Preliminary evaluation of anti-tuberculosis potential of siderophores against drug-resistant Mycobacterium tuberculosis by mycobacteria growth indicator tube-drug sensitivity test

Karuna Gokarn1,2* and Ramprasad B. Pal1

Abstract

Background: Alternative treatment strategies have become essential in overcoming the problem of drug-resistant Mycobacterium tuberculosis (Mtbb). In this preliminary in vitro study, the anti-tuberculosis (anti-TB) activity of exogenous iron chelators (xenosiderophores) such as Exochelin-MS (Exo-MS) and Deferoxamine-B (DFO-B) was evaluated against ten multi-drug-resistant (MDR) and seven pyrazinamide-resistant (PZA) Mtbb isolates.

Methods: Mycobacteria Growth Indicator Tube-Drug Susceptibility Test was used to assess the anti-TB effect of Exo-MS or DFO-B individually and their combinations with isoniazid (INH), rifampicin (RIF) and pyrazinamide (PZA).

Results: For the MDR-Mtbb isolates, Exo-MS alone inhibited two out of the five isolates tested. Whereas, DFO-B alone inhibited nine out of the ten MDR isolates tested. For PZA-resistant Mtbb isolates, both Exo-MS and DFO-B individually inhibited five out of the seven isolates. The MIC of Exo-MS in combination with INH, RIF and PZA remained the same. The MIC of DFO-B decreased when tested in combination with INH, RIF and PZA.

Conclusions: Exo-MS and DFO-B were shown to have activity against drug-resistant Mtbb isolates. Therefore, these xenosiderophores may be useful adjuncts to antibiotics in overcoming the problem of drug-resistant Mtbb in clinical setting.

Keywords: Exochelin-MS, Deferoxamine-B, Xenosiderophores, Mycobacteria growth indicator tube-drug sensitivity test, Tuberculosis

Background

Tuberculosis (TB) is one of the leading infectious diseases in the world today. Mycobacterium tuberculosis (Mtbb), the causative agent of TB, is mainly transmitted from person to person through aerosols. Infections caused by TB bacteria are usually treated with the first-line antituberculosis drugs, namely, isoniazid (INH), rifampicin (RIF), ethambutol (EMB), pyrazinamide (PZA), and streptomycin (STR).

However, Mtbb bacteria have acquired resistance over the years to many of these drugs due to inappropriate use, mutations in Mtbb, etc. These factors have resulted in the emergence of multi-drug-resistant TB (MDR-TB) and extensively-drug-resistant TB (XDR-TB), which have rendered current treatment strategies ineffective.

In MDR-TB, the organisms develop resistance to INH and RIF, the two most important primary drugs. In XDR-TB, the bacteria are resistant to the first-line as well as to the second-line anti-TB drugs. XDR-TB develops in about 9% of MDR-TB patients and is more challenging to treat [1]. In 2014, globally 4,80,000 cases of MDR-TB were reported with mortality of 1,90,000. XDR-TB patients have been identified in about 100
countries. As per this WHO report, India, along with other third world countries is a high-burden country for TB. With the aim of ending TB by 2035, WHO has initiated an “End TB Strategy” [2].

Iron is a vital nutrient for all living organisms necessary for life-sustaining cellular processes such as cell growth, DNA synthesis, electron transport, oxygen transport, etc. The mammalian host maintains low levels of iron using its iron-binding proteins; about 10−18 M of circulating free ferric (Fe3+) ions. Microorganisms synthesize small molecules called siderophores (Greek “iron-carrier”) which scavenge iron from the host to promote their own growth [3]. Siderophore-Fe3+ ion complexes that are formed are then transported intracellularly via specific outer membrane receptor proteins by bacteria.

If Fe3+ ion is complexed with xenosiderophores (not “self”) for a given microorganism, iron uptake may be affected due to the absence of specific receptors in most bacteria. A decrease in the availability of iron would probably be detrimental to the growth of pathogens including the drug-resistant ones. A phytosiderophore extracted from plant root washings has been reported to inhibit the in vitro growth of H37Ra strain of Mtb [4].

