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Expression of Organic Anion Transporting Polypeptides (OATPs) in
Cancer Cell Lines and Tissues

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1. ABSTRACT

Ovarian Cancer is one of the most common female genital tract cancers and has the worst prognosis of all gynecological malignancies. This poor prognosis is largely due to a late diagnosis and chemoresistance of these tumors. A major problem is that prognostic factors and tumor markers, especially CA-125, are not specific enough to make a diagnosis at early stage, when the tumor has not spread beyond the ovaries. Ewing's sarcoma, a small round-cell tumor, is prevalently arising in the bones of children. It is characterized by high membrane expression of CD99 and in 85% of cases an abnormal reciprocal translocation between chromosome 11 of the EWS gene and chromosome 22 of the fli1 gene resulting in the chimeric EWS-fli1 fusion gene. In both cancers an improvement of the therapeutic outcome is desirable. This could be achieved by an early detection using appropriate tumor markers and an increased efficiency to standard chemotherapeutic regimens. Therefore, transporters from the family of organic anion transporting polypeptides (OATPs), which mediate the transport of various anticancer drugs, may provide novel chemotherapeutic targets. Investigations of OATPs appear to be a promising approach to lead to novel targets for anticancer chemotherapy and this would help to improve the prognosis for these cancers. Therefore, the aim of the present thesis was to analyze the distribution of all 11 known OATPs, by comparing their expression pattern in cancer cell lines and in normal tissue. Quantitative real time PCR was applied to analyze the expression of all 11 known human OATPs in Ewing's sarcoma cell lines. Expression data were compared to that in human healthy tissue. Additionally colorectal cancer cell lines were applied as controls. Furthermore ovarian cancer cell lines transfected with cDNA of OATP1B1 and OATP1B3 were investigated for paclitaxel cytotoxicity by exposing them to different dilutions of paclitaxel. A number of OATPs were detected in ovarian and Ewing's sarcoma cell lines. Remarkably, the Ewing's sarcoma cell line TC-71 has exhibited significant higher expression rates of OATP5A1 than normal brain tissue. Functional studies showed that the paclitaxel-sensitive ovarian cancer cell line SK-OV-3 exhibited a higher grade of sensitivity with lower IC₅₀ values after transfection with OATP1B1 and OATP1B3. Taken together these data confirms that OATP expression is significant in ovarian and Ewing's sarcoma cell lines and these cell lines offer a good model to study the effect of anti-cancer drugs.

Das Ovarialkarzinom ist eine der häufigsten Krebsarten im weiblichen Genitaltrakt mit der schlechtesten Prognose aller gynäkologischen Erkrankungen. Die Ursache dafür liegt in der späten Diagnose und der Chemoresistenz. Ein sehr großes Problem dabei ist, dass prognostizierende Faktoren und Tumormarker, vor allem CA-125, nicht spezifisch genug sind um eine frühe Diagnose in Stadien, in denen sich der Tumor noch nicht über die Ovarien hinaus verbreitet hat, zu ermöglichen. Das „Ewing's Sarcoma“ ist ein kleinzelliger Tumor, der vorwiegend in den Knochen von Kindern lokalisiert ist. Er zeichnet sich durch hohe Expression von CD99 in den Zellmembranen aus und in 85% der Fälle durch eine reziproke Translokation des Chromosoms 11 des EWS Gens und des Chromosoms 22 des fli1 Gens. Diese Translokation resultiert in der Bildung eines chimären EWS-fli1 Fusionsproteins. In beiden Krebsarten ist eine Verbesserung der Therapie wünschenswert. Diese könnte durch eine frühe Diagnose mit geeigneten Tumormarkern und einer Erhöhung der Effektivität der Standard-Zytostatika erreicht werden. Deshalb könnten möglicherweise Transportproteine der Familie der OATPs, verantwortlich für den Transport verschiedener Zytostatika, neue Therapieansätze liefern. Untersuchungen von OATPs scheinen daher eine vielversprechende Möglichkeit zu sein neue Angriffsziele in der Therapie zu entwickeln, um die Prognose für diese Krebsarten zu verbessern. Daher war es Ziel dieser Diplomarbeit die Verteilung aller 11 bekannten humanen OATPs, durch einen Vergleich ihres Expressionsmusters in Krebszellen und in gesundem Gewebe, zu analysieren. „Quantitative real time RT-PCR“ wurde verwendet um die Expression aller 11 bekannten humanen OATPs im „Ewing's Sarcoma“ zu analysieren. Expressionsdaten wurden mit gesundem Gewebe und kolorektalen Krebszelllinien verglichen, die beide als Kontrolle dienten. Weiters wurden Ovarialkrebszellen mit den Genen OATP1B1 und OATP1B3 transfeziert um diese anschließend verschiedenen Konzentrationen des zytotoxischen Agens, Paclitaxel, auszusetzen. In den „Quantitativen real time RT-PCR“ Experimenten zeigte die „Ewing's Sarcoma“ Zelllinie TC-71 eine signifikant höhere Expressionsrate des OATP5A1-Gens verglichen mit normalem Gehirngewebe. Hinsichtlich des Zytotoxizitätstests waren Zellen der Paclitaxel-sensitiven Ovarialkarzinomzelllinie SK-OV-3 nach Transfektion mit den Genen OATP1B1 und OATP1B3 noch sensibler gegenüber Paclitaxel, gemessen an niedrigeren IC50-Werten. Zusammenfassend bestätigen diese Daten eine signifikante Expression von OATPs in beiden Krebsarten und ihre Zelllinien bieten sich als Model für weitere Zytostatikastudien an.

2. AIMS

The intracellular concentration of certain anticancer drugs is important for the success of a given cancer chemotherapy. The concentration is determined by the activity of drug efflux pumps and importantly, also by drug uptake transporters, e.g. member of the organic anion transporting polypeptide family (OATP). Activity of these transporters may limit the success of a given chemotherapy.

OATPs are sodium-independent transporters, which mediate the cellular uptake of many endogenous and exogenous chemicals including anticancer drugs. 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 play a critical role in drug absorption, distribution and excretion (Hagenbuch and Gui, 2008). However, data on their expression in malignant tumors is still rare. Therefore it was the aim of this thesis to investigate OATP expression in two types of malignancies, namely ovarian cancer and Ewing's sarcoma, for which the success of anticancer chemotherapy is still limited.

Ovarian Cancer is the fifth leading cause of cancer mortality in women. This cancer has the highest incidence in Caucasian women (Holzschneider and Berek, 2000) and accounts for 6% of all cancers in females. The overall lifetime risk of developing ovarian cancer is approximately 1 in 58-75 in industrialized countries and the median age at diagnosis is 59–63 years (Agarwal and Kaye, 2003). In clinical treatment, cytotoxic drugs like taxanes (paclitaxel) and platin derivatives (cisplatin) are applied in a first line therapy in these tumors (Agarwal and Kaye, 2003), but after an initial success, chemotherapy resistance is a major problem.

Ewing's sarcoma is the second most common malignant bone tumour occurring in children and young adults, and accounts for 10–15% of all primary bone tumours. However, the annual incidence is approximately 0.6/million total population and it usually occurs in adolescents and young adults (Burchill et al, 2003).

Therefore, the aim of this diploma thesis was to analyze the expression of SLCO-genes, encoding for OATP, in cancerous tissue in comparison with the expression in normal tissue. In particular OATP1B1/1B3 and OATP2B1, previously reported to be highly expressed in human liver, may be important for active uptake of cytotoxic agents into tumor cells. Studies will also be done in different cancerous cell lines from ovarian cancer and Ewing's sarcoma with colon cancer cell lines as controls.

An increasing knowledge about the expression pattern of OATPs in cancerous tissues should bring further advantages for the therapy of ovarian cancer, Ewing's sarcoma and various other cancers with highly elevated OATP levels. Hence, these knowledge should help to:

- overcome drug resistance of various anti-cancer agents, in particular paclitaxel,
- develop antineoplastic drugs by avoiding adverse effects and
- increase the effect of antineoplastic substances.

Furthermore, elucidating the expression pattern of OATPs could lead to the identification of novel tumor-markers. This would result in a great improvement and bring the early diagnosis of these cancers.

3. INTRODUCTION

3.1 Ovarian Cancer

Ovarian cancer is a malignant neoplasm arising from cells in the ovary. Depending on the tissue, different types of ovarian carcinoma can be distinguished: epithelial ovarian cancer (in the lining of the ovary), germ-cell cancer (egg-cell) and stromal ovarian tumors are the most common forms (Agarwal and Kaye, 2003).

3.1.1 Classification

The WHO (World Health Organization) classifies ovarian neoplasm in 46 different epithelial tumor types, 24 sex cord stromal types, 29 germ cell types, and 13 other categories. Considering the enormous variation of different tumor types, it is essential that histopathological assessment is exactly performed (Bartlett, 2000).

Epithelial ovarian cancer (EOC) is the most common type of ovarian cancer. At least 90% of tumors arise from the coelomic epithelium.

Table 1: *Classification of the epithelial ovarian cancer (EOC) (Bartlett, 2000).*

1.Serous tumors
1a Benign
1. Cystadenoma and papillary cystadenoma
2. Surface papilloma
3. Adenofibroma and cystadenofibroma
1b Of borderline malignancy (of low-malignant potential)
1. Cystic tumor and papillary cystic tumor
2. Surface papillary tumor
3. Adenofibroma and cystadenofibroma
1c Malignant
1. Adenocarcinoma, papillary adenocarcinoma, and papillary cystadenocarcinoma
2. Surface papillary adenocarcinoma
3. Adenocarcinofibroma and cystadenocarcinofibroma
2. Mucinous Tumors: endocervical-like and intestinal type
2a Benign
1. Cystadenoma
2. Adenofibroma and cystadenofibroma
2b Of borderline malignancy (of low-malignant potential)
1. Cystic tumor
2. Adenofibroma and cystadenofibroma

Table 1: Continuation

2c Malignant
1. Adenocarcinoma and cystadenocarcinoma
2. Adenocarcinofibroma and cystadenocarcinofibroma
3. Endometrioid tumors
3a Benign
1. Cystadenoma
2. Cystadenoma with squamous differentiation
3. Adenofibroma and cystadenofibroma
4. Adenofibroma and cystadenofibroma with squamous differentiation
3b Of borderline malignancy (of low-malignant potential)
1. Cystic tumor
2. Cystic tumor with squamous differentiation
3. Adenofibroma and cystadenofibroma
4. Adenofibroma and cystadenofibroma with squamous differentiation
3c Malignant
1. Adenocarcinoma and cystadenocarcinoma
2. Adenocarcinoma and cystadenocarcinoma with squamous differentiation
3. Adenocarcinofibroma and cystadenocarcinofibroma
4. Adenocarcinofibroma and cystadenocarcinofibroma with squamous differentiation
3d Epithelial-(endometrioid) stromal and (endometrioid) stromal
1. Adenosarcoma, homologous and heterologous
2. Mesodermal (müllerian) mixed tumor (carcinosarcoma), homologous and heterologous
3. Stromal sarcoma
4. Clear cell tumors
4a Benign
1. Cystadenoma
2. Adenofibroma and cystadenofibroma
4b Of borderline malignancy (of low-malignant potential)
3. Cystic tumor
4. Adenofibroma and cystadenofibroma
4c Malignant
1. Adenocarcinoma
2. Adenocarcinofibroma and cystadenocarcinofibroma
5. Transitional cell tumors
Brenner tumor
Brenner tumor of borderline malignancy (proliferating)

The most common and lethal malignant ovarian tumor, according to Auersperg et al (2008), is the “high grade serous ovarian carcinoma”. It has his origin in the fallopian tubes. The general assumption that the EOC arise from the coelomic epithelium, which surrounds the ovarian surface, is questioned and it is suggested that the tumor arise from tissue of the Müllerian ducts (Dubeau, 1999).

3.1.2 Tumor stage

The following table summarizes the International Federation of Gynecology and Obstetrics (FIGO) staging system for ovarian cancer:

Table 2: FIGO staging system (Staging Announcement: FIGO Cancer Committee, 1986).

