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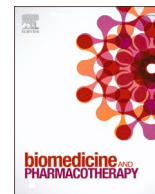
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## Polycationic phosphorous dendrimer potentiates multiple antibiotics against drug-resistant mycobacterial pathogens

Mohammad Imran<sup>a,d,1,2</sup>, Shriya Singh<sup>a,1,3</sup>, Mohammad Naiyaz Ahmad<sup>a,d,4</sup>, Pradip Malik<sup>a,d,5</sup>, Atri Mukhopadhyay<sup>a,d,6</sup>, Karan Singh Yadav<sup>b,d</sup>, Umesh D. Gupta<sup>e,7</sup>, Madhav N. Mugale<sup>b,d,8</sup>, Kalyan Mitra<sup>c,d,9</sup>, Kishore K. Srivastava<sup>a,d</sup>, Sidharth Chopra<sup>a,d,10</sup>, Serge Mignani<sup>f,g,\*,11</sup>, Evgeny Apartsin<sup>h,12</sup>, Jean-Pierre Majoral<sup>i,j,\*\*,13</sup>, Arunava Dasgupta<sup>a,d,\*\*\*,14</sup>

<sup>a</sup> Division of Molecular Microbiology & Immunology, CSIR-Central Drug Research Institute, Sitapur Road, Jankipuram Extension, Lucknow, Uttar Pradesh 226031, India

<sup>b</sup> Division of Toxicology & Experimental Medicine, CSIR-Central Drug Research Institute, Sitapur Road, Jankipuram Extension, Lucknow, Uttar Pradesh 226031, India

<sup>c</sup> Electron Microscopy Unit, CSIR-Central Drug Research Institute, Sitapur Road, 226031, India

<sup>d</sup> Academy of Scientific and Innovative Research (AcSIR), Ghaziabad 201002, India

<sup>e</sup> National JALMA Institute for Leprosy and Other Mycobacterial Diseases, Agra 282001, India

<sup>f</sup> Centre d'Etudes et de Recherche sur le Medicament de Normandie (CERMN), Université de Caen Normandie, Caen 14032, France

<sup>g</sup> Centro de Química da Madeira, MMRG, Campus da Penteada, Universidade da Madeira, Funchal 9020-105 19, Portugal

<sup>h</sup> Université de Bordeaux, CNRS, Bordeaux INP, CBMN, UMR 5248, Pessac F-33600, France

<sup>i</sup> Laboratoire de Chimie de Coordination du CNRS, 205 Route de Narbonne, BP 44099, 31077 Toulouse Cedex 4, France

<sup>j</sup> LCC-CNRS, Université de Toulouse, CNRS, Toulouse 31400, France

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### ABSTRACT

*Mycobacterium tuberculosis* (Mtb), causative agent of tuberculosis (TB) and non-tubercular mycobacterial (NTM) pathogens such as *Mycobacterium abscessus* are one of the most critical concerns worldwide due to increased drug-resistance resulting in increased morbidity and mortality. Therefore, focusing on developing novel therapeutics to minimize the treatment period and reducing the burden of drug-resistant Mtb and NTM infections are an urgent and pressing need. In our previous study, we identified anti-mycobacterial activity of orally bioavailable, non-cytotoxic, polycationic phosphorus dendrimer **2G0** against Mtb. In this study, we report ability of **2G0** to potentiate activity of multiple classes of antibiotics against drug-resistant mycobacterial strains. The observed synergy was confirmed using time-kill kinetics and revealed significantly potent activity of the combinations as compared to individual drugs alone. More importantly, no re-growth was observed in any tested

\* Correspondence to: UNICAEN, CERMN (Centre d'Etudes et de Recherche sur le Medicament de Normandie), 14032 Caen, France.

\*\* Corresponding author at: Laboratoire de Chimie de Coordination du CNRS, 205 Route de Narbonne, BP 44099, 31077 Toulouse Cedex 4, France

\*\*\* Correspondence to: Division of Molecular Microbiology and Immunology, CSIR-Central Drug Research Institute, Sitapur Road, Sector 10, Janakipuram Extension, Lucknow, Uttar Pradesh 226031, India.

E-mail addresses: [serge.mignani@staff.uma.pt](mailto:serge.mignani@staff.uma.pt) (S. Mignani), [jean-pierre.majoral@lcc-toulouse.fr](mailto:jean-pierre.majoral@lcc-toulouse.fr) (J.-P. Majoral), [a.dasgupta@cdri.res.in](mailto:a.dasgupta@cdri.res.in) (A. Dasgupta).

<sup>1</sup> These authors contributed equally to this work

<sup>2</sup> Orcid: 0000-0002-0738-0785

<sup>3</sup> ORCID: 0000-0003-0397-5498

<sup>4</sup> ORCID: 0000-0002-9300-6107

<sup>5</sup> ORCID: 0000-0001-9774-1371

<sup>6</sup> ORCID: 0000-0002-9665-4396

<sup>7</sup> ORCID: 0000-0002-0899-8560

<sup>8</sup> ORCID: 0000-0003-3138-3860

<sup>9</sup> ORCID: 0000-0002-7252-1716

<sup>10</sup> ORCID: 0000-0001-8823-6074

<sup>11</sup> ORCID: 0000-0002-1383-5256

<sup>12</sup> ORCID: 0000-0003-3334-0397

<sup>13</sup> ORCID: 0000-0002-0971-817X

<sup>14</sup> ORCID: 0000-0001-9014-1904

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combination. The identified combinations were further confirmed in intra-cellular killing assay as well as murine model of NTM infection, where **2G0** potentiated the activity of all tested antibiotics significantly better than individual drugs. Taken together, this nanoparticle with intrinsic antimycobacterial properties has the potential to represent an alternate drug candidate and/or a novel delivery agent for antibiotics of choice for enhancing the treatment of drug-resistant mycobacterial pathogens.

## 1. Introduction

In addition to tuberculosis (TB), infection caused due to drug-resistant NTM pathogens such as *Mycobacterium abscessus* also referred to as *Mycobacteroides abscessus* are rapidly emerging as a threat to healthcare systems worldwide due to increasing morbidity and mortality [1]. These pathogens are responsible for causing pulmonary and extra-pulmonary infections [2–4]. Number of reported cases has been increasing over the past two decades and the situation is more alarming in immune-compromised individuals as well as patient with cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) [5–9]. In addition, NTM infections are often misdiagnosed as TB resulting in vastly underestimation of these infections [10,11].

The treatment of drug resistant mycobacterial infections in general and *M. abscessus* in particular is extremely challenging and primarily reliant on drug-combinations but complete clearance from host is hampered by their intrinsic resistance to a wide variety of antimicrobials [12–14]. Hence, there is an urgent unmet need to develop novel agents for the comprehensive treatment of drug-resistant mycobacterial infections especially *M. abscessus* [15].

The unique structure and composition of the mycobacterial cell wall have historically created challenges for conventional drug discovery, making many antibiotics ineffective. In this context, dendrimers, wherein branched repeating units of unimolecular nanoparticles assembled to form synthetic molecules are often used as a drug carrier to increase drug efficacy as well as to reduce the toxicity of the drug, offer a novel solution. The polycationic dendrimers can disrupt the bacterial membrane by interacting with negatively charged bacterial cell membrane [16,17]. Interestingly, intrinsic antibacterial activity of many dendrimers has been studied in the last few years to explore their role as a novel drug molecule, although not much for phosphorus dendrimers. A recent study demonstrated the synergistic benefits of phosphorus dendrimer containing 96-end carboxylic groups with levofloxacin, resulting in a decreased antibiotic dose for treatment of bacterial infection [18, 19].

Our efforts to identify a novel antimycobacterial have previously identified an orally bio-available, water soluble polycationic phosphorus dendrimer **2G0** as a promising candidate against Mtb [20]. In this study, we further demonstrate its capability to synergize with several distinct antibiotic classes against drug-resistant mycobacterial pathogens. Most importantly, this nanoparticle demonstrated a remarkable ability to potentiate *in vitro* and *in vivo* activity of different antibiotics belonging to distinct classes against one of the most notoriously drug-resistant mycobacterial pathogens, *M. abscessus*, for which the clinical pipeline is almost empty [14].

## 2. Materials and methods

### 2.1. Chemicals and reagents

Bacterial culture media including Middlebrook 7H9 broth, Middlebrook 7H11 Agar; media supplements such as albumin-dextrose-catalase (ADC) and oleic acid-albumin-dextrose-catalase (OADC), FBS were purchased from Becton-Dickinson (NJ, USA). All other chemicals and antibiotics were purchased from Sigma-Aldrich (MO, USA).