*M. smegmatis* produces three siderophores: mycobactin S (S for *smegmatis*), carboxymycobactin S and Exochelin MS (Exo-MS; MS for *M. smegmatis*). Mtb expresses mycobactin T (T for *tuberculosis*) and carboxymycobactin T. However, it does not produce exochelin. Therefore, Exo-MS is a xenosiderophore for *Mtb*. Similarly, the commercially available Deferoxamine mesylate (DFO-B), originally extracted from *Streptomyces pilosus*, is also a xenosiderophore for *Mtb*. Desferri forms of Exo-MS and DFO-B were tested for their anti-TB potential alone and in combination with drugs against MDR and *PZA*β-Mtb isolates by the Mycobacteria Growth Indicator Tube – Drug Susceptibility Test (MGIT™-DST) [5].

MGIT-DST is a qualified test used for antmycobacterial susceptibility testing of *Mtb* developed by Becton Dickinson. A fluorescent oxygen-quenched sensor (Tris 4, 7-diphenyl-1, 10 phenanthroline ruthenium chloride pentahydrate) embedded in silicone is present at the base of a tube. The principle underlying the test is that the initial concentration of dissolved oxygen in the Middlebrook 7H9 broth quenches the emission of fluorescence from this sensor and therefore is not visualized under UV light. However, when actively growing *Mtb* bacilli consume the available oxygen, the sensor fluoresces and is visualized under UV light. The intensity of this fluorescence is directly proportional to the amount of oxygen consumed by the *Mtb* bacilli for growth. The amount of oxygen utilized by *Mtb* for growth in the medium is monitored by the BACTEC MGIT system which automatically interprets results as ‘S’(susceptible) or ‘R’ (resistant) based on the extent of fluorescence [5].

**Methods**

**Extraction and purification of Exo-MS from *M. smegmatis* mc²155**

*M. smegmatis* was grown in an iron-deficient medium [6, 7] for 10 days at 37 °C with aeration [For details of Exo-MS extraction, refer to Gokarn et al., 2017, BCAM DOI:10.1186/s12906-017-1657-8]. Briefly, Exo-MS was recovered from the culture supernatant using the benzyl alcohol extraction procedure [8]. Purification was carried out on an alumina column [9], which removed most of the hydrophobic impurities; Exo-MS was then eluted using a mixture of methanol and formic acid. HR-LCMS was carried out to determine the purity of Exo-MS.

**Determination of in vitro effect of Exo-MS and DFO-B on MDR and *PZA*-resistant Mtb isolates by MGIT-DST method**

i. **Clinical isolates**: To identify the drug-resistant status of the clinical *Mtb* isolates, MGIT-DST was carried out using multiple drugs that include streptomycin, INH, RIF and ethambutol (SIRE) along with PZA and other antibiotics. The isolates resistant to INH and RIF – termed as MDR isolates – were used in this study. Besides these, isolates showing resistance to *PZA* were also used. The revival of the *Mtb* isolates was done in Middlebrook 7H9 broth supplemented with MGIT PANTA (reconstituted with MGIT growth supplement).

Five MDR-Mtb isolates were used for testing the anti-TB activity of Exo-MS alone and its combination with *INH* and *RIF*. Ten MDR-Mtb isolates were used for testing the activity of DFO-B alone and its combination with *INH* and *RIF*. Five of the MDR-Mtb isolates were also resistant to *PZA*. These five and two non-MDR isolates resistant to *PZA* (making a total of seven *PZA*-resistant *Mtb* isolates) were used to evaluate the activity of Exo-MS and DFO-B individually and in combination with *PZA*.

ii. **Method**: MGIT-DST was used to determine the anti-TB activity of the siderophores and their combination with drugs. The test was conducted as per the standard protocol recommended in the BACTEC MGIT system manual [5].

iii. **Testing of siderophores and their combinations**: Exo-MS isolated in our laboratory was used for this study at a concentration of 19 mg/mL [Gokarn et al., 2017, BCAM DOI:10.1186/s12906-017-1657-8]. The concentration of Exo-MS and its combinations with *INH* and *RIF* used against MDR isolates are shown in Table 1. Similarly, its concentration alone and in combination with *PZA* against *PZA*-resistant isolates are shown in Table 2.
A working stock of DFO-B (160 mg/mL) was prepared from 250 mg/mL stock solution before use. The concentration of DFO-B and its combinations with INH and RIF used against MDR isolates are shown in Table 3. Similarly, its concentration alone and in combination with PZA against PZA-resistant isolates are shown in Table 4.

