Final report

ABCG2 and ABCB1 (P-glycoprotein, Pgp) are exporter type ABC proteins that can expel numerous chemically unrelated xeno- and endobiotics from cells. They are expressed in tissues with barrier function including the blood-brain barrier, blood-testis barrier, intestine, liver, placenta and kidneys. Because of their tissue localization and their broad substrate spectrum, they play an important role in the absorption, distribution, elimination, and toxicity (ADME-Tox) of chemotherapeutic drugs used for the treatment of various diseases. When expressed in tumor cells and tumor stem cells, they confer multidrug resistance, contributing to the failure of chemotherapy. On the other hand, genetic polymorphisms leading to their decreased expression and/or impaired function may result in altered chemotherapy responses, drug-related toxic reactions, as well as hyperuricemia and gout, since ABCG2 is also a key player of the intestinal uric acid elimination pathway. Therefore, understanding the molecular details of their duty cycle may help rational design to overcome multidrug resistance, improve drug delivery through pharmacological barriers (e.g., blood-brain barrier) or to treat gout.

The functional form of ABC transporters consists of two transmembrane domains (TMD) that can bind and translocate substrates through the plasma membrane and two nucleotide binding domains (NBDs), which bind and hydrolyse ATP. Although the recent boom of structural studies, especially the cryo-EM technique served high-resolution structures of their different conformers, the details of the structural and energetic interplay between the NBDs and TMDs remained unclear in case of both transporters. Since structures are static snapshots in the path of the full transport cycle, biochemical and biophysical analysis combined with molecular dynamics (MD) simulations can contribute to filling the gaps in our understanding of the transporter function.

1. Studying the catalytic cycle of human ABCG2

Based on structural studies, in absence of ATP ABCG2 adopts an inward-facing (IF) conformation, wherein the large substrate-binding pocket formed by the TMDs is accessible from the cytoplasm, while the ATP-bound ABCG2 is in an outward-facing (OF) state, allowing the release of substrates to the extracellular side. However, the molecular details orchestrating substrate translocation and ATP hydrolysis in ABCG2 remain elusive. Since ABC transporters are very sensitive to the composition of their plasma membrane environment, we have developed fluorescence-based methods to study intramolecular crosstalk in live cells or semi-

permeabilized cells, where ABCG2 molecules are present in their quasi-natural lipid environment and in an undisturbed context with other molecules.

Using the conformation-sensitive antibody 5D3, we showed that the switch from the inward-facing (IF) to the outward-facing (OF) conformation of ABCG2 is induced by nucleotide binding. The IF-OF transition is facilitated by substrates, and hindered by the inhibitor Ko143. The nucleotide induced IF to OF transition coincides with the high-to-low switch of substrate affinity, and this event precedes ATP hydrolysis. Low substrate binding persists in the post-hydrolysis state, indicating that dissociation of the ATP hydrolysis products is required to reset the high substrate affinity IF conformation of ABCG2. In live cells, only a small fraction of ABCG2 molecules is in a transient substrate-bound conformation ready to harvest the energy of ATP for transport, while depletion of ATP increases this fraction prolonging the waiting time before the IF to OF transition.

Cryo-EM studies of membrane proteins are readily performed in membrane mimetics, such as artificial liposomes or lipid nano-discs. In addition, the temperature shock induced by rapid plunge-freezing applied in cryo-EM studies can limit the conformational spectrum of the studied proteins. Therefore, alternative methods that allow the study of membrane transporters in their native plasma membrane environment have crucial importance and may serve as important control experiments to prove the biological relevance of cryo-EM structures. It is important to note that our functional studies are in good agreement with the results of recent cryo-EM studies from other labs.

By finishing this part of the project, we have accomplished one of the major goals of the OTKA proposal. The paper summarizing our results regarding the catalytic mechanism of ABCG2 had been published in eLife in 2023. The PhD student (Zsuzsanna Gyöngy) involved in this work finished her PhD thesis and her thesis is currently under review by the PhD defence committee.

We would like to exploit the fluorescence-based methods developed in this project for studying the interdomain communication using ABCG2 variants carrying point mutations in the NBD-NBD or NBD-TMD interfaces (the project proposal is already submitted to NKFIH).

