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NOTE

The ATP synthase inhibitor bedaquiline interferes with small-molecule efflux in *Mycobacterium smegmatis*

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In order to counteract the development of drug-resistant tuberculosis, new chemotherapy regimen based on novel anti-mycobacterial drugs are urgently needed. Bedaquiline (BDQ), formerly known as TMC207, showed strong potency in clinical trials,[1] and recently received approval by the US Food and Drug Administration and by the European Commission for treatment of multidrug-resistant tuberculosis. BDQ acts as specific inhibitor of mycobacterial ATP synthase,[2,3] an essential enzyme in mycobacterial energy metabolism[4] that uses the energy of a proton motive force across the bacterial plasmatic membrane for synthesis of ATP.[5]

It has been hypothesized that an inhibitor of ATP synthesis, such as BDQ, may interfere with drug efflux.[6–8] Active efflux mediated by efflux pumps has been implied for resistance to antibacterial drugs in *Mycobacterium tuberculosis*[9,10] and in the non-pathogenic model strain *M. smegmatis*.[11,12] Prevention of drug efflux therefore may significantly enhance the potency of anti-tubercular drugs.[9,10] Efflux pumps are membrane transporters that can extrude a broad range of potentially harmful small molecules from the bacterial cytoplasma or periplasma. These pumps utilize the energy of either ATP (primary transporters or ATP-dependent efflux pumps) or the proton motive force (secondary transporters) for drug extrusion. An inhibitor of ATP synthesis, which depletes cellular ATP reserves, may concomitantly indirectly inactivate ATP-dependent efflux pumps.

In the current paper, we report a proof-of-principle test of this hypothesis and investigate whether the ATP synthase inhibitor BDQ decreases efflux of a small molecule in a *Mycobacterium*.

M. smegmatis (strain mc^2 155) was kindly provided by B.J. Appelmelk, Department of Molecular Cell Biology and Immunology, VU University Medical Center Amsterdam. Verapamil, rifampin (RIF) and isoniazid (INH) were purchased from Sigma-Aldrich (Hamburg, Germany). BDQ was kindly provided by Janssen Infectious Diseases BVBA, Beerse, Belgium. Stock solutions used were BDQ (20 mM) in dimethyl sulphoxide, RIF (32 mg ml^{-1}) in absolute methanol, INH (40 mg ml^{-1}) in distilled water and verapamil (30 mg ml^{-1}) in distilled water. *M. smegmatis* mc^2 155 was grown in 7H9/OADC (Becton Dickinson, Breda, The Netherlands) supplemented medium (50 ml) at 37 °C to an OD_{600} of 0.8. The culture was aliquoted and treated with different concentrations of BDQ or other antibacterials as controls. After the treatment, the culture was centrifuged at 13 000 r.p.m. for 3 min, and the pellet was washed twice and re-suspended in phosphate-buffered saline. After adjusting the OD to 0.4, glucose and ethidium bromide were added to the bacterial suspension (final concentrations 0.4% and 1 μg ml^{-1}, respectively). Ethidium bromide fluorescence was measured for 45 min at excitation and emission wavelengths of 530 and 600 nm[11] using a Cary Eclipse Fluorescence spectrophotometer (Varian Inc., Midddelburg, The Netherlands) equipped with a plate heater set at 37 °C. Cellular ATP levels were determined using the luciferase bioluminescence method described previously.[13] In brief, 1.0 ml samples taken from bacterial cultures grown and treated with antibacterials as described above were centrifuged at 8000 *g* for 10 min. The pellets were re-suspended in 50 μl water, and a 10-fold volume of boiling 100 mM Tris–HCl and 4 mM EDTA (pH 7.75) was added. After incubation at 100 °C for 2 min, the samples were centrifuged (1000 *g*, 60 s) and the supernatants were transferred to fresh tubes. A quantity of 100 μl luciferase reagent (ATP Bioluminescence assay, Roche, Basel, Switserland) was added to 100 μl sample, and luminescence was measured with a Luminometer (LKB, Turku, Finland).

