Titel der Dissertation

Role of Organic Anion Transporting Polypeptides (OATPs) in the cellular accumulation of antitumor agents in lung, breast and liver cancer

verfasst von

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A drug therapy requires efficacy and safety which are not easy to predict. Therefore, absorption, distribution, metabolism and excretion of the drug are pharmacokinetic parameters which should be properly characterized as they determine the outcome. The aim of this thesis was to elucidate cellular uptake mechanisms and the way biotransformation influences drug uptake. We investigated transporters and metabolism in cells with a focus on OATPs (organic anion transporting polypeptides). OATPs are of special interest in cancer, since their expression pattern changes during tumor development. Besides OATPs, biotransformation can influence the efficacy of a treatment. Therefore, the role of phase II-metabolites in uptake and bioavailability of a drug was investigated.

As the liver is the main organ responsible for biotransformation and elimination processes, we investigated OATP expression in liver and hepatic cancer, with a special focus on OATP2A1, OATP3A1, OATP4A1 and OATP5A1. For that reason, mRNA levels of all eleven OATPs were determined in cancerous and adjacent non-cancerous specimens from 43 patients with hepatocellular carcinoma, cholangiocellular carcinoma, and liver metastases from colon tumors. We found out that all OATPs, apart from OATP1C1 and OATP6A1, are highly expressed in most samples. In contrast to a downregulation of OATP1B1, OATP1B3, OATP1A2 and OATP2B1 in tumors, OATP2A1, OATP3A1, OATP4A1 and OATP5A1 mRNA levels were increased. Immunofluorescent staining revealed OATP2A1, OATP3A1, OATP4A1 and OATP5A1 protein expression in plasma membranes and cytosol of hepatic tumor cells.

Another study focused on OATPs in lung cancer, where we investigated OATP expression in primary and metastatic small cell lung cancer (SCLC), lung carcinoid cell lines and cells from pleural effusions. The aim was to determine the influence of OATPs on tumor progression, chemotherapy resistance and the neuroendocrine markers chromogranin and synaptophysin. In immunohistochemistry experiments, OATPs were localized in paraffin-embedded SCLC samples. By detecting significant differences of OATP expression in the cell lines and tissues investigated we revealed a strong correlation between the OATP expression pattern and the origin of the cells, as cells from primary or metastatic SCLC, carcinoid tumors and pleural effusions differ with respect to OATP levels. OATP4A1 was found to be the most prominent OATP in all SCLC and carcinoid cell lines and in non-malignant lung tissue whereas
OATP5A1 appeared to be expressed in metastatic cells. OATP6A1 was present in SCLC cell lines at low levels only. Additionally, we detected that treatment with topotecan, etoposide and cisplatin caused significant changes in the expression patterns of OATP4A1, OATP5A1, OATP6A1, chromogranin and synaptophysin, also by comparing the expression pattern in an untreated SCLC tumor with late-stage chemoresistant tumor cell lines.

In further studies we concentrated on natural compounds and the role of OATPs in their transport. Resveratrol, a naturally occurring polyphenolic compound, provides various pharmacological activities despite its low bioavailability, which is particularly caused by extensive phase II metabolism. To find out, whether resveratrol and its metabolites can accumulate to bioactive levels in organs and tissues through protein mediated transport mechanisms, we investigated the role of OATPs in the cellular transport of resveratrol and its major glucuronides and sulfates. Uptake experiments in transfected Chinese Hamster Ovary (CHO) cells and ZR-75-1 hormone-dependent breast cancer cell lines revealed OATP1B1, OATP1B3 and OATP2B1 as transporters for resveratrol and resveratrol-3-O-4\'-O-disulfate as substrate of OATP1B1 and OATP1B3, while resveratrol-3-O-sulfate was only transported by OATP1B3. Interestingly, resveratrol-4\'O-sulfate, resveratrol-3-O-glucuronide and resveratrol-4\'O-glucuronide did not show any affinity for these OATPs. Furthermore, the involvement of OATPs in the uptake of flavopiridol, a selective inhibitor of cyclin dependent kinases and antitumor agent, was investigated in OATP1B1-, OATP1B3- and OATP2B1-expressing CHO cells and ZR-75-1 breast cancer cell lines. Flavopiridol was found to be a substrate of all three investigated OATPs with highest transport capacity for OATP1B3. By comparing ZR-75-1 wild-type and OATP1B1 knockdown cells, higher flavopiridol uptake led to several-fold decreased IC₅₀ values in the cytotoxicity assay. Furthermore, cell cycle profile also showed a stronger cell cycle arrest in the G2/M phase for ZR-75-1 wild-type cells, also indicating an active uptake via OATP1B1.

Additionally, we also observed the effect of various anthocyanidins and anthocyanins on the expression of OATPs. Anthocyanins are naturally occurring plant pigments that exhibit many biological effects and health benefits and are widely used in nutritional supplements which makes safety of those products highly important. In order to predict possible interactions, we examined the effects of anthocyanins on the expression of OATPs in primary cultures of human hepatocytes by quantifying the expression of OATP mRNA and confirming OATP protein. Overall, the investigated anthocyanins did
not extraordinarily induce the expression of OATP1B1 and OATP1B3 mRNA and protein which leads to the conclusion that within the range of common ingestion of anthocyanins in food and dietary supplements, any impact on OATP1B1 and OATP1B3 activity is rather not to be expected.

In summary, the present findings about the expression of OATPs in liver and lung cancer as well as in breast cancer cell lines provide the basis for further research regarding the transport of endobiotics and xenobiotics and their metabolites in normal and malignant tissue. This might help to better understand the complex processes of uptake and metabolism in vivo.
3 Zusammenfassung


OATPs sind speziell bei Krebs relevant, weil sich ihr Expressionsmuster während der Tumorgenese ändert. Abgesehen von OATPs untersuchten wir auch die Rolle von Phase 2-Metaboliten bei Aufnahme und Bioverfügbarkeit eines Arzneistoffs, denn auch dessen Biotransformation kann die Effizienz einer Behandlung beeinflussen.


In einer anderen Studie untersuchten wir OATPs in primärem und metastasierendem kleinzelligen Lungenkrebs (SCLC), karzinoiden Zelllinien und Zellen, die aus einem Pleuraerguss stammten. Unser Ziel war es, die neuroendokrinen Marker Chromogranin und Synaptophysin und den Einfluss von OATPs auf Tumorwachstum und Chemoresistenz zu untersuchen. Weiters lokализierten wir OATPs auf Proteinebene immunhistochemisch in Paraffin-Schnitten. Indem wir große Unterschiede in der OATP-Expression sowohl in Zelllinien als auch im Gewebe entdeckten, gelang es uns, das OATP-Expressionsmuster und die Herkunft der Zellen zu korrelieren. OATP4A1 stellte sich als wichtigster OATP-Vertreter in allen SCLC- und karzinoiden

In weiteren Studien konzentrierten wir uns auf Naturstoffe und darauf, welche Rolle OATPs bei deren Transport spielen. Das Polyphenol Resveratrol weist hohe pharmakologische Aktivität auf, obwohl es eine sehr niedrige Bioverfügbarkeit besitzt, was vor allem durch seine Phase 2-Metabolisierung bedingt wird. Um herauszufinden, ob Resveratrol und seine Metabolite in Organen und Geweben überhaupt mithilfe proteinbedingter Transportmechanismen in solchen Konzentrationen kumulieren können, um eine gewisse Bioaktivität zu erreichen, untersuchten wir den zellulären Transport von Resveratrol und dessen wichtigsten Glucuroniden und Sulfaten. Aufnahme-Experimente in Chinese Hamster Ovary (CHO)-Zellen und hormonabhängigen ZR-75-1 Brustkrebszelllinien stellten OATP1B1, OATP1B3 und OATP2B1 als Transporter für Resveratrol dar, und Resveratrol-3-O-4′-O-disulfat als Substrat für OATP1B1 und OATP1B3, während Resveratrol-3-O-sulfat nur von OATP1B3 transportiert wurde. Interessanterweise zeigten Resveratrol-4′-O-sulfat, Resveratrol-3-O-glucuronid und Resveratrol-4′-O-glucuronid keine Affinität zu OATPs.

Außerdem untersuchten wir die Auswirkung von OATPs auf die Aufnahme von Flavopiridol, einem selektiven Inhibitor Cyclin-abhängiger Kinasen mit Antitumor-Wirkung in OATP1B1-, OATP1B3- und OATP2B1-transfizierten CHO-Zellen und ZR-75-1-Brustkrebszelllinien. Obwohl Flavopiridol von allen drei untersuchten OATPs transportiert wurde, zeigte es die höchste Transportkapazität bei OATP1B3. Indem wir nicht transfizierte ZR-75-1-Zellen mit ausgeknockten OATP1B1 Zellen verglichen, fanden wir heraus, dass eine erhöhte Aufnahme von Flavopiridol verminderte IC₅₀-Werte in Zytotoxizitätsassays bedingte. Außerdem wurde ein stärkerer Verbleib in der Zellzyklus-Phase G2/M bei den nicht transfizierten Zellen gezeigt, was für einen aktiven OATP1B1-Transport spricht.

Zusätzlich untersuchten wir die Auswirkung von verschiedenen Anthocyanidinen und Anthocyaninen auf die OATP-Expression. Anthocyanine sind natürlich vorkommende Pflanzenpigmente, die viele physiologische Effekte und positive Gesund-
heitsaspekte besitzen. Aufgrund ihrer weiten Verbreitung in Nahrungsergänzungsmitteln ist besonderes Augenmerk auf ihre Unbedenklichkeit zu legen. Um mögliche Wechselwirkungen vorherzusagen, untersuchten wir den Effekt von Anthocyanen auf die OATP-Expression in primären Leberzellkulturen, indem wir OATPs auf mRNA- und Proteinebene quantifizierten. Im Großen und Ganzen konnte keine außergewöhnliche Induktion von OATPs festgestellt werden, weshalb innerhalb des Rahmens der normalen Aufnahme von Anthocyanen in Diä tetika kein besonderer Einfluss auf OATPs erwartet werden muss.

Zusammengefasst kann gesagt werden, dass die momentanen Ergebnisse bezüglich OATPs in Leber- und Lungenkrebs sowie in Brustkrebs-Zelllinien die Basis für weitere Forschung zum Transport endogener Stoffe und Xenobiotika und deren Metaboliten in normalem und malignem Gewebe bilden. Es bleibt zu hoffen, dass diese Arbeit dabei hilft, die komplexen Prozesse über Aufnahme und Metabolismus in vivo zu beleuchten.
4 Introduction

Efficacy and safety in a drug therapy are essential and determined by the drug`s pharmacokinetics for which absorption, distribution, metabolism and excretion (ADME) are major determinants. These parameters influence the exposure of the drug to the tissues and therefore influence pharmacological activity of the compound as a drug. After its administration to the body, usually the xenobiotic is taken up into the blood, directed to its target organ or tissue via circulation, altered by enzymes (redox reactions, conjugations etc.), and removed by renal or biliary excretion with urine and feces. The main factors for the efficacy of drugs are metabolism and transport. Since a major route for the removal and detoxification of endogenous and exogenous substances is their excretion into bile, the liver is the most responsible organ for biotransformation and elimination. The hepatic elimination process is divided into four phases including uptake of substrates in phase 0, biotransformation in phase 1 and 2 and excretion of metabolites in phase 3. Transport proteins localized at the basolateral and canalicular membrane of hepatocytes are responsible for uptake and excretion of metabolites.

4.1 Metabolism

4.1.1 Enzymes

Functions of enzymes that are involved in the elimination of xenobiotics and endogenous substances are cytochrome P450 (CYP)-dependent oxidations in Phase I and conjugations with UDP-glucosyltransferases (UGTs), sulfotransferases (SULTs) and glutathione S-transferases (GSTs) in Phase II.

4.1.1.1 Phase I

In phase I biotransformation more polar substrates are generated including alcohols, carbocyclic acids etc. by involving oxidations, reductions, hydrolysis, cyclization and decyclization reactions to facilitate their excretion. Oxidative reactions mainly take place in the liver involving CYPs, but they can also be found in the intestine. The cytochrome P450 family is the main responsible group of enzymes catalyzing Phase I
reactions, but flavin monooxygenases, dehydrogenases, amine oxidases, xanthine oxidases, peroxidases can also be involved. Usually, phase I reactions prepare compounds for subsequent phase 2 conjugation.\textsuperscript{2, 6} In phase I, metabolic enzymes of the superfamily of CYPs take part in more than 60 \% of biotransformation reactions.\textsuperscript{7} CYPs are found in the membrane of the endoplasmic reticulum (ER), but their catalytic site is located in the cytoplasm.\textsuperscript{8} Based on their percent amino acid sequence identity, CYPs are classified in several families and subfamilies. Enzymes with at least 40 per cent amino acid sequence homology are grouped in one family, defined by an Arabic numeral. CYPs exceeding more than 54 \% homology are defined by a letter. In humans, 18 CYP families can be identified and classified in 41 protein-coding subfamilies, encoding 57 genes. The CYP2, CYP3 and CYP4 families include much more genes than the other families. A reason for that might be that these three families possibly better respond to the environment and their genes are inducible, in contrast to the others.\textsuperscript{9} CYP3A4 is the most responsible enzyme for biotransformation of more than half of common drugs.\textsuperscript{10} Although predominantly expressed in the liver and the intestinal epithelium it can be found throughout the body.\textsuperscript{11} During the last decades, drug-drug interactions involving CYPs are being recognized, especially for CYP3A4.\textsuperscript{12}

4.1.1.2 Phase II

Phase II reactions for increasing hydrophilicity of molecules include hepatic sulfation, glucuronidation and glutathione conjugations. They prepare substrates for excretion into bile or basolateral transport for renal drug elimination. Sulfation is a process with high affinity that shows an overlapping substrate spectrum with glucuronidation, but affords lower substrate concentrations than glucuronidation reactions. Other phase II reactions like conjugation with amino acids, methylation and acetylation are of minor importance.\textsuperscript{6}

4.1.1.2.1 Sulfation

Sulfation as most important phase II reaction often follows phase I oxidations. Usually it is part of the physiologic detoxification process, but sometimes it actually activates hepatotoxins or drugs.\textsuperscript{6} After conjugation of sulfates with AMP (adenosine monophosphate) by ATP (adenosine triphosphate) sulfurylase and subsequent phosphory-
lation by APS (adenosine phosphosulfate) kinase to PAPS (3-phosphoadenosine-5-phosphosulfate), the transfer of sulfate as phase II conjugations takes place. Alterations in systemic sulfate concentrations lead to changes in hepatic sulfate and PAPS levels since there is a strong correlation between hepatic PAPS and circulating inorganic sulfates. Sulfation is often not only dependent on enzymes (SULTs) but also limited by the availability of circulating inorganic sulfates and hepatic PAPS. In vitro systems like single-pass isolated perfused liver and hepatocytes provide constant sulfate concentrations. Therefore linear conditions are achieved and only sulfotransferase maximal velocity is responsible for kinetic determinants. In mammals, SULTs are classified into membrane SULTs, situated in the Golgi apparatus and metabolizing macromolecules, and cytosolic SULTs, responsible for xenobiotics and small endogenous compounds.

In humans, sulfotransferases (SULTs) can be classified in three families, SULT1 (phenol sulfotransferase family), SULT2 (hydroxysteroid sulfotransferase family), and brain-specific SULT4. SULT1 is further subdivided into SULT1A, SULT1B, SULT1C, and SULT1E, while the SULT2 family consists of two subfamilies, namely SULT2A and SULT2B. The most important subfamilies mediating sulfation of xenobiotics are SULT1A and SULT1C. In humans, three isoforms of SULT1A can be found. Thermostable SULT1A1 and SULT1A2 are responsible for sulfation of hydroxyl groups on phenolic molecules and thermolabile SULT1A3 catalyzes sulfation of monoamine groups on aromatic molecules. SULT2A conjugates bile acids with sulfates. Despite overlapping substrate specificity occurring at the level of subfamilies and even families, substrate affinity can vary among them. Although being only a weakly inducible enzyme, steroids, and especially glucocorticoids, were found to induce SULTs in rats and humans, partly by activating the pregnane X receptor.

In a reverse reaction, sulfates can be deconjugated by sulfatases. Therefore, there always has to be considered an interplay between sulfotransferases and sulfatases, regulating levels of sulfates in vivo.

### 4.1.1.2.2 Glucuronidation

Uridine diphosphate glucuronosyltransferases (UGTs) catalyze the transfer of a glucuronic acid from uridine diphosphogluconic acid (UDP-GA) to its substrates, resulting in a more polar compound being easily biliary or renal eliminated. Glucuronid-
Uridine diphosphate (UDP) glucuronosyltransferases (UGTs) catalyze reactions that take place inside microsomal membranes by UGTs being attached to the membrane of the endoplasmic reticulum (ER).\(^6\)\(^28\)

Being mainly a hepatic enzyme, UGTs are also found in extrahepatic tissues as the gastrointestinal tract, kidney and brain. Until today, seventeen UGTs have been identified in humans that consist of two families, UGT1 and UGT2, which are further classified on the basis of sequence homology into the subfamilies UGT1A, UGT2A and UGT2B.\(^29\) While UGT1A subfamily isoenzymes contain a conserved C-terminal region responsible for UDP-GA binding and linking to the endoplasmic reticulum, the N-terminal region varies.\(^30\) Subfamily UGT1A comprises nine isoforms, among the most interesting is UGT1A1 because of its involvement in detoxification of bilirubin. Therefore, hyperbilirubinemia may also be caused by deficiency of UGT1A1.\(^31\)

Whereas UGT2A1 as only subfamily member, localized in olfactory tissues, UGT2B subfamily consists of seven isoenzymes.\(^32\),\(^33\) Especially UGT2B7 is known for its role in glucuronidation of morphine to form the pharmacologically active morphine-6-glucuronide.\(^34\) Similar to sulfation, expression of some UGTs can be regulated by ligands of pregnane X receptor, androstane receptor, glucocorticoid receptor, or peroxisome proliferator-activated receptor.\(^6\) A known inhibitor of glucuronidation is probenecid which inhibits competitively and prevents UDP-GA import into the ER (Endoplasmic reticulum).\(^35\)

Glucuronides are normally eliminated into bile, as a substrate of multidrug resistance-associated protein 2 (MRP2).\(^36\) Though, glucuronide conjugation is a process that can be reversed via glucuronidases in vivo. Since high activity of glucuronidases are detected in the intestines, removal of glucuronide metabolites can prolong bioavailability.\(^37\)

### 4.1.1.2.3 Glutathione Conjugation

Glutathione serves as electron donor and therefore provides antioxidant effects while it prevents substrates from covalently binding intracellular macromolecules. Among substrates of glutathione conjugation are parent compound electrophiles, but also some phase I and phase II metabolites. Although conjugation with glutathione can be spontaneously, it is often catalyzed by GSTs (glutathione S-transferases) and can take place in the cytosol or ER.\(^6\)


4.1.2 Drug Transporters

To get a substance in and out of cells, passive or active uptake mechanisms exist beside simple diffusion. Active transport requires energy. In primary active transport, energy is usually generated by hydrolysing ATP and the substrate leads to a conformational change of the transporter, making unidirectional transport possible. In secondary active transport, transport of a substrate is linked to the transport of a cosubstrate, in the same or opposite direction. An abundance of uptake and efflux transporters responsible for the translocation of a variety of substrates is already known. Transporters can be found throughout the body with distinct distribution patterns in various tissues and organs. Regulatory mechanisms that alter expression on mRNA or protein level are dependent on transcription factors, pathophysiological conditions, post-translational modifications, activation, sex and ontogeny.38, 39

4.1.2.1 Uptake Transporters

Several families of transporters mediating the uptake of substrates into the cell and thereby modulating metabolism have been detected. Most uptake transporters are part of the solute carrier (SLC) family, comprising organic anion transporting polypeptides (OATPs), organic anion transporters (OATs), organic cation transporters (OCTs) as well as organic cation/carnitine transporters, but also peptide transporters and concentrative and equilibrative nucleoside transporters. Also involved in drug uptake, but not members of SLC-family, are sodium-dependent taurocholate transporting polypeptides (NTCPs) and organic solute transporters (OST).38

Focusing on the liver as major organ for biotransformation, transport proteins which can be found in the basolateral hepatocyte plasma membrane are transporters that mediate the uptake of organic anions such as OATP1B1, OATP1B3, OATP2B140, and OAT2/SLC22A741, OAT7/SLC22A942, organic cation transporter, OCT1/SLC22A143 and bile salt transporter NTCP/SLC10A1.44

4.1.2.1.1 Organic Anion Transporting Polypeptides

OATPs as sodium-independent transporters are responsible for the cellular uptake of a large amount of drugs and endogenous substrates. In the OATP superfamily, eleven human OATP family members have been classified, occurring in multiple tissues
throughout the body, like intestine, liver, kidney, and brain. OATPs are involved in the processes of absorption, distribution and excretion of drugs. They have been detected in various tissues, transporting a large variety of substrates. Nevertheless, some OATPs are exclusively expressed in certain organs and show high substrate specificity.\textsuperscript{40, 45} Most prominent OATPs are OATP1B1\textsuperscript{46-48}, OATP1B3\textsuperscript{49, 50}, and OATP2B1,\textsuperscript{51, 52} which are found to be highly expressed in human liver and therefore play a crucial role in the hepatic uptake of many drugs.

### 4.1.2.1.2 General Information about the OATP-Superfamily

The OATP family consists of eleven human OATPs. They also occur in other species and were first detected in rats in 1994, called Oatps.\textsuperscript{53, 54} OATPs as sodium-independent transporters usually facilitate the uptake of a wide range of compounds, e.g. endobiotics like eicosanoids, hormones or bile acids, but also of xenobiotics including several, mainly anionic, drugs.\textsuperscript{40, 45} Although official nomenclature distinguishes between genes (using the term “SLCO”) and protein (“OATP”), often the term OATP is used for both to facilitate understanding.\textsuperscript{40}

In 2004, a new classification system has been established based on amino acid sequence homology. OATPs with over 40 % amino acid sequence homology are classified within the same family (OATP1-6), whereas OATPs with more than 60% identity are defined by the subfamily letter A-C.\textsuperscript{40} The largest OATP family OATP1 consists of OATP1A, OATP1B, OATP1C and members can be found in organs like intestine, liver, kidney or brain.\textsuperscript{55-57} Especially OATP1B1 and OATP1B3 were found to be liver specific\textsuperscript{46-48, 50} whereas OATP1C1, transporting thyroid hormones, was detected in testis, brain and the ciliary body.\textsuperscript{58, 59} Family OATP2 consists of only two members, prostaglandin transporter OATP2A1 and OATP2B1, and occurs in liver, kidney, lung and small intestine.\textsuperscript{51, 60, 61} Ubiquitously expressed OATP3A1, kidney specific OATP4C1 and little described OATP5A1 and OATP6A1 seem to be of minor importance so far.\textsuperscript{53}
**Table 1:** Tissue distribution human OATP transporters

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Organ distribution</th>
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<tr>
<td>SLCO1A2</td>
<td>OATP1A2</td>
<td>Blood-brain barrier, kidney, cholangiocytes, enterocytes</td>
</tr>
<tr>
<td>SLCO1B1</td>
<td>OATP1B1</td>
<td>Liver</td>
</tr>
<tr>
<td>SLCO1B3</td>
<td>OATP1B3</td>
<td>Liver</td>
</tr>
<tr>
<td>SLCO1C1</td>
<td>OATP1C1</td>
<td>Brain, testes, ciliary body</td>
</tr>
<tr>
<td>SLCO2A1</td>
<td>OATP2A1</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td>SLCO2B1</td>
<td>OATP2B1</td>
<td>Liver, placenta, heart, blood-brain barrier, enterocytes</td>
</tr>
<tr>
<td>SLCO3A1</td>
<td>OATP3A1</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td>SLCO4A1</td>
<td>OATP 4A1</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td>SLCO4C1</td>
<td>OATP 4C1</td>
<td>Kidney</td>
</tr>
<tr>
<td>SLCO5A1</td>
<td>OATP5A1</td>
<td>Lactiferous ducts in breast</td>
</tr>
<tr>
<td>SLCO6A1</td>
<td>OATP6A1</td>
<td>Testes</td>
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### 4.1.2.1.3 Structure of OATPs

OATPs consist of 643–722 amino acids that probably form twelve transmembrane domains (loops), yielding five intracellular and six extracellular loops with C- and the N-terminus facing the cytosol.\(^{40, 53, 63}\) N-glycosylations at various positions of the loops influence functional activity and localization of the transporter in the plasma membrane.\(^{40, 64}\) Ten cysteine residues were detected in extracellular loop 5 which are normally disulfide-bonded and to a large extent essential for proper transport function.\(^{65, 66}\) A C-terminal PDZ (P: post-synaptic protein, PSD-95, D: the Drosophila septate junction protein, Discs-large, Z: the tight junction protein, ZO-1) consensus sequence that is important for localization of some OATPs in the plasma membrane can also influence transport.\(^{67, 68}\)
Figure 2. Predicted structure of human OATP1B1 with 12 transmembrane domains. Conserved amino acids in black; conserved cysteine residues in grey. Three N-glycosylation sites marked as Y in extracellular loop E2 and E5.53

4.1.2.1.4 OATPs in Cancer

OATPs are known to change their expression pattern during tumor development and malignant transformation of cells. Therefore, distinct OATP expression patterns might be useful for diagnosis, predicting the effect on therapy and interactions.

