A-045

ICAAC IDSA 2008

Identification of the Efflux Transporter of Ciprofloxacin in Murine Macrophages: Studies with Ciprofloxacin-Resistant Cells

Béatrice Marquez, Nancy E. Caceres, Marie-Paule Mingeot-Leclercq, Paul M. Tulkens, Françoise Van Bambeke

Pharmacologie cellulaire et moléculaire, Université catholique de Louvain - Brussels – Belgium

Mailing address:

F. Van Bambeke
Pharmacologie cellulaire et moléculaire
UCL 73.70 av. Mounier 73
1200 Brussels - Belgium
francoise.vanbambeke@uclouvain.be

ABSTRACT

Background: Ciprofloxacin (CIP) is subject to active efflux in murine J774 macrophages, but the transporter, a member of the large family of Multidrug Resistance-related Proteins (MRP; see [1]) has not been identified so far.
Methods: We used cells made resistant to CIP obtained by long-term exposure to increasing CIP concentrations [2], and which show a ~ 87 % reduction of CIP accumulation vs. wild type cells. The expression of the 7 mouse *Mrp* genes (1-7) was examined by real-time PCR in comparison with wild-type cells. For overexpressed genes, (i) Western-blot analysis was performed for identification of the endoded protein; (ii) confocal microscopy was used to ascertain its localization in cells; (iii) transient silencing experiments with specific siRNA targeting known exons were made, and their impact on the corresponding RNA transcripts and protein contents, and CIP accumulation assessed.
Results: Real-time PCR showed a selective overexpression of *Mrp2* and *Mrp4* vs. wild-type cells, the expression of other *Mrp* remaining essentially unchanged. Western blots performed showed an overexpression of the corresponding proteins, and confocal microscopy confirmed their localization at the cell surface with, however, no or little colocalization. *Mrp4* transcripts were overwhelmingly predominant (45 [wild type cells] to 95 % [ciprofloxacin-resistant cells] of all *Mrp* transcripts tested). Silencing experiments with specific anti-*Mrp2* and anti-*Mrp4* siRNA showed a reduction of the transcripts and of the corresponding proteins in both cases, but only *Mrp4* silencing was associated with an increase in ciprofloxacin accumulation (in both ciprofloxacin-resistant and wild-type cells).
Conclusion: Our data identifies Mrp4 as the transporter of CIP in murine macrophages, but leaves open a possible common upregulation mechanism for both Mrp4 and Mrp2 upon chronic exposure of macrophages to CIP.

INTRODUCTION

- Fluoroquinolones are widely used, broad spectrum antibiotics, that accumulate into phagocytic cells. This property makes them active against intracellular infections [3].
- Active efflux is a well documented mechanism of resistance to fluoroquinolones in bacteria [4]. More recently, fluoroquinolones have also been shown to be substrates for eukaryotic efflux pumps belonging to the ATP-binding cassette (ABC) superfamily [5].
- Previous work of our laboratory showed that ciprofloxacin (CIP) is substrate for an ABC transporter belonging to the MRP (Multidrug Resistance-related Proteins) family in murine J774 macrophages [1]. Upon chronical exposure of wild-type (WT) cells to increasing CIP concentrations, we were able to select for CIP-resistant macrophages (RS), which accumulate CIP about 4-fold less than WT cells [2].

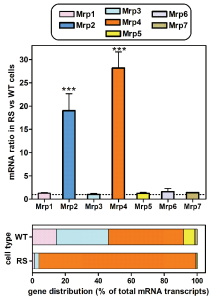
AIM OF THE STUDY

To identify the Mrp efflux pump involved in CIP efflux in murine macrophages using the following approaches:

- ✓ Quantification of the expression level of the 7 genes coding for the murine *Mrp* (real-time PCR), in wild-type and CIP-resistant macrophages.
- ✓ Determination of the expression level of the corresponding proteins in both cell lines (Western Blot).
- ✓ Specific silencing of the MRP overexpressed in CIP-resistant cells to evaluate their involvement in CIP transport (siRNA).