2. Elucidation of the functional relationship between the nucleotide binding sites (NBDs) in ABCB1

The human ABCB1 is a full transporter with two nucleotide binding domains (NBDs) and two pseudo-symmetric transmembrane domains (TMDs). The two NBDs form two symmetrically arranged composite nucleotide binding sites (NBSs). Each NBS is formed by the

A-loop, H-loop, Walker A, Walker B and Q-loop of one NBD, and the X-loop and signature sequence of the other NBD. The conserved tyrosine of the A-loop aligns the adenine ring of the bound ATP, contributing to nucleotide binding affinity through stacking interactions. The Walker A lysine interacts with the α and β phosphate of ATP. The two nucleotide binding domains of ABCB1 were shown to be functionally equivalent, and the integrity of both catalytic centers is generally believed to be needed for substrate transport. Widely accepted models based on experiments carried out using heterologous expression systems, such as Sf9 cells or yeasts, predicted that the two NBDs hydrolyze ATP in a strictly alternating order. To gain deeper insight into the functional relationship of NBDs in ABCB1, we have mutated certain key amino acids in the evolutionary conserved sequence motifs of one or both NBDs and expressed the mutant ABCB1 variants at high level in mammalian cells using the *Sleeping Beauty* transposon-based expression system. We have demonstrated that while ABCB1 variants carrying bilateral A-loop or Walker A mutations are completely inactive, the unilateral exchange of the A-loop tyrosine to alanine or the unilateral mutation of the Walker A lysine to methionine is compatible with both ATP hydrolytic activity and transport function. Characterization of the single mutants revealed a significant (about 10-fold) reduction of the apparent ATP binding affinity compared to wild-type ABCB1. Stabilization of the post-hydrolytic complex by phosphate mimicking anions, such as vanadate or BeFx also occurred at higher ATP concentrations compared to wild-type, supporting that the mutated site probably has an effect on the overall conformation of the NBD dimer. Although the basal catalytic activity was strongly reduced in accordance with the decreased ATP binding affinity of the unilateral mutants, the degree of ATPase stimulation by verapamil was almost identical to that of the wild-type, showing that drug-stimulation of the ATPase activity is preserved in the unilateral mutants. Location of the mutation in the N or C terminal NBD did not affect the extent of ATPase stimulation by verapamil further supporting the functional identity of the two NBDs. Taken together, our data indicate that, in contrast to prevailing views, single-site NBD mutant ABCB1 molecules retain a weak, but significant uphill transport activity, suggesting that the wild-type catalytic site can hydrolyze ATP in repeated cycles without hydrolysis at the other NBS. (A manuscript is in preparation from these data.)

While numerous ABC transporters, such as ABCB1 and ABCG2 possess two functionally symmetric nucleotide binding sites (NBSs), in several ABC exporters one of the NBSs is practically unable to hydrolyse ATP. A hallmark of this degenerate NBS is the lack of the catalytic glutamate in the Walker B motif. Importantly, unilateral exchange of the catalytic

glutamate in NBS1 (E556M/Q or E1201M/Q) renders human ABCB1 transport incompetent. In contrast, the closely related bile salt export pump ABCB11 (BSEP), which shares 49% identity with human ABCB1, naturally contains a methionine in place of the catalytic glutamate in NBS1. However, the NBD-NBD interface of ABCB11 differs from ABCB1 in another three amino acid residues, all within NBS1. We have shown that the catalytic glutamate (E556M) mutant ABCB1 variant regained its ATP-dependent substrate transport activity, when the additional three diverging NBS1 residues were also replaced. Our molecular dynamics (MD) simulations revealed that the catalytic glutamate mutant ABCB1 (E556M) cannot hydrolyse ATP in NBS1 leading to an arrest of the transport cycle, while introducing the additional three mutations into the NBS1 modifies the geometry of ATP binding allowing ABCB1 to evade a conformationally locked state. Taken together, these data also support that a single hydrolysis competent catalytic site is sufficient to promote transport in ABCB1, if formation of the ATP locked state in a non-canonical NBS is avoided. Our results challenge former catalytic cycle models proposing the continuous switching of ATP hydrolysis between NBS1 and NBS2 in ABCB1. The results of this study were published in Plos Genetics in 2020.

3. Synergistic inhibitory effect of polyphenols on ABCB1

In the framework of another project (GINOP-2.2.1-15-2017-00079) we have tested the interaction of 15 dietary polyphenols with ABCB1. We have found that 9 of them interacted with ABCB1 as substrates or inhibitors. Quercetin (QUR) and ellagic acid showed the strongest inhibitory effect on the transport and ATPase activity of ABCB1 and also increased the fraction of the UIC2-reactive inward facing (IF) ABCB1 conformers in the plasma membrane of live cells. On the other hand, tested polyphenols with weaker effects on the ATPase and transport function of ABCB1 did not affect the binding of the conformation selective UIC2 mAb. Combined treatment with verapamil, a known ABCB1 ligand, and certain polyphenols strongly inhibited the transport and ATPase activity of ABCB1 and facilitated the trapping of ABCB1 in the UIC2-reactive conformation. Although the exact mode of inhibitor binding to ABCB1 and the action mechanism of potential inhibitors is not known, it seems likely that numerous potent ABCB1 inhibitors act stabilizing the UIC2-reactive IF conformational state of the pump and consequently hinder the formation of the NBD sandwich dimer and thus inhibit ATP hydrolysis. (Results were published in *Pharmaceutics* **2021**, *13*, 2062).

