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In vitro evaluation of dinactin, a potent microbial metabolite against *Mycobacterium tuberculosis*

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ABSTRACT

Current long duration treatment options and the emergence of drug resistance in tuberculosis (TB) have led to renewed interest in discovery of novel anti-tubercular agents or the scaffolds exhibiting enhanced efficacy with current anti-TB drugs. Herein, dinactin, a potent bioactive macrotetrolide isolated from *Streptomyces puniceus* AS13, was evaluated against *Mycobacterium tuberculosis* H37Rv and other susceptible and drug-resistant clinical isolates of *M. tuberculosis*. *In vitro* pharmacological assays showed that dinactin is bactericidal against laboratory standard strain *M. tuberculosis* H37Rv (minimum inhibitory concentration [MIC] 1 μg/mL and minimum bactericidal concentration [MBC] 4 μg/mL). Dinactin also retained its activity against various clinical isolates, including multidrug-resistant strains of *M. tuberculosis*. Whole cell interaction assays with standard first- and second-line anti-TB drugs showed the synergistic interaction of dinactin with rifampicin or amikacin, reflecting its suitability for use in combination regimens. The killing kinetics studies of dinactin against *M. tuberculosis* H37Rv revealed that it has strong concentration-dependent anti-TB activity that is also dependent on time. The kill curve also showed dynamic killing capacity of dinactin as it exhibited bactericidal potential at all concentrations tested. Kill curve data demonstrated that dinactin, like isoniazid, exerts its strong tuberculocidal activity within the first two days of exposure. This evidence strongly supports further evaluation of dinactin as a new option in the treatment of TB.

1. Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, still accounts for 1.8 million global deaths annually [1,2]. About one-third of the global population is estimated to be latently infected with *M. tuberculosis*, which reflects a big reservoir for developing new active cases [3]. In recent years, the rise in numbers of multidrug-resistant (MDR) and extensively drug-resistant (XDR) isolates of *M. tuberculosis* have seriously hampered the treatment of disease [4]. The prevalence of human immunodeficiency virus (HIV)/acquired immune deficiency syndrome (AIDS) epidemic in addition to unwanted drug-drug interactions has further burdened the treatment of TB [5,6]. These factors highlight the urgency for discovering new antimicrobial scaffolds with a novel mechanism of action, or evaluating the alternative therapeutic entities to establish novel therapeutic combinations for curbing the rapid spread of drug-resistant TB [7].

The molecules derived from *Actinomycetales* have a long history in the treatment of TB. A breakthrough in TB therapy came after the discovery of streptomycin [8]. Many actinobacteria-derived antibiotics, particularly streptomycin and rifampicin (RIF), have played a significant role in the treatment of TB, but there are no new natural product leads for *M. tuberculosis* despite their dominance for other bacterial infections [9,10]. Many new

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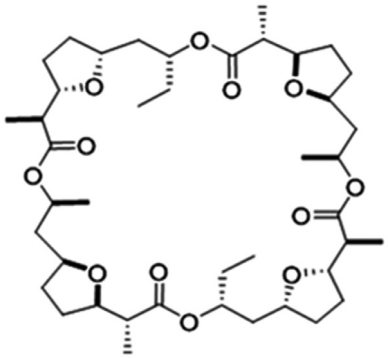

Fig. 1. Structure of dinactin (a macrotetrolide).

actinobacterial isolates containing antimicrobial natural products have been discovered but few have been evaluated specifically against TB [11,12]. Against this background, we recently carried out the bioprospection of actinobacterial extracts from rare habitats using whole cell-based phenotypic screening to identify potent anti-TB strains [13]. During this effort, dinactin (Fig. 1), a macrotetrolide compound isolated from *Streptomyces puniceus* AS13, was found to be highly active against *M. tuberculosis* H37Rv and to have antibacterial potential. Dinactin was also observed to have the least cytotoxicity (IC_{50} ~80 μM) in normal cells (HEK-293) [14]. These findings rationalise the present attempt to evaluate the anti-mycobacterial potential of the isolated lead molecule dinactin.