I Growth limited to the ovaries.
A Growth limited to one ovary; no ascites; no tumor on the external surface; capsule intact.
B Growth limited to both ovaries; no ascites; no tumor on the external surfaces; capsule intact.
C Tumor either Stage IA or IB, but with tumor on the surface of one or both ovaries; or with capsule ruptured; or with malignant cells in ascites or peritoneal washings.
II Growth involving one or both ovaries with pelvic extension.
A Extension and/or metastases to the uterus and/or tubes.
B Extension to other pelvic tissues.
C Tumor either Stage IIA or IIB, but with tumor on the surface of one or both ovaries; or capsule ruptured; or with malignant cells in ascites or peritoneal washings.
III Tumor involving one or both ovaries with peritoneal implants outside the pelvis and/or positive retroperitoneal or inguinal lymph nodes. Superficial liver metastasis equals Stage III. Tumor is limited to the true pelvis but with histologically verified malignant Extension small bowel or omentum.
A Tumor grossly limited to the true pelvis but with histologically confirmed microscopic of abdominal peritoneal surfaces.
B Tumor involving one or both ovaries with histologically confirmed implants of abdominal peritoneal surfaces, none exceeding 2 cm in diameter. Nodes are negative.
C Abdominal implants greater than 2 cm in diameter and/or positive retroperitoneal or inguinal nodes.
IV Growth involving one or both ovaries with distant metastases. If pleural effusion is present there must be positive cytology to allot a case to Stage IV. Parenchymal liver metastases equals Stage IV.

3.1.3 Epidemiology

The incident of ovarian cancer is remarkably low in Japan and developing countries, while a high incidence is seen in the USA, Northern Europe and Israel. Ovarian cancer is the fifth leading cause of cancer mortality in women (Holzschneider and Berek, 2000). It accounts for 6% of all cancers in the female. Because ovarian carcinomas are most detected at advanced stages at which the tumor is inoperable, the five-year survival rate is very poor (Herbst et al, 1994).

3.1.4 Pathogenesis

3.1.4.1 Etiology

The Etiology of ovarian cancer is not clear yet, but there are at least three risk-factors known:

1. Genetic predisposition

In women with mutations in the BRCA1- and BRCA-2 gene, a high incidence of ovarian- and mammary carcinomas is found. The risk for women with a mutation of BRCA1-gene for developing ovarian cancer amounts between 40-55% is significant higher than that of women carrying the BRCA2-gene (Wooster et al, 1995).

But ovarian cancer with these genetic predisposition accounts for less than 5%, as the vast majority of cases are sporadic, occurring among women with no family history of ovarian cancer (Amos et al, 1993).

2. Endocrinal factors

In accordance with two studies (Whittemore et al, 1992; Franceschi et al, 1991) a reduction of approximately 40% in the risk of ovarian cancer, with use of the oral contraceptive pills and a 5–10% risk reduction with every year of use, seem to be possible. Furthermore the protective effect lasts for at least 15–20 years.

In contrast the studies show an increasing risk of ovarian cancer with women having low parity.

Fathalla's (1971) hypothesis of "Incessant ovulation" postulates a relation of woman's risk of ovarian cancer to her lifetime frequency of ovulation. In his hypothesis ovulation causes trauma to the ovarian epithelium and raises the mitotic rate through oestrogen-rich follicular fluid leading to neoplasm.

3. Nutrition and environment

Frequent intake of saturated fat and a poor intake of vegetables may increase the risk of ovarian cancer (Banks et al, 1997).

3.1.4.2 Metastasis

There are two migration pathways for cancer cells to form metastases in the peritoneum and distant organs e.g. liver, lung etc.

- Intraabdominal migration through the peritoneal fluid leads to metastases in colon, liver etc.
- Lymphogenic migration leads to metastases in distant organs.

3.1.5 Screening at early stages

The detection of ovarian cancer is often without symptoms until the cancer reaches advanced stages, and thus the 5-year-survival rate is very poor.

CA-125 antigen is a serum marker commonly used to detect ovarian cancer. Although it is a Mullerian duct differentiation antigen and overexpressed in epithelial ovarian cancer cells, it is not absolutely specific on ovarian cancer. It is possible to have increased levels of CA-125 in other malignant cancers or benign neoplasm.

Therefore a combination of CA-125 and ultrasonography might be useful to improve the specificity screening at early stages of ovarian cancer.

Another approach of screening uses transvaginal ultrasound, which is established as first-line test because of higher sensitivity for early-stage disease.

Bimanual pelvic exam has been shown to be of no value in the early detection of ovarian cancer.

3.1.6 Treatment

On behalf of the European Society of Medical Oncology, S.Aebi and M.Castiglione (2008) elaborated recommendations, including treatment:

1. Early stage disease, FIGO 1 and 2a:

At FIGO 1a/1b with well differentiated and non clear cell histology a surgery is sufficient, whereas FIGO 1a/1b stage with poorly differentiated, clear cell histology and FIGO 1c/2A stage needs optimal surgery as well as adjuvant chemotherapy. This chemotherapy should contain either a combination of three cycles of carboplatin + paclitaxel or a monotherapy of six cycles of carboplatin or paclitaxel.

2. Advanced disease, FIGO 2b-3c:

Total abdominal hysterectomy and bilateral salpingo-oophorectomy is necessary at this stage of cancer. Further an administration of carboplatin \pm paclitaxel every 3 weeks for six cycles should be applied. Also an intraperitoneal chemotherapy is recommended.

3. Advanced disease, FIGO stage 4

The maximum surgery and chemotherapy as described for FIGO 2b-3c.

4. Recurrence disease

Patients, that with no event of relapse over 1-year surgery resection is recommended and for those with long intervals (>6 months) from initial chemotherapy a platinum-based therapy is first choice (e.g. paclitaxel + carboplatin). In cases with short treatment-free intervals and with second and later recurrences, palliative chemotherapy with pegylated liposomal doxorubicin, gemcitabine or topotecan might be suggestive.

3.1.7 Paclitaxel and the mechanism of resistance

By binding β -tubulin, paclitaxel stabilize the microtubule and stop mitosis in G₂-M-phase, which induces apoptosis. However paclitaxel modulates the “mitogen activated protein kinase” (MAPK), that leads to the dephosphorylation of the “proapoptotic protein” (BAD), the phosphorylation of B-cell lymphoma 2 (BCL2) and finally the induction of apoptosis (Agarwal and Kaye, 2003).

According to Agarwal and Kaye 2003, drug resistance originates in three main categories: Pharmacokinetic, tumor environmental and cancer-cell specific.

Pharmacokinetic: Differences in first-pass metabolism, conversion of prodrugs to active metabolites, renal clearance, hepatic drug metabolism and tumor vascularity between the patients are problematic for dose findings.

Tumor microenvironment: Hypoxia might leads to a larger number of cells arresting in the G₀-phase of the mitosis with reduced development of free radicals and enhanced drug detoxification. In consequence of the hypoxic stress, the hypoxia inducible factor 1 (HIF1)-mediated inhibition of apoptosis is suggested. Cytokines secreted from stroma cells can activate anti-apoptotic signalling pathways as well.

Cancer-cell specific factors:

- Cytotoxic agents are primarily effective against proliferating cells in rapid growing tumors. However, even in fast growing tumors, a number of cells are quiescent and stay in the G₀-phase (Shah and Schwartz, 2001).

- Membran efflux pumps such as P-glycoprotein (PGP), multidrug-resistance protein (MRP) and lung-resistance protein (LRP), which are members of the ABC transporter family, reduce the intracellular concentration of drugs and decrease the sensitivity to a certain drug (Borst, Evers, Kool and Wijnholds, 2000).
- Cisplatin acts cytotoxic by forming DNA-platinum adducts, those can be repaired by the nucleotide excision repair –pathway (NER). In vitro experiments have shown that an enhanced activation of the NER-pathway correlates with increasing resistance against cisplatin (Dijt, Fichtinger-Schepman, Berends and Reedijk, 1988).
- Taxane-resistant cells display a reduced concentration of tubulin as well as changes in the isotypes of tubulin subtypes. Also changes in the paclitaxel-mediated tubulin polymerization, by mutations of β tubulin, were observed (Giannakakou, P. et al, 1997).
- G H Shen et al (2000) have detected an overexpression of the vascular endothelial growth factor (VEGF) in ovarian carcinomas and their meaning for the angiogenesis of tumor and thus for resistance. Hence, VEGF can be recognized as a poor prognostic factor.

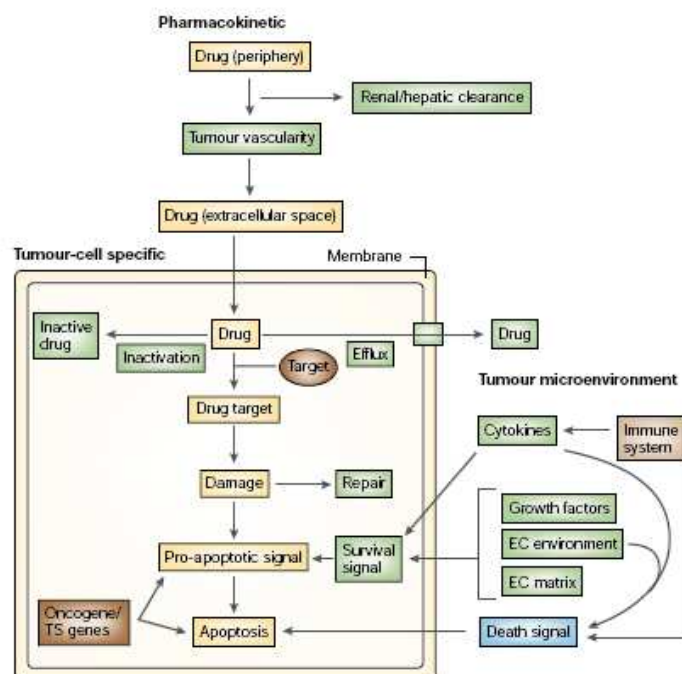


Figure 1: Drug resistance mechanisms, which generally leads to a reduction of the active intracellular drug concentration and are discussed previously (Agarwal and Kaye, 2003).

- Yellow: pathways of drug action.
- Blue: pathways of apoptosis.
- Green: pathways of drug resistance.
- Brown: pathways of pro- and anti-cytotoxic effects.

Resistance of paclitaxel:

In the liver, paclitaxel is inactivated by CYP-enzymes (CYP3A4, CYP 2A8) and excreted by P-glycoprotein (MDR-1 gene). Different polymorphisms in the multidrug resistance-1 (MDR-1) gene, encoding for the transport protein P-glycoprotein, also might be responsible for drug resistance. Mutations in β -tubulin, resulting in alterations of the paclitaxel-induced tubulin polymerization were declared as clinically unconfident (Sale et al, 2002). Smith NF et al (2005) observed the role of SLCO1B3, encodes for transport protein OATP1B3, in the hepatic uptake of paclitaxel.

According to Cicchillitti and Di Michele (2009) interactions of ERp57, a disulphide isomerase, with class III-tubulin (TUBB3) could be a possible pathway in the paclitaxel resistance in ovarian as well as other cancers. The interactions modify the attachment of microtubules to chromosomes.

In the study of Odonkor et al (2008), it was suggested that caspase-activation and activity levels plays the main role in paclitaxel-induced apoptosis in tumor cells.

Overcoming Drug resistance: (Agarwal and Kaye, 2003)**1. New strategies:**

- Intraperitoneal chemotherapy:

The peritoneal cavity is the main site of the disease, therefore directly application in peritoneum using a catheter is reasonable. A randomized clinical trial, performed by Armstrong et al (2006), has shown a significant higher survival time in patients with optimally debulked stage III ovarian cancer treated with intravenous paclitaxel plus intraperitoneal cisplatin than with intravenous paclitaxel plus cisplatin.

- Combination chemotherapy:

established first-line therapy with effective, non-cross resistant drugs.

2. Approaches of novel anticancer agents:

- Down-regulation of MMR (the DNA mismatch repair system, hMLH1 gene) by methylation. However, decitabine, a demethylating agent can lead to the reexpression of hMLH1 gene and increase therefore the sensitivity for agents.

- Buthionine sulphoximine (BSO), a synthetic amino acid, is a suppressor of the synthesis of glutathione. It inhibits the conjugation of cisplatin with glutathione, and thus the detoxification.
- Bevacizumab, a monoclonal antibody, inhibits angiogenesis, by suppressing all isoforms VEGF-A (vascular endothelial growth factor-A).

3.2 The Ewing's sarcoma

3.2.1 General features

Ewing's sarcoma was first described by James Ewing in 1921. It is a small round-cell tumour and typically arises in the bones of children. Second to osteosarcoma, Ewing's sarcoma is the most frequent malignant bone tumour, but overall is still a rare tumor, as there are only 225 new cases in North America per year (Bernstein et al, 2006). In 85% of cases an abnormal reciprocal translocation between chromosome 11 of *ews* gene and chromosome 22 of the *fli1* gene, which results in the chimeric *ews-fli1* fusion gene, is observed (Bernstein et al, 2006).

