Final report

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The role of uptake transporters (promoting the uptake of compounds into the cells) in determining the *in vivo* fate of drugs is increasingly recognized. The family of Organic Anion Transporting Polypeptides (OATPs) plays a pivotal role in determining the pharmacokinetics of medically relevant compounds. OATPs mediate the uptake of steroid hormones, bile acids and also various clinically applied drugs. Hence OATPs are renowned participants in ADME-Tox (Absorption, Distribution, Metabolism, Excretion and Toxicity) processes. Currently there is a need for sensitive assays for the prediction of drug-OATP interaction. Another challenge is that the substrate recognition pattern or even the functionality of many members of the OATP family is still unknown.

The main objective of the project was to unravel the functional role of human OATPs through the detailed biochemical characterization of dedicated models.

The specific aims of this project were the following:

- **Specific aim 1: Setting up various expression systems**
- **Specific aim 2: Defining the driving force of OATP-dependent transport**
- **Specific aim 3: Setting up *in vitro* functional assay(s)**
- **Specific aim 4: Model generation for pharmacokinetic studies**
- **Specific aim 5: Structure-function studies**

MAJOR ACHIEVEMENTS

1. Expression of human OATPs in various cellular models

The first step was to establish appropriate expression systems/model cells that allow the investigation of the entire human OATP family.

1.1. **We demonstrated that all 11 human OATPs can be expressed at high levels in the heterologous Sf9 insect cell system. We identified sodium fluorescein as a general OATP substrate, which allowed the functional characterization of the entire family. We showed that acidic extracellular pH greatly facilitates fluorescein uptake by all OATPs** (Specific aim 2), **and we found that fluorescein-methotrexate is transported by OATP1B1, 1B3, 1A2 and 2B1,** but not by the other OATPs. These studies demonstrate, for the first time, that the insect cell system is suitable for the functional expression of the entire human OATP family, and may be used to screen for drug-OATP interactions. Based on the developed assay, we managed to identify new molecular interactions between OATP2B1 and the bcr-abl inhibitor, Imatinib. In addition, we demonstrated interactions between OATP1C1, 3A1, 4C1, 5A1 and 6A1 and various hormones (Patik et al., 2015).

1.2. Compared to the transient expression in insect cells, stable cell lines provide additional means for transport measurements, localization studies, drug tests, and also co-expression with ABC multidrug transporters. **To generate stable mammalian cell lines overexpressing the given OATP, we chose the transposon based gene delivery method. In order to set up this method we first generated cell lines overexpressing human ABCG2 (Gal et al., 2015), a well-characterized drug transporter.** This cell line also served to generate a cellular model for pharmacological studies with OATP ABCG2 co-expression (see Section 3.).

2. Setting up *in vitro* functional assay(s)

Although fluorescein and fluorescein-methotrexate are applicable to measure OATP function (see above), due to their relatively high membrane permeability and pH sensitivity, they are not

necessarily ideal candidates for large scale drug screens. Therefore we performed a scan on a large set of commercially available, cell impermeable fluorescent dyes in order to develop a fluorescent assay with improved characteristics, lower passive cell permeability and increased OATP affinity. **By this approach, we identified a set of novel fluorescent molecules that are transported substrates of hepatic OATPs involved in drug ADME, OATP1B1, 1B3 and 2B1. We engineered the well-adherent human epidermoid carcinoma cell line, A431, for stable OATP overexpression.** Transport of the fluorescent substrates was tested and confirmed in cells overexpressing OATPs 1B1, 1B3 or 2B1. We **adapted the assay to a 96-well plate (semi high-throughput) format**. OATPs 1B1, 1B3 and 2B1 influence the pharmacokinetics of several drugs. Accordingly, the regulations by international agencies (U.S. Food and Drug Administration and the European Medicines Agency) require the test of the interaction between new drugs and these OATPs during drug development. Based on our results, the novel assay is **an excellent tool for OATP-drug interaction screens** (Figure 1). Furthermore, we found that one of the new fluorescent substrates is applicable for selective enrichment of OATP1B or 2B1 overexpressing cells, allowing **cell sorting based on OATP1B or 2B1 function**. A manuscript describing these results was submitted for publication in Molecular Pharmacology on Aug 30, 2017 (Patik, paper submitted). With the help of these cell lines we characterized the OATP-mediated uptake of the PET probe ^{11}C-erlotinib (see the details in the Additional achievements section, and in (Bauer et al., 2017)).

