Expression and functionality of ABC transporters in J774 macrophages selected by chronic exposure to ciprofloxacin or moxifloxacin, two fluoroquinolone antibiotics

C. Vallet, B. Marquez, N. Nhiri*, M-P Mingeot-Leclercq, P.M. Tulkens, E. Jacquet*, F. Van Bambeke

Unité de pharmacologie cellulaire et moléculaire, Université catholique de Louvain, Brussels, Belgium
*Institut de chimie des substances naturelles, Gif sur Yvette, France
Mailing address : F. Van Bambeke, Pharmacologie cellulaire et moléculaire, UCL Bat. Van Helmont, 7370 Av. E. Mounier,1200 Brussels, Belgium, francoise.vanbambeke@uclouvain.be

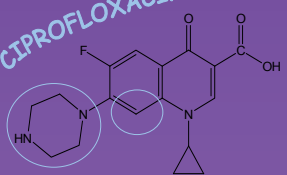
INTRODUCTION

Chronic and prolonged exposure of cells to xenobiotics can lead to a multidrug resistance phenotype associated with the overexpression of multidrug transporters from the ABC protein family.

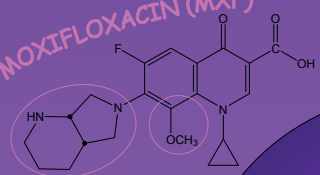
Previous work from our laboratory showed that the fluoroquinolone antibiotic ciprofloxacin (CIP) is substrate for Mrp4 (Abcc4) in mouse J774 macrophages, while moxifloxacin (MXF), another fluoroquinolone, is not [1]. Moreover, chronic exposure of J774 macrophages to CIP selects for a resistance phenotype characterized by the overexpression of Mrp4 [2].

The aim of this study is to compare cells made resistant to ciprofloxacin (**CIP-R cells**) or moxifloxacin (**MXF-R cells**) at the phenotypic, genotypic, and proteomic level.

CIPROFLOXACIN (CIP)



MOXIFLOXACIN (MXF)



MATERIALS AND METHODS

Cells : J774 mouse macrophages were gradually exposed to increasing concentrations (0.1 to 0.2mM [3]) of CIP or MXF, reaching a level that would kill wild-type cells.

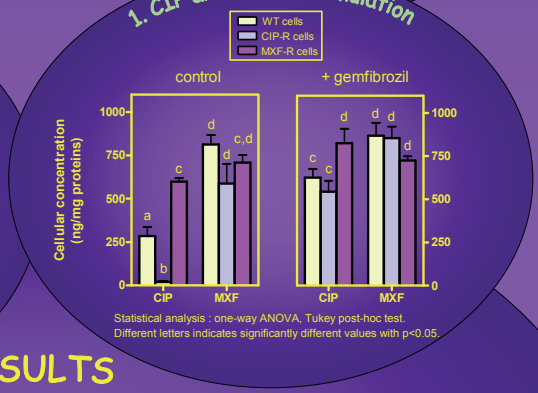
Accumulations : cells were incubated with 50µM of CIP or MXF, or with 10µM of rhodamine 123 (Rho = P-gp specific substrate), or with 0.1µM of Bodipy-Prazosin (BP = Bcrp specific substrate). Inhibitors used were gemfibrozil (Gem, 500µM), verapamil (V, 100µM), and fumitremorgin C (FTC, 10µM). Drug or substrate accumulation was measured by fluorimetry and expressed by reference to the total protein content in each sample.

CIP efflux : cells were incubated for 2h at 37°C with 50µM of CIP (in combination with Gem for CIP-R cells), and reincubated in CIP-free medium for up to 30 min. Results are expressed as the percentage of the CIP cell content measured before transfer to CIP-free medium.

Real Time PCR : 47 ABC transporters mRNA expression levels were quantified by TaqMan Low Density Array on a 7900HT Fast Real Time PCR System (Applied Biosystems). Values are the mean of duplicates from two biological samples, and two housekeeping genes (*Gapdh* and *Gusb*) were used to normalize gene expression as compared to WT cells.

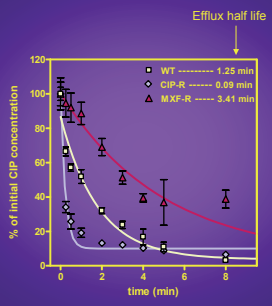
Western Blot : Mrp4 was detected in whole cell lysates with M_2I-10 monoclonal antibody (anti-actin antibody was used as control), while P-gp and Bcrp1 were detected in enriched membrane preparations with C219 and BXP-53 antibodies respectively (anti-prohibitin antibody was used as control).