3.2.2 Epidemiology, Sex and Age

Ewing's sarcoma is slightly more common in male which is reflected in a male:female ratio of 55:45. The most frequent time of diagnosis is childhood, which is important for the differential diagnosis to exclude other tumors. The Ewing's sarcoma affects more Caucasians than Asians and it is rarely diagnosed in Africans and African-Americans (Bernstein et al, 2006).

3.2.3 Localization

The Ewing's sarcoma predominantly appears either in the truncal skeleton or the long bones.

1. Truncal skeleton: pelvis, scapula, vertebral column, ribs and clavicle.
2. Long bones: femur, humerus, tibia and bones of the forearm.

Further the Ewing's sarcoma has a high probability for metastases with a predilection for lungs and bones (Iwamoto, 2007).

3.2.4 Symptoms

Early symptoms give useful information for the correct diagnosis. The first symptom is pain without defined trauma pain can be intermittent and variable in intensity and in some cases it is accompanied by paresthesia. Another symptom is a swelling of the affected site because of a progressive growth of the tumour. Other, but not specific symptoms for Ewing's sarcoma are fever, anemia, inflammation, increased LDH-levels (Iwamoto, 2007).

3.2.5 Diagnosis

Diagnosis is made by initial imaging investigation of the Ewing's sarcoma: Tumor-related osteolysis and new bone-mass in response to stimuli of the inflammatory periosteum surrounding the bone are signs for this these tumors. For more precisely investigations magnetic resonance imaging is usually done and biopsy is made (Bernstein et al, 2006).

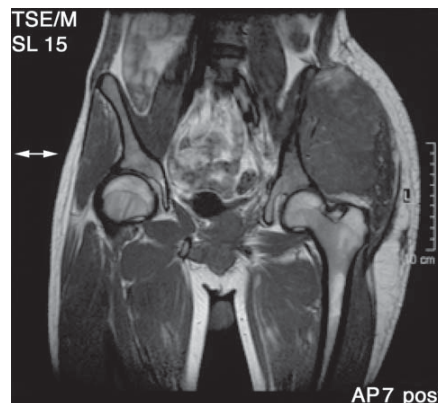


Figure 2: Magnetic resonance image of a pelvic Ewing's sarcoma (Bernstein et al, 2006).

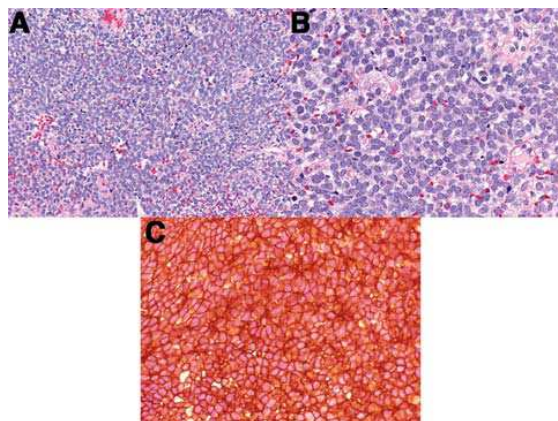


Figure 3: Histologic and immunohistochemical slices of Ewing's sarcoma/pPNET (Bernstein et al, 2006).

- (A): Classic Ewing's sarcoma is recognized as monotonous round cells.
 (B): Cells have little cytoplasm and round nuclei.
 (C): A strong, diffuse membrane staining is demonstrated.

3.2.6 Pathology

EWS-Fli1 fusion gene, the result of the chromosomal translocation, acts as an oncogene. It is assumed that this fusion gene is involved in the expression of cell cycle-regulatory proteins, which control the transition between the G₁- and S-phase (Iwamoto et al, 2007).

Expression of different cell cycle proteins is influenced

1. Up-regulation of the G₁-cyclins: cyclin D1 and E
2. Down-regulation of the CDK-inhibitors of the G₁-S transition: p21 and p27

The resulting imbalance between the G₁ cyclin-CDK-complex and the CDK-inhibitors may cause uncontrolled proliferation and may affect the pathway of the tumor suppressor genes Rb and p53. This would lead to the entry in the S-phase of the cell cycle (Iwamoto, 2007).

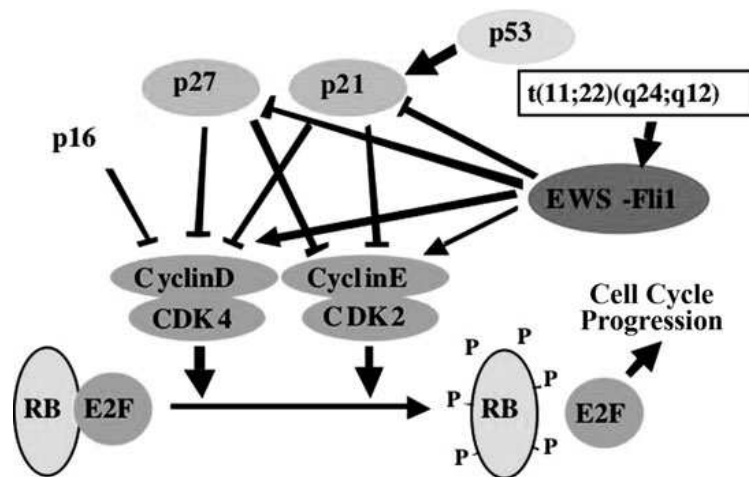


Figure 4: *Oncogenesis of Ewing's sarcoma (Iwamoto et al, 2007).*

- EWS-Fli1 fusion gene...Ewing's sarcoma-Friend leukaemia virus integration fusion gene
- p16, 27, 21...cyclin-dependent kinase inhibitors
- p53...tumor suppressor protein
- Cyclin D,E...cell cycle regulator proteins
- CDK...cyclin dependent kinase
- RB...retinoblastoma protein (tumor suppressor)
- E2F...cell cycle activator.

3.2.7 Treatment

The current treatment includes a combination of surgery, radiation therapy and chemotherapy. For the chemotherapeutical treatment a combination of anticancer drugs (doxorubicin, cyclophosphamide, vincristine, actinomycin-D, ifosfamide and etoposide) is applied. Treatment begins with an intensive chemotherapy to diminish the tumour and reduce the risk of metastasis during the surgery. After the resection of Ewing's sarcoma, the resected bone mass has to be reconstructed to recover the function. After surgery, a cycle of chemotherapy and irradiation is usually given (Iwamoto, 2007).

3.2.8 Prognostic factors

Unfavourable factors are an age over ten years, a tumour size over 200 ml, more central tumours and a low response to the chemotherapy. With the standard of current treatment, it is not possible to achieve more than approximately 20% chance of long-term survival in patients with bone metastases. However, it depends on the site of metastasis as the prognosis for patients with isolated lung metastasis is better than those with bone metastasis (Bernstein et al, 2006).

3.2.9 Future investigations

To establish new therapy strategies according to Iwamoto (2007), drugs affecting the pathway downstream of EWS-Fli1 are of mainly interest.

3.3 Drug resistance and drug transporters:

Organic Anion transporting Polypeptides

3.3.1 General features

First Oatp was identified and isolated in 1994 from rat liver performed by cloning (Jacquemin et al, 1994). The organic anion transporting polypeptides form a sodium independent transport system, which mediates the transmembrane transport of various amphiphatic endogenous as well as exogenous organic substrates (Hagenbuch and Meier, 2004; König et al, 2006). In human named "OATP" and derived from other species named "Oatp", both are members of the solute carrier organic anion transporter family = SLCO Gene family (Hagenbuch and Meier, 2004).

As a result of calculations OATPs are predicted to have twelve transmembrane domains with 6 extracellular loops, in which loop 2 and 5 have putative N-glycosylation sites (Hagenbuch and Meier, 2004). Additionally loop 5 contains ten conserved cysteine residues, which are disulfid bonds under normal condition and essential for the expression of a functional protein (Hänggi et al, 2006). Various OATPs (in human, rat, mouse and other species) have a C-terminal consensus sequence and for rat Oatp1a1 those sequence was observed to be essential for the localization in plasma membrane (Wang P et al, 2005).

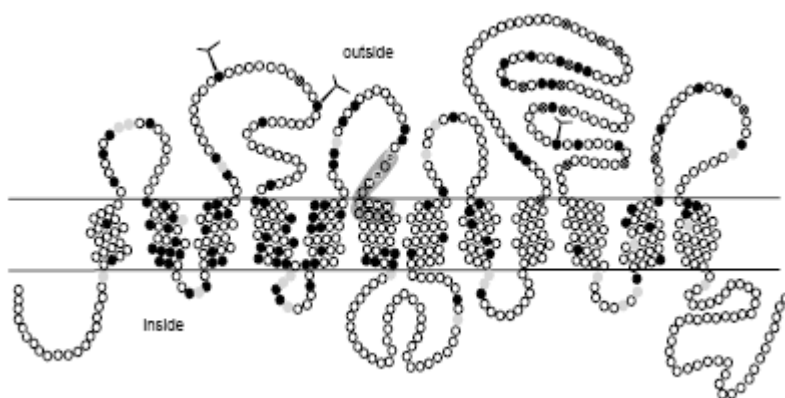


Figure 5: *Calculated structure of human OATP1B1 with twelve transmembrane domains and conserved (black) amino acids (Hagenbuch and Meier, 2003).*

3.3.2 Nomenclature and Classification

The new nomenclature and classification system, that is characterized by species independence and a significant similarity to the cytochrome P450-system and was accepted by the HUGO-gene Nomenclature Committee. Therefore the OATPs are classified based on amino acid sequence homology into families and subfamilies:

- **Amino acid sequence identity more than 40%:** OATP1, OATP2, OATP3, OATP4, OATP5 and OATP6 are grouped in the same family name.
- **Amino acid sequence identity more than 60%:** OATP1A, OATP1B and so on, are classified in subfamilies.
- **If there are several individual proteins within the same subfamily, they added a number based on the chronology of identification:** e.g. Oatp1a1 (Hagenbuch and Meier, 2004).

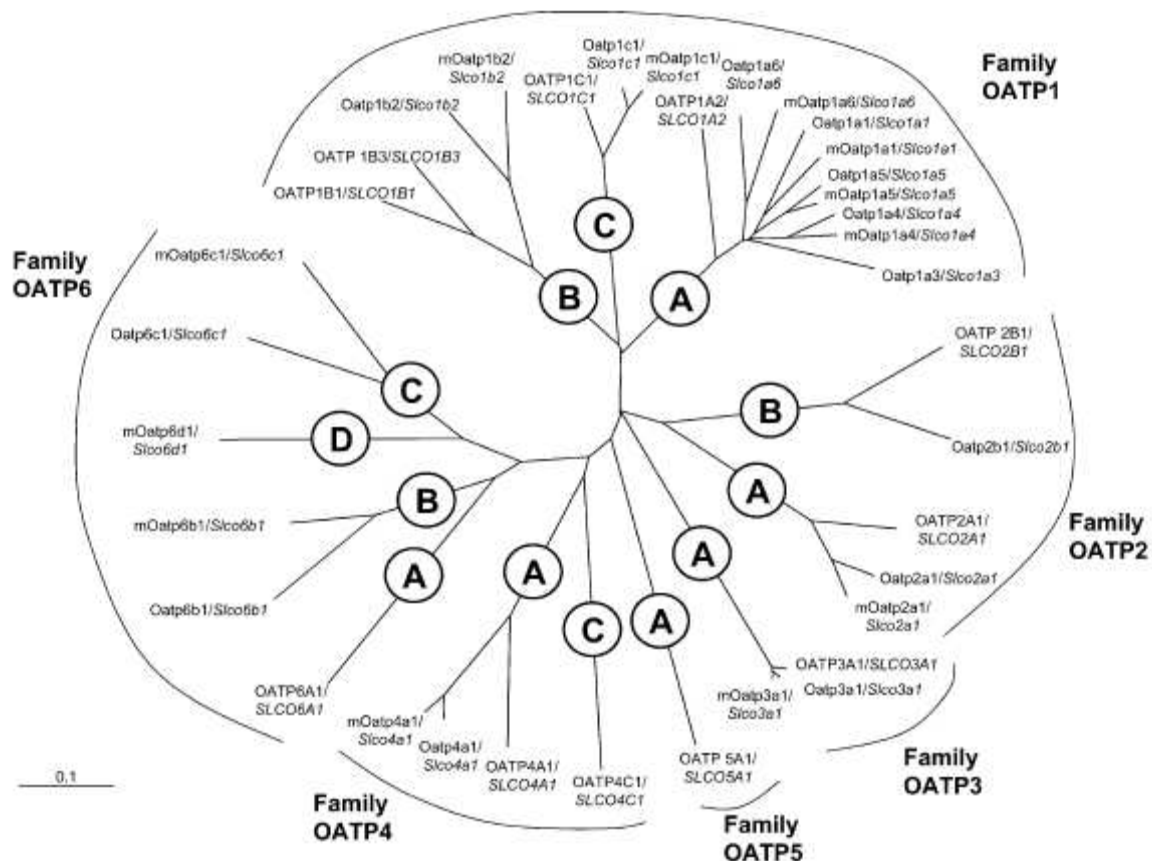


Figure 6: "The phylogenetic tree of the OATP-gene superfamily" (Hagenbuch and Gui, 2008).

