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“Verily in the Creation of the Heavens and the Earth, and in the Alteration of Night and Day, and the Ships which Sail through the Sea with that which is of used to Mankind, and the Water (Rain) which God sends down from the Sky and makes Earth Alive there with after its Death, and the moving Creatures of all kind that he has Scattered therein, and the Veering of Winds and Clouds which are held between the Sky and the Earth, are indeed Signs for Peoples of Understanding.”

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1. Summary

Curcumin is a yellowish orange polyphenol with pleiotropic activities. However, it has a poor bioavailability due to rapid metabolism in intestine and liver. Since, pharmacokinetics (absorption, distribution, metabolism and excretion) plays a determinant role in efficacy, toxicity and interaction of drugs; thus, the role of uptake transporters and metabolizing enzymes on the activity of curcumin was investigated.

In the first project, the metabolism of curcumin in human hormone-dependent ZR-75-1 and hormone independent MDA-MB-231 breast cancer cell lines was elucidated by using high-performance liquid chromatography (HPLC) coupled with UV and mass spectrometry which clearly showed the formation of cucumin sulfate as the main metabolite. Interestingly, sulfation was far more pronounced in ZR-75-1 cells compared to MDA-MB-231 cells leading to higher intracellular curcumin sulfate levels, thereby explaining the lower EC_{50} of curcumin in this cell line. For the very first time, our data also confirmed the formation of curcumin dimer as a novel minor curcumin metabolite in the breast cancer cells. The concentration of curcumin glucuronide and other metabolites was below of the detection limit.

In the second project, our focus was to investigate the role of organic anionic transporting polypeptides (OATPs) in the uptake of curcumin and its main metabolites curcumin sulfate, curcumin glucuronide and tetrahydrocurumin. This data may help to elucidate whether curcumin and its metabolites can accumulate to bioactive levels in tissues and organs. By using the OATP-transfected Chinese hamster ovarian cells (CHO), we found that OATP1B1, 1B3 and 2B1 were able to transport curcumin and its sulfated conjugate in CHO cell line. Tetrahydrocurcumin was only transported by OATP1B1 and 1B3 but not by OATP2B1. Moreover, all the three OATPs were not able to transport curcumin glucuronide. To further prove the importance of OATPs, OATP1B1-knockdown ZR-75-1 cells were incubated with curcumin which showed a decreased curcumin uptake compared to the OATP1B1 overexpressing wild-type cells. This leads to higher EC_{50} values and a decreased inhibition value of interleukin β-induced NF-κB reporter expression.

In the third project, we investigated the spasmolytic activity of curcumin, demethoxycurumin, bisdemethoxycurcumin, the major curcumin metabolite tetrahydrocurcumin and the non-enzymatic curcumin hydrolysis products ferulic acid, feruloyl methane and vanillin in isolated ileum, aorta and pulmonary artery of guinea pig. The

ionotropic and chronotropic activities of these compounds were also determined in papillary muscles and right atrium of guinea pig, respectively. The biological effects of turmeric powder have been already reported but the extent to which demethoxycurcumin and bisdemethoxycurcumin, tetrahydrocurcumin and the three main degradation products also contribute to pharmacological effects individually, was still unknown. Our results revealed that curcuminoids and tetrahydrocurcumin demonstrated significant spasmolytic activity in ileum. However, only demethoxycurcumin relaxed pulmonary artery significantly. All three curcuminoids, but not tetrahydrocurcumin, exhibited mild negative chronotropic activity. Interestingly, curcumin and demethoxycurcumin demonstrated mild positive ionotropic activity. In contrast, bisdemethoxycurcumin showed mild negative ionotropic activity. Ferulic acid, feruloyl methane and vanillin demonstrated no pharmacological activity at all in the various isolated organs. Moreover, we also analyzed the uptake of curcumin, demethoxycurcumin, bisdemethoxycurcumin and tetrahydrocurcumin into the various tissue samples where concentrations correlated with the pharmacological activity.

In conclusion, our results show for the first time that curcumin is sulfated in breast cancer cells and that intracellular curcumin sulfate levels inversely correlated with cytotoxicity. We also demonstrated that OATPs act as cellular uptake transporters for curcumin, tetrahydrocurcum and curcumin sulfate, but not for curcumin glucuronide. Moreover, our data also showed that demethoxycurcumin and bisdemethoxycurcumin demonstrated more pronounced spasmolytic and vasodilating activity than curcumin indicating that both the curcuminoids significantly contribute to the observed pharmacological effects of *Curcuma longa* which have to be considered in humans after oral intake of turmeric powder.

2. Zusammenfassung

Curcumin ist ein orangegelbes Polyphenol mit pleiotropischen Eigenschaften. Die Bioverfügbarkeit ist gering, bedingt durch sehr rasche Metabolisierung in Darm und Leber. Da die Pharmakokinetik (Resorption, Verteilung, Metabolismus und Ausscheidung) einen bestimmenden Einfluss auf Effektivität, Toxizität und Arzneistoffwechselwirkungen ausübt, wurde die Rolle von Aufnahmetransportern und metabolisierenden Enzymen auf die Aktivität von Cucumin untersucht.

In der ersten Studie wurde die Metabolisierung von Curcumin in der hormonabhängigen ZR-75-1 und der hormonunabhängigen MDA-MB-231 humanen Brustkrebszelllinie mittels Hochleistungsflüsigkeitschromatographie gekoppelt mit UV und Massenspektrometrie untersucht, wobei sich deutlich zeigte, dass die Bildung von Curcuminsulfat den Hauptmetabolisierungsweg darstellt. Interessanterweise war die Bildung von Curcuminsulfat in ZR-75-1-Zellen deutlich stärker ausgeprägt als in MDA-MB-231 Zellen was die geringeren IC_{50}-Werte von Curcumin in dieser Zelllinie erklärt. Zum ersten Mal bestätigten unsere Daten auch die Bildung eines neunen dimeren Curcumins in humanen Brustkrebszelllinien. Die Konzentration von Curcuminglucuronid und anderer Biotransformationsprodukte war unterhalb der Nachweisgrenze.

Der Fokus der zweiten Studie lag auf der Rolle von „organic anionic transporting polypeptides“ (OATPs) auf die zelluläre Aufnahme von Curcumin und dessen Hauptmetaboliten Curcuminsulfat, Curcuminglucuronid und Tetrahydrocurcumin. Diese Studie sollte bei der Beantwortung der Frage helfen, ob Curcumin und dessen Biotransformationsprodukte in Geweben und Organen angereichert werden können um dort bioaktive Spiegel zu erreichen. Unter Verwendung von OATP-transfizierten „Chinese hamster ovarian cells“ (CHO) konnten wir zeigen, dass Curcumin und Curcuminsufat mittels OATP1B1, 1B3 und 2B1 transportiert werden. Tetrahydrocurcumin wird hingegen nur von OATP1B1 und 1B3 transportiert, während Curcuminglucuronid kein Substrat für diese drei OATPs darstellt. Um die Bedeutung von OATPs für die Aufnahme von Curcumin zu unterstreichen wurden OATP1B1-knockdown ZR-75-1-Brustkrebszellen mit Curcumin inkubiert, wobei im Vergleich zu überexprimierten Wildtyp-Zellen ein verringerte zelluläre Aufnahme von Curcumin gefunden wurde. Dies führte zu höheren IC50-Werten und zu einer verringerten Expression von Interleukin β-induziertem NF-κB.

In einem letzten Projekt untersuchten wir die spasmolytische Aktivität von Curcumin, Demethoxycurcumin, Bisdemethoxycurcumin und Tetrahydrocurcumin, sowie der Curcuminhydrolyseprodukte Ferulasäure, Feroylmethan und Vanillin in isolierten Gewebsstücken des Ileums, der Aorta sowie der Lungenarterie von Meerschweinchen. Eine mögliche inotrope und chronotrope Aktivität wurde am isolierten Papillarmuskel und am rechten Vorhof untersucht. Die pharmakologische Wirkung von Curcumin wurde bisher nur für eine Gelbwurzmischung nachgewiesen nicht hingegen für reines Demethoxycurcumin, Bisdemethoxycurcumin und die drei nicht-enzymatischen Zerfallsprodukten. Unsere Ergebnisse zeigten erstmals anschaulich, dass alle drei Curcuminoide sowie Terahydrocurcumin eine ausgeprägte spasmolytische Aktivität am Ileum aufweisen, während Demethoxycurcumin nur die Pulmonalarterie relaxiert. Alle drei Curcuminoide, nicht jedoch Tetrahydrocurcumin, zeigten weiters eine geringe negative chronotrope Aktivität. Bisdemethoxycurumin hingegen zeigte eine geringe positive inotrope Aktivität. Interesanterweise konnte für Ferulasäure, Feroylmethan und Vanillin keine pharmakologische Aktivität festgestellt werden. In weiteren Versuchen analysierten wir auch die Aufnahme von Curcumin, Demethoxycurcumin, Bisdemethoxycurcumin und Tetrahydrocurcumin in die verschieden Gewebe und Organe, wobei deren Konzentration mit der pharmakologischen Wirkung korrelierte.

Zusammenfassend zeigten unsere Daten zum ersten Mal, dass Curcumin in humanen Brustkrebszellen zu Curcuminsulfat metaboliert wird und dass der intrazelluläre Spiegel von Curcuminsulfat eine inverse Korrelation mit der Cytotoxiziät aufweist. Wir konnten weiters nachweisen, dass OATPs für die zelluläre Aufnahme von Curcumin, Curcuminsulfat und Tetrahydrocurcumin, nicht jedoch von Curcuminglucuronid veranwortlich sind. Darüber hinaus konnten wir zeigen, dass Demethoxycurcumin, Bisdemethoxycurcumin eine gegenüber Curcumin eine ausgeprägtere spasmolytische, und vasodilatatorische Wirkung besitzen. Beide Curcuminoide dürften daher an der pharmakologischen Wirkung von *Curcuma longa* beteiligt sein, was nach peroraler Einnahme von Gelbwurzpulver bei Menschen berücksichtigt werden sollte.

3. Introduction

3.1 Curcumin

Turmeric powder (obtained from the rhizome of *Curcuma longa*) is a yellowish orange powder, used as a spice, colorant and medicine all over the world [1]. Since the ancient times, it is used in Indian and Chinese folk medicines with a history of 2000 years [2]. In Indian herbal medicines, its paste is topically applied against skin diseases (skin infection, acne, eczema, itching and ulcers), vertigo, swelling, bruises, inflammatory joints, diabetic wounds as well as insect and reptile bites [3]. Moreover, it is ingested orally for the treatment of respiratory diseases (common cold, bronchitis and asthma), for gastrointestinal problems (indigestion, gastritis, gastrointestinal reflex disease, diarrhea, flatulence, gut worm, intestinal spasm and hepatic diseases), anemia, urinary disorders and diabetic wounds [3,4]. Turmeric paste is applied on umbilical cord of the new born babies due to its antiseptic activity [3,5]. In Chinese medicine, turmeric powder is used to treat the symptoms caused by blood obstruction such as psychataxia and arthralgia [6]. It is also consumed traditionally to cure menstrual pain. In veterinary folk medicine, it is used against wounds, parasitic infections, and skin disorders [3].

Curcuminoids are polyphenols which are active ingredients of *Curcuma longa*, consisting of curcumin (77%), demethoxycurcumin (17%) and bisdemethoxycurcumin (6%) [5]. In modern science, it is reported that they have various biological effects including: wound healing, anti-inflammatory, anti-oxidant, anti-neoplastic, spasmolytic, anti-diabetic, anti-rheumatic and anti-microbial activities [3,7,8]. They also have gastrointestinal, hepatic-, cardio-, renal- and neuro-protective properties [3,9-11]. Recent studies have shown that curcumin plays beneficiary role against acute myocardial infarction, dyslipidemia, biliary disease, bronchial asthma, oral diseases, inflammatory bowel disease, obesity, Alzheimer's disease, depression and dermatitis [12,13]. Curcuma extracts showed spasmolytic effect on rabbit intestine by blocking the calcium channels [4], whereas intravenous administration of sodium curcuminate caused lowering of blood pressure and bradycardia in anesthetized cats and dogs [14]. Curcumin is also involved in relaxing pre-contracted aorta independent of nitric oxide synthesis [6]. These results demonstrate that curcumin is a potent spasmolytic agent which exerts its antispasmodic activity by blocking the calcium channels.

Curcumin is also a potent anti-cancer agent and has anti-proliferative activity against cancers of skin, stomach, head, neck, colon, breast, liver, pancreas, prostate, nasopharynx, lungs, ovaries and uterus [5,15,16]. Curcumin suppresses the expression of cyclin D1; modulating transcription factors such as NFκB, AP1, STAT3 and STAT5; modulates CAMP

response element binding proteins and electrophile response elements including Egr-1, PPAR-γ, β-catenin and Nrf-2 [5,17]. It is also responsible for downregulating expression of BCL2, BCL-XL, COX-2, MMP-9, EGFR, HER2 and TNF [5,18]. Recently, it is reported that curcumin is involved in reduction of angiogenesis and tumor growth in mouse xenograft of human breast cancer by influencing NF-κB. Furthermore, both *in vitro* and *in vivo* studies exhibit that curcumin and its metabolites are involved in apoptosis and anti-proliferative activity against hormone dependent and hormone independent breast tumors [19].

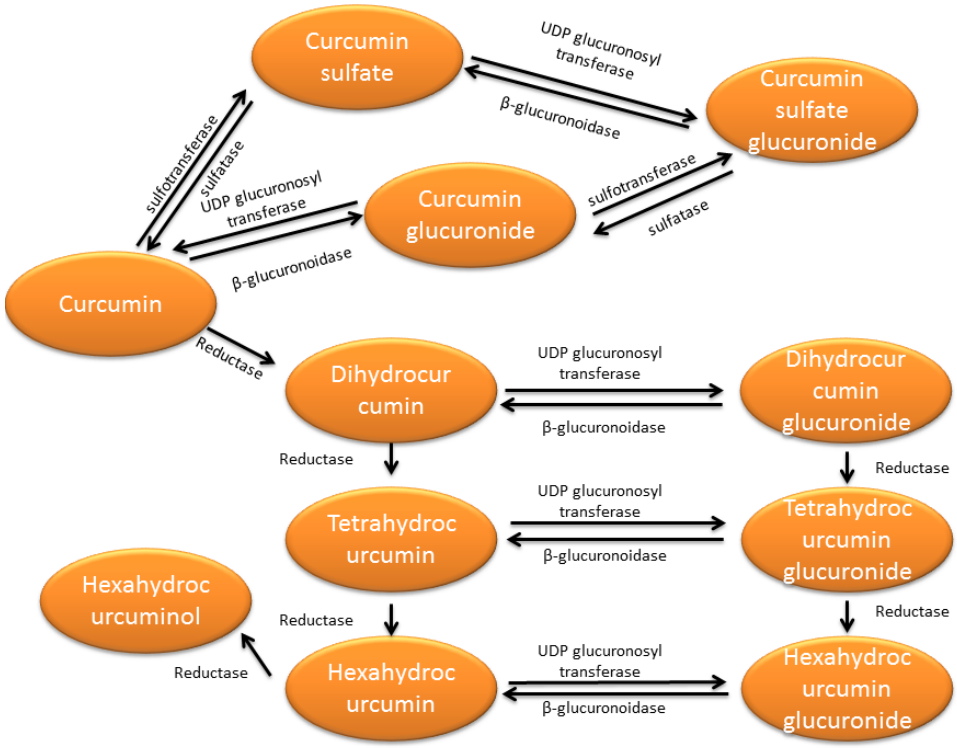

Fig 1: Metabolism of curcumin. Curcumin is actively metabolized by reductases into major metabolites including dihydrocurcumin, tetrahydrocurcumin and hexhydrocurcumin, while by sulfotransferase and UDP glucuronosyl transferase; it is converted into curcumin sulfate and curcumin glucuronide respectively, which are minor metabolites.

Despite these vast reported biological activities of the curcuminoids, they have not been transformed into a drug mainly due to low bioavailability which is due to low oral absorption and rapid metabolism. Curcumin is well tolerated after oral dose; 12g/day, [20,21] and upon ingestion of 10-12 g of curcumin in a single bolus dose, no free curcumin was detected after 30 min. However, its glucuronide and sulfated conjugates were detected in plasma samples, suggesting that curcumin is extensively metabolized after oral ingestion [21].

However, when curcumin was injected intravenously or intraperitoneally, it rapidly metabolized into dihydrocurcumin, tetrahydrocurcumin, hexahydrocurcumin and

hexahydrocurcuminol. Moreover, these phase-I metabolites further undergo sulfate and glucuronide conjugation, resulting in dihydrocurcumin glucuronide, tetrahydrocurcumin glucuronide, hexahydrocurcumin glucuronide, tetrahydrocurcumin sulfate and hexahydrocurcumin sulfate [22].

Interestingly, phase-I metabolites including tetrahydrocurcumin, hexahydrocurcumin and octahydrocurcumin are pharmacologically active compounds. Tetrahydrocurcumin exhibits anti-oxidant, anti-inflammatory, neuro protective, anti-aging, anti-diabetic and anti-cancer activities [23]. Tetrahydrocurcumin was more active compared to parent compound curcumin in modulating some biological targets such as P21, P53, cPLA2, GSK-3β, mTOR, GST, GPx, Hb, LPO, PI3K, JNK, SOD and catalase [24]. Similar to tetrahydrocurcumin, hexahydrocurcumin and octahydrocurcumin also exhibit anti-inflammatory and anti-oxidant activities [25-27]. Hexahydrocurcumin exhibited synergistic effects with 5-flurouracil in suppressing colon cancer [28]. Curcumin sulfate was also shown to inhibit prostaglandin E2 production *in vitro* and *in vivo* in rat model [26].

Taken together, curcumin, its congeners and its metabolites exhibit pleothora of biological activities. However, curcuminoids demonstrated erratic bioavailability due to pharmacokinetic issues; hence majorly research is now being focused on improving the bioavailability of curcuminoids, thus making them druggable substances. The bioavailability of any substance depends on multiple factors starting from absorption, tissue distribution and then metabolism. After oral intake, a drug or compound is absorbed into blood stream either by simple diffusion or with the help of primary or secondary transporters. The primary and secondary transporters, in addition to cardiac output, tissue blood perfusion rate, vascular permeability, plasma proteins and drug solubility, play an important role in the cellular uptake of the drugs. Thus, drug transporters are a key factor in drug bioavailability and determine efficacy and toxicity of a drug. After distribution and cellular uptake, a drug substance is metabolized. In humans, liver is the main organ for metabolism however; extrahepatic metabolism also takes place in the body. Metabolism is divided into phase-I and phase-II. The phase-I metabolism is involved in adding functional group, whereas phase-II metabolism is responsible for conjugation (sulfonation or glucuronidation). Metabolism is usually a detoxification process but sometimes active metabolites are also produced. Similar to transporters, metabolizing enzymes are also important in determining pharmacokinetics of a drug.

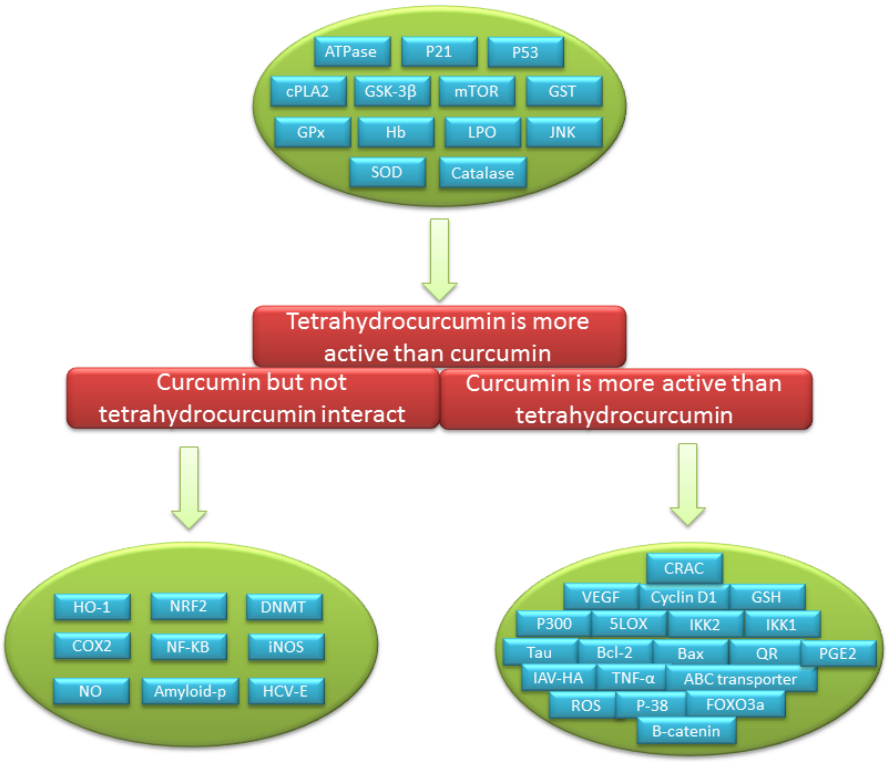

Fig 2: Targets of curcumin vs tetrahydrocurcumin. Different biological targets are modulated differentially by curcumin and tetrahydrocurmin, thus indicating differential affinity and potency of the both parent compound curcumin and its active metabolite tetrahydrocurcumin.

In our first manuscript, we focused on phase-II metabolism of curcumin in breast cancer cell lines and determined its role in cytotoxicity. We quantified curcumin sulfate and curcumin glucuronide after incubating the curcumin with ZR-75-1 and MDA-MB-231 cell lines in order to co-relate metabolism of curcumin with cytotoxicity. In the second manuscript, we described the role of organic anion transporting polypeptides (OATPs) in the cellular uptake of curcumin and its metabolites in OATP1B1, 1B3 and 2B1 transfected Chinese hamster ovarian (CHO) cell lines in addition to wild type and OATP1B1 transfected ZR-75-1 cell line to determine whether these compounds can accumulate in the cells to bioactive level. In our last manuscript, we determined the spasmolytic, ionotropic and chronotropic activities of all three curcuminoids and their major metabolite i.e., tetrahydrocurcumin in various isolated tissues of Guinea pigs. We also checked tissue levels of the curcuminoids and their active metabolite in respective tissue samples to establish a correlation in pharmacological activity with the respective tissue uptake. In the following part of introduction, we generally described metabolism followed by transporters and then animal models to study the drug effects.

3.2 Metabolism

Metabolism also called biotransformation performs a vital role in the clearance of xenobiotics with the help of enzymes, hence, protects the body from toxicity. Metabolism is mainly divided into two phases; Phase-I (functional group addition) and phase-II (conjugation) [29].

3.2.1 Phase-I

The phase-I metabolizing enzymes play a role in adding functional group to their substrates, resulting in more polar metabolites [29,30]. N- and O-dealkylation and oxidation, aliphatic and aromatic hydroxylation, reduction, cyclization, decyclization and deamination are important reactions performed by the phase-I enzymes [30]. Cytochrome P450 (CYP 450) is the major enzyme family responsible for phase-I metabolism with up to 60% of all drug metabolic reactions [31]. However, several other enzymes including aldehyde oxidase, monoamine oxidase, xanthine oxidase, dehydrogenase, flavin monooxygenase, peroxidase and hydrolase also play a role in the phase-I metabolism [29]. CYPs are majorly expressed in liver however they are also present in intestine, kidney and adrenal gland [29]. CYPs are membrane bound enzymes, localized in endoplasmic reticulum, however, its catalytic site is found in cytoplasm [32]. Cytochrome 450 is a large superfamily of enzymes, having 18 families and 41 protein coding subfamilies, encoded by 57 genes [33]. The enzymes having at least 40% sequence identity are classified into one family, denoted by the Arabic numeral, whereas CYPs having more than 54% sequence identity are placed into one subfamily, designated by alphabet letters [33]. CYP 1-4 are encoded by more genes as compared to other families. CYP1A2, 2B6, 2C8, 2C9, 2C19, 2E1 and 3A4 are the most predominant enzymes with 200 clinical drug substrates. CYP3A4 has the broadest range of substrates due to its flexible active site [34,35]. In short, CYPs are important enzymes for drug elimination, and metabolize a broad range of drugs; thus, their inhibition or induction may cause drug-drug interactions [35].

3.2.2 Phase-II

In contrast to the phase-I metabolism, the phase-II metabolism is involved in conjugation of xenobiotics and the phase-I metabolites by using glucuronidation, sulfation, acetylation, methylation, glutathione and amino acids conjugation reactions [30]. Hence, the phase-II metabolizing enzymes convert their substrates into more polar compounds, helping their excretion from biliary or renal route. Mostly, the phase-II metabolites are pharmacologically less active but there are some exceptions [29,30]. UDP-glucuronosyltransferases,

sulfotransferases and glutathione-S-transferases are majorly involved in the phase-II reactions, whereas N-acetyl transferases and methyl-transferases play a minor role [29].

3.2.2.1 UDP-glucuronosyltransferases

UDP-glucuronotransferases (UGTs) are membrane bound enzymes responsible for glucuronidation of their substrates, resulting in the formation of β-D-glucuronide metabolites that are easily excreted via biliary or renal route [30]. This enzyme superfamily plays a key role in the phase-II detoxification process, involving in conjugation of numerous endo- and xenobiotics including bilirubin, hormones (steroid and thyroxine), bile acids, fat soluble vitamins, various drugs, environmental pollutant, carcinogens as well as dietary constituents [36,37]. Approximately, 40-70% of drugs and their phase-I metabolites undergo glucuronidation in humans [38]. Four UGTs families: UGT1, UGT2, UGT3 and UGT8 are identified in humans with 22 members (UGT1A1, 1A2, 1A3, IA4, 1A5, 1A6, 1A7, 1A8, 1A9, 1A10; 2A1, 2A3; 2B4, 2B7, 2B10, 2B11, 2B15, 2B17, 2B28; 3A1, 3A2 and 8A1) [30]. UGT1 and UGT2 use uridine-diphospho-α glucuronic acid (UDPGA) as a cofactor for glucuronidation of various endo- and xenobiotics (aliphatic alcohols, phenols, thiols carboxylic acids as well as primary, secondary and tertiary alcohols) [30,39]. However, UGT8 is involved in the biosynthesis of substances for nervous system (glycosphingolipids and cerebosides) by converting UDP-galactose to UDP-galactosidate ceramides [30]. Recently, UGT3A1 is found responsible for N-acetylglucosaminyl transferation [40]. The UGT family share at least 40% homology at DNA level and classified by Arabic numbers. The subfamily has minimum 60% sequence identity of DNA and sub-classified by alphabet letters after first Arabic number whereas second Arabic number describe individual gene [41].

Generally, UGTs are localized in endoplasmic reticulum and it is proposed that a co-factor and the substrate binding sites are located at luminal side, whereas metabolites are effluxed from cytosolic side [42,43]. UGTs are widely distributed in various tissues but majorly localized in liver. Several UGTs (UGT1A1, 1A3, 1A4, 1A6, 1À9, 2B7 and 2B15) play a major role in hepatic glucuronidation [42,44]. Extrahepatic glucuronidation takes place in intestine, kidney, placenta, brain, pancreas and nasal epithelium [30,45,46]. UGT1A7, 1A8 and 1A10 are expressed in intestine and responsible for first pass effect resulting in reduced bioavailability of clinical drugs and dietary constituents [47-49].

3.2.2.2 Sulfotransferases

Sulfotransferases (SULT) are responsible for conjugation of their substrate with the help of 3'-phosphoadenosine 5'-phosphosulfate (PAPS); a co-factor (source of sulfonate group) [29,30]. PAPS is produced in all tissues, and sulfonate group from PAPS is transferred to the substrate, leaving behind 3'-phosphoadenosine-5'-phosphate (PAP) [50,51]. Broadly speaking, sulfotransferases are classified into two groups; Golgi apparatus membrane bound sulfotransferases are responsible for sulfation of several large molecules including proteins, peptides, glycosaminoglycans and lipids, resulting in important structural and functional modulation. The cytosol sulfotransferases (SULT) play an important role in sulfation of drugs and small endogenous molecules such as neurotransmitters, bile acids, hormones and peptides [52]. Four families (SULT1, SULT2, SULT4 and SULT6) are found in humans with 13 isozymes (SULT1A1, 1A2, 1A3, 1A4; 1B1; 1C1, 1C2, 1C4; 1E1; 2A1; 2B1; 4A1 and 6B1) [53]. The family shares 45% homology at genetic level and classified by Arabic numbers after word 'SULT', whereas subfamily shares the 60% homology for their members and sub-classified by alphabet letter while Arabic number after the alphabet indicates the gene [54]. Mostly, sulfotransferases detoxify the active substances by converting them into more polar compounds that can be excreted easily by biliary or renal route [55]. However, it is not always the case; some sulfated metabolites are more active or toxic than their parent compounds, for example, sulfated metabolites of N-hydroxy heterocyclic amine, N-hydroxyarylamine and hydroxyl heterocyclic amines are charged molecules which are carcinogenic and mutagenic [55]. Similarly minoxidil (a hair growth and antihypertensive agent), morphine (opioid), triamterene (diuretic) and cholecystokinin (a neuroendocrine peptide) are converted into more active metabolites upon sulfation [29,52]. In contrast to glucuronidation, sulfation is a low capacity and high affinity reaction which predominates at low concentrations while at high concentration glucuronidation is a more prominent reaction [53,56]. Sulfotransferases are not only involved in sulfation of the primary xenobiotics but also the metabolites from the phase-I reaction and glucuronides are substrates for SULTs [29]. SULTs are widely distributed in the body including liver, adrenal gland, lung, kidney, brain, intestine, endometrium, platelets, leukocytes, prostate and testis [30]. Interestingly, human fetus lacks glucuronidase enzyme until 20^{th} week of the gestation, during that period sulfation is mainly responsible for the metabolism and detoxification [57], particularly SULT1A1, 2A1, 1C1, 1C2 and 1C4 play a vital role [58].

Structure of cytosolic sulfotransferase

Sulfotransferases are globulins, consisting of single α/β domain. The α helices surrounds every side of 5 stranded parallel structure of β domain. The β domain structure is important functionally: as PAPS binding site and catalytic residues are located at β domain of SULTs [52,59]. The catalytic residues present at β domain is conserved in all SULTs (cytosolic and membrane bound) [59]. In addition, the PAPS binding site is almost same in all cytosolic SULTs [60]. It is important to know that conformational changes in 'third loop' and 'α14-15' helices of SULTs are needed for the PAPS binding to the enzyme [61,62]. Phosphate binding loop (PSB loop) and GxxGxxK motif at loop-3 in addition to residues 'Arg130' and 'Ser138' are important for the PAPS binding [63].

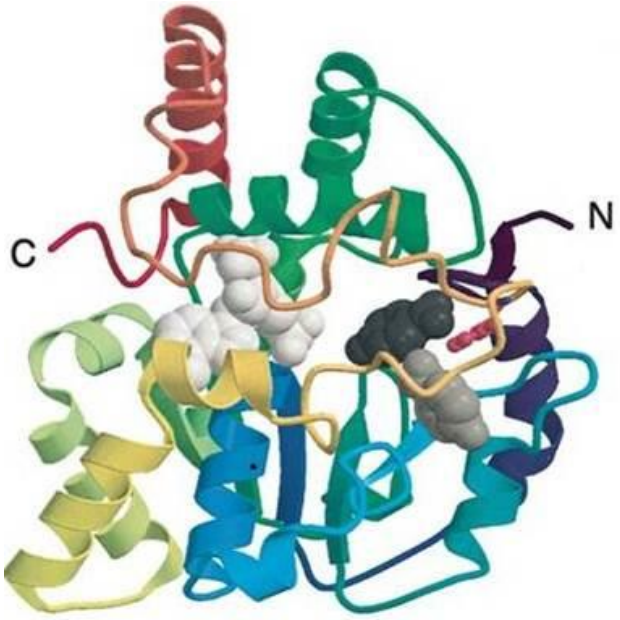

Fig 3: Model of human SULT1A1. C-terminus is illustrated by red color; N-terminus is shown by blue-color; Coils represent for helices; Spherical models indicate bound ligands: PAP is demonstrated in white [64].

PSB loop is a P-shaped loop having residues 45-TYPKSGTT-52 (SULT1A1) and is an important site for binding of 5'-phosphate of PAPS to SULT [59]. It plays a vital role in orientation of the co-factor (PAPS) for the transfer of in line SO_3- group to nucleophile substrate [59,61,65]. Hydrogen bonding is formed between 5'-phosphate of PAPS and last two residues of phosphate binding loop (PSB loop) [65] whereas, residues (257-259) from initial region of GxxGxxK motif and two additional residues (Arg130 and Ser138) are binding site of 3'-phosphate of PAPS [59]. In addition, four residues; Trp53, Tyr193, Thr227 and Phe229

play a determinant role for positioning of adenine ring of PAPS through staking, T-shaped interaction and hydrogen bonding [66]. Sulfotransferases have a wide range of substrates; however, numerous SULTs subfamilies prefer different substrates. Unlike the conserved PAPS binding site, enzymes from SULT family have variable substrate binding site [52].

SULT1A1 was previously known as thermostable phenol sulfotransferase-1 or phenol sulfotransferase, which conjugates a wide variety of drugs and other xenobiotics [30,67]. Besides liver, it is localized in brain [68], lung, kidney, gastrointestinal tract, breast, adrenal gland, platelets, endometrium cells of uterus and placenta, indicating their vital role in sulfation [69]. Many phenolic substances including benzyl alcohols, aromatic amines, hydroxylamines, monocyclic phenols, naphtols, 4 nitrophenols, N-hydroxy-2-amino-1-methyl-6phenylimidazo pyridine, dihydrostilboestrol, dopamine, adrenaline and iodothyronines are substrates of sulfotransferases [70]. As most of the therapeutic drugs are phenolic compounds hence, SULT1A1 is involved in sulfation of numerous drugs. Human liver exhibits the highest expression of SULT1A1 whereas SULT1A1 and 1A3 are responsible for 90% of the sulfation process in this organ [29,51].

SULT1A2 was previously called thermostable sulfotransferase-2 [30]. Its mRNA, but not the expressed protein, is detected in humans [52]. It is believed that SULT1A2 is involved in sulfation of various pro-carcinogens such as aromatic hydroxylamines, and hence converts them into carcinogens by adding charged species into its substrates. This process results in covalent binding of these substrates with DNA, and hence damaging it [30]. Furthermore, it is also responsible for metabolism of 2-naphthol, 4-nitrophenol and minoxidil in *in vitro* models [53].

SULT1A3 was termed as monoamine sulfotransferase or themolabile sulfotransferase and it is only identified in humans [52,67]. It has a considerable affinity for monocyclic phenols and is involved in sulfation of catecholamines, thus playing a determinant role in maintaining levels of neurotransmitters [71]. Noradrenaline, catechols, monocyclic phenols and aromatic molecules are substrates of SULT1A3 [52]. Interestingly, it is not localized in adult liver, however, it is distributed in other tissues with highest expression in intestine [72].

SULT1B1 was previously known as dopa/tyrosine sulfotransferase [73]. Thyroid hormones and small molecules including 1-naphtol, diethylstilbosterol, iodothyrinines and 4

nitrophenol are substrates of SULT1B1 [53,74]. Expression of SULT1B1 is found in white blood cells, liver, colon and small intestine. It is a dominant enzyme in intestine and both SULT1B1 and 1A3 are responsible for 67% of sulfation in intestine [51,55].

SULT1C1 is expressed mainly in heart, lungs, gastrointestinal tract and kidney of human fetus. Various iodothyroids are weak substrates of SULT1C1 [30,53,75].

SULT1C2 was detected in human fetus [75]. In addition, its low expressions are also detected in adult human gastrointestinal tract [76]. The substrates of SULT1C2 include 4-nitrophenols and N-hydroxyl-2-acetylaminoflureon [53].

SULT1C4 was identified in lung tissues of human fetus. High mRNA expression of SULT1C4 was detected in fetal kidney and lung, whereas low mRNA expressions are identified in heart [75]. *p*-nitreophenol [77] and N-hydroxyl-2-acetylaminoflourorene [75] are known substrate of SULT1C4. Similar to SULT1C2, adult human gastrointestinal tract also exhibits low expressions of SULT1C4 [76].

SULT1E1 was known as estrone sulfotransferase which exhibits highest affinity for estrogen compared to all of SULTs. It plays a vital role in metabolism of estrogen, thus regulates its activity [30]. Moreover, it also conjugates pregnenolone, genistein, 1-naphtol, equilenine, minoxidil, diethylstiboestrol, DHEA and 4-hydroxytemoxifin [52]. SULT1E1 also have good affinity for iodothyronines [78]. Lung demonstrates the highest expression for SULT1E1 [51]. In addition, expressions of SULT1E1 have also been detected in cytosol of jejunum and liver [52,79-81]. Furthermore, high level of SULT1E1 was also found in kidney, liver and lung of fetus [52,58].

SULT2A1 is involved in sulfation of hydroxysteroids, thereby it was also known as DHEA sulfotransferase. It exhibits broad specificity of substrates and is responsible for sulfation of cyclic amines, steroids, small peptides, planar molecules and various drugs [29]. DHEA, pregnenolone, androgen, testosterone, cholesterol, lithocholic acid, minoxidil, estrone and bile acids are some important substrates of SULT2A1. Interestingly, sulfate metabolites of DHEA and pregnenolone are active neurosteroids [55,82]. SULT2A1 is localized with high expression in adult human liver [81] whereas, its low expressions are also detected in human

adult and fetal adrenal gland, placenta, prostate, ovary, small intestine, stomach, colon and kidney [72,83-86].

SULT2B1 was also known as cholesterol sulfotransferase. DHEA, estrogen, androgens and bile acids are substrates of SULT2B1 [30,72]. In humans, two variants of SULT2B1 are detected that differ by their amino acid sequences at N-terminal; SULT2B1a is a shorter variant (15 amino acids less) compared to its 'b' variant [54,87]. SULT2B1a gives preference to the sulfation of 3β-hydroxysteroid such as pregnenolone, DHEA, androstenediol and epiandrosterone [88] whereas SULT2B1b prefers sulfation of cholesterol, oxysterols and 3β-hydroxysteroid hormones [72,87]. SULT2B1a is localized in liver, adrenal gland, placenta, lung, kidney, prostate, ovary and small intestine. However, the other variant exhibits wider distribution and is localized in lung, kidney, gastrointestinal tract, liver, spleen, thyroid, thymus, ovary, prostate, placenta and adrenal gland [89].

SULT4A1 is expressed particularly in human brain that is why it was initially called as "brain sulfotransferase like" (BR-STL) [90]. It is less identical (only 36%) to first two families of sulfotransferase [50]. However, no endogenous or xenobiotic substrate of SULT4A1 is identified till to date, probably due to missing PAPS binding site [91], thus making it an orphan enzyme [92].

SULT6B1 is localized in kidney [93] and testis [94]. Not much is known about the substrate of this enzyme [55]. However, a recent study described that recombinant mouse enzymes showed activity for thyroxine, bithionol and chlorinated bisphenol [93].

3.3 Cellular drug transport

Besides simple diffusion, active and passive transport are responsible for uptake and efflux of xenobiotics. In contrast to the passive transport, the active transport requires energy. The active transport is further divided into primary and secondary active transport. The primary active transport is unidirectional; consuming energy from ATP hydrolysis and leading to conformational changes in transporter. However, the secondary active transport is involved in transportation of a co-substance with xenobiotics in the same or opposite direction.

Drug transporters are membrane proteins that are distributed throughout the body, showing specific distribution in various organs. These transporters are liable for accumulation of drugs in various tissues, and hence, responsible for drug efficacy, toxicity and drug-drug

interactions [95]. Furthermore, it is also reported that various types of tumors exhibit over-expression of these transporters that leads to an increase in efflux of certain anti-cancer drugs, making resistant strains of tumors. This fact makes these transporters as a novel target for drug designing. In this instance, it is crucial to know which transporters are involved in the uptake and efflux of various drugs [96].

Drug transporters principally include ATP binding cassette (ABC transporters) and solute carrier (SLC) families [97]. ABC transporters are primary efflux transporters that transfer drugs and metabolites out of the cells. These require energy for this process; thus, these are primary active transporters. However, SLC transporters are mainly influx or uptake transporters and classified as secondary active transporters [97].