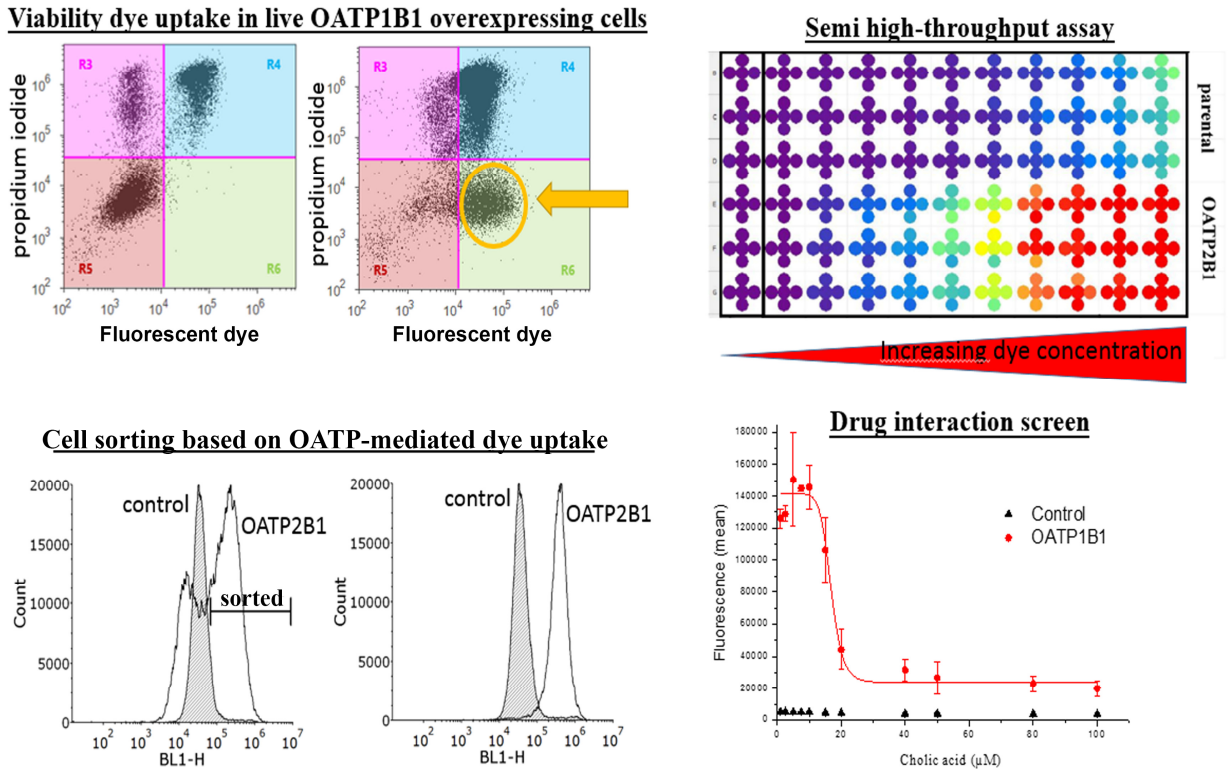

Figure 1. Graphical abstract from the submitted paper summarizing the relevance of the novel fluorescence-based assay

3. Generation of model cell lines and novel fluorescent assays for pharmacological studies

The major hepatic transporters important in ADME are ABCG2 and ABCC2 (MRP2) from the ABC (ATP Binding Cassette) transporter family, and OATP1B1 and 1B3 from the OATP family. These proteins have largely overlapping substrate specificities, and their concerted action (and also that of the cytoplasmic enzymes) is necessary for successful hepatic detoxification. Model cell lines with co-expression of these transporters are therefore generally accepted *in vitro* models for predicting drug interactions with these proteins. **In harmony with Specific aim 4, we have generated MDCKII cells overexpressing ABCG2 and OATP1B1, or MRP2 and OATP1B1. Moreover, we found that**

the novel fluorescent substrates for OATP1Bs and 2B1 identified in this project, are also transported by ABCG2 and MRP2. Using these cell lines and the fluorescent dyes, we managed to establish a novel fluorescence-based method to measure transcellular transport that provides a new assay for drug ABC and drug OATP interaction tests (Figure 2, *manuscript in preparation*).

