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# DISSERTATION

Titel der Dissertation

Altered expression of organic anion transporting polypeptides in human solid tumors: relevance for anticancer therapy

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Nihil fit sine causa

**FOR ANDREA**

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# TABLE OF CONTENTS

# ABBREVIATIONS

| | |
|---|---|
| ACE | Angiotensin converting enzyme |
| AhR | Aryl-hydrocarbon receptor |
| ARS | Arylsulfatase |
| cAMP | Cyclic adenosinmonophosphate |
| CAR | Constitutive androstane receptor |
| cDNA | Complementary desoxyribonucleic acid |
| DNP-SG | S-(2,4-dintriphenyl)-glutathione |
| FXR | Farnesoid X receptor |
| HCC | Hepatocellular carcinoma |
| HGF | Hepatocyte growth factor |
| HMG-CoA | 3-Hydroxy-3-methylglutaryl-coenzyme A |
| HNF | Hepatocyte nuclear factors |
| IL | Interleukin |
| $K_m$ | Michaelis Menten constant |
| LXR | Liver X receptor |
| mRNA | Messenger RNA |
| Ntcp | $Na^+$-dependent taurocholate cotransporting polypeptide |
| OATP | Organic anion transporting polypeptide |
| PCR | Polymerase chain reaction |
| PepT | Peptide transporter |
| PXR | Pregnane receptor |
| RNA | Ribonucleic acid |
| SLC | Solute linked carrier |
| Stat | Signal transducers and activators of transcription |
| STS | Steroidsulfatase |
| SULT | Sulfotransferase |
| $V_{max}$ | Maximal velocity |
| 17ß-HSD | 17β-Hydroxysteroid-dehydrogenas |

# 1 SUMMARY

Organic anion transporting polypeptides (OATPs) have been identified as $Na^+$-independent transport system for bile acids in rat and human liver. Until their discovery in the 1990s members of this transporter family were found to be present in various species, including mouse, rat, cow, horse, and quail. In humans, eleven OATP family members are known so far, which are classified based on their amino acid sequence homology into families and subfamilies. The human OATP-family occurs in a variety of different tissues, including liver, kidney, intestine, and brain. While some of them are ubiquitously expressed, others show a rather narrow expression pattern, like the OATP1B family members, OATP1B1 and 1B3, which are exclusively present in the basolateral membrane of hepatocytes in human liver, and OATP4C1 which has only been identified in the kidney. OATPs have been described to mediate the transport of endogenous compounds as well as xenobiotics, although only the members of the OATP1 family and OATP2B1 are one of the best-characterized OATPs concerning their substrate specificity. Until now no systematic studies are available, characterizing the substrate specificity of all known human OATPs. Furthermore, the requirements for molecules transported via the OATP family are not totally clarified. Presently, OATP substrates are defined as anionic amphipathic organic compounds with a high molecular weight (>450 Da), although neutral and cationic compounds have also been identified as OATP substrates. Bile acids and steroid/ thyroid hormones including their conjugates are typical endogenous OATP substrates. In addition, several clinically used drugs have been characterized as OATP substrates, including antibiotics, statins, and antihypertensives. Based on their localization and their function as uptake transporters OATPs play a critical role in the absorption, disposition and excretion of xenobiotics. Although some anticancer agents have been reported to be mediated via OATPs, the knowledge on the role of these transporters in malignancies is limited.

Therefore, the aim of this thesis was to identify the expression pattern of the human OATP family in selected cancer diseases. First the occurrence of OATP family members in breast cancer tissues was investigated. Real-time PCR analysis was performed for tissue samples derived from 13 patients, showing mRNA expression of six OATPs in this malignancy. Adjacent non-malignant samples were chosen as normal control, as it is known that expression pattern of transporter proteins show high interindividual variability as result of genetic, environmental and physiological factors.

Indeed, in the majority of investigated specimens clear differences were observed in the mRNA expression comparing malignant and non-malignant specimens, with higher mRNA levels in the non-malignant control samples. In addition, interindividual alterations in OATP expression were detected, indicating a possible role of these transporters in altered uptake rates of anticancer agents into tumor cells. OATPs in breast cancer may not only be important for the uptake of anticancer drugs into tumor cells, but also for the uptake of estrone-3-sulfate. This is of special interest as sulfatases in estrogen-dependent breast tumors are able to convert inactive estrone-3-sulfate into active estrogen. Contrary, the family of sulfotransferases (SULTs) can convert estrogen back into the inactive sulfated form. Therefore, the presence of three sulfatases (STS, ARS-B, ARS-A) and four sulfotransferases (SULT1A1, 1A2, 1A3, 1E1) was investigated in breast cancer tissue samples and their adjacent non-malignant specimens by real-time PCR analysis. Similar mRNA expression levels were revealed for the sulfotransferase family members SULT1A2, 1A3, and 1E1 comparing tumorous and non-malignant tissue samples. However, SULT1A1 expression was below the detection limit in all samples. Regarding mRNA expression of investigated sulfatases, steroid sulfatase (STS) showed higher mRNA levels in non-malignant specimens than in tumor tissue samples. Neither for arylsulfatases ARS-A nor for ARS-B, any differences in mRNA expression between malignant and non-malignant samples could be detected.

Paclitaxel, an anticancer drug used in the treatment of metastatic breast cancer, has been investigated as possible substrate for the liver-specific OATP1B1 and 1B3, identifying OATP1B3 as high affinity transporter for paclitaxel. Regarding our mRNA expression analysis in breast cancer tissues, this OATP family member could not be detected in the investigated specimens. However, as OATPs are known to exhibit overlapping substrate specificity, an involvement of other OATP family members in the transport of paclitaxel was suggested. Therefore, the present work initiated the first systematic characterization of all known human OATPs for their ability to transport paclitaxel. Thus, full length cRNAs for all eleven OATP family members were generated and injected into *Xenopus laevis* oocytes. OATP expressing oocytes were then incubated with radiolabeled paclitaxel for an initial time of 60 min, showing paclitaxel transport activity for OATP1B1 and 1B3. For all other nine OATP family members paclitaxel exhibited no substrate activity. Kinetic analysis of OATP1B1 and 1B3 mediated paclitaxel transport revealed $K_m$ values of 0.6 µM and 1.6 µM, respectively, identifying OATP1B1 as additional high affinity paclitaxel transporter.

Beside paclitaxel, methotrexate has been previously identified as substrate for OATP1A2. As this agent is used in the chemotherapy regimen of osteosarcomas and OATPs have not been investigated in this disease until now, the present thesis further show investigations on OATP distribution in bone tumors. Bone tumor specimens from 21 patients as well as cell lines derived from osteosarcomas (HOS and MG-63), normal human osteoblasts outgrowth cells (hOB) and bone marrow stromal cells (BMSC) were examined concerning mRNA expression pattern of all eleven human OATP family members by real-time PCR analysis. With the exception of the liver-specific OATP1B1 and 1B3 and the testes specific OATP6A1, all other members of this transporter family could be detected in the specimens investigated. However, the cell lines only exhibited detectable mRNA levels of OATP3A1 and 4A1.

The liver is known to play a key role in metabolism and elimination. The identification and localization of OATPs in the basolateral membrane of hepatocytes and their ability to mediate a broad spectrum of endogenous and exogenous compounds allows a more detailed insight and understanding for the physiologic function of the human liver but also for the occurrence of side effects in therapies. Although the role of OATPs in human liver is well studied, the knowledge of their expression pattern in liver malignancies is limited or focused on the expression of OATP1B3 and 1B1 in hepatocellular carcinoma. Therefore, the presence of all known OATP family members in liver malignancies of 22 patients was examined using real-time PCR. To clarify possible changes in mRNA expression between normal controls and tumors, again the adjacent non-malignant tissues of the same patients were included in real-time PCR analysis. With the exception of OATP6A1 and 1C1, which were only present in a minority of specimens, all other OATPs showed detectable mRNA expression. Interestingly, OATP1B1, 1B3, 2B1, 1A2, and 4C1 showed decreased mRNA levels in the malignant samples compared to the adjacent non-malignant controls. Otherwise, mRNA of OATP2A1, 3A1, 4A1, and 5A1 were increased in the malignant samples. Beside hepatocellular carcinomas, cholangiocarcinomas and liver metastases derived from tumors of other organs are also frequent malignant diseases. Therefore, the specimens investigated derived from these different types of liver malignancies. Indeed differences in mRNA expression pattern of present OATPs between hepatocellular-, cholangiocarcinomas and liver metastases were observed.

Summarized, various OATPs were identified to be present in bone, breast, and liver malignancies, showing interindividual mRNA expression differences and altered

expression pattern compared to non-malignant controls. These findings indicate a possible role of this transporter family in response rate variation of anticancer therapy when using drugs which are OATP substrates.

# 2 ZUSAMMENFASSUNG

Organische Anionen transportierende Polypeptide (OATPs) wurden als Natrium-unabhängige Transportsysteme für Gallensäuren in der Ratten- und Menschenleber identifiziert. Seit ihrer Entdeckung in den 90er Jahren wurde ihr Vorkommen in den verschiedensten Spezies nachgewiesen. Im Menschen konnten bisher elf verschiedene OATPs identifiziert werden, die nach ihrer Homologie in der Aminosäuresequenz in Familien und Unterfamilien eingeteilt werden und bisher in einer Vielzahl von Geweben nachgewiesen werden konnten. Während manche dieser OATPs in unterschiedlichen Geweben vertreten sind, zeigen andere, wie etwa OATP1B1 und 1B3 die bis dato nur in der Leber identifiziert wurden, Gewebsspezifität. OATP4C1 hingegen konnte nur in der Niere nachgewiesen werden. Die Familie der OATPs ist für die Aufnahme von endogenen als auch exogenen Substanzen in die Zelle verantwortlich. Bis heute wurden jedoch nur einige wenige Mitglieder dieser Transporterfamilie hinsichtlich ihrer Substratspezifität charakterisiert. Weiters sind die Voraussetzungen, welche eine Substanz erfüllen muss um von OATPs als Substrat transportiert zu werden, noch nicht vollständig geklärt. Bisherige Untersuchungen haben gezeigt, dass amphipathische, organische Anionen mit hohem Molekulargewicht (>450 Da) aber auch neutrale sowie kationische Substanzen Substrate von OATPs sind. Als typische Substrate wurden Gallensäuren, Steroid-/ Thyroidhormone sowie deren Konjugate identifiziert. Zu den Arzneistoffen, die als OATP Substrate gelten, gehören Vertreter der Antibiotika, Cholesterinsenker oder Antihypertensiva. Basierend auf ihrer Lokalisation im menschlichen Körper und ihrer Funktion als Aufnahmetransporter spielen OATPs eine wichtige Rolle in der Absorption, Disposition und Elimination von exogenen Substanzen. Obwohl schon bekannt ist, dass auch Zytostatika zu den OATP Substraten zählen, ist das Wissen über deren Rolle in malignen Tumoren limitiert. Aus diesem Grund war es Ziel dieser Dissertation, die Expression der OATP Familie in ausgesuchten Tumorarten zu untersuchen. Erst wurde mittels real-time PCR das Vorkommen aller elf im Menschen identifizierten OATPs in Brustkrebsgewebeproben von 13 Patientinnen untersucht. Sechs der elf bekannten OATPs konnten auf mRNA Ebene in diesen Proben nachgewiesen werden. Weiters sollte festgestellt werden, ob ein Unterschied in der mRNA Expressionsrate zwischen Normal- und Tumorgewebe besteht. Dazu wurde zu jeder der untersuchten Tumorproben das Normalgewebe derselben Patientin, welches aus dem sogenannten Resektionsrand entnommen wurde, als Kontrolle

untersucht. Es ist von großer Bedeutung, eine Probe derselben Patientin als Kontrollgewebe zur Verfügung zu haben, da das Expressionsmuster von Proteinen durch genetische, physiologische und umweltbedingte Faktoren interindividuell sehr unterschiedlich ausgeprägt sein kann. Wie erwartet konnte ein deutlicher Unterschied in der Expression der sechs präsenten OATPs zwischen malignem und normalem Gewebe festgestellt werden. In der Mehrzahl der untersuchten Tumorproben konnten höhere mRNA Raten verglichen mit dem Normalgewebe gezeigt werden. Weiters offenbarte sich auch eine große interindividuelle Schwankung in der Expression. Diese Ergebnisse weisen darauf hin, dass eine unterschiedliche Expression dieser Aufnahmetransporter für unterschiedliche Akkumulationsraten von Chemotherapeutika verantwortlich sein könnte. Weiters spielen OATPs eine wichtige Rolle bei der Aufnahme von Östron-3-sulfat, einem typischen Substrat dieser Transporterfamilie. Dies ist von besonderem Interesse, da für viele Brusttumore eine Östrogenabhängigkeit nachgewiesen wurde. Diese Tumore sind in der Lage, aufgenommenes Östron-3-sulfat mittels Sulfatasen in aktives Östrogen umzuwandeln. Hingegen können Enzyme der Familie der Sulfotransferasen aktives Östrogen wieder in die inaktive Ausgangsform Östron-3-sulfat konvertieren. Daher wurde weiters die mRNA Expression sowohl von Sulfatasen als auch Sulfotransferasen in malignen und korrespondierenden normalen Gewebeproben von Patientinnen mit Brustkrebs mittels real-time PCR untersucht. Drei der vier untersuchten Sulfotransferasen (SULT1A2, 1A3, 1E1) zeigten ähnliche mRNA Expressionsraten im Tumorgewebe verglichen mit dem Normalgewebe. SULT1A1 hingegen konnte weder im Normal- noch im Tumorgewebe nachgewiesen werden. Interessanterweise konnten für die Steroidsulfatase wesentlich höhere mRNA Expressionsraten im untersuchten Normalgewebe verglichen mit den Tumorproben gezeigt werden. Die beiden Arylsulfatasen A und B hingegen wiesen keinen Unterschied in ihren mRNA Expressionen zwischen malignen und nichtmalignen Proben auf.

Paclitaxel, ein in der Therapie von metastasierendem Brustkrebs häufig eingesetztes Zytostatikum, wurde hinsichtlich seiner Substrateigenschaften bisher nur für die leberspezifischen OATP Familienmitglieder- OATP1B1 und 1B3- untersucht. Diese Studie charakterisierte Paclitaxel als Substrat für OATP1B3. In den Untersuchungen dieser Dissertation zeigte sich allerdings, dass OATP1B3 in den getesteten Brusttumorproben nicht vorkommt. Da aber eine überlappende Substratspezifität der einzelnen OATP Familienmitglieder bekannt ist, liegt es nahe, dass auch andere

Mitglieder der OATP Familie als Transportproteine für Paclitaxel fungieren könnten. Deshalb wurden erstmals die Transporteigenschaften aller im Menschen bekannten OATPs für Paclitaxel charakterisiert. Für diese Untersuchung wurden cRNAs aller elf OATPs generiert und in Oozyten des südafrikanischen Krallenfrosches (*Xenopus laevis)* injiziert. Erst wurde die Aufnahme von radioaktiv markiertem Paclitaxel in den OATP exprimierenden Oozyten nach einer Inkubation von 60 Minuten untersucht. Diese zeigte, dass neben OATP1B3 auch OATP1B1 eine deutliche Transportaktivität für Paclitaxel aufweist. Für die restlichen der elf OATPs konnte kein Paclitaxeltransport festgestellt werden. Der OATP1B1- und OATP1B3-vermittelte Paclitaxeltransport wurde ebenfalls hinsichtlich seiner Kinetik charakterisiert und beschrieb eine 2.5-fach höhere Affinität von Paclitaxel zu OATP1B1 ($K_m$ OATP1B1: 0.6 µM; Km OATP1B3: 1.6 µM).
Neben Paclitaxel wurde auch Methotrexat als Substrat für OATP1A2 in vorhergehenden Studien identifiziert. Da Methotrexat ein wichtiger Bestandteil der Standardtherapie von Knochentumoren ist, wurde in dieser Dissertation auch die Expression von OATPs in Osteosarkomaproben von 21 Patienten untersucht. In weiterer Folge wurden Osteosarkomazelllinien (HOS und MG-63) sowie nicht maligne Zelllinien (hOB und BMSC) in die real-time PCR Analysen miteinbezogen. Mit Ausnahme der leberspezifischen Transporter OATP1B1 und 1B3 und des testisspezifischen OATP6A1, konnten alle anderen der elf bekannten OATPs in den Osteosarkomaproben nachgewiesen werden. In den Zelllinien hingegen war eine mRNA Expression nur für OATP3A1 und 4A1 nachweisbar.
Die Leber spielt eine wichtige Rolle in der Metabolisierung und Eliminierung endogener wie exogener Substanzen. Die Identifizierung und Lokalisierung von OATPs in der basolateralen Membran der Hepatozyten und deren Funktion als Aufnahmetransporter für eine Vielzahl von endo- und exogenen Stoffen ermöglicht uns ein besseres Verständnis der physiologischen Funktion dieses Organs aber auch der Ursachen von Interaktionen oder toxischen Nebenwirkungen von Arzneistoffen. Obwohl die Rolle der OATPs in der normalen, gesunden Leber gut untersucht ist, ist ihre Bedeutung in malignen Erkrankungen dieses Organs noch nicht vollständig geklärt beziehungsweise konzentrierten sich bisherige Untersuchungen auf die Expression der beiden leberspezifischen OATPs, OATP1B1 und 1B3, vor allem im hepatozellulärem Karzinom. Aus diesem Grund wurden abermals real-time PCR Analysen in Lebertumorgewebeproben von 22 Patienten durchgeführt und das Vorkommen der mRNA aller humanen OATPs untersucht. Auch bei dieser Studie wurden die nicht

malignen Gewebeproben derselben Patienten als Kontrolle in die mRNA Expressionsanalyse miteinbezogen. Mit der Ausnahme von zwei OATP-Familienmitgliedern- OATP6A1 und 4C1- konnte die mRNA aller im Menschen bisher bekannten OATPs nachgewiesen werden. Interessant war, dass fünf dieser OATPs (OATP1B1, 1B3, 2B1, 1A2, 4C1) geringere mRNA Expressionsraten in den malignen Gewebeproben, verglichen mit den jeweiligen Kontrollen, zeigten. Hingegen wurde für die anderen vier OATP Familienmitglieder eine erhöhte mRNA Expression in den malignen Gewebeproben festgestellt. Da neben dem hepatozellulären Karzinom sowohl Cholangiokarzinome als auch Lebermetastasen anderer Tumore weitverbreitete Erkrankungen darstellen, deckten die in dieser Arbeit untersuchten Gewebeproben alle drei Arten maligner Erkrankungen der Leber ab. Dadurch konnten auch mögliche Unterschiede im Expressionsmuster zwischen diesen verschiedenen Tumorarten untersucht und festgestellt werden.

Zusammenfassend konnte in der vorliegenden Arbeit das Vorhandensein von Mitgliedern der OATP Transporterfamilie in Tumorgeweben der Brust, des Knochens und der Leber nachgewiesen werden. Das Expressionsmuster auf mRNA Ebene zeigte nicht nur deutliche interindividuelle Unterschiede sondern auch veränderte Expressionsmuster in malignen Geweben. Diese Ergebnisse weisen auf eine wichtige Rolle der OATPs in Krebszellen hin, da unterschiedliche Expressionsraten ein Grund für interindividuell schwankende Ansprechraten auf Chemotherapien mit Zytostatika, die OATP Substrate sind, sein könnten.

# 3 INTRODUCTION

## 3.1 Organic Anion Transporting Polypeptides (OATPs)

### 3.1.1 History of discovery

In hepatocytes, transmembrane transport of organic anions, e.g. bile acids, bilirubin-glucuronide, and bromosulfophthalein has been shown to be mediated by $Na^+$-dependent and -independent transport systems.[1,2] One year after the characterization of the $Na^+$-dependent taurocholate cotransporting polypeptide (Ntcp) in mammalian liver in 1993,[3] the first member of a $Na^+$-independent transport system, the so-called Oatp family (Oatp1a1), could be identified after cloning of the transporter from a rat liver cDNA library. The transporter was characterized by size-fractioning and was expressed in *Xenopus laevis* oocytes for transport studies.[4] In 1995, the first human OATP family member, OATP1A2, was identified in human liver.[5] Since these early descriptions, genome-sequencing projects predict the existence of more than 160 OATPs/Oatps in about 25 animal species. However, only few of these putative transporters were structurally and functionally characterized until now.[6] In humans, eleven OATP family members have been identified so far.[7]

### 3.1.2 Classification and nomenclature

Since the discovery of the first OATP/Oatp family member, a number of OATPs/Oatps have been identified in humans and different animal species including rat, mouse,[8,9] cow[10], horse[11], and quail.[12] The identification of more and more OATP/Oatp family members by different groups, the use of various arbitrary names and the lack of consistent nomenclature resulted in confusion. Furthermore, the old classification system led to difficulties in the differentiation between OATPs/Oatps of different species, as sometimes the same name was accidently assigned for different family members in different species (rOatp1/ hOATP1 or rOatp2/mOatp2/hOATP2).[8]

To reach clarification, Hagenbuch and Meier (2004) established a species-independent and open-ended nomenclature, based on the classification system used for the cytochrome P450 family. This new nomenclature was approved by the HUGO Gene Nomenclature Committee (Table 1). Based on the amino acid sequences, the OATP/Oatp superfamily is divided into six families consisting of up to four subfamilies (Figure 1).The six OATP/Oatp families - OATP1, OATP2, OATP3, OATP4, OATP5, and

OATP6 - are designated by numbers from 1 to 6. Each family is characterized by an amino sequence homology of more than 40%. The members of the same subfamily show an amino acid sequence identity of more than 60%. Every subfamily is designated by a letter from A to D and followed by an additional numbering for each individual member. To facilitate differentiation between human and rodent members of this transporter family, the classification system provides designation of human OATP/SLCO proteins/genes by capitals (e.g. OATP1A2/SLCO1A2), while those of rat and mouse are given in small letters with an initial capital (e.g. Oatp1a1/Slco1a1). In this new nomenclature, the symbol “SLCO” is selected for gene classification, while the old protein symbol “OATP” is kept for the corresponding protein nomenclature.[8]

Table 1 New and old gene and protein nomenclature (modified from Hagenbuch and Meier 2004)[8]

| New Gene Name | Old Gene Name | New Protein Name | Old Protein Names |
|---|---|---|---|
| **SLCO1A2** | SLC21A3 | **OATP1A2** | OATP-A, OATP |
| **SLCO1B1** | SLC21A6 | **OATP1B1** | OATP-C, LST-1, OATP2 |
| **SLCO1B3** | SLC21A8 | **OATP1B3** | OATP8, LST-2 |
| **SLCO1C1** | SLC21A14 | **OATP1C1** | OATP-F, OATP-RP5 |
| **SLCO2A1** | SLC21A2 | **OATP2A1** | hPGT |
| **SLCO2B1** | SLC21A9 | **OATP2B1** | OATP-B, OATP-RP2 |
| **SLCO3A1** | SLC21A11 | **OATP3A1** | OATP-D, OATP-RP3 |
| **SLCO4A1** | SLC21A12 | **OATP4A1** | OATP-E, OATP-RP1 |
| **SLCO4C1** | SLC21A20 | **OATP4C1** | OATP-H |
| **SLCO5A1** | SLC21A15 | **OATP5A1** | OATP-J, OATP-RP4 |
| **SLCO6A1** | SLC21A19 | **OATP6A1** | OATP-I, GST |

In fact, all Oatps/OATPs together form a transporter gene superfamily, with homology between different species, resulting in a phylogenetic tree (Figure 1).[8] Regarding this phylogenetic tree and the distribution of the 36 identified OATPs/Oatps in human, rat and mouse, it can be recognized that some family members show a 1:1 relationship between different species like the OATP3 family. However, the OATP1A family exhibit only one member in humans but five in rats. Likewise the OATP1B family consists of two human members but have only one member in rat and mouse, respectively. This unequal distribution of OATP/Oatp family members in-between the different species may be due to gene duplication events after humans and rodents diverged.[13]

Although the official nomenclature provides the differentiation of genes and proteins by using the terms “SLCO/Slco” or “OATP/Oatp”, respectively, I will limit myself to the use of the term “OATP/Oatp” throughout this thesis to simplify understanding for people who are not so familiar with the classification system of the OATP family.

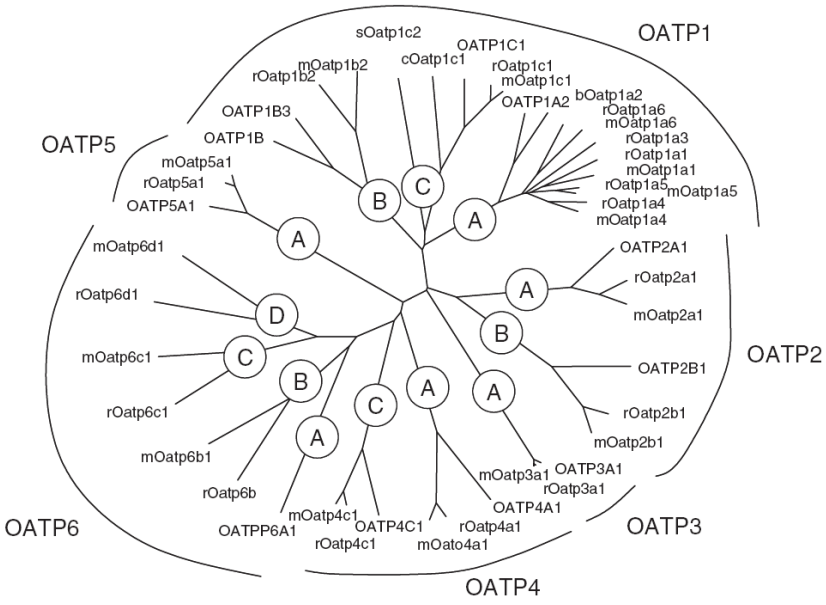


Figure 1 Phylogenetic tree of the OATP/Oatp superfamily in human and various animal species. b, bovine; c, chicken; h, human; m, mouse; r, rat; s, skate.[6]

### 3.1.3 Chromosomal location and tissue distribution

Table 2 Tissue distribution and chromosomal location of human OATPs.[14]

| OATP | Gene locus | Tissue distribution |
|---|---|---|
| OATP1A2 | 12p12 | Brain, kidney, liver, intestine, ciliary body |
| OATP1B1 | 12p12 | Liver |
| OATP1B3 | 12p12 | Liver |
| OATP1C1 | 12p12 | Brain, testes, ciliary body |
| OATP2A1 | 3q21 | Ubiquitous |
| OATP2B1 | 11q13 | Liver, placenta, intestine, heart, skin, pancreas, lung, colon, ovary, testes, kidney, spleen, skeletal muscle |
| OATP3A1 | 15q26 | Ubiquitous |
| OATP4A1 | 20q13.1 | Ubiquitous |
| OATP4C1 | 5q21 | Kidney |
| OATP5A1 | 8q13.1 | Heart, skeletal muscle, fetal brain[#] |
| OATP6A1 | 5q21 | Testis<br>Spleen, brain, fetal brain, placenta[#] |

[#], identified only on the mRNA level

As shown in Table 2, the location of OATP encoding genes is varying among the particular family members. While genes which are situated at the short arm of chromosome 12 are encoding the members of the OATP1 family, the genetic information for OATP4C1 and 6A1 proteins is located on chromosome 5. The genes encoding OATP2A1, 2B1, 3A1, and 5A1 are allocated on chromosome 3, 11, 15, and 8, respectively.[14]

While most of the OATP family members are expressed in a variety of different tissues, others of them are predominantly present in distinct organs.[8,15]

#### 3.1.3.1 OATP1A2

OATP1A2 was the first human OATP identified and cloned from human liver, where it has been shown to be located in cholangiocytes.[5,16] Beside its identification in human liver, this family member has been further detected in other tissues including brain, kidney, intestine, and ciliary body epithelium.[5,16,17,18,19] Investigations on the localization of OATP1A2 in tissue samples of the human brain, using either immunofluorescence or immunohistochemical staining, showed high expression of this transporter in microvessels and endothelial capillaries but no staining in neurons or astrocytes.[16,17] In human kidney, this OATP family member could be located in the apical domain of distal nephrons,[16] while in the intestine it is expressed in the apical brush-border membrane of intestinal enterocytes.[18] Regarding its location in enterocytes in more detail, OATP1A2 is situated along the whole crypt–villus axis showing the highest protein expression on the villus tip.[18] In human ocular tissues OATP1A2 was found to be present in the ciliary body epithelium were it is located in the basolateral membrane of the inner non-pigmented epithelium.[20]

#### 3.1.3.2 OATP1B1 and 1B3

Both members of the OATP1B subfamily, OATP1B1 and 1B3, were originally cloned from human liver, where they are found to be exclusively expressed under normal physiological conditions.[21,22,23,24,25] Therefore, these OATP family members are designated “liver-specific”. Both transporters are located in the basolateral membrane of human hepatocytes. Nevertheless, they differ in their exact localization. While OATP1B1 is situated in the basolateral membrane of hepatocytes throughout the liver lobe, the expression of OATP1B3 is more restricted around the central vein.[22,23,24]

#### 3.1.3.3 OATP1C1

OATP1C1 was originally cloned from human brain and later it was also identified in human testes and the ciliary body of the human eye.[20,26] In human brain, the expression of OATP1C1 was only investigated on mRNA level by Northern blot analysis using a multiple tissue expression array (Clontech Laboratories, Mountain View, CA), showing the presence of this transporter in a variety of brain regions, excepting in cerebellum and pons.[26] In human testis, however, OATP1C1 localization was investigated by immunohistochemical staining, showing its expression in the nests of Leydig cells.[26]

#### 3.1.3.4 OATP2A1

OATP2A1 was first cloned from human kidney in 1996.[27] It was later identified in a variety of different tissues at mRNA level.[28] Until now the protein of this transporter has been verified in human ocular tissue, gastrointestinal tract, endometrium, and brain.[29,30,31,32] Studies focusing on the protein expression of OATP2A1 in human ocular tissues detected this OATP family member in the choroid/retinal pigment epithelium as well as in cornea, conjunctiva, iris, and the ciliary body.[29] In the upper gastrointestinal tract OATP2A1 could be localized in the antrum as well as in the corpus and duodenal mucosa, showing highest protein expression in the antrum.[30] Investigations on the protein localization in human endometrium revealed that OATP2A1 is situated in luminal and glandular epithelial and stromal cells. Interestingly, differences in protein expression in the endometrium were seen during the menstrual cycle, showing a higher OATP2A1 expression during the proliferative and early secretory phase compared to the mid and late secretory phase.[31] In human brain, OATP2A1 is localized in neurons, microglia, and astrocytes.[32]

#### 3.1.3.5 OATP2B1

The coding sequence of OATP2B1 was first predicted in 1998 as a result of a cDNA sequencing project using size fractioned human brain cDNA libraries.[33] Two years later it was first isolated from human brain.[27] Thereafter, OATP2B1 mRNA was detected in numerous tissues including pancreas, liver, lung, colon, small intestine, ovary, testis, heart, placenta, kidney, and spleen. But highest expression levels were observed in human liver.[27,34,35] Protein expression in human liver could be confined to the basolateral membrane of hepatocytes.[34]

Various data are available focusing on the expression of the OATP2B1 protein in different tissues, illustrating that this transporter is widely distributed in the human body. In the small intestine, OATP2B1 expression is restricted to the apical surface of enterocytes but is not expressed in goblet cells.[36]

In human placenta, OATP2B1 was found to be located in the basal membrane of the syncytiotrophoblast.[37] A closer examination of its exact localization in human placenta showed clear expression in membranes of the cytotrophoblast and, after differentiation, on the basal surface of the syncytiotrophoblast throughout first, second and third trimester placenta. Interestingly, a lower expression of OATP2B1 was seen in the proliferating cytotrophoblasts of the first trimester placenta when compared to the differentiated syncytiotrophoblast.[38]

OATP2B1 was also identified in the mammary gland, where it is located to the contractile myoepithelial cells surrounding the ducts.[39] Furthermore, this OATP family member could be detected in the human skeletal muscle,[40] ocular tissue,[20,29] and keratinocytes.[41]

In human heart, it is located in the endothelium of capillaries, arteries, and veins.[42] Recent studies further identified OATP2B1 in human platelets and in megakaryocytes.[43]

#### 3.1.3.6 OATP3A1

For OATP3A1, two variants have been identified and designated OATP3A1_v1 and OATP3A1_v2. OATP3A1_v1, first isolated from human kidney in 2000, represents the wild-type form,[27] while OATP3A1_v2 is a splice variant and was first isolated from human brain (2007). The variant 2 is characterized by an additional splice donor site in exon 10, resulting in the lack of 18 amino acids at the COOH-terminal end.[44]

OATP3A1_v1 is known to be ubiquitously expressed in humans and mammalians.[20,27,40,41,44,45,46,47,48,49] Comparing the frequency of the two splice variants in the human body, OATP3A1_v1 exhibit a wider tissue distribution with higher mRNA levels, whereas the variant 2 seems to be predominantly present in human brain and testes.[44] Additionally, differences have been also observed for the exact localization of OATP3A1 variants in these two organs. For example, in the choroid plexus of the human brain, OATP3A1_v1 is situated at the basolateral, while OATP3A1_v2 is rather expressed at the apical plasma membrane domain. Furthermore, in brain parenchyma, OATP3A1_v1 was only detected in neuroglial cells of the gray matter, while OATP3A1_v2 showed additional expression in the white matter.[44] However, in testis,

the two OATP3A1 splice variants were determined in different developmental stages of germ cells with a more restricted expression pattern for OATP3A1_v1. Interestingly, OATP3A1_v2 was the only variant of this transporter present in sertoli cells.[44]

#### 3.1.3.7 OATP4A1

OATP4A1 was first isolated from human kidney[27] and brain[50] by two independent groups in 2000 and 2001. Like OATP3A1, this OATP family member is known to be present in a variety of different tissues.[27,50] On the mRNA level it was identified in mammary epithelial cells,[49] placenta,[48,50,51] human epidermal keratinocytes,[41] mammary gland,[40] fetal heart,[52] adipose tissue,[53] human jejunum,[46] heart, lung, liver, skeletal muscle, kidney, and pancreas.[27,50] However, information available on the expression of this transporter at the protein level is rare and, until now, verified only in the placenta, where it is located on the apical side of the villous membranes of the syncytiotrophoblasts,[51,54] and the ciliary body epithelial of the human eye.[20]

#### 3.1.3.8 OATP4C1

OATP4C1 was originally isolated from human kidney in 2004. Screening for OATP4C1 mRNA expression in several human tissues revealed a restricted appearance in the adult as well as the fetal kidney. However, in fetal liver very low mRNA levels were seen.[55] Immunohistochemical investigations on protein expression of this transporter in kidney have only been performed for the rat ortholog Oatp4c1, showing significant staining in the basolateral membrane of proximal tubule cells.[55]

#### 3.1.3.9 OATP5A1

The open reading frame sequence of OATP5A1 has been identified by the Health Mammalian Gene Collection Program of the National Cancer Institute in 2001.[56] However, until now, data on the tissue distribution of OATP5A1 is very limited and only real-time RT-PCR analysis data in a panel of normal tissues have been published showing high mRNA expression in skeletal muscle, heart, and fetal brain. Lower expression was seen in thymus, stomach, prostate, and adult brain.[57]

#### 3.1.3.10 OATP6A1

OATP6A1 has been first isolated from human testis.[58] Faint mRNA expression levels of this OATP-family member were further seen in spleen, brain, fetal brain, and placenta.[57,59]

### 3.1.4 Physiological function

Based on the characterization of OATP family members concerning their tissue distribution and functional properties it can be assumed that OATPs are uptake systems for endogenous and exogenous compounds, especially in organs with barrier functions, including liver, kidney, and the blood-brain barrier. Therefore, they are suggested to be important determinants in substrate absorption, distribution and excretion. However, further studies are needed to clarify their exact physiological function as until now, the best characterized family members include the OATP1 family (OATP1A2, 1B1, 1B3) and OATP2B1.[6,7,15,60] Based on the expression of OATP1A2 at the apical membrane of cells in the distal nephron, this OATP family member is suggested to reabsorb compounds which are secreted or filtered in the proximale tubule.[16] (Figure 2B) Additionally, its presence in the blood-brain barrier may enable the uptake of drugs into the brain.[17] Contrary, OATP1B1, 1B3, and 2B1 are known to be present in the basolateral membrane of the liver. (Figure 2A) In this organ they facilitate the uptake of potentially substrates from the blood into hepatocytes, bringing them to the metabolizing system and enable their biotransformation into more water-soluble metabolites for the excretion into the bile.[60]

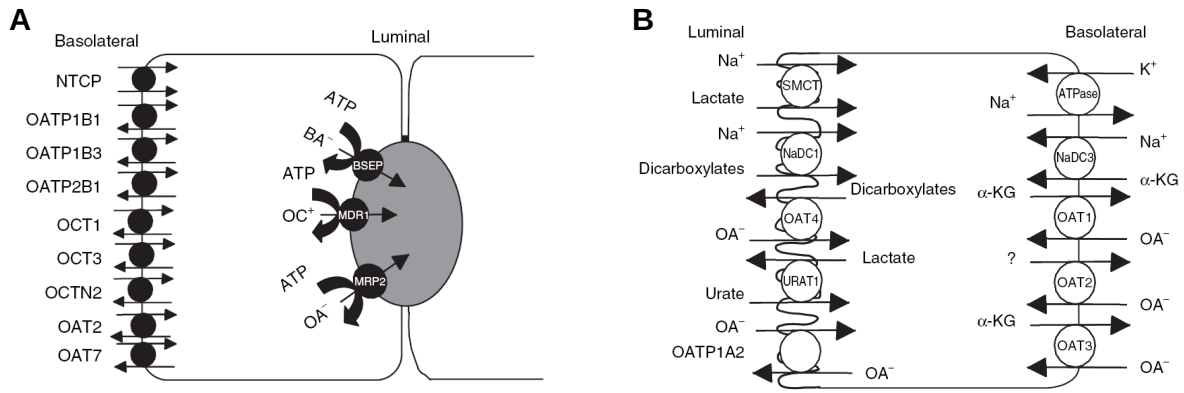


Figure 2 Characterized uptake systems in hepatocytes (A) and the proximal tubule (B). OATP, Organic anion transporting polypeptides; OCT, organic cation transporter; NTCP, $Na^+$/taurocholate cotransporting polypeptide; OAT, organic anion transporter; SMCT, sodium-monocarboxylate transporter; URAT, urate transporter; NaDC, sodium dependent dicarboxylate transporter.[60]

With respect to the localization of the two designated “liver-specific” OATPs, OATP1B1 and 1B3, in human hepatocytes it has been shown that OATP1B1 is present throughout the whole liver lobe, while 1B3 is restricted to the area surrounding the central vein. Therefore, OATP1B3 is assumed to function as a backup transporter for OATP1B1.[60] Although these OATPs are known to be present in several other tissues, their function there is still unknown. Furthermore, not all of the known human OATP family members

have been characterized concerning their tissue distribution and substrate specificity. Therefore, information on their specific role is lacking and requires additional investigations.