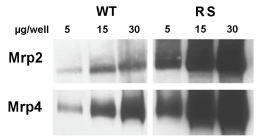
RESULTS

1. CIP-resistant macrophages over-express Mrp2 and Mrp4 at both mRNA and protein levels

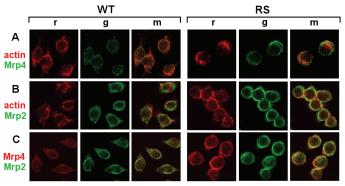


Upper panel: mRNA ratios shown are means ± SD (n=3). Statistical analysis (one-way ANOVA, Dunnett Multiple Comparisons Test): ***: p< 0.001 as compared to values in WT cells
Lower panel: mRNA transcripts (copy number as determined by real-time PCR) expressed as the percentage of the total number of *Mrp* transcripts detected starting from 1 µg purified total RNA for each cell type.
Note that the boxes corresponding to *Mrp2* and *Mrp5* are not visible because the values are smaller than the corresponding surrounding lines.

Western blot of membrane preparations



Confocal microscopy of permeabilized cells

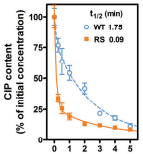


⇨ RS macrophages over-express *Mrp4* and *Mrp2* mRNA as compared to WT cells. *Mrp4* transcript is predominant among the 7 *Mrp* transcripts investigated, both in WT and RS cells (45 % and 95% of total Mrp transcripts respectively). *Mrp2* transcripts are much less abundant (< 0.1% and < 0.4% of the total in WT and RS cells respectively).

⇨ Both Mrp2 and Mrp4 proteins are overexpressed in RS macrophages and localized at the plasma membrane.

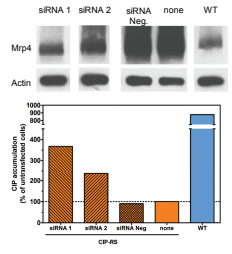
2. Only Mrp4 is involved in CIP faster efflux and lower accumulation in CIP-resistant macrophages

CIP efflux

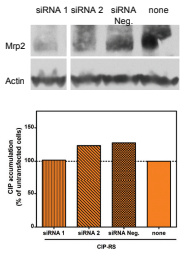


Cells were incubated for 2 h at 37°C with 50 µM CIP, alone for WT and with 200 µM gemfibrozil for RS cells, transferred to a CIP-free medium and reincubated for up to 30 min at 37°C. Results are expressed as the percentage of the CIP cell content measured before transfer to CIP-free medium. Data were best fitted to a two-phases exponential decay function.

Effects of *Mrp4* silencing



Effects of *Mrp2* silencing



RS macrophages were either transfected with specific siRNAs or with a non-targeting siRNA (siRNA Neg) at 25 nM, or left untreated (none).
Left panels: anti-*Mrp4* siRNA (# 284556, siRNA 1; #284555, siRNA 2); right panels: anti-*Mrp2* siRNA (# 161716, siRNA 1; #71770, siRNA 2).
Upper panels: Western blot analysis of whole cell extracts of the corresponding cells: left panel, 5 µg/well; right panel, 45 µg/well.
Lower panels: accumulation of CIP expressed as the percentage increase over untransfected cells. Cells were incubated with 50 µM CIP for 2 h at 37°C. Data are from a typical experiment (with measurements made in triplicate).

⇨ Silencing of *Mrp4* results in an increase in CIP accumulation in RS macrophages, whereas silencing of *Mrp2* has no effect in CIP accumulation.