2. Material and Methods

2.1. Drugs, Chemicals and Media

Isoniazid (INH), rifampicin (RIF), levofloxacin (LVX), ethambutol (EMB) and amikacin (AMK) were obtained from Sigma-Aldrich (USA). Middlebrook 7H11 Agar base, Middlebrook 7H9 broth base OADC (oleic acid, albumin, dextrose and catalase) and ADC (albumin, dextrose and catalase) supplements were obtained from Hi-Media (India). Tween 80 was purchased from Merck (India). Stock solutions of the test compounds (50 mg/mL) were prepared separately in dimethyl sulfoxide (DMSO), RIF in Methanol, and INH and LVX in Milli-Q water; all solutions were stored at -20°C. Working solutions were freshly prepared at the time of experiment in Middlebrook 7H9 broth.

2.2. Bacterial strains

M. tuberculosis H37Rv (ATCC 25177) were obtained from American Type Culture Collection (USA). *M. tuberculosis* clinical isolates C, H, J, A, B, D, SHE, L, L-53, 375 and E used in this study were previously well characterised in our laboratory. *M. tuberculosis* cultures were sub-cultured and grown in Middlebrook 7H9 broth supplemented with ADC to a cell density of 10^8 colony-forming units (CFU)/mL. Glycerol stocks were prepared and stored at -20°C in 1.0-mL aliquots. A single vial was thawed, cultured to mid-log phase and used each time for each experiment. This study was approved by the institutional bio-safety committee.

2.3. Determination of MIC in clinical isolates

MICs against *M. tuberculosis* strains were determined by the standard broth dilution method according to CLSI document M24-A. Briefly, stock solutions of the drugs were solubilised in Middlebrook 7H9 broth (supplemented with 10% ADC) without Tween 80. Two-fold serial dilutions of drug solutions were prepared in 96-well plates (Nest Biotech, China) to give concentrations of test compound ranging from 0.125 to 64 μg/mL, including a growth control and media control column (200 μL in each well). An equivalent volume (50 μL) of bacterial inocula containing mid-log phase grown *M. tuberculosis* was added to all columns except the media control column to give a final cell density in each well of approximately 1×10^5 CFU/mL. The plates were incubated at 37°C and read after three weeks by visual inspection. MIC tests were carried out three times in duplicate. MIC was the minimum concentration of molecules that completely inhibited the visible growth of bacteria.

2.4. Determination of minimum bactericidal concentration (MBC)

MBC was determined by a procedure reported previously, with slight modification [15]. Serial 2-fold dilutions of test molecule up to 6-fold of its MIC (1 to 32 μg/mL) and INH (0.039-2.5 μg/mL) were prepared in sterile conical screw cap tubes (20 mL) filled with 4.8 mL Middlebrook 7H9 broth. A drug-free control was also included in the tests. All tubes were inoculated with 0.2 mL mid-log phase bacterial inocula to give a final cell density of 1×10^5 CFU/mL in each tube. The tubes were incubated at 37°C for 16 days. MBC was determined by serial 10-fold dilution of these tubes using phosphate buffer saline (0.1 M, pH 7.4) as a diluent. Each dilution (0.5 mL) was plated in triplicate onto Middlebrook 7H10 agar supplemented with 10% OADC and incubated at 37°C. The plates were counted for CFU on day 21 and day 28 of incubation. MBC was taken as the lowest concentration that killed 99.99% of the initial *M. tuberculosis* inoculum.

