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Chloroquine enhances the antimycobacterial activity of isoniazid and pyrazinamide by reversing inflammation-induced macrophage efflux

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ABSTRACT

Mycobacterium tuberculosis (MTB) is notorious for persisting within host macrophages. Efflux pumps decrease intracellular drug levels, thus fostering persistence of MTB during therapy. Isoniazid (INH) and pyrazinamide (PZA) are substrates of the efflux pump breast cancer resistance protein-1 (BCRP-1), which is inhibited by chloroquine (CQ). In this study, BCRP-1 was found to be expressed on macrophages of human origin and on foamy giant cells at the site of MTB infection. In the current in vitro study, interferon-gamma (IFNγ) increased the expression of BCRP-1 in macrophages derived from the human monocytic leukaemia cell line THP-1. Using a BCRP-1-specific fluorescent dye and radioactively labelled INH, it was demonstrated that efflux from macrophages increased upon activation with IFNγ. CQ was able to inhibit active efflux and augmented the intracellular concentrations both of INH and the dye. In agreement, CQ and specific inhibition of BCRP-1 increased the antimycobacterial activity of INH against intracellular MTB. Although PZA behaved differently, CQ had comparable advantageous effects on the intracellular pharmacokinetics and activity of PZA. The adjunctive effects of CQ on intracellular killing of MTB were measurable at concentrations achievable in humans at approved therapeutic doses. Therefore, CQ, a widely used and worldwide available drug, may potentiate the efficacy of standard MTB therapy against bacteria in the intracellular compartment.

1. Introduction

With an estimated 1.5 million deaths from tuberculosis (TB) in 2013, it remains among the most important causes of death due to an infectious agent [1]. The duration of standard short-course treatment for susceptible TB with the combination of four antituberculous drugs, comprising isoniazid (INH), pyrazinamide (PZA), rifampicin and ethambutol, lasts ≥6 months. Unfortunately, exposure to antibiotics for 6 months results in considerable hurdles, including significant adverse drug events, poor patient adherence, high treatment costs and insecure access to drugs with logistic bottlenecks. In part, these shortcomings of standard TB treatment contributed to the development of multidrug-resistant TB [2]. Therefore, shortening TB therapy has become a research priority. Disappointingly, recent attempts to shorten the duration of treatment were not successful [3,4]. The difficulties encountered include mycobacterial factors, the host response and pharmacological compartmentalisation [5–7].

It is well described that *Mycobacterium tuberculosis* (MTB) survives intracellularly in macrophages by manipulating their defence system, thereby contributing to bacterial persistence [8]. Likewise, the intracellular localisation increases the complexity of pharmacodynamics by separating the bacterial population into two different subpopulations, i.e. extracellular and intracellular MTB [7]. Indeed, there is convincing evidence from animal models and observations in humans that macrophages provide an intracellular niche where MTB survives treatment [8–10].

Efflux pumps are localised on the plasma membrane and on endosomal membranes of macrophages. They are necessary to clear potentially toxic xenobiotics from the cell [11–13]. In the context of MTB infection, efflux pumps are thought to reduce the intracellular concentration of antibiotics, thus inadvertently providing an

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environment with reduced drug concentrations where bacteria can survive during antimicrobial treatment [8,9,14]. To deepen our understanding of active transport of anti-TB drugs, the interaction of anti-TB drugs with efflux pumps such as breast cancer resistance protein-1 (BCRP-1, ABCG2) is interesting. INH and PZA are predicted to be substrates for BCRP-1 based on their physical and chemical properties [15] and were proven to be substrates in a plasma membrane model (Anna Seelig, pers. comm.). Chloroquine (CQ) is a highly efficient inhibitor of BCRP-1 [16,17].

In this study, the interaction of two first-line anti-TB drugs (INH and PZA) with their specific efflux pump BCRP-1 was investigated. In particular, we hypothesised that CQ could increase the intracellular drug concentration and enhance the anti-TB activity of INH and PZA via inhibition of BCRP-1.

2. Materials and methods

2.1. Cells and flow cytometry

THP-1 cells (human monocytic leukaemia cell line) were cultured and differentiated to macrophages as previously described [18]. Cells were labelled with biotin-conjugated anti-BCRP-1 antibody (Abcam, Cambridge, UK) or an isotype control antibody provided by the manufacturer in 1% bovine serum albumin/phosphate-buffered saline (BSA/PBS) (Sigma-Aldrich, St Louis, MO) for 30 min at 4 °C. After washing, cells were incubated with streptavidin-conjugated phycoerythrin (Abcam) at 4 °C. Finally, cells were washed three times and were analysed with a CyAn™ ADP flow cytometer (Beckman Coulter, Munich, Germany).

2.2. Tissue samples and immunohistochemistry

Five formalin-fixed paraffin-embedded samples of lymph node TB were retrieved from the archives of the Department of Pathology of University Hospital Heidelberg (Heidelberg, Germany) and were transferred to the tissue bank of the National Center for Tumor Diseases (Heidelberg, Germany). Tissue samples were used in accordance with the regulations of the tissue bank and following approval of the Ethics Committee of Heidelberg University. Infection with mycobacteria was confirmed by microscopy (Ziehl–Neelsen staining) and subsequent PCR restriction enzyme analysis of the *hsp65* gene.

