

Carbonyl Cyanide 3-Chlorophenylhydrazone (CCCP) Exhibits Direct Antibacterial Activity Against *Mycobacterium abscessus*

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Objective: Treatment choices for *Mycobacterium abscessus* (*M. abscessus*) infections are very limited, and the prognosis is generally poor. Effective new antibiotics or repurposing existing antibiotics against *M. abscessus* infection are urgently needed. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP), a member of the lipophilic weak acid class, is known as an efflux pump inhibitor for *Mycobacterium tuberculosis*. The aim of this study was to determine the inhibitory activity of CCCP as a potential novel antibiotic against *M. abscessus*.

Methods: A total of 47 reference strains of different mycobacterial species and 60 clinical isolates of *M. abscessus* were enrolled. In vitro inhibitory activity of CCCP was accessed using microplates alamar blue method with the reference and clinical isolates. The activity of CCCP against intracellular *M. abscessus* residing within macrophage was also evaluated by intracellular colony numerating assay.

Results: CCCP exhibited good activity against *M. abscessus* clinical isolates in vitro, the minimum inhibitory concentration (MIC) ranged from 0.47 µg/mL to 3.75 µg/mL, with a MIC₅₀ of 1.875 µg/mL and MIC₉₀ of 3.75 µg/mL. At concentrations safe for the cells, CCCP exhibited highly intracellular bactericidal activities against *M. abscessus* and *M. massiliense* reference strains, with inhibitory rates of 84.8%±8.8% and 72.5%±13.7%, respectively. CCCP demonstrated bactericidal activity against intracellular *M. abscessus* that was comparable to clarithromycin, and concentration-dependent antimicrobial activity against *M. abscessus* in macrophages was observed. In addition, CCCP also exhibited good activities against most reference strains of rapidly growing mycobacterial species.

Conclusion: CCCP could be a potential candidate of novel antimicrobiological agent to treat *M. abscessus* infection.

Keywords: *Mycobacterium abscessus*, carbonyl cyanide 3-chlorophenylhydrazone, minimum inhibitory concentration, intracellular bactericidal activity

Introduction

Non-tuberculous mycobacteria (NTM) are opportunistic pathogens that exist ubiquitously in soil and water sources and usually cause serious lung infections in vulnerable population.¹ Human lung infections caused by NTM have rapidly increased globally in the past decades.^{2,3} Among NTM species, *Mycobacterium abscessus* (*M. abscessus*) is one of the most frequently identified species with clinical significance.⁴ *M. abscessus* belongs to the fast-growing group of NTM that cause skin and soft tissue infections beside lung infection. It is estimated that

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this species caused 5–20% of all non-*Mycobacterium tuberculosis* (Mtb) infections globally. Furthermore, this rate is likely to be an underestimation since most of the countries do not routinely report these infectious diseases. The treatment of *M. abscessus* caused infections requires combinational usage of some of the following antibiotics: macrolide antibiotics, amikacin, cefoxitin and other drugs. However, poor prognosis of treatment is frequently confronted, whereas the cure rate only reaches 25–58%.^{5,6} As a consequence, *M. abscessus* infection is often considered as “antibiotic nightmare”.⁷ Therefore, new or repurposed drugs are urgently needed to change this clinical predicament.

Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) functions as a proton carrier that reduces the transmembrane electrochemical gradient and causes an accumulation of antibiotics within bacterial cells. However, CCCP has generally been used only as an efflux pump inhibitors (EPIs).^{8–10} CCCP has been proven to reduce the minimum inhibitory concentration (MIC) of rifampicin, isoniazid for Mtb¹¹ and improved the activity of clarithromycin (CLA) against CLA-resistant *M. abscessus* clinical strains.¹² It is generally deemed that CCCP, as an EPIs, is more effective when used in combination with antibiotics.¹³ We identified that CCCP alone had antibacterial activity against *M. abscessus* and possessed the potential to be considered as an independent antibiotic. In this study, we determined the susceptibility profile of *M. abscessus* clinical isolates to CCCP, investigated its bactericidal activity against *M. abscessus* in macrophages, and also analyzed its cytotoxicity. To our knowledge, this is the first report on the independent anti-*M. abscessus* activity of CCCP as a novel candidate antibiotic.

Materials and Methods

Ethical Approval

The study was approved by the Ethics Committee of Beijing Chest Hospital, Capital Medical University. All the reference strains and clinical strains were collected from the Beijing biobank of Clinical Resources on Tuberculosis located in Beijing Chest Hospital, Capital Medical University (Beijing, China); therefore the informed consents were waived.

Bacterial Strains and Culture

The *M. abscessus* clinical isolates were originally isolated from sputa of patients with lung infections. The strains

were classified as NTM preliminarily with p-nitrobenzoic acid containing medium, and later identified into species by sequence alignments of *16S rRNA*, *hsp65*, *rpoB*, *16–23S rRNA* internal transcribed spacer in parallel. The reference strains including 29 rapidly growing mycobacteria (RGM) species, 18 slowly growing mycobacteria (SGM) species were obtained either from the American Type Culture Collection (ATCC) or from the German Collection of Microorganisms (DSM). All the bacterial strains studied were stored at –80°C in the Bio-bank in Beijing Chest Hospital, including reference strains and a total of 60 *M. abscessus* clinical isolates. *M. abscessus* clinical isolates consist of 40 *M. abscessus* subsp. *abscessus* and 20 *M. abscessus* subsp. *massiliense*. Bacterial suspensions were inoculated on the Middlebrook 7H10 (M7H10) agar plates or on the Lowenstein-Jensen (LJ) medium. The growth phenotypes of the clinical strains on the L-J medium were recorded.

