Effect of n-octanesulphonylacetamide (OSA) on ATP and protein expression in Mycobacterium bovis BCG

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Objective: To determine the effect on BCG of \(n\)-octanesulphonylacetamide (OSA), a novel compound of the class \(\beta\)-sulphonylcarboxamides, which has potent in vitro activity against pathogenic mycobacteria.

Methods and results: The effect of OSA in BCG was examined using two-dimensional protein electrophoresis. Treatment of BCG with OSA resulted in overexpression of two proteins identified as the \(\beta\)-subunit of ATP synthase (Rv1306) and a 17 kDa heat shock protein (Rv0251c). \([35S]\)Methionine pulse-labeling revealed that overexpression occurred within as little as 3.5 h post-exposure. These results were confirmed by RT–PCR. ATP levels decreased in OSA-treated BCG at 5 min, and 1, 3 and 24 h, with a 64%, 45%, 54% and 73% reduction in ATP, respectively. Only dicyclohexylcarbodiimide (DCCD), a known ATP synthase inhibitor, had a similar effect. No appreciable difference in ATP level was observed in BCG treated with standard antimycobacterial drugs, additional respiratory chain inhibitors or a fatty acid synthase inhibitor at a comparable time-point. Protein synthesis decreased within 5 min of exposure to OSA (56%), DCCD (74%) and thenoyltrifluoroacetone (TTFA) (77%). Ethanol (2.3%) potentiated the activity of OSA. In contrast, no synergic effect was observed with streptomycin and ethanol. Mycolic acid levels decreased 79% with DCCD, 46% with TTFA, a complex II inhibitor, and 43% with OSA compared with untreated controls.

Conclusions. Our results suggest that OSA may interfere directly or indirectly with ATP synthase and possibly other components of the mycobacterial respiratory chain. These effects may hinder energy production, leading to interruption in the synthesis of large macromolecules including proteins and mycolic acids.

Keywords: growth inhibition, mycobacterial respiration, mycolic acids, ethanol metabolism, 2-D protein electrophoresis

Introduction

Tuberculosis (TB) remains a significant health problem in the USA and globally. TB is the leading cause of death due to a single infectious agent in the world. Approximately 1.86 billion people or 32% of the world’s population are infected with Mycobacterium tuberculosis.\(^1\) There are about 8 million new active cases of TB per year and approximately 2 million deaths.\(^2\)–\(^5\) This translates into a mortality rate of 200 people every hour and 5000 people every day.\(^3\) Patients with HIV infection demonstrate a significantly increased susceptibility to \(M.\) tuberculosis with an \(\sim\)50-fold risk increase over patients without HIV.\(^6\)–\(^7\)

Similarly, the rate of progression of latent TB to active disease following initial infection is \(>40\)% compared with \(\sim5\)% in non-HIV-infected individuals. With the continued expansion of HIV globally, particularly in Asia and the Indian subcontinent, the incidence and mortality of TB can only be expected to increase.\(^2\)

In conjunction with the spreading epidemic of TB has been the emergence of drug resistance.\(^8\)–\(^10\) Increasing incidence of \(M.\) tuberculosis strains resistant to one or more of the standard first-line agents intensifies the need for the identification of novel targets and new drug development. Multidrug-resistant (MDR)-TB is difficult and expensive to treat, as well as being associated with significantly higher mortality rates than drug-susceptible

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TB.8,11,12 In the absence of effective prevention and therapeutic measures, MDR-TB will become an increasing and uncontrollable problem.

A significant need exists for improved TB drugs with reduced toxicity, activity against MDR-TB, alternative mechanisms of action and activity against latent disease. Several promising drug classes are under development including long-acting rifamycins, fluoroquinolones,13–15 oxazolidinones16 and nitrimidazoles.17 Given the success rate of new compounds coming to clinical use, ~0.5%, there is a need for discovery and identification of new unique mycobacterial targets for development. No new antimycobacterial drugs with novel mechanisms of action have been developed in the past 30 years.

n-Octanesulphonylacetamide (OSA) is a novel compound of the class β-sulphonylcarboxamides. As a class, these compounds have potent in vitro activity against pathogenic mycobacteria, including MDR-TB.18,19 β-Sulphonylcarboxamides were originally designed as inhibitors of the β-ketoacyl synthase step in fatty acid synthesis. However, earlier studies on the mechanism of action of OSA indicate that the highly specific, antimycobacterial activity of this β-sulphonylcarboxamide is not due primarily to fatty acid synthesis inhibition.19 In this study we examined additional OSA-mediated effects in Mycobacterium bovis BCG cultures using two-dimensional (2-D) gel electrophoretic protein profiles in response to various levels of OSA with concurrent sequencing of the overexpressed proteins and relevant biochemical assays. The OSA-mediated overexpression of two proteins, the b-subunit of ATP synthase (Rv1306) and hsp (Rv0251c) suggests a novel mechanism of action.

