found at various loading concentrations of B.

Table 13: *Specific transport of BG by selected transporters compared to previous ATPase assay (Zhang et al., 2007a) and transporter transfected MDCK cell monolayer assay results (Zhang et al., 2007a). ATPase assay (+/-): Activation/no activation observed). MDCK cell transport assay: + refers to transport observed. Transport and inhibition VT data represent the average of three experiments (± SD). *Inhibition VT and VT direct assays in BCRP-MCF7, MDR1-K562, MRP2-HEK293, MRP3-HEK293, MRP4-HEK293 cells. **ATPase assay was performed on BCRP-MCF7, MDR1-Sf9, MRP2-Sf9 and MRP3-Sf9 cells.*

ABC Transporter	***Inhibition of transport by BG in VT assay IC_{50} (± SD) (μM)****	***ATP dependent transport rate of BG (pmol/mg protein/min) (Starting BG concentration)****	***Activation by BG in ATPase assay (concentration of BG)*** (Zhang et al., 2007a)***	***Transport in transfected MDCKII cell modell (Li et al., 2012))***
BCRP	*3.41 ± 1.83*	*22.22 ± 2.07 (1 μM)*	*+ (20 μM)*	*Not available*
MDR1	*94.84 ± 31.10*	*Not significant (100 μM)*	*- (100 μM)*	*Not significant*
MRP2	*210.13 ± 110.49*	*64.17 ± 17.60 (100 μM)*	*+ (100 μM)*	*+ (2.17 μM)*
MRP3	*14.01 ± 2.51*	*65.89 ± 36.37 (10 μM)*	*+ (20 μM)*	*Not significant*
MRP4	*14.39 ± 5.69*	*5.09 ± 1.29 (10 μM)*	*Not available*	*Not available*

The only transport study published to date was a monolayer study using MDCKII transfectants (Li *et al.*, 2012). It was shown that MRP2 but not MDR1 transported BG. Interestingly MRP3 did not transport BG either in that study. As MRP3 is known to be a general transporter of phytoestrogen glucuronides (van de Wetering *et al.*, 2009) and BG activated the MRP3 ATPase (Zhang *et al.*, 2007a), the lack of transport in MDCKII-MRP3 cells must be due to inadequate uptake of BG or low activity of MRP3 in that cell line. Importantly, significantly greater basolateral than apical transport of BG was observed in Caco-2 cells upon treatment with B (Zhang *et al.*, 2007a). Also, using targeted metabolomics phytoestrogen glucuronides were found as major Mrp3 substrates in mice (van de Wetering *et al.*, 2009).

MRP4 is probably expressed on the basolateral side of enterocytes (Ming *et al.*, 2010). It is probable that after passive diffusion of B into the enterocytes, an intensive glucuronidation to BG takes place in the small intestinal wall (Zhang *et al.*, 2005a). BG will be effluxed to the mesenteric blood mainly by MRP3 and MRP4, located on the basolateral side. Part of BG is pumped back to the intestinal lumen by apically located MRP2 and BCRP. Similarly, MRP2 and BCRP are the major efflux transporters responsible for the biliary efflux of BG and MRP3 and MRP4 are responsible for the sinusoidal efflux of BG.

BG is a low passive permeability compound (Xu *et al.*, 2013) and cellular uptake may require an uptake transporter. BG was shown to inhibit OATP2B1 (Zhang *et al.*, 2011b), an uptake transporter expressed in the apical membranes of enterocytes (Oswald *et al.*, 2013) and sinusoidal membranes of hepatocytes (Schaefer *et al.*, 2012). If BG is an OATP2B1 substrate, this transporter can cooperate with MRP3 and MRP4 to enhance absorption of BG in the upper intestinal tract. Similarly, OATP2B1 and MRP2 or BCRP can mediate biliary excretion of BG.

6.3. Baicalin is a substrate of OATP2B1

Owing to sustained levels of BG in the systemic circulation as a result of absorption dynamics, enterohepatic recirculation of the BG may be considered as an important distribution phenomenon for BG. The extensive enterohepatic distribution has been confirmed after both oral and intravenous routes of dosing in rats (Xing *et al.*, 2005). Biliary excretion plays a major role in bringing the glucuronide and sulfate conjugates of B back to the small intestine where it undergoes hydrolytic cleavage through intestinal beta-glucuronidase (Liu *et al.*, 2010).

Because of its poor passive permeability, uptake transporters could be a key determinant in hepatobiliary excretion of BG.

In an interesting clinical study, the interaction potential of rosuvastatin, a substrate for hepatic uptake transporters OATP1B1, OATP1B3, OATP2B1 and OATP1A2, and BG, was investigated (Fan *et al.*, 2008). The long-term treatment of patients with BG resulted in a decrease in the exposure of rosuvastatin. Later, another study using using CHO-OATP1B1, CHO-OATP1B3 and MDCKII-OATP2B1 cells reported that 10 μM BG had inhibitory effect on the transport function of OATP2B1 and OATP1B3 by 83.7±15.4 % and 40.1±3.7% respectively, suggesting that BG might be substrate of both transporters. However, BG did not affect transport by OATP1B1. Moreover, probenecid, an OATP substrate, significantly reduced the biliary clearance of BG (Zhang *et al.*, 2011b). Another inhibitory study using HEK293-OATP1A2, HEK293-OATP1B1, HEK293-OATP1B3 and HEK293-OATP2B1 also showed inhibition of OATP1B3 and OATP2B1 transport by BG. BG did not affect transport by OATP1B1 and OATP1A2 (Xu *et al.*, 2013).

In a recent clinical study, after oral administration of B, total plasma concentrations of BG reached 6.77 μM. In that study, BG inhibited uptake by HEK293-OATP2B1 and HEK293-OATP1B3 cells with IC_{50} values of 5.6 ± 3.2 μM and 13.7 ± 3.6 μM respectively (Li *et al.*, 2014).

The purpose of the present study was to elucidate the role of OATP1B3 and OATP2B1 in the hepatic uptake of BG since BG inhibited transport by both transporters.

Accordingly, transport studies were carried out at 2 pharmacologically relevant concentrations (1μM and 10 μM) and 2 incubation time points (2 and 20 minutes), using MDCKII-OATP2B1 and HEK293-OATP1B3 cells to determine whether BG is a substrate of these transporters. BG exhibited an up to 23-fold greater, statistically highly significant uptake in OATP2B1 expressing cells compared to wild type MDCKII control cells. Meanwhile it was found that the magnitude of BG uptake was only 2-fold greater and statistically not significant, in OATP1B3-expressing cells compared to HEK293-Mock cells **(*Tables 11a and 11b*).**

Further experiments were carried out to elucidate time dependence of BG uptake by OATP2B1. This transport exhibited linear kinetics at time points up to 10 minutes (***Figure 12***).

Concentration dependence of OATP2B1-mediated uptake of BG was assessed at 1-100 μM, with an incubation time of 3 minutes (***Figure 13***).

BG transport by OATP2B1 was saturable with average K_m, V_{max} and intrinsinc clearance (Cl_{int}) values of 9.71 ± 3.56 μM, 1575 ± 674.58 pmol/mg protein min and 162.13 μl/min mg protein respectively.

According to these results, it can be concluded that OATP2B1 is the major transporter responsible for the uptake of BG into hepatocytes.

The liver is regarded as the most important organ for the disposition of various endogenous and exogenous substances in the body. Before hepatic metabolism and biliary excretion, drugs need to enter the hepatocytes first, either through passive diffusion or mediated by transporters. As for the hepatic disposition of glucuronide conjugate already existing in the circulation, the hepatic uptake of BG is critical due to its difficulty to traverse the basolateral cell membrane.

On the sinusoidal hepatocyte membrane, OATP1B1, OATP1B3 and OATP2B1 are major uptake transporters for exogenous anions. Among these transporters, OATP2B1 was found to be primarily responsible for the hepatic uptake of BG in the present study.

