*Ph.D. short thesis*

***Drosophila MRP, a high turnover model of the long human Multidrug Resistance-associated Proteins***

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## Introduction

ATP Binding Cassette (ABC) transporters are ubiquitous multidomain integral membrane proteins capable of transporting ligands across biological membranes, a process requiring the energy of ATP binding and hydrolysis and being critical for most aspects of cell physiology. Besides their physiological relevance, ABC transporters have clinical and economical significance as well. A number of ABC transporters provide resistance against antibiotics, pesticides and herbicides or underlie human genetic diseases. Several ABC transporters, such as Multidrug Resistance-associated Protein1 (ABCC1/MRP1), confer resistance against a wide range of cytotoxic agents resulting in the failure of the cancer therapy due to the acquired multidrug resistance phenotype of cancer cells.

The 48 human and 56 *Drosophila* ABC proteins are grouped into subfamilies based on phylogenetic analysis. The ABCC/MRP subfamily harbours a subset of proteins termed as “long MRPs”. The hallmark of these proteins is their unique domain structure involving three transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs). The intensively studied human long MRPs are organic anion transporters of a vast array of endo- and xenobiotics, thus having crucial role in either physiological or pathological conditions including chemo-resistance, regulated distribution of physiological compounds and multidrug resistance. Long MRPs are represented by five members in humans and by a single member in fruit fly, the Drosophila MRP (DMRP), the subject of our investigations. The functions and characteristics of DMRP at the beginning of our investigations were unrevealed.



## Aims/Objectives

Though human long MRPs are in the focus of intensive investigations, many of their substantial characteristics are far beyond understanding. One of the several reasons concealing behind this is their moderate activity in *in vitro* assays.

Our preliminary experiments indicated that DMRP, the fruit fly orthologue of all human long MRPs, exhibits outstanding activity. Therefore we decided to perform systematic comparative investigations aiming to reveal the characteristics of DMRP as a potential high turnover model of human long MRPs. To shed light on the characteristics of DMRP, we aimed to:

1. Reveal the evolutionary relationship of Drosophila and human ABCC proteins and propose an improved membrane topology model for DMRP.

2. Characterise its substrate/inhibitor profile in comparative functional assays towards well-established human MRP substrates and inhibitors.

3. Investigate the catalytic cycle by the study of its nucleotide binding and trapping properties and its vanadate-sensitive ATPase activity.

4. Study the endogenous expression and potential physiological role.

Providing the basis for our investigations, we needed the following tools:

a) Suitable expression of DMRP in Sf9-baculovirus system used by our laboratory to characterise human long MRPs.

b) An antibody specifically recognising DMRP. (Collaboration with Steven Robinow, University of Hawaii)

## Methods

### *In silico* analysis

Protein sequences were aligned using CLUSTALW (1.83) and subjected to neighbour-joining tree producing algorithm MEGA2. Predicted membrane topology of DMRP was proposed based on hMRP1 using the online membrane topology prediction programme, HMMTOP (www.enzim.hu/hmmtop/).

### Generation of pAcUW21L-dMRP vector construct

The dMRP ORF encoded by cDNA SD07655 was amplified with primers containing Not I and Sac I restriction sites. The amplified fragment was cloned into Not I and Sac I sites of pACUW21L baculovirus expression vector.

### Expression of DMRP in Sf9 cells

Sf9 (*Spodoptera frugiperda)* cells were co-transfected with the pAcUW21L- dMRP construct and linearized BaculoGold DNA (Pharmingen). Recombinant virus was amplified and cloned by end-point dilution yielding clones of suitable expression.

### Membrane preparation of Sf9 cells

(Sf9) cells were infected with recombinant virus. After 72 hours cells were harvested and homogenized. Membrane fraction was isolated with differential ultracentrifugation. Pellet was suspended to 7-12 mg/ml total protein concentration.

### Cyclodextrin treatment of Sf9 membrane vesicles

Sf9 membrane preparations were incubated with 13 different cyclodextrins for 1 hour on ice. Subsequently, membranes were washed and pellet was re-suspended.



### Preparation of *Drosophila* samples

Heads/bodies or intact flies were grained in a tissue homogeniser in DB on ice. Samples were filtered and homogenised.

### SDS-PAGE and electro-blotting to PVDF membranes

Total cell lysates/membrane preparations containing 30-80µg/2,5-5µg total protein/sample were separated by SDS-PAGE and blotted to PVDF membranes.

### Immunoblotting

PVDF membranes were blocked in milk and incubated in anti-DMRP, anti-hMRP1 or anti-hMRP2 antibody. Membranes were subsequently washed and incubated with HRP-conjugated secondary antibodies. The protein-antibody interaction was visualized by the ECL, bands were quantified by densitometry.

### Coomassie staining

Proteins were separated by SDS-PAGE and stained with Coomassie dye.

### Nucleotide binding and trapping

Isolated Sf9 membrane preparations were incubated for 5 min at 0˚C for binding or 37 °C for trapping in the presence/absence of trapping anions (orthovanadate or fluoroaluminate) in the presence of 8-$N_3$[$\alpha^{32}$P]ATP. Samples of binding experiments were irradiated with UV on ice for 5 min. In trapping experiments the reaction was stopped by ice-cold washing buffer containing excess ATP and trapping anions. The samples were than washed and the pellet was irradiated with UV on ice for 5 min. After UV irradiation samples were subjected to SDS-PAGE and autoradiography. Identity of the $^{32}$P-labelled bands was confirmed by immunostaining.