3.3.3 Biological functions

The largest and best-known family is the OATP1 family, which is further divided in OATP1A, OATP1B and OATP1C. In contrast of OATP1A2, which is expressed in various tissues, OATP1B1 and OATP1B3 have only been found at the basolateral membrane of hepatocytes, and hence are "liver-specific" OATPs (Kullak-Ublick et al, 1995; Lee et al, 2005; Glaeser et al, 2007; Abe et al, 1999, 2001; Hsiang et al, 1999; König et al, 2000).

OATPs catalyze the multispecific and sodium-independent transport of various amphiphatic organic anions, neutral compounds and also few cations.

- **Substrates: endobiotics:** bile acids, eicosanoids, steroid and thyroid hormones.
- **Substrates: xenobiotics:** anionic oligopeptides, organic dyes, several toxins and drugs. (Hagenbuch and Meier, 2004; König et al, 2006)

Many members of the ATP-binding cassette transporters (ABC transporter) play a important role in the elimination of endo- and xenobiotics (drugs and chemotherapeutics) and for some of these ABC transporters, multiple substrate binding sites were detected (Ambudkar et al, 2003; Karwatsky and Georges, 2004; Bakos and Homolya, 2007; Hardwick et al, 2007). Substances like digoxin, estradiol-17-glucuronid, leukotriene C₄, paclitaxel and methotrexate are transported by ABC transporters and are also substrates of OATPs. This suggests that OATPs might also contain multiple substrate binding sites (Hagenbuch and Meier, 2004; König et al, 2006; Hagenbuch and Gui, 2008).

3.3.4 Transport-mechanism:

Although the exact transport mechanism is not yet investigated, its anionic exchange might be occurs. Thereby, uptake of one anionic organic compounds is connected with the efflux of another organic compound (e.g. bicarbonate, glutathione and/or glutathione-S-conjugates) (Shi et al, 1995; Satlin et al, 1997; Li et al, 2000; Li et al, 1998).

All OATPs appear to mediate bidirectional transport and the direction of the transport depends on the substrates' local concentration gradients, so the transport appears to be pH-dependent and electroneutral (Nakao et al, 2006).

3.3.5 Description of human OATPs

11 human OATPs divided into 6 families (OATP1 – OATP6) were identified. OATP families are further divided into subfamilies (Hagenbuch and Gui, 2008).

OATP1A2 (synonym: OATP-A) (Hagenbuch and Meier, 2004)

The highest expression of OATP1A2 was found in brain (Kullak-Ublick et al, 1995; Gao et al 2000), but expression of OATP1A2 was also found in the liver, where it is located in cholangiocytes. It is expressed in the brush border membrane in the distal nephron (Lee et al, 2005) as well as in the apical membrane of enterocytes (Glaeser et al, 2007).

Substrates: several endogenous and exogenous, mainly amphipathic organic compounds (bile salts, hormones and conjugates, cyclic and linear peptides, toxins, organic cations, numerous drugs) (Hagenbuch and Gui, 2008). Like all

other human OATPS, OATP1A2 from the OATP1A subfamily has also homologous proteins in animal species (Hagenbuch and Meier, 2004).

OATP1B1 (synonym: OATP-C) (Hagenbuch and Meier, 2004)

As a liver-specific transporter (Abe et al, 1999; Hsiang et al, 1999; König et al, 2000) OATP1B1 is localized at the basolateral membrane of hepatocytes (König et al, 2000). It is widely assumed, that OATP1B1 is involved in the uptake of amphipathic organic substances into liver (König et al, 2000). Considering the wide range of drugs transported by OATP1B1 an effect on adverse drug-drug interactions might be possible (Hagenbuch and Gui, 2008).

Substrates: numerous of endogenous and exogenous substrates (bile salts, hormones and conjugates, eicosanoids, cyclic and linear peptides, toxins, numerous drugs incl. antibiotics, statines, AT-RA, ACE inhibitors, anticancer drugs) (Hagenbuch and Gui, 2008).

OATP1B3 (synonym: OATP8, LST-2) (Hagenbuch and Meier, 2004)

This transporter has 80% amino acid identity to OATP1B1 (Hagenbuch and Meier, 2003). It is also assumed as being liver-specific with high expression rates around central vein (Abe et al, 2001; König et al, 2000).

Substrates: similar to OATP1B1, but with some specific substrate like digoxin.

OATP1C1 (synonym: OATP-F) (Hagenbuch and Meier, 2004)

There is one human and one rodent member in the subfamily of OATP1C, respectively (Hagenbuch and Meier, 2004). Brain and testis are tissues with highest expression rates of OATP1C1 and with immunochemistry methods detected it was detected in the Leydig cells. Furthermore it is also located in the blood-brain barrier (Pizzagalli et al, 2002). Physiological experiments in *Xenopus laevis* oocytes and transfected Chinese hamster ovary cells (CHO) suggest that OATP1C1 have an important role in the transport of thyroid hormones to their targets (Hagenbuch et al, 2007).

Substrates: smaller numbers of substrates (thyroid hormones with highest affinity, BSP, oestradiol-17 β -glucuronide, oestrone-3-sulfate) but with higher affinities to their substrates in contrast of the multispecific transporters above (Hagenbuch and Gui, 2008).

OATP2A1 (synonym: PGT = prostaglandin transporter) (Hagenbuch and Meier, 2004)

OATP2A1, which has orthologues in animal species (Schuster et al, 2002; Hagenbuch and Meier, 2004) was originally cloned from adult kidney (Lu et al, 1996). Northern blot analysis shows an almost ubiquitary expression of OATP2A1 in human tissues. According to the physiological function, OATP2A1, which is a prostaglandin transporter, is involved in regulation of pericellular prostaglandin levels in cells expressing COX-2 (Schuster et al, 2002).

Substrates: eicosanoids, but no conventional substrates of the OATPs (Hagenbuch and Gui, 2008).

OATP2B1 (synonym: OATP-B) (Hagenbuch and Meier, 2004)

OATP2B1 was detected with orthologues in rat and mouse (Hagenbuch and Meier, 2004) and found in human kidney, lung, heart, liver, placenta, brain, spleen, testis, ovary, colon (Kullak-Ublick et al, 2001). Because of this ubiquitous expression in human tissue, OATP2B1 could play a role in the absorption and disposition of endo- and xenobiotics (Hagenbuch and Gui, 2008).

Substrates: only BSP, oestrone-3-sulfate, DHEAS at pH 7.4, further at an acid pH, taurocholate, bilirubin conjugates, fexofenadine, statins, glibenclamide, loop diuretics (M17055) (Hagenbuch and Gui, 2008).

OATP3A1 (synonym: OATP-D) (Hagenbuch and Meier, 2004)

These transporters also have orthologues in rat and mouse, with 97% amino acid identity to their human analogues and hence are the most conserved proteins within the OATP superfamily (Hagenbuch and Meier, 2004). Similar to the members of the OATP2 subfamily, OATP3A1 was found ubiquitous with highest levels in testis, brain, heart, lung, spleen, peripheral blood leukocytes and thyroid gland (Adachi et al, 2003; Huber et al, 2007). It is further postulated that these transporters are involved in the transport of neuron-active peptides (Hagenbuch and Gui, 2008).

Substrates: prostaglandins, thyroid hormones, BQ-123 (cyclic peptide, ETA-RA), vasopressin (Hagenbuch and Gui, 2008).

OATP4A1 (synonym: OATP-E) (Hagenbuch and Meier, 2004)

The OATP4A subfamily has one member in human as well as one member in rats and mice (Hagenbuch and Meier, 2004). The OATP4A1 transporter is mainly expressed in heart, placenta, lung, liver, skeletal muscle, kidney and pancreas (Tamai et al, 2000; Fujiwara et al, 2001).

Substrates: the specificity for oestrone-3-sulfate, oestradiol-17 β -glucuronide, benzylpenicillin, thyroid hormones and prostaglandin E2 was reported by Tamai et al (2000), but in case of prostaglandin E2 not confirmed by Fujiwara et al (2001).

OATP4C1 (synonym: OATP-H) (Hagenbuch and Meier, 2004)

Analogue to OATP4A, the OATP4C subfamily also have one member in human, rats and mice. The human transporter, OATP4A1 is found in kidney, where it is located at the basolateral membrane of the proximal tubule. It has 80% amino acid identity with the transporter expressed in rats (Hagenbuch and Meier, 2004; Mikkaichi et al, 2004). This transporter is assumed to be important for the thyroid hormone delivery to the kidney (Hagenbuch and Gui, 2008).

Substrates: digoxin, ouabain, thyroxine, methotrexate, cAMP, dipeptidyl peptidase-4 inhibitor sitagliptin (Hagenbuch and Gui, 2008).

OATP 5A1 and 6A1 (synonym: OATP-J and -I) (Hagenbuch and Meier, 2004)

OATP5A1 is marginal characterized (Hagenbuch and Gui, 2008). OATP6A1 shows high expression rates in the normal testis and low expression rates in spleen, brain, foetal brain and placenta (Suzuki et al, 2003; Lee et al, 2004). However, it is also found in several lung tumours, lung cancer cell lines, bladder and oesophageal tumours (Lee et al, 2004).

3.3.6 Substrates

The substrates of Oatp/OATP-family can be simplified characterized as anionic amphiphatic molecules with a molecular weight over 450 and subsequently are bound to albumin under physiological conditions (steroids or small linear and cyclic peptides) (Hagenbuch and Meier, 2004).

Table 3: Substrates of all 11 human OATPs (Hagenbuch and Meier,2004).

New nomenclature	Old nomenclature	Substrates	Tissue disstribution
OATP1A2	OATP-A	APD-ajmalinium, Atrasentan, Bamet-R2, Bamet-UD2, Bilirubin, BQ-123, BSP chlorambuciltaurocholate, cholate, ciprofloxacin, DHEAS, deltorphin 2, DPDPE, enoxacin estradiol-17 β -glucuronide, estrone-3-sulfate, fexofenadine, gatifloxacin, Gd-B20790, glycocholate, levofloxacin, lomefloxacin, methotrexate, microcystin, N-methylquinine, norfloxacin, ouabain, pitavastatin, PGE2, rT3, rocuronium, rosuvastatin, saquinavir, taurocholate, T4, TCDCA, TUDCA, TR-14035, unoprostone metabolite	brain,liver, kidney, intestine, lung, testis
OATP1B1	OATP-C, OATP2, LST-1	ACU154, arsenic, atorvastatin, atrasentan, bamet-R2, bamet-UD2, benzylpenicillin, bilirubin, bisglucuronosyl bilirubin, bosetan, BQ-123, BSP, caspofungin cerivastatin, CDCA-NBD, cholate, DADLE, DHEAS, demethylphalloin, DPDPE, enalapril, estradiol-17 β -glucuronide, estrone-3-sulfate, fluvastatin, glycocholate, GUDCA, LTC4, LTE4, methotrexate, microcystin, monoglucuronosyl bilirubin, olmesartan, phalloidin, pitavastatin, pravastatin, PGE2, rifampicin, Ro 48-5033, rosuvastatin, SN-38, taurocholate, TUDCA, TXB2, T3, T4, TR-14035, troglitazone sulfate, valsartan	Liver
OATP1B3	OATP-8	amanitin, atrasentan, bilirubin, bosentan, BQ-123, BSP, CDCA-NBD, cholate, CCK-8, DHEAS, deltorphin2, demethylphalloin, digoxin, docetaxel, DPDPE, enalapril, estradiol-17 β -glucuronide, estrone-3-sulfate, fexofenadine, fluvastatin, fluo-3, glutathione, glycocholate, GUDCA, LTC4, methotrexate, microcystin, monoglycucuronosyl bilirubin, olmesartan, ouabain, paclitaxel, phalloidin, pitavastatin, rifampicin, Ro 48-5033, rosuvastatin, taurocholate, TCDCA, TDCA, TUDCA, telmisartan, T3, T4, TR-14035, valsartan	Liver
OATP1C1	OATP-F	BSP, estradiol-17 β -glucuronide, estrone-3-sulfate, rT3, T3, T4	brain, testis
OATP2A1	hPGT	eicosanoids: PGD2, PGE1, PGE2, PGE2 α , TXB2	kidney, brain, colon, heart, liver, lung, ovary, pancreas, placenta, prostate, skeletal muscle, spleen, small intestine
OATP2B1	OATP-B	atorvastatin, benzylpenicillin, bosetan, BSP, CP-671,305, DHEAS, estrone-3-sulphate, fexofenadine, fluvastatin, glibenclamide, M17055, pravastatin, pitavastatin, pregnenolone sulphate, prostaglandin E2, rosuvastatin, taurocholate, unprotone metabolite	liver, placenta, heart, brain, eye, kidney, lung, spleen, testis, ovary, colon, small intestine
OATP3A1	OATP-D	<i>OATP3A1_v1</i> : benzylpenicillin, BQ-123, deltorphin, estrone-3-sulfate, PGE1, PGE2, PGF2 α , T4, vasopressin <i>OATP3A1_v2</i> : arachidonicacid, BQ-123, PGE1, PGE2, T4, vasopressin	testis, brain, heart, lung, spleen, peripheral blood, leucocytes, thyroid gland, kidney
OATP4A1	OATP-E	benzylpenicillin, estradiol-17 β -glucuronide, estrone-3-sulphate, PGE2, rT3, taurocholate, T4, T3, unprostone metabolite	heart, placenta, lung, liver, skeletal muscle, brain, kidney, pancreas
OATP4C1	OATP-H	cAMP, digoxin, methotrexate, ouabain, sitagliptin, T4, T3	kidney
OATP5A1	OATP-J	unknown	unknown
OATP6A1	OATP-I, GST	unknown	testis, brain, placenta