OATP1A2 mRNA was detected in colon, with a decrease in cancer tissue and polyps.69 Although OAT1A2 was found in endothelial cells at the barriers of blood-brain and the blood-tumor, it was not seen in the glioma cells.70 In breast carcinoma, results concerning OATP1A2 expression were inconsistent. While OATP1A2 mRNA and protein were detected in breast cancer tissue, but not in tumor surrounding healthy tissue,71, 72 its expression in breast cancer could not be confirmed by others.73

In contrast to normal colon, OATP1B1 mRNA levels were found to be elevated in colon polyps and cancer tissue.69 In a study, Lockhart et al. correlated OATP1B3 expression in colorectal cancer to clinical outcome.74 Reduced OATP1B1 and, in particular, OATP1B3 levels have been detected in liver cancer compared to healthy liver.75-78 79 Because of an increased expression in metastases, OATP1B3, beside OATP2B1, were found to be a possible biomarker for elevated prostate cancer-
specific mortality. While OATP1B3 normally is primarily found in liver, it was detected in cancers of ovary, lung, prostate, breast, colon, and pancreas. Reduced levels of OATP1B3 have been detected in cancers of liver. Interestingly, beside an upregulation, cytoplasmic staining of OATP1B3 was detected in colorectal adenocarcinoma tissues. Pressler et al. described differences in OATP expression of all liver-specific OATPs 1B1, 1B3 and 2B1 between normal and tumorous tissues in several cancers, such as thyroid, pancreatic, liver, colon, testicular and prostate cancer.

OATP1C1 seems to be of minor importance as its mRNA expression was only detected in osteosarcomas, and partly in kidney cancer metastasis or bone cysts. There was a tendency towards higher levels of OATP2A1 mRNA in non-malignant breast tissue compared to breast cancer samples. This trend could also be observed in liver cancer tissue where OATP2A1 was found in hepatocellular carcinoma, in cholangiocellular carcinoma and metastatic adenocarcinoma. Furthermore, OATP2A1 mRNA was highly expressed in bone metastases from kidney cancer. There was also reduced OATP2A1 expression in cancers of colon, stomach, ovary, lung, and kidney confirmed.

In another study, OATP2B1 mRNA levels were higher in malignant tissue compared to normal tissue and correlated with the grade and stage of the disease. This is in contrast to the work of Wlcek et al. who detected an elevated but not significant expression of OATP2B1 in non-malignant breast tissue, and Kindla et al. did not find any differences in the mRNA amount between normal and malignant breast tissue. In liver, OATP2B1 mRNA was reduced in hepatocellular carcinoma, in cholangiocellular carcinoma, and liver metastasis in colon.

In glioma, OATP2B1 expression was identified in endothelial cells at the blood-brain barrier and blood-tumor barrier. mRNA expression levels of OATP3A1 were also found to be increased in non-malignant breast tissue compared to tumorous one in most samples. Also, expression of OATP3A1, as well as OATP4A1, was increased in the non-malignant bone cyst samples compared to the osteosarcoma. In human breast, OATP4A1 mRNA expression was again found to be higher in non-malignant breast tissue samples. An up-regulation of SLCO4A1 was described in colon cancer.

For OATP4C1, a reduction in metastatic liver cancer was observed, but no difference was found concerning breast.
OATP5A1 mRNA was detected in normal and cancerous breast tissue and in bone tumors. In liver cancer, OATP5A1 was found to be elevated compared to normal liver tissue. OATP6A1, usually expressed exclusively in testis, was detected in tumors of lung, bladder, esophagus and brain. Interestingly, differences in the cellular localization of some OATPs between tumorous and non-tumorous tissue can be detected. Additionally to their usual localization in the plasma membrane in breast cancer, OATP2B1, OATP3A1 and OATP5A1 are predominantly expressed in the cytoplasm. In primary and metastatic liver cancer, OATP2A1, OATP3A1, OATP4A1 and OATP5A1 were also located in the membrane and the cytosol of cells. In normal breast, OATP2B1 has been detected in the myoepithelium around ductal epithelial cells whereas in breast cancer, ductal epithelial cells showed OATP2B1 positive staining. Since most OATPs have been detected in a majority of human cancers and are famous for their multispecificity in drug transport, they might be a promising target in cancer therapy.

4.1.2.2 ATP-dependent Efflux by ABC-Transporters

ATP-binding cassette (ABC) transporters mediate the ATP-dependent translocation of many endogenous end exogenous substrates across membranes out of the cell. Based on their amino acid sequences, there are 48 human ABC transporters, divided into seven subfamilies A-G. Most important members are P-glycoprotein/ABCB1 (P-gp), breast cancer resistance protein BCRP/ABCG2 and the multi-drug resistance proteins (MRPs) of the ABCC subfamily. The cellular membrane glycoprotein P-glycoprotein (ABCB1) plays an essential role in physiological detoxication by transporting mainly neutral and cationic, only partly anionic, organic substrates and lowers intercellular drug concentrations in liver, small intestine, colon mucosa cells, kidney and the pancreatic duct. BCRP (ABCG2), situated apically in liver, kidney, intestine, brain, testes, gall bladder, eye, lung, heart is especially responsible for the efflux of sulfated conjugates. ABC-transporter MRP2 (ABCC2), transporting the widest range of anionic substrates, can be found in the apical membrane of hepatocytes, kidney, gallbladder, intestine, placenta, whereas other MRP transporters MRP1, MRP3-6 are rather localized basolaterally.
4.1.3 Interplay of Transporters and Enzymes on Metabolism of Drugs

Transporters are essential modulators of pharmacokinetics and effects since drugs have to be transferred in and out of cells, in which biotransformation takes place. OATPs and OATs as uptake transporters for mainly anionic drugs and endogenous substances, often followed by Phase I cytochrome P450-dependent oxidation and Phase II conjugations, and ABC-transporters responsible for efflux, work in concert in the process of drug elimination.\textsuperscript{104, 105}

Figure 1 A shows basolaterally situated influx transporters (OATP, OCT, NTCP) which mediate uptake of substrates into liver cells. In the hepatocytes, biotransformation by cytochromes P450, UDP-glucuronosyltransferases and sulfotransferases take place. Efflux transporters (BCRP, MRP2 and P-gp) remove substrates into bile. In the enterocytes (B) OATPs located at the apical membrane are responsible for drug uptake from the intestinal lumen, whereas OCTs transport substrates from the blood stream into the cell. In general, intestinal efflux is mediated by BCRP, P-gp and MRP2 apically and MRP3 basolaterally.\textsuperscript{38, 105}
4.1.3.1 Interplay of Cytochrome P450 and OATPs

As uptake transporters mediate the influx of drugs into cells, they are responsible for the access of drugs to metabolic enzymes. As a result, any alterations in the function of the transporters could regulate metabolism without directly influencing the activity of enzymes. Organic anion transporting polypeptides OATP1B1, OATP1B3, and especially OATP2B1 are highly expressed in human liver and mediate the active hepatic uptake of various drugs. Inhibition of this active hepatic drug uptake can result in higher blood levels and severe side effects such as those already known for statins after co-administration with cyclosporine, an OATP inhibitor. In a clinical study, plasma concentrations of the HMG-Co-reductase inhibitor cerivastatin were significantly increased in kidney transplant patients treated with cyclosporine. Studies revealed that cyclosporine was shown to be only a weak inhibitor of cerivastatin metabolism, but it was a potent inhibitor of OATB1B1 mediated hepatic cerivastatin uptake indicating that the cerivastatin-cyclosporine interaction was the result of OATP1B1 inhibition. Also, interactions of atorvastatin with CYP3A4 inhibitors itraconazole and erythromycin have been observed, resulting in higher AUC values, whereas with rifampicin, an inducer of CYP3A4 and a potent inhibitor of several OATPs, the plasma concentrations of predominantly biliary excreted atorvastatin significantly increased. Similar interactions could be expected after co-administration of statins and OATP substrates like repaglinide caused by higher blood levels leading to side effects. In a perfused rat liver model, concentrations of digoxin, a CYP3A/ABCB1 substrate which is metabolized by CYP3A4 to a large extent, were significantly lowered when applied with OATP1B1 inhibitor rifampicin. This can be caused by rifampicin reducing the hepatic uptake of digoxin and resulting in decreased formation of biotransformation products.

4.1.3.2 Interplay of Cytochrome P450 and P-Glycoprotein

A functional interaction between P-glycoprotein, which prevents drugs from absorption and facilitates biliary and renal elimination, and CYP3A4, most responsible en-
zyme for Phase I metabolism, together forming a barrier against the oral absorption of drugs and xenobiotics can be found in the small intestine and in the liver. High interindividual differences in expression of P-gp and CYP3A, especially in human liver, can be found among humans. P-gp might regulate the exposure of drugs to metabolism via CYP3A isoenzymes by pumping drugs out of cells/enterocytes so that they can be taken up again, therefore increasing the probability of drugs being metabolized by repeated exposure to CYPs. An example for an interplay of cytochrome P450 and P-glycoprotein is Sirolimus which is a substrate of both, P-gp and CYP3A4. Inhibition of P-gp in the intestines would increase absorption by blocking efflux and therefore metabolism results in a significantly enhanced intestinal bioavailability. Furthermore digoxin, a CYP3A4 substrate, is also a substrate of P-gp in hepatocytes. Coadministration of P-gp inhibitor quinidine increased concentrations of digoxin and its metabolite in a perfusate by inhibiting P-gp from transporting digoxin into the bile.

### 4.1.3.3 Interplay of UDP-Glucuronosyltransferases and Multidrug Resistance Protein 2 (ABCC2)

As glucuronidation is important for activation or detoxification of many exogenous and endogenous substances, changes in the amount of renal and biliary glucuronide excretion via induction or inhibition of the isoenzymes or by competition or inhibition of transport mechanism responsible for their excretion can influence blood concentration and therapy. MRP2 as a major xenobiotic efflux pump on the canalicular membrane determines the biliary excretion of organic anions, including bilirubin-diglucuronide, glutathione conjugates, sulfated bile salts, as well as several drugs like sulfipyrazone, indomethacin, rifampicin, vinblastin, cisplatin and methotrexate. Bilirubin metabolism includes the cowork of OATP1B1 and OATP1B3, which are responsible for its uptake into hepatocytes. It is then turned by UGT1A1 as a glucuronic acid-conjugation product into a substrate of MRP2, which transports it into bile. An absence of functional MRP2 impairs glucuronide excretion into bile and leads to accumulation of glucuronides in blood, called Dubin-Johnson syndrome. Another significant interplay between UGT-catalyzed glucuronidation and the anticancer drug flavopiridol and MRP2 is described. Since flavopiridol is extensively
metabolised in the rat liver to two monoglucuronides, followed by biliary elimination, high glucuronide levels in plasma and enterohepatic circulation were detected.\textsuperscript{134} Because flavopiridol is excreted via conjugation in the same way as bilirubin, hyperbilirubinemia as side effect of flavopiridol treatment was noticed in 22 \% of patients.\textsuperscript{133, 135} As MRP2 plays a key role in the excretion of conjugated bilirubin into bile, it might contribute to active transport of flavopiridol glucuronides across the canalicular membrane into bile.\textsuperscript{133} In an isolated perfused rat liver model of MRP2-deficient TR\textsuperscript{−} rats, the biliary excretion of metabolites of flavopiridol was extremely reduced down to 4-5 \%, whereas flavopiridol was only reduced down to 50 \%.\textsuperscript{133}

Several interactions between MRPs and UGTs can be expected, since there are many endobiotics and drugs that are biliary eliminated after glucuronidation, such as mycophenolate mofetil and cyclosporine. After absorption of mycophenolate mofetil, it is converted to the active drug mycophenolic acid and gets glucuronidated by UGTs in liver rapidly, which then is followed by biliary elimination via MRP2.\textsuperscript{136} Patient studies have shown lower plasma levels of mycophenolic acid when simultaneously applying mycophenolic acid and the calcineurin inhibitor cyclosporine.\textsuperscript{136} By the use of MRP2 deficient rats it could be shown that cyclosporine-mediated inhibition of the biliary excretion of mycophenolic acid glucuronide by MRP2 is the mechanism of interactions between cyclosporine and mycophenolate mofetil.\textsuperscript{137}

Normally, the anticancer drug irinotecan is inactivated by glucuronidation. In MRP2 deficient rats biliary excretion of both, unglucuronidated and glucuronidated forms, was reduced. So, polymorphisms in MRP2 are likely to influence irinotecan metabolism.\textsuperscript{138} It was shown that patients having a certain genotype of MRP2 were associated with higher AUCs of irinotecan and that genetic variants of MRP2 therefore can influence irinotecan toxicity and success of therapy.\textsuperscript{139}

\subsection*{4.1.3.4 Interplay of UDP-Glucuronosyltransferases, Sulfotransferases and OATPs}

Resveratrol transport and metabolism includes the interplay of UDP-glucuronosyltransferases, sulfotransferases and OATPs as well as of efflux transporters.\textsuperscript{140, 141} Usually it is taken up into cells as resveratrol via OATP1B1, OATP1B3 or OATP2B1 and then metabolized by UDP-Glucuronosyltransferases and sulfotransferases primary to resveratrol-3-O-glucuronide, resveratrol-4\`-O-glucuronide,
resveratrol-3-O-sulfate, resveratrol-4′-O-sulfate and resveratrol-3-O-4′-O-disulfate, beside conjugation products including sulfuric and glucuronic acid. 141-143 We pointed out that,141 based on in vitro studies, resveratrol sulfates have been found to have comparable or greater potency than resveratrol against specific molecular targets, namely, COX 1 and 2, quinone reductase 1, nuclear factor κB as well as similar ability to scavenge free radicals.144-146 Furthermore, sulfates were also very recently shown to attenuate the Escherichia coli-LPS-induced IL-6 and TNF-α release.147 In contrast to resveratrol sulfates, the few published studies have shown that resveratrol glucuronides are ineffective in various human cell lines, macrophages and HIV-1 infection.147-151 However, the in vitro activity of resveratrol-metabolites may not necessarily reflect their in vivo function given that intracellular sulfatases or β-glucuronidases could easily convert the conjugates back to resveratrol.152 Recent data from our laboratory have indeed shown that intracellular resveratrol-3-O-sulfate is hydrolyzed in breast tissue samples by members of the sulfatase family to regenerate parent resveratrol.152 Monosulfate metabolites were also converted back to the parent compound in human colorectal cells.148 An active transport for resveratrol and its conjugates is also supported by several in vitro and in vivo studies showing that the multidrug resistance-associated proteins (MRPs), namely MRP2 and MRP3, are responsible for the efflux of resveratrol glucuronides whereas breast cancer-resistance protein has been found to be the major efflux pump for native resveratrol and resveratrol sulfates.153-155

As shown in a recent study from our lab, we demonstrated the importance of OATP1B1 for the uptake of resveratrol and its sulfates, hormone-dependent ZR-75-1 cells that were previously shown to express high levels of OATP1B1, but not OATP1B3 and OATP2B173, were incubated for 1 min with increasing concentrations of resveratrol and resveratrol-3-O-4′-O-disulfate.141 Indeed, the uptake of resveratrol by the ZR-75-1 OATP1B1-knockdown cells was significantly reduced compared to control cells. Concomitant with the decreased uptake detected in ZR-75-1 OATP1B1 knockdown cells, we also observed decreased formation of the metabolites resveratrol-4′-O-sulfate and resveratrol-3-O-sulfate after 72 h, which led to a higher IC\textsubscript{50} value in the cytotoxicity assay in OATP1B1-expressing wild-type cells. As expected, we also observed lower uptake of the OATP1B1 substrate resveratrol-3-O-4′-O-disulfate in the ZR-75-1 OATP1B1-knockdown cells, which was confirmed by decreased formation of resveratrol-3-O-sulfate.141
As demonstrated in a previous study, plasma concentrations of glucuronides and sulfates were up to 15-fold higher compared to the unconjugated compound. OATP-dependent cellular uptake might therefore support pharmacological activities of resveratrol.\textsuperscript{156}
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6 Aims of the Thesis

The outcome of a drug therapy depends on the absorption, distribution, metabolism, excretion and toxicity of a substance. Especially in cancer therapy, it is very reasonable to predict the outcome in order to save time, but also for economic reasons.

OATPs are known to mediate the uptake of many xenobiotics as well as of several endogenous substances, such as steroid hormones and bile acids. So far, eleven OATP family members have been identified. They are found throughout the body and localized in most organs, especially in liver, intestine and kidney which show enormous involvement in drug uptake and elimination. There still is limited information about the expression and function of OATPs in malignancies and how they might contribute to chemoresistance. Also, the expression of OATP mRNA and protein as biomarkers for malign diseases is of general interest. Moreover, OATPs providing distinct expression patterns in various types of cancer might help to foresee effects of therapies, if the chemotherapeutics are substrates of OATPs or interact with them.

For that reason, the aim of this thesis was to localize and to point out differences in OATP expression in normal and cancerous tissue of different tumor entities. Because of its major function as organ for metabolism and detoxification, we first investigated OATP expression in the liver. The hypothesis was to detect OATP expression shifts during tumour development, comparing hepatocellular carcinomas, liver metastases, cholangiocellular carcinoma with non-malignant liver tissue from patients.

In a second study, we also elucidated OATPs in various small cell lung cancer (SCLC) cell lines and patient samples, as lung cancer is still one of the major causes of death by cancer worldwide and often linked to chemoresistance.

In three other studies, we investigated for the first time the role of OATPs in natural, bioactive compounds and their metabolites in various transfected cell lines. Among these compounds were the anticancer compound flavopiridol, resveratrol and its main glucuronides and sulfates, and various anthocyanins.

If OATPs are indeed a key factor for drug disposition, the results of this thesis might be a further step towards personalized medicine, by determining the patient’s individual expression of drug metabolizing enzymes and transporters. This would not only create efficacy, but also avoid toxic side effects and drug interactions.
7 Results

7.1 Original Papers and Manuscripts


I performed immunofluorescence experiments.


I carried out lentiviral transfection and transport studies.


I was involved in western blot experiments and data analysis.
Resveratrol and its sulfated conjugates are substrates of organic anion transporting polypeptides (OATPs): impact on breast cancer cell growth


*Mol Nutr Food Res. 2014 Jul 3.*
Resveratrol and its major sulfated conjugates are substrates of organic anion transporting polypeptides (OATPs): Impact on growth of ZR-75-1 breast cancer cells

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Scope: Resveratrol is a naturally occurring polyphenolic compound with various pharmacological activities. These effects are observed despite its low bioavailability, which is particularly caused by extensive phase II metabolism. It is unknown whether resveratrol and its metabolites can accumulate to bioactive levels in organs and tissues through protein-mediated transport mechanisms. Because organic anion transporting polypeptides (OATPs) mediate the uptake of many clinically important drugs, we investigated their role in the cellular transport of resveratrol and its major glucuronides and sulfates.

Methods and results: Uptake experiments were performed with resveratrol and its glucuronides and sulfates in OATP-expressing Chinese hamster ovary (CHO) and breast cancer (ZR-75-1) cells. The uptake rates for resveratrol in OATP1B1, OATP1B3, and OATP2B1-transfected Chinese hamster ovary cells were four- to sixfold higher compared to wild-type cells. Resveratrol-3-O-4'-O-dissulfate was transported by OATP1B1 and OATP1B3, while resveratrol-3-O-sulfate was exclusively transported by OATP1B3. However, resveratrol-4'-O-sulfate, resveratrol-3-O-glucuronide, and resveratrol-4'-O-glucuronide did not show any affinity for these OATPs. OATP-dependent uptake of resveratrol was also confirmed in ZR-75-1 cells.

Conclusion: Our data revealed that OATPs act as cellular uptake transporters for resveratrol and its major sulfates, which must be considered in humans following oral uptake of dietary resveratrol.

Keywords:
Breast cancer / Metabolism / OATP/Resveratrol / Transport

Additional supporting information may be found in the online version of this article at the publisher's web-site

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Abbreviations: BSP, bromosulfophthalain; CHO, Chinese hamster ovary; OATP, organic anion transporting polypeptides; WT, wild-type

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1 Introduction

Resveratrol (trans-3,5,4'-trihydroxystilbene) is a naturally occurring compound found at low concentrations in grapes, berries, peanuts, and red wine [1]. Over the last decade, it has been shown that resveratrol exhibits a wide variety of biological and pharmacological properties. In several in vitro, animal models and human studies, resveratrol has been found to be active in the prevention and treatment of cancer, cardiovascular diseases, inflammation, ischemic injuries, and...
neurodegenerative diseases, and resveratrol may also act as an
antioxidant and antiaging compound [2]. The observed phar-
maceutical activities of resveratrol cannot be explained by
the low blood and tissue concentrations of unchanged resver-
atrol based on extensive metabolism in the gut and liver,
leading to five to tenfold higher circulating plasma con-
centrations of resveratrol glucuronides and resveratrol sulfates
compared with unconjugated resveratrol [3,4]. Currently, lim-
ited information is available regarding the possible bene-
fits of resveratrol metabolites. Based on in vitro studies, resver-
atrol sulfates have been found to have comparable or greater
potency than resveratrol against specific molecular targets,
namely, COX 1 and 2, quinone reductase 1, nuclear factor κB
as well as similar ability to scavenge free radicals [5–7]. Fur-
nthermore, sulfates were also very recently shown to attenuate
the Escherichia coli LPS-induced IL-6 and TNF-α release [8].
In contrast to resveratrol sulfates, the few published studies
have shown that resveratrol glucuronides are ineffective in
various human cell lines, macrophages, and HIV-1 infection
[8–12]. However, the in vivo activity of resveratrol metabolites
may not necessarily reflect their in vitro function given that
intracellular sulfatases or β-glucuronidases could easily convert
the conjugates back to resveratrol [13]. Recent data from our
laboratory have indeed shown that intracellular resveratrol-
3-O-sulfate is readily hydrolyzed in breast tissue samples
by members of the sulfatase family to regenerate parent resver-
atrol [13]. Monosulfate metabolites were also converted to
the parent compound in human colorectal cells [9]. The
observed induction of autophagy and senescence, however,
as was abrogated by inclusion of a sulfatase inhibitor, which
reduced intracellular resveratrol. Interestingly, cellular uptake
of resveratrol 3-O-sulfate and resveratrol 4′-O-sulfate into cy-
toplasm of human colon cancer cells was dependent on cell
line (HCFEG < HCA-7 < HT-29), strongly indicating uptake
transport mechanisms rather than passive diffusion [9]. An
active transport for resveratrol and its conjugates is also sup-
ported by several in vitro and in vivo studies showing that
the multidrug resistance-associated proteins (MRPs) namely
MRP2 and MRP3 are responsible for the efflux of resveratrol
glucuronides whereas breast cancer-resistance protein has
been found to be the major efflux pump for native resver-
atrol and resveratrol sulfates [14,15]. We therefore hypothesize
that resveratrol and its conjugates may accumulate to bioac-
tive levels in cells, organs, and tissues through active transport
mechanisms.