In summary, the current study examined mechanisms underlying the enterohepatic transport of BG. After its oral administration, BG will be hydrolyzed to B. After passive diffusion of B into the enterocytes, an intense glucuronidation to BG takes place in the small intestinal wall. OATP2B1 cooperates with MRP3 and MRP4 to enhance absorption of BG in the upper intestinal tract. The circulating BG would be expected to re-enter the gastrointestinal tract via the biliary excretion mechanism. In the liver, OATP2B1 mediates hepatic uptake from the blood, and MRP2 or BCRP mediate biliary excretion, whereas MRP3 and MRP4 are partly responsible for the sinusoidal efflux of BG back to the blood (***Figures 14 and 15***).

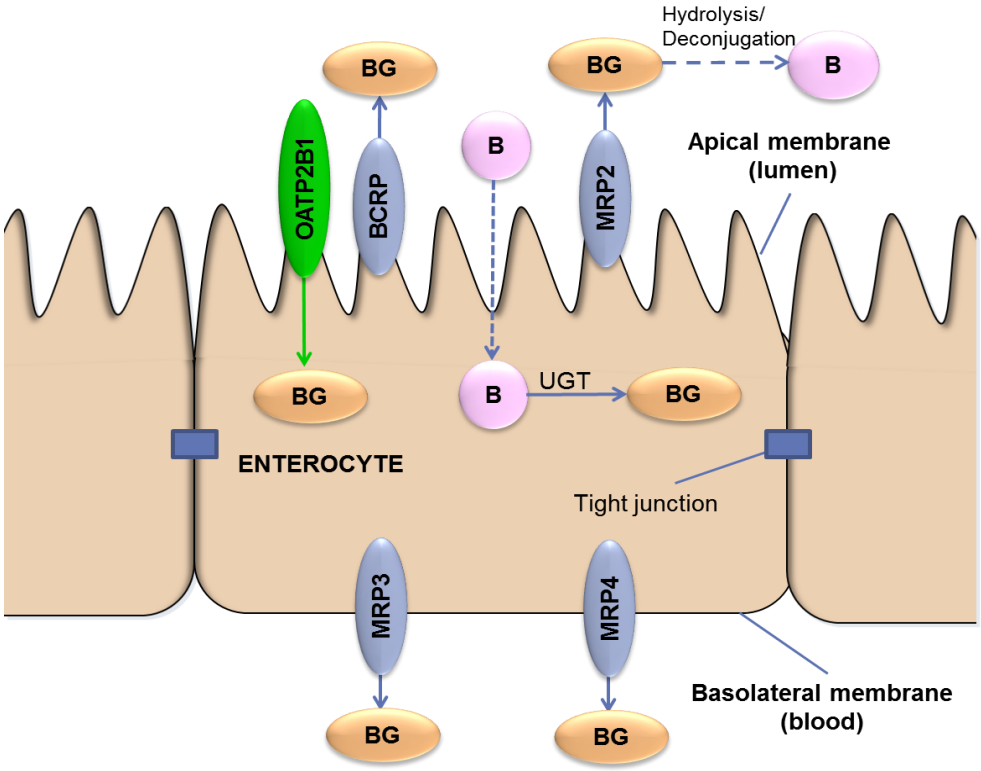

Figure 14*: Role of active and efflux transporters in transport of BG in enterocytes (edited by Kalapos-Kovács Bernadett)*

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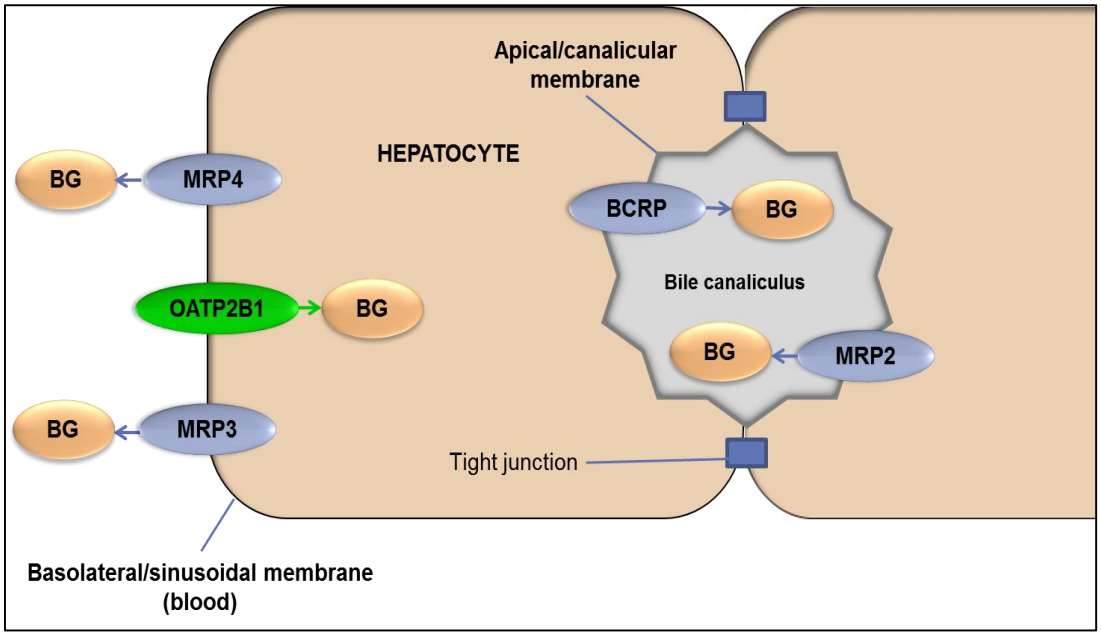

***Figure 15**: Role of active and efflux transporters in hepatic transport of BG (edited by Kalapos-Kovács Bernadett)*

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7. Conclusion

BG is a promising agent that has potential utility in the management of several diseases either given alone or by a combined use with another agent. The use of BG has been on the rise due its interesting pharmacological properties as evidenced by the scores of in vitro, preclinical and/or clinical data. Since BG and B have such enormous therapeutic potentials, a better understanding of their pharmacokinetics and bioavailability is necessary to specify clinical effects, developing clinical regimens and elucidating potential drug interactions.

Upon oral intake, BG is either directly absorbed from the upper intestinal tract or undergoes hydrolysis by intestinal glucuronidases or intestinal microflora to release its aglycone B, which will then be absorbed via passive diffusion. Concomitantly upon oral intake of B, B is absorbed via passive diffusion. It is probable that after passive diffusion of B into the enterocytes, an intensive glucuronidation to BG takes place in the small intestinal wall.

The present thesis was designed to identify the transporters responsible for the efflux and uptake of BG in enterocytes and hepatocytes and to explore potential drug-herb interactions.

We have shown that BG is a substrate of efflux transporters MRP2, MRP3, MRP4 and BCRP and substrate of uptake transporter OATP2B1. BG inhibited transport by MDR1, MRP2, MRP3, MRP4 and BCRP at a physiologically relevant concentration.

From our results, it can be concluded that BG is effluxed from enterocytes to the mesenteric blood mainly by MRP3 and MRP4, located on the basolateral side. OATP2B1 cooperates with MRP3 and MRP4 to enhance absorption of BG in the upper intestinal tract. Part of BG is pumped back to the intestinal lumen by apically located MRP2 and BCRP. The circulating BG would be expected to re-enter the gastrointestinal tract via the biliary excretion mechanism. In the liver, OATP2B1 mediates hepatic uptake from the blood, and MRP2 or BCRP mediate biliary excretion, whereas MRP3 and MRP4 are partly responsible for the sinusoidal efflux of BG back to the blood.

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By inhibiting efflux by ABC transporters, BG could interfere with the absorption and disposition of coadministered transporter substrate drugs, such as methotrexate, increasing the risk of therapeutic failure, adverse effects and toxicity.