### 2.2. Bacterial cultures and cell lines

*M. tuberculosis* H37Rv ATCC-27294, Isoniazid (INH)-resistant *M. tuberculosis* ATCC-35822, Rifampicin (RIF)-resistant *M. tuberculosis* ATCC-35838, Streptomycin (STR)-resistant *M. tuberculosis* ATCC-35820, Ethambutol (ETB)-resistant *M. tuberculosis* ATCC-35837, *Mycobacterium fortuitum* (*M. fortuitum*) ATCC-6841 and *M. abscessus* ATCC-19977 were procured from ATCC (VA, USA). The mycobacterial strains were cultured in 7H9-ADC broth supplemented with glycerol and 0.1% tyloxapol in a 37°C roller bottle incubator (ThermoFisher Scientific Inc., USA) at 20 rpm. J774A.1 was also procured from ATCC and maintained in RPMI 1640 (Gibco, Thermo Fisher Scientific, MA, United States) supplemented with 200 mM glutamine, 50 µg ml<sup>-1</sup> of neomycin, 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, MA, United States) at 37°C in the presence of 5% CO<sub>2</sub>.

### 2.3. Antibacterial susceptibility testing

MIC of **2G0** was determined by Microplate Alamar Blue Assay (MABA) utilizing broth microdilution assay according to CLSI guidelines as described previously [21]. Briefly, 10 mg/ml stock solutions of **2G0** and control drugs were prepared in DMSO and sterile water respectively. Bacterial cultures were inoculated in 7H9-ADC broth in such a way that the final OD<sub>600</sub> becomes ~10<sup>5</sup> colony-forming units (CFU)/ml. 2.5 µl of test and control compounds with varying concentration ranging from 0.03 to 64 µg/ml and 97.5 µl of bacterial suspension were added to each well of a 96-well plate. The plates were incubated at 37°C for 7 days for Mtb and 72 h for *M. fortuitum* and *M. abscessus*. The MIC is defined as the lowest concentration in which there is complete absence of visible growth. The MIC determinations were carried out independently three times in duplicate for each compound and the mean value is presented.

### 2.4. Bacterial time-kill kinetics

To determine whether **2G0** possesses bactericidal or bacteriostatic activity, time-kill analysis was performed as described previously [22]. Briefly, bacterial culture was diluted to ~10<sup>5</sup> CFU/ml, added to a 96-well plate along with **2G0** and control antibiotics at 1x, 2.5x and 5x MIC, followed by incubation at 37°C for 7 days for Mtb and 3 days for NTMs. For evaluating the reduction in CFU, samples (0.03 ml) was removed aseptically every 24 h (for Mtb) or 12 h (for NTMs), serially diluted in normal saline (0.27 ml) and plated (0.1 ml) on 7H11-OADC agar plates. The plates were incubated at 37°C for 72 h for NTMs, 4 weeks for Mtb and colonies were enumerated, log<sub>10</sub> CFU/ml values were plotted to construct kill curves. Each experiment was performed thrice in duplicate and the mean data was plotted by using GraphPad Prism software.

### 2.5. Study of synergistic effect of 2G0 with approved drugs

The interaction of **2G0** with other drugs was tested by the checkerboard method against Mtb, *M. fortuitum* and *M. abscessus* as described previously [21]. Briefly, **2G0** was two-fold diluted and added along the ordinate while the antibiotics were serially diluted along the abscissa in a 96-well plate. ~95 µl of bacterial culture was added to each well and plates were incubated at 37°C for 3 days for NTMs and 7 days for Mtb. The fractional inhibitory concentrations (ΣFIC) were calculated as depicted below:

$$\Sigma\text{FIC} = \text{FIC}_{\text{antibiotic}} + \text{FIC}_{2\text{G0}}$$

$$\text{or, } \Sigma\text{FIC} = \frac{\text{MIC of antibiotics in presence of } 2\text{G0}}{\text{MIC of antibiotics alone}} + \frac{\text{MIC of } 2\text{G0 in presence of antibiotics}}{\text{MIC of } 2\text{G0 alone}}$$

The combination is considered synergistic when the  $\Sigma\text{FIC}$  value is  $\leq 0.5$ , no interaction when the  $\Sigma\text{FIC}$  is  $>0.5-4$  and antagonistic when the  $\Sigma\text{FIC}$  is  $>4$  [23].

## 2.6. Intracellular killing activity of 2G0 against *M. abscessus*

To determine the ability of 2G0 to inhibit the growth of mycobacteria residing inside macrophage, intracellular killing assay was performed as described previously (12). Briefly, *M. abscessus* was cultured overnight in supplemented 7H9 Middlebrook broth. The bacterial inoculum was prepared from bacteria in mid-log phase and diluted to approximately  $\sim 10^7$  CFU/ml. Macrophage cell line J774A.1 cells were seeded at a density of approximately  $10^5$  cells per well in 12-well flat-bottom plates and after 24 hours cells were infected with *M. abscessus* at a multiplicity of infection (MOI) of 1:5. After 4 hours of infection, the cells were washed twice with 1x phosphate-buffered saline (PBS) and then replenished with fresh RPMI medium. The medium contained either 2G0 alone or in combination with rifampicin (RIF), bedaquiline (BDQ), linezolid (LZD), clofazimine (CFZ), and amikacin (AMK) as a control.

To determine the initial bacterial count, three wells were lysed 4 hours post-infection, and the lysate was serially diluted and plated on supplemented Middlebrook 7H11 agar plates. After 72 hours of incubation, another set of three wells was lysed, serially diluted, plated, and incubated at  $37^\circ\text{C}$  for 72 hours to estimate colony-forming units (CFU). Kill curves were generated by counting colonies from the plates and plotting CFU/ml. Each experiment was conducted three times in duplicate, and the mean data were plotted.

## 2.7. *In vitro* resistant mutant generation, resistant frequency determination and Mutant prevention concentration (MPC)

Determination of *in vitro* emergence of resistance to 2G0 and MPC were performed as reported earlier [12,24,25]. Briefly, *M. fortuitum* culture containing  $\sim 10^{10}$  CFU/ml was spread on 7H11-OADC agar plates containing either 8–256x MIC of 2G0, RIF, LZD and CFZ and combinations of 2G0 and respective antibiotics or without any antibiotics as control. The plates were incubated at  $37^\circ\text{C}$  and were observed daily from the 3rd–14th day for appearance of colonies [24]. The colonies that exhibited resistance from these plates were streaked onto new plates containing respective drugs and the time of appearance of colonies on these fresh plates were compared with the time of appearance of initial colonies in the previous stage. MPC was defined as the concentration which effectively prevented the appearance of any resistant mutant colonies [25]. The resistant frequency (Rf) was calculated as the ratio between number of resistant colonies on the drug plate ( $R_N$ ) and CFU of inoculum ( $T_N$ ). To rule out the possibility of emergence of resistant colonies due to degradation of drugs, similar experiment was performed on 14-day old drug-containing plates stored at  $37^\circ\text{C}$  and mutant generation frequency was compared with freshly prepared drug-containing plates.

## 2.8. Scanning electron microscopy

Scanning Electron Microscopy (SEM) was utilized to observe any topological and morphological alterations in *M. abscessus* following treatment with 2G0 alone and combinatorial treatment of 2G0 with RIF, BDQ, CFZ and LZD. The mid-log phase culture of *M. abscessus* ( $\text{OD}_{600} = 0.6-0.7$ ) were treated with 5x MIC of 2G0 alone and 2G0 in combination with RIF, BDQ, CFZ and LZD for 8 h, whereas untreated control was treated with vehicle control DMSO. After treatment, the cells were washed twice with phosphate buffer saline (PBS) pH 7.2 and processed as described previously [26]. Briefly, cells were treated with a solution containing 2.5% glutaraldehyde in 0.1 M phosphate buffer. After a rinse

in the same phosphate buffer, the cell suspensions were placed onto glass chips coated with poly-L-lysine, where they were allowed to attach for 10 minutes at room temperature. Subsequently, the samples were treated with 1%  $\text{OsO}_4$ , followed by a dehydration process using increasing concentrations of ethanol and dried using a critical point method. Finally, the samples were coated with an 80:20 mixture of Au-Pd using a Polaron E5000 sputter coater and observed under a FEI Quanta 250 SEM (Thermo Fisher, USA) at 30 kV using a secondary electron detector.

## 2.9. Animal experiments

Mice were procured from the National Laboratory Animal Facility of the CSIR-Central Drug Research Institute (CDRI), Lucknow. Experimental protocol and use of mice was approved by the Institutional Animal Ethics Committee at CSIR-CDRI. Animal experiments were performed per the guidelines provided by the Committee for Control and Supervision of Experiments on Animals (CPCSEA, Govt. of India).