One major cellular uptake mechanism is via members of
the organic anion transporting polypeptide (OATP) family
[16–18]. Among the 11 human OATPs, OATP1B1 and
OATP1B3 are highly expressed in the liver and mediate the
uptake of numerous drugs into hepatocytes. OATP2B1 shows
substantial expression in the apical membrane of enterocytes,
where it contributes to the intestinal absorption of many en-
dogenous compounds and clinically important drugs, thereby
affecting drug disposition [19,20]. Based on the structural
similarity of resveratrol 3-O-sulfate with estrone-3-sulfate, for
which OATP1B1-, OATP1B3-, and OATP2B1-mediated up-
take exists, we hypothesized that resveratrol and its conju-
gates might also be substrates of these OATPs. Any differ-
ences in the affinity of resveratrol glucuronides and resver-
atrol sulfates to these transporters might also explain the
observed differences in their pharmacological activity.

Recent data from our laboratory have also shown high
expression of various OATPs in human hormone recep-
tor-positive (MCF-7 and ZR-75-1) and negative (MDA-MB-231)
breast cancer cell lines [21,22]. As pronounced resveratrol
uptake into the cytoplasm was also observed in these cell
lines we hypothesized that members of the OATP family
may mediate intracellular resveratrol concentrations, thereby
affecting cell growth.

Therefore, in the present study, we investigated the
transport of resveratrol and its major metabolites, namely
resveratrol-3-O-glucuronide, resveratrol-4′-O-
glucuronide, resveratrol-3-O-sulfate, resveratrol-4′-O-sulfate,
and resveratrol-3-O′-4′-disulfate, in OATP1B1-, OATP1B3-,
and OATP2B1-transfected Chinese hamster ovary (CHO)
cells. In addition, the OATP-mediated transport of resveratrol
and its correlation with cytotoxicity were also investigated in
the human breast cancer cell line ZR-75-1.

2 Materials and methods

2.1 Chemicals

trans-Resveratrol was purchased from Sigma-Aldrich (Mun-
ich, Germany), and trans-resveratrol-3-O-glucuronide was
obtained from Santa Cruz Biotechnology (Santa Cruz,
CA, USA). trans-Resveratrol-3-O-sulfate, trans-resveratrol-
4′-O-sulfate, trans-resveratrol-3-O′-4′-disulfate, and trans-
resveratrol-4′-O-glucuronide were synthesized as described
previously [23, 24]. MeOH and water were of HPLC grade
(Merck, Darmstadt, Germany). All other chemicals and sol-
vents were commercially available and of analytical grade and
were used without further purification.

2.2 Cell culture

CHO cells that were stably transfected with OATP1B1,
OATP1B3, and OATP2B1 and wild-type (WT) CHO cells
were provided by the University of Zurich, Switzerland and
have been extensively characterized previously [25,26]. The
CHO cells were grown in DMEM supplemented with 10% FCS,
50 µg/mL l-proline, 100 µL/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 mamma-
lian 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 supple-
mented with 10% FCS, 100 U/mL penicillin, 100 µg/mL
streptomycin, and 1% GlutaMAX. The cells were grown in T-flasks with a 25 cm² growth area (BD Biosciences, Franklin Lakes, NJ, USA), maintained at 37°C under 5% CO₂ and 95% relative humidity. The cells were passaged once a week and were used up to passage 55 [22].

2.3 OATP1B1 knockdown in ZR-75-1 cells via lentiviral transduction with a short hairpin RNA

For 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 h, 250 μl of medium supplemented with 8 μg/ml polybrene (Sigma, H9268) were added. Transductions were performed by the addition of 10 μl of shRNA (Mission® Transduction Particles NM_006446, Sigma, TRCN0000043203, coding sequence CCGGGCTTCTAATGCTAAACTCTCAGA-GATGTAGCTTGTAGTCAAGGCTTGTGTGTTTGT). 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 h. The obtained silencing efficiency was evaluated after 3 weeks via real-time PCR and immunofluorescence.

2.4 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 micrograms 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 Biosystems, Warrington, UK) were purchased for human OATP1B1. The 18S gene was used as a reference gene as previously described [21]. 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’ exoribonuclease activity of DNA polymerase was measured with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). All samples were amplified in triplicate. To determine 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 1.9.1.1, Applied Biosystems). 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 pre-fabricated TaqMan® Gene Expression Assays (Applied Biosystems) containing inter-species primer HQ00722374_ml1 for OATP1B1.

2.5 Immunofluorescence

ZR-75-1 OATP1B1-knockdown cells and cells transfected with the empty vector were allowed to attach on culture slides overnight (8-Chamber Polylysine 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 (OATP1B1/131 mDMQ mouse mAb; Actis Antibodies, Herford, Germany) was diluted 1:100, and incubation was performed for 2 h. Optimal antibody concentrations were determined by titrating serial antibody dilutions. The applied dilutions corresponded to the minimum concentration necessary to produce a positive signal. WT and OATP1B1-transfected CHO cells were used as controls. Following incubation with the secondary antibody (1:1000 dilution; Alexa Fluor® 488 Goat Anti-Mouse IgG; Invitrogen, Carlsbad, CA, USA) for 30 min, cell nuclei were stained with 0.5 μg/ml Hoechst 33342 (Sigma-Aldrich, St. Louis, MO, USA). Thereafter, the slides were rinsed with distilled water before being mounted in Mowiol 4–88 (Carl Roth, Karlsruhe, Germany). Fluorescent staining was visualized with an AxioPlan 2 microscope (Carl Zeiss, Jena, Germany). Images were captured using an AxioCam HRc2 Color CCD digital camera and Axiovision 4.8 software (Carl Zeiss Vision GmbH, Aalen, Germany). 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.

2.6 Western blotting

Western blotting to confirm OATP1B1-, OATP1B3-, and OATP2B1-overexpression in transfected CHO cells was performed with membrane extracts (Fierce, Rockford, IL, USA) from these cells. WT CHO cell membrane extracts were used as controls. Protein content was determined with an assay kit from Bio-Rad Laboratories (Hercules, CA, USA) with BSA as the standard. For immunoblotting, 25 μg of protein were separated by SDS polyacrylamide gel 12% electrophoresis and transferred onto Hybond PVDF membranes. To control equal sample loading, protein on the membranes were stained.
with Ponceau S. In the immunoblot, 5% nonfat dry milk in TBS-0.1% Tween 20 was used for blocking. As primary antibodies an anti-OATP1B1/1B3 mouse mAb from Acris Antibodies (BM5542) and an anti-OATP2B1 rabbit mAb from Abcam Antibodies (ab83532, Cambridge, UK) diluted 1:500 in blocking solution were applied. Peroxidase-conjugated species-specific IgGs were used as secondary antibodies (dilution 1:2000). Detection was done with an ECL detection kit from Thermo Scientific (Portsmouth, NH, USA) and Amersham Hyperfilms (GE-Healthcare, Amersham, UK).

2.7 Uptake assays in CHO cells

Transport assays were performed on 12-well plates as described in detail elsewhere [25]. Briefly, CHO cells were seeded at a density of 350 000 cells per well on 12-well plates (BD Biosciences). 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) to induce nonspecific gene expression [28]. Resveratrol and its sulfates were dissolved in DMSO and diluted with uptake buffer pH 7.4 (final DMSO concentration of 0.5%, which was constant in all transport experiments) to 12.5-600 μM. Control experiments contained DMSO in the medium in place of resveratrol. Prior to the transport experiment, the cells were rinsed twice with 2 mL of prewarmed (37°C) uptake buffer (116.4 mM NaCl, 5.3 mM KCl, 1 mM NaH2PO4, 0.8 mM MgSO4, 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 by repeated (five 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 [22].

As most OATPs are stimulated by an acidic pH, uptake experiments were also performed at pH 6.5 under identical conditions as mentioned above except now using acidic uptake and washing buffers.

2.8 Uptake assays in ZR-75-1 cells

Transport assays were performed on 12-well plates as described above for CHO cells. Resveratrol and its sulfates were dissolved in DMSO and diluted with uptake buffer (pH 6.5; final DMSO concentration of 0.5%) to 12.5-400 μM. The experiments were performed in triplicate. Control experiments contained DMSO in the medium in place of resveratrol. Prior to the transport experiment, the cells were rinsed twice with 2 mL of prewarmed (37°C) uptake buffer. Uptake was initiated by adding 0.25 mL of uptake buffer containing the substrate. After 1 min at 37°C, uptake was stopped by removing the uptake solution and washing the cells five times with 2 mL of buffer. After trypsinization, cell membranes were disrupted by repeated shock freezing in liquid nitrogen. Following centrifugation, the supernatant was diluted with methanol/water, and aliquots (80 μL) were analyzed via HPLC as described above.

2.9 Inhibition analysis

For the inhibition experiments with rifampicin and bromosulfophthalein (BSP; Sigma-Aldrich), stock solutions of these compounds were prepared in DMSO containing the indicated concentrations. CHO cells grown on 12-well plates were first washed twice with prewarmed uptake buffer (pH 7.4) and incubated for 10 min at 37°C under 5% CO2 with 1 μM resveratrol or 10 μM reseratrol sulfates (resveratrol-3-O-sulfate and resveratrol-3'-O-sulfate) in the presence of the inhibitors ranging from 0.001 to 100 μM. Control experiments were performed without BSP and rifampicin under identical conditions as mentioned above.

2.10 Metabolism studies in ZR-75-1 cells

Cells were plated on six-well plates and allowed to attach overnight. Resveratrol was dissolved in DMSO and diluted with medium (final DMSO concentration <0.1%) to a concentration of 3–100 μM. The experiments were performed under each set of conditions in triplicate. Control experiments contained DMSO in the medium in place of resveratrol. After 72 h, 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 (five times) shock freezing in liquid nitrogen and thawing. Following centrifugation at 13 500 × g for 3 min, 80 μL of the supernatant (cytoplasm) was subsequently analyzed by HPLC [22]. Additionally, the cell pellets containing the membranes were extracted with 200 μL of methanol and analyzed by HPLC for their resveratrol content. The protein concentration in the cell pellets was determined using a bicinchoninic acid protein assay kit (Pierce Science, Rockford, IL, USA), with BSA as a standard.

2.11 Cytotoxicity assay

CellTiter-Blue (Promega, Southampton, UK) is a colorimetric and fluorescent assay used to measure cell viability via nonspecific 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 at 37°C under 5% CO2. For cytotoxicity assays, the cells were incubated with various
concentrations of resveratrol (5–400 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 multinoise plate reader (Tecan Austria GmbH, Groedig, Austria). The absorbance was also measured in CellTiter-Blue assays in blank wells (without resveratrol) 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.

2.12 Determination of protein concentrations

Total protein was determined using the colorimetric bicinchoninic acid protein assay kit (Pierce Science) with BSA 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).

2.13 HPLC analysis

Resveratrol and its glucuronidated and sulfated biotransformation products were quantified by HPLC as described previously [22, 29] using a Dionex UltiMate 3000 system (Sunnyvale, CA, USA) equipped with an I-7250 injector, an I-7100 pump, an I-7300 column oven (set at 35°C), a D-7000 interface and an I-7400 UV detector (Thermo Fisher Scientific, Waltham, MA, USA) set at a wavelength of 207 nm. 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 resveratrol, trans-resveratrol-3-O-sulfate, trans-resveratrol-4′-O-sulfate, trans-resveratrol-3-O-4′-O-disulfate, trans-resveratrol-3-O-glucuronide, and trans-resveratrol-4′-O-glucuronide to give a concentration range from 0.002 to 25.7 μM (average correlation coefficients: >0.999). For this method, the lower limit of quantification for resveratrol, resveratrol sulfates and resveratrol glucuronides was 11, 13, and 25 nM, respectively. Coefficients of accuracy and precision for these compounds were <15%.

2.14 Data analysis

Kinetic analysis of the uptake of resveratrol and metabolites was performed over a substrate concentration range of 1–600 μM. Prior to these experiments, the linearity of cellular uptake over time (1, 3, and 10 min) was individually determined for WT and OATP2B1-transfected CHO cells by using resveratrol (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 WT cells for each individual concentration. The data were fitted to the Michaelis–Menten model. Kinetic parameters were calculated using the Graph-Pad Prism Version 5.0 software program (GraphPad Software, San Diego, CA, USA) for Michaelis–Menten: 

\[
V = \frac{V_{\text{max}} \cdot S}{K_m + S},
\]

where \( V \) is the rate of the reaction; \( V_{\text{max}} \) 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 \( V_{\text{max}}/K_m \), quantifies the transport capacity. \( IC_{50} \) values were determined by plotting the log inhibitor concentration against the net uptake rate and nonlinear regression of the dataset using the equation:

\[
y = \frac{a}{1 + (1/(IC_{50}) + b)}
\]

in which \( y \) is the net uptake rate (pmol/μg protein/min), \( I \) is the inhibitor concentration (μM), \( s \) is the slope at the point of inversion, and \( a \) and \( b \) are the maximum and minimum values for cellular uptake. Net uptake was calculated for each inhibitor concentration as the difference in the uptake rates of the transporter-expressing and WT cell lines. Unless otherwise indicated, values are expressed as mean ± SD of three individual experiments. Significant differences from control values were determined using Student’s paired t-test at a significance level of \( p<0.05 \).

3 Results

3.1 Accumulation of resveratrol and metabolites in transfected CHO cells

To investigate whether resveratrol and its major conjugates are substrates of OATPs, uptake analysis was performed in OATP1B1, OATP1B3, and OATP2B1-transfected CHO cells which highly expressed these transporters in the membranes (Supporting Information Fig. 1). Uptake of resveratrol (12.5–400 μM) at pH 7.4 for all three OATPs was only linear for up to 1 min (Fig. 1). We therefore finalized all experiments at 1 min (initial linear phase). As shown in Table 1 and Fig. 2A, the initial net OATP1B1, OATP1B3, and OATP2B1-mediated accumulation rates (transfected-WT) for resveratrol followed Michaelis–Menten kinetics, with approximately twofold higher \( V_{\text{max}} \) values for OATP1B1 compared to OATP1B3 and OATP2B1 (\( V_{\text{max}} \): 1100 versus 818 versus 640 pmol/mg protein/min at pH 7.4). \( K_m \) values were the lowest for OATP1B3 (62.0 μM) and followed by OATP2B1 (88.1 μM) and OATP1B1-transfected cell lines (88.4 μM). The uptake of resveratrol-3-O-4′-O-disulfate (25–600 μM) in OATP1B1- and OATP1B3-transfected CHO cells, however, was less pronounced, showing \( V_{\text{max}} \) values of 100 and 107 pmol/mg protein/min, respectively (Fig. 2C). Interestingly, the affinity
of resveratrol-3-O-4'-O-disulfate for OATP1B1 and OATP1B3 was about twofold higher with $K_m$ values of 37.6 and 37.5 $\mu$M compared to resveratrol. Resveratrol-3-O-sulfate (25-600 $\mu$M) was only taken up by OATP1B3, again with low $K_m$ (101 $\mu$M) and $V_{\text{max}}$ (122 pmol/mg protein/min) values. The uptake of resveratrol-4'-O-sulfate, resveratrol-3-O-glucuronide, and resveratrol-4'-O-glucuronide by OATP1B1, OATP1B3, and OATP2B1-transfected and WT CHO cells was below the detection limit (13-25 nM). As it is well known that most OATPs are stimulated by an acidic extracellular pH [27], we also performed uptake experiments at pH 6.5 with WT and OATP-transfected CHO cells. Table 1 revealed that the $K_m$ values for the uptake of resveratrol and its sulfated conjugates were in general lower at pH 6.5 compared to pH 7.4 but only reached a level of significance for resveratrol-3-O-4'-O-disulfate by OATP1B1 and OATP1B3. $V_{\text{max}}$ values also showed a pH dependency which was, however, less pronounced and only significant for resveratrol uptake by OATP1B3 and OATP2B1.

### 3.2 Effect of OATP inhibitors on the accumulation of resveratrol and major sulfates in transfected CHO cells

To investigate whether known OATP inhibitors impact OATP1B1-, OATP1B3-, and OATP2B1-mediated resveratrol accumulation, resveratrol was quantified after incubation of 1 $\mu$M in OATP1B1-, OATP1B3-, and OATP2B1-expressing CHO cells at pH 7.4 in the absence and presence of increasing concentrations of BSP and rifampicin. The inhibition of resveratrol uptake by BSP in OATP1B1-expressing CHO cells was more potent ($K_i$ : 1.99 $\mu$M) compared to OATP1B1- and OATP2B1-expressing CHO cells ($K_i$ values: 1.19 and 1.47 $\mu$M, respectively). Rifampicin was an even more potent inhibitor of resveratrol uptake in OATP2B1 ($K_i$ : 0.48 $\mu$M) but not in OATP1B1- and OATP1B3-transfected CHO cells ($K_i$ : 2.14 and 1.34 $\mu$M, respectively). Uptake inhibition of 10 $\mu$M resveratrol-3-O-sulfate in OATP1B3-transfected CHO cells by BSP and rifampicin was also pronounced with $K_i$ values of 3.52 and 0.33 $\mu$M, respectively. Inhibition of resveratrol-3-O-4'-O-disulfate uptake by BSP and rifampicin resulted in even lower $K_i$ values for OATP1B1 and OATP1B3 (0.27 and 0.29 $\mu$M versus 0.25 and 0.23 $\mu$M).

### 3.3 OATP1B1 knockdown in ZR-75-1 cells

The PCR data from various lentiviral-transfected clones revealed an up to tenfold reduction of OATP1B1 expression in ZR-75-1 cells. The cells exhibiting the lowest expression

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pH 7.4</th>
<th>pH 6.5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$</td>
<td>$V_{\text{max}}$</td>
</tr>
<tr>
<td>Res</td>
<td>68.4 ± 17.3</td>
<td>1100 ± 67.9</td>
</tr>
<tr>
<td>Dis</td>
<td>37.6 ± 5.23*</td>
<td>100.3 ± 3.12*</td>
</tr>
<tr>
<td>OATP1B1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Res</td>
<td>62.0 ± 10.6*</td>
<td>816 ± 39.3*</td>
</tr>
<tr>
<td>SOS</td>
<td>101 ± 28.1*</td>
<td>122 ± 6.81</td>
</tr>
<tr>
<td>Dis</td>
<td>37.5 ± 5.87*</td>
<td>107.6 ± 3.76*</td>
</tr>
<tr>
<td>OATP1B3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Res</td>
<td>88.1 ± 17.8</td>
<td>640 ± 40.5*</td>
</tr>
</tbody>
</table>

Values are means ± SD of three 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 ($K_m$) equation with nonlinear regression. Res, resveratrol; SOS, resveratrol-3-O-sulfate; Dis, resveratrol-3-O-4'-O-disulfate; n.d., not determined. $K_m$ and $V_{\text{max}}$ values in bold and marked with an asterisk are significantly different (p < 0.05) between pH 6.5 and pH 7.4.
Figure 2. Uptake of resveratrol and its major sulfates in OATP- transfected and wild-type CHO cells. The uptake of resveratrol (12.5-400 µM) (A), resveratrol-3-O-sulfate (25-600 µM) (B), and resveratrol-3-O-4′-O-disulfate (25-600 µM) (C) by OATP1B1-, OATP1B3- and OATP2B1-transfected CHO cells and wild-type CHO cells was determined after 1 min at pH 7.4, 37°C. The data represent the mean ± SD of three individual determinations.

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 [21], the expression of the OATP1B1 protein was confirmed via immunofluorescence using a specific OATP1B1/1B3 mouse mAb. Figure 3 clearly shows normal expression (bright green fluorescence) of OATP1B1 in ZR-75-1 empty vector cells (A) and extremely low expression of this transporter in ZR-75-1 OATP1B1-knockdown cells (B). OATP1B1-transfected CHO cells (C) were used as positive control, compared to WT CHO cells (D) without fluorescence.

3.4 Resveratrol accumulation in WT and ZR-75-1 OATP1B1-knockdown cells

Based on the much higher OATP1B1 mRNA level found in the WT ZR-75-1 breast cancer cell line compared to the

Figure 3. Immunofluorescent characterization of OATP1B1 in ZR-75-1 and OATP1B1 knockdown ZR-75-1 cells. Cells were grown on culture slides and stained with an antibody against OATP1B1 (green) while nuclei were visualized by Hoechst 33342 staining to give a bright blue color (see Section 2). Immunofluorescence was performed in ZR-75-1 empty vector-transfected cells (A), ZR-75-1 OATP1B1 knockdown cells (B), OATP1B1-transfected CHO cells (C), and CHO wild-type cells (D). Bright green fluorescence was seen in ZR-75-1 empty vector-transfected cells (A) and OATP1B1-transfected CHO cells (C).

OATP1B1-knockdown clone, we expected that OATP1B1 expression might be directly correlated with intracellular resveratrol concentrations. For kinetic analysis, an incubation time of 1 min was selected in order to prevent cellular uptake from interference with cellular efflux mechanisms such as MRPs and breast cancer-resistance protein. Figure 4A depicts representative Michaelis–Menten plots for resveratrol uptake by WT and ZR-75-1 OATP1B1-knockdown cells, where significantly higher uptake rates and approximately twofold lower \( K_m \) values were found in the OATP1B1-expressing control (WT; 3360 versus 2412 pmol/mg protein/min, \( K_m \); 144
45

versus 193 μM). Resveratrol-3-O-4'-O-dissulfate, a substrate of OATP1B1, also showed higher uptake rates and a lower affinity for this transporter in ZR-75-1 cells transfected with the empty vector compared with the OATP1B1-knockdown clone (V_max: 870 versus 734 pmol/mg protein/min; K_m: 194 versus 234 μM). However, incubation with resveratrol-3-O-sulfate, which is a substrate of OATP1B3 but not of OATP1B1, did not show any differences in uptake in the two cell lines, thus strongly indicating the impact of OATP1B1 on resveratrol transport.

3.5 Cytotoxicity of resveratrol and resveratrol disulfate in ZR-75-1 OATP1B1-knockdown cells

The cytotoxicity of resveratrol in WT 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, resveratrol exhibited an approximately twofold lower IC_{50} value in control ZR-75-1 cells (37.7 μM) compared to the OATP1B1 knockdown clone (58.0 μM), supporting the importance of OATP1B1-dependent resveratrol uptake. This trend was also true for resveratrol-3-O-4'-O-dissulfate, for which the IC_{50} value was lower in WT cells (6.97 μM) compared to OATP1B1-knockdown cells (9.15 μM). Due to the overall low cytotoxicity of resveratrol-3-O-4'-O-dissulfate, we could not determine IC_{50} values in either cell line.

3.6 Differences in the metabolism of resveratrol and resveratrol disulfate in ZR-75-1 cells

To further evaluate the differences in resveratrol biotransformation, WT and OATP1B1-knockdown ZR 75-1 breast cancer cells were incubated for 72 h with resveratrol and
As observed in the cytoplasm, resveratrol-3-O-sulfate was the major biotransformation product in the cellular supernatant (14,000 ± 561 pmol/h/mg protein in WT cells versus 8992 ± 1022 pmol/h/mg protein in ZR-75-1 OATP1B1-knockdown cells). Similarly, the concentrations of resveratrol-4'-O-sulfate and resveratrol-3-O-4'-O-disulfate were lower in ZR-75-1 OATP1B1-knockdown cells compared to WT cells (553 ± 731 versus 5888 ± 627 pmol/h/mg protein; and 849 ± 79 versus 496 ± 83 pmol/h/mg protein).

