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DISSERTATION

Titel der Dissertation

NOVEL INSIGHTS INTO XENOBIOTIC TRANSPORT BY ORGANIC ANION TRANSPORTING POLYPEPTIDES (OATPs) AND OATP-EXPRESSION PROFILING IN OVARIAN CARCINOMA AND OTHER SOLID TUMORS

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1. Summary

Organic anion transporting polypeptides (called OATPs in humans and Oatps in other species) are members of the *SLCO* (solute carrier organic anion transporting polypeptides) gene family belonging to the SLC superfamily of transporters. They represent a group of putative 12 transmembrane domain (TMD) transport proteins embedded in the plasma membrane where they are responsible for the cellular uptake of a broad range of substances independently from ATP and Na^+, Cl^-, or K^+ gradients.

OATPs/Oatps have been described in several species including human, mouse, rat, cow, horse, quail, skate, and dog. To date, 11 members of the OATP family have been identified in humans. Multi-substrate specificity and broad tissue distribution are common characteristics of many OATPs (e.g., OATP3A1, OATP4A1, OATP2B1, and OATP2A1), but some OATPs, such as OATP1B1 and OATP1B3, seem to exhibit unique cellular expression patterns. These proteins are found on the basolateral membrane of human hepatocytes. OATP6A1 is another organ-specific OATP that is expressed exclusively in the testis. To date, no data are available concerning the distribution of OATP5A1 within the human body as well as substrate specifity.

Typical endogenous OATP substrates include bile acids, bilirubin, steroid conjugates, thyroid hormones, eicosanoids, and oligopeptides. In addition, a wide range of exogenous substances serve as OATP substrates, including antibiotics, statins, fexofenadine, and glibenclamide, as well as cytotoxic anticancer agents such as methotrexate, paclitaxel, docetaxel, and the small targeted inhibitor imatinib. Nevertheless, only a few OATPs have been thoroughly characterized with regard to their substrate specificity. For instance, the substrates for OATP5A1 and OATP6A1 are currently completely unknown.

The aim of this thesis was to investigate the possible role of OATPs present in cancer tissues.

To this end, the expression and distribution of OATPs in malignant tissues, as well as non-malignant tissues, deriving from breast, bone, liver and ovarian cancers were studied. Furthermore, uptake studies using radiolabeled paclitaxel as a substrate were performed on ovarian cancer cell lines and on cRNA microinjected *X. laevis* oocytes in order to elucidate the active transporters of this taxane among the OATP family members. In the cancer cell lines, expression of OATPs was assessed with

respect to paclitaxel transport. Finally, these findings could have a profound impact on the use of taxane-based chemotherapy regimens.

Ovarian cancer, also known as the “silent killer” or “the disease that whispers”, is usually diagnosed at an advanced stage because early symptoms are inconspicuous and reliable diagnostic biomarkers are not available. As a result, it is the most lethal of all the gynecologic malignancies and paclitaxel represents an important first-line anticancer agent in the treatment of ovarian cancer.

By systematically investigating the transport properties of paclitaxel for all 11 OATPs using cRNA microinjected *X. laevis* oocytes, OATP1B1 was identified as an additional uptake transporter for paclitaxel. Paclitaxel uptake followed Michaelis-Menten saturation kinetics with a K_m value of 0.6 µM, while OATP1B3 displayed a K_m of 1.6 µM, which indicates that 1B1 has a higher affinity than 1B3 for this taxane. Moreover, upon exposing OATP5A1 and OATP6A1 cRNA-injected *X. laevis* oocytes to the prototypical OATP substrate estrone-3-sulfate (E3S), only OATP5A1-mediated transport of E3S was observed. Thus, OATP1B1 was found to be a novel, high-affinity and active paclitaxel transporter in transfected *X. laevis* oocytes and the same may be true for the uptake of this compound in human liver and ovarian cancer cells.

Assessment of the mRNA expression profile by TaqMan® real-time RT-PCR in the ovarian cancer cell lines OVCAR-3 and SK-OV-3 revealed a higher expression of the paclitaxel transporting OATP1B3 and other OATPs, including OATP1B1 in the SK-OV-3 cells. Indeed, higher initial uptake rates for this taxane were also measured in the SK-OV-3 cells than in the OVCAR-3 cells. In a subsequent study, the mRNA expression profile of the 11 OATPs and of the ABC transporters, which were previously associated with drug resistance in ovarian carcinomas, was assessed in a panel of 191 clinically defined ovarian cancer patients (treatment, response to chemotherapy, disease-free survival, and overall survival) by TaqMan® real-time RT-PCR. It is important to note that of all the OATPs, only OATP3A1 expression was shown to be significantly correlated with the tumor (FIGO) stage. Furthermore, by implementing a Cox regression model, a highly significant predictor for the disease-free survival of ovarian cancer patients was found by combining the expression levels of three candidate genes, namely *SLCO6A1* and two ABC-transporters, with the drug-efflux pump *ABCC2* (MRP2) and *ABCB2* (TAP1), a transporter associated with antigen processing. Analysis of these transporters is now being carried out on the

protein level using immunofluorescence staining methods and a quantitative evaluation of the immunostaining pattern.

In subsequent studies, a comparative investigation involving malignant and adjacent non-malignant tissues derived from 13 breast cancer patients was performed. The investigation of the OATP expression at the mRNA level in the different tissues and cell lines was accomplished using TaqMan® real-time RT-PCR. Indeed, large differences in the expression of six OATPs were found between cancerous and non-cancerous samples, with higher mRNA levels of most OATPs in the latter group. However, no significant correlation of the expression levels of any OATPs in non-malignant and malignant breast tissue samples with the patient's age, tumor size, hormonal receptor, or HER-2 status could be detected, possibly due to the small sample size. Nevertheless, OATPs might play an important role in breast cancer progression because at least seven OATPs are known to mediate estrone-3-sulfate (E3S) entry into cells. E3S is not an active steroid hormone but represents an important storage form, and it has been shown that hormone-dependent breast cancer cells are able to convert E3S to active estrogen via the steroid sulfatase enzyme. Moreover, OATPs might be important determinants for the efficacy of certain anticancer drugs. However, the known paclitaxel transporter, OATP1B3, was found not to be expressed in breast cancer tissue in this study.
Similar results were obtained in a study examining the expression of all 11 OATPs in malignant and benign bone tumors using TaqMan® real-time RT-PCR. That study showed that mRNA levels of OATPs were generally reduced in the malignant specimens derived from osteosarcomas and bone metastases when compared to benign bone lesions from 21 patients. Distinct differences were also observed between the MG-63 and HOS bone cancer cell lines and primary bone cells (i.e., human osteoblast outgrowth cells and bone marrow stromal cells). OATPs can have diverse effects on bone and bone disease progression. Sex steroid hormones are important regulators of bone turnover and have effects on the activity of osteoblasts and osteoclasts. In addition to E3S, dehydroepiandrosterone sulfate (DHEAS) can also be taken up into bone cells via OATPs and be converted into active 17β-estradiol. Moreover, the OATP substrate prostaglandin E2 (PGE2) is a potent inducer of osteolysis, but once it is taken up into cells, it can be rapidly inactivated via oxidation. Several OATPs are also involved in the transport of methotrexate, an

antifolate drug used in the treatment of osteosarcoma. Thus, OATPs could be an important influence on bone tumor growth and progression by providing important regulators of bone homeostasis and by influencing the efficacy of chemotherapy regimens in terms of the uptake of certain anticancer drugs.

The liver is the most important detoxification organ and functions by removing toxic endogenous metabolites and xenobiotic substances from circulating blood. OATPs are known to play an important role in this process. Hepatocellular carcinoma (HCC) is currently the third most common cause of cancer-related death in the world. Thus, differences in OATP expression in HCC, cholangiocellular carcinoma (CCC) and metastases from primary tumors in the liver were compared to non-malignant adjacent tissues from 22 patients. The most pronounced reduction in “liver-specific” OATP1B1 and OATP1B3 mRNA expression was observed in CCC using TaqMan® real-time RT-PCR and may at least partly reflect the replacement of hepatocytes by malignant cells transformed from cholangiocytes and their precursors. It also became evident that mRNA expression of OATP2A1 and OATP5A1 is upregulated in primary and secondary hepatic tumors. This observation was confirmed at the protein level by immunofluorescence staining. Moreover, OATP4A1 upregulation was restricted to liver metastases, which was shown at the mRNA level as well as by protein immunofluorescence staining. The distinct expression pattern of individual OATPs suggests their specific functions in tumor cells, which may involve the transport of molecules important for cellular signaling and the transport of drugs used in therapy.

In summary, the present results regarding the expression of various OATP transporters in breast cancer, bone cancer, and liver cancer, as well as ovarian cancer, provide the basis for further investigations into the transport processes for endogenous and exogenous compounds in malignant and non-malignant disease. These results may also be important for further investigations regarding the efficacy of a given treatment in cancer therapy.

2. Zusammenfassung

"Organic Anion Transporting Polypeptides" (beim Menschen OATPs, bei anderen Spezies Oatps) gehören zur Überfamilie der SLC-Transporter, worin sie die SLCO (Solute Carrier OATP) Genfamilie bilden. Sie repräsentieren eine Gruppe von in die Plasmamembran eingebetteten Transport-Proteinen mit vermutlich 12 Transmembran-Domänen (TMD). OATPs/Oatps sind verantwortlich für die zelluläre Aufnahme einer breiten Palette von Stoffen, unabhängig von ATP bzw. Na^+, Cl^- oder K^+ Gradienten. OATPs/Oatps wurden in mehreren Spezies, darunter Mensch, Maus, Ratte, Rind, Pferd, Wachteln, Rochen und Hund beschrieben. Bisher wurden beim Menschen elf Mitglieder der OATP-Familie identifiziert. Häufige Merkmale vieler OATPs sind Multisubstrat-Spezifität und eine breite Verteilung im Gewebe (zB. OATP3A1, OATP4A1, OATP2B1 und OATP2A1), aber einige OATPs, zB. OATP1B1 und OATP1B3, scheinen spezifische zelluläre Expressionsmuster aufzuweisen, da sich beide ausschließlich auf der basolateralen Membran von Hepatozyten befinden. Ein weiterer organspezifischer OATP ist OATP6A1, dessen Expression ausschließlich im Hodengewebe gezeigt wurde. Derzeit liegen keine Daten über die Verteilung von OATP5A1 innerhalb des menschlichen Körpers vor. Typische endogene OATP Substrate bestehen aus Gallensäuren, Bilirubin, Steroid-Konjugaten, Schilddrüsenhormonen, Eicosanoiden und Oligopeptiden. Darüber hinaus dienen eine Vielzahl von exogenen Substanzen als Substrate für OATPs, darunter auch verschiedene Medikament wie zB. Antibiotika, Statine, Fexofenadin, Glibenclamid, aber auch Krebsmedikamente wie die Zytostatika Methotrexat, Paclitaxel, Docetaxel aber auch Imatinib. Dennoch ist nur eine geringe Anzahl von OATPs hinsichtlich ihrer Substratspezifität gut charakterisiert, zB. sind die Substrate für OATP5A1 und OATP6A1 derzeit nicht bekannt. Das Ziel dieser Arbeit war es, die mögliche Rolle von OATPs in verschiedenen Tumorentitäten zu untersuchen. Dabei wurde die Expression und die Verteilung der OATPs in malignen als auch nicht-malignen Geweben der weiblichen Brust untersucht sowie auch in Knochen-, Leber- und Eierstockkrebsgewebe. Darüber hinaus wurden Aufnahme-Studien mit Ovarialkarzinom-Zelllinien sowie mit cRNA-mikroinjizierten *X. laevis* Oozyten durchgeführt wobei radioaktiv markiertes Paclitaxel als Substrat diente, um aktive Transporter dieses Taxans unter den OATP-Familienmitgliedern zu identifizieren.

Diese Erkenntnisse könnten schließlich wichtige Auswirkungen auf den Erfolg der Chemotherapie mit Taxanen haben.

Eierstockkrebs, auch als "leiser Killer" oder "die Krankheit, die flüstert“ bekannt, wird in der Regel erst in einem fortgeschrittenen Stadium diagnostiziert, da frühe Symptome unauffällig und zuverlässige diagnostische Biomarker nicht verfügbar sind. Daher ist es die tödlichste aller gynäkologischen Malignomen. Paclitaxel ist ein wichtiges “first line“ Therapiemittel in der Behandlung dieser Krebsart. Untersuchung der mRNA Genexpression mittels TaqMan® real-time RT-PCR in den Ovarialkarzinomzelllinen OVCAR-3 und SK-OV-3 zeigte eine höhere Expression des Paclitaxeltransporters OATP1B3 aber auch von anderen OATPs, unter anderem OATP1B1 in den SK -OV-3 Zellen. In der Tat wurden auch höhere Aufnahmeraten für dieses Taxan in der SK-OV-3 Zelllinie, verglichen mit OVCAR-3, gemessen. Durch die systematische Untersuchung der Transporteigenschaften von Paclitaxel für alle elf OATPs mittels cRNA mikroinjizierten *X. laevis* Oozyten, konnte OATP1B1 als zusätzlicher Aufnahmetransporter für Paclitaxel identifiziert werden. In der durchgeführten Michaelis-Menten Kinetik-Analyse, wurde für den OATP1B1-vermittelteten Paclitaxel-Transport ein K_m-Wert von 0,6 µM bzw. für OATP1B3 ein K_m-Wert von 1,6 µM gemessen was auf eine höhere Affinität zu Paclitaxel von OATP1B1 im Vergleich zu OATP1B3 hinweist. Darüber hinaus wurde mit dem prototypischen OATP Substrat Östron-3-Sulfat (E3S) in OATP5A1 und OATP6A1 cRNA injizierten *X. laevis* Oozyten nur OATP5A1 vermittelter Transport von Estron-3-Sulfat (E3S) beobachtet. Zusammenfassend konnte OATP1B1 als neuer hochaffiner Paclitaxel-Transporter in transfizierten *X. laevis* Oozyten bestimmt werden, und dies kann auch für die Aufnahme von Paclitaxel in die Leber und in Ovarialkarzinomzellen zutreffen.

In einer nachfolgenden Studie wurde die mRNA Genexpression aller elf OATPs und verschiedener ABC-Transporter in einem Panel von 191 klinisch genau bewerteten Ovarialkarzinom-Patientinnen mit Hilfe von TaqMan® real-time RT-PCR bestimmt. Als ein erstes Ergebnis konnte OATP3A1 als einziges Kandidatengen signifikant mit dem Stadium der FIGO-Klassifizierung korreliert werden. Darüber hinaus wurde durch die Implementierung eines Cox-Regressionsmodell ein hoch signifikanter Prädiktor für das krankheitsfreie Überleben von Patientinnen mit Ovarialkarzinom durch die Kombination der Expression von drei Kandidatengenen gefunden, nämlich *SLCO6A1* und die ABC-Transporter *ABCC2* (MRP2) und *ABCB2* (TAP1). TAP1 ist

ein Transporter, der mit Antigen-Präsentation assoziiert ist. In derzeit laufenden Studien werden diese Transporter auch auf der Proteinebene durch Immunfluoreszenzfärbung untersucht.

In einer weiteren Studie wurde eine vergleichende Untersuchung zwischen malignen und nicht-malignen angrenzenden Gewebeproben von 13 Brustkrebspatientinnen durchgeführt. Die Analyse der OATP Expression auf mRNA-Ebene in den verschiedenen Geweben und Zelllinien wurde mit Hilfe von TaqMan® real-time RT-PCR durchgeführt. Tatsächlich konnten große Unterschiede in der Expression von sechs OATPs zwischen Brustkrebs- und Kontroll-Gewebeproben mit höherem mRNA Gehalt in den nicht-malignen Proben nachgewiesen werden. Allerdings konnte keine signifikante Korrelation mit Patientenalter, Tumorgröße, Hormon-Rezeptor- oder HER-2-Status und der Expression von OATPs in nicht-malignen und malignen Brustgewebe Proben nachgewiesen werden, möglicherweise aufgrund der geringen Fallzahl. Dennoch kann davon ausgegangen werden, daß OATPs eine wichtige Rolle bei der Progression von Brustkrebs spielen könnten da es von mindestens sieben OATPs bekannt ist, E3S in Zellen zu transportieren. E3S ist kein aktives Steroidhormon, stellt allerdings eine wichtige Östrogen-Speicherform dar und es hat sich gezeigt, daß hormonabhängige Brustkrebszellen in der Lage sind über das Enzym Steroidsulfatase E3S in aktives Östrogen umwandeln zu können. Außerdem könnten OATPs wichtige Determinanten für die Wirksamkeit bestimmter Krebsmedikamente sein. Unter anderem wurde OATP1B3 als hochaffiner Paclitaxel-Transporter identifiziert, ein Wirkstoff der in der Behandlung von metastasierendem Brustkrebs Anwendung findet. Obwohl Expression von OATP1B3 in Brustkrebsgewebe in dieser Studie nicht festgestellt wurde, ist es wahrscheinlich, daß andere OATPs an der Paclitaxel-Aufnahme beteiligt sind, bedingt durch die typischerweise überlappende Substratspezifität, die ein gemeinsames Merkmal der OATPs darstellt.

Ähnliche Ergebnisse wurden in einer Arbeit über die Untersuchung der Expression von sämtlichen OATPs in gutartigen und malignen Knochentumoren mittels TaqMan® real-time-RT-PCR gewonnen. Dabei konnte in Übereinstimmung mit der vorangegangenen Arbeit gezeigt werden, daß die Expression von OATPs auf mRNA-Ebene im Vergleich zu gutartigen Läsionen des Knochens in malignen Proben aus Osteosarkomen und Knochenmetastasen im Allgemeinen reduziert war. Deutliche Unterschiede wurden auch zwischen den Knochenkrebs-Zelllinien MG-63 und HOS

und primären Knochenzellen (“human osteoblast outgrowth cells“, Stromazellen des Knochenmarks) festgestellt. OATPs können verschiedene Auswirkungen auf den Knochen bzw. den Verlauf von Knochenerkrankungen haben. Sex-Steroidhormone sind wichtige Regulatoren des Knochenstoffwechsels, die Auswirkungen auf die Aktivität von Osteoblasten und Osteoklasten haben.

Neben E3S kann auch Dehydroepiandrosteronsulfat (DHEAS) über OATPs in Knochenzellen aufgenommen und in aktives 17*β*-Östradiol umgewandelt werden. Darüber hinaus ist das OATP-Substrat Prostaglandin E2 (PGE2) ein potenter Induktor von Osteolyse und nach Aufnahme in die Zelle kann es schnell durch Oxidation inaktiviert werden. Mehrere OATPs können auch Methotrexat, ein Antifolat-Medikament das Verwendung in der Behandlung von Osteosarkomen findet, transportieren. Indem OATPs wichtige Regulatoren des Kochenstoffwechsels zur Verfügung stellen und möglicherweise die Wirksamkeit einer bestimmten Chemotherapie durch die Aufnahme von Krebsmedikamenten beeinflussen, könnten OATPs von Bedeutung für das Wachstum von Knochentumoren sein.

Die Leber ist das wichtigste Entgiftungsorgan indem sie schädliche endogene Metaboliten und Xenobiotika aus dem Blutkreislauf entfernt und OATPs sind dafür bekannt, eine wichtige Rolle in diesem Prozess zu spielen. Derzeit ist das hepatozelluläre Karzinom (HCC) die dritthäufigste Ursache der weltweit durch Krebs verursachten Todesfälle. In der vorliegenden Arbeit wurden die Unterschiede in der OATP Expression in HCC, cholangiozellulärem Karzinom (CCC) und Metastasen von Primärtumoren in der Leber mit Proben von nicht-malignem angrenzenden Gewebe in 22 Patienten verglichen. Die stärkste Reduzierung der "leberspezifischen" OATP1B1 und OATP1B3 mRNA-Expression konnte im CCC mit Hilfe von TaqMan® real-time RT-PCR festgestellt werden und dieses Ergebnis könnte zumindest teilweise den Ersatz von Hepatozyten durch maligne Zellen aus Cholangiozyten und deren Vorstufen widerspiegeln. Ansonsten wurde deutlich, daß die mRNA-Expression von OATP2A1 und OATP5A1 in primären und sekundären Lebertumoren, was auch auf Protein-Ebene durch Immunfluoreszenzfärbung bestätigt werden konnte, hochreguliert ist. Darüber hinaus konnte sowohl auf mRNA- sowie auf Protein-Ebene gezeigt werden, daß die Hochregulation von OATP4A1 auf die Gruppe der Lebermetastasen beschränkt blieb.