### 3.1.5 Substrates

While some of the OATP family members (e.g. OATP1A2, 1B1, 1B3) demonstrate a broad and overlapping substrate preference, others of them (e.g. OATP1C1, 2A1, 4C1) show a rather narrow substrate specificity. However, the substrate specificity of two family members, namely OATP5A1 and 6A1, is still unknown.[15] Although various publications summarize the substrate characteristics of several OATP family members,[6,7,15,60] no systematic studies are available investigating the chemical and structural requirements for OATP substrates. However, the existing knowledge concludes that anionic amphipathic organic compounds of high molecular weight beyond 450 Da are candidates for OATP mediated transport.[7] Especially molecules with steroidal structure components (e.g. bile salts, steroid hormones and their conjugates), but also linear (e.g. cholecystokinin-8, deltorphin II) and cyclic peptides (e.g. [D-Pen$^{2,5}$]-Enkephalin) which are normally bound to proteins (e.g. albumin) were characterized as OATP substrates.[7] (Table 3)

Furthermore, neutral compounds and even some cations (e.g. N-methylquinidine) have been described as OATP substrates.[8] Typical endogenous substrates include bile acids, steroid and thyroid hormones as well as their conjugates and eicosanoids.[6,7] The role of OATPs in the absorption, distribution and excretion of endogenous compounds aroused the interest in the investigation of their transport ability for xenobiotics. Thus, various drugs have been identified as OATP substrates so far, including HMG-CoA reductase inhibitors, antibiotics, ACE inhibitors, endothelin receptor antagonist, thrombin inhibitor, opioid receptor agonists, and angiotensin II antagonists.[7,15]

Table 3 Substrates of human OATPs.[6,7,15]

| | | |
|---|---|---|
| **OATP1A2** | endogenous | Bilirubin, cholate, dehydroepiandrosteron-3-sulfate, deltorphine II, [D-Pen$^{2,5}$]-enkephalin, estradiol-17β-glucuronide, estrone-3-sulfate, glycholate, prostaglandin $E_2$, taurocholate, taurochenodeoxycholate, tauroursodeoxycholate, thyroxin, triiodothyronine, reverse triiodothyronine |
| | exogenous | ADP-ajmalinium, atrasentan, bamet-R2, bamet-UD2, BQ123, bromosulfophthalein, chlorambuciltaurocholate, ciprofloxacin, CRC220, enoxacin, erythromycin, fexofenadine, gatifloxacin, Gd-B20790, imatinib, levofloxacin, lomefloxacin, methotrexate, microcystin-LR, N-Methyl-quinidine, N-methylquinin, norfloxacin, ouabain, pitavastatin, rocuronium, rosuvastatin, saqinavir, TR-14035, unoprostone |
| **OATP1B1** | endogenous | Bilirubin, bisglucuronosyl bilirubin, chenodeoxycholyl-(Nε-NBD)-lysine, cholate, dehydroepiandrosteron-3-sulfate, [D-Pen$^{2,5}$]-enkephalin, [D-Ala2,D-Leu5]-enkephalin, estradiol-17β-glucuronide, estrone-3-sulfate, glycholate, glycoursodeoxycholate, leukotriene $C_4$, leukotriene $E_4$, monoglucuronosyl bilirubin, prostaglandin $E_2$, taurocholate, tauroursodeoxycholate, thromboxane $B_2$, thyroxin, triiodothyronine |
| | exogenous | ACU154, arsenic (arsenite, arsenate), atorvastatin, atrasentan, bamet-R2, bamet-UD2, benzylpenicillin, bosentan, bosentan metabolite (Ro 48-5033), BQ123, bromosulfophthalein, capsofungin, cerivastatin, demethylphalloin, enalapril, ezetimib glucuronide, fexofenadine, fluvastatin, methotrexate, microcystein-LR, olmesartan, phalloidin, pitavastatin, pravastatin, repaglinide, rifampicin, rosuvastatin, SN-38, temocapril, TR-14035, troglitazone sulfate, valsartan |
| **OATP1B3** | endogenous | Bilirubin, chenodeoxycholyl-(Nε-NBD)-lysine, cholate, cholecystokinin-8, dehydroepiandrosteron-3-sulfate, deltorphin II, [D-Pen$^{2,5}$]-enkephalin, estradiol-17β-glucuronide, estrone-3-sulfate, glutathione, glycholate, glycoursodeoxycholate, leukotriene $C_4$, monoglucuronosyl bilirubin, taurochenodeoxycholate, taurocholate, taurodeoxycholate, tauroursodeoxycholate, thyroxin, triiodothyronine |
| | exogenous | Amanitin, atrasentan, bosentan, bosentan metabolite (Ro 48-5033), BQ123, bromosulfophthalein, demethylphalloin, digoxin, docetaxel, enalapril, erythromycin, fexofenadine, fluo-3, fluvastatin, imatinib, methotrexate, microcystin-LR, olmesartan, ouabain, paclitaxel, phalloidin, pitavastatin, pravastatin, rifampicin, rosuvastatin, SN-38, telmisartan, TR-14035, valsartan |
| **OATP1C1** | endogenous | Estradiol-17β-glucuronide, estrone-3-sulfate, thyroxin, triiodothyronine, reverse triiodothyronine |
| | exogenous | Bromosulfophthalein |

Table 3 continued

| | | |
|---|---|---|
| **OATP2B1** | endogenous | Dehydroepiandrosteron-3-sulfate, estrone-3-sulfate, pregnenolone sulfate, prostaglandin $E_2$, taurocholate |
| | exogenous | Atorvastatin, benzylpenicillin, bosentan, bromosulfophthalein, CP-671305, fexofenadine, fluvastatin, glibenclamide, M17055, pitavastatin, pravastatin, rosuvastatin, unoprostone |
| **OATP3A1_v1** | endogenous | Deltorphin, estrone-3-sulfate, prostaglandin $E_1$, prostaglandin $E_2$, prostaglandin $F_{2\alpha}$, thyroxin, vasopressin |
| | exogenous | Benzylpenicillin, BQ123 |
| **OATP3A1_v2** | endogenous | Arachidonic acid, estrone-3-sulfate, prostaglandin $E_1$, prostaglandin $E_2$, thyroxin, vasopressin |
| | exogenous | Benzylpenicillin |
| **OATP4A1** | endogenous | cAMP, estradiol-17β-glucuronide, estrone-2-sulfate, prostaglandin $E_2$, reverse triiodothyronine, taurocholate, thyroxin, triiodothyronine |
| | exogenous | Benzylpenicillin, digoxin, methotrexate, ouabain, unoprostone |
| **OATP4C1** | endogenous | Thyroxin, triiodothyronine |
| | exogenous | Digoxin, methotrexate, ouabain, sitagliptin |
| **OATP5A1** | | Not verified |
| **OATP6A1** | | Not verified |

BQ123, endothelin receptor antagonist; CRC220, thrombin inhibitor; TR-14035, integrin dual antagonist; ACU154, metabolite of PKI166 a tyrosin kinase inhibitor; M17055, diuretic candidate; CP-671305, phosphodiesterase 4 inhibitor

A possible explanation for the multispecificity of some OATP family members is the existence of multiple binding sites, which allows the recognition of such a variation of structurally different compounds as substrates.[6] For OATP1B1 a high- and a low-affinity binding site has been identified for estrone-3-sulfate transport, resulting in biphasic kinetics of this substrate.[61] Different binding sites have been further demonstrated for OATP1B3, OATP2B1, and 4C1 in inhibition/ stimulation studies.[9,62,63] Although progesterone is not mediated by OATP2B1, it shows a stimulatory effect on estrone-3-sulfate transport increasing the $K_m$ value about four fold. Contrary, higher concentrations of progesterone decreased the affinity of estrone-3-sulfate again. These findings indicate the existence of a high and a low affinity binding site for progesterone, causing a stimulative or inhibitory effect on estrone-3-sulfate transport, respectively.[63] Similar observations have been described for OATP4C1, which mediated the uptake of

digoxin and triiodothyronine. But when coadministered these two substrates, no inhibition could be observed, supporting the hypothesis of different binding sites for these substrates.[9] Furthermore, depending on the OATP1B3 mediated substrate, clotrimazol showed an inhibitory or stimulatory effect indicating an interaction of the substrates with different binding sites.[62]

### 3.1.6 Molecular structure

OATPs are transmembrane transporter proteins encoded by genes consisting of 643-722 amino acids.[7] Computer based hydropathy calculations predict a 12 transmembrane general molecular structure (Figure 3) for mammalian OATPs/Oatps, but no experimental data are available to verify these predictions.[6]

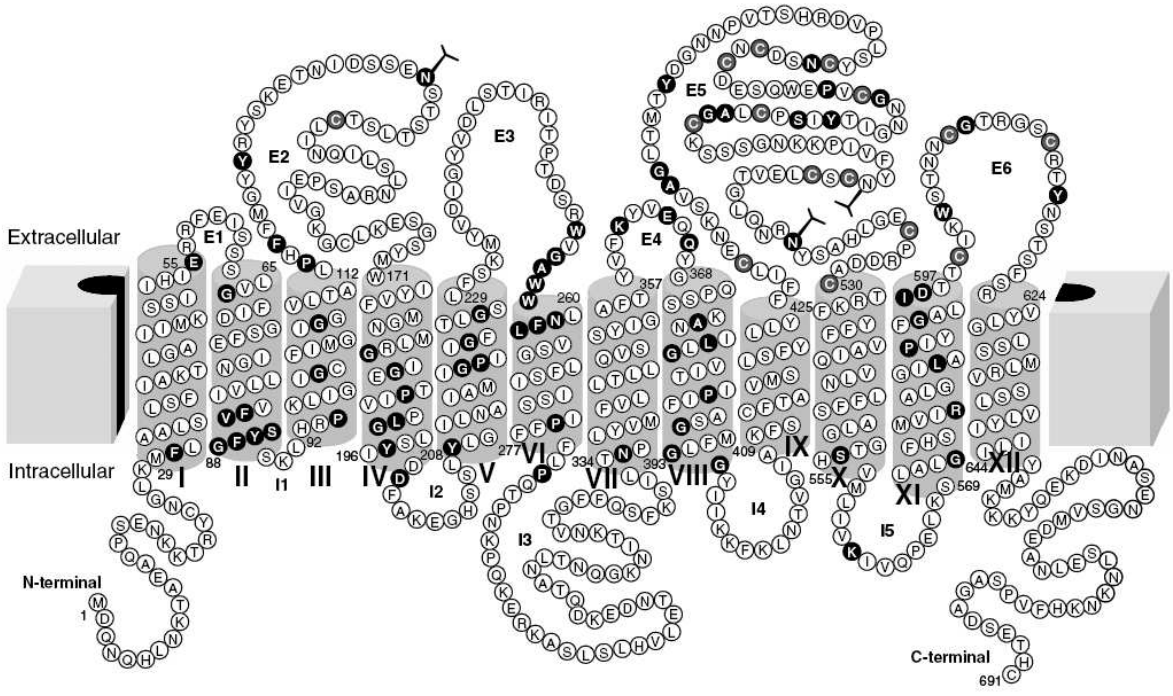


Figure 3 Model of human OATP1B1. Black dots indicate amino acids which are conserved in 77 out of 97 mammalian OATPs/Oatps, while greys mark cysteine residues. In extracellular loops 2 and 5 (E2, E5) three N-glycosilation sites are marked as Y.[6]

Regarding the calculated model, the large extracellular loop 5 (E5) between the transmembrane domains IX and X with ten cysteine residues is one of the predicted structural characteristic of the OATP/Oatp family.[7]

Further common features of the OATP molecular structures are several assumed N-glycosylation sites in extracellular loops E2 and E5 and the “superfamily signature” overlapping the border between extracellular loop E3 and the transmembrane domain

VI. This signature is formed by the highly conserved consensus sequence D-X-RW-(I,V)-GAWW-XG-(F,L)-L (X, any amino acid) including three tryptophane residues and which is used for the identification of OATP/Oatp family members in other species by database searching.[8]

### 3.1.7 Transcriptional Regulation

The exact regulation mechanism of OATP expression is unknown, but evidences for possible regulation pathways are given in some studies. Some OATPs have been identified as targets for the hepatocyte growth factor (HGF), which is a ligand of the tyrosine kinase receptor c-met and has been shown to be involved in the regulation of cytochrome P450 expression.[64,65,66,67] The treatment of human hepatocytes with HGF resulted in a down-regulation of OATP1B1 and 2B1 expression in a dose-dependent manner.[68]

Other studies suggest the involvement of the proinflammatory cytokines IL-1β and IL-6 in the regulation of OATP expression as they cause decreasing expression of some OATPs.[69,70]

The activation of proteinkinase C may be another possible regulation mechanism, because its activation resulted in the phosphorylation of OATP2B1 and subsequently in a reduction of transport activity which may be due to OATP2B1 internalization.[71]

Nuclear receptors like LXR (liver X receptor), FXR (farnesoid receptor) and PXR (pregnane receptor) as well as other xenobiotic receptors including Ahr (aryl-hydrocarbon receptor) and CAR (constitutive androstane receptor) are further hypothesized to be involved in the regulation of altered OATP expression.[72,73,74,75,76,77]

In addition it has been reported that prolactin and growth hormone induced OATP1B3 expression via stat5 (the signal transducers and activators of transcription 5), as mutated promoter regions encoding the stat5 binding site showed no hormonal induction of OATP1B3 expression.[78] Regarding the binding sites for transcription factors, characterization of the promoter region is available for the OATP1A2, 1B1, and 4C1 family members describing the existence of recognition sites for ubiquitous (activator protein AP1 and AP3, octamer binding site Oct-1, transcription initiation factor TFIID, CAAT element, nuclear factor NF-1) as well as liver-enriched transcription factors (hepatocyte nuclear factors HNF1, HNF3, HNF4, HNF5, CCAAT-enhancer binding protein CEBP-β).[79,80,81] Regarding the liver-specific presence of OATP1B1, HNF1α is suggested to play an essential role in hepatocyte-specific expression.[80]

### 3.1.8 Transport mechanism

Little is known about the exact transport mechanism of OATPs/Oatps. There are some studies dealing with potential driving forces of OATP/Oatp mediated transport, but no systematic investigations are available for all known OATP/Oatp members. While some examinations were performed for human OATPs, others especially focused on the family members in rats. But based on the amino acid homology (31%-82%)[7] and common structural features of mammalian OATPs/Oatps, it can be suggested that they exhibit similar transport mechanisms. Available studies on the transport mode of the OATP/Oatp family examined on the one hand side the influence of extra- and intracellular conditions including pH-changes and ion-gradients on transport function. On the other side, they investigate the importance of structural features on the transport activity of OATPs/Oatps as they show conserved structural identities (see 3.1.6.).

#### 3.1.8.1 Influence of extra- and intracellular conditions on transport function

*Ion-gradients*

The OATP/Oatp superfamily has been identified as a $Na^+$-independent transport system for bile acids in the liver. More detailed investigations on the transport mode of this family showed that the transport mediated by Oatps/OATPs is furthermore independent of $K^+$ and $Cl^-$ gradients[5,4,82,83,84] and the replacement of $Na^+$ with other cations like $K^+$, $Li^+$, $Rb^+$, choline or N-methyl-D-glucamine had only little effect on the transport.[46]

*ATP levels and membrane potential*

OATP mediated transport has been reported to be ATP-independent.[85] Furthermore, membrane potential seems not to be a driving force for the transport activity of Oatp/OATP family members as depolarization did not effect OATP1B1/ 1B3 mediated estrone-3-sulfate transport.[86]

*pH-sensitivity*

There is evidence for a pH-sensitive OATP mediated transport. With the exception of OATP2A1, 5A1, and 6A1, for all other OATPs, studies are available investigating the effect of the pH in the extracellular medium on OATP-mediated substrate transport. Although Mahagita et al. (2007) reported no effect of pH changes on OATP1B1 or 1B3 mediated estrone-3-sulfate uptake,[86] a stimulating effect of a more acidic pH (pH 5.0-

6.5) on the transport activity of OATPs expressed in *Xenopus laevis* oocytes was shown for OATP1A2, 1B1, 1B3, 2A1, 2B1, 3A1_v1, 3A1_v2, 4A1, and 4C1.[87,88,89]

Interestingly, OATP1C1-mediated transport of substrates like taurocholate, estrone-3-sulfate, prostaglandine $E_2$, and thyroxin was not affected by changes of the extracellular pH, leading to the hypothesis that the transport mechanism of this family member differs from that of the other OATPs.[87] Further experiments should clarify the effect of different pH values on the kinetic parameters of OATP1B1-, 1B3- and 1C1- mediated estrone-3-sulfate and thyroxin transport. Again, with the exception of OATP1C1 transport which left unaffected, a decrease of $K_m$ values at acidic extracellular pH was revealed. However, the $V_{max}$ values were not significantly changed, suggesting that lowering the pH results in a higher affinity of the substrate to the respective transporter.[87] To explain the observed different transport characteristics of OATP1C1, the amino acid sequence of OATP1C1 was compared with that of OATP1B1 and 1B3, which exhibited pH-sensitivity, showing the replacement of a highly conserved histidine residue by a glutamine residue in the transmembrane domain III, which faces the extracellular space.[87]

Remarkably, it has been shown that histidine residues are important for pH-sensitive transport mechanisms in other transporter families as well. This was shown for the peptide transporters PepT1 and PepT2,[90,91] folate transporters,[92] $Na^+/H^+$ exchanger[93,94] or the organic cation/$H^+$ antiporter.[95] As the pKa value for histidine in proteins is 6.9,[96] protonation of the imidazole ring and a positive charge is caused by shifting the extracellular pH to acidic conditions, and this could explain the importance of this amino acid for the pH-sensitivity of transporters. In case of OATP1C1, an exchange of the glutamine residue in position 130 by histidine led to a significant increase of the transport activity for the typical OATP1C1 substrate thyroxin at acidic pH (pH 6.5) compared to the transport activity at pH 8.0.[87]

In accordance to these findings and supporting the hypothesis that the histidine residue in the transmembrane domain III is important for pH-dependent stimulation of Oatp/OATP mediated transport, the rat Oatp1a1 mutant His107Gln showed no increased substrate uptake at lower pH value.[87]

*Exchange with bicarbonate and proton gradient*

The finding of a pH related transport activity further proposed an association of OATP-mediated transport either with an anion ($HCO_3^-$ or $OH^-$) exchange or an $H^+$ cotransport.

A possible involvement of bicarbonate exchange in the transport mechanism was studied for the uptake of the typical substrate estrone-3-sulfate in OATP1B3 or 2B1 transfected CHO cells. Cells were preloaded with $HCO_3^-$ and intracellular pH was measured after the addition of the substrate to the extracellular medium, showing an higher intracellular acidification rate in the OATP-transfected cells compared to the control without $HCO_3^-$ preload.[87] This finding indicates an exchange of estron-3-sulfate and bicarbonate for these human transporters and is in accordance to the observations for rat Oatp mediated transport.[83,97]

Beside the observed exchange of bicarbonate, an involvement of an $H^+$-gradient in OATP/Oatp transport mechanism has been revealed for OATP2B1. For estrone-3-sulfate uptake studies in OATP2B1 transfected cells an inwardly directed $H^+$-gradient was generated by the adjustment of the intracellular pH to 7.4 and the extracellular pH to 6.0, resulting in an overshoot phenomenon of substrate accumulation. In absence of the $H^+$-gradient or an additional treatment with the $H^+$-ionophore carbonylcyanide-p-trifluoromethoxyphenylhydrazone the lack of the overshoot phenomenon was observed, indicating that the uptake of estrone-3-sulfate via OATP2B1 is partially driven by a proton gradient.[46,98]

*Involvement of glutathione in OATP mediated transport*

Experiments for rat Oatp members indicate that glutathione efflux is coupled to Oatp mediated uptake of various substrates. At high intracellular glutathione concentrations, stimulation of taurocholate or digoxin uptake could be observed for rat Oatp1a1 and 1a4 expressed in *Xenopus laevis* oocytes.[99,100] Detailed investigations on the stoichiometry of the glutathione efflux/ taurocholate uptake for rat Oatp1a1 showed an 1:1 anion exchange.[100]

However, reducing the physiological gradient between the intra- and the extracellular glutathione concentration by addition of glutathione to the extracellular medium showed only minimal stimulative effect on taurocholate and digoxin uptake via Oatp1a4. This indicates that uptake by Oatp1a4 is not driven by an outwardly directed glutathione transmembrane gradient, but the presence of high intracellular glutathione concentrations may favor the uptake.[99] This asymmetric transport mechanism is also supported by an observed interaction of Oatp1a4 with the glutathione- conjugate S-(2,4-dintriphenyl)-glutathione (DNP-SG). Although transport of DNP-SG is not mediated by

Oatp1a4, its intracellular presence stimulates the uptake of taurocholate, which is a known substrate for this transporter.[99]

Whether human OATPs work as glutathione exchangers has only been investigated for a limited number of OATP family members and results are controversial. Briz et al (2006) showed that OATP1B3 expressed in *Xenopus laevis* oocytes transport bile acids with higher rates when glutathione or oxidized glutathione was added to the extracellular medium, while bicarbonate was not able to activate transport of taurocholic acid under these conditions.[101] Interestingly, an inwardly directed glutathione gradient inhibited the efflux of cholic acid by OATP1B3, excluding the possibility of an exchange of cholic acid with glutathione as transport mechanism.[101] Further investigations showed that glutathione itself is transported by OATP1B3 and kinetic analysis of bile acid transport in the presence of glutathione showed increasing $V_{max}$ but unchanged $K_m$ values. This suggests symport of anions and glutathione in the ratio of 1:2 by OATP1B3.[101] Controversial results were presented in another study, showing neither cis- nor trans-stimulation of OAT1B3 mediated transport by glutathione or oxidized glutathione, excluding the suggestion that OATPs function as glutathione symporter or exchanger.[86] OATP1B1 and 2B1 mediated transport is further described to be independent of glutathione.[98,101]

*Bidirectional transport*

Investigations on bidirectional transport ability of OATP1B3 revealed that OATP1B3 expressed in *Xenopus laevis* oocytes is able to mediate efflux of intracellular loaded cholic acid.[101] This OATP1B3 mediated efflux was further trans-stimulated by the addition of taurocholic acid to the extracellular medium.[101]

#### 3.1.8.2 Molecular structural requirements for OATP-mediated transport

*The extracellular loop 5*

The large extracellular loop 5 between the transmembrane domains IX and X with ten cysteine residues seem to be responsible for the expression of these transporters in cell membranes and therefore for their transport activity as this is reported for OATP2B1.[102] Hänggi et al (2006) generated nine cystein-to-alanin substitutions in the extracellular loop 5 and mutants were investigated concerning their cellular localization and transport activity for estrone-3-sulfate in transfected CHO-K1 cells.[102] With exception of the wild-type OATP2B1 and the mutant Cys493Ala, all other mutant proteins as well as the

truncated OATP2B1$_{\Delta 489\text{-}557}$ showed intracellular localization. Additionally, the mutants Cys493Ala and Cys557Ala achieved 60% and 30% of estrone-3-sulfate transport compared to the OATP2B1 wild type, respectively. All other OATP2B1 mutants showed a 85% decrease in estron-3-sulfate transport rate. These data demonstrate that the cysteine residues in extracellular loop 5 seem to be necessary for the expression of the transporter on the cell surface and therefore for its ability to transport estrone-3-sulfate.[102]

*PDZ domain*

In addition, an oligomerization of OATPs/Oatps with a PDZ domain is suggested to be essential for proper subcellular localization and therefore its function.[103]

PDZ domains consist of approximately 90 amino acids and are one of the most represented protein domains in sequenced genomes. The acronym “PDZ” is composed of the first three identified proteins containing PDZ domains.[104] (Table 4)

Table 4 Composition of the acronym “PDZ“.[104]

| | |
|---|---|
| **P**SD-95/SAP90 | Postsysnaptic protein |
| **D**iscs-large | *Drosophila* septate junction protein |
| **Z**O-1 | Tight junction protein |

PDZ domains mediate protein-protein interactions by binding to the C-terminus of the target protein. Therefore they assemble protein complexes, which have been reported to organize signaling pathways, cell polarity and subcellular location.[104]

Several OATPs/Oatps have been identified to exhibit a PDZ consensus binding site at their C-terminal, including the human OATP1A2 and 2B1 and the rat Oatp1a1, 1a4, 1b2, and 2b1.[103] Sequence alignment analysis of the C-terminus sequences of the human OATPs 1A2, 1B1, 1B3, 1C1, 2B1, 3A1, and 4A1 revealed consistence with the binding motif of Class I PDZ domains, characterized by a X(S/T)XΦ sequence (X, any amino acid possible; Φ, hydrophobic amino acid), for OATP1A2, 3A1, and 4A1.[103,104,105] Additional yeast two-hybrid analysis verified a positive interaction of human OATP1A2, 1C1, and 3A1 with PDZ proteins.[105] Detailed investigations on the involvement of protein interaction with PDZ domains in the localization of OATPs/Oatps showed that in contrast to a basolateral membrane localization of Oatp1a1 in wild type mice shifted to a predominantly intracellular distribution when PDZ was knocked-out.[103] Whether PDZ-

interaction is necessary for transmembrane localization of human OATPs has to be evaluated.

*The transmembrane domains 8 and 9*

The importance of the molecular structure on OATP transport has been further investigated in another study using HEK293 cell lines transfected with chimeric OATP1B1 transporters by Miyagawa et al (2009).[106] Chimeric OATP1B1 mutants were generated by the substitution of different OATP1B1 transmembrane domains with those of OATP1B3. After transfection, kinetic analyses were performed for the typical OATP1B1 substrate estrone-3-sulfate and for estradiol-17β-D-glucuronide, a substrate accepted by both- OATP1B1 and 1B3- transporters. While transport kinetics of estradiol-17β-D-glucuronide were not changed by the substitution of the transmembrane domain 9 of OATP1B1 with that of OATP1B3, a significant increase (7.4-fold) of the $K_m$ values for estrone-3-sulfate was observed for this OATP1B1 chimer. Contrary, the exchange of the OATP1B1 transmembrane domain 8 with that of OATP1B3 caused an increased $K_m$ value for estrone-3-sulfate (18-fold) but showed no transport activity for estradiol-17β-D-glucuronide. These results indicate an important role of the transmembrane domain 8 and 9 for OATP1B1 mediated transport activity.[106]

*The transmembrane domain 10*

In addition, such chimeric proteins between OATP1B1 and 1B3 were further generated and transfected into HEK293 cells in another study, investigating cholecystokinin uptake by different chimeric OATP1B1-1B3 proteins.[107] This study showed that cholecystokinin uptake was dramatically reduced when the transmembrane domain 10 of OATP1B3 was replaced by that from OATP1B1, indicating an important role of the transmembrane domain 10 in the transport of cholecystokinin. More detailed investigations identified three key residues in position Y537, S545, and T550 in this transmembrane domain of OATP1B3. Replacing these amino acids in OATP1B3 with the corresponding ones of OATP1B1 resulted in similar reduced cholecystokinin uptake as observed after the replacement of the whole transmembrane domain 10. Therefore, it is suggested that the three amino acids Y537, S545 and T550 in OATP1B3 are important for the uptake of cholecystokinin via this transporter.[107]

*Rocker-switch type of transport mechanism*

Furthermore, structural models of OATP1B3 and 2B1 predict the presence of a central positively charged pore, which seems to be functionally important as the positive electrostatic potential and the size of this pore is consistent with the shape of typical OATP/Oatp substrates. However, this model has to be experimentally verfied.[13]

Moreover, investigations of known polymorphisms in the OATP1B and 2B family correspond with this proposed rocker-switch type of transport mechanism. Reported polymorphisms in OATP1B1[108] and 2B1[88] are indeed associated with changes in $K_m$ and $V_{max}$ values for e.g. estrone-3-sulfate transport. This supports the suitability of the predicted model, particularly as related amino acid residues face the suggested central pore. This may therefore be important for substrate recognition and binding.[13]

## 3.2 OATPs and malignancies

Although it has been demonstrated that drugs used in the treatment of cancer are substrates of OATP family members, the current knowledge on the distribution and functions of individual OATPs in malignant tissues is limited.

OATP1A2 has been shown to mediate the uptake of methotrexate, while the 1B3 family member was identified as high affinity paclitaxel transporter.[109,110] In addition, OATPs may be important in cancer diagnosis and prognosis as OATP1B1 and 1B3 are suggested to be histological markers in the diagnosis of hepatocellular carcinomas and additionally OATP1B3 has further been shown to be a prognostic factor in invasive ductal carcinomas of the breast.[111, 112]

### 3.2.1 OATPs in breast cancer

Worldwide 14% of female cancer deaths are attributed to breast cancer and every year up to 5% increasing rates in breast cancer incidences are predicted.[113] Estrogens are known to be important determinants in the progression of estrogen-dependent breast cancers. The sources of active estrogens are circulating androstendione and estrone-3-sulfate which are converted to active estrogens in tumor cells either in the aromatase or sulfatase pathway (Figure 4).[114] As sulfatases showed a higher activity in breast cancer patients than aromatase, they are likely to be the main estrogen producing enzymes.[115]

In contrast to the estrogen-sulfate hydrolyzing sulfatases, which produce biological active 17ß-estradiol, enzymes belonging to the sulfotransferase family (SULT) are able to convert active estrogens, particularly 17ß-estradiol with help of the ß-

hydroxysteroide-dehydrogenase, back into the inactive sulfoderivate estrone-sulfate. Both, sulfatases and SULTs have been reported to be highly expressed in breast tissue.[116,117]

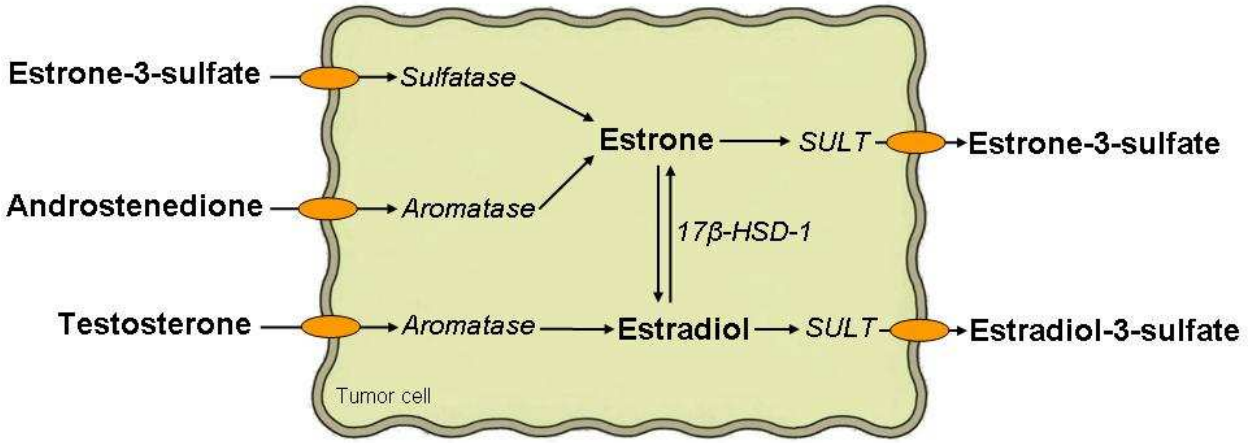


Figure 4 Production of active estrogens in human breast cancer cells. 17ß-HSD-1, 17ß-hydroxysteroid-dehydrogenase type 1; SULT, sulfotransferases. Modified from Pasqualini.[117]

While estrogen converting pathways are well studied, the uptake mechanisms of the estrogen precursors into breast tumors are still not well characterized. Estrone-3-sulfate is the estrogen present at highest concentration in human serum and is a substrate for many OATPs.[118] Therefore, it is hypothesized that OATPs play an important role in the transport of this conjugated estrogen into tumor cells and, thereby, influence estrogen-dependent proliferation.

Until now, data on the presence and localization of OATP family members in breast cancer tissue is limited. However, some of them have been studied in various breast cancer cell lines showing mRNA expression of several OATPs.

OATP1A2 mRNA was reported to be present in T-47D, MCF-7, and ZR-75-1 cells, but absent in MDA-MB-231 and MDA-MB-468 cells.[40,119] However, OATP1B1, 1B3, and 1C1 mRNA was not present, neither in T-47D nor in MCF-7 cells, when determined with conventional PCR,[120,121] but OATP1C1 was detectable in HS578T cells.[119] Additionally, the liver specific OATP1B3 was shown to be present in MDA-MB-231 and HS578T cells.[119] OATP2A1 mRNA could be detected in BT-549, HS578T, and MCF-7 cells,[119] while expression of OATP2B1 mRNA was undetectable in T-47D, MCF-7 or MDA-MB-453 cell lines.[120,121] mRNA of OATP3A1 and 4A1 has been detected in T-47D, MCF-7, and MDA-MB-453 breast cancer cell lines by conventional and real-time PCR.[40,122,123] OATP4C1 mRNA was found in MCF-7, BT-549, and MDA-MB-231 cells.[119]

However, only the expression of OATP2B1 was confirmed at the protein level so far and immunofluorescence staining experiments showed localization in epithelial cells in invasive ductal carcinoma.[40]

For human breast cancer tissue, knowledge on OATP mRNA expression and OATP protein localization is even more limited. Both, OATP1A2 and 2B1 mRNA have been identified to be expressed in tumorous breast tissues.[72.124] A study investigating 102 specimens of invasive ductal carcinomas of the breast showed protein expression of OATP1B3. As its expression levels were associated with tumor size, OATP1B3 was regarded as a prognostic factor in breast cancer.[112]

### 3.2.2 OATPs in liver malignancies

The different tumor types occurring in the human liver are categorized by their place of origin. While the hepatocellular carcinoma arouses from hepatocytes, cholangiocarcinomas derive from cells in the bile duct (bile ductular cells, cholangiocytes). In addition to these malignancies which are derived from cells of the liver itself, metastatic tumors deriving from malignancies of other organs often occur in the liver.[125]

Under physiologic conditions, some OATPs are important influx transporters in the liver, mediating the uptake of compounds into hepatocytes. Today, our information on differences in the OATP expression pattern in hepatic malignancies is limited, as studies generally focus on the two liver-specific OATP1B1 and 1B3.