Notably, the concentrations of resveratrol metabolites were approximately threefold higher in the medium compared to the cytosolic samples, thereby strongly indicating the occurrence of rapid cellular efflux after formation. As shown in Fig. 6B, the major metabolite following the uptake of 50 μM resveratrol-3-O-4'-O-disulfate was the deconjugation product, resveratrol-3-O-sulfate, followed by resveratrol-4'-O-sulfate. Parent resveratrol was below the detection limit of 11 nM.

4 Discussion

To identify the relevance of uptake transporters to the in vivo activity and to identify the human OATP isoforms responsible for the hepatic uptake of resveratrol and its glucuronidated and sulfated conjugates, we employed cells that stably expressed these OATPs. As shown in Fig. 2 and Table 1, resveratrol exhibited saturable uptake kinetics at pH 7.4 for OATP1B1, OATP1B3, and OATP2B1. The highest affinity was observed for OATP1B3, with a K_{m} of 62 μM, whereas the affinities for OATP1B1 and OATP2B1 were lower.

Interestingly, resveratrol-3-O-sulfate was only transported by OATP1B1 with a low affinity (K_{m}: 101 μM), while resveratrol-3-O-4'-O-disulfate was transported with equally high affinities by OATP1B1 (K_{m}: 37.6 μM) and OATP1B3 (K_{m}: 37.5 μM). The K_{m}/V_{max} value for the OATP1B1-mediated uptake of resveratrol-3-O-disulfate was similar to that for OATP1B3 (2.67 versus 2.87 μL/min/μg) (see Table 2). Resveratrol-4'-O-sulfate did not show any affinity for any of the three OATPs. Notably, there was also no uptake of resveratrol-3-O-glucuronide or resveratrol-4'-O-glucuronide, indicating that resveratrol sulfates, and not glucuronides, provide the intracellular pools for resveratrol generation [9].

Based on Michaelis–Menten parameters, however, the exact contribution of OATP2B1 in the gut and of OATP1B1, OATP1B3, and OATP2B1 in the liver to the overall uptake of resveratrol and its major sulfates in humans cannot be determined because of high interindividual variability (up to tenfold) in OATP levels as determined by quantitative proteomics or by Western blotting [30–32]. Our data also suggest that OATP1B1, OATP1B3, and OATP2B1 are low-affinity transporters. Because of the low bioavailability, the portal concentration of resveratrol is probably lower than the K_{m} values. Indeed, administration of resveratrol (5.9 g/day) for up to 28 days to healthy volunteers showed peak plasma concentrations of 4 μM and 13 μM [33] for unconjugated resveratrol.
Table 2. pH dependency of Michaelis–Menten parameters in stably transfected CHO cells

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pH 7.4 V_{max}/K_m (µM/min/µg)</th>
<th>pH 6.5 V_{max}/K_m (µM/min/µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OATP1B1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ros</td>
<td>12.4 ± 3.07</td>
<td>14.4 ± 3.88</td>
</tr>
<tr>
<td>3OS</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>DIS</td>
<td>2.67 ± 0.46</td>
<td>4.05 ± 1.93</td>
</tr>
<tr>
<td>OATP1B3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ros</td>
<td>13.2 ± 2.79</td>
<td>15.2 ± 5.82</td>
</tr>
<tr>
<td>3OS</td>
<td>1.21 ± 0.44</td>
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</tr>
<tr>
<td>DIS</td>
<td>2.87 ± 0.55</td>
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<tr>
<td>OATP2B1</td>
<td></td>
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</tr>
<tr>
<td>Ros</td>
<td>7.26 ± 1.86*</td>
<td>12.9 ± 4.07*</td>
</tr>
<tr>
<td>3OS</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>DIS</td>
<td>n.d.</td>
<td>n.d.</td>
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</table>

Values are means ± SD of three 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 (see Table 1). Ros, resveratrol; 3OS, resveratrol-3-O-sulfate; DIS, resveratrol-3-O-4'-disulfate; n.d., not determined. V_{max}/K_m values in bold and marked with an asterisk are significantly different (p < 0.05) between pH 6.5 and pH 7.4.

and resveratrol-3-O-sulfate, respectively. However, concentrations in the colon tissue after administration of resveratrol (10.0 g/day) for up to 8 days to patients with colorectal cancer were significantly higher with mean values of 674 µM for resveratrol and 67 µM for resveratrol-3-O-sulfate [34] and presumably also resveratrol-3-O-4'-disulfate, a metabolite recently shown to be present in human plasma at the same level as resveratrol-3-O-sulfate [35].

Interestingly, affinity of resveratrol and its sulfates to OATPs was overall higher at pH 6.5 compared to 7.4. This is in accordance with literature data [27] also showing pH differences in the uptake of OATP substrates. As OATP2B1 is expressed in the upper part of the gut, which also shows a slightly acidic microenvironment [36] and as several literature data have shown an acid pH in the tumor environment [37, 38], pH dependency of Michaelis–Menten parameters for OATPs have to be considered in vivo.

To further demonstrate the importance of OATP1B1 for the uptake of resveratrol and its sulfates, hormone-dependent ZR-75-1 cells that were previously shown to express high levels of OATP1B1, but not OATP1B3 and OATP2B1 [21], were incubated for 1 min with increasing concentrations of resveratrol and resveratrol-3-O-4'-disulfate. Indeed, the uptake of resveratrol by the ZR-75-1 OATP1B1-knockdown cells was significantly reduced compared to control cells, as indicated by higher K_m and lower V_{max} values (Fig. 4 and Table 3). Comitant with the decreased uptake detected in ZR-75-1 knockdown cells, we also observed decreased formation of the metabolites resveratrol-4'-O-sulfate and resveratrol-3-O-sulfate after 72 h, which led to a lower IC_{50} value in the cytotoxicity assay in OATP1B1-expressing WT cells (37.7 versus 58 µM; Fig. 5). As expected, we also observed lower uptake of the OATP1B1 substrate resveratrol-3-O-4'-disulfate in the ZR-75-1 OATP1B1-knockdown cells, which was confirmed by decreased formation of resveratrol-3-O-sulfate. Due to its several-fold lower cytotoxicity in breast cancer cell lines [24], the IC_{50} values for resveratrol-3-O-4'-disulfate could not be determined in the ZR-75-1 control and OATP1B1-knockdown cells. However, the IC_{50} values were again lower in WT cells (6.97 µM) compared to the OATP1B1-knockdown cells (9.15 µM). As demonstrated in a previous study, metabolic concentrations in the cellular medium were up to 15-fold higher compared to the cytoplasm, supporting the important role of ABCG2 in resveratrol uptake [22].

Table 3. Michaelis–Menten parameters determined in ZR-75-1 cells

<table>
<thead>
<tr>
<th>Substrate</th>
<th>ZR-75-1</th>
<th>OATP1B1 knockdown ZR-75-1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K_m (µM)</td>
<td>V_{max} (pmol/mg/min)</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>144 ± 34.4*</td>
<td>3360 ± 281*</td>
</tr>
<tr>
<td>Resveratrol-3-O-4'-disulfate</td>
<td>194 ± 40.7*</td>
<td>870 ± 66.8*</td>
</tr>
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</table>

Values are means ± SD of three individual determinations. V_{max}/K_m values in bold and marked with an asterisk are significantly different (p < 0.05) between ZR-75-1 and OATP1B1 knockdown ZR-75-1 cells.
transporter-mediated drug-drug interactions. Our data demonstrated that BSP and rifampicin effectively inhibited resveratrol uptake in CHO cells, mediated by OCTP1B1, OATP1B3, and OATP2B1 (IC50 values: 0.48–1.47 μM). Additional potential inhibitors include clarithromycin, erythromycin, and roxithromycin, which inhibit the uptake of pravastatin in OATP1B1- and OATP1B3–transfected HEK293 cells (showing IC50 values of 32–37 μM). Moreover, cyclosporin A significantly decreases the OATP1B1- and OATP1B3–mediated uptake of bosentan [26] and fenofibrate [40] in HEK293 and CHO cells. In addition to clinically applied drugs, naturally occurring flavonoids also interfere with the OATP uptake of dehydroepiandrosterone, thus indicating that they constitute a novel class of OATP1B1 modulators [12]. Ongoing studies are verifying the interactions of drugs and dietary supplements with the OATP1B1, OATP1B3, and OATP2B1–mediated uptake of resveratrol and its sulfates.

Whether other transporters such as OATP2B1 and OATP4C1 which are expressed in ZR-75-1 WT cells [23] are also involved in the uptake of resveratrol and its conjugates is not known yet. Possible candidates may be organic anion transporters (OATs). Besides numerous clinically used drugs, OATs are also involved in the transport of polyphenol conjugates clearly showing substrate specificity. While OAT1-overexpressing human embryonic kidney 293H cells demonstrated enhanced uptake for sulfated and glucuronide conjugates, such as quercetin-3-O-sulfate, dihydroxy-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 [41,42]. Another candidate protein might be the sodium-dependent glucose transporter SGLT1 which was shown to be responsible for the cellular uptake of resveratrol glycoside (transpiceid), but not of trans-resveratrol in Caco-2 cells [13]. In vascular endothelial HUVEC cells, however, Chen et al. could very recently demonstrate that resveratrol uptake involves both passive diffusion and, at least partly, an SGLT1-mediated process [49]. Active mediated uptake of resveratrol was also supported by data from Maier-Salomon et al. [44] and from Delmas and Lin [45], clearly showing that in Caco-2 and hepatoblastoma cells, resveratrol uptake was significantly higher (around 50%) at 37°C than at 4°C when protein-mediated transport should be minimal.

Contrary to previous data in Caco-2 and HUVEC cells, we could not confirm any passive diffusion mechanism for the uptake of resveratrol as uptake kinetics in WT and OATP1B1–knockout ZR-75-1 cells were saturable, strongly indicating active transport. As passive diffusion and active transport work 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 resveratrol, resveratrol-3-O-sulfate, and resveratrol-3-O-4′-O-dinsulfate but not for resveratrol-4′-O-sulfate and resveratrol glucuronides. The OATP-dependent uptake of resveratrol sulfates in concert with intracellular sulfatases, which rapidly dealignuate sulfates to the pharmacologically active parent compound, represents a key factor explaining the observed pharmacological activity of resveratrol. Future in vivo studies should focus not only on the concentration of resveratrol and its conjugates in target tissues but also on the expression levels of OATPs.

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The authors have declared no conflicts of interest.

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The analysis of organic anion transporting polypeptide (OATP) mRNA and protein in primary and metastatic liver cancer


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The analysis of organic anion transporting polypeptide (OATP) mRNA and protein patterns in primary and metastatic liver cancer

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Key words: OATP, SLCO, liver, hepatocellular carcinoma, cholangiocellular carcinoma, colon metastases, cholangiocytes, hepatocytes, transporter, immunofluorescence microscopy

Abbreviations: OATP, organic anion transporting polypeptides; HCC, hepatocellular carcinoma; CCC, cholangiocellular carcinoma; MLT, metastatic liver tumors

Organic anion transporting polypeptides (OATP, SLCO genes) mediate the uptake of endobiotics and drugs. Thus, their expression levels and pattern could be of relevance for cancer therapy. This prompted us to investigate the expression of poorly characterized OATPs, namely OATP2A1, OATP3A1, OATP4A1 and OATP5A1 in hepatic cancer of different origin. First, mRNA levels of all OATPs were determined in paired (cancerous and adjacent non-cancerous) specimens from 43 patients with primary liver cancer (hepatocellular carcinoma, HCC; cholangiocellular carcinoma, CCC) and liver metastases from colon tumors (MLT). Real-time RT-PCR analysis revealed that all OATPs, except OATP1C1 and OATP6A1, are extensively expressed in nearly all samples. In contrast to downregulated OATP1B1, OATP1B3, OATP1A2 and OATP2B1 in cancerous vs. non-cancerous samples, an increase in OATP2A1, OATP3A1, OATP4A1 and OATP5A1 mRNA levels was seen in tumors (up to 40-fold for OATP5A1 in the MLT group). Therefore, OATP2A1, OATP3A1, OATP4A1 and OATP5A1 were further investigated by immunofluorescence microscopy on paraffin-embedded cancerous and non-cancerous sections (seven per group). OATP-derived immunoreactivity was observed in plasma membranes and cytosol of hepatic tumor cells, and additionally, in various cytoplasmic and positive bile ducts. An increased percentage of immunoreactive cells and a higher staining intensity in cancerous vs. non-cancerous paraffin sections paralleled higher mRNA levels of OATP2A1, OATP3A1, OATP4A1 and OATP5A1 in cancerous tissues of HCC, CCC and MLT patients. The extensive expression of OATP2A1, OATP3A1, OATP4A1 and OATP5A1 in hepatic tumors of different origin suggests that these transporters might be further exploited for the discovery of novel anticancer agents.

Introduction

Eleven members of the organic anion transporting family (OATP family) have been identified in humans so far. This group of transporters mediates the sodium-independent transmembrane transport of a broad spectrum of substrates, including hormones and their conjugates, prostaglandins, xenobiotics and therapeutic drugs.1 Although the majority of human OATPs show a wide tissue distribution, some members exhibit unique expression in distinct organs. The best studied members of the OATP family are the "liver-specific" transporters OATP1B1 and OATP1B3.2,3 These OATPs are located in the basolateral membrane of hepatocytes,2 where they mediate the uptake of endogenous substrates and drugs into cells and play a critical role in the first step of the elimination process through the liver.2,4 Expression of other OATPs, e.g., OATP2A1,5 OATP3A1 and OATP4A1,6 was first investigated by northern blotting and conventional PCR analysis, and in more recent studies, by real-time RT-PCR in normal human liver.7,10 However, data showing their expression at the protein level in liver are rare. With the exception of OATP1A2, which was localized to bile duct cells,11 and OATP2B1, which was detected in hepatocytes by western blotting,12 the expression pattern of non liver-specific human OATPs at the protein level and their localization in hepatic cells has not been fully elucidated.13

Studies of OATPs in liver cancer have mainly focused on the two liver-specific OATP family members, OATP1B1 and OATP1B3, which were shown to be downregulated in hepatic carcinomas.14,15 These data indicate that malignant transformation of cells alters the expression pattern of some OATPs, including OATP1B1 and OATP1B3. This has been further demonstrated...
Figure 1. Heat map showing mRNA expression of all 11 human OATPs in non-cancerous and cancerous tissue samples from 43 patients. The clustered image map shows the pattern of OATP gene expression as determined by real-time RT-PCR relative to the expression of CYC. UBC, ATP5B and 18S selected as most appropriate reference genes. Data represent upregulation (red) or downregulation (green) of OATP mRNA compared to the median expression level (black) of all samples. For the hierarchical clustering, the average linkage algorithm with 1 - r as the metric distance (r = Pearson correlation coefficient) after subtraction of column means was applied. Note that there are two clusters for the predominantly "non-cancerous" (N) and "cancerous specimens" (T), respectively. Non-cancerous samples: black number with H for the HCC, C for the CCC, and M for the MLT group, respectively. Cancerous samples: red numbers with respective H, C or M.

for other OATP family members in a recent study from our lab, in which OATP2B1, OATP3A1 and OATP4A1 were shown to be differentially expressed in breast cancer as compared with non-malignant tissue. Similarly, differences in OATP mRNA expression were also seen in primary and metastatic bone tumors. In addition, a number of OATPs such as OATP1A2, OATP1C1, OATP2B1 and OATP4A1 have been described in other human tumors, e.g., gliomas. These data suggest that besides the "liver-specific" OATPs, as well as OATP1A2 and OATP2B1, OATPs from other families might also be expressed in hepatic tumors and their expression pattern may differ from that of normal liver.

Therefore, the main goal of this study was to elucidate the expression of less characterized OATPs in hepatic cancers that respond poorly to conventional chemotherapy regimens. First, the expression pattern of all eleven OATP family members in primary and metastatic liver tumors was investigated using paired cancerous and non-cancerous samples from 43 patients suffering from hepatocellular carcinoma (HCC), cholangiocarcinoma (CCC) and liver metastases of solid tumors in colon (MLT). The cellular localization of OATPs, which are upregulated at the mRNA level in hepatic tumors, namely OATP2A1, OATP3A1, OATP4A1 and OATP5A1, and which have not been studied thoroughly in liver tumors yet, was assessed by indirect immunofluorescence microscopy on seven formaldehyde-fixed paraffin-embedded sections from each group.

Results

OATP mRNA expression in human malignant and non-malignant liver samples. To investigate the possible changes in the expression pattern of human OATPs in hepatic malignancies, paired cancerous and adjacent non-cancerous samples from primary (HCC, n = 11; CCC, n = 9) and metastatic liver tumors (MLT, n = 23) were analyzed for mRNA expression of all eleven human OATPs using real-time RT-PCR.

mRNA expression of nine OATPs (OATP1A2, OATP1B1, OATP1B3, OATP2A1, OATP2B1, OATP5A1, OATP4A1, OATP5A1, OATP4C1) was clearly detectable in cancerous and non-cancerous specimens. Data are depicted in Figure 1 showing a hierarchical cluster analysis of OATP mRNA expression in individual samples. The applied algorithm allows the discrimination between non-cancerous (black numbers) and cancerous (red numbers) samples revealing a non-cancerous (N, left) and cancerous (T, right) cluster. Red and green color spots indicate high and low expression relative to the median expression of a given transporter across all samples. In the "cancerous" cluster, decreased mRNA expression (green) was preferentially observed for "liver-specific" OATP1B1 and OATP1B3, as well as for OATP1A2, OATP2B1 and OATP4C1. On the other hand, expression of OATP2A1, OATP3A1, OATP4A1 and OATP5A1 was increased in the great majority of cancerous samples (red) grouped in the "cancerous" cluster. Exceptions to this pattern are four cancerous specimens, namely samples H1, H5, M16 and M11 from HCC and MLT tumors (see Table 1) that fit into the "non-cancerous" cluster. Interestingly, for the MLT tumor sample M11, the OATP expression pattern is even nearly identical in the cancerous and non-cancerous specimen, although the samples show clear differences in the histo-pathological picture.

On the opposite, C6 and H11 cancerous samples and matched C6 and H11 non-cancerous samples from patients with CCC
and HCC, respectively, also show a nearly identical “cancerous” OATP pattern belong to the “cancerous” cluster. This sometimes-overlapping pattern in cancerous and non-cancerous samples indicates that alterations in the OATP expression can occur earlier than obvious pathological changes. This may also be the case for other non-cancerous specimens found in the “cancerous” cluster, namely specimens M3, M15, H10, M18 and M20 from the non-cancerous part of MLT and HCC tumors, respectively.

mRNA expression levels of two OATPs, namely OATP1C1 and OATP6A1, were below the detection limit in the majority of non-cancerous liver samples. Only low levels of these OATPs were detectable in a few specimens. These OATPs were not studied further.

A more detailed analysis revealed relative quantitative differences in OATP levels between the group of HCC, CCC and MLT samples (Fig. 2). For this analysis, two MLT samples from an unknown primary tumor were excluded to get a more uniform sample group. Calculating logarithmic fold changes of mRNA in matched cancerous vs. non-cancerous samples in each group we found that expression of “liver-specific” OATP1B1 and OATP1B3 as well as OATP2B1 and OATP1A2 is significantly repressed, particularly in the CCC group (up to 60-fold, $p < 0.01$). Importantly, mRNA expression of OATP2A1, OATP3A1 and OATP5A1 is upregulated in tumors, particularly in the MLT group and, with the exception of OATP2A1, also in the CCC group. Expression levels of these OATPs were up to 40-fold higher (OATP5A1 in the MLT group, $p < 0.001$) than in the matched control liver. Although in the HCC group (and for OATP2A1 also in the CCC group), the levels for these OATPs were generally higher in the cancerous than in the non-cancerous specimens, differences did not reach significance, because of the high variability in individual samples.

Interestingly, a 22-fold upregulation ($p < 0.002$) was observed for OATP4A1 in the MLT group, in which patients suffered from metastases of adenocarcinomas in the colon, whereas levels in HCC and CCC were similar or lower than that of the non-malignant controls. Upregulation of OATP4A1 in the MLT group might reflect increased OATP4A1 mRNA expression in colon cancer, as suggested by previous array data and from our studies, in which we found significant upregulation of OATP4A1 in malignant samples from colon cancer patients with mean expression levels (calculated in relation to the calibrator sample) of 2.44-fold ± 0.39 (range 1.95–3.28-fold) in cancerous samples and mean expression levels of 0.46-fold ± 0.72 (range 0.16–0.93) in matched non-cancerous samples ($n = 24$, $p < 0.05$).

For OATP4C1, mRNA expression levels were highly variable, particularly, in primary liver tumors, but were significantly reduced in the MLT group ($p < 0.02$).

**Immunohistochemistry and OATP1C1 identification**. Uregulated OATP2A1, OATP3A1, OATP4A1 and OATP5A1 in cancerous and non-cancerous liver tumor samples. Uregulated OATP2A1, OATP3A1, OATP4A1 and OATP5A1 in HCC, CCC and/or MLT specimens were studied at the protein level in non-cancerous and cancerous liver by immunofluorescence staining of formaldehyde-fixed, paraffin-embedded and frozen sections from selected tumor specimens.

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<tr>
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H = HCC (hepatocellular carcinoma); C = CCC (cholangiocellular carcinoma); MLT = metastatic liver tumor; P = primary tumor not specified.
(n = 7 of each group). Representative pictures for the immunolocalization of these OATPs are shown in HCC, CCC and MLT sections from different patients in comparison to normal liver (patients’ numbers are given in Figs. 3–6). Antibodies and their dilutions are shown in Table 2.

To prove the specificity of the antibodies applied in the immunofluorescence, western blot analysis was done on samples showing either high or low mRNA expression levels of these OATPs in both cancerous and non-cancerous tissues. OATP1B1 and OATP1B3 expression was used as a control. For OATP2A1, OATP3A1 and OATP4A1 immunoreactive bands were found at 70, 80 and 85 kDa, respectively, according to the proposed amino acid sequence by these OATPs (data not shown).

In the immunofluorescence microscopy, application of the antibody against OATP2A1 (Fig. 3) yielded a rather weak green immunofluorescence staining of hepatocytes located in the lobule close to the portal fields. However, in the whole liver sections (approx. 1.5 cm²), a few areas with intensive staining for OATP2A1 were found that were surrounded by regions, without a specific staining. Such heterogeneity in the distribution in the liver was also seen for the other OATPs investigated. The green OATP2A1 associated fluorescence overlaps with the red CK19 staining to a faint orange to yellow color visible in the two bile ducts located central in the portal field. This overlap indicates that OATP2A1 is expressed in cells in the bile ducts of normal liver. Similarly, in the HCC, CCC and MLT samples, intensive green immunofluorescence staining for OATP2A1 is seen on the plasma membrane and in the cytosol of tumor cells and, frequently, in bile duct cells. In the HCC sample, tumor cells are surrounded by fibrotic stroma, in which many small bile ducts are embedded. Like in normal liver, the bright green color for OATP2A1 overlaps with the red CK19 fluorescence to give a yellow to orange color. Similarly, in the CCC specimen, OATP2A1 staining in green is overlapping with the red CK19 staining in ductal cells, and many tumor cells are positive for OATP2A1. This is also seen in the MLT specimen, but here, green OATP2A1 staining is mostly seen in tumor cells negative for the ductal marker CK19.