This complex study has shown BG and B have such enormous therapeutic potentials, a better understanding of their pharmacokinetics and bioavailability is necessary to specify clinical effects, developing clinical regimens and elucidating potential drug interactions.

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8. Summary

A number of mechanistic studies were performed to understand the absorption, distribution, metabolism, and excretion profiles of baicalin, an emerging phytomedicine with various pharmacological activities. Baicalein, the aglycone formed by hydrolysis of baicalin in the intestine, is well absorbed by passive diffusion but subjected to extensive intestinal glucuronidation. Transport of baicalin, the low passive permeability glucuronide of baicalein from enterocytes likely depends on a carrier-mediated transport, since baicalin is found in the circulation. The circulating baicalin would be expected to re-enter the gastrointestinal tract via the biliary excretion mechanism, suggesting a potential role of uptake transporters in the hepatic uptake of baicalin in the liver. The present study was designed to explore potential drug-herb interaction by investigating the inhibitory effect of baicalin on the transport of reporter substrates by efflux transporters and to identify the transporters responsible for the uptake and efflux of baicalin from enterocytes and hepatocytes. The interaction of baicalin with specific ABC transporters, believed to play a role in the disposition and bioavailability of baicalin, was studied using membrane vesicles from cells overexpressing the transporters of interest. The inhibitory effect on transport and direct transport of baicalin by selected efflux transporters on these membranes was investigated. In addition, by using mammalian cells stably expressing the hepatic uptake transporters of interest, hepatic uptake of baicalin was also studied.It is probable that after passive diffusion of B into the enterocytes, an intensive glucuronidation to BG takes place in the small intestinal wall. BG will be effluxed to the mesenteric blood mainly by MRP3 and MRP4, located on the basolateral side. OATP2B1 cooperates with MRP3 and MRP4 to enhance absorption of BG in the upper intestinal tract. Part of BG will be pumped back to the intestinal lumen by apically located MRP2 and BCRP. The circulating BG would be expected to re-enter the gastrointestinal tract via the biliary excretion mechanism. In the liver, OATP2B1 mediates hepatic uptake from the blood, and MRP2 or BCRP mediate biliary excretion, whereas MRP3 and MRP4 are partly responsible for the sinusoidal efflux of BG back to the blood. Moreover, via interaction with transporters at a physiologically relevant concentration, baicalin could interfere with the absorption and disposition of drugs, e.g. methotrexate, increasing the risk of therapeutic failure, adverse effects and toxicity.

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9. Összefoglalás

A baikalin egy olyan flavonoid, melynek számos farmakológiai, pl. daganatellenes, gyulladásgátló, antioxidáns, antivirális, neuroprotektív és antiallergén hatása ismert. A baikalin a bélben hidrolizálódik és aglikonja jól abszorbeál passzív diffúzióval, de a bélhámsejtben visszaalakul baikalinná. Mivel az így kialakult baikalin nem abszorbál passzív diffúzióval, viszont a vérkeringésben megjelenik, feltételezhető, hogy a bélhámsejtből történő transzportjában fontos szerepet játszanak az efflux transzporterek. A felszívódott baikalin az epével ürül a gasztrointesztinális traktusba, amelynek feltétele a májba történő bejutás. Így valószínűthető, hogy a hepatikus felvételben uptake transzporterek, míg az epébe történő ürülésben az efflux transzporterek játszanak fontos szerepet. Célunk a baikalin enterohepatikus transzportjának feltérképezése, valamint a baikalin efflux transzporter szubsztrátokkal való kölcsönhatásának vizsgálata volt.

Vizsgálataink során gátlás illetve direkt vezikuláris transzport tesztet végeztünk kiválasztott efflux transzporterekkel overexpresszált sejtekből kinyert membrán vezikulumokon. Továbbá uptake transzporterekkel overexpresszált sejteken vizsgáltuk, hogy mely uptake transzporter felel a baikalin májba történő felvételéért.

Valószínűsíthető, hogy a bélhámsejben kialakuló baikalin kiáramláért a bél lumenbe az apikális oldalon az MRP2 és BCRP felel, míg az OATP2B1 együttműködik az MRP3 és MRP4 el a baikalin vérbe történő felszívódásáért. A felszívódott baikalint az OATP2B1 juttatja be a májba, az MRP2 és BCRP pedig az epe felé ürítik, miközben az MRP3 és MRP4 visszapumpálják a baikalint a véráramba. Fontos megemlíteni, hogy a baikalint gyakran alkalmazzák egyéb gyógyszerekkel együtt is, amely esetekben a transzportereknél kölcsönhatás léphet fel a flavonoid és a gyógyszermolekula között, modulálva ez utóbbi ADME tulajdonságait. Ezeket a hatásokat fontos figyelembe venni a gyógyszeres terápia megtervezésénél.

10. Bibliography

Abe K, Inoue O Yumioka E. (1990) Biliary excretion of metabolites of baicalin and baicalein in rats. Chem Pharm Bull, 38**:** 209-211.

Akao T, Hanada M, Sakashita Y, Sato K, Morita M Imanaka T. (2007) Efflux of baicalin, a flavone glucuronide of Scutellariae, Radix, on Caco-2 cells through multidrug resistance-associated protein 2. Journal of Pharmacy and Pharmacology, 59**:** 87-93.

Akao T, Kawabata K, Yanagisawa E, Ishihara K, Mizuhara Y, Wakui Y, Sakashita Y Kobashi K. (2000) Baicalin, the predominant flavone glucuronide of scutellariae radix, is absorbed from the rat gastrointestinal tract as the aglycone and restored to its original form. J Pharm Pharmacol, 52**:** 1563-1568.

Akao T, Sakashita Y, Hanada M, Goto H, Shimada Y Terasawa K. (2004) Enteric excretion of baicalein, a flavone of Scutellariae Radix, via glucuronidation in rat: involvement of multidrug resistance-associated protein 2. Pharm Res, 21**:** 2120-2126.

Akao T, Sato K Hanada M. (2009) Hepatic contribution to a marked increase in the plasma concentration of baicalin after oral administration of its aglycone, baicalein, in multidrug resistance-associated protein 2-deficient rat. Biol Pharm Bull, 32**:** 2079-2082.

Akao T, Sato K, He JX, Ma CM Hattori M. (2013) Baicalein 6-O-beta-D-glucopyranuronoside is a main metabolite in the plasma after oral administration of baicalin, a flavone glucuronide of scutellariae radix, to rats. Biol Pharm Bull, 36**:** 748-753.

Anthwal A, Singh K, Rawat MS, Tyagi AK, Haque A, Ali I Rawat DS. (2016) Synthesis of 4-piperidone Based Curcuminoids with Anti-inflammatory and Anti-Proliferation Potential in Human Cancer Cell Lines. Anticancer Agents Med Chem, 16**:** 841-851.

Awad R, Arnason JT, Trudeau V, Bergeron C, Budzinski JW, Foster BC Merali Z. (2003) Phytochemical and biological analysis of skullcap (Scutellaria lateriflora L.): a medicinal plant with anxiolytic properties. Phytomedicine, 10**:** 640-649.

Bailey DG, Arnold JM, Munoz C Spence JD. (1993) Grapefruit juice--felodipine interaction: mechanism, predictability, and effect of naringin. Clin Pharmacol Ther, 53**:** 637-642.

Bailey DG, Spence JD, Edgar B, Bayliff CD Arnold JM. (1989) Ethanol enhances the hemodynamic effects of felodipine. Clin Invest Med, 12**:** 357-362.

Barrington RD, Needs PW, Williamson G Kroon PA. (2015) MK571 inhibits phase-2 conjugation of flavonols by Caco-2/TC7 cells, but does not specifically inhibit their apical efflux. Biochem Pharmacol, 95**:** 193-200.