**Vesicular transport of radiolabelled substrates**

Sf9 membrane vesicles were incubated with radiolabelled substrates in the presence of MgATP /AMP at 0, 23 or 37°C. The samples were filtered, and filter-bound radioactivity was determined in a ß-counter.

**Vesicular transport of fluorescent substrates**

Vesicular transport measurements with fluorescent substrates were performed similarly. Fluorescence intensity was detected by flow cytometry.

**ATPase activity measurements**

Vanadate-sensitive ATPase activity was measured by colorimetric detection of inorganic phosphate liberation (Sarkadi et al, 1992) in the presence or absence of modulators at various temperatures and incubation times.

**Transition-state thermodynamic analysis of the catalytic cycle**

Apparent activation parameters of the rate-limiting step of the catalytic cycle were determined from ATPase activity measurements performed as a function of temperature, in the 17˚C to 37˚C temperature range with 4˚C increments, in the absence or in the presence of substrates. The thermodynamic parameters of the transition-state were obtained using the linearized Eyring equation.

**Linear Free Energy Relationship analysis**

Initial velocity of the vanadate-sensitive ATPase activity was measured at two different temperatures (25 and 37˚C), in the presence/absence of various concentrations of $LTC_4$ and $E_2$17ßDG. Log turnover numbers of ATPase activities detected at 37˚C were plotted as a function of the corresponding data for 25˚C.



## Results

1. We have shown that DMRP is the unique fruit fly orthologue of the long human MRPs, MRP1-3 and 6, and proposed a new domain structure model for DMRP, resembling the $TMD_0$-$L_0$-$TMD_1$-$ABC_1$-$TMD_2$-$ABC_2$ membrane topology of its human orthologues.

2. We expressed DMRP in the Sf9-baculovirus system at an expression level suitable for investigating its function in comparison with the previously expressed and functionally characterised human orthologues.

3. In systematic and comprehensive functional assays we have determined the transport kinetic parameters of DMRP for established human long MRP substrates, such as the inflammatory mediator $LTC_4$, the estrogen metabolite $E_2$17ßD-glucuronide, the fluorescent calcium indicators calcein and fluo3 and the fluorescent indicator of reactive oxygen species, carboxidichlorofluorescein. Our comparative analysis revealed that DMRP possessed the highest turnover rate ever-detected for all the above mentioned human long MRP substrates, characterised by 1-2 magnitudes higher activities than those of the relevant human transporters measured in parallel experiments.

4. In comparative functional assays we found that the inhibitory profile of DMRP overlaps with those of the human long MRPs.

5. The observed high turnover of DMRP prompted us to study the catalytic cycle of the protein. Therefore we performed nucleotide-binding experiments and studied the formation of a transition-state nucleotide intermediate in nucleotide-trapping experiments. We have not found any marked difference in the nucleotide-binding and -trapping properties of DMRP as compared to human long MRPs.

6. To further analyse the catalytic cycle of DMRP, we investigated its ATP hydrolytic capacity in vanadate-sensitive ATPase activity experiments. We have found a high-level vanadate-sensitive basal ATPase activity for DMRP in the Sf9 membranes markedly different from that of the human long MRPs. This basal ATPase activity could be stimulated in the presence of the uricosuric drug, probenecid, and the artificial N-ethylmaleimide-glutathione conjugate NEM-GS, compounds known to interact with human long MRPs.

7. Substrates are known to stimulate the ATPase activity of MRPs as a consequence of the tight coupling of ATP hydrolysis to substrate transport. However, in our ATPase activity measurements characterised DMRP substrates surprisingly inhibited the basal ATPase activity of DMRP in a concentration dependent manner. Therefore, we postulated a hypothesis for this unexpected phenomenon. We hypothesised the presence of an endogenous modifier (an endogenous substrate or an allosteric activator) in the Sf9 membrane capable of stimulating the intrinsic ATPase activity of DMRP. We postulated that the exogenous substrates competed with the endogenous modifier resulting in an apparent activation of the "basal" ATPase activity. We have shown supporting experimental data for the above hypothesis.

8. Taking advantage of the unique high turnover of DMRP we investigated its transition-state thermodynamic parameters referring to the formation of the rate limiting-intermediate of the full catalytic cycle. Our analysis revealed two distinct rate-limiting steps, characterised by distinct activation enthalpy and entropy terms, referring to the catalytic cycle in the presence or in the absence of exogenous substrates. However, the change in the activation free energy in the presence or in the absence of exogenous substrates resulted in a similar value.



9. We showed the endogenous expression of dMRP in S2 embryonic cells and adult flies on the protein level providing supporting evidence for the ubiquitous expression of DMRP.

10. We identified two potential pesticide substrates of DMRP having agricultural and economical relevance.

## Conclusions

1. In comparative functional assays we demonstrated that DMRP is a suitable model protein of human long MRPs, being a functional orthologue in terms of substrate specificity and inhibitor profile, whereas possessing outstanding activity.

2. Taking into consideration the elevated level of basal ATPase activity and the inhibitory character of the transported substrates in ATPase experiments, we have hypothesised and experimentally supported the presence of an endogenous modifier in the Sf9 cell membrane preparations.

3. We were the first to present data concerning the thermodynamics of an MRP-type ABC-transporter. Our data suggests that, independently of the previously published differences between the catalytic cycle of MDR and MRP-type proteins, the steady-state formation of the catalytic intermediates of these proteins might be similar with respect to their thermodynamic features.

4. In addition, potential pesticide substrates of DMRP were identified indicating the role of insect and human long MRPs in the elimination of these toxins, and we confirmed the ubiquitous expression pattern of DMRP at the protein level.

## Bibliography of the candidate's publications

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