Die speziellen Expressionsmuster der verschiedenen OATP Transporter unterstreichen ihre möglichen spezifischen Funktionen in Tumorzellen, die den

Transport von wichtigen Signaltransduktionsmolekülen sowie von in der Tumortherapie verwendeten Medikamenten beinhalten kann.
Zusammenfassend ist festzustellen, daß die vorliegenden Ergebnisse der häufigen Expression verschiedener OATP-Transporter in Brust-, Knochen-, Leber- und Eierstockkrebs die Grundlage für weitere Untersuchungen liefern bezüglich Transportprozesse für endogene und exogene Verbindungen in malignen und nicht-malignen Erkrankungen. Dies könnte auch für weitere Untersuchungen hinsichtlich der Wirksamkeit einer bestimmten Behandlung in der Krebstherapie von Bedeutung sein.

3. Introduction

3.1. Membrane Transport

3.1.1 The Plasma Membrane

One important prerequisite of life is the appearance of barriers that are capable of separating order from randomness (i.e., the cell's interior from the outside environment). Therefore, compartmentalization is necessary in order to assure the undisturbed course of life-maintaining functions under defined chemical and physiological conditions.
This function is accomplished in all living cells by the plasma membrane, which forms a permeability barrier between the inside of the cell and the outside environment. In the case of eukaryotic cells, cell organelles like the nucleus and mitochondria are additionally surrounded by intracellular membranes. Moreover, all biomembranes are composed of noncovalently associated building blocks called phospholipids. The structures at the two ends of these molecules are very different. One end consists of a hydrophobic tail containing fatty acid chains while the other end has a hydrophilic head that includes polar groups such as phosphate. Both groups are linked by an organic molecule such as glycerol. Because of the differences in polarity throughout the molecule, phospholipids are considered to be amphipathic molecules. Their specific physical properties allow the formation of the typical sheet-like membrane structure. Under suitable conditions, phospholipids spontaneously form a bilayer

structure with the polar head facing outward, thereby shielding the hydrophobic fatty acid groups from water. The three main constituents of a mammalian cell membrane are phosphoglycerides, sphingolipids and cholesterol. Cholesterol in particular is highly abundant in the membranes of mammalian cells but is, for instance, absent from all plant cells. Cholesterol intercalates between phospholipids and serves an important structural role in membranes.

A cellular membrane consisting of pure phospholipids would be impermeable to virtually all ions, amino acids, sugars and other important water-soluble molecules, and while it is tempting to think of the cellular membrane as a solid, impermeable wall, nothing could be farther from the truth. Indeed, the plasma membrane has the seemingly contradictory roles of confining and protecting the intracellular space and of also allowing the efficient and specific passage of structurally highly diverse endo- and xenobiotics across the membrane in both directions. Furthermore, the transfer of intercellular communication signals has to be assured. For this purpose, different classes of transport and receptor proteins are embedded in the phospholipid bilayer and are arranged in an amphipathic manner with the polar groups protruding out from the phospholipid bilayer into the aqueous space and the nonpolar groups largely buried in the hydrophobic interior of the membrane. This fluid mosaic model for the structure of membranes was first suggested by Singer & Nicolson [SINGER & NICOLSON, 1972].

3.1.2. Mechanisms of Membrane Transport

As mentioned above, the pure phospholipid bilayer represents an effective barrier, as only a small fraction of substances, such as the gases O_2 and CO_2, small hydrophobic molecules, and small uncharged polar molecules like ethanol may cross the membrane by simple diffusion.

Other molecules are dependent on integral membrane proteins embedded in the plasma membrane and other cellular membranes for movement across the phospholipid bilayer. In order to achieve this movement, they either travel “downhill” along a concentration gradient from higher to lower concentrations, which does not involve consumption of energy, or they are moved “uphill” against a concentration gradient, which represents a thermodynamically unfavorable process that requires an external source of energy. An appropriate energy source is provided by the opposing concentration gradients of Na^+ and K^+ ions generated by an ATP-powered pump, the

so called Na^+/K^+ ATPase, in the plasma membrane. This pump uses the energy-releasing hydrolysis of ATP to ADP + P_i to pump Na^+ out of the cell and K^+ into the cell. In addition to the chemical concentration gradient, an electrical gradient or potential is generated by the movement of ions. For example, positively charged K^+ ions are released from the cell via K^+ channels. The energy that is provided by ion concentration gradients and the electric potential are known as the electrochemical gradient, and can be used for "uphill" transport by different transport proteins to mediate the uptake of a broad range of molecules.

3.1.3. Classes of Transport Proteins

Most molecules would be incapable of crossing membranes without the assistance of specialized transport proteins and even the passage of substances usually able to diffuse through the cell membrane is frequently accelerated by specific proteins because their natural rate of diffusion is not fast enough to meet cellular needs. A common feature of all membrane transport proteins is the presence of multiple membrane-spanning elements that are thought to form a protein duct through the phospholipid bilayer, thereby avoiding contact of the hydrophilic substrates with the hydrophobic interior of the cell membrane (LODISH et al., 2008).
Generally, membrane transport proteins can be divided into three classes:

1. ATP-powered pumps
2. Ion channels
3. Transporters

3.1.4. ATP-powered Pumps

These transport proteins are characterized by the hydrolysis of ATP to ADP + P_i that releases the energy required to move ions or small molecules "uphill" against a concentration gradient. This process is considered to be "active transport" or "primary active transport" because these proteins work against a concentration gradient and are additionally providing the energy necessary for this process. An example would be the above-mentioned Na^+/K^+ ATPase. The class of ATP-powered pumps can be further divided into the four subclasses: P-class pumps, V-class proton pumps, F-class proton pumps, and the ABC (ATP-binding cassette) superfamily. The ABC superfamily represents the most interesting subclass among the ATP-powered

pumps with regard to the transport of drugs across cell membranes. Forty-eight ABC transporters with diverse functions are known in humans [DEAN et al., 2001]. The first eukaryotic ABC protein was discovered because it conferred resistance to several drugs in different cell lines [KARTNER, RIORDAN & LING, 1983]. This multidrug-resistance (MDR) protein P-glycoprotein is the product of the *ABCB1* gene and is frequently amplified in MDR cells, where it extrudes a large variety of drugs from the cell interior. Additionally, it was shown that the two copper-transporting P-class pumps, ATP7A and ATP7B, transport platinum compounds out of the cell, thereby mediating drug resistance [KATANO et al., 2003; SAMIMI et al., 2004; KOMATSU et al., 2000].

3.1.5. Ion Channels

Ion channels enable the passage of ions (Na^+, K^+, Ca^{2+}, and Cl^-), water, and small hydrophilic molecules through the phospholipid bilayer down their concentration gradients via facilitated transport or facilitated diffusion by forming a hydrophilic passageway through the phospholipid bilayer. The previously mentioned membrane K^+ channel is a good example. Movement of ions through channels can occur at very high rates and channels are highly selective for the type of ion they transport.

3.1.6. Transporters

Transporters (solute carriers) have the ability to move a broad range of structurally unrelated compounds across cellular membranes. This class, the SLC (*So*l*u*te *c*arrier) superfamily, is, along with ABC transporters, involved in drug uptake. While ABC transporters are considered to be primary active transporters, SLC transporters are responsible for secondary active transport because they are dependent on established concentration and/or electrical gradients like that of the Na^+ ion as an energy source for the “uphill” movement of their respective substrates.

3.2. OATP – Organic Anion Transporting Polypeptides

3.2.1. General Description of OATPs/Oatps

Organic anion transporting polypeptides (OATPs in humans, Oatps in other species) belong to the SLC superfamily of transporters where they are members of the *SLCO* (solute carrier organic anion transporting polypeptides) gene family (Fig. 1). The first

Oatp was cloned in 1994 from rat liver [JACQUEMIN et al., 1994] and it has became evident that transport mediated by this Oatp was independent from Na^+ as a cotransport [KULLAK-UBLICK et al., 1995; KÖNIG et al., 2000a]. Since then, OATPs/Oatps became a widely studied group of transporters and exhibit a broad range of substrate specifity. OATPs/Oatps catalyze the multispecific and sodium-independent transport of various amphipathic organic anions, neutral compounds and also few cations including endogenous, amphipathic organic compounds like bile salts, thyroid hormones, prostaglandins, and steroid conjugates. In recent years, OATPs/Oatps raised attention as they revealed to play a critical role in the bioavailability of drugs, as they mediate the excretion of endogenous and exogenous compounds including drugs in clinical use [HAGENBUCH & GUI, 2008].

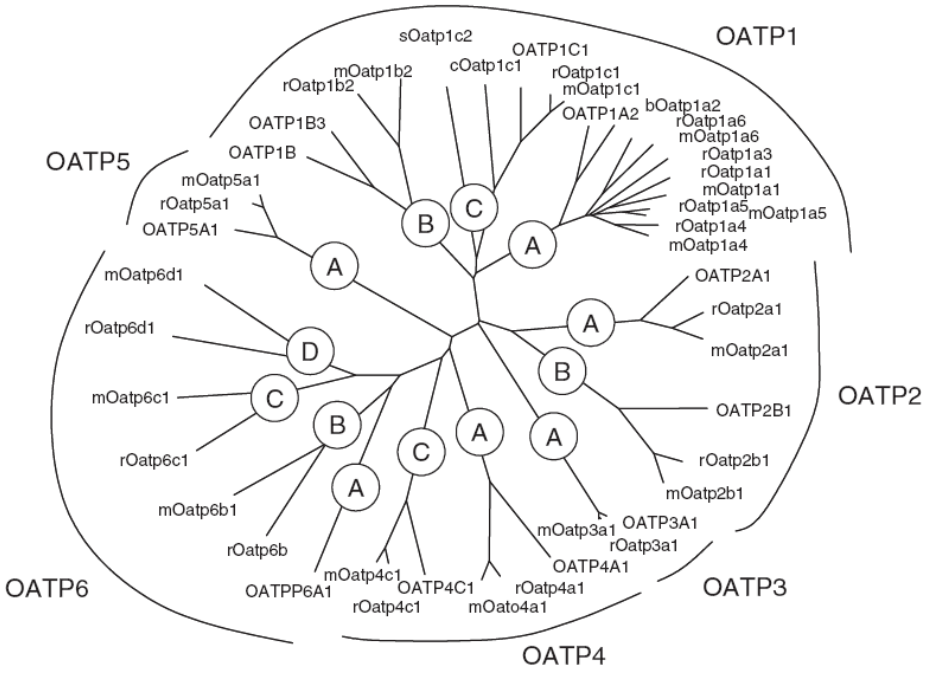

Fig. 1 Phylogenetic tree of the OATP-gene superfamily. OATPs/Oatps with more than 40% amino acid sequence identity are classified in the same family (OATP1, OATP2, OATP3, OATP4, OATP5, and OATP6). Members with more than 60% are classified in the same sub-family (e.g. OATP1A, OATP1B, and OATP1C). Human OATPs are given in all capitals, all other species are indicated with a small letter preceding the protein symbol Oatp. b, Bovine; c, chicken; m, mouse; r, rat; s, skate. [HAGENBUCH & GUI, 2008]

3.2.2. Structure of OATPs/Oatps

The first high resolution structure of an integral transmembrane protein by X-ray crystallography was published in 1985 [DEISENHOFER et al., 1985] and it has become clear that the determination of high-resolution structures is a bottleneck in

the characterization of membrane protein function because of difficulties in the purification, expression and crystallization of integral membrane proteins. Whereas membrane proteins make up about 20-30% of all open reading frames (ORFs) in fully sequenced genomes, only approximately 70 high-resolution membrane protein structures exist as compared to the thousands of solved structures of water-soluble proteins [NAM, JEON & KIM, 2009].

OATPs/Oatps are transmembrane proteins made up of 643-722 amino acids [HAGENBUCH & MEIER, 2004] and no 3-D structure of an OATP/Oatp based on X-ray crystallography has been generated thus far. Topological models including 3-D structures [MEIER-ABT et al., 2005] are based on computational methods and predict these proteins to have 9-12 transmembrane domains based on the general assumption that OATPs/Oatps contain 12 transmembrane-spanning regions (Fig. 2). This assumption is supported by experimental evidence regarding Oatp1a1 [WANG et al., 2008]. The 12-transmembrane structure results in the formation of six extracellular and five intracellular loops with both the N-terminal and C-terminal ends of the polypeptide chain located within the cell's interior. It should be noted that extracellular loops 2 and 5 contain putative N-glycosylation sites that are conserved in most OATP/Oatp family members [HAGENBUCH & MEIER, 2004]. Post-translational modification by N-glycosylation is important for OATPs/Oatps because it seems that this modification is responsible for both its functional activity and its delivery to the cell membrane [LEE et al., 2003]. According to the study of Lee et al., four asparagine residues at positions 62, 124, 135, and 492 are used in Oatp1a1 for N-glycosylation. This finding is in conflict with the study of Wang and coworkers, who suggested that only residues 124, 135, and 492 are used for glycosylation and that residue 62 is inaccessible for glycosylation due to its location within a transmembrane domain [WANG et al., 2008]. This discrepancy may be at least partially explained by differences in the experimental model systems like non mammalian *X. laevis* oocytes and mammalian cell lines. Moreover, Oatp1a1 expressed in transfected yeast cells was entirely unglycosylated [LEE et al., 2003]. Differences were even reported within mammalian cell lines, as glycosylated and unglycosylated OATP1B1 were detected in transfected MDCK cells while only the glycosylated form was detected in HEK293 cells [KÖNIG et al., 2000b].

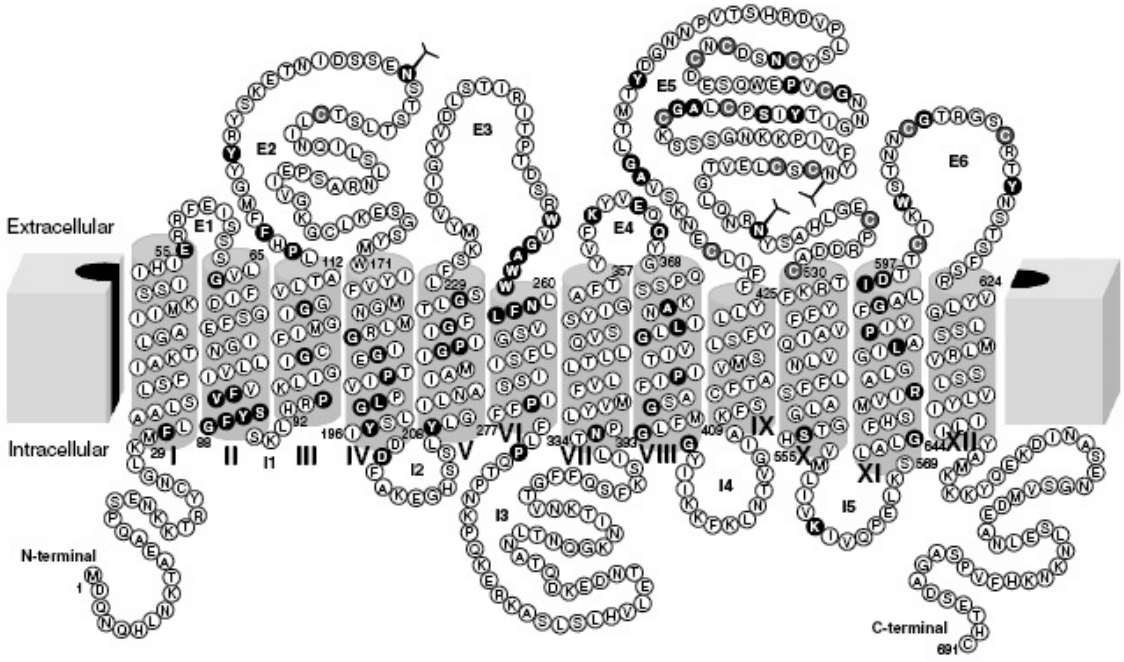

Fig. 2 Predicted topological model of human OATP1B1 with twelve transmembrane domains. Amino acids conserved in 77 out of 97 mammalian OATPs/Oatps are indicated in black. Conserved cysteine residues are given in grey. Three N-glycosylation sites are indicated (Y) in extracellular loops E2 and E5. [HAGENBUCH & GUI, 2008]

3.2.3. Plasma Membrane Sorting of OATPs/Oatps

PDZ (PSD-95/Discs-large/ZO-1) domains in proteins are generally composed of 80-90 amino acids. They have been shown to influence drug transport by recruiting and stabilizing drug transporter proteins in the outer cellular membrane and by modulating their function via direct interaction with amino acids located at the C-terminus of drug transporters [KATO, WATANABE & TSUJI, 2006]. It should be noted that PDZ motifs are generally conserved between humans and mice with the exception of PEPT1 and the OATP/Oatp family members [KATO, WATANABE & TSUJI, 2006]. Specific interactions with PDZ proteins were identified for several xenobiotic transporters, including OATP1A2, OATP3A1, and OATP1C1, using the yeast two-hybrid system [KATO et al., 2004]. Furthermore, it has been demonstrated with *PDZK1* knock-out mice that an interaction with *PDZK1* is required for the expression of rat Oatp1a1 on the intravenous surface of hepatocytes [WANG et al., 2005]. While Oatp1a1 was expressed at high levels in rat hepatocytes, the protein remains largely in the intracellular space. This result was also confirmed *in vivo* when PDZK1 knock-out mice showed reduced clearance of bromosulfophthalein (BSP) after administration. Several OATPs lack putative PDZ motifs, including OATP2A1,

which is predominantly localized intracellularly [BAO et al., 2002], and the two liver specific OATPs, OATP1B1 and OATP1B3, which are expressed on the basolateral membrane of human hepatocytes.

3.2.4. Transport Mechanisms of OATPs/Oatps

Since the discovery of the first Oatp in 1994 [JACQUEMIN et al., 1994], the underlying driving forces of OATP/Oatp-mediated transport have remained controversial. It is generally agreed that OATPs/Oatps act independently of ATP and Na^+, Cl^-, or K^+ gradients, a finding confirmed by several studies [JACQUEMIN et al., 1994; KULLAK-UBLICK et al., 1995; NOE et al., 1997; NOZAWA et al., 2004; MAHAGITA et al., 2007]. The first *in vitro* evidence for the assumption that OATPs/Oatps function as anion exchangers provided rat Oatp1a1, where extrusion of HCO_3^- was observed coupled to the influx of taurocholate in stably transfected HeLa cells but investigations if HCO_3^- can also serve as an counterion in vivo have not been performed [SATLIN et al., 1997]. Moreover, as intracellular pH is lower than extracellular pH, HCO_3^- gradients are unlikely to energize the transport of organic anions. Indeed, HCO_3^--driven transport has only been observed when the pH gradient was reversed and occurs opposite to the normal physiological condition. In another study, reduced glutathione (GSH) was reported as an Oatp1a1 counterion in cRNA injected *X. laevis* oocytes. This anionic tripeptide is present in virtually all cells at high concentrations (~10 mM) relative to its concentration in plasma (~10 µM) and this strong chemical gradient could provide a significant driving force for Oatp1a1-mediated transport [LI et al., 1998]. Cotransport of GSH was also reported for the uptake of bile acids in *X. laevis* expressing OATP1B3 [BRIZ et al., 2006]. However, this result was not confirmed in another study [MAHAGITA et al., 2007]. These data support the hypothesis that OATP1B1 and OATP1B3 function as bidirectional facilitated diffusion transporters that are energized by the electrochemical substrate gradient rather than by exchangers or cotransporters that are directly coupled to the simultaneous movement of another substrate across the membrane [MAHAGITA et al., 2007]. The contribution of bicarbonate ion and GSH gradients to the OATP/Oatp driving force was also not supported by data from another study [NOZAWA et al., 2004].

A common transport mechanism has been proposed for all OATPs/Oatps, in which substrates are translocated through a central, positively charged pore in a rocker-

switch-type mechanism [MEIER-ABT et al., 2005]. However, it is unclear whether this transport mode involves the coupled movement of another solute across the membrane or occurs by facilitated diffusion through the putative central pore.