Blach-Olszewska Z, Jatczak B, Rak A, Lorenc M, Gulanowski B, Drobna A Lamer-Zarawska E. (2008) Production of cytokines and stimulation of resistance to viral infection in human leukocytes by Scutellaria baicalensis flavones. J Interferon Cytokine Res, 28**:** 571-581.

Bodo A, Bakos E, Szeri F, Varadi A Sarkadi B. (2003) Differential modulation of the human liver conjugate transporters MRP2 and MRP3 by bile acids and organic anions. J Biol Chem, 278**:** 23529-23537.

Borst P, Evers R, Kool M Wijnholds J. (2000) A family of drug transporters: the multidrug resistance-associated proteins. J Natl Cancer Inst, 92**:** 1295-1302.

Brand W, Schutte ME, Williamson G, van Zanden JJ, Cnubben NH, Groten JP, van Bladeren PJ Rietjens IM. (2006) Flavonoid-mediated inhibition of intestinal ABC transporters may affect the oral bioavailability of drugs, food-borne toxic compounds and bioactive ingredients. Biomed Pharmacother, 60**:** 508-519.

Brekhman, II, Grinevitch MA Kyu KB. (1981) Oriental medicine: a computerized study of complex recipes and their components: herbs most frequently used in traditional Japanese and Korean medicine. Am J Chin Med, 9**:** 134-143.

Cai Y, Li S, Li T, Zhou R, Wai AT Yan R. (2016) Oral pharmacokinetics of baicalin, wogonoside, oroxylin A 7-O-beta-d-glucuronide and their aglycones from an aqueous extract of Scutellariae Radix in the rat. J Chromatogr B Analyt Technol Biomed Life Sci, 1026**:** 124-133.

Cao F, Zhang H, Guo J Ping Q. (2008) Mrp2-related efflux of scutellarin in the intestinal absorption in rats. Pharmazie, 63**:** 75-80.

Chandra P Brouwer KL. (2004) The complexities of hepatic drug transport: current knowledge and emerging concepts. Pharm Res, 21**:** 719-735.

Chao JI, Su WC Liu HF. (2007) Baicalein induces cancer cell death and proliferation retardation by the inhibition of CDC2 kinase and survivin associated with opposite role of p38 mitogen-activated protein kinase and AKT. Mol Cancer Ther, 6**:** 3039-3048.

Chen CH, Huang LL, Huang CC, Lin CC, Lee Y Lu FJ. (2000) Baicalein, a novel apoptotic agent for hepatoma cell lines: a potential medicine for hepatoma. Nutr Cancer, 38**:** 287-295.

Chen H, Gao Y, Wu J, Chen Y, Chen B, Hu J Zhou J. (2014) Exploring therapeutic potentials of baicalin and its aglycone baicalein for hematological malignancies. Cancer Lett, 354**:** 5-11.

Chen XW, Serag ES, Sneed KB, Liang J, Chew H, Pan SY Zhou SF. (2011) Clinical herbal interactions with conventional drugs: from molecules to maladies. Curr Med Chem, 18**:** 4836-4850.

Chiu YW, Lin TH, Huang WS, Teng CY, Liou YS, Kuo WH, Lin WL, Huang HI, Tung JN, Huang CY, Liu JY, Wang WH, Hwang JM Kuo HC. (2011) Baicalein inhibits the

migration and invasive properties of human hepatoma cells. Toxicol Appl Pharmacol, 255**:** 316-326.

Choi YH Yu AM. (2014) ABC transporters in multidrug resistance and pharmacokinetics, and strategies for drug development. Curr Pharm Des, 20**:** 793-807.

Chu M, Xu L, Zhang MB, Chu ZY Wang YD. (2015) Role of Baicalin in Anti-Influenza Virus A as a Potent Inducer of IFN-Gamma. Biomed Res Int, 2015**:** 263630.

Conseil G, Baubichon-Cortay H, Dayan G, Jault JM, Barron D Di Pietro A. (1998) Flavonoids: a class of modulators with bifunctional interactions at vicinal ATP- and steroid-binding sites on mouse P-glycoprotein. Proc Natl Acad Sci U S A, 95**:** 9831-9836.

Cooray HC, Janvilisri T, van Veen HW, Hladky SB Barrand MA. (2004) Interaction of the breast cancer resistance protein with plant polyphenols. Biochem Biophys Res Commun, 317**:** 269-275.

CP. (2005) The Pharmacopeia Commission of P. R. China. Pharmacopeia of the People's Republic of China, 1**:** 211.

Crowley E, McDevitt CA Callaghan R. (2010) Generating inhibitors of P-glycoprotein: where to, now? Methods Mol Biol, 596**:** 405-432.

Dai J-Y, Yang J-l Li C. (2008) Transport and metabolism of flavonoids from Chinese herbal remedy Xiaochaihu-tang across human intestinal Caco-2 cell monolayers. Acta Pharmacologica Sinica, 29**:** 1086-1093.

de Oliveira MR, Nabavi SF, Habtemariam S, Erdogan Orhan I, Daglia M Nabavi SM. (2015) The effects of baicalein and baicalin on mitochondrial function and dynamics: A review. Pharmacol Res, 100**:** 296-308.

Dean M, Rzhetsky A Allikmets R. (2001) The human ATP-binding cassette (ABC) transporter superfamily. Genome Res, 11**:** 1156-1166.

Ding XM, Pan L, Wang Y Xu QZ. (2016) Baicalin exerts protective effects against lipopolysaccharide-induced acute lung injury by regulating the crosstalk between the CX3CL1-CX3CR1 axis and NF-kappaB pathway in CX3CL1-knockout mice. Int J Mol Med, 37**:** 703-715.

Doige CA Sharom FJ. (1992) Transport properties of P-glycoprotein in plasma membrane vesicles from multidrug-resistant Chinese hamster ovary cells. Biochim Biophys Acta, 1109**:** 161-171.

Dou YQ, Xue Y, Chen HZ Zhao ML. (2007) [Study on anti-endotoxin of baicalin]. Hua Xi Kou Qiang Yi Xue Za Zhi, 25**:** 169-172.

Dreiseitel A, Oosterhuis B, Vukman KV, Schreier P, Oehme A, Locher S, Hajak G Sand PG. (2009) Berry anthocyanins and anthocyanidins exhibit distinct affinities for the efflux transporters BCRP and MDR1. Br J Pharmacol, 158**:** 1942-1950.

Du G, Han G, Zhang S, Lin H, Wu X, Wang M, Ji L, Lu L, Yu L Liang W. (2010) Baicalin suppresses lung carcinoma and lung metastasis by SOD mimic and HIF-1alpha inhibition. Eur J Pharmacol, 630**:** 121-130.

Ellens H, Deng S, Coleman J, Bentz J, Taub ME, Ragueneau-Majlessi I, Chung SP, Heredi-Szabo K, Neuhoff S, Palm J, Balimane P, Zhang L, Jamei M, Hanna I, O'Connor M, Bednarczyk D, Forsgard M, Chu X, Funk C, Guo A, Hillgren KM, Li L, Pak AY, Perloff ES, Rajaraman G, Salphati L, Taur JS, Weitz D, Wortelboer HM, Xia CQ, Xiao G, Yamagata T Lee CA. (2013) Application of receiver operating characteristic analysis to refine the prediction of potential digoxin drug interactions. Drug Metab Dispos, 41**:** 1367-1374.

Fan L, Zhang W, Guo D, Tan ZR, Xu P, Li Q, Liu YZ, Zhang L, He TY, Hu DL, Wang D Zhou HH. (2008) The effect of herbal medicine baicalin on pharmacokinetics of rosuvastatin, substrate of organic anion-transporting polypeptide 1B1. Clinical Pharmacology & Therapeutics, 83**:** 471-476.

Fasinu PS, Bouic PJ Rosenkranz B. (2012) An overview of the evidence and mechanisms of herb-drug interactions. Front Pharmacol, 3**:** 69.