For each isolate, one set of tubes was supplemented with excess ferric ammonium citrate as control to ascertain whether the growth inhibition was solely due to iron deprivation. All the tubes were mixed by inverting several times and then placed in set carriers before transferring them into the MGIT instrument maintained at 37 °C. The first tube in the set carrier is always the Growth Control (GC) tube. In MGIT-DST, the GC should have a Growth Unit (GU) value of 400 or more. If the GU value of the drug-containing tube is <100, the interpretation “S” is read by the MGIT instrument. If the GU value of the drug-containing tube is ≥ 100, the reading “R” is given by the instrument [5]. When the GU value of the GC was 400 or greater, the tubes were removed, scanned and a report was generated.

iv Test for bactericidal or bacteriostatic effect of the siderophores: Determination of minimum bactericidal concentration was carried out to test whether the concentration of the siderophores used had a bacteriostatic (inhibiting), or bactericidal (killing) effect. For this test, 0.5 mL aliquots from the tubes showing GU value <100 after 14 days were re-inoculated into fresh Middlebrook 7H9 broth without any siderophores and antibiotics; the tubes were incubated in the MGIT instrument for 35 days.

Results

Extraction and purification of Exo-MS from M. smegmatis mc²155

HR-LC/MS analysis confirmed the presence of Exo-MS [Gokarn et al., 2017, BCAM DOI:10.1186/s12906-017-1657-8]. Since Exo-MS was used to chelate iron, it was isolated in desferri form, i.e., not saturated with iron. DFO-B was obtained in desferri form.

Table 1: Protocol for MGIT-DST of MDR-Mtb with Exo-MS alone and in combination with INH and RIF

| No | Drug     | Initial Concentration of Drug | Volume added to MGIT for Test | Final Concentration in MGIT tube except GC | No. of Middle Brook tubes |
|----|----------|-------------------------------|-------------------------------|-------------------------------------------|---------------------------|
| 1  | INH      | 8.3 μg/mL                     | 100 μL                        | 0.1 μg/mL                                 | 1                         |
| 2  | RIF      | 83 μg/mL                      | 100 μL                        | 1.0 μg/mL                                 | 1                         |
| 3  | Exo-MS + INH | 19 mg/mL + 8.3 μg/mL        | 50 μL + 100 μL                | 0.125 mg/mL and 0.1 μg/mL                 | 1                         |
| 4  | Exo-MS + INH | 19 mg/mL + 8.3 μg/mL        | 100 μL + 100 μL               | 0.25 mg/mL and 0.1 μg/mL                 | 1                         |
| 5  | Exo-MS + INH | 19 mg/mL + 8.3 μg/mL        | 200 μL + 100 μL               | 0.5 mg/mL and 0.1 μg/mL                 | 1                         |
| 6  | Exo-MS + RIF | 19 mg/mL + 8.3 μg/mL        | 50 μL + 100 μL                | 0.125 mg/mL and 1.0 μg/mL               | 1                         |
| 7  | Exo-MS + RIF | 19 mg/mL + 8.3 μg/mL        | 100 μL + 100 μL               | 0.25 mg and 1.0 μg/mL                 | 1                         |
| 8  | Exo-MS + RIF | 19 mg/mL + 8.3 μg/mL        | 200 μL + 100 μL               | 0.5 mg and 1.0 μg/mL                 | 1                         |
| 9  | Exo-MS    | 19 mg/mL                      | 50 μL                         | 0.125 mg/mL                              | 1                         |
| 10 | Exo-MS    | 19 mg/mL                      | 100 μL                        | 0.25 mg/mL                               | 1                         |
| 11 | Exo-MS    | 19 mg/mL                      | 200 μL                        | 0.5 mg/mL                                | 1                         |
| 12 | Exo-MS + FeCl₃ | 19 mg/mL + 80 mg/mL    | 200 μL + 50 μL                | 0.5 mg/mL and 0.5 mg/mL                 | 1                         |