OATP/Oatp transport activity may also be influenced by pH gradients, using the proton gradient across the membrane as the driving force. This process has been demonstrated for the transport of BSP by rat liver Oatp in transiently transfected HeLa cells [KANAI et al., 1996]. Furthermore, the uptake of pravastatin by OATP2B1 was significantly increased in an acidic extracellular pH compared to neutral pH in transiently transfected HEK293 cells [KOBAYASHI et al., 2003]. In accordance with the previous study, significantly increased transport of estrone-3-sulfate (E3S) by OATP2B1 was seen at pH 5.0 compared to pH 7.4. As expected, transport of E3S was reduced by the proton ionophore FCCP only at acidic pH [NOZAWA et al., 2004]. Interestingly, the opposite phenomenon was observed, where acidification of the intracellular compartment of HepG2 cells resulted in the increased uptake of glycocholic acid. These findings do not support a role for the pH-driven transport of glycocholic acid in these cells [MARIN et al., 2003]. A recent study showed that the transport activity of OATPs/Oatps is generally stimulated by an acidic extracellular environment and, additionally, that OATP/Oatp substrate transport leads to the stimulation of bicarbonate efflux, further supporting the theory that OATPs/Oatps act as anion exchangers [LEUTHOLD et al., 2009].

These seemingly contrary data demonstrate that the ultimate driving force(s) for OATP-mediated transport as well as the manner in which its mechanism relates to the physiological role of OATPs is not yet clearly established.

3.3. OATPs in Humans

11 members of the OATP family have been identified in humans. They are classified in the six different superfamilies OATP1, OATP2, OATP3, OATP4, OATP5, and OATP6 [HAGENBUCH & MEIER 2004]. Human OATPs are expressed in a variety of tissues including intestine, liver, kidney, and brain, and are known to play a critical role in drug absorption, distribution, and the excretion of endogenous and exogenous compounds including drugs in clinical use [HAGENBUCH & GUI 2008]. While several OATP members like OATP3A1, OATP4A1, OATP2B1, and OATP2A1 could be identified in different tissues, some members exhibit unique cellular expression in distinct organs [HAGENBUCH & MEIER 2004; KÖNIG 2006]. Whereas OATP4C1 is

considered to be kidney-specific [MIKKAICHI et al., 2004], are OATP1B1 [ABE et al., 1999; KÖNIG et al., 2000b] and OATP1B3 [KÖNIG et al., 2000a] exclusively expressed in human liver and involved in the hepatic clearance of a multitude of structurally unrelated compounds from the portal blood. Furthermore, five additional OATPs could be identified in this organ to date, either on protein or mRNA level, namely OATP1A2 [KULLAK-UBLICK et al., 1995], OATP2A1 [LU et al., 1996], OATP2B1 [TAMAI et al.. 2000], OATP3A1 [TAMAI et al., 2000] and OATP4A1 [TAMAI et al., 2000]. Nevertheless, the tissue distribution and function of two OATP members, namely OATP5A1 and OATP6A1, is still less clear and needs to be further characterized. Only two studies about OATP6A1 on mRNA level are available suggesting a testis specific expression of human OATP6A1 [LEE et al., 2004; SUZUKI et al., 2003].

3.3.1. The OATP Superfamily of Uptake Transporters

A new nomenclature was introduced in the year of 2004 for the systematic classification of OATP/Oatp protein and gene names [HAGENBUCH & MEIER, 2004]. Therefore, old protein names for the human OATPs are given in parentheses as they are still appearing in literature, especially when published before 2004.

OATP1A2 (OATP, OATP-A) was isolated in 1995 from a human liver cDNA library [KULLAK-UBLICK et al., 1995]. In liver, OATP1A2 expression could be localized to the cholangiocytes but not to hepatocytes [LEE et al., 2005]. Other tissues with confirmed OATP1A2 expression include small intestine [GLAESER et al., 2007], the brush border membrane in the distal nephron [LEE et al., 2005], and the blood brain barrier [GAO et al., 2000]. OATP1A2 consists of 670 amino acids and can be found in liver in its glycosylated form with a molecular mass of 85 kDa [KULLAK-UBLICK et al., 1997a]. In contrast, the mass of OATP1A2 in brain comprises only approximately 60 kDa, due to incomplete glycosylation [GAO et al., 2000]. Transport of endogenous substrates include the thyroid hormones triiodothyronine (T3) and thyroxin (T4) [FUJIWARA et al., 2001], the steroids E3S [LEE et al., 2005] and DHEAS [KULLAK-UBLICK et al., 1998], the bile acids cholate and taurocholate [KULLAK-UBLICK et al., 1995], and bilirubin [BRIZ et al., 2003]. Among OATP1A2 transported xenobiotics are BSP [KULLAK-UBLICK et al., 1995], but also several drugs like the antibiotics ciprofloxacin and levofloxacin [MAEDA et al., 2007], the statins pitavastatin [FUJINO

et al., 2005] and rosuvastatin [HO et al., 2006], and the anticancer agents methotrexate [BADAGNANI et al., 2006] and imatinib [HU et al., 2008].

OATP1B1 (LST-1, OATP2, OATP-C) was independently cloned by three different groups in 1999 from human liver cDNA libraries [ABE et al., 1999; HSIANG et al., 1999; KÖNIG et al., 2000b]. OATP1B1 is considered to be liver specific and protein expression is restricted to the basolateral membrane of hepatocytes where it is supposed to play an important role in the clearance of organic anions from the blood [KÖNIG et al., 2000b]. OATP1B1 consists of 693 amino acids with an approximate molecular mass of 58 kDa when unglycosylated and 84 kDa after glycosylation [KÖNIG et al., 2000b]. Furthermore, OATP1B1 has been extensively studied in the past and represents the best characterized OATP regarding substrate specificities. OATP1B1 transports a broad range of endogenous and exogenous substances which is in part overlapping with the substrate spectrum of OATP1B3. Endogenous substrates of OATP1B1 are hormones and their conjugates like E3S [CUI et al., 2001], estradiol-17*β*-glucuronide [ABE et al., 1999], DHEAS [ABE et al., 1999], T3 and T4 [ABE et al., 1999], bile acids like cholate [CUI et al., 2001], glycocholate [KULLAK-UBLICK et al., 2001] and glycoursodeoxycholate [MAEDA et al., 2006], bilirubin [BRIZ et al., 2003], and the eicosanoid prostaglandin E2 (PGE2) [ABE et al., 1999]. Many xenobiotics are transported by OATP1B1 including BSP [CUI et al., 2001], the antibiotics benzylpenicillin [TAMAI et al., 2000] and rifampicin [VAVRICKA et al., 2002], and the statins fluvastatin [KOPPLOW et al., 2005], pitavastatin [HIRANO et al., 2004] and pravastatin [HSIANG et al., 1999].
Due to the broad substrate specifity, high expression levels of OATP1B1 at the basolateral membrane of hepatocytes and its involvement into uptake of endogenous substances and xenobiotics it can significantly contribute to interindividual differences in drug effects. Several polymorphisms in the *SLCO1B1* gene have been described affecting either protein function or localization *in vitro* [TIRONA et al., 2001]. Moreover, impacts of *SLCO1B1* polymorphisms *in vivo* have been reported. In a study including 41 healthy volunteers, individuals with a 512TC genotype had on average a 106 % higher pravastatin plasma concentration compared to the 521TT genotype group [NIEMI et al., 2004]. In addition, similar to the *in vivo* finding with pravastatin, the AUC (area under curve) for repaglinide in subjects with the 521CC genotype was 107 % and 188 % higher, respectively, compared to individuals with

521TC and 521CC genotype in a study with 56 healthy volunteers [NIEMI et al., 2005].

The second member of the human OATP1 sub-family is OATP1B3 (LST-2, OATP-8). OATP1B3 was independently cloned by two different groups from liver cDNA libraries [ABE et al., 2001; KÖNIG et al., 2000a]. Similar to OATP1B1, OATP1B3 expression is liver specific but in contrast to OATP1B1 which is expressed throughout the liver lobe, OATP1B3 expression is found to be condensed around the central vein [ABE et al., 2001]. OATP1B3 is a glycoprotein of 702 amino acids and a molecular weight of approximately 120 kDa in human liver which is reduced to approximately 65 kDa when unglycosylated. It shares 80 % amino acid identity with OATP1B1. OATP1B3 belongs to the group of well characterized OATPs and many substrates for OATP1B3 have been defined using mammalian cell lines or the *X. laevis* expression system. OATP1B3 transports a broad range of different substrates which is partly overlapping with the one of OATP1B1. Endogenous substrates of OATP1B3 are T3 and T4 [KULLAK-UBLICK et al., 2001], E3S [KULLAK-UBLICK et al., 2001], estradiol-17*β*-glucuronide [CUI et al., 2001], cholate and taurocholate [BRIZ et al., 2006], and bilirubin [BRIZ et al., 2003]. Various xenobiotics are transported by OATP1B3, like BSP [KULLAK-UBLICK et al., 2001], the cytotoxic drugs methotrexate [ABE et al., 2001], docetaxel and paclitaxel [SMITH et al., 2005] and mycophenolic acid (MPA) -7-O-glucuronide (MPAG) [PICARD et al., 2010], a conjugate of MPA, which is the active moiety of the immunosuppressive drug mycophenolate mofetil (MMF). It could be furthermore demonstrated in the work of Picard et al. that the pharmacokinetics of MPA are significantly influenced by the common T334G polymorphism in the *SLCO1B3* gene in renal transplant patients receiving MMF in a cyclosporine-free immunosuppressive regimen.

OATP1C1 (OATP-F) was cloned from a human brain cDNA library [PIZZAGALLI et al., 2002]. Highest expression levels were found in brain, where it seems to be expressed at the blood-brain barrier [PIZZAGALLI et al., 2002] and furthermore in Leydig cells in the testis [PIZZAGALLI et al., 2002]. OATP1C1 consists of 712 amino acids with a calculated mass of approximately 78 kDa [PIZZAGALLI et al., 2002]. OATP1C1 is thought to have a narrower substrate specificity compared to the other members of the OATP1 sub-family but it could be shown that OATP1C1 transports

thyroid hormones (reverse T3 and T4) with high affinity [PIZZAGALLI et al., 2002]. However, it also transports the common OATP substrates E3S, estradiol-17*β*-glucuronide and BSP [PIZZAGALLI et al., 2002]. In an interesting recent study it could be demonstrated that OATP1C1 transport activity is insensitive to extracellular acidic pH due to the lack of a conserved His in the third TMD at the position 130 [LEUTHOLD et al., 2009].

OATP2A1 (PGT) was originally cloned from a human kidney cDNA library [LU et al., 1996]. In contrast to the members of the OATP1 sub-family, OATP2A1 is considered to be widely expressed in the human body [LU et al., 1996]. OATP2A1 is a protein of 643 amino acids with a calculated molecular mass of approximately 70 kDa. Interestingly, OATP2A1 has a quite unique spectrum of substrates as it transports prostaglandins and other eicosanoids but not the typical OATP substrates like BSP, E3S, and estradiol-17*β*-glucuronide.

OATP2B1 (OATP-B) was cloned from a human kidney cDNA library [TAMAI et al., 2000]. OATP2B1 expression could be determined in a variety of different tissues including liver [KULLAK-UBLICK et al., 2001], placenta [ST.-PIERRE et al., 2002], and heart [GRUBE et al., 2006]. Moreover, OATP2B1 might represent the OATP family member with the best defined distribution of protein expression at the cellular level. OATP2B1 expression was determined at the sinusoidal membrane of hepatocytes [KULLAK-UBLICK et al., 2001], at the basal membrane of the syncytiotrophoblasts [ST.-PIERRE et al., 2002], furthermore in the endothelial cells of capillaries, small arteries and veins of the heart [GRUBE et al., 2006], at the luminal membrane of brain endothelial cells [BRONGER et al., 2005], at the basolateral membrane of the non-pigmented epithelium in the ciliary body [GAO et al., 2005], at the brush border membrane of the small intestine [KOBAYASHI et al., 2003], and at the apical membrane of Caco2 cells [SAI et al., 2006]. OATP2B1 is a glycoprotein consisting of 709 amino acids with different molecular masses depending on the organ in which it is expressed. Whereas the molecular mass in liver is approximately 85 kDa [KULLAK-UBLICK et al., 2001], and approximately 95 kDa in ciliary body [GAO et al., 2005], two different bands at 85 and 95 kDa were observed in brain [BRONGER et al., 2005] possibly due to different states of glycosylation. Concerning substrate specificity, OATP2B1 was firstly characterized having a rather narrow

range of substrates because with assays performed at pH 7.4 only the “classic” OATP substrates BSP [KULLAK-UBLICK et al., 2001], E3S and DHEAS [PIZZAGALLI et al., 2003] could be identified. However, if performed at acidic pH, a broader substrate range for OATP2B1 could be observed including taurocholate, fexofenadine, pravastatin [NOZAWA et al., 2004], and glibenclamide [SATOH et al., 2005].

OATP3A1 (OATP-D) is the single member of the human OATP3 sub-family. Interestingly, two different splice variants, OATP3A1_v1 and OATP3A1_v2 could be identified which are expressed in a tissue dependent pattern. OATP3A1_v1 was originally cloned from a human kidney cDNA library [TAMAI et al., 2000] and OATP3A1_v2 was cloned from a human brain cDNA library [HUBER et al., 2007]. OATP3A1 is ubiquitously expressed on mRNA level with highest levels found in testis, brain, heart, and spleen [ADACHI et al., 2003; HUBER et al., 2007]. At the protein level, the two splice variants could be detected in different tissues [HUBER et al., 2007]. OATP3A1_v1 consists of 710 amino acids and OATP3A1_v2 of 692 with calculated molecular weights of approximately 76 and 74 kDa, respectively. The two proteins only differ at their C-terminal end. It should be noted that OATP3A1 shares about 97 % amino acid identity with the rat and mouse ortholog making it the most conserved member within the OATP family [HAGENBUCH & MEIER, 2004]. Both splice variants reveal a similar range of transported substrates when expressed in *X. laevis* oocytes and CHO cells, including prostaglandin E1 (PGE1), PGE2, and T4 [HUBER et al., 2007].

OATP4A1 (OATP-E) was originally cloned from a human kidney cDNA library [TAMAI et al., 2000] and a human hippocampus cDNA library [FUJIWARA et al., 2001]. Expression of OATP4A1 is widely and could be confirmed at the mRNA level by northern blot or RT-PCR analysis in a variety of different tissues including placenta, lung, liver, heart, skeletal muscle, kidney, and pancreas [TAMAI et al., 2000; FUJIWARA et al., 2001]. Moreover, it could be detected at the protein level on the apical membrane of syncytiotrophoblasts in the placenta [SATO et al., 2003]. The OATP4A1 protein contains 722 amino acids with a molecular mass of approximately 65 kDa in brain and placenta [SATO et al., 2003]. The substrate range of OATP4A1 is relatively narrow, with E3S, estradiol-17β-glucuronide, taurocholate,

benzylpenicillin, T3, and T4 amongst its substrates [TAMAI et al., 2000; FUJIWARA et al., 2001].

OATP4C1 (OATP-H) was isolated from a human kidney cDNA library [MIKKAICHI et al., 2004]. Based on northern blot analysis, OATP4C1 was demonstrated to be kidney specific and the rat ortholog Oatp4c1, sharing 80 % amino acid identity, could be localized on the basolateral membrane of the proximal tubule cells by immunohistochemistry [MIKKAICHI et al., 2004]. The human OATP4C1 is composed of 724 amino acids with a calculated mass of 79 kDa. Like OATP4A1, OATP4C1 exhibits a narrow spectrum of transported compounds including cyclic AMP (cAMP), methotrexate, T3, and T4 [MIKKAICHI et al., 2004].

OATP5A1 (OATP-J) mRNA expression has been identified using real-time RT-PCR in various organs including thymus, heart, skeletal muscle, prostate, and fetal brain [OKABE et al., 2008] but the protein expression and function has not been thoroughly characterized so far. An expression profile of OATP5A1 protein can be viewed at http://www.proteinatlas.org/tissue_profile.php?antibody_id=25062 with strongest expression in cortical cells from the adrenal gland and follicle cells of the ovary. The putative OATP5A1 protein contains 848 amino acids with a calculated molecular mass of 92 kDa.

OATP6A1 (OATP-I, GST, OATPY) was cloned from a human testis cDNA library [SUZUKI et al., 2003]. It has been shown by RT-PCR that OATP6A1 mRNA is predominantly expressed in testis but weak expression could also be detected in spleen, brain, fetal brain, and placenta [SUZUKI et al., 2003; LEE et al., 2004]. OATP6A1 is a predicted protein of 719 amino acids with a calculated molecular mass of approximately 79 kDa. Like OATP5A1, the protein expression and function of OATP6A1 needs further characterization.

3.4. The Relevance of OATPs in Cancer

Although further investigations are needed to define the physiological role of the OATP family of transporters in healthy tissues, it is already well established that numerous OATPs are aberrantly expressed under pathophysiological conditions.

Hepatocellular Carcinoma (HCC):

The first studies of OATPs in malignant disease focused on the expression of OATP1A2 in the HepG2 hepatoblastoma cell line and its ability to act as a specific bile acid uptake system [KULLAK-UBLICK et al., 1996] that could be used to deliver bile acid-coupled anticancer drugs like chlorambucil-taurocholate into cancer cells [KULLAK-UBLICK et al., 1997b]. In HCC, it was demonstrated by western blot analysis that the liver-specific OATPs, OATP1B1 and OATP1B3, are downregulated relative to the surrounding normal liver tissue and that the expression of the two liver-specific OATPs are absent in the HepG2 cell line. The study was accomplished using a self-generated antibody that recognized both OATP family members and which could be useful as part of an optimized marker panel suitable for the identification of HCCs in diagnostically problematic cases [CUI et al., 2003]. Findings of reduced OATP1B1 expression in HCC reported by Zollner and coworkers [ZOLLNER et al., 2005] taken together with the finding that OATP1B3 shows decreased expression in HCC tissue [CUI et al., 2003] indicate that the strategy of targeting liver cancer cells via bile acid-coupled chemotherapeutic drugs needs further careful evaluation.

Gastrointestinal Cancers:

In contrast to HCC, overexpression of the liver specific OATP1B3 was demonstrated in gastrointestinal cancers of the stomach, colon, and pancreas by immunohistochemistry [ABE et al., 2001]. Interestingly, OATP1B3 expression by primary colon cancer was also maintained in a metastatic lesion of the lymph node. Moreover, it was shown that the anticancer agent methotrexate is transported by OATP1B3 and that the expression of OATP1B3 in gastrointestinal cancer cell lines like KatoIII and PK-8 is accompanied by increased sensitivity to this drug [ABE et al., 2001].

Colorectal Cancer (CRC):

The OATP1B3 mRNA and protein are significantly overexpressed in colorectal cancer but no difference in expression was observed for the closely related OATP1 family members OATP1A2 and OATP1B1 between tumor and normal colon tissue [LEE et al., 2008]. Remarkably, the staining pattern of OATP1B3 in the colorectal tumor tissue showed a primarily cytoplasmic pattern that is very different from the membrane staining in liver, which served as control tissue. Unexpectedly, OATP1B3-

expressing colon cancer cells (RKO, HCT-8) exhibited a survival advantage relative to empty vector-transfected control cells when both are treated with the chemotherapeutic drug camptothecin. It seems that OATP1B3 overexpression interferes with the transcriptional activity of p53 and its downstream targets [LEE et al., 2008]. Moreover, the antiapoptotic effect of OATP1B3 seems to be dependent on its transporter function because overexpression of an OATP1B3 variant with a loss-of-function point mutation did not affect p53 activity [LEE et al., 2008]. Additionally, increased expression of OATP4A1 could be observed in CRC by micro-array analysis [ANCONA et al., 2006]. Compatible with this result is a study of OATP expression in inflammatory bowel disease (IBD), where a highly significant overexpression of OATP2B1 and OATP4A1 mRNA was detected in specimens collected from inflamed ulcerative colitis (UC) patients compared to specimens from non-inflamed UC [WOJTAL et al., 2009]. Patients with long-standing IBD have a higher risk in developing CRC [XIE & ITZKOWITZ, 2008]. In another study, the role of the prostaglandin uptake transporter OATP2A1 was investigated in CRC [HOLLA et al., 2008]. It was demonstrated that OATP2A1 is downregulated in CRC relative to normal mucosa and that the PGE2-extruding pump MRP4 is upregulated in CRC [REID et al., 2003]. PGE2 in particular has been identified as an important mediator of cancer progression in CRC [SHENG et al., 2001]. Prostaglandins can act via G-coupled membrane receptors or via nuclear peroxisome proliferator-activated receptors (PPARs) [GUPTA et al., 2000]. The import of PGE2 into cancer cells is of importance as it facilitates the removal of PGE2 from the extracellular milieu, which is followed by inactivation via intracellular 15-hydroxyprostaglandin dehydrogenase (*15-PGDH*) [NOMURA et al., 2004].