FDA. (2012) Drug Interaction Studies —Study Design, Data Analysis, Implications for Dosing, and Labeling; Recommendations.

Fekete Z, Rajnai Z, Nagy T, Jakab KT, Kurunczi A, Gemes K, Heredi-Szabo K, Fulop F, Toth GK, Czerwinski M, Loewen G Krajcsi P. (2015) Membrane Assays to Characterize Interaction of Drugs with ABCB1. J Membr Biol, 248**:** 967-977.

Feng B, LaPerle JL, Chang G Varma MV. (2010) Renal clearance in drug discovery and development: molecular descriptors, drug transporters and disease state. Expert Opin Drug Metab Toxicol, 6**:** 939-952.

Fong SY, Wong YC, Xie C Zuo Z. (2015) Herb-drug interactions between Scutellariae Radix and mefenamic acid: Simultaneous investigation of pharmacokinetics, anti-inflammatory effect and gastric damage in rats. J Ethnopharmacol, 170**:** 106-116.

Gabrielska J, Oszmianski J, Zylka R Komorowska M. (1997) Antioxidant activity of flavones from Scutellaria baicalensis in lecithin liposomes. Z Naturforsch C, 52**:** 817-823.

Gaire BP, Moon SK Kim H. (2014) Scutellaria baicalensis in stroke management: nature's blessing in traditional Eastern medicine. Chin J Integr Med, 20**:** 712-720.

Gao C, Chen X Zhong D. (2011a) Absorption and disposition of scutellarin in rats: a pharmacokinetic explanation for the high exposure of its isomeric metabolite. Drug Metab Dispos, 39**:** 2034-2044.

Gao C, Zhang H, Guo Z, You T, Chen X Zhong D. (2012) Mechanistic studies on the absorption and disposition of scutellarin in humans: selective OATP2B1-mediated hepatic uptake is a likely key determinant for its unique pharmacokinetic characteristics. Drug Metab Dispos, 40**:** 2009-2020.

Gao J, Morgan WA, Sanchez-Medina A Corcoran O. (2011b) The ethanol extract of Scutellaria baicalensis and the active compounds induce cell cycle arrest and apoptosis including upregulation of p53 and Bax in human lung cancer cells. Toxicol Appl Pharmacol, 254**:** 221-228.

Gao L, Duan DD, Zhang JQ, Zhou YZ, Qin XM Du GH. (2016) A Bioinformatic Approach for the Discovery of Antiaging Effects of Baicalein from Scutellaria baicalensis Georgi. Rejuvenation Res, 10.1089/rej.2015.1760DOI: 10.1089/rej.2015.1760.

Giacomini KM, Huang SM, Tweedie DJ, Benet LZ, Brouwer KL, Chu X, Dahlin A, Evers R, Fischer V, Hillgren KM, Hoffmaster KA, Ishikawa T, Keppler D, Kim RB, Lee CA, Niemi M, Polli JW, Sugiyama Y, Swaan PW, Ware JA, Wright SH, Yee SW, Zamek-Gliszczynski MJ Zhang L. (2010) Membrane transporters in drug development. Nat Rev Drug Discov, 9**:** 215-236.

Gillet JP Gottesman MM. (2011) Advances in the molecular detection of ABC transporters involved in multidrug resistance in cancer. Curr Pharm Biotechnol, 12**:** 686-692.

Glavinas H, Krajcsi P, Cserepes J Sarkadi B. (2004) The role of ABC transporters in drug resistance, metabolism and toxicity. Curr Drug Deliv, 1**:** 27-42.

Glavinas H, Mehn D, Jani M, Oosterhuis B, Heredi-Szabo K Krajcsi P. (2008) Utilization of membrane vesicle preparations to study drug-ABC transporter interactions. Expert Opin Drug Metab Toxicol, 4**:** 721-732.

Gong X Sucher NJ. (2002) Stroke therapy in traditional Chinese medicine (TCM): prospects for drug discovery and development. Phytomedicine, 9**:** 478-484.

Heredi-Szabo K, Kis E Krajcsi P. (2012) The vesicular transport assay: validated in vitro methods to study drug-mediated inhibition of canalicular efflux transporters ABCB11/BSEP and ABCC2/MRP2. Curr Protoc Toxicol, Chapter 23**:** Unit 23 24.

Heredi-Szabo K, Palm JE, Andersson TB, Pal A, Mehn D, Fekete Z, Beery E, Jakab KT, Jani M Krajcsi P. (2013) A P-gp vesicular transport inhibition assay - optimization and validation for drug-drug interaction testing. Eur J Pharm Sci, 49**:** 773-781.

Horio M, Gottesman MM Pastan I. (1988) ATP-dependent transport of vinblastine in vesicles from human multidrug-resistant cells. Proc Natl Acad Sci U S A, 85**:** 3580-3584.

Hou YC, Lin SP, Tsai SY, Ko MH, Chang YC Chao PD. (2011) Flavonoid pharmacokinetics and tissue distribution after repeated dosing of the roots of Scutellaria baicalensis in rats. Planta Med, 77**:** 455-460.

Hu Q, Noor M, Wong YF, Hylands PJ, Simmonds MS, Xu Q, Jiang D, Hendry BM Xu Q. (2009) In vitro anti-fibrotic activities of herbal compounds and herbs. Nephrol Dial Transplant, 24**:** 3033-3041.

Hu Z, Yang X, Ho PC, Chan SY, Heng PW, Chan E, Duan W, Koh HL Zhou S. (2005) Herb-drug interactions: a literature review. Drugs, 65**:** 1239-1282.

Jani M, Ambrus C, Magnan R, Jakab KT, Beery E, Zolnerciks JK Krajcsi P. (2014a) Structure and function of BCRP, a broad specificity transporter of xenobiotics and endobiotics. Arch Toxicol, 88**:** 1205-1248.

Jani M Krajcsi P. (2014b) In vitro methods in drug transporter interaction assessment. Drug Discov Today Technol, 12**:** e105-112.

Jiang RH, Su WC, Liu HF, Huang HS Chao JI. (2010) Opposite expression of securin and gamma-H2AX regulates baicalein-induced cancer cell death. J Cell Biochem, 111**:** 274-283.

Jung HS, Kim MH, Gwak NG, Im YS, Lee KY, Sohn Y, Choi H Yang WM. (2012) Antiallergic effects of Scutellaria baicalensis on inflammation in vivo and in vitro. J Ethnopharmacol, 141**:** 345-349.

Kalapos-Kovacs B, Magda B, Jani M, Fekete Z, Szabo PT, Antal I, Krajcsi P Klebovich I. (2015) Multiple ABC Transporters Efflux Baicalin. Phytother Res, 29**:** 1987-1990.

Kang MJ, Ko GS, Oh do G, Kim JS, Noh K, Kang W, Yoon WK, Kim HC, Jeong HG Jeong TC. (2014) Role of metabolism by intestinal microbiota in pharmacokinetics of oral baicalin. Arch Pharm Res, 37**:** 371-378.

Kantamreddi VS, Parida S, Kommula SM Wright CW. (2009) Phytotherapy used in Orissa state, India for treating malaria. Phytother Res, 23**:** 1638-1641.

Kennedy DA Seely D. (2010) Clinically based evidence of drug-herb interactions: a systematic review. Expert Opin Drug Saf, 9**:** 79-124.

Kim H. (2005) Neuroprotective herbs for stroke therapy in traditional eastern medicine. Neurol Res, 27**:** 287-301.

Kim SJ Lee SM. (2012) Effect of baicalin on toll-like receptor 4-mediated ischemia/reperfusion inflammatory responses in alcoholic fatty liver condition. Toxicol Appl Pharmacol, 258**:** 43-50.

Kis E, Nagy T, Jani M, Molnar E, Janossy J, Ujhellyi O, Nemet K, Heredi-Szabo K Krajcsi P. (2009) Leflunomide and its metabolite A771726 are high affinity substrates of BCRP: implications for drug resistance. Ann Rheum Dis, 68**:** 1201-1207.