Anti-BRCP-1 (Abcam; clone BXP-21, dilution 1:200) was used for immunohistochemistry. For the detection of bound primary antibody, a DAKO Real Detection Multilink System (Agilent Technologies, Santa Clara, CA) with goat anti-mouse, anti-rabbit and anti-sheep antibodies was used. After antigen retrieval (citrate buffer, pH 6 in a steam pot), sections were blocked for endogenous avidin/biotin activity (Linaris, Dossenheim, Germany). Subsequently, sections were incubated for 30 min at room temperature with the primary antibody, were washed and were incubated with the respective secondary antibodies for 20 min at room temperature. Sections were then incubated with horseradish peroxidase for 12 min at room temperature and were counterstained with haematoxylin. Immunohistochemical staining was evaluated by a pathologist.

2.3. Dye efflux measurements

Following differentiation, cells were incubated with interferon-gamma (IFNγ) (BioLegend, San Diego, CA) at a concentration of 50 ng/mL. Subsequently, cells were washed with PBS and were incubated with 16.5 μM Hoechst 33342 fluorescent dye (Life Technologies, Carlsbad, CA) for 30 min. After washing in PBS, cells were re-incubated in dye-free medium for 30 min in the absence or presence of the BCRP-1 inhibitor YHO-13177 (Calbiochem, San Diego, CA)) or CQ (Sigma-Aldrich) at 1 μM. After 30 min, supernatants were removed and the extracellular dye was measured by spectrofluorometry (excitation 350 nm/emission 461 nm) using the default settings of SoftMax® Pro v.5 software (Molecular Devices, Sunnyvale, CA).

2.4. Accumulation of [^{14}C]-labelled drugs

Changes in intracellular concentrations of INH and PZA in the presence of different concentrations of BCRP-1 inhibitor or CQ were determined using [^{14}C]-PZA and [^{14}C]-INH purchased from ANAWA Trading SA (Zurich, Switzerland) [19]. Differentiated and activated THP-1 macrophages were incubated for 60 min with [^{14}C]-INH and unlabelled INH or with [^{14}C]-PZA and unlabelled PZA to achieve the following final radioactivity concentrations: for INH, 0.01 μCi/mL and 0.05 μCi/mL with a mass concentration of 1 mg/L; and for PZA, 0.05 μCi/mL with a mass concentration of 30 mg/L.

Following incubation, cells were washed twice with ice-cold NaCl 0.9% and were then re-suspended in liquid scintillation cocktail (Ultima Gold™ solution; PerkinElmer, Waltham MA). Radioactivity was measured in a liquid scintillation counter (WALLAC 1410; PerkinElmer Wallac Inc., Turku, Finland). Experiments were performed in triplicate.

2.5. Bacterial strains and growth conditions

Mycobacterium tuberculosis H37Rv #1424, a derivative of *M. tuberculosis* H37Rv carrying a non-restrictive *rpsL* mutation (RpsL, 42 Lys→Arg) conferring streptomycin resistance [20], was grown on Middlebrook 7H10 agar or in Middlebrook 7H9 liquid broth (BD Difco, Sparks, MD) supplemented with oleic acid–albumin–dextrose–catalase (OADC) (BD BBL, Sparks, MD). Middlebrook 7H9 liquid broth was supplemented with Tween 80 (0.05% v/v) to avoid clumping. For expression of green fluorescent protein (GFP) in *M. tuberculosis* H37Rv #1424, the vector pOLYG-Pr-GFP was transformed by electroporation (see Supplementary methods). Transformants were selected and propagated in the presence of hygromycin B (25 μg/mL) (Invitrogen, Carlsbad, CA).

2.6. Drug susceptibility testing of Mycobacterium tuberculosis

Two-fold microdilution chequerboard plates were produced with the robotic platform Freedom EVO 100 (Tecan, Männedorf, Switzerland) in 384-well plates containing 20 μL of the combinations INH/CQ (concentrations, INH 0.004–1.6 μg/mL; CQ 0.08–40 μg/mL) and INH/BCRP-1 inhibitor (concentration, BCRP-1 inhibitor 0.008–4 μM) as well as PZA/CQ (concentration, PZA 0.1–480 μg/ml) and PZA/BCRP-1 inhibitor.

Mycobacterium tuberculosis H37Rv #1424 harbouring the pOLYG-Pr-GFP vector was grown in a roller bottle at 37 °C. When mid-exponential phase was reached, the bacterial suspension was diluted to an optical density at 600 nm of 0.040 ± 0.005 in Middlebrook 7H9 broth. Then, 20 μL of this bacterial suspension was added to the 384-well microdilution chequerboard plates. Subsequently, GFP intensity was measured on a Bio-Tek Synergy™ HT microplate reader (Bio-Tek Instruments, Winooski, VT) with filter sets of 485 ± 20 nm excitation and 528 ± 20 nm emission to determine the initial fluorescence intensity. After 10 days of incubation at 37 °C, fluorescence intensity was measured to determine growth.

2.7. Intracellular growth experiments

Differentiated THP-1 macrophages were grown in 24-well plates (TPP, Trasadingen, Switzerland) at 7×10^5 cells per well and 2 mL of RPMI 1640 (Gibco, Life Technologies, Paisley, UK) per well supplemented with 10% foetal bovine serum (Gibco, Life Technologies). Bacterial suspensions were prepared using cultures grown to