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Investigation of the Anti-Mycobacterial Mechanism of Action of 7-Methyljuglone*

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ABSTRACT

Objectives: Although the naphthoquinone, 7-methyljuglone (7-MJ), is active against *Mycobacterium tuberculosis* (MTB) *in vitro*, neither the cellular site nor mechanism of anti-mycobacterial action of this agent has been identified. The primary objective of the current study was to investigate the mycobacterial outer membrane as a potential target of 7-MJ by measuring the effects of this agent (0.023 - 1.5 mg/L) on microbial ATP levels and uptake of K^+. **Methods:** Bioluminescence and radiometric (uptake of $^{86}Rb^+$) procedures were used to assay microbial ATP levels and K^+ transport respectively. **Results:** Exposure of MTB (strain H37Rv) to 7-MJ for 60 min resulted in dose-related decreases in both microbial ATP levels and uptake of $^{86}Rb^+$ which achieved statistical significance ($P < 0.05$) at concentrations of 0.4 and 0.1 mg/L respectively. **Conclusions:** These observations are compatible with the mycobacterial membrane as being the putative site of action of 7-MJ, targeting microbial energy metabolism and K^+ transport.

Keywords: Adenosine Triphosphate; Cell Membrane; Energy Metabolism; Potassium

1. Introduction

Tuberculosis (TB) remains a major world health problem, in particular since the incidence of multi-drug-resistant tuberculosis has increased in many countries. Consequently, discovery of new drugs with novel modes of action represents a major challenge [1].

Euclea natalensis is a shrub or small to medium size tree, which occurs in a variety of habitats, including coastal and inland forests, as well as bushveld in southern Africa. The roots of *E. natalensis* are used by indigenous people of southern Africa to treat various bacterial infections. Powdered root bark of this species is used as an ingredient in medicines to treat urinary tract infections, venereal diseases and dysmenorrhoea [2]. The Zulus, a South African tribe, use the root bark to treat respiratory diseases such as TB, bronchitis, pleurisy and asthma [3]. Several secondary metabolites such asterpenoids, naphthoquinones etc. have been isolated from *E. natalensis* [3]. According to previous studies, the minimum inhibitory concentration (MIC) of a naphthoquinone isolated from the roots of the plant, methyljuglone (7-MJ), against a drug-sensitive strain of *Mycobacterium tuberculosis* (MTB) was very significant and comparable to some of the existing antituberculosis drugs (MIC: 0.5 mg/L) [4].

The primary objective of the current study was to investigate the mycobacterial outer membrane as a potential target of 7-MJ by measuring the effects of this agent on microbial ATP levels and uptake of potassium (K^+).

2. Materials and Methods

2.1. 7-Methyljuglone (7-MJ)

7-MJ was dissolved in dimethyl sulphoxide (DMSO) and used at a final concentration range of 0.023 - 1.5 mg/L in the assays described below. Appropriate solvent controls (0.15% DMSO) were included in all experiments.

2.2. *Mycobacterium tuberculosis*

MTB strain H37Rv, ATCC 26518 was provided by the

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Inflammation and Immunity Research Unit of the South African Medical Research Council (Pretoria, South Africa). MTB was grown for 7 days at 37˚C in OADC (oleic acid, albumin dextrose, catalase)-supplemented Middlebrook 7H9 nutrient broth (Difco, Detroit, MI, USA) containing 0.05% Tween 80.

2.3. Potassium (K^+) Uptake

$^{86}Rb^+$ (rubidium-86 chloride, 37 MBq, PerkinElmer Radiochemicals, Boston, MA, USA) was used as a surrogate tracer for measurement of uptake of K^+ by MTB as previously described [5]. Briefly, following 7 days of culture, MTB was harvested from the bacteriological culture medium by centrifugation (2500 g/15 min), washed and resuspended in glucose- and K^+-free minimal medium (K_0N_0 buffer, pH 7.4) to deplete intracellular K^+ [5]. The bacterial suspension was adjusted turbidometrically to a concentration of 1×10^7 colony forming units (cfu)/mL. Approximately 2×10^6 cfu/mL of MTB were then treated with 7-MJ (0.023 - 1.5 mg/L) and incubated at 37˚C for 30 min, then pelleted by centrifugation, and resuspended in 2 mL K_0N_0 containing $^{86}Rb^+$ (2 mCi/L) and glucose (22 mM) and a further incubation period of 90 min at 37˚C. The reaction was terminated by the addition of ice-cold phosphate buffered saline (PBS, 0.15 mM, pH 7.4, 100 mM cold K^+), and washed twice with ice-cold PBS. The pellets were disrupted by adding 0.4 mL warm 5% trichloroacetic acid (Merck, Darmstadt, Germany). Radioactivity was assayed using a liquid scintillation spectrometer (Tri-Carb 2100 TR, Packard Instrument Co, Meriden, CT, USA) and net uptake of $^{86}Rb^+$ expressed as the difference in radioactive counts per minute (cpm) between tubes incubated at 37˚C and negative controls kept on ice.

2.4. Adenosine Triphosphate (ATP)

Mycobacterial ATP concentrations were determined using a sensitive luciferase chemiluminescence procedure (BacTiterGlo™ Microbial Cell Viability Assay, Promega, Madison, WI, USA). Briefly, the bacteria at a concentration of 1×10^7 cfu/mL in 10 mL K_0N_0 were incubated for 60 min at 37˚C without and with 7-MJ (0.023 - 1.5 mg/L). The bacteria were then concentrated by centrifugation and mixed with an equal volume of BacTiterGlo™ solution, which contains the bacteriolytic constituent, for 5 min at room temperature. The lysates were then assayed for ATP using a $20/20^n$ chemiluminometer (Turner Biosystems Inc., Sunnyvale, CA, USA) and the results expressed as relative light units (rlu).