Similar to OATP2A1, OATP3A1 derived green fluorescence is less intensive in normal hepatocytes arranged in the lobule in the non-cancerous sample (control) as compared to the bright green fluorescence associated with the tumor cells in the HCC, CCC and MLT samples (Fig. 4). Also, CK19 positive bile ductal cells contain OATP3A1 as visible by yellow to orange staining obtained from the overlap of green OATP3A1 and the red CK19 fluorescence staining. In the HCC sample, the tumor cells are arranged in plates that are separated by a large mass of connective tissue. In the tumor cells arranged in large plates, a pronounced green staining for OATP3A1 is visible on the plasma membrane and the cytosol. Similarly, in this HCC sample, the green OATP2A1 staining overlaps with the red CK19 in the large bile duct surrounded by connective tissue. The co-localization of OATP3A1 with CK19 is clearly given in the CCC and the MLT specimen. In the latter, a bright yellow color of the luminal membrane is visible in the large ductal structure.

In contrast to OATP2A1 and OATP3A1, OATP4A1 is detectable in hepatocytes, but is usually absent from bile ducts as shown in the section of normal (control) and HCC liver (Fig. 5). A clear overlap between CK19 and OATP4A1 staining, however, is seen in the CCC and MLT samples, indicating that OATP4A1 is present in ductal cells in these tumors. In the MLT sample, overlapping staining between OATP4A1 and CK19 is particularly pronounced at the luminal membrane of CK19 positive cells in the ductal structures of the MLT sample.
For OATP5A1 (Fig. 6) the immunofluorescence staining of hepatocytes gives a rather faint green signal in the control liver. No reactivity for this OATP is seen in CK19 positive bile ducts in normal liver and an overlap of CK19 and OATP5A1 is only occasionally seen in the HCC, CCC and MLT sample. In the latter, the green OATP5A1 stained tumor cells are surrounding the tumor center, where small fat droplets appear as tiny yellow spots.

Immunofluorescence staining experiments were also done on cryosections from cancerous and non-cancerous samples. The almost identical distribution pattern of OATP2A1, OATP3A1, OATP4A1 and OATP5A1 in normal and cancerous liver on cryo- and paraffin sections indicated that the fixation process does not influence the distribution pattern of these OATPs. Because of the better structural preservation of paraffin-embedded tissues, immunofluorescence images of OATPs on paraffin sections were chosen for a quantitative evaluation of cellular OATP levels.

Visual analysis and automatic image analysis under application of the TissueQuest software (TissueGnostics),\textsuperscript{12} were applied to quantitate the immunoreactivity in individual tumors. As not all areas on a liver section were uniformly stained for the OATPs, the intensity and the extent of the staining of cancerous and non-cancerous samples was assessed on representative fields of view. The degree of immunoreactivity was calculated for individual OATPs using a scale of 0–4 arbitrary units (U). Mean immunoreactivity in non-cancerous samples was generally poor and in a scale of 0–4 U, where absence of immunoreactive staining or low staining intensity in less than 5% of cells is regarded 0, while low to moderate staining in <20% of tumor cells is considered as 1, values did not exceed 1. In cancerous samples, immunoreactivity values for OATP2A1, OATP3A1 and OATP4A1 were generally higher but showed considerable interindividual variability. However, all mean values for the tumor groups were >1 U with 1.19 U ± 1.5 for OATP5A1 in the MLT, and 2.85 U ± 1.1 for OATP3A1 in the CCC group (Fig. 7). These pronounced immunoreactivity parallels higher mRNA expression level of these OATPs in the cancerous vs. non-cancerous parts.

Discussion
We show that out of eleven human OATPs, all but OATPI1 and OATP6A1 are considerably expressed in human cancerous and adjacent non-cancerous samples from hepatic tumors.

Figure 3. Immunolocalization of OATP2A1 and the bile duct marker CK19 in liver cancer. Double immunofluorescence staining experiments were done on paraffin sections from representative samples from HCC, CCC and MLT specimens for patients H8, C4 and M2, respectively and normal liver (control) using antibodies against OATP2A1 (green fluorescence) and CK19 (red fluorescence). Bile ducts are identified by the bright red color of the CK19 staining. Nuclei were stained with Hoestcht 33342 to give a bright blue color. Overlay of OATP2A1 and CK19 derived fluorescence is seen in bile ducts. Magnification x100.
Figure 4. Immunolocalization of OATP3A1 and the bile duct marker CK19 in liver cancer. In double-immunofluorescence staining experiments with antibodies against OATP3A1 and CK19, immunolocalization of OATP3A1 was investigated in HCC, CCC and MLT samples from patients H7, C1 and M12, respectively, as well as normal liver. Magnification x100.

Although, the expression levels of these transporters vary in cancerous and non-cancerous samples as well as in different tumor groups (HCC, CCC and MLT), a common pattern of OATPs being either down- (OATPs of family 1 and OATP2B1) or upregulated (OATP2A1, OATP3A1, OATP4A1 and OATP5A1) in hepatic cancers as compared to non-cancerous controls was found. Since all OATPs may facilitate the intracellular accumulation of various compounds in liver, the latter OATPs might be important with respect to the discovery of novel cancer agents.

In accordance to previously published data in references 15 and 16, we demonstrate decreased expression of OATP1B1 and OATP1B3 in hepatic cancer compared to non-cancerous liver tissues. Moreover, we show a diverse pattern of downregulation of OATP1B1 and OATP1B3 mRNA expression in different liver tumor types as only OATP1B1, an OATP, which is exclusively expressed in liver, was significantly reduced in primary and metastatic tumors. The most pronounced reduction in OATP1B1 and OATP1B3 mRNA expression in CCC may at least partly reflect the replacement of hepatocytes by malignant cells derived from different precursors. Dedifferentiation of cells during tumor progression may also explain the downregulation of OATP1A2, a transporter located in cholangiocytes in healthy human liver.22,24

Similar to our results on the reduced expression of OATP2B1 mRNA in cancerous liver, downregulation of OATP2B1, whose mRNA expression levels in normal liver are higher than that of other OATPs,25 was recently shown in liver cirrhosis caused by hepatitis C infection.26 Therefore, downregulation of these OATPs in liver cancer may follow a common mechanism to which expression of proinflammatory cytokines such as TNFα, interleukin 6 and interferon γ may contribute.2226

On the other hand, we show for the first time that transporters for prostaglandins, steroid and thyroid hormones, drugs and xenobiotics, namely OATP2A1, OATP3A1 and OATP4A1, as well as OATP5A1, an OATP with a yet unknown function,1 are upregulated at the mRNA level in the majority of liver cancer specimens as compared to the apparently normal counterparts. Regarding the differences in expression levels for “liver-specific” transporters, as well as OATP2B1 on one hand and OATP2A1, OATP3A1, OATP4A1 and OATP5A1 on the other, it might be speculated that expression of e.g., inflammatory cytokines could have distinct effects on OATP expression by decreasing expression of some and increasing that of other OATPs.

We also show that the mean levels of OATP2A1, OATP3A1, OATP4A1 and OATP5A1 assessed by immunofluorescence...
microscopy and visual and automatic image quantification analysis on paraffin-embedded tissue sections were generally higher in the cancerous sections of the HCC, CCC and MLT groups than in the non-cancerous counterparts, but show high inter-individual variability. In cancerous samples, immunonegativity for these OATPs was visible on plasma membranes and in the cytosol of cancer cells. This staining pattern seems to apply also for other tumors, as in a recent study, membrane and cytosolic staining was shown for OATP3A1 and OATP5A1 in breast cancer cells.29

We further found frequent co-localization of OATP2A1, OATP3A1 and OATP4A1 with the biliary/progenitor cell marker CK19, which expression in liver tumors is associated with a poor prognosis.30 This indicates presence of these OATPs in cells of proliferating bile ducts, where they may contribute to the hepatobiliary excretion of endogenous compounds (bile acids, steroid hormone conjugates) as well as drugs and xenobiotics. However, the role of these OATPs in healthy and diseased liver or in any other organ has not been elucidated yet, and it is unclear whether their transport function might be related to malignant transformation and/or tumor progression. At least for OATP2A1, a role in the termination of prostaglandin signaling may exist. This would be similar to the proposed role of this OATP in colon cancer, where OATP2A1 mediates the cellular accumulation of pro-inflammatory prostaglandin E2 as a prerequisite for its degradation by intracellular 15-prostaglandin-dehydrogenase.28 Whether OATP3A1, OATP4A1 and OATP5A1 have a role in the transport of endogenous compounds including steroid hormones, bilirubin and its degradation products requires further investigations.

In summary, we could show that OATP2A1, OATP3A1, OATP4A1 and OATP5A1 are highly expressed in primary and/or metastatic hepatic tumors, where they are located in the plasma membrane and in the cytosol of cancer cells. The broad expression of OATP2A1, OATP3A1, OATP4A1 and OATP5A1 in hepatic tumors might be important for cellular accumulation of various endogenous and exogenous compounds and may lead to further studies in order to find novel drugs against difficult-to-treat liver cancers.

Patients and Methods

Patient samples. Liver tumor samples and the adjacent non-cancerous tissues were obtained from 43 patients undergoing routine surgery for cancer in the liver. Specimens were only taken from tumors, where the non-cancerous parts showed a normal liver structure without cirrhosis and/or inflammation. Tumor specimens were obtained at the time of surgery before any chemo- and radiotherapy. Informed consent was obtained from
all patients, and permission for the study was obtained from the Ethical Committee of the Institution.

Hospital patient records were used to obtain information regarding tumor stage at diagnosis, tumor grade and age. The total number of tumor specimens was 43 with an overall gender distribution between female (n = 18) and male (n = 25). Twenty of the specimens were collected from primary liver cancer (HCC, n = 11 and CCC, n = 9) and 23 from metastatic liver tumors (MLT). Twenty-one samples were from patients with a primary tumor in the colon that had formed metastases in liver, while for the remaining two samples the primary tumor was not specified. Detailed patient characteristics are given in Table 1.

RNA extraction. Total RNA was extracted from liver tissue and cell lines using TRI Reagent™ (Applied Biosystems, Foster City) according to the manufacturer’s instructions. The concentration, purity, and integrity of RNA samples were determined by UV absorbance and electrophoresis. RNA from cancerous and non-cancerous colon samples was a kind gift from Dr. E. Kallay (Department of Pathophysiology & Allergy Research, Medical University, Vienna).83

Reverse transcription and real-time RT-PCR. Two µg of total RNA was reverse transcribed to cDNA with the high capacity cDNA reverse transcription kit (Applied Biosystems), using random hexamer primers as recommended by the manufacturer. TaqMan® Gene Expression Assays (Applied Biosystems) were purchased for all 11 human OATPs. To evaluate the appropriate reference genes, the expression of 12 different human housekeeping genes was analyzed in malignant and adjacent non-malignant tissue samples using a geNorm10 housekeeping gene selection kit with PerfectProbe™ (PrimerDesign Ltd., South Hampton, UK). CYC1, UBC, ATP5B and HSS were selected as acceptable reference genes for TaqMan® real-time RT-PCR analysis of the tissue samples. Real-time RT-PCR was performed as previously shown in our lab (reaction volume of 10 µl).13,26 The target gene amplification mixture contained 5 µl 2x TaqMan® Gene Expression PCR Master Mix, 0.5 µl of the appropriate Gene Expression Assay, 10 ng template cDNA diluted in 2.5 µl nuclease free water and 2 µl nuclease free water. Thermal cycling conditions were as follows: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C. Fluorescence generation from TaqMan® probe cleavage by the 5′→3’ exonuclease activity of the DNA polymerase was measured with the StepOnePlus system (Applied Biosystems). All samples were amplified in duplicates. Results were imported into Microsoft Excel for further analysis and comparable cDNA amounts in the experimental samples were calculated according to Hellemans et al.25

Real-time RT-PCR was performed with the following prefabricated TaqMan® Gene Expression Assays (Applied Biosystems):
containing intron-spanning primers: OATP1A2: Hs00245360_m1, OATP1B1: Hs00272374_m1, OATP1B3: Hs00251986_m1, OATP1C1: Hs00121974_m1, OATP2A1: Hs00319554_m1, OATP2B1: Hs00200670_m1, OATP3A1: Hs00203184_m1, OATP4A1: Hs0020583_m1, OATP4C1: Hs00606225_m1, OATP5A1: Hs00229597_m1, OATP6A1: Hs00542846_m1 and the endogenous controls 36B4 Part # 4310893E (Applied Biosystems), CYCI, UBC and ATP5B (HK-3D-hu-300, Primer Design Ltd.).

**Real-time RT-PCR data analysis.** Real-time RT-PCR data are presented as a heat map with hierarchical clustering analysis using average linkage algorithms and distance measures based on standard Pearson correlation (centered correlation) using Gene Cluster 3.0 and Java TreeView.26,27

Immunofluorescence microscopy. Antibodies applied in the indirect immunofluorescence staining experiments and their dilutions are stated in Table 2. In double-immunofluorescence staining experiments, for primary antibodies against OATPs generated in mouse, a rabbit anti-CK-19 antibody was applied, while for the OATP antibodies generated in rabbits and goat, a mouse anti-CK-19 antibody was used.

Formalin-fixed, paraffin-embedded sections (4 μm) were deparaffinized in xylene, and rehydrated with decreasing concentration of ethanol according to standard procedures. Antigen retrieval was performed by boiling the sections in 10 mM citric acid (pH 6.0) for 10 min in a microwave oven. Slides were allowed to cool down for 1 h, followed by a washing and the blocking step. The incubation with the primary antibodies was done overnight. In negative controls, primary antibodies were replaced by non-reactive immunoglobulins. Optimal antibody concentrations were determined by titrating serial antibody dilutions. The applied dilutions correspond to the minimum concentration necessary to produce a positive signal (Table 2). OATP1B1/OATP1B3, previously shown to be located at the basolateral membrane of liver parenchymal cells in normal liver and in the cytosol and membrane of HCCs were used as positive controls (data not shown).28 After incubation with the secondary antibody for 0.5–1 h, cell nuclei were stained with 0.5 μg/ml Hoechst 33342 (Sigma-Aldrich, St. Louis, MO).

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**Table 2.** Primary and secondary antibodies used in OATP immunofluorescence staining experiments on paraffin-embedded sections from liver tumors and normal liver.

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<th>Primary antibodies</th>
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<td>Atlas Antibodies (Stockholm, Sweden)</td>
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<tr>
<td>OATP5A1</td>
<td>Anti-SLC05A1; rabbit</td>
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<td>Atlas Antibodies</td>
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<tr>
<td>CK-19</td>
<td>Keratin19 Ab-1 (Clone AS5-B/A 2.26); mouse</td>
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<td>NeoMarkers (Thermo Fisher, Fremont, CA)</td>
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<tr>
<td>CK-19</td>
<td>Anti-Cytokeratin 19; rabbit</td>
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**Conjugate**

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|戈文 | Alexa Fluor® 488 | Goat Anti-Rabbit IgG | 1:1000 | Invitrogen (Carlsbad, CA) |
|Alexa Fluor® 488 | Goat Anti-Mouse IgG | 1:1000 | Invitrogen |
|Alexa Fluor® 568 | Goat Anti-Mouse IgG | 1:1000 | Invitrogen |
|Alexa Fluor® 568 | Goat Anti-Rabbit IgG | 1:300 | Invitrogen |

**Figure 7.** Quantitative evaluation of OATP2A1, OATP3A1, OATP4A1 and OATP5A1 immunoreactivity in hepatic tumor groups. The mean immunoreactivity (±SD, n = 7) was calculated from the fluorescence intensity and percentage of stained cells. It is given in arbitrary units (U) and is depicted on the y-axis.
Thereafter, slides were rinsed with distilled water before they were mounted in Mowiol 4-88 (Carl Roth, Karlsruhe, DE).

Experiments were also done on cryosections prepared from frozen specimens. Before fixation with acetone at -20°C, sections were air-dried for 1 h at room temperature. The subsequent immunofluorescence staining steps were done as described above.

Fluorescent staining was visualized with an Axioskop 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). In order to minimize background signals and to make the signal intensity and extension in different samples comparable, the exposure times for the individual antibody staining were evaluated and kept constant between the samples.

Quantitative evaluation of the OATP staining pattern.

Cancerous and corresponding non-cancerous tissue sections from the three tumor groups (n = 7, each) were analyzed for the extent and intensity of the fluorescence staining of the respective OATP first, visually, by two independent researchers and subsequently, by an automated immunofluorescence imaging system Tissue FAXS® and the TissueQuest® v.2.2 software (TissueGnostics GmbH, Vienna, Austria).21,22 In a scale of 0–4 arbitrary units (U), absence of immunoreactive staining or low staining intensity in less than 5% of cells was regarded as 0 U, low to moderate staining in >20% of tumor cells as 1 U, moderate staining intensity in >40 or <80% as 2 U, moderate to strong staining intensity in up to 60% of tumor cells as 3 U. Strong staining intensity in >60% of tumor cells was considered as 4 U. Antibodies against OATP2A1, OATP3A1 and OATP4A1 were tested in western blots on membrane fractions from malignant and non-malignant liver tissue specimens, which were prepared with the Proteojet™ Membrane Extraction Kit (Thermo Fisher Scientific, St. Leon-Rot, DE). Antibodies against OATP1B1 and OATP1B3 were used in control experiments. The antibody against OATP5A1 (Arias, Stockholm, SW) was not suitable for western blotting. Immunoreactive proteins were detected with the Super Signal West Pico Chemiluminescent Substrate (Pierce).

Statistical analyses. Comparisons of significant differences in the expression of OATP's between cancerous and adjacent non-cancerous tissue specimens were performed using a paired t-test (non-cancer vs. cancerous samples). Significance was defined as p < 0.05.

OATP nomenclature. Official nomenclature differs between genes and protein using the terms SLCO and OATP, respectively.34 To facilitate understanding, OATP was used for both, genes and proteins in this study.

Acknowledgements

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References


The effect of organic anion-transporting polypeptides 1B1, 1B3 and 2B1 on the antitumor activity of flavopiridol against breast cancer cells


The effect of organic anion-transporting polypeptides 1B1, 1B3 and 2B1 on the antitumor activity of flavopiridol in breast cancer cells

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Abstract. The contribution of organic anion transporting polypeptides (OATPs) to the cellular uptake of flavopiridol was investigated in OATP1B1- and OATP1B3- and OATP2B1-expressing Chinese hamster ovary (CHO) cells. Uptake of flavopiridol into these cells showed typical Michaelis-Menten kinetics with much higher transport capacity for OATP1B3 compared to OATP1B1 and OATP2B1 (Km 33.9 vs. 8.84 and 2.41 μM, respectively). The predominant role of OATPs was further supported by a dramatic inhibition of flavopiridol uptake in the presence of the OATP substrate rifampicin. Uptake of flavopiridol by OATPs also seems to be an important determinant in breast cancer cells. The much higher mRNA level for OATP1B1 found in wild-type compared to ZR-75-1 OATP1B1 knockdown cells correlated with higher flavopiridol initial uptake leading to 4.6-fold increased IC50 values in the cytotoxicity assay (IC50 1.45 vs. 6.64 μM). Cell cycle profile also showed a clear incidence for a stronger cell cycle arrest in the G2/M phase for ZR-75-1 wild-type cells compared to OATP1B1 knockdown cells, further indicating an active uptake via OATP1B1. In conclusion, our results revealed OATP1B1, OATP1B3 and OATP2B1 as up-transporters for flavopiridol in cancer cells, which may also apply in patients during cancer therapy.

Introduction

Flavopiridol (Alvocidib, NCI-649890, (5,7-dihydroxy-2-(2-chlorophenyl)-8[4-(3-hydroxy-1-methyl) piperedidinyl]-4H-benzopyran-4-on) is a selective inhibitor of cyclin dependent kinases (cdk1, cdk2, cdk4 and cdk7) thereby blocking cell cycle progression at the G1 to S and G2 to M interface (1). Flavopiridol, therefore, exerts pronounced antitumor activity in a variety of cell types including human breast, prostate, hematopoietic, lung, head and neck cancer cells, and also in human tumor xenograft models including colon and prostate carcinomas (1-4). Clinical trials with flavopiridol as a single agent or in combination with anticancer drugs, including taxanes and gemcitabine (5,6), also showed tumor responses in most phase I (7,8) and phase II (9,10) studies on different types of progressive tumors refractory to conventional treatment. Furthermore, the overall response rate could be also increased when flavopiridol was administered as a single agent using a pharmacokinetically-directed schedule (8,10). However, still there was a high amount of variability in pharmacokinetics, response and toxicity which could not be explained by demographic, patient’s and disease characteristics but might be caused by an altered flavopiridol accumulation in cancer cells. One factor strongly affecting anticancer drug concentrations thereby leading to altered response rates is metabolism. Indeed, recent data from our lab showed extensive glucuronidation of flavopiridol to the 5- and 7-hydroxy position in human liver microsomes by uridine diphosphate glucuronosyltransferase isoforms UGT1A1 and UGT1A9, respectively (11). Polymorphic UGTs may therefore affect the extent of glucuronidation as well as flavopiridol disposition, activity and toxicity in a manner similar to irinotecan, a drug which shows pronounced glucuronidation (12-14). In fact, patients with diarrhea after flavopiridol treatment had a lower metabolic ratio (flavopiridol glucuronide/flavopiridol) than patients without diarrhea, indicating that systemic glucuronidation of flavopiridol is inversely associated with the risk of developing diarrhea. It is well established that overexpression of ATP-powered efflux pumps such as P-glycoprotein (P-gp), MDRI and ABCB1, the breast cancer resistance protein BCRP (ABCG2) and the multidrug resistance protein MRP2 (ABCC2) have a great impact on intracellular concentrations of various anticancer agents. Indeed, flavopiridol was less...
toxic in CHO cells expressing higher levels of P-gp (15) and in acute leukemia patients with high BCRP mRNA expression in the blasts (16). MRP2 might indirectly also contribute to cellular drug concentrations as it is the main efflux transporter for flavopiridol glucuronides into bile where they can be cleaved by β-glucuronidase and reabsorbed (17). However, uptake mechanisms into tumor cells might be even more important than efflux transporters for the efficacy of anticancer drugs because they are determinants for intracellular drug concentration (18). One of the most important cellular drug uptake mechanisms in humans is via members of the organic anion-transporting polypeptide family (OATP) (19, 20). To facilitate readability and understanding of this report, “OATP” is used for both genes and proteins. OATPs are expressed in a variety of tissues (21) and tumors (22-24), where they mediate the transport of endogenous and xenobiotic compounds, including drugs (19,20,25). Studies have shown that uptake transporters can confer sensitivity to anticancer agents (26-30) such as the OATP1B3 substrates methotrexate and paclitaxel (26,31). This may be therapeutically important because expression of OATP varies greatly among tumor cell lines (32). Cellular uptake of flavopiridol is facilitated by OATP1B1 in transiently transfected HEK-293 and MDDC2-II cells (33). Furthermore, Niu and coworkers (33) also identified OATP1B1 rs1045819 as a polymorphic OATP1B1 variant associated with improved flavopiridol response in relapsed chronic lymphocytic leukemia patients. However, the authors did not investigate the kinetic parameters for the cellular uptake of flavopiridol in OATP1B1-transfected cells nor did they elucidate the impact of OATP expression on flavopiridol cytotoxicity in cancer cells. As OATPs exhibit overlapping substrate specificity, we hypothesized that additional OATPs may also contribute to the uptake of flavopiridol. In the present study, we therefore investigated the time and concentration-dependent transport of flavopiridol in stable OATP1B1-, OATP1B3- and OATP2B1-transfected CHO cells. Furthermore, the impact of OATP expression on cytotoxicity and cell cycle progression of flavopiridol-treated human breast cancer cells ZR-75-1 was also investigated.

Materials and methods

Materials. Flavopiridol (2-(2-chlorophenyl)-5,7-dihydroxy-8-(38,4R)-3-hydroxy-1-methyl-4-[piperidinyl]-4-chromene) was obtained from Sigma-Aldrich (Munich, Germany). Acetonitril and water were of HPLC grade (Merck, Darmstadt, Germany). All other chemicals and solvents were commercially available, of analytical grade and used without further purification.