3.3.7 Significance for the drug response

1. Genetic factors:

Previous studies showed the influence of genetic polymorphisms in OATPs on their subcellular location, function, activity and specificity. According to Niemi et al (2005) genetic polymorphism in SLCO1B1 is related to pharmacokinetic differences for repaglinide and this might be important for the administration of this drug. For example, a reduced uptake of pravastatin, a HMG-CoA-reductase inhibitor, through a mutated OATP1B1 might be associated with a reduction of its cholesterol-lowering effects (Niemi et al, 2005).

2. Drug-drug interactions:

Decreased expression of OATPs, and, subsequently, reduced uptake into the liver might lead to a decreased bioavailability. This could further lead to more side-effects side effects as well as poor therapeutic effects (Tirona and Kim, 2002).

Shitara et al (2003, 2004) has demonstrated that ciclosporin A is an inhibitor of human OATP1B1, and OATP1B3, which causes relevant drug-drug interactions by inhibition of these OATPs.

Rifampicin, however, predominantly inhibits human OATP1B3. It is considerable that specific OATP-inhibitors might be essential to increase the concentration of drugs with a strong first-pass elimination induced by hepatic OATPs (Hagenbuch and Meier, 2004).

3. Drug-nutrition interactions:

Table 4 shows several nutrition-caused interactions for OATPs. For example, flavonoids influence the plasma-concentration of clinically relevant drugs which are transported by OATPs.

Table 4: *The effect of nutrition caused interactions.*

OATP	Substrate	Nutrition	Effect	IC 50	References
OATP 1A2	fexofenadine	fruit juice	↓		Dresser et al, 2002
OATP 1A2,	fexofenadine	Naringin (flavonoid)	↓	3.6 µM	Bailey et al, 2007
OATP 1A2	fexofenadine	hesperidin (flavonoid)	↓	2.7 µM	Bailey et al, 2007
OATP 1B1	DHEAS	biochanin A (flavonoid)	↓	11.3 µM	Wang X et al, 2005
OATP 1B1	DHEAS	genistein (flavonoid)	↓	14.9 µM	Wang X et al, 2005
OATP 1B1	oestrone-3- sulfate	Glycyrrhizin	↓	15.9 µM	Ismair et al, 2003
OATP 1B3	oestrone-3- sulfate	Glycyrrhizin	↓	12.3 µM	Ismair et al, 2003
OATP 2B1	oestrone-3- sulfate	extracts of green tea	↓	21.9 µM	Fuchikami et al. 2006
OATP 2B1	oestrone-3- sulfate	extracts of green tea	↓	11.2 µM	Fuchikami et al. 2006

3.3.8 OATPs and cancer

Certain oatps /OATPs show a more restricted expression pattern in normal tissues (OATP1B1, OATP1B3), while others are ubiquitarily expressed and might have housekeeping functions. (Hagenbuch and Meier, 2003).

The majority of OATPs are expressed in normal tissue as well as in cancerous tissue (Hagenbuch and Gui, 2008): In cancer, there is a special OATP expression pattern and certain tumors are characterized by the expression of a certain OATP:

- breast cancer: OATP1A2, OATP3A1, OATP4A1, OATP1B3, OATP2B1 (invasive ductal carcinoma in mammary tissue)
- blood-tumour barrier: OATP1A2, OATP2B1
- colon cancer: OATP4A1
- lung tumour, lung cancer: OATP6A1, OATP1B3
- bladder tumour, oesophageal tumour: OATP6A1
- liver, gastrointestinal cancers: OATP1B3

Investigations by Smith et al (2005) have detected that various anticancer drugs, including paclitaxel, docetaxel and others, are substrates, at least, for OATP1B3, which have the significant role in the uptake into tumor cells.

Two studies (Miki et al, 2006; Sarabaki et al, 2006) with quantitative real time RT-PCR has shown the expression of OATP1A2 and OATP2B1 in human breast carcinoma, in which OATP2B1 was also found in normal breast tissue, but OATP1A2 in tumor samples only.

Recently findings by Van de Steeg et al (2008) have showed in vivo data, which might attributes human OATP1B1 a rate-limited role in methotrexate elimination, by using transgenetic mice.

4. MATERIALS and METHODS

4.1 Cell culture

Cell lines were selected because earlier studies in our lab showed that these cell lines express high concentrations of OATP5A1 (Ewing sarcoma cell lines) and OATP1B1/1B3 (ovarian and colon carcinoma cell lines), respectively.

4.1.1 Thawing of cells

Cells were stored in fluid nitrogen (-196 °C). Before putting them into cell culture, they had to be thawed using a 37°C water bath, before a centrifugation step to remove the medium was carried out. After the centrifugation at 1000rpm for 3min. at room temperature and the removal of the supernatant, the cell pellet had to be resuspended in 4ml medium (RPMI-1640, containing 10% FBS and 1% P/S) and transferred into a culture flask. Thereafter, they were stored in the incubator (37°C).

4.1.2 Changing the medium

To provide an environment for an uncomplicated growth of cells, changing the medium 2-3x per week is recommended. Therefore the old medium was removed and fresh medium was added to the cells under sterile conditions in the laminar air flow. Afterwards cells were kept for growing in the incubator at 37°C.

4.1.3 Splitting of cells

First the medium had to be removed again by vacuum-pump. After that the dead cells were removed by short shaking with 4ml PBS (25 cm³). Then 1ml of trypsin was added for approximately 3min. Thereafter, cells were detached from the culture-flask. For the necessary inactivation of trypsin and the separation of the cells, cells in the culture-flask (25cm³) were washed with 4ml fresh medium. Finally a new culture-flask had to be filled with 4ml medium and 3 drops of the cell-suspension from the old culture-flask were put in. Culture was done in the incubator at 37°C.

4.1.4 Freezing of cells

It is necessary to passage/split the cells between 70 and 80% confluence. The freezing medium has to be prepared in advance. Then 1ml trypsin was given to solve them in medium and flushed with 12ml PBS. After the centrifugation at 1000

rpm for 3min. the supernatant was removed and the pellet resuspended in 6ml freezing medium (half amount of PBS). The cell-suspension was divided in 5 cryotubes; as quick as possible (DMSO causes differentiation in cell lines). At last the cryotubes were stored 1 day at -20°C before final storage at -180°C in fluid nitrogen.

4.1.5 Mycoplasma-Testing

Materials

Agarose Gel Electrophoresis

- Agarose UltraPure™ (Invitrogen, Carlsbad, CA USA)
- 10x Tris-acetate EDTA (TAE) buffer pH 7.2
 - TRIS (Merck, Darmstadt, GER) 96.8g
 - Sodium acetate (Merck) 54.5g
 - EDTA (Merck) 7.6g
 - ddH₂O ad 2 l
- Ethidium bromide (5 mg/ml)
- Gene Ruler™ 100bp DANN Ladder (MBI Fermentas, Vilnius Lithuania)
- 6x Loading dye solution (MBI Fermentas)
- Transluminator and camera Herolab E.A.S.Y. 429K (Herolab, Wiesloch Germany)
- Gel electrophoresis unit HE 133 (Hoefer, San Francisco USA)
- Power supply Power Pac 3000 (Biorad Laboratories, Hercules CA USA)
- Microwave oven MIK 4600 (Elin, Austria)

Method:

The cells were tested on mycoplasma contamination, because mycoplasmas have no cell walls, and therefore are resistant against our commonly used medium-antibiotics, which target cell wall synthesis. The small size of mycoplasmas (<1µm) inhibits the detection on the microscope and so the performance was done by using Myco Sensor® PCR Assay Kit. Of course the testing has to be done before the working with the cell-culture starts:

1. 100µl from each cell-line was taken into an Eppendorf
2. Heated at 95°C for 5 min
3. Added 10µl Strata Clean against impurities -> vortex and centrifugation

4. 30µl of the supernatant has to be transferred in a new Eppendorf and stored by -20°C
5. Master-Mix preparation:

Table 5: Master-Mix preparation (compounds and concentrations).

Master Mix for a single PCR reaction		MM halved amounts	
PCR water	fill to 50 µl	fill to 25 µl	
MgCl ₂ (2.0 nM)	depends on conc. µl	depends on conc. µl	
10x Taq buffer	5,0 µl	2,5 µl	
dNTP mix	1,0 µl	0,5 µl	
Mycoplasma primer mix	2,0 µl	1 µl	
internal control template	4,0 µl	2 µl	
Taq Polymerase DNA (5 U/µl)	0,5 µl	0,25 µl	
sample (or positive control)	5,0 µl	2,5 µl	
total volume of reaction	50,0 µl	25,0 µl	

MgCl₂ stock solution: $25\text{nM}/25\mu\text{l} = 1\text{nM}/1\mu\text{l} \Rightarrow 2\mu\text{l}$ MgCl₂ are to add for the required concentration.

According to the instruction manual:

- Taq DNA polymerase (5 U/µl)...0.5 µl = 2.5 U/µl
- half the amount of reaction0.25 µl = 1.25 U/µl
- available concentration 2 U/µl....1.25/2 = 0.63 µl

Table 6: Master-Mix preparation (compounds and concentrations).

Master Mix for a single PCR reaction		
PCR water	13,9	µl
MgCl ₂ (2.0 nM)	2,0	µl
10x Taq buffer	2,5	µl
dNTP mix	0,5	µl
Mycoplasma primer mix	1,0	µl
internal control template	2,0	µl
Taq Polymerase DNA (5 U/µl)	0,63	µl
sample (or positive control)	2,5	µl
total volume of reaction	25,0	µl

6. PCR:

PCR-parameters for PCR-cycler:

- Numbers of cycles: 40
- 94°C...30sec

- 55°C...1min
- 72°C...1min

7. Agarose-Gel

Acquired compounding:

- Agarose...0.450g
- Buffer...360ml
- Ethidium Bromid...1.5µl

The results of the mycoplasma testing were all negative; hence none of our cell lines were contaminated.

4.2 Protein purification

For further experiments, membrane-proteins and cytosolic-proteins were isolated from cells. The membrane fraction is of particular importance as OATPs are transmembrane proteins.