3.3.1 ATP binding cassette-Transporters

ATP binding cassette transporters (ABC transporters), are comprised of P-glycoproteins (P-gp/ABCB1), breast cancer resistant protein (BCRP/ ABCG2) and multidrug resistant protein-1(MRP1/ABCC1). It is a major transporter family that is responsible for transportation of endogenous compounds and their metabolites from inside to outside of the cells in eukaryotes, thus, principally protecting cells from cytotoxic activities of various xenobiotics and responsible for drug resistance during cancer and antimicrobial treatments [98,99]. The fact that ABC transporters are main determinants of pharmacokinetics and pharmacodynamics of various anticancer drugs which makes these transporters an important target in resistant cancer therapy [97]. Human ABC transporters consist of seven families that are encoded by 49 genes [100,101].

3.3.1.1 P-glycoprotein

P-glycoprotein (Pgp) is the first member that was discovered in ABC transporters family in 1986 [102]. It is encoded by *MDR1*gene [101]. It eliminates a large number of substances from the cells that are structurally unrelated to each other by consuming ATP energy [103]. P-glycoproteins play an important role as detoxifier under normal physiological conditions and protect vital organ and tissues from accumulating xenobiotics, toxins and carcinogens [101,103]. Additionally, P-glycoproteins are also involved in synthesis and secretion of various steroids such as cortisol [104-106]. They extrude various anticancer drugs such as colchicine, vinca alkaloids, anthracyclines, taxanes, epipodophyllotoxins and tyrosine kinase inhibitors from tumors and cancerous tissues, causing multidrug resistance that leads to failure

of therapy [101]. This makes P-glycoprotein as an important target in cancer therapy, especially multidrug resistant therapy.

3.3.1.2 Breast cancer resistant protein

As shown by name, breast cancer resistant protein is the efflux transporter pump that was first discovered in MCF-7 a breast cancer cell line. However, it is also expressed in placenta, intestine, liver, uterus, adrenal gland, prostate, colon, testis, ovaries, lungs, endothelium of brain and central nervous system under normal physiological conditions [100,107]. It is encoded by *ABCG2* gene. Expression of BCRP has been reported in several malignancies and tumors including acute lymphoblastic leukemia, acute myelogenous leukemia, chronic myeloid leukemia, intestinal adenocarcinoma, gastric carcinoma, endometrial carcinoma, hepatocellular carcinoma, small cell lung cancer, non-small cell lung cancer, colon cancer and melanoma [108,109]. Over 200 xenobiotics, including several anticancer agents such as methotrexate, various tyrosine kinase inhibitors, camptothecin derivatives, anthracyclines and flavopiridol are substrates of BCRP, indicating that BCRP can cause various resistant cancers [108,109].

3.3.1.3 Multi drug resistant proteins

Multi drug resistant proteins are the efflux pumps consisting of 9 subfamilies, MRP1-MRP9; these are responsible for resistance of several anticancer agents [100,110]. These are widely distributed in several organs including liver, kidney tubules, ileum, duodenum, colon, gut, pancreas, gallbladder, skeleton muscles, brain, salivary gland, prostate, spleen and testes [111-118]. It is important to know that breast cancer shows high level of MRP8 and MRP9 expression [119,120]. In normal physiological state, MRPs mostly play protective and detoxification roles in the body [100,121-123]. Substrates of MRPs include leukotrienes, glutathione, glucuronide and sulfate conjugates, reduced folate, cAMP, cGMP, bile acids, various anticancer drugs and other xenobiotics [100,124-127]. Mostly, MRPs exhibit high expression in various cancers and cause resistance to several anticancer drugs including methotrexate, anthracyclines, camptothecins, imatinib, vinca alkaloids, epipodophyllotoxins, cisplatin, taxanes, cyclic and acyclic nucleoside monophosphate as well as their analogs [110,118,125,128-133]. Moreover, they are involved in resistance against PEMA, dideoxycytidine and ganciclovir [134,135].

3.3.2 Solute carrier- Transporters

Human solute carrier transporters are secondary active transporters that are principally responsible for the uptake of various xenobiotics. They are expressed in various important organs such as liver, kidney, intestine and brain; suggesting their determinant role in pharmacokinetics of various xenobiotics including several anti-cancer agents. This family plays a key role in maintaining homeostasis and any malfunction may result into a disease [96]. SLC is an important and large family, consisting of 52 subfamilies and more than 400 members; most of them are localized in cell membrane [136-139]. Organic anion transporting polypeptides (OATPs), organic anionic transporters (OATs) and organic cationic transporters (OCTs/OCTNs) are the pivotal subfamilies of solute carrier transporters that are responsible for transportation of diverse endogenous and exogenous substrates including anions, cations and zwitterionic substances [96,140]. Other important SLC proteins are peptide transporters and concentrative and nucleoside transporters that are involved in the uptake of various drugs [97,141]. In addition, crucial non-SLC transporters involved in the uptake of several drugs are sodium dependent taurocholate transporting proteins (NTCPs) and organic solute transporters (OST) [141]. Indeed, SLC transporters are important target for drug development in order to achieve critical goals such as improvement in compliance and adherence by alleviating in adverse effects and augmenting the chemo sensitivity [139,142].

3.3.2.1 Organic anionic transporting polypeptides

Organic anionic transporting polypeptides (OATPs) are encoded by *SLCO* gene and responsible for Na^+ independent uptake of large hydrophobic anions through the cell membranes, generally more than 300kDa [96,143,144]. Human OATPs are classified into six families (OATP1-6) and eleven subfamilies based on their amino acid identity; OATPs having more than 40% sequence identity are classified into the same family and members that share more than 60% similarity belongs to same subfamily [143,145,146]. They are not only extensively expressed throughout the body in normal tissues, especially in liver, kidney, intestine and brain but also exhibit altered expression in various cancers [96,143]. OATPs have a wide variety of endogenous and xenobiotic substrates; important endogenous substrates are bile acids, estrone-3-sulfate, thyroid hormones, eicosanoids, prostaglandins, steroids and their conjugates while methotrexate, imatinib, statins and HIV protease inhibitors are some examples of xenobiotic substrates of OATPs [96].

Structure of OATPs

Human OATPs consists of 643-724 amino acids, the only exception is OATP5A1 that has 848 amino acids [140]. OATPs have twelve transmembrane (TM) domains with N- and C-terminal [96,97,143]. At C-terminal of several OATPs, a PDZ motif is located that may be involved in transport activity, ion channel signaling and other signal transductions [147,148]. It is reported that TM 3, 6, 9 and 12 are majorly embedded in membrane, whereas TM 1, 2, 4 and 5 from N-terminal as well as 7, 8, 10 and 11 from C-terminal mostly interact with substrates [97]. OATPs also have five intracellular (ICL) and six extracellular loops (ECL). The large fifth ECL is important as all the ten disulfide bonds in cysteine residues present here are vital for the surface expression of OATPs and any mutation in these cysteine residues may lead to misfolded and easily degraded OATPs [96,140,143,145]. Furthermore, OATPs have several conserved N-glycosylation sites in the second and fifth loop which is important for OATPs function [96,140,149].

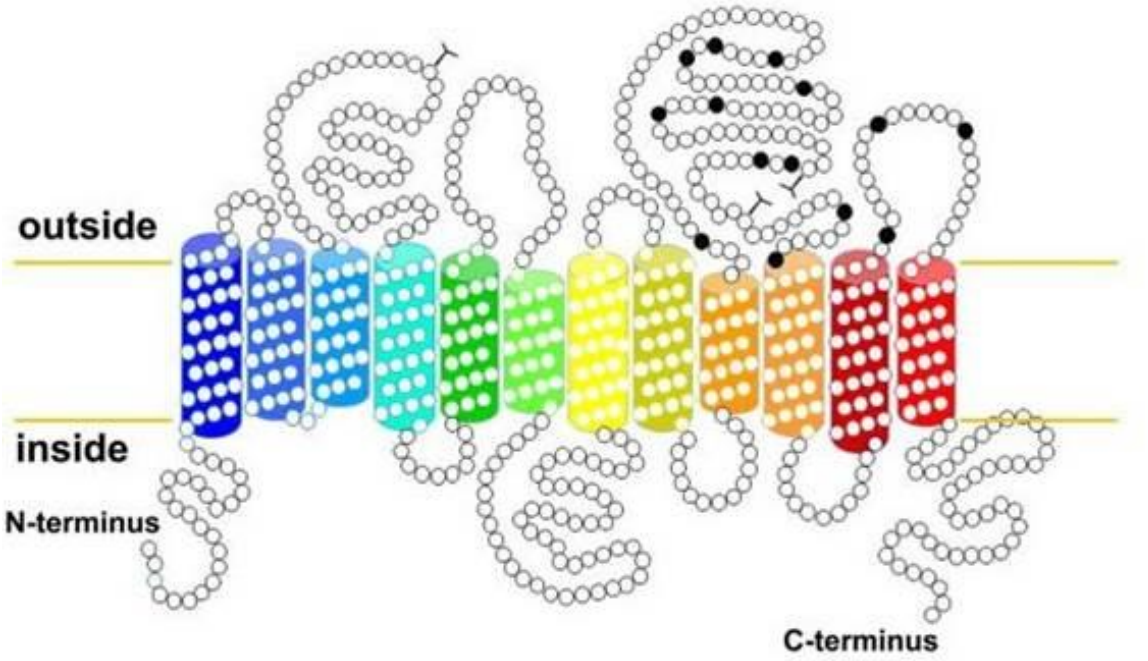

Fig 4: Model of human OATP1B1; transmembranes are depicted by cylinders; conserved cysteine residues in extracellular loops are shown in black circles [145]

OATPs and cancer

It is interesting to know that not only altered expression of OATPs is detected in various cancers and tumors but also OATPs play a vital role in cancer development by facilitating the uptake of estrone-3-sulfate, estradiol-17β-glucuronide, testosterone and

dehydroepiandrosterone sulfate (DHEA-S) to the cancer tissues [143,150]. Seven out of eleven OATPs are involved in the uptake of estrone-3-phosphate [150] and OATP1B3 is responsible for the uptake of testosterone [151,152]. Therefore, OATPs can prove an important target for cancer detection and therapy as well as for drug interactions [139,150].

An alteration in the expression of OATP1A2 was identified in gliomas and various cancers including breast, bone, colon and prostate cancers [143,150]. Colon cancer tissues show decreased expression of OATP1A2 as compares to healthy colon tissues [153]. Human gliomas exhibit the expression of different subtypes of OATP1A2 [154]. High expression of OATP1A2 was detected in T47-D and ZR-75-1 breast cancer cell lines while low expression was observed in MCF-7, MDA-MB-231 and MDA-MB-468 breast cancer cell lines [143]. This was further confirmed in human breast tissue samples showing that cell membrane and cytoplasm of breast carcinoma cells express elevated OATP1A2 in comparison to healthy tissues particularly in stage-I and stage-IIA cancer [155,156] . A recent study on mouse xenograft reported that OATP1A2 is involved in regulation of estrone-3-phosphate and causes proliferation of hormone dependent breast cancer [157]. In addition, human prostate cancer cell lines; LNCaP and 22RV1 expressing OATP1A2 promote the uptake of DHEA-S, thus, resulting in growth of cancer cells in androgen depleted conditions [158]. Increased levels of OATP1A2 were detected in bone metastasis from human kidney cancer as well as in human osteosarcoma cell lines; HOS and MG-63 [159].

OATP1B1 and OATP1B3 are liver specific transporters under normal conditions; however, they are also detected in various cancers and cancer cell lines. Several studies reported reduced expression of OATP1B1 in hepatocellular carcinoma (HCC) tissue samples and cell lines in compassion to normal hepatocytes [160-164]. Expression of OATP1B1 is also detected in colon cancer and polyps [153] as well as in ovarian cancer cell line (SK-OV-3) [165]. OATP1B1 may facilitate the uptake of paclitaxel in ovarian cancer [165]. Reduced expressions of OATP1B3 are also observed in primary and metastasized HCC and hepatic adenocarcinoma [163,166]. Various cancers and cancer cell lines frequently express OATP1B3; including cell lines from stomach (KatoIII), pancreas (MIA-Paca2, BXPC-1, PK-8, PK-9 and PK-45P), gall bladder (HuCCT-1, OcuchLM1, and TFK-1), colon (DLD-1, MIP101, Clone A and CX-1), lung (A549) and glioblastoma (A172) as well as clinical samples from gastric, colorectal, pancreatic, breast, prostate and testis cancers [151,152,167-169]. In clinical samples taken from breast and prostate cancer, expression of OATP1B3 was found in 50 and 56% of samples respectively, and it was linked to better prognosis and clinical outcomes as well as reduced reoccurrence in androgen depleted prostate and non-

estrogen dependent breast cancers [168,169]. High expression of OATP1B3 was also detected in the early stage and low grade colorectal tumors [169]. Moreover, expression of OATP1B3 mRNA is markedly increased in non-small-cell lung cancer [162]. In contrast to OATP1B1 and 1B3, mRNA of OATP1C1 is only detected in metastasized renal cancer and osteosarcomas having highest expression in aneurysmal bone cyst [159].

Altered expression of OATP2A1 is found in various cancer and cancer cell lines. For instance, expression of OATP2A1 mRNA is detected in breast cancer cell lines including MCF-7, MDA-MB-231 and ZR-751 cell lines [170] and elevated expression of this transporter are also observed in breast cancer, hepatocellular carcinoma, cholangiocarcinoma, liver cancer metastasized from colon cancer and bone cancer metastasized from kidney cancer [159,170,171]. In contrast, tumors from colon, stomach, lung, ovary, and kidney exhibit reduced expressions of OATP2A1 at mRNA and protein level [172].

A change in expression of OATP2B1 was also detected in several tumors and cell lines of tumors. Expression of OATP2B1 mRNA was detected in CX-1 (colon cancer cell line) [173] and its expression was found higher in bone cysts in comparison to osteosarcoma [159]. Both clinical samples (normal and tumor breast specimens) as well as breast cancer cell lines express OATP2B1 [174]. A direct correlation of tumor grade and expression of OATP2B1was reported [174]. Malignant samples of breast tumors exhibit higher expression of OATP2B1 as compared to non-malignant specimens [170]. In contrast, lower expressions of OATP2B1 mRNA were quantified in liver and pancreas cancer in comparison to surrounding non-malignant tissues [175]. In human gliomas, expression of OATP2B1 was localized in endothelial cells of blood brain barrier and blood tumor barrier [154].

Expression of both OATP3A1 and 4A1 mRNA are elevated in numerous cancerous cell lines such as; HOS, MG-63 (osteocarcinoma) [159]; GI-101 (breast carcinoma) [173]; GI-102 (ovarian carcinoma) [173]; GI-103 (pancreatic carcinoma); CX-1, GI-112 (colon adenocarcinoma) [173]; LX-1, GI-117 (lung carcinoma) [173]; T-47D (breast ductal carcinoma) [176,177] and MCF-7 (breast adenocarcinoma) [178]. At protein level, expression of OATP3A1 and 4A1 are markedly higher in aneurismal bone cyst compared to osteosarcoma [159]. Both transporters are over-expressed in primary as well as in metastatic liver cancers [171]. Elevated expression of OATP4A1 mRNA is reported in colorectal cancer samples and is associated with reduced sensitivity of cyclic nucleotides in colorectal neoplasia [179]. Moreover, expression of OATP4A1 is detected both in normal tissues as well as in breast tumors [170], whereas OATP3A1, OATP4C1 and OATP5A1 are localized in membrane and cytoplasm of malignant breast cancer tissues only [180]. Additionally, mRNA

and protein expression of OATP5A1 are up-regulated in liver cancer [171] and in small cell lung cancer [181]. Transfection of HEK-293 cells with OATP5A1 resulted in resistance of satraplatin therapy [181]. In short, frequent alterations of OATPs in several cancers are reported, indicating that they can act as a promising tool for detection and therapy of respective cancers.

OATP1A2 is the first cloned human OATP and is widely expressed throughout the body with highest expression in brain [96,140]. Besides luminal membrane of endothelial cells of brain, it is also localized in apical membrane of distal nephrons, enterocytes of duodenum, apical membrane of cholangiocytes of liver, epithelium of retinal pigment, lung, testis and placenta [96,140,143,182,183]. Localization of OATP1A2 in critical organs enable it to regulate absorption, distribution and excretion of drugs [140]. Under normal physiological condition, OATP1A2 regulates drug secretion into bile and urine as well as permeation of xenobiotics by blood brain barrier. It also mediates absorption of xenobiotics in intestine and testis. Estrone-3-sulfate, estradiol 17β glucuronide, dehydro epiandrosterone sulfate (DHEA-S), T_3, T_4, prostaglandin E_2 and bile acids are some classic endogenous substances transported by OATP1A2 [143]. However, typical drug substrates of OATP1A2 include β-blockers, statins, fexofenadine, fluoroquinolones, HIV protease inhibitors, labetalol, sotalol and anti-cancer agents; methotrexate, imatinib, paclitaxel, docetaxel and doxorubicin.[143,150,184-188]

OATP1B1 is a liver specific transporter and localized in basolateral membrane of hepatocyte throughout the lobes of liver [96,140]. It is involved in transportation of amphiphilic organic substances into liver in Na^+ and ATP independent fashion [96]. Several endogenous substances including bile salts (conjugated and unconjugated), bilirubin (conjugated and unconjugated), estradiol 17β glucuronide, estrone-3-sulfate, DHEA-S, leukotrienes, prostaglandin E_2, thromboxane B_2 and various thyroid related substrates are transported by OATP1B1 [143,145]. Whereas, statins, antiviral agents, ACE inhibitors, sartans, bosentan, caspofungin, cefazolin, ezetimibe, rifampicin, troglitazone, and various anticancer agents including methotrexate, paclitaxel, docetaxel, doxorubicin, rapamycin, are some examples of drugs transported by OATP1B1 [143,150].

OATP1B3 is also a liver specific transporter and localized at basolateral membrane of hepatocyte near central vein [143] however, mRNA of OATP1B3 has also been detected in

retina recently [189]. OATP1B3 shares 80% of structure similarity with OATP1B1 [185]. Thus, it is responsible for the uptake of same endogenous substances that is transported by OATP1B1, in addition to gastrointestinal peptide cholecystokinin-8 (CCK-8); that is the only OATP1B3 specific substrate [143,145,190]. Exogenous substances transported by OATP1B3 are similar to OATP1B1. However, digoxin is transported by OATP1B3 only [143,150,185].

Table 1: Substrates of human OATPs transporters

Protein	**Gene**	**Endogenous substrates**	**Exogenous substrates**
OATP1A2	*SCLO1A2*	Estrone-3-sulfate, Estradiol 17βglucuronide, DHEA-S, T_3, T_4, PGE_2, Bile acids[143]	β blockers, Statins, Fexofenadine, Fluoroquinolones, HIV protease inhibitors, *Methotrexate, Imatinib, Paclitaxel, Docetaxel, Doxorubicin* [143]
OATP1B1	*SLCO1B1*	Bile salt, bilirubin, Estradiol 17β glucuronide, Estrone-3-sulfate, PGE_2, DHEA-S, Leukotrienes, thromboxane B_2, thyroids [143,145]	Statins, Antiviral agents, ACE inhibitors, Sartans, Bosentan, Caspofungin, Cefazolin, Ezetimibe, Rifampicin, Troglitazone, *Methotrexate, Paclitaxel, Docetaxel, Doxorubicin, Rapamycin* [143,150]
OATP1B3	*SCLO1B3*	Similar to OATP1B1 and CCK-8 [143,190]	Similar to OATP1B1 and Digoxin [143,185]
OATP1C1	*SCLO1C1*	Taurocholate, Estradiol 17β-glucuronide, Estrone -3-sulfate, T3, rT_3, T_4, thyroxine sulfate [150,191,192]	Not known
OATP2A1	*SCLO2A1*	Prostaglandins, Thromboxane B2 [193,194]	Latanoprost [193]
OATP2B1	*SCLO2B1*	Estrogen-3-sulfate, DHEA-S, Thyroxin, Bile acid salts, PGE_2 [143,195]	Statins, Ezetimibe, Glibenclamide, Montelukast and Talinolol [143,186]
OATP3A1	*SCLO3A1*	Estrone-3-sulfate, $PGE_{1,2}$, Throxine, Vasopressin [191,196]	Benzylpenicillin, BQ-123, Deltorphin [196]
OATP4A1	*SCLO4A1*	Estradiol-17β-glucuronide, Estrone-3-sulfate, T_3, T_4,	Benzylpenicillin, Unoprostone metabolite [173,198]

		rT_3, Taurocholate, PGE_2 [173,197]	
OATP4C1	*SCLO4C1*	cAMP, Estrone-3-sulfate, T_4, rT_3, Bile salts [145]	Cardiac glycosides, *Methotrexate* [145]
OATP5A1	*SCLO5A1*	Not known	Not known
OATP6A1	*SCLO6A1*	Not known	Not known

Anti-cancer agents are represented in *italics* in the table

OATP1C1 is detected in brain and testes [199] while protein expression of OATP1C1 is localized in placenta [96], basolateral membrane of choroid plexus epithelium cells [200], glial cells of hypothalamus [201], pars para of pigmented ciliary body epithelium [202] and leydig cells of testes [199]. OATP1C1 shows high affinity for rT_3 and T_4 and is responsible for their transportation in brain [150]. Endogenous substances transported by OATP1C1 includes; taurocholate, [191] estradiol 17β-glucuronide, estrone-3-sulfate, T3, rT_3, T_4, [199] thyroxine sulfate [192] and BSP [143]. Till to date, no anti-cancer drug is reported to be transported by OATP1C1.

OATP2A1 is an example of ubiquitously expressed transporter [203,204]. Expression of OATP2A1 mRNA is detected in various tissues and organs, for instance: brain, heart, liver, kidney, colon, pancreas, spleen, small intestine, skeleton muscle, ovary, placenta and prostate [205]. Protein expression of OATP2A1 is localized in epithelial cells of retina as well as epi- and endothelial cell membrane of various eye tissues, for example in ciliary body [193]. Moreover, it is also distributed in glandular and luminal epithelium of endometrium, [206] in neurons of the frontal gyrus of brain [207] as well as in pyloric cells antrum and parietal cells of gastric corpus [208]. OATP2A1 is also called human prostaglandin transporter and it is involved in transportation of various prostaglandins including prostaglandin E1, E2, F2α, H2, D2 and 8-iso-prostaglandin F2α [193,194]. In addition to prostaglandins, OATP2A1 also transports thromboxane B2 [194]. Furthermore, latanoprost is only known drug that is transported by OATP2A1 into eye [193].

OATP2B1 mRNA is detected in various organs with highest levels in liver [143,209]. Whereas, protein expression of this transporter is localized in basolateral membrane of liver cells [209], brush border membrane of enterocyte of small intestine [210], in luminal membrane of blood brain barrier [154], in endothelial cells of heart [211], in myoepithelium

of mammary gland duct [176], in syncytiotrophoblast of placenta [212], in keratinocytes [213], in pars plicata of ciliary body [193,198], in platelets [214] and skeleton muscles [215]. It is responsible for transportation of estrogen-3-sulfate, DHEA-S, thyroxin, bile acid salts and prostaglandin E_2 [143,195]. Moreover, statins [186,211,216], ezetimibe, glibenclamide, montelukast and talinolol are some examples of drugs that are transported by OATP2B1 [143].

OATP3A1 is an abundantly expressed transporter and highest levels of its mRNA are detected in brain, testes, lung and heart followed by spleen**,** peripheral blood leukocytes and thyroid gland [196,217]. At protein level, two splice variants of OATP3A1 are detected in humans, usually localized either in different cells or in different membrane of same cell [196]. For instance, OATP3A1 variant 1 and variant 2 are expressed in germ cells and sertoli cells of testes respectively. Estrone-3-sulfate [191], prostaglandin E_1 and E_2 [196], thyroxine [191] and vasopressin are some endogenous substrates transported by both variants of OATP3A1. In contrast, arachidonic acid is only transported by variant 2 [196]. OATP3A1 is involved in the uptake of drugs and drug candidates such as; benzylpenicillin [173], BQ-123 and deltorphin [196].

OATP4A1 is another example of ubiquitously expressed transporter [143] having highest levels of mRNA in heart and placenta, followed by lung, liver, skeleton muscles, kidney and pancreas [173,197]. However, OATP4A1 protein is localized in apical membrane of syncytiotrophoblast of placenta [218,219], epithelium of human ciliary body [198], adult and fetal cerebral cortex [220] and mammary gland [176]. Endogenous substrates of this transporter include estradiol-17β-glucuronide, estrone-3-sulfate, T_3, T_4, rT_3, taurocholate and prostaglandin E_2 [173,197]. Thus far, benzylpenicillin [173] and unoprostone metabolites [198] are known drugs transported by OATP4A1.

OATP4C1 was considered a kidney specific transporter and it is localized in sinusoidal membrane of proximal tubules [221]. However, a recent microarray report shows that it is also expressed in liver [222] and at low levels in normal breast tissues [170]. OATP4C1 is responsible for the uptake of cAMP, estrone-3-sulfate, T_4, rT_3, bile salts, cardiac glycosides (digoxin, auabain), methotrexate and sitagliptin and uremic acid toxins [145].

OATP5A1 mRNA was found in fetal and adult brain [222,223], skeletal muscles, prostate, thymus [222] and heart [223]. The protein of OATP5A1 is localized at plasma membrane of epithelial lining of lactiferous ducts in breast [180]. Substrates of OATP5A1 is still poorly understood [96].

OATP6A1 mRNA is detected in testes. In addition, its weak expression is also found in spleen, fetal and adult brain as well as in placenta [224]. Thus far, a little is known about its function and substrate [96]

As OATPs are distributed in numerous organs and localized in important tissues of the body as well as responsible for transportation of wide variety of endogenous substances and drugs so, they play a determinant role in pharmacokinetics, drug-drug and drug food interaction of several drugs.

3.4 Cell and animal experimental models

Cell and animal experimental models are the major tool for evaluation of both pharmacokinetics and pharmacodynamics of drugs and xenobiotics. Several kinds of models including cell culture, isolated animal tissues or organs, whole animals, transgenic and humanized animals are used in biomedical and pharmaceutical studies, depending upon the specific research question. Since, every model has merits, demerits and limitations, thus, usually more than one model is combined to confirm the research findings.

3.4.1 Cell culture

After development of immortal cell lines, mammalian cell culture has become a popular model in biomedical research as it is a convenient, comparatively cheap and easy to handle. In addition, established cell lines are very homogeneous and experiments are performed in strictly controlled and replicable environment [225]. Both human and animal cell lines of several organs including intestine, liver, lung, breast, intestine, ovaries, heart and spleen are available for biomedical and pharmaceutical studies. In addition, several diseases especially cancer cell lines of various organs are now developed to understand the underline mechanisms and impact of different drugs and xenobiotics on these diseases. Breast cancer (MDA-MB-231, MCF-7, and ZR-75-1), liver cancer (HepG2, SNU-182, SNU-423, and ALM-12), pancreatic cancer (BXPC3, AsPC-3), lung cancer (A-549 VIM RFP, NCI-H128) kidney cancer (HEK293, CaKi-1, CaKi-2), colon cancer (HT-29, SW-480, LS-180) cell lines are some examples of cancer cell lines that are developed for cancer studies [226].

With the advancement of biomedical and molecular research, it became possible to transfect or knock out specific genes from cell lines and it is much easier to transfect the cell lines in comparison to whole animal. Thus, cell lines are important tool for understanding the role of different proteins including drug transporters and enzymes. OATP, MRP, sulfotransferase and UDP-glucuronsyltransferase, transfected and knock out cell lines are commonly used in uptake and metabolism studies [227]. The 3D cell culture model is an advanced type of cell culture, allowing cell to grow in three dimensional direction to achieve more relevant physiological behavior including apical basal polarization of cells, formation of lumen, more relevant RNA and proteins expression and representation of better hallmark of *in v*ivo tissues [228,229]. Thus, 3D cell culture is used to co-culture the cells (combining the cell lines in such a way that they represent the tissues and organs), to control the fluids spatially by microfluid system, to elucidate the angiogenesis and tumor invasion assay and to develop vasculature models [228].

Since, human normal and cancer cell lines are derived from human beings, thus, these cell lines are close to the human models. However, as cell lines are not the whole organs, thus it does not give the answer of some questions.

3.4.2 Isolated organs

Isolated organs of animal and humans also play an important role in biomedical and pharmaceutical studies. The diseased or normal organs are used in these studies. Intestine, heart, arteries, muscles and liver are important organs used for biomedical studies [230-232]. Usually surgically removed diseased organs or tumors from humans, whereas healthy as well as chemical or germ induced diseased organs from animals are used in these studies, either to elucidate the effect of xenobiotics or to identify transporters, enzymes or other cancer hallmarks [227]. Isolated organs are the better experimental models for certain types of studies such as studying heart rate, ionotropic, chronotropic, spasmolytic and identifying transporters, metabolites and cancer hallmarks in the samples. However, isolated organs are not much suitable for the complex studies involving several organs and immune systems.

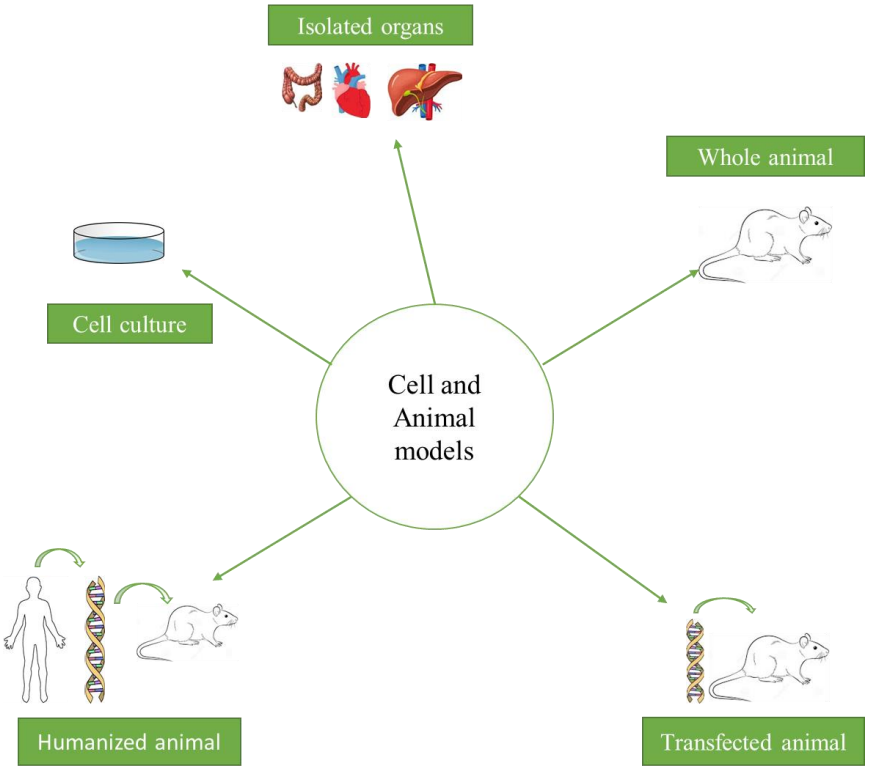

Fig 5: Cells and animal experimental model; Cell culture and isolated animal organs are considered *in vitro* studies; whole animal, transfected and humanized animals are considered *in vivo* studies

3.4.3 Whole animal

With the development of identical breeds, the use of animals in life sciences field is much increased. Guinea pig, mice, rats and primates are commonly used in biomedical research [233]. Usually drug or xenobiotics are fed to the healthy animals and impact of substances is studied. However, with the advancement of science, it is also possible to induce the disease in animals by using germs or substances. For example, mice with liver cancer can be produced by using liver toxins, whereas mice with cystic fibrosis or pneumonia can be induced with respective germs [234].

While using the whole animal model, one should be really very careful in choosing the animal because the course of disease may be different in human and the animal. For example, cystic fibrosis in mice can develop intestinal disease similar to human but fails to develop pulmonary complications similar to human beings. However, Swine model can replicate the intestinal as well as pulmonary complications [235]. We should also be careful in doing extrapolation of animal studies to the humans because drug transporters and metabolism enzymes may be different in animals compared to humans. For example, study

conducted for metabolism of curcumin both in rat and human models reveals that sulfation of curcumin is five folds in rat intestine as compared to human intestine [22].

After reorganization of impact of GIT and dermal flora on research, the germ free mice or mice having one or two defined micro-organism strains is possible to develop with the help of Caesarian birth, flexible film isolated cage and irradiated food [236].

3.4.4 Transgenic animals

Transgenic animal having knock in or knock out genes can act as an excellent model when traditional models fails to answer the research question [233]. With the advancement of genetics, transgenic rats, mice, chicken, cats, dogs, rabbit, sheep, goat, cattle, pigs, zebra fish and non-human primates are developed to study the role of certain proteins or enzymes [233,237].

3.4.5 Humanized animals

Humanized animals are the transfected animals carrying human genes. Rats expressing human major histocompatibility locus, HLA-B27 are an example of such models. In addition, introduction of mutated human genes in animals have enabled the scientist to mimic the genetic diseases of humans in those animals [233]. For example, creation of human immune system in mice by implanting the human fetal lymphoid tissues or peripheral blood leukocytes allowed researchers to investigate hematopoiesis, basic immunology, infectious disease and autoimmune related studies [238]. Similarly, animals having human liver are important source of drug metabolism and viral hepatitis related studies [233,239].

Thus, it is concluded that cells and animals plays a key role in bio medical and pharmaceutical field.

3.5 References

1. Metzler M, Pfeiffer E, Schulz SI, Dempe JS. Curcumin uptake and metabolism. BioFactors (Oxford, England) 2013; 39: 14-20
2. Hatcher H, Planalp R, Cho J, Torti FM, Torti SV. Curcumin: from ancient medicine to current clinical trials. Cellular and molecular life sciences : CMLS 2008; 65: 1631-1652
3. Sasikumar B. Genetic resources of Curcuma: diversity, characterization and utilization. Plant Genetic Resources 2007; 3: 230-251
4. Gilani AH, Shah AJ, Ghayur MN, Majeed K. Pharmacological basis for the use of turmeric in gastrointestinal and respiratory disorders. Life Sci 2005; 76: 3089-3105
5. Goel A, Kunnumakkara AB, Aggarwal BB. Curcumin as "Curecumin": from kitchen to clinic. Biochem Pharmacol 2008; 75: 787-809
6. Sasaki Y, Goto H, Tohda C, Hatanaka F, Shibahara N, Shimada Y, Terasawa K, Komatsu K. Effects of curcuma drugs on vasomotion in isolated rat aorta. Biological & pharmaceutical bulletin 2003; 26: 1135-1143
7. Devassy JG, Nwachukwu ID, Jones PJ. Curcumin and cancer: barriers to obtaining a health claim. Nutr Rev 2015; 73: 155-165
8. Prasad S, Tyagi AK, Aggarwal BB. Recent developments in delivery, bioavailability, absorption and metabolism of curcumin: the golden pigment from golden spice. Cancer Res Treat 2014; 46: 2-18
9. Heger M, van Golen RF, Broekgaarden M, Michel MC. The molecular basis for the pharmacokinetics and pharmacodynamics of curcumin and its metabolites in relation to cancer. Pharmacological reviews 2014; 66: 222-307
10. Trujillo J, Chirino YI, Molina-Jijon E, Anderica-Romero AC, Tapia E, Pedraza-Chaverri J. Renoprotective effect of the antioxidant curcumin: Recent findings. Redox biology 2013; 1: 448-456
11. Anand P, Kunnumakkara AB, Newman RA, Aggarwal BB. Bioavailability of curcumin: problems and promises. Molecular pharmaceutics 2007; 4: 807-818
12. Anand P, Thomas SG, Kunnumakkara AB, Sundaram C, Harikumar KB, Sung B, Tharakan ST, Misra K, Priyadarsini IK, Rajasekharan KN. Biological activities of curcumin and its analogues (Congeners) made by man and Mother Nature. Biochemical pharmacology 2008; 76: 1590-1611
13. Pulido-Moran M, Moreno-Fernandez J, Ramirez-Tortosa C, Ramirez-Tortosa M. Curcumin and Health. Molecules (Basel, Switzerland) 2016; 21: 264

14. Rao TS, Basu N, Seth SD, Siddiqui HH. Some aspects of pharmacological profile of sodium curcuminate. Indian journal of physiology and pharmacology 1984; 28: 211-215
15. Bandyopadhyay D. Farmer to pharmacist: curcumin as an anti-invasive and antimetastatic agent for the treatment of cancer. Frontiers in chemistry 2014; 2: 113
16. Bimonte S, Barbieri A, Leongito M, Piccirillo M, Giudice A, Pivonello C, de Angelis C, Granata V, Palaia R, Izzo F. Curcumin anticancer studies in pancreatic cancer. Nutrients 2016; 8:
17. Shishodia S, Chaturvedi MM, Aggarwal BB. Role of curcumin in cancer therapy. Current problems in cancer 2007; 31: 243-305
18. AGGARWAL BB, KUMAR A, BHARTI AC. Anticancer Potential of Curcumin: Preclinical and Clinical Studies. ANTICANCER RESEARCH 2003; 23: 363-398
19. Lv ZD, Liu XP, Zhao WJ, Dong Q, Li FN, Wang HB, Kong B. Curcumin induces apoptosis in breast cancer cells and inhibits tumor growth in vitro and in vivo. Int J Clin Exp Pathol 2014; 7: 2818-2824
20. Kunnumakkara AB, Bordoloi D, Padmavathi G, Monisha J, Roy NK, Prasad S, Aggarwal BB. Curcumin, the golden nutraceutical: multitargeting for multiple chronic diseases. Br J Pharmacol 2016, DOI: 10.1111/bph.13621:
21. Vareed SK, Kakarala M, Ruffin MT, Crowell JA, Normolle DP, Djuric Z, Brenner DE. Pharmacokinetics of curcumin conjugate metabolites in healthy human subjects. Cancer Epidemiol Biomarkers Prev 2008; 17: 1411-1417
22. Ireson CR, Jones DJ, Orr S, Coughtrie MW, Boocock DJ, Williams ML, Farmer PB, Steward WP, Gescher AJ. Metabolism of the cancer chemopreventive agent curcumin in human and rat intestine. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology 2002; 11: 105-111
23. Wu J-C, Tsai M-L, Lai C-S, Wang Y-J, Ho C-T, Pan M-H. Chemopreventative effects of tetrahydrocurcumin on human diseases. Food Funct 2013; 5: 12-17
24. Aggarwal BB, Deb L, Prasad S. Curcumin differs from tetrahydrocurcumin for molecular targets, signaling pathways and cellular responses. Molecules (Basel, Switzerland) 2014; 20: 185-205
25. Pan MH, Lin-Shiau SY, Lin JK. Comparative studies on the suppression of nitric oxide synthase by curcumin and its hydrogenated metabolites through down-

regulation of IkappaB kinase and NFkappaB activation in macrophages. Biochem Pharmacol 2000; 60: 1665-1676

26. Ireson C, Orr S, Jones DJL, Verschoyle R, Lim C-K, Luo J-L, Howells L, Plummer S, Jukes R, Williams M, Steward WP, Gescher A. Characterization of Metabolites of the Chemopreventive Agent Curcumin in Human and Rat Hepatocytes and in the Rat in Vivo, and Evaluation of Their Ability to Inhibit Phorbol Ester-induced Prostaglandin E₂ Production. Cancer Research 2001; 61: 1058-1064
27. Li F, Nitteranon V, Tang X, Liang J, Zhang G, Parkin KL, Hu Q. In vitro antioxidant and anti-inflammatory activities of 1-dehydro-[6]-gingerdione, 6-shogaol, 6-dehydroshogaol and hexahydrocurcumin. Food chemistry 2012; 135: 332-337
28. Srimuangwong K, Tocharus C, Yoysungnoen Chintana P, Suksamrarn A, Tocharus J. Hexahydrocurcumin enhances inhibitory effect of 5-fluorouracil on HT-29 human colon cancer cells. World journal of gastroenterology 2012; 18: 2383-2389
29. Antzelevitch C, Nesterenko V, Shryock JC, Rajamani S, Song Y, Belardinelli L. The role of late I Na in development of cardiac arrhythmias. Handb Exp Pharmacol 2014; 221: 137-168
30. Jancova P, Anzenbacher P, Anzenbacherova E. Phase II drug metabolizing enzymes. Biomedical papers of the Medical Faculty of the University Palacky, Olomouc, Czechoslovakia 2010; 154: 103-116
31. Venkatakrishnan K, Von Moltke LL, Greenblatt DJ. Human drug metabolism and the cytochromes P450: application and relevance of in vitro models. Journal of clinical pharmacology 2001; 41: 1149-1179
32. Guengerich FP, Waterman MR, Egli M. Recent Structural Insights into Cytochrome P450 Function. Trends in pharmacological sciences 2016; 37: 625-640
33. Nebert DW, Wikvall K, Miller WL. Human cytochromes P450 in health and disease. Philosophical transactions of the Royal Society of London Series B, Biological sciences 2013; 368: 20120431
34. Rendic S, Guengerich FP. Survey of Human Oxidoreductases and Cytochrome P450 Enzymes Involved in the Metabolism of Xenobiotic and Natural Chemicals. Chemical research in toxicology 2015; 28: 38-42
35. Sychev DA, Ashraf GM, Svistunov AA, Maksimov ML, Tarasov VV, Chubarev VN, Otdelenov VA, Denisenko NP, Barreto GE, Aliev G. The cytochrome P450 isoenzyme and some new opportunities for the prediction of negative drug interaction in vivo. Drug design, development and therapy 2018; 12: 1147-1156