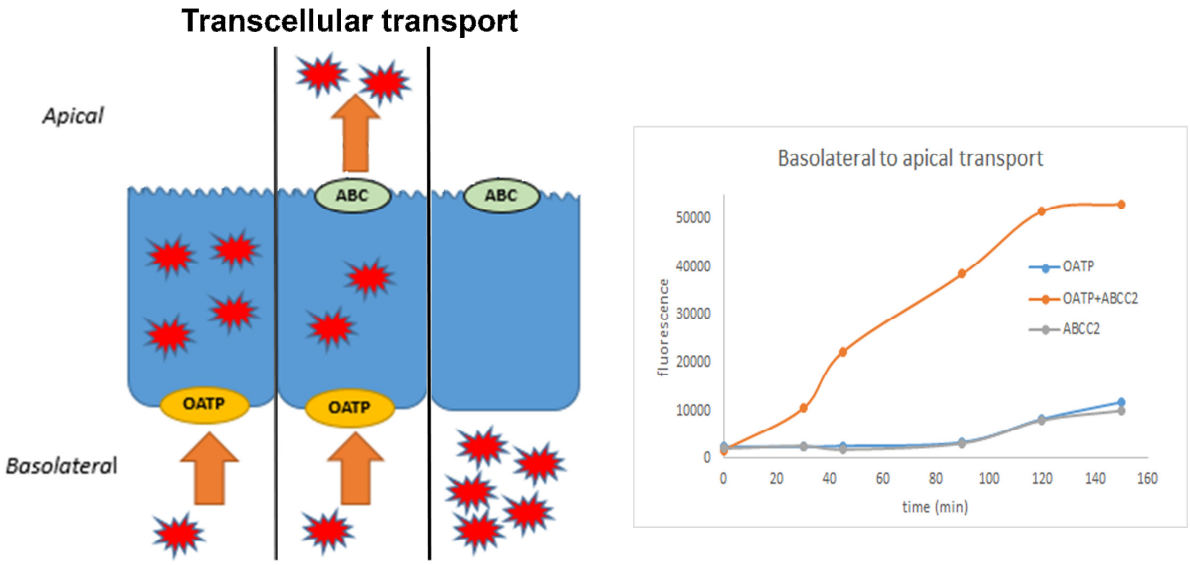

Figure 2. The novel fluorescent OATP substrates can be used to monitor OATP1B1 and ABCC2 function simultaneously. The cell impermeable dye shows transcellular transport only when both OATP1B1 (localized basolaterally) and ABCC2 (found in the apical membrane) are present and active.

4. Structure-function studies

Little is known about the structural determinants of OATP function. Our first goal was to evaluate the relevance of single amino acids and larger amino acid segments through the analysis of naturally occurring OATP SNPs (single nucleotide polymorphisms) and transcript variants.

4.1. SNPs: We designed oligos to detect SNPs in the genes (termed as SLCO) of OATP1C1, 2B1, 4A1 and 6A1 by RFLP. In a collaboration with the Medical University of Vienna (Theresia Thalhammer and Walter Jaeger) we investigated the relation of the two most frequent SNPs (R70Q and V78I) to the expression levels of SLCO4A1 in colon cancer patients. We observed that colon cancer cells isolated from patients harboring the R70Q polymorphism show decreased OATP4A1 expression, however the relevance of this finding needs further investigations. These data were presented as a poster (2nd Conference of the Hungarian Molecular Life Sciences, see the publication list).

4.2. Isoforms: From the 11 human OATP genes (SLCOs), due to alternative transription or alternative splicing various isoforms can be formed. However, the existence and function of these additional OATP isoforms has not been systematically investigated. With an exhaustive search of the databases (Aceview, NCBI, Ensemble, Uniprot) **we collected all the reported transcript variants of human OATPs**. Most of these are only predicted mRNAs, their existence has not been proven experimentally. Based on the length and topology of the isoforms and considering exon assembly **we identified 5 isoforms that are potentially coding functional proteins** (see Figure 3). Of these 5 isoforms **we were able to detect and clone 3 novel OATP transcripts from healthy tissue RNA libraries.** After successful cloning and expression in model cell lines, we **confirmed the functionality of these novel protein variants.** Detailed functional characterization to reveal differences in transport or substrate specificity is underway. Also, immunohistochemistry studies have been initiated to study the relevant site and time of expression in healthy and diseased tissues. To this end, we launched the generation of isoform-specific