## 2.10. *In vivo* efficacy of 2G0 in murine bacteremia model

Animal experiments were performed on neutropenic *M. fortuitum* and *M. abscessus* infection models to mimic clinical conditions since the majority of NTM infections occur in immuno-compromised conditions [27,28]. Neutropenia was induced in 5–6 week-old BALB/c mice by intraperitoneally administering 150 mg/kg cyclophosphamide 4 days and 100 mg/kg 1 day before infection. Neutropenic mice were then infected with  $\sim 5 \times 10^6$  CFU/ml of *M. fortuitum* or *M. abscessus* through intravenous route as described [12] and divided randomly into 12 groups: 11 groups had six mice each while the sham group had 8 mice. The drug treatment was initiated 1-day post infection with intraperitoneal administration of amikacin (AMK) (100 mg/kg in water) as a control drug and oral administration of 0.1 ml of 2G0 (25 mg/kg in water), LZD (125 mg/kg), RIF (10 mg/kg), BDQ (10 mg/kg), CFZ (50 mg/kg) with 2G0 and alone while sham group mice received normal saline only. After 10 days, all the mice were sacrificed; the lung, spleen and kidney were resected and homogenized in 5 ml of normal saline; serially diluted and spread on 7H11-OADC agar plates for bacillary load enumeration. After incubation for 2–3 days at  $37^\circ\text{C}$ , CFU were enumerated and the data was averaged across experiments and mean data was plotted.

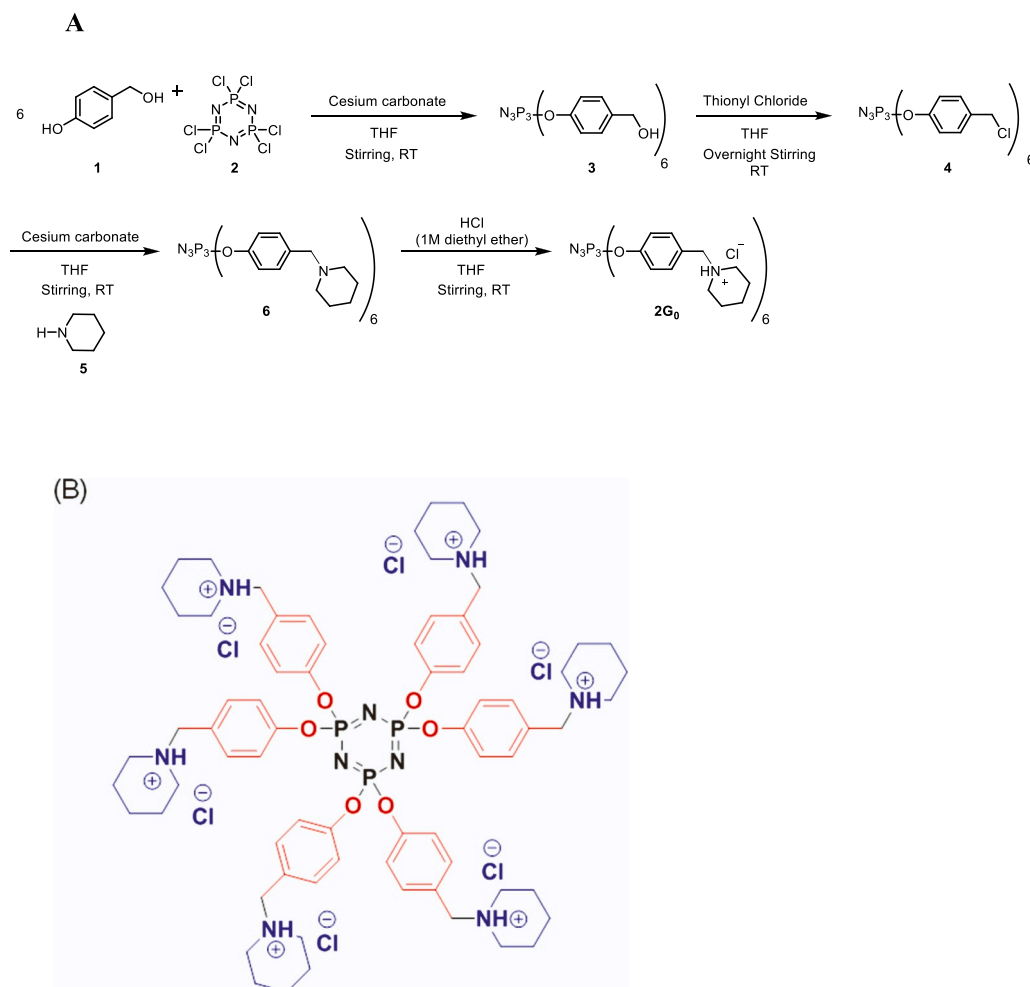
## 2.11. Statistical analysis

For statistical analysis, GraphPad Prism 9.0 software (GraphPad Software, La Jolla, CA, USA) was used. One-way ANOVA and post hoc Tukey's multiple comparisons tests were performed for comparison between three or more groups. P values of  $<0.05$  were considered to be significant.

## 3. Results

### 3.1. 2G0 is a stable bioactive low generation polycationic phosphorus dendrimer

2G0 was synthesized in 4 straightforward steps as reported earlier [20]. The first two steps involve the substitution of the six P-Cl bonds of the hexachlorocyclotriphosphazene **2** with 4 hydroxy benzyl alcohol **1** leading to the compound **3** followed by chlorination of the hydroxy groups with thionyl chloride as the chlorinated agent. Substitution of the terminal chloride of **4** with piperidine allows obtaining the neutral small phosphorus dendrimer **6**. Then protonation of the piperidine end groups of **6** leads to the cationic phosphorus dendrimer, 2G0 with total 80% yield. Remarkably, 2G0 is highly soluble in water and is stable at room temperature for more than 2 years without any detected chemical degradation. The detailed reaction conditions and the chemical



**Fig. 1.** Schematic diagram of the reaction conditions for synthesis of **2G0**, a polycationic, phosphorus dendrimer of low-generation (Fig. 1A) and its chemical structure (Fig. 1B).

**Table 1**

MIC ( $\mu\text{g/ml}$ ) of **2G0** and control drugs against various clinically relevant mycobacterial pathogens.

Bacterial strain		MIC ( $\mu\text{g/ml}$ )												
		2G0	RIF	INH	STR	ETB	MPM	AMK	CLR	LZD	MXF	LVF	BDQ	CFZ
Mtb	H37Rv ATCC-27294	2	0.015	0.03	0.5	1	4	0.25	2	0.5	0.06	0.25	0.03	0.06
	RIF-resistant ATCC-35838	2	64	0.03	1	0.5	4	0.12	NT	NT	NT	0.12	NT	0.06
	INH-resistant ATCC-35822	2	0.03	>64	1	0.5	2	0.25	NT	NT	NT	0.25	NT	0.06
	ETB-resistant ATCC-35837	2	0.03	0.03	1	64	2	0.12	NT	NT	NT	0.12	NT	0.06
	STR-resistant ATCC-35820	2	0.03	0.03	>64	1	4	0.12	NT	NT	NT	0.25	NT	0.06
NTM	<i>M. abscessus</i> ATCC-19977	16	16	>64	64	64	8	8	1	4	1	2	0.12	1
	<i>M. fortuitum</i> ATCC-6841	16	8	32	16	8	4	1	1	2	0.03	0.06	0.06	2
	RIF-resistant <i>M. fortuitum</i>	16	>256	NT	NT	NT	8	2	4	NT	0.03	0.06	NT	NT
	<i>M. chelonae</i> ATCC-35752	8	2	32	64	4	2	1	0.5	NT	0.5	0.12	NT	NT

\*RIF: Rifampicin, INH: Isoniazid, STR: Streptomycin, ETB: Ethambutol, MPM: Meropenem, AMK: Amikacin, CLR: Clarithromycin, LZD: Linezolid, MXF: Moxifloxacin, LVF: Levofloxacin, BDQ: Bedaquiline, CFZ: Clofazimine, NT: Not tested