Antimicrobial Susceptibility Testing

CCCP (CAS#555-60-2) and CLA (CAS#81103-11-9) were purchased from Sigma-Aldrich Co. (St Louis, MO, USA) or Solarbio (Beijing, China), respectively. Both drugs were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 2400 µg/mL. MICs were determined according to the guidelines from the Clinical and Laboratory Standards Institute (CLSI).¹⁴ Cation-adjusted Mueller-Hinton broth (CAMHB) (BD, USA) enriched with 5% OADC was used for SGM, while CAMHB without OADC was used for RGM. CCCP was serially diluted (1:2) with final concentrations ranging from 0.115 to 60 µg/mL. NTM cultures were scraped from LJ media, homogenized and then adjusted to McFarland 0.5 with sterile saline, and then diluted 200-fold for inoculation. One hundred µL of diluted inoculum was added into each well. The plates were incubated at 37 °C for 3 to 4 days for RGM and 7 to 8 days for SGM until visible growth of mycobacteria in the control wells without CCCP. Twenty µL of alamarBlue (Invitrogen) and 50 µL of 5% Tween 80 were added to each well and the plates were re-incubated at 37°C for additional 24 h before assessing color change. Blue color indicated no mycobacterial growth and pink color indicated mycobacterial growth. The MIC was defined as the lowest drug concentration that inhibited the bacterial growth, observed by no color change from blue to pink. The MIC testing for each strain was performed in triplicate.

Minimum Bactericidal Concentration (MBC) Against *M. abscessus*

The reference strains and three clinical isolates for each of *M. abscessus* subsp. *abscessus* or subsp. *massiliense* were randomly selected for the assay. After 4 days' incubation with CCCP at different concentrations, the wells in which the drug concentrations were higher than the MIC were resuspended and 100 μ L medium of each well was inoculated onto 7H10-OADC plate. The plates were incubated at 37°C for 5 days, followed by counting the number of colonies. The MBC value was defined as the minimum drug concentration with no colony growth. An antibiotic was considered bactericidal when the MBC/MIC ratio was ≤ 4 ; otherwise, it was considered bacteriostatic.¹⁵

Cytotoxicity Assay

The toxicity of CCCP was assessed using THP-1 and U937 cells which were purchased from the Wuhan Procell Life Science&Technology Co., Ltd. Cells were seeded into 24-well plates and differentiated into macrophages with 100 nM PMA. After 24 h, cells were washed once and cultured in fresh RPMI medium (Gibco) with 10% fetal bovine serum (RPMI complete medium). CCCP solution was added into the wells with final concentrations ranging from 0.78 to 25 μ g/mL, and incubated for additional 48 h. The same volume of DMSO was used to replace CCCP as control. The cytotoxicity of different concentrations of CCCP was monitored using a lactate dehydrogenase (LDH) cytotoxicity detection kit (Beyotime, Shanghai, China). The LDH activity in the cell culture supernatant were analysed and the absorbance value (Abs) under 490nm was recorded using Multiskan Go microplate reader (Thermo Fisher, USA). The cell survival rate (%), at each concentration was determined as follows: cell survival rate = $(1 - (\text{Abs}_{490} \text{ of treated cells} - \text{Abs}_{490} \text{ of control cells}) / (\text{Abs}_{490} \text{ of total lysis cells} - \text{Abs}_{490} \text{ of control cells})) \times 100\%$.

Intracellular Killing and Concentration-Kill Assay

U937 cells were seeded at 2×10^5 cells/well in a 24-well plate and differentiated with PMA. Cells were infected with *M. abscessus* reference strain (ATCC 19977) and *M. massiliense* reference strain (CCUG 48898) at a multiplicity of infection (MOI) of 1 or 10. At 2 h post-infection, the cells were gently washed three times with pre-warmed phosphate-buffered saline (PBS) to remove

the extracellular bacteria. RPMI complete medium containing 250 μ g/mL amikacin were added to kill the remaining extracellular bacteria for an additional 2 h.¹⁶ For intracellular killing assay, RPMI complete medium containing CCCP (6 μ g/mL) or CLA (2 μ g/mL) were added. Meanwhile, RPMI complete medium containing different concentrations of CCCP were supplied for the concentration killing assay. The culture medium with vehicle (DMSO) was included as growth controls. At 48 h post-infection, macrophages were extensively washed with PBS and lysed with 0.1% Tween 80, and serially diluted with 0.05% Tween 80. The number of CFUs was quantified by plating serial dilutions of lysates on M7H10 agar plates. The bacterial survival rate was calculated using the formula: viability = CFU of bacteria under the treatment of CCCP or CLA / CFU of bacteria treated with vehicle DMSO $\times 100\%$.

Statistical Analysis

Data were analyzed using SPSS 24.0 software and GraphPad Prism 8.0 software. For antimicrobial susceptibility assay, non-parametric test was used to determine significant differences between groups. For cytotoxicity assay and intracellular bactericidal assay, one-way analysis of variance (ANOVA), followed by post hoc test, was used to determine significant differences between the groups. Differences were considered to be statistically significant for p value of < 0.05 .