Klukovits A Krajcsi P. (2015) Mechanisms and therapeutic potential of inhibiting drug efflux transporters. Expert Opin Drug Metab Toxicol, 11**:** 907-920.

Kumagai T, Muller CI, Desmond JC, Imai Y, Heber D Koeffler HP. (2007) Scutellaria baicalensis, a herbal medicine: anti-proliferative and apoptotic activity against acute lymphocytic leukemia, lymphoma and myeloma cell lines. Leuk Res, 31**:** 523-530.

Lai MY, Hsiu SL, Tsai SY, Hou YC Chao PD. (2003) Comparison of metabolic pharmacokinetics of baicalin and baicalein in rats. J Pharm Pharmacol, 55**:** 205-209.

Laki S, Kalapos-Kovacs B, Antal I Klebovich I. (2013) [Importance of drug interactions with smoking in modern drug research]. Acta Pharm Hung, 83**:** 107-120.

Li-Weber M. (2009) New therapeutic aspects of flavones: the anticancer properties of Scutellaria and its main active constituents Wogonin, Baicalein and Baicalin. Cancer Treat Rev, 35**:** 57-68.

Li C, Lin G Zuo Z. (2011a) Pharmacological effects and pharmacokinetics properties of Radix Scutellariae and its bioactive flavones. Biopharm Drug Dispos, 32**:** 427-445.

Li C, Zhou L, Lin G Zuo Z. (2009) Contents of major bioactive flavones in proprietary traditional Chinese medicine products and reference herb of radix Scutellariae. J Pharm Biomed Anal, 50**:** 298-306.

Li CR, Zhang L, Zhou LM, Wo SK, Lin G Zuo Z. (2012) Comparison of Intestinal Absorption and Disposition of Structurally Similar Bioactive Flavones in Radix Scutellariae. Aaps Journal, 14**:** 23-34.

Li M, Shi A, Pang H, Xue W, Li Y, Cao G, Yan B, Dong F, Li K, Xiao W, He G, Du G Hu X. (2014) Safety, tolerability, and pharmacokinetics of a single ascending dose of baicalein chewable tablets in healthy subjects. J Ethnopharmacol, 156**:** 210-215.

Li N, Je YJ, Yang M, Jiang XH Ma JH. (2011b) Pharmacokinetics of baicalin-phospholipid complex in rat plasma and brain tissues after intranasal and intravenous administration. Pharmazie, 66**:** 374-377.

Li Y Paxton JW. (2013) The effects of flavonoids on the ABC transporters: consequences for the pharmacokinetics of substrate drugs. Expert Opin Drug Metab Toxicol, 9**:** 267-285.

Liu L, Deng Y-X, Liang Y, Pang X-Y, Liu X-D, Liu Y-W, Yang J-S, Xie L Wang G-J. (2010) Increased Oral AUC of Baicalin in Streptozotocin-Induced Diabetic Rats due to the Increased Activity of Intestinal beta-Glucuronidase. Planta Medica, 76**:** 70-75.

Liu Z Hu M. (2007) Natural polyphenol disposition via coupled metabolic pathways. Expert Opin Drug Metab Toxicol, 3**:** 389-406.

Liu Z, Zhang X, Wu H, Li J, Shu L, Liu R, Li L Li N. (2011) Preparation and evaluation of solid lipid nanoparticles of baicalin for ocular drug delivery system in vitro and in vivo. Drug Dev Ind Pharm, 37**:** 475-481.

Lu T, Song J, Huang F, Deng Y, Xie L, Wang G Liu X. (2007) Comparative pharmacokinetics of baicalin after oral administration of pure baicalin, Radix scutellariae extract and Huang-Lian-Jie-Du-Tang to rats. J Ethnopharmacol, 110**:** 412-418.

Luo XQ Yang JQ. (2010) [Prescription design and dissolution evaluation of self-emulsifying drug delivery systems of baicalin]. Zhong Yao Cai, 33**:** 1157-1159.

Magda B, Marta Z, Imre T, Kalapos-Kovacs B, Klebovich I, Fekete J Szabo PT. (2015) Unexpected retention behavior of baicalin: Hydrophilic interaction like properties of a reversed-phase column. J Pharm Biomed Anal, 111**:** 119-125.

Miao Q, Wang Z, Zhang Y, Miao P, Zhao Y, Zhang Y Ma S. (2016) In vitro potential modulation of baicalin and baicalein on P-glycoprotein activity and expression in Caco-2 cells and rat gut sacs. Pharm Biol, 10.3109/13880209.2015.11077441-9.

Ming X Thakker DR. (2010) Role of basolateral efflux transporter MRP4 in the intestinal absorption of the antiviral drug adefovir dipivoxil. Biochem Pharmacol, 79**:** 455-462.

Moghaddam E, Teoh BT, Sam SS, Lani R, Hassandarvish P, Chik Z, Yueh A, Abubakar S Zandi K. (2014) Baicalin, a metabolite of baicalein with antiviral activity against dengue virus. Sci Rep, 4**:** 5452.

Mohamed ME Frye RF. (2011) Effects of herbal supplements on drug glucuronidation. Review of clinical, animal, and in vitro studies. Planta Med, 77**:** 311-321.

Moon YJ, Wang X Morris ME. (2006) Dietary flavonoids: effects on xenobiotic and carcinogen metabolism. Toxicol In Vitro, 20**:** 187-210.

Mu J, Liu T, Jiang L, Wu X, Cao Y, Li M, Dong Q, Liu Y Xu H. (2016) The Traditional Chinese Medicine Baicalein Potently Inhibits Gastric Cancer Cells. J Cancer, 7**:** 453-461.

Mu X, He G, Cheng Y, Li X, Xu B Du G. (2009) Baicalein exerts neuroprotective effects in 6-hydroxydopamine-induced experimental parkinsonism in vivo and in vitro. Pharmacol Biochem Behav, 92**:** 642-648.

Muller F Fromm MF. (2011) Transporter-mediated drug-drug interactions. Pharmacogenomics, 12**:** 1017-1037.

Nabekura T, Kamiyama S Kitagawa S. (2005) Effects of dietary chemopreventive phytochemicals on P-glycoprotein function. Biochem Biophys Res Commun, 327**:** 866-870.

Nguyen MA, Staubach P, Tamai I Langguth P. (2015) High-dose short-term administration of naringin did not alter talinolol pharmacokinetics in humans. Eur J Pharm Sci, 68**:** 36-42.

Nobili S, Landini I, Giglioni B Mini E. (2006) Pharmacological strategies for overcoming multidrug resistance. Curr Drug Targets, 7**:** 861-879.

Noh K, Kang Y, Nepal MR, Jeong KS, Oh do G, Kang MJ, Lee S, Kang W, Jeong HG Jeong TC. (2016) Role of Intestinal Microbiota in Baicalin-Induced Drug Interaction and Its Pharmacokinetics. Molecules, 21**:** 337.

Oga EF, Sekine S, Shitara Y Horie T. (2012) P-glycoprotein mediated efflux in Caco-2 cell monolayers: the influence of herbals on digoxin transport. J Ethnopharmacol, 144**:** 612-617.

Oswald S, Groer C, Drozdzik M Siegmund W. (2013) Mass spectrometry-based targeted proteomics as a tool to elucidate the expression and function of intestinal drug transporters. AAPS J, 15**:** 1128-1140.

Pal A, Mehn D, Molnar E, Gedey S, Meszaros P, Nagy T, Glavinas H, Janaky T, von Richter O, Bathori G, Szente L Krajcsi P. (2007) Cholesterol potentiates ABCG2 activity in a heterologous expression system: improved in vitro model to study function of human ABCG2. J Pharmacol Exp Ther, 321**:** 1085-1094.