Cell culture. Chinese hamster ovary (CHO) cells that were stably transfected with OATP1B1, OATP1B3 or OATP2B1 and wild-type CHO cells were provided by the University of Zurich, Switzerland and have been extensively previously characterized (34,35). The CHO cells were grown in Dulbecco’s modified Eagle’s 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) (66). All the media and supplements were obtained from Invitrogen (Karlsruhe, Germany). The mammary 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-cm² growth area (BD Biosciences, Franklin Lakes, NJ, USA), maintained at 37°C under 5% CO² and 95% relative humidity. The cells were passaged once a week and were used up to passage 55 (37).

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 h, 250 μl of medium supplemented with 8 μg/ml polybrene (HB268, Sigma) was added. Transductions were performed by the addition of 10 μl of shRNA (Mission™ transduction particlesNM_000646 Sigma, TRCN0000004320, coding sequence CCGCGCCCTTCATCTCAAGCTACA TTCTCGAGATGTTAGCCTTAGATGGAAGGCTTTTTTG). Twenty-four hours after the transduction, the cell culture medium was changed, and 1 ml of growth medium supplemented with 1 or 5 μg/ml of puromycin (P9620, Sigma) was added to select infected cells after an additional 24 h. The obtained silencing efficiency was evaluated after 3 weeks via real-time PCR and immunofluorescence (38).

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. Total RNA (2 μg) were reverse transcribed to cDNA using random hexamer primers and the ReverTra Aid™ H Minus M-MuLV Reverse Transcriptase system (Fermentas, St. Leon-Rot, Germany), as recommended by the manufacturer. TaqMan® Gene Expression assays (Applied Biosystems, Warrington, UK) were purchased for human OATP1B1. The 18S gene was used as a reference gene, as previously described (23). 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 sec at 95°C and 1 min at 60°C. Fluorescence generation due to TaqMan® probe cleavage via the 5′-3′ exonuclease activity of TaqMan® probe cleavage was measured with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). 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 the Sequence Detection Software (SDS 1.9.1, Applied Biosystems). 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 120.
the endogenous control. Real-time RT-PCR was performed with the following pre-fabricated TaqMan® Gene Expression Assays (Applied Biosystems) containing intron-spanning primer Hs00272374_ml for OATP1B1.

Immunofluorescence. ZR-75-1 OATP1B1-knockdown cells and cells transduced with the empty vector were allowed to attach on culture slides overnight (8-Chamber Poly styrene 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 (OATP1B1/103 mMDQ mouse monoclonal antibody, Alexis Antibodies, Herford, Germany) was diluted 1:100, and incubation was performed for 2 h. 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 controls. Following incubation with the secondary antibody (1:1,000 dilution; Alexa Fluor® 488 goat anti-mouse IgG; Invitrogen, Carlsbad, CA, USA) for 30 min, cell nuclei were stained with 0.5 μg/ml Hoechst 33342 (Sigma-Aldrich). Thereafter, the slides were rinsed with distilled water before being mounted in Mowiol 4-88 (Carl Roth, Karlsruhe, Germany). Fluorescent staining was visualized with an Axioplan 2 microscope (Carl Zeiss, Jena, Germany). Images were captured using a AxioCam MRc2 Color CCD digital camera and AxioVision 4.8 software (Carl Zeiss Vision GmbH, Aalen, Germany). 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 (38).

Cellular uptake. Transport assays were performed on 12-well plates as described in detail elsewhere (34). Briefly, CHO cells were seeded at a density of 350,000 cells/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) to induce non-specific gene expression (39). Flavipiridol was dissolved in DMSO and was diluted with uptake buffer (pH 7.4, final DMSO concentration of 0.5%) to 25-800 μM. Control experiments contained DMSO in the medium in place of flavipiridol. Prior to the transport experiment, the cells were rinsed twice with 2 ml of prewarmed (37°C) uptake buffer (116.4 mM NaCl, 5.3 mM KCl, 1 mM NaH2PO4, 0.8 mM MgSO4, 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 x 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 (37).

Inhibition analysis. For the inhibition experiments with rilpamycin and bromosulfophthalein (BSP; Sigma-Aldrich), stock solutions of these compounds were prepared in DMSO containing the indicated concentrations. CHO cells grown on 12-well plates were first washed twice with pre-warmed uptake buffer (pH 7.4) and incubated for 10 min at 37°C under 5% CO2 with 1 μM flavipiridol in the presence of the inhibitors ranging from 0.0001 to 100 μM. Control experiments were performed without BSP and rilpamycin under identical conditions as mentioned above.

Cytotoxicity assay. CellTiter-Blue (Promega, Southampton, UK) is a type of a colorimetric 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 h at 37°C under 5% CO2. For cytotoxicity assays, the cells were incubated with various concentrations of flavipiridol (5-400 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 (570 nm). The assay results were measured on a Tecan M200 multimode plate reader (Tecan Austria GmbH, Groeding, Austria). The absorbance was also measured in CellTiter-Blue assays in blank wells (without reseratrol) and subtracted from the values from experimental wells. The viability of the treated cells was expressed as a percentage of the viability of the control corresponding cells. All experiments were repeated at least three times.

Cell cycle distribution analyses by fluorescence activated cell sorting (FACS). ZR-75-1 wild-type and OATP1B1 knockdown cells were plated on 6-well plates at a concentration of 1x10⁶ cells/ml and allowed to attach overnight. After 24- and 48-h incubation at 37°C cells were trypsinized by the addition of 100 μl of trypsin, transferred into 15-ml tubes and centrifuged (4°C, 800 rpm, 5 min) (40). The supernatant was discarded and the cell pellet washed with cold PBS (phosphate-buffered saline, pH 7.4), centrifuged (4°C, 800 rpm, 5 min), resuspended in 1 ml cold ethanol (70%) and fixed for 30 min at 4°C. After two washing steps with cold PBS, the cell pellet was resuspended in 500 μl cold 100% PBS and transferred into a 5-ml polyethylene round bottom tube. RNAse A and propidium iodide were added to a final concentration of 50 μg/ml and incubated for 1 h at 4°C. The final cell number was adjusted between 0.5 and 1x10⁶ cells in 500 μl. Cells were analyzed by the FACSCalibur flow cytometer (BD Biosciences). Cell cycle distribution was calculated with ModFit LT software (Verity Software House, Topsham, ME, USA).

Determination of protein concentrations. Total protein was determined using the colorimetric biuretichem 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 UVProbe software (version 2.31, Shimadzu). The protein concentrations were consistent among the plates (0.150±0.005 mg/well).
Table I. Uptake kinetic parameters for fluvastatin in OATP-transfected CHO and ZR-75-1 cells.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Km (µM)</th>
<th>Vmax (pmol/mg/min)</th>
<th>Vmax/Km (µl/min/µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OATP1B1 tf. CHO</td>
<td>66.4±10.3</td>
<td>583±19.8</td>
<td>8.6±0.30</td>
</tr>
<tr>
<td>OATP1B3 tf. CHO</td>
<td>66.8±21.3</td>
<td>256±156</td>
<td>33.9±0.94</td>
</tr>
<tr>
<td>OATP2B1 tf. CHO</td>
<td>175±25.8</td>
<td>422±19.9</td>
<td>2.4±0.03</td>
</tr>
<tr>
<td>ZR-75-1 w.t.</td>
<td>80.8±14.1</td>
<td>1876±74.0</td>
<td>23.2±0.90</td>
</tr>
<tr>
<td>OATP1B1 k.d. ZR-75-1</td>
<td>99.0±24.0</td>
<td>758±44.9</td>
<td>7.6±0.13</td>
</tr>
</tbody>
</table>

Values are means ± SD of 3 individual determinations. The net OATP-mediated uptake values were calculated by subtracting the values obtained with the wild-type (w.t.) CHO cells from those obtained with the stably transfected cells. Kinetic parameters were calculated by fitting the data to the Michaelis-Menten (Km) equation with non-linear regression, t.f., transfected; w.t., wild-type; k.d., knockdown.

**HPLC chromatography.** The determination of fluvastatin was performed using a Merck 'La Chrom' System (Merck, Darmstadt, Germany) equipped with an L-7250 injector, an L-7100 pump, an L-7300 column oven (set at 37°C), a D-2500 interface, and a L-7400 UV detector (set at a wavelength at 264 nm). Chromatographic separation of fluvastatin was performed on a Hypersil BDS C18 Column (5 µM, 250 x 4.6 mm I.D.; Thermo Electron Corp.), preceded by a Hypersil BDS C18 precolumn (5 µM, 10 x 4.6 mm I.D.) at a flow rate of 1 ml/min. The mobile phase consisted of a continuous linear gradient, mixed from 10 mM ammonium acetate/acetate acid buffer, pH 7.4 (mobile phase A), and acetonitril (mobile phase B), to elute fluvastatin according to their lipophilicity. The mobile phase was filtered through a 0.45 µM filter (HVLP04700; Millipore, Vienna, Austria). The gradient ranged from 10% acetonitril (0 min) to 40% B at 15 min and linearly increased to 80% B at 17 min where it remained constant for 23 min. Subsequently, the percentage of acetonitril was decreased within 3 min to 10% in order to equilibrate the column for 9 min before application of the next sample. The sample injection volume was 80 µl. Calibration of the chromatogram was accomplished using the external standard method by spiking drug-free cell culture medium with standard solutions of fluvastatin to give a concentration range of 0.005-1 µg/ml (average correlation coefficient: >0.999). For this method the lower limit of quantification for fluvastatin was 10 ng/ml. Coefficients of accuracy and precision for this compound were <9.8%.

Data analysis. Kinetic analysis of the uptake of fluvastatin was performed over a substrate concentration range of 25-800 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 fluvastatin (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 GraphPad Prism Version 5.0 software program (GraphPad Software, San Diego, CA, USA) for Michaelis-Menten: \( V = V_{max} \frac{S}{K_m + S} \), 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 determined by plotting the log inhibitor concentration against the net uptake rate and non-linear regression of the dataset using the equation:

\[
y = \frac{a}{1 + [I/(IC_{50})] + b}
\]

in which \( y \) is the net uptake rate (pmol/µg protein/min), \( I \) is the inhibitor concentration (µM), \( s \) is the slope at the point of inversion, and \( a \) and \( b \) are the maximum and minimum values for cellular uptake. Net uptake was calculated for each inhibitor concentration as the difference in the uptake rates of the transporter-expressing and wild-type cell lines. Unless otherwise indicated, values are expressed as mean ± SD of 111 three individual experiments. Significant differences from control values were determined using a Student's paired t-test at a significance level of P<0.05.

**Results**

**Uptake kinetics of fluvastatin in OATP transfected CHO cells.** To investigate whether OATPs other than OATP1B1 contribute to fluvastatin uptake respective studies were...
Figure 2. Uptake of flavopiridol in OATP-transfected and wild-type CHO cells. The uptake of flavopiridol (25-800 μM) by (A) OATP1B1-, (B) OATP1B3- and (C) OATP2B1-transfected CHO cells and wild-type CHO cells was determined after 1 min at pH 7.4, 37°C. After the uptake into wild-type cells was subtracted, net uptake into OATP1B1, OATP1B3 and OATP2B1-mediated uptake is included to the Michaelis-Menten equation to calculate K_m and V_max values. The data represent the mean ± SD of 3 individual determinations.

Figure 3. Inhibition of flavopiridol uptake into OATP-transfected and wild-type CHO cells by rifampicin. (A) OATP1B1-, (B) OATP1B3- and (C) OATP2B1-transfected CHO cells were co-incubated with 1 μM flavopiridol and increasing concentrations of rifampicin (0.0001-10 μM) at 37°C for 1 min (Materials and methods). Values are expressed as a percentage of vehicle control; each value represents the mean ± SD of three independent experiments.

Effect of OATP inhibitors on the accumulation of flavopiridol in transfected CHO cells. To investigate whether known OATP inhibitors impact OATP1B1-, OATP1B3- and OATP2B1-mediated flavopiridol accumulation, flavopiridol was quantified after treatment of OATP1B1-, OATP1B3- and OATP2B1-overexpressing CHO cells with 1 μM flavopiridol in the absence and presence of increasing concentrations of the known OATP inhibitors bromosulfophthalein (BSP) and rifampicin (41,42). As shown in Fig. 3, rifampicin was a potent inhibitor for flavopiridol uptake in OATP1B3- followed by OATP2B1- and OATP1B1-transfected CHO cells (IC_{50} values, 1.00, 1.36 and 2.06 μM, respectively). BSP, however, did not inhibit but rather stimulated OATP-dependent flavopiridol uptake at concentrations up to 100 μM.

OATP1B1 knockdown in ZR-75-1 cells. The cells exhibiting the lowest expression of OATP1B1 (relative mRNA expression was reduced from 14.8±0.26 to 1.19±0.02) were chosen for...
Figure 4. Immunofluorescent characterization of OATP1B1 in ZR-75-1 and OATP1B1 knockdown ZR-75-1 cells. Cells were grown on culture slides and stained with an antibody against OATP1B1. (Materials and methods). Immunofluorescence was performed in ZR-75-1 empty vector-transfected cells (A) and ZR-75-1 OATP1B1 knockdown cells (B). Bright green fluorescence was seen in ZR-75-1 empty vector-transfected cells.

Figure 5. Uptake of flavopiridol in ZR-75-1 and OATP1B1 knockdown ZR-75-1 cells. The uptake of flavopiridol (25-600 µM) in ZR-75-1 empty vector-transfected and OATP1B1 knockdown ZR-75-1 cells was determined after 1 min at pH 7.4, 37°C and the OATP1B1-mediated uptake was fitted to the Michaelis-Menten equation to calculate Km and Vmax values. Data represent the mean ± SD of triplicate determination.

Figure 6. Cytotoxicity of flavopiridol to ZR-75-1 and OATP1B1 knockdown ZR-75-1 cells. After incubation for 72 h with 5-600 µM flavopiridol at 37°C percent viable cells were determined (Materials and methods). Cytotoxicity curves were obtained by non-linear curve fitting using the GraphPad Prism 5.0 program. The data represent the mean ± SD of 3 independent determinations.

Further experiments. Because ZR-75-1 cells express OATP1B1 (23) but not OATP1B3 and OATP2B1, the expression of the OATP1B1 protein was furthermore confirmed by immunofluorescence. Fig. 4 clearly shows constitutive expression (bright green fluorescence) of OATP1B1 in ZR-75-1 control cells (transfected with empty vector) (Fig. A) and suppressed protein levels of this transporter in ZR-75-1 OATP1B1 knockdown cells (Fig. B).

Uptake kinetics of flavopiridol in wild-type and OATP1B1 knockdown ZR-75-1 cells. Based on much higher OATP1B1 expression levels in wild-type ZR-75-1 breast cancer cell lines compared to OATP1B1 knockdown ZR-75-1 cells, we expected increased intracellular flavopiridol levels in the wild-type cells. For kinetic analysis, an incubation time of 1 min was chosen in order to prevent cellular uptake from interference with cellular efflux mechanisms such as MRPs and BCRP. Fig. 5 depicts representative Michaelis-Menten kinetics for a significantly higher flavopiridol uptake (2.5-fold) by wild-type compared to OATP1B1 knockdown ZR-75-1 cells (Vmax, 1876 pmol/mg vs. 758 protein/min). Affinity of flavopiridol to OATP1B1, however, was comparable in wild-type and OATP1B1 knockdown ZR-75-1 cells (Km, 30.8 µM±14.1 and 99.0 µM±24.0) indicating that OATP1B1 knockdown did not unmask other transporters for flavopiridol.

Cytotoxicity of flavopiridol in wild-type and OATP1B1 knockdown ZR-75-1 cells. The cytotoxicity of flavopiridol against ZR-75-1 and OATP1B1 knockdown ZR-75-1 breast cancer cells was quantified using the CellTiter-Blue test kit. As shown in Fig. 6, flavopiridol was significantly less toxic in OATP1B1 knockdown (IC50, 6.64 µM) than in wild-type ZR-75-1 cells (IC50, 1.45 µM) underscoring that OATP1B1 is important for flavopiridol uptake.

Effect of flavopiridol on the cell cycle of ZR-75-1 wild-type and OATP1B1 knockdown cells. ZR-75-1 cells were incubated with 5 µM flavopiridol for 8, 24 and 48 h and then subjected to FACS analyses. In the absence of flavopiridol both cell lines showed a nearly identical distribution of cells in the different phases of the cell cycle (Fig. 7). Addition of flavopiridol, however, exhibited distinct effects dependent on OATP1B1 expression.
While the cell cycle of wild-type cells was inhibited in G1 and G2/M phase, OATP1B1-knockdown cells were inhibited only in G1 phase at the expense of G2/M- and S-phase cells after 24 and 48 h of flavopiridol treatment. Notably, reduced uptake of flavopiridol in OATP1B1-knockdown cells was also associated with a decreased proportion of cells in sub-G1 indicating decreased induction of apoptosis observed after 48 h of flavopiridol incubation (14.5% compared to 18.2% for wild-type cells).

**Discussion**

In the present study, we identified the human OATP isoforms responsible for the cellular uptake of flavopiridol and demonstrated the relevance of flavopiridol uptake for its anticancer activity. To date, the only OATP that has been characterized, by Ni et al. (33), as an uptake transporter for flavopiridol is the OATP1B1. Furthermore, expression of the polymorphic variant OATP1B1_rsl1045819 was associated with improved flavopiridol response in patients with chronic lymphatic leukemia (33). By systematically investigating the transport properties of flavopiridol for other OATPs using the transfected CHO cells as an in vitro model, we were able to identify OATP1B3 and OATP2B1 as additional uptake transporters for flavopiridol. As shown in Fig. 2 and Table I, flavopiridol exhibited saturable uptake kinetics for OATP1B1, OATP1B3, and OATP2B1. The affinity of flavopiridol for OATP1B1 and OATP1B3 was similar (Km, 66.0 and 66.8 µM) but 2.6-fold lower for OATP2B1 (Km, 175 µM). Furthermore, uptake of flavopiridol into OATP1B3-transfected CHO cells was up to 5.3-fold higher (Vlim, 2263 pmol/mg/min) leading to an 3.2- and 14-fold increased transport capacity for OATP1B3 compared to OATP1B1 and OATP2B1 (Vlim/Kms, 33.9 vs. 8.84 and 2.41 µM/min/mg protein, respectively; Table I) indicating that OATP1B3 might be the most important uptake transporter for flavopiridol followed equally by OATP1B1 and OATP2B1.

Our data also suggest that OATP1B1, OATP1B3, and OATP2B1 are low affinity transporters and that the blood concentration of flavopiridol is considerably lower than the Km values. Indeed, administration of the standard dose of flavopiridol (50 mg/m²) as a 4 h infusion dose to patients with relapsed, symptomatic CLL or small lymphocytic lymphoma (SLL) in phase I and II studies showed peak plasma concentrations of ~3 µM (45). Despite this low substrate concentration relative to the Km value, uptake of flavopiridol into cancer cells is most likely pharmacodynamically effective but slow. It should be kept in mind that the local concentrations at the cancer cells are unknown, and other parameters like local pH (36) may also affect the transport rate of the OATPs expressed in cancer cells. In addition, extrapolating *in vitro* results to *in vivo* should be done with care as the absolute amount of OATP transporters in cancer cells may vary and as it is not known yet whether other transporters like OATP2A1 and OATP4C1 which are expressed in ZR-75-1 wild-type cells (23) are also involved in the uptake of flavopiridol. Other possible candidates may be organic anion transporters (OATs). Besides numerous clinically used drugs, OATs are also involved in the transport of polyphenol conjugates (44).

To further prove the importance of OATP1B1 for the uptake of flavopiridol, hormone-dependent ZR-75-1 cells that were previously shown to express high levels of OATP1B1, but not OATP1B3 and OATP2B1 (45), were incubated for 1 min with increasing concentrations of flavopiridol. Indeed, the uptake of flavopiridol by the ZR-75-1 OATP1B1-knockdown cells was significantly reduced compared to control cells, as indicated by lower Vmax values (Fig. 5 and Table I). Concomitant with the decreased uptake of flavopiridol detected in OATP1B1-knockdown cells, its cytotoxicity decreased 4.6-fold (Fig. 6). The pan-CDK inhibitor flavopiridol blocks the ATP pocket of CDKs and inhibits MCF-7 and MDA-MB-468 breast cancer cells simultaneously in the G1 and G2/M phases (46). Also wild-type ZR-75-1 breast cancer cells were inhibited in G1 and G2/M, whereas knockdown cells were only sensitive in the G1 phase upon flavopiridol treatment. This indicated that G1-specific CDKs, such as CDK4/6, were inhibited at lower flavopiridol concentrations (which was the case in OATP1B1 knockdown cells) compared to CDKs that are specific for the G2/M phase or required for both G1 and G2 transition (i.e. CDK1, CDK2, and CDK12, respectively). Furthermore, we also observed a significantly decreased proportion of cells in the sub-G1 phase (a marker for cell debris occurring throughout cell death) after 48 h of flavopiridol treatment in OATP1B1 knockdown cells. As apoptosis is known to be induced by flavopiridol (47) decreased apoptosis rates again support the role of OATP1B1-dependent flavopiridol uptake for cytotoxicity.

OATP1B1, OATP1B3, and OATP2B1-mediated flavopiridol transport may be of clinical importance, as all three transporters are expressed in various tumor entities including colorectal, liver, ovarian, pancreatic and prostate cancer tissues (48). Any variations in OATP expression may significantly alter the uptake of flavopiridol into targeted cells and tissues, thereby strongly affecting the efficacy of treatment. Patients with low expression of wild-type OATP1B1, OATP1B3 and OATP2B1 or patients carrying polymorphic OATP alleles may therefore show decreased response. Concomitant administration of OATP inhibitors may also...
interfere with the uptake of flavopiridol, leading to transporter-mediated drug-drug interaction. Our data demonstrated that ifosfamide selectively inhibited flavopiridol uptake in CHO cells, mediated by OATP1B1, OATP1B3 and OATP2B1 (IC50 values: 0.48-1.47 μM). Additional potential inhibitors include clathrin/mycin, erythromycin and roxithromycin, which inhibit the uptake of pravastatin in OATP1B1- and OATP1B3-transfected HEK293 cells (showing IC50 values of 32-37 μM) (49). Moreover, cyclosporine A significantly decreases the OATP1B1- and OATP1B3-mediated uptake of bosentan (35) and felodipine (50) in HEK293 and CHO cells. In addition to clinically applied drugs, naturally occurring flavonoids also interfere with the OATP uptake of dehydroepiandrosterone (DHEA-S), thus indicating that they constitute a novel class of OATP1B1 modulators (51). Whether all these potential OATP-dependent inhibitors interfere with the flavopiridol uptake in tumor cells is not yet known, however, care should be taken if patients use these drugs in combination with flavopiridol.

Ongoing studies are verifying the interactions of drugs and dietary supplements with the OATP1B1, OATP1B3- and OATP2B1-mediated uptake of flavopiridol.

In conclusion, our data revealed that OATP1B1, OATP1B3 and OATP2B1 act as transporters for flavopiridol; this role may also apply for the uptake of this compound into human cancer cells. Future in vivo studies should focus not only on the concentration of flavopiridol in target tissues but also on the expression levels of OATPs.