Breast Cancer:

The role of OATP uptake transporters is of special interest in hormone-responsive cancers because many OATPs are known to conduct hormones and their conjugates across cell membranes. In normal breast tissue, OATP2B1 is expressed at high levels in cells from the myoepithelium. However, in tumor-bearing tissue, OATP2B1 was most apparent in areas of invasive ductal carcinoma and lost its association with myoepithelial cells [PIZZAGALLI et al., 2003]. As OATP2B1 was identified as steroid-sulfate carrier [PIZZAGALLI et al., 2003], it could be an important factor that contributes, together with elevated levels of 17*β*-hydroxysteroid dehydrogenase I and

aromatase enzymes, to high intratumoral levels of active 17*β*-estradiol (E2) [ARIGA et al., 2000; MIYOSHI et al., 2001]. Furthermore, it has been demonstrated that the liver-specific OATP1B3 is expressed in breast cancer tissue and has been identified as a potent prognostic marker in a study of 102 specimens of invasive ductal breast carcinoma using immunohistochemistry [MUTO et al., 2007]. A recent study conducted in our laboratory revealed that the expression of OATP3A1, OATP4A1 and, OATP2B1 is significantly altered at the mRNA level between malignant and adjacent non-malignant breast cancer specimens [WLCEK et al., 2008]. All of these transporters were identified as having E3S as a substrate, which is highly abundant in plasma and can be converted within the tumor cells to active estrogen.

Bone Cancer:

In another study examining OATP expression patterns in bone tumors and benign bone cysts we were able to show the expression of all OATPs except OATP1B1, OATP1B3 and OATP6A1 in bone tumors. OATP3A1 and OATP4A1 were most widely found. Furthermore, mRNA levels of OATPs were generally reduced in malignant specimens derived from osteosarcomas and bone metastases compared to benign bone lesions from 21 patients [LIEDAUER et al., 2009]. Distinct differences were also observed between the MG-63 and HOS bone cancer cell lines and primary bone cells (i.e., human osteoblast outgrowth cells and bone marrow stromal cells). OATPs may have diverse effects on bone and bone disease progression as many of them are known to transport sex steroid hormones, which are important regulators of bone turnover that influence the activity of osteoblasts and osteoclasts [SVOBODA, HAMILTON & THALHAMMER, 2010]. As a result, OATPs may also play an important role in malignant bone disease. Moreover, the OATP substrate PGE2 was identified as a potent promoter of bone resorption [HIKIJI et al., 2008]. Several OATPs are also involved in the transport of methotrexate, an antifolate drug used in the treatment of osteosarcoma (see section 2.4.1). Thus, OATPs may be of importance in the regulation of bone tumor growth and progression by providing important regulators of bone homeostasis and by influencing the efficacy of chemotherapy regimens through the uptake of certain anticancer drugs.

Brain Tumors:
Altered expression patterns of OATPs were also studied in brain tumors (gliomas). The expression of OATP1A2 and OATP2B1 was confirmed in the blood-brain barrier but was not found in glioma cells, suggesting that the uptake of drugs proceeds through the endothelial cells but that entry into glioma cells may be impaired [BRONGER et al., 2005]. Moreover, expression of the testis-specific OATP6A1 was also identified in medulloblastomas, and its localization to the membrane and its limited expression in normal tissues suggest that OATP6A1 could be a target for antibody-based immunotherapy [OBA-SHINJO et al., 2008].

Lung Cancer:
Serological analysis of recombinant expression libraries (SEREX) has led to the identification of OATP6A1 as a cancer/testis (CT) antigen expressed in lung cancer [LEE et al., 2004]. Therefore, OATP6A1 could represent a potential target for monoclonal antibody-based cancer therapy, as it should be associated with cell surface expression, which is not the case for most other CT antigens [LEE et al., 2004].

Prostate Cancer:
In a recent study, SLCO1B3 was found to be markedly overexpressed in prostate cancer compared to normal prostate or benign prostate hyperplasia tissue and a statistically significant association of a specific SLCO1B3 haplotype with survival of patients with prostatic cancer was identified. Furthermore, it could be shown that OATP1B3 is mediating testosterone uptake into cells and enhanced testosterone uptake may therefore shorten the time to androgen independence in prostate cancer [HAMADA et al., 2008].

3.4.1. OATPs as Transporters for Chemotherapeutic Agents

It is well known that the overexpression of ATP-powered efflux pumps, such as P-glycoprotein (P-gp) coded by the *ABCB1* gene, induces drug resistance for cytotoxic agents including paclitaxel [BAEKELANDT et al., 2000]. However, drug uptake mechanisms into tumor cells might also be important determinants for therapy efficacy because they ensure a sufficient intracellular concentration of chemotherapeutic agents. In addition to the mode whereby drugs enter cells mostly

via passive diffusion at a rate related to their lipophilicity, there is increasing evidence that xenobiotics, and therefore also cytotoxic agents, utilize a mechanism of "hitchhiking" with carriers that act on natural, often unknown, endogenous substrates [DOBSON & KELL, 2008]. This idea suggests that uptake transporters might also have a profound impact on the success of a particular chemotherapeutic treatment.

In one of the first investigations of this concept, it was shown that the cytostatic agent chlorambucil is transported by OATP1A2 when coupled to a bile acid. This strategy was designed to specifically target hepatocellular carcinoma cells [KULLAK-UBLICK et al., 1997b]. Furthermore, the transport of Bamet-R2 and Bamet-UD2 by OATP1A2 and OATP1B1 was demonstrated using cRNA injected *X. laevis* oocytes [BRIZ et al., 2002]. Bamet-R2 and Bamet-UD2 are compounds that consist of the anticancer agent cisplatin coupled to bile acid side chains. It should be noted that uncoupled cisplatin does not seem to be transported by OATPs [BRIZ et al., 2002]. The antifolate drug methotrexate was identified as a substrate for the liver specific OATPs, OATP1B1 and OATP1B3, which may help to explain methotrexate-induced liver dysfunction [ABE et al., 2001], OATP1A2 [BADAGNANI et al., 2006], and the kidney specific OATP4C1 [MIKKAICHI et al., 2004] also showed methotrexate uptake activity. The liver specific OATP1B3 was also found to be a high affinity transporter of the microtubule stabilizing agents paclitaxel and docetaxel in studies using cRNA-injected *X. laevis* oocytes [SMITH et al., 2005]. This finding is indirectly supported by the work of Gui et al., who could show inhibition of OATP1B3 mediated uptake of estradiol-17*β*-glucuronide into stably transfected CHO cells by paclitaxel and, interestingly, this inhibition effect was also observed when using OATP1B1 transfected cells [GUI et al., 2008]. OATP1B3-mediated paclitaxel transport could not be confirmed in a different study using stably transfected HEK293 cells [YAMAGUCHI et al., 2008].

In conclusion, inter-individual differences in OATP expression may help explain the varied responses of cancer patients treated with OATP substrates and suggests the need for systematic investigations of all OATP family members in regard to tumor expression and their ability to transport chemotherapeutic agents, which could help to avoid giving toxic therapies to patients unlikely to benefit from them.

3.5. References

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4. Aims of the Thesis

The “gold standard” treatment for solid tumors is the removal of the tumor load by surgery. However, surgical removal alone is often not sufficient to prevent tumor recurrence and metastasis. Therefore, chemotherapeutic treatment and/or radiation therapy are additionally applied. However, the efficacy of those approaches is reaching its limits, particularly in cases of advanced tumors.
The efficacy of chemotherapy is highly dependent on the intracellular concentration of the chemotherapeutic agent, which is determined by the rate of the drug’s entry into the target cells, by the rate of biotransformation via drug metabolizing enzymes, and by the extrusion of the drug or its metabolites via specific drug efflux pumps.

The aim of this thesis was to evaluate the role of a family of plasma membrane transporters, the organic anion transporting polypeptides (OATPs), in the delivery of endogenous and xenobiotic compounds into solid tumors from the ovary, mammary glands, liver, and bone. Some members of the OATP family have already been shown to be capable of transporting anticancer agents as well as steroid hormones and their conjugates, which could be important determinants for tumor progression in hormone dependent cancers like cancers of the breast or ovary. However, despite the great progress made concerning the role of OATPs in physiology since their discovery in 1994, only piecemeal information is available concerning the expression of the 11 human OATP family members in cancers of the breast, bone, liver and ovary. Therefore, their expression should be assessed in malignant tissue samples and compared to adjacent normal tissue and benign tumors in order to identify aberrant patterns of OATP expression. Moreover, the whole OATP family should be characterized with regard to its capability to transport the anticancer agent paclitaxel, which is frequently used to treat ovarian and metastatic breast cancer. This will be accomplished by studying the uptake of radiolabeled paclitaxel into OATP-cRNA microinjected *X. laevis* oocytes. In a final study, the impact of OATP expression will be investigated in a large cohort of ovarian cancer patients and correlated with the patients’ survival.
As a result of the development of drug resistance and toxic side effects, the success of different chemotherapeutic approaches is still not ideal. The studies planned in this thesis will contribute to a more personalized therapy by determining if the presence

or absence of certain drug uptake transporters has an impact on the success of these therapies. This contribution will allow patients to avoid therapies unlikely to benefit them and will prevent toxic effects caused by drug-drug interactions.

5. Results

Original papers and manuscripts

Martin Svoboda, Katrin Wlcek, Barbara Taferner, Steffen Hering, Bruno Stieger, Dan Tong, Robert Zeillinger, Theresia Thalhammer, Walter Jäger. Paclitaxel transport by organic anion transporting polypeptides (OATPs) in the ovarian cancer cell lines OVCAR-3 and SK-OV-3. (Submitted for publication to *Cancer Letters*.)

(MS helped to prepare the cRNA for microinjection into oocytes, contributed to the oocyte uptake and western blot experiments, did all cell culture work and real-time RT-PCR investigations and was responsible for writing most of the manuscript.)

Katrin Wlcek, Martin Svoboda, Theresia Thalhammer, Franz Sellner, Georg Krupitza and Walter Jaeger. Altered expression of organic anion transporter polypeptide (OATP) genes in human breast carcinoma. *Cancer Biol Ther. 7 (2008) 1450-1455.*

(MS participated in the real-time RT-PCR experiments and data analysis.)

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(MS contributed to the RNA isolation and cDNA synthesis, designed and validated real-time RT-PCR primers and probes and participated in the conduction of the real-time RT-PCR experiments and data analysis.)

Katrin Wlcek, Martin Svoboda, Juliane Riha, Gabriele Stefanzl, Ulrike Olszewski, Zdenek Dvorak, Franz Sellner, Walter Jäger, Theresia Thalhammer. Analysis of organic anion transporting polypeptide (OATP) mRNA and protein reveals differences in primary and metastatic liver cancer title. (In revision at *Cancer Biology & Therapy*.)

(MS contributed to the real-time RT-PCR experiments and data analysis, did the HepG2 cell culture work and participated in the writing of the manuscript.)

Martin Svoboda, Katrin Wlcek, Dietmar Pils, Gudrun Hager, Dan Tong, Robert Zeillinger, Walter Jäger, Theresia Thalhammer. mRNA expression of *SLCO3A1*, *SLCO6A1*, *ABCB2*, and *ABCC2* in Ovarian Carcinoma is associated with Tumor Staging and Patients' Survival. (Manuscript in preparation for the submission to *Clinical Cancer Research*.)

(MS performed the cDNA synthesis, the entire real-time RT-PCR investigation and wrote the raw manuscript.)

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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 °C 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 °C 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 °C, 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 °C for 30 s, followed by 25 cycles of 98 °C for 10 s, 62 °C for 20 s, and 72 °C 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 °C. Subsequently, 9 µl of reverse transcription 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 °C for 1 h, followed by 70 °C for 10 min. The sequence of OATP6A1 was amplified 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 °C for 30 s, followed by 40 cycles of 98 °C for 10 s, 68.5 °C for 20 s and 72 °C for 1 min on a Bio-Rad MyCycler™. The conditions for OATP5A1 and OATP6A1 were identical except for annealing at 66 °C. The purified cDNA was then cloned 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 °C for 30 s, 25 cycles of 98 °C for 15 s, 55 °C for 20 s, and 72 °C for 1 min on a Bio-Rad MyCycler™. The obtained 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 °C for 48 hours before performing 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 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 NM_006672.3
MDR1	hs00184500_m1	AGACATGACCAGGTATGCCTATTAT	NM_000927.3
MRP2	hs00166123_m1	CACCTCCAACAGGTGGCTTGCAATT	NM_000392.3
MRP3	hs00358656_m1	CAGCTGCTCAGCATCCTGATCAGGT	NM_001144070.1 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 °C 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 °C 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.

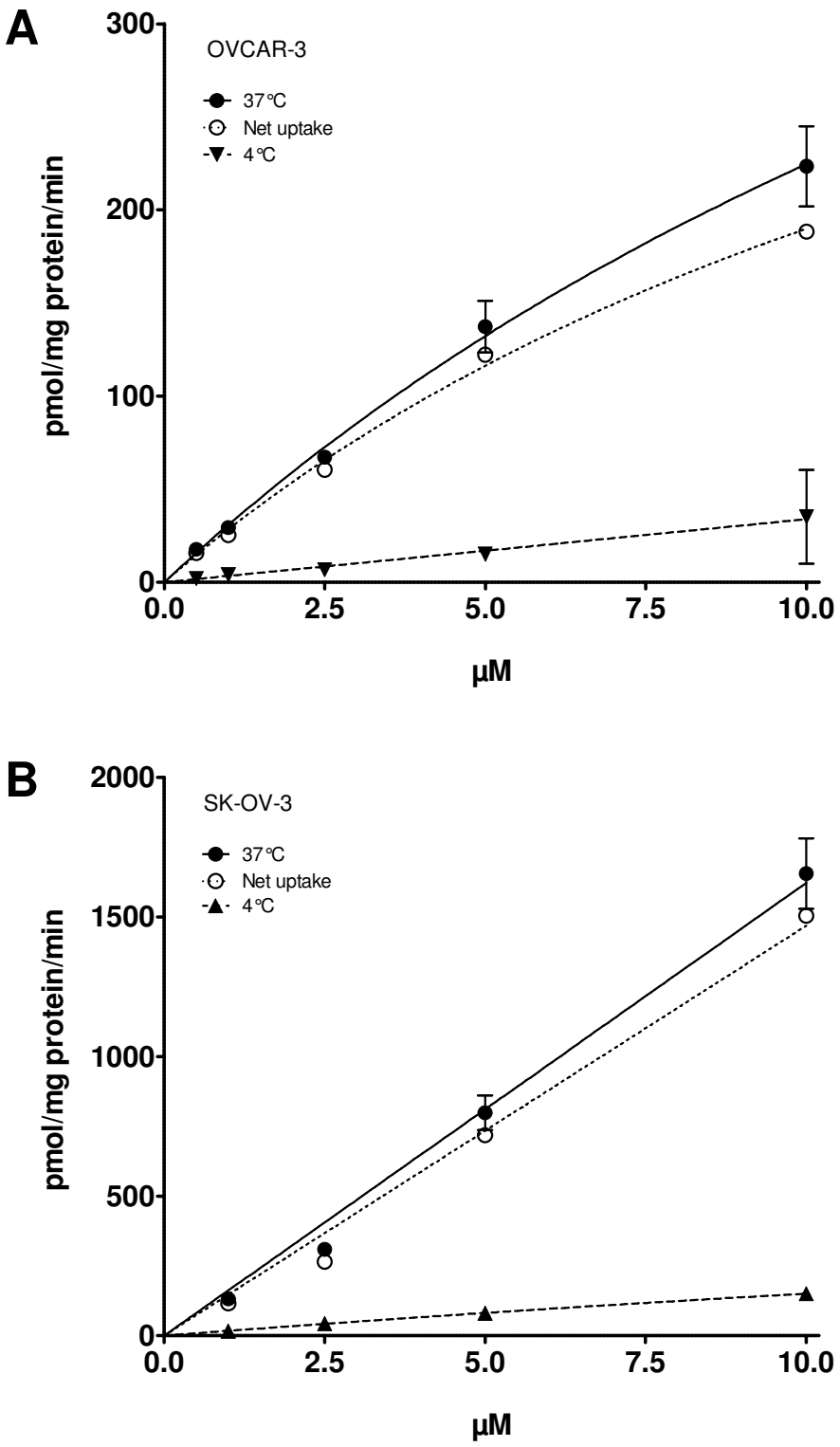

A
OVCAR-3
37°C
Net uptake
4°C
pmol/mg protein/min
300
200
100
0
0.0
2.5
5.0
7.5
10.0
µM
B
SK-OV-3
37°C
Net uptake
4°C
pmol/mg protein/min
2000
1500
1000
500
0
0.0
2.5
5.0
7.5
10.0
µM

Fig. 2.

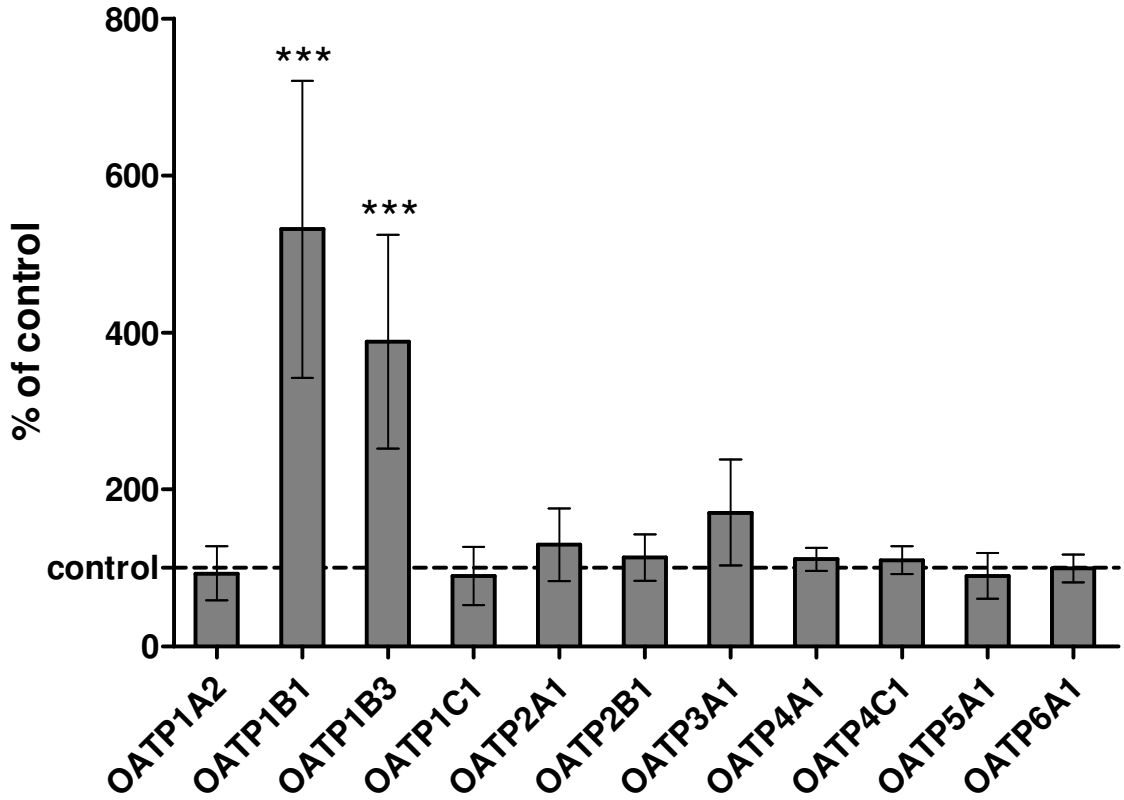

800
600
400
200
control
0
% of control
***
***
OATP1A2
OATP1B1
OATP1B3
OATP1C1
OATP2A1
OATP2B1
OATP3A1
OATP4A1
OATP4C1
OATP5A1
OATP6A1