OATP1B1 as well as OATP1B3 could be detected in hepatocellular carcinomas either on mRNA and protein level.[126,127] More detailed investigations observed a decreased expression of OATP1B3 in hepatocellular carcinoma (HCC) specimens compared to the non-malignant tissue samples, but data on OATP1B1 expression are controversial. While some studies observed a reduced mRNA and protein expression of OATP1B1 in HCC tumor samples,[126,127,128] others revealed no significant decrease of this transporter in tumorous specimens, neither on the mRNA nor on the protein level.[129] Our knowledge of OATP distribution in cholangiocarcinomas and metastases is limited to the information that OATP1B1 and 1B3 could not be detected by immunofluorescence staining in these malignancies.[126]

### 3.2.3 OATPs in tumors of other organs

OATPs have further been detected in other cancer types originated from lung, blood cells, brain, pancreas and the gastrointestinal tract.[130,131,132,133,134,135,136] In colon cancers OATP1A2, 1B1, 1B3, and 4A1 have been identified so far.[130,131,132] OATP1B3 was additionally characterized in gastric and pancreatic cancer.[131] The ubiquitous distributed OATP3A1 was also present in cell lines deriving from blood cell malignancies (promyelocytic leukemia, chronic myelogenous leukemia, lymphoblastic leukemia, Burkitt's lymphoma) and various solid tumors, including cervical cancer, colorectal adenocarcinoma, lung carcinoma, and melanoma.[133] In human brain tumors, OATP1A2 and 2B1 showed localization at the blood-tumor barrier.[134] However, in lung cancers, eight OATP family members have been identified so far, including OATP1A2, 1B3, 1C1, 2A1, 2B1, 3A1, 4A1, and the less characterized OATP6A1.[135,136] The latter has further been detected in bladder and esophageal tumors.[136]

Although OATPs have been identified in these different types of tumors and they are reported to mediate the transport of some anticancer agents, no systematic studies are available. Taken together, it can be hypothesized that this transporter family may contribute to uptake of anticancer agents into cancer cells and subsequently to efficacy of cancer therapy. However, further studies are required to get more insight in the role of OATP family members in malignancies.

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# 5 AIMS OF THE THESIS

Organic anion transporting polypeptides (OATPs) have been reported to mediate the uptake of endogenous and exogenous compounds, including bile acids steroid hormones and their conjugates as well as antibiotics, statins, and antihypertensive drugs. Until now eleven family members have been identified in humans, exhibiting distribution in a variety of different tissues. The localization of OATPs in organs like intestine, liver, kidney, and brain indicate a key role in absorption, disposition and elimination of exogenous compounds. Indeed, studies in OATP-expressing *X. laevis* oocytes and cancer cell lines show that OATPs mediate the influx of xenobiotics, including anticancer agents into cells, suggesting an important role of these transporters also for the uptake of anticancer drugs into tumor cells. Nevertheless, the knowledge on OATP expression and function in malignancies is limited and their possible role in chemoresistance of tumor cells is still unknown. To account for these problems, the aim of this thesis was to investigate the presence of this transporter family in selected cancer types. A potential difference in mRNA expression rates between malignant and non-malignant tissues should be investigated. First, real-time PCR analyses should detect mRNA expression of all known human OATPs in three common used breast cancer cell lines and one non-malignant breast cell line. To verify whether these cell lines are useful *in vitro* models for human tissues, data from OATP mRNA expression analyses in cell lines were compared to OATP mRNA expression pattern in breast cancer tissue samples from 13 patients. To examine possible variations in the mRNA expression levels between tumorous and normal tissue, adjacent non-malignant specimens from the same patient were included into real-time PCR analyses.

Estrogen-dependent tumor progression is a severe problem in many patients with breast cancer. Particularly after menopause, adrenal-derived inactive estrone-3-sulfate is effectively hydrolyzed by sulfatases in breast cancer cells to provide active 17ß-estradiol leading to the proliferation of hormone-dependent tumor cells. However, counteracting sulfotransferases (SULTs) are able to convert estrogens back into its inactive precursor estrone-3-sulfate. To get a better insight in the expression levels of sulfatases and SULTs in breast cancer cells, mRNA expression of these enzymes were further investigated in malignant and non-malignant breast cancer tissue samples.

Another topic of this thesis is the investigation on the anticancer agent paclitaxel, used in the treatment of metastatic breast cancer, regarding its substrate activity for

OATP1B1 and 1B3. However, whether all other OATP family members, identified in humans, show substrate specificity for paclitaxel, has not been studied yet. The next aim was to characterize all eleven human OATPs concerning their ability to transport paclitaxel in the *Xenopus laevis* oocytes expression model. Therefore, full length cRNAs of all eleven OATP family members were generated, injected into *Xenopus laevis* oocytes, and the uptake of radiolabeled paclitaxel was measured. Furthermore, kinetic analysis of paclitaxel transport mediated by the OATPs identified as paclitaxel transporters was performed.

Beside paclitaxel, other anticancer agents including methotrexate have been identified as OATP substrates so far. As methotrexate is commonly used in the treatment of osteosarcomas, another aim of this study was to elucidate the expression pattern of this transporter family in specimens derived from 21 patients suffering from osteosarcomas using real-time PCR. In addition, this project should examine mRNA levels of all OATPs in common used osteosarcoma cell lines and non-malignant bone cells.

In addition to the uptake of xenobiotics into target organs, metabolism and elimination are other important determinants for an effective anticancer chemotherapy with minor side- or toxic effects. Most part of metabolism reactions take place in the liver, where two members of the OATP family (1B1 and 1B3) are situated in the basolateral membrane of hepatocytes, facilitating the uptake of drugs and bringing them to the metabolizing system. Although the importance of some OATPs in human liver has been well investigated, the overall role of all OATPs in liver malignancies is still unclear. Therefore, another aim of this thesis was to investigate the expression of all known human OATP family members in tumorous liver tissues from 22 patients by real-time PCR. Again, the corresponding non-tumorous tissue samples from the same patients were also examined to detect a possible change in mRNA expression rates comparing tumor and normal specimens. To cover the three most frequent malignant tumors in human liver, the samples derived from patients suffering from hepatocellular-, cholangiocarcinomas or metastases from tumors of other organs were investigated for expression all OATPs and studied differences in the OATP mRNA expression pattern between these liver tumors.

The results from these studies should allow a better understanding in the role of OATPs in frequent human cancer, e.g. breast and liver cancer. This would contribute to better understand mechanisms leading to the sensitivity of tumors against chemotherapeutic drugs.

# 6 RESULTS

## 6.1 Original papers and manuscripts

**Wlcek K**, Svoboda M, Thalhammer T, Sellner F, Krupitza G, Jaeger W. Altered expression of organic anion transporter polypeptide (OATP) genes in human breast carcinoma. Cancer Biol Ther. 2008; 7:1450-5.

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I carried out RNA isolation of selected bone tumor specimens.

Miksits M, **Wlcek K**, Svoboda M, Thalhammer T, Ellinger I, Stefanzl G, Falany CN, Szekeres T, Jaeger W. Expression of sulfotransferases and sulfatases in human breast cancer: impact on resveratrol metabolism. Cancer Lett. 2010; 289:237-45.

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Svoboda M, **Wlcek K**, Taferner B, Hering S, Stieger B, Tong D, Zeillinger R, Thalhammer T, Jäger W. Paclitaxel transport by organic anion transporting polypeptides (OATPs) in the ovarian cancer cell lines OVCAR-3 and SK-OV-3. Cancer Lett. Submitted May 2010.

I carried out Plasmid/cRNA generation and transport studies

**ALTERED EXPRESSION OF ORGANIC ANION TRANSPORTER POLYPEPTIDE (OATP) GENES IN HUMAN BREAST CARCINOMA**

**Wlcek K**, Svoboda M, Thalhammer T, Sellner F, Krupitza G, Jaeger W

Cancer Biol Ther. 7:1450-5, 2008

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Research Paper

# Altered expression of organic anion transporter polypeptide (OATP) genes in human breast carcinoma

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**Abbreviations:** OATP, organic anion transporter polypeptide; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase PCR; HER-2, human epidermal growth factor receptor 2; FBS, fetal bovine serum; E3S, estrone-3-sulfate; DHEAS, dehydroepiandrosterone sulfate

**Key words:** OATP, uptake transporter, breast cancer, mRNA expression, quantitative real-time RT-PCR

**Organic anion transporter polypeptides (OATPs) mediate the transmembrane uptake of endogenous compounds and clinically important drugs in various tissues thereby effecting drug disposition and tissue penetration. OATPs have also been identified in gastric, pancreatic and colon carcinomas but little is known about their expression in breast carcinoma. We therefore analyzed the expression pattern of all 11 known OATPs in three breast cancer cell lines (MCF-7, ZR-75-1, MDA-MB-231) and one immortalized breast epithelial cell line (MCF-10A) using quantitative real-time RT-PCR. Transcripts of 7/11 OATP genes with heterogeneity in their expression profile were detected in control and/or cancer cell lines. Of these seven OATPs, five were also expressed in breast tumor and adjacent non-tumorous specimens from 13 patients. OATP2B1, not found in the analyzed cell lines, was verified in the tissue samples. Interestingly, mRNA expression of OATP2B1, OPATP3A1 and OATP4A1 was significantly higher ($p < 0.022$) in non-malignant specimens as compared to tumor tissue samples.**

## Introduction

Organic anion transporting polypeptides (OATPs) form a superfamily of sodium-independent transport systems and mediate the cellular uptake of many endogenous and exogenous chemicals including drugs in clinical use. Eleven members of the OATP family have currently been identified in humans. They are expressed in a variety of different tissues including intestine, liver, kidney and brain, and they play a critical role in drug absorption, distribution and excretion. Although multi-specificity and wide tissue distribution are common characteristics of many OATPs, some members have a high substrate specificity and exhibit unique cellular expression in distinct organs.[1,2]

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OATP1B1, 1B3 and OATP2B1, in particular, are highly expressed in human liver and are involved in the active hepatic uptake of various drugs. This active hepatic drug uptake process can be subject to inhibition resulting in higher blood levels and in a range of severe side effects, such as those reported for statins after co-administration with the OATP inhibitors cyclosporine A and gemfibrozil.[2-7]

Recent findings have demonstrated that several anticancer agents, including paclitaxel, docetaxel, actinomycin D, mitoxantrone and SN-38, are substrates, at least, for OATP1B3, indicating a major role in the uptake of anticancer drugs into tumor cells.[8,9] While OATP1B3 was also found to be expressed by solid digestive organ neoplasms, including gastric, pancreatic and colon cancer,[10] the expression of OATPs in breast carcinomas is still poorly characterized and results are controversial.

Transcripts of OATP1A2, 3A1 and 4A1 genes have recently been identified in hormone-dependent T-47D and MCF-7 breast cancer cell lines by conventional RT-PCR,[11,12] but the expression of OATP3A1 and 4A1, only, could be confirmed by quantitative real-time RT-PCR in both cell lines by Pizzagalli and coworkers.[13] Very recent data from Miki et al. however, was able to demonstrate OATP1A2 mRNA expression in T-47D, MCF-7, ZR-75-1, MDA-MB-231 and MDA-MB-468 cells, with highest expression levels in T47D and ZR-75-1 cells.[14]

Presently, the expression of only two members of the OATP-family, namely OATP1A2 and OATP2B1, has been demonstrated in human breast carcinoma by quantitative real-time RT-PCR.[14,15] While OATP2B1[15] was expressed in both tumor and normal breast specimens, OATP1A2[14] was found only in the tumor tissue samples. Alcorn and coworkers also identified OATP1A2, 2B1, 3A1 and 4A1 mRNA expression in human mammary epithelial cells prepared from mammoplasty tissues using quantitative real-time RT-PCR.[16]

Based on these data and the importance of OATPs for cellular drug uptake, we did the first investigation into mRNA expression of all 11 known human OATPs in malignant and non-malignant breast cell lines by TaqMan® quantitative real-time RT-PCR. The early stage and hormone receptor positive breast cancer cell line MCF-7, the invasive hormone receptor negative breast cancer cell line MDA-MB-231, the hormone-dependent and HER-2 expressing breast cancer cell line ZR-75-1, and the immortalized normal breast

tissue cell line MCF-10A, were chosen in order to represent different types of breast tumors. To better understand the biological characteristics of breast tumors and their response to chemotherapy, we also explored the expression of these OATPs in the breast tumor and its corresponding peritumoral tissue on tissue sections from 13 patients. Any differences in the expression of OATP between malignant and adjacent non-malignant breast tissue may play a key role in the sensitivity or insensitivity of breast cancer tissue to chemotherapeutic OATP substrates.

## Results

**OATP expression in human breast cell lines.** We measured the relative mRNA levels of all known OATP family members in two hormone-dependent (MCF-7, ZR-75-1) and one independent (MDA-MB231) breast cancer cell lines by quantitative real-time RT-PCR. The immortalized non-malignant mammary epithelial MCF-10A line was also included in the analyses. We identified the mRNA expression of 7/11 OATPs, namely OATP1B1, OATP1B3, OATP2A1, OATP3A1, OATP4A1, OATP4C1 and OATP5A1 in at least one cell line (Table 2). Heterogeneity in OATP patterns and expression levels was observed. In all four cell lines, mRNA expression of OATP1A2, OATP1C1, OATP2B1 and OATP6A1 was below the detection limit.

As shown in Table 2, MCF-7 and ZR-75-1 revealed 3.6- and 3.4-fold higher expression levels for OATP2A1 as compared to control MCF-10A cells. Interestingly, mRNA expression in MDA-MB-231 cells was only 10% of control levels. In contrast to OATP2A1, OATP3A1 mRNA expression was highest in MDA-MB-231 and MCF-10A cells. Significantly lower expression levels (30% and 7% of control) were seen in both hormone-dependent MCF-7 and ZR-75-1 cell lines.

OATP4A1 mRNA expression was highest in non-malignant MCF-10A cells, whereas in MCF-7 and ZR-75-1 cells, expression was significantly reduced to 40% and 6%. Expression of OATP4A1 in MDA-MB-231 cells was hardly detectable. A 3.4-fold higher mRNA expression of OATP5A1, as compared to control, was observed in MCF-7 cells. Expression values of this polypeptide in ZR-75-1 cells were reduced to 40% of control levels and below the detection level in the MDA-MB-231 cell line.

Expression of OATP1B1 mRNA was highest in ZR-75-1 cells; only moderate levels were seen in the MDA-MB-231 cell line. In MCF-7 and non-tumorous MCF-10A cells, mRNA expression of this OATP was not detectable. OATP4C1 was detected in all three breast cancer cell lines with highest expression levels in MCF-7 followed by MDA-MB-231 and ZR-75-1 cells. No expression could be observed in non-tumorous MCF-10A cells.

Interestingly, in comparison to the other six expressed OATPs, OATP1B3 mRNA expression was only detected in the hormone-independent MDA-MB-231 cells.

**OATP expression in human tissue samples.** A total of 13 breast cancer samples were chosen and matched with 13 corresponding non-malignant breast tissue specimens to perform quantitative real-time RT-PCR analysis for all known human OATPs. Our investigations revealed mRNA expression of six OATPs, namely OATP2A1, OATP2B1, OATP3A1, OATP4A1, OATP4C1 and OATP5A1, in tumorous and corresponding normal breast tissue samples. In all specimens, expression of OATP1A2, OATP1B1, OATP1B3, OATP1C1 and OATP6A1 was below the detection limit.

**Table 1 Tumor characteristics of tissue samples**

| Patient no. | Tumor size[a] | Lymph node status[a] | Hormone receptor status | HER-2 status | Tumor-differentiation |
|---|---|---|---|---|---|
| 1 | T3 | + | ER -<br>PR - | - | G3 |
| 2 | T2 | + | ER -<br>PR - | - | G3 |
| 3 | T1 | + | ER -<br>PR - | - | G2 |
| 4 | T2 | + | ER +<br>PR - | - | G3 |
| 5 | T2 | + | ER +<br>PR - | - | G3 |
| 6 | T1 | + | ER +<br>PR - | - | G2 |
| 7 | T4 | - | ER +<br>PR - | - | G3 |
| 8 | T2 | - | ER -<br>PR - | - | G3 |
| 9 | T2 | x | ER +<br>PR - | - | G2 |
| 10 | T2 | + | ER -<br>PR + | - | G2 |
| 11 | T1 | - | ER -<br>PR - | - | G2 |
| 12 | T2 | x | ER +<br>PR + | - | G2 |
| 13 | T2 | - | ER -<br>PR + | + | G3 |

[a]According to the Tumor, Node, Metastasis (TNM) classification from the American Joint Committee on Cancer (AJCC). T1, tumor size up to 2 cm. T2, tumor size between 2 and 5 cm. T3, tumor size greater than 5 cm. T4 tumor of any size with skin involvement. x, could not be evaluated. ER, estrogen receptor. PR, progesterone receptor.

As seen in Table 3, the expression levels of OATP2A1 mRNA were up to 9.6-fold higher ($p < 0.05$) in eight of thirteen non-malignant tissue samples in comparison with the tumorous one. In three tumor samples, expression of OATP2A1 was slightly higher (up to 62%) as compared to control tissues; No difference in OATP2A1 expression between tumor and control tissues was seen in two patients (No. 7 and 8). For OATP3A1, we found elevated (1.8- to 6.9-fold) mRNA expression in 11 of 13 normal breast specimens. In two tumorous samples (No. 7 and 8) we measured a significant increase of OATP3A1 expression as compared to the control tissue.

OATP4A1 mRNA expression levels vary over a wide range. In 10/13 of the control breast samples, we detected up to a 14.5-fold increased OATP3A1 mRNA expression as compared to the paired tumorous tissues. For three patients (No. 1, 8 and 11), we found a slightly (1.1- to 1.2-fold) higher expression of this specific OATP in the cancer samples. Again, mRNA expression of OATP5A1 showed high variability. Control tissue samples from 8/13 patients showed significantly higher levels of OATP5A1 expression than found in the malignant sample. Three patients (No. 4, 8 and 11), however, expressed OATP5A1 mRNA in the tumorous specimens to a higher amount (2.3–10.4-fold) while only a very low difference in expression of OATP51 was seen in samples No. 1 and 3.

**Table 2 OATP mRNA expression levels in malignant (MCF-7, MDA-MB-231, ZR-75-1) and non-malignant (MCF-10A) breast cell lines**

| Cell line | OATP2A1 | OATP3A1 | OATP4A1 | OATP5A1 | OATP1B1 | OATP1B3 | OATP4C1 |
|---|---|---|---|---|---|---|---|
| MCF-10A | 1.00 ± 0.25 | 1.00 ± 0.10 | 1.00 ± 0.12 | 1.00 ± 0.20 | n.d. | n.d. | n.d. |
| MCF-7 | **3.64 ± 0.17*** | **0.30 ± 0.11*** | **0.40 ± 0.17*** | **2.99 ± 0.19*** | n.d. | n.d. | **8.16 ± 0.32*** |
| MDA-MB-231 | **0.10 ± 0.20*** | 1.17 ± 0.13 | **0.003 ± 0.005*** | n.d.* | 1.00 ± 0.22* | 1.00 ± 0.12* | **3.14 ± 0.28*** |
| ZR-75-1 | **3.38 ± 0.22*** | **0.07 ± 0.12*** | **0.06 ± 0.08*** | **0.40 ± 0.26*** | **14.78 ± 0.26*** | n.d. | 1.00 ± 0.44* |

n.d., not detected. Values in bold indicate a 2-fold more increase or decrease compared to the calibrator. Relative mRNA expression levels are given as the n-fold change to the calibrator ± SD. *significantly different from non-malignant MCF-10A cells ($p < 0.05$).

OATP2B1 expression levels were significantly increased in 8 normal samples as compared to their paired tumor tissues. A higher expression in tumorous samples could be detected in three specimens (No. 4, 6 and 11). No differences in expression levels of OATP2B1 could be detected between control and tumorous samples of patient No. 2 and 7. For OATP4C1, we observed higher expression levels in seven normal specimens. In three patients (No. 10, 12 and 13) the expression of this uptake transporter was below the detection limit in control samples but not in tumorous ones.

As shown in Table 3 mRNA expression of OATP2B1, OPATP3A1 and OATP4A1 were significantly higher ($p < 0.022$) in non-malignant specimens as compared to tumor tissue samples. OATP4C1 and OATP2A1 mRNA expression was also higher in the majority of specimens but did not reach statistical significance. Interestingly, OATP expression may be influenced by tumor size, as patient No. 7 with a tumor size of T4 (tumor of any size with direct extension to chest wall) showed very low expression of OATPs as compared to patients No. 3, 6 and 11 with tumor sizes of 2.0 cm or less (T1).

## Discussion

In the present study we elucidated the transcriptional expression for all known human OATPs in human malignant and non-malignant breast cell lines and tissue samples using quantitative real-time RT-PCR. First, we investigated OATP mRNA expression in three commonly used breast cancer cell lines. To cover the most important different types of breast tumors we choose the hormone-dependent, HER-2 negative MCF-7, the hormone-dependent, HER-2 positive ZR-75-1, and the hormone-independent MDA-MB-231 cell lines. The immortalized non-malignant mammary epithelial MCF-10A line was included in the analyses as a control to determine differences in the expression profiles of OATPs between cancerous and control cells. Consequently, we observed the expression of seven OATP family members with a high variability in expression levels (Table 2). In accordance with the results of Pizzagalli et al. we detected mRNA of OATP3A1 and 4A1 in MCF-7 cells.[13] However, contrary to Miki et al. expression levels of OATP1A2 were below detection limit in the four cell lines investigated.[14] In addition to OATP3A1 and 4A1, we also identified for the first time the expression of OATP1B1, 1B3, 2A1, 4C1 and 5A1 in these cell lines by quantitative real-time RT-PCR. Our data also showed that the expression levels of OATPs differ between the cancer and control cell lines. MCF-7 cells express the majority of the detected OATPs at higher levels compared to MDA-MB-231 and ZR-75-1 cells. MCF-7 cells may therefore be more sensitive to OATP-specific anticancer drugs. Altered uptake of OATP-substrates may also influence cellular drug metabolism especially in breast cancer cells with high enzyme activity as very recently shown for the sulfation of resveratrol in ZR-75-1 but not in MDA-MB-231 cells.[17]

Differences in cancer and control cells also suggest that alterations in mRNA expression levels may also be observed in tumor tissue samples from patients. As it is known that the activity and the expression of transport proteins may vary greatly between individuals, as a function of genetic, environmental and physiological factors, adjacent breast peritumoral tissues were chosen as references for human breast tumoral tissues.[18,19] Indeed, we also demonstrated great differences in the expression of six OATPs, namely, OATP2A1, OATP2B1, OATP3A1, OATP4A1, OATP4C1 and OATP5A1, in 13 breast cancer and control tissue samples (Table 3). Among these polypeptides, expression of OATP2A1, OATP3A1, OATP4A1, OATP4C1 and OATP5A1 has not previously been detected in breast cancer tissue. In accordance with literature data, we could also detect OATP2B1 expression in breast tumor and normal tissues.[15,16] However, contrary to Al Sarakbi et al. who used non-matched breast tumor and control tissue samples, we could not detect higher expression levels of OATP2B1 in malignant tissue samples as compared with the non-malignant ones.[15] These differing results might be caused by the high inter-individual variability in the expression levels of OATPs in matched malignant and adjacent non-malignant tissue samples as shown in our study. OATP3A1 and 4A1 expression in non-malignant mammary tissues were in line with literature data as Alcorn et al. also showed the expression of these two OATPs in human mammary epithelial cells isolated from mammoplasty tissue.[16] In contrast to data from a previous study with non-matched material,[14] OATP1A2 mRNA expression was below the detection limit in all analyzed breast tumor tissue samples.

OATP expression may negatively correlate with tumor size, as in one patient (No. 7) with a T4 tumor lower expression of all six expressed OATPs were found when compared to three patients with T1 tumors (patients No. 3, 6 and 11). Otherwise, no significant correlation in the expression levels of all OATPs in non-malignant and malignant breast tissue samples with patient's age, tumor size, hormonal receptor, or HER-2 status could be obtained, possibly based on the small sample size. Although not verified in our study, OATP genes might correlate with the grade and stage of breast cancer as recently reported by Al Sarakbi et al. for OATP2B1. However, analogous to our study, Al Sarakbi and coworkers could also not show any correlation of OATP2B1 expression with estrogen receptor status, HER-2 status and clinical outcome.[15]

Major differences between the OATP mRNA expression in breast cancer cells and tissues were seen in the OATP1B subfamily of

**Table 3 OATP mRNA expression levels in malignant and non-malignant tissue samples**

| Patient | Tissue | OATP2A1 | OATP3A1* | OATP4A1* | OATP5A1 | OATP2B1* | OATP4C1 |
|---|---|---|---|---|---|---|---|
| 1 | M | 1.06 ± 0.04 | 1.00 ± 0.18 | 41.30 ± 0.09 | 1.89 ± 0.06 | 1.00 ± 0.03 | 2.64 ± 0.06 |
| | NM | **3.69 ± 0.09** | **5.88 ± 0.12** | 36.71 ± 0.08 | 1.92 ± 0.08 | **3.37 ± 0.12** | 2.48 ± 0.10 |
| 2 | M | 2.66 ± 0.04 | 2.36 ± 0.05 | 3.04 ± 0.08 | 4.11 ± 0.08 | 2.54 ± 0.05 | 2.58 ± 0.11 |
| | NM | 1.93 ± 0.06 | **6.05 ± 0.17** | **13.82 ± 0.07** | 6.69 ± 0.13 | 2.67 ± 0.17 | 3.90 ± 0.24 |
| 3 | M | 1.18 ± 0.03 | 3.89 ± 0.08 | 2.61 ± 0.07 | 5.43 ± 0.06 | 4.17 ± 0.08 | 1.00 ± 0.16 |
| | NM | **7.51 ± 0.05** | **17.11 ± 0.07** | **20.03 ± 0.09** | 5.57 ± 0.10 | 8.17 ± 0.07 | **10.84 ± 0.14** |
| 4 | M | 1.83 ± 0.04 | 1.96 ± 0.09 | 11.98 ± 0.04 | **4.47 ± 0.02** | 4.07 ± 0.09 | 1.54 ± 0.10 |
| | NM | 3.55 ± 0.10 | **4.55 ± 0.16** | 16.75 ± 0.15 | 1.85 ± 0.19 | 2.97 ± 0.16 | **3.95 ± 0.11** |
| 5 | M | 1.68 ± 0.04 | 1.21 ± 0.03 | 1.00 ± 0.11 | 1.00 ± 0.10 | 1.62 ± 0.03 | 6.48 ± 0.05 |
| | NM | **5.32 ± 0.12** | **6.90 ± 0.24** | **14.46 ± 0.22** | **2.15 ± 0.19** | **3.96 ± 0.24** | **32.07 ± 0.13** |
| 6 | M | 1.00 ± 0.10 | 1.93 ± 0.07 | 15.74 ± 0.08 | 1.93 ± 0.05 | 4.77 ± 0.07 | 1.43 ± 0.10 |
| | NM | **9.58 ± 0.10** | **13.44 ± 0.11** | 16.96 ± 0.08 | 3.33 ± 0.19 | 4.53 ± 0.11 | **10.47 ± 0.09** |
| 7 | M | 2.92 ± 0.14 | 2.95 ± 0.07 | 2.80 ± 0.12 | 1.56 ± 0.16 | 2.16 ± 0.07 | 1.37 ± 0.28 |
| | NM | 2.71 ± 0.10 | 1.78 ± 0.06 | 5.26 ± 0.21 | 2.08 ± 0.11 | 2.31 ± 0.06 | 2.52 ± 0.07 |
| 8 | M | 3.34 ± 0.08 | 11.49 ± 0.03 | 12.59 ± 0.10 | **6.83 ± 0.03** | 3.47 ± 0.03 | **13.69 ± 0.06** |
| | NM | 3.38 ± 0.14 | 7.02 ± 0.03 | 11.36 ± 0.09 | 2.99 ± 0.20 | 6.49 ± 0.03 | 6.36 ± 0.07 |
| 9 | M | 6.30 ± 0.06 | 4.52 ± 0.06 | 9.15 ± 0.10 | 1.93 ± 0.37 | 3.77 ± 0.06 | 1.59 ± 0.27 |
| | NM | 4.61 ± 0.14 | **13.92 ± 0.37** | **36.99 ± 0.17** | 3.67 ± 0.19 | 5.08 ± 0.37 | **5.74 ± 0.24** |
| 10 | M | 4.57 ± 0.10 | 4.22 ± 0.27 | 53.21 ± 0.19 | 3.82 ± 0.18 | 2.84 ± 0.27 | **2.81 ± 0.21** |
| | NM | 7.56 ± 0.07 | **19.41 ± 0.17** | 84.40 ± 0.10 | **15.9[illegible] ± 0.18** | **6.41 ± 0.17** | n.d. |
| 11 | M | 6.50 ± 0.11 | 11.13 ± 0.10 | 47.78 ± 0.27 | **9[illegible] ± 0.10** | 7.12 ± 0.10 | 9.32 ± 0.17 |
| | NM | 7.68 ± 0.05 | **22.98 ± 0.10** | 40.80 ± 0.16 | 9.47 ± 0.18 | 6.85 ± 0.10 | 5.86 ± 0.15 |
| 12 | M | 1.69 ± 0.12 | 9.03 ± 0.21 | 58.90 ± 0.15 | 1.12 ± 0.21 | 3.16 ± 0.21 | 1.64 ± 0.20 |
| | NM | **5.40 ± 0.15** | **35.92 ± 0.15** | **125.32 ± 0.13** | **3.55 ± 0.47** | **6.81 ± 0.15** | n.d. |
| 13 | M | 11.59 ± 0.24 | 17.69 ± 0.42 | 46.43 ± 0.21 | 8.45 ± 0.15 | 5.22 ± 0.42 | **5.35 ± 0.17** |
| | NM | 7.12 ± 0.10 | 32.59 ± 0.81 | 75.64 ± 0.07 | 13.57 ± 0.03 | 7.47 ± 0.41 | n.d. |

M, malignant. NM, adjacent non-malignant tissue sample. n.d., not detected. Values in bold indicate a 2-fold or more increase compared to the corresponding tissue sample. Relative mRNA expression levels are given as the n-fold change to the calibrator ± SD. *indicates significant differences in the expression levels of tissue specific OATP between all 13 malignant and adjacent non-malignant tissue samples ($p < 0.05$).



these transporters. While OATP1B3 was found in MDA-MB 231 cells, this transporter was absent in all tissue samples. Additionally, OATP1B1 mRNA expression was undetectable in the non-malignant and malignant tissues but was expressed in MDA-MB-231 and ZR-75-1 cells. Analogously, OATP2B1 was expressed in the tissues but not in any cell lines investigated.

Any variations in OATP expression, however, may significantly alter uptake of anticancer drugs into tumors cells thereby strongly affecting efficacy of treatment. This is especially true for the anticancer agents paclitaxel and docetaxel, which have recently been identified as substrates of OATP1B3.[8] Along with the identification of OATP1B3 as taxane transporter by Smith and coworkers[8] in OATP1B3 transfected *Xenopus laevis* oocytes, it is most likely that other OATPs may also contribute to intracellular paclitaxel and docetaxel uptake. Furthermore, estrone sulfate, with 5–10 times higher circulating plasma concentration than that of unconjugated estrogens,[11] and which is cleaved within the tumor cells by sulfatases to active estrogen, has also been shown to be a substrate for at least seven OATPs, namely OATP1A2, OATP1B1, OATP1B3, OATP1C1, OATP2B1, OATP3A1 and OATP4A1.[2]

The uptake of taxanes by OATPs has clinical relevance as patients with low or no detectable expression of OATPs in breast cancer tissues may also show a decreased response rate or even no response to docetaxel and paclitaxel. Furthermore, other OATP specific drugs concomitantly prescribed during breast cancer therapy may interfere with the uptake of taxanes leading to transporter-mediated drug-drug interactions. Clarithromycin, erythromycin and roxithromycin, for example, inhibited the uptake of sulfobromophthalein and pravastatin in OATP1B1 and OATP1B3-transfected HEK293 cells ($IC_{50}$ values of 32–37 μM)[20] whereas cyclosporin A and rifampicin significantly decreased the OATP1B1 and OATP1B3-mediated uptake of bosentan[21] and fexofenadine[22] in HEK293 and CHO cells with $IC_{50}$ values below their effective plasma concentrations in humans. Additionally, Noé et al. observed that the OATP1B1 inhibitor gemfibrozil potently reduces the uptake of fluvastatin, pravastatin and simvastatin.[7] It is also reported that testosterone inhibits the OATP2B1-mediated uptake of estrone-3-sulfate (E3S) and dehydroepiandrosterone sulfate (DHEAS), whereas progesterone stimulates the E3S and DHEAS uptake.[23] A recent study also demonstrated that there might be potential functional interaction between OATP2B1 and the breast cancer resistance protein ABCG2 in transepithelial transport of E3S in the human placenta[24] which may also apply for human breast cancer tissue. Beside clinically used drugs, also naturally occurring flavonoids interfere with the OATP-uptake of DHEAS, indicating that they are a novel class of OATP1B1 modulators.[25] Whether all of these potential OATP dependent inhibitors interfere with the taxane uptake in tumor cells is not yet known, however, care should be taken in patients using these drugs in combination with docetaxel and paclitaxel. Ongoing studies are meant to verify the importance of all seven OATPs expressed in breast cancer tissue samples for paclitaxel and docetaxel uptake.

Taken together, our results elucidate the OATP expression in breast cancer cell lines and tissues. Still to be evaluated is whether inter-individual differences in OATP expression may explain altered response rates in breast cancer patients after therapy with OATP substrates.

## Materials and Methods

**Patient samples.** Thirteen samples of breast tumorous and normal tissue were obtained from patients undergoing routine surgery for breast tumors. Informed consent was obtained from all patients and permission for the study was obtained from the Ethical Committee of the Institution.

Hospital tumor history records were used to obtain information regarding stage at diagnosis, tumor grade, as well as hormone receptor and HER-2 status. All patients were between 48 and 69 years of age at the time of diagnosis. Clinical data of breast cancer patients and histological characteristics of their tumors are summarized in Table 1.

**Cell culture.** MCF-7, MDA-MB-231, ZR-75-1 and MCF-10A cell lines were purchased from the American Tissue Culture Collection (LGC Promochem, Wesel, Germany) and were incubated in a humidified atmosphere at 37°C with 5% $CO_2$/95% air. MCF-7, MDA-MB-231 and ZR-75-1 cells were grown in phenol-red free RPMI 1640 medium (PAN Biotech, Aidenbach, Germany) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics (100 U of penicillin/ml and 100 µg of streptomycin/ml; Invitrogen, Paisley, United Kingdom). MCF-10A cells were maintained according to the supplier's directions.

**RNA extraction.** Total RNA was extracted from tissue samples and cell lines using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The concentration, purity and integrity of RNA samples were determined by UV absorbance and electrophoresis.

**Quantitative real-time RT-PCR.** We reverse transcribed 2 µg of total RNA to cDNAs using random hexamer primers and the RevertAid™ H Minus M-MuLV Reverse Transcriptase system (Fermentas, St.Leon-Rot, Germany) as recommended by the manufacturer. We purchased TaqMan® Gene Expression Assays (Applied Biosystems, Warrington, United Kingdom) for all 11 human OATPs. In order to evaluate an appropriate reference gene, we analyzed 13 different human housekeeping genes for tissue samples and cell lines and selected β-actin as an acceptable reference gene for the tissue samples and 18S as housekeeping gene for the cell lines using a geNorm kit (PrimerDesign Ltd., South-Hampton, United Kingdom). 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 2X TaqMan® Universal PCR Master Mix, 1 µl of the appropriate Gene Expression Assay, 1 µl TaqMan®, endogenous control (human β-actin or 18S assay, respectively), 10 ng template cDNA diluted in 5 µl nuclease free water, and 3 µl nuclease free water. Thermal cycling conditions comprised two minutes at 50°C, 10 min at 95°C followed by 40 cycles of 15 s at 95°C and one minute at 60°C. Fluorescence generation due to TaqMan® probe cleavage by the 5'→3' exonuclease activity of the DNA polymerase 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 that included our amount of target mRNA, a standard curve of six serial dilutions from 50 ng to 500 pg of pooled cDNA was analyzed by Sequence Detection Software (SDS 1.9.1., Applied Biosystems). Results were imported into Microsoft Excel for further analysis. Comparable cDNA amounts 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. The tissue sample with the lowest detectable target gene expression was arbitrarily applied as the calibrator and obtained results were calculated in relation to the calibrator's expression level. In the case of cell lines, we chose the non-malignant MCF-10A cell line as calibrator to calculate relative expression levels.

Quantitative real-time RT-PCR was performed with the following prefabricated TaqMan® Gene Expression Assays (Applied Biosystems) containing intron-spanning primers: OATP1A2: Hs00245360_m1, OATP2B1: Hs00200670_m1, OATP1B1: Hs00272374_m1, OATP1B3: Hs00251986_m1, OATP1C1: Hs00213714_m1, OATP2A1: Hs00194554_m1, OATP3A1: Hs00203184_m1, OATP4A1: Hs00249583_m1, OATP4C1: Hs00698884_m1, OATP5A1: Hs00229597_m1, OATP6A1: Hs00542846_m1, and the endogenous control's β-Actin: Part # 4326315E and 18S Part # 4310893E.

**Statistical analyses.** Comparisons of significant differences in the expression of OATPs between breast cancer cell lines and the non-malignant control were performed using an unpaired t-test. A paired t-test was used to calculate different OATP expressions between breast tumorous and non-malignant tissues. The expression of identified OATPs was compared with patient's age, tumor size, hormonal receptor, and HER-2 status; and the significance of associations was determined with the Mann-Whitney U-test. Significance was defined as $p < 0.05$.