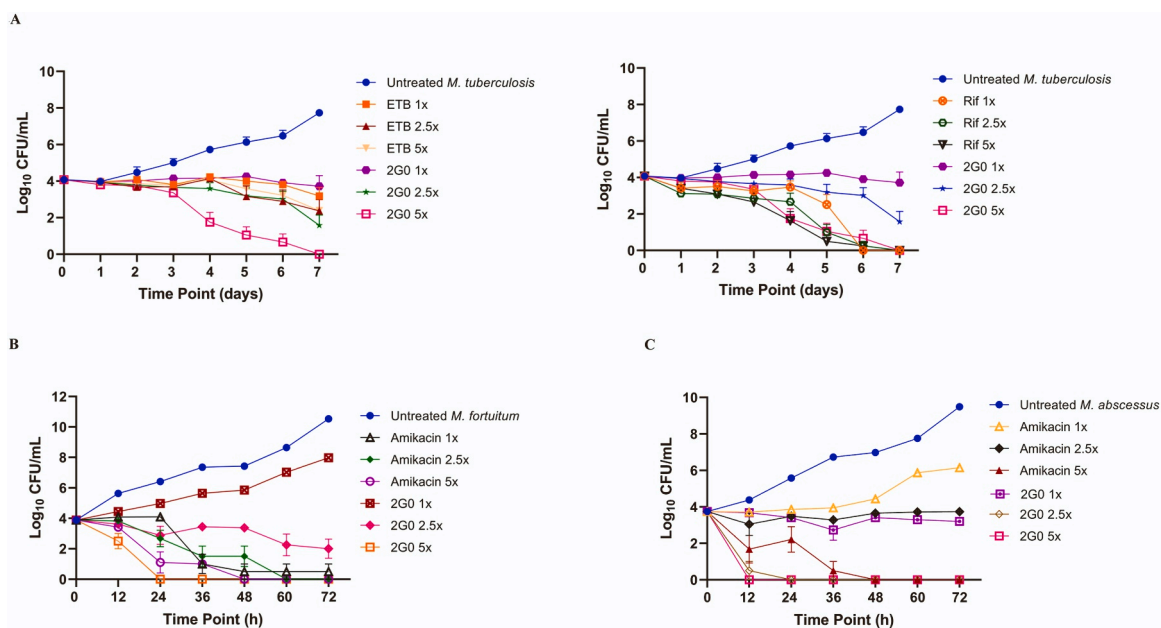
structure of **2G0** are depicted in Fig. 1A & 1B.

### 3.2. **2G0** is active against various mycobacterial pathogens

The MIC of **2G0** was determined against Mtb H37Rv ATCC-27294, *M. fortuitum* ATCC-6841, *M. chelonae* ATCC-35752 and *M. abscessus* ATCC-19977 along with comparator antibiotics. **2G0** exhibits promisingly low MIC (2–16 mg/L) against Mtb and NTMs and most interestingly, it is equipotently active against different isogenic single drug-resistant (SDR) Mtb including INH-resistant (ATCC-35822), RIF-

resistant (ATCC-35838), STR-resistant (ATCC-35820) and ETB-resistant (ATCC-35837) Mtb strains, indicating a possible new mechanism of action which is distinct from existing drug resistance mechanisms (Table 1). Moreover, MIC of **2G0** against NTMs including *M. abscessus* and RIF-resistant *M. fortuitum* is comparable to meropenem (MPM), which is clinically utilized for their treatment. This is significantly encouraging as *M. abscessus* is one of the most notorious drug-resistant mycobacterial pathogens against which chemotherapeutic options is very limited [29,30].





**Fig. 2.** Time kill kinetics of 2G0 and comparator antibiotics against (A) *M. tuberculosis* H37Rv, (B) *M. fortuitum* and (C) *M. abscessus*. Time kill kinetics of 2G0 and comparator drugs (ETB and RIF) was done in the same set of experiments and the results were shown in two separate panels for clarity (Fig. 2A). The log<sub>10</sub> CFU/ml values of 2G0 (1x, 2.5x and 5x MIC) and untreated control are same in two panels. The error bars in all the panels denote the standard errors of mean values obtained from duplicate samples from two independent set of experiments.

**3.3. 2G0 expresses concentration-dependent bactericidal activity against various mycobacterial pathogens**

To determine the killing kinetics of 2G0 against Mtb and NTMs, 2G0 was treated at 1x, 2.5x and 5x MIC against Mtb H37Rv, *M. fortuitum* and *M. abscessus* along with control drugs and plotted in Fig. 2A-C.

When tested against Mtb H37Rv, 1x MIC of 2G0 led to a reduction of ~4 log<sub>10</sub> CFU/ml in 7 days as compared to untreated, which is comparable to ETB, one of the front-line drugs used for the treatment of TB. Interestingly, 5x MIC of 2G0 caused a reduction of ~7.7 log<sub>10</sub> CFU/ml which is comparable to RIF and significantly outperformed ETB at 5x MIC in 7 days with no re-growth observed afterwards (Fig. 2A).

When tested against *M. fortuitum*, 1x MIC of 2G0 does not show significant reduction of bacterial burden. In contrast, 2.5x MIC of 2G0 significantly reduced bacterial count (~8.5 log<sub>10</sub> CFU/ml) as compared to the untreated control in 72 h. Most strikingly, the kill kinetics of 2G0 at 5x MIC was comparable to AMK in 24 h with no re-growth observed (Fig. 2B).

In contrast, *M. abscessus* was far more susceptible to 2G0, as 2G0 at

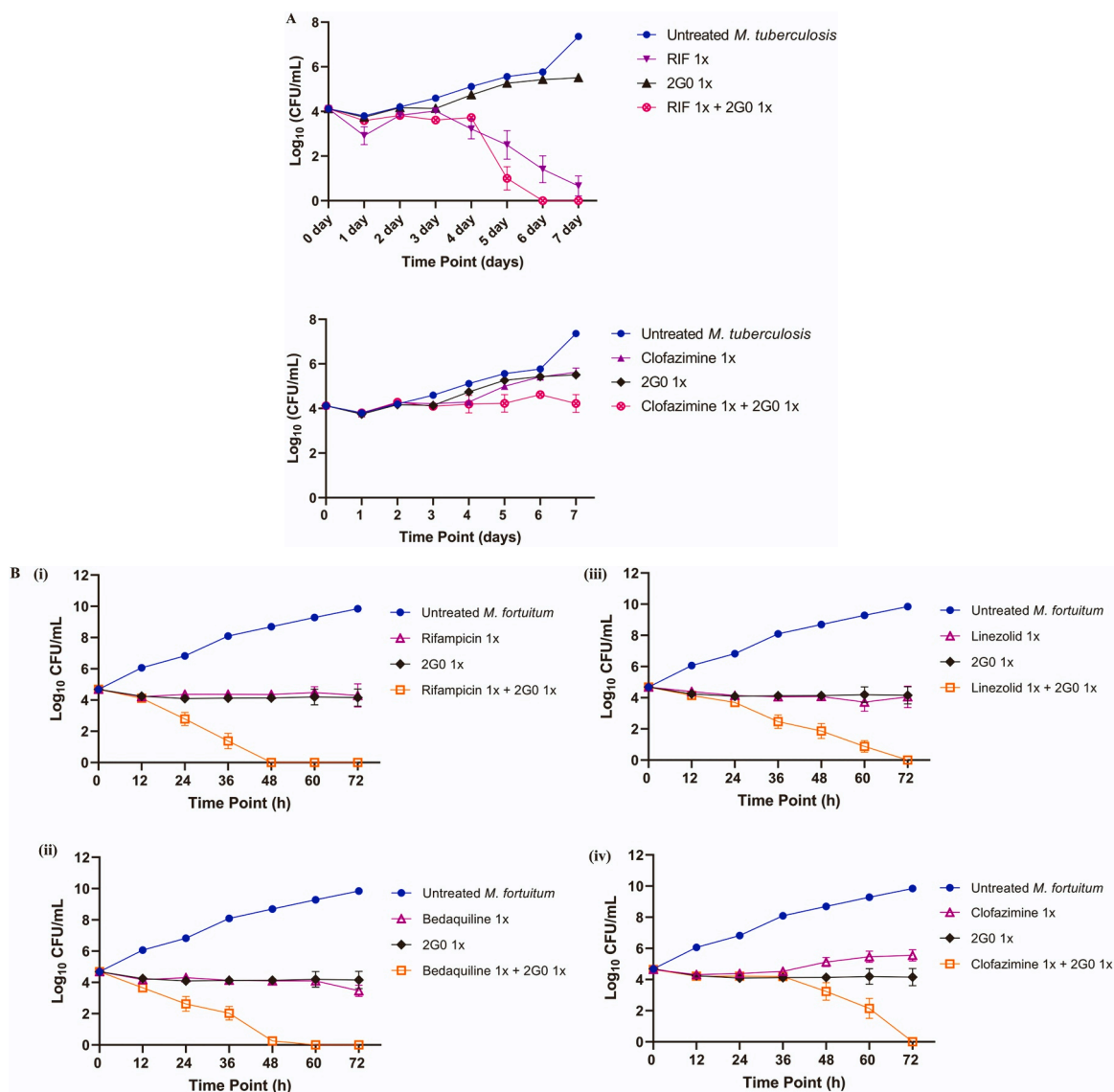
1x MIC eliminated all bacterial culture within 48 h with no observed re-growth. At 2.5x MIC of 2G0, eliminated culture (~4.0 log<sub>10</sub> CFU/ml) within 24 h and at 5x MIC of 2G0 eliminated culture (~6.0 log<sub>10</sub> CFU/ml) within 12 h. This concentration-dependent killing against *M. abscessus* is better than AMK, drug of choice for the treatment of NTM infections, which at 1x MIC demonstrates static activity, while at 2.5x MIC, reduced ~2.4 log<sub>10</sub> CFU/ml with continuous growth and similar pattern observed at 5x MIC compared to untreated control in 72 h (Fig. 2C).