In further experiments, when QUR and cyanidin-3O-sophoroside (C3S) were applied together, both a stronger ATPase inhibition and a robust decrease of substrate transport were

observed, supporting the synergistic ABCB1 inhibitory effect of the two polyphenols. In addition, co-treatment with QUR and C3S shifted the conformational equilibrium to the IF conformer of ABCB1, as it was detected by the conformation-selective UIC2 mAb. To gain deeper insight into the molecular details of ligand-ABCB1 interactions molecular docking experiments and MD simulations were also carried out. Our *in silico* studies supported that QUR and C3S can bind simultaneously to ABCB1. The most favourable ligand-ABCB1 interaction is obtained when C3S binds to the central substrate binding site and QUR occupies the "access tunnel". Our results also highlight that the strong ABCB1 inhibitory effect of the combined treatment with QUR and C3S may be exploited in chemotherapy protocols for the treatment of multidrug-resistant tumors or for improving drug delivery through pharmacological barriers. The manuscript was submitted to *International Journal of Molecular* Sciences. In the first round of reviewing process Reviewers asked longer MD simulations and some minor modifications. The modified manuscript has been re-submitted and it is currently under review.

In these studies, we successfully combined biochemical/biophysical tools and *in silico* experiments, such as molecular docking and molecular dynamics (MD) simulations to understand ligand-protein interactions in case of ABCB1. The PhD student (Kuljeet Singh) involved in this part of the project will defend his PhD thesis in the near future.

4. Methodical achievements/conclusions of our project

- We have adopted state of the art fluorescence-based methods including fluorescence co-localization and Fluorescence Correlation Spectroscopy (FCS) to exploit them for studying the substrate binding affinity of ABC transporters at different steps of their duty cycle.

- We applied FCS technique for the first time to study substrate binding by ABC transporters in the plasma membrane of live cells.

- We exploited the conformation selective 5D3 monoclonal anti-ABCG2 antibody for studying the kinetics of the IF-OF transition and demonstrated that substrate binding accelerates this transition.

- Combining thorough biochemical/biophysical analysis with *in silico* studies we could contribute to the detailed, atomic level understanding of the ABC transporters' working mechanism.

Published papers and manuscripts referring the OTKA project:

1. ***Zsuzsanna Gyöngy***, Gábor Mocsár, Éva Hegedűs, Thomas Stockner, ***Zsuzsanna Ritter***, László Homolya, Anita Schamberger, Tamás I. Orbán, Judit Remenyik, ***Gergely Szakács***

and ***Katalin Goda***: Nucleotide binding is the critical regulator of ABCG2 conformational transitions. eLife Elife 2023 Feb 10;12:e83976. doi: 10.7554/eLife.83976)

2. ***Katalin Goda***, Yaprak Dönmez-Cakil, ***Szabolcs Tarapcsák***, Gábor Szalóki, Daniel Szöllősi, Zahida Parveen, Dóra Türk, ***Gergely Szakács***, Peter Chiba, Thomas Stockner: Human ABCB1 with an ABCB11-like degenerate nucleotide binding site maintains transport activity by avoiding nucleotide occlusion. PLoSGenet 16(10):e1009016. https://doi.org/10.1371/ journal.pgen.1009016

3. ***K. Singh***, **Sz. *Tarapcsák***, ***Zs. Gyöngy***, ***Zs. Ritter***, Gy. Batta, R. Bosire, J. Remenyik and ***K. Goda***: Effects of Polyphenols on P-glycoprotein (ABCB1) Activity. Pharmaceutics 2021 Dec 2;13(12):2062. doi: 10.3390/pharmaceutics13122062

4. J. Káldy, E. Patakiné Várkonyi, G. L. Fazekas, Z. Nagy, Zs. J. Sándor, K. Bogár, Gy. Kovács, M. Molnár, B. Lázár, ***K. Goda, Zs. Gyöngy, Zs. Ritter***, P. Nánási Jr., Á. Horváth, U. Ljubobratović: Effects of hydrostatic pressure treatment of newly fertilized eggs on the ploidy level and karyotype of pikeperch (*Stizostedion lucioperca Linnaeus, 1758*)

5. ***Kuljeet Singh***, Rajesh Patil, ***Vikas Patel***, Judit Remenyik, Tamás Hegedűs and ***Katalin Goda***: Synergistic inhibitory effect of quercetin and cyanidin-3O-sophoroside on ABCB1. (The revised manuscript is under review in International Journal of Molecular Sciences)

<u>Conference lectures based on the OTKA project:</u>

1. ***Goda Katalin*.** A P-glikoprotein működési mechanizmusának vizsgálata nukleotid-kötőhely mutánsok segítségével. 48. Membrán-Transzport Konferencia, Sümeg 2018. május 15-18.