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**DIFFERENT EXPRESSION PATTERNS OF ORGANIC ANION TRANSPORTING POLYPEPTIDES IN OSTEOSARCOMAS, BONE METASTASES AND ANEURYSMAL BONE CYSTS**

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# Different expression patterns of organic anion transporting polypeptides in osteosarcomas, bone metastases and aneurysmal bone cysts

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**Abstract.** Organic anion transporting polypeptides (OATP) were identified as transmembrane transporters for various endo- and exogenous organic compounds (hormones, prostaglandins, anticancer drugs). OATP expression had been shown in different tissues, but not in bone tumors. Therefore, the expression pattern of all known eleven human OATPs was analyzed by quantitative RT-PCR in 21 human bone tumor specimens (osteosarcomas, bone metastases and benign aneurysmal bone cysts). Transcriptional expression of OATP1A2, 1C1, 2A1, 2B1, 3A1, 4A1, 4C1 and 5A1, but not of OATP1B1, 1B3 and 6A1 was observed in malignant and non-malignant tumor specimens at varying level. Importantly, OATP3A1, 4A1, 2B1 and 1C1 mRNA levels were significantly higher in aneurysmal bone cysts as compared to osteosarcomas. Elevated mRNA levels of OATP2A1, 1A2, and 4C1 in metastases from kidney cancer and of OATP5A1 in prostate cancer suggest that the OATP expression pattern in metastases is comparable to that of the primary tumors. Different to tissue, OATP expression in osteosarcoma cell lines HOS and MG-63, normal human osteoblast outgrowth cells (hOB) and bone marrow stromal cells (BMSC) is limited to OATP3A1 and OATP4A1. High OATP expression levels, particularly in benign bone tumors, suggest an important role of these transporters for providing hormones, their conjugates, prostaglandins and drugs in bone cells. Thereby, they may influence bone resorption and formation.

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*Key words:* organic anion transporting polypeptide, drug uptake, bone tumor, multidrug-resistance

## Introduction

Osteosarcomas arising from osteoblasts are among the most frequent solid tumors in teenage cancer patients and in young adults. Surgical resection of lesions combined with radiotherapy, neoadjuvant and adjuvant chemotherapy greatly improved the prognosis of these malignant tumors, particularly, if diagnosis is made at an early stage. Current chemotherapy regimens for all stages include standard anticancer drugs, e.g. methotrexate and doxorubicin in combination with other anticancer drugs (1). In young adults, osteosarcomas, but also benign tumors, e.g. the cartilage-derived chondroma, are often associated with the formation of osteolytic aneurysmal bone cysts, caused by hemodynamic disturbances, post-traumatic events, and reactive vascular malformation. However, aneurysmal bone cysts are also found as primary tumors with a genetical predisposition (2). In older adults, the most common malignant bone tumors are bone metastases derived from primary solid tumors in prostate, breast, kidney and lung. For these bone malignancies, the standard therapy regimen is based on the systemic chemotherapy for the primary tumor (3).

Despite recent progress in the treatment of bone malignancies, the success of a given therapy with respect to an improvement in the quality of life and overall survival, is still hampered by intrinsic or acquired resistance of bone tumor cells to a broad number of anticancer drugs (multidrug resistance, MDR) (4,5). A well established mechanism for MDR is overexpression of efflux transporters such as ABCB1 (P-glycoprotein, Pgp), which can be induced by activation of the chemosensitizing pregnane X receptor (PXR) through many drugs and xenobiotics. In tumor cells, an enhanced efflux of Pgp substrates such as doxorubicin causes an insufficient intracellular drug accumulation leading to a reduced cytotoxic effect (6). While induction of MDR by ABC-efflux pumps is thoroughly studied, less is known about transporters for the uptake of anticancer drugs into the cells, which may determine net intracellular accumulation and efficacy of drugs (7,8).

In the last decade, it has also became clear that intracellular accumulation of many hormones, prostaglandins and drugs is not simply due to passive diffusion, but evidence is growing that carriers are needed for drug accumulation in certain tissues

(9). Indeed, members of the organic anion transporting polypeptide (OATP) family encoded by the *SLCO* genes were identified as sodium-independent transport proteins for various amphipathic organic compounds such as hormones and their conjugates, prostaglandins and others, recently shown to be important in the regulation of bone homeostasis (10). Also xenobiotics including the anticancer drug methotrexate are OATP substrates.

Eleven OATP family members have been identified in humans (11). Some of these OATPs are highly expressed in excretory organs, e.g. liver, kidney, intestine, but others are found at elevated levels in brain, placenta and testes as well as in malignant tissues and cancer cell lines. For example, OATP1A2, known for its wide substrate specificity, is highly expressed in excretory organs, while elevated levels of OATP1C1, a high-affinity thyroid hormone transporter, are found in brain, testes and kidney (12). The prostaglandin transporter OATP2A1 (13) and OATP2B1, transporting steroid hormones such as dehydroepiandrosterone-sulfate (DHEAS) are expressed in a wide number of tissues (14). Ubiquitous tissue distribution was also shown for OATP3A1 and 4A1, for which hormones, drugs and prostaglandins were identified as substrates (15,16). A more restrictive localization was shown for OATP4C1, a kidney-specific transporter for endobiotics and drugs and for two OATPs, OATP6A1 and 5A1, less characterized for their substrate specificity. OATP6A1 was detected in normal testes, some cancer cell lines and malignant tissues, e.g. oesophagus (17-19), and, only recently in our lab (20), OATP5A1 in breast tumors and breast cancer cell lines.

Often an altered OATP expression pattern is seen in malignant tissues and tumor cells as compared to normal tissue (11). For example, OATP1B1 and OATP1B3 were first regarded as liver-specific (7,8,21) but were later also found in intestinal tumors (22) and breast cancer tissues (20).

The wide tissue distribution of various OATPs in normal and malignant tumor cells suggests that OATPs may also be expressed in bone tumors. In bone cells, their activity could influence the local concentration of e.g. estrogen-conjugates, thyroid hormones and prostaglandin E2 (PGE2), which had been shown to regulate bone resorption and formation (osteolytic and osteoblastic processes) (23-25).

Based on these considerations, we investigated mRNA expression of all eleven OATPs in specimens from human osteosarcoma and compared it to that in bone metastases and benign bone tumors by quantitative TaqMan® RT-PCR. We also assessed whether two osteosarcoma cell lines (HOS and MG-63), normal human osteoblast outgrowth cells (hOB) and bone marrow stromal cells (BMSC) might offer suitable models for further investigations on these transporters in bone cells.

## Materials and methods

*Samples from bone tumor patients*. Patient characteristics are given in Table I. In total, 21 samples from malignant (seven grade 3 osteosarcomas and seven bone metastases from prostate, breast, kidney and lung) and non-malignant bone tumors (six aneurysmal bone cysts and a chondroma, underlying an aneurysmal bone cyst) were analyzed. OATP mRNA expression data for the latter specimen was not incorporated into statistical analysis of OATP mRNA expression in bone tumor groups.

All human tissue samples were obtained during routine surgery at the Department of Orthopaedic Surgery, Medical University of Vienna. Informed consent from all patients was obtained and the study was approved by the Ethics Committee of the institution. Pathological inspection was done by the Department of Clinical Pathology, Medical University of Vienna, Austria.

*Cell lines*. Two human osteosarcoma cell lines, derived from a female (HOS, ATCC CRL 1543) and a male (MG-63, ATCC CRL 1427) teenage osteosarcoma patient were chosen. These cell lines were originally derived from the American Tissue Culture Collection (ATCC, Manassas, VA). Cells were routinely cultured in RPMI-1640 medium supplemented with 10% FCS and 1% penicillin/streptomycin under standard culture conditions.

Human osteoblast outgrowth cells (hOB) were isolated from human cancellous bone obtained during orthopaedic surgery (hip replacement); bone marrow derived stromal cells (BMSC) were isolated from an aspirate of human bone marrow, derived from the superior iliac crest of the pelvis. Both bone cell types were regarded normal and cultured, as described previously (26).

*Expression analysis*. Total RNA was isolated from 21 frozen tissue samples, cancer cell lines grown to subconfluency and cultured hOB and BMSC cells using the TRIzol reagent (Invitrogen Life Technologies, Paisley, Scotland). One microgram of total RNA per sample was reverse transcribed to cDNA using the RevertAid™ H Minus M-MuLV Reverse Transcriptase (Fermentas Life Sciences, Vilnius, Lithuania). The purity of extracted RNA was assessed by determination of the $A_{260}/A_{280}$ ratio and by separation on a 2% agarose gel. To quantify the expression of SLCO/OATP, Pgp and PXR mRNA, multiplex realtime (RT)-PCR, using TaqMan gene expression assays (Applied Biosystems, Foster City, CA) was performed, as described previously (20). Following TaqMan assays were used: Hs00245360_m1 (OATP1A2), Hs00272 374_m1 (OATP1B1), Hs00251986_m1 (OATP1B3), Hs00213714_m1 (OATP1C1), Hs00194554_m1 (OATP2A1), HS00200670_m1 (OATP2B1), Hs00203184_m1 (OATP3A1), Hs00249583_m1 (OATP4A1), Hs00698884_m1 (OATP4C1), Hs00229597_m1 (OATP5A1), Hs00542846_m1 (OATP6A1), IIs00184500_m1 (Pgp), IIs00243666_m1 (PXR). All samples were tested in triplicates, using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Thermal cycling conditions comprised 2 min at 50˚C, 10 min 95˚C followed by 40 cycles of 15 sec at 95˚C and 1 min at 60˚C. Sequence Detection Software (SDS 1.9.1, Applied Biosystems) results were imported into the software Q-gene for further processing and calculation of mean normalized expression (MNE) levels according to the equation: $MNE = (E_{reference})^{CT_{reference, mean}}/(E_{target})^{CT_{target, mean}}$, where $E_{target}$ and $E_{reference}$ = efficiency of PCR amplification of the target and the reference, respectively; $CT_{target, mean}$ and $CT_{reference, mean}$ are calculated means for the target and the reference, respectively (27). RT-data were normalized to the most stably expressed housekeeping gene, cytochrome C-1 (CYC1), identified using

Table I. Characteristics of tumor patients and tumors.[a]

| Osteosarcomas | # | Type | Age | Gender | Location |
|---|---|---|---|---|---|
| | 1 | Osteoblastic | 16 | M | Distal femur (r) |
| | 2 | Osteoblastic/anablastic | 15 | M | Proximal humerus (l) |
| | 3 | Osteoblastic/chondroblastic | 16 | M | Proximal tibia (r) |
| | 4 | Osteoblastoma-like | 34 | F | Proximal femur (r) |
| | 5 | Chondroblastic | 53 | F | Distal femur (r) |
| | 6 | Osteoblastic | 15 | F | Proximal humerus (r) |
| | 7 | Parosteal | 36 | F | Distal femur (r) |
| Metastases | # | Primary tumor | Age | Gender | Location |
| | 8 | Prostate | 83 | M | Femoral head |
| | 9 | Prostate | 74 | M | Humerus (r) |
| | 10 | Mamma | 36 | F | Femoral neck |
| | 11 | Mamma | 65 | F | Femur (l) |
| | 12 | Kidney | 54 | M | Femoral head |
| | 13 | Kidney | 65 | M | Femoral head |
| | 14 | Lung | 58 | M | Os ilium |
| Benign tumors | # | Type of lesion | Age | Gender | Location |
| | 15 | Aneurysmal bone cyst | N/A | F | N/A |
| | 16 | Aneurysmal bone cyst | 11 | M | Humerus (l) |
| | 17 | Aneurysmal bone cyst | 12 | F | Os ilium (l) |
| | 18 | Aneurysmal bone cyst | 19 | M | Acetabulum |
| | 19 | Aneurysmal bone cyst | 58 | M | Femoral neck |
| | 20 | Aneurysmal bone cyst | 17 | M | Femur (l) |
| | 21 | Ahondroma | 19 | M | Os sacrum |

[a]All osteosarcoma specimens were derived from histological grade (G) 3 tumors. All osteosarcoma patients received neoadjuvant chemotherapy according to the COSS regimen (33).

the geNorm™ housekeeping gene selection kit (PrimerDesign Ltd.). The following intron-spanning primers and the TaqMan probe for CYC1 were designed using the software Primer Express™ V1.5 (Applied Biosystems): CYC1-f. p.: 5'-GTT TGA CGA TGG CAC CCC AG-3'; r. p.: 5'-CTT GAG CCC CAT GCG TTT T-3'; TaqMan probe: Tamra-5'-CCC AGA TAG CCA AGG ATG TGT GCA CCT-3'-Blackhole quencher 2.

*Data analysis*. RT-PCR data were statistically analyzed by one-way ANOVA; P-values of ≤0.05 were considered statistically significant.

## Results

*Transcriptional expression of OATPs in human bone tumor specimens*. Quantitative RT-PCR was used to determine the mRNA expression pattern of all eleven OATPs in samples from malignant and non-malignant bone tumors. As demonstrated in Fig. 1, eight OATPs, namely OATP1A2, 1C1, 2A1, 2B1, 3A1, 4A1, 4C1 and 5A1 were detected in a collective of 21 bone tumor specimens, consisting of seven poorly differentiated osteosarcomas (specimens #1-7), seven bone metastases (specimens #8-14) from solid tumors in the prostate, breast, kidney (n=2, each) and lung (n=1) and seven specimens from benign bone lesions (specimens #15-21). Data for individual patients are given in Table I: Eight specimens were from female, 13 from male patients with a female to male ration of 4/3 in osteosarcoma, 2/5 in metastases and 2/5 benign bone tumors, respectively. Osteosarcoma and benign bone tumor patients were of similar age: mean age: 26.4±14.9 and 22.7±17.6 years, respectively, while patients in the metastatic tumor group were older: mean age 62.1±15.0 years (P≤0.05).

In all tumor samples, mRNA expression of three transporters, OATP1C1, 2A1 and 4A1, was present at a considerable level. For two other transporters, namely OATP2B1 and OATP3A1, mRNA expression was found in all but one sample. While OATP2B1 was undetectable in a prostate cancer metastasis (#8), OATP3A1 mRNA levels were below the detection limit in a metastasis from breast cancer (#11).

As demonstrated in Fig. 1A and Table II, mean OATP mRNA expression levels of four transporters (OATP1C1, 2B1, 3A1, and 4A1) were 2-3-fold higher (P<0.001-0.05) in the aneurysmal bone cyst group than in the osteosarcoma group.

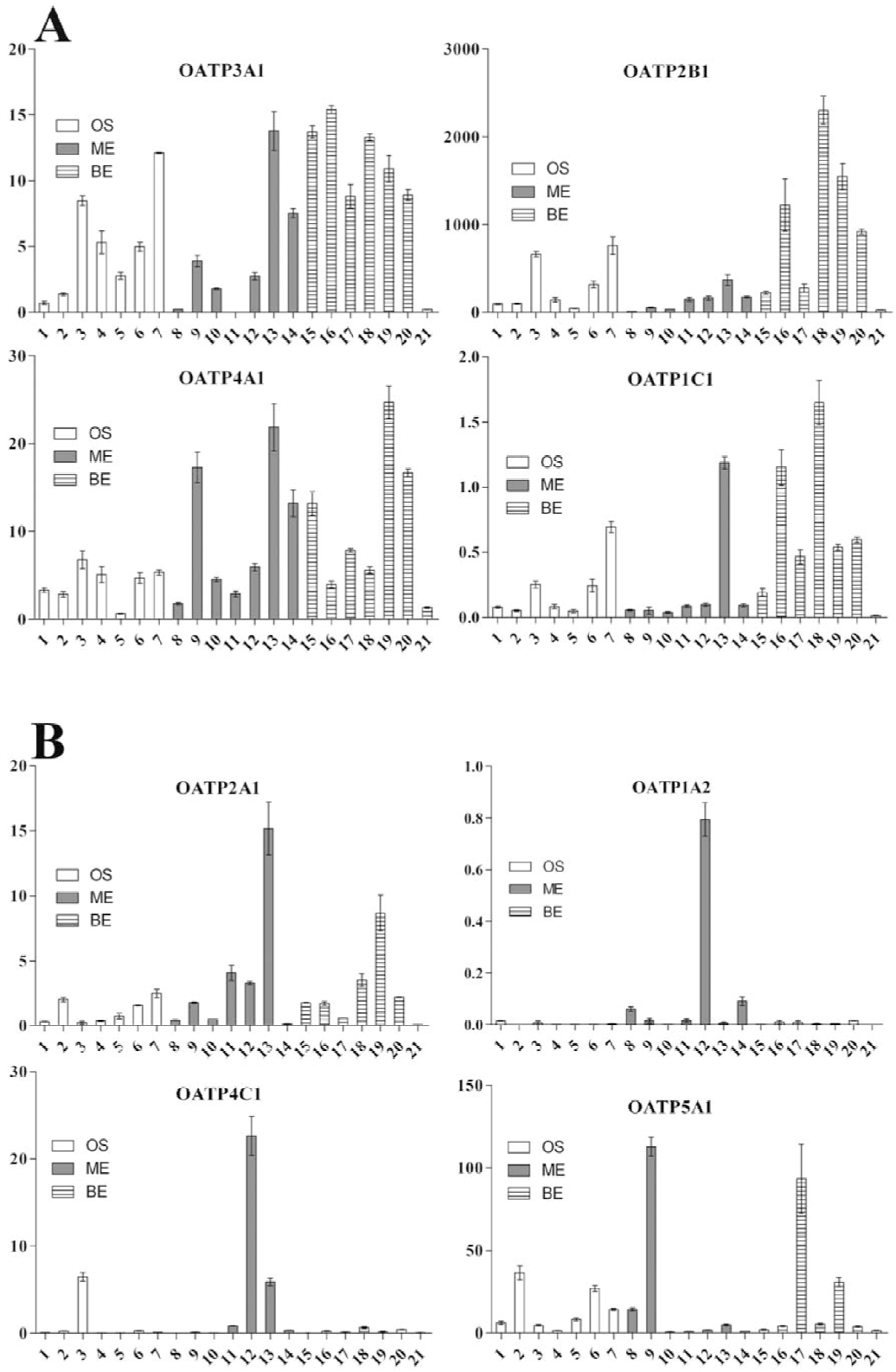


Figure 1. OATP mRNA expression in human bone tumor specimens. mRNA expression of 11 OATPs was assessed in specimens from osteosarcomas (OS; #1-7), metastases (ME) from prostate (#8,9), breast (#10,11), kidney (#12,13) and lung (#14) tumors and benign bone tumors (BE; aneurysmal bone cyst: #15-20, chondroma: #21*); mRNA expression of eight OATPs, namely OATP1A2, 1C1, 2A1, 2B1 (A), 3A1, 4A1, 4C1 and 5A1 (B) was detected, while other OATPs (OATP1B1, 1B3 and 6A1) were below the detection limit in all specimens. Expression levels are given as mean normalized expression (MNE), calculated as described in the Materials and methods section.

Table II. Mean MNE (mean normalized expression) levels of OATP-, Pgp- and PXR-mRNA (mean ± SD) in bone tumor specimens.

| | Osteosarcoma | Bone metastases | Aneurysmal bone cysts |
|---|---|---|---|
| OATP1A2 | 0.004±0.005 | 0.14±0.29 | 0.006±0.006 |
| OATP1C1 | **0.21±0.23**[a] | 0.23±0.42 | 0.66±0.57 |
| OATP2A1 | 1.10±0.92 | 3.63±5.31 | 2.65±2.87 |
| OATP2B1 | **300.9±295.1**[a] | **134.29±122.99**[b] | 929.8±825.9 |
| OATP3A1 | **5.11±4.1**[b] | **4.28±4.90**[b] | 10.18±5.04 |
| OATP4A1 | **4.07±2.01**[a] | 9.63±7.83 | 10.46±8.22 |
| OATP4C1 | 1.04±2.40 | 4.27±8.37 | 0.25±0.22 |
| OATP5A1 | 14.11±12.97 | 19.55±41.41 | 20.23±33.86 |
| Pgp | 3.93±3.87 | 3.50±6.82 | 3.14±2.68 |
| PXR | 1.36±0.92 | 0.92±0.71 | 1.01±0.63 |

mRNA expression of different OATPs, Pgp and PXR was analyzed using TaqMan® gene expression assays as described in the Materials and methods section. Mean normalized expression levels were calculated using the software Q-gene; statistically significant differences were calculated by one-way ANOVA: [a]P<0.05 vs. bone cysts group; [b]P<0.01 vs. bone cysts group. Bold, significantly higher expression levels in benign vs. malignant tumors.

For OATP2B1 and 3A1 mRNA expression levels, significant differences (P<0.001) were additionally found between aneurysmal bone cysts and the metastatic bone tumor group. Up to 6-fold higher levels were seen for OATP2B1 in aneurysmal bone cysts as compared to metastases, while for OATP3A1 differences were ~2-fold.

Generally, OATP mRNA expression levels, given as mean normalized expression (MNE), varied in individual patients (Fig. 1A and B). Only for OATP3A1and 4A1 mRNA expression, a more uniform pattern was observed. Similar levels with an up to 20- and 30-fold enrichment were observed in individual samples, particularly in the benign bone cyst group. Highest mRNA expression levels of all OATPs detected in bone tumors were seen for OATP2B1 which showed a maximal enrichment of 2.5-fold in an aneurysmal bone cysts (#18). OATP1C1 mRNA expression levels were generally low and reached a maximal enrichment of only 1.8-fold, also found in sample #18. Despite the enormous differences in the expression levels, the MNE pattern for these two OATPs were similar in individual specimens from malignant and benign primary bone tumors.

As indicated in Fig. 1B and Table II, for OATP2A1, mean mRNA expression levels were higher in some aneurysmal bone cyst and metastases samples, but mean MNEs did not differ significantly between the groups. Highest (16-fold) expression was found in the kidney metastasis (#13). For three other transporters, OATP1A2, 4C1 and OATP5A1, mRNA expression levels were particularly high in kidney (OATP1A2 and 4C1 in specimens #12 with a 0.8-fold and 22-fold enrichment) and prostate (OATP5A1 in specimen #9 with a 110-fold enrichment) metastases. While OATP4C1 mRNA was detected only in one of the osteosarcoma specimens (#3), OATP5A1 mRNA was additionally found in a number of aneurysmal bone cysts and osteosarcoma specimens.

For three other OATPs, namely OATP 1B1, 1B3 and 6A1, mRNA expression levels were below the detection limit in all bone tumor specimens and cells investigated.

*OATP expression in osteosarcoma cell lines and in normal hOB and BMSC.* To compare the OATP expression pattern in malignant bone tumor samples with that in isolated bone cells, we studied OATP mRNA expression in malignant MG-63 and HOS osteosarcoma cell lines, originating from a male and a female patient. As a model for normal bone cells, we used normal human osteoblast outgrowth cells (hOB) and did additional studies in bone marrow stromal cells (BMSC).

As shown in Fig. 2, mRNA expression of only three OATPs, namely OATP1A2, 3A1 and 4A1, was detected in the HOS and MG-63 cells at MNE levels similar to that in the bone tumors. OATP1A2 and 4A1 mRNA expression were even higher (~1.4- and 1.7-fold) in MG-63 cells than in HOS cells.

In hOB and BMSC, only OATP3A1 and OATP4A1 mRNA were expressed at similar levels in both cell types. However, in hOB and BMSC cells, OATP3A1 levels were 5-6 times higher than in the osteosarcoma cell lines. In contrast to bone tumors, OATP4A1 mRNA expression levels were 5-6 times lower in normal cells as compared to the osteosarcoma cell lines.

*Expression of Pgp and PXR mRNA in bone tumors and bone cells.* We also assessed whether mRNA expression of ABCB1 coding for the MDR efflux pump Pgp, and for the nuclear receptor PXR, might be correlated with a specific OATP pattern in malignant and non-malignant bone tumors and in bone cell lines.

As demonstrated in Fig. 3, Pgp mRNA expression was detected in all osteosarcoma specimens, bone metastases and benign bone tumors. As shown in Table II, mean MNEs were similar in aneurysmal bone cysts as compared to metastases and osteosarcomas. Highest Pgp mRNA expression levels were found in a renal cancer metastasis (#12) and in an osteosarcoma (#1) specimen (MNEs: 18- and 11-fold, respectively). On the other hand, Pgp mRNA expression was near the detection limit in the metastases from prostate (#8) and breast (#10) cancer as well as in the chondroma (#21) specimen.

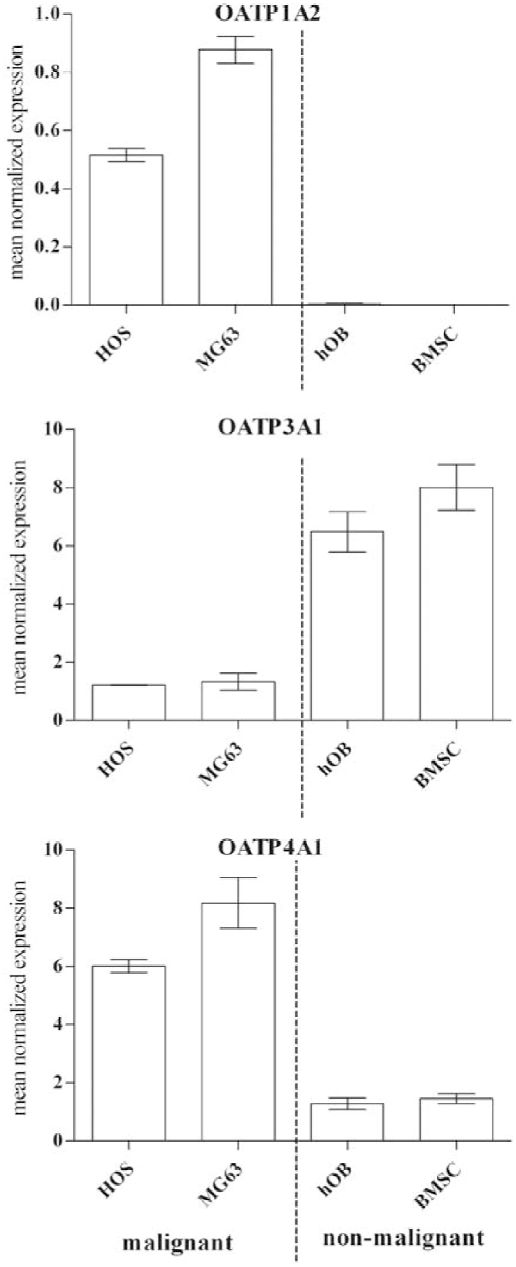


Figure 2. OATP mRNA expression in malignant and non-malignant bone cells. mRNA expression of 11 OATPs was assessed in the osteosarcoma cell lines HOS and MG-63 and in the normal human bone marrow derived osteoblast-outgrowth cells (hOB) and bone marrow stromal cells (BMSC) by quantitative PCR; mRNA expression of three OATPs, namely OATP1A2, 3A1 and 4A1 was detected, while other OATPs (OATP1B1, 1B3, 1C1, 2A1, 2B1, 4C1, 5A1 and 6A1) were below the detection limit in all cell lines and bone cells.

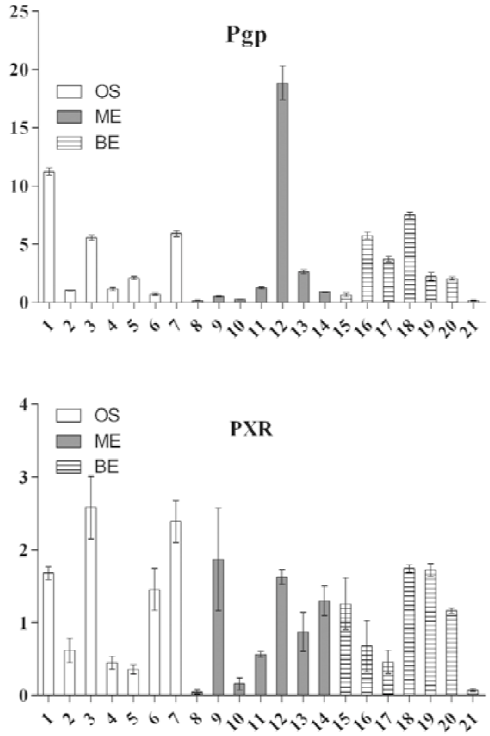


Figure 3. Pgp and PXR mRNA expression in human bone tumor specimens. mRNA expression of the MDR efflux pump P-glycoprotein (Pgp) and the nuclear receptor PXR was assessed in specimens from osteosarcomas (OS; #1-7), metastases (ME) from prostate (#8,9), breast (#10,11), kidney (#12,13) and lung (#14) tumors and benign bone tumors (BE; aneurysmal bone cyst: #15-20, chondroma: #21*). Pgp mRNA was detected in all specimens, with highest expression levels in a metastasis from renal cancer (#12) and an osteosarcoma specimen (#1).

Contrary to bone tumor specimens, Pgp mRNA expression levels were below the detection limit in osteosarcoma cell lines, hOB and BMSC.

Similarly to Pgp, PXR mRNA expression levels did not differ between the bone tumor groups. Maximal enrichment of PXR mRNA in individual samples was lower than that of Pgp. Up to 2.8-fold higher PXR mRNA levels were found in two osteosarcoma specimens, one from a responder to chemotherapy (#3) and one from a non-responder (specimen #7).

In the two osteosarcoma cell lines, however, PXR mRNA expression was also near the detection level, while that in normal hOB and BMSC were similar to that in bone tumors (~0.9-fold).

## Discussion

This is the first demonstration of transcriptional expression of eight of eleven known human OATPs, namely OATP1A2, 1C1, 2A1, 2B1, 3A1, 4A1, 4C1 and 5A1, in human bone tumors. Most widely expressed were OATP 3A1 and 4A1, which were found at considerable levels in nearly all bone tumor specimens, HOS and MG-36 osteosarcoma cell lines, normal hOB cells and BMSCs. Osteosarcoma cell lines (MG-63 and HOS) and normal bone-derived cells (hOB and BMSC) are therefore not suitable models for studying OATP-mediated transport processes as OATP expression levels were considerably lower compared to bone tumor samples.

We found differences in the expression pattern between non-malignant and malignant bone tumors, as mRNA expression levels of OATP3A1, 4A1, 2B1 and 1C1 were significantly higher in the bone cyst group than in the osteo-

sarcoma group. This is in accordance with previous data from our lab (20), showing that OATP3A1, 4A1 and 2B1 are higher expressed in benign vs. malignant breast tissue. Ancona *et al* (28) also found higher OATP4A1 levels in normal vs. malignant colon suggesting that OATP3A1, 4A1, 2B1 and possibly, also 1C1 may play an important role in providing endogenous OATP substrates e.g. hormones to cells. For example, thyroid hormones are substrates for many OATPs, e.g. OATP3A1, 4A1, and OATP1C1 (12), which have the highest affinity for these hormones. We found high levels of OATP1C1 in osteolytic aneurysmal bone cysts and kidney metastases, which would fit with the bone resorptive effect of thyroid hormones (29). On the other hand, this OATP was also highly expressed in the osteoblastic prostate metastases.

Another important substrate is estrone-sulfate, which is present at high levels in plasma. It could be taken up into bone cells by OATP2B1 and other OATPs, e.g. 3A1. In bone cells, it is further metabolized to 17ß-estradiol (E2) by estrogen-metabolizing enzymes (26) providing the active estrogen. Furthermore, OATP2B1 also transports DHEAS, which is a precursor for E2. Indeed, E2 derived from DHEAS was found to stimulate bone formation (30).

Prostaglandins, e.g. PGE2, are substrates for many OATPs, e.g. OATP1A3 and 4A1, but OAT2A1, highly expressed in many organs, e.g. brain, colon, kidney, has the highest specificity for these mediators (31). PGE2 is not only important for tumor development, but it is also a potent inducer of osteolysis (25). Although biological effects of PGE2 are mediated by binding to its plasma membrane receptors, transport of PGE2 into the cell by OATP2A1 leads to rapid inactivation by oxidation in the cell interior (13).

Our data show, particular high OATP2A1 levels in an osteolytic kidney metastasis. This seems to reflect the function of OATP2A1 in the primary tumor of the kidney, where its expression is regulated by electrolytes, e.g. high sodium (32).

We further demonstrated in this pilot study that the 'kidney-specific OATP4C1' (18) is almost exclusively expressed in kidney metastases, however, low levels are also found in prostate and lung metastases. This further suggests that the OATP expression pattern in metastases, at least in that from renal cancer, is comparable to that of the primary tumor. Whether OATPs might be used as biomarkers to define the primary tumor of metastases is not known yet and has to be investigated. In breast cancer metastases, however, OATP expression did not correlate with primary tumor as OATP2B1 and 4A1, considerably expressed in breast cancer tissue (20), were found only at low expression levels in the breast metastases.

In our study, all osteosarcoma patients had received neoadjuvant chemotherapy based on doxorubicin and methotrexate (33). On the other hand, from the OATPs previously found to transport methotrexate (OATP1A2, 1B1, 1B3, 4C1), only OATP 4C1 was expressed at a considerable level in the osteosarcoma specimen #3, derived from a responder to chemotherapy. This suggests that other not yet identified transporters could also be important for the cellular uptake of methotrexate into osteosarcoma cells. This might also apply for the uptake of doxorubicin.

In addition to the OATP-mediated uptake, net cellular accumulation of drugs and endogenous compounds is determined by their efflux from the cells. So did recent data showing that doxorubicin used for treatment of osteosarcomas could also induce overexpression of ABC-efflux transporters such as ABCB1 (Pgp). Our data, however, could not show any induction of Pgp in samples from doxorubicin-treated patients vs. untreated aneurysmal bone cysts patients.

Gender, tumor localization and age do not seem to be related to OATP and Pgp expression. Furthermore, the OATP expression pattern in individual samples did not correlate with Pgp and PXR, although transcriptional regulation of OATP1A2 and 2B1 by PXR activation was previously shown in liver (34).

In summary, our data revealed that 8 out of 11 OATPs (OATP1A2, 1C1, 2A1, 2B1, 3A1, 4A1, 4C1 and 5A1) are expressed in human bone tumors with differences in the expression levels between osteosarcomas, bone metastases and benign bone tumors. The distinct pattern of expression indicates that OATPs could be important to provide important regulators of bone homeostasis and affect tumor growth and progression. Additionally, OATP-mediated uptake of certain anticancer drugs may also influence the efficacy of chemotherapy regimens in which OATP substrates are applied.

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**EXPRESSION OF SULFOTRANSFERASES AND SULFATASES IN HUMAN BREAST CANCER: IMPACT ON RESVERATROL METABOLISM**

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# Expression of sulfotransferases and sulfatases in human breast cancer: Impact on resveratrol metabolism

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ABSTRACT

Resveratrol is a naturally occurring anticancer compound present in grapes and wine that undergoes pronounced metabolism in human intestine and liver. In order to determine whether resveratrol is also bio-transformed in human breast carcinoma, metabolism experiments were conducted in breast tumor and adjacent non-tumorous specimens from 13 patients. Resveratrol was metabolized in cytosolic tissue fractions to resveratrol-3-*O*-sulfate: the formation rates were up to 33.5-fold higher in cancer samples than in peritumoral tissue. Further quantitative real-time RT-PCR analysis revealed similar expression of sulfotransferases *SULT1A2*, *1A3*, and *1E1* in the paired control and tumor tissues. Sulfotransferase *SULT1A1* expression was below the detection limit in all samples. Interestingly, mRNA expression of steroid sulfatase *STS*, but not of arylsulfatases *ARS-A* and *ARS-B*, was significantly higher ($p < 0.0017$) in non-malignant specimens than in tumor tissue samples, which might explain the higher resveratrol-3-O-sulfate concentrations in breast cancer specimens. Cellular localization of SULT1A3 and STS was also assessed by indirect immunofluorescence on paraffin-embedded sections from control and malignant breast tissue clearly showing a correlation of qRT-PCR data with protein expression of these two enzymes. Our data elucidate the metabolism of resveratrol in malignant and non-malignant breast tissue, which must be considered in humans after oral uptake of dietary resveratrol as a chemopreventive agent.



## 1. Introduction

Breast cancer is a major cause of cancer death in women worldwide. Chemoprevention in combination with anticancer treatment is therefore important to reduce morbidity and mortality. Evidence from epidemiological and experimental studies indicates that natural constituents of the diet may well act as chemopreventive agents and inhibit mammary carcinogenesis [1]. One of such compounds is resveratrol (3,4′,5-trihydroxy-*trans*-stilbene), which is produced by several plants, berries, fruits and is mainly found in the skin of grapes and red wine [2]. The antiproliferative property of resveratrol has been demonstrated *in vitro* against hormone-dependent and hormone-independent breast cancer cells due to the induction of apoptosis via down-regulation of NF-kappa B, Bcl-2, inhibition of ribonucleotide reductase, and DNA polymerase [3]. In addition, resveratrol is an excellent antioxidant and radical scavenger, a fact that plays a crucial role in the anticancer effects of stilbene derivates [4]. Resveratrol was also shown to inhibit cyclooxygenases (COX-1, COX-2) [5], which partly explains why this compound also reduces the occurrence of colon cancer and breast cancer. In addition to these *in vitro* data, recent experiments have showed significantly less tumor growth in human

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breast cancer xenografts *in vivo*, supporting the use of this polyphenol as a potential chemotherapeutic agent. Furthermore, a recent human study analyzing the relationship between dietary intake of resveratrol and breast cancer risk using data from a case control-study on 369 cases and 602 controls also showed a significant inverse association for this polyphenol [6]. Interestingly, resveratrol is active against tumor cells but has not shown any organ-specific cytotoxicity in animal models [7].