2.5. Drug synergy assay

In vitro drug interaction study was performed as described previously [16]. Briefly, synergistic/additive/antagonist interactions of test molecule with known anti-TB drugs against *M. tuberculosis* H37Rv (INH, RIF, EMB, AMK and LVX) were evaluated by determining the MICs of the test molecule and anti-TB drugs alone and in combination in 96-well plates. Each combination was prepared so the initial concentration of each molecule equalled its MIC (i.e., both the drugs were at their MIC). Serial dilutions were made in subsequent wells. 50 μL of a log-phase culture of *M. tuberculosis* H37Rv was added to each well to give a bacterial density of approximately 1×10^5 CFU/mL in each well. The plates were sealed and incubated at 37°C and then read by visual inspection for the next 16 days. MICs of each drug alone and in combination were described where the lowest concentrations showing no visible growth of *M. tuberculosis* were considered inhibitory concentrations. The combinatorial reductions in MICs were used to calculate the fractional inhibitory concentration (FIC). Fractional inhibitory concentration indices (ΣFIC) were interrupted as follows: ≤0.5, synergism; >0.5-4.0, addition or indifference; and >4.0, antagonism.

2.6. Kill kinetic studies

The kill cure assay was performed as described previously, with slight modification [17,18]. *M. tuberculosis* H37Rv cultures with cell density 1×10^5 CFU/mL (confirmed by quantities plate counts) were prepared in Middlebrook 7H9 broth in screw cap tubes. The test molecule was added to each mycobacterial suspension to give concentrations of 1 × MIC, 2 × MIC, 4 × MIC, 8 × MIC, 16 × MIC and 32 × MIC in the tubes. Growth control with no drug was also included. All the tubes were incubated at 37°C under shaking (100 rpm) for 20 days. Aliquots (1.0 mL) from these drug-treated mycobacterial suspensions at specified time intervals (0, 2, 4, 8, 12, 16 and 20 days) were diluted in PBS to 10^{-1} to 10^{-5} to prevent drug carry-over effects and were plated onto Middlebrook 7H11

Table 1
Anti-mycobacterial activities of dinactin against drug-susceptible and resistant *M. tuberculosis* clinical isolates.

Clinical isolate no.	Susceptibility	Dinactin MIC (μg/mL)[b]
H37Rv*	Susceptible	1
1	Susceptible	1
2	Susceptible	2
3	Susceptible	1
4	INH-R	16
5	INH-R	4
6	INH-R	16
7	INH-R; EMB-R	8
8	RIF-R	4
9	MDR	16
10	MDR	16
11	MDR	8

Susceptible - clinical isolate susceptible to first-line antituberculosis drugs. RIF: Rifampicin; INH: Isoniazid; EMB: Ethambutol; AMK: Amikacin; LVX: Levofloxacin; DA: Dinactin, MDR: Multidrug-resistant; INH-R: Isoniazid resistant; RIF-R: Rifampin resistant; EMB-R: Ethambutol resistant.

* Standard laboratory strain of *M. tuberculosis* H37Rv, ATCC: American Type Culture Collection (USA).[a]1 to 11 are the *M. tuberculosis* clinical isolates with their resistance patterns as shown in the table.

[b] Determined by serial-2-fold Broth Microdilution Assay

Table 2
Pairwise interaction of dinactin with first- and second-line anti-TB drugs against *M. tuberculosis* H37Rv.

Drug combination	MIC[a] (μg/mL)		FIC[b]	ΣFIC*	Remarks**
	Alone	Combination			
INH	0.312	0.039	0.125	0.25	Synergism
RIF	0.078	0.009	0.125		
DA	1	0.5	0.5	1	Additive
INH	0.312	0.156	0.5		
DA	1	1	1	2	Additive
EMB	2.5	2.5	1		
DA	1	0.25	0.25	0.5	Synergism
RIF	0.078	0.019	0.25		
DA	1	0.5	0.5	1	Additive
LVX	2.5	1.25	0.5		
DA	1	0.0019	0.0019	0.0039	Synergism
AMK	2.5	0.0049	0.0019		

[a] The MIC in the combination was determined using a checkerboard method.RIF: Rifampicin; INH: Isoniazid; EMB: Ethambutol; AMK: Amikacin; LVX: Levofloxacin; DA: Dinactin.

* ΣFIC= Fractional inhibitory index are calculated as combinatorial reductions in MICs.

[b] Fractional inhibitory concentration (FIC); FIC of drug A = MIC of A in combination with B/MIC of A alone, FIC of drug B = MIC of drug B in combination with A/MIC of drug B alone. ΣFIC is calculated as the FIC of drug A+ FIC of drug B.