36. Kiang TK, Ensom MH, Chang TK. UDP-glucuronosyltransferases and clinical drug-drug interactions. Pharmacology & therapeutics 2005; 106: 97-132
37. Cashman JR, Perotti BY, Berkman CE, Lin J. Pharmacokinetics and molecular detoxication. Environ Health Perspect 1996; 104 Suppl 1: 23-40
38. Wells PG, Mackenzie PI, Chowdhury JR, Guillemette C, Gregory PA, Ishii Y, Hansen AJ, Kessler FK, Kim PM, Chowdhury NR, Ritter JK. Glucuronidation and the UDP-glucuronosyltransferases in health and disease. Drug Metab Dispos 2004; 32: 281-290
39. Fisher MB, Paine MF, Strelevitz TJ, Wrighton SA. The role of hepatic and extrahepatic UDP-glucuronosyltransferases in human drug metabolism. Drug metabolism reviews 2001; 33: 273-297
40. Mackenzie PI, Rogers A, Treloar J, Jorgensen BR, Miners JO, Meech R. Identification of UDP glycosyltransferase 3A1 as a UDP N-acetylglucosaminyltransferase. The Journal of biological chemistry 2008; 283: 36205-36210
41. Burchell B, Brierley CH, Rance D. Specificity of human UDP-glucuronosyltransferases and xenobiotic glucuronidation. Life Sci 1995; 57: 1819-1831
42. Rowland A, Miners JO, Mackenzie PI. The UDP-glucuronosyltransferases: their role in drug metabolism and detoxification. The international journal of biochemistry & cell biology 2013; 45: 1121-1132
43. Miners JO, Smith PA, Sorich MJ, McKinnon RA, Mackenzie PI. Predicting human drug glucuronidation parameters: application of in vitro and in silico modeling approaches. Annual review of pharmacology and toxicology 2004; 44: 1-25
44. Harbourt DE, Fallon JK, Ito S, Baba T, Ritter JK, Glish GL, Smith PC. Quantification of human uridine-diphosphate glucuronosyl transferase 1A isoforms in liver, intestine, and kidney using nanobore liquid chromatography-tandem mass spectrometry. Analytical chemistry 2012; 84: 98-105
45. Sneitz N, Court MH, Zhang X, Laajanen K, Yee KK, Dalton P, Ding X, Finel M. Human UDP-glucuronosyltransferase UGT2A2: cDNA construction, expression, and functional characterization in comparison with UGT2A1 and UGT2A3. Pharmacogenetics and genomics 2009; 19: 923-934
46. Sutherland L, Ebner T, Burchell B. The expression of UDP-glucuronosyltransferases of the UGT1 family in human liver and kidney and in response to drugs. Biochem Pharmacol 1993; 45: 295-301

47. Gregory PA, Lewinsky RH, Gardner-Stephen DA, Mackenzie PI. Regulation of UDP glucuronosyltransferases in the gastrointestinal tract. Toxicology and applied pharmacology 2004; 199: 354-363
48. Tukey RH, Strassburg CP. Genetic multiplicity of the human UDP-glucuronosyltransferases and regulation in the gastrointestinal tract. Molecular pharmacology 2001; 59: 405-414
49. Cheng Z, Radominska-Pandya A, Tephly TR. Studies on the substrate specificity of human intestinal UDP- lucuronosyltransferases 1A8 and 1A10. Drug Metab Dispos 1999; 27: 1165-1170
50. Strott CA. Sulfonation and molecular action. Endocrine reviews 2002; 23: 703-732
51. James MO, Ambadapadi S. Interactions of cytosolic sulfotransferases with xenobiotics. Drug metabolism reviews 2013; 45: 401-414
52. Gamage N, Barnett A, Hempel N, Duggleby RG, Windmill KF, Martin JL, McManus ME. Human Sulfotransferases and Their Role in Chemical Metabolism. Toxicological Sciences 2006; 90: 5-22
53. Lindsay J, Wang LL, Li Y, Zhou SF. Structure, function and polymorphism of human cytosolic sulfotransferases. Current drug metabolism 2008; 9: 99-105
54. Blanchard RL, Freimuth RR, Buck J, Weinshilboum RM, Coughtrie MW. A proposed nomenclature system for the cytosolic sulfotransferase (SULT) superfamily. Pharmacogenetics 2004; 14: 199-211
55. Coughtrie MWH. Function and organization of the human cytosolic sulfotransferase (SULT) family. Chemico-biological interactions 2016; 259: 2-7
56. Falany JL, Azziz R, Falany CN. Identification and characterization of cytosolic sulfotransferases in normal human endometrium. Chemico-biological interactions 1998; 109: 329-339
57. Suiko M, Kurogi K, Hashiguchi T, Sakakibara Y, Liu MC. Updated perspectives on the cytosolic sulfotransferases (SULTs) and SULT-mediated sulfation. Bioscience, biotechnology, and biochemistry 2017; 81: 63-72
58. Coughtrie MW. Sulfation through the looking glass--recent advances in sulfotransferase research for the curious. The pharmacogenomics journal 2002; 2: 297-308
59. Kakuta Y, Petrotchenko EV, Pedersen LC, Negishi M. The sulfuryl transfer mechanism. Crystal structure of a vanadate complex of estrogen sulfotransferase and mutational analysis. The Journal of biological chemistry 1998; 273: 27325-27330

60. Dong D, Ako R, Wu B. Crystal structures of human sulfotransferases: insights into the mechanisms of action and substrate selectivity. Expert opinion on drug metabolism & toxicology 2012; 8: 635-646
61. Pedersen LC, Petrotchenko E, Shevtsov S, Negishi M. Crystal structure of the human estrogen sulfotransferase-PAPS complex: evidence for catalytic role of Ser137 in the sulfuryl transfer reaction. The Journal of biological chemistry 2002; 277: 17928-17932
62. Rehse PH, Zhou M, Lin SX. Crystal structure of human dehydroepiandrosterone sulphotransferase in complex with substrate. The Biochemical journal 2002; 364: 165-171
63. Chiba H, Komatsu K, Lee YC, Tomizuka T, Strott CA. The 3'-terminal exon of the family of steroid and phenol sulfotransferase genes is spliced at the N-terminal glycine of the universally conserved GXXGXXK motif that forms the sulfonate donor binding site. Proceedings of the National Academy of Sciences of the United States of America 1995; 92: 8176-8179
64. Gamage NU, Duggleby RG, Barnett AC, Tresillian M, Latham CF, Liyou NE, McManus ME, Martin JL. Structure of a human carcinogen-converting enzyme, SULT1A1. Structural and kinetic implications of substrate inhibition. The Journal of biological chemistry 2003; 278: 7655-7662
65. Dajani R, Cleasby A, Neu M, Wonacott AJ, Jhoti H, Hood AM, Modi S, Hersey A, Taskinen J, Cooke RM, Manchee GR, Coughtrie MW. X-ray crystal structure of human dopamine sulfotransferase, SULT1A3. Molecular modeling and quantitative structure-activity relationship analysis demonstrate a molecular basis for sulfotransferase substrate specificity. The Journal of biological chemistry 1999; 274: 37862-37868
66. Yoshinari K, Petrotchenko EV, Pedersen LC, Negishi M. Crystal structure-based studies of cytosolic sulfotransferase. Journal of biochemical and molecular toxicology 2001; 15: 67-75
67. Weinshilboum R. Phenol sulfotransferase inheritance. Cellular and molecular neurobiology 1988; 8: 27-34
68. Richard K, Hume R, Kaptein E, Stanley EL, Visser TJ, Coughtrie MW. Sulfation of thyroid hormone and dopamine during human development: ontogeny of phenol sulfotransferases and arylsulfatase in liver, lung, and brain. The Journal of clinical endocrinology and metabolism 2001; 86: 2734-2742

69. Gamage N, Barnett A, Hempel N, Duggleby RG, Windmill KF, Martin JL, McManus ME. Human sulfotransferases and their role in chemical metabolism. Toxicological sciences : an official journal of the Society of Toxicology 2006; 90: 5-22
70. Glatt H, Meinl W. Pharmacogenetics of soluble sulfotransferases (SULTs). Naunyn Schmiedebergs Arch Pharmacol 2004; 369: 55-68
71. Brix LA, Barnett AC, Duggleby RG, Leggett B, McManus ME. Analysis of the substrate specificity of human sulfotransferases SULT1A1 and SULT1A3: site-directed mutagenesis and kinetic studies. Biochemistry 1999; 38: 10474-10479
72. Javitt NB, Lee YC, Shimizu C, Fuda H, Strott CA. Cholesterol and hydroxycholesterol sulfotransferases: identification, distinction from dehydroepiandrosterone sulfotransferase, and differential tissue expression. Endocrinology 2001; 142: 2978-2984
73. Sakakibara Y, Takami Y, Zwieb C, Nakayama T, Suiko M, Nakajima H, Liu MC. Purification, characterization, and molecular cloning of a novel rat liver Dopa/tyrosine sulfotransferase. The Journal of biological chemistry 1995; 270: 30470-30478
74. Pai TG, Sugahara T, Suiko M, Sakakibara Y, Xu F, Liu MC. Differential xenoestrogen-sulfating activities of the human cytosolic sulfotransferases: molecular cloning, expression, and purification of human SULT2B1a and SULT2B1b sulfotransferases. Biochimica et biophysica acta 2002; 1573: 165-170
75. Sakakibara Y, Yanagisawa K, Katafuchi J, Ringer DP, Takami Y, Nakayama T, Suiko M, Liu MC. Molecular cloning, expression, and characterization of novel human SULT1C sulfotransferases that catalyze the sulfonation of N-hydroxy-2-acetylaminofluorene. The Journal of biological chemistry 1998; 273: 33929-33935
76. Teubner W, Meinl W, Florian S, Kretzschmar M, Glatt H. Identification and localization of soluble sulfotransferases in the human gastrointestinal tract. The Biochemical journal 2007; 404: 207-215
77. Yoshinari K, Nagata K, Shimada M, Yamazoe Y. Molecular characterization of ST1C1-related human sulfotransferase. Carcinogenesis 1998; 19: 951-953
78. Kester MH, Kaptein E, Roest TJ, van Dijk CH, Tibboel D, Meinl W, Glatt H, Coughtrie MW, Visser TJ. Characterization of human iodothyronine sulfotransferases. The Journal of clinical endocrinology and metabolism 1999; 84: 1357-1364
79. Falany CN, Krasnykh V, Falany JL. Bacterial expression and characterization of a cDNA for human liver estrogen sulfotransferase. The Journal of steroid biochemistry and molecular biology 1995; 52: 529-539

80. Forbes KJ, Hagen M, Glatt H, Hume R, Coughtrie MW. Human fetal adrenal hydroxysteroid sulphotransferase: cDNA cloning, stable expression in V79 cells and functional characterisation of the expressed enzyme. Molecular and cellular endocrinology 1995; 112: 53-60
81. Riches Z, Stanley EL, Bloomer JC, Coughtrie MW. Quantitative evaluation of the expression and activity of five major sulfotransferases (SULTs) in human tissues: the SULT "pie". Drug Metab Dispos 2009; 37: 2255-2261
82. Baulieu EE. Neurosteroids: of the nervous system, by the nervous system, for the nervous system. Recent progress in hormone research 1997; 52: 1-32
83. Luu-The V, Dufort I, Paquet N, Reimnitz G, Labrie F. Structural characterization and expression of the human dehydroepiandrosterone sulfotransferase gene. DNA and cell biology 1995; 14: 511-518
84. Otterness DM, Wieben ED, Wood TC, Watson WG, Madden BJ, McCormick DJ, Weinshilboum RM. Human liver dehydroepiandrosterone sulfotransferase: molecular cloning and expression of cDNA. Molecular pharmacology 1992; 41: 865-872
85. Tashiro A, Sasano H, Nishikawa T, Yabuki N, Muramatsu Y, Coughtrie MW, Nagura H, Hongo M. Expression and activity of dehydroepiandrosterone sulfotransferase in human gastric mucosa. The Journal of steroid biochemistry and molecular biology 2000; 72: 149-154
86. Barker EV, Hume R, Hallas A, Coughtrie WH. Dehydroepiandrosterone sulfotransferase in the developing human fetus: quantitative biochemical and immunological characterization of the hepatic, renal, and adrenal enzymes. Endocrinology 1994; 134: 982-989
87. Fuda H, Lee YC, Shimizu C, Javitt NB, Strott CA. Mutational analysis of human hydroxysteroid sulfotransferase SULT2B1 isoforms reveals that exon 1B of the SULT2B1 gene produces cholesterol sulfotransferase, whereas exon 1A yields pregnenolone sulfotransferase. The Journal of biological chemistry 2002; 277: 36161-36166
88. Meloche CA, Falany CN. Expression and characterization of the human 3 beta-hydroxysteroid sulfotransferases (SULT2B1a and SULT2B1b). The Journal of steroid biochemistry and molecular biology 2001; 77: 261-269
89. Geese WJ, Raftogianis RB. Biochemical characterization and tissue distribution of human SULT2B1. Biochemical and biophysical research communications 2001; 288: 280-289

90. Falany CN, Xie X, Wang J, Ferrer J, Falany JL. Molecular cloning and expression of novel sulphotransferase-like cDNAs from human and rat brain. The Biochemical journal 2000; 346 Pt 3: 857-864
91. Allali-Hassani A, Pan PW, Dombrovski L, Najmanovich R, Tempel W, Dong A, Loppnau P, Martin F, Thornton J, Edwards AM, Bochkarev A, Plotnikov AN, Vedadi M, Arrowsmith CH. Structural and chemical profiling of the human cytosolic sulfotransferases. PLoS biology 2007; 5: e97
92. Sakakibara Y, Suiko M, Pai TG, Nakayama T, Takami Y, Katafuchi J, Liu MC. Highly conserved mouse and human brain sulfotransferases: molecular cloning, expression, and functional characterization. Gene 2002; 285: 39-47
93. Takahashi S, Sakakibara Y, Mishiro E, Kouriki H, Nobe R, Kurogi K, Yasuda S, Liu MC, Suiko M. Molecular cloning, expression and characterization of a novel mouse SULT6 cytosolic sulfotransferase. Journal of biochemistry 2009; 146: 399-405
94. Freimuth RR, Wiepert M, Chute CG, Wieben ED, Weinshilboum RM. Human cytosolic sulfotransferase database mining: identification of seven novel genes and pseudogenes. The pharmacogenomics journal 2004; 4: 54-65
95. Shitara Y, Horie T, Sugiyama Y. Transporters as a determinant of drug clearance and tissue distribution. European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences 2006; 27: 425-446
96. Zhou F, Zhu L, Wang K, Murray M. Recent advance in the pharmacogenomics of human Solute Carrier Transporters (SLCs) in drug disposition. Advanced Drug Delivery Reviews, DOI: http://dx.doi.org/10.1016/j.addr.2016.06.004:
97. Hong M. Biochemical studies on the structure–function relationship of major drug transporters in the ATP-binding cassette family and solute carrier family. Advanced Drug Delivery Reviews, DOI: http://dx.doi.org/10.1016/j.addr.2016.06.003:
98. Yu XQ, Xue CC, Wang G, Zhou SF. Multidrug resistance associated proteins as determining factors of pharmacokinetics and pharmacodynamics of drugs. Current drug metabolism 2007; 8: 787-802
99. Mo W, Zhang JT. Human ABCG2: structure, function, and its role in multidrug resistance. International journal of biochemistry and molecular biology 2012; 3: 1-27
100. Choudhuri S, Klaassen CD. Structure, Function, Expression, Genomic Organization, and Single Nucleotide Polymorphisms of Human ABCB1 (MDR1), ABCC (MRP), and ABCG2 (BCRP) Efflux Transporters. International Journal of Toxicology 2006; 25: 231-259

101. Efferth T, Volm M. Multiple resistance to carcinogens and xenobiotics: P-glycoproteins as universal detoxifiers. Arch Toxicol 2017; 91: 2515-2538
102. Roninson IB, Chin JE, Choi KG, Gros P, Housman DE, Fojo A, Shen DW, Gottesman MM, Pastan I. Isolation of human mdr DNA sequences amplified in multidrug-resistant KB carcinoma cells. Proceedings of the National Academy of Sciences of the United States of America 1986; 83: 4538-4542
103. Chufan EE, Sim HM, Ambudkar SV. Molecular basis of the polyspecificity of P-glycoprotein (ABCB1): recent biochemical and structural studies. Advances in cancer research 2015; 125: 71-96
104. Thiebaut F, Tsuruo T, Hamada H, Gottesman MM, Pastan I, Willingham MC. Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. Proceedings of the National Academy of Sciences of the United States of America 1987; 84: 7735-7738
105. Sugawara I, Kataoka I, Morishita Y, Hamada H, Tsuruo T, Itoyama S, Mori S. Tissue distribution of P-glycoprotein encoded by a multidrug-resistant gene as revealed by a monoclonal antibody, MRK 16. Cancer Res 1988; 48: 1926-1929
106. Borst P, Elferink RO. Mammalian ABC transporters in health and disease. Annual review of biochemistry 2002; 71: 537-592
107. Peña-Solórzano D, Stark SA, König B, Sierra CA, Ochoa-Puentes C. ABCG2/BCRP: Specific and Nonspecific Modulators. Medicinal Research Reviews 2016; 37: 987-1050
108. Mao Q. Role of the breast cancer resistance protein (ABCG2) in drug transport. The AAPS journal 2005; 7: E118-E133
109. Mao Q, Unadkat JD. Role of the breast cancer resistance protein (BCRP/ABCG2) in drug transport--an update. The AAPS journal 2015; 17: 65-82
110. Kruh GD, Belinsky MG. The MRP family of drug efflux pumps. Oncogene 2003; 22: 7537-7552
111. Schaub TP, Kartenbeck J, Konig J, Spring H, Dorsam J, Staehler G, Storkel S, Thon WF, Keppler D. Expression of the MRP2 gene-encoded conjugate export pump in human kidney proximal tubules and in renal cell carcinoma. Journal of the American Society of Nephrology : JASN 1999; 10: 1159-1169
112. Cha SH, Sekine T, Fukushima JI, Kanai Y, Kobayashi Y, Goya T, Endou H. Identification and characterization of human organic anion transporter 3 expressing predominantly in the kidney. Molecular pharmacology 2001; 59: 1277-1286

113. Chan LM, Lowes S, Hirst BH. The ABCs of drug transport in intestine and liver: efflux proteins limiting drug absorption and bioavailability. European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences 2004; 21: 25-51
114. Lee K, Klein-Szanto AJ, Kruh GD. Analysis of the MRP4 drug resistance profile in transfected NIH3T3 cells. Journal of the National Cancer Institute 2000; 92: 1934-1940
115. Kool M, de Haas M, Scheffer GL, Scheper RJ, van Eijk MJ, Juijn JA, Baas F, Borst P. Analysis of expression of cMOAT (MRP2), MRP3, MRP4, and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1), in human cancer cell lines. Cancer Res 1997; 57: 3537-3547
116. Kool M, van der Linden M, de Haas M, Baas F, Borst P. Expression of human MRP6, a homologue of the multidrug resistance protein gene MRP1, in tissues and cancer cells. Cancer Res 1999; 59: 175-182
117. Scheffer GL, Hu X, Pijnenborg AC, Wijnholds J, Bergen AA, Scheper RJ. MRP6 (ABCC6) detection in normal human tissues and tumors. Laboratory investigation; a journal of technical methods and pathology 2002; 82: 515-518
118. Hopper E, Belinsky MG, Zeng H, Tosolini A, Testa JR, Kruh GD. Analysis of the structure and expression pattern of MRP7 (ABCC10), a new member of the MRP subfamily. Cancer Lett 2001; 162: 181-191
119. Bera TK, Iavarone C, Kumar V, Lee S, Lee B, Pastan I. MRP9, an unusual truncated member of the ABC transporter superfamily, is highly expressed in breast cancer. Proceedings of the National Academy of Sciences of the United States of America 2002; 99: 6997-7002
120. Bera TK, Lee S, Salvatore G, Lee B, Pastan I. MRP8, a new member of ABC transporter superfamily, identified by EST database mining and gene prediction program, is highly expressed in breast cancer. Molecular medicine (Cambridge, Mass) 2001; 7: 509-516
121. Borst P, Evers R, Kool M, Wijnholds J. A family of drug transporters: the multidrug resistance-associated proteins. Journal of the National Cancer Institute 2000; 92: 1295-1302
122. Cole SP, Bhardwaj G, Gerlach JH, Mackie JE, Grant CE, Almquist KC, Stewart AJ, Kurz EU, Duncan AM, Deeley RG. Overexpression of a transporter gene in a

multidrug-resistant human lung cancer cell line. Science (New York, NY) 1992; 258: 1650-1654

123. Paulusma CC, Oude Elferink RP. The canalicular multispecific organic anion transporter and conjugated hyperbilirubinemia in rat and man. Journal of molecular medicine (Berlin, Germany) 1997; 75: 420-428

124. Jedlitschky G, Leier I, Buchholz U, Hummel-Eisenbeiss J, Burchell B, Keppler D. ATP-dependent transport of bilirubin glucuronides by the multidrug resistance protein MRP1 and its hepatocyte canalicular isoform MRP2. The Biochemical journal 1997; 327 (Pt 1): 305-310

125. Kawabe T, Chen ZS, Wada M, Uchiumi T, Ono M, Akiyama S, Kuwano M. Enhanced transport of anticancer agents and leukotriene C4 by the human canalicular multispecific organic anion transporter (cMOAT/MRP2). FEBS letters 1999; 456: 327-331

126. Madon J, Eckhardt U, Gerloff T, Stieger B, Meier PJ. Functional expression of the rat liver canalicular isoform of the multidrug resistance-associated protein. FEBS letters 1997; 406: 75-78

127. Hirohashi T, Suzuki H, Takikawa H, Sugiyama Y. ATP-dependent transport of bile salts by rat multidrug resistance-associated protein 3 (Mrp3). The Journal of biological chemistry 2000; 275: 2905-2910

128. Hinoshita E, Uchiumi T, Taguchi K, Kinukawa N, Tsuneyoshi M, Maehara Y, Sugimachi K, Kuwano M. Increased expression of an ATP-binding cassette superfamily transporter, multidrug resistance protein 2, in human colorectal carcinomas. Clinical cancer research : an official journal of the American Association for Cancer Research 2000; 6: 2401-2407

129. Koike K, Kawabe T, Tanaka T, Toh S, Uchiumi T, Wada M, Akiyama S, Ono M, Kuwano M. A canalicular multispecific organic anion transporter (cMOAT) antisense cDNA enhances drug sensitivity in human hepatic cancer cells. Cancer Res 1997; 57: 5475-5479

130. Cui Y, Konig J, Buchholz JK, Spring H, Leier I, Keppler D. Drug resistance and ATP-dependent conjugate transport mediated by the apical multidrug resistance protein, MRP2, permanently expressed in human and canine cells. Molecular pharmacology 1999; 55: 929-937

131. Kool M, van der Linden M, de Haas M, Scheffer GL, de Vree JM, Smith AJ, Jansen G, Peters GJ, Ponne N, Scheper RJ, Elferink RP, Baas F, Borst P. MRP3, an organic

anion transporter able to transport anti-cancer drugs. Proceedings of the National Academy of Sciences of the United States of America 1999; 96: 6914-6919

132. Robbins BL, Connelly MC, Marshall DR, Srinivas RV, Fridland A. A human T lymphoid cell variant resistant to the acyclic nucleoside phosphonate 9-(2-phosphonylmethoxyethyl)adenine shows a unique combination of a phosphorylation defect and increased efflux of the agent. Molecular pharmacology 1995; 47: 391-397
133. Schuetz JD, Connelly MC, Sun D, Paibir SG, Flynn PM, Srinivas RV, Kumar A, Fridland A. MRP4: A previously unidentified factor in resistance to nucleoside-based antiviral drugs. Nature medicine 1999; 5: 1048-1051
134. Adachi M, Sampath J, Lan LB, Sun D, Hargrove P, Flatley R, Tatum A, Edwards MZ, Wezeman M, Matherly L, Drake R, Schuetz J. Expression of MRP4 confers resistance to ganciclovir and compromises bystander cell killing. The Journal of biological chemistry 2002; 277: 38998-39004
135. Guo Y, Kotova E, Chen ZS, Lee K, Hopper-Borge E, Belinsky MG, Kruh GD. MRP8, ATP-binding cassette C11 (ABCC11), is a cyclic nucleotide efflux pump and a resistance factor for fluoropyrimidines 2',3'-dideoxycytidine and 9'-(2'-phosphonylmethoxyethyl)adenine. The Journal of biological chemistry 2003; 278: 29509-29514
136. Perland E, Fredriksson R. Classification Systems of Secondary Active Transporters. Trends in pharmacological sciences 2017; 38: 305-315
137. César-Razquin A, Snijder B, Frappier-Brinton T, Isserlin R, Gyimesi G, Bai X, Reithmeier Reinhart A, Hepworth D, Hediger Matthias A, Edwards Aled M, Superti-Furga G. A Call for Systematic Research on Solute Carriers. Cell 2015; 162: 478-487
138. Rives ML, Javitch JA, Wickenden AD. Potentiating SLC transporter activity: Emerging drug discovery opportunities. Biochem Pharmacol 2017; 135: 1-11
139. El-Gebali S, Bentz S, Hediger MA, Anderle P. Solute carriers (SLCs) in cancer. Molecular Aspects of Medicine 2013; 34: 719-734
140. Roth M, Obaidat A, Hagenbuch B. OATPs, OATs and OCTs: the organic anion and cation transporters of the SLCO and SLC22A gene superfamilies. British journal of pharmacology 2012; 165: 1260-1287
141. Klaassen CD, Aleksunes LM. Xenobiotic, bile acid, and cholesterol transporters: function and regulation. Pharmacological reviews 2010; 62: 1-96
142. Lin L, Yee SW, Kim RB, Giacomini KM. SLC transporters as therapeutic targets: emerging opportunities. Nature reviews Drug discovery 2015; 14: 543-560

143. Obaidat A, Roth M, Hagenbuch B. The expression and function of organic anion transporting polypeptides in normal tissues and in cancer. Annual review of pharmacology and toxicology 2012; 52: 135-151
144. Hagenbuch B, Gui C. Xenobiotic transporters of the human organic anion transporting polypeptides (OATP) family. Xenobiotica 2008; 38: 778-801
145. Hagenbuch B, Stieger B. The SLCO (former SLC21) superfamily of transporters. Mol Aspects Med 2013; 34: 396-412
146. Gui C, Miao Y, Thompson L, Wahlgren B, Mock M, Stieger B, Hagenbuch B. Effect of pregnane X receptor ligands on transport mediated by human OATP1B1 and OATP1B3. European journal of pharmacology 2008; 584: 57-65
147. Lee HJ, Zheng JJ. PDZ domains and their binding partners: structure, specificity, and modification. Cell communication and signaling : CCS 2010; 8: 8
148. Wang P, Wang JJ, Xiao Y, Murray JW, Novikoff PM, Angeletti RH, Orr GA, Lan D, Silver DL, Wolkoff AW. Interaction with PDZK1 is required for expression of organic anion transporting protein 1A1 on the hepatocyte surface. The Journal of biological chemistry 2005; 280: 30143-30149
149. Stieger B, Hagenbuch B. Organic anion-transporting polypeptides. Current topics in membranes 2014; 73: 205-232
150. Thakkar N, Lockhart AC, Lee W. Role of Organic Anion-Transporting Polypeptides (OATPs) in Cancer Therapy. The AAPS journal 2015; 17: 535-545
151. Hamada A, Sissung T, Price DK, Danesi R, Chau CH, Sharifi N, Venzon D, Maeda K, Nagao K, Sparreboom A, Mitsuya H, Dahut WL, Figg WD. Effect of SLCO1B3 haplotype on testosterone transport and clinical outcome in caucasian patients with androgen-independent prostatic cancer. Clinical cancer research : an official journal of the American Association for Cancer Research 2008; 14: 3312-3318
152. Wright JL, Kwon EM, Ostrander EA, Montgomery RB, Lin DW, Vessella R, Stanford JL, Mostaghel EA. Expression of SLCO transport genes in castration-resistant prostate cancer and impact of genetic variation in SLCO1B3 and SLCO2B1 on prostate cancer outcomes. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology 2011; 20: 619-627
153. Ballestero MR, Monte MJ, Briz O, Jimenez F, Gonzalez-San Martin F, Marin JJ. Expression of transporters potentially involved in the targeting of cytostatic bile acid derivatives to colon cancer and polyps. Biochem Pharmacol 2006; 72: 729-738

154. Bronger H, Konig J, Kopplow K, Steiner HH, Ahmadi R, Herold-Mende C, Keppler D, Nies AT. ABCC drug efflux pumps and organic anion uptake transporters in human gliomas and the blood-tumor barrier. Cancer Res 2005; 65: 11419-11428
155. Miki Y, Suzuki T, Kitada K, Yabuki N, Shibuya R, Moriya T, Ishida T, Ohuchi N, Blumberg B, Sasano H. Expression of the steroid and xenobiotic receptor and its possible target gene, organic anion transporting polypeptide-A, in human breast carcinoma. Cancer Res 2006; 66: 535-542
156. Meyer zu Schwabedissen HE, Tirona RG, Yip CS, Ho RH, Kim RB. Interplay between the nuclear receptor pregnane X receptor and the uptake transporter organic anion transporter polypeptide 1A2 selectively enhances estrogen effects in breast cancer. Cancer Res 2008; 68: 9338-9347
157. Banerjee N, Fonge H, Mikhail A, Reilly RM, Bendayan R, Allen C. Estrone-3-sulphate, a potential novel ligand for targeting breast cancers. PloS one 2013; 8: e64069
158. Arakawa H, Nakanishi T, Yanagihara C, Nishimoto T, Wakayama T, Mizokami A, Namiki M, Kawai K, Tamai I. Enhanced expression of organic anion transporting polypeptides (OATPs) in androgen receptor-positive prostate cancer cells: possible role of OATP1A2 in adaptive cell growth under androgen-depleted conditions. Biochem Pharmacol 2012; 84: 1070-1077
159. Liedauer R, Svoboda M, Wlcek K, Arrich F, Ja W, Toma C, Thalhammer T. Different expression patterns of organic anion transporting polypeptides in osteosarcomas, bone metastases and aneurysmal bone cysts. Oncology reports 2009; 22: 1485-1492
160. Cui Y, Konig J, Nies AT, Pfannschmidt M, Hergt M, Franke WW, Alt W, Moll R, Keppler D. Detection of the human organic anion transporters SLC21A6 (OATP2) and SLC21A8 (OATP8) in liver and hepatocellular carcinoma. Laboratory investigation; a journal of technical methods and pathology 2003; 83: 527-538
161. Zollner G, Wagner M, Fickert P, Silbert D, Fuchsbichler A, Zatloukal K, Denk H, Trauner M. Hepatobiliary transporter expression in human hepatocellular carcinoma. Liver international : official journal of the International Association for the Study of the Liver 2005; 25: 367-379
162. Monks NR, Liu S, Xu Y, Yu H, Bendelow AS, Moscow JA. Potent cytotoxicity of the phosphatase inhibitor microcystin LR and microcystin analogues in OATP1B1- and OATP1B3-expressing HeLa cells. Mol Cancer Ther 2007; 6: 587-598

163. Libra A, Fernetti C, Lorusso V, Visigalli M, Anelli PL, Staud F, Tiribelli C, Pascolo L. Molecular determinants in the transport of a bile acid-derived diagnostic agent in tumoral and nontumoral cell lines of human liver. The Journal of pharmacology and experimental therapeutics 2006; 319: 809-817
164. Vander Borght S, Libbrecht L, Blokzijl H, Faber KN, Moshage H, Aerts R, Van Steenbergen W, Jansen PL, Desmet VJ, Roskams TA. Diagnostic and pathogenetic implications of the expression of hepatic transporters in focal lesions occurring in normal liver. The Journal of pathology 2005; 207: 471-482
165. Svoboda M, Wlcek K, Taferner B, Hering S, Stieger B, Tong D, Zeillinger R, Thalhammer T, Jager W. Expression of organic anion-transporting polypeptides 1B1 and 1B3 in ovarian cancer cells: relevance for paclitaxel transport. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie 2011; 65: 417-426
166. Vavricka SR, Jung D, Fried M, Grutzner U, Meier PJ, Kullak-Ublick GA. The human organic anion transporting polypeptide 8 (SLCO1B3) gene is transcriptionally repressed by hepatocyte nuclear factor 3beta in hepatocellular carcinoma. Journal of hepatology 2004; 40: 212-218
167. Abe T, Unno M, Onogawa T, Tokui T, Kondo TN, Nakagomi R, Adachi H, Fujiwara K, Okabe M, Suzuki T, Nunoki K, Sato E, Kakyo M, Nishio T, Sugita J, Asano N, Tanemoto M, Seki M, Date F, Ono K, Kondo Y, Shiiba K, Suzuki M, Ohtani H, Shimosegawa T, Iinuma K, Nagura H, Ito S, Matsuno S. LST-2, a human liver-specific organic anion transporter, determines methotrexate sensitivity in gastrointestinal cancers. Gastroenterology 2001; 120: 1689-1699
168. Muto M, Onogawa T, Suzuki T, Ishida T, Rikiyama T, Katayose Y, Ohuchi N, Sasano H, Abe T, Unno M. Human liver-specific organic anion transporter-2 is a potent prognostic factor for human breast carcinoma. Cancer science 2007; 98: 1570-1576
169. Lockhart AC, Harris E, Lafleur BJ, Merchant NB, Washington MK, Resnick MB, Yeatman TJ, Lee W. Organic anion transporting polypeptide 1B3 (OATP1B3) is overexpressed in colorectal tumors and is a predictor of clinical outcome. Clinical and experimental gastroenterology 2008; 1: 1-7
170. Wlcek K, Svoboda M, Thalhammer T, Sellner F, Krupitza G, Jaeger W. Altered expression of organic anion transporter polypeptide (OATP) genes in human breast carcinoma. Cancer Biol Ther 2008; 7: 1450-1455
171. Wlcek K, Svoboda M, Riha J, Zakaria S, Olszewski U, Dvorak Z, Sellner F, Ellinger I, Jager W, Thalhammer T. The analysis of organic anion transporting polypeptide

(OATP) mRNA and protein patterns in primary and metastatic liver cancer. Cancer Biol Ther 2011; 11: 801-811

172. Holla VR, Backlund MG, Yang P, Newman RA, DuBois RN. Regulation of prostaglandin transporters in colorectal neoplasia. Cancer prevention research (Philadelphia, Pa) 2008; 1: 93-99
173. Tamai I, Nezu J, Uchino H, Sai Y, Oku A, Shimane M, Tsuji A. Molecular identification and characterization of novel members of the human organic anion transporter (OATP) family. Biochemical and biophysical research communications 2000; 273: 251-260
174. Al Sarakbi W, Mokbel R, Salhab M, Jiang WG, Reed MJ, Mokbel K. The role of STS and OATP-B mRNA expression in predicting the clinical outcome in human breast cancer. Anticancer Res 2006; 26: 4985-4990
175. Pressler H, Sissung TM, Venzon D, Price DK, Figg WD. Expression of OATP family members in hormone-related cancers: potential markers of progression. PloS one 2011; 6: e20372
176. Pizzagalli F, Varga Z, Huber RD, Folkers G, Meier PJ, St-Pierre MV. Identification of steroid sulfate transport processes in the human mammary gland. The Journal of clinical endocrinology and metabolism 2003; 88: 3902-3912
177. Nozawa T, Suzuki M, Takahashi K, Yabuuchi H, Maeda T, Tsuji A, Tamai I. Involvement of estrone-3-sulfate transporters in proliferation of hormone-dependent breast cancer cells. The Journal of pharmacology and experimental therapeutics 2004; 311: 1032-1037
178. Nozawa T, Suzuki M, Yabuuchi H, Irokawa M, Tsuji A, Tamai I. Suppression of cell proliferation by inhibition of estrone-3-sulfate transporter in estrogen-dependent breast cancer cells. Pharmaceutical research 2005; 22: 1634-1641
179. Kleberg K, Jensen GM, Christensen DP, Lundh M, Grunnet LG, Knuhtsen S, Poulsen SS, Hansen MB, Bindslev N. Transporter function and cyclic AMP turnover in normal colonic mucosa from patients with and without colorectal neoplasia. BMC gastroenterology 2012; 12: 78
180. Kindla J, Rau TT, Jung R, Fasching PA, Strick R, Stoehr R, Hartmann A, Fromm MF, Konig J. Expression and localization of the uptake transporters OATP2B1, OATP3A1 and OATP5A1 in non-malignant and malignant breast tissue. Cancer Biol Ther 2011; 11: 584-591

181. Olszewski-Hamilton U, Svoboda M, Thalhammer T, Buxhofer-Ausch V, Geissler K, Hamilton G. Organic Anion Transporting Polypeptide 5A1 (OATP5A1) in Small Cell Lung Cancer (SCLC) Cells: Possible Involvement in Chemoresistance to Satraplatin. Biomarkers in cancer 2011; 3: 31-40
182. Chan T, Zhu L, Madigan MC, Wang K, Shen W, Gillies MC, Zhou F. Human organic anion transporting polypeptide 1A2 (OATP1A2) mediates cellular uptake of all-trans-retinol in human retinal pigmented epithelial cells. British journal of pharmacology 2015; 172: 2343-2353
183. Gao B, Vavricka SR, Meier PJ, Stieger B. Differential cellular expression of organic anion transporting peptides OATP1A2 and OATP2B1 in the human retina and brain: implications for carrier-mediated transport of neuropeptides and neurosteriods in the CNS. Pflügers Archiv - European Journal of Physiology 2015; 467: 1481-1493
184. Eechoute K, Franke RM, Loos WJ, Scherkenbach LA, Boere I, Verweij J, Gurney H, Kim RB, Tirona RG, Mathijssen RH, Sparreboom A. Environmental and genetic factors affecting transport of imatinib by OATP1A2. Clinical pharmacology and therapeutics 2011; 89: 816-820
185. Kalliokoski A, Niemi M. Impact of OATP transporters on pharmacokinetics. British journal of pharmacology 2009; 158: 693-705
186. Shirasaka Y, Suzuki K, Shichiri M, Nakanishi T, Tamai I. Intestinal absorption of HMG-CoA reductase inhibitor pitavastatin mediated by organic anion transporting polypeptide and P-glycoprotein/multidrug resistance 1. Drug Metab Pharmacokinet 2011; 26: 171-179
187. Hartkoorn RC, Kwan WS, Shallcross V, Chaikan A, Liptrott N, Egan D, Sora ES, James CE, Gibbons S, Bray PG, Back DJ, Khoo SH, Owen A. HIV protease inhibitors are substrates for OATP1A2, OATP1B1 and OATP1B3 and lopinavir plasma concentrations are influenced by SLCO1B1 polymorphisms. Pharmacogenetics and genomics 2010; 20: 112-120
188. Badagnani I, Castro RA, Taylor TR, Brett CM, Huang CC, Stryke D, Kawamoto M, Johns SJ, Ferrin TE, Carlson EJ, Burchard EG, Giacomini KM. Interaction of methotrexate with organic-anion transporting polypeptide 1A2 and its genetic variants. The Journal of pharmacology and experimental therapeutics 2006; 318: 521-529
189. Okabe M, Szakacs G, Reimers MA, Suzuki T, Hall MD, Abe T, Weinstein JN, Gottesman MM. Profiling SLCO and SLC22 genes in the NCI-60 cancer cell lines to identify drug uptake transporters. Mol Cancer Ther 2008; 7: 3081-3091