Materials

- Protease inhibitor cocktail P8340 (Sigma, St. Louis USA)
- PBS (Merck, Darmstadt Germany)
- 1.5ml reaction tubes (Biozym Biotech Trading GmbH, Vienna Austria)
- Plastic vials Polyvials® (Zinsser Analytic GmbH, Frankfurt Germany)
- Eppendorf centrifuge 5415D (Eppendorf AG, Hamburg Germany)
- Lysis buffer (see below)
- Scraper
- Ultracentrifuge
- Fluid nitrogen

Method:

In contrast to cell culture the whole procedure was done under normal laboratory conditions. Cells were flushed in culture-flask with PBS and scraped gently from the flask. After the centrifugation at 1000rpm for 3min., the supernatant was detached from the pellet, which was then solved 1ml lysis-buffer and 1µl Protease-inhibitor. Then 0.5ml of the suspension was vortexed and transferred in a new Eppendorf-tube. To crack the cell-membrane the tube was hold in fluid nitrogen and frozen for at least 3x to raise membrane-fraction in the following measurement. Centrifugation at

3000rcf for 10min. at 4°C was done, before the supernatant was exposed an ultracentrifugation at 28000rpm for 60min. Finally the supernatant was discarded (cytosolic proteins), the pellet (membrane proteins) resuspended with 50µl lysis-buffer and both fractions were stored at -20°C.

4.3. RNA purification

Careful RNA isolation is very important as the enzyme RNase, which catalyses RNA-fragmentation, is highly resistant to inactivation and ubiquitously present. RNase is characterized through high stability that even overcomes autoclaving. All cells produce RNase, which hydrolysis RNA-phosphodiester-bonds. Therefore, to isolate RNA, special materials with an exclusive application in RNA experiments (RNA-pipettes, -tubes and RNase-free water) are required.

Materials:

- TriFast™ (Peq-Lab, Erlangen Germany)
- PBS
- RNase/Dnase free pipette filter tips (Biozym Biotech Trading GmbH, Vienna, Austria)
- RNase/Dnase free reaction tubes 1.5ml (Biozym Biotech Trading GmbH, Vienna, Austria)
- RNase-free water (Invitrogen GmbH, Lofer Austria)
- Chloroform
- Isopropanol

Single-step method by (Chomczynski und Sacchi 2006):

RNA was isolated with peqGOLD TriFast ®. It was prepared one-phase solution of phenol and guanidinisoithiocyanat.

Homogenisation:

The medium from cell-culture was detached and cells were washed carefully with ice-cold PBS. Then cells were lyzed directly in the cell culture flask (25cm³) with 2ml TriFast® under constant shaking on ice for 10min.

Phase separation:

One ml of each homogenate was transferred in one 1.5ml reaction tube. After incubation at room temperature for 5min. for the dissociation of nucleotide complexes, 0.2 ml chloroform was added in each Eppendorf-tube. These tubes were centrifuged at 12000g for 5min. at 4°C and 3 phases received:

- 1) Bottom (organic) phase = red chloroform-phenol phase, containing DNA and proteins.
- 2) Middle phase = inter phase, containing DNA and proteins.
- 3) Top (aqueous) phase = transparent-diluted phase, containing RNA.

RNA-precipitation

The top phase was transferred in a new tube and 0.5ml isopropanol to each ml TriFast (-> 0.5 *2ml -> 1ml isopropanol) was added. After incubation for 10min. on ice the tube was centrifuged at 12000g for 10min. at 4°C and then a RNA-precipitate-gel, at the bottom of the tube, was sustained. Carefully the supernatant was removed from the RNA-pellet. For purification this pellet was given 1ml 75% ethanol and subsequently vortexed and centrifuged at 12000g for 10min. This performance had to be repeated several times. Then the RNA-pellet was dissolved with RNase free water and dried carefully at 55°C.

Agarose gel-electrophoresis

In order to verify the correct isolation it is important to scan the 28S and 18S-rRNA bands on the gel. A positive result means no digestion by RNase.

4.4 Real time PCR

4.4.1 General Information

The instrument to perform real-time PCR and all chemistries used are products of Applied Biosystems Inc., Foster City, CA USA)

Real-time PCR allows the monitoring in the progress of PCR as it occurs. Data are collected throughout the process revolutionizing the PCR-based quantification of DNA and RNA. Higher starting concentrations of copy numbers of the cDNA enable an earlier significant increase in fluorescence level indication DNA amplification.

4.4.2 Phases of PCR

PCR can be divided into 3 phases:

First the exponential phase including the exact doubling of product and accumulation at each cycle (assuming a 100% efficiency of reaction). The exponential phase is very specific and precise.

Second is the linear phase, which displays high variability. From one cycle to the next components are consumed, thus the reaction is slowing down and the products begin to degrade.

The last phase is the plateau. The reaction has come to its end and products will now begin to degrade when left long enough. No further product is being made at this final stage.

4.4.3 Performance of real-time RT-PCR

We purchased Gene Expression Assays (Applied Biosystems) for all eleven human OATPs. TaqMan[®] real time RT-PCR was performed in a reaction volume of 10µl. The target gene amplification mixture contained 5µl 2x TaqMan[®] Gene Expression PCR Master Mix, 0.5µl of the appropriate Gene Expression Assay, 10ng template cDNA diluted in 2.5µl nuclease free water, and 2 µl nuclease free water. Thermal cycling conditions were as follows: 2min. at 50°C, 10min. at 95°C, 40 cycles at 95°C and 1min. at 60°C. Fluorescence generation from Taq Man[®] 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.

4.4.4 Data Analysis

For the calculation of the results the *delta C_T method* (Livak and Schmittgen, 2001) was applied:

The amount of the target is normalized to an endogenous reference and set relative to a calibrator, and thus described as:

$$2^{-\Delta\Delta C_T}$$

4.5 Indirect Immunofluorescence

To investigate localisation of OATP1B1/1B3 in the colon carcinoma cell line, their cellular localization was studied in cell line HT-29, and A2780ADR cells (negative control) by indirect-immunofluorescence experiments. Staining of α -tubulin was included as positive control.

Procedure for both cell lines: HT-29 cell growing on 8-well chamber slides (NUNC®, Thermo Electron LED GmbH, Langenselbold Germany) were fixed with acetone and blocked with 5% BSA in PBS. Incubation with the primary antibody against OATP1B3/1B1 (BM5541, Acris Antibodies GmbH, 32052 Herford Germany) and α -tubulin (T-5168,) was done for 2h at room temperature. Dilutions for primary antibodies were 1:100 and 1:2000, respectively. After washing, sections were incubated with Alexa Fluor® IgG (Invitrogen, Carlsbad, CA) using the following dilutions: Alexa Fluor® 488 anti-mouse IgG (1:2000) and (1:200), and Alexa Fluor® 568 anti-mouse IgG (1:1000). Cell nuclei were stained with bisbenzimidazole in PBS (Hoechst 33342, Sigma, Munich, Germany) at a 1:5000 dilution. The sections were mounted in Mowiol 4-88 (Carl Roth, Karlsruhe, Germany) and, afterwards, fluorescent staining was visualized with an Axioplan 2 microscope (Carl Zeiss, Jena, Germany). Subsequently images were captured by using AxioCam HRc2 Color CCD digital camera and Axiovision 4.6 software (Carl Zeiss Vision GmbH, Aalen, Germany).

4.6 Cytotoxicity Test with paclitaxel on SK-OV-3 and A2780 cell lines

4.6.1 Aims of these experiments

To compare whether paclitaxel treated cells transiently transfected with OATP1B1/1B3 cDNA inserted into the transient expression vector pCMV6-XL4 (OriGene, 9620 Medical Center Dr. Rockville USA) differ in cell viability. In control experiments, cells were transfected with an empty vector only. Fugene® HD (Roche Diagnostics, Basel Suisse) was used as a transfection reagent for OATP1B1/1B3 genes (SLCOs) as well as for the empty vector. For cytotoxicity measurements, a CellTiter-Glo® Luminescent Cell Viability Assay (Promega Corp., Wisconsin USA) was performed.

4.6.2 Cell Viability Assays

CellTiter-Glo[®] Luminescent Cell Viability Assay (Promega) was used to determine the percentage of viable cells in culture. Detection is based on using the luciferase reaction to measure the amount of ATP in viable cells. After loss of the membrane integrity, cells are no longer able to synthesize ATP, thus the levels of ATP decrease rapidly, which correlates with cell viability. In the CellTiter-Glo[®] Reagent Kit, components to lyse cell membranes for the release of ATP, for inhibiting endogenous ATPases, luciferin and luciferase for ATP detection, are provided. The signal can be recorded with a luminometer, CCD camera or modified fluorometer and generally has a half-life of five hours, providing a consistent signal across large batches of plates. The luminescent signal can be detected as soon as 10 minutes after adding reagent, and persist for several hours later. This is important to provide flexibility for batch processing of plates. The CellTiter-Glo[®] Assay is extremely sensitive and can detect as few as 10 cells.

4.6.3 Method:

Transfection with Fugene on 6-well plate:

On the day of the transfection the cell lines should be 50-80 % confluent.

First Fugene was diluted in the transfection medium (Optimem) and incubated for 5min. at room temperature. Dropwise the diluted Fugene was added to a tube, containing the DNA-plasmide, and gently tapped and incubated for 15min. at room temperature. This mixture was given to the cells, which were portioned in the 6-well plate, and then placed into the incubator for 24 hours.

Table 7: *Demonstration of all compounds in the 6-well plate.*

Approximately 3 mio cells of A2780, OATP1B1 , Fugene, Optimem, ampicillin-free medium	Approximately 3 mio. cells of A2780, OATP1B3 , Fugene, Optimem, ampicillin-free medium	Approximately 3 mio cells of A2780, Empty vector , Fugene, Optimem, ampicillin-free medium
Approximately 3 mio cells of SK-OV-3, OATP1B1 , Fugene, Optimem, ampicillin-free medium	Approximately 3 mio. cells of SK-OV-3, OATP1B3 , Fugene, Optimem, ampicillin-free medium	Approximately 3 mio cells of SK-OV-3, Empty vector , Fugene, Optimem, ampicillin-free medium

Following steps for each well after 24 hours:

After medium was removed the cells were washed with PBS and trypsinized for 7min. To receive single cells it was needful to pipette up and down several times.

Then a centrifugation for 3min. at 1000rpm was done, the supernatant was detached and resuspended with 1ml medium.

Distribution on 96-well plate after 48 hours:

Appropriated dilutions of the cells were made in order to plate 2500 cells per well in 100µl medium on 96-well plates. These dilutions were portioned in 3 tubes each cell line [(e.g. an 115-fold dilution (A2780, OATP1B1) by a final volume of 15ml each plate means 0.13ml = 130µl cell suspension and 15ml medium for this tube (A2780, OATP1B1)]. So there were 3 tubes: OATP1B1/1B3, EV (empty vector) each cell line and each tube was distributed on one 96-well plate, this results in 6 (96-well) plates.

Dilution series of paclitaxel

50mM stock solution of paclitaxel in DMSO was available. Hence we created a dilution series: 0.01nM, 0.10nM, 0.50nM, 1.00nM, 2.00nM, 3.00nM, 4.00nM, 6.00nM, 10.00nM and 50.00nM.

Eight wells were treated with the appropriate concentration of paclitaxel or solvent alone (0.1% DMSO) as control.

Cell viability assay

Materials:

- CellTiter-Glo® Luminescent Cell Viability Assay (Cat.# G7570, G7571, G7572, G7573) and protocol #TB288 (www.promega.com/tbs/tb288/tb288.html)
- opaque-walled multiwell plates adequate for cell culture
- 8-channel electronic multipipette (Biohit Oyj, Laippatie 1 Helsinki Finland)
- plate shaker, for mixing multiwell plates
- Victor³™ Multilabel Counter (Perkin-Elmer, Inc., MA USA)

Method for each plate:

First the plate and its contents had to be equilibrated to room temperature for approximately 30 minutes. Then a volume of CellTiter-Glo® Reagent, equal to the volume of cell culture medium present in each well, was added (100µl reagent to 100µl of medium). Next, the contents were mixed for 2 minutes in orbital shaker to induce cell lysis, before the plate was incubated at room temperature for 10 minutes to stabilize the luminescent signal. And finally the luminescence was recorded.

5. RESULTS and DISCUSSION

Organic anion transporting polypeptides (OATPs) mediate the transmembrane uptake of endogenous and exogenous substrates in various tissues and OATP action subsequently effects the penetration of drugs in tissue and their availability. To investigate the difference in the expression pattern of all 11 known OATPs between cancerous cell line and normal tissue, we have used TaqMan[®] real time RT-PCR.

In addition to these studies, the sensitivity of cancerous cell lines against cytotoxic agents was tested. We transfected the cell lines A2780 and SK-OV3 with the genes OATP1B1 and OATP1B3. These cell lines were exposed to different dilutions of paclitaxel and expected an increased sensitivity (decreased IC₅₀) in OATP1B1 and OATP1B3 transfected cells.