antibodies (21st Century Biochemicals, USA). Unfortunately, despite repated immunizations and several rounds of antibody purification, the company has failed to deliver an isoform-specific antibody. Therefore we have initiated a collaboration with Dr. Bruno Stieger (University of Zürich), who has previously produced antibodies against various members of the OATP family. With more data on the expression of the potein isoforms, we expect to complete the work on the novel variants and submit our manuscript to the Journal of Physiology (current IF: 5.03).

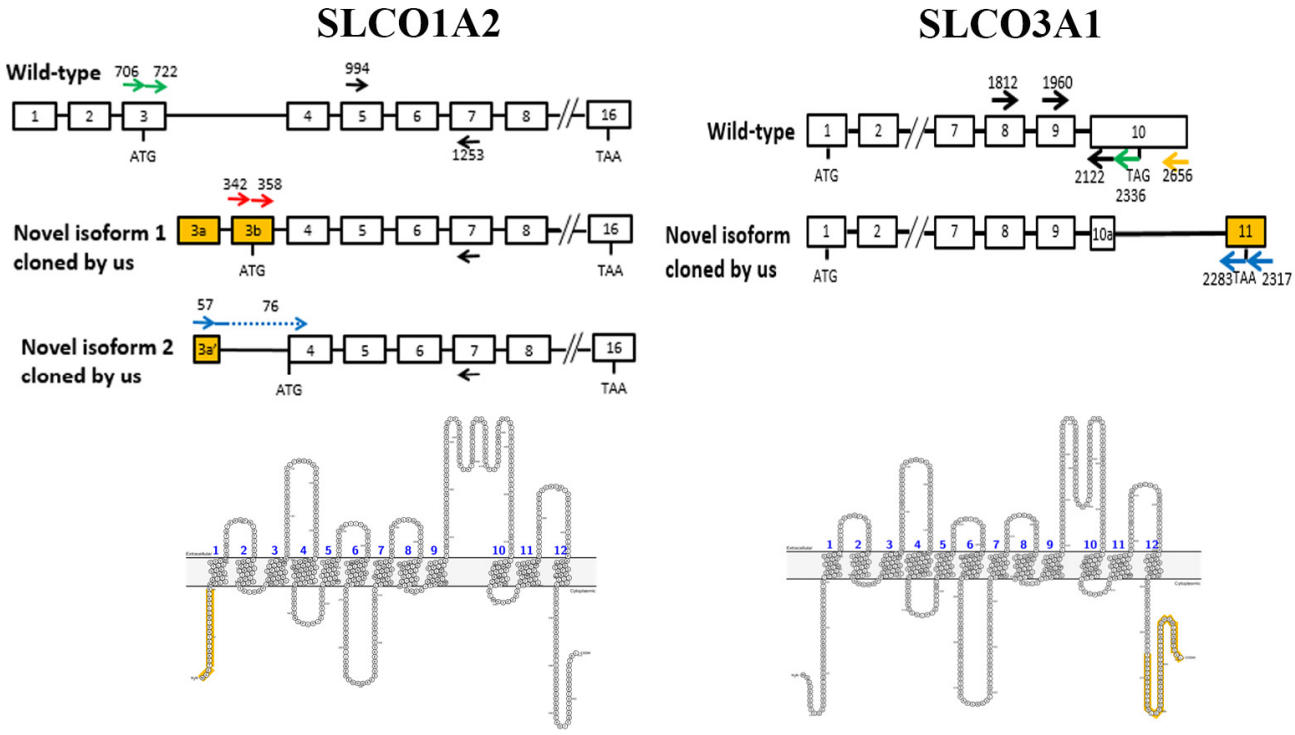

Figure 3: Exon assembly and predicted membrane topology of the novel isoforms. Arrows indicate the position of oligos designed to detect the isoforms by PCR. Yellow rectangles and protein fragments indicate the exons and protein sequences, respectively different between the variants.