190. Ismair MG, Stieger B, Cattori V, Hagenbuch B, Fried M, Meier PJ, Kullak-Ublick GA. Hepatic uptake of cholecystokinin octapeptide by organic anion-transporting polypeptides OATP4 and OATP8 of rat and human liver. Gastroenterology 2001; 121: 1185-1190
191. Leuthold S, Hagenbuch B, Mohebbi N, Wagner CA, Meier PJ, Stieger B. Mechanisms of pH-gradient driven transport mediated by organic anion polypeptide transporters. American journal of physiology Cell physiology 2009; 296: C570-582
192. van der Deure WM, Hansen PS, Peeters RP, Kyvik KO, Friesema EC, Hegedus L, Visser TJ. Thyroid hormone transport and metabolism by organic anion transporter 1C1 and consequences of genetic variation. Endocrinology 2008; 149: 5307-5314
193. Kraft ME, Glaeser H, Mandery K, Konig J, Auge D, Fromm MF, Schlotzer-Schrehardt U, Welge-Lussen U, Kruse FE, Zolk O. The prostaglandin transporter OATP2A1 is expressed in human ocular tissues and transports the antiglaucoma prostanoid latanoprost. Investigative ophthalmology & visual science 2010; 51: 2504-2511
194. Lu R, Kanai N, Bao Y, Schuster VL. Cloning, in vitro expression, and tissue distribution of a human prostaglandin transporter cDNA(hPGT). J Clin Invest 1996; 98: 1142-1149
195. Nozawa T, Imai K, Nezu J, Tsuji A, Tamai I. Functional characterization of pH-sensitive organic anion transporting polypeptide OATP-B in human. The Journal of pharmacology and experimental therapeutics 2004; 308: 438-445
196. Huber RD, Gao B, Sidler Pfandler MA, Zhang-Fu W, Leuthold S, Hagenbuch B, Folkers G, Meier PJ, Stieger B. Characterization of two splice variants of human organic anion transporting polypeptide 3A1 isolated from human brain. American journal of physiology Cell physiology 2007; 292: C795-806
197. Fujiwara K, Adachi H, Nishio T, Unno M, Tokui T, Okabe M, Onogawa T, Suzuki T, Asano N, Tanemoto M, Seki M, Shiiba K, Suzuki M, Kondo Y, Nunoki K, Shimosegawa T, Iinuma K, Ito S, Matsuno S, Abe T. Identification of thyroid hormone transporters in humans: different molecules are involved in a tissue-specific manner. Endocrinology 2001; 142: 2005-2012
198. Gao B, Huber RD, Wenzel A, Vavricka SR, Ismair MG, Reme C, Meier PJ. Localization of organic anion transporting polypeptides in the rat and human ciliary body epithelium. Experimental eye research 2005; 80: 61-72

199. Pizzagalli F, Hagenbuch B, Stieger B, Klenk U, Folkers G, Meier PJ. Identification of a novel human organic anion transporting polypeptide as a high affinity thyroxine transporter. Molecular endocrinology (Baltimore, Md) 2002; 16: 2283-2296
200. Roberts LM, Woodford K, Zhou M, Black DS, Haggerty JE, Tate EH, Grindstaff KK, Mengesha W, Raman C, Zerangue N. Expression of the thyroid hormone transporters monocarboxylate transporter-8 (SLC16A2) and organic ion transporter-14 (SLCO1C1) at the blood-brain barrier. Endocrinology 2008; 149: 6251-6261
201. Alkemade A, Friesema EC, Kalsbeek A, Swaab DF, Visser TJ, Fliers E. Expression of thyroid hormone transporters in the human hypothalamus. The Journal of clinical endocrinology and metabolism 2011; 96: E967-971
202. Gao B, Hagenbuch B, Kullak-Ublick GA, Benke D, Aguzzi A, Meier PJ. Organic anion-transporting polypeptides mediate transport of opioid peptides across blood-brain barrier. The Journal of pharmacology and experimental therapeutics 2000; 294: 73-79
203. Nomura T, Lu R, Pucci ML, Schuster VL. The two-step model of prostaglandin signal termination: in vitro reconstitution with the prostaglandin transporter and prostaglandin 15 dehydrogenase. Molecular pharmacology 2004; 65: 973-978
204. Nomura T, Chang HY, Lu R, Hankin J, Murphy RC, Schuster VL. Prostaglandin signaling in the renal collecting duct: release, reuptake, and oxidation in the same cell. The Journal of biological chemistry 2005; 280: 28424-28429
205. Schuster VL. Prostaglandin transport. Prostaglandins & other lipid mediators 2002; 68-69: 633-647
206. Kang J, Chapdelaine P, Parent J, Madore E, Laberge PY, Fortier MA. Expression of human prostaglandin transporter in the human endometrium across the menstrual cycle. The Journal of clinical endocrinology and metabolism 2005; 90: 2308-2313
207. Choi K, Zhuang H, Crain B, Dore S. Expression and localization of prostaglandin transporter in Alzheimer disease brains and age-matched controls. Journal of neuroimmunology 2008; 195: 81-87
208. Mandery K, Bujok K, Schmidt I, Wex T, Treiber G, Malfertheiner P, Rau TT, Amann KU, Brune K, Fromm MF, Glaeser H. Influence of cyclooxygenase inhibitors on the function of the prostaglandin transporter organic anion-transporting polypeptide 2A1 expressed in human gastroduodenal mucosa. The Journal of pharmacology and experimental therapeutics 2010; 332: 345-351

209. Kullak-Ublick GA, Ismair MG, Stieger B, Landmann L, Huber R, Pizzagalli F, Fattinger K, Meier PJ, Hagenbuch B. Organic anion-transporting polypeptide B (OATP-B) and its functional comparison with three other OATPs of human liver. Gastroenterology 2001; 120: 525-533

210. Kobayashi D, Nozawa T, Imai K, Nezu J, Tsuji A, Tamai I. Involvement of human organic anion transporting polypeptide OATP-B (SLC21A9) in pH-dependent transport across intestinal apical membrane. The Journal of pharmacology and experimental therapeutics 2003; 306: 703-708

211. Grube M, Kock K, Oswald S, Draber K, Meissner K, Eckel L, Bohm M, Felix SB, Vogelgesang S, Jedlitschky G, Siegmund W, Warzok R, Kroemer HK. Organic anion transporting polypeptide 2B1 is a high-affinity transporter for atorvastatin and is expressed in the human heart. Clinical pharmacology and therapeutics 2006; 80: 607-620

212. St-Pierre MV, Hagenbuch B, Ugele B, Meier PJ, Stallmach T. Characterization of an organic anion-transporting polypeptide (OATP-B) in human placenta. The Journal of clinical endocrinology and metabolism 2002; 87: 1856-1863

213. Schiffer R, Neis M, Holler D, Rodriguez F, Geier A, Gartung C, Lammert F, Dreuw A, Zwadlo-Klarwasser G, Merk H, Jugert F, Baron JM. Active influx transport is mediated by members of the organic anion transporting polypeptide family in human epidermal keratinocytes. The Journal of investigative dermatology 2003; 120: 285-291

214. Niessen J, Jedlitschky G, Grube M, Bien S, Schwertz H, Ohtsuki S, Kawakami H, Kamiie J, Oswald S, Starke K, Strobel U, Siegmund W, Rosskopf D, Greinacher A, Terasaki T, Kroemer HK. Human platelets express organic anion-transporting peptide 2B1, an uptake transporter for atorvastatin. Drug Metab Dispos 2009; 37: 1129-1137

215. Knauer MJ, Urquhart BL, Meyer zu Schwabedissen HE, Schwarz UI, Lemke CJ, Leake BF, Kim RB, Tirona RG. Human skeletal muscle drug transporters determine local exposure and toxicity of statins. Circulation research 2010; 106: 297-306

216. Ho RH, Tirona RG, Leake BF, Glaeser H, Lee W, Lemke CJ, Wang Y, Kim RB. Drug and bile acid transporters in rosuvastatin hepatic uptake: function, expression, and pharmacogenetics. Gastroenterology 2006; 130: 1793-1806

217. Adachi H, Suzuki T, Abe M, Asano N, Mizutamari H, Tanemoto M, Nishio T, Onogawa T, Toyohara T, Kasai S, Satoh F, Suzuki M, Tokui T, Unno M, Shimosegawa T, Matsuno S, Ito S, Abe T. Molecular characterization of human and

rat organic anion transporter OATP-D. American journal of physiology Renal physiology 2003; 285: F1188-1197

218. Sato K, Sugawara J, Sato T, Mizutamari H, Suzuki T, Ito A, Mikkaichi T, Onogawa T, Tanemoto M, Unno M, Abe T, Okamura K. Expression of organic anion transporting polypeptide E (OATP-E) in human placenta. Placenta 2003; 24: 144-148
219. Loubiere LS, Vasilopoulou E, Bulmer JN, Taylor PM, Stieger B, Verrey F, McCabe CJ, Franklyn JA, Kilby MD, Chan SY. Expression of thyroid hormone transporters in the human placenta and changes associated with intrauterine growth restriction. Placenta 2010; 31: 295-304
220. Chan SY, Martin-Santos A, Loubiere LS, Gonzalez AM, Stieger B, Logan A, McCabe CJ, Franklyn JA, Kilby MD. The expression of thyroid hormone transporters in the human fetal cerebral cortex during early development and in N-Tera-2 neurodifferentiation. The Journal of physiology 2011; 589: 2827-2845
221. Mikkaichi T, Suzuki T, Onogawa T, Tanemoto M, Mizutamari H, Okada M, Chaki T, Masuda S, Tokui T, Eto N, Abe M, Satoh F, Unno M, Hishinuma T, Inui K, Ito S, Goto J, Abe T. Isolation and characterization of a digoxin transporter and its rat homologue expressed in the kidney. Proceedings of the National Academy of Sciences of the United States of America 2004; 101: 3569-3574
222. Bleasby K, Castle JC, Roberts CJ, Cheng C, Bailey WJ, Sina JF, Kulkarni AV, Hafey MJ, Evers R, Johnson JM, Ulrich RG, Slatter JG. Expression profiles of 50 xenobiotic transporter genes in humans and pre-clinical species: a resource for investigations into drug disposition. Xenobiotica 2006; 36: 963-988
223. Isidor B, Pichon O, Redon R, Day-Salvatore D, Hamel A, Siwicka KA, Bitner-Glindzicz M, Heymann D, Kjellen L, Kraus C, Leroy JG, Mortier GR, Rauch A, Verloes A, David A, Le Caignec C. Mesomelia-synostoses syndrome results from deletion of SULF1 and SLCO5A1 genes at 8q13. American journal of human genetics 2010; 87: 95-100
224. Lee SY, Williamson B, Caballero OL, Chen YT, Scanlan MJ, Ritter G, Jongeneel CV, Simpson AJ, Old LJ. Identification of the gonad-specific anion transporter SLCO6A1 as a cancer/testis (CT) antigen expressed in human lung cancer. Cancer immunity 2004; 4: 13
225. Daniel CR, Labens R, Argyle D, Licka TF. Extracorporeal perfusion of isolated organs of large animals - Bridging the gap between in vitro and in vivo studies. Altex 2018; 35: 77-98

226. Jamil QUA, Jaerapong N, Zehl M, Jarukamjorn K, Jager W. Metabolism of Curcumin in Human Breast Cancer Cells: Impact of Sulfation on Cytotoxicity. Planta Med 2017; 83: 1028-1034
227. Miksits M, Wlcek K, Svoboda M, Thalhammer T, Ellinger I, Stefanzl G, Falany CN, Szekeres T, Jaeger W. Expression of sulfotransferases and sulfatases in human breast cancer: impact on resveratrol metabolism. Cancer Lett 2010; 289: 237-245
228. van Duinen V, Trietsch SJ, Joore J, Vulto P, Hankemeier T. Microfluidic 3D cell culture: from tools to tissue models. Current opinion in biotechnology 2015; 35: 118-126
229. Ravi M, Paramesh V, Kaviya SR, Anuradha E, Solomon FD. 3D cell culture systems: advantages and applications. Journal of cellular physiology 2015; 230: 16-26
230. Xie YL, Zhou M, Ma HH, Wang X, Liu JJ. Vasorelaxation effect of gastrodin on isolated thoracic aorta rings of rats. Chinese journal of integrative medicine 2015; 21: 944-948
231. Wong YL, Lautenschlager I, Dombrowsky H, Zitta K, Bein B, Krause T, Goldmann T, Frerichs I, Steinfath M, Weiler N, Albrecht M. Hydroxyethyl starch (HES 130/0.4) impairs intestinal barrier integrity and metabolic function: findings from a mouse model of the isolated perfused small intestine. PloS one 2015; 10: e0121497
232. Brunhofer-Bolzer G, Gabriel M, Studenik CR, Erker T. Discovery of small molecules with vasodilating characteristics and adjustable hydrolytic behavior. Bioorg Med Chem 2015; 23: 4710-4718
233. Ericsson AC, Crim MJ, Franklin CL. A brief history of animal modeling. Missouri medicine 2013; 110: 201-205
234. McNally PR. Chapter 25 - Drug-Induced Liver Disease. In: McNally PR ed, GI/Liver Secrets (Fourth Edition). Philadelphia: Mosby; 2010: 180-185
235. Stoltz DA, Meyerholz DK, Pezzulo AA, Ramachandran S, Rogan MP, Davis GJ, Hanfland RA, Wohlford-Lenane C, Dohrn CL, Bartlett JA, Nelson GAt, Chang EH, Taft PJ, Ludwig PS, Estin M, Hornick EE, Launspach JL, Samuel M, Rokhlina T, Karp PH, Ostedgaard LS, Uc A, Starner TD, Horswill AR, Brogden KA, Prather RS, Richter SS, Shilyansky J, McCray PB, Jr., Zabner J, Welsh MJ. Cystic fibrosis pigs develop lung disease and exhibit defective bacterial eradication at birth. Science translational medicine 2010; 2: 29ra31

236. Licht TR, Madsen B, Wilcks A. Selection of bacteria originating from a human intestinal microbiota in the gut of previously germ-free rats. FEMS microbiology letters 2007; 277: 205-209
237. Sasaki E, Suemizu H, Shimada A, Hanazawa K, Oiwa R, Kamioka M, Tomioka I, Sotomaru Y, Hirakawa R, Eto T, Shiozawa S, Maeda T, Ito M, Ito R, Kito C, Yagihashi C, Kawai K, Miyoshi H, Tanioka Y, Tamaoki N, Habu S, Okano H, Nomura T. Generation of transgenic non-human primates with germline transmission. Nature 2009; 459: 523-527
238. Shultz LD, Brehm MA, Garcia-Martinez JV, Greiner DL. Humanized mice for immune system investigation: progress, promise and challenges. Nature reviews Immunology 2012; 12: 786-798
239. Yoshizato K, Tateno C, Utoh R. Mice with liver composed of human hepatocytes as an animal model for drug testing. Current drug discovery technologies 2012; 9: 63-76

4. Aims of the Thesis

Curcumin is a yellowish orange polyphenol demonstrating several pharmacological effects. However, the major hurdle to use it as a therapeutic drug is its poor bioavailability because of its low uptake and rapid metabolism. Organic anion transporting proteins (OATPs) are the secondary active transporters involved in the uptake of several xenobiotics and endogenous compounds. Their distribution throughout the body especially in liver, kidney and intestine describes their vital role in drug uptake, distribution and elimination. However, limited data is available about expression and function of OATPs in tumor cells and their role in chemo-resistance. Thus, role of OATPs in the uptake of curcumin and its metabolites may advance the understanding of curcumin uptake process. Moreover, it is documented that after oral intake curcumin is metabolized to curcumin sulfate and curcumin glucuronide by phase-II metabolizing enzymes; sulfotransferases and glucuronidases. However, limited information was present to describe how curcumin is metabolized in breast cancer cells. Furthermore, all three curcuminoids as well as their major metabolite; tetrahydrocurcumin are pharmacologically active compounds; however, the exact contribution of these compounds in pharmacological effect and correlation of their activity with tissue uptake is still unknown. Thus, the main aims of thesis are to elucidate

i. Metabolic fate of curcumin in breast cancer cell lines and impact of metabolism on cytotoxicity.
ii. The impact of OATPs on transport of curcumin and its metabolites.
iii. The *in vitro* activities of curcuminoids and its metabolite on isolated organs of guinea pig and correlation of uptake with their activities.

This thesis consists of three manuscripts. The first manuscript describes the role of sulfotransferases on cytotoxicity of curcumin in hormone dependent ZR-75-1 and hormone independent MDA-MB-231 cell line. The second manuscript illustrates the differential role of OATP1B1, 1B3 and 2B1 in the uptake of curcumin in Chinese hamster ovarian (CHO) cell line. In addition, it also elaborates the role of OATP1B1 on the uptake of curcumin by using wild type and OATP1B1-knockdown ZR-75-1 breast cancer cell line. The third manuscript explains the differential spasmolytic activities of curcumin, its derivatives and metabolites on ileum, aorta and pulmonary artery of guinea pig while chronotropic and ionotropic activities on papillary muscle and right atrium respectively. It also describes the correlation of described activities with the tissue uptake.

5. Results

5.1 Original papers and manuscripts

5.1.1 Manuscript 1

Qurratul Ain Jamil, Natharath Jaerapong, Martin Zehl, Kanokwan Jarukamjorn, Walter Jäger; Metabolism of curcumin in human breast cancer cells: impact of sulfation on cytotoxicity; Planta Medica, (impact factor 2.494) Aug; 2017; 83(12/13) 1028-1034 DOI: 10.1055/s-0043-107885.

I performed transport and metabolism studies, HPLC analysis, cytotoxicity assay, and determination of protein concentration.

5.1.2 Manuscript 2

Qurratul Ain Jamil, Shahid Muhammad Iqbal, Walter Jaeger, Christian Studenik; Vasodilating, spasmolytic, ionotropic and chronotropic activities of curcuminoids from *Curcuma Longa* in isolated organs preparations of guinea pig; Journal of Physiology and Pharmacology (impact factor 2.478), manuscript accepted

I performed HPLC quantification of curcumin, demethoxycurcumin, bisdemethoxycurcumin and tetrahydrocurcumin in respective tissue samples, data analysis and manuscript preparation.

5.1.3 Manuscript 3

Nattharath Jaerapong, **Qurratul Ain Jamil**, Juliane Riha, Daniela Milovanovic, Geoge Krupitza, Bruno Stieger, Kanokwan Jarukomjorn, Walter Jaeger; The contribution of organic anion-transporting polypeptides 1B1, 1B3 and 2B1 to the antitumor activity of curcumin and its main metabolites against human breast cancer cells; International Journal of Oncology (impact factor 3.3), manuscript submitted

I collected, analysed and interpreted the data for OATP uptake experiments and cytotoxicity in CHO and ZR-75-1 cells.

Thieme

Metabolism of Curcumin in Human Breast Cancer Cells: Impact of Sulfation on Cytotoxicity*

Authors

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Key words

curcumin, human breast cancer cells, metabolism, sulfation, cytotoxicity

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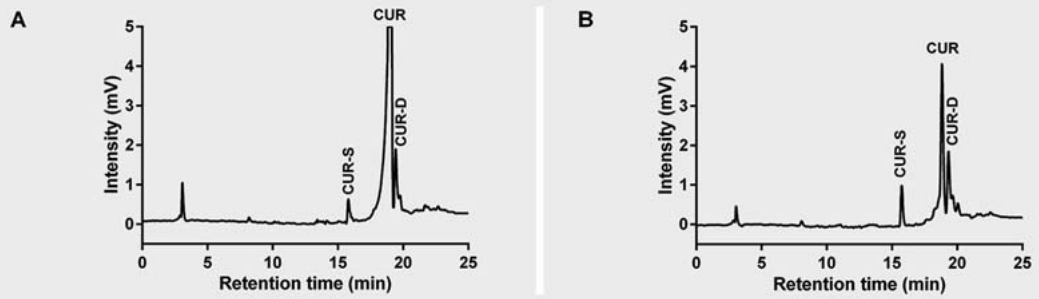
ABSTRACT

Curcumin is a natural polyphenol with promising anticancer properties that undergoes pronounced metabolism in humans. In order to determine whether metabolism of curcumin also occurs in tumor cells and whether biotransformation has any impact on cytotoxicity, metabolism experiments were conducted with hormone-dependent ZR-75-1 and hormone-independent MDA-MB-231 human breast cancer cells. By using HPLC-ESI-Qq-TOF-MS, it was possible to identify one main metabolite, namely curcumin sulfate, in both cell lines. Its concentration in the cytoplasm and culture medium was 1.6- to 1.7-fold higher in ZR-75-1 cells than in MDA-MB-231 cells, concomitant with a 2-fold higher IC_{50} value in the ZR-75-1 cell line (14 µM compared to 7.3 µM). The net result of sulfation seems to lower the intracellular concentration of curcumin, thereby decreasing its growth inhibitory activity. Interestingly, for the first time, we also found the formation of a curcumin dimer in the cytoplasm but not in the cellular medium of both cell lines. Compared to curcumin sulfate, however, its maximal intracellular concentrations were up to 4-fold lower, indicating only a minor contribution to the overall curcumin clearance. In conclusion, our data elucidated the metabolism of curcumin in breast cancer cells, which must be considered in humans following oral uptake of dietary curcumin as a chemopreventive agent.

Introduction

Breast cancer is the most common cancer for women and the second leading cause of cancer-related deaths among females worldwide. Chemoprevention in combination with anticancer treatment is therefore crucial for reducing the incidence and the mortality of this disease. Evidence from epidemiological and experimental studies indicates that natural constituents present in the diet can act as chemopreventive agents to inhibit mammary carcinogenesis [1, 2]. One of these compounds is curcumin, a bright yellow colored polyphenol extracted from the rhizome of the plant *Curcuma longa* L. (Zingiberaceae). Tumeric has been used for over 2000 years as a traditional medicine in China and India in

* Dedicated to Professor Dr. Max Wichtl in recognition of his outstanding contribution to pharmacognosy research.



▶ **Fig. 1** Representative HPLC chromatograms of curcumin (CUR) and its metabolites curcumin sulfate (CUR-S) and curcumin dimer (CUR-D) in the cytoplasm of MDA-MB-231 (**A**) and ZR-75-1 cells (**B**) taken 3 h after incubation. For details, see Materials and Methods.

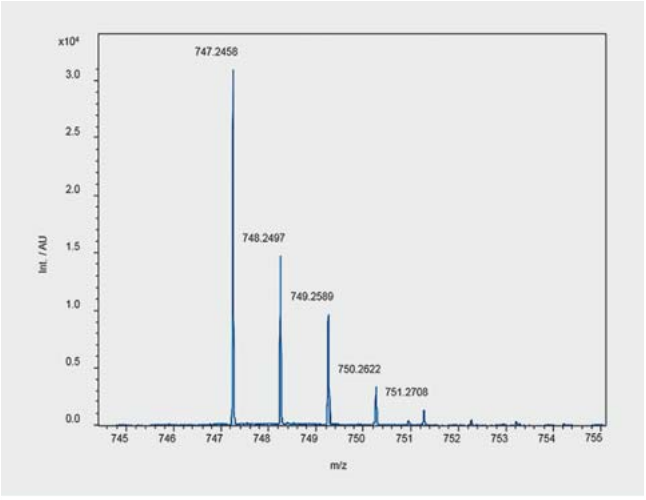
the prevention and treatment of various human diseases [3]. The breast cancer preventing activity of curcumin has been very recently first demonstrated in a mouse xenograft breast cancer model, which showed that the consumption of a curcumin supplemented diet inhibited tumor growth and angiogenesis [4]. The antiproliferative property of curcumin has been shown *in vitro* and *in vivo* against hormone-dependent and hormone-independent human breast cancer cells due to induction of apoptosis via modulating multiple intracellular signaling pathways including the transcriptional factors STAT3, NF*k*B, and AP-1, the receptors HER2 and CXCR4, the kinases EGFR, ERK, and JAK, and the cytokines TNF, IL, and MP [5–7]. Curcumin also reduces the expression of major matrix metalloproteinases (MMPs) due to reduced NF*k*B activity and transcriptional downregulation of AP-1 [8]. Reduced NF*k*B/AP-1 activity and MMP expression also lead to a significantly lower number of lung metastasis in immunodeficient mice after intercardiac injection of ER-negative human breast cancer MDA-MB-231 cells [8].

Curcumin also shows a synergistic effect *in vitro* and *in vivo* with other anticancer drugs. A very recent study demonstrated a synergistic effect of paclitaxel in combination with curcumin against human MCF-7 and MDA-MB-231 cells [9]. Improved clinical responses were also observed in a phase I clinical trial of docetaxel plus curcumin in patients with advanced and metastatic breast cancer, indicating curcumin as a favorable agent in combination with other anticancer drugs [10]. Most importantly, many clinical studies confirmed that the consumption of curcumin is safe. The European Food Safety Authority (EFSA) established an acceptable daily intake (ADI) of 3 mg/kg body weight per day based on the NOAEL (no observed adverse effect level) of 250–320 mg/kg body weight per day [11]. Studies conducted in laboratory animals have reported an extensive biotransformation of curcumin in the small intestine and liver mainly to curcumin sulfate and curcumin glucuronide [12], explaining its very low bioavailability (< 1 %) after peroral administration. Curcumin bioavailability is also poor in humans, as indicated by a pilot study of a standardized curcuma extract in colorectal cancer patients [13]. Curcumin undergoes metabolic phase II conjugation to curcumin glucuronide and curcumin sulfate, and phase I bioreduction to tetrahydrocurcumin, hexahydrocurcumin, octahydrocurcumin, and hexahydrocurcuminol [14, 15]. Reduced curcumins are also extensively biotransformed to glucuronides and sulfates [14]. Based on the rapid metabolic reduction and conjugation, free curcumin is undetectable in plasma even at high oral doses of 10 or 12 g to human volunteers. Curcumin glucuronide and sulfate, however, were easily quantified in the plasma samples of all 12 human volunteers with maximal concentrations in the very low microgram/mL range. Interestingly, glucuronidation was favored over sulfation with a ratio of glucuronide to sulfate of 1.9 : 1 [11].

It is still unknown how curcumin is metabolized in human breast cancer cells and whether biotransformation has any impact on cytotoxicity. Data from our lab showed resveratrol, like curcumin a natural polyphenol, is almost exclusively metabolized to its sulfate in breast cancer cells, whereas glucuronide formation was below the detection limit [16]. We therefore hypothesized that this might also be true for curcumin. Therefore, the aim of the present study was to investigate the metabolic fate of cucumin in hormone-dependent ZR-75-1 and hormone-independent MDA-MB-231 breast cancer cell lines. Furthermore, the chemical structures of biotransformation products should be identified by LC-MS/MS and by using identical standards. The consequences of concentration- and time-dependent metabolism in combination with cellular uptake should then be correlated with cytotoxicity in order to better explain curcumin efficacy against breast cancer.

Results

Exponentially growing estrogen-dependent ZR-75-1 and estrogen-independent MB-MDA-MB-231 breast cancer cell lines were incubated with curcumin (10–100 µM) for up to 24 h. The cytoplasm and the cellular medium were subsequently analyzed by HPLC for curcumin and its metabolites. Typical HPLC chromatograms from these experiments are shown in ▶ **Fig. 1 A, B** for MDA-MB-231 and ZR-75-1 breast cancer cells, respectively. In addition to curcumin, two biotransformation products could be detected in the cytoplasm, but only one in the medium samples of both cell lines. Analysis of these samples by HPLC-ESI-Qq-TOF-MS showed an $[M - H]^-$ ion at *m/z* 447, which showed a characteristic loss of 80 Da (SO_3) upon collision-induced dissociation



▶ **Fig. 2** HRMS showing the $[M - H]^-$ ion of a new curcumin metabolite tentatively identified as dimer of curcumin with the sum formula $C_{43}H_{40}O_{12}$.

in agreement with the molecular weight of curcumin monosulfate (data not shown). Based on an identical retention time (t_r = 15.79 min) and negative ion mass spectra compared to a commercial standard, the structure of this metabolite was confirmed as curcumin sulfate. Further analysis of the samples showed a series of nearly coeluting metabolites whose $[M - H]^-$ ions were detected in the range of *m/z* 650–760 (data not shown). The most abundant of these metabolites, with a retention time of t_r = 19.35 min, was assigned the sum formula $C_{43}H_{40}O_{12}$, which is similar but not identical to previously published curcumin dimers ($C_{42}H_{38}O_{12}$) (▶ **Fig. 2**). Thus, we assume that this biotransformation product originates from dimerization of curcumin by a different mechanism than published previously [17], which is not surprising considering that the formerly reported dimers originated from chemical oxidation in nonaqueous media. The formation of other known main curcumin metabolites, namely curcumin glucuronide and tetrahydrocurcumin, was below the detection limit in all cytoplasm and medium samples.

To further evaluate differences in the formation of curcumin sulfate and curcumin dimer in the breast cancer cell lines, MDA-MB-231 and ZR-75-1 cells were incubated for 24 h with increasing concentrations of curcumin (10–100 µM) and the cytoplasm and cellular supernatant were assayed for both biotransformation products by HPLC. In accordance with literature data [18], we found that curcumin was quite unstable at pH 7.4, leading to several degradation products like vanilin, ferulic acid, and feruloyl methane. We therefore terminated all incubations after 3 h. At this time, intracellular concentrations of unconjugated curcumin increased from 4.9 ± 1.9 pmol/h/mg protein at 10 µM curcumin to 3765 ± 608 pmol/h/mg protein at 100 µM curcumin in MDA-MB-231 cells (▶ **Fig. 3 A**). In ZR-75-1 cells, however, intracellular amounts of curcumin were significantly lower, especially at the higher curcumin concentration (472 ± 94 pmol/h/mg protein for ZR-75-1 at 100 µM) (▶ **Fig. 3 B**). Curcumin content in the cellular membranes was low (< 5 %) compared to the concentrations in the cytoplasm.

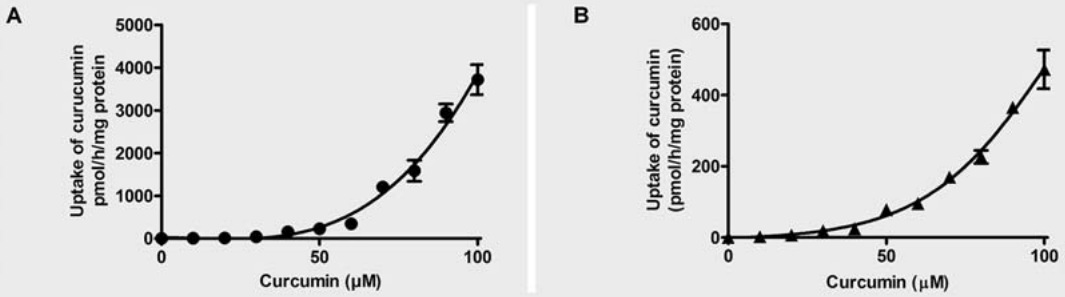
As shown in ▶ **Fig. 4 A** and ▶ **Table 1**, the kinetics of curcumin sulfation at 10–100 µM in ZR-75-1 and MDA-MB-231 cells fitted both to the Michaelis-Menten kinetics resulting in slightly higher V_{max} values (34.1 ± 2.8 pmol/h/mg protein and 27.1 ± 4.2 pmol/h/mg protein) but significantly lower K_m values (40.6 ± 8.0 µM and 72.5 ± 21.4 µM) for ZR-75-1 cells. Clearance rates (V_{max}/K_m) were therefore 2.3-fold higher in ZR-75-1 compared to MDA-MB-231 cells (0.84 ± 0.15 µL/h/mg protein and 0.37 ± 0.04 µL/h/mg protein, respectively). In order to investigate differences in the efflux of this metabolite, we also analyzed the cellular medium for curcumin sulfate. Efflux of curcumin sulfate was fast, indicating an active and rapid transport system out of the cells into the medium. Concentration-dependent efflux followed classical Michaelis-Menten kinetics with 2.2-fold higher V_{max} values for ZR-75-1 than for MDA-MB-231 cells (▶ **Fig. 4 B**). Clearance rates (V_{max}/K_m) were 1.4-fold higher in ZR-75-1 compared to MDA-MB-231 cells (7.91 ± 1.16 µL/h/mg protein and 5.61 ± 1.04 µL/h/mg protein, respectively).

Interestingly, we also found, for the first time, the formation of several curcumin dimers in the cytoplasm, but not in the cellular medium of both cell lines. The Michaelis Menten kinetics of the most abundant one is shown in ▶ **Fig. 5** and ▶ **Table 2**. Analogous to curcumin sulfate, V_{max} was more than 2-fold higher in ZR-75-1 compared to MDA-MB-231 cells leading to about a 1.9-fold higher cellular clearance (V_{max}/K_m) of 0.28 ± 0.04 versus 0.15 ± 0.05 µL/h/mg protein. Compared to curcumin sulfate, however, V_{max} values were up to 4-fold lower, indicating a less pronounced formation of this biotransformation product.

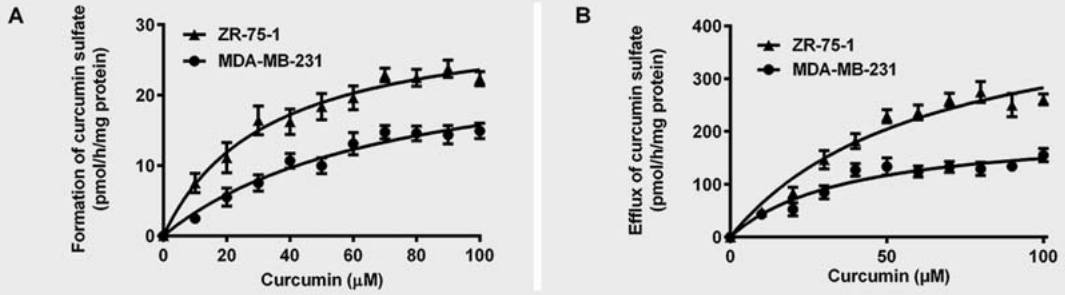
The cytotoxicity of curcumin to the human hormone-dependent ZR-75-1 and hormone-independent MDA-MB-231 breast cancer cell lines was quantified by using a CellTiter-Blue assay. As shown in ▶ **Fig. 6**, curcumin exhibited significantly lower IC_{50} values towards MDA-MB-231 cells (7.3 ± 0.59 µM) compared to ZR-75-1 cells (14.0 ± 1.2 µM).

Discussion

In the present study, uptake and metabolism of curcumin was investigated in hormone-dependent ZR-75-1 and hormone-independent MDA-MB-231 breast cancer cells with increasing concentrations of curcumin (10–100 µM). The main metabolite in both cell lines is curcumin sulfate, in which the formation in ZR-75-1 was about 2-fold higher than in MDA-MB-231 cells. Surprisingly, curcumin glucuronide, which is the major biotransformation product in human liver and intestines, could not be detected. Preferential formation of sulfates over glucuronides in breast cancer cells is in accordance with literature data and has already been shown for other compounds, such as estrogen, 2-methoxyestradiol, genistein, and resveratrol [16, 19, 20]. Curcumin sulfate concentration in the cellular medium was up to 12-fold higher compared to the cytoplasm, indicating a not yet identified active transport system for this metabolite. A likely candidate for the cellular transport into the medium is the breast cancer resistance protein (BCRP, ABCG2), which is distributed in several tissues,



▶ **Fig. 3** Concentration-dependent cellular uptake of curcumin into the cytoplasm of MDA-MB-231 (**A**) and ZR-75-1 (**B**) breast cancer cells after 3 h of incubation. Error bars indicate means ± SD of three independent experiments.



▶ **Fig. 4** Kinetics of curcumin sulfate formation in the cytoplasm (**A**) and its concentration-dependent efflux into the medium (**B**) of ZR-75-1 and MDA-MB-231 breast cancer cells after 3 h of incubation. Error bars indicate means ± SD of three independent experiments.

▶ **Table 1** Kinetic parameters of curcumin sulfate formation in and its efflux out of breast cancer cells.

	Intracellular formation of sulfate			Efflux of sulfate into medium		
Cell lines	**K_m (μM)**	**V_{max} (pmol/h/mg protein)**	**V_{max}/K_m (μL/h/mg protein)**	**K_m (μM)**	**V_{max} (pmol/h/mg protein)**	**V_{max}/K_m (μL/h/mg protein)**
ZR-75-1	40.6 ± 8.01*	34.1 ± 2.76	0.84 ± 0.15*	62.4 ± 14.6	459.5 ± 53.3*	7.91 ± 1.16*
MDA-MB-231	72.5 ± 21.4	27.1 ± 4.22	0.37 ± 0.04	37.9 ± 11.0	205.6 ± 23.5	5.61 ± 1.04

Parameters are calculated using data obtained after 3 h of incubation and presented as the mean ± SD. *Significant difference from MDA-MB-231 cells

such as placenta, small intestine, colon, and the hepatic canalicular membrane, but also in breast ductal cells, and plays an important role in the efflux of sulfated conjugates of steroids and xenobiotics [21]. Interaction of curcumin with BCRP has already been documented and may also apply to its sulfate [22].

Higher formation rates of curcumin sulfate in ZR-75-1 cells as compared with rates in MDA-MB-231 cells prompted us to suggest that sulfation may be important in the intrinsic drug activity in breast cancer cell lines. Indeed, curcumin inhibited the growth of ZR-75-1 cells with an IC_{50} value of 14 μM. In contrast, MDA-MB-231 cells are more sensitive to growth inhibition by curcumin with an IC_{50} value of 7.3 μM. MDA-MB-231 cell lysates contained lower amounts of curcumin sulfate as determined by HPLC. The majority (91 %) of this metabolite was found in ZR-75-1 cell media, maintaining significantly lower concentrations of curcumin in ZR-75-1 cells. In contrast, since less pronounced metabolism occurs in MDA-MB-231 cells, intracellular concentrations of curcumin were about 8-fold higher in this cell line. These data suggest that the differential sensitivity of ZR-75-1 and MBA-MB-231 cells to curcumin growth inhibition could be due to the intracellular formation of curcumin sulfate and its excretion from ZR-75-1 cells. Our results are in line with recent experiments from our lab

also demonstrating lower IC_{50} values for resveratrol in the hormone-independent breast cancer cell line MDA-MB-231 cells compared to the hormone-dependent ZR-75-1 one [16]. Up to now, it is not known which of the SULT enzymes are responsible for the observed differences in curcumin sulfation between the two breast cancer lines. A very recent work showed that SULT1A3, SULT1C4, and SULT1E1 are the major isoforms catalyzing sulfation of curcumin; SULT1A1 and SULT2A1 demonstrated lower formation rates [23]. RT-PCR analysis in our lab revealed that only SULT1A1 mRNA levels were significantly higher in ZR-75-1 cells whereas SULT1A3 is equally expressed. SULT1E1 and SULT2A1 mRNA levels were below the detection limit in both cell lines [20]. As SULT1C4 mRNA only has been found at high levels in human fetal lung, liver, small intestine, and kidney, and at low levels in adult kidney, ovary, and spinal cord but not in breast tissue [24], we hypothesized that SULT1A1 may mainly contribute to curcumin sulfation in ZR-75-1 and MDA-MB-231 cells leading to decreased anticancer activity. Our hypothesis is further supported by data of Irison and coworkers who indeed showed that curcumin sulfate has less biological activities compared to curcumin [11].

For the first time, we could also demonstrate the intracellular formation of curcumin dimers. The main dimer followed Michaelis-Menten formation, but its maximal concentrations in the cytoplasm were up to 4-fold lower compared to curcumin sulfate, indicating only a minor contribution to the overall curcumin clearance. It is not known whether curcumin dimers exhibit antitumor activities. However, a pharmacological activity is very likely, as gnetin, a resveratrol dimer, demonstrated cytotoxicity values comparable with resveratrol [25].

In conclusion, we found that curcumin is metabolized in human breast cancer cells mainly to curcumin sulfate. This biotransformation product does not remain inside the cells, but is rapidly excreted into the cellular medium. The net result of this action is to lower the intracellular concentration of curcumin, thereby decreasing its growth inhibitory activity as observed in ZR-75-1 cells. Curcumin sulfation may therefore also be present in breast cancer tissue in patients following oral uptake of dietary curcumin.

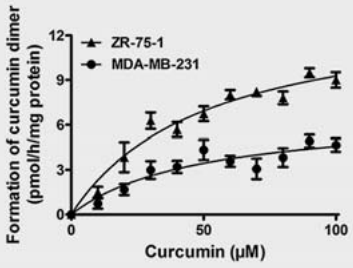
Materials and Methods

Chemicals

Curcumin (98% pure) and tetrahydrocurcumin (95% pure) were purchased from Sigma; curcumin sulfate and curcumin glucuronide was obtained from TLC Pharmaceutical Standards Ltd.; MeOH and water were of HPLC grade (Merck). All other chemicals and solvents were commercially available and of analytical grade and were used without further purification.

Cell culture

Human malignant MDA-MB-231 and ZR-75-1 breast cancer cell lines were purchased from the ATCC and were maintained in RPMI medium supplemented with 10% FCS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 1% GlutaMAX (Life Technologies). Cells were grown in T-flasks with a 25-cm^2 growth area (BD Biosciences), maintained at 37 °C under 5% CO_2 and 95% relative humidity. The cells were passaged once a week and were used up to passage 55.



