PROTEOMIC ANALYSIS OF CIPROFLOXACIN-RESISTANT MURINE MACROPHAGES

M. Aerts[1], N. Caceres[2], J. Van Beeumen[1], P.M. Tulkens[2], F. Van Bambeke[2] and B. Devreese[1]
[1] L-ProBE, Ghent University, Belgium;
[2] Unité de Pharmacologie cellulaire et moléculaire, Université catholique de Louvain, Brussels, Belgium

INTRODUCTION: A general mechanism by which eukaryotic cells protect themselves from potentially harmful molecules is active efflux. Unfortunately, these mechanisms also compromise intracellular drug treatments. In this context, ciprofloxacin-resistant J774 macrophages (CIP-J774) from mouse were generated by gradually increasing the antibiotic's concentration in the growth medium[1]. Increased efflux and decreased intracellular concentration of ciprofloxacin were measured in the resistant cells and it was shown that in *Listeria monocytogenes*-infected resistant cells, a 12-fold higher extracellular concentration was needed for a bacteriostatic affect similar to that of wild-type macrophages. The ATP-dependency of the efflux and the fact that resistant cells revert slowly to the wild-type situation might indicate that overexpression mechanisms of certain ATP-energized multidrug transporters are involved. For prove and more insights in the general efflux mechanisms a SILAC-based GeLC-MS/MS-proteomic analysis was set up where ciprofloxacin-resistant and wild-type macrophages were grown in the presence of $^{12}C_6$-Lys, $^{12}C_6$-Arg and $^{13}C_6$-Lys, $^{13}C_6$-Arg respectively (**Fig.1**). Identification and quantification were performed in a Thermo LTQ-FT ICR MS system.

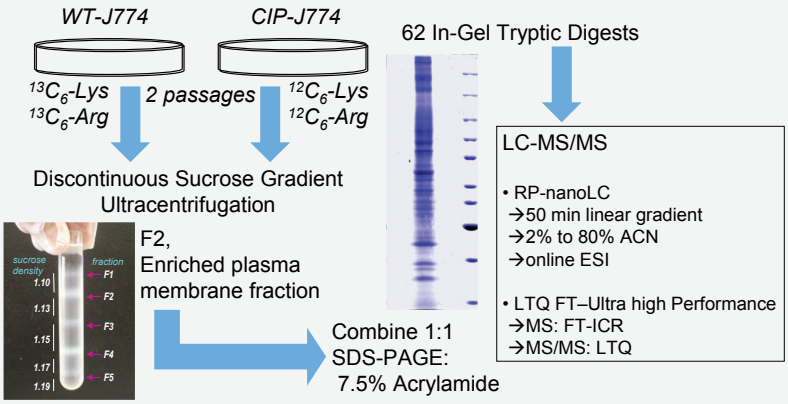

Fig.1: SILAC-based GeLC-MS/MS-proteomic analysis Flow chart

PROTEIN IDENTIFICATION:

STEP 1: Identification MS/MS spectra for each single LC-MS/MS experiment using the SEQUEST algorithm.
→ mouse protein database (uniprot-taxonomy-10090, 59.560 entries) was concatenated with the shuffled decoy database generated by the Decoy Database Builder software tool[2].

STEP 2: Peptide validation using the PeptideProphet tool from the Trans-Proteomic-Pipeline (TPP)[3].
→ identifications with a probability ≥ 0.70 were retained

STEP 3: Protein validation using the ProteinProphet tool from TPP.

STEP 4: The identified proteins from each individual LC experiment were grouped using an in-house written VBA macro.

PROTEIN QUANTIFICATION:

Peptides were quantified using the XPRESS and ASAPRatio software tools from TPP. The mass interval was set to 0.01Da, Arginine and Lysine were checked as possibly labeled amino acids with a 6.02013 mass difference. Peptide abundance ratios were manually checked and if necessary adjusted or deleted.

RESULTS: In total 98,051 MS/MS spectra were searched from 62 digests, resulting in 798 proteins identified with at least 2 spectra. Using the concatenated decoy database, we calculated a false positive rate of less than 1%. **Table 1** and **Table 2** summarize the 20 down (<0.50 ratio CIP:WT) and 22 up (>2.0 ratio CIP:WT) regulated gene products respectively.