Taken together, 2G0 expresses concentration dependent bactericidal activity with no-regrowth against various mycobacterial pathogens and the activity is comparable with several standard of care antibiotics. This is extremely important since incomplete clearance of bacterial load and induced resistance to various antimicrobials, especially macrolides, is a hallmark of drug-resistant *M. abscessus* [14,29,30].

**Table 2**  
Determination of interaction of 2G0 with approved antibiotics against *M. tuberculosis*, *M. fortuitum* & *M. abscessus* .

Target	Drug	<i>M. tuberculosis</i>		<i>M. fortuitum</i>		<i>M. abscessus</i>	
		ΣFIC	Analysis	ΣFIC	Analysis	ΣFIC	Analysis
Protein synthesis	Rifampicin	0.3125	Synergy	0.375	Synergy	0.375	Synergy
	Linezolid	0.75	No interaction	0.375	Synergy	0.31	Synergy
	Streptomycin	1.5	No interaction	1	No interaction	1.25	No interaction
	Amikacin	1.5	No interaction	0.75	No interaction	1.25	No interaction
	Clarithromycin	0.75	No interaction	0.75	No interaction	1.5	No interaction
Cell wall	Isoniazid	0.75	No interaction	NT*	-	NT*	-
	Ethambutol	0.625	No interaction	NT*	-	NT*	-
	Delamanid	0.73	No interaction	NT*	-	NT*	-
	Pretomanid	1.25	No interaction	NT*	-	NT*	-
	Meropenem	0.75	No interaction	1.125	No interaction	1	No interaction
DNA gyrase	Levofloxacin	1.5	No interaction	1.5	No interaction	1.12	No interaction
	Moxifloxacin	1.5	No interaction	1.5	No interaction	1.25	No interaction
ATP synthesis	Bedaquiline	1.25	No interaction	0.18	Synergy	0.27	Synergy
Unknown	Clofazimine	0.5	Synergy	0.375	Synergy	0.28	Synergy

\* NT: Not tested



**Fig. 3.** Time kill kinetics of 2G0 in combination with (A) (i) RIF and (ii) CFZ against *M. tuberculosis*, (B) (i) RIF, (ii) BDQ (iii) LZO, (iv) CFZ against *M. fortuitum* and (C) (i) RIF, (ii) BDQ, (iii) LZO, (iv) CFZ against *M. abscessus*. All the time kill kinetics experiments against particular mycobacteria were done in single set of experiment [Fig. 3A (i and ii)] [Fig. 3B (i-iv)] and [Fig. 3C (i-iv)] and the results were shown in different panels for clarity. The log<sub>10</sub> CFU/ml values of 2G0 (1x MIC) and untreated control against particular mycobacteria are thus same across the above mentioned panels. The error bars in all the panels denote the standard errors of mean values obtained from duplicate samples from two independent set of experiments.

**3.4. 2G0 synergizes and potentiates multiple antibiotics against mycobacterial pathogens**

The treatment therapy for all mycobacterial infections is based on combinations of various antibiotics, which reduces the emergence of drug-resistance as well as leads to increased clearance of pathogens. To validate the synergistic effects of any new drug molecule, it needs to be tested for its ability to synergize with clinically utilized antibiotics [31, 32]. In this context, the synergistic effects of 2G0 with approved drugs that are utilized for the treatment of mycobacterial infections were determined by chequerboard method and data are summarized in Table 2.

As can be seen in Table 2, 2G0 strongly synergized with RIF with  $\Sigma$ FIC value of 0.312 and moderately synergized with CFZ against Mtb H37Rv ( $\Sigma$ FIC 0.5) whereas it did not interact with INH, ETB, Delamanid, Pretomanid, MPM, STR, CLR, LZD, MXF and BDQ.

On the other hand, 2G0 synergized with RIF, BDQ, LZD and CFZ

against *M. fortuitum* as well as against *M. abscessus* (Table 2). These results demonstrate the capability of 2G0 to synergize the action of several important classes of antibiotics against mycobacterial pathogens including against drug-resistant *M. abscessus*.

These observed synergistic interactions were further confirmed by time-kill assay at 1xMIC of all and the results were plotted in Fig. 3A-C. The combination of 1x MIC of RIF and 2G0 was more efficacious than 1x MIC of either RIF or 2G0 alone and reduced ~7.3 log<sub>10</sub> CFU/ml in 7 days against Mtb whereas there was no significant reduction in bacterial counts for the combination of 2G0 and CFZ (Fig. 3A).

When tested against *M. fortuitum*, the combinatorial effect of 2G0 and RIF (1x MIC) was more efficacious than either RIF or 2G0 alone and no CFU was observed in 48 h as compared to untreated with no-growth (Fig. 3B). Similarly, combinations of 1x MICs of 2G0 with BDQ, LZD and CFZ were significantly more effective than individual drugs alone (Fig. 3B).

A similar effect was observed against *M. abscessus*, where the

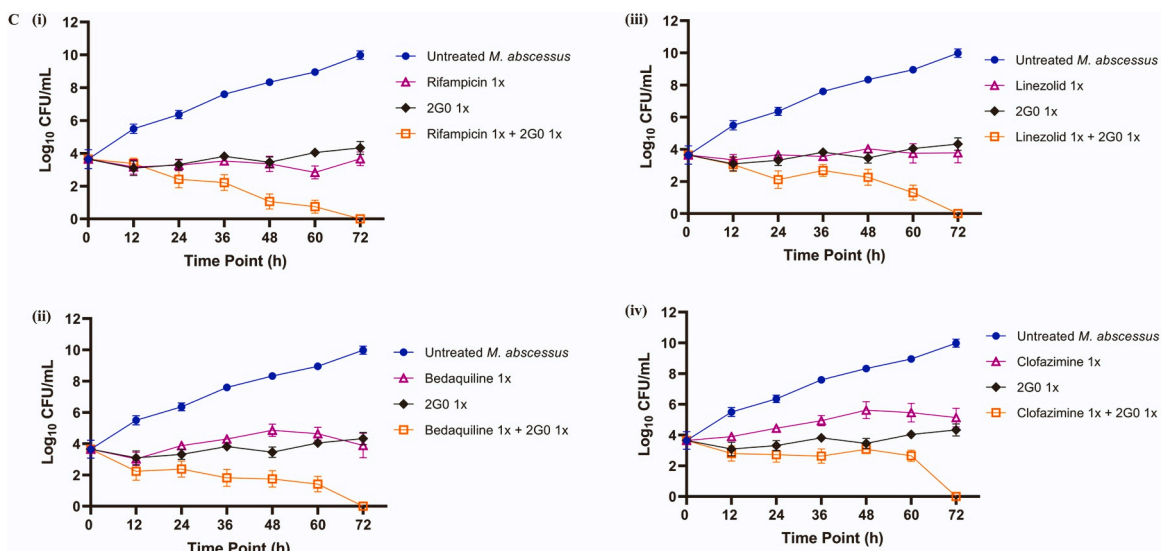


Fig. 3. (continued).