** ΣFIC values ≤0.5, >0.5-4.0 and>4.0 reflect synergistic, additive or indifferent and antagonistic interactions, respectively [35,36].

agar plates. All the plates were incubated at 37°C for 40 days. CFU counting was conducted on day 21 and day 40 of incubation. Any new CFU observed on day 40 were added to those already scored on day 21. Kill curve was generated by plotting Log_{10} CFU/mL values against time.

2.7. MIC determination in the presence of serum/albumin

MIC determination in the presence of serum/albumin was performed using a previously described procedure, with slight modification [19]. Broth microdilution method (as discussed above) was adopted and MICs against *M. tuberculosis* H37Rv were determined without protein enrichment, in the presence of 4% bovine serum albumin (BSA), and in the presence of 10% foetal bovine serum (FBS).

3. Results

3.1. Determination of minimal bactericidal concentration (MBC)

Recently, we conducted whole cell-based screening of crude extracts from selected actinobacteria against *M. tuberculosis* H37Rv using the standard broth microdilution method [13]. We reported that d-inactin, a macrotetrolide from *Streptomyces puniceus,* has promising MIC against *M. tuberculosis* (1 μg/mL), and has least cytotoxicity (IC_{50} ~80 μM) in normal cells (HEK-293 cells) [13,14]. In the current study, bactericidal activity (killing capacity) of dinactin was determined against *M. tuberculosis* H37Rv. The results revealed that dinactin is strongly bactericidal at 4-times its MIC. Dinactin was found to have an MBC of 4 μg/mL, where MBC was taken as the lowest concentration of compound that clears 99.9% of CFU of initial *M. tuberculosis* inoculum.

3.2. Activity against clinical MDR tuberculosis isolates

The anti-TB potential of d-inactin was further investigated by surveying against clinical isolates of *M. tuberculosis*, including multidrug-resistant (MDR) strains. Dinactin was active against a panel of clinical drug-susceptible and MDR isolates (Table 1). Dinactin retained its activity against clinical isolates that included three susceptible isolates, three INH-resistant isolates, an EMB-INH resistant isolate, an RIF-resistant isolate and three other previously characterised multidrug-resistant isolates. The activity against these strains was reflected in low MIC values (1-16 μg/mL).

3.3. Anti-TB potential of dinactin in combination with first- and second-line anti-TB drugs

To examine the *invitro* efficacy of dinactin in combination with other TB drugs, drug-drug interaction assays with drugs of current anti-TB regimens were performed. MICs of RIF, INH, EMB, LVX, AMK and dinactin against *M. tuberculosis* were 0.078, 0.312, 2.5, 2.5, 2.5 and 1 μg/mL respectively. Combinations of INH and RIF showed strongest synergism (ΣFIC=0.25) (Table 2), which was in accordance with previous reports [20,21]. Dinactin exhibited synergistic interactions with RIF and AMK with ΣFIC values of 0.5 and 0.0039 respectively against *M. tuberculosis* H37Rv. Dinactin and RIF in combination showed 4-fold reduction in MIC compared with the individual MIC values, while lowest MIC values were observed with the dinactin and AMK combination (reduced 512-fold compared to their individual MICs). In contrast to these results, additive effects were observed in combination of d-inactin with INH, EMB and LVX with ΣFIC values of 1, 2 and 1, respectively (Table 2). Therefore, dinactin is suitable for use in combination treatments as it can either enhance efficacy of the companion drug or at least not cause any antagonistic/negative effect on the co-administered drugs.