Results

Activity Spectra of CCCP Against NTM Reference Strains

The MICs of CCCP against the 47 reference strains are shown in Table 1. CCCP showed excellent activities against most of the RGM reference strains. However, four RGM species had MICs of 3.75 μ g/mL and 7.5 μ g/mL, and 24 other RGM species had MICs of ≤ 1.875 μ g/mL. Additionally, all of the 18 enrolled SGM species had MICs of ≥ 7.5 μ g/mL.

MICs and MBCs of CCCP Against *M. abscessus* Isolates

A total of 60 *M. abscessus* clinical isolates including 40 *M. abscessus* subsp. *abscessus* and 20 *M. abscessus* subsp. *massiliense*, were studied. The morphological observation indicated that 22 out of the 40 isolates of *M. abscessus* subsp. *abscessus* had smooth morphotypes, while 18

Table 1 MICs of CCCP for Reference Strains of 29 RGM Species and 18 SGM Species

| Strain by Type | Mycobacterium Species (Strain) | MIC ($\mu\text{g/mL}$) |
|--------------------|--------------------------------|--------------------------|
| RGM species | | |
| ATCC 19977 | <i>M. abscessus</i> | 0.94 |
| CCUG 48898 | <i>M. massiliense</i> | 0.94 |
| ATCC 33464 | <i>M. austroafricanum</i> | 0.94 |
| ATCC 700731 | <i>M. septicum</i> | 1.875 |
| ATCC 35796 | <i>M. senegalense</i> | 0.94 |
| ATCC 27406 | <i>M. agri</i> | 0.47 |
| ATCC 19527 | <i>M. thermoresistibile</i> | 0.94 |
| ATCC 27278 | <i>M. chubuense</i> | 0.94 |
| ATCC 35154 | <i>M. pulveris</i> | 0.94 |
| ATCC 19627 | <i>M. chitae</i> | 0.94 |
| ATCC 43271 | <i>M. peregrinum</i> | 1.875 |
| ATCC 19686 | <i>M. parafortuitum</i> | 0.47 |
| ATCC 15483 | <i>M. vaccae</i> | 7.5 |
| ATCC 11758 | <i>M. phlei</i> | 0.47 |
| ATCC 14474 | <i>M. flavescens</i> | 0.47 |
| ATCC 19340 | <i>M. diernhoferi</i> | 0.94 |
| ATCC14472 | <i>M. chelonae</i> | 0.94 |
| ATCC 6841 | <i>M. fortuitum</i> | 3.75 |
| ATCC 25795 | <i>M. neoaurum</i> | 1.875 |
| ATCC 33776 | <i>M. porcinum</i> | 1.875 |
| DMS 44124 | <i>M. mucogenicum</i> | 1.875 |
| ATCC BAA-955 | <i>M. goodii</i> | 1.875 |
| ATCC 43909 | <i>M. gilvum</i> | 0.23 |
| ATCC 44829 | <i>M. cosmeticum</i> | 3.75 |
| ATCC 19420 | <i>M. smegmatis</i> | 7.5 |
| ATCC 23366 | <i>M. aurum</i> | 0.94 |
| ATCC 27280 | <i>M. aichiense</i> | 0.94 |
| ATCC 27023 | <i>M. obuense</i> | 0.94 |
| ATCC 35219 | <i>M. fallax</i> | 1.875 |
| SGM species | | |
| ATCC 27294 | Mtb H37Rv | 15 |
| ATCC 19422 | <i>M. microti</i> | 15 |
| ATCC 15754 | <i>M. gastri</i> | 15 |
| ATCC13950 | <i>M. intracellulare</i> | 7.5 |
| ATCC 14470 | <i>M. gordonae</i> | 7.5 |
| ATCC 12478 | <i>M. kansasii</i> | 7.5 |
| ATCC 35799 | <i>M. szulgai</i> | 15 |
| ATCC 25291 | <i>M. avium</i> | 15 |
| DSM 44648 | <i>M. parascrofulaceum</i> | 15 |
| ATCC 51131 | <i>M. celatum</i> | 15 |
| ATCC 19981 | <i>M. scrofulaceum</i> | 7.5 |
| ATCC 27726 | <i>M. gadium</i> | 7.5 |
| ATCC 19250 | <i>M. xenopi</i> | 15 |
| ATCC 27024 | <i>M. rhodesiae</i> | 30 |
| ATCC 19530 | <i>M. nonchromogenicum</i> | 60 |
| ATCC 25276 | <i>M. asiaticum</i> | 15 |
| ATCC 15755 | <i>M. terrae</i> | 15 |
| ATCC 23292 | <i>M. triviale</i> | 7.5 |