Table 2: Protocol for MGIT-DST for PZA°-Mtb with Exo-MS alone and in combination with PZA

| No | Drug     | Initial Concentration of Drug | Volume added to MGIT for Test | Final Concentration in MGIT tube except GC | No. of PZA tubes |
|----|----------|-------------------------------|-------------------------------|-------------------------------------------|-----------------|
| 1  | PZA      | 8 mg/mL                       | 100 μL                        | 0.1 mg/mL                                 | 1               |
| 2  | Exo-MS + PZA | 19 mg/mL + 8 mg/mL           | 50 μL + 100 μL                | 0.125 mg/mL and 0.1 mg/mL                 | 1               |
| 3  | Exo-MS + PZA | 19 mg/mL + 8 mg/mL           | 100 μL + 100 μL               | 0.25 mg/mL and 0.1 mg/mL                 | 1               |
| 4  | Exo-MS + PZA | 19 mg/mL + 8 mg/mL           | 200 μL + 100 μL               | 0.5 mg/mL and 0.1 mg/mL                 | 1               |
| 5  | Exo-MS    | 19 mg/mL                      | 50 μL                         | 0.125 mg/mL and 0.1 mg/mL               | 1               |
| 6  | Exo-MS    | 19 mg/mL                      | 100 μL                        | 0.25 mg/mL                              | 1               |
| 7  | Exo-MS    | 19 mg/mL                      | 200 μL                        | 0.5 mg/mL                                | 1               |
| 8  | Exo-MS + FeCl₃ | 19 mg/mL + 80 mg/mL   | 200 μL + 50 μL                | 0.5 mg/mL and 0.5 mg/mL                 | 1               |
Inhibitory effect of Exo-MS alone and in combination with INH and RIF on MDR-Mtb isolates in vitro

Table 5 shows the GU values of Exo-MS, Exo-MS + INH, and Exo-MS + RIF combinations on five different isolates of MDR-Mtb. For two of the five MDR-Mtb isolates, MIC of Exo-MS alone was 0.5 mg/mL. No concentration below this was inhibitory in combination with INH or RIF.

Inhibitory effect of DFO-B alone and in combination with INH and RIF on MDR-Mtb isolates in vitro

Table 6 shows the GU values of DFO-B and DFO-B + INH, DFO-B + RIF combinations against the ten different isolates of MDR-Mtb isolates. For four MDR isolates, MIC of DFO-B alone was 0.5 mg/mL. For five other MDR isolates, MIC of DFO-B alone was 1.0 mg/mL. For one of these five isolates, MIC of DFO-B + INH and DFO-B + RIF combinations decreased to 0.5 mg/mL.

So, a total of nine out of ten MDR-Mtb isolates were inhibited by DFO-B alone (and its combinations).

Inhibitory effect of Exo-MS and DFO-B alone and in combination with PZA on PZA-resistant Mtb isolates in vitro

Table 7 shows the GU values of Exo-MS and Exo-MS + PZA combination on seven PZA-resistant isolates. For the two non-MDR PZA-resistant isolates, MIC of Exo-MS alone was 0.125 mg/mL. For two MDR PZA-resistant isolates, the MIC was 0.25 mg/mL. For one MDR PZA-resistant isolate, the MIC was 0.5 mg/mL.

Thus, of the seven PZA-resistant Mtb isolates tested, five were inhibited by Exo-MS alone. Exo-MS + PZA combination did not change the MIC.

Table 8 shows the GU values of DFO-B and DFO-B + PZA combination on seven PZA-resistant isolates. For the two non-MDR PZA-resistant isolates, MIC of DFO-B alone was 0.5 mg/mL and it decreased to 0.25 mg/mL for DFO-B + PZA combination. For three of the five MDR PZA-resistant isolates, MIC of DFO-B alone was 1.0 mg/mL. For two of these isolates, MIC of DFO-B + PZA combination decreased to 0.25 mg/mL. Thus, of the seven isolates of PZA-resistant Mtb, five were susceptible to DFO-B and DFO-B + PZA.