In order to elucidate the impact of BDQ on small-molecule efflux, we determined the intracellular levels of ethidium bromide in *M. smegmatis* during the initial days of BDQ treatment. *M. smegmatis* displays similar susceptibility to BDQ as *M. tuberculosis* (MIC_{90} is 0.007 mg l^{-1} for *M. smegmatis* and 0.03 mg l^{-1} for *M. tuberculosis*[2]). Ethidium bromide is widely used as fluorescent reporter for assessment of efflux function in bacteria.[11,12] For bacteria that were grown in the absence of BDQ, the ethidium bromide fluorescence increased with time until saturation, when equilibrium was reached between ethidium bromide influx and efflux (Figure 1a). As a control, addition of the efflux pump inhibitor verapamil to the assay mixture increased cellular ethidium bromide levels (Figure 1a), as reported previously.[11] *M. smegmatis* treated with BDQ for 2 days also displayed enhanced ethidium bromide fluorescence (Figure 1a). The increase

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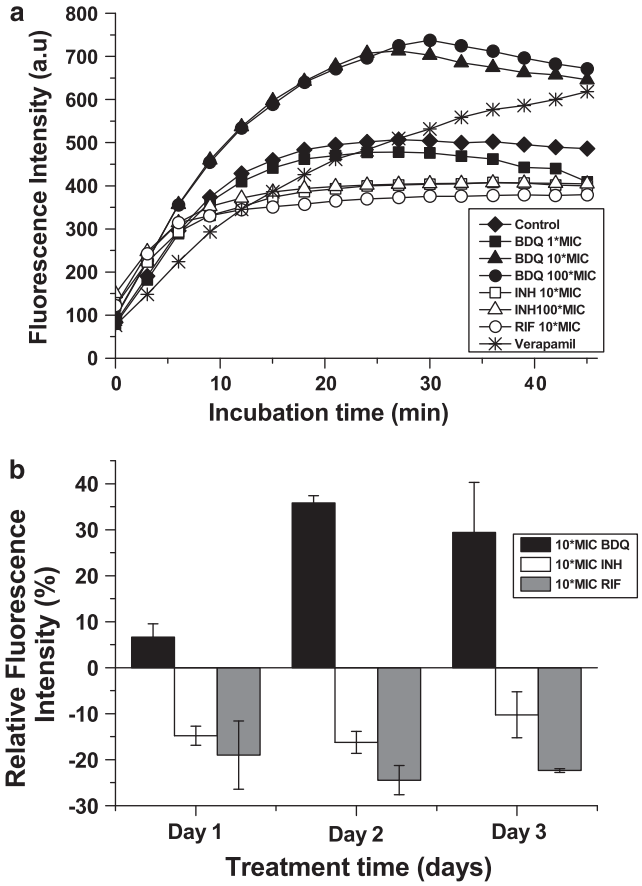

Figure 1 Effect of bedaquiline (BDQ) on the accumulation of ethidium bromide in *M. smegmatis*. (**a**) Dependency of cellular ethidium bromide levels on the applied BDQ dose. Assays were performed at 37 °C using *M. smegmatis* pretreated for 2 days with the indicated concentrations of BDQ. Rifampin (RIF) and isoniazid (INH) were used as controls, for RIF no experiments with 100× MIC were done owing to insufficient drug solubility. Verapamil was included in the assay mixture as control of efflux inhibition. Full diamonds: untreated control; full squares: BDQ 1× MIC; full triangles: BDQ 10× MIC; full circles: BDQ 100× MIC; open squares: INH 10× MIC; open triangles: INH 100× MIC; open circles: RIF 10× MIC; and asterisks: verapamil (150 mg l^{-1}). Three independent experiments were performed, representative results are shown. (**b**) Dependency of cellular ethidium bromide levels on the duration of antibacterial treatment. Assays were performed at 37 °C using *M. smegmatis* pretreated for the indicated time with antibacterials (10× MIC): BDQ (black bars), INH (white bars) and RIF (gray bars). The ethidium bromide fluorescence intensity at 45 min time point relative to untreated controls is shown. Bars represent the mean values and s.d. from three independent experiments.