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References


Specific expression of OATPs in primary small cell lung cancer (SCLC) cells as novel biomarkers for diagnosis and therapy


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Specific expression of OATPs in primary small cell lung cancer (SCLC) cells as novel biomarkers for diagnosis and therapy

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Abstract

The expression of organic anion transporting polypeptides (OATPs) was elucidated in cell lines from small cell lung cancer (SCLC) and lung carcinoids and in paraffin-embedded samples from primary and metastatic SCLCs. We found a strong relationship between OATP expression and the origin of the cells, as cells from primary or metastatic SCLC and carcinoid tumors differ with respect to OATP levels. OATP4A1 is most prominent in non-malignant lung tissue and in all SCLC and carcinoid cell lines and tissues, OATP7A1 is most prominent in metastatic cells, and OATP8A1 is most prominent in SCLC cell lines and tumors. Treatment with topotecan, etoposide and cisplatin caused significant changes in the expression patterns of OATP4A1, OATP5A1, OATP6A1, chongramatin and synaptophysin. This effect was also evident in GLC-14 cells from an untreated SCLC patient before chemotherapy compared to GLC-14-15 chemoresistant tumor cells from this patient after therapy. mRNA expression of OATP4A1, 5A1 and 8A1 correlates with protein expression as confirmed by quantitative microscopic image analysis and Western blots. OATPs might be novel biomarkers for tumor progression and the development of metastasis in SCLC patients.

Introduction

Lung cancer is the leading cause of cancer-related death in western countries and small-cell lung cancer (SCLC) accounts for 15–20% of all lung cancer types [1]. SCLC is characterized by rapid tumor doubling time, a high growth fraction and the development of widespread metastases, especially to the brain, at a rather early stage [2]. In contrast to the majority of non-small cell lung cancers (NSCLC), SCLCs express neuroendocrine markers (e.g., chromogranin A, synaptophysin) and are thought to originate from lung neuronal precursor cells, from which other neuroendocrine lung tumors (e.g., carcinoids) are also derived [3,4]. Importantly, SCLC cells, but not carcinoids, are usually highly responsive to initial chemotherapy, usually with a platin derivative-containing regimen in combination with etoposide. However, a subsequent relapse of SCLC mostly occurs within 2 years. Recurrent tumors are than often resistant or has only a mild response to further treatment [5,6]. Only 10% of patients with advanced tumors survive longer than 2 years, leading to a five-year survival rate of only 3–7% [7–9]. Therefore, clues to a more effective treatment for primary SCLC and its metastases are of utmost interest. In lung cancer, the role of transporters in drug absorption, distribution and elimination processes as well as in drug–drug interactions is increasingly being recognized [10]. One of the most important cellular uptake mechanisms for anticancer drugs and endogenous compounds is via the organic anion transporting polypeptides (OATP proteins/SLC10 genes) [11,12]. The term OATP is used for both genes and proteins throughout the manuscript [13–18]. Eleven human OATPs, divided into six distinct subfamilies (OATP-6) [19], were found in human tissues mainly at biological barriers with a specific pattern [16,20]. Some OATPs, such as OATP4A1, are widely expressed in the human body and were also identified at the mRNA level in bronchial epithelial cell models and might contribute to the uptake of solutes into lung cells [21–23]. The malignant transformation of cells is known to alter the OATP expression pattern in organs. Indeed, the gonad-specific OATP6A1 was identified as a testis-specific antigen in lung tumors and lung tumor cell lines. The altered uptake of OATP substrates including anticancer drugs may lead to changes in the activity of drugs...
and may therefore play a role in the chemosensitivity of cancer cells.

For example, OATP1A1 seems to play a role in the resistance of lung cancer cell lines to satraplatin [20,24]. Otherwise, treatment with chemotherapeutic agents alters the expression pattern of OATPs in that cells, which again influences the response to specific cancer therapeutics. Therefore, this study aimed to investigate the OATP expression of SCLC cell lines. We used cell lines from primary and metastatic SCLC tumors as well as pulmonary carcinoid cells and performed immunofluorescence and immunohistochemistry on paraffin-embedded SCLC samples. Furthermore, the time in RNA expression of OATP5A1, OATP1A1 and OATP1A2 was assessed in cell lines that were exposed to the chemotherapeutic drugs cisplatin, etoposide and topotecan, which are all applied in SCLC therapy [10,21,23].

Chromogranin A and synaptophysin were applied as markers for neuroidendocrine differentiation of the tumor cells, and catherin-1 was applied as a marker for the epithelial origin of cells [25,26].

Materials and methods

Reagents

Cisplatin, etoposide, and topotecan were obtained from Etbio (Vienna, Austria).

Other reagents and solvents (if not otherwise stated), obtained from Sigma (Munich, Germany), were of analytical grade.

Cancer cell lines and treatment

NCI-H417 cells, the NSCLC cell line A549 [25] and the two pulmonary carcinoid cell lines NCI-H727 and NCI-H858 [26] were obtained from ATCC (Rockville, MD, USA). Other SCLC cell lines were obtained from the European Collection of Cell Cultures (Porton Down, Salisbury, UK).

The NCI-H417 cell line was originally derived from a human lung tumor of a patient with lung cancer treated with cisplatin and etoposide. It was obtained from Dr. R. Pedersen, Department of Radiation Biology, The Institute of Cancer Research, National University Hospital, Copenhagen, Denmark [27]. The NCI-H417 and NCI-H417-L5 cells were originally established from untreated SCLC patients. The NCI-H417-L5 cells were obtained from a patient with lung cancer treated with cisplatin and etoposide [28]. NCI-H417-L5 was derived from a bone marrow metastasis of an untreated SCLC patient [29]. NCI-H417-L5 cells were established from the pleural effusion of an untreated SCLC patient [24]. NCI-H727 and NCI-H858 were derived from lung carcinoid patients prior to therapy [30]. Cells were grown in RPMI-1640 bicarbonate medium (Serenomy, Berlin, Germany) supplemented with 10% fetal bovine serum (Serenomy), 4 mM glutamine and antibiotics (penicillin/streptomycin). Cells were cultured in RPMI-1640 medium (Serenomy). Cell cultures, stocks were prepared in DMEM (0.1%), which was used as the control. 1 x 10^6 cells in six-well plates were incubated with the compound for 3 days and washed with PBS before RNA isolation.

RNA extraction

RNA was extracted from cells using TRI Reagent® (Applied Biosystems, Foster City, CA, USA). The concentration, purity and integrity of the RNA samples were determined by UV absorbance and electrophoresis. RNA from normal human lung tissue (5 donor pairs, obtained from EORC (Halle, Germany)), RNA from human bronchial epithelial (HBE) cells was obtained from SciCell Research Lab (Carlsbad, CA).

Reverse transcription and real-time RT-PCR

One microgram of total RNA was reverse transcribed to cDNA with a high capacity cDNA reverse transcription kit (Life Technologies, Carlsbad, CA, USA). For reference genes, expression of 12 housekeeping genes was analyzed using a geNorm reference gene selection kit with PerfectProBESTM (PrimerDesign Ltd., Southamp ton, UK). GAPDH, glyceraldehyde-3-phosphate dehydrogenase, YWHAZ (14-3-3 protein zeta/delta) and TBP (DNA topoisomerase I) were used for validation of real-time RT-PCR analysis. Real-time RT-PCR was performed [38] using the TaqMan® Gene Expression Assays containing tampon spanning primers: OATP1A1: Hs00625590_m1, OATP1B1: Hs00272274_m1, OATP1B3: Hs0021986_m1, OATP1C1: Hs0013714_m1, OATP2A1: Hs01004195_5_m1, OATP2B1: Hs00081060_m1, OATP1A1: Hs00201184_m1, OATP1A2: Hs00265853_m1, OATP1A3: Hs01058684_m1, and the endogenous controls: GUSB, YWHAZ and TBP (PrimerDesign Ltd., Southamp ton, UK).

Immunochemistry and immunohistochemistry in cancer specimens

Double immunofluorescence staining was performed as previously described [40]. For antigen retrieval, 10 mM citrate-acid (pH6.0) was used. Antibodies: Rabbit anti-SCLC antigen 4-111 (A10 Antibodies, Stockholm, Sweden) 1:50, anti-SCLCO1A1 (Nexcel Molecular, Littleton, CO, USA) 1:1000 and for double immunofluorescence, mouse polyvalent antibody Anti-CD4 (Lab Vision - Neo Markers, Fremont, CA, USA) as endothelial cell marker were used (1:50). In negative controls, OATP antibodies were replaced by non-immunogenic IgG. Thereafter, samples were incubated with a secondary Alexa Fluor® 488 antibody (Invitrogen, Carlsbad, CA, USA) and the cell nuclei were stained with 1 μg/mL Hoechst 33342 (Sigma-Aldrich, St. Louis, MO). The slides were monitored on a TissueFAXS System with a Zeiss Fluorescence Microscope [41]. For immunohistochemical staining, sections were processed with the Eosin System-HRP (Dako, Glostrup, Denmark). Immunostaining intensity was first evaluated by two experienced researchers. Thereafter, quantitative evaluation of the percentage of stained cells and the intensity of staining per area given as immunoreactive score (IRS) was performed by using the HistoQuest program (TissueGnostics, Vienna, Austria) as described in detail by Roumit et al. [42].

Results

OATP mRNA expression studies in lung tumor cell lines

The relative mRNA expression of OATPs was assessed in cells from primary lung tumors (DMS-114, NCI-H417, SCLC metastatic tumors (DMS-153, NCI-526), carcinoid cell lines (NCI-H727, NCI-H858) and cells isolated from pleural SCLC effusions (NCI-H69, SCLC26A) (Fig. 1A and 1B). Normal lung tissue, human bronchial epithelia (HBE) and the NSCLC cell line A549 [43] were used for comparison. We showed that the OATP4A1 gene was the most abundant OATP in normal lung tissue and in cancer cells (Fig. 1B, Supplementary Table S1). In normal lung, the relative OATP4A1 mRNA expression levels were 2.9-fold higher than that of the housekeeping gene YWHAZ.

High levels were also found for the OATP2A1 gene (2.2-fold). The OATP1A2, OATP2B1, OATP3A1, OATP4C1, and OATP5A1 genes were also clearly expressed, but the levels were lower than that of YWHAZ (ranging between 0.33-fold for OATP2B1 and 0.005-fold for OATP5A1). Compared to normal lung, OATP gene expression was greatly reduced in non-malignant HBE. While OATP4A1 mRNA expression was reduced by 66%, a 99% reduction was observed for OATP2A1. Therefore, OATP4A1 was still the highest expressed OATP gene in these cells (Fig. 1B). A rather uniform expression pattern for OATP4A1 was observed in all tumor cell lines although the OATP4A1 gene levels were lower than in lung (up to 13%). Similarly, OATP2A1, OATP2B1,

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Fig. 1. (A and B) Relative mRNA expression of all known 11 OATPs in SCLC cell lines. 1 μg RNA isolated from non-malignant lung tissue, human bronchial epithelia (HBE), and cell lines from primary SCLC tumours and SCLC metastasis, lung carcinoid cell lines, and a non-small cell lung cancer cell line was reverse transcribed. qPCR studies were performed using TaqMan Assays as described in the Materials and Methods section. SCLC cell lines were either derived from biopsy specimens or from pleural effusions.

mRNA expression levels of OATP1A2, OATP1B1, OATP1B3, OATP1C1, OATP2A1 OATP2B1 (A) and OATP3A1, OATP4A1, OATP4C1, OATP5A1 and OATP6A1 (B) are expressed as n-fold changes related to the most stably expressed reference genes YWHAZ.

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and OATP1A1 were expressed at lower levels in SCLC and carcinoid tumor cell lines compared to normal lung tissue, but higher expression levels in SCLC cell lines than in normal human lung were observed for OATP1A2, OATP1B3, OATP4C1, OATP5A1 and OATP6A1. Cells also differed according to whether they originated from primary and metastatic SCLC tumors or from lung carcinoids. Particularly, OATP5A1 levels were highest in DMS-153 cells from metastatic tumors (approximately 2.5-fold higher than in normal lung tissue). Although OATP5A1 was also expressed in SCLC-32A cells and in the carcinoid cell line NCI-H835, it was expressed at lower levels than in the metastatic cell lines (0.13- and 0.23-fold of YWHAZ, respectively). Similar to OATP5A1, OATP1A2 transcripts were found at 2-fold elevated levels in NCI-H526 and 3.7-fold higher levels in NCI-H835 cells compared to normal lung (0.028- and 0.038-fold over YWHAZ).

Additionally, OATP1A1 gene expression was highest in these two cell lines and in SCLC-32A cells, in which its expression was approximately 10-fold over normal lung tissues (0.084- vs. 0.007-fold of YWHAZ). In the primary SCLC cell lines, NCI-H417 and DMS-114, the mRNA expression of two OATPs, namely OATP4C1 and OATP6A1, was evident. This finding is remarkable as OATP6A1, which was identified as a cancer-testis antigen, was also proposed as a lung cancer marker [45]. Compared to SCLC cells, the NSCLC cell line A549 showed a contrasting OATP expression pattern. In SCLC cells highly expressed OATP genes (e.g., the OATP4A1 gene) were expressed at low levels in A549 cells. The highest mRNA levels (0.218 x YWHAZ) were observed for OATP1B3 in NCI-H777 cells. Whereas OATP8B1 mRNA is considerably expressed in normal lung and A549 cells, it is reduced in SCLC and carcinoid cell lines (apart from metastatic NCI-H526 SCLC cells).

**OATP and neuroendocrine marker mRNA expression in cell lines in response to chemotherapy**

To study the changes in the OATP mRNA expression pattern during development of resistance to chemotherapy and subsequent tumor progression, GCL-14, GCL-16 and GCL-19 cells were assessed [44]. GCL cells expressed OATP1A2, 2A1, 2B1, 3A1, 4A1, 4C1, 5A1 and OATP1A4 genes. Relative values of OATP gene expression (related to lung) are shown in Table 1. OATP4A1 gene expression was upregulated by 120% and 80% in later stage GCL-16 and GCL-19 cell lines, respectively, compared to chemosensitive GCL-14 cell lines, while all other OATP genes were down-regulated. GCL cell lines expressed the epithelial cell marker cadherin-1 and both neuroendocrine markers chromogranin and synaptophysin. The cadherin-1 gene expression was increased by approximately 3-fold in GCL-19 cells compared to GCL-14 cells. Chromogranin expression was also increased by 25% in GCL-16 and by 13% in GCL-19. Synaptophysin levels were increased by 58% in GCL-19 cells. We also studied whether changes in the mRNA levels of neuroendocrine peptides might be related to altered OATP gene expression. We selected OATP1A1 (high levels in normal lung, low in tumor cells), OATP5A1 (low levels in primary SCLC cell lines, high in metastatic SCLC), and OATP6A1 (expressed in SCLC cells, but not found in normal lung tissue) to study the effect of in vitro treatment with chemotherapeutic agents on primary SCLC cell lines (NCI-H417) and the metastatic DMS-153 cells (Fig. 2), both expressing chromogranin and synaptophysin.

**OATP protein expression**

Western blotting was also performed to confirm OATP4A1, OATP5A1, and OATP6A1 expression in GCL-14, GCL-16 and GCL-19 cells from the SCLC patient. We found a clear correlation between protein and mRNA expression during tumor progression. While OATP4A1 was strongly induced, OATP5A1 and OATP6A1 decreased in the later stage GCL-16 and GCL-19 cell lines (see Fig. 3). As topotecan was most effective to modulate OATP mRNA expression, we further studied cellular protein levels of OATP4A1, OATP5A1 and OATP6A1 in topotecan treated NCI-H417 and DMS-153 cells. Up-regulation was again observed for OATP4A1 and in NCI-H417 cells after treatment with 1.25 μM and 2.5 μM topotecan. In DMS-153 cells, OATP4A1 was also up-regulated whereas OATP6A1 protein expression was down-regulated in both cell lines. This corresponds to PCR-data shown in Fig. 2 and Table 1.

**Studies in lung tumor specimens**

The cellular localization of OATP4A1, OATP5A1 and OATP6A1 was studied in paraffin-embedded SCLC specimens first by immunohistochemistry and then by immunofluorescence with positive staining for the transporters in non-malignant lung tissue and in primary and metastatic SCLC tumor specimens. Fig. 4 shows a representative picture of SCLC metastatic tissue, in which brown cytoplasmic and membrane staining of OATP4A1, OATP5A1 and OATP6A1 is visible in tumor cells. For quantitative analysis of the OATP levels in different samples, we used the immunohistochimical stained sections for which the TissueQuest program was found to be suitable [42]. Data from five non-malignant lung tissues, SCLC primary and metastatic tumors, respectively, revealed that the pattern of OATP4A1, OATP5A1 and OATP6A1.
Fig. 2. mRNA expression of OATP4A1, OATP5A1, and OATP6A1 in NCi-H417 and DMS-153 SCLC cell lines treated with anticancer agents. NCi-
H417 and DMS-153 cell lines were treated with cisplatin (0.6 and 1.25 μM), etoposide (0.6 and 1.25 μM), and topotecan (1.25 and 2.5 μM), respectively, for 48 h. The RNA was isolated. Then, 1 μg RNA was reverse-transcribed and TaqMan Assays were applied in the quantitative PCR. Expression levels in untreated cells were set to 100% and changes in the expression levels were calculated as % of control (*p < 0.05; n = 3).

Table 2

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Fig. 3. Effect of anticancer chemotherapy on cellular OATP4A1, OATP5A1 and OATP6A1 levels in SCLC cancer cell lines. Protein extracts (50 μg) isolated from GLC-14 (before), GLC-16 (during) and GLC-10 (after therapy) cell lines derived from one SCLC patient, and in the NCi-H417 and the DMS-153 SCLC cancer cell line treated with topotecan (1.25 μM and 2.5 μM), respectively, were separated on a 12% PAGE and proteins were blotted onto nitrocellulose membranes. Blots were probed with antibodies against OATP4A1, OATP5A1 and OATP6A1, respectively. GAPDH was used as loading control.
are reduced (by approximately 50%) in the tumor sections compared to the non-cancerous tissue. OATP5A1 levels are highest in the metastatic tumors, while OATP6A1 levels are near the detection limit in the non-cancerous tissue, but its levels are up to 10 times higher in the primary tumors.

Immunofluorescence microscopy of these OATPs on paraffin-embedded sections from SCLC tumors and non-malignant lung tissue confirms these findings (representative images are shown in Fig. 5A and B). In Fig. 5A, the green OATP staining is visible while in the merged image (Fig. 5B) green OATP, red CD34 (for endothelial cells) and blue nuclei are present. Bright green immunofluorescence staining of OATP4A1 was observed in the samples from healthy lung tissue. The OATP5A1 fluorescence staining was clearly visible in the metastases, while for OATP6A1, a rather diffuse staining in all tissue samples was visible as expected from the mRNA expression pattern in the cell lines. Additional experiments investigating whether OATP4A1, OATP5A1, and OATP6A1 might also be expressed in blood vessels were performed. By applying an antibody against CD34 together with antibodies against OATP4A1, OATP5A1, and OATP6A1, no co-staining of CD34-positive vascular endothelial cells in the lung tissue was observed indicating absence of these OATP isoforms in these cells (Fig. 5B).

Discussion

In the present study we elucidated the mRNA expression pattern of all 11 OATPs in primary and metastatic SCLC and lung carcinoid tumor cell lines in comparison to normal non-malignant lung tissue and non-malignant human broncho-epithelial cells. Then we evaluated the effect of chemotherapeutic agents on the expression of selected OATPs. OATP4A1 was most abundant in normal lung tissue and expressed in lung cancer cell lines derived from SCLC, carcinoid and NSCLC tumors. Similar to other OATPs, it was significantly reduced in non-malignant HBE. Expression of this OATP was also previously found in lung adenocarcinomas [21], epithelial BEAS-2B cells [23], and the adenocarcinoma cell line Calu-3 [23]. Interestingly, while OATP1A2, OATP1B3, OATP4C1, OATP5A1 and OATP6A1 demonstrated higher mRNA expression levels in SCLC cell lines compared to normal lung tissue, other OATP genes, namely OATP2A1, OATP2B1, and OATP3A1 were reduced in SCLC and carcinoid tumor cell lines. Furthermore, our data also showed distinct differences in the mRNA expression pattern of OATPs between metastatic and primary tumor cell lines. This applies to OATP4A1 in which expression levels were high in cell lines derived from metastatic tumors (DMS-153 and NCI-H526) and in carcinoid cell line NCI-H835. On the other hand, OATP6A1 mRNA was found in the primary cell lines NCI-H417 and DMS-114 and in the pleural effusion cell line NCI-H69. Contrary to SCLC cell lines, the NSCLC cell line A549 showed a unique OATP expression pattern with preferentially OATP1B3 gene expression. This suggests possible differences in the

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uptake of OATP substrates e.g., anticancer drugs between the two tumor entities [11].

In accordance with data from the cell lines on mRNA expression and on OATP4A1, OATPS1A and OATPS4A1 levels in Western blot, OATP4A1 was more prominent in paraffin embedded sections from non-malignant lung tissue than in primary and metastatic tumors. However, its levels in the investigated tumor tissues and cell lines were still higher than those of other OATPs (OATPS1A, OATPS4A1). The prominent expression of OATP4A1 in all lung samples (SCLC, carcinoid cell lines, tissue sections from primary and metastatic SCLC samples) suggests an important role for OATP4A1 in the uptake of specific substrates, such as thyroid hormones, estrone-sulfate, and prostaglandins into lung cancer cells. Prostaglandins are important regulators in lung cells under physiological and pathological conditions, as they control smooth muscle airway contraction [46] and modulate the inflammatory process [47]. Whether OATP4A1 might work together with lung-cell expressed OATP2A1, which is a specific uptake transporter for prostaglandins (including prostaglandin E2) [48] for their intracellular inactivation [49,50], is still unknown. Other OATP4A1 substrates are thyroid hormones and they are known to modulate tumor progression via binding the nuclear thyroid hormone receptor [50]. Thyroid hormones are the only known substrates for another OATP, namely OATP1C1, whose expression is particularly high at the blood–brain barrier [51,52]. mRNA of this OATP is also expressed at a low level in lung tissue and in lung adenocarcinoma A549 cell line, but not in SCLC tumors and cell lines. Additionally, estrone-sulfate uptake via OATP4A1 was shown [53]. This might be important because estrone sulfate can be converted via steroid sulfatase to active 17β-estrone, which was found to influence pulmonary inflammation and lung cancer progression [54]. In addition to OATP4A1, other OATPs are also active in the uptake of steroid precursors, including OATP family 1 members and OATP2B1 [55,56]. The latter is also present in normal lung tissue, and in contrast to A549 cells, it is only rarely expressed in SCLC cells. Similar to OATP2B1, OATP1B3 known as tumor marker in cancer of various organs including gastrointestinal tract, breast, and prostate, was rather low expressed in SCLC. Interestingly, higher OATPS1A mRNA levels were observed in metastatic SCLC cell lines, carcinoid- and pleural effusion-derived cells, compared to normal lung tissue. OATPS1A immunoreactivity was also more pronounced in lung cancer metastases from SCLC patients, suggesting a role for this OATP in tumor metastasis. This assumption is supported by a recent study showing that expression of Hela cells with OATPS1A induces expression of several genes involved in the regulation of differentiation and migration [24,57]. We also showed that the cancer-testis antigen OATPS1A is present in SCLC tumor cells and demonstrated its presence in better than primary SCLC and metastases, indicating that it may also be considered as a tumor marker for this lung cancer subtype [45]. To date, no substrate for human OATP6A1 has been identified, although the rat homologue was found to transport DHEA sulfate [58]. Therefore, its function is still unclear.