Data from our laboratory revealed that resveratrol is metabolized in the human breast cancer cell lines MDA-MB-231 and ZR-75–1 to resveratrol-3-*O*-sulfate, dependent on sulfotransferase (*SULT*) *1A1* expression [8]. Using recombinant enzymes we were also able to show that sulfation of resveratrol is catalyzed by SULT1A1 and to a lesser extent by SULT1A2, 1A3 and 1E1 [9].

Only a few studies conducted thus far have been focused on *SULT* expression in breast cancer tissues. While Suzuki and coworkers demonstrated the expression of *SULT1E1* by real-time PCR in breast cancer tissues derived from laser capture microdissection [10], a study conducted by Aust et al. revealed the expression of *SULT1A1*, *SULT1A2*, *SULT1A3*, and *SULT1E1* in malignant and non-malignant breast tissue using conventional RT-PCR [11]. Expression of SULT1A1 and SULT1A3, but not SULT1E1, was confirmed in mammary tissue-derived cytosol from 24 healthy persons by Western blot analysis [12].

Whether resveratrol is also sulfated in breast tumor is not yet known. The aim of the present study was to investigate the metabolism of resveratrol in malignant human breast cancer specimens and its adjacent non-cancerous tissue. Adjacent breast tissues were selected as reference for human breast cancer samples because the activity and expression of drug-metabolizing enzyme systems may vary greatly between individuals due to genetic, environmental and physiological factors [13]. A further aim of the study was to identify the chemical structure of isolated biotransformation products by liquid chromatography/mass spectrometry (LC/MS) and NMR. Furthermore, the mRNA expression of the major isoenzymes responsible for the observed metabolism and the corresponding hydrolytic enzymes in human breast cancer in comparison to normal tissue were investigated by quantitative real-time PCR and correlated with protein expression on paraffin-embedded sections from the same patients as measured by indirect immunofluorescence.

Our results demonstrated that resveratrol sulfation was significantly higher in breast cancer specimens compared to non-malignant samples as a result of significantly higher expression of *STS* in control tissue specimens. This information is important as intracellular biotransformation may affect anticancer activity of resveratrol.

## 2. Materials and methods

### 2.1. Materials

Resveratrol (3,4′,5-trihydroxy-*trans*-stilbene), sulfatase type V: from Limpets (Patella vulgata), adenosine-3′-phosphate-5′-phosphosulfate (PAPS, 78% purity) and dithiothreitol (DTT) were obtained from Sigma–Aldrich (Munich, Germany). Methanol and water were of HPLC grade and purchased from Merck, Darmstadt, Germany. The Pierce Micro BCA Protein Assay Reagent Kit (Rockford, IL, USA) was used to determine protein content. All other chemicals and solvents were commercially available, of analytical grade and used without further purification.

### 2.2. Patient samples

Breast tumor and surrounding tumor-free tissue samples were obtained from 13 female breast cancer patients (aged between 48 and 69 years; 59.9 ± 7.8 years, mean ± SD) who had undergone routine surgery for breast tumor at the Kaiser-Franz-Josef-Spital, Vienna, Austria. Half of each sample was snap-frozen and stored at -80C until required for metabolism assays and for RNA extraction while the rest was subjected to histological examinations. Histopathological examinations were done to differentiate normal and malignant tissues. Paraffin-embedded specimens were used for the investigation of SULT1A3, and STS localization.

Informed consent of all patients, histopathological and clinical data were provided. The permission for the study was obtained from the Ethical Committee of the Institution. None of the breast cancer patients had received chemotherapy prior to surgery or had a previous history of cancer. Clinical data of breast cancer patients and histological characteristics of their tumors are summarized in Table 1.

### 2.3. Preparation of human mamma cytosol

For the preparation of cytosol for metabolism assays, frozen breast tissue was weighed (100–150 mg) and placed in 2-ml tubes on ice that contained tissue disruption beads and the sixfold amount of chilled extraction buffer. The extraction buffer consisted of 50 mM potassium phosphate (pH 7.4) and 10% glycerol. The tissue was lysed at 4 °C with the FastPrep apparatus (Carlsbad, CA) using 2 × 30 s bursts and 1 min cooling between bursts. Samples were centrifuged at 10,000g for 20 min at 4 °C to remove tissue fragments and beads. Cytosol was prepared by subsequent centrifugation of the supernatant at 100,000g for 60 min at 4 °C. Protein concentrations were determined for each sample by the BCA method (Pierce), using bovine serum albumin as the standard.

### 2.4. Metabolism of resveratrol in human breast cancer and adjacent non-cancerous tissue samples

Owing to the high fat content of breast tissues it was difficult to obtain sufficient protein concentrations for analysis. Therefore, the enzyme kinetics of resveratrol sulfation was achieved with 0.5–100 μM resveratrol only for three of 13 breast cancer tissue samples. For the remaining breast cancer and adjacent non-malignant specimens, metabolism was investigated using 1 μM of resveratrol. In addition to resveratrol, the reaction mixture contained 50 μM PAPS, 7.4 mg/ml DTT, 0.05 M potassium phosphate (pH 6.5) and human mamma cytosol (11.36 μg/ml of pro-

**Table 1**
Clinicopathological characteristics of 13 breast cancer patients.

| Patient No. | Histology | Tumor size[a] | Nodal status[a] | ER status | PR status | HER-2 status | Tumor differentiation[b] |
|---|---|---|---|---|---|---|---|
| 1 | ID | T3 | + | – | – | – | G3 |
| 2 | IL | T2 | + | – | – | – | G3 |
| 3 | ID | T1 | + | – | – | – | G2 |
| 4 | ID | T2 | + | + | | | G3 |
| 5 | ID | T2 | + | + | – | – | G3 |
| 6 | ID | T1 | + | + | – | – | G2 |
| 7 | IM | T4 | – | + | – | – | G3 |
| 8 | ID | T2 | – | – | – | – | G3 |
| 9 | IL | T2 | × | + | – | – | G2 |
| 10 | ID | T2 | + | – | + | – | G2 |
| 11 | ID | T1 | – | – | – | – | G2 |
| 12 | ID | T2 | × | + | + | – | G2 |
| 13 | ID | T2 | – | – | + | + | G3 |

[a] According to the tumor, node, metastasis (TNM) classification of the American Joint Committee on Cancer (AJCC). T1: tumor size up to 2 cm; T2: tumor size between 2 and 5 cm; T3: tumor size greater than 5 cm; T4: tumor of any size with skin involvement; ×: could not be evaluated; ER: estrogen receptor; PR: progesterone receptor. ID: invasive ductal carcinoma; IL: invasive lobular carcinoma; IM: invasive mucinous carcinoma.
[b] Histologic tumor grade according to the Bloom–Richardson method [28].

tein) in a final volume of 1000 µl. PAPS and DTT stock solutions were freshly prepared each day. Reactions were initiated by adding PAPS, incubated for 60 min at 37 °C, and terminated by placement of the reaction vessels to −80 °C for 60 min. Next, the lyophilization process was performed for 24 h under vacuum (Christ, ALPHA I/6, Germany), according to the manufacturer's protocol. Once lyophilized, the samples were stored at −80 °C until analysis. Each lyophilized sample was reconstituted at ambient temperature by adding the corresponding volume of HPLC grade methanol (600 µl), mixed and centrifuged at 13,000g for 5 min and 80 µl of the supernatant was injected onto the HPLC column. Control experiments in the absence of PAPS were run in parallel.

### 2.5. Chromatographic conditions

Resveratrol and its sulfates were determined by HPLC as described previously [8]. Calibration of the chromatogram was accomplished using the external standard method. Linear calibration curves were performed from the peak area of resveratrol and resveratrol-3-*O*-sulfate to the external standards resveratrol and resveratrol-3-*O*-sulfate using standard solutions of these compounds to give a concentration range of 0.1 µg/ml to 100 µg/ml. Standard curve correlation coefficients ($r^2$) were $\geqslant 0.99$.

### 2.6. Structural identification of resveratrol metabolites

After a 60-min incubation of human mammary cytosol, 2 µl of sulfatase (20 U) was added to the corresponding reaction mixtures and further incubated at 37 °C for 24 h. The reactions were stopped by the addition of methanol and the samples analyzed as mentioned above. Control samples were incubated in the absence of enzyme. In order to distinguish between mono- and disulfation of resveratrol, liquid chromatography–mass spectrometry (LC/MS) measurements were performed using an HPLC system fitted with an LC200 pump, a 235C DAD detector, and a 200 autosampler (PerkinElmer Sciex Instruments, Wellesley, MA). The system was coupled to an API 150Ex mass selective detector (PerkinElmer Sciex) fitted with an atmospheric pressure ionization source for electrospray ionization in the negative mode. Operating conditions were as follows: capillary voltage, −4.00 kV; orifice voltage, −40 V; gas temperature 400 °C. Column, mobile phase, gradient, flow rate, and injection volume were identical to those used in the analytical HPLC assay (see above). The confirmation of resveratrol metabolites in human cytosol samples was based on their retention times and ion fragmentations in the MS mode, as compared with standards (purity >95%) isolated from rat bile [14].

### 2.7. Quantitative real-time RT-PCR

Total RNA was extracted from tissue samples using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The concentration, purity and integrity of RNA samples were determined by UV absorbance and electrophoresis. 2 µg of total RNA were reverse transcribed to cDNAs using random hexamer primers and the RevertAid™ H Minus M-MuLV Reverse Transcriptase system (Fermentas St.Leon-Rot, Germany) in accordance with the manufacturer's instructions. TaqMan® Gene Expression Assays (Applied Biosystems) were purchased for *SULT1A1*, *SULT1A2*, *SULT1A3*, *SULT1E1*, *ARS-A*, *ARS-B* and *STS*. In order to find an appropriate reference gene, 13 different human housekeeping genes were analyzed and β-actin was selected as the acceptable reference gene for the tissue samples. Multiplex real-time PCR was performed in an amplification mixture volume of 20 µl. The target gene amplification mixture contained 10 µl 2 × TaqMan® Universal PCR Master Mix, 1 µl of the appropriate Gene Expression Assay, 1 µl TaqMan® endogenous control (β-actin), 10 ng template cDNA diluted in 5 µl nuclease-free water, and 3 µl nuclease-free water. *SULT1A1* amplification was determined in single plex reactions containing 10 µl 2 × TaqMan® Universal PCR Master Mix, 1 µl *SULT1A1* Gene Expression Assay, 10 ng template cDNA diluted in 5 µl-nuclease-free water, and 4 µl nuclease-free water. Endogenous control human β-actin amplifi-

cation was performed in an analogous manner. Thermal cycling conditions were as follows: 2 min at 50 °C, 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 by the 5′ → 3′ exonuclease activity of the DNA polymerase was measured using the StepOnePlus system (Applied Biosystems). All samples were amplified in triplicate. To cover the range of anticipated $C_t$ values that included our quantity of target mRNA, a standard curve of six serial dilutions from 50 ng to 500 pg of pooled cDNA was analyzed by the Sequence Detection Software (SDS 1.9.1., Applied Biosystems), and the results were transferred to Microsoft Excel for further analysis. Comparable cDNA amounts in the experimental samples were calculated, depending on the amplification efficiency of the target and reference gene, according to the $2^{-\Delta\Delta C_t}$ method [15] or according to Pfaffl [16]. Relative gene expression data are given as the *n*-fold change in transcription of target genes normalized to the endogenous control. The sample with the least detectable target gene expression was arbitrarily used as the calibrator, and the obtained data were calculated in relation to the calibrator's expression level.

### 2.8. Primers and probes

Real-time PCR was performed with the following prefabricated TaqMan® Gene Expression Assays (Applied Biosystems) containing intron-spanning primers: *SULT1A1*: Hs00738644_m1, *SULT1A2*: Hs02340929_g1, *SULT1A3*: Hs00413970_m1, *SULT1E1*: Hs00193690_m1, *STS*: Hs00165853_m1, *ARS-A*: Hs00166646_m1, *ARS-B*: Hs00940966_m1 and the endogenous controls β-actin: Part #4326315E and 18S Part #4310893E.

### 2.9. Immunofluorescence studies

Paraffin-embedded tissue sections from mamma specimens from selected patients were deparaffinized in xylene, and rehydrated with decreasing concentrations of ethanol according to standard methods. Antigen retrieval was performed by boiling the sections with 10 mM citric acid (pH 6.0). For detection of SULT1A3, and STS, sections were blocked with phosphate buffer saline containing 5% goat-serum and 0.05% saponin for 15 min. Slides were incubated for 60 min at room temperature in a humidifier chamber with the polyclonal anti-human SULT1A3 and anti-human placental STS antibodies, respectively, followed by incubation with an Alexa Fluor® 546 conjugated goat anti-rabbit IgG. Nuclei were stained with Hoechst 33342 dye (Roche, Mannheim, Germany). After a final washing step, slides were rinsed with distilled water, dried and mounted with permanent Fluoromont G Mounting Medium (Southern Biotech, Inc., Aurora, OH). Acquisition of more than 50 fields-of-view per section was performed with the TissueFAXS system (TissueGnostics, Vienna) using a 20× objective and identical exposure times for all samples.

### 2.10. Antibodies

The polyclonal rabbit anti-human SULT1A3 antibody was obtained from Proteintech Group, Inc (Chicago, IL, USA) and used at recommended dilutions. STS was kindly provided by Charles N. Falany (University of Alabama at Birmingham, AL, USA) and applied at a final dilution of 1:50.

### 2.11. Sulfatase assay

STS was purified from human full-term placenta using the method of Hernandez-Guzman et al. [17] as described previously [18]. STS activity was evaluated as reported by Cook and coworkers [19]. To determine the sensitivity of resveratrol-3-*O*-sulfate to STS hydrolysis, resveratrol-3-*O*-sulfate was generated using cytosolic extracts prepared from *Escherichia coli* containing cDNA for human SULT 1A1 * 1. Assays were performed in duplicate in a final volume of 100 µl containing 0.05 M potassium phosphate (pH 6.5), resveratrol (50 µM, final concentration), PAPS (50 µM), and enzyme protein (0.25 mg/ml). Reactions were incubated at 37 °C for 60 min. The concentration of resveratrol as well as the SULT isoform chosen to generate resveratrol-3-*O*-sulfate was based on the reactivity of the human SULT isoform with this compound as described previously [9]. For the STS assay, duplicate 40 µl aliquots were removed from the reaction mixtures to fresh tubes. Human STS (2 µg) was added to one tube while the other tube received the phosphate buffer only as control. The reactions were again incubated at 37 °C for the appropriate time (15–60 min) and terminated by the addition of methanol. The reaction mixtures were centrifuged at 13,000*g* for 5 min, and 80 µl of the clear supernatant applied onto an HPLC column as mentioned above.

### 2.12. Data analysis

Each incubation was performed at least in triplicate and the results were expressed as means ± SD. The data were fitted to Michaelis–Menten (hyperbolic) and substrate inhibition models. Kinetic parameters were estimated using the Enzyme kinetics 1.0, SigmaPlot 2000 (version 6.01, SPSS Inc., Chicago, IL, USA) for Michaelis–Menten (Eq. (1)) and substrate inhibition kinetics (Eq. (2)).

$$V = V_{max} \cdot S/(K_m + S) \quad (1)$$

$$V = V_{max}/(1 + K_m/S + S/K_i) \quad (2)$$

where $V$ is the rate of reaction, $V_{max}$ is the maximum velocity, $K_m$ is the Michaelis constant, $S$ is the substrate concentration and $K_i$ is the inhibition constant. Enzymatic efficacy, which is defined as the $V_{max}/K_m$ ratio, quantifies the sulfation capacity and corresponds to the intrinsic clearance. Statistical differences from control values were determined using the Student's paired *t*-test, at a significance level of $p < 0.05$. Comparisons of significant differences in the expression of identified *SULTs* between breast cancer tissue and non-malignant control tissue were performed using a paired *t*-test. The level of significance was set at $p < 0.05$.

## 3. Results

### 3.1. Metabolism of resveratrol in human breast cancer tissue samples

Cytosol from 13 individual human breast tumor and adjacent non-tumor specimens were incubated with 1 μM resveratrol for 60 min at 37 °C and subsequently analyzed by HPLC. In the presence of PAPS, one biotransformation product was identified ($t_r$ = 28.4) in addition to resveratrol ($t_r$ = 31.5 min). No metabolite formation was seen in the absence of PAPS (data not shown). Treatment of samples with sulfatase prior to HPLC analysis led to the disappearance of this metabolite. The concomitant increase of parent resveratrol indicates sulfation of the metabolite (data not shown). Negative ion mass spectra of this biotransformation product showed a stable molecular ion at $m/z$ = 307 amu, with subsequent loss of 80 amu (sulfuric acid moiety) in agreement with the molecular weight of resveratrol monosulfate (data not shown). Based on identical retention times and negative ion mass spectra compared to an isolated standard, the structure of the metabolite was confirmed as trans-resveratrol-3-O-sulfate. As shown in Fig. 1, resveratrol sulfation was significantly higher ($p$ < 0.001) in 12/13 breast cancer tissue samples; these showed up to 33.5-fold higher formation rates compared to their non-tumor counterparts with the highest values observed in patients Nos. 8, 10 and 11 (30.2 ± 3.67, 25.1 ± 3.05, and 24.8 ± 2.78 pmol/mg/min, respectively). More significantly, the formation of resveratrol-3-O-sulfate was below the detection limit in five of 13 corresponding tissue specimens. Interestingly, resveratrol sulfation in patient No. 7 was below detection limit in the malignant as well as the non-malignant tissue samples (Fig. 1).

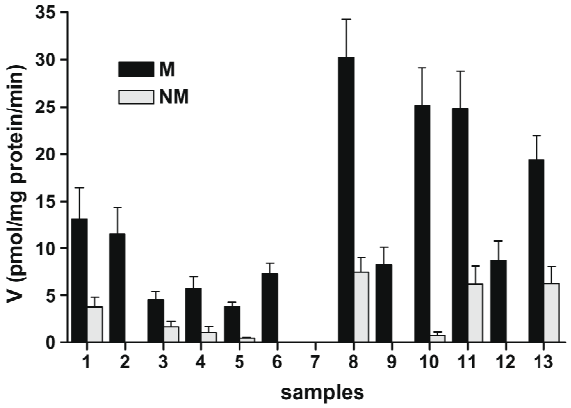


**Fig. 1.** Formation rates of *trans*-resveratrol-3-O-sulfate in human malignant (M) and non-malignant (NM) breast cancer tissue samples at 1 μM resveratrol. Data represent the mean ± SD of triplicate determinations.

### 3.2. Kinetics of resveratrol sulfation in human breast cancer and adjacent non-cancerous tissue

Owing to the limited quantity of tissue specimen, concentration-dependent enzyme kinetics of resveratrol sulfate formation in human breast cancer tissue could be investigated only in three of 13 breast tumor samples (patients Nos. 5, 8, and 9). Formation of *trans*-resveratrol-3-O-sulfate was linear with time, up to 120 min (data not shown), and the kinetic constants for these reactions were estimated using resveratrol concentrations ranging from 0.5 to 100 μM (see Table 2).

As shown in Fig. 2A and B (patients Nos. 5, 8), the kinetics of resveratrol sulfation at 0.5–25 μM resveratrol fitted to the Michaelis–Menten kinetics (Eq. (1): $r^2$ = 0.977, $r^2$ = 0.996, respectively). However, when resveratrol concentration exceeded 25 μM, resveratrol sulfate gradually decreased and apparent enzyme kinetic parameters were estimated by fitting to the substrate inhibition Eq. (2) ($r^2$ = 0.952, $r^2$ = 0.985, respectively). The enzyme kinetics for sulfate formation in patient No. 9 (Fig. 2C) showed a Michaelis–Menten kinetic profile ($r^2$ = 0.954) at 0.5–10 μM resveratrol and again substrate inhibition ($r^2$ = 0.907) with velocity values of 0.5–100 μM resveratrol.

### 3.3. Expression of sulfotransferases and sulfatases in human breast cancer and adjacent non-cancerous tissue

Thirteen breast cancer samples were chosen and matched with corresponding non-malignant breast tissue specimens to perform quantitative real time RT-PCR analysis for the sulfotransferases *SULT1A1*, *1A2*, *1A3*, *1E1* and the sulfatases *ARS-A*, *ARS-B*, *STS*. As seen in Table 3, *SULT1A2* mRNA was up to 19.2-fold higher in nine of 13 non-malignant samples compared to the paired tumor tissue, but did not achieve statistical significance ($p$ < 0.750). For three patients (Nos. 2, 5, 13) we found a slightly (1.1- to 1.5-fold) higher expression of *SULT1A2* in the cancer samples. *SULT1A2* expression in patient No. 8, however, was significantly higher in breast cancer tissue than in the adjacent peritumoral tissue ($p$ < 0.0002). *SULT1A3* mRNA expression levels vary over a wide range. In nine of 13 control tissue specimens we found greater (1.3- to 7.8-fold) mRNA expression compared to tumor tissue while patients Nos. 1, 4, 8, and 9 demonstrated significantly higher mRNA expression (up to 3.9-fold) of this conjugating enzyme in tumor samples as compared to peritumoral ones. Notably, *SULT1E1* mRNA expression levels could only be detected in three of 13 malignant specimens and in one of the control samples. *SULT1A1* mRNA expression was below the detection limit in all tissue samples.

Differences in the expression pattern between tumor tissue and the corresponding non-malignant tissue were also observed for the sulfatases *ARS-A*, *ARS-B*, and *STS* (see Table 4). Peritumoral tissue samples from eight of 13 patients showed insignificantly higher *ARS-A* mRNA expression levels ($p$ < 0.134) than the tumor specimens. In four tumor samples (Nos. 1, 7, 8, 11) we found increased *ARS-A* expression (up to 1.93-fold) as compared to control tissue while only a very slight difference in the expression of *ARS-A* was seen in sample No. 9. As shown in Table 4, there was no significant difference between the expression levels of *ARS-B* mRNA in non-malignant tissue samples compared to the corresponding tumor specimens ($p$ < 0.481). Nevertheless, seven of 13 breast cancer tissue samples (Nos. 1, 2, 4, 7, 8, 11, 13) showed slightly higher *ARS-B* expression levels

**Table 2**
Kinetic parameters of trans-resveratrol-3-O-sulfate formation in human breast cancer tissue samples[a].

| Patient No. | Model | $K_m$ (μM) | $V_{max}$ (pmol/mg protein/min) | $V_{max}/K_m$ (μl/min/mg) | $K_i$ (μM) |
|---|---|---|---|---|---|
| 5 | Michaelis–Menten[b] | 0.29 ± 0.08 | 4.88 ± 0.21 | 17.0 ± 5.03 | n.a. |
| | Substrate Inhibition[c] | 0.36 ± 0.13 | 5.24 ± 0.41 | 14.6 ± 8.14 | 144.5 ± 56.1 |
| 8 | Michaelis–Menten[b] | 2.94 ± 0.35 | 135.8 ± 4.52 | 46.2 ± 5.58 | n.a. |
| | Substrate Inhibition[c] | 5.75 ± 1.44 | 200.1 ± 26.8 | 34.8 ± 12.1 | 50.1 ± 13.9 |
| 9 | Michaelis–Menten[b] | 0.43 ± 0.16 | 12.5 ± 0.93 | 29.1 ± 6.28 | n.a. |
| | Substrate Inhibition[c] | 0.65 ± 0.28 | 14.4 ± 1.68 | 22.2 ± 12.3 | 91.0 ± 40.8 |

n.a. = not applicable.
[a] Data shown are means ± SD from three determinations.
[b] Kinetic parameters were calculated by Eq. (1).
[c] Kinetic parameters were calculated by Eq. (2).

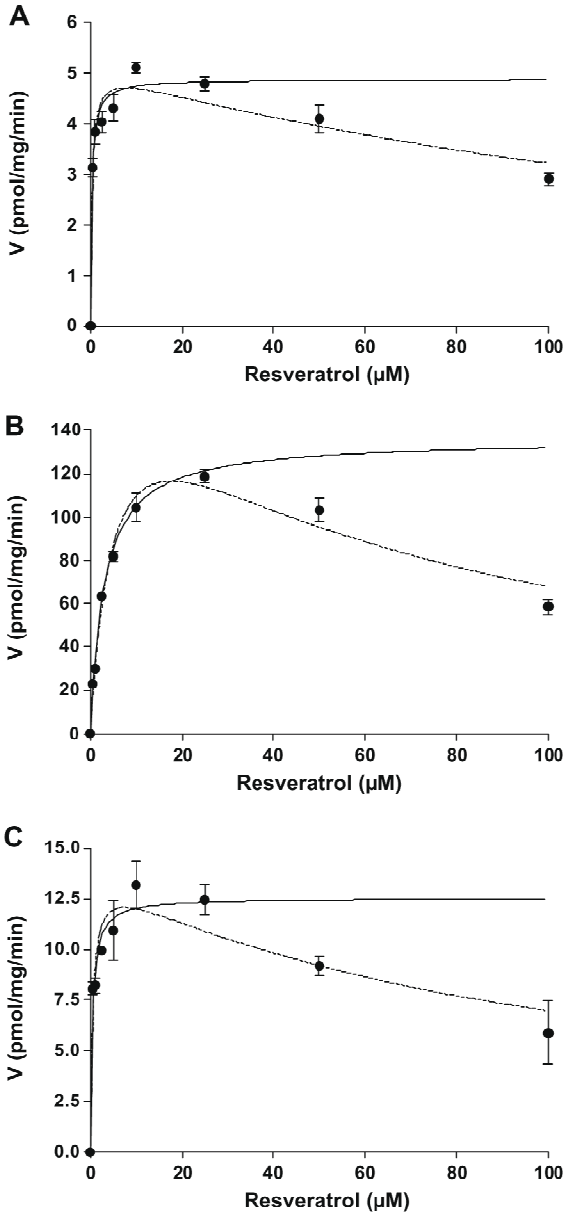


**Fig. 2.** Kinetics of *trans*-resveratrol-3-*O*-sulfate formation in human breast cancer tissue samples of patients No. 5 (A), No. 8 (B), and No. 9 (C) normalized to protein content as a function of resveratrol concentration. Data are expressed as means ± SD ($n = 3$) individual preparations. The solid line represents the curve fitting to the Michaelis–Menten Eq. (1); the dotted line represents the fitting curve to the substrate inhibition Eq. (2).

than the corresponding peritumoral specimens. Notably, *STS* mRNA expression was significantly higher ($p < 0.0017$) in 12 of 13 non-malignant specimens as compared to tumor tissue samples. Only patient No. 2 showed 1.7-fold higher levels of this enzyme in the cancerous counterpart.

### 3.4. Immunofluorescence staining of SULT1A3 and STS in human breast cancer and adjacent non-cancerous tissue

Cellular localization of SULT1A3 and STS was assessed by indirect immunofluorescence on paraffin-embedded sections from breast cancer tissue (Fig. 3). Data shown are representative for all tumors. In the malignant part of tumors from patients Nos. 1 and 8, bright red cytoplasmic staining for SULT1A3 was found in the majority of tumor cells, particularly in the grade 3 tumor from patient No. 1 (Fig. 3C) and to a lesser amount in patient No. 8 (Fig. 3D). In the normal tissue, however, staining for SULT1A3 in sections from both patients is poor and mainly localized in the cytosol in cells around the ductuli (Fig. 3A and B). Contrary to SULT1A3, immunofluorescence staining for STS is higher in normal (Fig. 3E and F) than in tumor specimens from these two patients (Fig. 3G and H). Again, STS staining is seen in the cytosol mainly in ductal epithelial cells (normal tissue) and in tumor cells (malignant sections).

**Table 3**
*SULT* and sulfatase mRNA expression levels in malignant (M) and non-malignant (NM) tissue samples.

| Patient No. | Tissue | *SULT1A2* | *SULT1A3* | *SULT1E1* |
|---|---|---|---|---|
| 1 | M | **0.36** ± 0.35 | **12.37 ± 0.68** | n.d. |
| | NM | **1.47 ± 0.09** | 3.15 ± 0.07 | n.d. |
| 2 | M | 2.28 ± 0.33 | 4.60 ± 0.48 | **3.91 ± 0.16** |
| | NM | 1.49 ± 0.04 | 6.85 ± 0.66 | n.d. |
| 3 | M | 0.27 ± 0.58 | 2.10 ± 0.08 | n.d. |
| | NM | **3.03 ± 0.07** | **16.38 ± 1.16** | n.d. |
| 4 | M | 0.43 ± 0.24 | **9.92 ± 0.28** | n.d |
| | NM | **5.23 ± 0.23** | 4.49 ± 0.66 | 1.01 ± 0.13 |
| 5 | M | 9.57 ± 0.07 | 3.64 ± 0.37 | n.d. |
| | NM | 6.23 ± 0.39 | 6.00 ± 1.29 | n.d. |
| 6 | M | 1.74 ± 0.24 | 9.76 ± 0.13 | n.d. |
| | NM | 3.39 ± 0.20 | 16.14 ± 1.74 | n.d. |
| 7 | M | 0.26 ± 0.27 | 2.03 ± 0.36 | n.d. |
| | NM | **0.88 ± 1.10** | 3.49 ± 0.30 | n.d. |
| 8 | M | **15.55 ± 1.09** | 27.36 ± 2.22 | n.d. |
| | NM | 0.83 ± 0.77 | 14.71 ± 3.03 | n.d. |
| 9 | M | 0.26 ± 1.36 | 10.36 ± 0.98 | n.d. |
| | NM | **4.98 ± 0.20** | 8.95 ± 0.70 | n.d. |
| 10 | M | 4.50 ± 0.32 | 9.44 ± 1.58 | n.d. |
| | NM | 6.65 ± 0.51 | 12.13 ± 1.48 | n.d. |
| 11 | M | 0.44 ± 0.81 | 12.06 ± 1.32 | **7.83 ± 1.29** |
| | NM | **5.00 ± 0.45** | 16.75 ± 1.04 | n.d. |
| 12 | M | 1.47 ± 0.37 | 4.86 ± 1.14 | n.d. |
| | NM | **5.12 ± 0.54** | **17.08 ± 3.86** | n.d. |
| 13 | M | 14.14 ± 0.38 | 12.84 ± 1.45 | **19.86 ± 4.31** |
| | NM | 13.08 ± 0.18 | 18.49 ± 1.54 | n.d. |

n.d., Not detected. Relative gene expression data are given as the *n*-fold change in transcription of target genes normalized to the endogenous control ± SD. The sample with the lowest detectable target gene expression was arbitrarily applied as the calibrator and the obtained data were calculated in relation to the calibrator's expression level. Values in bold indicate a 2-fold or greater increase compared to the corresponding tissue sample.

### 3.5. Resveratrol desulfation by STS

After 60 min of incubation with STS, almost all of resveratrol-3-*O*-monosulfate produced by recombinant SULT1A1 was hydrolyzed, and only approximately 17% of the monosulfate remained (data not shown). This indicates that STS may also catalyze the hydrolysis of resveratrol monosulfate to resveratrol in breast tissue.

## 4. Discussion

In the present study we investigated the metabolism of resveratrol in breast cancer samples and corresponding non-malignant breast tissue specimens. Incubation of res-

**Table 4**
*SULT* and sulfatase mRNA expression levels in malignant (M) and non-malignant (NM) tissue samples.

| Patient No. | Tissue | *ARS-A* | *ARS-B* | *STS*[*] |
|---|---|---|---|---|
| 1 | M | **1.96** ± 0.22 | 1.28 ± 0.33 | 1.01 ± 0.12 |
| | NM | 1.58 ± 0.07 | 0.75 ± 0.44 | **3.16 ± 0.17** |
| 2 | M | 1.35 ± 0.09 | 1.24 ± 0.19 | 5.31 ± 1.06 |
| | NM | 1.85 ± 0.02 | 0.67 ± 0.53 | 3.14 ± 0.22 |
| 3 | M | 1.14 ± 0.08 | 1.33 ± 0.43 | 1.71 ± 0.12 |
| | NM | **2.43 ± 0.09** | **2.68 ± 0.35** | **9.48 ± 1.02** |
| 4 | M | 1.90 ± 0.09 | 1.24 ± 0.28 | 1.79 ± 0.32 |
| | NM | 2.85 ± 0.28 | 1.01 ± 0.45 | **3.94 ± 0.40** |
| 5 | M | 2.34 ± 0.24 | 0.98 ± 0.24 | 1.31 ± 0.04 |
| | NM | 3.33 ± 0.43 | **2.99 ± 0.43** | **5.71 ± 0.79** |
| 6 | M | 1.41 ± 0.16 | 1.33 ± 0.28 | 1.57 ± 0.14 |
| | NM | **4.00 ± 0.40** | 2.40 ± 0.35 | **13.90 ± 1.67** |
| 7 | M | 1.54 ± 0.08 | 1.41 ± 0.30 | 3.95 ± 0.28 |
| | NM | 1.28 ± 0.07 | 0.97 ± 0.39 | 4.95 ± 0.18 |
| 8 | M | 4.06 ± 0.40 | **3.36 ± 0.15** | 2.31 ± 0.34 |
| | NM | 2.10 ± 0.15 | 1.09 ± 0.14 | **4.96 ± 0.45** |
| 9 | M | 2.09 ± 0.17 | 1.62 ± 0.52 | 1.91 ± 0.29 |
| | NM | 1.94 ± 0.20 | 1.78 ± 0.60 | **6.86 ± 1.33** |
| 10 | M | 2.05 ± 0.50 | 1.13 ± 0.11 | 6.35 ± 1.47 |
| | NM | 3.92 ± 0.31 | **2.34 ± 0.55** | **13.46 ± 1.75** |
| 11 | M | 3.99 ± 0.07 | 1.85 ± 0.26 | 5.13 ± 0.64 |
| | NM | 2.37 ± 0.07 | 1.19 ± 0.14 | **12.02 ± 1.49** |
| 12 | M | 1.89 ± 0.41 | **1.32 ± 0.23** | 2.10 ± 0.31 |
| | NM | **5.27 ± 0.41** | 3.96 ± 0.16 | **12.83 ± 1.93** |
| 13 | M | 2.54 ± 0.24 | 1.26 ± 0.11 | 11.06 ± 0.75 |
| | NM | 4.39 ± 0.42 | 1.00 ± 0.24 | 11.71 ± 0.73 |

n.d., Not detected. Relative gene expression data are given as the n-fold change in transcription of target genes normalized to the endogenous control ± SD. The sample with the lowest detectable target gene expression was arbitrarily applied as the calibrator and the obtained data were calculated in relation to the calibrator's expression level. Values in bold indicate a 2-fold or greater increase compared to the corresponding tissue sample.
[*] Indicates significant differences in the expression levels of *STS* between all 13 malignant and adjacent non-malignant tissue samples ($p = 0.0017$).

veratrol in cytosolic tissue fractions revealed the presence of resveratrol-3-*O*-sulfate, whose formation is strongly dose-dependent. While sulfation clearly prevailed in the lower substrate concentrations (0.5–10 μM), an increase of the initial applied dose (25–100 μM) led to a dramatic reduction of resveratrol sulfation by up to 55.3% after 60 min of incubation. Kinetic analysis further showed non-competitive substrate inhibition to be the main mechanism of the decreased sulfate formation at higher resveratrol concentrations. The data obtained in the present study are in good agreement with previous findings from our lab, showing that in breast cancer cell lines MDA-MB-231 and ZR-75–1 as well as in human intestinal Caco-2 cells and human liver, sulfation is also a main metabolic pathway for resveratrol; it is shown best by the substrate inhibition model [8,9,20].

Interestingly, significantly greater sulfation of resveratrol occurred in tumor tissue than in adjacent non-malignant tissue. These higher formation rates of resveratrol-3-*O*-sulfate led to the assumption that resveratrol metabolism may correlate with tissue expression of *SULT1A1*, *1A2*, *1A3* and *1E1* isoenzymes, which were previously shown in our laboratory to catalyze resveratrol sulfation. By using quantitative real-time RT-PCR, we could indeed demonstrate mRNA expression of *SULT1A2* and *SULT1A3* in breast cancer and control tissue samples, whereas *SULT1E1* expression was only observed in three malignant and one non-malignant specimens. *SULT1A1* mRNA expression was below the detection limit in all analyzed samples. In contrast to our study, Aust and coworkers observed substantial expression of *SULT1A1* mRNA in malignant as well as non-malignant breast tissue [11]. However, contrary to the quantitative real-time RT-PCR used in our study, Aust et al. employed conventional RT-PCR, which may also detect unspecific traces of final amplified products at the end-point.