General problems and difficulties:

- Although various mechanisms of OATP-mediated transport have been suggested, often with controversial results, many studies demonstrated that an acidic extracellular pH triggers OATP-mediated transport. In the frame of this project, in harmony with Specific aim 2, we have investigated the role of pH in various cellular assays. Based on these results, we found that although an acidic pH usually triggers OATP-mediated transport, this activating effect depends on the OATP investigated and also can vary for the same transporter when using different substrates (Patik et al., 2015). Therefore a proton gradient driven OATP transport is not a general rule, other mechanisms may exist. Our further efforts to investigate this issue more thoroughly by using isolated membrane vesicles failed. In spite of numerous vesicle preparation methods, we could not produce membrane vesicles with convincing OATP transport activity.
- The publication of the cloning and functional characterization of the novel OATP isoforms is hindered by the lack of isoform specific antibodies. Although the immunization was launched more than a year ago it was not successful. Now, a different approach is under way (as described above).
- In the frame of this project, novel fluorescent OATP substrates were identified. This finding could have been patented. However, due to financial constrains this plan was abandoned. Still this procedure resulted in a significant delay in publication of the novel assay (described in Section 2, page 2).

Additional achievements:

1. ***In vitro* validation of OATP-mediated uptake of the PET probe ^{11}C-erlotinib.** There is significant need for cell lines with high levels of OATP overexpression, e.g in OATP-drug interaction tests. However, generation of such cell lines is not a straightforward task. The function-based sorting method developed in this project, described in Section 2 (page 2), allows the enrichment of OATP1B and 2B1 overexpressing cells. In collaboration with Oliver Langer (MU Vienna, AIT), we found that the PET probe ^{11}C-erlotinib is increasingly accumulated in a transporter-mediated way in the liver of healthy humans. Using the cell lines developed in the framework of this project, we showed that OATP2B1 is in fact responsible for the hepatic enrichment of the PET probe ^{11}C-erlotinib observed in humans (Bauer et al., 2017).
2. **OATPs can sensitize cells toward chemotherapeutics:** Several, multispecific members of the OATP family (OATP1A2, 1B1, 1B3 and 2B1) recognize clinically applied chemotherapeutics. Moreover, these transporters are overexpressed in cancers of the breast, lung and/or colon, therefore they are good candidates for targeted delivery of chemotherapeutics. However, evidence about the ability of OATPs to sensitize cells toward anti-cancer agents is still lacking. In order to address this important issue **we performed a screen with a set of clinically used anti-cancer drugs in OATP2B1 overexpressing cells. In these experiments increased sensitivity in cells overexpressing OATP2B1 was observed**, underlining the potential of OATP-mediated targeted tumor treatment (Figure 4). A manuscript summarizing these results is in preparation.

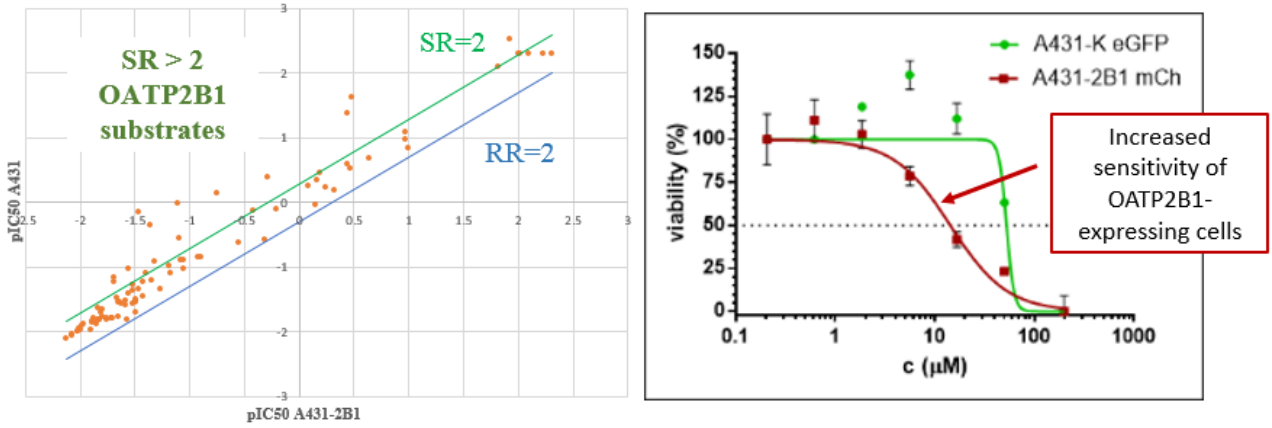

Figure 4. Screening of an anticancer drug set identifies potential OATP2B1 substrates. Dots above the green line represent chemotherapeutic compounds selectively killing cells with OATP2B1 expression (left panel).Cells overexpressing OATP2B1 are more sensitive to the chemotherapeutic agent etoposide (right panel).