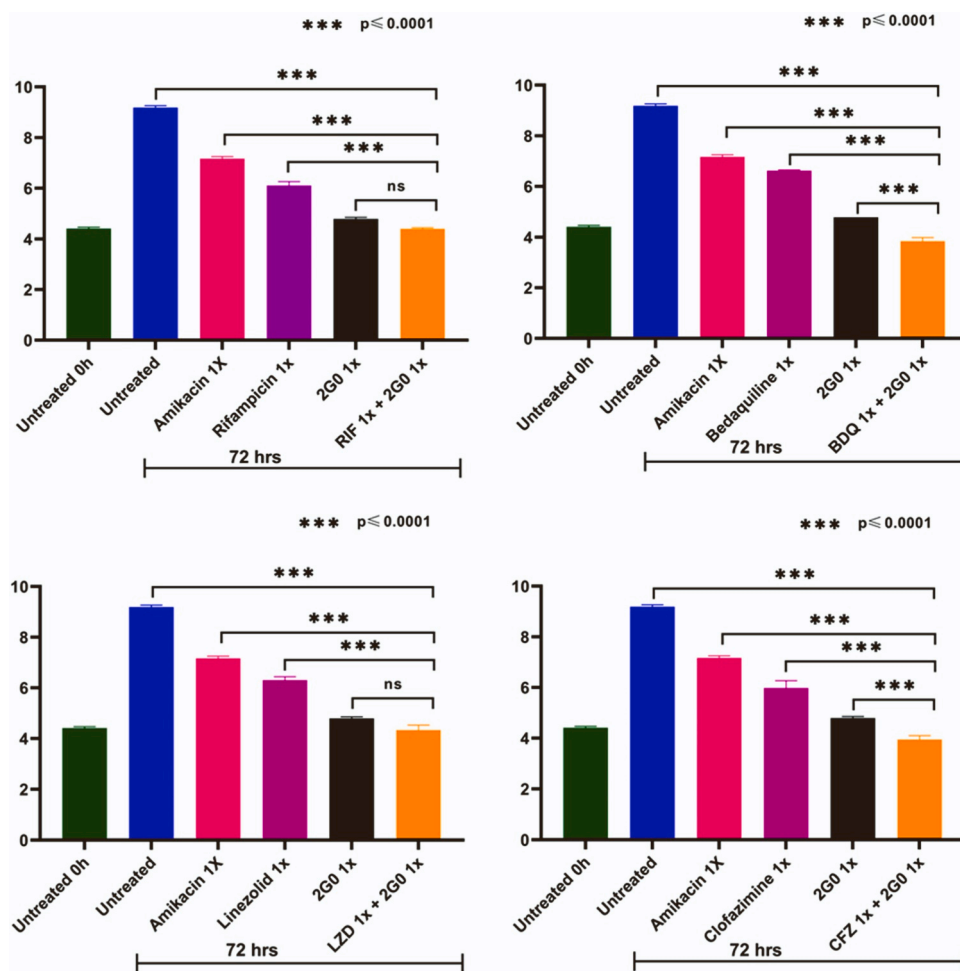


Fig. 4. Intracellular activity of 2G0 alone and in combination with RIF, BDQ, LZD, CFZ. J774A.1 cells were infected with *M. abscessus* ATCC 19977 at a MOI of 1:5 for 4 h prior to treatment with 1x MICs of respective drugs and combinations. AMK (8 mg/L) was used as control. CFU were enumerated after 72 h. Each experiment was performed in a single set and the results were shown in different panels for ease in comparison. The experiment was performed in triplicate and the data were presented as mean  $\pm$  s.d. (\*\*\*) equals  $\leq 0.0001$ .

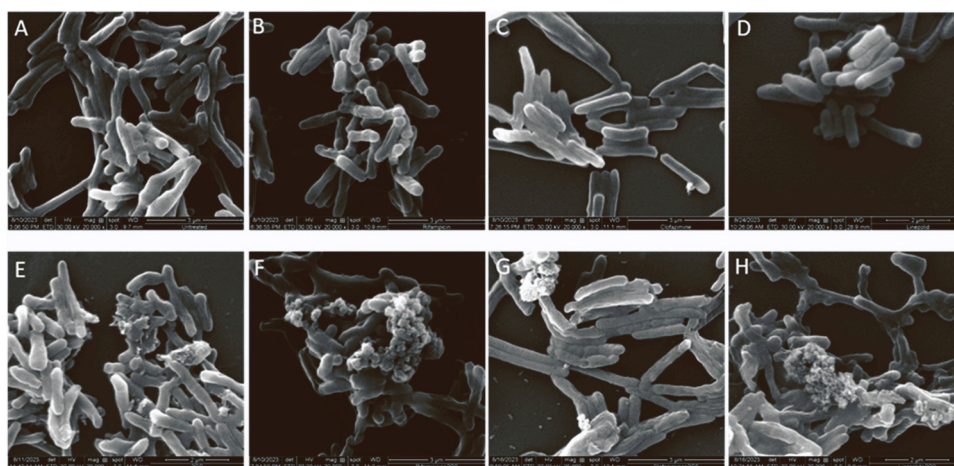


**Table 3**

Determination of frequency of spontaneous resistance mutants and Mutant prevention concentration (MPC) for individual drugs, **2G0** and drug-combinations against *M. fortuitum*.

Drugs	MIC (mg/l)	Mutant Frequency at fold MIC (mg/L)						Mutant prevention concentration (MPC)	$\Delta$ fold change in MPC wrt to Antibiotic/ <b>2G0</b> alone
		8x	16x	32x	64x	128x	256x		
<b>2G0</b>	16	TNTC	5.7 $\times 10^{-9}$	0	0	0	0	32x MIC	-
<b>RIF</b>	8	TNTC	TNTC	TNTC	TNTC	6 $\times 10^{-9}$	0	256x MIC	-
<b>RIF + 2G0</b>	8+16	8.7 $\times 10^{-4}$	0	0	0	0	0	16x MIC	16/2
<b>LZD</b>	8	TNTC	6.5 $\times 10^{-6}$	0	0	0	0	32x MIC	-
<b>LZD + 2G0</b>	8+16	0	0	0	0	0	0	8x MIC	4/4
<b>CFZ</b>	2	TNTC	TNTC	7.2 $\times 10^{-5}$	1.3 $\times 10^{-5}$	0	0	128x MIC	-
<b>CFZ + 2G0</b>	2+16	9.8 $\times 10^{-4}$	0	0	0	0	0	16x MIC	8/2

\* TNTC: Too numerous to count



**Fig. 5.** Representative SEM images showing morphological effects of various drugs at 5x MIC for 8 h treatment (alone and in combination) on *M. abscessus*. RIF, CFZ and LZD do not induce visible damage and the bacterial morphology and is similar to untreated cells (A – D). **2G0** induces topological alterations and its damaging effects are visible in (E). **2G0** in combination with RIF, CFZ and LZD induces more severe damage and alterations compared to treatment with **2G0** alone (F-H).

combination with 1x MIC of BDQ and **2G0** was more potent than individual drugs alone and reduced  $\sim 9.9 \log_{10}$  CFU/ml in 72 h. Similarly, combinatorial efficacy of 1x MIC of **2G0** and LZD reduced  $\sim 8 \log_{10}$  CFU/ml, combination of RIF and **2G0** reduced  $\sim 9.9 \log_{10}$  CFU/ml in 72 h while the combination of CFZ and **2G0** reduced  $\sim 8.4 \log_{10}$  CFU/ml in 72 h, more than individual drugs alone as compared to untreated (Fig. 3C). In all time kill kinetics, the individual drugs exhibit bacteriostatic activity, whilst the combinations exhibit cidal activity. More importantly, none of the combinations exhibited re-growth after treatment in any combinations, further demonstrating the value of drug-combinations. Collectively, these studies indicated that identified combinations have the potential to be a part of a multi-drug treatment regimen for the treatment of drug-resistant mycobacterial infections.

### 3.5. **2G0** is potently active against intracellular *M. abscessus* individually and in combination

The efficacy of **2G0** alone and in combination with RIF, BDQ, LZD, and CFZ in clearing intracellular *M. abscessus* was assessed using J774A.1 cells infected with a MOI of 1:5. The 1x MIC of **2G0** alone and in combination with RIF, BDQ, LZD, and CFZ and AMK were added into the culture, followed by cell lysis and plating on OADC supplemented Middlebrook 7H11 agar to quantify CFU. The results depicted in the figure demonstrated that **2G0** alone as well as in combination with

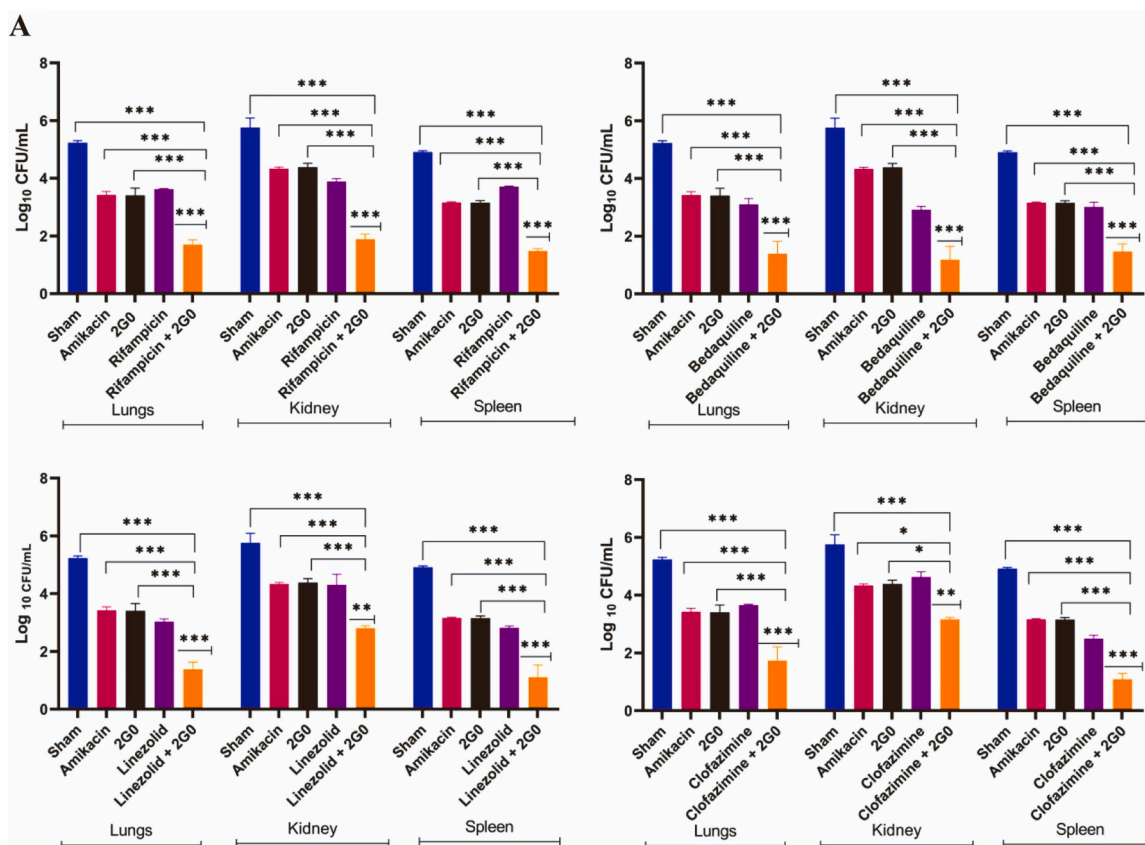
different drugs lead to a significant reduction ( $> 4 \log_{10}$ ) in the bacterial load as compared with individual drug alone (Fig. 4).