### Table 3 Protocol for MGIT-DST of MDR-Mtb with DFO-B alone and in combination with INH and RIF

| No | Drug          | Initial Concentration of the drug preparation | Volume added to MGIT for Test | Final Concentration in MGIT tube except GC | No. of Middle Brook tubes |
|----|---------------|-----------------------------------------------|-------------------------------|--------------------------------------------|---------------------------|
| 1  | INH           | 8.3 μg/mL                                      | 100 μL                        | 0.1 μg/mL                                   | 1                         |
| 2  | RIF           | 83 μg/mL                                       | 100 μL                        | 1.0 μg/mL                                   | 1                         |
| 3  | DFO-B + INH   | 160 mg/mL + 8.3 μg/mL                         | 125 μL and 100 μL             | 0.25 mg/mL and 0.1 μg/mL                   | 1                         |
| 4  | DFO-B + INH   | 160 mg/mL + 8.3 μg/mL                         | 25 μL and 100 μL              | 0.5 mg/mL and 0.1 μg/mL                    | 1                         |
| 5  | DFO-B + INH   | 160 mg/mL + 8.3 μg/mL                         | 50 μL and 100 μL              | 1.0 mg/mL and 0.1 μg/mL                    | 1                         |
| 6  | DFO-B + RIF   | 160 mg/mL + 8.3 μg/mL                         | 125 μL and 100 μL             | 0.25 mg/mL and 1.0 μg/mL                   | 1                         |
| 7  | DFO-B + RIF   | 160 mg/mL + 8.3 μg/mL                         | 25 μL and 100 μL              | 0.5 mg/mL and 1.0 μg/mL                    | 1                         |
| 8  | DFO-B + RIF   | 160 mg/mL + 8.3 μg/mL                         | 50 μL and 100 μL              | 1.0 mg/mL and 1.0 μg/mL                    | 1                         |
| 9  | DFO-B         | 160 mg/mL                                      | 125 μL                        | 0.25 mg/mL                                 | 1                         |
| 10 | DFO-B         | 160 mg/mL                                      | 25 μL                         | 0.5 mg/mL                                  | 1                         |
| 11 | DFO-B         | 160 mg/mL                                      | 50 μL                         | 1.0 mg/mL                                  | 1                         |
| 12 | DFO-B + FeCl₃ | 160 mg/mL + 80 mg/mL                          | 50 μL + 50 μL                 | 1.0 mg/mL and 0.5 mg/mL                    | 1                         |

### Table 4 Protocol for MGIT-DST for PZA-resistant Mtb with DFO-B alone and in combination with PZA

| No | Drug         | Initial Concentration of the drug preparation | Volume added to MGIT for Test | Final Concentration in MGIT tube except GC | No. of PZA tubes GC PZA₆ |
|----|--------------|-----------------------------------------------|-------------------------------|--------------------------------------------|---------------------------|
The control tubes with ferric citrate added showed GU value of 400 for all the isolates.

Bacteriostatic or bactericidal effect of the siderophores in vitro
When the inhibited isolates showing GU value of <100 were re-inoculated into fresh siderophore-free and drug-free Middlebrook 7H9 medium, the distinction between bacteriostatic and bactericidal effect of the siderophores (and their combinations) could be inferred based on the GU values. If the GU value of re-inoculated tube was <100, the original siderophore-drug combination was considered bacteriostatic. If the GU value of re-inoculated tube was 400, the original siderophore-drug combination was considered bactericidal.

After 14 days of re-inoculation into fresh medium, the GU values were <100 for two isolates inhibited by Exo-MS + INH, three of the nine isolates inhibited by DFO-B + RIF, and three of the five isolates inhibited by DFO-B + PZA. However, at the end of 35 days, all concentrations of siderophores and their combinations with drugs were found to be bacteriostatic for all isolates, with GU values of 400.

Discussion
Tubercle bacilli acquire iron from a mammalian host for their growth. Excess iron in vivo promotes Mtb infection and may impair macrophage function affecting innate immunity. A study using female Balb/C mouse infected with Mtb showed that iron overloading significantly reduced the bactericidal activity of INH and completely neutralized the bacteriostatic activity of ethambutol [10].