SUMMARY

The OTKA grant has allowed me to launch a novel research project. In spite of the unforeseen difficulties most of the objectives were successfully met. Moreover, the minor alteration from the plan resulted in additional achievements. The results were presented both at domestic and international conferences, in the form of 4 original papers (Bauer et al., 2017; Gal et al., 2015; Patik et al., 2015; Patik, paper submitted) and 2 invited review papers (Kovacsics et al., 2017; Rizner et al., 2017). Moreover at least 2 additional original research articles with the support of the current grant are planned to be published in the next half year.

This grant support also promoted the involvement of young students in the research. 5 undergraduate students of which 2 continued their work as PhD students in the research group participated in the project. With their contribution parts of this work were successfully presented on the Conference for Undergraduate Students (3rd and 1st prize on Faculty and National conferences, respectively). Additionally, these talented students involved in the current research were awarded with various

prizes (National Excellence Programme, Ujvári János diploma thesis prize and the Stephen Kuffler Scholarship).

Finally, our *in vitro* models and the established fluorescence-based assays paved the way for novel collaborations. We have established a fruitful collaboration with the Medical University of Vienna to investigate the role of OATPs in tumors, and in the uptake of imaging probes. Based on our achievements, we were invited to participate in a stage-2 proposal to be submitted to the IMI call "Unlocking the Solute Carrier Gene-Family for Effective New Therapies (Unlock SLCs)", https://ec.europa.eu/research/participants/portal/desktop/en/opportunities/h2020/topics/imi2-2016-10-06.html).

Publications with the OTKA support:

Bauer M, Matsuda A, Wulkersdorfer B, Philippe C, Traxl A, **Ozvegy-Laczka C**, Stanek J, Nics L, Klebermass EM, Poschner S, Jager W, Patik I, Bakos E, Szakacs G, Wadsak W, Hacker M, Zeitlinger M and Langer O (2017) Influence of OATPs on hepatic disposition of erlotinib measured with positron emission tomography. *Clinical pharmacology and therapeutics in press.*

Gal Z, Hegedus C, Szakacs G, Varadi A, Sarkadi B and **Ozvegy-Laczka C** (2015) Mutations of the central tyrosines of putative cholesterol recognition amino acid consensus (CRAC) sequences modify folding, activity, and sterol-sensing of the human ABCG2 multidrug transporter. *Biochimica et biophysica acta* **1848**(2): 477-487.

Kovacsics D, Patik I and **Ozvegy-Laczka C** (2017) The role of organic anion transporting polypeptides in drug absorption, distribution, excretion and drug-drug interactions. *Expert opinion on drug metabolism & toxicology* **13**(4): 409-424.

Patik I, Kovacsics D, Nemet O, Gera M, Varady G, Stieger B, Hagenbuch B, Szakacs G and **Ozvegy-Laczka C** (2015) Functional expression of the 11 human Organic Anion Transporting Polypeptides in insect cells reveals that sodium fluorescein is a general OATP substrate. *Biochemical pharmacology* **98**(4): 649-658.

Rizner TL, Thalhammer T and **Ozvegy-Laczka C** (2017) The Importance of Steroid Uptake and Intracrine Action in Endometrial and Ovarian Cancers. *Frontiers in pharmacology* **8**: 346.

Submitted:

Patik I, Székely, V, Német, O, Szepesi, Á, Kucsma, N, Várady, Gy, Szakács, G, Bakos, É, and **Özvegy-Laczka, C** Identification of fluorescent amine reactive dyes as novel substrates of Organic Anion Transporting Polypeptides OATP1B1, 1B3 and 2B1 for screening transporter function and drug interactions. *Molecular pharmacology* (*submitted)*