Materials and methods

Mycobacteria and culture conditions

*M. tuberculosis* (H37Rv) and *M. bovis* BCG (BCG, Pasteur strain, ATCC 35734) were used in this study. Strains were maintained on Lowenstein–Jensen agar slants or Middlebrook 7H10 agar plates (Difco, Detroit, MI, USA). For all assays, *M. bovis* BCG cultures were grown at 37°C in Middlebrook 7H9–ADC–Tween broth (Difco) on a rotary shaker to mid-log phase (OD600 0.3–0.4).

Compounds

Stock and working solutions of OSA (Craig Townsend, Johns Hopkins University, Baltimore, MD, USA), dicyclohexylcarbodi-imide (DCCD; an ATP synthase-specific inhibitor; ICN, Costa Mesa, CA, USA), thienyltrifluoroacetone (TTFa; a respiratory complex II inhibitor; ICN), rotenone (a respiratory complex I inhibitor; ICN), dicumarol (an alternative dehydrogenase inhibitor; ICN) and cerulenin (a fatty acid synthase inhibitor; Sigma–Aldrich, St Louis, MO, USA) were made up in dimethyl sulphoxide (DMSO; Sigma). Mixtures containing cells and lysis solution were bead-beaten at maximum speed for 60 s in a Biospec Mini-8 beadbeater using 200–300 μm glass beads (Sigma).

Protein determinations

Protein assays were performed according to established protocols. Briefly, *M. bovis* BCG cultures were grown in Middlebrook 7H9–ADC–Tween broth to early log phase (OD600 0.3–0.4). At this time, either DMSO (diluent), or OSA (MIC 6.25 mg/L; 16× MIC 100 mg/L), cerulenin (24 mg/L), DCCD (15 mg/L) or isoniazid (1.0 mg/L) was added to 20 mL control and treated broth cultures, respectively. Following additional incubation for 24 h under the same conditions, cells were harvested by centrifugation (13 000 g for 30 s), the supernatant removed and the process repeated once using 1 × PBS. The cellular contents of each tube were divided into two or three Eppendorfs (3–5 OD units per tube), and 250 mL of a lysis solution containing 3 M urea (Sigma), 0.5% Triton X-100 (Sigma), 500 mg dithiothreitol (DTT; Gibco BRL, Life Technologies, Gaithersburg, MD, USA) and 500 mL Pharmalyte (Pharmacia Biotech, Piscataway, NJ, USA) was added. Subsequently, phenylmethylsulphonyl fluoride (PMSF; Sigma) (100 mg/mL) and leupeptin (2 mg/mL) (Sigma) were also added. Mixtures containing cells and lysis solution were centrifuged (13 000 g) for a minimum of 30 s and the protein-containing supernatant removed. Protein concentration was determined using a standardized colorimetric assay (Coomassie Plus; Pierce, Rockford, IL, USA) and BSA standards (Pierce) in a Shimadzu UC-1201 spectrophotometer. Quantified samples were aliquoted and frozen at −70°C.

For time-course studies *M. bovis* BCG broth cultures (500 mL) were grown as described above (OD600 0.3–0.4) and split into 150 mL aliquots and either DMSO or OSA (100 mg/L) was added to respective control and treated cultures followed by incubation and aeration at 37°C for 1 h. Aliquots (20 mL) were removed from each culture at hourly intervals during the first 4 h after addition of compound, with extra time-points at 16, 24 and 48 h. Cells were harvested by low-speed centrifugation, washed once in sterile distilled water and frozen at −70°C. For determination of protein synthesis BCG was grown at 37°C, with shaking, to mid-log phase (OD600 0.3–0.4) and split into 150 mL aliquots. At this time, either DMSO, or OSA (100 mg/L), DCCD (100 mg/L) or TFFa (100 mg/L), respectively, were added to control and treated cultures as outlined above. Subsequently, 5.0 mCi/mL of [35S]methionine was added to each culture followed by incubation at 37°C. Aliquots (40 mL) were removed from each culture at 5 min, 1 and 3 h, the proteins extracted and quantified as described above. [35S]Methionine incorporation was examined by scintillation counting of quantified protein samples and expressed as cpm/mg protein.