exhibited rough morphotypes. Similarly, 11 of the 20 *M. abscessus* subsp. *massiliense* isolates exhibited smooth colonies and 9 exhibited rough colonies. The MICs of CCCP against *M. abscessus* (ATCC19977) and *M. massiliense* (CCUG48898) were both 0.94 $\mu\text{g/mL}$. The MICs of CCCP against *M. abscessus* clinical isolates ranged from 0.47 to 3.75 $\mu\text{g/mL}$, the majority of these ranged from 0.9375 to 1.875 $\mu\text{g/mL}$ (Figure 1A). The MIC₅₀ and MIC₉₀ of CCCP against *M. abscessus* subsp. *abscessus* were both 1.875 $\mu\text{g/mL}$. The MIC₅₀ and MIC₉₀ of CCCP against *M. abscessus* subsp. *massiliense* were 1.875 and 3.75 $\mu\text{g/mL}$, respectively. In contrast with *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense* showed greater MIC to CCCP ($P < 0.05$) (Figure 1C). Additionally, the MICs of CCCP against *M. abscessus* rough morphotype were greater than these of *M. abscessus* smooth morphotype ($P < 0.05$) (Figure 1D). Specifically, the *M. abscessus* subsp. *abscessus* with smooth morphotype was the most sensitive group whereas the *M. abscessus* subsp. *massiliense* with rough morphotype was the most tolerant group to CCCP exposure (Figure 1B). The MBC/MIC ratio of CCCP against *M. abscessus* reference strains and 6 clinical isolates ranged from 4 to 8 (Table 2). Furthermore, 4 strains had MBC/MIC ratios > 4 , one strain belonged to *M. abscessus* subsp. *abscessus* with smooth morphotype and another one belonged to *M. abscessus* subsp. *massiliense* with rough morphotype had MBC/MIC ratios = 4.

Cytotoxicity Assay of CCCP in the THP-1 and U937 Cells

When the concentration of CCCP was lower than 6.25 $\mu\text{g/mL}$, the survival rates for both THP-1 and U937 cells were almost 97%. However, with the concentration of CCCP at 12.5 $\mu\text{g/mL}$ or greater, the cells survival rates were less than 80% (Figure 2).

Intracellular Bactericidal Activity of CCCP Against *M. abscessus* in Macrophages

Bacterial survival rate was quantitated as shown in Figure 3. Both at MOI=1:1 and MOI=1:10, obvious reductions were observed in the colony number of *M. abscessus* (CFU/mL) treated with CCCP group (6 $\mu\text{g/mL}$) compared to the DMSO group. The intracellular growth inhibition rates of CCCP against both *M. abscessus* and

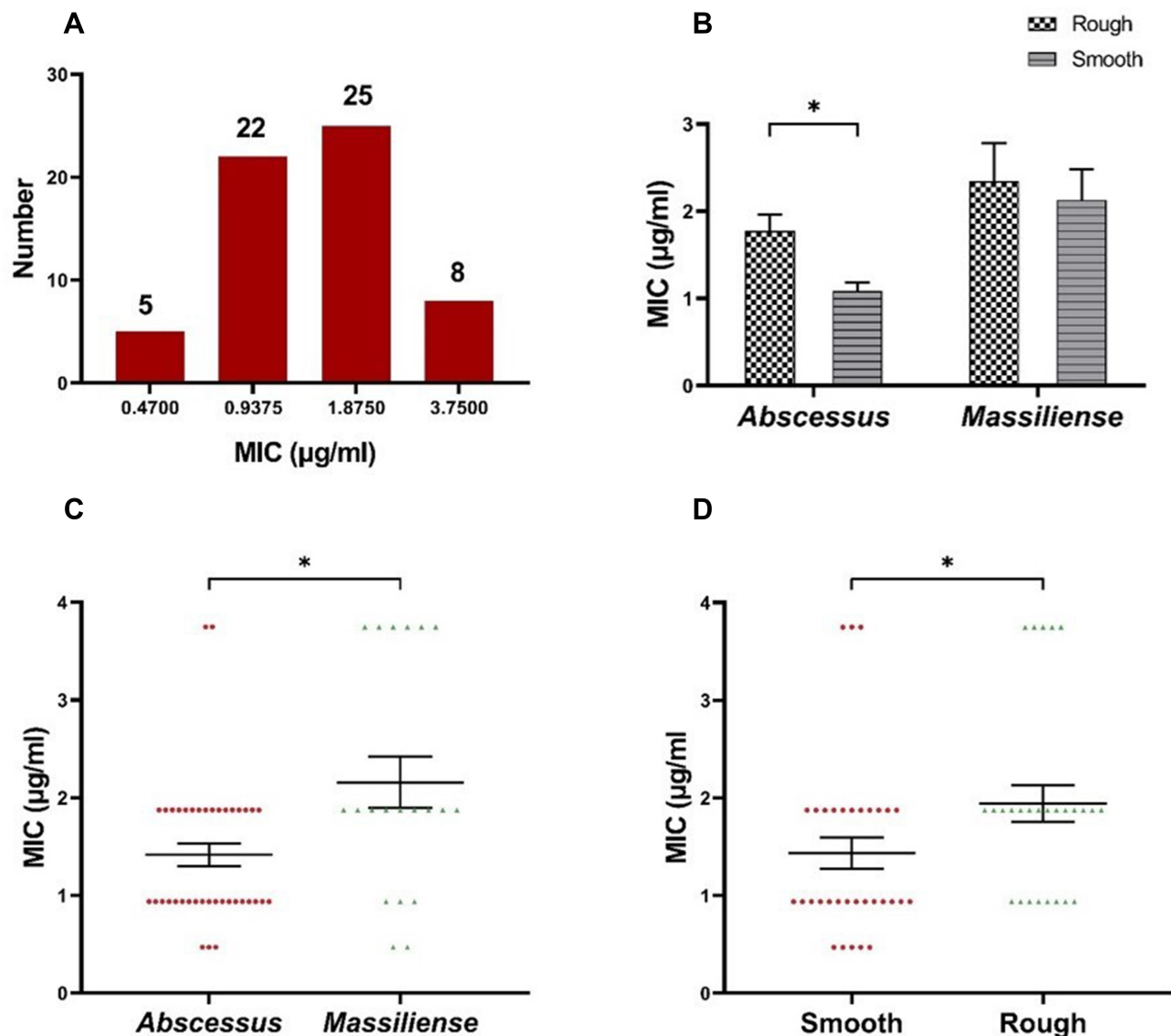


Figure 1 The MIC of CCCP against 60 clinical *M. abscessus* isolates. **(A)** The MIC distribution of the *M. abscessus* isolates. **(B)** The MICs of CCCP in rough morphotype and smooth morphotype. **(C)** The MIC distribution of the isolates of *M. abscessus* subspecies *abscessus* and *M. abscessus* subspecies *massiliense*. **(D)** The MIC distribution of the isolates with smooth morphotype or rough morphotype. Error bars represent the standard errors of the data points. * $P < 0.05$.