5.1 Characterization of the cancer cell lines

A short description of cell lines used in this study is given below:

HT-29 is an adherent human colon adenocarcinomas cell line (DSMZ no.: ACC 299) originally established from the primary tumour of a 44-year-old Caucasian woman with colon adenocarcinomas. TC-71 is a loosely adherent human Ewing's sarcoma cell line (DSMZ no.: ACC 516) established from the tumour of a 22 -year-old man with metastatic Ewing' sarcoma. SK-N-MC is an adherent human neuroblastoma cell line (DSMZ no.: ACC 203) established from the supraorbital metastasis of a neuroblastoma of a 14-year-old girl (see ATCC HTB 10). Although described in the original paper as neuroblastoma, SK-N-MC is now widely regarded as having originated from the morphologically similar Askin's tumor related to Ewing's sarcoma.

SK-OV-3 is an adherent human ovarian adenocarcinoma cell line (ATCC no.: HTB 77) derived from the ascitic fluid from a 64 year old caucasian female with an ovarian tumor. A2780 cells are an adherent human ovarian cancer cell line established from tumor tissue from an untreated patient.

5.2 Assessment of selected OATP genes (SLCOs) by quantitative real-time RT-PCR

The following figures, four groups of samples were marked with different colours to emphasize their meaning in context of the results:

- Ewing's sarcoma cell line (blue),
- colon- and colorectal cancer cell lines (pink),
- healthy tissue (green) as positive control and
- healthy brain tissue (red) used as calibrator.

The obtained data from real time RT-PCR were calculated with “delta Ct-method” and data from the “calibrator” brain were set to the value of 1. Thus the expression rates have to be understood as a relative multiple of healthy brain tissue.

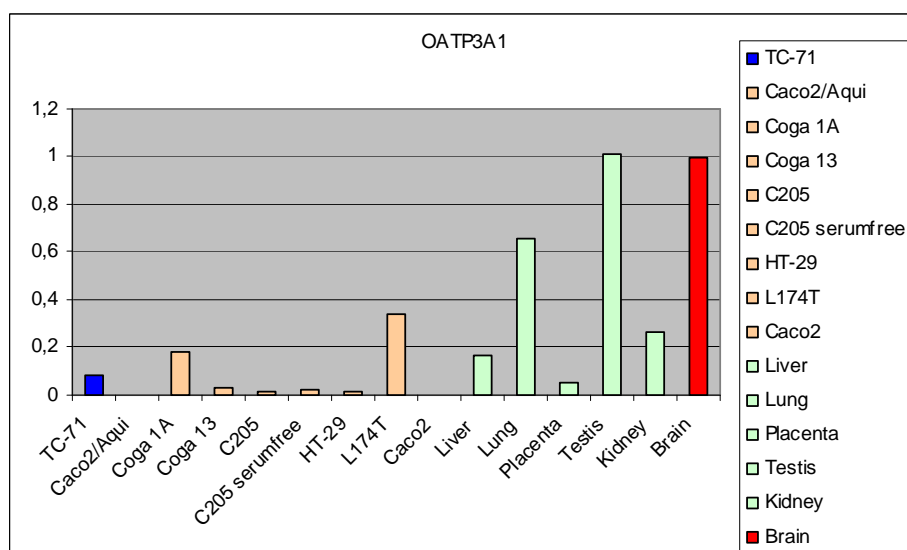


Figure 7: Expression rates of OATP3A1. Studies were done using samples from tissues and various cell lines with TaqMan PCR.

As expected from preliminary studies done in this laboratory, there is a high expression of OATP3A1 in testis (1.01 fold), brain and low expression in kidney (0.26 fold), liver (0.16 fold) and placenta (0.05 fold). In the colon carcinoma cell line L174T, a 1.3 fold higher expression rate than in kidney and 2.1 fold higher expressions than in liver is observed. Also in Coga1A cells, OATP3A1 is increased expressed and consequently 2.25 fold higher than in the Ewing's sarcoma cell line, TC-71.

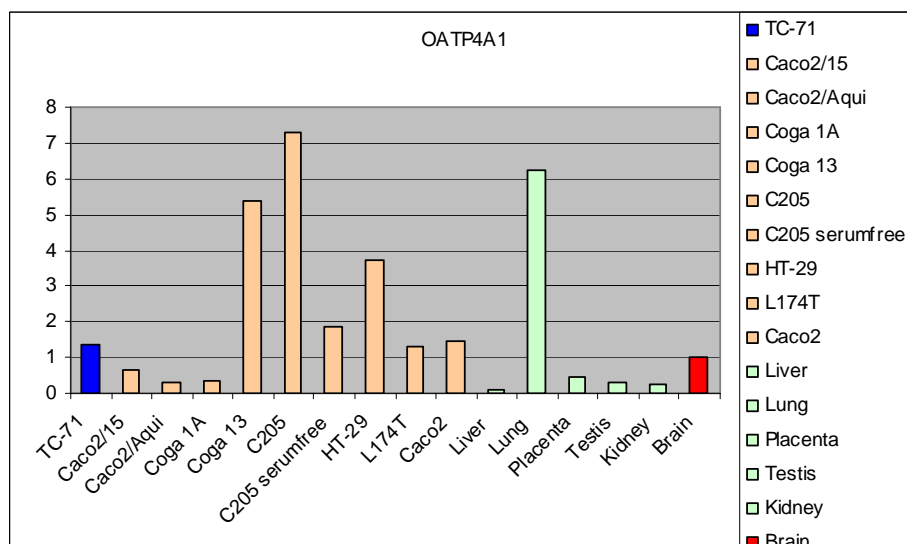


Figure 8: Expression rates of OATP4A1. Studies were done using samples from tissues and various cell lines with TaqMan PCR.

As demonstrated in Figure 8 the most impressive results for OATP4A1 were observed in the colon carcinoma cell lines, as a high expression rate of this OATP was detected in most colon- and colorectal carcinoma cell lines, including Coga13 (5.4 fold), C205 (7.3 fold), HT-19 (3.7 fold), L174T (1.3 fold) and Caco2 (1.4 fold) cells. The OATP4A1 expression rates exceed the expression rates of OATP4A1 in brain. Similar to colon- and colorectal cell lines, TC-71 displays a moderate enhancement (1.36) of this OATP compared to brain. In relation to healthy tissue, there is a high expression of OATP4A1 genes in lung (6.22 fold), but a low expression in placenta, kidney and liver.

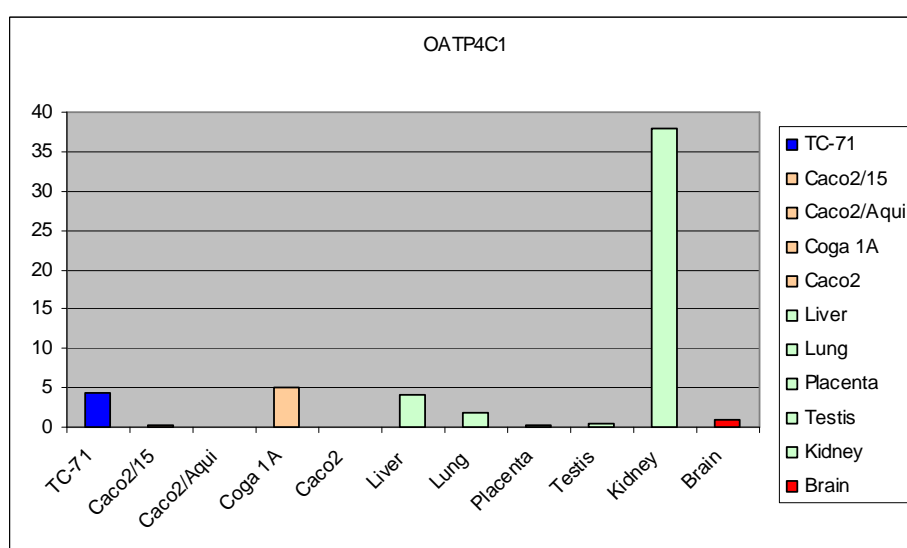


Figure 9: Expression rates of OATP4C1. Studies were done using samples from tissues and various cell lines with TaqMan PCR.

Figure 9 demonstrates high expression rates for OATP4C1 in kidney (38.1 fold), TC-71 cells (4.2 fold), liver (4.1 fold) and Coga1A cells (5.1 fold) show almost similar considerable expression rates of OATP4C1.

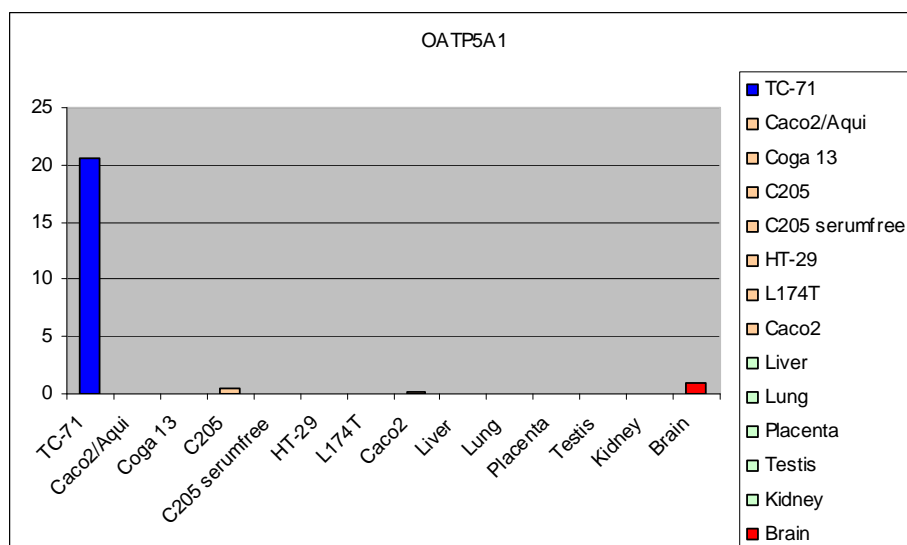


Figure 10: Expression rates of OATP5A1. Studies were done using samples from tissues and various cell lines with TaqMan PCR.

Figure 10 represents data on the expression rates of OATP5A1, which is the most unknown and uncharacterized of the 11 known OATP-transporters. Remarkably, there is almost no expression in healthy tissue except brain, where expression is also low. Low expression was seen in colon- and colorectal carcinoma cell lines, but an amazingly 20.5 fold expression of OATP5A1 in TC-71 cells compared to brain was found.

Because of these interesting results, I further investigated the expression of OATP5A1 in Ewing's sarcoma cell lines. Therefore, I started new real time RT-PCR experiments with the cell lines TC-252 and SK-N-MC. However, it has to be mentioned that the cell line SK-N-MC does not originate from Ewing's sarcoma, but from a neuroectodermal Askin's tumour, which is morphological similar and has the same pathological changes like Ewing's sarcoma (Iwamoto, 2007).

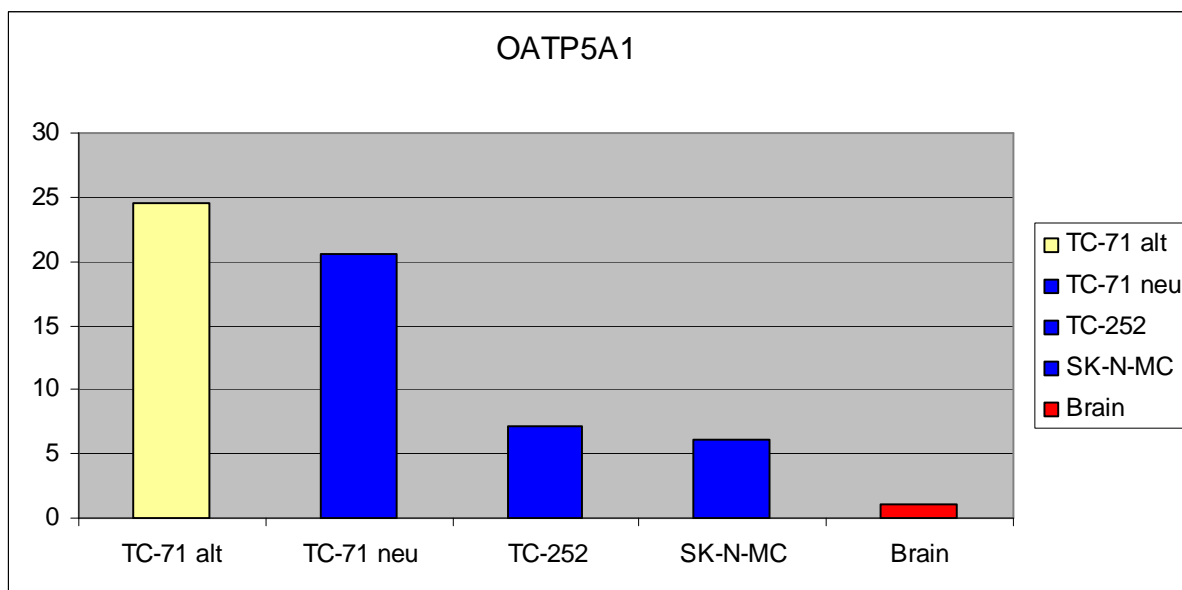


Figure 11: Expression rates of OATP5A1 (Ewing's sarcoma). Studies were done using samples from brain tissue and Ewing's sarcoma related cell lines with TaqMan PCR. TC-71a was done earlier, while TC-71 is part of this experiment.

