

# Multidrug resistance testing with in vitro ABC transporter assay

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## BACKGROUND

Although in most cases chemotherapeutics are given in combination as resistance to single agents occurs almost universally, tumors are often characterized by resistance to a broad spectrum of structurally unrelated cytotoxic drugs (i.e. multidrug resistance – MDR). This phenomenon usually results from the expression of ATP-binding cassette (ABC) transporters, such as the ABCB1 (MDR1 and P-gp), ABCC1 (MRP1), and ABCG2 (MXR and BCRP), which are known to function as drug efflux pumps to cause drug resistance by actively extruding multiple anticancer drugs with expenses of ATP. This multidrug resistance (MDR) seriously limits the conventional pharmaceutical treatment of cancer patients.

Since the beginning of the 90's several studies have confirmed the role of multidrug transporter proteins in chemoresistance in a variety of hematological malignancies. The studies focused on rapidly progressing acute leukaemia's, with the emphasis being on acute myeloid leukaemia (AML).

We used two major forms of ABC transporter assays to investigate the MDR phenomenon: dye efflux assays using whole cells expressing the transporter of interest and ATPase (Ref.) and vesicular transport (VT) assays using purified membrane vesicles. ABC (ATP-binding cassette) membrane transporters mediate the transport of substrates against a concentration gradient. This transport activity requires the energy of ATP hydrolysis, controlled by drug interaction, and coupled to the actual substrate translocation.

Aims of this study were to test the interactions several drugs and ABC efflux transporters and to measure function of these transporters on the cell lines. We have intended to develop a new fluorescent assay, in order to extend it for the measurement of BCRP/ MXR activity as well. This assay allows more accurate prediction of drug resistance than quantifying the expression of the transporters at mRNA or at protein level.

Ref: Sarkadi B, et al: Expression of the human multidrug resistance cDNA in insect cells generates a high activity drug-stimulated membrane ATPase. *J Biol Chem* 1992; 267; 4854-4858

## RESULTS

### Characterization of drugs with ATPase, VT and dye efflux assays on membrane preparations of three transporters

	MDR1			MRP1			MXR				
	ATPase assay		Calcein assay	ATPase assay		Calcein assay	VT	ATPase assay		Hoechst assay	VT
	EC50 (µM)/% max. activation	IC50 (µM)/% max. inhibition	IC50 (µM)/% max. inhibition	EC50 (µM)/% max. activation	IC50 (µM)/% max. inhibition	IC50 (µM)/% max. inhibition	IC50 (µM)/% max. inhibition	EC50 (µM)/% max. activation	IC50 (µM)/% max. inhibition	IC50 (µM)/% max. inhibition	IC50 (µM)/% max. inhibition
Actinomycin-D	0	3/120	15/60	0	3/140	3/95	30/70	0	100/50	0	30/75
Carboplatin	0	0	0	0	0	0	0	0	90/50	0	0
Camptothecin	0	0	0	40/17	0/34	0/27	0	3/68	0/26	0	4/41
Chlorambucil	30/15	0	0	0	0	0/29	0/17	0	0/38	0	0
Cisplatin	0	0	0/25	0	0	30/61	0/14	0	80/50	0	0/40
Dacarbazine	0	0	0	0	0	0	0	0/39	0	0	0/27
Daunorubicin	0	50/60	0	0	40/63	0	0/31	20/40	100/50	0	20/63
Doxorubicin	0	10/50	toxic effect	0	0	toxic effect	100/50	0	0	0	40/56
Etoposide	0	0.4	0	0	0	0	0	0	0	0	0
Flavopiridol	0	0	0	8/22	0	0.4/147	0	0	0	0	5/60
Fluor-uracil	0	0/34	0	20/23	0	0	0	0	0	0	0/26
Hydroxylurea	0	0	0	0	0	0	0	0	0	0	0
Melphalan	0	40/72	0	30/37	0/37	25/61	0/13	0	80/50	0	0/43
Mitoxantron	30/15	0	toxic effect	0	0	toxic effect	0	0	0	0	50/50
Taxol	0	1.5/100	10/67%	0	10/100	15/70	100/50	0	10/69	0/23	50/50
Topotecan	0	0	toxic effect	0	0/39	0	50/50	8/65	0	0/27	25/70
Verapamil	3/100	0	6/100	0	0	20/100	0	1/31	0	0	0/22
Vinblastine	0.2/39	0	12/100	0	15/88.5	30/63	0/14	0	9/100	0	0/30
Vincristine	0	0	0	0	0	0	0	0	0/28	0	0/38
Dexamethasone	0	0	0	0	0	0	0	0	0	0	0/17
Indomethacin	0	0	0	5/66	0	5/72	0	8/22	0/24	0	0/18
Azathioprine	0	0	0	0	0	0	0	0	0	0	0/15
Cyclophosphamid	0	0	0	20/19	0	0	0	0	0	0	0
Cyclosporin A	0	2/100	2/125	0	10/100	8/150	50/50	0	20/134	0/13	25/83
Leflunomide	0	0	0	0	0	0	0	5/130	0	15/97	10/70
Methotrexate	0	0	0	0	0	0	0/27	1.5/43	0/21	0/23	0
Sulfasalazin	0	0	0	4/30	0/28	0	0	5/100	0	0	<5/100

