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Activity of DSA against anaerobically adapted *Mycobacterium bovis* BCG *in vitro*

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Summary

DSA is a β -sulfonylacetamide with *in vitro* activity against pathogenic mycobacteria. Although the enzymatic target(s) of DSA has not been identified, studies to date suggest that this class of compounds may interfere directly or indirectly with ATP synthase and other components of the mycobacterial respiratory chain. In this study we further evaluated the *in vitro* activity of DSA against anaerobically adapted BCG using two established models. DSA killed BCG in the anaerobic Wayne model. Bactericidal activity ranged from >99% to 60%. DSA killed rifampin-tolerant persisters with a reduction in viable counts of 1.5 log₁₀ versus controls. Conclusive identification of the DSA-specific target(s) will permit a better understanding of the unique mechanism of action of this class of compounds against both aerobically growing and anaerobically adapted bacilli *in vitro*.

Keywords

Mycobacterium tuberculosis; latency; Wayne model; rifampin tolerant persisters

A significant obstacle for the eradication of tuberculosis has been the ability of the organism to transition to a non-replicating (latent) state in which metabolism is reduced to an extremely low basal level.¹ Currently available antibiotics utilized for the treatment of tuberculosis are not effective in eliminating latent or persistent bacilli in infected patients except with extended treatment courses. Current drug regimens require a combination of first-line drugs for a minimum of 6 months. In the case of latency, lengthy treatment regimens are also attributed to a sub-population of “persistent” bacilli.² This sub-population is tolerant rather than resistant to extremely high concentrations of rifampin (100 μ g/ml) as well as other antimycobacterial drugs. Tolerance in this case is due to inactivity of the enzymatic target rather than mutation or alteration of the target gene. Upon re-entry into aerobic, vegetative growth, this population regains susceptibility to RIF with minimal inhibitory concentrations often $\leq$ 1.0 μ g/ml. Two *in vitro* models have been developed for the evaluation of antibiotic activity against mycobacteria in the latent or non-replicating state. The Wayne model is based on the gradual depletion of oxygen through utilization by an actively growing culture, and has been used to screen drugs for their ability to kill *Mycobacterium tuberculosis* (*M. tb.*) and the closely related *M. bovis* BCG (BCG) while in a state of anaerobic latency.³ Hu and coworkers established an

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in vitro “sterilizing” model for screening new drugs for the treatment of TB for their ability to kill rifampin-tolerant persisters.² The sterilizing model predicts that compounds found to have effective activity against dormant to semi-dormant organisms may provide a means to shorten the course of therapy *in vivo*.

n-decanesulfonylacetamide (DSA) is a lead compound of the class β -sulfonylacetamides with *in vitro* activity against actively growing, pathogenic mycobacteria, including multi-drug resistant tuberculosis (MDR-TB).^{4, 5, 6} Studies to date suggest that the mechanism of action of this class of compounds involves disruption of energy production through interference with ATP synthase and possibly other components of the mycobacterial respiratory chain.^{5, 6} Downstream effects of this inhibition include significant reduction in both protein and mycolic acid synthesis.^{5, 6} Although the activity of this class of compounds has been well characterized against aerobically growing bacilli, no studies existed in which activity was assessed against anaerobically adapted organisms. In this study we further evaluated the *in vitro* activity of DSA using the established Wayne and Sterilizing models to determine the activity of DSA against both latent and rifampin-tolerant populations of BCG *in vitro*.

BCG Pasteur (ATCC 35734) was used in this study. Isoniazid (INH), rifampin (RIF), and metronidazole (MTZ) were purchased from Sigma, St. Louis, Missouri. DSA was synthesized according to established methods.⁴ All assays were performed in duplicate.

The *in vitro* Wayne model was established according to published protocols.^{1, 3} Cultures of BCG were grown in Dubos Tween-albumin broth (Difco, Detroit, MI.). Oxygen was gradually depleted as previously described from aerobic, exponentially growing cultures ($\sim 10^6$ CFU/ml) by aeration at 250 rpm's for a period of approximately 10 to 12 days using Hungate type anaerobic culture tubes (Bellco Glass, Inc., Vineland, N.J.). Optical density and colony forming units were determined to monitor the progression of the cultures from aerobic growth through non-replicating persistence stages 1 and 2.^{1, 3} Methylene blue (Sigma) was used to detect the presence of oxygen in the culture. Complete decolorization was used as an indicator for anaerobiosis. Once anaerobiosis had been established, varying concentrations of DSA, INH, RIF or MTZ were added to respective test cultures and further incubated for an additional 48 hours. INH (0.1 μ g/ml to 0.4 μ g/ml) and RIF (0.06 μ g/ml to 0.1 μ g/ml) were used as negative controls since neither drug is active against latent bacilli and have been shown to be ineffective in killing BCG in this model.³ MTZ (12.5 μ g/ml) was used as a positive control since it is only active under anaerobic conditions. For each culture, serial dilutions were made and plated to M7H10 agar. Following ~ 15 days of incubation, the CFU/ml were determined for each culture condition and compared to the untreated and positive and negative controls.