Table 1
Down regulated proteins

Swiss-Prot Accession number		Protein Abundance Ratio[a,b]
P58681	Toll-like receptor 7 precursor	0.07
Q3TNE7	hypothetical Arginine-rich region profile containing protein	0.13
Q3UQJ7	DnaJ (Hsp40) homolog, subfamily C, member 13	0.17
Q8BNL1	hypothetical protein	0.19
P24063	Integrin alpha-L precursor	0.20
P62814	Vacuolar ATP synthase subunit B, brain isoform	0.25
Q9Z1G3	Vacuolar proton pump subunit C 1	0.25
P50518	Vacuolar proton pump subunit E 1	0.26
P50516	Vacuolar ATP synthase catalytic subunit A	0.27
P20152	Vimentin	0.27
Q8BVE3	Vacuolar proton pump subunit H	0.27
A1L314	Mpeg1 protein	0.30
Q69ZN7	Myoferlin	0.31
O89001	Carboxypeptidase D precursor	0.35
P56480	ATP synthase subunit beta, mitochondrial precursor	0.36
P57746	Vacuolar proton pump subunit D	0.37
P47738	Aldehyde dehydrogenase, mitochondrial precursor	0.39
Q80VQ0	Aldehyde dehydrogenase 3B1	0.46
Q7TPR4	Alpha-actinin-1	0.47
P17439	Glucosylceramidase precursor	0.49

Table 2
Up regulated proteins

Swiss-Prot Accession number		Protein Abundance Ratio[a,b]
Q3TZN9	ATP-binding cassette, sub-family C (CFTR/MRP), member 4	453.4
P63085	Mitogen-activated protein kinase 1	21.1
Q91YW3	DnaJ homolog subfamily C member 3	14.0
P03975	IgE-binding protein	4.4
A2AR26	Solute carrier family 2 (Facilitated glucose transporter), member 6	4.2
Q810U4	Neuronal cell adhesion molecule precursor	4.1
P01900	H-2 class I histocompatibility antigen, D-D alpha chain precursor	4.1
P97333	Neuropilin-1 precursor	3.0
Q3UT74	integrin alpha 9	2.9
P26955	Cytokine receptor common beta chain precursor	2.8
Q921H8	3-ketoacyl-CoA thiolase A, peroxisomal precursor	2.7
Q3TXY2	clone:K530044I02 product:testis expressed gene 264	2.6
P12265	Beta-glucuronidase precursor	2.6
Q9JLZ8	Single Ig IL-1-related receptor	2.5
P01902	H-2 class I histocompatibility antigen, K-D alpha chain precursor	2.4
Q3TYD6	Serine/threonine-protein kinase LMTK2 precursor	2.3
P31996	Macrosialin precursor	2.3
Q924S8	Sprouty-related, EVH1 domain-containing protein 1	2.3
Q05910	ADAM 8 precursor	2.2
Q9D620	Rab11 family-interacting protein 1	2.2
Q9DC29	Mitochondrial ATP-binding cassette sub-family B member 6	2.1
O54782	Epididymis-specific alpha-mannosidase precursor	2.1

[a]Average ASAPRatio ratio after normalization
[b]Light:Heavy; CIP:WT

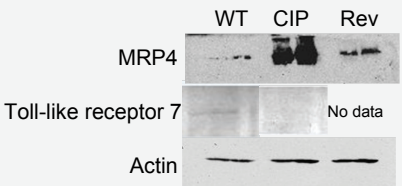

Fig.2: Western blot confirming the up and down regulation of MRP4 and Toll-like receptor 7 in WT and ciprofloxacin resistant cells. Negative control experiments were performed on reverted cells who lost resistance.

DISCUSSION:

1. MRP4, a member of the C subfamily of ABC transporters, has in previous studies already been detected as a possible candidate for ciprofloxacin efflux[1]. In this experiment the same MRP4 was found highly up regulated which is not only important as it confirms the efflux mechanism, but also validated the total SILAC GeLC-MS/MS experiment (**Fig.2**).
2. Noticeable are the down regulated subunits of a lysosomal ATPase which might indicate energy conservation mechanisms.
3. Perhaps most interesting are the identification of an up regulated Rab-11 interacting protein and lemur tyrosine kinase 2, two proteins involved in protein recycling. Together with the down regulated DNAJ (Hsp40) homolog, a protein identified as a possible inhibitor of this protein recycling pathway[4], these findings indicate that besides efflux pump up regulation, also protein recovery might play an important role in ciprofloxacin resistance.

CONCLUSION:

Here, we present two possible mechanisms for ciprofloxacin resistance. First, we confirmed up regulation of the MRP4 efflux pump, as was previously suggested[1]. Secondly, we found some indications that protein recycling and degradation also contribute to drug resistance, albeit as a secondary effect so efflux pumps are kept in the plasma membrane. Although, some findings are already confirmed in different studies using alternative techniques, further experiments to prove our hypothesis's are recommended.

References:

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2.Reidegeld, K. *et al.*, *Proteomics* 2008, **8**, 1129.
3.Hwang, S-I. *et al.*, *Mol. Cell. Proteomics* 2006, **5**, 1131.
4.Girard, M., *et al.*, *J. Biol. Chem.* 2008, **280**, 40135.