M. massiliense reference strains at MOI of 10 were significantly higher than MOI of 1. Specifically, at MOI of 10, CCCP inhibited $84.8\% \pm 8.8\%$ of intracellular bacterial growth against *M. abscessus* and $72.5\% \pm 13.7\%$ of intracellular bacterial growth against *M. massiliense*. Similarly, compared with DMSO group, a pronounced decrease was observed in the colony number of *M. abscessus* treated with CLA group ($2 \mu\text{g/mL}$) (with inhibitory rates of $92.0\% \pm 4.0\%$ in *M. abscessus* and $76.4\% \pm 11.7\%$ in *M. massiliense*, respectively) and CCCP + CLA group ($3 \mu\text{g/mL}$ CCCP and $1 \mu\text{g/mL}$ CLA) (with inhibitory rates of $90.4\% \pm 5.6\%$ in *M. abscessus* and $79.4\% \pm 4.9\%$ in *M. massiliense*, respectively). By gradually increasing the

CCCP concentration, the intracellular bactericidal activity enhanced linearly, and uniform tendencies were observed at the MOI of 1 and 10 (Figure 4).

Discussion

Due to the intrinsic resistance of *M. abscessus* to most antibiotics, treatment of *M. abscessus* infections is highly challenging and often results in failure.¹⁷ Previous studies have shown that CCCP as an EPI can reduce the drug tolerance level of Mtb and NTM.^{11–13} However, antibacterial activity of CCCP as an independent antibiotic has never been reported. In this study, we evaluated the in vitro efficacy of CCCP against 46 NTM reference

Table 2 MIC, MBC Values and Antibacterial Activities of CCCP Against *M. abscessus* Complex

| Isolate | Subspecies | Morphotype | MBC/MIC Ratio | Antibacterial Activity |
|------------|--------------------|------------|---------------|------------------------|
| ATCC19977 | <i>abscessus</i> | Smooth | 8 | Bacteriostatic |
| 164 | <i>abscessus</i> | Rough | 8 | Bacteriostatic |
| 170 | <i>abscessus</i> | Rough | 8 | Bacteriostatic |
| 276 | <i>abscessus</i> | Smooth | 4 | Bactericidal |
| CCUG 48898 | <i>massiliense</i> | Smooth | 8 | Bacteriostatic |
| 131 | <i>massiliense</i> | Rough | 8 | Bacteriostatic |
| 122 | <i>massiliense</i> | Rough | 4 | Bactericidal |
| 274 | <i>massiliense</i> | Smooth | 8 | Bacteriostatic |

strains and observed that CCCP presented good activities against most of the RGM reference strains, including the notorious *M. abscessus* species. Then, we specifically tested more clinical *M. abscessus* isolates and surprisingly found that CCCP showed excellent intracellular bactericidal activity against *M. abscessus*. Moreover, variable inhibitory activities were presented against different subspecies or different morphotypes of *M. abscessus*. The *M. abscessus* subsp. *abscessus* with smooth morphotype was the most sensitive group to CCCP exposure while the *M. abscessus* subsp. *massiliense* was the vice versa. The MBC/MIC ratio of CCCP for *M. abscessus* is mainly indicative of bacteriostatic activity, although bactericidal activity might also be possible for the minority population strains of these species. The most important finding of our study is that CCCP has a strong intracellular

bactericidal activity and this activity is concentration-dependent. The above results indicate that CCCP could be a promising drug candidate for the treatment of *M. abscessus* and worthy of further investigation.

In this study, RGM and SGM showed very different susceptibility profiles to CCCP treatment in vitro. CCCP presented good inhibitory activities against most of the enrolled RGM reference strains, but evidently weaker inhibitory activities against the enrolled SGM reference species. We speculate that the different growth and metabolism characteristics of RGM and SGM might cause this discrepancy. However, the mechanism underlying this phenomenon remains to be elucidated. Among the other enrolled RGM reference strains, *M. chelonae* had MIC to CCCP at 0.94 $\mu\text{g/mL}$, whereas *M. fortuitum*, another frequently isolated RGM species with clinical significance,