For 2-D gels and sequencing ~250 mg of each protein sample was mixed with a solution containing 8 M urea, 0.5% Triton X-100, Pharmalyte 3–10, DTT and a few grains of Bromophenol Blue (Sigma) (240 mL total volume/sample). This mixture was used to rehydrate commercially prepared acrylamide pH strips (gradient 4–7) overnight at room temperature using the manufacturer’s standard protocols (Pharmacia Biotech). Completely rehydrated strips were subsequently subjected to a 2-D protein gel system according to standardized protocols (Pharmacia Biotech) [first dimension: 16 h at 20°C, followed by equilibration of individual gel strips in a solution containing Tris–HCl (pH 6.8), urea (8 M), glycerol (30%) (Sigma), SDS (1 mg/mL) (Sigma) and either DTT or iodoacetamide (Sigma)]. Following equilibration, strips were applied to commercially prepared acrylamide gels (245 × 110 × 0.5 mm, gradient 8–18%; Pharmacia) and run for 2 h at 150 V until the manufacturer’s standardized protocols (Pharmacia). Molecular weight standards (size range: 14–200 kDa) were purchased from Gibco BRL, Life Technologies and 10 μL loaded per gel. Proteins were visualized by Coomassie Blue (Sigma) staining of gels overnight. Excess stain was removed with two or three washes of methanol/water/acetic acid (9:9:2; Sigma). Proteins of interest from four gels were pooled and the excised gel fragments were washed twice in 50%
Reverse transcriptase–PCR

Total RNA was extracted with 1 mL of Trizol reagent (Invitrogen, Carlsbad, CA, USA) from 15 mL cultures of BCG treated for 15 min with either DMSO (diluent) or OSA (20 mg/L). Subsequently, bacterial cells were homogenized in a mini-beadbeater for 30 s (twice) and chloroform was added to the bacterial lysate. Total RNA was precipitated with iso-propanol, washed with ethanol and resuspended in distilled water (DNase, RNase free; Invitrogen). Total RNA (0.5–1.0 μg) was digested with two units of DNase I (Qiagen, Valencia, CA, USA) and purified with RNeasy mini-kit (Qiagen). Reverse transcription to cDNA was carried out according to the manufacturer’s instructions using 2 μg of RNA and Super Script II, RNase H-reverse transcriptase (Invitrogen).

Forward and reverse primers were designed using MacVector software (Oxford Molecular Ltd, Oxford, UK) for the b-subunit of ATP synthase (atpF, Rv1306) and hsp (Rv0251c). They are as follows: atpF, 5′ primer, 25185-GGATCCATGGGTGAAGGG-25202; 3′ primer, 25700-CTCGAGTTACCTCGTCGC-25702; hsp, 5′ primer, 15471-CGGTTGCTGGTTGGAAACTG-15490; 3′ primer, 15191-CTTCGTGATGGCGATGCG-15172. Primers for the constitutively expressed control gene MT10Sa, which encodes a synthase-specific inhibitor; and dicumarol (7 mg/L), an alternative dehydrogenase inhibitor.

Activity of OSA in the presence of ethanol

The in vitro activity of OSA in the presence of ethanol, a respiratory substrate, was determined using a modification of the standard Bactec radiometric growth procedure. Briefly, inocula were prepared from M. bovis BCG and M. tuberculosis cultures maintained on Lowenstein–Jensen agar slants (Difco) using glass beads and commercially available diluting fluid (Becton Dickinson, Sparks, MD, USA). Mycobacterial suspensions were vortexed with glass beads and allowed to settle for 30 min. The supernatant was adjusted to a 1.0 McFarland standard and inoculated (0.1 mL) into each Bactec 12B bottle. OSA was added to individual bottles to the following final concentrations: 1.5, 3.0, 6.25, 12.5 and 25.0 mg/L. The final ethanol concentration in both controls and that used for combination testing was 2.3%. Combinations of streptomycin (0.05, 1.0 and 2.0 mg/L) and ethanol were also tested to determine whether synergic effects observed for OSA were compound specific or generalizable to an alternative antimycobacterial drug. All bottles were incubated at 37°C, and the growth index of each bottle recorded daily.