The observed differences in resveratrol sulfation between tumor and adjacent control tissue samples, however, did not correlate with the mRNA expression of *SULT1A2*, *SULT1A3* and *SULT1E1*. As resveratrol sulfate may be readily hydrolyzed in tissue samples by members of the sulfatase family, thus regenerating parent resveratrol, we also investigated the mRNA expression of three human sulfatases, namely *ARS-A*, *ARS-B* and *STS*. Intracellularly, STS is localized in the rough endoplasmic reticulum, while ARS-A and B are found predominantly in endosomes and lysosomes [21]. Using quantitative real-time RT-PCR, our studies showed significantly higher *STS* mRNA levels in normal breast tissue ($p < 0.0017$), possibly explaining the observed higher resveratrol sulfate concentrations in breast cancer samples than in control breast specimens. These data were confirmed by immunofluorescence staining of paraffin-embedded tissue section of selected patients also showing a more prominent localization of STS in the adjacent normal tissue. Higher *STS* mRNA levels in non-malignant tissue samples, however, are in contrast to the data reported by Utsumi et al. and Miyoshi et al., who both detected higher *STS* expression levels in malignant breast tissues than in normal tissue samples [22,23]. These differences might have been due to the high inter-individual variability in the expression levels of *STS* in matched malignant and adjacent non-malignant tissue samples as shown in our study.

In contrast to *STS*, *ARS-A* and *ARS-B* were approximately equally expressed in malignant and non-malignant tissue samples. Notably, the expression of *ARS-A* and *ARS-B* has not previously been detected in breast cancer specimens. Bhattacharyya and coworkers, however, showed equivalent thresholds for *ARS-B* expression between non-malignant mammary myoepithelial cells (MEC) and hormone-dependent MCF-7 breast cancer cells using real-time PCR [24]. Interestingly, Türkmen et al. evaluated ARS-A activity in leukocytes of patients with breast cancer and benign breast disease where ARS-A activity was significantly higher compared to healthy women [25].

Whether resveratrol itself can accumulate to bioactive levels in human breast tissue is not known yet. A recent study from Juan et al. demonstrated that after intravenous application to rats, resveratrol distributes into various organs like brain, liver, kidney, lungs and testis strongly indicating a possible uptake also into breast tissue [26]. Furthermore, in all organs analyzed conjugated metabo-

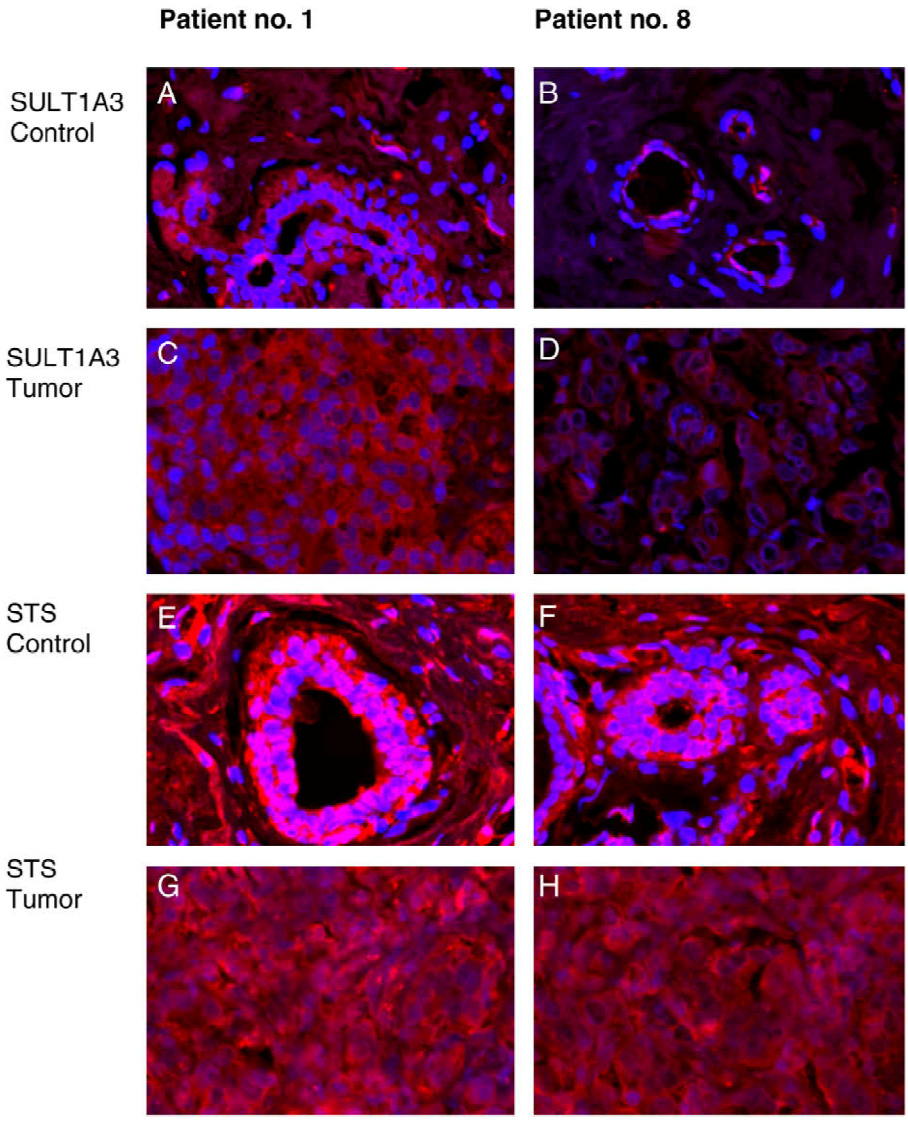


**Fig. 3.** Immunofluorescence analysis of SULT1A3 (A–D) and STS (E–H) for single tissue slices in representative specimens of control and malignant breast tissue from patients Nos. 1 and 8, respectively. See Section 2 for the exact procedure. Note, that the bright red staining for SULT1A3 was more pronounced in the majority of tumor cells while red staining for STS was higher in control specimens.

lites were detected at concentrations higher than that of the parent resveratrol. This is in line with data from our study also showing resveratrol sulfation in most human malignant and non-malignant breast tissue samples. Importantly, resveratrol sulfate has decreased anticancer activity against MCF-7, MDA-MB-231 and ZR-75-1 cells as compared to resveratrol [27]. However, the *in vitro* activity of resveratrol sulfate may not necessarily reflect its *in vivo* function, given the fact that ARS-A, ARS-B and STS could easily convert the metabolite back to resveratrol.

Notably, formation of resveratrol sulfate greatly differs between tissue specimens based on high inter-individual variation of sulfotransferases and sulfatases expression. Uptake of dietary resveratrol in patients with low (patients Nos. 3–5) or no detectable metabolism (patient No. 7) may have increased resveratrol concentrations in breast tumor tissue.

In conclusion, we found that resveratrol is metabolized in human breast cancer tissue to resveratrol-3-*O*-sulfate, which is formed to a significantly greater extent in tumor tissue than in adjacent non-malignant specimens, as a result of increased *STS* expression in control tissue samples. Although the sample size used in this study was small, our data may be predictive as we used tumor specimens and control samples from the same patients. However, long-term *in vivo* studies are highly warranted to deter-

mine the preventive and therapeutic efficacy of resveratrol as dietary supplement or in combination therapies on tumor development and progression.

## Conflicts of interest

All authors (Michaela Miksits, Katrin Wlcek, Martin Svoboda, Theresia Thalhammer, Isabella Ellinger, Gabriele Stefanzl, Charles N. Falany, Thomas Szekeres and Walter Jaeger) have disclosed no financial relationship and conflict of interest for the submitted manuscript entitled: "Expression of sulfotransferases and sulfatases in human breast cancer: impact on resveratrol metabolism".

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**ANALYSIS OF ORGANIC ANION TRANSPORTING POLYPEPTIDE (OATP) mRNA AND PROTEIN REVEALS DIFFERENCES IN PRIMARY AND METASTATIC LIVER CANCER**

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**TITLE PAGE**

# Analysis of organic anion transporting polypeptide (OATP) mRNA and protein reveals differences in primary and metastatic liver cancer

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**KEY WORDS**

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## ABBREVIATIONS

OATP, organic anion transporting polypeptides; HCC, hepatocellular carcinoma; CCC, cholangiocarcinoma; MLT, metastatic liver tumors

## CONFLICT OF INTEREST

All authors have disclosed no financial relationships and conflict of interest.

**ABSTRACT**

Transmembrane transporters for endobiotics and drugs from the organic anion transporting polypeptide (OATP) family are expressed in the liver and may be altered in cancer. Thus, we investigated mRNA expression of all eleven OATPs in paired cancerous and adjacent non-cancerous specimens (n=22) from hepatocellular (HCC), cholangiocellular carcinomas (CCC) and liver metastases (MLT). TaqMan® real-time RT-PCR analysis revealed that all OATPs except OATP1C1 and 6A1 are expressed in the majority of tissues samples. With the exception of OATP1A2, they are also expressed in primary hepatocytes and HepG2 cells. In comparing cancerous and non-cancerous specimens, we found that mRNA expression levels were reduced for OATP1B1, 1B3, 1A2 and 2B1 and increased for OATP2A1, 3A1 and 5A1 in cancer samples. Differences were also seen between different tumor types. While downregulation of OATP1A2 and 1B3 mRNA expression was most pronounced in CCC, marked up-regulation of OATP4A1 and 5A1 mRNA was observed in MLT. Immunofluorescence studies for OATP2A1, 5A1 and 4A1 revealed immunoreactive plasma membrane staining in cancer cells. Staining of cytokeratin-19 positive cells in proliferating bile ducts was observed for OATP2A1 and 4A1. Further studies in HepG2 cells revealed reduced mRNA levels of all OATPs except OATP4A1 and 4C1 when compared to human hepatocytes. Treatment of HepG2 cells with Aza-dC increased OATP2A1 and 5A1 mRNA expression to the level in hepatocytes, indicating that hypermethylation inhibits their expression. Distinct regulation of OATP expression in primary and metastatic hepatic tumors may alter uptake of drugs, hormones and prostaglandins, thereby influencing the efficacy of a given therapy.

**Running title:** Altered OATP expression in liver cancer

## INTRODUCTION

Eleven members of the organic anion transporter family (OATP-family) have been identified in humans so far. They represent a group of sodium-independent transmembrane transporters for a broad spectrum of substrates, including hormones and their conjugates, prostaglandins, xenobiotics, and therapeutic drugs.[1] Although the majority of human OATPs show wide tissue distribution, some members exhibit unique expression in distinct organs. The best studied of these are the "liver–specific" members OATP1B1[2,3] and 1B3.[4] These OATPs are located in the basolateral membrane of hepatocytes where they mediate the uptake of endogenous substrates and drugs into the cell and play a critical role in the first step of the elimination process through the liver.[5,6,7] In addition to OATP1B1 and 1B3, expression of the OATPs OATP1A2,[8] 2A1,[9] 2B1, 3A1 and 4A1[10] was first investigated by Northern blotting and conventional PCR analysis, and in more recent studies, by real-time RT-PCR in normal liver.[11,12] While robust expression of OATP1A2 and low levels of OATP3A1 and 4A1 mRNA were consistently reported, OATP2A1 was detected only by Northern blotting[9] but not by conventional PCR analysis.[10]

Investigations of OATP protein levels in liver are only available for a limited number of OATPs. Immunohistochemical studies showed that OATP1B1, 1B3 and OATP2B1 are located in hepatocytes,[2,3,4,13] and OATP1A2 was detected in bile duct cells (cholangiocytes).[14]

It is known that expression of some OATPs such as OATP1B3 is altered by malignant transformation of cells. In addition, confined expression to specific organs as seen in normal tissue can be lost.[1] In a recent study from our lab, we show that OATP2B1, 3A1 and 4A1 are differently expressed in breast cancer as compared to non-malignant tissue.[15] Similarly, differences in OATP mRNA expression are also seen for primary and metastatic bone tumors,[16] and a number of OATPs such as OATP1A2, 1C1, 2B1 and 4A1 are expressed in human gliomas.[17]

For liver cancers, studies have mainly focused on the two liver-specific OATP family members, OATP1B1 and 1B3.[18,19] These transporters are down-regulated in the hepatoma cell line HepG2[20,11] and in liver cancer (hepatocellular carcinoma).[19] However, expression of other OATPs such as the prostaglandin transporter OATP2A1, the ubiquitously expressed OATP4A1, and the less characterized OATP5A1 has not been investigated in liver tumors as of yet.[1]

As OATPs regulate the uptake of metabolites, drugs, and xenobiotics, expression of OATPs in malignant liver disease may influence tumor progression as well as the response to chemotherapeutic interventions. Therefore, we investigated the presence of all eleven OATP family members in primary and metastatic liver tumors using paired cancerous and non-cancerous samples from 22 patients with the primary liver cancers hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCC) and metastases of solid tumors (MLT), the most frequent tumors in the liver.[21] Additionally, we assessed OATP mRNA expression in HepG2 cells treated with the demethylating agent 5-aza-2´-deoxycytidine (Aza-dC) and compared it to that of hepatocytes derived from nine human donors.

Differential expression of individual OATPs in cancerous and normal liver might alter the uptake of signaling molecules including hormones, prostaglandins and anticancer drugs into hepatic cells, thus affecting the proliferation of tumor cells.

## RESULTS

**OATP mRNA expression in human malignant and non-malignant liver samples.** To study possible changes in the expression pattern of all human OATPs in hepatic malignancies, paired cancerous (HCC (n=7), CCC (n= 5) and MLT (n=10)) and adjacent non-cancerous samples were analyzed for mRNA expression of all known human OATPs using real-time RT-PCR.

The OATP mRNA expression profile is depicted as a heat map in Figure 1, showing a cluster of non-cancerous ("normal") and of cancerous samples with distinct expression of OATPs. Exceptions were seen for HCC samples #1 and #17 and sample #2 from the non-cancerous part of an MLT, which fit into the normal and cancer clusters, respectively.

Decreased mRNA expression in most cancerous samples was observed for the liver-specific transporters OATP1B1 and 1B3, and other OATPs, OATP2B1, 1A2 and 4C1. However, expression of OATP2A1, 3A1 and 4A1 and the less characterized OATP5A1 was increased in most of the malignant samples as compared to the non-cancerous specimens. OATP1C1 and OATP6A1 mRNA levels were below the detection limit in all but three samples, which showed only marginal levels of these transporters.

Further analysis revealed relative quantitative differences in OATP levels between the HCC, CCC and MLT groups (Figure 2). Remarkably, OATP1B1, showed a 44- and 36-fold decrease in CCC and MLT, respectively. Pronounced reduction of 1A2 and 1B3 was also observed in the CCC group (113- and 75-fold, respectively). Decreases in OATP1B1 mRNA levels in HCC and OATP1B3 in HCC and MLT samples were moderate, up to 4-fold ($p<0.05$). Reduced expression was also seen for OATP2B1 in the CCC and MLT groups (4- to 7-fold).

In contrast, an up to 34-fold increase was seen for OATP2A1, 3A1 and 5A1 mRNA expression in HCC, CCC and MLT samples. Interestingly, a 20-fold upregulation was observed for OATP4A1 in the MLT group, while levels in HCC and CCC did not differ compared to the nonmalignant controls. This may be related to an upregulation in OATP4A1 expression in colon cancer as suggested from previous array data[22] and in our control samples (6-fold upregulation, n=24, data not shown). Expression of OATP4C1 mRNA was highly variable but similar to that in the non-cancerous samples.

**Analysis of cellular localization of OATP2A1, 4A1 and 5A1 by immunofluorescence.** Localization of OATP2A1, 4A1 and 5A1, which have not been previously studied at the protein level in hepatic tumors, were analyzed by

immunofluorescence staining for their cellular localization on cryosections of tumors. In accordance with the real-time RT-PCR results, immunofluorescent staining of OATP2A1 was more intense in the cancerous samples than in the non-cancerous controls. However, OAT2A1 staining was rather diffuse in the cytoplasm of liver parenchymal cells, and mostly membranous staining was visible in the cancer section, suggesting activation of the transporter in these tumors (Figure 3A).

Only background fluorescence was observed in hepatocytes in the non-cancerous HCC and MLT samples with anti-OATP5A1 and 4A1 antibodies, respectively (Figure 3A). In the cancerous sections, OATP5A1 and 4A1 staining is clearly membrane associated.

To investigate whether OATP2A1, 4A1 and 5A1 may also be expressed in cells deriving from proliferating bile ducts, we performed double staining with cytokeratin 19 (CK-19) as a marker for biliary/progenitor cells.[23] Remarkably, while OATP2A1 and 4A1 is clearly co-localized with CK-19 immunofluorescence in cells surrounding the bile ducts, OATP5A1 staining is barely apparent in CK-19 positive cells. Representative HCC and MLT samples are shown in Figure 3B and C, respectively.

**OATP mRNA expression in human hepatocytes and HepG2 cells.** As HepG2 cells are a widely used model for the study of liver cancer, we also investigated the OATP mRNA expression pattern in this cell line (Figure 5) and compared it to that in human hepatocytes derived from nine human donors (Figure 4). mRNA expression of eight OATPs (OATP1B1, 1B3, 2A1, 2B1, 3A1, 4A1, 4C1 and 5A1) was observed in all hepatocyte samples and HepG2 cells. OATP1A2 was detectable in only one hepatocyte sample, but not in HepG2 cells.

In hepatocytes, interindividual variations for OATP1B3, 2B1, 4C1 and 5A1 comprised a rather small range of 2-fold, but expression of OATP2A1, 3A1, and 4A1 mRNA varied considerably. Expression levels ranged from nearly undetectable to 9-fold for OATP2A1, 4-fold for OATP3A1 and 6-fold for OATP4A1.

In HepG2 cells, pronounced downregulation by was seen for OATP1B1, 1B3, and 5A1 mRNA expression compared to hepatocytes ($p<0.05$) (Figure 5). In addition, OATP2A1 and 3A1 mRNA levels were 11- and 43-fold lower, respectively, than the average expression in the hepatocytes. However, because of the high interindividual variability of these OATPs in hepatocytes (Figure 4), differences did not reach significance. OATP4A1 and 4C1 were the only OATPs that were expressed at higher levels in HepG2 cells than in human hepatocytes.

To investigate whether downregulation of OATP mRNA might be due to hypermethylation in the promoter region, we analyzed changes in OATP expression after the treatment of HepG2 cells with the demethylating agent Aza-dC (Figure 5). Expression of OATP2A1 and 5A1 mRNA was vastly increased, and their expression reached levels similar to that observed in hepatocytes (OATP5A1: 1.1.0±0.16 and OATP2A1: 2.54±1.05; Means±SEM). Interestingly, mRNA expression of OATP4A1 and 4C1 in the HepG2 cells was decreased after Aza-dC treatment.

## DISCUSSION

We show that of the eleven human OATPs, all but OATP1C1 and OATP6A1 are expressed in human cancerous and non-cancerous liver samples. With the exception of OATP1A2, these transporters are also detectable in hepatocytes and HepG2 cells.

However, the expression patterns differ between non-cancerous samples and HCC, CCC and MLT specimens. Previous data demonstrated decreased expression of OATP1B1 and 1B3 in benign hyperplasia and malignant hepatocellular lesions as compared to normal liver,[18,19,24] indeed, we also show different degrees of down-regulation of these transporters in HCC, CCC and MLT as compared to non-cancerous adjacent tissue. The most pronounced reduction in OATP1B1 and 1B3 mRNA expression was observed in CCC and may at least partly reflect replacement of hepatocytes by malignant cells transformed from cholangiocytes and their precursors. Dedifferentiation of these cells may also explain downregulation of OATP1A2, a transporter expressed in cholangiocytes in healthy human liver.[25]

We further show that expression of OATP2A1, 3A1, 4A1 and 5A1, an OATP with an unknown function,[1] are detectable in most non-cancerous liver specimens. This was also seen in isolated normal hepatocytes, although the expression of OATP5A1 was highly variable between individuals. High interindividual variability may also explain why the expression of OATP2A1, 3A1 and 4A1 was not consistently observed in all studies of human liver using RNA from different sources.[10,11,12]

Overall, our real-time RT-PCR data clearly show that in all tumor groups, OATP2A1, 3A1 and 5A1 are expressed at higher levels in cancer versus non-cancerous tissues. Furthermore, increased mRNA levels of OATP2A1 correlated with a clear membrane-located staining of cancer cells in HCC and MLT samples. Additionally, an overlapping pattern with the biliary/progenitor cell marker cytokeratin [1926] was observed in CCC and MLT. Similar to colon cancer, the prostaglandin transporter OATP2A1 may also be important in the termination of prostaglandin signaling in liver cancer.[27,28] In addition, immunofluorescent staining of OATP4A1 and 5A1 was detected on the cell membranes in cancerous sections. However, bile duct derived cytokeratin 19-positive cells were only positive for OATP4A1. This is interesting, as expression of this cytokeratin is associated with a poor prognosis in liver cancer.[29] In addition, we observed higher mRNA and protein expression of OATP4A1 only in the MLT group. This may reflect up-regulation of OATP4A1 mRNA in samples isolated from colon tumors, confirming

previous array data.[22] This would warrant further investigations of OATP4A1 as a possible diagnostic marker for colon carcinoma derived MLTs.

We further demonstrate that all OATPs expressed in hepatic tumors, except OATP1A2, are also present in HepG2 cells. However, expression of most OATPs was downregulated in the hepatoma cell line. Epigenetic silencing of OATP expression may be important for the regulation of the expression of some OATPs. Indeed, this was recently demonstrated for OATP1B3 in HepG2 cells[30] and for OATP2A1 in the colon cancer cell lines HT-29 and LS174.[28] In line with these findings, we showed that Aza-dC effectively restored OATP2A1 and 5A1 mRNA expression in HepG2 cells to levels similar to those observed in hepatocytes.

In conclusion, we show that in contrast to the “liver-specific” OATP1B1 and 1B3, mRNA expression of OATP2A1 and 5A1 is upregulated in primary and secondary hepatic tumors. Moreover, OATP4A1 mRNA upregulation is restricted to the MLT group. The distinct patterns and an epigenetic regulation for individual OATPs suggest specific functions for these factors in tumor cells, which may involve transport of molecules important for cellular signaling as well as drugs used in therapy.

## PATIENTS AND METHODS

**Patient samples.** Samples of liver cancer and the adjacent non-cancerous tissue were obtained from patients undergoing routine surgery for liver tumors. Informed consent was obtained from all patients and permission for the study was obtained from the Ethical Committee of the Institution.

Hospital tumor history records were used to obtain information regarding stage at diagnosis, tumor grade and age. The total number of tumor specimens was 22 with a nearly equal gender distribution of ten male (n=10) and twelve (n=12) female samples. Twelve of the specimens were collected from HCC (n=7), CCC (n=5) and MLT (n=10) patients. Eight metastases were from patients with a primary tumor in the colon with the remainder of the primary tumors unknown. Detailed patient characteristics are shown in Table 1.

**Human hepatocytes.** Expression of OATPs in hepatocytes from nine individuals was studied using real-time RT-PCR. Two females and seven males with a mean age of 50.9±11.8 (S.D.) years served as liver donors. Hepatocytes were isolated as described previously.[31]

**Cell culture (HepG2 cell line).** The HepG2 cell line was originally obtained from ATCC and was maintained in phenol-red free RPMI 1640 medium (PAN Biotech, Aidenbach, Germany) containing 10% heat-inactivated fetal bovine serum (FBS) and antibiotics (100 U of penicillin/ml and 100 µg of streptomycin/ml; Invitrogen, Paisley, United Kingdom) in a humidified atmosphere at 37℃ with 5% $CO_2$/ 95% humidity.

**5-aza-2´-deoxycytidine (Aza-dC) treatment.** HepG2 cells were seeded in 6-well plates. After 24 h incubation in serum-free medium, cells were treated with either 5 µM Aza-dC (Sigma, Deissenkirchen, Germany) dissolved in medium containing 0.1% DMSO or medium containing 0.1% DMSO only. After 72 h of treatment, total RNA from cells was isolated as described below.

**RNA extraction.** Total RNA was extracted from liver tissue and cell lines using TRI Reagent® (Applied Biosystems, Warrington, UK) 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.[32]

**Quantitative real-time RT-PCR.** We reverse transcribed 2 µg of total RNA to cDNA using the high capacity cDNA reverse transcription Kit (Applied Biosystems) using random hexamer primers as recommended by the manufacturer. We purchased

TaqMan® Gene Expression Assays (Applied Biosystems) for all eleven human OATPs. In order to evaluate the appropriate reference genes, we analyzed the expression of 12 different human housekeeping genes in malignant and adjacent non-malignant tissue samples using a geNorm™ housekeeping gene selection kit with PerfectProbe™ (PrimerDesign Ltd., South-Hampton, UK). We selected CYC1, UBC, ATP5B and 18S as acceptable reference genes for TaqMan® real-time analysis of the tissue samples. For the human hepatocytes and HepG2 cells, we tested a panel of eight housekeeping genes and HPRT was chosen as the acceptable reference gene. Real-time RT-PCR was performed in a reaction volume of 10 µl. 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℃, 10 min at 95℃, 40 cycles of 15 s at 9 5℃ and 1 min at 60℃. 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.[33]

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: Hs00213714_m1, OATP2A1: Hs00194554_m1, OATP2B1: Hs00200670_m1, OATP3A1: Hs00203184_m1, OATP4A1: Hs00249583_m1, OATP4C1: Hs00698884_m1, OATP5A1: Hs00229597_m1, OATP6A1: Hs00542846_m1 and the endogenous controls 18S Part # 4310893E (Applied Biosystems), HPRT Part # 4310890E (Applied Biosystems), CYC1, UBC and ATP5B (HK-DD-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).

**Immunofluorescence.** For immunofluorescence staining, 4 µm sections were generated with a Cryostat-Microtome HM 500 OM (Microm, Heidelberg, Germany) from frozen liver samples (stored at -80℃). Tissue sect ions were fixed with acetone and blocking was performed with 5% BSA in PBS. Incubation with the primary antibody was

done overnight. Dilutions for primary antibodies were 1:100 for OATP2A1 (ab160200, Cayman Chemical, Ann Arbor, MI) and OATP5A1 (HPA 025062, Atlas Antibodies, Stockholm, Sweden), 1:20 for OATP4A1 (sc-51169, Santa Cruz Biotechnology, CA), and 1:500 for cytokeratin 19 (clone A53-B/A2.26, Lab Vision-Neo Markers, Fremont, CA, USA). After washing, sections were incubated with Alexa Fluor® IgG (Invitrogen, Carlsbad, CA) using the following dilutions: Alexa Fluor® 488 anti-rabbit IgG (1:2000) and anti-goat IgG (1:200), and Alexa Fluor® 568 anti-mouse IgG (1:1000). Cell nuclei were counterstained with bisbenzimide in PBS (Hoechst 33342, Sigma, Munich, Germany) at a 1:5000 dilution. Sections were mounted in Mowiol 4-88 (Carl Roth, Karlsruhe, Germany) and 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 4.6 software (Carl Zeiss Vision GmbH, Aalen, Germany).

**Statistical analyses.** Comparisons of significant differences in the expression of OATPs between cancerous and adjacent non-cancerous tissue specimens were performed using the Mann-Whitney U test. 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 usedfor both genes and proteins.

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**Table 1 Patient's characteristics**

| Sample | Gender | Age | Carcinoma type | Grade |
|---|---|---|---|---|
| 1 | Male | 43 | HCC | 3 |
| 2 | Male | 54 | MLT | 3 |
| 3 | Male | 57 | MLT | 3 |
| 4 | Male | 56 | MLT | 2 |
| 5 | Female | 58 | CCC | 2 |
| 6 | Male | 62 | HCC | 2 |
| 7 | Male | 48 | HCC | 2-3 |
| 8 | Male | 22 | MLT[†] | 3 |
| 9 | Female | 41 | CCC | 2 |
| 10 | Female | 42 | MLT | 2 |
| 11 | Female | 16 | HCC | 3 |
| 12 | Female | 83 | CCC | 3 |
| 13 | Female | 74 | CCC | 2 |
| 14 | Female | 78 | CCC | 3 |
| 15 | Female | 75 | MLT[†] | 3 |
| 16 | Male | 62 | MLT | 3 |
| 17 | Male | 75 | HCC | 2 |
| 18 | Female | 66 | MLT | 2 |
| 19 | Female | 75 | MLT | 3 |
| 20 | Female | 66 | MLT | 3 |
| 21 | Female | 77 | HCC | 3 |
| 22 | Male | 44 | HCC | 2-3 |

HCC, hepatocellular carcinoma; CCC, cholangiocarcinoma; MLT, metastatic liver tumors; [†], primary tumors not specified

## FIGURE LEGENDS

**Figure 1. Heatmap showing mRNA expression of all 11 human OATPs in non-cancerous (N) and cancerous (T) liver tissue samples of 22 patients.** Data represent upregulation (red) or downregulation (green) of OATP mRNA compared to the median expression level (black) of all samples.

**Figure 2. mRNA expression of human OATPs in different types of liver cancer compared to the non-cancerous controls.** Data represent the logarithmic fold regulation of mRNA in the cancerous samples compared to the adjacent non-cancerous ones. Data are given as means±SEM. HCC, hepatocellular carcinoma; CCC, cholangiocarcinomas; MLT, metastatic liver tumors of the colon. Asterisks indicate statistical significance ($p<0.05$).

**Figure 3. Immunolocalization of OATP2A1, 4A1 and 5A1 in liver cancer.**

A Immunofluorescence staining of tissue sections from non-cancerous and adjacent cancerous sections were probed with antibodies against OATP2A1, 4A1 and 5A1. (inserts without nuclei staining). Preferential green staining of plasma membranes is seen in the cancerous sections, only.

B Double immunofluorescence staining of OATP2A1 and 5A1 (green) with the biliary/progenitor cell marker cytokeratin 19 (red) and the merged OATP/CK-19 image in an HCC sample.

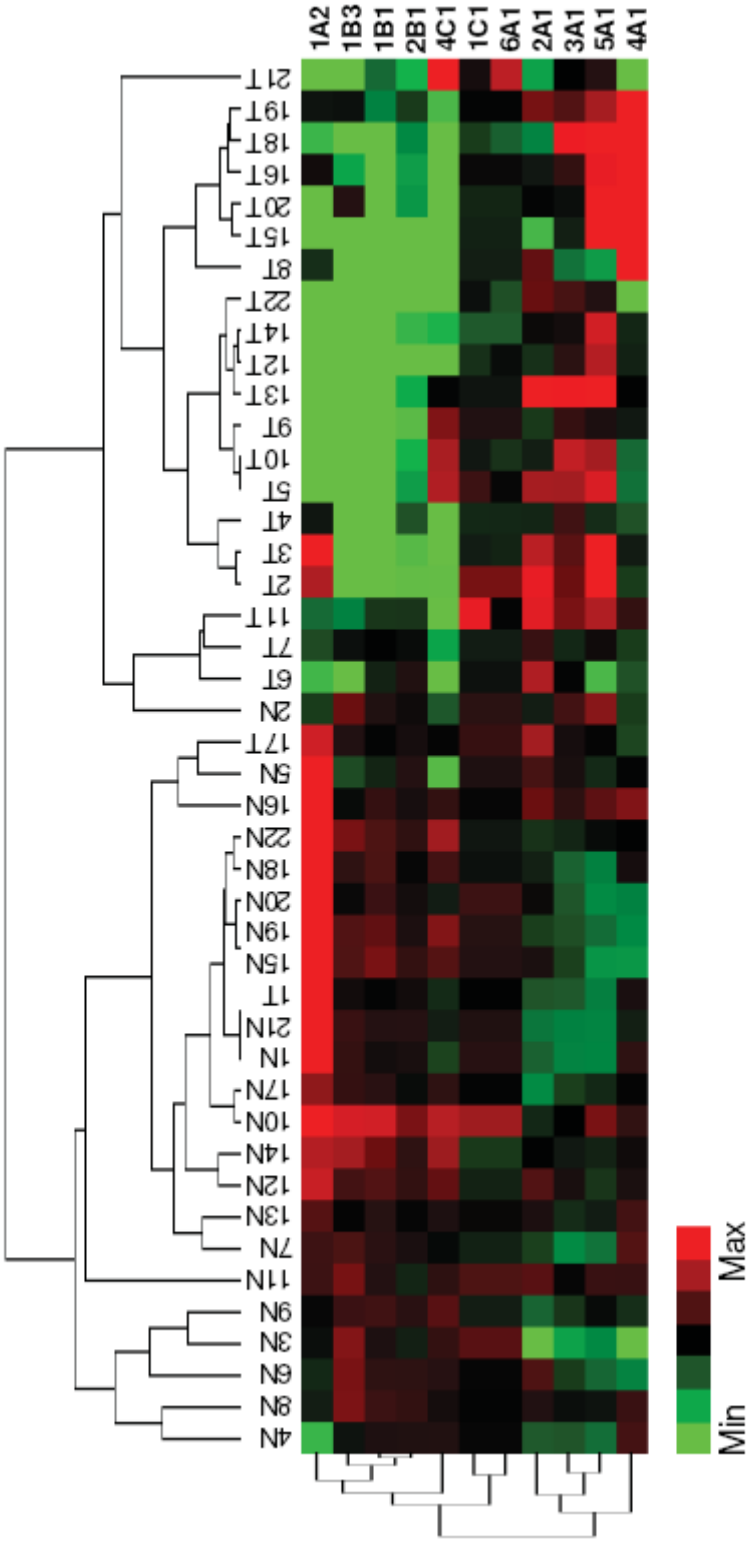
C Double immunofluorescence staining of OATP2A1 and 4A1 (green) with the biliary/progenitor cell marker cytokeratin 19 (red) and the merged OATP/CK-19 in an MLT sample.

**Figure 4. Expression of OATPs in human hepatocytes.** Total RNA was isolated and reverse transcribed from hepatocytes prepared from livers of nine healthy individuals. OATP mRNA expression was then investigated by real-time RT-PCR analysis. Values from each of the seven analyzed hepatocyte populations are indicated. Line, mean of the mRNA values from the nine hepatocyte donors.

**Figure 5. Expression of OATP mRNAs in HepG2 cells and restoration of OATP2A1 and 5A1 mRNA expression after Aza-dC treatment.** Total RNA was isolated and reverse transcribed from HepG2 cells either treated with solvent (0.1% DMSO) or with 5

µM 5-Aza-2’-deoxycytidine for 72 h. mRNA expression levels of individual OATPs are given as the logarithmic fold regulation of mRNA in the HepG2 cells compared to the human hepatocytes. Data are given as means±SEMs.