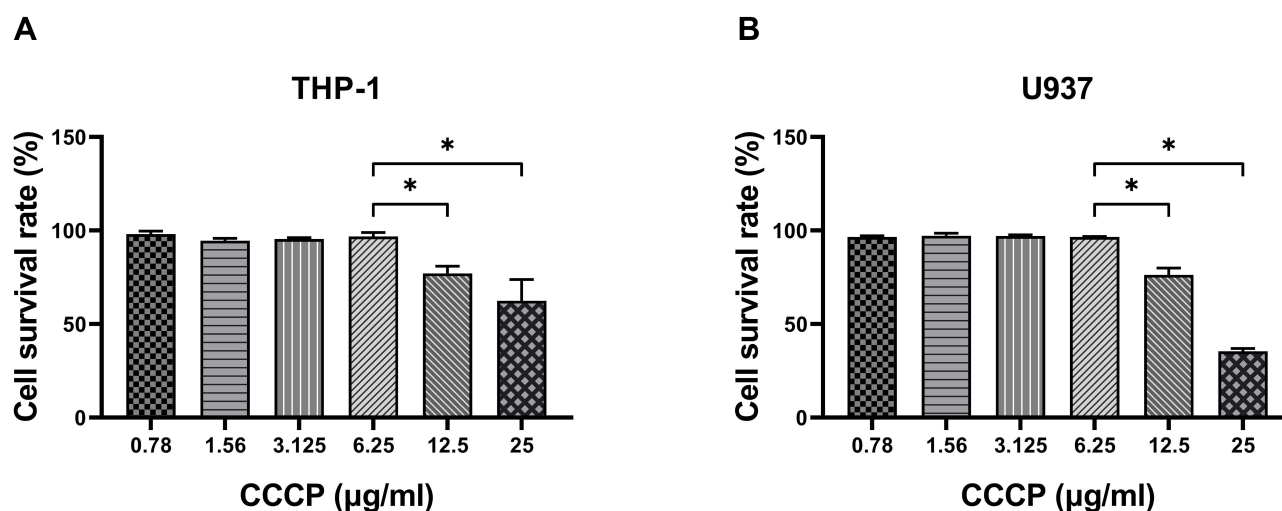


Figure 2 Cytotoxicity assay of CCCP in the THP-1 and U937 cells. THP-1 cells (A) and U937 cells (B) were treated with different concentrations of CCCP and LDH activity in the cell culture supernatant were analysed. The experiments were carried out in quintuplicate. All data are shown as the means \pm SD (n = 5). Results shown are from one representative experiment, *P < 0.05.

