Phenotypic, genotypic and proteomic characterization of J774 macrophages upon chronic exposure to fluoroquinolone antibiotics

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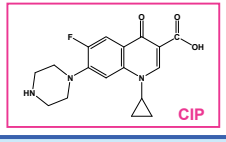
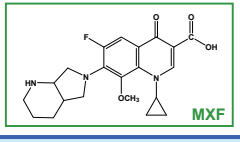
INTRODUCTION

Active efflux is a well known mechanism of resistance to chemotherapy. Overexpression of multidrug transporters can indeed be selected through exposure to sublethal concentrations of drugs substrates.

We have shown that the fluoroquinolone ciprofloxacin (CIP) is substrate for Abcc4 (a multidrug ABC transporter, belonging to the MRP subfamily) in J774 macrophages [1] while a closely-related but more lipophilic molecule, moxifloxacin (MXF), is not affected [2].

Our aim was to compare the changes in phenotype, gene expression and membrane proteome induced in J774 macrophages by exposure to a drug efflux pump substrate (CIP) or to a non-substrate (MXF). For this purpose, J774 cells were exposed to increasing concentrations of either CIP or MXF. We studied the resulting cells lines in comparison with wild-type (WT) cells.





MATERIALS & METHODS

Cells: we used wild-type J774 murine macrophages (WT), and CIP- and MXF-resistant cells obtained by long-term exposure to CIP (0.1 mM to 0.2 mM) [3] or MXF (0.1 mM to 0.18 mM) respectively.

Fluoroquinolone accumulation & efflux: cells were incubated for 2h at 37°C with 20 µg/ml of CIP or MXF, in the absence or presence of a MRP inhibitor, gemfibrozil (500 µM), then collected to measure the cell drug content by fluorimetry. For each sample, cell drug content was expressed by reference to its total protein content [1-3]. For CIP efflux, cells were incubated for 2 h at 37°C with 20 µg/ml of CIP, alone for WT and MXF-resistant cells, and with 200 µM gemfibrozil for CIP-resistant cells, transferred to CIP-free medium, reincubated for up to 30 min at 37°C and collected at various time points. Results are expressed as the percentage of the CIP cell content measured before transfer to CIP-free medium.

Real time PCR: ABC transporters mRNA expression was quantified by TaqMan Low Density Array on a 7900HT Fast Real-Time PCR System (Applied Biosystems). Samples have been tested in duplicate from two biological samples. Ten housekeeping genes have been tested and the two more stable (*Gapdh* and *Gusb*, as determined with GeNorm and NormFinder), were used to normalise gene expression as compared to WT cells.

Western Blot: Abcc2 and Abcc4 proteins were detected in whole cell extracts from WT, CIP- and MXF-resistant macrophages, respectively with M_2III-5 and M_4I-10 monoclonal antibodies. Anti-actin polyclonal antibody was used as a control. DnajC3 and Tlr7 were detected in membrane samples from WT and CIP-resistant macrophages with antiserum to mouse DnajC3 [4] and a polyclonal antibody to Tlr7 (IMG-581A); polyclonal antibody against prohibitin (H-80) was used as a control.

SILAC-based GeLC-MS/MS-proteomic analysis: WT and CIP-resistant cells were grown respectively in media with $^{13}C_6$-Lys and $^{13}C_6$-Arg or $^{12}C_6$-Lys and $^{12}C_6$-Arg. Cells homogenates (Dounce B homogenizer, 25 strokes) were separated on a discontinous sucrose gradient (sucrose solutions with densities: 1.10, 1.13, 1.15, 1.17 and 1.19) and ultracentrifuged. The resulted visible bands at interphases 1 or 2 from WT and CIP-resistant cells were collected, pooled at a 1:1 ratio and separated by SDS-PAGE. In-Gel tryptic digests were submitted to LC-MS/MS analysis. Peptides were identified with the SEQUEST algorithm, and proteins were validated and quantified with tools from the Trans-Proteomic-Pipeline (TPP).

CONCLUSIONS

- Expression of the CIP transporter **Abcc4** is differentially affected upon fluoroquinolone long-term exposure:
  - exposure to **CIP** leads to its overexpression, which is responsible for a lower accumulation and faster efflux of CIP than in WT cells;
  - exposure to **MXF** leads to a reduction of the Abcc4 protein expression that accounts for a higher accumulation and a slower efflux of CIP (the rather small reduction at the mRNA level suggests that post-transcriptional mechanisms might be involved).
- On the contrary, **Abcc2** expression is equally affected by both fluoroquinolones (increase of expression), suggesting independant regulatory mechanisms for both efflux pumps.
- Moreover, **CIP** exposure induces changes in expression of membrane proteins, involved in different pathways, which might account for an adaptative response to the antibiotic pressure.