Fig. 3

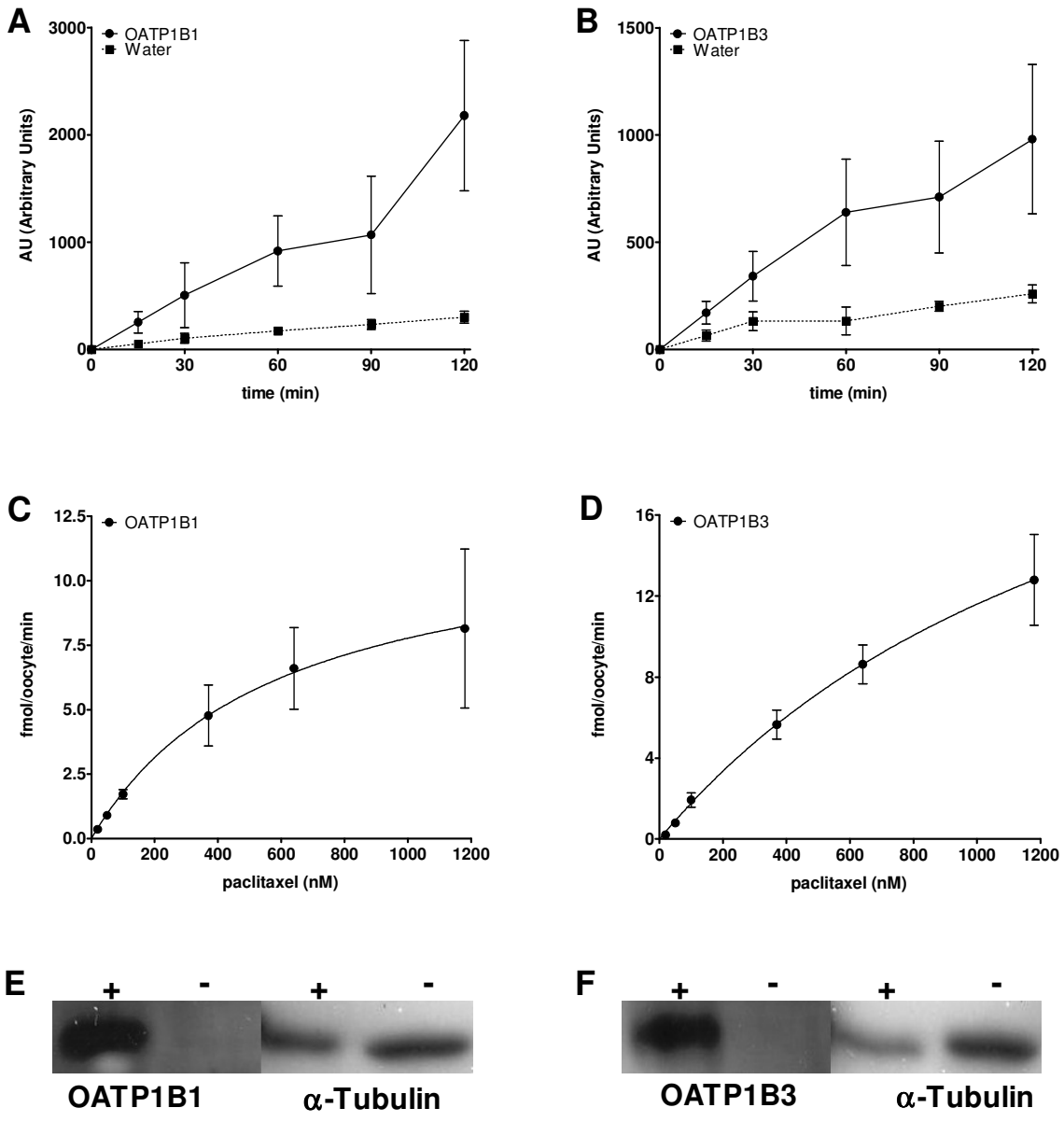
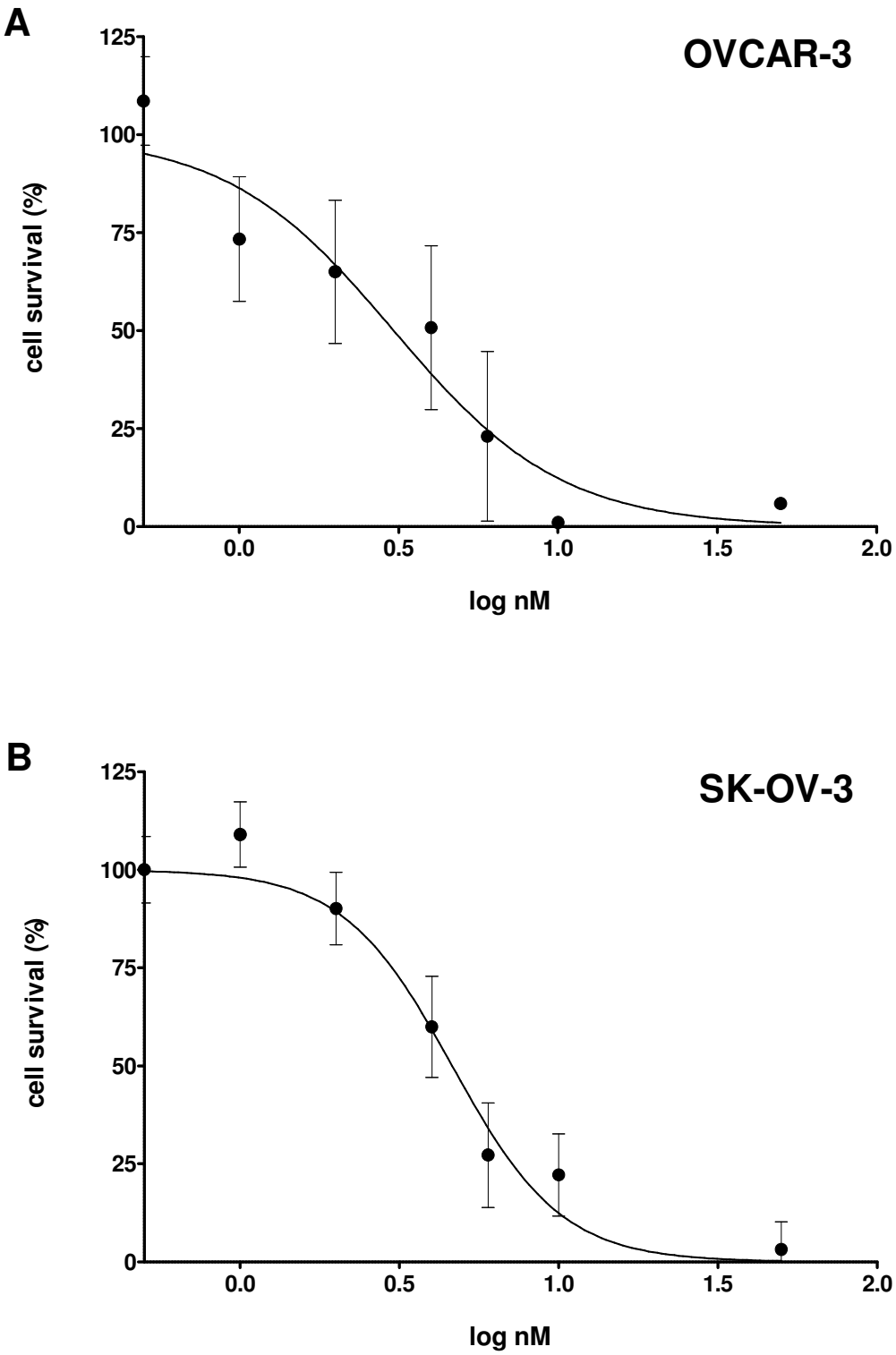

A
OATP1B1
Water
AU (Arbitrary Units)
time (min)
B
OATP1B3
Water
AU (Arbitrary Units)
time (min)
C
OATP1B1
fmol/oocyte/min
paclitaxel (nM)
D
OATP1B3
fmol/oocyte/min
paclitaxel (nM)
E
+ - + -
OATP1B1
α-Tubulin
F
+ - + -
OATP1B3
α-Tubulin

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

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

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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 3.0-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 - PR -	-	G3
2	T2	+	ER - PR -	-	G3
3	T1	+	ER - PR -	-	G2
4	T2	+	ER + PR -	-	G3
5	T2	+	ER + PR -	-	G3
6	T1	+	ER + PR -	-	G2
7	T4	-	ER + PR -	-	G3
8	T2	-	ER - PR -	-	G3
9	T2	x	ER + PR -	-	G2
10	T2	-	ER - PR +	-	G2
11	T1	-	ER - PR -	-	G2
12	T2	x	ER + PR +	-	G2
13	T2	-	ER - 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	**15.20 ± 0.18**	**6.41 ± 0.17**	n.d.
11	M	6.50 ± 0.11	11.13 ± 0.10	47.78 ± 0.27	**98.75 ± 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 a 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 controls 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), Hs00272374_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), Hs00184500_m1 (Pgp), Hs00243666_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 Figs. 1 and 2, 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.

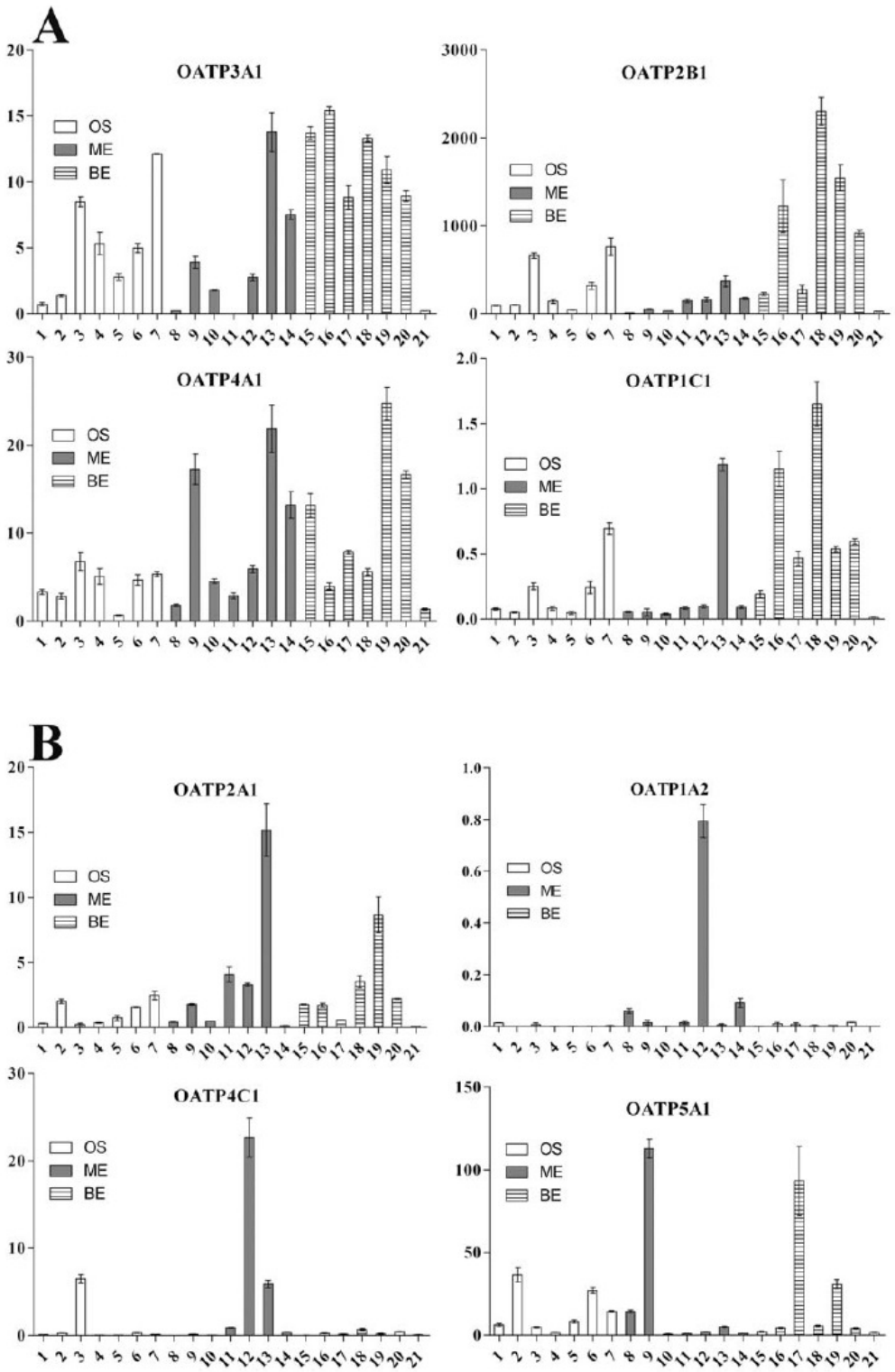

Figure 1. OATP mRNA expression in human bone tumor specimens. mRNA expression of 11 OATPs was assessed in specimens from osteosarcomas (OS; #1-7), metastases (ME) from prostate (#8,9), breast (#10,11), kidney (#12,13) and lung (#14) tumors and benign bone tumors (BE; aneurysmal bone cyst: #15-20, chondroma: #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. *Data from specimen #21 were not used for the calculation of mean MNEs in the benign bone tumor group (given in Table II).

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 2.500-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.

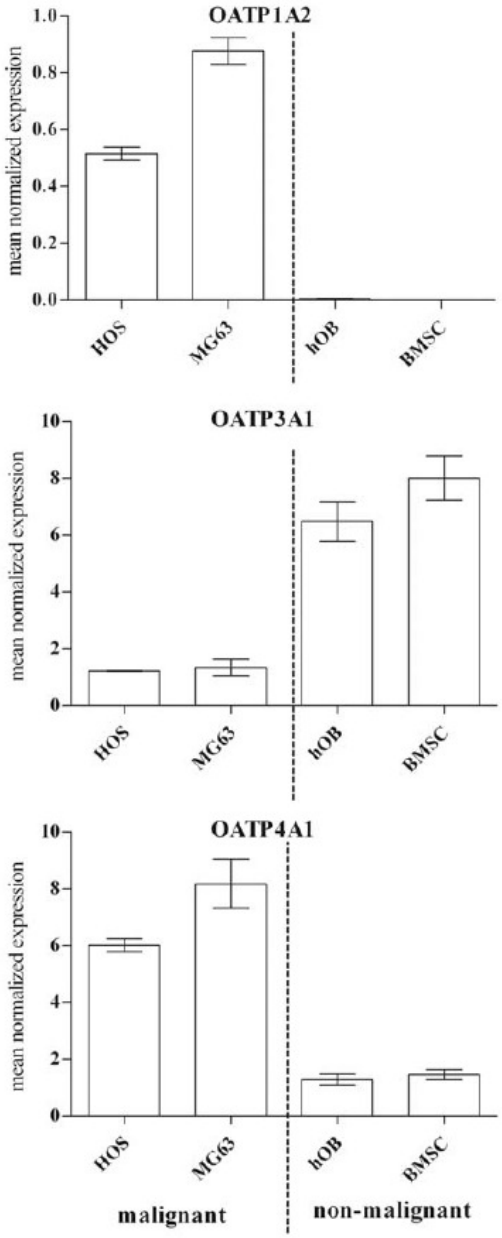

Figure 2. OATP mRNA expression in malignant and non-malignant bone cells. mRNA expression of 11 OATPs was assessed in the osteosarcoma cell lines HOS and MG-63 and in the normal human bone marrow derived osteoblast-outgrowth cells (hOB) and bone marrow stromal cells (BMSC) by quantitative PCR; mRNA expression 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.

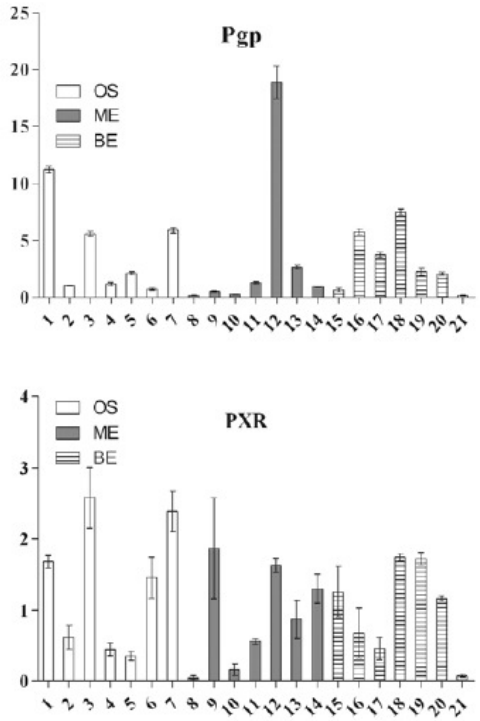

Figure 3. Pgp and PXR mRNA expression in human bone tumor specimens. mRNA expression of the MDR efflux pump P-glycoprotein (Pgp) and the nuclear receptor PXR was assessed in specimens from osteosarcomas (OS; #1-7), metastases (ME) from prostate (#8,9), breast (#10,11), kidney (#12,13) and lung (#14) 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). *Data from specimen #21 were not used for the calculation of mean MNEs in the benign bone tumor group.

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

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KEY WORDS

OATP, liver, hepatocellular carcinoma, cholangiocarcinomas, colon metastases, cholangiocytes, hepatocytes, transporter, HepG2, PCR, immunofluorescence

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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 19[26] 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°C 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°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C. Fluorescence generation from TaqMan® probe cleavage by the 5'→3' exonuclease activity of the DNA polymerase was measured with the

StepOnePlus system (Applied Biosystems). All samples were amplified in duplicates. Results were imported into Microsoft Excel for further analysis and comparable cDNA amounts in the experimental samples were calculated according to Hellemans et al.[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°C). Tissue sections 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 used for both genes and proteins.

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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.

Fig. 1

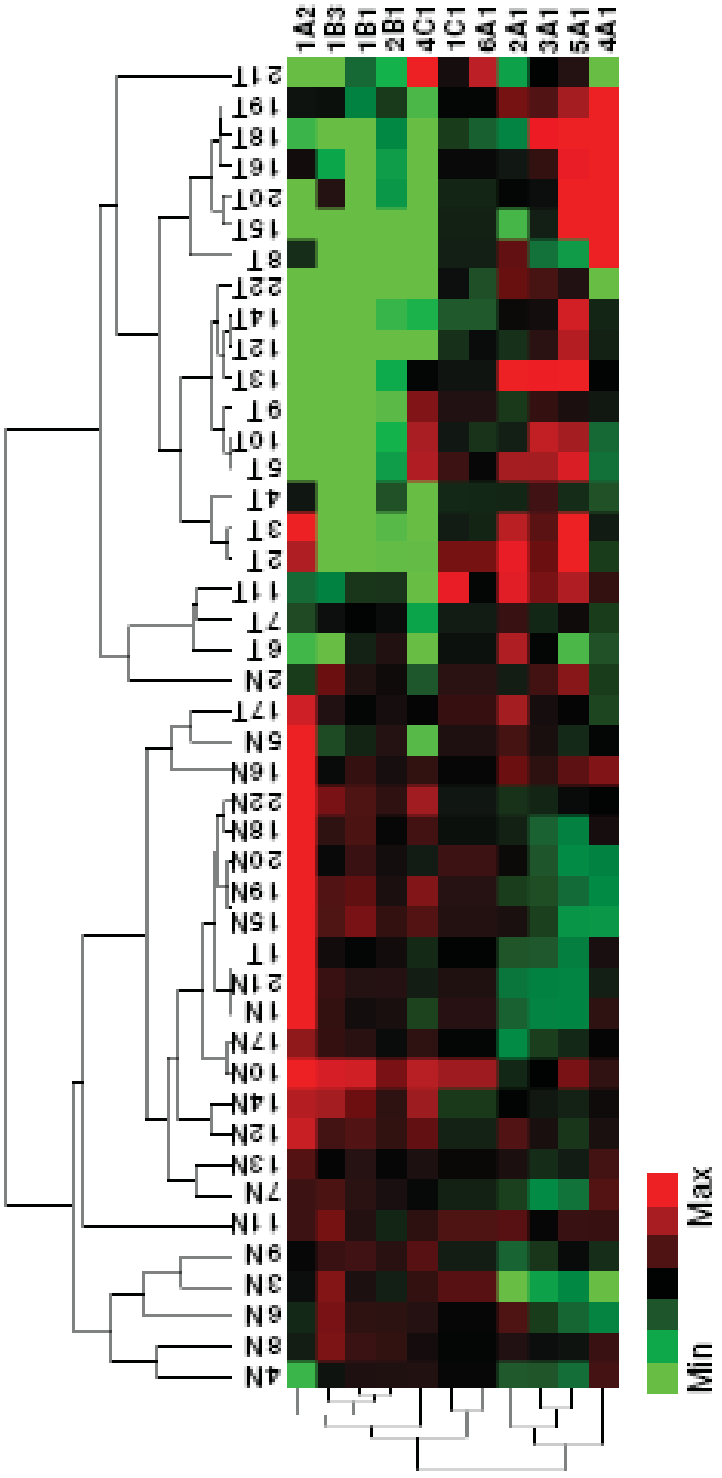
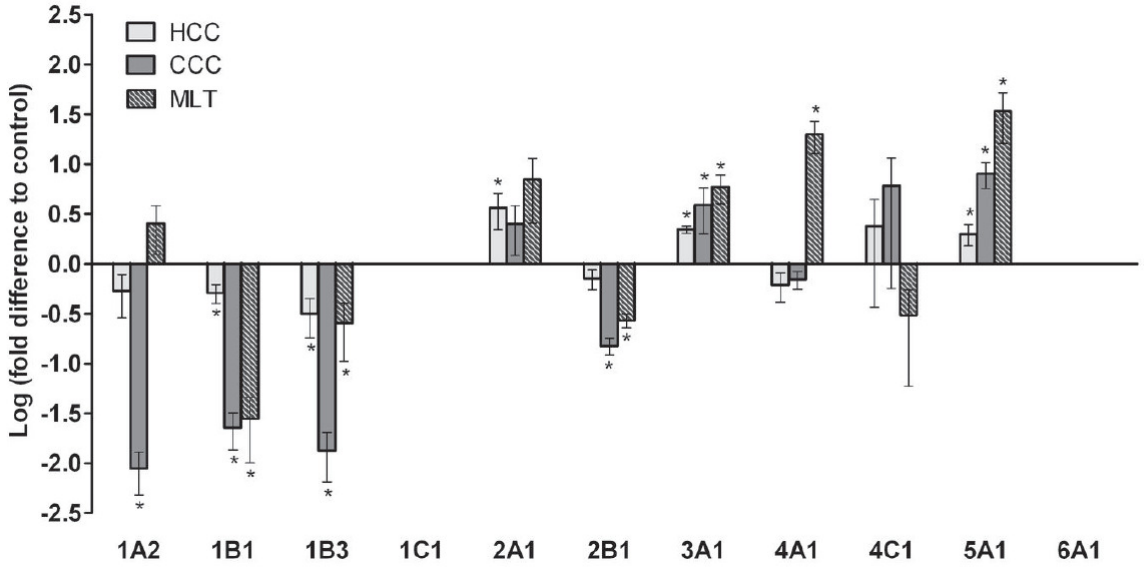