2.5. Statistical Analysis

The results are expressed as the mean ± standard deviation (SD) for 3 experiments, with at least 3 replicates for each concentration of the test agents or control systems in each experiment. Levels of statistical significance were calculated using the Student's paired *t*-test. Differences were considered significant if the probability value (*P*) was less than 0.05.

3. Results

3.1. Effects of 7-MJ on Uptake of $^{86}Rb^+$ by MTB

Exposure of MTB to 7-MJ resulted in dose-related inhabitation of the uptake of $^{86}Rb^+$ which achieved statistical significance at a concentration of 0.094 mg/L, with complete inhibition observed at >0.375 mg/L of this agent (**Figure 1**).

3.2. Effects of 7-MJ on Mycobacterial ATP Levels

Exposure of MTB to 7-MJ at the lowest concentration tested (0.023 mg/L) resulted in a significant ($P < 0.05$) increase in microbial ATP levels, followed by a progressive decline, being almost undetectable at 7-MJ concentrations of ≥0.375 mg/L (**Figure 2**).

4. Discussion

The antimicrobial activity of 1,4-naphthoquinones is well recognised and appears to result from the electrophilic addition of these agents to nucleic acids and proteins, as well as by intracellular redox cycling mechanisms, resulting in the generation of toxic reactive oxygen species (ROS) such as superoxide and hydrogen peroxide (H_2O_2) [6,7]. Although the relative lack of selectivity of these agents for prokaryotes presents a challenge in respect of

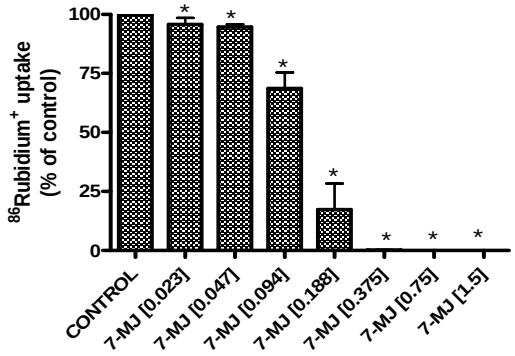

Figure 1. The effect of 7-methyljuglone (7-MJ) on the uptake of K^+ by the H37Rv strain of MTB. The results are from one experiment with five replicates for each concentration and are representative of 3 different experiments showing similar trends in each. The results are expressed as the mean percentages for uptake of $^{86}Rb^+$ of the corresponding compound-free control systems ± SD; the absolute value of the control system is 100,859 counts per minute. $^*P$ values < 0.05 when compared to the solvent control system.

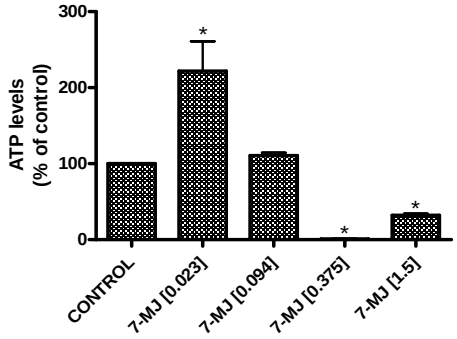

Figure 2. The effect of 7-methyljuglone (7-MJ) on the levels of ATP in the H37Rv strain of MTB. The data shown are that of one experiment with five replicates for each concentration and are representative of 3 different experiments showing similar trends. The results are expressed as the mean percentages of the corresponding compound-free control systems ±SD; the absolute value of the control system is 2,306,410 relative light units. ****P* values < 0.05 when compared to the solvent control.

clinical development, 7-MJ is a noteworthy exception [4]. The MIC value of this agent for MTB is 0.5 mg/L, which is significantly lower than its IC_{50} value (30.0 mg/L) for eukaryotic cell lines *in vitro* [4]. Nonetheless, relatively little is known about either the primary targets or rapidity of onset of anti-mycobacterial activity of this agent.

In the current study, a relatively brief exposure (30 - 60 min) of MTB to 7-MJ resulted in significant, dose -related inhibition of both microbial energy metabolism and uptake of K^+, which, in both cases was maximal at concentrations close to the MIC value. In the case of ATP levels, exposure of MTB to 7-MJ at a concentration of 0.0263 mg/L resulted in a significant increase in ATP, with a progressive, dose-related decrease at higher concentrations. The increase may represent a stress response to moderate oxidative trauma caused by low concentrations of 7-MJ as described for other types of antimicrobial agents [6,7], while at higher concentrations, irreversible ROS-mediated toxicity predominates. Alternatively, albeit speculatively, 7-MJ may interfere with the activity of mycobacterial type 2 NADH: quinone oxidoreductase, an early step in the mycobacterial respiratory chain [8].

Inhibition of mycobacterial K^+ transport by 7-MJ closely paralleled interference with microbial energy metabolism, and is probably secondary to ATP depletion. MTB possesses two major K^+ uptake systems. These are the Kdp and Trk A/B systems, driven by ATP and proton motive force, respectively [5]. The experimental conditions used in the current study (low K^+ medium) are likely to favour preferential utilisation of the inducible, high-affinity, Kdp system, accounting for the susceptibility of K^+ transport to ATP depletion, which in turn may lead to inactivation of the Trk A/B system due to dissipation of the membrane potential.

In conclusion, 7-MJ appears to target mycobacterial energy metabolism, leading to secondary membrane dysfunction and inhibition of bacterial growth. This agent may serve as a prototype for the development of novel naphthoquinone-based anti-tuberculosis agents.

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