Table 5: MGIT-DST of MDR-Mtb with Exo-MS alone and in combination with INH and RIF

| Isolate | Exo-MS (mg/mL) | Exo-MS (mg/mL) + INH 0.1 µg/mL | Exo-MS (mg/mL) + RIF 1.0 µg/mL | Ferric citrate + Exo-MS (mg/mL) |
|---------|----------------|-------------------------------|-------------------------------|-------------------------------|
|         | 0.125 0.25 0.5 | 0.125 0.25 0.5 | 0.125 0.25 0.5 | 0.5 |
| 1       | 400 400 400    | 400 400 400                    | 400 400 400                    | 400 |
| 2       | 400 400 0      | 400 400 0                      | 400 400 0                      | 400 |
| 3       | 400 400 0      | 400 400 0                      | 400 400 0                      | 400 |
| 4       | 400 400 400    | 400 400 400                    | 400 400 400                    | 400 |
| 5       | 400 400 253    | 400 400 105                    | 400 400 131                    | 400 |
| INFERENCE | R R S n = 2 | R R S n = 2 | R R S n = 2 | R |

GU value of GC for all five isolates was 400. GU value in INH- and RIF-containing medium for all isolates was also 400. R resistant, S susceptible. If the GU value of the drug-containing tube is ≥100, the interpretation is Resistant by the MGIT instrument. If the GU value of the drug tube is <100, the interpretation is Susceptible (n = no. of isolates susceptible).

Inhibitory effect of Exo-MS, Exo-MS + INH, Exo-MS + RIF on five clinical isolates of MDR-Mtb (INH-, RIF- and PZA-resistant). The medium used was Middlebrook 7H9 broth, pH = 7 incubated at 37 °C for 14 days in the MGIT instrument

Table 6: MGIT-DST of MDR-Mtb with DFO-B alone and in combination with INH and RIF

| Isolate | DFO-B (mg/mL) | DFO-B (mg/mL) + INH 0.1 µg/mL | DFO-B (mg/mL) + RIF 1.0 µg/mL | Ferric citrate + DFO-B (mg/mL) |
|---------|----------------|-------------------------------|-------------------------------|-------------------------------|
|         | 0.25 0.5 1.0   | 0.25 0.5 1.0                  | 0.25 0.5 1.0                  | 1.0 |
| 1       | 400 400 0      | 400 400 0                     | 400 400 0                     | 400 |
| 2       | 400 400 0      | 400 400 0                     | 400 400 0                     | 400 |
| 3       | 400 0 0        | 400 0 0                       | 400 0 0                       | 400 |
| 4       | 400 0 0        | 400 0 0                       | 400 0 0                       | 400 |
| 5       | 400 400 400    | 400 400 400                   | 400 400 400                   | 400 |
| 6       | 400 400 0      | 400 400 0                     | 400 400 0                     | 400 |
| 7       | 400 400 0      | 400 400 0                     | 400 400 0                     | 400 |
| 8       | 400 0 0        | 400 0 0                       | 400 0 0                       | 400 |
| 9       | 400 0 0        | 400 0 0                       | 400 0 0                       | 400 |
| 10      | 400 400 0      | 400 400 0                     | 400 400 0                     | 400 |
| INFERENCE | R S n = 4 | S n = 9 | R S n = 5 | S n = 9 |

GU value of GC for all ten isolates was 400. GU value in INH- and RIF-containing medium for all isolates was also 400. R resistant, S susceptible. If the GU value of the drug-containing tube is ≥100, the interpretation is Resistant by the MGIT instrument. If the GU value of the drug tube is <100, the interpretation is Susceptible (n = no. of isolates susceptible).

Inhibitory effect of DFO-B, DFO-B + INH, DFO-B + RIF on ten different clinical isolates of MDR-Mtb (INH-, RIF- and PZA-resistant). The medium used was Middlebrook 7H9 broth, pH = 7 incubated at 37 °C for 14 days in the MGIT instrument.
There are various strategies that the human body has adapted to safeguard the iron. Iron retention by the reticuloendothelial system [11] and mild anemia which is a common occurrence in TB patients are examples of host mechanisms to ensure iron-deficient conditions in the body [12, 13]. Though these natural processes exist, restricting mechanisms to ensure iron-deficient conditions in the body were susceptible to its combination with INH and RIF. Due to limited availability of Exo-MS, only five isolates of MDR-\(Mtb\) could be tested with it.

When the inhibitory effect of DFO-B on MDR-\(Mtb\) isolates was determined, nine out of ten isolates of MDR-\(Mtb\) tested were found to be susceptible to DFO-B. The same numbers were susceptible to its combination with INH and RIF, though at a lower DFO-B concentration for some.