Mycolic acid studies

BCG (50 mL) was grown aerobically at 37°C in M7H9–ADC–Tween to early log phase (OD600 0.2). At this time, 1 mCi/mL of [1,2,14C]acetate (Amersham, Arlington Heights, IL, USA) and either diluent (DMSO), OSA (100 mg/L), DCCD (100 mg/L) or TTFA (100 mg/L), was added to the cultures and incubated under the same conditions for 10 min. Cultures were immediately placed on ice and cells were harvested by centrifugation at 3000 g for 15 min at 4°C.

Mycolic acid extraction was performed as described previously. Briefly, polar and non-polar extractable lipids were removed from equal volumes of cells (60 mg wet weight) according to established protocols. The resulting defatted cells containing bound mycolic acids were subjected to alkaline hydrolysis in methanol (1 mL), 30% KOH (1 mL) and toluene (0.1 mL) at 75°C overnight and subsequently cooled to room temperature. The mixture was then acidified to pH 1 with 3.6% HCl and extracted three times with diethyl ether. Combined extracts were dried under N2. Fatty acid methyl esters of mycolic acids were prepared by mixing dichloromethane (1 mL), a catalyst solution (1 mL) and isodamethane (25 mL), for 30 min, centrifuging and discarding the upper phase. The lower phase was dried under N2. Incorporation of [14C]acetate into mycolic acids was determined by scintillation counting (Beckman LS6500 multi-purpose scintillation counter) and values expressed as a percentage of untreated controls.

Results

Initially, the qualitative and quantitative biological effects of OSA were examined using 2-D gel electrophoretic protein...
profiles following 24 h exposure in BCG. Previous investigators successfully used a similar approach to determine the effects of isoniazid in *M. tuberculosis*. As shown in Figure 1 (right), treatment with OSA resulted in significant overexpression of two relatively small proteins with approximate molecular weights of 17–18 kDa. Both proteins were undetectable in the corresponding unexposed controls (Figure 1, left). Expression of both proteins occurred at both the MIC of OSA (6.25 mg/L) and concentrations up to 16 × MIC (100 mg/L) in a dose-dependent manner (complete series of gels not shown). A separate time-course experiment using [35S]methionine pulse-labelling demonstrated that both proteins were expressed rapidly (4 h) post-OSA exposure. In contrast, treatment of BCG with cerulenin or isoniazid, two potent mycobacterial inhibitors, did not result in overexpression of either protein at concentrations of up to 16 × MIC. However, DCCD, an ATP synthase inhibitor, yielded a similar 2-D protein profile with two proteins consistent in molecular weight and pI with those present on the OSA gels (complete series of gels not shown).

Protein sequencing of pooled 2-D gel fragments containing each of the two proteins demonstrated the more prominent species (B in Figure 1) to be a small heat shock protein (hsp, Rv0251c) of 17 786 Da with an isoelectric point (pI) of 5.0. The second protein (A in Figure 1) was identified as the b-subunit of ATP synthase (Rv1306); (B) hsp (Rv0251c). These were the only two proteins that were consistently overexpressed in the presence of OSA.

Effect of OSA on *M. bovis* BCG samples demonstrated that incorporation of [35S]methionine was markedly reduced at all three time-points examined. Subjectively, the decrease in protein synthesis was reflected in the 2-D gels. These decreases were 56%, 79% and 76% at 5 min, 1 h and 3 h, respectively. Protein synthesis was also inhibited following treatment of *M. bovis* BCG with the ATP synthase inhibitor DCCD and the complex II inhibitor TTFA at all three of the corresponding time-points. These decreases were 74%, 97% and 99% for DCCD and 77%, 73% and 57% for TTFA.