Orange: To differ from references

Yellow: To fit to references

## CONCLUSIONS:

- ATPase and VT assay are suitable *in vitro* membrane based methods to detect interactions between ABC transporters and substrates molecules. We determined EC50/IC50 values to characterize nature of these interactions. For example daunorubicin and doxorubicin interact each transporter, in case of some analysed drug we observed more interactions what earlier data suggested. Actinomycin-D, melphalan and vinblastine inhibited BCRP transporter in ATPase and VT assay, although these inhibitions could not be detected in Hoechst assay.
- MultiDrugQuant kit provides a separate measure of multidrug resistance for Pgp/ MRP/ BCRP
- Detection of the majority of drug resistant cases with the MDQ kit could support clinical decision-making and satisfy an unmet diagnostic need with significant economical and therapeutic impact.

## METHODS

ATPase and VT assays were performed on membrane preparations from ABC transporters overexpressing cells which show a basal ATPase activity.

In the ATPase **activation** assay, ABC transporters with various concentrations of test compound and the effect on basal ATPase activity. EC50 is defined as the concentration of the test compound needed to reach 50% of the activator's own maximal efficacy. In **inhibition** assay, the compound is tested for its ability to reduce the stimulatory effect of the control drugs on the respective ABC transporter. IC50 is defined as the concentration of the test compound that inhibits the maximally stimulated ATPase activity by 50%.

In the VT assay rapid filtration of the membrane suspension through a filter that retains membrane vesicles allows us to remove substrate molecules that are „outside“ leaving the membrane vesicles with transported molecules trapped „inside“ on the filter. In the indirect assay radiolabelled or fluorescent reporter substrate is used allowing for measurement of transported substrate by liquid scintillation counting or with a fluorimeter. IC50 is defined as the concentration required to inhibit the transport of the reporter substrate by 50%.

Dye efflux assays are based on determining fluorescence intensity differences in a flow cytometer after a short in-vitro incubation of the cell suspension with a fluorescent dye such as the calcein-acetoxymethyl ester (calcein AM) for MDR1 and MRP1 with or without the addition of selective inhibitors of MDR1 and MRP1 and Hoechst 33342 (internal standardization). As the MDQ kit utilizes mitoxantrone as BCRP substrate the compound set will be retested using mitoxantrone as a substrate. However, it is likely that the two assays will yield similar data as Ko134 and novobiocin inhibited the transport of the Hoechst dye as well as mitoxantrone with the same potency.

- We have characterized ABC transporters overexpressing cell lines (HL60,K562/MDR+; HL60/MRP+; PLB/MXR+) by flow cytometry with several substrates (eg. Verapamil, mitoxantron, Hoechst) and selective inhibitors (eg. KO134, novobiocin, PSC833)
- We calculated from the fluorescence intensity the multidrug activity factor according to the following equations:

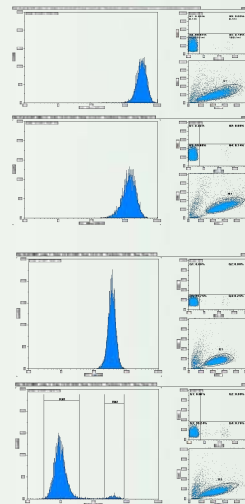
$$MAF_{Total} = 100 \times (F_{max} - F_0) / F_{max}$$

$$MAF_{MRP1} = 100 \times (F_{MRP} - F_0) / F_{max}$$

$$MAF_{MDR1} = MAF_{Total} - MAF_{MRP1}$$

$$MAF_{BCRP} = 100 \times (F_{MX} - F_0) / F_{MX}$$

$F_{max}$ : fluorescence with inhibitor  
 $F_0$ : fluorescence without inhibitor



- PLB-MXR cell line has 55% MAF value
- Inhibition of the BCRP function with KO134 and novobiocin was similarly
- Histograms indicate the mitoxantrone accumulation on the PL negative and R1 gated population
- We found lower fluorescence intensity on the HL60-MDR1 cell line with inhibitors: for example PSC 833, Verapamil
- We observed 3% non-resistant RN2 population (lower left histogram)
- We detected 97% MAF value on the HL-60/ MDR1 cell line

### MultiDrugQuant™ Assay Kit Carries All Positive Features of the Calcein Assay Concept

- Provides quantitative multidrug resistance activity factors (MAF), selectively for MDR1, MRP1 and BCRP functions
- Designed as a simple routine laboratory flow cytometry method
- Sensitivity highly surpasses other methods

- (1) First clinical study completed: "Calcein assay for multidrug resistance reliably predicts therapy response and survival rate in acute myeloid leukemia" Karácsi É., et al., British Journal of Haematology 2001, 112 (2), pp. 308-314
- (2) Haematological application launched in April 2001 for research use only
- (3) Clinical trials start soon with a new, refurbished version of the kit for haematology and inflammatory diseases