⇨**Long-term exposure to fluoroquinolones modulates proteins expression, among which Abcc4, the CIP transporter, plays a major role in phenotype, but other (membrane) proteins might be involved in resistance.**

REFERENCES

[1] Marquez *et al.* (2009) Identification of the efflux transporter of the fluoroquinolone antibiotic ciprofloxacin in murine macrophages: studies with ciprofloxacin-resistant cells. Antimicrob. Agents Chemother. 53: 2410-2416.
[2] Michot *et al.* (2005) Influence of efflux transporters on the accumulation and efflux of four quinolones (ciprofloxacin, levofloxacin, garenoxacin, and moxifloxacin) in J774 macrophages. Antimicrob. Agents Chemother. 49: 2429-2437.
[3] Michot *et al.* (2006) Cellular accumulation and activity of quinolones in ciprofloxacin-resistant J774 macrophages. Antimicrob. Agents Chemother. 50: 1689-1695.
[4] Oyadomari *et al.* (2006) Cotranslocational degradation protects the stressed endoplasmic reticulum from protein overload. Cell 126: 727-739.

Acknowledgments

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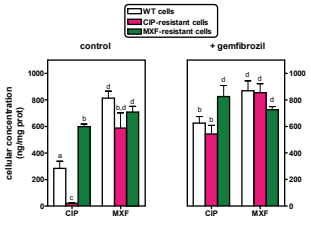
RESULTS

Effects of fluoroquinolone exposure on cell phenotype and multidrug transporters expression

Fluoroquinolone accumulation and efflux



Statistical analysis (one-way ANOVA, Tukey post-hoc test): different letters indicate significantly different values with p<0.05

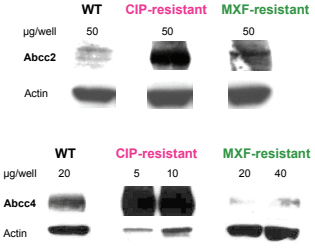
Cell type	CIP efflux $T_{1/2}$ (min)	
	control	+ gemfibrozil
WT	1.25 – 1.75	4.49
CIP-resistant	0.09	4.35
MXF-resistant	3.41	nd

Multidrug transporters expression: Abcc2 and Abcc4

Cell type	mRNA expression ratio*	
	Abcc2	*Abcc4*
WT	1	1
CIP-resistant	9.25	14.59
MXF-resistant	5.09	-1.82

* as determined by real-time PCR.

Western-blot from whole cell lysates



⇨ **Changes in CIP accumulation and efflux rate are due to variations in Abcc4 expression between cell lines.**

⇨ **MXF accumulation is not affected by Abcc4 expression level.**

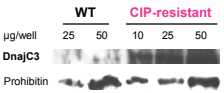
Effects of ciprofloxacin exposure on membrane proteome

⇨ Among 735 identified proteins, 25 proteins showed a higher expression and 26 proteins, a lower expression in CIP-resistant macrophages as compared to WT cells (**~ 7% of proteins with a change in expression**).

10 most over-expressed proteins in CIP-resistant cells versus WT cells

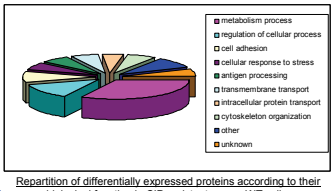
Gene name	Accession n°	Name	Log2(Ratio)
Abcc4	Q3TZN9	ATP-binding cassette, sub-family C (CFTR/MRP), member 4	5,6526
DnajC3	Q91YW3	DnaJ (Hsp40) homolog, subfamily C, member 3	3,8331
H2-D1	P01899	H-2 class I histocompatibility antigen, D-B alpha chain (H-2D^b)	2,3238
Nrcam	Q810U4	neuron-glia-CAM-related cell adhesion molecule	2,2837
Slc2a6	A2AR26	solute carrier family 2 (facilitated glucose transporter), member 6	2,0953
Iap	P03975	IgE-binding protein	1,9874
H2-D1	P01900	H-2 class I histocompatibility antigen, D-D alpha chain (H-2D^b)	1,8025
Itga9	Q3UT74	Integrin alpha 9	1,6662
H2-K1	P01901	H-2 class I histocompatibility antigen, K-B alpha chain (H-2K^b)	1,5051
Csf2rb	P26955	Colony stimulating factor 2 receptor, beta, low-affinity	1,4822
Nrp1	P97333	Neuropilin 1	1,3830

Western-blot from membrane proteins



10 most lower expressed proteins in CIP-resistant cells versus WT cells

Gene name	Accession n°	Name	Log2(Ratio)
Tlr7	P58681	toll-like receptor 7	-3,8917
DnajC13	Q3TNE7	DnaJ (Hsp40) homolog, subfamily C, member 13	-2,5450
Itgal	P24063	integrin alpha L	-2,3692
Atp6v1b2	P62814	ATPase, H+ transporting, lysosomal V1 subunit B2	-2,0039
Vim	P20152	vimentin	-1,9352
Atp6v1a	P50516	ATPase, H+ transporting, lysosomal V1 subunit A	-1,9189
Atp6v1e1	P50518	ATPase, H+ transporting, lysosomal V1 subunit E1	-1,9127
Mpeg1	A1L314	macrophage expressed gene 1	-1,7664
Fer1l3	Q69ZN7	myoferlin	-1,7581
Atp6v1d	P57746	ATPase, H+ transporting, lysosomal V1 subunit D	-1,4864



Repartition of differentially expressed proteins according to their biological function in CIP-resistant versus WT cells.

⇨ **Changes in proteins expression detected with the SILAC GeLC-MS/MS experiment were validated by WB (Abcc4, DnajC3, Tlr7).**

⇨ **CIP-resistant cells show complex modifications of their membrane proteome, with proteins involved in metabolism and regulatory pathways being the most affected.**