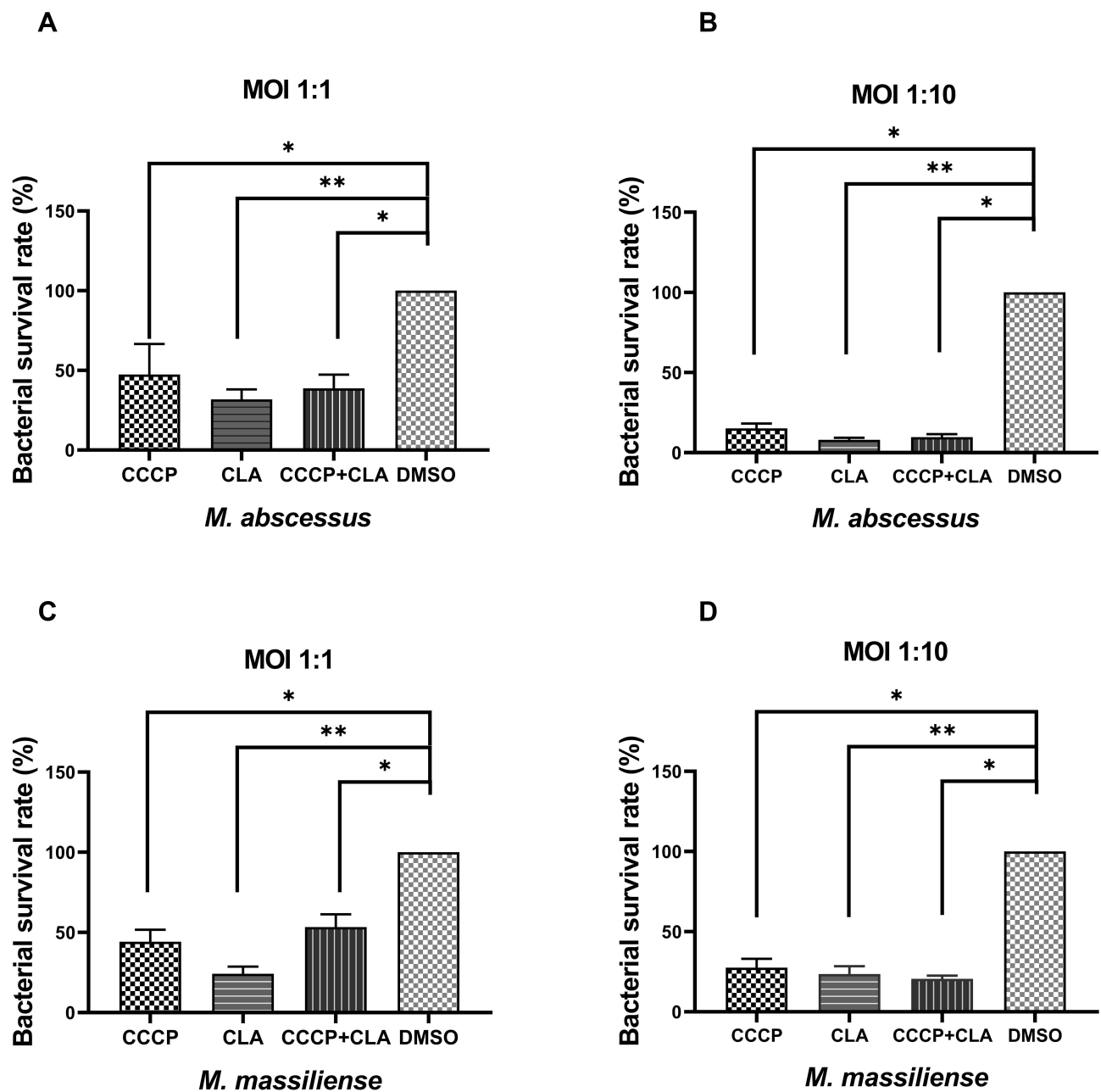


Figure 3 Intracellular bacterial survival rate of *M. abscessus* (ATCC 19977) (A and B) and *M. massiliense* (CCUG 48898) (C and D) with CCCP treatment. CCCP group: infected macrophage treated with CCCP (6 $\mu\text{g/mL}$); CLA group: infected macrophage treated with CLA (2 $\mu\text{g/mL}$); CCCP+CLA group: infected macrophage treated with CCCP (3 $\mu\text{g/mL}$) and CLA (1 $\mu\text{g/mL}$). The CFU were enumerated in triplicates. All data are shown as the means \pm SD ($n = 3$). * $P < 0.05$; ** $P < 0.001$.

had MIC at 3.75 $\mu\text{g/mL}$. This diversity within RGM is also worthy of further study.

Our study focused on evaluation of the antimicrobial activity against *M. abscessus*, which is one of the most frequently isolated NTM species with treatment difficulty. CCCP demonstrated good antimicrobial activities against both the reference strains and clinical strains of *M. abscessus*. A narrow MIC distribution range was obtained (0.47–3.75 $\mu\text{g/mL}$) and only four 2-fold diluted

concentrations were covered. 36.67% (22/60) and 41.67% (25/60) of the clinical isolates had MIC at 0.93 $\mu\text{g/mL}$ or 1.83 $\mu\text{g/mL}$, respectively. A study using CCCP as an experimental control previously occasionally detected the MIC value of CCCP against *M. abscessus* ATCC19977 at 0.4 $\mu\text{g/mL}$,¹⁸ which was in accordance with our results. In this study, both the in vitro and intracellular testing demonstrated that CCCP has good and concentration-dependent antimicrobial activity against *M. abscessus*.