On the basis of the protein data, which suggested a possible connection to ATP synthase via interaction with the b-subunit, time-course studies over 24 h were performed to examine the effect of OSA (100 mg/L) on ATP levels in *M. bovis* BCG. As shown in Figure 4, ATP[M] levels decreased rapidly (64%) in OSA-treated *M. bovis* BCG (*P* = 0.001) compared with unexposed controls at 5 min post-exposure. This was the earliest time-point for which we could obtain reproducible values using these methods. Following this initial rapid loss, a slight recovery
occurred at 30 min (9%) and 1 h (17%), suggesting a compensatory mechanism for stabilization of the cellular ATP concentration. However, by 3 h cellular ATP concentration had declined to 44% and to 73% at 24 h when compared with unexposed controls.

To determine whether the rapid and dramatic decrease in cellular ATP observed following exposure to OSA could be the result of a generalized stress response in *M. bovis* BCG, a variety of respiratory chain inhibitors and antimycobacterial agents was evaluated. The respiratory chain inhibitors included rotenone, specific for NADH dehydrogenase (respiratory complex I); TTFA, a respiratory complex II inhibitor; DCCD, an ATP synthase-specific inhibitor; and dicumarol, an alternative dehydrogenase inhibitor. The remaining compounds evaluated included isoniazid, rifampicin, streptomycin, ethambutol and cerulenin, a fatty acid synthase inhibitor. The antimycobacterial agents were tested, as with OSA, at 16× MIC. As shown in Figure 5, isoniazid, dicumarol, rotenone, and cerulenin resulted in an increase in cellular ATP at 5 min post-exposure ranging from 1.9% for cerulenin to 20.7% for rotenone. In contrast, DCCD, TTFA, OSA, streptomycin, rifampicin and the combination of dicumarol and rotenone all resulted in a decrease in cellular ATP. The rapid loss of ATP demonstrated by OSA (63.7%, *P* < 0.001) was most closely mimicked by DCCD (92.4%, *P* < 0.001), the specific ATP synthase inhibitor.

Owing to the possible involvement of ATP synthase and other components of the respiratory chain, studies were performed with OSA in the presence of ethanol (2.3%). Ethanol is a respiratory substrate and has been used by several investigators to study cellular respiration.25–26 As shown in Figure 6, 2.3% ethanol potentiated the effects of OSA on growth inhibition, reducing the MIC for *M. bovis* BCG from 6.25 to 3.0 mg/L. This potentiation was greater for *M. tuberculosis* H37Rv in which the MIC of OSA was reduced from 6.25 to < 1.5 mg/L (data not shown). No potentiation of activity was observed between ethanol and streptomycin (data not shown).

Previously we reported that treatment of *M. bovis* BCG with OSA resulted in a decrease in mycolic acids, with no apparent effect on intermediates in this pathway.19 A significant decrease in available cellular ATP could potentially have direct or indirect deleterious effects on the biosynthesis of other macromolecules, including mycolic acids of the cell wall. To investigate the role of ATP synthesis and respiration in mycolic acid production, inhibitors of ATP synthase (DCCD) and respiratory complex II (TTFA) were evaluated and compared with OSA and unexposed controls in *M. bovis* BCG. A short time interval of 10 min post-exposure was selected to ensure that inhibition of mycolate synthesis was not due to cell death. As shown in Figure 7, total mycolic acid levels decreased 79% with DCCD

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**Figure 4.** Time-course of the effect of OSA (100 mg/L) on ATP in *M. bovis* BCG. ATP[M] was calculated per cfu of treated versus untreated cells. Colony counts (cfu/mL) were determined for each culture using standard plating procedures. Relative ATP[M] was then calculated by normalizing treated values to controls and expressed as percentage change. Error bars represent the standard error for two separate experiments. Statistical significance was calculated using a two-tailed Student’s *t*-test. *P* < 0.01, **P** = 0.001.

**Figure 5.** Comparative effects of respiratory inhibitors and antimycobacterial antibiotics on ATP concentration in *M. bovis* BCG. OSA, 100 mg/L; DCCD, 100 mg/L; TTFA, 100 mg/L; isoniazid (INH), 1.6 mg/L; rifampicin (RIF), 32 mg/L; streptomycin (STR), 32 mg/L; ethambutol (EMB), 32 mg/L; rotenone (ROT), 25 mg/L; dicumarol (DC), 7 mg/L; and cerulenin (CER), 24 mg/L. ATP[M] was calculated per cfu of treated versus untreated cells. Colony counts (cfu/mL) were determined for each culture using standard plating procedures. Relative ATP[M] was then calculated by normalizing treated values to controls and expressed as percentage change. Error bars represent the standard error for two separate experiments. Statistical significance was calculated using a two-tailed Student’s *t*-test. *P* < 0.001.