MATERIALS & METHODS

Cells: we used wild-type (WT) and CIP-resistant (RS) J774 macrophages. The latter were obtained by chronical exposure of WT macrophages to increasing concentrations of CIP (0.1 mM to 0.2 mM) [2].
Real time PCR: *Mrp* mRNA expression was quantified by real-time PCR using SYBR Green detection and *Ywhaz* and *Rpl13A* as housekeeping genes. Results are expressed as a ratio of expression in RS cells versus WT cells (expression set to 1).
Western Blot: Mrp2 and Mrp4 proteins were detected in membrane preparations from WT and RS macrophages, respectively with M_2III-5 and M_4I-10 monoclonal antibodies. Cells treated with siRNA were lysed and the same antibodies were used to detect Mrp2 and Mrp4 in whole cell extracts; anti-actin polyclonal antibodies were used as a control.
Confocal microscopy: Mrp2 and Mrp4 proteins were detected in cells fixed with 0.8 % formaldehyde in acetone and permeabilized with saponin [1]. We used a polyclonal anti-mouse Mrp2 antibody [6] and M_4I-10 for Mrp4; actin was stained with rhodamine-labeled phalloidin.
Silencing of gene expression: specific siRNA targeting either *Mrp2* (#71770 and #161716) or *Mrp4* (#284555 and #284556) (Silencer® Pre-designed siRNA, Ambion) were transfected into RS macrophages with INTERFERin™. Cells were incubated at 37°C for 24h, then washed and further incubated 24h before performing CIP accumulation experiment [1,2] or Western Blot.

CONCLUSIONS

- ✓ Chronical exposure of mouse macrophages to CIP selects for resistant cells in which CIP accumulation is markedly decreased. This phenotype is due to a faster efflux of CIP.
- ✓ CIP-RS macrophages overexpress Mrp2 and Mrp4, but the expression of Mrp4 is largely predominant in both cell lines.
- ✓ Specific silencing of *Mrp4* but not of *Mrp2* causes an increase in the accumulation of CIP in RS cells.
- ✓ P-gp and Bcrp are not involved in CIP efflux in our model as there is no change in CIP accumulation in the presence of verapamil or elacridar, two known inhibitors of these pumps (data not shown).
- ⇨ **Mrp4 is the efflux transporter for CIP in murine macrophages and its expression is increased upon selection with CIP.**

REFERENCES

[1] Michot *et al.* (2004) Active Efflux of Ciprofloxacin from J774 Macrophages through an MRP-Like Transporter. Antimicrob. Agents Chemother. 48:2673-2682.
[2] Michot *et al.* (2006) Cellular accumulation and activity of quinolones in ciprofloxacin-resistant J774 macrophages Antimicrob. Agents Chemother. 50:1689-1695.
[3] Carryn *et al.* (2003) Intracellular pharmacodynamics of antibiotics. Infect Dis Clin North Am 17: 615-634.
[4] Van Bambeke *et al.* (2000) Antibiotic efflux pumps. Biochem. Pharmacol. 60:457-470.
[5] Alvarez *et al.* (2008) Fluoroquinolone efflux mediated by ABC transporters. J Pharm.Sci. 97:3483-93.
[6] Soontornmalai *et al.* (2006) Differential, strain-specific cellular and subcellular distribution of multidrug transporters in murine choroid plexus and blood-brain barrier. Neuroscience 138: 159-169.

ACKNOWLEDGEMENTS

We thank J.-M. Fritschy (Institute of Pharmacology and Toxicology, University of Zurich, Zurich, Switzerland) for the kind gift of the polyclonal anti-mouse Mrp2 antibody.
This research was supported by the *Fonds de la Recherche Scientifique Médicale* (grants no. 3.4.542.02, 3.4.639.04, 3.4.597.06, and 3.4.583.08), the *Fonds de la Recherche Scientifique* (grant no. 1.5.195.07), the Belgian Federal Science Policy Office (Research project P5/33 [research action P5] and P6/19 [research action P6]), and a grant-in-aid from the *Association Mucoviscidose: ABCF.*

This poster will be available for download after the meeting at: http://www.facm.ucl.ac.be/posters.htm