Inhibitory effect of Exo-MS and DFO-B on \(PZA\)-resistant \(Mtb\) isolates was determined in a modified Middlebrook 7H9 medium with pH 5.9. The standard growth medium used for testing INH and RIF is not used for \(PZA\), since \(PZA\) requires acidic pH for activity in vitro. Five of the seven \(PZA\)-resistant \(Mtb\) isolates were susceptible to Exo-

### Table 7 MGIT-DST of \(PZA^R\)-\(Mtb\) with Exo-MS alone and in combination with \(PZA\)

| \(Mtb\) Isolate | Exo-MS (mg/mL) | Exo-MS (mg/mL) + \(PZA\) 100 \(\mu g/mL\) | Ferric citrate + Exo-MS (mg/mL) |
|-----------------|---------------|-------------------------------------|---------------------------------|
|                 | 0.125         | 0.25                                | 0.5                             | 0.125                          |
| \(PZA^R\) isolate 1 | 0             | 0                                    | 0                               | 0                              |
| \(PZA^R\) isolate 2 | 0             | 0                                    | 0                               | 0                              |
| MDR + \(PZA^R\) isolate 3 | 400           | 400                                  | 0                               | 0                              |
| MDR + \(PZA^R\) isolate 4 | 400           | 25                                   | 0                               | 0                              |
| MDR + \(PZA^R\) isolate 5 | 400           | 317                                  | 0                               | 0                              |
| MDR + \(PZA^R\) isolate 6 | 400           | 400                                  | 0                               | 0                              |
| MDR + \(PZA^R\) isolate 7 | 0             | 400                                  | 400                             | 0                              |
| INFERRENCE      | \(S \ n = 2\) | \(S \ n = 4\)                        | \(S \ n = 5\)                    | \(S \ n = 5\)                  |

**GU value of GC for all seven isolates was 400. GU value in \(PZA\)-containing medium for all isolates was also 400. R resistant, S susceptible. If the GU value of the drug-containing tube is \(\geq\)100, the interpretation is Resistant by the MGIT instrument. If the GU value of the drug tube is <100, the interpretation is Susceptible \((n = \text{no. of isolates susceptible})\)**

### Table 8 MGIT-DST of \(PZA^R\)-\(Mtb\) with DFO-B alone and in combination with \(PZA\)

| \(Mtb\) Isolate | DFO-B (mg/mL) | DFO-B (mg/mL) + \(PZA\) 100 \(\mu g/mL\) | Ferric citrate + DFO-B (mg/mL) |
|-----------------|---------------|-------------------------------------|---------------------------------|
|                 | 0.125         | 0.25                                | 0.5                             | 0.25                           |
| \(PZA^R\) isolate 1 | 209           | 41                                   | 0                               | 0                              |
| \(PZA^R\) isolate 2 | 129           | 0                                    | 0                               | 0                              |
| MDR + \(PZA^R\) isolate 3 | 400           | 400                                  | 0                               | 0                              |
| MDR + \(PZA^R\) isolate 4 | 400           | 400                                  | 0                               | 0                              |
| MDR + \(PZA^R\) isolate 5 | 400           | 400                                  | 0                               | 0                              |
| MDR + \(PZA^R\) isolate 6 | 400           | 400                                  | 0                               | 0                              |
| MDR + \(PZA^R\) isolate 7 | 400           | 400                                  | 0                               | 0                              |
| INFERRENCE      | \(R \ n = 2\) | \(S \ n = 4\)                        | \(S \ n = 5\)                    | \(S \ n = 5\)                  |

**GU value of GC for all seven isolates was 400. GU value in \(PZA\)-containing medium for all isolates was also 400. R resistant, S susceptible. If the GU value of the drug-containing tube is \(\geq\)100, the interpretation is Resistant by the MGIT instrument. If the GU value of the drug tube is <100, the interpretation is Susceptible \((n = \text{no. of isolates susceptible})\)**

Inhibitory effect of DFO-B and DFO-B + \(PZA\) on seven \(PZA^R\) isolates: The medium used was Middlebrook 7H9 broth, pH = 5.9 incubated at 37 °C for 14 days in the MGIT instrument.
MS alone. There was no change in the MIC in combination with PZA. Similarly, there were five of these isolates were susceptible to DFO-B alone and also to DFO-B + PZA. For four of these isolates, the MIC of DFO-B decreased to half when used in combination with PZA.