▶ **Fig. 5** Kinetics of curcumin dimer in the cytoplasm of ZR-75-1 and MDA-MB-231 breast cancer cells after 3 h of incubation. Error bars indicate means ± SD of three independent experiments.

Transport and metabolism studies

MDA-MB-231 and ZR-75-1 cells (10^6 cells, each) were plated on 6-well plates and allowed to attach overnight. Curcumin was dissolved in DMSO and diluted with medium (final DMSO concentration < 0.5%) to a concentration of 10–100 µM. The experiments were performed under each set of conditions in triplicate. Control experiments contained DMSO in the medium in place of curcumin. After 3 h, the medium (1.0 mL per well) was aspirated via suction, and aliquots (100 µL) were subsequently analyzed through HPLC. The cells were then trypsinized by the addition of 100 µL of trypsin, washed three times with phosphate buffered saline, and lysed by repeated (5 times) shock freezing in liquid nitrogen and thawing. Following centrifugation at 13 500 *g* for 5 min, 80 µL of the supernatant (cytoplasm) was subsequently analyzed by HPLC. Additionally, the cell pellets containing the membranes (cell pellets) were extracted with 200 µL of methanol and analyzed by HPLC for their curcumin content. The protein concentration in the cell pellets was determined using a BCA assay kit (Pierce Science), with bovine serum albumin as a standard.

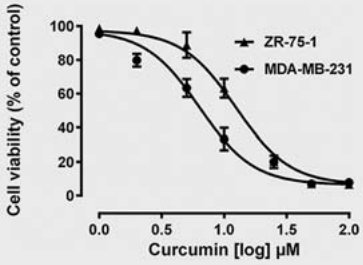
HPLC analysis

Curcumin and its biotransformation products were quantified by HPLC using a Dionex UltiMate 3000 system equipped with an L-7250 injector, an L-7100 pump, an L-7300 column oven (set at 35 °C), a D-7000 interface, and an L-7400 UV detector (Thermo Fisher Scientific) set at a wavelength of 420 nm (for tetrahydrocurcumin: 280 nm). Separation of curcumin and its metabolites was carried out at 35 °C using a Hypersil BDS-C18 column (5 µm, 250 × 4.6 mm I. D., Thermo Fisher Scientific), preceded by a Hypersil BDS-C18 precolumn (5 µm, 10 × 4.6 mm I. D.). The mobile phase consisted of a continuous linear gradient, mixed from 10 mM ammonium acetate/acetic acid buffer, pH 5.0 (mobile phase A) and methanol (mobile phase B). The flow rate was kept at 1 mL/min. The filtration of the mobile phase was performed through a 0.45-µM filter (HVLP04700; Millipore). The gradient range was from 10% methanol (0 min) to 90% methanol at 17 min followed by another increase to 95% at 18 min where it remained constant until 25 min. Subsequently, the percentage of

▶ **Table 2** Kinetic parameters of curcumin dimer formation in breast cancer cells.

Cell lines	K_m (μM)	V_{max} (pmol/h/mg protein)	V_{max}/K_m (μL/h/mg protein)
ZR-75-1	50.8 ± 11.5	13.9 ± 1.43*	0.28 ± 0.04*
MDA-MB-231	48.3 ± 21.0	6.76 ± 1.31	0.15 ± 0.05

Parameters are calculated using data obtained after 3 h of incubation and presented as the mean ± SD. *Significant difference from MDA-MB-231 cells



▶ **Fig. 6** Cytotoxicity of curcumin to human breast cancer ZR-75-1 and MDA-MB-231 breast cancer cells. Dose-response curves were obtained by nonlinear curve fitting using the GraphPad Prism 6.0 program. Note that the concentration is shown as a logarithmic function. Error bars indicate means ± SD of three independent experiments.

methanol was decreased within 2 min to 10% in order to equilibrate the column for 8 min before application of the next sample. Calibration of the chromatogram was accomplished using the external standard method. Linear calibration curves were performed by spiking drug-free cell culture medium with standard solutions of curcumin and curcumin sulfate to give a concentration range from 0.01 to 10 μg/mL (average correlation coefficients: > 0.999). Coefficients of accuracy and precision for these compounds were < 11%. Because a standard for curcumin dimer was not available in adequate amounts, quantification of this metabolite was based on the assumption that the molar extinction coefficient of curcumin dimer is similar to that of curcumin.

Structure identification of curcumin metabolites

HPLC-ESI-Qq-TOF-MS measurements of diluted cytoplasm samples (20 μL plus 80 μL water) were performed using an UltiMate 3000 RSLC-series system (Dionex/Thermo Scientific) coupled to a maXis HD ESI-Qq-TOF mass spectrometer (Bruker Daltonics). Column, mobile phase, gradient, flow rate, and injection volume were identical to those used in the analytical HPLC assay (see above). After passing the DAD, the eluate flow was split approximately 1 : 8 and the following ESI ion source settings were applied: capillary voltage: ± 4.5 kV, nebulizer: 2.0 bar (N_2), dry gas flow: 8.0 L/min (N_2), and dry temperature: 200 °C. Full scan mass spectra were recorded in the range of *m/z* 55–1000 in positive and negative ion modes. The sum formulas of the detected ions were determined using Bruker Compass Data Analysis 4.2 based on the mass accuracy ($\Delta m/z \leq 3$ ppm) and isotopic pattern matching (SmartFormula algorithm).

Cytotoxicity assay

CellTiter-Blue (Promega) is a type of a colorimetric and fluorescent assay used to measure cell viability via nonspecific redox enzyme activity (reduction from resazurin to resorufin by viable cells). MDA-MB-231 and ZR-75-1 cells (50 000 cells, each) were seeded into 96-well flat-bottomed plates and incubated for 24 h at 37 °C under 5% CO_2. For cytotoxicity assays, the cells were incubated with various concentrations of curcumin (1–100 μM) for 72 h. The CellTiter-Blue (20 μL) reagent was added to the wells, and the plate was incubated for 2 h, protected from light. The absorbance was recorded for resazurin (605 nm) and resorufin (573 nm). The assay results were measured on a Tecan M200 multimode plate reader (Tecan). The absorbance was also measured in CellTiter-Blue assays in blank wells (without curcumin) and deducted from the values from experimental wells. The viability of the treated cells was expressed as a percentage of the viability of the corresponding control cells. All experiments were repeated at least three times.

Determination of protein concentrations

Total protein was determined using the colorimetric bicinchoninic acid protein (BCA) assay kit (Pierce Science) with bovine serum albumin as a standard and quantification at a wavelength of 562 nm on a spectrophotometer (UV-1800; Shimadzu). Raw data were analyzed using UVProbe software (version 2.31; Shimadzu). The protein concentrations were consistent among the plates (0.150 ± 0.005 mg/well).

Data analysis

Kinetic analysis of curcumin metabolite formation was performed over a substrate concentration range of 10 to 100 μM for 3 h. The data were fitted to the Michaelis-Menten model. Kinetic parameters were calculated using the GraphPad Prism Version 6.0 software program for Michaelis-Menten: $V = V_{max} \cdot S/(K_m + S)$, where V is the rate of the reaction, V_{max} is the maximum velocity, K_m is the Michaelis constant, and S is the substrate concentration. The intrinsic clearance, which is defined as the ratio V_{max}/K_m, quantifies the transport capacity. IC_{50} values were calculated by fitting dose-response curves using the GraphPad Prism Version 6.0 software program. Significant differences of the kinetic values between both cells lines were determined using a Student's paired t-test at a significance level of $p < 0.05$.

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Conflict of Interest

The authors declare no conflict of interest.

References

[1] Pan MH, Chiou YS, Chen LH, Ho CT. Breast cancer chemoprevention by dietary natural phenolic compounds: specific epigenetic related molecular targets. Mol Nutr Food Res 2015; 59: 21–35

[2] Aqil F, Jeyabalan J, Munagala R, Ravoori S, Vadhanam MV, Schultz DJ, Gupta RC. Chemoprevention of rat mammary carcinogenesis by Apiaceae spices. Int J Mol Sci 2017; 18: pii: E425

[3] Hatcher H, Planalp R, Cho J, Torti FM, Torti SV. Curcumin: from ancient medicine to current clinical trials. Cell Mol Life Sci 2008; 65: 1631–1652

[4] Bimonte S, Barbieri A, Palma G, Rea D, Luciano A, D'Aiuto M, Arra C, Izzo F. Dissecting the role of curcumin in tumour growth and angiogenesis in mouse model of human breast cancer. Biomed Res Int 2015; 2015: 878134

[5] Lv ZD, Liu XP, Zhao WJ, Dong Q, Li FN, Wang HB, Kong B. Curcumin induces apoptosis in breast cancer cells and inhibits tumor growth *in vitro* and *in vivo*. Int J Clin Exp Pathol 2014; 7: 2818–2824

[6] Wang Y, Yu J, Cui R, Lin J, Ding X. Curcumin in treating breast cancer: a review. J Lab Autom 2016; 2: 723–731

[7] Kumar G, Mittal S, Sak K, Tuli HS. Molecular mechanisms underlying chemopreventive potential of curcumin: current challenges and future perspectives. Life Sci 2016; 148: 313–328

[8] Bachmeier BE, Nerlich AG, Iancu CM, Cilli M, Schleicher E, Vené R, Dell'Eva R, Jochum M, Albini A, Pfeffer U. The chemopreventive polyphenol curcumin prevents hematogenous breast cancer metastases in immunodeficient mice. Cell Physiol Biochem 2007; 19: 137–152

[9] Quispe-Soto ET, Calaf GM. Effect of curcumin and paclitaxel on breast carcinogenesis. Int J Oncol 2016; 49: 2569–2577

[10] Bayet-Robert M, Kwiatkowski F, Leheurteur M, Gachon F, Planchat E, Abrial C, Mouret-Reynier MA, Durando X, Barthomeuf C, Chollet P. Phase I dose escalation trial of docetaxel plus curcumin in patients with advanced and metastatic breast cancer. Cancer Biol Ther 2010; 9: 8–14

[11] EFSA. Scientific opinion on the re-evaluation of curcumin (E 100) as a food additive. EFSA J 2010; 8: 1679

[12] Ireson C, Orr S, Jones DJL, Verschoyle R, Lim CK, Luo JL, Howells L, Plummer S, Jukes R, Williams M, Steward WP, Gescher A. Characterization of metabolites of the chemopreventive agent curcumin in human and rat hepatocytes and in the rat *in vivo*, and evaluation of their ability to inhibit phorbol ester-induced prostaglandin E2 production. Cancer Res 2001; 61: 1058–1064

[13] Sharma RA, McLelland HR, Hill KA, Ireson CR, Euden SA, Manson MM, Pirmohamed M, Marnett LJ, Gescher AJ, Steward WP. Pharmacodynamic and pharmacokinetic study of oral Curcuma extract in patients with colorectal cancer. Clin Cancer Res 2001; 7: 1894–1900

[14] Prasad S, Tyagi AK, Aggarwal BB. Recent developments in delivery, bioavailability, absorption and metabolism of curcumin: the golden pigment from golden spice. Cancer Res Treat 2014; 46: 2–18

[15] Stanić Z. Curcumin, a compound from natural sources, a true scientific challenge – a review. Plant Foods Hum Nutr 2017; 72: 1–12

[16] Murias M, Miksits M, Aust S, Spatzenegger M, Thalhammer T, Szekeres T, Jaeger W. Metabolism of resveratrol in breast cancer cell lines: impact of sulfotransferase 1A1 expression on cell growth inhibition. Cancer Lett 2008; 261: 172–182

[17] Masuda T, Toi Y, Bando H, Maekawa T, Takeda Y, Yamaguchi H. Structural identification of new curcumin dimers and their contribution to the antioxidant mechanism of curcumin. J Agric Food Chem 2002; 50: 2524–2530

[18] Esatbeyoglu T, Huebbe P, Ernst IM, Chin D, Wagner AE, Rimbach G. Curcumin–from molecule to biological function. Angew Chem 2012; 51: 5308–5332

[19] Spink BC, Katz BH, Hussain MM, Pang S, Connor SP, Aldous KM, Gierthy JF, Spink DC. SULT1A1 catalyzes 2-methoxyestradiol sulfonation in MCF-7 breast cancer cells. Carcinogenesis 2000; 21: 1947–1957

[20] Peterson TG, Coward L, Kirk M, Falany CN, Barnes S. The role of metabolism in mammary epithelial cell growth inhibition by the isoflavones genistein and biochanin A. Carcinogenesis 1996; 17: 1861–1869

[21] Tian Y, Bian Y, Jiang Y, Qian S, Yu A, Zeng S. Interplay of breast cancer resistance protein (BCRP) and metabolizing enzymes. Curr Drug Metab 2015; 16: 877–893

[22] Ebert B, Seidel A, Lampen A. Phytochemicals induce breast cancer resistance protein in Caco-2 cells and enhance the transport of benzo[a]pyrene-3-sulfate. Toxicol Sci 2007; 96: 227–236

[23] Lu X, Jiang K, Han L, Zhang M, Zhou Y, Ma Y, Zhou Y, Meng S. Sulfonation of curcuminoids: characterization and contribution of individual SULT enzymes. Mol Nutr Food Res 2015; 59: 634–645

[24] Hui Y, Luo L, Zhang L, Kurogi K, Zhou C, Sakakibara Y, Suiko M, Liu MC. Sulfation of afimoxifene, endoxifen, raloxifene, and fulvestrant by the human cytosolic sulfotransferases (SULTs): a systematic analysis. J Pharmacol Sci 2015; 128: 144–149

[25] Narayanan NK, Kunimasa K, Yamori Y, Mori M, Mori H, Nakamura K, Miller G, Manne U, Tiwari AK, Narayanan B. Antitumor activity of melinjo (*Gnetum gnemon* L.) seed extract in human and murine tumor models *in vitro* and in a colon-26 tumor-bearing mouse model *in vivo*. Cancer Med 2015; 4: 1767–1780

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VASODILATING, SPASMOLYTIC, INOTROPIC AND CHRONOTROPIC ACTIVITIES OF CURCUMINOIDS FROM *CURCUMA LONGA* IN ISOLATED ORGAN PREPARATIONS OF GUINEA PIGS

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SUMMARY

Turmeric is a yellowish orange spice, widely used in Asian cuisine and obtained from the rhizome of *Curcuma longa*. It is a mixture of three curcuminoids namely, curcumin, demethoxycurcumin and bisdemethoxycurcumin. Turmeric has been used as a medicinal substance since ancient times for respiratory and gastrointestinal problems. The aim of the present study was to investigate which curcuminoid contributes to the observed pharmacological activities, all three curcuminoids, the major curcumin metabolite tetrahydrocurcumin, and the non-enzymatic curcumin hydrolysis products ferulic acid, feruloyl methane and vanillin were analyzed for spasmolytic, inotropic and chronotropic activity. Furthermore, their uptake in respective tissue samples was also investigated and correlated with activity. Spasmolytic activity was determined in guinea pig ileum, aorta and pulmonary artery. Inotropic and chronotropic activity was determined on guinea pig papillary muscles and right atrium respectively, while tissue uptake was quantified by using high-performance liquid chromatography (HPLC). All the curcuminoids exhibited significant spasmolytic activity with highest EC_{50} values for bisdemethoxycurcumin (5.8 ± 0.6 µM) followed by curcumin (12.9 ± 0.7 µM), demethoxycurcumin (16.8 ± 3 µM) and tetrahydrocurcumin (22.9 ± 1.5 µM). While only demethoxycurcumin was able to significantly relax the pulmonary artery with EC_{50} value of 15.78 ± 0.85 µM. All three curcuminoids showed mild negative chronotropic effects in the isolated right atrium; tetrahydrocurcumin demonstrated no activity. Curcumin and bisdemethoxycurcumin also showed mild positive inotropic effect whereas demethoxycurcumin and tetrahydrocurcumin exhibited weak negative inotropic one.

Interestingly, ferulic acid, feruloyl methane and vanillin demonstrated no pharmacologicical activity at all in the various isolated organs. All three curcuminoids and tetrahydrocurcumin

showed high uptake into the various tissues where concentrations correlated with pharmacological activity. The results indicate pronounced differences in the in vitro pharmacological activities of curcumin, demethoxycurcumin, bisdemethoxycurcumin and tetrahydrocurcumin which have to be considered in humans after per-oral intake of turmeric powder.

Key words: curcumin, demethoxycurcumin, bisdemethoxycurcumin, tetrahydrocurcumin, spasmolytic activity, guinea pig

INTRODUCTION

Turmeric is a yellowish-orange spice obtained from the rhizome of *Curcuma longa* (*C. longa*). It is a mixture of three phenolic compounds collectively called curcuminoids, which consist of mainly curcumin and smaller amounts of demethoxycurcumin and bisdemethoxycurcumin (1). Around the world turmeric is used as a spice, colorant, flavorant and condiment. Beside its culinary value it is also widely used by traditional healers since the ancient times for anemia, cough, fever, pain, jaundice, wound healing, insect bite, itching, eczema, liver disorders, urinary diseases and joint problems (2, 3). In Chinese herbal medicine curcuma is traditionally used against various syndromes caused by the obstruction of blood circulation like psychataxia and arthralgia (4). It is also used against diarrhea, flatulence, gastritis, gastroesophageal reflux disease, asthma, cough and cold (2, 5-7). These traditional uses indicate antispasmodic and/or smooth muscle relaxant effects of curcuma drugs which are well corroborated by pharmacological studies. Curcuma extracts from different species relaxed pre-contracted aorta independent of NO synthesis (4), while the extract prepared from the rhizomes of *C. longa* exhibited spasmolytic effect on rabbit intestinal preparation with the indications for calcium channel blocking activity (2). Sodium salt of curcumin reduced blood pressure and heart rate in anesthetized dogs and cats when administered intravenously. This hypotensive and bradycardic effect was not antagonized by pretreatment with propranolol, mepyramine, atropine or bilateral vagotomy thus excluding β-adrenergic, histaminergic, muscarinic or vagal nerve involvement (8). The blood pressure lowering effect and bradycardia was also reported in conscious rats by methanolic extract of *C. longa* which was attributed to calcium channel blockade (9). It also exerted spasmolytic effect on smooth muscles of guinea pig vas deferens and intestine of dogs (8).

Since, crude curcumin is a mixture of three curcuminoids and it is still unknown which specific compound contribute to the spasmolytic activity. Curcumin itself is sensitive to oxygen, UV and visible light and quite unstable at physiological pH, (10). When curcumin was added to 0.1 M phosphate buffer, pH 7.4, curcumin was stable after 1 h and then started degrading gradually. Almost 50% and 90% of curcumin is degraded after 3 and 8h of incubation, mainly forming ferulic acid, feruloyl methane and vanillin due to non-enzymatic hydrolysis (*Fig. 1*) (11). In comparison to curcumin, demethoxycurcumin and bisdemethoxycurcumin are much more stable (1). Interestingly, the instability was dependent on the concentration of the curcuminoids and was most pronounced at low concentrations (11). Moreover, curcumin have extensive intestinal metabolism (12) and in body it is rapidly metabolized by alcohol dehydrogenase into dihydrocurcumin, octahydrocurcumin (minor metabolites) and tetrahydrocurcumin, (major metabolite). These reductive metabolites are extensively conjugated with glucuronic acid and sulfuric acid and rapidly excreted into feces (1). Up to now, only few studies have reported about the spasmolytic activity of a turmeric extract containing curcumin, demethoxycurcumin and bisdemethoxycurcumin (13) Whether curcumin is the only active compound or demothoxycurcumin and bisdemethoxycurcumin, the main curcumin metabolite tetrahydrocurcumin as well as the curcumin degradation products ferulic acid, feruloyl methane and vanillin may also contribute to spasmolytic activity is not known yet. Thus, in the present study we investigated for the first time the spasmolytic activity of curcumin, desmethoxy curcuminoids, tetrahydrocurcumin and the three curcumin degradation products on guinea pig aorta, ileum, papillary muscle, pulmonary artery and right atria. As pharmacological activity is strongly dependent on tissue concentration, we also determined for the first time the tissue uptake of these compounds into different tissue preparations by

a sensitive high-performance liquid chromatography (HPLC) assay and correlated uptake with observed pharmacological effects.

MATERIALS AND METHODS

Chemicals

Curcumin, demethoxycurcumin, bisdemethoxycurcumin, tetrahydrocurcumin, ferulic acid, vanillin and feruloyl methane were purchased from Sigma-Aldrich (Munich, Germany (purity: ≥98.0%). Methanol and water were of HPLC grade and obtained by Merck, Darmstadt, Germany. All other chemicals and solvent were of analytical grade, commercially available, and used without further purification.

Experiments on isolated tissue preparations

Guinea pigs of either sex weighing 340 - 480 g were obtained from the Department of Laboratory Zoology and Genetics, Medical University, Himberg, Austria. Animals were kept in air-conditioned room at a temperature of 22 - 24 °C and relative humidity 50 - 60% with 12 hour photo period. On the day of experiments animal was sacrificed by a blow on the neck followed by, animal heart, aorta, pulmonary artery and ileum were surgically excised and kept in Krebs-Henseleit solution (NaCl 144.9 mM, KCl 4.73 mM, $CaCl_2$ 3.2 mM, $MgSO_4$ 1.18 mM, $NaHCO_3$ 24.9 mM, KH_2PO_4 1.18 mM and glucose 10 mM; pH 7.2 - 7.4), continually aerated with 95% O_2 and 5% CO_2. Papillary muscles were dissected from the right ventricle of heart and cleared from Purkinje fibers to avoid spontaneous activity. We used muscles having diameter less than 0.87 mm to ensure proper oxygen supply. The right atrium was also dissected to check the chronotropic activity. Both aorta and pulmonary artery were cleaned and rings of 5 mm were cut while ileum was cut from the terminal portion into pieces of 1 - 2 cm. One end of the dissected tissues was tied with silver wire for attachment

with tissue holder while the other end was connected with force transducer (Transbridge™, 4-Channel Transducer Amplifier, World Precision Instruments, Sarasota, FL, USA). Terminal ileum was contracted by 60 mM KCl while pulmonary artery and aorta rings with 90 mM KCl solution which produce sustained contractions in respective tissues. The test conditions might be therefore stated as "vasodilatory effect during high K-pre-constricted state". Papillary muscles were electrically stimulated by an Anapulse Stimulator model 301-T and an Isolation UnitModel 305-1 (WPI, Hamden, CT, USA) with rectangular pulses of 3 ms at a frequency of 1 Hz. The amplitude of stimulation pulse was kept 10% above the threshold level. To obtain maximum contractility from the respective tissues, a constant resting tension of 3.9 mN for papillary muscle, 4.9 mN for terminal ileum, 10.4 mN for right atrium and 19.6 mN for aorta and pulmonary artery rings was applied throughout the experiment. After a control period of 15 min, different concentrations of test compounds were applied cumulatively in a bath solution every 30 min until steady effect was obtained. The responses were recorded by a chart recorder (BD 112 Dual Channel, Kipp & Zonen) and evaluated later. Stock concentrations for test compounds were made with distilled water and where required with DMSO. To exclude the effect of DMSO, experiments were performed with solvent only and observed effect was subtracted from the response of the test compounds.

Tissue uptake and HPLC analysis

For uptake experiments tissue samples were incubated in Krebs-Henseleit at 37°C containing 100 µM curcumin, desmethoxycurcumin and bisdesmethoxycurcumin and tetrahydrocurcumin, respectivity. After 30 min, tissue samples were washed 5 times with ice cold Phosphate buffer saline (PBS) and subsequently homogenized by ULTRA TURAX® homogenizer and diluted three times with PBS followed by centrifugation at 13,500 g for 10 min (4 °C). Supernatant was carefully collected for detection and quantification of

compounds on HPLC as described previously with some minor modifications (14). For HPLC analysis a Dionex UltiMate 3000 system equipped with an L-7250 injector, an L-7100 pump, an L-7300 column oven (set at 35 °C), a D-7000 interface, and an L-7400 UV detector (Thermo fischer Scientific) set at the wavelength of 420nm for curcumin, demethoxycurcumin, bisdemethoxycurcumin and 280 nm for tetrahydrocurcumin was used. Separation of curcuminoids and their metabolite tetrahydrocurcumin was carried out at 35°C with the help of Hypersil BDS-C18 column (5 µm, 250 × 4.6 mm I.D., Thermo Fischer Scientific), followed by Hypersil BDS Precolumn (5 µm, 10 × 4.6 mm I.D.). The mobile phase consisted of a continuous linear gradient, mixed from 10 mM ammonium acetate/acetic acid buffer, pH 5.0 (mobile phase A) and methanol (mobile phase B), having a flow rate of 1 mL/min. A filter (0.45µM, HVLP04700; Millipore) was used for mobile phase filtration. The gradient was ranged from 10 - 90% methanol from 0 - 17 min followed by another increase at 18 min, and then it remained constant till 25 min. Subsequently, the percentage of methanol was decreased within 2 min to 10% for equilibrating the column for 8 min before administration of the next sample. External standard method was used for calibration of chromatogram. Linear calibration curves were performed with standard solution of the compounds, with a concentration range of 0.01 - 10 µg/ml (average correlation coefficients >0.999). Coefficients of accuracy and precision of these compounds were <11%.

Statistical analysis

For statistical analysis mean and standard error of mean (SEM) was calculated for “n” experiments and significance was determined by applying student’s t-test for paired values (Sigma Plot version 9.0). Two-way ANOVA was used in order to evaluate statistical significance in a group and between groups of tissue samples from different organs for uptake studies by using GraphPad Prism 7.

RESULTS

Spasmolytic activity on Guinea pig ileum

In order to identify and compare the spasmolytic activity of curcumin, demethoxycurcumin, bisdemethoxycurcumin, tetrahydrocurcumin, ferulic acid, feruloyl methane and vanillin we used guinea pig ileum, pre-contracted by 60 mM KCl solution. All curcuminoids but not the degradation products ferulic acid, feruloyl methane and vanillin exhibited significant spasmolytic activity in a concentration dependent manner with EC_{50} values of 12.9 ± 0.7 µM for curcumin, 16.8 ± 3 µM for demethoxycurcumin, 5.8 ± 0.6 µM for bisdemethoxycurcumin and 22.9 ± 1.5 µM for tetrahydrocurcumin (*Fig. 2A*). To rule out the involvement of NO in curcuminoid mediated spasmolytic activity (15), we inhibited endothelial nitric oxide synthase (eNOS) in pre-contracted terminal ileum by incubating with 100 µM nitro-L-arginine (L-NNA) for 45 min. After this incubation period curcuminoids and their metabolite tetrahydrocurcumin was administered in a bolus dose of 20 µM to investigate any effect on spasmolytic activity. The inhibition of eNOS does not exhibit any significant effect on spasmolytic activity (*Fig. 2B*).

Vasodilating activity on pulmonary artery and aorta

The vasodilating potency of curcuminoids and their metabolite was assessed on pulmonary artery and aorta pre-contracted by 90 mM KCl solution. Demethoxycurcumin significantly alleviated the spasm of pre-contracted pulmonary artery with EC_{50} value of 15.78 ± 0.85 µM. Contrary to demethoxycurcumin, curcumin, bisdemethoxycurcumin and tetrahydrocurcumin only modestly dilated the pulmonary artery with a relative vasodilating potency of 38.5 ± 2.8%, 24.3 ± 6.8% and 37.6 ± 8.9%, respectively, indicating that demethoxycurcumin is primarily responsible for relaxation of pulmonary artery (*Fig. 3A*). In

pre-contracted aorta tissue rings, demethoxycurcumin, bisdemethoxycurcumin and tetrahydrocurcumin declined the spasm by 35.1 ± 9.6%, 18.6 ± 5.6% and 27.9 ± 7.2% at 100 µM concentration, while no effect was observed for curcumin (*Fig. 3B*), ferulic acid, feruloyl methane and vanillin.

Inotropic activity

Inotropic activity was performed on papillary muscles. Demethoxycurcumin and tetrahydrocurcumin showed negative ionotropic activity of 23.5 ± 8.9% and 29.2 ± 2.6% respectively, curcumin demonstrated a positive ionotropic effect in papillary muscles of 28.9 ± 8.9%. Interestingly, bisdemethoxycurcumin initially showed ionotropic activity of about 20% at 30 µM which was reduced to 15.2 ± 5.6% at 100 µM (*Fig. 4*). The degradative products ferulic acid, feruloyl methane and vanillin did not exhibit any activity.

Chronotropic activity

Chronotropic activity was determined on isolated guinea pig right atrium. Curcumin, demethoxycurcumin and bisdemethoxycurcumin exhibited a negative chronotropic activity with a reduction of 27.6 ± 4.5%, 35.5 ± 5.2% and 13.4 ± 5.6% in beating frequency at 100 µM (*Fig. 5*). In contrast, tetrahydrocurcumin, ferulic acid, feruloyl methane and vanillin did not exhibit any chronotropic activity.

Tissue Uptake

For uptake experiments tissue samples of the terminal ileum, aorta, pulmonary artery, right atria and papillary muscles were incubated at 37°C with 100 µM curcumin, demethoxycurcumin and bisdemethoxycurcumin tetrahydrocurcumin, respectively. After 30 min tissue samples were washed 5 times with PBS and immediately homogenized and

analyzed by HPLC. As shown in Fig. 6, our HPLC assay allowed the clear separation of curcumin (tr = 20.63 min), desmethoxycurcumin (tr = 19.60 min), bisdesmethoxycurcumin (tr = 18.39 min) and tetrahydrocurcumin (tr = 17.74 min). Furthermore, all three curcuminoids and tetrahydrocucumin demonstrated sufficient stability at physiological pH at least for 30 min as no further peaks from metabolites or degradation products showed up in the chromatograms (*Fig. 6*). The two peaks at 3.2 and 3.8 min in the chromatogram of tetrahydrocurcumin are impurities from the incubation medium and only seen at 280 nm and not at 420 nm, which was used for the detection of the other three curcuminoids.

Uptake of curcuminoids and tetrahydrocurcumin strongly differs in the various tissue samples (*Fig. 7*). While bisdemethoxycurumin concentration was highest in the terminal ileum, desmethoxycurcumin showed the highest uptake by the pulmonary artery. In the aorta, demethoxycurcumin and bisdemethoxycurcumin levels were high and at about in the same amount. This was also true for the papillary muscle and right atrium which also showed the highest uptake for demethoxycurcumin (*see Fig. 7 and Table 1*). As ferulic acid, feruloyl methane and vanillin did not show any activity in the isolated organ model no uptake experiments were carried out.

DISCUSSION

Turmeric powder is a commonly used spice in Asian cuisine and obtained from *C. longa*. It contains 40.36 mg/g of curcuminoids with the relative proportion of each compound is approximated as 4.18 - 22.8 mg/g of curcumin, 1.08 - 9.26 mg/g of demethoxycurcumin and 0.40 - 9.50 mg/g of bisdemethoxycurcumin (16). In Southeast Asia daily dietary consumption of turmeric powder is approximated as 1.5 g/day. Turmeric powder is also available as a popular over-the-counter food supplement with the high doses

up to 10 g/day. Many of these supplements use piperine in combination with turmeric powder which can increase bioavailability of curcumin in humans up to 2000% (17). Curcuminoids are relatively safe compounds as observed in dose escalation studies, where a consumption of single dose 12 g curcumin by healthy human volunteers did not exhibit any serious side effects (18). Up to now, there are only few data in the literature about the pharmacological activities of the three curcuminoids. Recent in vitro studies showed that the potency of demethoxycurcumin and bisdemethoxycurcumin to modulate inflammatory- and cell-proliferating signaling via suppression of tumor necrosis factor (TNF)-induced nuclear factor-kappaB (NF-kappaB) activation was only slightly lower than curcumin in various human cancer cell lines (19) . Tetrahydrocurcumin, a major metabolite of curcumin, was also shown to possess various biological activities. For example, tertrahydrocurcumin inhibited lipoxygenase to the same extent as curcumin (IC_{50}: 1 μM), (20) and was more than 3-fold more active than curcumin in relieving the sciatic nerve injury of rats (21).

The three curcumin degradation products ferulic acid, feruloyl methane and vanillin were also shown to demonstrate pharmacological activity. So was the free radical scavenging properties of ferulic acid, which was about 7-fold higher compared to curcumin (22). Vanillin is capable of attenuating cancer metastasis by modulating angiogenesis in A549 lung cancer cells (23) and has been shown to have minor anti-inflammatory effects via inhibition of cyclooxygenase 2 (COX-2) (24).

Therefore, in the present work, we investigated the spasmolytic activity of three curcuminoids and their major metabolite tetrahydrocurcumin along with degradative products ferulic acid, feruloyl methane and vanillin on guinea pig ileum, aorta and pulmonary artery. We also evaluated the chronotropic and inotropic activities of these compounds on isolated right atrium and papillary muscles respectively. Beside these

biological activities we quantified the uptake of curcuminoids and tetrahydrocurcumin in respective tissue samples and compared it with biological activities.

In isolated organ preparations of ileum all compounds exhibited significant spasmolytic activity (*Fig. 2A*), thus confirming the beneficial effects of turmeric powder against gastrointestinal spasm like irritable bowel syndrome (25, 26). In a pilot study of 105 irritable bowel syndrome patients consuming 144 mg of turmeric extract for 8 weeks, abdominal pain and discomfort was reduced in 25% of the patients (27). As nitric oxide has been described to modulate gastrointestinal movements (28, 29), we assessed the effect of eNOS blockade by L-NNA on curcuminoid mediated spasmolytic activity. However, our experiments demonstrated that blockade of eNOS does not affect the spasmolytic activity of curcuminoids and their metabolite (*Fig. 2B*). The observed spasmolytic effect of curcuminoids may be therefore attributed to a blockade of calcium influx from voltage-gated calcium channels (9, 30, 31), since plant materials relaxing high potassium induced contractions possess calcium channel blocking activity (32-35). In a further experimental setting, curcumin, bisdemethoxycurcumin and tertrahydrocurcumin only modestly whereas demethoxycurcumin significantly relaxed KCl-induced contractions in pulmonary artery with EC_{50} value of 15.78 ± 0.85 µM (*Fig. 3A*). These finding indicates that demethoxycurcumin and not the other curcuminoids are mainly responsible for the vasorelaxation of *C. longa* extracts (9). This effect of demethoxycurcumin on pulmonary artery can be additionally attributed to the inhibition of phosphodiesterase-5 as observed in rat pulmonary artery where only demethoxycurcumin inhibited phosphodiesterase-5 and produced strong vasorelaxation while curcumin and bisdemethoxycurcumin produced mild vasorelaxation by interfering with calcium ion movement (36). In aorta tissue preparation, curcumin showed mild vasoconstriction whereas demethoxycurcumin, bisdemethoxycurcumin and

tetrahydrocurcumin exhibited vasorelaxant effect (*Fig. 3B*). This explains the previously reported dual effect of *C. longa* crude extract on aorta rings, where it showed weak vasoconstriction in the absence of any agonists and vasorelaxation in agonist- induced contractions (2, 31). Recent data demonstrated that low concentrations of curcumin (<5 µM) stimulates the expression of COX-2 mRNA and protein in human coronary artery endothelial cells (37). Furthermore, it also increased the expression of prostaglandin I2 (PGI2) and prostaglandin E2 (PGE2) synthase mRNA with resultant enhancement of the production of PGE2 and PGI2 when adequate amounts of arachidonic acid were present (37). As PGI2 is a potent vasodilator (38), PGI2 might contribute, at least partly, to the observed vasorelaxant property of demethoxycurcumin and bisdemethoxycurcumin in the isolated aorta rings of guinea pigs. Crude extract of *C. longa* has been reported to exhibit a variable response including both a hypotension and hypertension on arterial blood pressure in anesthetized rats (2). So, we also screened for both inotropic and chronotropic effect of individual curcuminoids and their metabolite tetrahydrocurcumin in right atrium and papillary muscles. All the compounds mildly suppressed rate of atrial contractions thus exhibiting a bradycardic effect (*Fig. 5*). However, in papillary muscle preparation both curcumin and bisdemethoxycurcumin exhibited mild positive inotropic effect whereas demethoxycurcumin and tetrahydrocurcumin showed mild negative inotropic effect (*Fig. 4*). This explains why previously a variable response both (hypotensive and hypertensive) was observed by crude extract of *C. longa* (2). Our results are also in line with a more recent study which observed that curcumin lead to a short time (3 ± 1 min) hypotensive response in non-anesthetized rats and a more prolonged (15 ± 1 min) bradycardic effect (9). This indicates the antagonizing effect of curcuminoids may be beneficial in normalizing the blood pressure. However, pharmacokinetic interactions have been reported after concomitant use of curcuminoids

with cardiovascular drugs. Cautions should be therefore taken especially after a high dose of curcuminoids (39, 40).

Uptake of curcuminoids and of tetrahydrocurcumin into various tissue samples correlated with pharmacological activities. Bisdemethoxycurcumin not only demonstrated the most potent spasmolytic activity in the ileum, its concentration in this organ was also far highest. This was also true for the uptake of demethoxycurcumin into the pulmonary artery and in the aorta where highest concentration was well correlated with the vasodilating activity. Chronotropic activity in the right atrium also correlated with far highest concentrations of demethoxycurcumin in the right atrium while tetrahydrocurcumin with negligible tissue levels showed no activity. A correlation with tissue levels was also true for demethoxycurcumin and tetrahydrocurcumin which both showed the most pronounced inotropic activity. Besides tissue levels we also observed substrate specificity at least for curcumin which selectively showed a positive inotropic effect. The higher uptake of demethoxycurcumin and bisdemethoxycurcumin in different organ preparations might be due to increased stability at physiological pH preventing their degradation to non-active ferulic acid, feruloyl methane and vanillin (41). Also tetrahydrocurcumin is considered much more stable compared to curcumin both in plasma and 0.1M phosphate buffer, irrespective to pH (42).

To summarize, our data showed that demethoxycurcumin and bisdemethoxycurcumin showed more pronounced spasmolytic, vasodilating and negative inotropic activity than curcumin indicating that both curcuminoids significantly contributed to the observed pharmacological effects of *C. longa* extract. Enriched *C. longa* extracts with a higher content of demethoxycurcumin and bisdemethoxycurcumin is therefore highly favorable leading to more therapeutic efficacy. Unfortunately, there are no data in the

literature about the bioavailability and pharmacokinetics of demethoxycurcumin, bisdemethoxycurcumin and tetrahydrocurcumin. Further studies are therefore highly warranted to elucidate bioavailability and pharmacokinetics of these compounds in animal models and humans.

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Conflict of interest

The authors declare no conflict of interest.

REFERENCES

1. Metzler M, Pfeiffer E, Schulz SI, Dempe JS. Curcumin uptake and metabolism. *Biofactors* 2013; 39(1): 14-20.

2. Gilani AH, Shah AJ, Ghayur MN, Majeed K. Pharmacological basis for the use of turmeric in gastrointestinal and respiratory disorders. *Life Sci* 2005; 76(26): 3089-105.

3. Chander MP, Kartick C, Vijayachari P. Ethnomedicinal knowledge among Karens of Andaman & Nicobar Islands, India. *J Ethnopharmacol* 2015; 162: 127-33.

4. Sasaki Y, Goto H, Tohda C*, et al.* Effects of curcuma drugs on vasomotion in isolated rat aorta. *Biol Pharm Bull* 2003; 26(8): 1135-43.

5. Eigner D, Scholz D. *Ferula asa-foetida* and *Curcuma longa* in traditional medical treatment and diet in Nepal. *J Ethnopharmacol* 1999; 67(1): 1-6.

6. Islam MK, Saha S, Mahmud I*, et al.* An ethnobotanical study of medicinal plants used by tribal and native people of Madhupur forest area, Bangladesh. *J Ethnopharmacol* 2014; 151(2): 921-30.

7. Neamsuvan O, Phumchareon T, Bunphan W, Kaosaeng W. Plant materials for gastrointestinal diseases used in Chawang District, Nakhon Si Thammarat Province, Thailand. *J Ethnopharmacol* 2016; 194: 179-87.

8. Rao TS, Basu N, Seth SD, Siddiqui HH. Some aspects of pharmacological profile of sodium curcuminate. *Indian J Physiol Pharmacol* 1984; 28(3): 211-5.

9. Adaramoye OA, Anjos RM, Almeida MM*, et al.* Hypotensive and endothelium-independent vasorelaxant effects of methanolic extract from *Curcuma longa* L. in rats. *J Ethnopharmacol* 2009; 124(3): 457-62.