### 3.6. **2G0** and combinations reduce the formation of resistant mutants

The clinical utilization of drug combinations is also reported to reduce resistance and this was further confirmed for our identified combinations by calculating the resistant mutant frequency of individual drugs and combinations thereof. The data is presented in Table 3. As can be seen, mutant prevention concentration of **2G0** is determined as 32x MIC whilst for all combinations, there is an 4–16 fold drop in MPC as compared to antibiotic alone and 2–4 fold drop in MPC compared to **2G0** alone (Table 3). This is very promising and further emphasizes on the clinical potential of these identified drug combinations.

### 3.7. **2G0** in combination with drugs induces more severe morphological alterations in *M. abscessus*

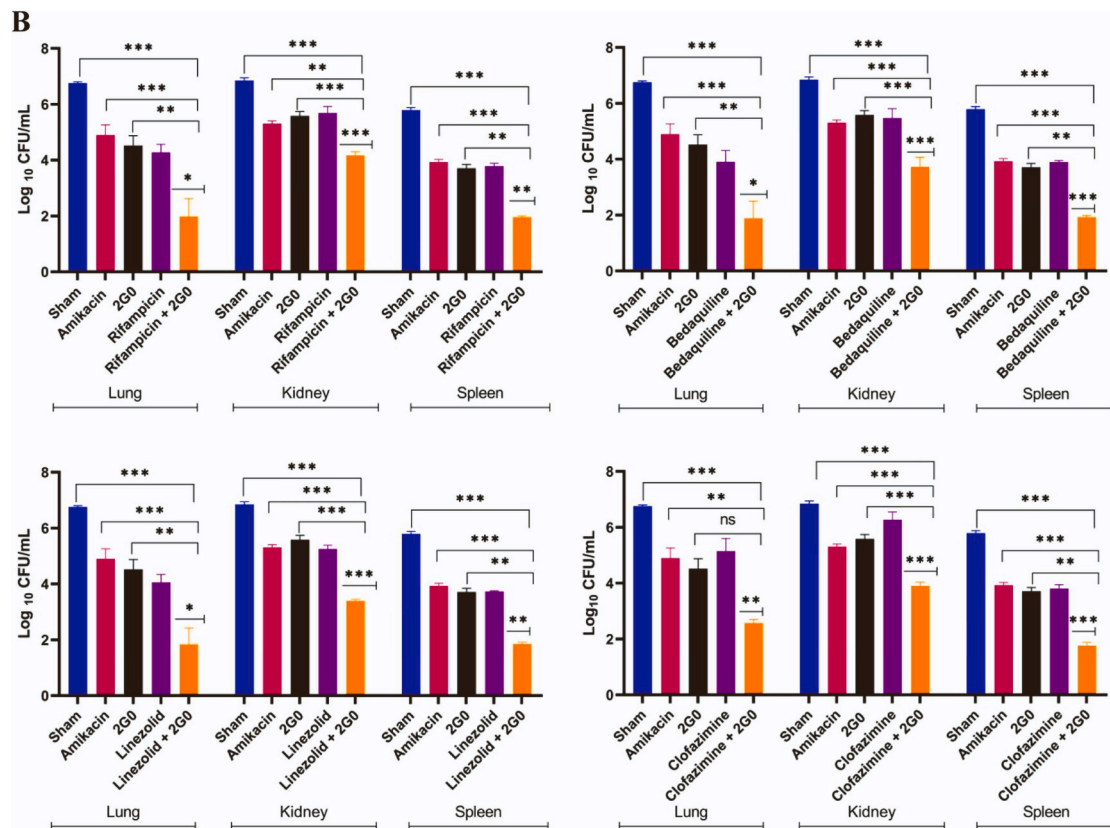
SEM experiments were performed to visualize the morphological and topological effects of **2G0**, RIF, CFZ, LZD and **2G0** in combination with these drugs on *M. abscessus* (Fig. 5). It was observed that RIF, CFZ and LZD do not induce visible morphological damage and the bacterial morphology is similar to untreated cells. In contrast, topological alterations and bacterial damage were observed in cells treated with **2G0** and



**Table 4A** (*M. fortuitum*)

Drug Combinations (A)	Treatment groups (B)	$\Delta \log_{10} \text{CFU/ml (B - A)}$		
		Lung	Kidney	Spleen
RIF + 2G0	Sham	2.2 (5.2 - 1.7)	3.9 (5.7 - 1.8)	3.5 (4.9 - 1.4)
	Amikacin	1.7 (3.4 - 1.7)	2.5 (4.3 - 1.8)	1.7 (3.1 - 1.4)
	2G0 alone	1.7 (3.4 - 1.7)	2.5 (4.3 - 1.8)	1.7 (3.1 - 1.4)
	RIF alone	1.9 (3.6 - 1.7)	2.0 (3.8 - 1.8)	2.3 (3.7 - 1.4)
LZD + 2G0	Sham	3.9 (5.2 - 1.3)	2.9 (5.7 - 2.8)	3.8 (4.9 - 1.1)
	Amikacin	2.1 (3.4 - 1.3)	1.5 (4.3 - 2.8)	2.0 (3.1 - 1.1)
	2G0 alone	2.1 (3.4 - 1.3)	1.5 (4.3 - 2.8)	2.0 (3.1 - 1.1)
	LZD alone	1.7 (3.0 - 1.3)	1.5 (4.3 - 2.8)	1.7 (2.8 - 1.1)
BDQ + 2G0	Sham	3.9 (5.2 - 1.3)	4.6 (5.7 - 1.1)	3.5 (4.9 - 1.4)
	Amikacin	2.1 (3.4 - 1.3)	3.2 (4.3 - 1.1)	1.7 (3.1 - 1.4)
	2G0 alone	2.1 (3.4 - 1.3)	3.2 (4.3 - 1.1)	1.7 (3.1 - 1.4)
	BDQ alone	1.8 (3.1 - 1.3)	1.8 (2.9 - 1.1)	1.6 (3.0 - 1.4)
CFZ + 2G0	Sham	3.5 (5.2 - 1.7)	2.6 (5.7 - 3.1)	3.9 (4.9 - 1.0)
	Amikacin	1.7 (3.4 - 1.7)	1.2 (4.3 - 3.1)	2.1 (3.1 - 1.0)
	2G0 alone	1.7 (3.4 - 1.7)	1.2 (4.3 - 3.1)	2.1 (3.1 - 1.0)
	CFZ alone	1.9 (3.6 - 1.7)	1.5 (4.6 - 3.1)	1.4 (2.4 - 1.0)

**Fig. 6.** *In vivo* efficacy of 2G0 alone and in combination with drugs against *M. fortuitum* (Fig. 6A) and *M. abscessus* (Fig. 6B) in murine neutropenic bacteremia model. The mice were infected with  $\sim 5 \times 10^6$  CFU/ml and treated with 2G0 (25 mg/kg) in combination with RIF (10 mg/kg), BDQ (10 mg/kg), LZD (125 mg/kg), CFZ (50 mg/kg) and with AMK (100 mg/kg). *In vivo* efficacy of 2G0 alone and in combination with different drugs was done in two set of experiments, one against *M. fortuitum* (Fig. 5A) and another against *M. abscessus* (Fig. 5B) and the result were shown separate panels for clarity. The  $\log_{10}$  CFU/ml values of Sham, AMK and 2G0 treated groups are same in respective organs in all the combination tested. All experiments were done in triplicates and data were presented as mean  $\pm$  s.d. \*\*\*equals  $< 0.001$ . The quantitative difference in bacterial load ( $\Delta \log_{10}$  CFU/ml) of Figs. 6A and 6B is tabulated in Table 4A for *M. fortuitum* and in Table 4B for *M. abscessus* respectively.