Fig. 2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CCC
MLT
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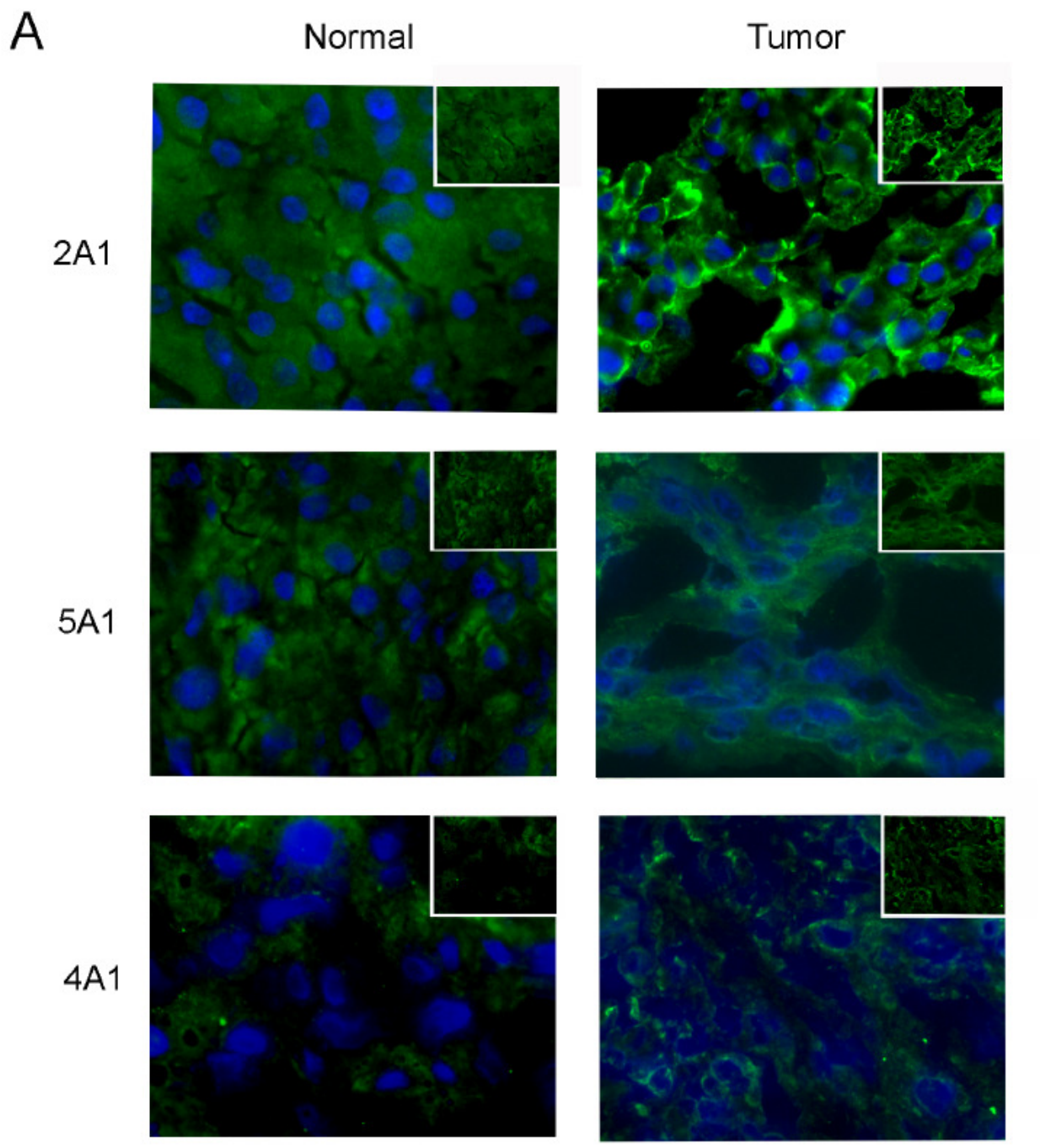
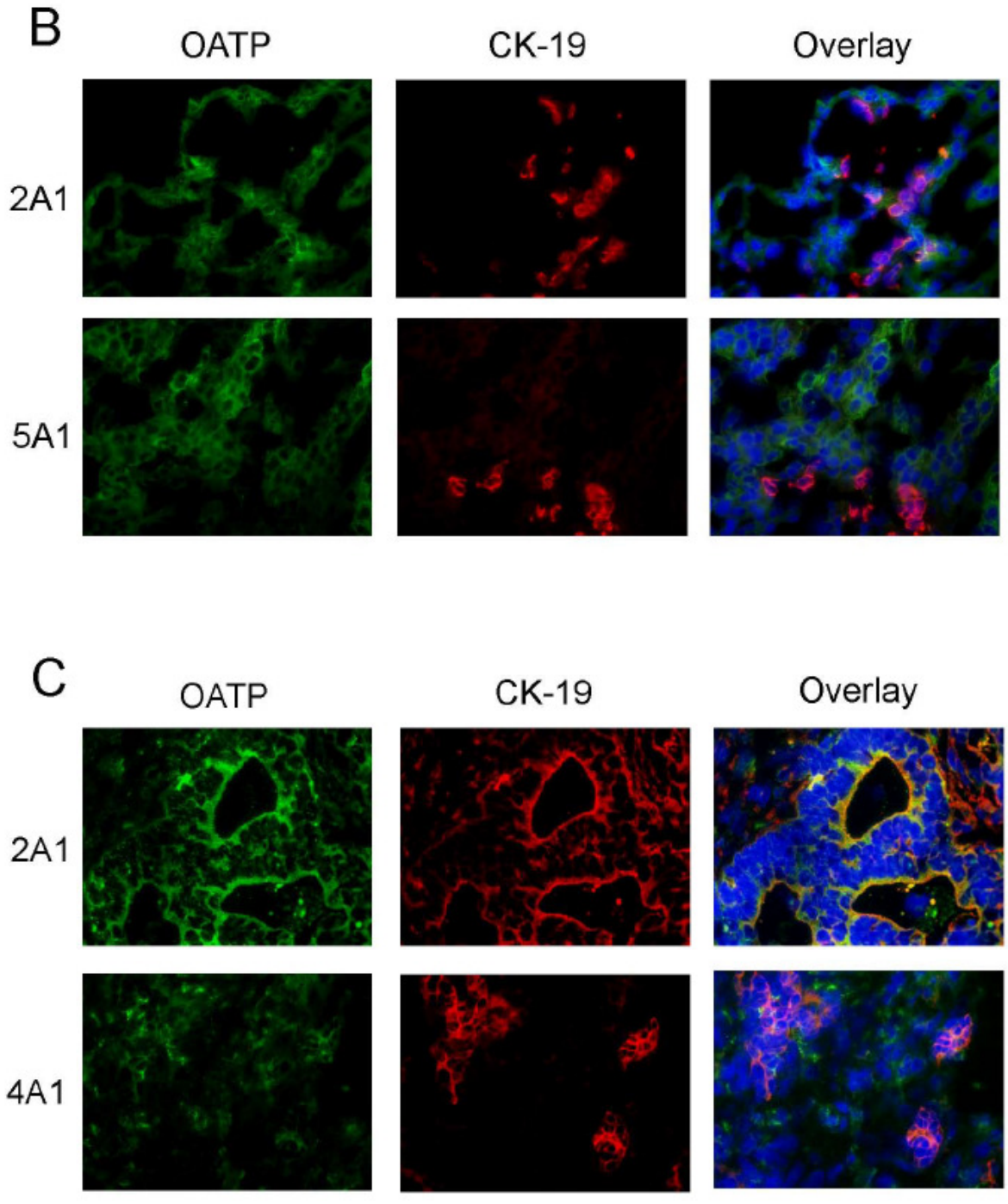
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
1A2
1B1
1B3
1C1
2A1
2B1
3A1
4A1
4C1
5A1
6A1

Fig. 3

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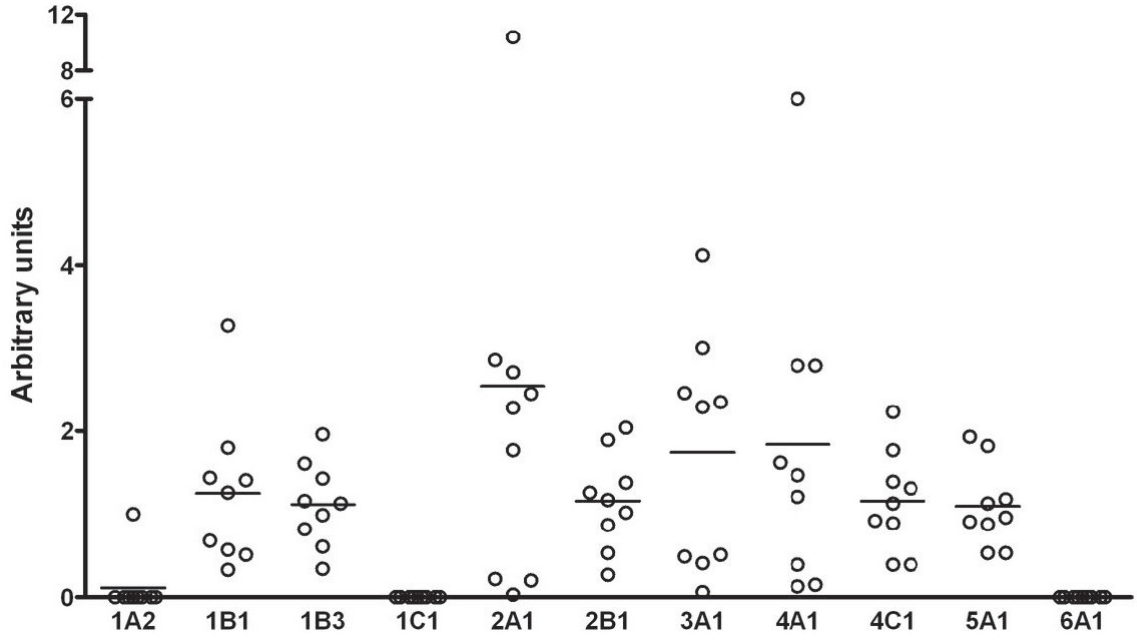
Overlay
2A1
5A1
C
OATP
CK-19
Overlay
2A1
4A1

Fig. 4


Arbitrary units
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8
6
4
2
0
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4A1
4C1
5A1
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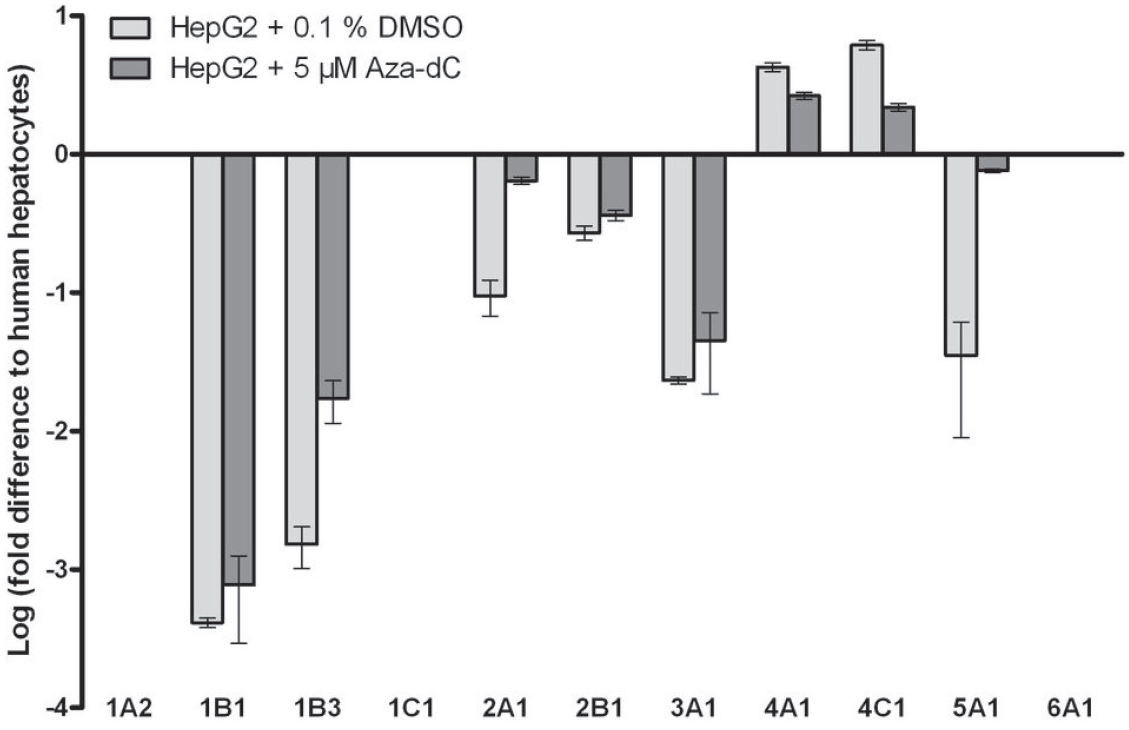
Fig. 5


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

Tab. 1

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

mRNA Expression of *SLCO3A1*, *SLCO6A1*, *ABCB2*, and *ABCC2* in Ovarian Carcinoma is associated with Tumor Staging and Patients' Survival

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Running Title: *SLCO*-Transporters in Ovarian Carcinoma

Statement of Translational Relevance:

The identification of markers for the progression and chemotherapy resistance of ovarian cancer is of high interest. OATPs/*SLCO*s proteins are capable of transporting a broad range of endogenous compounds including prostaglandins, steroid conjugates as well as anticancer drugs and were found to have relevance as cancer progression markers and in drug resistance. However, investigations of *SLCO* expression in malignant disease of the ovaries were restricted to ovarian cancer cell lines. In the present study, we systematically investigated the mRNA distribution of all 11 human *SLCO* and of four ABC-transporter genes, known to influence drug resistance in malignant disease, in a panel of 191 specimens of ovarian carcinomas. Indeed, we could show significant associations of *SLCO3A1* with FIGO staging and of *SLCO6A1* with the patients' outcome prompting further investigations on the role of *SLCO3A1* and *SLCO6A1* in the progression and chemotherapy resistance of ovarian cancer.

Abstract

Ovarian cancer has a poor prognosis as it is generally diagnosed at an advanced stage and tumors are often resistant to standard chemotherapy regimens. While the role of ABC-efflux transporters in chemoresistance of ovarian cancer is well established, data on OATP/*SLCO* drug uptake transporters in ovarian cancer are greatly missing. Therefore, we characterized the mRNA expression profiles of all *SLCO* family members together with four ABC-pumps related to anticancer drug resistance (*ABCB1*/P-glycoprotein, *ABCC2*/MRP2, *ABCC3*/MRP3, *ABCB2*/TAP1) in specimens from 191 ovarian cancer patients and correlated their expression pattern to clinicopathological parameters of the patients. *SLCO3A1* levels showed differences in FIGO stage (P = 0.03) and might be a marker for tumor progression.

Additionally, higher *ABCB2* levels were associated with a decreased residual tumor load (P = 0.015). In the univariate survival analysis, increased *ABCB2* expression was associated with a significantly lower relative risk of receiving a relapse (P = 0.005). Moreover, expression of *SLCO6A1*, *ABCB2* and *ABCC2*; minimal residual disease, FIGO stage and tumor grade, were found to be associated with the relative risk for relapse using a multivariate Cox proportional hazard model. The obtained predictor values were dichotomized (25 % percentile) for identifying a low and high risk group with significant differences in the disease free survival ($P < 0.0001$) and overall survival ($P = 0.012$). This is the first report showing *SLCO* transporters together with ABC-efflux pumps as potential markers in ovarian cancer.

Introduction

Ovarian cancer is generally diagnosed at an advanced stage as early symptoms are inconspicuous and reliable diagnostic biomarkers are not available [DiSAIA et al., 2002]. This contributes to a poor 5 year overall survival rate of < 40% and a high death rate [PERMUTH-WEY & SELLERS, 2009]. After initial surgery on the tumor, standard chemotherapy with platinum derivatives and taxanes like paclitaxel [ROWINSKY & DONEHOWER, 1995] is applied. 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 and this contributes to the overall poor survival [AGARWAL & KAYE, 2003] [MARKMAN, 2008].

An important factor in the development of anti-cancer drug resistance is an insufficient intracellular accumulation of cytotoxic agents. It is well established that several ABC-transporters including MDR1/P-glycoprotein (P-gp) encoded by the *ABCB1* gene [AMBUDKAR et al., 2003], MRP2 (*ABCC2*), MRP3 (*ABCC3*) are associated with chemoresistance in ovarian cancer by mediating the rapid efflux of

cytotoxic drugs [NANIWA et al., 2007; BAEKELANDT et al., 2000] [MATERNA et al., 2004; SUROWIAK et al., 2006; O'BRIEN et al., 2008]. In addition, TAP1 and TAP2 coded by the *ABCB2* and *ABCB3* gene, respectively, are involved in the major histocompatibility complex class I-restricted antigen processing machinery (APM), [LEHNER & CRESSWELL, 2004]. Indeed, *ABCB3* was found to be down-regulated in ovarian cancer at the mRNA level [AUNER et al., 2010]. Furthermore, it was suggested that TAP may contribute to MDR, in particular in cells overexpressing MRPs [IZQUIERDO et al., 1996].

Besides efflux, uptake into the cells is also important to determine intracellular drug concentration [CVETKOVIC et al., 1999]. One of the most important group of transport proteins is the organic anion transporting polypeptide family/solute liquid carriers (OATP/*SLCO*), which are highly expressed in tissues such as liver, intestine, kidney, brain, and breast and in various tumors and are candidates for anticancer drug uptake transporters [HAGENBUCH & GUI, 2008]. OATPs are capable of transporting a broad range of endogenous, amphipathic organic compounds including bile salts, thyroid hormones, prostaglandins, steroid conjugates, as well as anticancer drugs. Therefore, they play an important role in drug absorption, disposition and excretion [KIM RB, 2003; MIKKAICHI et al., 2004]

To date, 11 OATP/*SLCO* members grouped according to their amino acid homology in the OATP families 1-6, have been identified in various normal and tumorous tissues [HAGENBUCH & MEIER, 2004]. For example, high expression levels for *SLCO1B3*, mediating cellular uptake of paclitaxel [SMITH et al., 2005], were detected in tumors of the gastrointestinal tract, and breast cancer, while *SLCO6A1*, originally identified as a testis specific antigen, was found to be expressed in lung tumors [ABE et al., 2001; MUTO et al., 2007; LEE et al., 2004]. Thus far, in ovarian cancer, data on the expression of individual *SLCO*s are still limited. *SLCO2A1*, *SLCO3A1*, and

SLCO4A1 were first detected by Tamai and coworkers in the ovary and ovarian cancer cell lines by conventional RT-PCR [TAMAI et al, 2000]. Later, expression of various *SLCO*s, including *SLCO5A1,* was confirmed by real-time PCR in normal ovary and ovarian cancer cell lines of the NCI-60 panel [OKABE et al., 2008].