2. ***Gyöngy Zsuzsanna***, Szakács Gergely, ***Goda Katalin***. Az ABCG2 konformáció változásainak vizsgálata permeabilizált sejteken. 48. Membrán-Transzport Konferencia, Sümeg 2018. május 15-18. **(The poster received a poster award and it was presented as a short lecture in a dedicated section)**

3. ***Zsuzsanna Gyöngy***, Gábor Szalóki, ***Gergely Szakács*** and ***Katalin Goda***, Elucidation of the catalytic cycle of ABCG2 in permeabilized cells**.** FEBS Special Meeting 2020, ATP-Binding Cassette (ABC) Proteins: From Multidrug Resistance to Genetic Disease. 1-7 March 2020. Innsbruck, Austria **(poster presentation and also selected for short oral presentation)**

4. ***Goda Katalin***. ABCB1 és ABCG2: hasonlítanak vagy különböznek? 51. Membrán-Transzport Konferencia, Sümeg, 2022 május 17-20

5. ***Katalin Goda***: Crosstalk between nucleotide and substrate binding in ABCB1 and ABCG2. **9**th FEBS Special Meeting on ABC Proteins - ABC2023 in Innsbruck, Austria. 26 Feb – 3 Mar 2023.

<u>Posters based on the OTKA project:</u>

1. ***Szabolcs Tarapcsák, Zsuzsanna Gyöngy***, Beatrix Ágics, Dóra Türk, Gábor Szabó, ***Gergely Szakács, Katalin Goda.*** Studying the catalytic cycle of P-glycoprotein using Walker B and

A-loop mutants. “ATP-Binding Cassette (ABC) Proteins: From Multidrug Resistance to Genetic Disease” FEBS Special meeting, 6-12 March, 2018. Innsbruck, Austria

2. ***Tarapcsák Szabolcs***, Bársony Orsolya, Bacsó Zsolt, Szabó Gábor, ***Goda Katalin***. A membrán mikrokörnyezet és az aktin citoszkeleton hatása a P-glikoprotein (ABCB1) működésére. Membrán-Transzport Konferencia, Sümeg 2018. május 15-18.

3. ***Z. Gyöngy***, G. Szalóki, ***G. Szakács***, ***K. Goda***. Elucidation of the conformational changes of ABCG2 in permeabilized cells. “ATP-Binding Cassette (ABC) Proteins: From Multidrug Resistance to Genetic Disease” FEBS Special meeting, 6-12 March, 2018. Innsbruck, Austria

4. ***Z. Gyöngy***, G. Szalóki, ***G. Szakács***, ***K. Goda***. Elucidation of the conformational changes of ABCG2 in permeabilized cells. FEBS Advanced Course on Biochemistry of Membrane proteins. Structure, Trafficking, Regulation. 25-30 August, 2019. Budapest, Hungary

5. ***Katalin Goda***, Szabolcs Tarapcsák, ***Zsuzsanna Gyöngy***, Dóra Türk, ***Gergely Szakács*** Studying the catalytic cycle of Pgp and ABCG2 transporters. BioMedical Transporters 2019, Membrane transporters and channels: From basic research to drug development and clinical application. Lucerne, Switzerland, August 4–8, 2019**.**

6. ***Katalin Goda, Szabolcs Tarapcsák, Zsuzsanna Gyöngy***, Dóra Türk, ***Gergely Szakács*** Coupling between the ATP hydrolysis cycle and the substrate translocation in human P-glycoprotein. FEBS Special Meeting 2020, ATP-Binding Cassette (ABC) Proteins: From Multidrug Resistance to Genetic Disease. 1-7 March 2020. Innsbruck, Austria

7. ***Gyöngy, Zsuzsanna***; Mocsár, Gábor; ***Ritter, Zsuzsanna***; Stockner, Thomas; ***Szakacs, Gergely***; ***Goda, Katalin***. „Crosstalk between nucleotide and substrate binding in ABCG2. NCCR TransCure Final Conference, Bern, Switzerland, August 17-19, 2022

8. ***Zsuzsanna Ritter***, Szabolcs Tarapcsák, ***Zsuzsanna Gyöngy***, Orsolya Bársony, Nimrah Ghaffar, Thomas Stockner, ***Gergely Szakács***, and ***Katalin Goda***. Analysis of unilateral Walker A and A-loop mutants indicate that a single active catalytic site is sufficient to promote transport in ABCB1. 9th FEBS Special Meeting on ABC Proteins - ABC2023**,** Innsbruck, Austria. 26 Feb – 3 Mar 2023.