A significant observation was that the inhibitory activity of the siderophores was abolished when excess iron was added in the medium. This proves that the anti-TB effect of the siderophores is solely due to their ability to deprive pathogens of iron. This makes it imperative to use the desferri form of siderophores in such studies.

For all isolates, the siderophore concentrations used were found to be bacteriostatic. This could be beneficial in restricting multiplication of the pathogen in vivo, thus participating in controlling the infection.

The tubercle bacilli down-regulate iron-containing proteins during iron-deficiency [14]. Therefore, iron-deprivation by Exo-MS and DFO-B may result in inhibition or inactivation of proteins and enzymes involved in vital functions required for drug resistance such as cell wall integrity. Perhaps Exo-MS and DFO-B may act as facilitators for antibiotics across the cell membrane due to increased cell permeability. Therefore, in the presence of exogenous iron chelators, drug-resistant Mtb could once again become susceptible to the same drugs.

Middlebrook 7H9 medium contains high levels of iron which may not represent true physiological concentrations of ferric ions. It could be one of the reasons why the MIC of the siderophores was high in vitro. Such susceptibility testing predicts the possible antimicrobial agent for the treatment of an infection. The clinical outcome may not be the same due to factors such as host physiology or other interventions, which cannot be simulated in laboratory tests. In vivo studies are required to validate these most promising in vitro results. While DFO-B is already approved for use to treat iron overload in thalassemic patients, safety of Exo-MS for therapeutic use needs to be determined.

We have shown in another study that Exo-MS and DFO-B do not have any cytotoxic effects in vitro on normal mammalian cell lines, such as human embryonic kidney cell line HEK-293 and mouse fibroblast cell line NIH/3 T3 [Gokarn et al., 2017, BCAM DOI:10.1186/s12906-017-1657-8]. From the results of the anti-TB activity as well as the effect on normal mammalian cells, it can be concluded that Exo-MS at 0.5 mg/mL and DFO-B at 1.0 mg/mL selectively inhibit Mtb without significantly harming normal mammalian cells.

Conclusions
Our study is the first ever investigation done to evaluate siderophores as potential agents against drug-resistant Mtb by MGIT-DST method. Since the work was exploratory in nature, few drug-resistant isolates of Mtb were used. Nonetheless, it has provided a “proof of concept” that exogenous siderophores such as Exo-MS and DFO-B could be valuable additions to fight drug-resistant M. tuberculosis. In view of the encouraging preliminary results, new in vitro study with large number of M. tuberculosis isolates can be carried out.
8. Howard DH, Rafie R, Tiwari A, Faull KF. Hydroxamate Siderophores of Histoplasma capsulatum. Infect Immun. 2000;68(4):2338–43.
9. Barclay R, Ratledge C. Iron-binding compounds of Mycobacterium avium, M. intracellulare, M. scrofulaceum, and mycobactin-dependent M. paratuberculosis and M. avium. J Bacteriol. 1983;153:1138–46.
10. Lounis N, Maslo C, Truffot-Penot C, Grosset J, Boelaert R. Impact of iron loading on the activity of isoniazid or ethambutol in the treatment of murine tuberculosis. Int J Tuberc Lung Dis. 2003;7(6):575–9.
11. Cronje L, Bomman L. Iron overload and tuberculosis: a case for iron chelation therapy. Int J Tuberc Lung Dis. 2005;9:2–9.
12. Lee SW, Kang YA, Yoon YS, Um SW, Lee SM, Yoo CG, Kim YW, Han SK, Shim YS, Yim JJ. The Prevalence and Evolution of Anemia Associated with Tuberculosis. J Korean Med Sci. 2006;21:1028–32.
13. Isanaka S, Mugusi F, Urassa W, Willett WC, Bosch RJ, Villamor E, Spiegelman D, Duggan C, Fawzi WW. Iron Deficiency and Anemia Predict Mortality in Patients with Tuberculosis. J Nutr. 2012;142:350–7.
14. De Voss JJ, Rutter K, Schroeder BG, Barry CE. Iron Acquisition and Metabolism by Mycobacteria. J Bacteriol. 1999;181(15):4443–51.