**Table 4B** (*M. abscessus*)

Drug Combinations (A)	Treatment groups (B)	$\Delta \log_{10} \text{CFU/ml (B - A)}$		
		Lung	Kidney	Spleen
RIF + 2G0	Sham	4.8 (6.7 - 1.9)	2.7 (6.8 - 4.1)	3.8 (5.7 - 1.9)
	Amikacin	3.0 (4.9 - 1.9)	1.2 (5.3 - 4.1)	2.0 (3.9 - 1.9)
	2G0 alone	2.6 (4.5 - 1.9)	1.4 (5.5 - 4.1)	1.8 (3.7 - 1.9)
	RIF alone	2.3 (4.2 - 1.9)	1.5 (5.6 - 4.1)	1.8 (3.7 - 1.9)
LZD + 2G0	Sham	4.9 (6.7 - 1.8)	3.5 (6.8 - 3.3)	3.9 (5.7 - 1.8)
	Amikacin	3.1 (4.9 - 1.8)	2.0 (5.3 - 3.3)	2.1 (3.9 - 1.8)
	2G0 alone	2.7 (4.5 - 1.8)	2.2 (5.5 - 3.3)	1.9 (3.7 - 1.8)
	LZD alone	2.2 (4.0 - 1.8)	1.9 (5.2 - 3.3)	1.9 (3.7 - 1.8)
BDQ + 2G0	Sham	4.9 (6.7 - 1.8)	3.1 (6.8 - 3.7)	3.8 (5.7 - 1.9)
	Amikacin	3.1 (4.9 - 1.8)	1.6 (5.3 - 3.7)	2.0 (3.9 - 1.9)
	2G0 alone	2.7 (4.5 - 1.8)	1.8 (5.5 - 3.7)	1.8 (3.7 - 1.9)
	BDQ alone	2.1 (3.9 - 1.8)	1.7 (5.4 - 3.7)	2.0 (3.9 - 1.9)
CFZ + 2G0	Sham	4.2 (6.7 - 2.5)	2.9 (6.8 - 3.9)	4.0 (5.7 - 1.7)
	Amikacin	2.4 (4.9 - 2.5)	1.4 (5.3 - 3.9)	2.2 (3.9 - 1.7)
	2G0 alone	2.0 (4.5 - 2.5)	1.6 (5.5 - 3.9)	2.0 (3.7 - 1.7)
	CFZ alone	2.6 (5.1 - 2.5)	2.3 (6.2 - 3.9)	2.1 (3.8 - 1.7)

**Fig. 6.** (continued).

more severe damage and morphological alterations were observed in combination of 2G0 with RIF, CFZ and LZD.

**3.8. 2G0 is potently active in vivo against *M. fortuitum* and *M. abscessus* individually and in combination**

Since 2G0 exhibited potent *in vitro* activity against Mtb and NTMs

and demonstrated its safe as well as significant *in vivo* efficacy in murine chronic model against Mtb H37Ra [19], we checked the *in vivo* efficacy in combination against NTMs. For this, murine neutropenic bacteremia model was used as it resembles clinical aspects of NTM infections [12, 22,28]. The data are plotted in Fig. 6A and B and quantitative differences of bacterial load in log<sub>10</sub> CFU/ml ( $\Delta \log_{10} \text{CFU/ml}$ ) between drug combinations with individual treatments of Fig. 5A and B are shown in

Table 4A and B respectively.

Remarkably, the combination of 2G0 with the drugs tested lead to a highly significant reduction in the bacterial counts in all organs as compared with any of the individual treatments (sham, AMK, 2G0 alone, or respective drug alone) for *M. fortuitum* (Fig. 6A, Table 4A).

We further tested *in vivo* activity of 2G0's against notoriously drug-resistant *M. abscessus* in a murine neutropenic bacteremia model where 2G0 in combination with RIF, BDQ, LZD and CFZ demonstrated a highly significant reduction in bacterial counts compared to treatment with individual drugs in all the organs tested (Fig. 6B, Table 4B). Moreover, there were significant differences in lung histopathology in the combinatorial treatment groups compared to sham treated mice. Although there were areas of cellular infiltration resembling granulomatous structure observed in the treatment groups but they were more diffused and disorganized compared to the sham treated mice. Additionally, there were less thickening of alveolar septa in the lungs of mice treated with 2G0 plus antibiotics compared to the lungs of sham treated mice (data not shown). These experiments further confirm the ability of 2G0 to potentiate the activity of several antibiotics belonging to different classes.

#### 4. Discussion

The current regimen for treatment of TB and other NTM infections consists of an extensive multi-drug therapy, depending upon the drug-susceptibility status. The alarming intrinsic drug-resistance profile and lack of diagnostics for NTM infections have negatively impacted morbidity and mortality. To lessen the chemotherapeutic dose, improve patient compliance and reliably eradicate the infection *in vivo*, new drug regimens for drug resistant TB and NTM infections are the need of the hour [28]. In addition, there is also urgent need for novel anti-mycobacterial drugs against drug-resistant (MDR and XDR) TB [12, 33]. In this context, we have demonstrated that a combination of low-generation, polycationic dendrimer (2G0) acts synergistically and potentiates the activity of several classes of antibiotics including rifampicin, bedaquiline, linezolid and clofazimine against a diverse group of mycobacteria including antibiotic nightmare *M. abscessus*. The synergistic approach is one of the most crucial options for treating mycobacterial diseases in the context of antibiotic resistance [34,35]. In our previous study, we demonstrated the activity of polycationic phosphorous dendrimers as an anti-TB molecule including *in vitro* and *in vivo* activity against Mtb. These dendrimers have huge clinical possibilities as a nanomedicine due to their high solubility and stability [36]. These characteristics highlight 2G0's potential as a promising, novel anti-mycobacterial molecule targeting unmanageable drug-resistant mycobacterial infections with a unique mode of action that is not effected by existing resistance mechanisms as well as its inability to induce resistance [37].

Taken together, 2G0 is a novel orally bio-available, non-cytotoxic, polycationic phosphorous dendrimer demonstrating strong *in vitro* and *in vivo* activity against Mtb and NTMs including notoriously drug-resistant *M. abscessus*. Interestingly, this dendrimer showed potent *in vitro* activity against single drug-resistant Mtb pathogens as well as rifampicin-resistant, isogenic *M. fortuitum*. 2G0 exhibited concentration-dependent bactericidal activity in time-kill assay against Mtb, *M. fortuitum* and *M. abscessus* and synergized with RIF and CFZ against *M. tuberculosis*, RIF, BDQ, LZD and CFZ against *M. fortuitum* and *M. abscessus*, respectively. Intriguingly, the combinatorial efficacy of 2G0 with several antibiotics belonging to diverse chemical groups significantly reduced bacterial burden in infected mice as compared to individual treatment suggesting 2G0's remarkable ability to potentiate the activity of diverse classes of antibiotics. Further research is underway to decipher the molecular mechanism of antibiotic potentiation.

#### 5. Conclusions

In conclusion, this work fully demonstrated the unique properties of 2G0 a water soluble first in class phosphorus dendrimer with low generation (generation 0) and opens new perspectives of development in nanoscience in general and in nanomedicine in particular because such a compound as well as other phosphorus dendrimers of low generation offer many advantages. Indeed, they are prepared in a few steps with cheap starting materials used for their synthesis, they are highly soluble in water, stable for more than two years, they can be prepared in GMP conditions in large quantities and moreover, it is easy to modify the nature and the number of terminal groups as well as can be conjugated with different antibiotics according to precise need of developing novel antibacterial or novel delivery agent for antibiotics of choice targeting drug resistant mycobacteria.

#### CRedit authorship contribution statement

**Mohammad Imran:** Writing – original draft, Investigation, Data curation. **Shriya Singh:** Writing – original draft, Investigation, Data curation. **Mohammad Naiyaz Ahmad:** Writing – original draft, Investigation, Data curation. **Pradip Malik:** Investigation, Data curation. **Atri Mukhopadhyay:** Investigation, Data curation. **Karan Singh Yadav:** Investigation, Data curation. **Umesh D Gupta:** Writing – review & editing, Supervision, Formal analysis. **Madhav N Mugale:** Writing – review & editing, Supervision, Investigation, Data curation. **Kalyan Mitra:** Writing – review & editing, Supervision, Investigation, Data curation. **Kishore K Srivastava:** Writing – review & editing, Conceptualization. **Sidharth Chopra:** Writing – review & editing, Writing – original draft, Supervision, Formal analysis, Conceptualization. **Serge Mignani:** Writing – review & editing, Supervision, Formal analysis, Conceptualization. **Evgeny Apartsin:** Writing – review & editing, Investigation, Conceptualization. **Jean-Pierre Majoral:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Formal analysis, Conceptualization. **Arunava Dasgupta:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Formal analysis, Conceptualization.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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