**Figure 6.** Potentiation of the inhibitory activity of OSA with 2.3% ethanol. Standard Bactec (12B) radiometric media was supplemented with 2.3% ethanol for both control (CT) and OSA-treated cultures. The OSA concentration was half the MIC, or 3.0 mg/L.
acetate, mycolic acids extracted, followed by scintillation counting. Neither protein profiles were carried out in the presence of cerulenin, a stress response to cell injury. To evaluate this possibility 2-D bars represent the standard error for two separate experiments. CT, untreated control.

(100 mg/L), 46% with TTFA (100 mg/L) and 43% with OSA (100 mg/L) compared with untreated controls.

Discussion

In this study we further evaluated the effects of OSA in M. bovis BCG. Initial 2-D protein gel electrophoresis and subsequent sequencing of proteins demonstrated the overexpression of two relatively small proteins (14 kDa): the b-subunit of ATP synthase (F1F0 ATPase) encoded by the atpF gene and a small heat shock protein, hsp (Rv0251c). RT–PCR confirmed an increase in the level of hsp expression and to a lesser extent that of the b-subunit of ATP synthase, a pattern consistent with that observed on the 2-D gels. Initially, it was thought that overexpression of these two proteins could represent a generalized stress response to cell injury. To evaluate this possibility 2-D protein profiles were carried out in the presence of cerulenin, a potent antimycobacterial compound, and isoniazid. Neither cerulenin nor isoniazid treatment resulted in overexpression of either protein in BCG. However, exposure to DCCD, an ATP synthase inhibitor, resulted in a similar protein profile, yielding overexpression of two proteins consistent in molecular weight and pl with AtFp and hsp.

Overexpression of the b-subunit of ATP synthase suggested the possible involvement of ATP synthase, whether direct or indirect, following exposure of M. bovis BCG to OSA. Based on these observations, single time-point and time-course experiments were undertaken to determine cellular ATP[M] in the presence of OSA compared with DCCD, a known ATP synthase inhibitor. ATP[M] decreased significantly following OSA and DCCD treatment at all time-points tested. This decrease was reproducible for both compounds and occurred very rapidly. To determine whether this effect was compound specific or the result of a generalized stress response, additional antimycobacterial drugs and inhibitors of respiration were tested for their ability to affect ATP[M] at corresponding time-points. No appreciable decrease in ATP[M] was detected at 5 min post-exposure for any of the antibiotics or respiratory inhibitors tested, with the exception of TTFA, a specific inhibitor of complex II, which demonstrated a moderate decrease in ATP[M] at 5 min. This is not surprising since this inhibitor specifically targets succinate dehydrogenase (complex II), which is an integral part of the TCA cycle. Without this enzyme complex the TCA cycle is severely impaired. As a result, aerobic respiration becomes impaired, slowing the production of ATP.

In this study, OSA mimicked the effect of the well-documented ATP synthase inhibitor DCCD. In comparison, other antimycobacterial agents failed to elicit a similar effect. These findings suggest that OSA-mediated inhibition of ATP synthesis is specific in nature and not the result of cell death. These effects may involve direct or indirect interaction with the b-subunit of ATP synthase or other accessory or regulatory components of the complex. ATP synthases (F1F0 ATPase) utilize energy from the proton motive force to generate ATP. This enzyme complex consists of transmembrane (F0) and cytosolic (F1) sectors.

The relative importance of the b-subunit to ATP synthase structure and function has been demonstrated by several laboratories. It has been suggested by several lines of investigation that the b-subunit may play a role in the coupling of proton translocation to catalysis. The b-subunit of F0 would play both a structural and a functional role in the operation of F1F0 ATPase. Inhibition of this complex could occur through direct interaction with the b-subunit or a membrane-associated component of F0, resulting in significant impairment of ATP generation. Alternatively, the OSA-mediated decrease in cellular ATP may involve currently unidentified regulatory components involved in energy production.