3.4. Concentration- and time-dependent activity of dinactin

Killing kinetics studies are an important part of anti-TB drug development and the aim of these studies is to achieve the maximum efficacy of the drug by estimating the optimal effect of various drug exposures. *In vitro* killing kinetics of new compounds has been considered essential for their effective usage and suppression of drug resistance [22]. To investigate the killing kinetics of dinactin, 20-day kill-curve studies were performed using serial 2-fold dilutions of dinactin from 1xMIC to 32xMIC at six different time points against *M. tuberculosis* H37Rv. The kinetic kill-curve of dinactin was generated by plotting log_{10} CFU vs. time at all concentrations and is presented in Fig. 2. Dinactin displayed bactericidal effects at all tested concentrations with ~1.5 log CFU killing at MIC/2xMIC and culture sterilisation at 16xMIC (Fig. 2). From

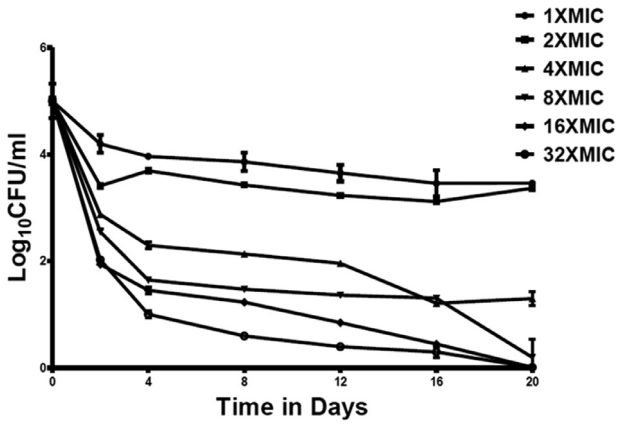

Fig. 2. Concentration- and time-dependent killing effect of dinactin on H37Rv strain of *M. tuberculosis*. Cultures of *M. tuberculosis* (1×10^5 CFU/mL) were exposed to dinactin at 2-fold increasing concentrations ($1\times$, $2\times$, $4\times$, $8\times$, $16\times$ and $32\times$ MIC) for 20 days at 37°C. At 0, 2, 4, 8, 12, 16 and 20 days of exposure, quantitative CFU were performed on drug-free plates.

Fig. 2 it is clear that at all time points (2-20 days), the *M. tuberculosis* killing by dinactin is strongly concentration dependent. At MIC and 2xMIC, the *M. tuberculosis* killing by dinactin did not improve significantly with time after day two up to the last time point tested. At 16xMIC and 32xMIC, *M. tuberculosis* killing improved significantly at all time points throughout the study, though initial rapid killing was observed at the first two time points (days 2 and 4). Thus, at all tested concentrations, dinactin showed an initial rapid killing (1.5 to 4 $\log_{10}$ CFU) within the first two days of exposure and hence resembles the standard anti-TB drug INH, which is the strongest known bactericidal anti-TB drug. Overall, at MIC and 2xMIC, dinactin exhibited monophasic kill curve and at higher concentrations it exhibited biphasic kill curve with culture sterilisation at 16 μg/mL. These data clearly reflect the bactericidal potential of dinactin and its potential for intensive TB treatment.

3.5. MIC in the presence of serum/albumin

MICs of dinactin and the standard anti-TB drug, INH, in unsupplemented media, media supplemented with 10% FBS and 4% BSA were determined to explore its protein binding capacity. MIC of dinactin reduced to 0.25 μg/mL in unsupplemented media from 1 μg/mL as in standard culture medium (containing 0.5% BSA). MICs of dinactin in the presence of 10% FBS and 4% BSA (a physiologically equivalent concentration of albumin) were 4- and 2-fold higher, respectively, than those in standard media. MICs of INH also increased 4- and 8-fold, respectively, under these supplementations. Thus, protein binding percentage of dinactin was comparable to that of INH.