We also found that resistance to chemotherapy is accompanied by altered OATP mRNA expression at least in the SCLC patient treated with surgical removal of the tumor, radiation and anticancer drug therapy. In the GLC-14 cell line, isolated before therapy, only OATP4A1, but not OATP1A1 and OATP2B1, was lower than in the late stage GLC-16 and GLC-19 cell lines from the same patient. These changes were related to an increase in neurogenic differentiation as shown by the increased levels in neurogenic peptides synapsin 1 and chromogranin and the epithelial marker catherin-1. Based on this interesting finding, we also studied the expression of these OATPs in NCI-H147 and DMS-153 cells after treatment with cisplatin, etoposide and topotecan. Generally, the effects were more pronounced in the primary NCI-H1-47 cells than in the metastatic cell line. Cisplatin was active to induce OATP4A1, OATPS1A and OATPS4A1 together with the neuroendocrine markers chromogranin and synaptophysin in NCI-H147 cells. In contrast to cisplatin and etoposide, topotecan induced OATP4A1 in both cell lines, while OATP6A1 was reduced. Therefore, we further analyzed topotecan treated cell lines in Western blots. In the GLC cell lines and in the two cancer cell lines NCI-H147 and DMS-153 treated with topotecan, mRNA expression of OATP4A1, OATPS1A and OATPS4A1 correlates with protein expression. This was confirmed by quantitative microscopic image analysis of the immunohistological staining pattern for these OATPs in SCLC tumor samples from SCLC patients (see Fig. 4 and Table 2). So far we know that this is the first report on the protein expression of these particular OATPs in SCLC tumors.

Taken together, our data revealed a specific OATP expression pattern, with OATP4A1 being the most prominent OATP in SCLC and carcinoid cell lines and SCLC tumors. We also confirmed that OATPS1A is highly expressed in metastatic tumor cells while OATP6A1 is present in SCLC cell lines and the tumors. Whether OATP modulation might also be observed in SCLC patients during chemotherapy is not known yet, but OATPs might function as novel biomarkers for tumor progression and the development of metastasis.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2014.09.025.

References

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Effects of anthocyanins on the expression of organic anion transporting polypeptides (OATPs) in primary human hepatocytes

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Running Title:
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Abstract

Anthocyanins (anthocyanins and their aglycones anthocyanidins) are colorful pigments, naturally occurring in berry fruits. They exhibit many biological effects and have potent health benefits. Anthocyanins are widely used as dietary supplements and the safety of products containing them is of great importance. To investigate whether anthocyanins influence the expression of hepatic uptake transporters belonging to the organic anion transporting polypeptide (OATP) family, we carried out studies on primary cultures of human hepatocytes. The hepato-cellular accumulation of widely used drugs such as statins and antibiotics is mediated by the liver-specific OATP1B1 and OATP1B3 transporters, thus any interference with expression of these particular transporters might influence therapeutic outcomes. We evaluated the effects of 21 anthocyanins and their corresponding 6 anthocyanidins on the levels of OATP1B1/OATP1B3 mRNA by RT-qPCR. Changes in OATP protein levels were confirmed by western blotting. Our data show that OATP1B1 responds differently to anthocyanins compared with OATP1B3. We observed the induction of OATP1B1 mRNA and protein in four hepatocyte samples by the anthocyanins malvin chloride, malvidin-3-O-galactoside chloride and cyanidin-3-O-sophoroside chloride. For OATP1B3, a reduction in the expression levels was seen with the anthocyanidin pelargonidin, and the anthocyanin delphin chloride. Although the values varied considerably between primary hepatocyte isolates from different individuals, a mean induction of OATP1B1 up to 60% and reduction of OATP1B3 gene expression by less than 25% were detected. We propose that the effects of anthocyanins derived from high dose dietary supplements may have to be taken into account in patients undergoing a therapy with drugs transported by OATP1B1 and OATP1B3.

Key Words:

Anthocyanins, anthocyanins, anthocyanidins, phytochemicals, OATP1B1, OATP1B3, OATP expression, human hepatocytes
Introduction

Anthocyanins are a sub-group of flavonoids that exist in all tissues of higher plants as water-soluble pigments, responsible for the red, blue or purple colors of berries, grapes, apples, corn and many vegetables.\textsuperscript{1} The broad term “anthocyan” encompasses both glycosides (termed anthocyanins) and aglycones (termed anthocyanidins). In higher plants, only anthocyanins are found, and in these, the pigment is linked to one or more sugars, often glucose, galactose, arabinose and xylose, but also to rather rare sugars including rhamnose, sophorose or sambubioside.\textsuperscript{2} The most common anthocyanidins are cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and petunidin.\textsuperscript{1} Many studies have demonstrated that anthocyanins exhibit anti-proliferative, anti-apoptotic, anti-tumor, anti-mutagenic, anti-oxidant, anti-inflammatory and nitric oxide inhibitory effects \textit{in vitro} that may be linked to their ability to confer important health benefits. Furthermore they are reported to reduce the incidence of diabetes, cardiovascular disease, arthritis and cancer.\textsuperscript{1,3-6}

For the last years, public interest in the cancer chemopreventive properties of dietary constituents has increased as people have sought effective and safe diet-derived alternatives to pharmaceuticals.\textsuperscript{7} In a study, the daily intake of anthocyanins in individuals in the U.S. has been estimated to be approximately 180-215 mg/day.\textsuperscript{8} Anthocyan-containing dietary supplements are available as juices, dried juice, dried fruits or water extracts, and are claimed to offer such an alternative.\textsuperscript{9} Importantly, the dose of anthocyanins in certain dietary supplements is extraordinarily high, e.g. in over-the-counter drugs taken for the treatment against diarrhea (blueberry extracts, daily doses of 100-200 mg anthocyanins) or against urinary tract infections (cranberry extracts, 60-225 mg per dose). However, there are no clinical studies investigating their effectiveness or potential side effects due to interactions with drug transport. For a critical review of the literature on anthocyanins in dietary supplements see Espin et al., 2007.\textsuperscript{10}

Anthocyanins are metabolised in the liver,\textsuperscript{5,11} and therefore food–drug interactions might be expected. It is of special interest to us to discover how these compounds may interact with the transport proteins that mediate the uptake of endogenous and exogenous compounds into liver cells. Two proteins, OATP1B1 and OATP1B3, members of the SLC superfamily of uptake transporters (SLC family 21) play an important role in the hepatic uptake of bilirubin, bile acids, conjugated steroids, eicosanoids and thyroid hormones, as well as xenobiotics, phytochemicals and drugs. There is considerable substrate overlap between these two OATPs, and if their substrates are co-administered, they may interfere with their transport capacity. This is especially important for drugs with a narrow
therapeutic index, such as statins, for which high concentrations in blood plasma can result in severe myopathy.\textsuperscript{12,13} In addition to the consequences of altered substrate competition, altered levels of expression of these OATPs might also change the uptake of these therapeutics.

It has been suggested that the expression of OATP1B1 and OATP1B3 can be modified by drugs, xenobiotics and natural compounds.\textsuperscript{14} Studies in rodents showed that activation of nuclear receptors, such as the pregnane X receptors (PXR) by natural compounds, for example the polyphenol hyperforin, and synthetic drugs such as the antibiotic rifampicin modulate the expression of OATPs.\textsuperscript{15} However, a recent study using human hepatocytes revealed that rifampicin, although an effective stimulator of PXR, has little effect on OATP1B1 and OATP1B3 expression.\textsuperscript{16}

To the best of our knowledge, there are no reports describing the effect of anthocyanins on the expression of any OATPs in human liver cells, and so we investigated whether the levels of expression of OATP1B1 and OATP1B3 mRNA and protein are modified by common anthocyanes (21 anthocyanins and their corresponding six anthocyanidins) in human hepatocytes isolated from four healthy donors.

**Materials and methods**

**Chemicals**

Dimethyl sulfoxide (DMSO) and rifampicin (RIF) were purchased from Sigma-Aldrich (Prague, Czech Republic). The following anthocyanins and anthocyanidins were purchased from Extrasynthese (Lyon, France): peonidin-3-O-glucoside chloride, peonidin-3-O-rutinoside chloride, pelargonidin-3,5-di-O-glucoside chloride, pelargonidin-3-O-rutinoside chloride, delphinidin-3-O-glucoside chloride, delphinidin-3-O-rutinoside chloride, delphinidin-3,5-di-O-glucoside chloride, delphinidin-3-O-sambubioside chloride, delphinidin-3-O-rhamnoside chloride, malvidin-3-O-glucoside chloride, malvidin-3,5-di-O-glucoside chloride, malvidin-3-O-galactoside chloride, cyanidin-3-O-glucoside chloride, cyanidin-3-O-rutinoside chloride, cyanidin-3,5-di-O-glucoside chloride, cyanidin-3-O-sophoroside chloride, cyanidin-3-O-arabinoside chloride, cyanidin-3-O-rhamnoside chloride, cyanidin-3-O-galactoside chloride, cyanidin-3-O-sambubioside chloride, cyanidin-3-O-lathyroside chloride, cyanidin chloride, delphinidin chloride, malvidin chloride, peonidin chloride, petunidin chloride, and pelargonidin chloride. All other chemicals and solvents were commercially available, of analytical grade, and used without further purification.
Human hepatocytes

Human hepatocytes were isolated from human liver obtained from multiorgan donors LH45 (M, 46 years), LH46 (M, 37 years), LH47 (M, 47 years) and LH49 (M, 38 years). The tissue acquisition protocol was in accordance with the requirements stated by the local ethical commission in the Czech Republic. Long-term preserved human hepatocytes were obtained from Biopredic International (Rennes, France) as monolayer batch HEP220670 (F, 64 years).

All hepatocytes were treated in a serum-free medium for 24 h with the tested compounds and/or vehicle (DMSO; 0.1% v/v). Cultures were maintained at 37°C and 5% CO₂ in a humidified incubator.3,17

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Total RNA was isolated using TRI Reagent® and cDNA was synthesized according to the common protocol, using M-MLV Reverse Transcriptase F-572 (Finnzymes, Thermo Scientific, Portsmouth, NH, USA) and random hexamers 3801 (Takara, Saint-Germain-en-Laye, France).18 TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) were purchased for OATP1B1 and OATP1B3 (containing intron-spanning primers: OATP1B1: Hs00272374_m1, OATP1B3: Hs00251986_m1). To select appropriate reference genes, the expression levels of 12 different human housekeeping mRNAs were analyzed using a geNorm reference gene selection kit with PerfectProbeTM (PrimerDesign Ltd., Southampton, UK). GAPDH (glyceraldehyde-3 phosphate dehydrogenase), YWHAZ (14-3-3 protein zeta/delta) and TOP1 (DNA topoisomerase 1) were selected as acceptable reference genes for TaqMan® q-PCR analysis of the samples as previously described.18 Data were analyzed by the delta-delta Ct method. Results are expressed as fold-induction over DMSO-treated cells. Data were visualized as a heat map using Java TreeView.19

Western blotting

Total protein extracts were prepared as described elsewhere.20 After SDS-PAGE separation and Western blot transfer, filters were probed with rabbit polyclonal antisera against OATP1B1 (rabbit polyclonal; LS-C161285; LifeSpan BioSciences, Seattle, WA, USA) or OATP1B3 (rabbit polyclonal; LS-C159033; LifeSpan BioSciences). Chemiluminescent detection was performed using a horseradish peroxidase-conjugated secondary antibody and an ECL detection kit both from Thermo Scientific (Portsmouth, NH, USA). A VersaDoc 4000MP Imaging System (Bio-Rad Laboratories
Inc., California, US) was used to capture images. As a loading control, the blots were probed to detect β-actin (data not shown).

Results

Effects of anthocyanidins on OATP expression

Initially, we elucidated the effect of the aglycones, namely the anthocyanidins cyanidin, peonidin, petunidin, pelargonidin, delphinidin, and malvidin on OATP1B1 and OATP1B3 mRNA levels in LH45, LH46, and LH47 cells. For comparison, we also used long-term preserved HEP220670 (HEP) cells derived from one donor. Cells were incubated for 24h with anthocyanidins (50 μM), and the vehicle (DMSO, 0.1% v/v), before mRNA and protein were isolated and OATP1B1 and OATP1B3 expression levels were determined.

As shown in Table 2, treatment with pelargonidin reduced OATP1B3 mRNA levels by approximately 25%. In HEP cells, no significant alterations in the mRNA expression levels were seen with any anthocyanidin.

Effects of anthocyanins on OATP expression

In the next series of experiments, the effects of 21 anthocyanins (50 μM) were tested on four different human hepatocyte cultures (LH45, LH46, LH47, LH49). The induction pattern of OATP1B1 and OATP1B3 (see heat map in Fig. 2) was highly variable between hepatocytes obtained from different donors and the effect of anthocyanins on OATP1B3 mRNA expression was more variable between the LH-cultures than it was on OATP1B1. The majority of anthocyanins increased the level of OATP1B1 mRNA in LH49 cells, whereas hardly any induction of OATP1B3 mRNA was observed in these cells.

By contrast, on using LH45 cells, OATP1B3 mRNA levels increased in response to many anthocyanins, and strong induction of OATP1B1 mRNA expression was observed after cyanidin-3-O-sophoroside chloride exposure, with more-moderate increases for other anthocyanins. In LH46-hepatocytes, malvin chloride caused a pronounced increase of OATP1B1 and OATP1B3 mRNA levels. In LH47 cells, increased levels of OATP1B1, but not of OATP1B3 mRNA occurred in response to cyanidin-3-O-sambubioside chloride, malvidin-3-O-galactoside chloride and pelargonidin-3-O-rutinoside chloride.

The mean values and minimum and maximum values for OATP1B1 and OATP1B3 mRNA levels in the LH cells are summarized in Table 3. The data indicate that the effects of anthocyanins on OATP
mRNA expression are rather moderate, revealing a maximal induction of 1.6-fold (mean values) of OATP1B1 mRNA with malvidin-3-O-galactoside chloride. A small, but significant increase in the mean levels of OATP1B1 mRNA was also produced by two other anthocyanins: malvin chloride (1.51-fold) and cyanidin-3-O-sophoroside chloride (1.38-fold). Protein levels corresponded with the mRNA response pattern in hepatocyte samples. Higher levels of immunoreactive OATP1B1 after malvidin chloride and malvidin-3-O-galactoside chloride treatment reflected the higher mRNA levels. Incubation with pelargonin chloride and pelargonidin-3-O-rutinoside chloride, which had no effect on OATP1B1 mRNA, also did not alter OATP1B1 protein levels (Fig. 3).

In contrast to OATP1B1, mean OATP1B3 mRNA expression in LH45, LH46, LH47 and LH49 cells was not significantly increased by anthocyanins (Table 3). Notably, OATP1B3 mRNA expression was reduced by nearly 20% (mean value 0.817-fold) after delphin chloride incubation. Again, OATP1B3 protein levels corresponded with the mRNA levels.

In control experiments in the hepatocytes, we also noticed that rifampicin does not induce the expression of either OATP1B1 or OATP1B3 (n-fold induction 0.74-0.99). This is not surprising, as in a previous study, rifampicin derivatives, known to be common inducers of some drug metabolising P450 enzymes (CYPs) and transporters \(^{15,16,21}\), did also not show any specific upregulation of OATP mRNA.\(^{16}\)

**Discussion**

Our investigation into the effects of 27 anthocyanins on OATP1B1 and OATP1B3 expression in primary human hepatocytes revealed that these phytochemicals can indeed influence the expression of these liver-specific transporters. Interestingly, we observed induction of OATP1B1 expression, but reduction of OATP1B3 expression with some anthocyanins.

In all hepatocyte cell samples tested, increases of OATP1B1 mRNA and protein levels of at least 10% occurred in response to the anthocyanins malvin chloride, malvidin-3-O-galactoside chloride, and cyanidin-3-O-sophoroside chloride. For OATP1B3 a reduction in the expression levels was seen with one anthocyanidin, pelargonidin, and one anthocyanin, delphin chloride (Table 2 and 3). Only the glycosides but not the corresponding aglycones were able to affect increases in expression, indicating that the molecule together with the sugar moiety is necessary for the stimulation. The transporters OATP1B1 and OATP1B3 mediate the intracellular accumulation of widely used drugs such as statins and chemotherapeutic agents, so our findings are important because altered expression of OATP1B1 and OATP1B3 by these dietary constituents might change the efficacy of a drug
therapy. However, it must be considered that the OATP expression pattern varies considerably between hepatocytes from individual donors\textsuperscript{22} and those variations may have a more severe impact on the OATP-mediated cellular accumulation of drugs in patients. It is also important that in the liver, OATP1B1 is expressed in all hepatocytes throughout the lobules, whereas OATP1B3 expression is highest in hepatocytes located around the central vein\textsuperscript{23}. This implicates that their expression in liver is regulated differently, which could also account for the different reaction of OATP1B1 and OATP1B3 to anthocyanins.

In general, our data concur with previous studies on the effect of anthocyanins on biotransformation pathways that showed only small effects of anthocyanins on selected enzymes. Kamenickova et al. recently reported the effects of cyanidin, delphinidin, malvidin, peonidin, petunidin, pelargonidin and their most common anthocyanins on the aryl hydrocarbon receptor (AhR)–cytochrome P450 CYP1A1 pathway.\textsuperscript{3,17} They showed, using human hepatocytes and HepG2 cells, that pelargonidin is a weak ligand/agonist of the AhR and of AhR-dependent gene expression. Pelargonidin moderately induced CYP1A1 mRNA expression in all of their primary hepatocyte cultures. Among the anthocyanins, pelargonidin-3-O-rutinoside chloride and cyanin chloride were weak inducers of CYP1A1 mRNA expression.\textsuperscript{3,17} Studies of the effect of anthocyanins on expression of CYP2C9, CYP2A6, CYP2B6, and CYP3A4 in human hepatocytes and liver microsomes did not reveal significant alterations of mRNA or protein levels.\textsuperscript{1}

In summary, our data demonstrate that some anthocyanins are capable of altering the expression of OATP1B1 and OATP1B3 in cultured primary human hepatocytes. However, the effects varied considerably among individual cell isolates. Therefore, we conclude that, within the range of commonly ingested nutritional supplements, possible effects on OATP1B1 and OATP1B3 expression may not present major problems. However, in patients under therapy with OATP substrates (e.g. statins) consequences of interaction between these OATP transporters and anthocyanins taken at high doses in dietary supplements cannot be excluded.

Conflict of interest

The authors declare that they have no conflict of interest.
Acknowledgements

Our laboratories are supported by a grant GACR 303/12/G163 from the Grant Agency of the Czech Republic. This research was also supported by a “BioProMotion” Bioactivity and Metabolism grant from the University of Vienna, Austria.
**Table 1** Chemical structures of anthocyanins

![Chemical structure](image)

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<th>R4</th>
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**Anthocyanidins**

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Table 2 Effects of anthocyanidins on OATP1B1 and OATP1B3 mRNA levels in primary human hepatocytes treated for 24 h with tested compounds. Results are expressed as n-fold change compared to DMSO-treated cells (control). Data, normalized to the reference genes, are expressed as mean ± SD (n=3) for HEP220670 and for LH45, LH46, LH47 as means (min-max values), calculated from two separate determinations. Values in bold are significantly different (p<0.05) as assessed with the Mann-Whitney U test.

<table>
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<th>LH45, LH46 and LH47</th>
<th>HEP220670</th>
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<tr>
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<td>OATP1B1</td>
<td>OATP1B3</td>
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<tr>
<td>Control</td>
<td>1.151 (0.77-1.62)</td>
<td>1.006 (1.00-1.01)</td>
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<td>Cyanidin</td>
<td>1.027 (0.90-1.29)</td>
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<td>0.916 (0.54-1.39)</td>
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<td>Malvidin</td>
<td>0.991 (0.66-1.36)</td>
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<td>Peonidin</td>
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<td>Petunidin</td>
<td>0.947 (0.67-1.10)</td>
<td>1.189 (0.70-1.97)</td>
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Table 3
Effects of anthocyanins on the levels of OATP1B1 and OATP1B3 mRNA in primary human hepatocytes LH45, LH46, LH47 and LH49 treated for 24 h with the indicated compounds. Results are expressed as n-fold changes compared to vehicle-controls. Data are mean ± SD from triplicate measurements.
Values in bold are significantly different (p<0.05), determined by Mann-Whitney U test.

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<th>Changes in mRNA expression</th>
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<td>1.193 (0.91-1.56)</td>
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<td>1.066 (0.92-1.20)</td>
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<td>Pelargonin chloride</td>
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References


Chemical structures of the anthocyanins malvin chloride, malvidin-3-O-galactoside chloride and cyanidin-3-O-sophoroside chloride.
339x331mm (150 x 150 DPI)
Heat map showing mRNA expression levels for OATP1B1 and OATP1B3 in LH45, LH46, LH47 and LH49 human hepatocyte cultures. The image map (Java TreeView 1.9) shows the pattern of OATP gene expression as determined by TaqMan RT-PCR. Data shows upregulation (red) or downregulation (blue) of OATP mRNA after incubation with anthocyanins, compared to the median expression level (black) of DMSO-treated hepatocyte cultures.

227x136mm (150 x 150 DPI)
Effects of selected anthocyanins on the expression of OATP1B1 and OATP1B3 proteins in human hepatocytes. Primary human hepatocyte cultures (LH45, LH46, LH47, LH49) were incubated for 24 h with pelargonin chloride (50 μM), pelargonidin-3-O-rutinoside chloride (50 μM), malvidin chloride (50 μM), malvidin-3-O-galactoside chloride (50 μM) and DMSO (0.1% v/v) as a vehicle control. Western blots show analyses of OATP1B1 and OATP1B3 proteins in pooled samples LH45-49.
8 Curriculum Vitae

Personal data

Name: Juliane Riha
Date of birth: 17.01.1984
Place of birth: St. Pölten, Austria
Nationality: Austria

Education

1990-1994: Primary School, Nußdorf ob der Traisen
1994-2002: Grammar School, Krems
07.06.2002: Final exam
10/2002-03/2007: Studies of pharmacy, University of Vienna
09/2006-03/2007: Diploma thesis at the Department of Pharmaceutical Technology, University of Vienna. Title: “Saures Polysaccharid aus Hyptis suaveolens: Präbiotikum und protektives Hydrogel”
27.03.2007: Graduation - Academic degree: Mag. pharm.
Since 10/2009: PhD studies at the Department of Clinical Pharmacy and Diagnostics, University of Vienna (Advisor: Prof. Dr. Walter Jäger)
Professional experience

2007: Tutor for undergraduate students of pharmacy
("Grundpraktikum Pharmazeutische Technologie") at
the Department of Pharmaceutical Technology, University of Vienna

04/2007-04/2008: Practical training year in a pharmacy

21.03.2008: Final examination - “Fachprüfung für den Apotheker-
beruf”

Since 2008: Partial employment in a pharmacy, Apotheke Triller-
park, 1210 Wien

05/2010-12/2011: University assistant ("Prae doc") at the Department of
Clinical Pharmacy and Diagnostics, University of Vienna

Since 2010: Tutor for undergraduate students of “FH-
Studienlehrgang Biotechnologie” ("Praktikum zur
allgemeinen, anorganischen und analytischen
Chemie") at the Department of Clinical Pharmacy and
Diagnostics, University of Vienna

Since 2010: Lecturer for undergraduate students of pharmacy
("Qualitative pharmazeutische Analytik") at the De-
partment of Clinical Pharmacy and Diagnostics, Uni-
versity of Vienna
9 List of Publications

9.1 Authorship in Peer-reviewed Journals


Stefan Brenner, Lukas Klameth, Juliane Riha, Madeleine Schölm, Gerhard Hamilton, Erika Bajna, Christoph Ausch, Angelika Reiner, Walter Jäger, Theresia Thalhammer,


9.2 Poster Presentations

2010
Differences in mRNA and protein expression of Organic Anion Transporting polypeptides (OATPs) in malignant and non-malignant human liver samples. 9th International ISSX Meeting, September 4-8, 2010, Istanbul, Turkey

2012
Resveratrol and its sulphated conjugates are substrates of Organic Anion Transporting polypeptides (OATPs): Impact on resveratrol disposition. 12th European Regional ISSX Meeting, June 17-21, 2012, Noordwijk aan Zee, The Netherlands

2013
Expression of Organic Anion-Transporting Polypeptides (OATP) 2A1, 4A1, 5A1 and 6A1 as possible biomarkers for human ovarian cancer. 10th International ISSX Meeting, September 29–October 3, 2013, Toronto, Canada