10. Vijaya Saradhi UV, Ling Y, Wang J*, et al.* A liquid chromatography-tandem mass spectrometric method for quantification of curcuminoids in cell medium and mouse plasma. *J Chromatogr B Analyt Technol Biomed Life Sci* 2010; 878(30): 3045-51.

11. Pfeiffer E, Hohle S, Solyom AM, Metzler M. Studies on the stability of turmeric constituents. *J Food Eng* 2003; 56(2-3): 257-9.

12. Ravindranath V, Chandrasekhara N. In vitro studies on the intestinal absorption of curcumin in rats. *Toxicology* 1981; 20(2-3): 251-7.

13. Itthipanichpong C, Ruangrungsi N, Kemsri W, Sawasdipanich A. Antispasmodic effects of curcuminoids on isolated guinea-pig ileum and rat uterus. *J Med Assoc Thai* 2003; 86 Suppl 2: S299-309.

14. Jamil QUA, Jaerapong N, Zehl M, Jarukamjorn K, Jager W. Metabolism of Curcumin in Human Breast Cancer Cells: Impact of Sulfation on Cytotoxicity. *Planta Med* 2017; 83(12-13): 1028-34.

15. Wang TT, Zhou ZQ, Wang S*, et al.* Mechanisms of vasorelaxation induced by total flavonoids of Euphorbia humifusa in rat aorta. *J Physiol Pharmacol* 2017; 68(4): 619-28.

16. Li R, Xiang C, Ye M, Li HF, Zhang X, Guo DA. Qualitative and quantitative analysis of curcuminoids in herbal medicines derived from Curcuma species. *Food Chem* 2011; 126(4): 1890-5.

17. Shoba G, Joy D, Joseph T, Majeed M, Rajendran R, Srinivas PS. Influence of piperine on the pharmacokinetics of curcumin in animals and human volunteers. *Planta Med* 1998; 64(4): 353-6.

18. Lao CD, Ruffin MT, Normolle D*, et al.* Dose escalation of a curcuminoid formulation. *BMC Complement Altern Med* 2006; 6(1): 10.

19. Sandur SK, Pandey MK, Sung B*, et al.* Curcumin, demethoxycurcumin, bisdemethoxycurcumin, tetrahydrocurcumin and turmerones differentially regulate anti-inflammatory and anti-proliferative responses through a ROS-independent mechanism. *Carcinogenesis* 2007; 28(8): 1765-73.

20. Novaes JT, Lillico R, Sayre CL*, et al.* Disposition, Metabolism and Histone Deacetylase and Acetyltransferase Inhibition Activity of Tetrahydrocurcumin and Other Curcuminoids. *Pharmaceutics* 2017; 9(4).

21. Greeshma N, Prasanth KG, Balaji B. Tetrahydrocurcumin exerts protective effect on vincristine induced neuropathy: Behavioral, biochemical, neurophysiological and histological evidence. *Chem Biol Interact* 2015; 238: 118-28.

22. Mancuso C, Santangelo R. Ferulic acid: pharmacological and toxicological aspects. *Food Chem Toxicol* 2014; 65: 185-95.

23. Lirdprapamongkol K, Sakurai H, Kawasaki N*, et al.* Vanillin suppresses in vitro invasion and in vivo metastasis of mouse breast cancer cells. *EUR J PHARM SCI* 2005; 25(1): 57-65.

24. Murakami Y, Hirata A, Ito S*, et al.* Re-evaluation of cyclooxygenase-2-inhibiting activity of vanillin and guaiacol in macrophages stimulated with lipopolysaccharide. *Anticancer Res* 2007; 27(2): 801-7.

25. Annaházi A, Róka R, Rosztóczy A, Wittmann T. Role of antispasmodics in the treatment of irritable bowel syndrome. *World J Gastroenterol* 2014; 20(20): 6031-43.

26. Rahimi R, Abdollahi M. Herbal medicines for the management of irritable bowel syndrome: a comprehensive review. *World J Gastroenterol* 2012; 18(7): 589-600.

27. Bundy R, Walker AF, Middleton RW, Booth J. Turmeric extract may improve irritable bowel syndrome symptomology in otherwise healthy adults: a pilot study. *J Altern Complement Med* 2004; 10(6): 1015-8.

28. Konturek SK, Konturek PC. Role of nitric oxide in the digestive system. *Digestion* 1995; 56(1): 1-13.

29. Li CG, Rand MJ. Nitric oxide and vasoactive intestinal polypeptide mediate non-adrenergic, non-cholinergic inhibitory transmission to smooth muscle of the rat gastric fundus. *Eur J Pharmacol* 1990; 191(3): 303-9.

30. Kim K, Kim JJ, Jung Y*, et al.* Cyclocurcumin, an Antivasoconstrictive Constituent of *Curcuma longa* (Turmeric). *J Nat Prod* 2017; 80(1): 196-200.

31. Micucci M, Aldini R, Cevenini M*, et al. Curcuma longa* L. as a therapeutic agent in intestinal motility disorders. 2: Safety profile in mouse. *PLoS One* 2013; 8(11): e80925.

32. Iqbal SM, Jamil Q, Jamil N, Kashif M, Mustafa R, Jabeen Q. Antioxidant, antibacterial and gut modulating activities of *Kalanchoe Laciniata*. *Acta Pol Pharm* 2016; 73(5): 1221-7.

33. Iqbal SM, Mushtaq A, Jabeen Q. Antimicrobial, antioxidant and calcium channel blocking activities of *Amberboa divaricata*. *Bangladesh J Pharmacol* 2014; 9(1): 29-36.

34. Janbaz KH, Qayyum A, Saqib F, Imran I, Zia-Ul-Haq M, de Feo V. Bronchodilator, vasodilator and spasmolytic activities of Cymbopogon martinii. *J Physiol Pharmacol* 2014; 65(6): 859-66.

35. Kitic D, Brankovic S, Radenkovic M*, et al.* Hypotensive, vasorelaxant and cardiodepressant activities of the ethanol extract of Sideritis raeseri spp. raeseri Boiss & Heldr. *J Physiol Pharmacol* 2012; 63(5): 531-5.

36. Kruangtip O, Chootip K, Temkitthawon P*, et al.* Curcumin analogues inhibit phosphodiesterase-5 and dilate rat pulmonary arteries. *J Pharm Pharmacol* 2015; 67(1): 87-95.

37. Tan X, Poulose EM, Raveendran VV, Zhu BT, Stechschulte DJ, Dileepan KN. Regulation of the expression of cyclooxygenases and production of prostaglandin I(2) and E(2) in human coronary artery endothelial cells by curcumin. *J Physiol Pharmacol* 2011; 62(1): 21-8.

38. Vane JR, Botting RM. Pharmacodynamic profile of prostacyclin. *The American journal of cardiology* 1995; 75(3): 3A-10A.

39. Bahramsoltani R, Rahimi R, Farzaei MH. Pharmacokinetic interactions of curcuminoids with conventional drugs: A review. *J Ethnopharmacol* 2017; 209: 1-12.

40. Hu CW, Sheng Y, Zhang Q*, et al.* Curcumin inhibits hERG potassium channels in vitro. *Toxicol Lett* 2012; 208(2): 192-6.

41. Hoehle SI, Pfeiffer E, Solyom AM, Metzler M. Metabolism of curcuminoids in tissue slices and subcellular fractions from rat liver. *J Agric Food Chem* 2006; 54(3): 756-64.

42. Pan MH, Huang TM, Lin JK. Biotransformation of curcumin through reduction and glucuronidation in mice. *Drug Metab Dispos* 1999; 27(4): 486-94.

Fig. 1. Chemical structures of the three curcuminoids curcumin, demethoxycurcumin, bisdesmethoxycurcumin, the curcumin metabolite tetrahydrocurcumin and the curcumin degradation products ferulic acid, feruloyl methane and vanillin.

Fig. 2. Spasmolytic activity of curcuminoids and their metabolite. (A), concentration response curves for curcumin, demethoxycurcumin, bisdemethoxycurcumin and tetrahydrocurcumin determined on isolated guinea pig terminal ileum. Data is presented as Mean ± SEM (n = 4 - 5). (B), bar graph representing the spasmolytic activity of curcumin (CUR), demethoxycurcumin (DMC), bisdemethoxycurcumin (BDMC) and tetrahydrocurcumin (THC), after 45 min incubation with L-NNA. Data is presented as Mean ± SEM (n = 4) and significance was determined by applying 2-way ANOVA followed by Tukey´s posttest.

Fig. 3. Vasodilatory activity of curcuminoids and their metabolite. Concentration response curve for curcumin (CUR), demethoxycurcumin (DMC), bisdemethoxycurcumin (BDMC) and tetrahydrocurcumin (THC), determined on guinea pig (A) pulmonary artery (n = 5), and (B) Aorta (n = 5 - 6). Data is represented as Mean ± SEM.

Fig. 4. Inotropic activity of curcuminoids and their metabolite. Concentration response curve for curcumin (CUR), demethoxycurcumin (DMC), bisdemethoxycurcumin (BDMC) and tetrahydrocurcumin (THC), determined on papillary muscle from guinea pig right ventricle. Data is represented as Mean ± SEM (n = 4).

Fig. 5. Chronotropic activity of Curcuminoids and their metabolite. Concentration response curves for curcumin (CUR), demethoxycurcumin (DMC), bisdemethoxycurcumin (BDMC) and tetrahydrocurcumin (THC), determined on guinea pig right atria. Data is represented as Mean ± SEM (n = 4).

Fig. 6. Representative HPLC chromatograms of curcumin (CUR) (A), demethoxycurcumin (DMC) (B), bisdemethoxycurcumin (BDMC) (C) and terahydrocurcumin (THC) (D) in the pulmonary artery after 30 min at 37°C with 100 μM of the pure compounds.

Fig. 7. Tissue uptake experiments for curcuminoids and their metabolite. Bar graphs representing uptake of (A), curcumin (B), demethoxycurcumin (C), bisdemethoxycurcumin (D), tetrahydrocurcumin in respective tissue samples. Data is presented as Mean ± SEM (n = 3)

Figure 1

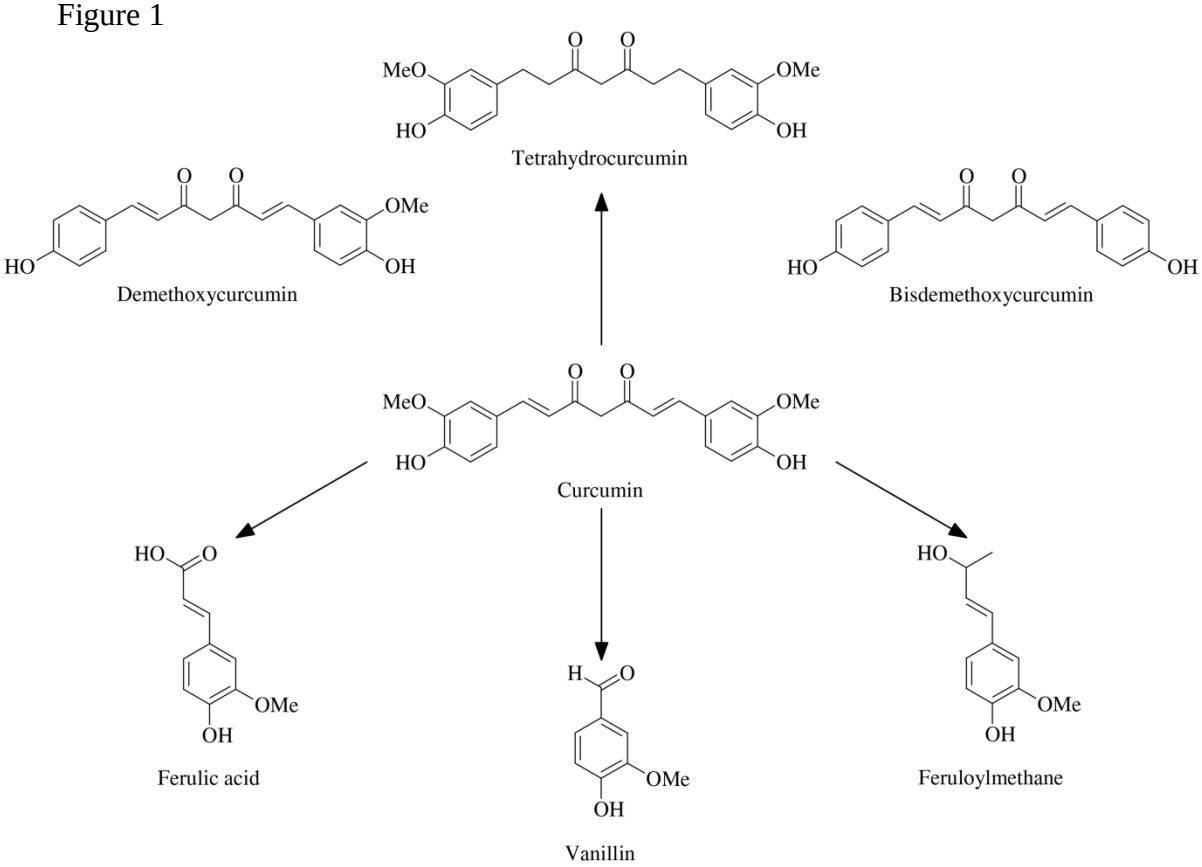
Tetrahydrocurcumin
Demethoxycurcumin
Bisdemethoxycurcumin
Curcumin
Ferulic acid
Vanillin
Feruloylmethane

Figure 2

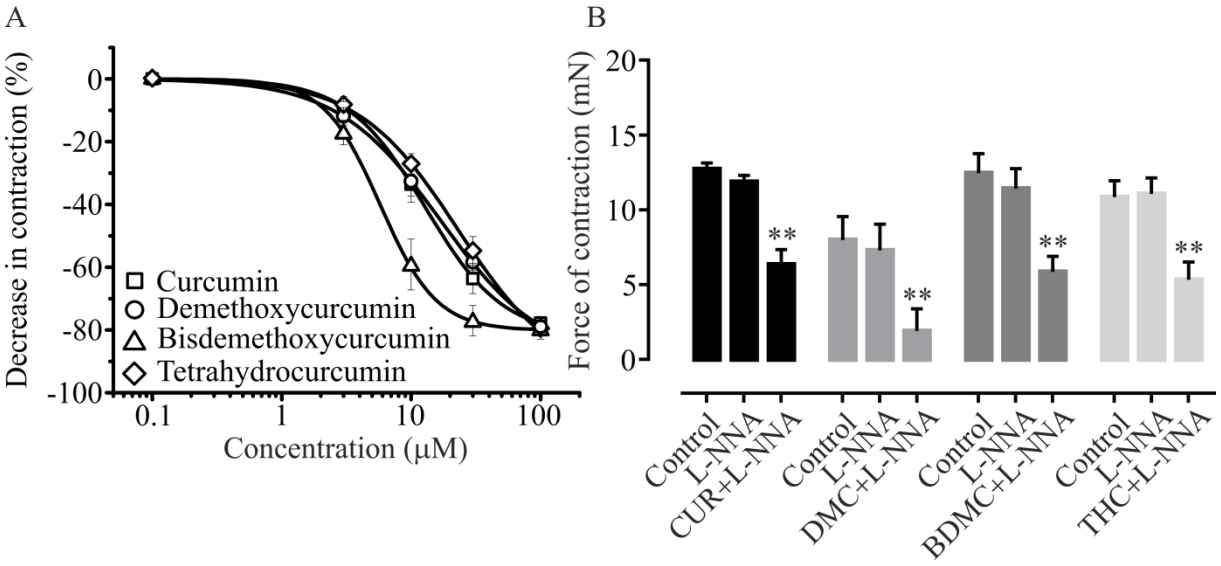
A
Decrease in contraction (%)
0
-20
-40
-60
-80
-100
0.1
1
10
100
Concentration (μM)
Curcumin
Demethoxycurcumin
Bisdemethoxycurcumin
Tetrahydrocurcumin
B
Force of contraction (mN)
20
15
10
5
0
**
Control
L-NNA
CUR+L-NNA
DMC+L-NNA
BDMC+L-NNA
THC+L-NNA

Figure 3

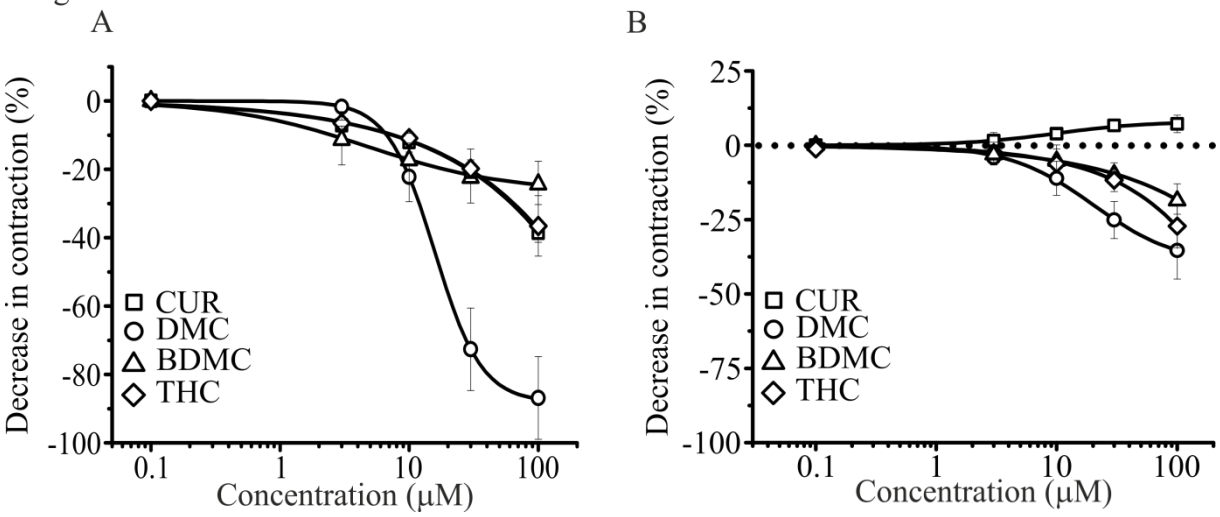
A
B
Decrease in contraction (%)
0
-20
-40
-60
-80
-100
25
-25
-50
-75
CUR
DMC
BDMC
THC
0.1
1
10
100
Concentration (μM)

Figure 4

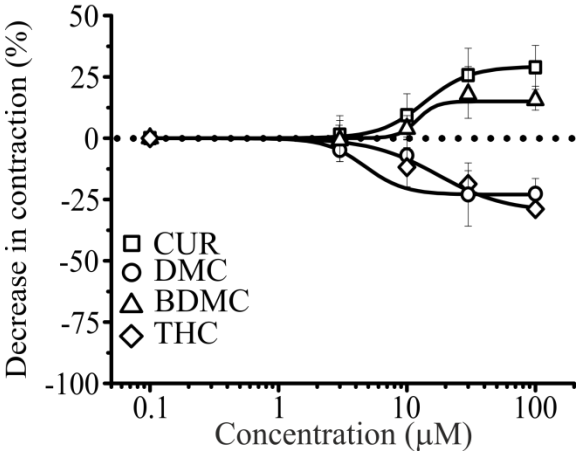
50
25
0
-25
-50
-75
-100
Decrease in contraction (%)
CUR
DMC
BDMC
THC
0.1
1
10
100
Concentration (μM)

Figure 5

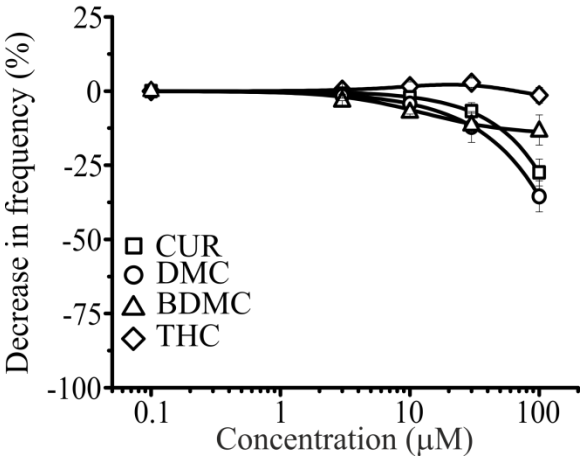
25
0
-25
-50
-75
-100
Decrease in frequency (%)
CUR
DMC
BDMC
THC
0.1
1
10
100
Concentration (μM)

Figure 6

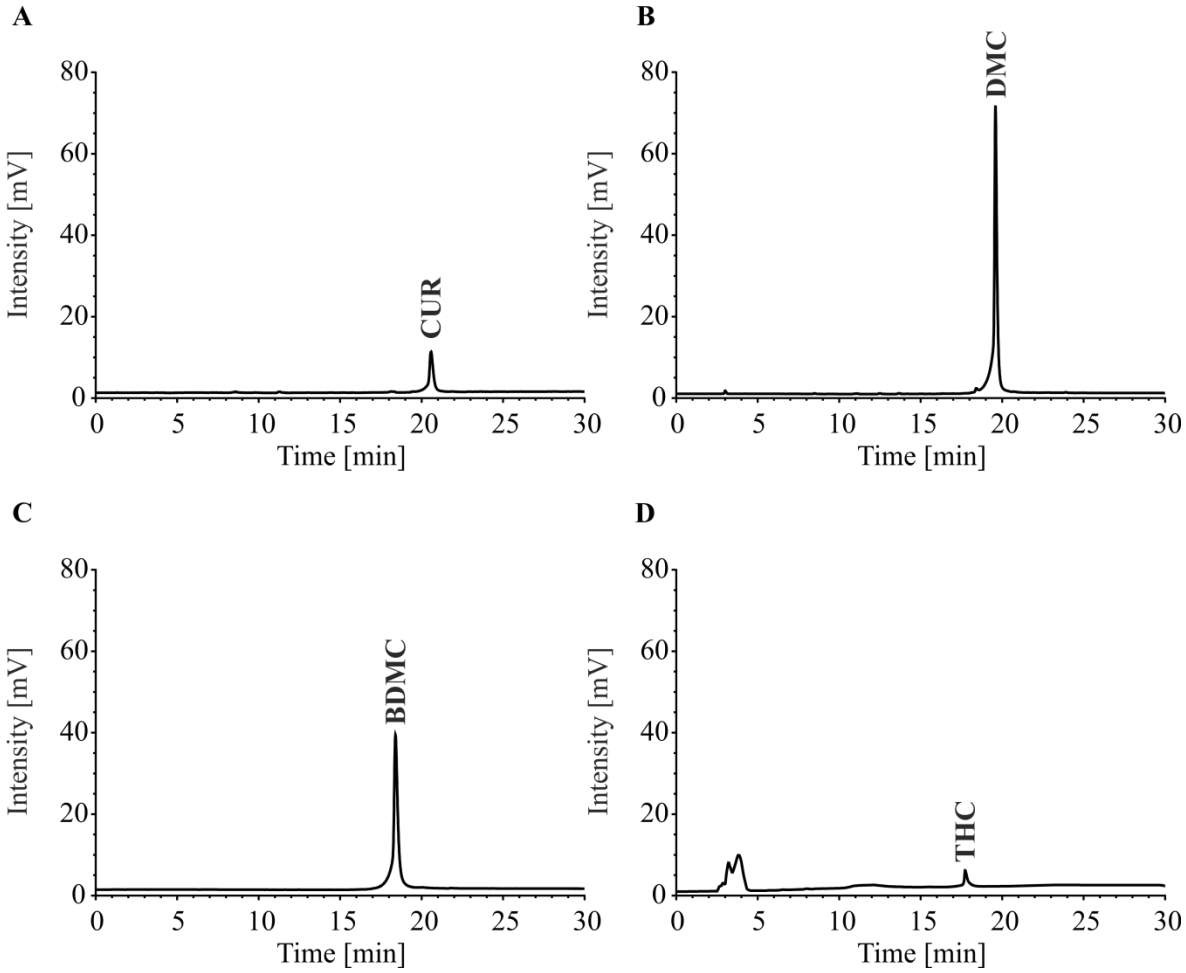
A
B
C
D
Intensity [mV]
Time [min]
CUR
DMC
BDMC
THC
0
20
40
60
80
0
5
10
15
20
25
30

Figure 7

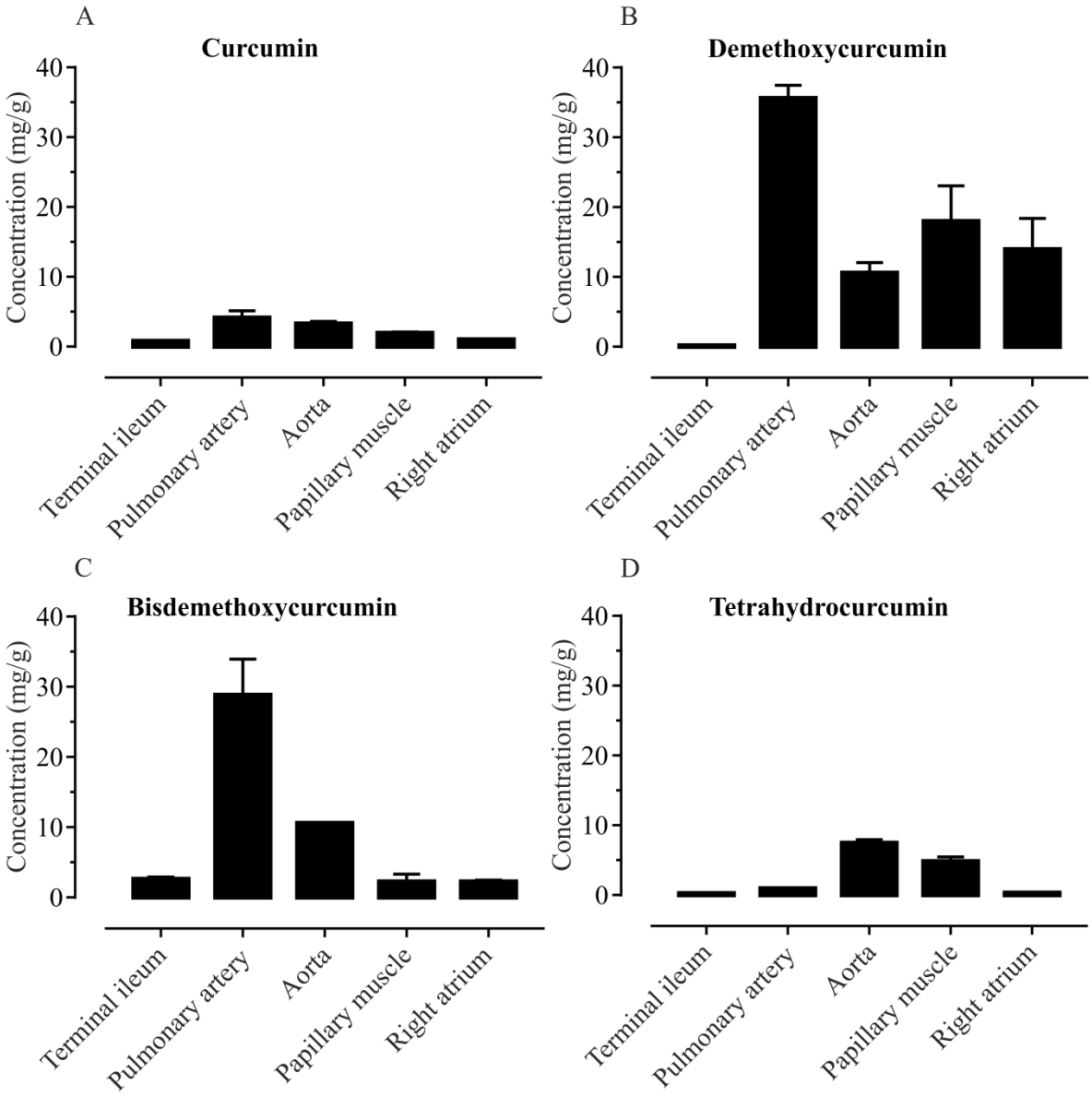
A
Curcumin
Concentration (mg/g)
0
10
20
30
40
Terminal ileum
Pulmonary artery
Aorta
Papillary muscle
Right atrium
B
Demethoxycurcumin
Concentration (mg/g)
0
10
20
30
40
Terminal ileum
Pulmonary artery
Aorta
Papillary muscle
Right atrium
C
Bisdemethoxycurcumin
Concentration (mg/g)
0
10
20
30
40
Terminal ileum
Pulmonary artery
Aorta
Papillary muscle
Right atrium
D
Tetrahydrocurcumin
Concentration (mg/g)
0
10
20
30
40
Terminal ileum
Pulmonary artery
Aorta
Papillary muscle
Right atrium

Table 1. Relative uptake of curcumin, demethoxycurcumin, bisdemethoxycurcumin and tetrahydrocurcumin in respective tissue samples. Each data point represents Mean ± SEM of three experiments performed on different tissue samples.

Tissue	Curcumin	Demethoxycurcumin	Bisdemethoxycurcumin	Tetrahydrocurcumin
	Mean ± SEM (mg/g)	Mean ± SEM (mg/g)	Mean ± SEM (mg/g)	Mean ± SEM (mg/g)
Ileum	0.79 ± 0.07	0.18 ± 0.04	2.61 ± 0.28	0.23 ± 0.00
Aorta	3.26 ± 0.36	10.56 ± 1.50	10.58 ± 0.01	7.48 ± 0.47
Right Atrium	1.00 ± 0.12	13.96 ± 4.44	2.23 ± 0.25	0.31 ± 0.01
Papillary Muscles	1.93 ± 0.17	17.99 ± 5.05	2.233 ± 1.09	4.82 ± 0.63
Pulmonary Artery	4.13 ± 1.00	35.62 ± 1.83	28.83 ± 5.12	0.94 ± 0.01

The contribution of organic anion-transporting polypeptides 1B1, 1B3 and 2B1 to the antitumor activity of curcumin and its main metabolites against human breast cancer cells

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Abstract. Curcumin is a natural polyphenolic compound with pronounced anticancer properties. These effects are observed despite its low bioavailability, which is particularly caused by extensive phase II metabolism. It is unknown whether curcumin and its metabolites can accumulate to bioactive level in organs and tissues through protein-mediated transport mechanism. Because organic anion transporting polypeptides (OATPs) mediate the uptake of many clinically important drugs, we investigated their role in the cellular transport of curcumin and its major glucuronides and sulfates in OATP-expressing Chinese hamster ovary (CHO) and breast cancer (ZR-75-1) cells. The uptake rates for curcumin in OATP1B1-, OATP1B3- and OATP2B1-transfected CHO cells were 2- to 3 - fold higher compared to wild-type cells. Curcumin sulfate again was transported by all three OATPs, however, to a much lesser extent, while uptake of tetrahydrocurcumin was only demonstrated by OATP1B1 and OATP1B3. Interestingly, curcumin glucuronide did not show any affinity for these OATPs. The much higher mRNA levels for OATP1B1 is found in wild-type compared to OATP1B1 knockdown ZR-75-1 cells, which are correlated with higher initial uptake leading to decreased IC_{50} values and a slightly more pronounced inhibition of NF-κB. In conclusion, our data reveal that OATPs act as cellular uptake transporters for curcumin and its major metabolites, this may also be apply to human, following oral uptake of dietary curcumin.

Introduction

Breast cancer is the most predominant form for female and the secondary leading cause of the disease related mortality among females globally. Chemoprevention in association with anticancer treatment is crucial for reduction in the incident and the mortality of this disease. Evidence from epidemiological and experimental studies shows that natural components available in the food can act as chemopreventive factors to prevent mammary carcinogenesis (1,2). One of these constituents is curcuma, a bright-yellow-colored polyphenol got from rhizome of the crop *Curcuma longa* L. (Zingiberaceae). Tumeric has been in use for over two millennia as an herbal therapy in China and India in the prevention and cure of different human diseases (3). The breast cancer preventing activity of curcumin has been recently demonstrated in a mouse xenograft breast cancer model, which proved that the intake of a curcumin-supplemented meal prevented tumor growth and angiogenesis (4). The anti-proliferative feature of curcumin has been elucidated *in vitro* and *in vivo* against hormone-reliant and hormone-free human breast cancer cells. Curcumin was found to interfere with apoptosis through regulating many intracellular showing routes as well as the transcriptional factors STAT3, NFkB, and AP-1, the receptors HER2 and CXCR4, the kinases EGFR, ERK, and JAK, and the cytokines TNF, IL and MP (5-7). Curcumin also attenuates the expression of major matrix metalloproteinases (MMPs) owing to diminished NFkB operation and transcriptional down regulation of AP-1 (8). Abridged NFkB/AP-1 function and MMP performance results in sizeably lower number of lung metastasis in immunodeficient mice after intracardiac injection of estrogen receptor (ER)-negative human breast cancer MDA-MB-231 cells (8).

Curcumin also shows a synergistic effect *in vitro* and *in vivo* with other anticancer drugs. The most current research revealed a synergistic result of paclitaxel in combination with curcumin against human MCF-7 and MDA-MB-231 cells (9). Better clinical responses were also noticed in a phase I clinical trial of docetaxel plus curcumin in patients with prolonged and metastatic breast cancer showing curcumin as a favourable agent in combination with other anticancer drug (10). Studies carried out in laboratory animals have reported extensive biotransformation of curcumin in the small intestine and liver yielding mainly curcumin sulfate and curcumin glucuronide (11) thereby explaining its very low bioavailability (<1%) after per oral administration. Curcumin bioavailability is also negligible in

humans, as shown by a pilot study of standardized curcuma-derived extract in colorectal cancer patients (12). Curcumin undergoes metabolic phase II conjugation to curcumin glucuronide and curcumin sulfate and phase I bio-reduction majorly to tetrahydrocurcumin and to a minor part to hexahydrocurcumin, octahydrocurcumin, and hexahydrocurcuminol (13,14). Reduced curcumins are subsequently further conjugated to glucuronides and sulfates (13). Based on the rapid metabolism free curcumin is not noticed in plasma even after large oral doses of 10 g or 12 g to human volunteers. Curcumin glucuronide and sulfate, nevertheless, was easily measured in the plasma derived from all human 12 participants with maximal concentrations in the very low microgram/ml range (15).

Current experiments in our lab confirmed the formation of curcumin sulfate as the major metabolite in hormone-dependent ZR-75-1 and hormone-independent MDA-MB-231 breast cancer cells; curcumin glucuronide concentrations were below the detection limit (16). Interestingly, curcumin sulfate concentration in the cellular medium was up to 12-fold higher compared to the cytoplasm indicating a not yet identified active efflux system for this metabolite. A likely candidate for the cellular transport from the cytoplasm into the medium is the breast cancer resistance protein (BCRP, ABCG2), which is expressed in numerous tissues, such as placenta, small intestine, colon, and the hepatic canalicular membrane, but also in breast ductal cells, and performs a significant function in the efflux of sulfated conjugates of steroids and xenobiotics (17). Interplay of curcumin with BCRP has already been described and may also be apply for its sulfate (18).

The observed pharmacological activities of curcumin cannot be explained by the very low blood and tissue concentrations of unchanged curcumin leading to pronounced levels of conjugates. Currently, limited information is available regarding the possible benefits of curcumin metabolites. In vitro data, however, suggest that the main metabolites curcumin sulfate and curcumin glucuronide are less potent than curcumin against various tumor cell lines. Though, the in vitro work of curcumin metabolites may not particularly reflect their in vivo function given that intracellular sulfatases or β-glucuronidases might quickly change the conjugates back to curcumin.

Uptake mechanisms into tumor cells might be therefore even more significant than efflux movement for the efficiency of curcumin because they are factors for intracellular drug concentration. One main cellular uptake mechanism is via members

of the organic anion transporting polypeptide (OATP) family (19-21). Among the 11 human OATPs, OATP1B1 and OATP1B3 are highly expressed in the liver and mediate the uptake of several drugs into hepatocytes. OATP2B1 is expressed in liver and in addition demonstrates substantial expression in the apical membrane of enterocytes, where it contributes to the intestinal absorption of many endogenous compounds and clinically important drugs, thereby affecting drug disposition (22-23). Data from our laboratory have also shown a high expression of various OATPs in human hormone receptor-positive (MCF-7) and negative (MDA-MB-231) breast cancer cell lines (24). As pronounced curcumin uptake into the cytoplasm was also observed in these two cell lines (16), we hypothesized that members of the OATP family may mediate intracellular curcumin concentrations, thereby affecting cell growth. In the present study, we therefore investigated the time and concentration-dependent transport of curcumin, curcumin sulfate, curcumin glucuronide and tetrahydrocurcumin in stably OATP1B1-, OATP1B3- and OATP2B1-transfected CHO cells. Furthermore, the impact of OATP 1B1 on cytotoxicity and NF-κB inhibition of curcumin-treated human breast cancer cells ZR-75-1 was also investigated.

Materials and methods

Materials. Curcumin (98% pure) and tetrahydrocurcumin (95% pure) were purchased from Sigma; curcumin sulfate and curcumin glucuronide were obtained from TLC Pharmaceutical Standards Ltd; MeOH and water were of HPLC grade (Merck). All other chemicals and solvents were commercially available and of analytical grade and were used without further purification.

Cell culture. Chinese hamster ovary (CHO) cells that were stably transfected with OATP1B1, OATP1B3 and OATP2B1 and wild-type CHO cells were provided by the Department of Clinical Pharmacology and Toxicology, University Hospital Zurich, Switzerland, which have been extensively characterized previously (25,26). The CHO cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FCS, 50 µg/ml L-proline, 100 U/ml penicillin and 100 µg/ml streptomycin. The selective medium for stably transfected CHO cells additionally contained 500 µg/ml geneticin sulfate (G418) (27). All of the media and supplements were obtained from Invitrogen (Karlsruhe, Germany). The mammalian ZR-75-1 breast cancer cell line

was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) and was maintained in RPMI medium supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 1% GlutaMAX. The cells were grown in T-flasks with a 25-cm^2 growth area (BD Biosciences, Franklin Lakes, NJ, USA), maintained at 37°C under 5% CO_2 and 95% relative humidity. The cells were passaged once a week and were used up to passage 55 (21).

OATP1B1 knockdown in ZR-75-1 cells. For lentiviral transduction, ZR-75-1 cells were plated in 24-well tissue culture plates at a density of 40,000 cells/well in 0.5 ml of growth medium. After 24 hours, 250 µl of medium supplemented with 8 µg/ml polybrene (Sigma, H9268) was added. Transductions were performed by the addition of 10 µl of shRNA (Mission® Transduction Particles NM_006446, Sigma, TRCN0000043203 coding sequence: CCGGGCCTTCATCTAAGGCTAACATCTCG-AGATGTTAGCCTTAGATGAAGGCTTTTTG). Twenty-four hours after transduction, the cell culture medium was changed, and 1 ml of growth medium supplemented with 1 or 5 µg/ml of puromycin (Sigma, P9620) was added to select infected cells after an additional 24 hours. The obtained silencing efficiency was evaluated after 3 weeks via real-time PCR and immunofluorescence.

Real-time RT-PCR. Total RNA was extracted from cell lines using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The concentration, purity, and integrity of the RNA samples were determined through UV absorbance and electrophoresis. Two µg of total RNA were reverse transcribed to cDNA using random hexamer primers and the RevertAid™ H Minus M-MuLV Reverse Transcriptase system (Fermentas, St. Leon-Rot, Germany), as recommended by the manufacturer. TaqMan® Gene Expression Assays (Applied Bio-systems, Warrington, United Kingdom) were purchased for human OATP1B1. The 18S gene was used as a reference gene as previously described (24). Multiplex quantitative real-time RT-PCR was performed in an amplification mixture with a volume of 20 µl. The target gene amplification mixture contained 10 µl of 2X TaqMan® Universal PCR Master Mix, 1 µl of the appropriate Gene Expression Assay, 1 µl of the TaqMan® endogenous control (human β-actin or 18S), 10 ng of template cDNA diluted in 5 µl of nuclease-free water and 3 µl of nuclease-free water. The thermal cycling conditions were as follows: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at

95°C and 1 min at 60°C. Fluorescence generation due to TaqMan® probe cleavage via the 5'→3' exonuclease activity of DNA polymerase was measured with the ABI PRISM 7700 Sequence Detection System (Applied Bio-systems). All samples were amplified in triplicate. To cover the range of expected Ct values for the target mRNA, a standard curve of six serial dilutions from 50 ng to 500 pg of pooled cDNA was analyzed using Sequence Detection Software (SDS 1.9.1., Applied Bio-systems). The results were imported into Microsoft Excel for further analysis. Comparable cDNA contents in the experimental samples were calculated according to the standard curve method. Relative gene expression data are given as the n-fold change in transcription of target genes normalized to the endogenous control. Real-time RT-PCR was performed with the following prefabricated TaqMan® Gene Expression Assays (Applied Bio-systems) containing intron-spanning primer Hs00272374_m1 for OATP1B1.