Mycobacterial regulation of ATP synthesis and energy production is poorly understood at this time. In this study, the OSA-associated decrease in ATP concentration was accompanied by overexpression of hsp (Rv0251c). This suggests the possibility that the heat shock response may be linked to energy sensing/regulation in mycobacteria. This hypothesis is further strengthened by the similar response seen by 2-D protein profiles following DCCD treatment. Hsp (Rv0251c) encodes a relatively small protein of 159 amino acids and is a member of the Hsp20 or a-crystallin family of small heat shock proteins. Recently, Stewart et al. demonstrated that hsp (termed acr2 by the authors) was the most heat-inducible gene in the mycobacterial genome. hsp is also arranged in an apparent operon with two adjacent genes (Rv0250c and Rv0249c). Regulation of hsp involves the heat shock repressor, HspR and an ECF sigma factor σE. The latter is also up-regulated during oxidative or detergent stress and bears similarity to the a-crystallin (acr) (14 kDa antigen) of M. tuberculosis (41% identity over 98 amino acids). The heat shock response is ubiquitous and allows cells to survive under both normal and deleterious stress conditions. Emerging evidence has identified the existence of enzyme-specific chaperones, which are essential for the formation of specific enzyme complexes. Examples of enzyme-specific chaperones include the yeast ATP10, ATP11 and ATP12 genes, which encode proteins required for ATP synthase assembly. Additionally, some molecular chaperones are subject to redox regulation. The complete functional role of the mycobacterial hsp is largely unknown. However, the possibility exists that this heat shock protein may play a role in enzyme-specific assembly/regulation of ATP synthase or other associated complexes in the respiratory chain. Hsp may also represent a mycobacterial version of a redox-regulated heat shock protein.
The potential of OSA-mediated interference in central energy metabolism was further strengthened by the potentiation of activity with ethanol. It is known that mycobacteria can utilize low concentrations of ethanol and other short chain alcohols as carbon sources. Ethanol is a respiratory substrate, which is reversibly oxidized to acetaldehyde with the concomitant reduction of NAD by alcohol dehydrogenase. Subsequent oxidation of acetaldehyde yields acetic acid, which is then converted to acetyl-CoA in an ATP-dependent reaction. Acetyl-CoA is a critical molecule in central metabolism. Oxidation of acetyl-CoA via the TCA cycle drives the production of cellular energy. Thus, ethanol metabolism and respiration are interconnected. Previous investigators have shown that ethanol increases the rate of ATP synthesis in mammalian mitochondria as a result of increased production of NADH + H+, which leads to elevated proton flux through the respiratory chain. ATP:O ratios increase following addition of ethanol, which indicates an increased energetic conversion between respiration and ATP synthesis. It is unlikely that ethanol and OSA share the same target. However, ethanol metabolism resulting in the generation of oxidized NAD in the absence of a functioning respiratory chain for its reduction would disrupt the essential NAD:NADH + H+ ratios required for cellular metabolism. In such a case, potentiation of OSA and ethanol would be possible.

Inhibition of ATP synthesis and interference with cellular respiration could produce multiple downstream effects. These include a decrease in the energy-dependent synthesis of other macromolecules, such as mycolic acids and proteins. Previously, we reported that OSA decreased mycolic acid levels in M. bovis BCG, with no apparent effect on intermediates in this biosynthetic pathway. This observation was in contrast to the pattern of mycolate inhibition observed with thiacetomycin and cerulenin, known fatty acid synthase inhibitors. These findings suggested that inhibition of mycolic acid synthesis by OSA and other β-sulphonylcarboxamides could involve a mechanism other than fatty acid synthase inhibition. In this study, we demonstrated that inhibitors of ATP synthase and respiratory complex II could duplicate the effects of OSA on mycolic acid synthesis. Inhibition of energy production can result in a rapid non-specific decrease in mycolic acid synthesis. A similar effect could be expected on protein synthesis, which, as demonstrated in this study, decreased in parallel with ATP.

Conclusive identification of the target and mechanism of action of the β-sulphonylcarboxamides will answer these questions and provide a basis for rational, structure–function analysis, and guide the design and synthesis of new drugs for the treatment of TB.

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Transparency declarations

Under a licensing agreement between FASgen and the Johns Hopkins University (JHU), Drs Dick and Parrish are entitled to a share of royalties received by the university for potential sales of OSA. Dr Dick and the university own FASgen stock, which is subject to certain restrictions under university policy. Drs Dick and Parrish are also paid consultants to FASgen. The terms of this arrangement are being managed by JHU under the guidelines established in its conflict of interest policy.

References