The Sterilizing model was performed according to published protocols.² Briefly, cultures of BCG ($\sim 10^6$ CFU/ml) were grown in Middlebrook 7H9 broth supplemented with 0.05% Tween 80 and 10% (wt/vol) albumin-oleic acid-dextrose-catalase complex for 100 days at 37°C without shaking in Hungate anaerobic tubes. Cultures were then vortexed with glass beads (2 mm) followed by sonication (VWR Model 50T sonicator) for 5 minutes and RIF (100 μ g/ml) or diluent added to initial control and treated cultures, respectively. Initial RIF-containing cultures are used to isolate the population of drug tolerant persisters present with the corresponding controls used as a comparator. All cultures were incubated an additional 5 days. No growth was detected after day one of incubation on M7H10 plates inoculated from the RIF containing cultures. On day 6, all initial cultures were washed twice by centrifugation in phosphate-buffered saline and resuspended in fresh RIF-free M7H9 broth containing either diluent or DSA (1.5 μ g/ml to 50 μ g/ml). Following an additional 7 days of incubation under normal aerobic conditions, dilutions were prepared from each culture and plated to Middlebrook 7H10 agar (Difco). Plates were incubated at 37°C in 5% CO₂ and viable counts determined at approximately 15 days.

As shown in Figure 1, DSA, at concentrations ranging from 1.5 µg/ml (~MIC) to 50 µg/ml, killed anaerobically adapted, 'latent' BCG *in vitro*. The bactericidal activity was non-linear, a finding consistent with the known pharmacodynamics of this class of compounds which exhibit time rather than concentration dependent killing (data not shown). Activity was greatest (>99%) with the highest concentration of DSA tested (50 µg/ml), whereas other currently used agents, INH and RIF, had little effect (<10%). Lower concentrations of DSA resulted in bactericidal activity ranging from 59.8% (1.5 µg/ml) to 74.4% (3.0 µg/ml).

Figure 2 shows the sub-population of RIF-tolerant persisters present in the 100 day old cultures. These RIF tolerant bacilli were resistant to RIF at 100 µg/ml but were susceptible to DSA at all concentrations tested (1.5 to 50 µg/ml). As noted above, the bactericidal activity was non-linear in relation to concentration. Treatment with DSA resulted in a decrease in viable counts of 'persisters' of 1.5 logs. This decrease was observed at all concentrations tested.

M. tb. can persist in the host for prolonged periods of time due to the ability of the organism to enter into alternate metabolic states rendering the bacilli refractory to currently used antibiotics which require active metabolic processes such as cell wall synthesis for activity. For this reason common first-line antimycobacterial agents such as INH and RIF are frequently ineffective in eliminating these differentially adapted organisms. Latent infection is present in one-third of the world's population. This population provides the reservoir for reactivation disease, with current treatment regimens unable to guarantee clearance of latent organisms. Any effective approach to controlling TB must include the development of new drugs capable of killing these anaerobically adapted bacilli.

In this study, DSA demonstrated *in vitro* activity against anaerobically adapted BCG in both the Wayne and Sterilizing models. This suggests that the DSA target(s) may involve a metabolic pathway common in both aerobic and anaerobic growth. Energy production is certainly necessary under both conditions. Inhibition of the ATP synthase or a component of the respiratory chain would be consistent with these findings. Elucidation of the DSA-specific target(s) will provide the basis for understanding the unique mechanism of action responsible for the observed activity against aerobically growing and anaerobically adapted bacilli *in vitro*.

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Figure 1.

Activity of DSA in the Wayne model of non-replicating persistence. Abbreviations: INH, isoniazid, RIF, rifampin, DSA, *n*-decanesulfonyacetamide, MET, metronidazole. Values shown for all drugs and DSA are in $\mu\text{g/ml}$. The actual log decrease in CFU/ml: INH and RIF, 0.1, DSA (1.5 $\mu\text{g/ml}$), 1.5, DSA (3.0 $\mu\text{g/ml}$ to 12.5 $\mu\text{g/ml}$), 2.4, DSA (50.0 $\mu\text{g/ml}$), 2.5, MET, 2.5.

Figure 2.

Activity of DSA in the "sterilizing" model against rifampin 'tolerant' persisters. Abbreviations: DSA, *n*-decanesulfonyacetamide. Values shown for DSA are in $\mu\text{g/ml}$.