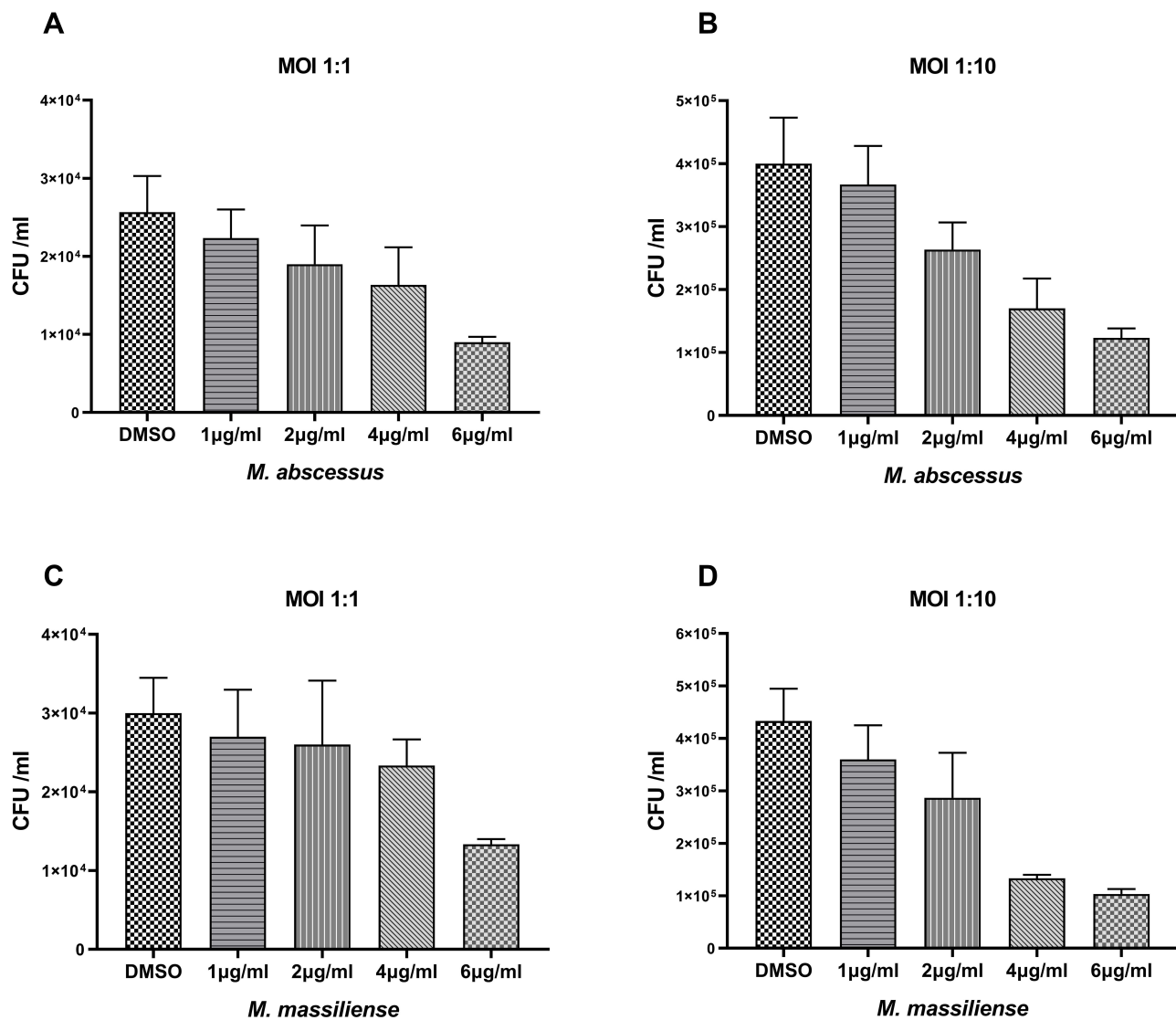


Figure 4 Intracellular bactericidal activities of different CCCP concentrations against *M. abscessus* and *M. massiliense* in macrophages. The number of *M. abscessus* (ATCC 19977) in macrophages treated with different concentrations of CCCP at a MOI of 1 (**A**) and a MOI of 10 (**B**); The number of *M. massiliense* (CCUG 48898) in macrophages treated with different concentrations of CCCP at a MOI of 1 (**C**) and MOI of 10 (**D**). All data are shown as the means \pm SD (n = 3).

Cytotoxicity assays indicated that THP-1 cells and U937 cells had high viability (>97%) when exposed to CCCP ≤ 6.25 $\mu\text{g/mL}$. The highest safe concentration was much greater than the greatest MIC of the enrolled *M. abscessus* clinical strains to CCCP, which favors the possibility of CCCP as a new antimicrobial agent. In the intracellular bactericidal experiments, CCCP plus CLA presented reasonable inhibitory activity in contrast to each of the drugs at doubled concentration. We presumed that the inhibition was mainly an outcome of the cumulative effect, while the possibility of the synergistic effect due to the canonical EPI function of CCCP was hard to be determined by our assay. We speculated that since the reference strains of *M. abscessus* were relatively susceptible to clarithromycin,

which makes the efflux pump inhibition effect less obvious when it combined with CCCP.

M. abscessus, which consists of the three subspecies ie, *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense* and *M. abscessus* subsp. *bolletii*, displays two colony morphology types: smooth and rough morphotypes. Previous studies reported that these three *M. abscessus* subspecies have different antibiotic resistance profiles.¹⁹ For example, *M. abscessus* subsp. *massiliense* was sensitive to doxycycline, but *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii* were resistant to it. In addition, the sensitivities of the three subspecies to clarithromycin were not identical either. The major difference between *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp.

massiliense is that the latter does not have an intact *erm(41)* gene and thus does not have inducible macrolide resistance. Therefore, the treatment response may be better among patients infected with *M. abscessus* subsp. *massiliense*.²⁰ Our study found that *M. abscessus* subspecies responded discrepantly when exposed to CCCP, the MIC for *M. abscessus* subsp. *massiliense* was generally one fold greater than this of *M. abscessus* subsp. *abscessus*. Since we do not have isolates of *M. abscessus* subsp. *bolletii*, we could not evaluate the activity of CCCP against this species.

Both smooth morphotype and rough morphotype can be found in subjects with *M. abscessus* infections.²¹ The smooth morphotype expresses glycopeptidolipid (GPL) on its cell surface while the rough morphotype expresses minimal amount of GPL on the cell wall.^{22–24} Biofilm formation and different colony morphotypes contribute to the persistence of *M. abscessus* in lung diseases. Correlations between morphotypes of *M. abscessus* and severity of disease were demonstrated in experimental and clinical studies, while rough morphotype has been considered more aggressive.^{25,26} However, the correlation between phenotype and drug susceptibility remains obscure. Gillian et al found that rough morphotype was more aggressive and formed biofilm-like aggregates, implying it was significantly more tolerant than smooth morphotype to acidic pHs and treatment with amikacin or azithromycin.²⁷ Whereas several other studies did not find the colony morphology of *M. abscessus* having association with susceptibilities to clarithromycin, amikacin, or cefoxitin, and one study even found that the MIC of smooth morphotype was higher than rough morphotype to tigecycline.²⁸ Our results showed that both morphotypes were susceptible to CCCP while smooth morphotype was more susceptible than rough morphotype. It would be an interesting study on whether CCCP has a stronger inhibitory activity against smooth morphotype resulting in a better therapeutic effect in vivo.

Our study has some limitations. Firstly, all the clinical isolates studied were obtained from a single institution. It may not represent all the characteristics of the *M. abscessus* isolates. Secondly, the bactericidal activity of CCCP against *M. abscessus* was only investigated in vitro and in macrophages, therefore the present result might not reflect the real reaction in vivo. Animal model study, pharmacokinetic/pharmacodynamic (PK/PD) studies and synergy of CCCP with other compounds should be further conducted to explore the antibacterial activity of CCCP against *M. abscessus* infection. Thirdly, although our result showed that the highest safe concentration was much greater than the greatest MIC value of the enrolled *M. abscessus* clinical strains, the toxicity of it

is still a big concern.^{29–31} Due to its interfering with mitochondrial depolarization which would induce mitophagy process, applying CCCP in human should be extremely cautious.

In conclusion, CCCP has a strong antimicrobial activity against *M. abscessus* in vitro and in macrophages with less cytotoxicity. CCCP may be a promising drug candidate for *M. abscessus* treatment, for which very limited effective drug choice available nowadays.

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Disclosure

The authors declare no conflicts of interest.

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