Figure 1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1B1
2B1
4C1
1C1
6A1
2A1
3A1
5A1
4A1
21T
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18T
16T
20T
15T
8T
22T
14T
12T
13T
9T
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2T
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17T
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16N
22N
18N
20N
19N
15N
1T
21N
1N
17N
10N
14N
12N
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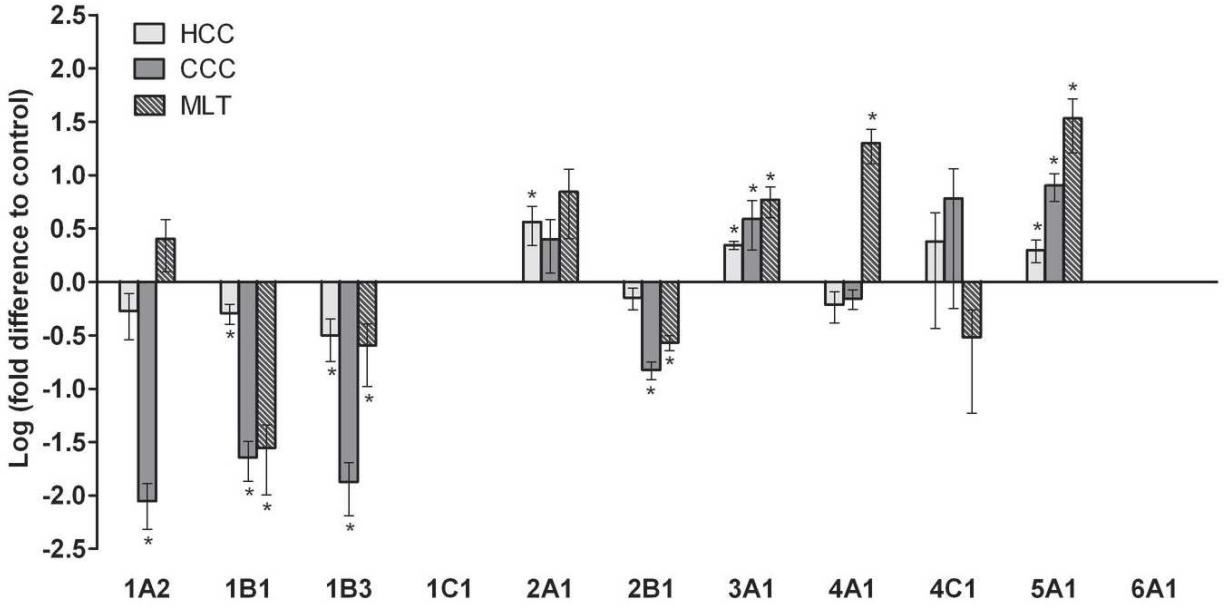
7N
11N
9N
3N
6N
8N
4N
Max
Min

Figure 2


HCC
CCC
MLT
Log (fold difference to control)
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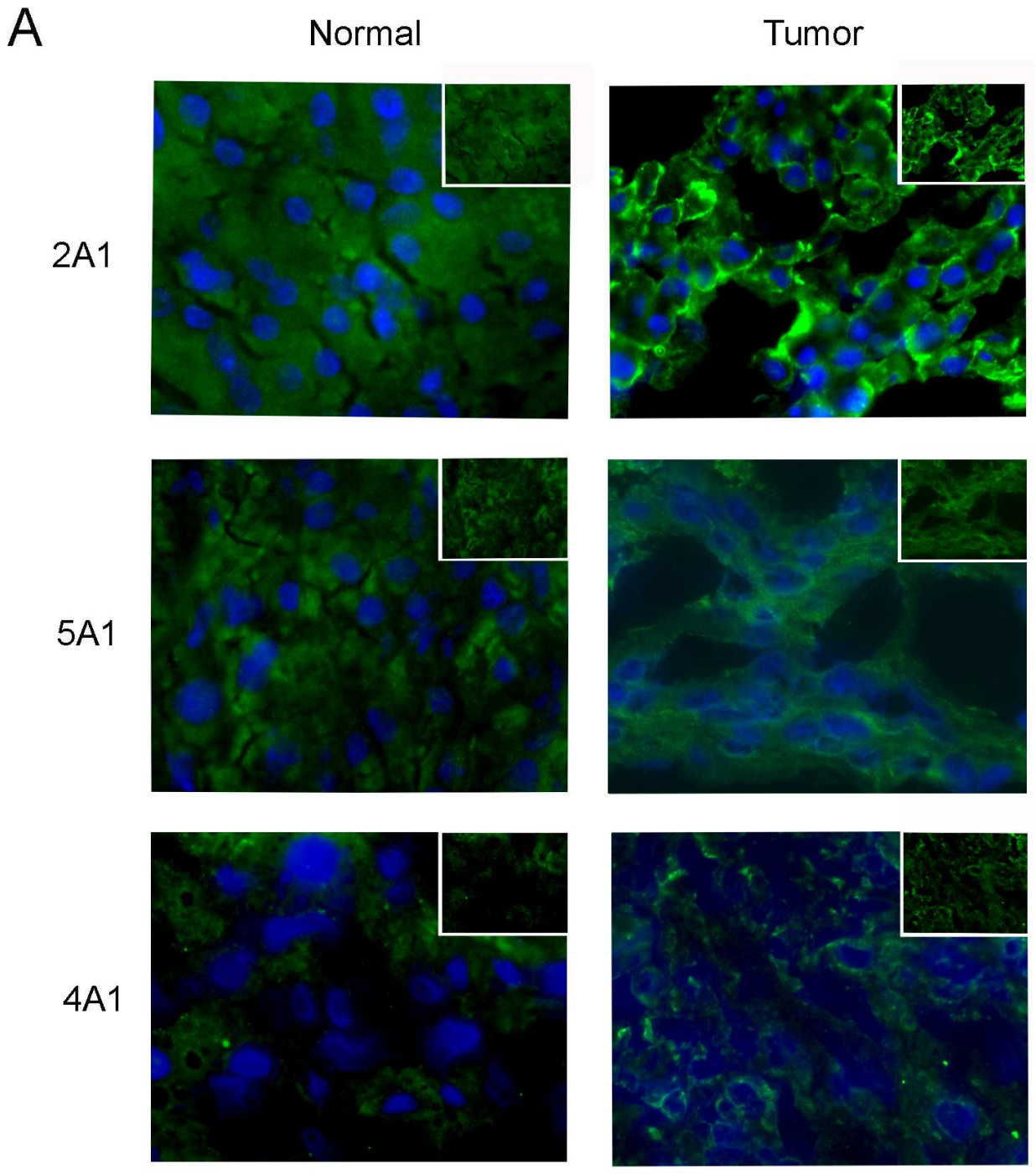
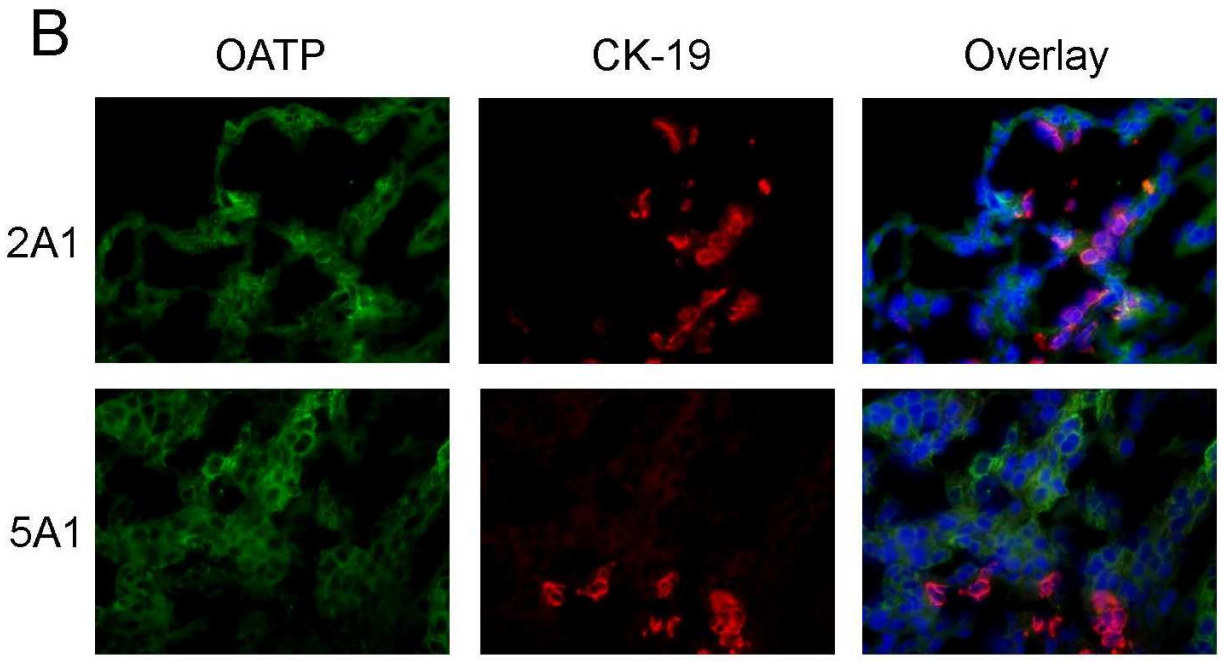
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
1A2
1B1
1B3
1C1
2A1
2B1
3A1
4A1
4C1
5A1
6A1

Figure 3




B
OATP
CK-19
Overlay
2A1
5A1

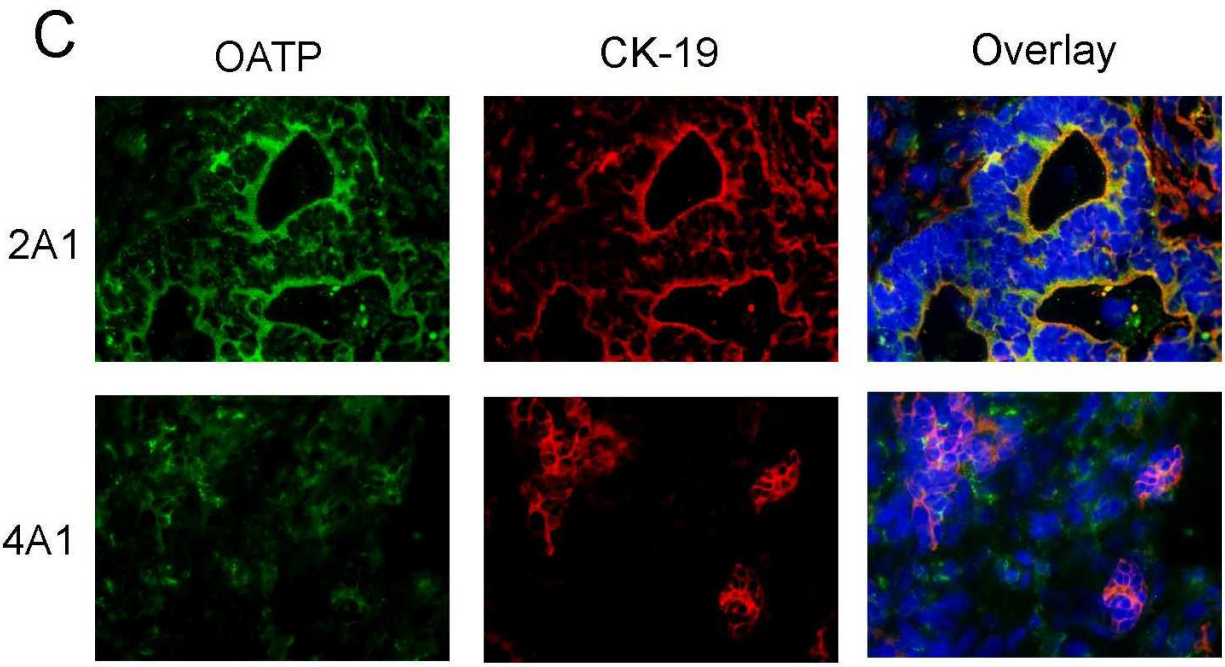
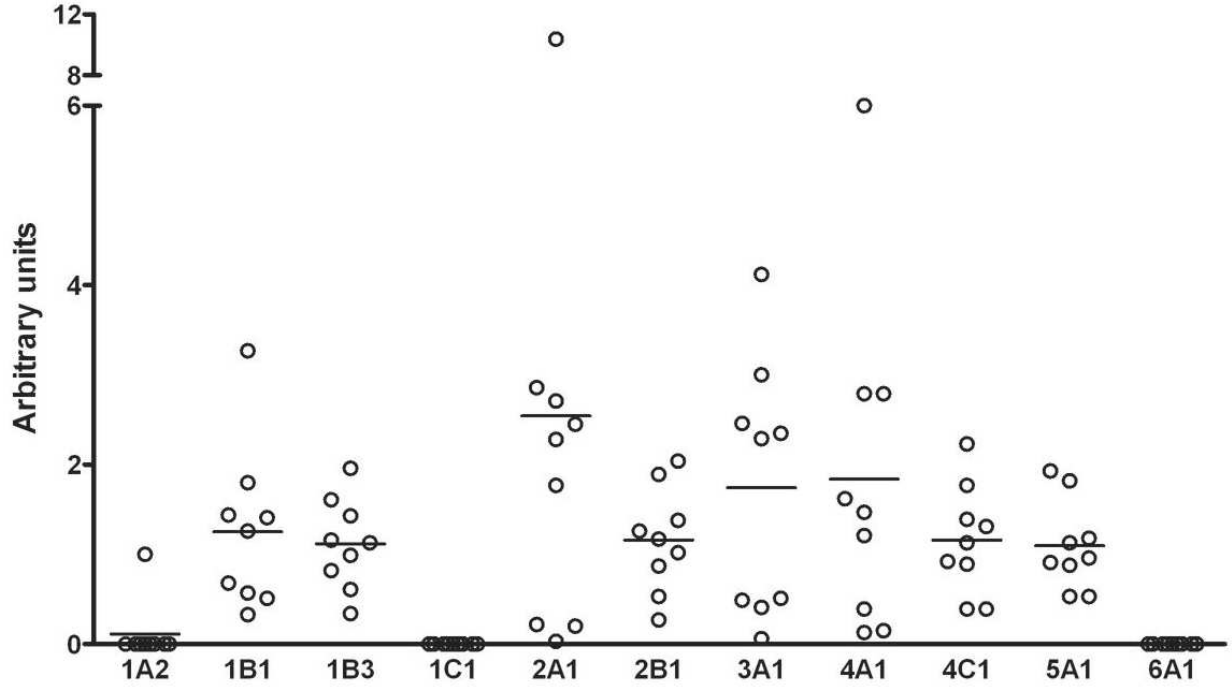

C
OATP
CK-19
Overlay
2A1
4A1

Figure 4


Arbitrary units
12
8
6
4
2
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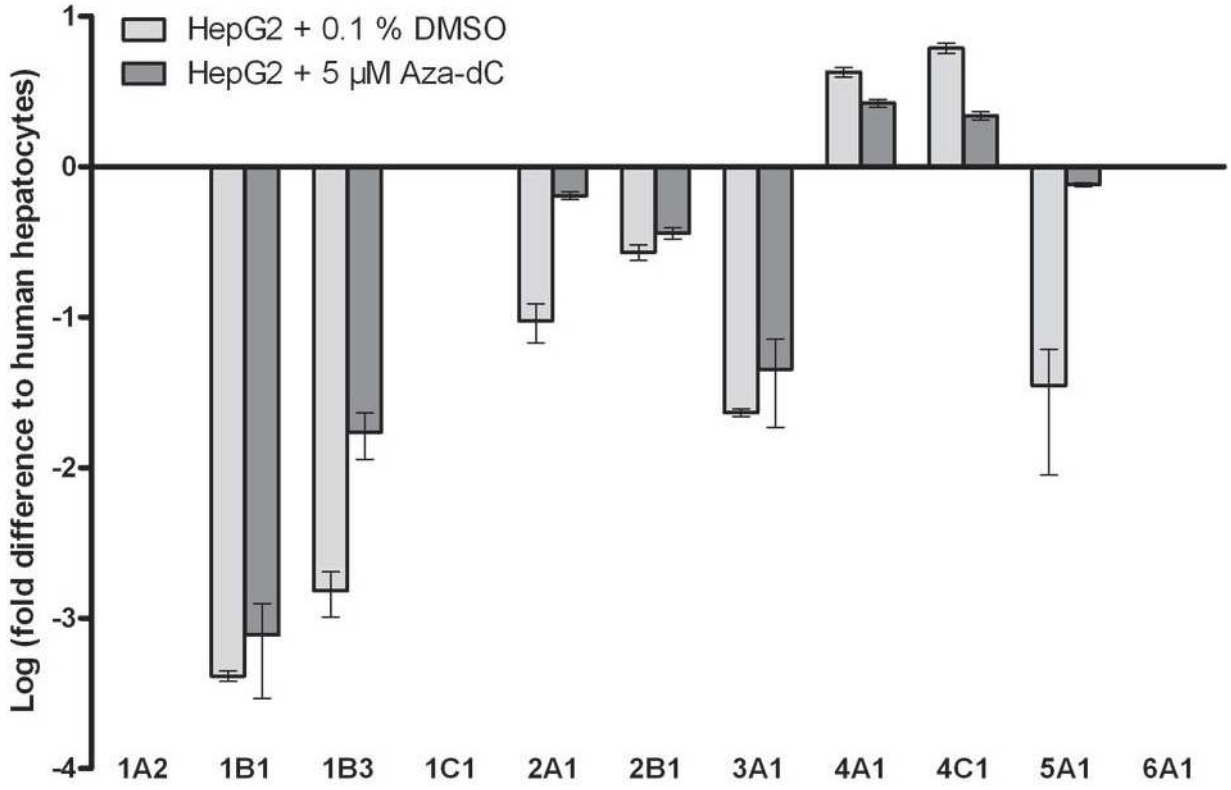
1B3
1C1
2A1
2B1
3A1
4A1
4C1
5A1
6A1

Figure 5


HepG2 + 0.1 % DMSO
HepG2 + 5 µM Aza-dC
Log (fold difference to human hepatocytes)
1
0
-1
-2
-3
-4
1A2
1B1
1B3
1C1
2A1
2B1
3A1
4A1
4C1
5A1
6A1

**PACLITAXEL TRANSPORT BY ORGANIC ANION TRANSPORTING POLYPEPTIDES (OATPS) IN THE OVARIAN CANCER CELL LINES OVCAR-3 AND SK-OV-3**

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# Paclitaxel transport by organic anion transporting polypeptides (OATPs) in the ovarian cancer cell lines OVCAR-3 and SK-OV-3

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**Abstract**

Paclitaxel is an anti-tumour agent used for the treatment of ovarian cancer with pronounced tumour accumulation. The diverse pharmacokinetics and variable efficacy of this substance are attributable to the fact that several ATP-binding cassette transporters are involved in the cellular efflux of the compound. To investigate whether organic anion transporter polypeptides (OATPs) may also act as cellular carriers for paclitaxel, we studied the mRNA expression of all known human OATPs in the two ovarian carcinoma cell lines OVCAR-3 and SK-OV-3. TaqMan® real-time RT-PCR analysis revealed expression of several OATPs in both cell lines with the highest mRNA levels for OATP1B1 and 1B3 in the SK-OV-3 cells, which was correlated with higher intracellular paclitaxel concentration in this cell line. Further studies using cRNA microinjected *Xenopus laevis* oocytes revealed OATP1B1 and OATP1B3 as high affinity transporters of paclitaxel with apparent $K_m$ values of 0.6 µM and 1.6 µM, respectively. Therefore, OATB1B1 and OATP1B3 may play a key role in the uptake of paclitaxel into ovarian cancer cells during therapy.

Keywords: Ovarian cancer, paclitaxel, OATPs, PCR, SK-OV-3, OVCAR-3

## 1. Introduction

Ovarian cancer is often diagnosed at an advanced stage because early symptoms are inconspicuous and reliable diagnostic biomarkers are not available [1]. This contributes to a poor 5-year overall survival rate (< 40%) and a high death rate [2]. After initial surgery on the tumour, a combination of paclitaxel together with platinum derivatives is applied in standard chemotherapy [3]. Although high response rates to this initial regimen are observed, a relapse is seen in two-thirds of patients due to the rapid development of drug resistance [4, 5], which is often caused by a reduced accumulation of the drug in cancer cells.

It is well-established that overexpression of ATP-powered efflux pumps such as P-glycoprotein (P-gp) or MDR1 coded by the *ABCB1* gene induces drug resistance for cytotoxic agents, including paclitaxel [6]. Indeed, taxane resistance due to overexpression of P-glycoprotein (MDR1) has been shown in ovarian cancer [7]. Furthermore, overexpression of the multidrug-resistance-related proteins MRP2 (*ABCC2*), MRP3 (*ABCC3*) and MRP7 (*ABCC10*) have been identified as paclitaxel-resistance factors in various cell lines and tumour tissues, including HER-2-amplified breast carcinoma and non-small-cell lung cancer [8, 9, 10]. However, uptake mechanisms into tumour cells might be even more important for the efficacy of anticancer drugs because they are important determinants for intracellular drug concentration [11].

One of the most important cellular drug uptake mechanisms in humans is via members of the organic anion transporting polypeptide family (OATP) [12, 13]. Official nomenclature differentiates between genes and protein using the term “*SLCO*” and “OATP”, respectively [14]. To facilitate readability and understanding of the manuscript, “OATP” is used for both genes and proteins. OATPs are expressed in a variety of

tissues [15] and tumours [16, 17] mediating the transport of endogenous and exogenous compounds and clinically used drugs [12, 13, 18]. Studies have shown that uptake transporters can confer sensitivity to anticancer agents [19, 20, 21, 22, 23, 24] as shown for the OATP1B3 substrate methotrexate [19]. This may be important for therapy because expression of OATP greatly varies in tumour cell lines [25].

Cellular uptake of paclitaxel is known to be facilitated by OATP1B3 at least in X*enopus laevis* oocytes [26] and by the organic anion transporter 2 (OAT2; gene symbol: *SLC22A7*) [27] expressed predominantly in human liver and kidney [28]. However, whether OAT2 is also expressed in human cancer cells is unknown. As OATPs exhibit overlapping substrate specificity, we hypothesised that additional OATPs may also contribute to the uptake of paclitaxel. To gain further insight into the possible role of OATP transporters in ovarian cancer, we characterised the mRNA expression profiles of all 11 known transporters and studied the uptake of paclitaxel in the estrogen responsive OVCAR-3 [29] and the estrogen-independent SK-OV-3 [30] ovarian cancer cell lines. Moreover, we investigated the specific paclitaxel transport activity of all 11 known OATPs by injecting *X. laevis* oocytes with their corresponding cRNA. Our results demonstrated that paclitaxel uptake in OVCAR-3 and SK-OV-3 cells correlates with OATP1B1 and OATP1B3 mRNA expression, indicating an important role of these transporters in tumour therapy.

## 2. Material and Methods

### *2.1. Materials*

[$^{3}$H]paclitaxel (740 GBq/mmol) was purchased from American Radiolabelled Chemicals (St. Louis, MO). The ovarian carcinoma cell lines OVCAR-3 and SK-OV-3 were obtained from the ATCC (Manassas, VA). Foetal Calf Serum (FCS) was obtained from Invitrogen (Carlsbad, CA). The ovarian carcinoma cell lines were maintained in phenol-red-free RPMI-1640 medium supplemented with L-glutamine (PAN-Biotech GmbH, Aidenbach, Germany), 10% FCS and 1% penicillin (10.000 U/ml)/streptomycin (10 mg/ml) solution (Invitrogen) in a humidified atmosphere at 37 ℃ with 5% $CO_2$.

### *2.2. TaqMan® real-time RT-PCR*

Total RNA from OVCAR-3 and SK-OV-3 cancer cells was isolated using the RNeasy® Mini kit (Qiagen, Hilden, Germany), and the RNA was treated with RNase free DNase (Qiagen) to remove possible genomic DNA. The concentration, purity and integrity of RNA samples were determined on a Nanodrop ND-1000 (Kisker-Biotech, Steinfurt, Germany) and agarose gel electrophoresis. A total of 1 µg of total RNA was reverse transcribed in 20 µl reactions using the High Capacity cDNA RT kit (Applied Biosystems, Foster City, CA) with the provided random hexamer primers and RNase inhibitor (Applied Biosystems) according to the manufacturer's instructions. TaqMan® Gene Expression Assays (Applied Biosystems) were purchased for all 11 human OATPs and four transporters from the ABC family (Table 1). Control assays for the *ACTB* (PN 4326315E) and *HPRT* (PN 4310890E) genes were also purchased from Applied Biosystems. Prefabricated primers and probes for the endogenous control genes *GAPDH* and *RPL13A* were obtained from PrimerDesign (PrimerDesign Ltd.,

Southampton, UK). TaqMan® real-time RT-PCR was performed in an amplification mixture volume of 10 µl containing 5 µl 2x TaqMan® Gene Expression PCR Master Mix (Applied Biosystems), 0.5 µl of the appropriate Gene Expression Assay, 10 ng template cDNA diluted in 4 µl nuclease free water, and 0.5 µl nuclease free water. Thermal cycling conditions were 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 on the 7900HT Sequence Detection System (Applied Biosystems) equipped with a 96-well fast cycling block. Results were imported into DataAssist™ 2.0 software (Applied Biosystems) for automated data analysis using the comparative Ct (ΔΔCt) method [31]. Thus, a normalisation factor (NF) was calculated by averaging the Ct values of the four endogenous control genes *ACTB*, *GAPDH*, *HPRT*, and *RPL13A* via the geometric mean. Relative quantities (RQ) for every sample were then calculated by the formula $RQ = 2^{(-\Delta Ct) / 2(-\Delta Ct_{reference})}$, where ΔCt = Average Ct – NF. The standard deviation (SD) was calculated for Ct values of the technical replicates and was used to calculate the $RQ_{Min}$ and $RQ_{Max}$:

$RQ_{Min} = 2^{(-\Delta Ct - SD) / 2(-\Delta Ct_{reference})}$

$RQ_{Max} = 2^{(-\Delta Ct + SD) / 2(-\Delta Ct_{reference})}$

according to the DataAssist™ v2.0 Software User Instructions (Applied Biosystems).

*2.3. Transport experiments in cell lines*

Cells that were 80-90% confluent were washed three times and incubated for 15 min (37 °C) in 12-well plates with 1 ml Krebs-Henseleit buffer (KHB, 118 mM NaCl, 23.8 mM $NaHCO_3$, 4.8 mM KCl, 1.0 mM $KH_2PO_4$, 1.2 mM $MgSO_4$, 12.5 mM HEPES, 5.0 mM glucose, and 1.5 mM $CaCl_2$, pH 7.4) [32]. Subsequently, cells were treated with 1 ml/well KHB containing 18.5 nM [$^3$H]paclitaxel. Unlabelled paclitaxel was added to a final concentration of 5 µM. Uptake was measured after 1, 2, 4, 6 and 10 min or,

alternatively, at graded concentrations (0.5, 1.0, 2.5, 5.0 and 10.0 µM paclitaxel) after 1 min of incubation, which had been determined to be within the linear range of uptake. Control studies were performed at 4 ℃ to determine temperature-insensitive paclitaxel accumulation. Paclitaxel transport was stopped by adding ice-cold KHB, followed by three washes. The amount of paclitaxel in the cells was determined by first adding 300 µl 0.2 N NaOH to each well. After 12 h at 4 ℃, 150 µl 0.4 N HCl and 4 ml Emulsifier-Safe™ scintillation cocktail (PerkinElmer) were added, and [$^{3}$H]paclitaxel was measured in an LS6500 scintillation counter (Beckman Coulter, Fullerton, CA). Protein determination was performed using a BCA™ Protein Assay (Pierce, Rockford, IL) with bovine serum albumin as the standard.

*2.4. Cytotoxicity assay*

A total of 1000 cells/well were seeded on 96-well plates. After 48 h, cells were treated with graded concentrations of paclitaxel (0.5, 1.0, 2.0, 4.0, 6.0, 10.0 and 50.0 nM) or 0.1% solvent dimethyl-sulfoxide alone. Viability was measured after 48 h using the CellTiter-Glo® Assay (Promega, Madison, WI) and a Victor™ microplate reader (PerkinElmer, Waltham, MA). Corresponding $IC_{50}$ values were calculated by non-linear regression analysis using GraphPad Prism 5 software (GraphPad, La Jolla, CA).

*2.5. Preparation of OATP cDNA for the expression in X. laevis oocytes*

The plasmids containing the human full-length cDNA from OATP1B1 (pSPORT1) and OATP1B3 (pSPORT1) were originally cloned from a human liver cDNA library, and OATP2B1 (pCMV6-XL4) was cloned from a human brain cDNA library [33]. OATP1A2 (pDRIVE, IHS1382-8428120), OATP2A1 (pOTB7, EHS1001-6793541), and OATP4C1 (pENTR223.1, OHS4559-99620240) full-length cDNA clones were purchased from

Open Biosystems (Thermo Fisher Scientific, Huntsville, AL). The lacking stop codon in the OATP4C1 clone was reconstructed by PCR using 0.5 mM dNTPs, 12.5 pmol of each primer, 0.5 U Hot Start Phusion® DNA polymerase (Finnzymes, Espoo, FI) and 1x HF buffer (Finnzymes) in a final volume of 25 µl. The following primer sequences were used (start codon in bold): forward: 5'-CC ACC **ATG** AAG AGC GC-3' and reverse: 5'-TCA CCC TTC TTT TAC TAT TTT GTT G-3'. Thermal cycling conditions were 98 ℃ for 30 s, followed by 25 cycles of 98 ℃ for 10 s, 62 ° C for 20 s, and 72 ℃ for 1 min on a MyCycler™ (Bio-Rad, Hercules, CA). The obtained full-length OATP4C1 PCR fragment was subcloned into the pJET1.2 vector (Fermentas, St.Leon-Rot, Germany) using blunt-end cloning and the CloneJET PCR cloning kit (Fermentas) according to the manufacturer's instructions. OATP1C1 (pBluescriptR, IRAKp961I0832Q) and OATP4A1 (pOTB7, IRALp962P0734Q) plasmids were purchased from Imagenes (Berlin, Germany). To obtain full-length cDNA sequences of OATP3A1 and OATP5A1 we purchased total RNA from normal human ovary (MVP™, Stratagene, La Jolla, CA). Total testis RNA (Clontech Laboratories, Inc., Mountain View, CA) for the cloning of OATP6A1 was donated by Dr. D. Mechtcheriakova (Department of Pathophysiology, Medical University of Vienna, Austria). Reverse transcription was performed by preincubation of 0.4 µg total RNA with 0.5 µg oligo(dT) primers in a volume of 11 µl for 5 min at 65 ℃. Subsequently, 9 µl of reverse transcr iption master mix was added, containing 0.5 mM of dNTPs (Fermentas), 20 U of RNaseOUT™ (Invitrogen, Carlsbad, CA), 200 U of RevertAid™ H Minus M-MuLV reverse transcriptase (Fermentas) and 1x reaction buffer in a final volume of 20 µl and incubation at 45 ℃ for 1 h, followed by 70 ℃ for 10 min. The sequence of OATP6A1 was amplifie d from 50 ng of testis cDNA and the sequence of OATP3A1 and OATP5A1 from 50 ng of ovary cDNA using 0.5 mM of dNTPs, 12.5 pmol of each primer, 0.75 µl DMSO, 0.5 U Hot Start Phusion® DNA

polymerase (Finnzymes, Espoo, FI) and 1x GC buffer (Finnzymes) in a final volume of 25 µl. Primer sequences were the following (start codons are in bold): OATP3A1, forward: 5'-A AGG **ATG** CAG GGG AAG AAG C-3', reverse: 5'-GCC CTC CTT TAG TCA CTA TAA AAC GG-3'; OATP5A1, forward: 5'-TGA ATT CTA AGC GCC **ATG** GAC GAA-3', reverse: 5'-TCT TCC ATT TTC AAG CTT CAG GAG G-3'; and OATP6A1, forward: 5'-A GCC **ATG** TTC GTA GGC GTC GC-3', reverse: 5'- ATC ACA ATG ATG ATC CAG TTA CAA GTC AG-3'. PCR conditions for OATP3A1 were 98 ℃ for 30 s, followed by 40 cycles of 98 ℃ for 10 s, 68.5 ℃ fo r 20 s and 72 ℃ for 1 min on a Bio-Rad MyCycler™. The conditions for OATP5A1 and OATP6A1 were identical except for annealing at 66 ℃. The purified cDNA was then clon ed into the pJET1.2 vector using the CloneJET PCR cloning kit (Fermentas). Sequences of OATP3A1, 5A1 and 6A1 were checked by forward and reverse full-length sequencing (MWG, Ebersberg, Germany), and compared to the appropriate reference sequences (OATP3A1 transcript variant 1: NM_013272.3; OATP5A1: NM_030958.1; OATP6A1: NM_173488.3). In OATP3A1, we found a polymorphism for 1083G>C, resulting in the amino acid exchange of Glu294Asp. For OATP5A1, we determined an SNP at 1406C>G affecting the amino acid exchange of Leu264Val. No polymorphisms were found in OATP6A1.

*2.6. Linearisation, template preparation, and in vitro transcription of OATP cRNA*

All plasmid inserts were verified for correct sequence and orientation by sequencing (MWG). For the in vitro synthesis of cRNA, the cDNA clone of OATP4A1 was linearised with BglII, and MluI was used for the linearisation of OATP1B1 and OATP1B3 plasmids. For OATP3A1, OATP4C1, OATP5A1, and OATP6A1 we used XbaI for linearisation. OATP1C1 was linearised with Acc65I and OATP2A1 with DraI. All restriction enzymes were obtained from Fermentas. After linearisation, proteinase K and 0.5% SDS

treatment, plasmid DNA was purified by phenol/chloroform extraction and ethanol precipitation. OATP1A2 and OATP2B1 sequences were amplified from plasmid using 0.5 mM of dNTPs, 12.5 pmol of each primer, 0.75 µl DMSO, 0.5 U Hot Start Phusion® DNA polymerase (Finnzymes) and 1x GC buffer (Finnzymes) in a final volume of 25 µl. Standard vector primers (M13) were used for amplification: forward: 5'- TGT AAA ACG ACG GCC AGT-3' and reverse: 5'- CAG GAA ACA GCT ATG ACC-3'. Thermal cycling conditions were as follows: 98 ℃ for 30 s, 25 cycl es of 98 ℃ for 15 s, 55 ℃ for 20 s, and 72 ℃ for 1 min on a Bio-Rad MyCycler™. The obt ained OATP1A2 and OATP2B1 PCR products were gel-purified and used as templates for in vitro transcription. 5' capped cRNAs were transcribed in vitro using 1 µg of template DNA and either T7 or SP6 mMESSAGE mMACHINE® kit (Ambion, Austin, TX) according to the manufacturer's instructions. Subsequently, the cRNAs were polyA-tailed using the polyA tailing kit (Ambion) according to the protocol. The polyA cRNA was then purified using the MEGAClear™ kit (Ambion). The concentration, purity, and integrity of cRNA samples were determined on a Nanodrop ND-1000 (Kisker-Biotech) and by denaturing gel electrophoresis.

*2.7. Paclitaxel transport studies in Xenopus laevis oocytes*

Preparation of stage V-VI oocytes from *X. laevis* was performed as previously described [[34]]. Briefly, *X. laevis* frogs (NASCO, USA) were anaesthetised by exposing them for 15 min to a 0.2% solution of MS-222 (methane sulfonate salt of 3-aminobenzoic acid ethyl ester; Sandoz Company) before surgical removal of parts of the ovaries. Follicle membranes from isolated oocytes were enzymatically digested with 2 mg/ml collagenase (Type 1A, Sigma). One day after isolation, the oocytes were injected with 10-50 nl of water either alone, to determine non-specific uptake of paclitaxel, or

containing approximately 200 ng/µl cRNA of each OATP. Injected oocytes were incubated in ND96 buffer (90 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 5 mM HEPES, pH adjusted to 7.4, supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin) at 18 ℃ for 48 hours before performi ng transport experiments with buffer exchange every day. Uptake studies were carried out in single oocytes using at least 12 oocytes per data point. Oocytes were washed three times with 3 ml substrate-free uptake buffer (100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, pH adjusted to 7.4 with TRIS) and incubated with 400 µl uptake buffer containing 20 nM [$^3$H]paclitaxel and 1% ethanol at room temperature for the appropriate time points. To avoid unspecific binding of [$^3$H]paclitaxel to the surfaces of laboratory plastic wares, all solutions containing [$^3$H]paclitaxel were manipulated using glass ware. For kinetic analysis, the uptake of radiolabelled paclitaxel was measured over a range of concentrations from 20 to 1180 nM using 60 min incubations, which had been determined to be within the linear component of uptake. Uptake was corrected for non-specific influx using water-injected oocytes from the same batch in each experiment. Uptake was stopped by the addition of 3 ml ice-cold uptake buffer, and oocytes were washed three times with buffer before being placed in scintillation vials for dissolution with 10% SDS overnight. Radioactivity was measured in a liquid scintillation counter (LS-6500; Beckman, California, USA).

*2.8. Western blot analysis of OATP1B1 and 1B3 in X. laevis oocytes*

Oocyte protein expression and membrane localisation of human OATP1B1 and 1B3 were confirmed by western blot using membrane and cytosolic protein fractions from OATP1B1- and 1B3-injected oocytes prepared according to Bianchi and Driscoll [35]. A total of 10 µg of protein was separated on 10% separating gels, blotted on PVDF

membranes and probed using an antibody against OATP1B1/1B3 (#BM5541, Acris, Herford, Germany), diluted 1:250 in TBS-T (20 mM Tris, 145 mM NaCl, 0.05% Tween 20, pH 7.6) containing 5% milk powder [36]. On control blots, an antibody to α-tubulin (Sigma, T-5168) was diluted 1:8000 in TBS-T containing 5% milk powder. The HRP-conjugated secondary antibody (#315-036-003, Jackson ImmunoResearch, Suffolk, UK) was used at a dilution of 1:10000 in TBS-T.

*2.9. Data analysis*

For kinetic analysis, data were fitted to the Michaelis-Menten (hyperbolic) model. Kinetic parameters were estimated using Prism 5 software (GraphPad) for Michaelis-Menten kinetics:

$$V=V_{max}\cdot S/(K_m+S)$$

Where $V$ is the rate of reaction, $V_{max}$ is the maximum velocity, $K_m$ is the Michaelis constant, and $S$ is the substrate concentration. Unless otherwise indicated, values are expressed as means ± SD of at least 12 individual oocytes per data point. Statistical differences from control values were evaluated using a Students' t-test, and the level of statistical significance was set at $P < 0.05$.

## 3. Results

*3.1. mRNA expression of OATPs and OAT2 in ovarian cancer cells*

To assess the role of OATP transporters for paclitaxel uptake, we investigated the mRNA expression of all 11 human OATPs in the human ovarian carcinoma cell lines OVCAR-3 and SK-OV-3. Interestingly, expression of the liver-specific OATP1B3 was up to 204-fold higher in SK-OV-3 cells compared to OVCAR-3 cells (Table 2). Even more pronounced was the difference for OATP1B1 expression, as this transporter was only

found in SK-OV-3 cells. Furthermore, a different expression pattern was also observed for OATP2B1, which was 71-fold higher in SK-OV-3 cells, and for OATP5A1, which was 85-fold higher in the OVCAR-3 cell line. OATP mRNA of 1A2, 2A1, 3A1, 4A1, 4C1, could be detected in OVCAR-3 and SK-OV-3, although the observed distribution was more balanced. Expression of OATP1C1, OATP6A1, and OAT2 mRNA was below the detection limit in both cell lines.