Immunofluorescence. ZR-75-1 OATP1B1-knockdown cells and ZR-75-1 cells transfected with the empty vector were allowed to attach on culture slides overnight (8-Chamber Polystyrene Vessel Tissue Culture-Treated Glass Slides, BD Falcon). Formalin fixation was followed by a washing step and a blocking step (by 5% BSA). The primary antibody against OATP1B1 (OATP1B1/1B3 mMDQ mouse monoclonal antibody; Acris Antibodies, Herford, Germany) was diluted 1:100, and incubation was performed for 2 hours. Optimal antibody concentrations were determined by titrating serial antibody dilutions. The applied dilutions corresponded to the minimum concentration necessary to produce a positive signal. Wild-type and OATP1B1-transfected CHO cells were used as negative and positive controls, respectively. Following incubation with the secondary antibody (1:1,000 dilution; Alexa Fluor® 488 Goat Anti-Rabbit IgG; Invitrogen, Carlsbad, CA) for 30 min, cell nuclei were stained with 0.5 µg/ml Hoechst 33342 (Sigma-Aldrich, St. Louis, MO). Thereafter, the slides were rinsed with distilled water before being mounted in Mowiol 4-88 (Carl Roth, Karlsruhe, DE). Fluorescent staining was visualized with an Axioplan 2 microscope (Carl Zeiss, Jena, DE). Images were captured using an AxioCam HRc2 Color CCD digital camera and Axiovision 4.8 software (Carl Zeiss Vision GmbH, Aalen, DE). To minimize background signals and to make the signal intensity and extension in different samples comparable, the exposure times for the individual antibodies were evaluated and kept constant between the samples.

Cellular uptake. Transport assays were performed on 12-well plates as described in detail elsewhere (28). Briefly, OATP-transfected CHO cells were seeded at a density of 350,000 cells per well on 12-well plates (BD Biosciences, Franklin Lakes, NJ, USA). Uptake assays were generally performed on day 3 after seeding when the cells had grown to confluence. Twenty-four hours before starting the transport experiments, the cells were additionally treated with 5 mM sodium butyrate (Sigma-Aldrich, St. Louis, MO, USA) to induce nonspecific gene expression (29). Curcumin and its metabolites were dissolved in DMSO and were diluted with uptake buffer (pH 7.4; final DMSO concentration of 0.5%, which was constant in all transport experiments) to 25–600 µM. The experiments run under each set of conditions were performed in triplicate. Control experiments contained DMSO in the medium in place of curcumin and its biotransformation products, respectively. Prior to the transport experiment, the cells were rinsed twice with 2 ml of pre-warmed (37°C) uptake buffer (116.4 mM NaCl, 5.3 mM KCl, 1 mM NaH_2PO_4, 0.8 mM $MgSO_4$, 5.5 mM D-glucose and 20 mM Hepes; pH adjusted to 7,4). Uptake was initiated by adding 0.25 ml of uptake buffer containing the substrate. After the indicated time period at 37°C, uptake was stopped by removing the uptake solution and washing the cells five times with 2 ml of buffer (pH 7.4). The cells were then trypsinized by the addition of 100 µl of trypsin and transferred into test tubes. Next, the cell membranes were disrupted via repeated (5 times) shock freezing in liquid nitrogen and thawing. Following centrifugation at 13,500 g for 5 min, 100 µl of the supernatant was diluted with methanol/water (2:1; v/v), and aliquots (80 µl) were analyzed via HPLC.

Transport of curcumin in wild-type ZR-75-1 and OATP1B1-knockdown ZR-75-1 cells. Cells were plated on 6-well plates and allowed to attach overnight. Curcumin was dissolved in DMSO and diluted with medium (final DMSO concentration < 0.1%) to a concentration of 25-200 µM. The experiments were performed under each set of conditions in triplicate. Control experiments contained DMSO in the medium in place of curcumin. After 72 hours, the medium was aspirated via suction, and aliquots (100 µl) were subsequently analyzed through HPLC. The cells were then trypsinized by the addition of 100 µl of trypsin, washed three times with phosphate buffered saline, and lysed by repeated (5 times) shock freezing in liquid nitrogen and thawing. Following centrifugation at 13,500 g for 5 min, 80 µl of the supernatant (cytoplasm)

was subsequently analyzed by HPLC. Additionally, the cell pellets containing the membranes were extracted with 200 µl of methanol and analyzed by HPLC for their curcumin content. The protein concentration in the cell pellets was determined using a BCA assay kit (Pierce Science, Rockford, IL, USA), with bovine serum albumin as a standard.

Cytotoxicity assay. CellTiter-Blue (Promega, Southampton, UK) is the type of a colorimetric and fluorescent assay used to measure cell viability via non-specific redox enzyme activity (reduction from resazurin to resorufin by viable cells). ZR-75-1 cells (50,000 cells/ml) were seeded into 96-well flat-bottomed plates and incubated for 24 hours at 37°C under 5% CO_2. For cytotoxicity assays, ZR-75-1 wild-type and OATP1B1-knockdown ZR-75-1 cells were incubated with various concentrations of curcumin (2.5-100 µM) for 72 hours. The Cell Titer-Blue (20 µL) reagent was added to the wells, and the plate was incubated for 2 hours, protected from light. The absorbance was recorded for resazurin (605 nm) and resorufin (573 nm). The assay results were measured on a Tecan M200 multimode plate reader (Tecan Austria GmbH, Groedig, Austria). The absorbance was also measured in Cell Titer-Blue assays in blank wells (without curcumin) and deducted from the values from experimental wells. The viability of the treated cells was expressed as a percentage from the viability of the corresponding control cells. All experiments were repeated at least three times.

Determination of protein concentrations. Total protein was determined using the colorimetric bicinchoninic acid protein (BCA) assay kit (Pierce Science, Rockford, IL, USA) with bovine serum albumin as a standard and quantification at a wavelength of 562 nm on a spectrophotometer (UV-1800, Shimadzu). Raw data were analyzed using UV Probe software (version 2.31, Shimadzu). The protein concentrations were consistent among the plates (0.150 ± 0.005 mg/well).

HPLC analysis. Curcumin and its biotransformation products were quantified by HPLC using a Dionex UltiMate 3000 system equipped with an L-7250 injector, an L-7100 pump, an L-7300 column oven (set at 35°C), a D-7000 interface and an L-7400 UV detector (Thermo Fisher Scientific) set at a wavelength of 420 nm (for tetrahydrocurcumin: 280 nm). Separation of curcumin and its metabolites was carried

out at 35°C using a Hypersil BDS-C18 column (5 µm, 250 x 4.6 mm I.D., Thermo Fisher Scientific), preceded by a Hypersil BDS-C18 precolumn (5 µm, 10 x 4.6mm I.D.). The mobile phase consisted of a continuous linear gradient, mixed from 10 mM ammonium acetate/acetic acid buffer, pH 5.0 (mobile phase A) and methanol (mobile phase B). The flow rate was kept at 1mL/min. The filtration of the mobile phase was performed through a 0.45µM filter (HVLP04700, Millipore, Austria). The gradient range was from 10% methanol (0 min) to 90% methanol at 17 min followed by another increase to 95% at 18 min where it remained constant until 25 min. Subsequently, the percentage of methanol was decreased within 2 min to 10% in order to equilibrate the column for 8 min before application of the next sample. Calibration of the chromatogram was accomplished using the external standard method. Linear calibration curves were performed by spiking drug-free cell culture medium with standard solutions of curcumin, curcumin sulfate, curcumin glucuronide, and tetrahydrocurcumin, respectively, to give a concentration range from 0.01 to 10 µg/mL (average correlation coefficients: >0.999). Coefficients of accuracy and precision for these compounds were < 11%.

NF-κB-luciferase reporter assay. 100,000 ZR-75-1 wild-type and OATP1B1-knockdown ZR-75-1 cells were seeded in 24-well plates and grown to 70% confluency for transfection. Simultaneous transfection with pTAL-NF-κB (NF-κB response element - *Firefly* luciferase reporter; Clonetech, Paolo Alto, CA, USA) and pRL-TK (Control - *Renilla* luciferase; Promega, Mannheim, Germany) was performed with Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA; Cat No.: 11668) according to the manufacturer's protocol. NF-κB was blocked for 30 min with 10 µM BAY11-7082 or 100 µM curcumin respectively. Then 10 ng/ml interleukin 1β (IL-1β) was added and incubated for 90 min, immediately followed by luciferase assay (Promega, Mannheim, Germany; Cat No.: E1910). Briefly, cells were lysed with lysis buffer and incubated with the first substrate to measure the *Firefly* luciferase signal. Upon addition of the second substrate signals for *Renilla* luciferase were measured.

Data analysis and statistics. Kinetic analysis of the uptake of curcumin and its metabolites was performed over a substrate concentration range of 25 to 600 µM. Prior to these experiments, the linearity of cellular uptake over time (1, 3 and 10 min) was individually determined for wild-type and OATP-transfected CHO cells by using

curcumin (50 µM) as a substrate. Cellular uptake rates are presented after normalization for the incubation time and total protein content. Net uptake rates were calculated as the difference in the uptake rate of the transfected and wild-type cells for each individual concentration. The data were fitted to the Michaelis–Menten model. Kinetic parameters were calculated using the Graph-Pad Prism Version 6.0 software program (GraphPad Software, San Diego, CA) for Michaelis–Menten: $V = Vmax * S / (Km + S)$, where V is the rate of the reaction; Vmax is the maximum velocity; Km is the Michaelis constant and S is the substrate concentration. The intrinsic clearance, which is defined as the ratio Vmax/Km, quantifies the transport capacity. The same software package was also used for all statistical analyses. All values were expressed as the mean ± SD of three independent biological replicates and one-way ANOVA combined with Tukey's post-test were used to compare differences between control samples and treatment groups. The statistical significance threshold was defined as $P<0.05$ for all calculations.

Results

Accumulation of curcumin and metabolites in transfected CHO cells. To investigate whether curcumin and its major conjugates are substrates of OATPs, uptake analyzes were performed in OATP1B1-, OATP1B3- and OATP2B1-transfected CHO cells which show high expression of these transporters in the plasma membrane (data not shown). CHO cells only transfected with the vector were used as controls. Uptake of curcumin (25-200 µM) for all three OATPs was linear for up to 1 min (data not shown). We, therefore, finalized all experiments at 1 min (initial linear phase). As shown in Table 1 and Fig. 1A-C, the initial (OATP-transfected CHO cells – CHO cells only transfected with the vector) OATP1B1-, OATP1B3- and OATP2B1-mediated accumulation rates for curcumin followed Michaelis-Menten kinetics, with higher Vmax values for OATP1B1 compared to OATP1B3 and OATP2B1 (Vmax: 310 vs. 205 vs. 167 pmol/mg protein/). Km-values were similar for all three OATPs and in the range of 46.9 – 51.9 µM. The uptake of curcumin sulfate (25-300 µM) in OATP1B1-OATP1B3- and OATP2B1-transfected CHO cells, however, was less pronounced, showing Vmax values of only 45.0, 33.9 and 24.3 pmol/mg protein/min, respectively (Table 1 and Fig. 2A-C). It's affinity, however, for OATP1B1 and OATP1B3 but not for OATP2B1 was 1.9- and 1.4-fold higher with Km values of 89.1 and 67.7 compared to

curcumin. Tetrahydrocurcumin was taken up by OATP1B1 and OATP1B3 with higher Vmax values (872 and 493 pmol/mg protein/min, respectively); Km values were 38.6 µM and 83.7 µM (Table 1 and Fig. 3A-C). The uptake of curcumin glucuronide by OATP1B1-, OATP1B3- and OATP2B1-transfected and wild-type CHO cells was below the detection limit.

OATP1B1-knockdown in ZR-75-1 cells. The PCR data from various lentiviral-transfected clones revealed an up to ten-fold reduction of OATP1B1 expression in ZR-75-1-cells. The cells exhibiting the lowest expression of OATP1B1 (relative mRNA expression was reduced from 14.78 ± 0.26 to 1.19 ± 0.02 based on the change to the calibrator) were chosen for further experiments. Because ZR-75-1 cells do not express OATP1B3 and OATP2B1 but do express OATP1B1 (24), the expression of the OATP1B1 protein was confirmed via immunofluorescence using a specific OATP1B1/1B3 mouse monoclonal antibody (data not shown).

Curcumin accumulation in wild-type and ZR-75-1 OATP1B1-knockdown cells. Based on the much higher OATP1B1 mRNA level found in the wild-type ZR-75-1 breast cancer cell line compared to the OATP1B1-knockdown clone, we expected that OATP1B1 expression might be directly correlated with intracellular curcumin concentrations. For kinetic analysis, an incubation time of 1 min was selected in order to prevent cellular uptake from interference with cellular efflux mechanisms like MRPs and BCRP. Fig. 4 depicts representative Michaelis-Menten plots for curcumin uptake by ZR-75-1wild-type and ZR-75-1 OATP1B1-knockdown cells, where significantly higher uptake rates and Km values were found in the OATP1B1-expressing control cells (Vmax: 3,535 vs. 1741 pmol/mg protein/min; Km: 85.1 vs. 56.7 µM), thus strongly indicating the impact of OATP1B1 curcumin transport.

Cytotoxicity of curcumin in ZR-75-1 OATP1B1-knockdown cells. The cytotoxicity of curcumin in ZR-75-1 wild-type and OATP1B1-knockdown ZR-75-1 breast cancer cells was quantified using the CellTiter-Blue test kit from Promega, as described above. As shown in Fig. 5, curcumin exhibited a lower IC_{50} value in wild-type ZR-75-1 cells (12.4 µM) compared to the OATP1B1 knockdown clone (15.2 µM), supporting the importance of OATP1B1-dependent curcumin uptake.

Inhibition of NF-κB-luciferase by curcumin in ZR-75-1 OATP1B1-knockdown cells. To further evaluate the OATP1B1-dependent differences in the activity of curcumin, WT, and OATP1B1-knockdown ZR-75-1 breast cancer cells were simultaneously transfected with an NF-κB promoter sequence connected to a luciferase reporter. Prior NF-κB reporter induction by 10 ng/ml IL-1β for 90 min, cells were treated with curcumin or BAY11-7082 for 30 min, and the luciferase signals measured. As shown in Fig. 6, curcumin significantly inhibited IL-1β-induced NF-κB reporter expression by 30.9 ± 10.13% in WT and by 40.12 ± 7.78% in OATP1B1 knockdown cells. Notably, curcumin was almost as potent as the known NF-κB inhibitor BAY11-7082, used as a positive control.

Discussion

To identify the relevance of uptake transporters to the in vivo activity and to elucidate the human OATP isoforms responsible for the hepatic uptake of curcumin and its major metabolites curcumin sulfate, curcumin glucuronide and tetrahydrocurcumin we employed cells that stably expressed these OATPs. As indicated in Fig. 1 and Table 1, curcumin displays saturable uptake kinetics for OATP1B1, OATP1B3, and OATP2B1 with similar Km values range from 46.9-51.9 µM indicating high affinity to the transporter. The affinity for curcumin sulfate was in the similar range for OATP2B1 but higher for OATP1B3 and OATP1B1 (Km values: 50.0, 67.7 and 89.1 µM, respectively). Interestingly, tetrahydrocurcumin was only transported by OATP1B1 and OATP1B3 with a reduced affinity for OATP1B1 (Km: 83.7 µM) but with an increased affinity (Km: 38.6 µM) for OATP1B3. Curcumin glucuronide was not transported by any of these three OATPs. Interestingly, OATP-dependent uptake was compound specific. While the transport capacity (Vmax/Km) for curcumin sulfate was low for all three OATPs (0.51, 0.50 and 0.48 µl/min/mg protein, respectively),the uptake of curcumin into OATP1B1, OATP1B3 and OATP2B1-transfected cells were 6.3-, 8.7- and 13.7-fold higher. Uptake of tetrahydrocurcumin by OATP1B1 was even more pronounced and 44.3-fold higher compared to curcumin. These data show that OATP1B1 could be the far most significant uptake transporter for tetrahydrocurcumin, whereas the three OATPs are equally significant for the cellular uptake of curcumin and curcumin sulfate. Nevertheless, based on the Michaelis-Menten parameters, the actual contribution of OATP2B1 in the gut and of OATP1B1, OATP1B3 and OATP2B1 in the liver to the overall uptake of curcumin and its major metabolites in

humans cannot be determined. This is the consequence of a great inter individual variability (up to 10 fold) of OATP protein levels as determined by quantitative proteomics or by Western blotting (30-32). Because of the low bioavailability, the portal concentration of curcumin is probably lower than the Km values. In fact, application of 3.63 g curcumin daily for up to twelve weeks to six patients with colorectal cancer resulted in only 4.31 ng/ml (11.68 nM), 5.84 ng/ml (10.65 nM) and 3.3 ng/ml (7.38 nM) mean plasma concentrations of curcumin, curcumin glucuronide, and curcumin sulfate, respectively, 1h after administration (15). But, total tissue concentrations of conjugates (curcumin sulfate + curcumin glucuronide) are much higher in mice 1 h after i.p. administration of curcumin (0.1 g/kg) leading to concentrations of 26.1 µg/g (52.6 µM) and 26.9 µg/g (54.3 µM) and 117 µg/g (236 µM) in the spleen, liver, and intestine, respectively (33). As found in plasma, the concentration of unconjugated curcumin in these tissue samples was very low. However, it should be kept in mind that other criteria such as local pH (36) may also affect the transport rate of the OATPs expressed in cancer cells.

To further proof the significance of OATB1 for the uptake of curcumin and its sulfates, hormone-dependent ZR-75-1 cells that were previously shown to express high levels of OATP1B1, but not OATP1B3 and OATP2B1 (24), were incubated for 48 hours with increasing concentrations of curcumin. Indeed, the intake of curcumin by the ZR-75-1 OATP1B1-knockdown cells was significantly reduced compared to control cells, as shown by higher Km and lower Vmax values (Fig. 4 and Table 2). Concomitant with the reduced uptake found in ZR-75-1 knockdown cells, we also observed non-significant higher IC_{50} values in the cytotoxicity assay compared to OATP1B1-expressing wild-type cells (15.2 vs 12.4 µM; Fig. 5). The decrease uptake of curcumin by the ZR-75-1 OATP1B1-knockdown cells also resulted to a decreased inhibition of IL-1β-activated NF-κB reporter expression, which was, however, not significant (Fig. 6). As the pro-inflammatory transcription factor NF-κB is highly expressed in breast cancer, thereby facilitating growth and progression (34), a considerable number of studies on cancer prevention at different stages have indicated curcumin as a favorable agent for cancer chemoprevention, used either on its own, or in combination with other anticancer drugs (34-35).

Any variations in OATP expression can greatly change the uptake of curcumin, curcumin sulfate, and tetrahydrocurcumin into specified cells and tissues, thereby strongly impacting on the effectiveness of treatment. Patients with little or no

detectable expression of OATP1B1, OATP1B3, and OATP2B1 may, as a result exhibit decreased response rates or even no response to curcumin and its primary metabolites. Concomitant administration of OATP inhibitors may also interfere with the uptake of curcumin and its major metabolites, resulting in transporter-mediated drug/drug interactions. Potential inhibitors include clarithromycin, erythromycin and roxithromycin, which inhibit the intake of pravastatin in OATP1B1- and OATP1B3-transfected HEK293 cells (showing IC_{50} values of 32-37 µM) (31). Moreover, cyclosporin A significantly decreases the OATP1B1- and OATP1B3-dependent uptake of bosentan (26) and fexofenadine (36) in HEK293 and CHO cells. In addition to clinically applied drugs, naturally occurring flavonoids also interfere with the OATP-dependent uptake of DHEAS, thus indicating that they constitute a novel class of group of OATP1B1 modulators (37). Ongoing studies are verifying the interaction of drugs and dietary supplements with the OATP1B1-, OATP1B3- and OATP2B1-mediated uptake of curcumin, curcumin sulfate, and tetrahydrocurcumin. Whether other transporters like OATP2A1 and OATP4C1 which are expressed in ZR-75-1 wild type cells (24) are also involved in the uptake of curcumin and its main metabolites is not yet known. Potential candidates may be organic anion transporters (OATs) which were also shown to be involved in the transport of polyphenol conjugates clearly showing substrate specificity. While OAT1-overexpressing human embryonic kidney 293H cells demonstrated improved uptake for sulfate and glucuronide conjugates, such as quercetin-3'-O-sulfate, daidzein-7-O-glucuronide, genistein-7-O-glucuronide and quercetin-3'-O-glucuronide, OAT3 seems to have a higher affinity for sulfates such as quercetin-3'-O-sulfate but not for the isoflavone glucuronides (38,39). Most important, we could not confirm any passive diffusion mechanism for the uptake of curcumin, curcumin sulfate, and tetrahydrocurcumin as uptake kinetics in wild-type and OATP1B1-knockdownZR-75-1 cells was saturable and thus strongly indicating protein mediated transport. Uptake and efflux transport works in concert it was not possible to discriminate each part from another in our breast cancer cell model.

In conclusion, our data revealed that OATPs act as transporters for curcumin, curcumin sulfate, and tetrahydrocurcumin but not for curcumin glucuronide. The OATP-dependent uptake of curcumin sulfate in concert with intracellular sulfatases, which rapidly deconjugate sulfates to the pharmacologically active parent compound, represents a key factor explaining the observed pharmacological activity of curcumin.

Future in vivo studies should focus not only on the concentration of curcumin and its conjugates in target tissues but also on the expression levels of OATPs.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

NJ performed experiments and analyzed the data (uptake experiments, cytotoxicity and statistical analysis). QAJ collected and analyzed/interpreted the data (uptake experiments and cytotoxicity). JR designed and supervised the uptake experiments. DM performed and interpreted the NF-κB experiments. GK wrote the section of NF-κB and interpreted the data. BS was involved in the study conception, interpretation of data and proofreading of the manuscript. KJ interpreted the data of the uptake experiments and was involved in the proofreading of the manuscript. WJ interpreted the data, and wrote and edited the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interest.

References

1. Pan MH, Chiou YS, Chen LH, Ho CT: Breast cancer chemoprevention by dietary natural phenolic compounds: specific epigenetic related molecular targets. Mol Nutr Food Res 59:21-35, 2015.

2. Aqil F, Jeyabalan J, Munagala R, Ravoori S, Vadhanam MV, Schultz DJ, Gupta RC: Chemoprevention of rat mammary carcinogenesis by Apiaceae spices. Int J Mol Sci 18: pii: E425, 2017.
3. Hatcher H, Planalp R, Cho J, Torti FM, Torti SV: Curcumin: from ancient medicine to current clinical trials. Cell Mol Life Sci 65:1631-1652, 2008.
4. Bimonte S, Barbieri A, Palma G, Rea D, Luciano A, D'Aiuto M, Arra C, Izzo F. Dissecting the role of curcumin in tumor growth and angiogenesis in a mouse model of human breast cancer. Biomed Res Int 2015: 878134, 2015.
5. Lv Z-D, Liu X-P, Zhao W-J, Dong Q, Li F-N, Wang H-B, Kong B: Curcumin induces apoptosis in breast cancer cells and inhibits tumor growth in vitro and in vivo. Int J Clin Exp Pathol 7: 2818-2824, 2014
6. Wang Y, Yu J, Cui R, Lin J, Ding X. Curcumin in treating breast cancer: a review. J Lab Autom 2: 723-731, 2016.
7. Kumar G, Mittal S, Sak K, Tuli HS: Molecular mechanisms underlying chemopreventive potential of curcumin: current challenges and future perspectives. Life Sci 148: 313-328, 2016.
8. Bachmeier BE, Nerlich AG, Iancu CM, Cilli M, Schleicher E, Vené R, Dell'Eva R, Jochum M, Albini A, Pfeffer U: The chemopreventive polyphenol Curcumin prevents hematogenous breast cancer metastases in immunodeficient mice. Cell Physiol Biochem 19: 137-152, 2007.
9. Quispe-Soto ET, Calaf GM: Effect of curcumin and paclitaxel on breast carcinogenesis. Int J Oncol 49: 2569-2577, 2016.
10. Bayet-Robert M, Kwiatkowski F, Leheurteur M, Gachon F, Planchat E, Abrial C, Mouret-Reynier MA, Durando X, Barthomeuf C, Chollet P: Phase I dose escalation trial of docetaxel plus curcumin in patients with advanced and metastatic breast cancer. Cancer Biol Ther 9: 8-14, 2010.
11. Ireson C, Orr S, Jones DJL, Verschoyle R, Lim C-K, Luo J-L, Howells L, Plummer S, Jukes R, Williams M, Steward WP, Gescher A: Characterization of metabolites of the chemopreventive agent curcumin in human and rat hepatocytes and in the rat in vivo, and evaluation of their ability to inhibit phorbol ester-induced prostaglandin E2 production Cancer Res 61: 1058-1064, 2001.
12. Sharma RA, McLelland HR, Hill KA, Ireson CR, Euden SA, Manson MM, Pirmohamed M, Marnett LJ, Gescher AJ, Steward WP: Pharmacodynamic and

pharmacokinetic study of oral Curcuma extract in patients with colorectal cancer. Clin Cancer Res 7:1894-1900, 2001.

13. Prasad S, Tyagi AK, Aggarwal BB: Recent developments in delivery, bioavailability, absorption and metabolism of curcumin: the golden pigment from golden spice. Cancer Res Treat 46: 2-18, 2014.
14. Stanić Z: Curcumin, a compound from natural sources, a true scientific challenge – a review. Plant Foods Hum Nutr 72:1-12, 2017
15. Vareed SK, Kakarala M, Ruffin MT, Crowell JA, Normolle DP, Djuric Z, Brenner DE: Pharmacokinetics of curcumin conjugate metabolites in healthy human subjects. Cancer Epidemiol Biomarkers Prev 17: 1411-1417, 2008.
16. Jamil Q, Jaerapong, Zehl M, Jarukamjorn K, Jäger W: Metabolism of curcumin in human breast cancer cells: impact of sulfation on cytotoxicity. Planta Med 2017 83:1028-1034, 2017.
17. Tian Y, Bian Y, Jiang Y, Qian S, Yu A, Zeng S: Interplay of breast cancer resistance protein (BCRP) and metabolizing enzymes. Curr Drug Metab 16:877-893, 2015.
18. Ebert B, Seidel A, Lampen A: Phytochemicals induce breast cancer resistance protein in Caco-2 cells and enhance the transport of benzo[a]pyrene-3-sulfate.Toxicol Sci 96:227-236, 2007.
19. Hagenbuch B, Gui, C: Xenobiotic transporters of the human organic anion transporting polypeptides (OATP) family. Xenobiotica 38: 778-801, 2008.
20. Kim RB: Organic anion-transporting polypeptide (OATP) transporter family and drug disposition. Eur J Clin Invest 33 (Suppl 2):1-5, 2003.
21. Gui C, Obaidat A, Chaguturu R, Hagenbuch B: Development of a cell-based high-throughput assay to screen for inhibitors of organic anion transporting polypeptides 1B1 and 1B3. Curr Chem Genomics 4: 1-8, 2010.
22. Obaidat A, Roth M., Hagenbuch, B., The expression and function of organic anion transporting polypeptides in normal tissues and in cancer. Annu Rev Pharmacol Toxicol 52: 135-151, 2012.
23. Svoboda M, Riha J, Wlcek K, Jaeger W, Thalhammer T: Organic anion transporting polypeptides (OATPs): regulation of expression and function. Curr Drug Metab 12: 139-153, 2011.

24. Wlcek K, Svoboda M, Thalhammer T, Sellner F, Krupitza G, Jaeger W: Altered expression of organic anion transporter polypeptide (OATP) genes in human breast carcinoma. Cancer Biol Ther 7: 1452-1457, 2008.
25. Gui C, Miao Y, Thompson L, Wahlgren B, Mock M, Stieger B, Hagenbuch B: Effect of pregnane X receptor ligands on transport mediated by human OATP1B1 and OATP1B3. Eur J Pharmacol 584: 57-65, 2008.
26. Treiber A, Schneiter R, Hausler S, Stieger B: Bosentan is a substrate of human OATP1B1 and OATP1B3: inhibition of hepatic uptake as the common mechanism of its interactions with cyclosporin A, rifampicin, and sildenafil. Drug Metab Dispos 35: 1400-1407, 2007.
27. Leuthold S, Hagenbuch B, Mohebbi N, Wagner CA, Meier PJ, Stieger B: Mechanisms of pH-gradient driven transport mediated by organic anion polypeptide transporters. Am J Physiol Cell Physiol 296: C570-582, 2009.
28. Brenner S, Riha J, Giessrigl B, Thalhammer T, Grusch M, Krupitza G, Stieger B, Jäger W: The effect of organic anion-transporting polypeptides 1B1, 1B3 and 2B1 on the antitumor activity of flavopiridol in breast cancer cells. Int J Oncol 46:324-332, 2015.
29. Palermo DP, DeGraaf ME, Marotti KR, Rehberg E, Post LE: Production of analytical quantities of recombinant proteins in Chinese hamster ovary cells using sodium butyrate to elevate gene expression. J Biotechnol, 19: 35-47, 1991.
30. Otsuka S, Schaefer, O, Kawakami H, Inoue T, Lehner S, Saito A, Ishiguro N, Kishimoto W, Ludwig-Schwellinger E, Ebner T, Terasaki T: Simultaneous absolute protein quantification of transporters, cytochromes P450, and UDP-glucuronosyltransferases as a novel approach for the characterization of individual human liver: comparison with mRNA levels and activities. Drug Metab Dispos 40: 83–92, 2012.
31. Prasad B, Evers R, Gupta, Hop CE, Evers R, Gupta A, Hop CE, Salphati L, Shukla S, Ambudkar SV, Unadkat JD: Interindividual variability in hepatic organic anion-transporting polypeptides and P-glycoprotein (ABCB1) protein expression: quantification by liquid chromatography-tandem mass spectroscopy and influence of genotype, age, and sex. Drug Metab Dispos 42: 78–88, 2014.
32. Nies AT, Niemi M, Burk O, Winter S, Zanger UM, Stieger B, Schwab M, Schaeffeler E: Genetics is a major determinant of expression of the human

hepatic uptake transporter OATP1B1, but not of OATP1B3 and OATP2B1. Genome Med 5: 1, 2013.

33. Pan MH, Huang TM, Lin JK: Biotransformation of curcumin through reduction and glucuronidation in mice. Drug Metab Dispos 27:486-494, 1999.
34. Wang W, Nag SA, Zhang R: Targeting the NFκB signaling pathways for breast cancer prevention and therapy. Curr Med Chem 22:264-289, 2015.
35. Sen GS, Mohanty S, Hossain DM, Bhattacharyya S, Banerjee S, Chakraborty J, Saha S, Ray P, Bhattacharjee P, Mandal D, Bhattacharya A, Chattopadhyay S, Das T, Sa G: Curcumin enhances the efficacy of chemotherapy by tailoring p65NFκB-p300 cross-talk in favor of p53-p300 in breast cancer. J Biol Chem 286: 42232-42247, 2011.
36. Matsushima, S., Maeda, K., Ishiguro, N., Igarashi, T., Sugiyama, Y., Investigation of the inhibitory effects of various drugs on the hepatic uptake of fexofenadine in humans. Drug metabolism and disposition: the biological fate of chemicals 2008, 36, 663-669.
37. Wang X, Wolkoff AW, Morris ME: Flavonoids as a novel class of human organic anion-transporting polypeptide OATP1B1 (OATP-C) modulators. Drug Metab Dispos 33:1666-1672, 2005.
38. Wong CC, Botting NP, Orfila C, Al-Maharik N, Williamson G: Flavonoid conjugates interact with organic anion transporters (OATs) and attenuate cytotoxicity of adefovir mediated by organic anion transporter 1 (OAT1/SLC22A6). Biochem Pharmacol 81:942–949, 2011.
39. Wong CC, Akiyama Y, Abe T, Lippiat JD, Orfila C, Williamson G: Carrier-mediated transport of quercetin conjugates: involvement of organic anion transporters and organic anion transporting polypeptides. Biochem Pharmacol 84: 564–570, 2012.

Figure legends:

Figure 1. Concentration dependence of curcumin uptake in OATP1B1- (A), OATP1B3- (B) and OATP2B1- (C) transfected CHO cells. 350,000 cells were seeded in 12-well plates and allow to grow to confluence. Then cells were incubated with curcumin (25-200 µM) for 1 min at pH 7.4, 37°C and the cytoplasm analyzed for curcumin by HPLC. The data represent the mean ± SD of 3 individual determinations.

Figure 2. Concentration dependence of curcumin sulfate uptake in OATP1B1- (A), OATP1B3- (B) and OATP2B1- (C) transfected CHO cells. 350,000 cells were seeded in 12-well plates and allow to grow to confluence. Then cells were incubated with curcumin sulfate (25-300 µM) for 1 min at pH 7.4 (37°C) and the cytoplasm analyzed for curcumin sulfate by HPLC. The data represent the mean ± SD of 3 individual determinations.

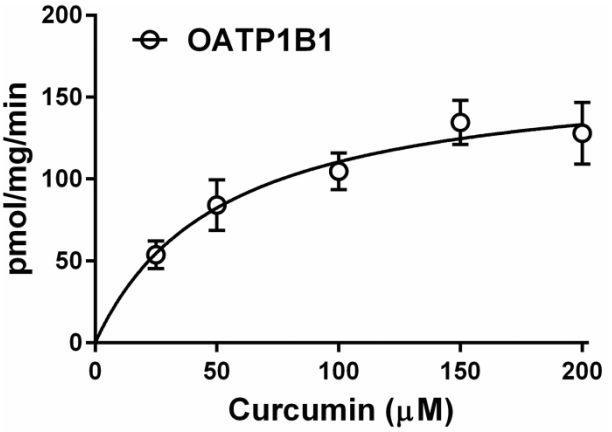
Figure 3. Concentration dependence of tetrahydrocurcumin uptake in OATP1B1- (A), OATP1B3- (B) and OATP2B1- (C) transfected CHO cells. 350,000 cells were seeded in 12-well plates and allow to grow to confluence. Then cells were incubated with tetrahydrocurcumin (50-600 µM) for 1 min at pH 7.4 (37°C) and the cytoplasm analyzed for tetrahydrocurcumin by HPLC. The data represent the mean ± SD of 3 individual determinations.

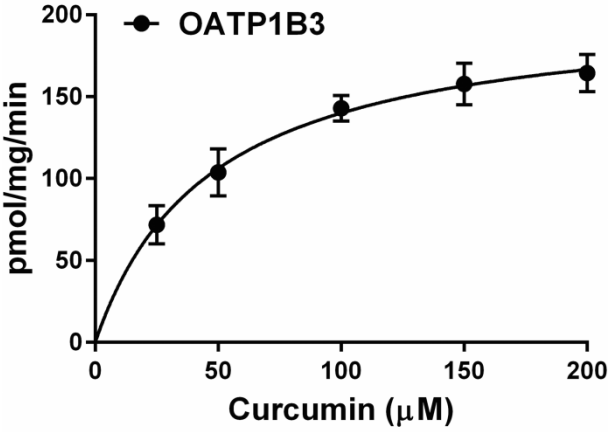
Figure 4. Concentration dependent uptake rates of curcumin in ZR-75-1 empty vector-transfected cells compared to ZR-75-1 OATP1B1-knockdown cells. 1×10^6 cells were seeded in 6-well plates and allowed to grow to confluence. The cells were incubated with curcumin (25- 200 µM) for 1 min at 37°C and the cytoplasm analyzed for curcumin by HPLC. The data represent the mean ± SD of 3 individual determinations.

Figure 5. Cytotoxicity of curcumin to ZR-75-1 and OATP1B1 knockdown ZR-75-1 cells. After incubation of 50,000 cells for 72 h with 2.5-100 µM curcumin at 37°C viable cells were determined. Dose response curves were obtained by nonlinear curve fitting using GraphPad Prism 6.0 program. The data represent the mean ± SD of three individual determinations.

Figure 6. Inhibition of NF-kB activity by curcumin. 100,000 ZR-75-1 wild type and ZR-75-1 OATP1B1-knockdown cells were seeded in 24-well plates and allowed to grow to 70% confluence. Cells were then pre-treated with 10 µM Bay11-7082 (BAY) or 100 µM curcumin (CUR) for 30 min or with solvent (DMSO; Co). Thereafter, where indicated, cells were stimulated with interleukin-1β (10 ng/ml IL-1β for 90 min), when cells were lysed and Firefly luciferase activity was determined, which was normalized to Renilla luciferase activity (measured subsequently; RLU: relative light unit). Experiments were done in triplicate, error bars indicate +/- SD and asterisks significance between the IL-1β-induced positive controls and the IL-1β-induced BAY and CUR treatment groups (significance: $P<0.05$; t-test).