This could be important as some *SLCO*s are discussed as markers for tumor progression e.g. *SLCO1B3* in breast cancer and *SLCO1B3* and *SLCO4A1* in colon cancer [MUTO et al., 2007; LEE et al., 2008; ANCONA et al., 2006].

To gain further insight into the possible role of membrane transporters in ovarian cancer, we characterized the mRNA expression profiles of all 11 known transporters of the OATP/*SLCO* families 1-6, and of four members from the ABC family known to be involved in drug resistance in specimens from 191 patients with ovarian cancer. The expression pattern of the transporters was then correlated to patients' survival.

Materials and Methods

Patients

A total of 191 patients were included into the present multicenter study (Department of Obstetrics and Gynecology, Medical University Innsbruck, Innsbruck, Austria: n = 7; Department of Obstetrics and Gynecology, Charité/Campus Virchow-Klinikum, University Medicine of Berlin, Berlin, Germany: n = 71; Institute of Tumorbiology, University Clinic Hamburg-Eppendorf, Hamburg, Germany: n = 30; Division of Gynecologic Oncology, University Hospitals Leuven, Leuven, Belgium: n = 61; Department of Obstetrics and Gynecology, Medical University Vienna, Vienna, Austria: n = 22). The tumor samples were collected in the course of the EU-project Ovarian Cancer Diagnosis (OVCAD, www.ovcad.eu, 2006-2010). Informed consent for the scientific use of biological material was obtained from all patients in

accordance to the requirements of the ethical committee of the individual institutions. Clinicopathological parameters of the patients are displayed in Table 1.

RNA extraction

Total RNA was isolated using the ABI 1600 nucleic acid prepstation (Applied Biosystems, Foster City, CA, USA) following the instructions of the manufacturer. Shortly, 20 – 30 mg of frozen tissue were pulverized using a micro-dismembrator (B. Braun Biotech International, Melsungen, Germany). The obtained tissue powder was dissolved in 1 ml of provided lysis solution. Thereafter, the specimens were digested with 10 µl proteinase K / ml of lysate for one hour at room temperature followed by RNA isolation using the ABI 1600 nucleic acid prep station including a DNase I digestion protocol. The eluted RNA was precipitated with sodium acetate and 100 % ethanol at -20 °C overnight. The RNA pellet was washed with 70 % ethanol, dried, and resuspended in nuclease free water. The integrity of the total RNA was confirmed by lab-on-a-chip technology using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA). The RNA concentration was assessed using a spectrophotometer.

Reverse Transcription and real-time RT-PCR

We reverse transcribed 1 µg of total RNA in 20 µl reactions using the High Capacity cDNA RT kit (Applied Biosystems) with the provided random primers and RNase inhibitor (PN N808-0119, Applied Biosystems) according to the instructions of the manufacturer. The reverse transcription mix contained 2 µl of 10x RT buffer, 0.8 µl of 25x dNTP mix (100mM), 2 µl 10x random primer mix, 1 µl RNase inhibitor, 1µl Multiscribe™ reverse transcriptase, 1 µg total RNA in nuclease-free water and nuclease-free water was added to a final volume of 20 µl. The thermal cycling

program for the reverse transcription reaction was 10 min 25°C, 120 min 37°C, and 5 s 85°C using a MyCycler™ thermocycler (Bio-Rad, Hercules, CA, USA). As it has been shown in the past, the choice of reference genes as internal normalization factors can have a profound effect on the experimental results in real-time RT-PCR [DHEDA, 2005]. For accurate normalization of mRNA between ovarian cancer tissue specimens we determined *ACTB*, *TOP1*, *UBC*, and *YWHAZ* out of a panel of 12 housekeeping genes (the others: *18S*, *ATP5B*, *B2M*, *CYC1*, *EIF4A2*, *GAPDH*, *RPL13A*, *SDHA*) as appropriate reference genes in specimens from ovarian cancer patients using a geNorm kit (PrimerDesign Ltd., Southampton, UK) and the geNorm software [VANDESOMPELE et al., 2002; VANDESOMPELE et al., 2004]. Gene expression assays (Applied Biosystems, Foster City, CA) were purchased for all 11 human *SLCO*s and the four ABC transporter genes (Table 2). The endogenous control assay for *ACTB* (PN 4326315E, Genbank Acc. No. NM_001101.2) was obtained from Applied Biosystems and prefabricated hydrolysis probe assays for the endogenous control genes *TOP1* (Genbank Acc. No. NM_003286), *UBC* (Genbank Acc. No. NM_021009), and *YWHAZ* (Genbank Acc. No. NM_003406) were purchased from PrimerDesign Ltd. Real-time RT-PCR using hydrolysis probes was performed in an amplification mixture of 10 µl containing 5 µl 2x TaqMan® Gene Expression Master Mix (Applied Biosystems), 0.5 µl of the appropriate Gene Expression Assay, 5 ng template cDNA diluted in 4 µl nuclease free water, and 0.5 µl nuclease free water. Thermal cycling conditions were 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 hydrolysis probe cleavage was measured with the ABI 7900HT Fast real-time PCR system equipped with SDS 2.3 software (Applied Biosystems). The real-time RT-PCR reactions were carried out in duplicates using 96 well format plates (Applied Biosystems) and adhesive optical films (Applied Biosystems). Since the total number

of samples exceeded the number of samples that could be analyzed during a single run the samples had to be split on multiple plates and a qPCR reference total RNA (Cat. No. 636690, Clontech Laboratories Inc., Mountain View, CA, USA) was used as an inter-run calibrator (IRC) to compensate between plates for technical inter-run variations. The IRC was analyzed in quadruplicates on every plate. Raw Ct values were exported into Microsoft Excel and results were calculated using the ΔΔCt method [LIVAK & SCHMITTGEN, 2001] as relative quantities (RQ) normalized to the geometric mean of the four endogenous control genes [VANDESOMPELE et al., 2002] and with the IRC assigned as calibrator. For the statistical analysis we transformed the obtained RQ values by log2(RQ).

Statistical Analysis

In order to compare *SLCO*- and ABC-transporter expression between two or more groups a T-test or one-way ANOVA, respectively, was performed using the log2-transformed RQ value as independent variable. The potential influence of *SLCO*- and ABC-transporter expression on disease free and overall survival is presented in plots of the corresponding Kaplan-Meier estimates and quantified using the Cox proportional hazards regression model. OVCAD criteria were used to define time points for disease progression. Univariate Cox estimates were used to approximate the influence of known prognostic factors and the potential new prognostic factors. The final Cox regression model including clinical parameters and candidate genes with relative risk < 0.95 and > 1.05 was built using the backward conditional procedure (the removal term was set to 0.1). All statistical analysis was performed with the IBM SPSS statistical package for PC (version 18.0; SPSS Inc., an IBM company, Chicago, IL, USA). Multiple-testing correction was done by the Bonferroni-

Holm procedure. Two-sided P-values ≤ 0.05 were considered as statistically significant.

Results

Expression of S*LCO*- and *ABC*-transporters in ovarian carcinoma

The investigation of *SLCO* and *ABC* mRNA in specimens from 191 patients with ovarian carcinoma was done using real-time RT-PCR and is displayed as $fold_{mean}$ up- or down-regulation together with the lowest ($fold_{min}$) and highest ($fold_{max}$) observed values compared to a commercially available reference RNA (Clontech) (Table 3). Consistent expression in all samples was seen for five *SLCOs (SLCO2A1, SLCO2B1, SLCO3A1, SLCO4A1 and SLCO5A1)* and all investigated ABC-transporters. Significant expression of *SLCO1A2, SLCO1B1, SLCO1B3, SLCO1C1, SLCO4C1* and *SLCO6A1* could only be detected in subsets of samples.

Significant relationships of *SLCO3A1* and *ABCB2* tumor expression with clinicopathologic parameters.

Using one-way ANOVA with log2-transformed RQ values as independent variable we found that the expression of the *SLCO3A1* gene, coding for the OATP3A1 uptake transporter, was significantly ($P = 0.030$) different in tumors with FIGO stage II (n = 9, $RQ_{mean} = 0.18$), III (n = 151, $RQ_{mean} = 0.33$) and stage IV (n = 30, $RQ_{mean} = 0.39$) (Figure 1 and Table 4). Furthermore, a significant connection between the expression of the *ABCB2* gene and the presence of residual tumor mass was observed. We detected higher *ABCB2* expression in tumors of patients where complete tumor resection was possible (n = 90, $RQ_{mean} = 1.04$ RQ) compared to patients with postoperative tumor mass (n = 96, $RQ_{mean} = 0.70$, $P = 0.015$) after debulking surgery

was performed (Figure 2 and Table 4). No other statistical significant relationships were observed between *SLCO*- or ABC-transporter gene expression concerning patients' age, FIGO stage, grade, histology, or residual tumor load.

Influence of *SLCO6A1*, *ABCB2*, and *ABCC2* on disease free survival and overall survival.

Univariate survival analysis revealed that higher *ABCB2* levels (P = 0.005) were found to be statistically significant to identify patients with longer disease free survival. From all *SLCO*-transporters, only *SLCO1B1* showed a tendency to improve disease free survival (P = 0.06). As expected, patients with lower FIGO stage (P = 0.009), better grading (P = 0.01), and no residual disease (P = 0.004) had a better outcome regarding disease-free survival (Table 5).

When all terms were analyzed by the multivariate Cox proportional hazard model in a backward elimination process, two parameters were predictive for a prolonged disease free survival, namely increased expression of the transporters *SLCO6A1* (RR = 0.90, P = 0.043), and *ABCB2* (RR = 0.81, P = 0.004). Associated with decreased disease free survival was a higher FIGO stage (RR = 1.30, P = 0.026), higher tumor grading (RR = 1.57, P = 0.025), and *ABCC2* transporter expression (RR = 1.20, P = 0.041) (Table 5). Residual disease, however, was not found to be statistically significant. The obtained predictor values were dichotomized at the 25 % percentile resulting in a low and high risk group with high significance in the disease free survival analysis (P < 0.0001, log-rank test) as shown in the corresponding Kaplan-Meier survival plot (Fig. 3). Additionally, a significant relationship was also observed in the overall survival analysis curve (P = 0.012, log-rank test) (Fig. 4).

Discussion

In the present study, we systematically investigated the mRNA distribution of all 11 human *SLCO* genes and of four ABC-transporter genes, known to influence drug resistance in malignant disease, in a panel of 191 specimens of ovarian carcinomas. Thus far, investigations of *SLCO* expression in malignant disease of the ovaries were restricted to ovarian cancer cell lines [TAMAI et al., 2000; OKABE et al., 2008]. We identified *SLCO2A1*, *SLCO2B1*, *SLCO3A1*, *SLCO4A1*, and *SLCO5A1* genes to be ubiquitously expressed in carcinomas of the ovaries. mRNA expression of *SLCO1A2*, *SLCO1B1*, *SLCO1B3*, *SLCO1C1*, *SLCO4C1*, and *SLCO6A1* was only detected in a limited number of tissue samples which expression levels did not correlate to any clinical parameters. These data are in accordance to previous studies also reporting aberrant expression of *SLCO*s in various cancer entities [ABE et al. 2001; MUTO et al., 2007; LEE et al., 2004; LEE et al. 2008].

We were able to show significantly different mRNA levels of the *SLCO3A1* gene (coding for the OATP3A1 protein) in tumors classified as FIGO stage II, III and IV (one-way ANOVA, $P = 0.03$). *SLCO3A1*-specific anticancer drugs have not been described so far, but functional analysis in *X. laevis* oocytes identified thyroid hormones, peptides and prostaglandin as substrates for this transporter [ADACHI et al., 2003, HUBER et al. 2007]. At least for another prostaglandin-transporting *SLCO*, namely *SLCO2A1,* it was shown that it is important for the removal of prostaglandin E_2 (PGE_2) from the extracellular space where it acts via G-protein coupled plasma membrane receptors [BREYER et al., 2001]. After *SLCO*-mediated transport into the cytosol PGE_2 is further inactivated via the intracellular 15-hydroxyprostaglandin dehydrogenase [NOMURA et al., 2004]. This could be important as PGE_2 was shown to increase cell invasiveness in ovarian cancer cells [LAU et al., 2010]. Increased expression might be related to tumor metabolism and a decreased extracellular pH

caused by anaerobic glycolysis in hypoxic tumor cells may stimulate the OATP activity [LEUTHOLD et al., 2009]. This would be important as an acidic tumor environment promotes a more aggressive and invasive phenotype [ANNIBALDI & WITTMAN, 2010].

Interestingly, decreased mRNA levels of *ABCB2/*TAP1 demonstrated a significant association with the presence of residual tumor load ($P = 0.015$). These findings are in line with the observation that impaired antigen presentation can lead to immunoescape in cancers [YIGIT et al., 2010]. TAP1 is located on the membrane of the endoplasmic reticulum (ER) where it passages short peptides, generated by ubiquitin/proteasome-mediated degradation, from the cytoplasm into the ER lumen. Subsequently, the peptides are assembled on MHC class I-β_2-microglubulin heterodimers which migrate to the cell membrane where they can be recognized by $CD8^+$ T-cells [PROCKO & GAUDET, 2009].

In our study, we found a significant beneficial influence of ABCB2 on the disease free survival by univariate as well as multivariate survival analysis supporting the findings of Han and coworkers where intact expression of APM components was strongly associated with prolonged survival of ovarian cancer patients [HAN et al., 2008].

A beneficial factor for patients' survival was also associated with increased expression of *SLCO6A1*. In healthy tissue, *SLCO6A1* is predominately expressed in testis but not in ovary [SUZUKI et al., 2003, OKABE 2008]. However, it is aberrantly expressed in lung cancer, lung cancer cell lines as well as in samples from bladder, esophageal and brain tumors [LEE et al., 2004; OBA-SHINJO et al, 2007]. The frequent expression of OATP6A1 in tumors as cancer/testis surface antigen may allow this transporter to be a potential target for monoclonal antibodies therapy. As

no substrates of *SLCO6A1* are known, further experiments have to clarify its role in physiology and cancerogenesis.

In contrast, the expression of the drug efflux pump *ABCC2* was significantly associated with an unfavorable prognosis in the multivariate Cox proportional hazard model. This is in line with the findings of Suroviak et al. (2006) who correlated nuclear membrane association of *ABCC2* with resistance to cisplatin and clinical outcome in ovarian cancer [SUROWIAK et al., 2006]. Furthermore, Auner and coworkers could also demonstrate a significant difference in the expression of *ABCC2* between primary and recurrent carcinoma indicating a possible role of *ABCC2* as mediator of multiple drug resistance in ovarian carcinoma [AUNER et al., 2010].

In conclusion, we investigated for the first time the expression of all *SLCO* gene family members in a large cohort of ovarian carcinoma patients and report significant associations of *SLCO3A1* with FIGO staging and of *SLCO6A1* with the patients' outcome prompting further investigations on the role of *SLCO3A1* and *SLCO6A1* in the progression and chemotherapy resistance of ovarian cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Legends to the figures

Figure 1 Box plot for the correlation of SLCO3A1 with FIGO stage.

Expression levels of SLCO3A1 in ovarian tumor samples were plotted against the number of patients with FIGO stage II (n = 9), III (n = 151) and IV (n = 30). The box is bounded above and below by the 25 % and 75 % percentiles, the median is the line in the box. There is a significant difference between the three groups (FIGO stages II, III, and IV) by one-way ANOVA ($P = 0.030$).

Figure 2 Box plot showing correlation of *ABCB2* expression with residual disease.

Expression of ABCB2 in ovarian cancer samples was plotted against number of patients with (n = 96) or without (n = 90) residual disease. The box is bounded above and below by the 25 % and 75 % percentiles, the median is the line in the box. A significant correlation of lower levels of *ABCB2* (TAP1) and presence of residual disease was observed by T-test ($P = 0.015$).

Figure 3 Kaplan-Meier survival curve for disease free survival (DFS).

The predictor values for disease free survival obtained by the multivariate Cox regression model were dichotomized at the 25 % percentile resulting in a high and low risk group ($P < 0.0001$, log-rank test).

Figure 4 Kaplan-Meier survival curve for overall survival.

The predictor values for DFS obtained by the multivariate Cox regression model showed to be also predictive for the overall survival ($P < 0.012$, log-rank test).

Figure 1

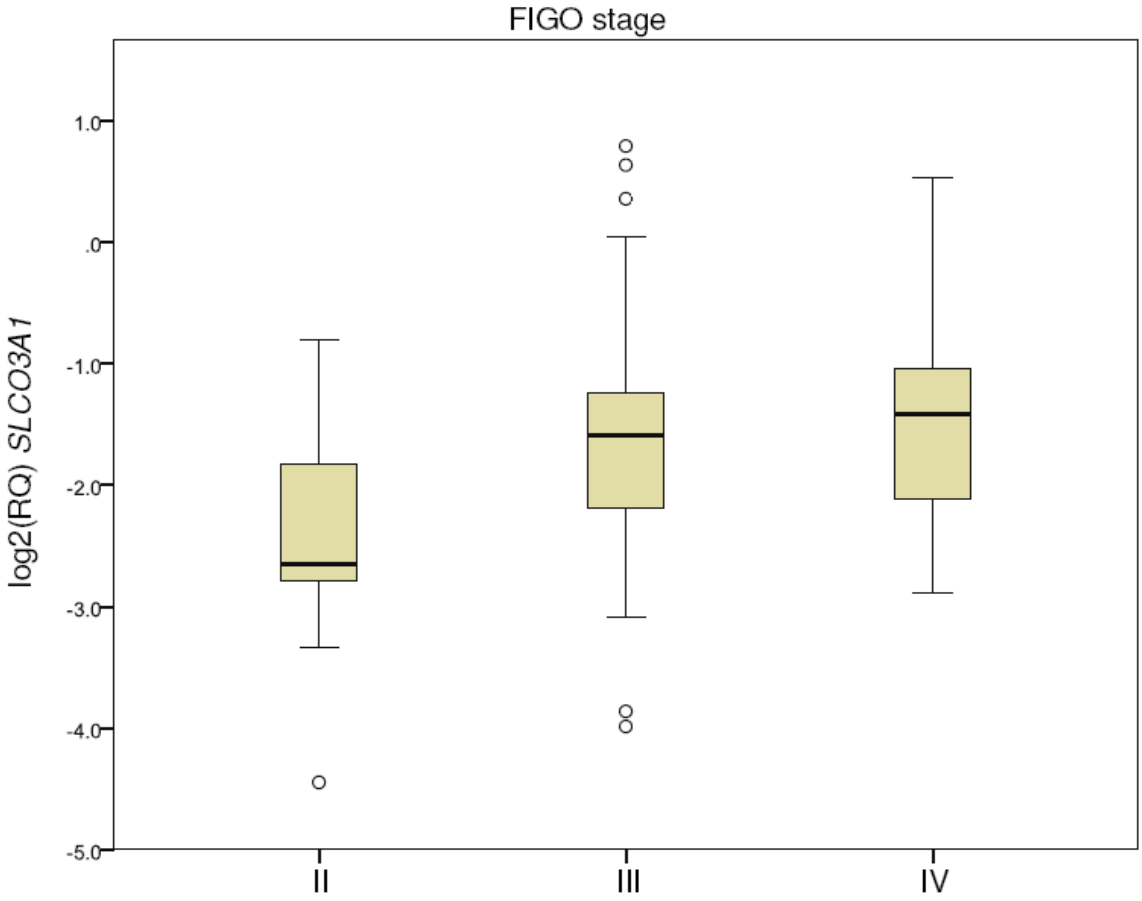

Figure 2

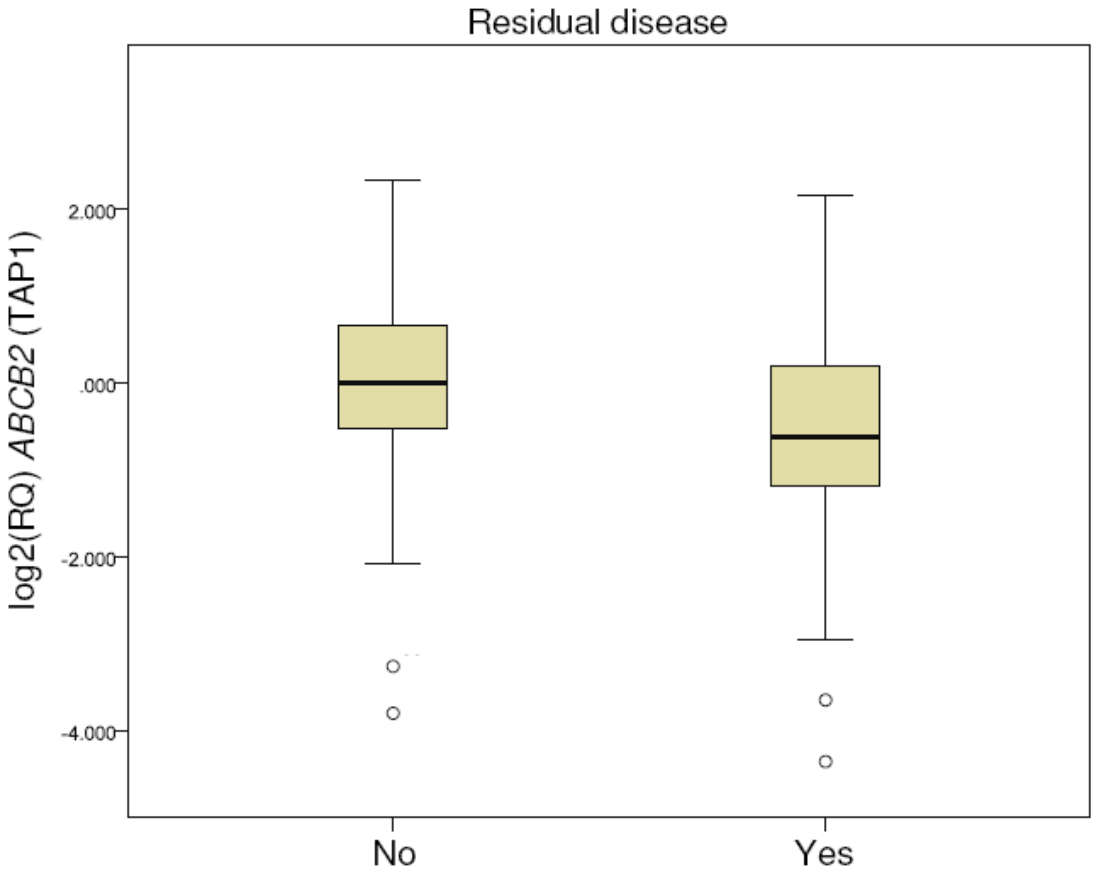
Residual disease
log2(RQ) ABCB2 (TAP1)
2.000
.000
-2.000
-4.000
No
Yes