*3.2. Transport of paclitaxel in ovarian cancer cell lines*

Based on the different expression of OATPs in OVCAR-3 and SK-OV-3 cells, we also expected altered intracellular paclitaxel levels. In both cell lines, uptake of paclitaxel was linear with time, up to 90 sec, and with respect to 5 µM paclitaxel concentrations (data not shown). For kinetic analysis, an incubation time of 1 min was therefore chosen, showing a typical Michaelis-Menten kinetic for the uptake of paclitaxel into the OVCAR-3 cells with a $V_{max}$ of 517.5 ± 86.2 pmol/mg protein/min and a $K_m$ value of 17.2 ± 4.1 µM (Fig. 1A). However, transport of paclitaxel into the SK-OV-3 cell line was far more pronounced, showing an uptake rate of 1660 ± 126 pmol/mg protein/min at a concentration of 10 µM. Based on the limited solubility of this drug in serum-containing cell culture medium, saturation kinetics could not be observed (Fig. 1B)

*3.3. Paclitaxel transport studies in X. laevis oocytes*

To investigate whether other OATPs besides OATP1B3 contribute to paclitaxel uptake into OVCAR-3 and SK-OV-3 cells, uptake studies were performed for all 11 known human OATPs in *X. laevis* oocytes injected with cRNA coding for each of the OATP family members. Using radiolabelled paclitaxel (20 nM for 60 min), uptake in OATP1B1- and 1B3-expressing oocytes was significantly higher compared to the water control

(5.3- and 3.9-fold, respectively; $P < 0.0001$), while no other OATP favoured paclitaxel uptake (Fig. 2). The time course of [$^3$H]paclitaxel uptake by OATP1B1- and 1B3-expressing oocytes was linear with time for up to 60 min (Figs. 3A and B). The kinetic constants for this uptake revealed saturable transport in OATP1B1-expressing oocytes, with a $V_{max}$ of 12.3 ± 0.4 fmol/oocyte/min and a $K_m$ value of 0.6 ± 0.04 µM. OATP1B3-injected oocytes showed a 2.4-fold higher $V_{max}$ for paclitaxel (30 ± 1.6 fmol/oocyte/min) and a 2.7-fold higher $K_M$ value of 1.6 ± 0.13 µM compared to OATP1B1 (Figs. 3C and D). OATP1B1 and 1B3 protein expression in *X. laevis* oocytes was also confirmed by western blot analysis (see Figs. 3E and F).

*3.4. Paclitaxel cytotoxicity*

Despite differences in uptake kinetics, both ovarian carcinoma cell lines showed a similar sensitivity to paclitaxel. After treatment of cells with various concentrations of paclitaxel for 48 h, an $IC_{50}$ of 3.05 ± 1.18 and 4.63 ± 1.08 nM was observed for OVCAR-3 and SK-OV-3 cell lines, respectively (Figs. 4A and B).

*3.5. Expression of paclitaxel efflux transporters in ovarian cancer cell lines*

Given that differences in the expression of OATP1B3 and 1B1 between the two cell lines did not correlate with the observed similar paclitaxel cytotoxicity, we further investigated the expression of four major paclitaxel efflux transporters in these two cells lines. We observed up to 18-fold higher mRNA expression levels for MRP2 and MRP3 in SK-OV-3 cells (Table 3), indicating a higher efflux of paclitaxel out of the cytoplasm into the cellular supernatant. MDR1 and MRP7 were approximately similar in distribution between the two cell lines.

## 4. Discussion

In the present study, we investigated OATPs and their correlation with paclitaxel uptake in the two ovarian cancer cell lines OVCAR-3 and SK-OV-3 and in cRNA-microinjected *Xenopus laevis* oocytes.

By investigation of all 11 known OATPs by TaqMan® real-time RT-PCR, we found that OATP1B1, 1B3 and 2B1 were far more significantly expressed in SK-OV-3 cells, whereas OATP1A2, 4C1 and 5A1 were primarily present in OVCAR-3 cells. The two transporters OATP1C1 and OATP6A1 were not detectable in either cell line. It should be noted that expression of OAT2, which was previously shown to mediate the uptake of paclitaxel in *X. laevis* oocytes, was below the detection limit in both cell lines. Our data are consistent with the previous work of Okabe and coworkers, who also showed similar differences of OATP expression in OVCAR-3 and SK-OV-3 cells [25]. However, contrary to their study, we were unable to confirm expression of OATP6A1 in the OVCAR-3 cell line.

Based on these differences in OATP expression, we further investigated the concentration-dependent uptake of paclitaxel in these two cell lines. Indeed, SK-OV-3 cells showed about 3-fold higher uptake rates at 10 µM paclitaxel (1660 ± 126 pmol/mg protein/min) compared to OVCAR-3 cells (518 ± 86 pmol/mg protein/min). Differences between the two cell lines were also seen in the uptake kinetics. Whereas paclitaxel uptake was linear in SK-OV-3 cells, uptake in OVCAR-3 cells followed classical Michaelis-Menten kinetics with a $K_M$ value of 17.25 ± 4.12 µM.

To date, only OATP1B3 has been confirmed as a high-affinity paclitaxel transporter [26] within the OATP family. Furthermore, uptake of paclitaxel was significantly reduced in the OATP1B3 expressing cell line HepG2 but not in OATP1B3-deficient PR-HepG2 cells by the OATP1B3 substrate and inhibitor bromosulfophtalein

[37]. By systematically investigating the transport properties of paclitaxel for all 11 OATPs using the *X. laevis* oocytes expression system, we were able to identify OATP1B1 as an additional uptake protein for paclitaxel. Indirect involvement of OATP1B1 in taxane transport has been reported earlier by Gui et al., who showed inhibition of the OATP1B1 substrate estradiol-17β-glucuronide by paclitaxel in stable transfected CHO cells [38]. An involvement of OATP1B1 in hepatic clearance of paclitaxel may explain the finding of a pharmacogenetic investigation of variants of the SLCO1B3 gene. In the study of Smith et al. [39], differences of paclitaxel pharmacokinetics did not associate with different OATP1B3 variants, one of which has recently been shown to influence the pharmacokinetics of mycophenolate [40].

The far higher uptake rates of paclitaxel in SK-OV-3 cells prompted us to hypothesise that this could correlate with cytotoxicity. However, paclitaxel inhibited the growth of OVCAR-3 and SK-OV-3 cells with comparable $IC_{50}$ values (3.1 nM and 4.6 nM), indicating that increased paclitaxel uptake in SK-OV-3 cells might be counteracted by efflux mechanisms. Several ABC transporters have been shown to successfully extrude paclitaxel from the cells. Importantly, it has been demonstrated that MDR1 and MRP2 as well as MRP3 and MRP7 mediate paclitaxel transport [7, 8, 9, 10]. By using TaqMan® real-time RT-PCR, we identified higher expression levels of MRP2 and MRP3 in SK-OV-3 cells, whereas differences of MDR1 and MRP7 mRNA expression were moderate. MRP2 and MRP3 may therefore facilitate increased efflux of paclitaxel in SK-OV-3 cells.

Our data suggest that, in addition to OATP1B3, OATP1B1 might be an even more important determinant of paclitaxel uptake in ovarian cancer cells based on its lower $K_m$ value in *X. laevis* oocytes (0.6 µm vs. 1.6 µM), which is in the range of the mean plasma concentration in patients (0.85 ± 0.21 µM) after 24 h of infusion [41].

OATP1B1- and 1B3-mediated paclitaxel transport is of clinical importance, as both transporters share a wide, overlapping substrate spectrum that includes HMG-CoA reductase inhibitors such as fluvastatin and pitavastatin, the antibiotic rifampicin, and the endothelin receptor antagonist BQ123 [42]. The hepatic uptake rate of both transporters might be reduced by drug competition, thus resulting in increased blood levels. Therefore, inter-individual expression levels of OATP1B1 and 1B3 in the basolateral membrane of hepatocytes might be responsible for differences in the occurrence of toxic side effects such as neutropenia, neuropathy and cardiac effects during paclitaxel therapy. Such drug-drug interactions have been reported for the OATP1B1 and 1B3 substrates pravastatine and bosentan [43, 44] when concomitantly administered with clarithromycin and cyclosporine or rifampicin, respectively.

In conclusion, we found that OATP1B1 and 1B3 are high affinity and active paclitaxel transporters in transfected *X. laevis* oocytes, which may also be true for the uptake of this compound in human liver and ovarian cancer cells.

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**Table 1**

Overview of the applied gene expression assays (ABI) used for real time RT-PCR analysis

| Protein name | Assay ID[a] | Context sequence (5'→3') | Accession number |
|---|---|---|---|
| OATP1A2 | hs00245360_m1 | ACCACCTTCAGATACATCTACCTCG | NM_134431.2<br>NM_021094.2 |
| OATP1B1 | hs00272374_m1 | ATTCCACATCATTTTCAAGGGTCTA | NM_006446.3 |
| OATP1B3 | hs00251986_m1 | CTAACTTTTTGTTGGGAATCATAAC | NM_019844.2 |
| OATP1C1 | hs00213714_m1 | CTCCTACCAAGGAACCAAACCTGTC | NM_017435.3 |
| OATP2A1 | hs00194554_m1 | GCGCTTGCTGGCCTGGCTGCCATCT | NM_005630.1 |
| OATP2B1 | hs00200670_m1 | CTGCCAGGAAGGGCAAGGACTCTCC | NM_007256.2 |
| OATP3A1 | hs00203184_m1 | CTCCTCGCTCTATATAGGAATCCTG | NM_013272.2 |
| OATP4A1 | hs00249583_m1 | CAGAGACCTGCCTCTCTCCATCTGG | NM_016354.3 |
| OATP4C1 | hs00698884_m1 | TGGGAGAAAGCACTGATGTCACTGA | NM_180991.4 |
| OATP5A1 | hs00229597_m1 | TCATTGGAAACTGGTGGAGTGGATT | NM_030958.1 |
| OATP6A1 | hs00542846_m1 | CTACACTTGCAGGACTTGTTTTAAT | NM_173488.3 |
| OAT2 | hs00198527_m1 | AGCCTCTGGTGGGTGCCTGAGTCTG | NM_153320.2<br>NM_006672.3 |
| MDR1 | hs00184500_m1 | AGACATGACCAGGTATGCCTATTAT | NM_000927.3 |
| MRP2 | hs00166123_m1 | CACCTCCAACAGGTGGCTTGCAATT | NM_000392.3 |
| MRP3 | hs00358656_m1 | CAGCTGCTCAGCATCCTGATCAGGT | NM_001144070.1<br>NM_003786.3 |
| MRP7 | hs00375716_m1 | CCCAGCTCAGATCCCAGTTGGCTAT | NM_033450.2 |

[a]Applied Biosystems

**Table 2**

Expression levels of OATP mRNA in ovarian cancer cell lines[a]

| Protein name | OVCAR-3 | | | SK-OV-3 | | |
|---|---|---|---|---|---|---|
| | **RQ** | **RQ-$RQ_{Min}$** | **$RQ_{Max}$-RQ** | **RQ** | **RQ-$RQ_{Min}$** | **$RQ_{Max}$-RQ** |
| OATP1A2 | **1.00** | **0.1** | **0.06** | 0.07 | 0.00 | 0.00 |
| OATP1B1 | ND | ND | ND | **1.00** | **0.01** | **0.01** |
| OATP1B3 | 1.00 | 0.00 | 0.00 | **203.93** | **6.87** | **7.11** |
| OATP1C1 | ND | ND | ND | ND | ND | ND |
| OATP2A1 | **1.00** | **0.08** | **0.09** | 0.45 | 0.04 | 0.05 |
| OATP2B1 | 1.00 | 0.00 | 0.00 | **70.57** | **0.99** | **1.01** |
| OATP3A1 | 1.00 | **0.05** | **0.05** | 0.18 | 0.00 | 0.00 |
| OATP4A1 | 1.00 | 0.03 | 0.03 | **6.29** | **0.05** | **0.05** |
| OATP4C1 | **1.00** | **0.02** | **0.03** | 0.07 | 0.00 | 0.00 |
| OATP5A1 | **1.00** | **0.00** | **0.00** | 0.01 | 0.00 | 0.00 |
| OATP6A1 | ND | ND | ND | ND | ND | ND |

[a]mRNA expression levels relative to OVCAR-3; Maximum values are in bold; ND, transcripts were below the detection limit

**Table 3**

Expression levels of ABC transporter mRNA in ovarian cancer cell lines[a]

| Protein name | OVCAR-3 | | | SK-OV-3 | | |
|---|---|---|---|---|---|---|
| | **RQ** | **RQ-$RQ_{Min}$** | **$RQ_{Max}$-RQ** | **RQ** | **RQ-$RQ_{Min}$** | **$RQ_{Max}$-RQ** |
| MDR1 | 1.00 | 0.00 | 0.00 | **1.73** | **0.02** | **0.02** |
| MRP2 | 1.00 | 0.01 | 0.01 | **18.31** | **1.15** | **1.23** |
| MRP3 | 1.00 | 0.04 | 0.05 | **5.30** | **0.28** | **0.29** |
| MRP7 | 1.00 | 0.01 | 0.02 | 0.93 | 0.00 | 0.00 |

[a]mRNA expression levels relative to OVCAR-3; Maximum values are in bold; ND, transcripts were below the detection limit

**Legends to the Figures**

Figure 1: Time- and concentration-dependent uptake of paclitaxel in the ovarian cancer cell lines OVCAR-3 and SK-OV-3. The time-dependent uptake of 5 µM paclitaxel was measured in OVCAR-3 (A) and SK-OV-3 (B) cells at 1, 2, 4, 6, and 10 min. Concentration-dependent paclitaxel uptake for OVCAR-3 (C) and SK-OV-3 (D) cells was evaluated after an incubation time of 1 min at 37 ℃ with paclitaxel concentrations ranging from 0.5 to 10 µM. Uptake values were corrected (net values; dotted line) for temperature-insensitive paclitaxel accumulation measured at 4 ℃ after 1 min of incubation for each concentration (dashed line). Kinetic constants were calculated by non-linear least-square analysis. Data represent the mean ± SD of triplicate determinations.

Figure 2: Paclitaxel uptake in *X. laevis* oocytes. The uptake of [$^3$H]paclitaxel was measured in *X. laevis* oocytes injected with human OATP cRNA after 60-min incubation with 20 nM [$^3$H]paclitaxel. Data are given as percent of controls ± SD of at least 12 oocytes per data point. OATPs showing significantly higher (***$P < 0.001$) uptake rates compared to water-injected oocytes (control) are marked with asterisks.

Figure 3: Time course and concentration-dependent uptake of paclitaxel by OATP1B1- and OATP1B3-expressing *X. laevis* oocytes. The time-dependent uptake of 20 nM [$^3$H]paclitaxel was measured for OATP1B1 (A) and OATP1B3 (B) cRNA-injected oocytes. Water-injected *X. laevis* oocytes were included as a negative control. Concentration-dependent paclitaxel uptake for OATP1B1- (C) and OATP1B3-expressing oocytes (D) was evaluated after an incubation time of 60 min with various

paclitaxel concentrations ranging from 20 to 1180 nM. Data represent the mean ± SD of triplicate determinations. Protein expression of OATP1B1 (E) and 1B3 (F) *in X. laevis* oocytes was confirmed by western blot analysis of cRNA injected (+) and water injected (-) oocytes.

Figure 4: Cytotoxic effect of paclitaxel. An ATP-based detection assay was used to assess paclitaxel cytotoxicity in OVCAR-3 (A) and SK-OV-3 (B) cells. Eight measurements were performed for each dilution point (mean ± SD).

**Fig. 1.**

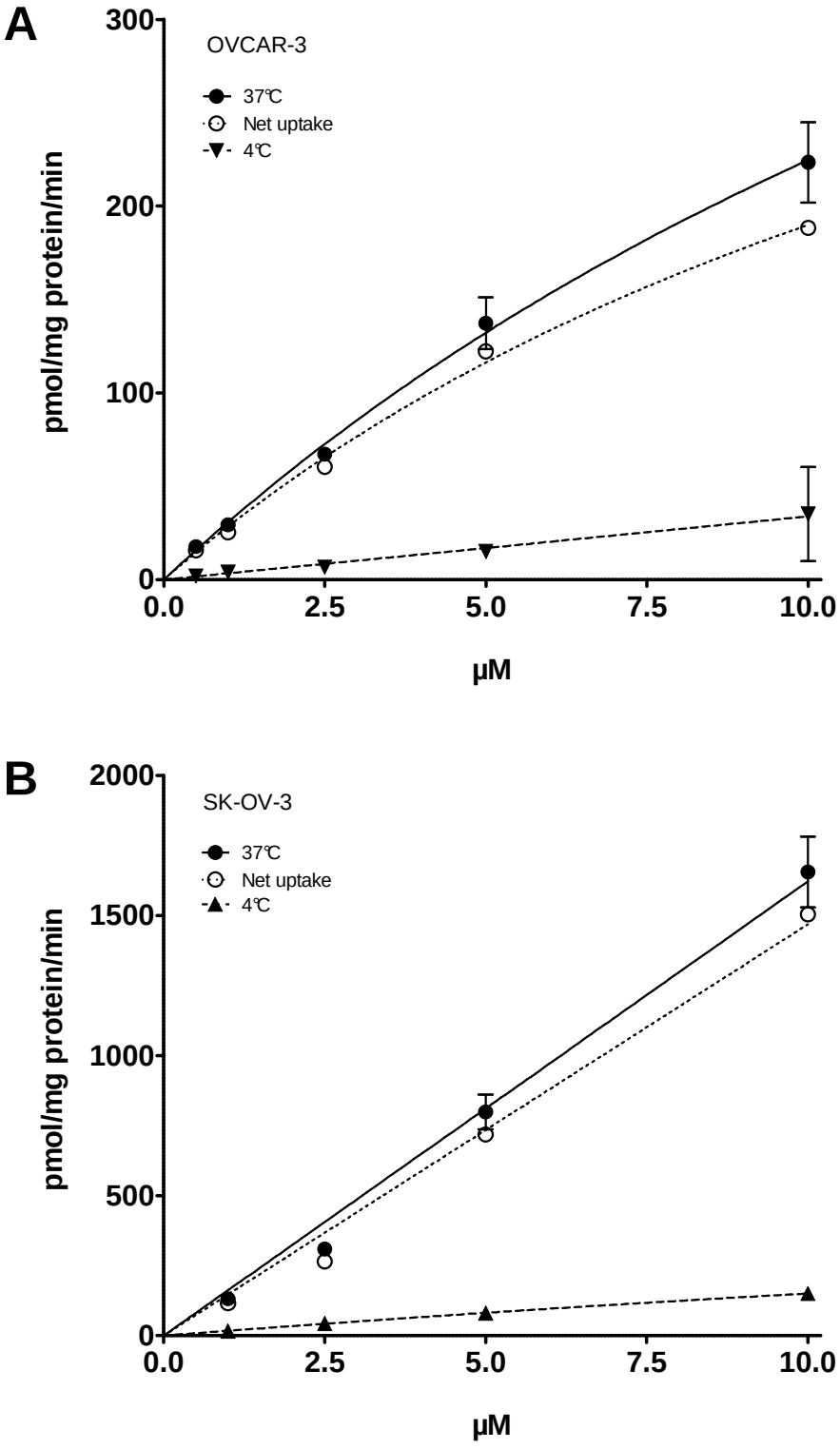

A
OVCAR-3
37℃
Net uptake
4℃
pmol/mg protein/min
300
200
100
0
0.0
2.5
5.0
7.5
10.0
μM
B
SK-OV-3
37℃
Net uptake
4℃
pmol/mg protein/min
2000
1500
1000
500
0
0.0
2.5
5.0
7.5
10.0
μM

**Fig. 2.**

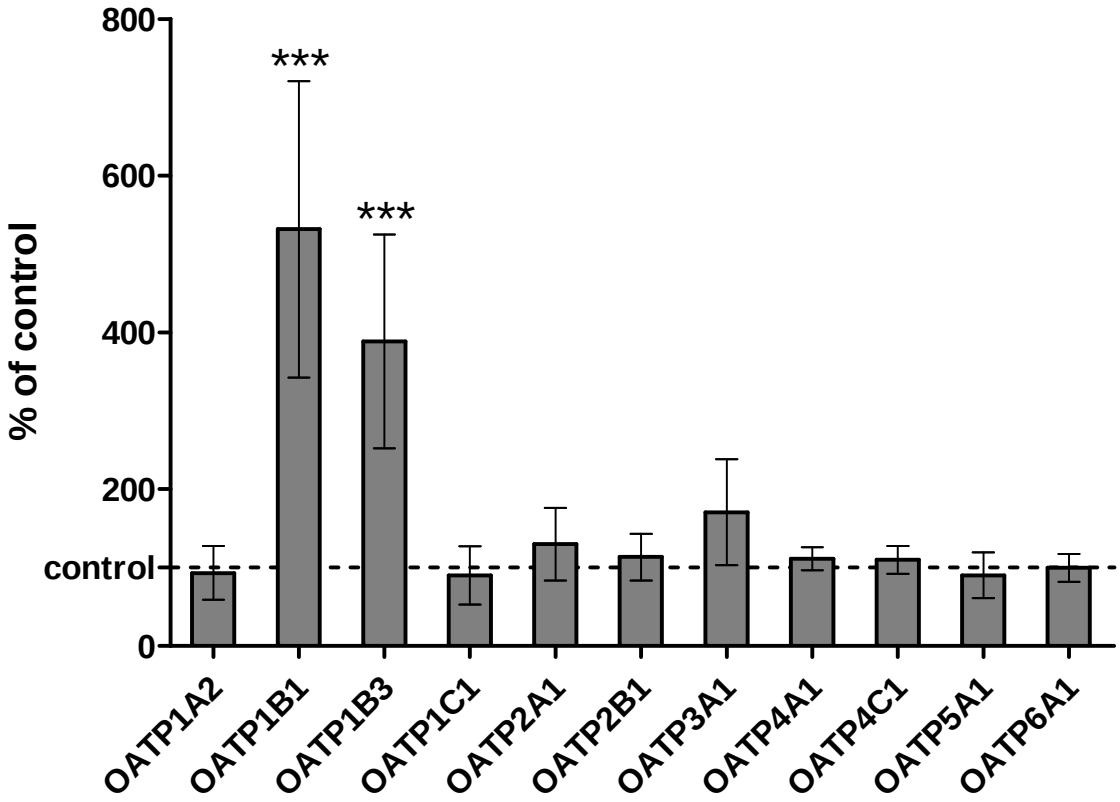

800
600
400
200
control
0
% of control
***
***
OATP1A2
OATP1B1
OATP1B3
OATP1C1
OATP2A1
OATP2B1
OATP3A1
OATP4A1
OATP4C1
OATP5A1
OATP6A1

**Fig. 3**

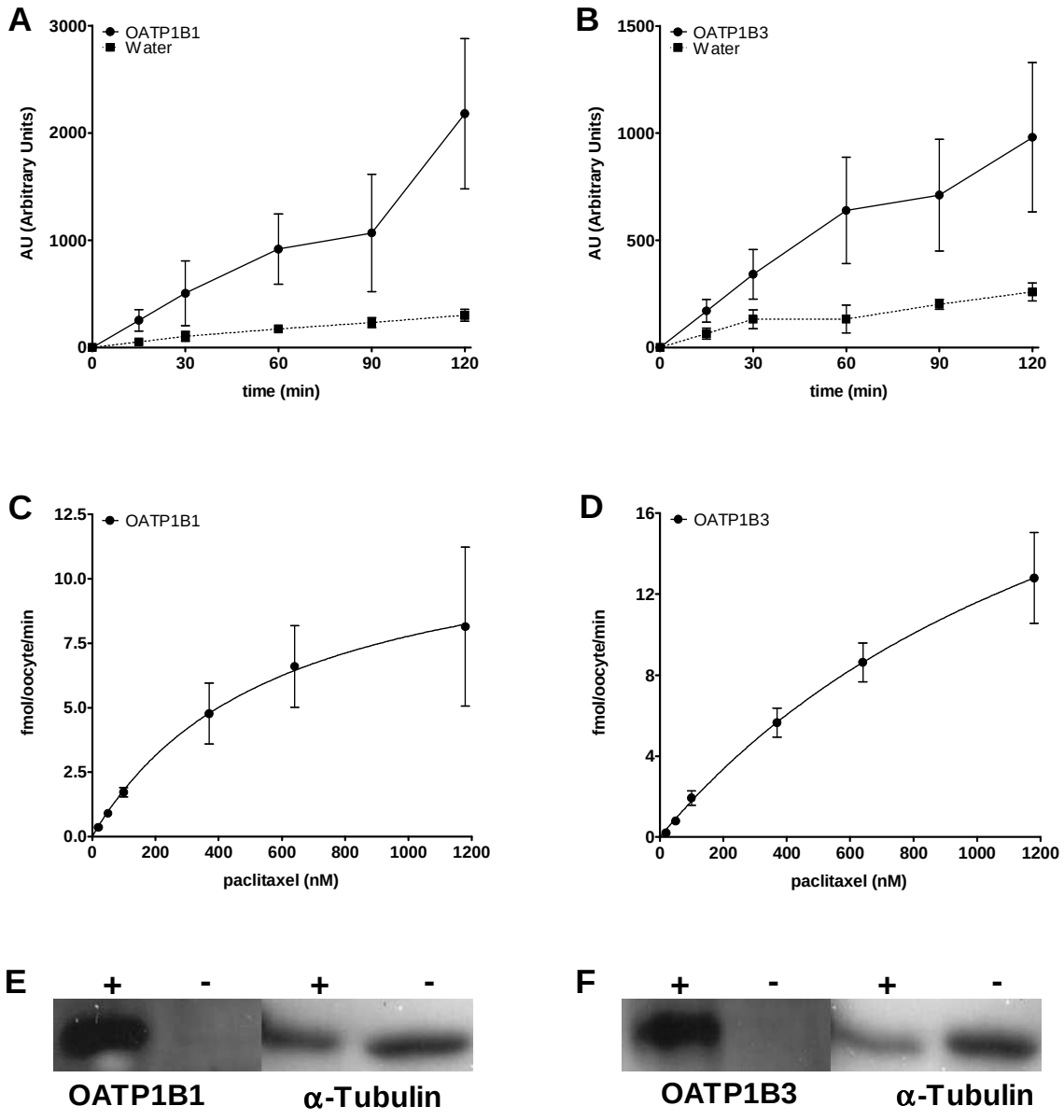
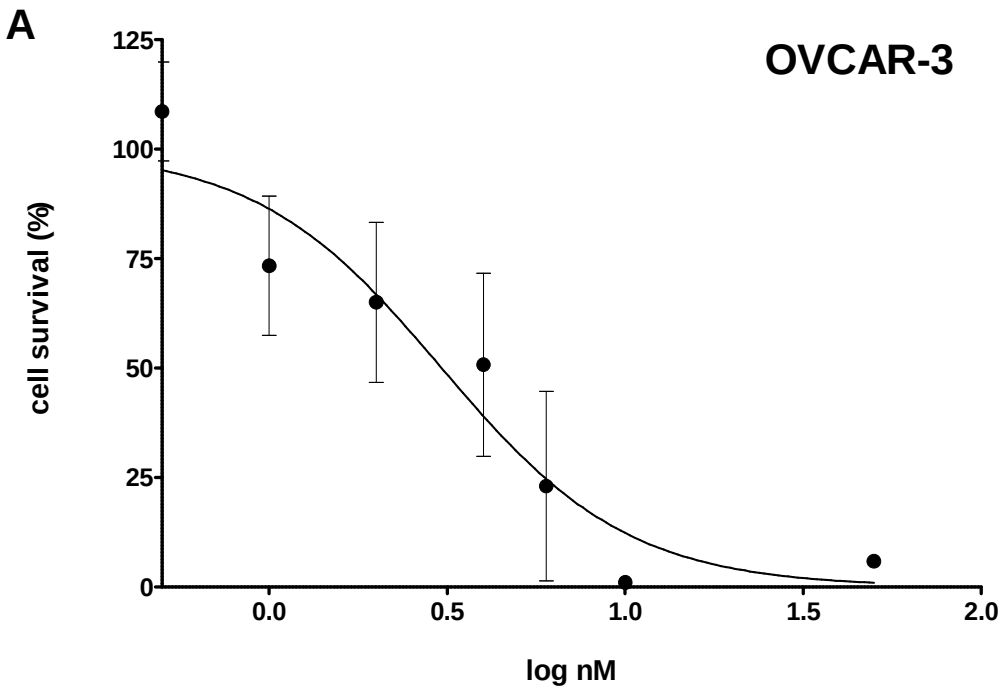
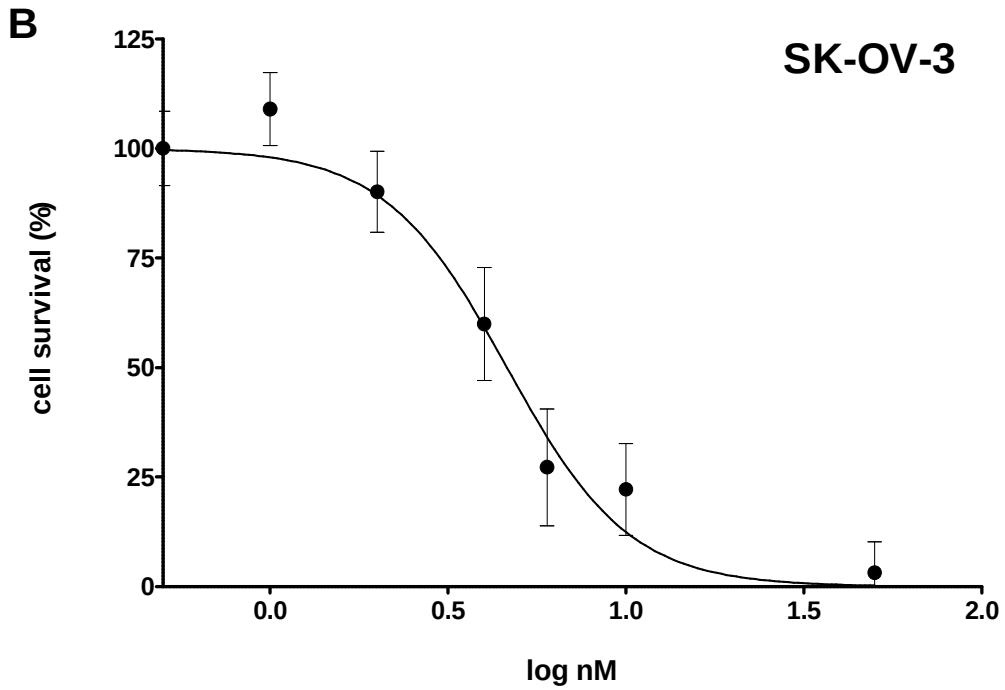

A
OATP1B1
Water
AU (Arbitrary Units)
time (min)
B
OATP1B3
Water
AU (Arbitrary Units)
time (min)
C
OATP1B1
fmol/oocyte/min
paclitaxel (nM)
D
OATP1B3
fmol/oocyte/min
paclitaxel (nM)
E
+ - + -
OATP1B1
α-Tubulin
F
+ - + -
OATP1B3
α-Tubulin

**Fig. 4.**


A
OVCAR-3
cell survival (%)
125
100
75
50
25
0
0.0
0.5
1.0
1.5
2.0
log nM


B
SK-OV-3
cell survival (%)
125
100
75
50
25
0
0.0
0.5
1.0
1.5
2.0
log nM

# 7 CURRICULUM VITAE

## Personal data

| | |
|---|---|
| **Name:** | Katrin Wlcek |
| **Date of birth:** | 07.04.1980 |
| **Place of birth:** | Vienna, Austria |
| **Nationality:** | Austria |

## Education

| | |
|---|---|
| **1986-1990:** | Primary School, Vienna |
| **1990-1998**: | Grammar School, Vienna |
| **09.07. 1998**: | Final exam |
| **10/1998-07/2004:** | Studies of pharmacy, University of Vienna |
| **03/2004-06/2004:** | Diploma thesis at the Department of Clinical Pharmacy and Diagnostics, University of Vienna and in collaboration with the Institute of Pathophysiology, Medical University of Vienna. Title: “Einfluss von Melatonin auf die Expression von CFTR in humanen Pankreas-karzinomzellinien: Bedeutung für die Behandlung der zystischen Fibrose” |
| **29.07. 2004:** | Graduation- Academic degree: Mag. pharm. |
| **Since 12/2005:** | PhD studies at the Department of Clinical Pharmacy and Diagnostics, University of Vienna and in collaboration with the Institute of Pathophysiology, Medical University of Vienna. (Advisor: Prof. Dr. Walter Jäger). |

My thesis was carried out with financial support of:

Austrian Academy of Sciences (DOC-fFORTE; 02/2007-01/2009)

Austrian National Bank 02/2009-05/2010

## Professional experience

| | |
|---|---|
| **11/2004-11/2005:** | Practical training year in a pharmacy (“Aspirantenjahr”)* |
| **17.11. 2005:** | Final examination- „Fachprüfung für den Apothekerberuf“ |
| **11/2005-02/2007:** | Partial employment in a pharmacy, Germania Apotheke, 1150 Wien |
| **2006/ 2007:** | Tutor for undergraduate student of „FH- Studienlehrgang Biotechnologie“ („Praktikum zur allgemeinen, anorganischen und analytischen Chemie“) at the Department of Clinical Pharmacy and Diagnostics, University of Vienna |
| **2006:** | Tutor for undergraduate students of pharmacy („Qualitative pharmazeutische Analytik“), at the Department of Clinical Pharmacy and Diagnostics, University of Vienna |
| **Since 2006:** | Lecturer for undergraduate students of pharmacy („Qualitative pharmazeutische Analytik“), at the Department of Clinical Pharmacy and Diagnostics, University of Vienna |

* Graduates have to complete one year of practical training in a pharmacy followed by a final examination in order to be qualified as pharmacist.

# 8 LIST OF PUBLICATIONS

## 8.1 Research articles

**Wlcek K**, Svoboda M, Thalhammer T, Sellner F, Krupitza G, Jaeger W. Altered expression of organic anion transporter polypeptide (OATP) genes in human breast carcinoma. Cancer Biol Ther. 2008; 7(9):1450-5.

Miksits M, **Wlcek K**, Svoboda M, Kunert O, Haslinger E, Thalhammer T, Szekeres T, Jäger W. Antitumor Activity of Resveratrol and its Sulfated Metabolites against Human Breast Cancer Cells. Planta Med. 2009; 75:1-4.

Liedauer R, Svoboda M, **Wlcek K**, Arrich F, Jäger W, Toma C, Thalhammer T. Different expression patterns of organic anion transporting polypeptides in osteosarcomas, bone metastases and aneurysmal bone cysts. Oncol Rep. 2009; 22:1485-92.

Miksits M, **Wlcek K**, Svoboda M, Thalhammer T, Ellinger I, Stefanzl G, Falany CN, Szekeres T, Jaeger W. Expression of sulfotransferases and sulfatases in human breast cancer: impact on resveratrol metabolism. Cancer Lett. 2010; 289:237-45.

**Wlcek K**, Svoboda M, Riha J, Stefanzl G, Olszewski U, Dvorak Z, Sellner F, Jäger W, Thalhammer T. Analysis of organic anion transporting polypeptide (OATP) mRNA and protein reveals differences in primary and metastatic liver cancer. Cancer Biol Ther. Submitted April 2010.

Svoboda M, **Wlcek K**, Taferner B, Hering S, Stieger B, Tong D, Zeillinger R, Thalhammer T, Jäger W. Paclitaxel transport by organic anion transporting polypeptides (OATPs) in the ovarian cancer cell lines OVCAR-3 and SK-OV-3. Cancer Lett. Submitted May 2010.

## 8.2 Poster presentations

**2007**

“Expression of OATP drug uptake transporters in human breast tumor and peritumoral tissues.” 8th International ISSX Meeting, October 8-12, 2007, Sendai, Japan.

“Sulfation of Resveratrol in human breast tumors and peritumoral tissues: role of sulfotransferases and sulfatases” 8th International ISSX Meeting, October 8-12, 2007, Sendai, Japan.

“Organic anion transporters (SLCO/OATP) in paclitaxel sensitive and resistant ovarian cancer cell lines A2780 and A2780ADR” 3rd PhD-Symposium, Medical University of Vienna, June 21–22, 2007, Vienna, Austria.

“Paclitaxel Resistance in the Adriamycin-resistant Cell Line A2780ADR: Role of SLCO Uptake Transporters” 5th International symposium on targeted anticancer therapies, March 8–10, 2007, Amsterdam, the Netherlands.

**2008**

“In vitro sulfation of piceatannol by human liver cytosol and human recombinant sulfotransferases” 10th European ISSX Meeting, May 18-21, 2008, Vienna, Austria.

“OATP-Uptake Transporters in Ovarian Cancer Cells: Implications for the Uptake of Drugs and Estrogens” AACR Annual Meeting, April 12-16, 2008, San Diego, USA.

**2009**

“Detection of Organic Anion Transporter Polypeptides (OATPs) mRNA in Malignant and Non-malignant Human Breast and Liver Tissue Samples.” BioMedical Transporters 2009, August 9-13, 2009, Thun, Switzerland.

“mRNA Expression of Organic Anion Transporter Polypeptides (OATPs) in Malignant and Non-malignant Human Liver.” AAPS Annual Meeting and Exposition 2009, November 8–12, 2009, Los Angeles, USA.

## 8.3 Oral presentations

“Expression of organic anion transporter polypeptides (OATPs) in human breast carcinoma” 21. Wissenschaftliche Tagung der ÖPhG, April 16-18, 2009, Vienna, Austria.