1. CIP and MXF accumulation



Statistical analysis : one-way ANOVA, Tukey post-hoc test. Different letters indicates significantly different values with p<0.05.

2. CIP efflux



3. ABC transporters with modification in mRNA level

	gene	CIP-R cells	MXF-R cells
	Abca8b	1.39	76.2
	Abca9	(-17.45)	(-10.48)
P-gp →	Abcb1a	-1.63	80.38
	Abcb9	6.08	9.83
	Abcc2	(9.25)	(5.09)
Mrp4 →	Abcc4	14.59	-1.82
	Abcc8	(1.22)	(7.74)
Bcrp1 →	Abcg2	1.14	103.94

mRNA variation expression ratios as compared to WT cells.
Red background: ABC transporters with mRNA over-expression (and Ct<30)
No background: no difference in mRNA level between resistant and WT cells, and/ or mRNA with low expression (Ct>30): value in parenthesis.

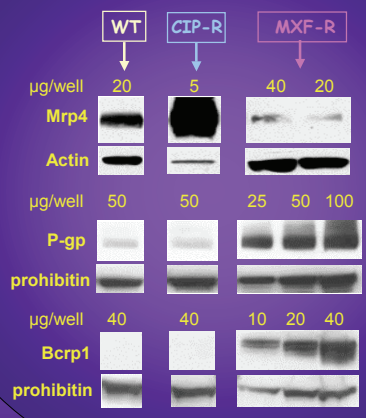
RESULTS

1 & 2 : **MXF** accumulation is high and remains unchanged in the 3 cell types, and is not affected by the presence of Gem. On the contrary, **CIP** accumulation is different in the 3 cell lines , as it is decreased in CIP-R cells and increased in MXF-R cells, as compared to WT cells. This correlates with a faster (CIP-R cells) or a slower (MXF-R cells) efflux of the drug.

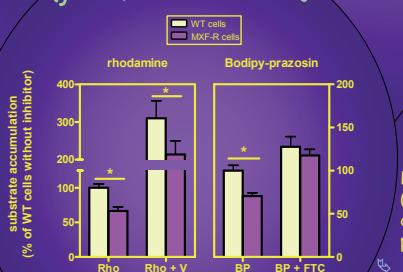
3 & 4 : CIP-R cells over-express **Mrp4** (mRNA and protein), whereas MXF-R cells show a lower expression of the **Mrp4** protein (almost no change at mRNA level). MXF-R cells show also change in the expression of other ABC transporters at mRNA level, and this was confirmed at the protein level for 2 MDR transporters **P-gp** (Abcb1a) and **Bcrp1** (Abcg2).

5 : **P-gp** and **Bcrp1** are functional in MXF-R cells : Rho and BP accumulations are decreased in MXF-R cells as compared to WT cells, and increased in the presence of specific inhibitors (verapamil for P-gp and fumitremorgin C for Bcrp1).

4. Mrp4, P-gp and Bcrp1 proteins expression



5. P-gp and Bcrp1 activities in MXF-R cells



Statistical analysis : one-way ANOVA, Tukey post-hoc test. * indicates significantly different values between 2 bars with p<0.05.

CONCLUSION

Chronic exposure of WT macrophages to a Mrp4 substrate like **CIP** or a non substrate like **MXF** induces opposite changes in the expression of Mrp4 (the CIP transporter).

Both **CIP** and **MXF** induce similar change for some ABC transporters (Abca9, Abcb9, Abcc2), but additionally **MXF** induces other specific changes affecting MDR transporters (P-gp and Bcrp1) or other ABC pumps (Abca8b).

➧ CIP-R cells are mainly characterized by Mrp4 over-expression, responsible for the decreased accumulation and faster efflux of **CIP**.

➧ MXF-R cells show tremendous modifications in their ABC transporters expression profile. They show a lower expression of the Mrp4 protein, accounting for the increased accumulation and slower efflux of **CIP** (sequencing of the Mrp4 ORF showed no mutation which could have altered CIP transport). Moreover, they over-express 2 functional MDR efflux pumps, namely: P-gp and Bcrp1. However these 2 transporters do not affect **MXF** accumulation (data not shown). Resistance of these cells to **MXF** seems therefore not to be mediated by efflux.

☞ **2 closely related fluoroquinolone antibiotics, CIP and MXF, can differentially affect ABC transporters expression, suggesting complex regulatory mechanisms** which will be the object of future investigations.

REFERENCES

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[2] 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.
[3] Michot *et al.* (2006) Cellular accumulation and activity of fluoroquinolones in ciprofloxacin-resistant J774 macrophages. Antimicrob. Agents Chemother. 50:1689-1695.

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