Figure 11 shows the expression rate of OATP5A1 in TC-71b (20.5 fold) in comparison with the former measurement of TC-71a performed in the laboratory (24.5 fold). Also TC-252 (7.1 fold) and SK-N-MC (6.1 fold) have remarkable mRNA levels as well. This suggests a relation between the expression OATP5A1 and the Ewing's sarcoma.

5.3 Cytotoxicity-test in SK-OV-3 and A2780 cell lines after treatment with paclitaxel

Data were obtained from the measured luminescence by using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega). High luminescence means a high percentage of viable lysed cells that are able to perform an ATP-dependent metabolic reaction with the added enzyme luciferase. Needless to say that in contrast low luminescence equates a low percentage of viable cells. In the following graphs the axis of ordinates shows the measured percentage of viable cells treated with various dilutions of paclitaxel controlled with untreated cells with 0.1% DMSO alone. The axis of abscissae displays the log concentration of paclitaxel. Therefore the IC50 was determined as the concentration of paclitaxel (x-axis) at 50% viable cells (y-

axis). It was expected to achieve lower values of the IC₅₀ in the cells transfected with OATP1B1 and OATP1B3 than in cells transfected with the empty vector (EV).

5.3.1 Result of the SK-OV-3 cell line

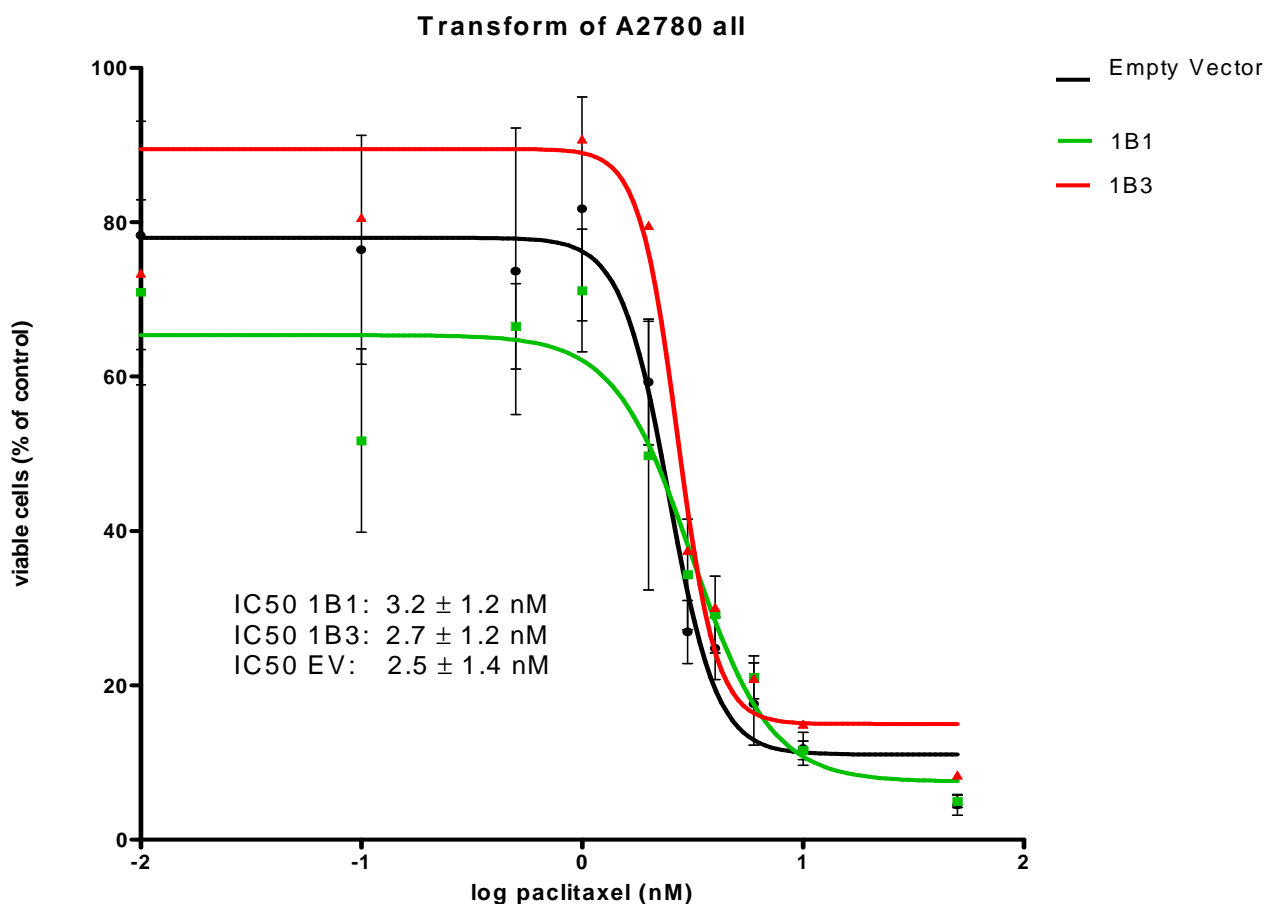


Figure 12: Cytotoxicity assay of SK-OV-3 cell line including the calculation of the IC₅₀.

Figure 12 demonstrates the differences of viability in the cell line SK-OV-3 including cells transfected with OATP1B1, OATP1B3 or with the EV. In the OATP1B1 and OATP1B3 transfected cells there is clearly a left shift to lower concentrations of paclitaxel observable in comparison with cells transfected with EV. The IC₅₀ of 3.5 (log 0.54) in the OATP1B1- and 3.9 (log 0.59) in the OATP1B3 transfected cells are almost equal and the difference to the IC₅₀ of 5.9 (log 0.77) in the EV cells is remarkable.

5.3.2 Results of the A2780 cell line

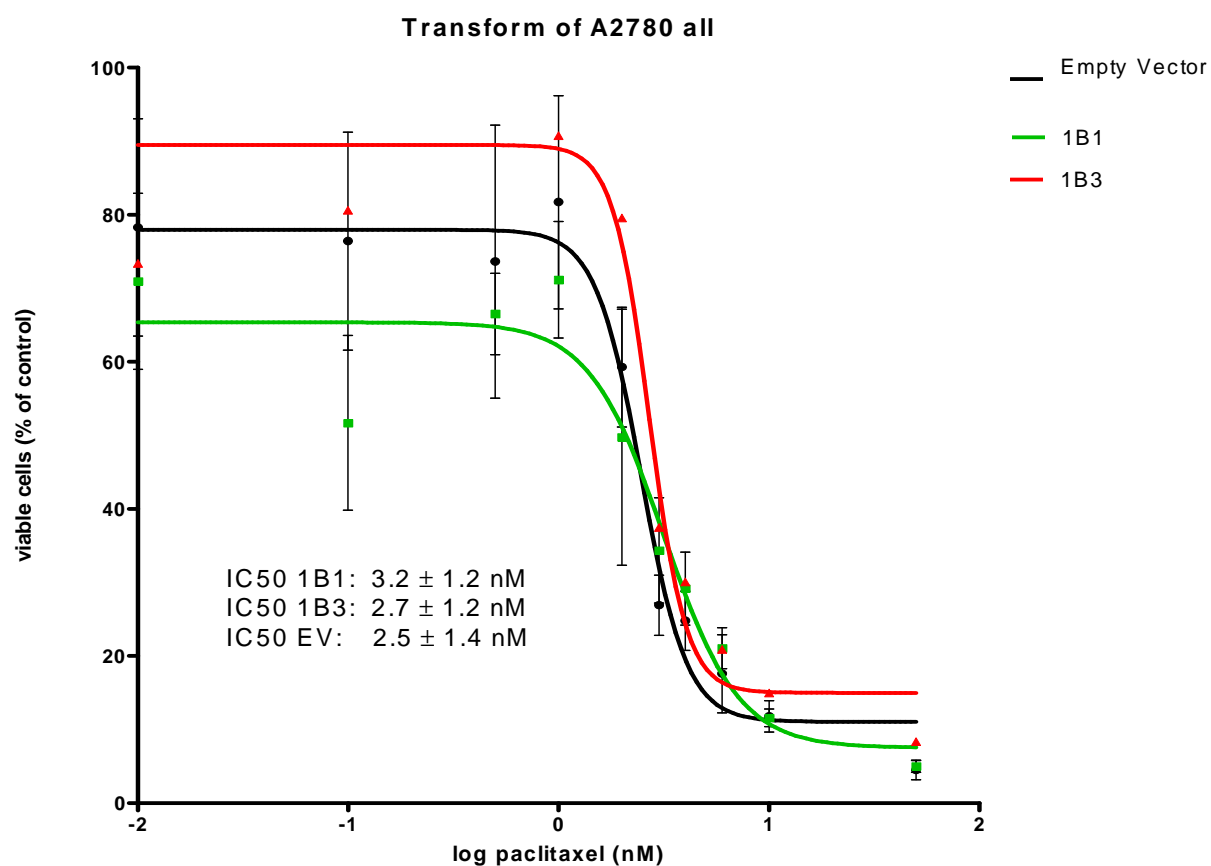


Figure 13: Cytotoxicity assay of A2780 cell line including the calculation of the IC₅₀.

Figure 13 exhibits the cells from the A2780 cell line, which did not correlate with the results obtained from SK-OV-3 cell lines. On the level of the IC₅₀ this graph does not show the expected left shift to lower concentrations of paclitaxel in OATP1B1 and OATP1B3 transfected cells like in the previous test, which also can be recognized by similar levels of the IC₅₀ in OATP1B1 (3.2), OATP1B3 (2.7) and EV (2.5).

6. SUMMARY

The most interesting cell line tested was the TC-71 cell line regarding the expression of OATPs. Moderate expression rates of OATP3A1, OATP4A1, OATP4C1 were found, while OATP5A1 was high. In contrast to TC-71 cells, in colon- and colorectal cancer cell lines OATP3A1, OATP4A1 and OATP4C1 are expressed at higher levels, in particular OATP4A1. High expression of OATP4A1 was previously seen in colon cancer in a gene array (Alsesso) and the high expression of OATP4C1 in kidney is in accordance with the proposed function of this transporter in the kidney where it might play a physiological role in drug excretion (Hagenbuch and Gui, 2008). Nevertheless, the highest value was obtained by the expression of OATP5A1 in TC-71. Considering the marginal knowledge on OATP5A1 expression and function, the enhanced expression rates in cell lines derived from Ewing's sarcoma and tissue from the Ewing's sarcoma familiar (SK-N-MC) could be interesting for further studies. It may bring out pathways to establish a better diagnosis for early stage tumors and this might improve the patients' prognosis. Transfection of the SK-OV-3 cell line with OATP1B1 and OATP1B3 leading to overexpression of functional proteins increased the uptake of paclitaxel and this is accompanied by an increased sensitivity against this drug. In contrast, sensitivity in A2780 cell line was not changed after transfection with these OATPs. This might either be caused by a complete lack of functional OATP1B1 and OATP1B3 in this cell line or as suggested (Liang et al, 2003; Knostman et al 2007) OATPs might not be located in the plasma membrane but may remain in the cytoplasm. In the latter, missorted OATPs could not contribute to paclitaxel uptake. Therefore, for further studies necessary to identify properties of OATP1B1 and OATP1B3-mediated uptake of anticancer drugs, SK-OV-3 cells, overexpressing OATP1B1 and OATP1B3, might be an appropriate model.

Taken together, investigations on OATP expression and their functions done in cancer tissue and cancer cell lines are important to gain further insight in resistance to anticancer chemotherapy.

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