Pick A, Muller H, Mayer R, Haenisch B, Pajeva IK, Weigt M, Bonisch H, Muller CE Wiese M. (2011) Structure-activity relationships of flavonoids as inhibitors of breast cancer resistance protein (BCRP). Bioorg Med Chem, 19**:** 2090-2102.

Rajnarayana K, Reddy MS, Vidyasagar J Krishna DR. (2004) Study on the influence of silymarin pretreatment on metabolism and disposition of metronidazole. Arzneimittelforschung, 54**:** 109-113.

Roth M, Obaidat A Hagenbuch B. (2012) OATPs, OATs and OCTs: the organic anion and cation transporters of the SLCO and SLC22A gene superfamilies. Br J Pharmacol, 165**:** 1260-1287.

Sahebkar A. (2012) Baicalin as a potentially promising drug for the management of sulfur mustard induced cutaneous complications: a review of molecular mechanisms. Cutan Ocul Toxicol, 31**:** 226-234.

Sahi J. (2005) Use of in vitro transporter assays to understand hepatic and renal disposition of new drug candidates. Expert Opin Drug Metab Toxicol, 1**:** 409-427.

Sarkadi B, Price EM, Boucher RC, Germann UA Scarborough GA. (1992) Expression of the human multidrug resistance cDNA in insect cells generates a high activity drug-stimulated membrane ATPase. J Biol Chem, 267**:** 4854-4858.

Schaefer O, Ohtsuki S, Kawakami H, Inoue T, Liehner S, Saito A, Sakamoto A, Ishiguro N, Matsumaru T, Terasaki T Ebner T. (2012) Absolute quantification and differential expression of drug transporters, cytochrome P450 enzymes, and UDP-glucuronosyltransferases in cultured primary human hepatocytes. Drug Metab Dispos, 40**:** 93-103.

Sesink AL, Arts IC, de Boer VC, Breedveld P, Schellens JH, Hollman PC Russel FG. (2005) Breast cancer resistance protein (Bcrp1/Abcg2) limits net intestinal uptake of quercetin in rats by facilitating apical efflux of glucuronides. Mol Pharmacol, 67**:** 1999-2006.

Shang X, He X, He X, Li M, Zhang R, Fan P, Zhang Q Jia Z. (2010) The genus Scutellaria an ethnopharmacological and phytochemical review. J Ethnopharmacol, 128**:** 279-313.

Sithisarn P, Michaelis M, Schubert-Zsilavecz M Cinatl J, Jr. (2013) Differential antiviral and anti-inflammatory mechanisms of the flavonoids biochanin A and baicalein in H5N1 influenza A virus-infected cells. Antiviral Res, 97**:** 41-48.

Sjogren E, Abrahamsson B, Augustijns P, Becker D, Bolger MB, Brewster M, Brouwers J, Flanagan T, Harwood M, Heinen C, Holm R, Juretschke HP, Kubbinga M, Lindahl A, Lukacova V, Munster U, Neuhoff S, Nguyen MA, Peer A, Reppas C, Hodjegan AR, Tannergren C, Weitschies W, Wilson C, Zane P, Lennernas H Langguth P. (2014) In vivo methods for drug absorption - comparative physiologies, model selection, correlations with in vitro methods (IVIVC), and applications for formulation/API/excipient characterization including food effects. Eur J Pharm Sci, 57**:** 99-151.

Srinivas NR. (2010) Baicalin, an emerging multi-therapeutic agent: pharmacodynamics, pharmacokinetics, and considerations from drug development perspectives. Xenobiotica, 40**:** 357-367.

Stieger B, Fattinger K, Madon J, Kullak-Ublick GA Meier PJ. (2000) Drug- and estrogen-induced cholestasis through inhibition of the hepatocellular bile salt export pump (Bsep) of rat liver. Gastroenterology, 118**:** 422-430.

Suzuki H Sugiyama Y. (2000) Role of metabolic enzymes and efflux transporters in the absorption of drugs from the small intestine. Eur J Pharm Sci, 12**:** 3-12.

Taiming L Xuehua J. (2006) Investigation of the absorption mechanisms of baicalin and baicalein in rats. J Pharm Sci, 95**:** 1326-1333.

Takahashi H, Chen MC, Pham H, Angst E, King JC, Park J, Brovman EY, Ishiguro H, Harris DM, Reber HA, Hines OJ, Gukovskaya AS, Go VL Eibl G. (2011) Baicalein, a component of Scutellaria baicalensis, induces apoptosis by Mcl-1 down-regulation in human pancreatic cancer cells. Biochim Biophys Acta, 1813**:** 1465-1474.

Tamai I Tsuji A. (2000) Transporter-mediated permeation of drugs across the blood-brain barrier. J Pharm Sci, 89**:** 1371-1388.

Tan KW, Cooney J, Jensen D, Li Y, Paxton JW, Birch NP Scheepens A. (2014) Hop-derived prenylflavonoids are substrates and inhibitors of the efflux transporter breast cancer resistance protein (BCRP/ABCG2). Mol Nutr Food Res, 58**:** 2099-2110.

Tan KW, Li Y, Paxton JW, Birch NP Scheepens A. (2013) Identification of novel dietary phytochemicals inhibiting the efflux transporter breast cancer resistance protein (BCRP/ABCG2). Food Chem, 138**:** 2267-2274.

Teodori E, Dei S, Martelli C, Scapecchi S Gualtieri F. (2006) The functions and structure of ABC transporters: implications for the design of new inhibitors of Pgp and MRP1 to control multidrug resistance (MDR). Curr Drug Targets, 7**:** 893-909.

Tian XJ, Yang XW, Yang X Wang K. (2009) Studies of intestinal permeability of 36 flavonoids using Caco-2 cell monolayer model. Int J Pharm, 367**:** 58-64.

Tseng YP, Wu YC, Leu YL, Yeh SF Chou CK. (2010) Scutellariae radix suppresses hepatitis B virus production in human hepatoma cells. Front Biosci (Elite Ed), 2**:** 1538-1547.

Valdameri G, Pereira Rangel L, Spatafora C, Guitton J, Gauthier C, Arnaud O, Ferreira-Pereira A, Falson P, Winnischofer SM, Rocha ME, Tringali C Di Pietro A. (2012) Methoxy stilbenes as potent, specific, untransported, and noncytotoxic inhibitors of breast cancer resistance protein. ACS Chem Biol, 7**:** 322-330.

van de Wetering K, Feddema W, Helms JB, Brouwers JF Borst P. (2009) Targeted metabolomics identifies glucuronides of dietary phytoestrogens as a major class of MRP3 substrates in vivo. Gastroenterology, 137**:** 1725-1735.

Vasiliou V, Vasiliou K Nebert DW. (2009) Human ATP-binding cassette (ABC) transporter family. Hum Genomics, 3**:** 281-290.

Waisundara VY, Siu SY, Hsu A, Huang D Tan BK. (2011) Baicalin upregulates the genetic expression of antioxidant enzymes in Type-2 diabetic Goto-Kakizaki rats. Life Sci, 88**:** 1016-1025.

Wang CZ, Zhang CF, Chen L, Anderson S, Lu F Yuan CS. (2015) Colon cancer chemopreventive effects of baicalein, an active enteric microbiome metabolite from baicalin. Int J Oncol, 47**:** 1749-1758.

Wang Y, Yang J, Li X Wang J. (2012) The metabolism of baicalin in rat and the biological activities of the metabolites. Evid Based Complement Alternat Med, 2012**:** 404529.

Woo AY, Cheng CH Waye MM. (2005) Baicalein protects rat cardiomyocytes from hypoxia/reoxygenation damage via a prooxidant mechanism. Cardiovasc Res, 65**:** 244-253.

Wu B, Kulkarni K, Basu S, Zhang S Hu M. (2011a) First-pass metabolism via UDP-glucuronosyltransferase: a barrier to oral bioavailability of phenolics. J Pharm Sci, 100**:** 3655-3681.