Figure 3

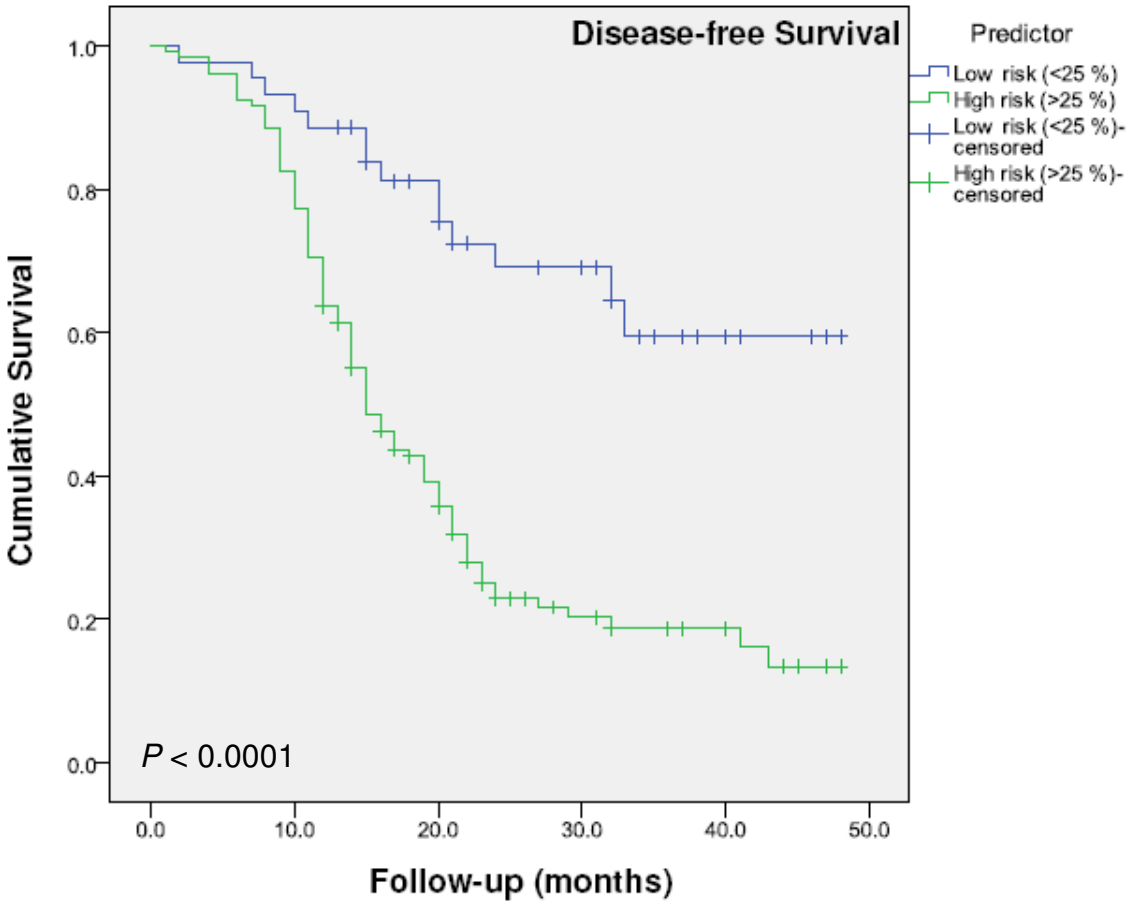
Disease-free Survival
Predictor
Low risk (<25 %)
High risk (>25 %)
Low risk (<25 %)-censored
High risk (>25 %)-censored
Cumulative Survival
1.0
0.8
0.6
0.4
0.2
0.0
P < 0.0001
0.0
10.0
20.0
30.0
40.0
50.0
Follow-up (months)

Figure 4

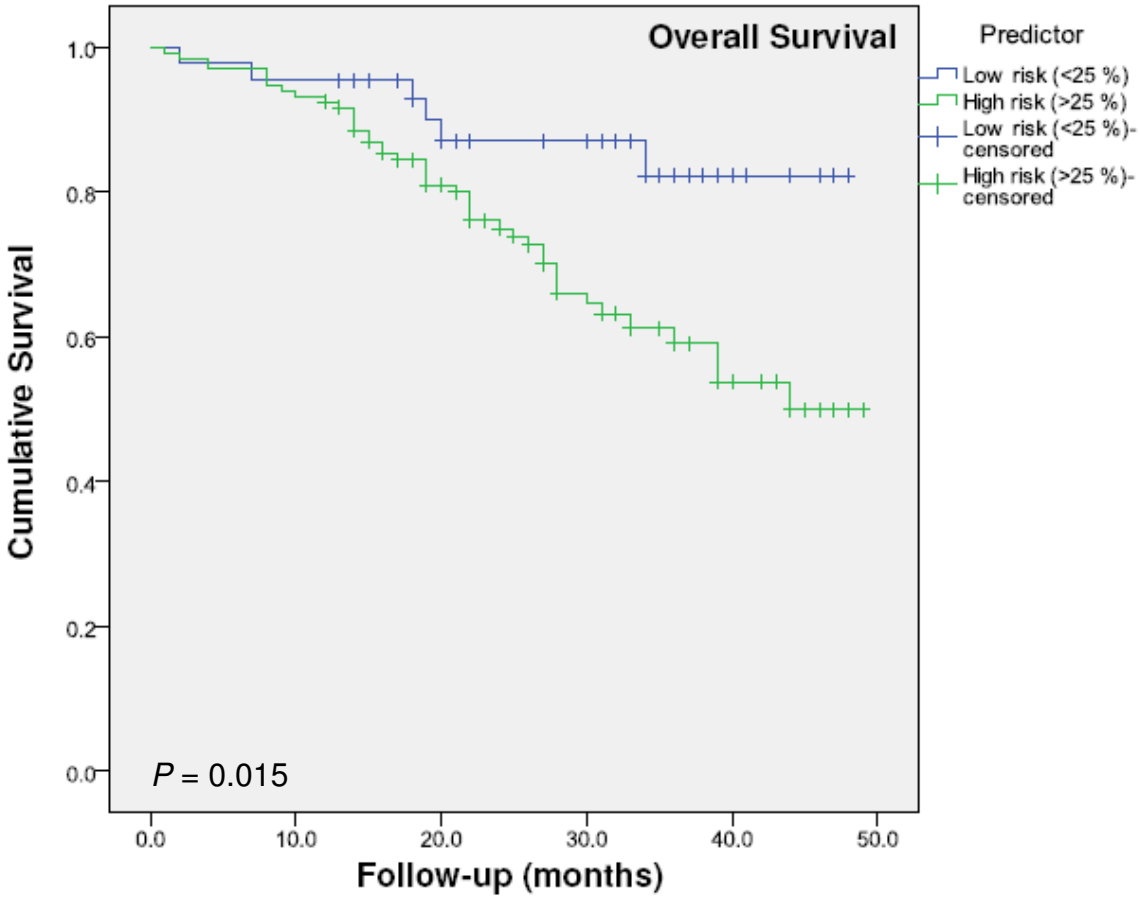
Overall Survival
Predictor
Low risk (<25 %)
High risk (>25 %)
Low risk (<25 %)-censored
High risk (>25 %)-censored
Cumulative Survival
1.0
0.8
0.6
0.4
0.2
0.0
P = 0.015
0.0
10.0
20.0
30.0
40.0
50.0
Follow-up (months)

Table 1. Clinicopathologic Parameters of Patients

Patients	**191 (100.0%)**
Age at diagnosis [years]	
Median	57 (26 -85)
Disease free survival [months]	
Median	17 (1 - 48)
Number of recurrencies	121 (63.4)
Overall survival [months]	
Median	25 (1 - 49)
Number of deaths	53 (27.7)
Histology	
Serous	169 (88.5)
Endometrioid	6 (3.1)
Mucinous	3 (1.6)
Mixed epithelial tumor	7 (3.7)
Undifferentiated carcinoma	6 (3.1)
FIGO[§] (1 [0.5%] missing)	
II	9 (4.8)
III	151 (79.0)
IV	30 (15.7)
Grading (9 [4.7%] missing)	
1	6 (3.1)
2	40 (20.9)
3	136 (71.3)
Residual disease after initial surgery (5 [2.6%] missing)	
None	90 (47.2)
≤ 1cm	29 (15.1)
> 1cm	67 (35.1)
Type of chemotherapy	
Adjuvant	147 (77.0)
Neoadjuvant	26 (13.6)
Intraperitoneal	18 (9.4)
First line chemotherapy	
Carboplatin and paclitaxel	137 (71.7)
Carboplatin and other	22 (11.5)
Carboplatin	13 (6.8)
Cisplatin and paclitaxel	8 (4.2)
Other	11 (5.8)
Response to first-line chemotherapy	
Responder	147 (77.0)
Non-Responder	44 (23.0)

[§]International Federation of Gynecologists & Obstetricians

Table 2. Gene Expression Assays used for real-time RT-PCR (Applied Biosystems)

Gene Symbol	Assay ID[a]	Amplicon size (bp)	Accession number
SLCO1A2	hs00245360_m1	57	NM_134431.2 NM_021094.2
SLCO1B1	hs00272374_m1	77	NM_006446.3
SLCO1B3	hs00251986_m1	69	NM_019844.2
SLCO1C1	hs00213714_m1	92	NM_017435.3
SLCO2A1	hs00194554_m1	87	NM_005630.1
SLCO2B1	hs00200670_m1	113	NM_007256.2
SLCO3A1	hs00203184_m1	89	NM_013272.2
SLCO4A1	hs00249583_m1	80	NM_016354.3
SLCO4C1	hs00698884_m1	77	NM_180991.4
SLCO5A1	hs00229597_m1	60	NM_030958.1
SLCO6A1	hs00542846_m1	63	NM_173488.3
ABCB1	hs00184500_m1	67	NM_000927.3
ABCC2	hs00166123_m1	75	NM_000392.3
ABCC3	hs00358656_m1	98	NM_00114407(NM_003786.3
ABCB2	hs00184465_m1	61	NM_000593.5

[a]Applied Biosystems

Table 3. SLCO- and ABC-Transporter Genes expressed in Ovarian Carcinoma‡

Gene Name	$Fold_{mean}$	$Fold_{min}$	$Fold_{max}$
SLCO1A2	-76.5	ND	2.2
SLCO1B1	-55.1	ND	3.1
SLCO1B3	-25.9	ND	40.2
SLCO1C1	-286.4	ND	-4.8
SLCO2A1	-9.1	-64.4	5.7
SLCO2B1	-3.5	-27.9	2.0
SLCO3A1	-3.1	-21.9	1.7
SLCO4A1	-4.3	-643.6	21.3
SLCO4C1	-130.1	ND	1.6
SLCO5A1	-5.4	-626.0	21.6
SLCO6A1	-3014.2	ND	31.6
ABCB1	-11.2	-100.4	2.9
ABCB2	-1.2	-20.5	5.0
ABCC2	-22.6	-188.7	2.2
ABCC3	-2.4	-91.8	11.2

‡Indicated as fold up- or downregulation compared to human reference RNA (Clontech); ND, not detectable.

Table 4. Comparison[§] of the mean log2(RQ) Values of SLCO and ABC Transporter Expression between clinicopathologic Parameters

	N	SLCO1A2	SLCO1B1	SLCO1B3	SLCO1C1	SLCO2A1	SLCO2B1	SLCO3A1	SLCO4A1	SLCO4C1	SLCO5A1	SLCO6A1	ABCB1	ABCB2	ABCC2	ABCC3
Age (y)[♦]																
< 50	55	-6.87	-5.51	-4.02	-7.95	-3.17	-1.70	-1.51	-1.78	-7.75	-2.44	-11.68	-3.17	-0.09	-4.33	-1.05
> 50	136	-6.01	-5.90	-4.97	-8.25	-3.19	-1.83	-1.65	-2.22	-6.73	-2.45	-11.51	-3.61	-0.33	-4.57	-1.31
P =		*1.000*	*0.663*	*1.000*	*1.000*	*1.000*	*1.000*	*1.000*	*1.000*	*0.420*	*0.993*	*1.000*	*0.405*	*1.000*	*1.000*	*1.000*
FIGO (1 missing)[†]																
II	9	-4.91	-4.68	-3.71	-8.02	-3.09	-1.97	-2.48	-2.80	-6.77	-3.25	-11.76	-3.64	0.03	-4.96	-1.55
III	151	-6.21	-5.83	-4.58	-8.20	-3.25	-1.76	-1.59	-2.03	-7.08	-2.26	-11.40	-3.42	-0.25	-4.44	-1.17
IV	30	-6.73	-5.85	-5.42	-7.97	-2.87	-1.89	-1.37	-2.24	-6.92	-3.23	-12.22	-3.79	-0.35	-4.60	-1.35
P =		*1.000*	*0.350*	*1.000*	*1.000*	*1.000*	*1.000*	***0.030***	*1.000*	*0.928*	*1.000*	*1.000*	*1.000*	*1.000*	*1.000*	*1.000*
Grade (9 missing)[†]																
1	6	-8.38	-5.18	-3.26	-8.47	-3.26	-1.47	-1.67	-2.52	-9.08	-1.51	-10.92	-3.05	-1.03	-4.15	-0.11
2	40	-6.80	-5.75	-4.52	-8.25	-3.20	-1.86	-1.68	-2.17	-7.31	-2.79	-11.88	-3.01	-0.52	-4.61	-1.52
3	136	-6.11	-5.82	-4.76	-8.16	-3.24	-1.84	-1.59	-2.04	-6.81	-2.46	-11.48	-3.69	-0.17	-4.50	-1.22
P =		*1.000*	*1.000*	*1.000*	*1.000*	*0.978*	*1.000*	*1.000*	*1.000*	*1.000*	*1.000*	*1.000*	*0.075*	*1.000*	*1.000*	*1.000*
Histology[♦]																
serous	169	-6.21	-5.82	-4.67	-8.17	-3.19	-1.86	-1.60	-2.17	-7.09	-2.46	-11.60	-3.50	-0.29	-4.50	-1.34
non-serous	22	-6.64	-5.50	-4.90	-8.11	-3.19	-1.29	-1.68	-1.53	-6.48	-2.34	-11.24	-3.37	-0.06	-4.48	-0.45
P =		*1.000*	*1.000*	*1.000*	*1.000*	*0.999*	*0.168*	*1.000*	*1.000*	*1.000*	*1.000*	*1.000*	*1.000*	*1.000*	*1.000*	*0.120*
Residual tumor load (5 missing)[♦]																
negative	90	-6.03	-5.75	-4.88	-8.17	-3.36	-1.71	-1.63	-1.75	-7.00	-1.96	-11.75	-3.38	0.06	-4.41	-0.98
positive	96	-6.37	-5.80	-4.43	-8.19	-3.05	-1.86	-1.55	-2.40	-7.00	-2.93	-11.40	-3.63	-0.52	-4.55	-1.37
P =		*1.000*	*1.000*	*1.000*	*1.000*	*1.000*	*1.000*	*1.000*	*0.084*	*0.994*	*0.732*	*1.000*	*1.000*	***0.015***	*1.000*	*0.793*

[§]Relative to human qPCR reference RNA (Clontech); [♦]analyzed using independent T-test; [†]analyzed with one-sided ANOVA; Bold, statistically significant

Table 5. Univariate and Multiple Cox Proportional Hazards Analysis

	Disease free survival			
	Univariate		Multiple	
	Relative risk$_{(95\% CI)}$ ‡	*P*	Relative risk$_{(95\% CI)}$ ‡	*P*
Age at diagnosis	1.01 (0.99 - 1.02)	0.267	♦	
Histology (non-serous *v* serous)†	0.95 (0.54 - 1.67)	0.864	♦	
FIGO stage	**1.38** (1.08 - 1.76)	0.009	**1.30** (1,03 - 1,64)	0.026
Grading	**1.70** (1.14 - 2.54)	0.010	**1.57** (1,06 - 2,32)	0.025
Residual disease (yes *v* no)	**1.71** (1.12 - 2.47)	0.004	1.43 (0,96 - 2,12)	0.077
SLCO1A2	0.99 (0,94 - 1,04)	0.753	♦	
SLCO1B1	0.86 (0,73 - 1,01)	0.060	∞	
SLCO1B3	0.99 (0,95 - 1,04)	0.734	♦	
SLCO1C1	0.98 (0,84 - 1,15)	0.800	♦	
SLCO2A1	0.96 (0,83 - 1,11)	0.572	♦	
SLCO2B1	0.93 (0,78 - 1,11)	0.413	∞	
SLCO3A1	0.96 (0,78 - 1,18)	0.706	♦	
SLCO4A1	0.97 (0,87 - 1,08)	0.584	♦	
SLCO4C1	1.03 (0,97 - 1,10)	0.289	♦	
SLCO5A1	1.00 (0,95 - 1,05)	0.875	♦	
SLCO6A1	0.92 (0,83 - 1,02)	0.106	**0.90** (0,81 - 1,00)	0.043
ABCB1	0.94 (0,81 - 1,09)	0.402	∞	
ABCB2	**0.82** (0,71 - 0,94)	0.005	**0.81** (0,70 - 0,93)	0.004
ABCC2	1.11 (0,94 - 1,23)	0.216	**1.20** (1,01 - 1,42)	0.041
ABCC3	1.03 (0,90 - 1,17)	0.674	♦	

‡CI, confidence interval; †categorical variable; ♦not implied for model building; ∞not selected in the backward selection procedure; bold, statistically significant.

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Authorship in peer-reviewed journals:

Martin Svoboda, Katrin Wlcek, Dietmar Pils, Gudrun Hager, Dan Tong, Robert Zeillinger, Walter Jäger, Theresia Thalhammer. mRNA expression of SLCO3A1, SLCO6A1, ABCB2, and ABCC2 in Ovarian Carcinoma is associated with Tumor Staging and Patients' Survival. (Manuscript in preparation for the submission to *Clinical Cancer Research*.)

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2009

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2008

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2007

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2006

Estrogen homeostasis in human bone and soft tissue tumors: Inactivation of estrogen by sulfonation. International Conference on Progress in Bone and Mineral Research, November 16–18 2006. Vienna, Austria.

2005

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