Figure 1


OATP1B1
pmol/mg/min
200
150
100
50
0
0
50
100
150
200
Curcumin (μM)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pmol/mg/min
200
150
100
50
0
0
50
100
150
200
Curcumin (μM)

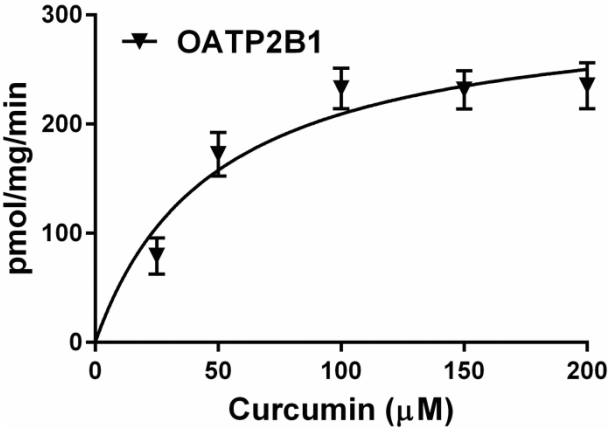
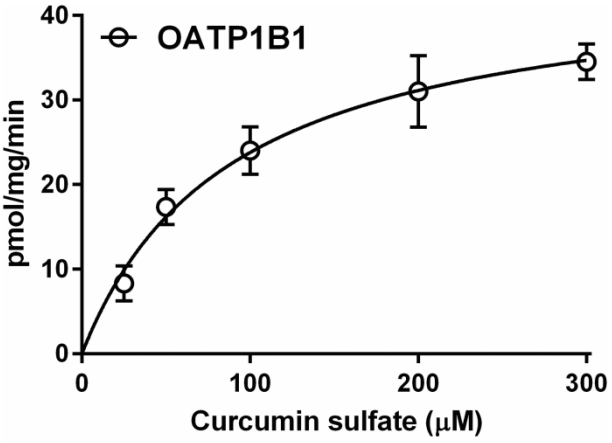
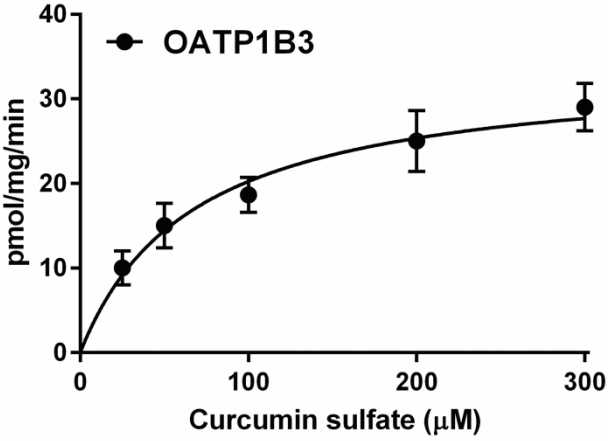

OATP2B1
pmol/mg/min
300
200
100
0
0
50
100
150
200
Curcumin (μM)

Figure 2


OATP1B1
pmol/mg/min
0
10
20
30
40
0
100
200
300
Curcumin sulfate (μM)


OATP1B3
pmol/mg/min
0
10
20
30
40
0
100
200
300
Curcumin sulfate (μM)

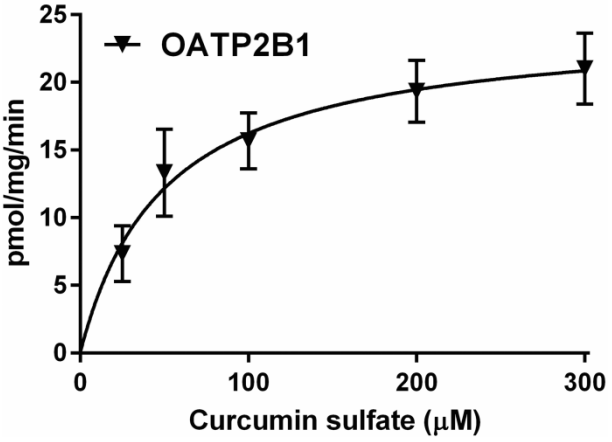
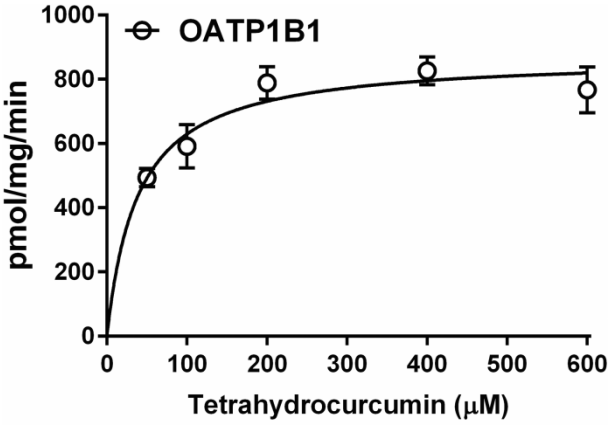
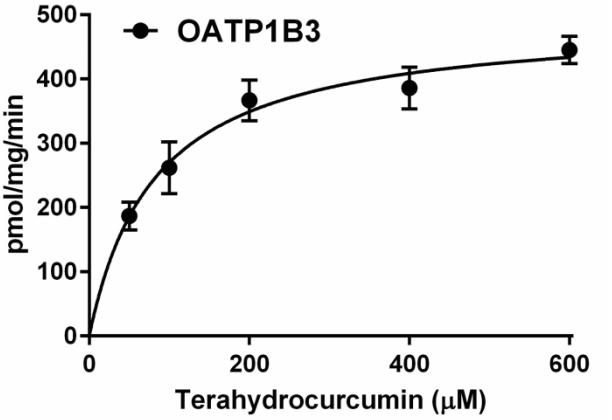

OATP2B1
pmol/mg/min
0
5
10
15
20
25
0
100
200
300
Curcumin sulfate (μM)

Figure 3


OATP1B1
pmol/mg/min
1000
800
600
400
200
0
0
100
200
300
400
500
600
Tetrahydrocurcumin (μM)


OATP1B3
pmol/mg/min
500
400
300
200
100
0
0
200
400
600
Terahydrocurcumin (μM)

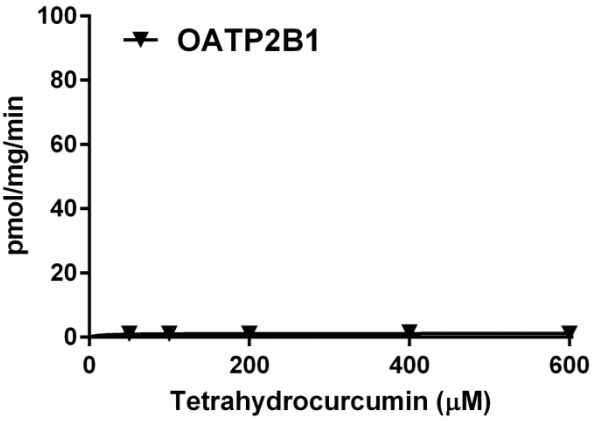

OATP2B1
pmol/mg/min
100
80
60
40
20
0
0
200
400
600
Tetrahydrocurcumin (μM)

Figure 4

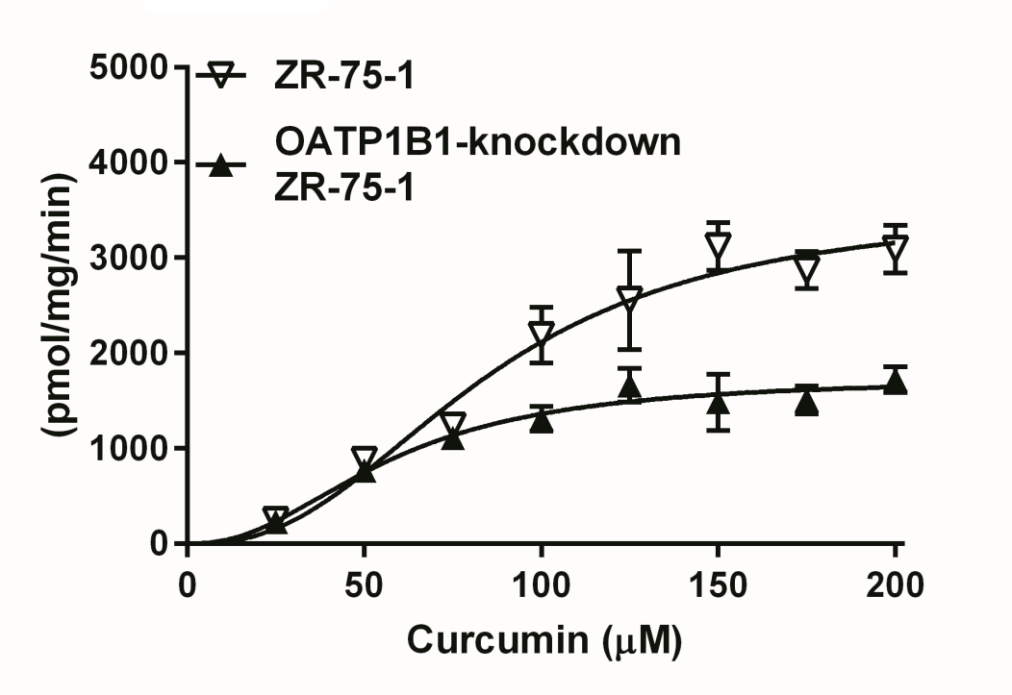

ZR-75-1
OATP1B1-knockdown ZR-75-1
(pmol/mg/min)
5000
4000
3000
2000
1000
0
0
50
100
150
200
Curcumin (μM)

Figure 5

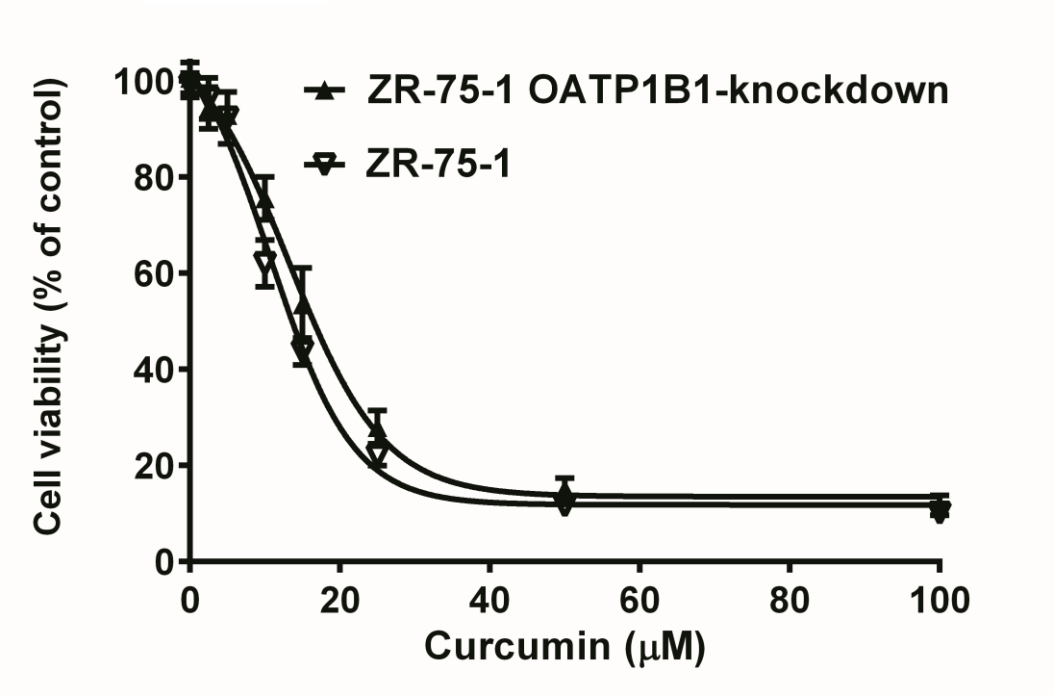

ZR-75-1 OATP1B1-knockdown
ZR-75-1
Cell viability (% of control)
100
80
60
40
20
0
0
20
40
60
80
100
Curcumin (μM)

Figure 6

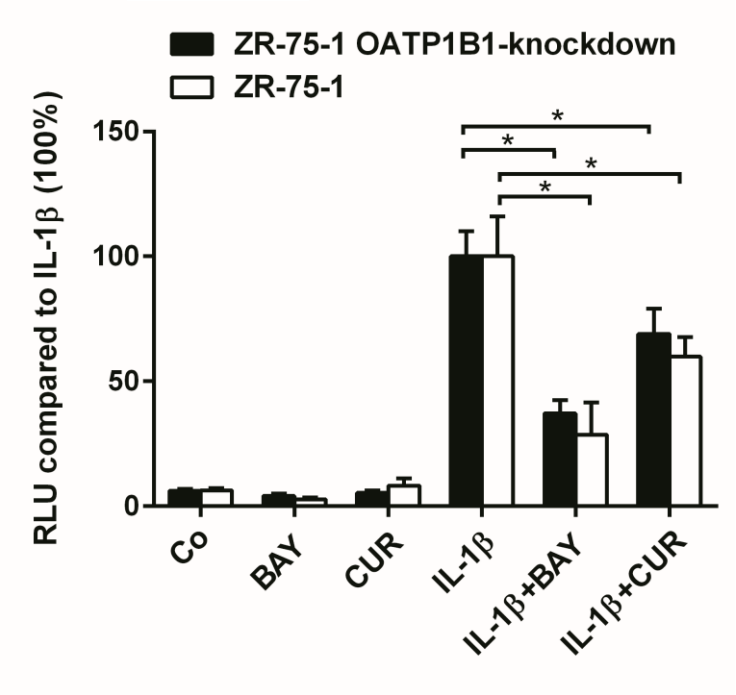

ZR-75-1 OATP1B1-knockdown
ZR-75-1
RLU compared to IL-1β (100%)
150
100
50
0
*
*
*
*
Co
BAY
CUR
IL-1β
IL-1β+BAY
IL-1β+CUR

Table 1. Michaelis-Menten parameters for the uptake of curcumin, curcumin sulfate, tetrahydrocurcumin and curcumin glucuronide in OATP-transfected CHO cells

Substrate	Km [µM]	Vmax [pmol/mg/min]	Vmax/Km [µl/min.µg]
OATP1B1			
Cur	51.9 ± 13.6	167 ± 14.8	3.21 ± 0.64
Cur-S	89.1 ± 16.4	45.0 ± 3.34	0.51 ± 0.06
TH-cur	38.6 ± 7.9	872 ± 35.6	22.6 ± 3.86
Cur-G	n.d.	n.d.	n.d.
OATP1B3			
Cur	46.9 ± 7.5	205 ± 10.7	4.37 ± 0.48
Cur-S	67.7 ± 15.3	33.9 ± 2.76	0.50 ± 0.076
TH-cur	83.7 ± 13.4	493 ± 22.5	5.89 ± 0.69
Cur-G	n.d.	n.d.	n.d.
OATP2B1			
cur	48.6 ± 12.7	310 ± 26.6	6.37 ± 1.27
Cur-S	50.0 ± 12.6	24.3 ± 1.87	0.48 ± 0.102
TH Cur	n.d.	n.d.	n.d.
Cur-G	n.d.	n.d.	n.d.

Values are means ± SE of 3 individual determinations. The net OATP-mediated uptake values were calculated by subtracting the values obtained with the wild-type CHO cells from those obtained with the stably-transfected cells. Kinetic parameters were calculated by fitting the data to the Michaelis-Menten equation with nonlinear regression. Cur, curcumin; Cur-S, curcumin sulfate; TH-cur; tetrahydrocucumin; Cur-G, curcumin glucuronide; nd., not determined

Table 2. Michaelis-Menten parameters of curcumin uptake determined in ZR-75-1 wild-type and OATP1B1-knockdown ZR-75-1 cells

Substrate	ZR-75-1		OATP1B-knockdown ZR-75-1	
	Km [µM]	Vmax [pmol/mg/min]	Km [µM]	Vmax [pmol/mg/min]
Curcumin	85.± 9.1	3535 ± 342	56.7± 5.8	1741 ± 122

6. Discussion and future aspects

The aim of the thesis was to elucidate the impact of cellular uptake and metabolism of curcumin on its activity. To investigate the role of phase-II metabolizing enzymes, we used hormone dependent ZR-75-1 and hormone independent MDA-MB-231 cell lines to detect the metabolic fate of curcumin. Our results showed that curcumin sulfate is a major metabolite in breast cancer cell lines. Interestingly, curcumin glucuronide was not detected in the breast cancer cells despite that it is a major metabolite in liver and intestine [1]. Preference of sulfate conjugation over glucuronidation in breast cancer cells are in line with the previous studies and several xenobiotics including resveratrol, genistein, 2-methoxy estradiol and estrogen which also display the same metabolic fate [2-4]. Since, curcumin sulfate is less active than curcumin and devoid of anti-cancer activity [5], so, we also quantified the concentration of curcumin sulfate produced by both the cell lines and analyzed the cytotoxicity in the respective cells. We found that ZR-75-1 cells are able to produce double amount of curcumin sulfate as compared to MDA-MB-231. Furthermore, we also quantified the concentration of curcumin sulfate in cellular medium and determined that concentration of curcumin sulfate was 12 times higher in medium as compared to the cytoplasm, suggesting that curcumin sulfate is immediately effluxed by some unidentified transporters. Breast cancer resistant protein (BCRP), multi drug resistance protein (MRP) and p-glycoprotein (P-gp) can play an important role in this efflux [6,7]. BCRP is expressed in several tissues including breast ductal cells and involved in efflux of several drugs or xenobiotics [8]. It is already reported that BCRP is responsible for the efflux of curcumin and this may also be true for its sulfate conjugate [9]. Furthermore, MRP8 and MRP9 exhibits high expressions in human breast cancer cells [10,11]; however, their interaction with curcumin or its sulfate metabolite is yet not identified and can be a future direction for further research work. By comparing both the cells lines, it was also revealed that effluxed curcumin sulfate was also double in ZR-75-1 cell line as compared to MDA-MB-231.

This formation and greater efflux of curcumin sulfate in ZR-75-1 cells resulted in significantly lower accumulation of unconjugated curcumin in the cytoplasm, leading to higher EC_{50} value (14µM) exhibited by curcumin in ZR-75-1 cells. The concentration of unconjugated curcumin was 8 times higher in MDA-MB-231 cells due to reduced formation of curcumin sulfate in this cell line that could be the reason for high sensitivity and lower EC_{50} value of curcumin exhibited in MDA-MB-231 cells. This data clearly indicates that cellular sulfation plays a major role in cytotoxic activity of curcumin in breast cancer cells.

Our results are in line with previous data of our lab regarding resveratrol [2]. Till to date, it is unknown which individual isoform is responsible for this differential sulfation of curcumin in breast cancer cells. However, a very recent study reveals that SULT1A3, 1C4 and 1E1 are major enzymes involved in conjugation of curcumin; whereas SULT1A1 and 2A1 also demonstrate lower sulfation rate of curcumin [12]. Moreover, RT-PCR studies conducted in our lab revealed that mRNA of SULT1A1 has higher expression in ZR-75-1 cell line in comparison to MDA-MB-231 cells whereas SULT1A3 has same expression levels in both cell lines [2]. Additionally, 1E1 and 2A1 were below detection limit in both the cell lines [2]. SULT1C4 is highly expressed in fetal liver, kidney and small intestine [13]. Thus, we hypothesized that SULT1A1is responsible for this differential sulfation of curcumin in breast cancer cell lines. However in future, our hypothesis may be confirmed experimentally.

For the very first time, we also detected the formation of curcumin dimers in breast cancer cells. However, its V_{max} value was 4 times lower than sulfation, suggesting that these dimers play a minor role in growth inhibition activity of curcumin. In short, our study revealed that sulfation of curcumin in breast cancer cells is main the biotransformation pathway.

In our second manuscript, we used stably transfected OATP expressing Chinese hamster ovarian (CHO) cell line in order to determine the role of OATP1B1, 1B3 and 2B1 on the cellular uptake of curcumin, its phase-I metabolite, tetrahydrocurcumin and phase-II conjugates curcumin sulfate and glucuronide. Till now, no study was conducted to elucidate OATP mediated transportation of curcumin. However, we identified that OATP1B1, 1B3, and 2B1 are involved in active transportation of curcumin and its sulfated conjugates into the cells. OATP2B1 exhibited highest affinity for curcumin with K_m value 35.3µM, whereas OATP1B1 and 1B3 demonstrated 1.6 and 2.7 fold lower affinity for curcumin. Interestingly, OATP1B1 and 1B3 have similar affinities for curcumin sulfate, whereas its uptake by SULT2B1 exhibits far lower affinity. In addition, V_{max}/k_m value for OATP1B1 and 1B3 mediated uptake of curcumin sulfate is also close to each other (0.4 and 0.6 µl/min/µg respectively).

It is worth noting that curcumin glucuronide is not transported by any of the three OATPs, suggesting that curcumin sulfate, instead of curcumin glucuronide, supplies the intracellular pool for curcumin generation. This fact is further supported by already documented reports that sulfate metabolites of several polyphenols can be easily converted back to their parent compounds with the help of sulfatases [14]. Moreover, tetrahydrocurcumin was only transported by OATP1B1 and 1B3, not by OATP2B1 with K_m

value of 24.38µM and 84.24µM respectively. As tetrahydrocurcumin is pharmacologically active compound, thus the uptake of tetrahydrocurcumin is very important and may play an important role in curcumin activity.

Furthermore, quantitative protein analysis and Western blot results revealed high inter-individual variability of OATPs, thus it is difficult to estimate exact role of OATP1B1 and OATP1B3 in liver as well as OATP2B1 in gut for the uptake of curcumin and its metabolites based upon Michaelis-Menten equation [15,16]. According to our results, OATP1B1, 1B3 and 2B1 are low affinity transporters, having plasma concentrations significantly lower than K_m values. The oral administration of 3.6 g of curcumin daily up to 4 months to human volunteers, demonstrated peak plasma concentration of 3.6µM for unconjugated curcumin [17]. Moreover, the phase-I clinical trial after 3.6g oral dosing of curcumin up to 28 days daily in colorectal patients reveals that maximum plasma concentrations for curcumin, curcumin sulfate and curcumin glucuronide are 11, 16 and 9 nmol/L respectively [18]. In spite of the fact, that curcumin shows low plasma concentration with respect to its K_m value, curcumin uptake into cancer cells is slow but pharmacodynamically effective. It is already reported that OATP mediated transportation is higher at low pH [19]. Since, OATP2B1 is localized in upper gut where the pH is relatively low and the literature data indicates local pH of cancerous tissues is also acidic, this similarity suggests that pH dependency of OATP mediated uptake of curcumin and its conjugates should be considered during *in vivo* therapy especially in cancer treatment [20].

In order to confirm the role of OATP1B1 for the uptake of curcumin in hormone dependent ZR-75-1 breast cancer cell line, we incubated the increasing concentrations of curcumin in wild type and OATP1B1 knockdown ZR-75-1 cell line for 1 min. Notably, it is already documented that ZR-75-1 cell line shows high expression for OATP1B1 but not for OATP1B3 and 2B1 [21]. As expected, we found out that OATP1B1 knockout cells transported significantly lower curcumin in comparison to wild type with higher K_m and lower V_{max} values (V_{max} 1840 for OATP1B1 knockdown versus 3377 pmol/mg/min for WT ZR-75-1 cells). This trend was also seen in curcumin mediated interleukin inhibition activity in both cell lines.

Curcumin uptake mediated by OATP1B1, 1B3 and 2B1 may prove to be clinically significant due to expression of these transporters in various tumors such as in colorectal, pancreatic, liver, ovarian and prostate cancer cells [22]. Any alteration in OATP expression may considerably change the transportation of curcumin and its metabolites into targeted cells and tissues, thus influencing the treatment efficacy. Indeed, patients with reduced expression

of wild type OATP1B1, 1B3 and 2B1 or patients carrying polymorphic OATP allele may thereby demonstrate lower response for therapy. In addition, transporter mediated drug-drug interactions have also been observed after co-administration of OATP inhibitors with curcumin. As rifampicin is already reported specific inhibitor of OATP [23], and contaminant administration of rifampicin and curcumin may demonstrate drug-drug interaction. The other potential OATPs inhibitors are clarithromycin, erythromycin and roxithromycin that are involved in inhibition of pravastatin transport in OATP1B1 and 1B3 transfected HEK293 cells with IC_{50} value 32-37μM [24]. In addition, cyclosprin-A also considerably inhibits the OATP1B1 and 1B3 mediated uptake of fexofenadine [25] and bosentan [26] in CHO and HEK293 cell line. It is still unknown whether all OATPs inhibitors influence the curcumin uptake in tumor cells but care should be taken in administrating OATPs inhibitors in combination of curcumin. Ongoing studies are confirming drug as well as dietary supplement interaction with OATP1B1, 1B3 and 2B1 mediated uptake of curcumin and its metabolites.

We have elucidated the role of OATP1B1, OATP1B3 and OATP2B1 for the uptake of curcumin and its metabolites; however, several other OATPs transporters including OATP2A1 and OATP4C1 are also expressed in wild type ZR-75-1 breast cancer cell line. Thus, it is still unknown about their role in transportation of curcumin and its metabolites. Furthermore, a very recent study reveals that organic anion transporters (OATs) such as OAT1 and OAT3 are involved in the uptake of curcumin in HEK293 cells. However, curcumin glucuronide is only transported by OAT3, [27] indicating that OATs, in addition to OATPs, also contribute in transportation of curcumin and its metabolites into the cells. The role of OATP2A1 and 4C1 in ZR-75-1 cell line can be a next step for further studies. In addition, a study can be conducted using increasing concentration of curcumin and its metabolites in OATs expressing cells to establish the Michaelis-Menten plot, determining K_m and V_{max} for curcumin and its metabolites.

In short, we demonstrated that OATPs act as transporter for curcumin, tetrahydrocurcumin and curcumin sulfate but not for curcumin glucuronide. This OATP mediated transported curcumin sulfate is de-conjugated into curcumin by sulfatase, explaining the observed pharmacological effects of curcumin. Thus, we suggest that future studies should also consider the expression levels of OATPs in addition to concentration of curcumin and its metabolites into the target tissues.

In our third manuscript we determined the spasmolytic effect of all three curcuminoids and their metabolite; tetrahydrocurcumin on ileum, aorta and pulmonary artery. In addition, we also investigated ionotropic and chronotropic activities on isolated papillary muscles and

right atrium of guinea pig, respectively. In addition to pharmacological activities, we also quantified uptake of these compounds in respective tissue samples and correlated them with their observed pharmacological effects.

All the four compounds demonstrated significant antispasmodic effect in isolated ileum, thereby, confirming the beneficial effects of turmeric powder in gastrointestinal problems such as in irritable bowel disease [28]. As observed in a pilot study where 144 mg of turmeric extract was given to 105 patients suffering from irritable bowel syndrome, 25% of them felt reduced abdominal pain and discomfort [29]. Since, nitric acid is already reported to be involved in modulation of gastrointestinal movements; hence, we used L-NNA to evaluate the effect of eNOS blockage on spasmolytic activity of curcuminoids. However, we did not observe any effect by eNOS blockade on curcumin mediated spasmolytic activity. So, the observed spasmolytic activity of curcuminoids may be attiributed to the blockade of calcium influx from voltage gated calcium channels since it relaxed high-K^+ pre-contracted tissues [30]. Curcumin, bismethoxycurcumin and tetrahydrocurcumin were able to modestly relax KCl-contracted pulmonary artery whereas demethoxycurcumin significantly relaxed pre-contracted pulmonary artery with EC_{50} value 15.78µM, suggesting that demthoxycurcumin was responsible for *Curcuma longa* extract mediated vasodilatory effect. It was reported previously that *Curcuma longa* extract demonstrated mild vasoconstriction in the absence of any agonist [31]. However, it showed vasorelaxation against agonist induced contractions [31,32]. This enigma of dual activity of *Curcuma longa* extract on aorta samples is solved by our results that curcumin demonstrated mild vasoconstriction whereas other two curcuminoids and tetrahydrocurcumin showed vasorelaxation in isolated aorta samples.

Curcuma longa extract also exhibits variable effect on arterial pressure of anesthetized mice, demonstrating both hypotension and hypertension. Thus, we determined both ionotropic and chronotropic activity of all compounds in right atrium and papillary muscles respectively. All three curcuminoids mildly attenuated atrial contraction rate, leading to bradycardia. However, these compounds showed variable ionotropic activity; curcumin and bismethoxycurcumin demonstrated mild positive ionotropic activity, whereas demethoxycurcumin and tetrahydrocurcumin showed mild negative ionotropic activity, explaining the reason behind variable response by crude extract.

As previously reported, curcumin has wide distribution after oral intake[33] , thus we also determined the tissue uptake of curcumin in different organs. Our results demonstrated that intestine is the rate limiting organ for curcumin uptake. The uptake of curcuminoids and tetrahydrocurcumin was somehow correlated with the activity. Bisdemethoxycurcumin not

only showed the most potent spasmolytic activity in ileum but also exhibited highest concentration in this tissue. Similarly, demethoxycurcumin has highest concentration in pulmonary artery which is well correlated with its vasodilating effect. Highest concentration of demethoxycurcumin was observed in pulmonary artery, whereas lowest concentration of tetrahydrocurcumin was found in right atria. This trend was also observed in chronotropic activities caused by them; demethoxycurcumin showed highest chronotropic activity, whereas tetrahydrocurcumin demonstrated negligible effects. Furthermore, highest ionotropic activities shown by demethoxycurcumin and tetrahydrocurcumin were well correlated to their highest tissue concentration. We observed that demethoxycurcumin and bisdemethoxycurcumin have higher tissue uptake than curcumin; this may be due to the higher stability of these compounds at physiological pH, preventing them from degradation into non active freulic acid, feruloyl methane and vanillin. Furthermore, tetrahydrocurcumin is also reported of having better stability than curcumin.

In short, demethoxycurcumin and bismethoxycurcumin exhibited higher spasmolytic, vasodilating and ionotropic activities than curcumin, suggesting their contribution to observed effects. As, ileum is found main rate limiting organ in our studies, thus, in future, studies may be conducted to identify the drug transporters responsible for uptake and efflux of curcuminoids and its metabolites from ileum. This may improve our knowledge about these transporters and help in increasing bioavailability of curcuminoids.

6.1 References

1. Metzler M, Pfeiffer E, Schulz SI, Dempe JS. Curcumin uptake and metabolism. BioFactors (Oxford, England) 2013; 39: 14-20
2. Murias M, Miksits M, Aust S, Spatzenegger M, Thalhammer T, Szekeres T, Jaeger W. Metabolism of resveratrol in breast cancer cell lines: Impact of sulfotransferase 1A1 expression on cell growth inhibition. Cancer Lett 2008; 261: 172-182
3. Spink BC, Katz BH, Hussain MM, Pang S, Connor SP, Aldous KM, Gierthy JF, Spink DC. SULT1A1 catalyzes 2-methoxyestradiol sulfonation in MCF-7 breast cancer cells. Carcinogenesis 2000; 21: 1947-1957
4. Peterson TG, Coward L, Kirk M, Falany CN, Barnes S. The role of metabolism in mammary epithelial cell growth inhibition by the isoflavones genistein and biochanin A. Carcinogenesis 1996; 17: 1861-1869
5. Ireson C, Orr S, Jones DJL, Verschoyle R, Lim C-K, Luo J-L, Howells L, Plummer S, Jukes R, Williams M, Steward WP, Gescher A. Characterization of Metabolites of the Chemopreventive Agent Curcumin in Human and Rat Hepatocytes and in the Rat in Vivo, and Evaluation of Their Ability to Inhibit Phorbol Ester-induced Prostaglandin E₂ Production. Cancer Research 2001; 61: 1058-1064
6. Yu XQ, Xue CC, Wang G, Zhou SF. Multidrug resistance associated proteins as determining factors of pharmacokinetics and pharmacodynamics of drugs. Current drug metabolism 2007; 8: 787-802
7. Mo W, Zhang JT. Human ABCG2: structure, function, and its role in multidrug resistance. International journal of biochemistry and molecular biology 2012; 3: 1-27
8. Choudhuri S, Klaassen CD. Structure, Function, Expression, Genomic Organization, and Single Nucleotide Polymorphisms of Human ABCB1 (MDR1), ABCC (MRP), and ABCG2 (BCRP) Efflux Transporters. International Journal of Toxicology 2006; 25: 231-259
9. Ebert B, Seidel A, Lampen A. Phytochemicals induce breast cancer resistance protein in Caco-2 cells and enhance the transport of benzo[a]pyrene-3-sulfate. Toxicological sciences : an official journal of the Society of Toxicology 2007; 96: 227-236
10. Bera TK, Iavarone C, Kumar V, Lee S, Lee B, Pastan I. MRP9, an unusual truncated member of the ABC transporter superfamily, is highly expressed in breast cancer. Proceedings of the National Academy of Sciences of the United States of America 2002; 99: 6997-7002

11. Bera TK, Lee S, Salvatore G, Lee B, Pastan I. MRP8, a new member of ABC transporter superfamily, identified by EST database mining and gene prediction program, is highly expressed in breast cancer. Molecular medicine (Cambridge, Mass) 2001; 7: 509-516
12. Lu X, Jiang K, Han L, Zhang M, Zhou Y, Ma Y, Zhou Y, Meng S. Sulfonation of curcuminoids: Characterization and contribution of individual SULT enzymes. Mol Nutr Food Res 2015; 59: 634-645
13. Sakakibara Y, Yanagisawa K, Katafuchi J, Ringer DP, Takami Y, Nakayama T, Suiko M, Liu MC. Molecular cloning, expression, and characterization of novel human SULT1C sulfotransferases that catalyze the sulfonation of N-hydroxy-2-acetylaminofluorene. The Journal of biological chemistry 1998; 273: 33929-33935
14. Bojarova P, Williams SJ. Sulfotransferases, sulfatases and formylglycine-generating enzymes: a sulfation fascination. Current opinion in chemical biology 2008; 12: 573-581
15. Nies AT, Niemi M, Burk O, Winter S, Zanger UM, Stieger B, Schwab M, Schaeffeler E. Genetics is a major determinant of expression of the human hepatic uptake transporter OATP1B1, but not of OATP1B3 and OATP2B1. Genome medicine 2013; 5: 1
16. Prasad B, Evers R, Gupta A, Hop CE, Salphati L, Shukla S, Ambudkar SV, Unadkat JD. Interindividual variability in hepatic organic anion-transporting polypeptides and P-glycoprotein (ABCB1) protein expression: quantification by liquid chromatography tandem mass spectroscopy and influence of genotype, age, and sex. Drug Metab Dispos 2014; 42: 78-88
17. Cheng AL, Hsu CH, Lin JK, Hsu MM, Ho YF, Shen TS, Ko JY, Lin JT, Lin BR, Ming-Shiang W, Yu HS, Jee SH, Chen GS, Chen TM, Chen CA, Lai MK, Pu YS, Pan MH, Wang YJ, Tsai CC, Hsieh CY. Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. Anticancer Res 2001; 21: 2895-2900
18. Sharma RA, Euden SA, Platton SL, Cooke DN, Shafayat A, Hewitt HR, Marczylo TH, Morgan B, Hemingway D, Plummer SM, Pirmohamed M, Gescher AJ, Steward WP. Phase I clinical trial of oral curcumin: biomarkers of systemic activity and compliance. Clin Cancer Res 2004; 10: 6847-6854

19. Leuthold S, Hagenbuch B, Mohebbi N, Wagner CA, Meier PJ, Stieger B. Mechanisms of pH-gradient driven transport mediated by organic anion polypeptide transporters. American journal of physiology Cell physiology 2009; 296: C570-582
20. Riha J, Brenner S, Bohmdorfer M, Giessrigl B, Pignitter M, Schueller K, Thalhammer T, Stieger B, Somoza V, Szekeres T, Jager W. Resveratrol and its major sulfated conjugates are substrates of organic anion transporting polypeptides (OATPs): impact on growth of ZR-75-1 breast cancer cells. Mol Nutr Food Res 2014; 58: 1830-1842
21. Wlcek K, Svoboda M, Thalhammer T, Sellner F, Krupitza G, Jaeger W. Altered expression of organic anion transporter polypeptide (OATP) genes in human breast carcinoma. Cancer Biol Ther 2008; 7: 1450-1455
22. Buxhofer-Ausch V, Secky L, Wlcek K, Svoboda M, Kounnis V, Briasoulis E, Tzakos AG, Jaeger W, Thalhammer T. Tumor-specific expression of organic anion-transporting polypeptides: transporters as novel targets for cancer therapy. Journal of drug delivery 2013; 2013: 863539
23. Asaumi R, Toshimoto K, Tobe Y, Hashizume K, Nunoya KI, Imawaka H, Lee W, Sugiyama Y. Comprehensive PBPK Model of Rifampicin for Quantitative Prediction of Complex Drug-Drug Interactions: CYP3A/2C9 Induction and OATP Inhibition Effects. CPT: pharmacometrics & systems pharmacology 2018; 7: 186-196
24. Seithel A, Eberl S, Singer K, Auge D, Heinkele G, Wolf NB, Dorje F, Fromm MF, Konig J. The influence of macrolide antibiotics on the uptake of organic anions and drugs mediated by OATP1B1 and OATP1B3. Drug Metab Dispos 2007; 35: 779-786
25. Matsushima S, Maeda K, Ishiguro N, Igarashi T, Sugiyama Y. Investigation of the inhibitory effects of various drugs on the hepatic uptake of fexofenadine in humans. Drug Metab Dispos 2008; 36: 663-669
26. Treiber A, Schneiter R, Hausler S, Stieger B. Bosentan is a substrate of human OATP1B1 and OATP1B3: inhibition of hepatic uptake as the common mechanism of its interactions with cyclosporin A, rifampicin, and sildenafil. Drug Metab Dispos 2007; 35: 1400-1407
27. Zhou X, Zhang F, Chen C, Guo Z, Liu J, Yu J, Xu Y, Zhong D, Jiang H. Impact of curcumin on the pharmacokinetics of rosuvastatin in rats and dogs based on the conjugated metabolites. Xenobiotica 2016, DOI: 10.1080/00498254.2016.1183060: 1-9

28. Annahazi A, Roka R, Rosztoczy A, Wittmann T. Role of antispasmodics in the treatment of irritable bowel syndrome. World journal of gastroenterology 2014; 20: 6031-6043
29. Bundy R, Walker AF, Middleton RW, Booth J. Turmeric extract may improve irritable bowel syndrome symptomology in otherwise healthy adults: a pilot study. Journal of alternative and complementary medicine (New York, NY) 2004; 10: 1015-1018
30. Adaramoye OA, Anjos RM, Almeida MM, Veras RC, Silvia DF, Oliveira FA, Cavalcante KV, Araujo IG, Oliveira AP, Medeiros IA. Hypotensive and endothelium-independent vasorelaxant effects of methanolic extract from Curcuma longa L. in rats. Journal of ethnopharmacology 2009; 124: 457-462
31. Gilani AH, Shah AJ, Ghayur MN, Majeed K. Pharmacological basis for the use of turmeric in gastrointestinal and respiratory disorders. Life Sci 2005; 76: 3089-3105
32. Micucci M, Aldini R, Cevenini M, Colliva C, Spinozzi S, Roda G, Montagnani M, Camborata C, Camarda L, Chiarini A, Mazzella G, Budriesi R. Curcuma longa L. as a therapeutic agent in intestinal motility disorders. 2: Safety profile in mouse. PloS one 2013; 8: e80925
33. Matabudul D, Pucaj K, Bolger G, Vcelar B, Majeed M, Helson L. Tissue distribution of (Lipocurc) liposomal curcumin and tetrahydrocurcumin following two- and eight-hour infusions in Beagle dogs. Anticancer Res 2012; 32: 4359-4364

7. Conclusion

Curcumin is a polyphenol having numerous biological activities however, it has poor bioavailability. The uptake of curcumin takes place with the help of OATP1B1, 1B3 and 2B1 in the Chinese hamster ovarian cell line whereas in ZR-75-1 cell line, OATP1B1 plays an important role in the uptake of curcumin. Curcumin is metabolized into curcumin sulfate, not to curcumin glucuronide, by the breast cancer cell lines; ZR-75-1 and MDA-MB-231. Thus, sulfotransferases are vital for the phase-II metabolism of curcumin in case of breast cancer. Our experimental results improve the understanding of the cellular uptake of curcumin and metabolism of curcumin in breast cancer, which may help to improve bioavailability of the compound in case of breast cancer. By taking one step further, we also determined that ileum is the main barrier for the uptake of curcumin. Furthermore, curcumin is found to possess a potent spasmolytic activity on ileum. Further studies are required to elucidate the role of other uptake transporters and metabolizing enzymes and interplay of uptake transporters with metabolizing enzyme to illustrate the complete picture of the fate of curcumin in cancer and normal tissues.

8. List of abbreviations

HPLC	High performance liquid chromatography
UV	Ultra violet
OATPs	Organic anionic transporting polypeptides
CHO	Chinese hamster ovarian cells
IC_{50}	Half maximal inhibitory concentration
NFκB	Nuclear factor kappa B
AP1	Activator protein 1
STAT3	Signal transducer and activator of transcription 3
STAT5	Signal transducer and activator of transcription 5
CAMP	A hemolysis factor
Egr-1	Early growth response protein 1
PPAR-γ	Peroxisome proliferator activated receptors
Nrf-2	Nuclear factor 2
BCL-XL	B-cell lymphoma-extra large
COX-2	Cyclooxygenase-2
MMP-9	Matrix metalloproteinase-9
EGFR	Epidermal growth factor receptor
HER2	Human epidermal growth factor receptor 2
TNF	Tumor necrosis factor
P21	Name of protein; also called Cyclin dependent kinase interacting protein 1
P53	Name of protein
cPLA2	cytosolic phospholipase A2
GSK-β3	Glycogen synthase kinase β3

mTOR	Mammalian target of rapamycin
GST	Glutathione S transferase
GPx	Glutathione peroxidase
Hb	Hemoglobin
LPO	Lipid hydro peroxidase
PI3K	Phosphoinositide 3 kinase
JNK	c-Jin NH2-terminal kinase
SOD	Superoxide dimutase
HO-1	Heme oxygenase-1
DNMT	DNA methyltransferase 1
iNOS	Inducible nitric oxide synthase
CRAC	Calcium release-activated calcium
VEGF	Vascular endothelial growth factor
GSH	glutathione
P300	Name of protein
5LOX	5-lipooxygenases
IKK2/IKK1	IκB kinase 1/2
Bcl-2	B cell lymphoma 2
Bax	Bcl-2 associated X protein
PGE2	Prostaglandin 2
IAV-HA	Influenza A viruse -hemaglutinin
ROS	Reactive oxygenase species
p-38	Name of protein

FOXO3a	Forkhead transcription factor 3a
CYP450	Cytochrome 450
UGTs	UDP glucurotransferases
UDPGA	Uridine-diphospho-α glucuronic acid
SULT	Sulfotransferases
PAPS	3'-phosphoadenosine 5' phosphosulfate
PAP	3'-phosphoadenosine-5'-phosphate
Arg	Arginine
Ser	Serine
SO_3-	Sulfonate group
PSB	Phosphate binding loop
Thr	Threonine
Phe	Phenylalanine
DNA	Deoxyribonucleic acid
mRNA	Messenger ribonucleic acid
GIT	Gastro intestinal tract
DHEA	Dehydroepiandosrerone
C terminal	Carboxyl terminal
N terminal	Amino acid terminal
BR-STL	Brain sulfotransferase like
ATP	Adenosine triphosphate
ABC transporters	ATP binding cassette transporters
SLC family	Solute carrier family

P-gp	P-glycoproteins
ABCB1	Gene encoding P-glycoproteins
BCRP	Breast cancer resistant protein
ABCG2	Gene encoding breast cancer resistant protein
MRP1	Multidrug resistant protein-1
ABCG2	Gene encoding multidrug resistant protein-1
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
PEMA	Phenylethylmalonamide
OATs	Organic anionic transporters
OCTs/OCTNs	Organic cationic transporters
NTCPs	Sodium dependent taurocholate transporting proteins
OST	Organic solute transporters
SLCO	Gene encoding OATPs
HIV	Human immunodeficiency virus
TM	Transmembrane
ECL	Extracellular loops
ICL	Intracellular loops
T_3	Triiodothyronine
rT_3	Reverse-triiodothyronine
T_4	Thyroxine
3D	Three dimensional