Wu B, Li J, Huang D, Wang W, Chen Y, Liao Y, Tang X, Xie H Tang F. (2011b) Baicalein mediates inhibition of migration and invasiveness of skin carcinoma through Ezrin in A431 cells. BMC Cancer, 11**:** 527.

Wu H, Long X, Yuan F, Chen L, Pan S, Liu Y, Stowell Y Li X. (2014) Combined use of phospholipid complexes and self-emulsifying microemulsions for improving the oral absorption of a BCS class IV compound, baicalin. Acta Pharm Sin B, 4**:** 217-226.

Xing J, Chen XY Zhong DF. (2005) Absorption and enterohepatic circulation of baicalin in rats. Life Sciences, 78**:** 140-146.

Xing S, Wang M, Peng Y, Chen D Li X. (2014) Simulated gastrointestinal tract metabolism and pharmacological activities of water extract of Scutellaria baicalensis roots. Journal of Ethnopharmacology, 152**:** 183-189.

Xu F, Li Z, Zheng J, Gee Cheung FS, Chan T, Zhu L, Zhuge H Zhou F. (2013) The inhibitory effects of the bioactive components isolated from Scutellaria baicalensis on the cellular uptake mediated by the essential solute carrier transporters. J Pharm Sci, 102**:** 4205-4211.

Xu H, Kulkarni KH, Singh R, Yang Z, Wang SW, Tam VH Hu M. (2009) Disposition of naringenin via glucuronidation pathway is affected by compensating efflux transporters of hydrophilic glucuronides. Mol Pharm, 6**:** 1703-1715.

Xu J, Huang R, Yang YJ, Jin SJ Zhang JF. (2011) [Effects of baicalin on apoptosis in rats with autoimmune encephalomyelitis]. Zhongguo Dang Dai Er Ke Za Zhi, 13**:** 665-668.

Xue X, Liu H, Qi L, Li X, Guo C, Gong D Qu H. (2014) Baicalein ameliorated the upregulation of striatal glutamatergic transmission in the mice model of Parkinson's disease. Brain Res Bull, 103**:** 54-59.

Yang J, Yang X, Chu Y Li M. (2011) Identification of Baicalin as an immunoregulatory compound by controlling T(H)17 cell differentiation. PLoS One, 6**:** e17164.

Yu CP, Hsieh YC, Shia CS, Hsu PW, Chen JY, Hou YC Hsieh YW. (2016a) Increased Systemic Exposure of Methotrexate by a Polyphenol-Rich Herb via Modulation on Efflux Transporters Multidrug Resistance-Associated Protein 2 and Breast Cancer Resistance Protein. J Pharm Sci, 105**:** 343-349.

Yu HY, Yin ZJ, Yang SJ, Ma SP Qu R. (2016b) Baicalin Reverses Depressive-Like Behaviours and Regulates Apoptotic Signalling Induced by Olfactory Bulbectomy. Phytother Res, 30**:** 469-475.

Yu Y, Pei M Li L. (2015) Baicalin induces apoptosis in hepatic cancer cells in vitro and suppresses tumor growth in vivo. Int J Clin Exp Med, 8**:** 8958-8967.

Yuan J, Wong IL, Jiang T, Wang SW, Liu T, Wen BJ, Chow LM Wan Sheng B. (2012) Synthesis of methylated quercetin derivatives and their reversal activities on P-gp- and BCRP-mediated multidrug resistance tumour cells. Eur J Med Chem, 54**:** 413-422.

Zamek-Gliszczynski MJ, Hoffmaster KA, Humphreys JE, Tian X, Nezasa K Brouwer KL. (2006) Differential involvement of Mrp2 (Abcc2) and Bcrp (Abcg2) in biliary excretion of 4-methylumbelliferyl glucuronide and sulfate in the rat. J Pharmacol Exp Ther, 319**:** 459-467.

Zhang L, Li C, Lin G, Krajcsi P Zuo Z. (2011a) Hepatic metabolism and disposition of baicalein via the coupling of conjugation enzymes and transporters-in vitro and in vivo evidences. AAPS J, 13**:** 378-389.

Zhang L, Li CR, Lin G, Krajcsi P Zuo Z. (2011b) Hepatic Metabolism and Disposition of Baicalein via the Coupling of Conjugation Enzymes and Transporters-In Vitro and In Vivo Evidences. Aaps Journal, 13**:** 378-389.

Zhang L, Lin G, Chang Q Zuo Z. (2005a) Role of intestinal first-pass metabolism of baicalein in its absorption process. Pharmaceutical Research, 22**:** 1050-1058.

Zhang L, Lin G, Kovacs B, Jani M, Krajcsi P Zuo Z. (2007a) Mechanistic study on the intestinal absorption and disposition of baicalein. European Journal of Pharmaceutical Sciences, 31**:** 221-231.

Zhang L, Lin G Zuo Z. (2007b) Involvement of UDP-glucuronosyltransferases in the extensive liver and intestinal first-pass metabolism of flavonoid baicalein. Pharm Res, 24**:** 81-89.

Zhang S, Wang X, Sagawa K Morris ME. (2005b) Flavonoids chrysin and benzoflavone, potent breast cancer resistance protein inhibitors, have no significant effect on topotecan pharmacokinetics in rats or mdr1a/1b (-/-) mice. Drug Metab Dispos, 33**:** 341-348.

Zhang S, Yang X Morris ME. (2004) Combined effects of multiple flavonoids on breast cancer resistance protein (ABCG2)-mediated transport. Pharm Res, 21**:** 1263-1273.

Zhang XW, Li WF, Li WW, Ren KH, Fan CM, Chen YY Shen YL. (2011c) Protective effects of the aqueous extract of Scutellaria baicalensis against acrolein-induced oxidative stress in cultured human umbilical vein endothelial cells. Pharm Biol, 49**:** 256-261.

Zhang Y, Li X, Ciric B, Ma CG, Gran B, Rostami A Zhang GX. (2015) Therapeutic effect of baicalin on experimental autoimmune encephalomyelitis is mediated by SOCS3 regulatory pathway. Sci Rep, 5**:** 17407.

Zhang Y, Luo R, Chen Y, Ke X, Hu D Han M. (2014) Application of carrier and plasticizer to improve the dissolution and bioavailability of poorly water-soluble baicalein by hot melt extrusion. AAPS PharmSciTech, 15**:** 560-568.

Zhou QB, Ju XN, Wang XY, Wang MH, Kong F, Sun C Bi JZ. (2016) Pretreatment with baicalin attenuates hypoxia and glucose deprivation-induced injury in SH-SY5Y cells. Chin J Integr Med, 22**:** 201-206.

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11. Biobliography of the candidate's publications

1. **Kalapos-Kovács Bernadett**, Magda Balázs, Jani Márton, Fekete Zsolt, Szabó Pál, Antal István, Krajcsi Péter, Klebovich Imre. (2015) Multiple ABC Transporters Efflux Baicalin. Phytother Res, 12: 1987-1990.
2. Magda Balázs, Márta Zoltán, Imre Tímea, **Kalapos-Kovács Bernadett**, Klebovich Imre, Fekete Jenő, Szabó Pál.(2015) Unexpected retention behavior of baicalin: Hydrophilic interaction like properties of a reversed-phase column. J Pharm Biomed Anal, 111: 119-125
3. Laki Szilvia, **Kalapos-Kovács Bernadett**, Antal István, Klebovich Imre. (2013) Importance of drug interactions with smoking in modern drug research. Acta Pharm Hun, **4**: 107-120.
4. Zhang Li, Lin Ge, **Kovács Bernadett**, Jani Márton, Krajcsi Péter, Zuo Zhong. (2007) Mechanistic study on the intestinal absorption and disposition of baicalein. Eur J Pharm Sci, 3-4: 221-231.

DOI:10.14753/SE.2017.2271

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