4. Discussion

Antibacterial drug discovery is a costly venture with a very limited probability of success [23]. In the case of TB, *M. tuberculosis* is resistant to most antibiotics; therefore, new drugs with novel modes of action are urgently required but such compounds are rare and difficult to identify [24]. However, to introduce any new TB therapy (particularly for MDR or XDR), it is crucial to ascertain that the new compound is equal to or better than current treatment options. The promising MIC (1 μg/mL) of dinactin against *M. tuberculosis* H37Rv in liquid culture is better than that for some of the first- and second-line TB drugs. Despite its potency, dinactin was also relatively non-toxic in a normal cell line (HEK-293) [14]. The activity profile of dinactin against a panel of MDR-TB clinical isolates was also strongly effective. Taken together, these findings warranted further evaluation of dinactin as a potential molecule for TB. As monotherapy in TB is associated with emergence of drug resistance as a consequence of selective pressure in bacteria-burdened lungs, combination drug regimens involving multiple drugs with distinct properties and bioactivities have been investigated in different clinical studies [25,26]. Some studies recommend that the many chemical entities that do not inhibit *M. tuberculosis* at clinically relevant concentrations could be involved in TB therapy, if administered in a synergistic combination with standard anti-TB drugs [27]. Therefore, interaction index or fractional inhibitory concentration of dinactin in combination with drugs of current TB therapy was also validated in the study. Interestingly, subinhibitory concentrations of dinactin induced a strong synergistic effect when tested in combination with RIF and AMK, which highlights the significant potential of dinactin for TB therapy. These observations are crucial for optimal dose designing to reduce drug dosing frequency. Synergism is known to prevent emergence of drug-resistance and is also recognised to enable reduced drug dosage and thereby decreased toxicity [28,29].

The current thrust in anti-TB treatment regimens indicates rationalisation of optimal dose strength and reduction of treatment time for standard and MDR therapies, which can be effectively achieved by studying activity dynamics of the drugs. The US Food and Drug Administration and the European Medicines Evaluation Agency, both emphasize on the use of various *in vitro* models to characterise the pharmacokinetic/pharmacodynamic properties of antibiotics to calculate the efficacy of candidate compounds in the very early stages of drug development [30,31]. The pharmacodynamic observations of *in vitro* killing assays enable an understanding of the degree of killing (concentration dependence) and rate of killing (time dependence), which are two significant parameters required for development of an anti-TB drug. The results of these assays are in concordance with therapeutic results obtained in studies involving TB patients [32]. The anti-TB activities of INH and RIF are concentration- and time-dependent, respectively [18,32], and the activity of pyrazinamide is driven by both in *in vitro* conditions [16,33]. The dynamic killing capacity of dinactin at different concentrations and time points provides an understanding for further development of this molecule. Concentration-dependent drugs are required in the initial phase of TB treatment and time-dependent drugs in the continuation phase, which requires sterilisation effects. Dinactin showed concentration-dependent killing activity with strong time-dependence, and may have implications in both phases of TB treatment. The kinetic kill assay of dinactin also revealed other important characteristics of this compound: at various concentrations within two days of exposure there were 1.5-4 $\log_{10}$ CFU/mL reductions in bacterial counts – this indicates the bactericidal nature of d-inactin and that killing resembles the first line anti-TB, INH [18,34]. Minimum concentration with maximum effect (E_{max}) of dinactin was observed to be 16 μg/mL. Thus, only a 16-fold higher concentration than MIC was required to eliminate all bacteria, whereas for standard drugs, like RIF and INH, much higher fold concentrations than their MIC are required to eliminate all bacteria. Further functional protein binding via MIC shift through the use of serum supplements showed that protein binding percentage of dinactin was comparable to that of INH. Thus, the present study highlights the anti-TB potential of dinactin and warrants further research, including pharmacokinetic, safety and efficacy studies in animal models, and *in vitro* studies focussing on target identification and mechanism of action.

In conclusion, this study showed for the first time that dinactin has a potent anti-mycobacterial action against drug-sensitive and drug-resistant *M. tuberculosis* strains. Dinactin displayed favourable synergy with first- and second-line anti-TB drugs, and demonstrated strong concentration-dependent anti-mycobacterial activity

that is also driven by time. Dinactin displays kill curve kinetics against *M. tuberculosis* that resemble the first-line anti-TB drug, INH. Collectively, these results are of sufficient interest to warrant further investigations for the development of dinactin as a novel anti-TB drug.

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Conflict of Interest

All authors declare that there is no conflict of interest.

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Ethical Approval

Not required.

Is your submission a Randomised Controlled Trial?

Not applicable.

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