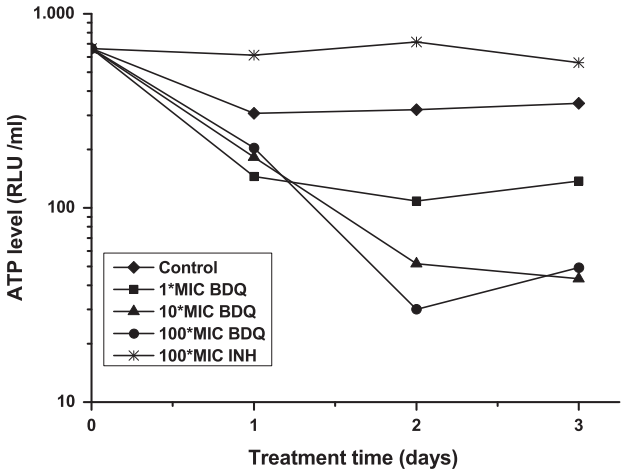

Figure 2 Cellular ATP levels of *M. smegmatis* during the initial days of bedaquiline (BDQ) treatment. Assays were performed at 37 °C using *M. smegmatis* treated for the indicated duration with different concentrations of BDQ. ATP levels were determined using the bioluminescence method and are displayed as relative luminescence units (RLUs). Diamonds: untreated control; squares: BDQ 1× MIC; triangle: BDQ 10× MIC; circles: BDQ 100× MIC; and asterisks: INH 100× MIC. Representative results from two experiments are shown.

was dose dependent, and bacteria treated with 10× or 100× MIC showed significantly elevated ethidium bromide levels (>50% fluorescence enhancement at 45 min time point), whereas samples at 1× MIC BDQ resembled the untreated control (Figure 1a). This result indicates that BDQ interferes with ethidium bromide efflux. The effect is BDQ specific, as typical front-line antibacterials such as RIF (10× MIC) and INH (10× and 100× MIC) did not cause any efflux inactivation under the same conditions (Figure 1a; MICs of *M. smegmatis* for RIF and INH are 32 and 4 mg l^{-1}, respectively).

We investigated whether inactivation of ethidium bromide efflux depends on the duration of BDQ treatment. Ethidium bromide efflux in *M. smegmatis* treated with BDQ (10× MIC) decreased with increasing duration of treatment. While only a minor efflux decrease was observed after 1 day, the effect was significantly more pronounced after 2 and 3 days of treatment (Figure 1b). In controls, INH and RIF did not decrease efflux on any of the 3 days investigated (Figure 1b). The results are consistent with the view that the decline of ethidium bromide efflux is caused by slow inactivation of efflux pumps. ATP-driven efflux pumps may become inactivated because of draining their energy source upon inhibition of ATP synthase by BDQ. Alternatively, potential downregulation of efflux pumps, as observed in a recent proteome study for *M. tuberculosis* in response to BDQ,[14] may contribute to the observed efflux decrease. Depletion of cellular ATP pools in *M. tuberculosis* occurs gradually within the initial days of BDQ treatment.[14] We tested whether BDQ has a similar effect on *M. smegmatis* and whether the reduction of bacterial ATP pools in the initial days of treatment correlates with the efflux decrease. As depicted in Figure 2, BDQ induced approximately a 10-fold decline of cellular ATP levels in *M. smegmatis* after 2 days of treatment at 10× or 100× MIC (Figure 2). As control, INH, an inhibitor of cell envelope biosynthesis, did not decrease bacterial ATP levels (Figure 2). Cellular ATP levels in mycobacteria typically are in the range of 2–5 mM.[13,15] Reduction of ATP levels by ~10-fold may thus well interfere with the activity of ATP-dependent efflux pumps, which typically display K_M values of 0.1–1 μM.[16,17] For this reason, we regard it as likely that the inactivation of efflux in the presence of BDQ is at least in part caused by inactivation of ATP-driven efflux pumps owing to depletion of their energy supply.

This inactivation of drug efflux may in part explain why BDQ can suppress the emergence of resistance to front-line anti-tuberculosis drugs.[18] Other drugs targeting components of energy metabolic pathways in mycobacteria, such as the phenothiazines or imidazopyridines,[8,19,20] may display similar properties to BDQ in terms of interfering with efflux by depleting bacterial energy reserves. This principle may also be applicable in non-mycobacterial pathogenic strains, as recently derivates of BDQ with potency against Gram-positive bacterial pathogens have been found.[6]

CONFLICT OF INTEREST

CV, KA and AK are the employees of Janssen. The remaining authors declare no conflict of interest.

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