Efficacious In Vitro and In Vivo Effects of Dihydrosphingosine—Ethambutol Analogues Against Susceptible and Multi-drug-resistant Mycobacterium tuberculosis

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Background and Aims. Tuberculosis (TB) is a major worldwide health problem in part due to the lack of new drugs and the emergence of multidrug-resistant strains (MDR). The aim of this study was to select anti-tuberculosis drug candidates from a collection of 69 synthetic sphingosine-ethambutol analogues through in vitro and in vivo evaluations.

Methods. The 69 compounds were evaluated in vitro against two Mycobacterium tuberculosis strains, a drug susceptible (H37Rv) and a MDR clinical isolate (CIBIN 99). Four selected compounds, those that exhibited the highest potency in vitro, were tested in vivo using a model of progressive TB in BALB/c mice infected with the drug susceptible strain, either alone or combined with conventional chemotherapy, as well as in mice infected with the MDR strain. The acute toxicity was evaluated on male and female adult BALB/c mice.

Results. Ten of the evaluated compounds resulted more potent in vitro than ethambutol. The experimental compound 2b (2-aminopalmitol benzyl ether) was the most efficacious and also showed additive effects in combination with conventional chemotherapy. It did not exhibit toxicity (LD50 > 2000 mg/kg).

Conclusions. Compound 2b can be considered as a new drug candidate to continue its development against M. tuberculosis MDR strains. © 2016 IMSS. Published by Elsevier Inc.

Key Words: Tuberculosis, MDR Mycobacterium tuberculosis, Dihydrosphingosine analogues, Ethambutol, Marine model, In vitro evaluation.

Introduction

Tuberculosis (TB) is a worldwide health problem. The World Health Organization (WHO) informed that there were 9.6 million new active cases and 1.5 million deaths during 2014 (1). Indeed, Mycobacterium tuberculosis is highly infectious, considering that one third of the world’s population is latently infected and 10% of this population will develop active disease. An additional problem is the association with HIV infection, an estimated 1.2 million (12.5%) of the 9.6 million people who developed TB in 2014 were HIV-positive (1).

Although TB can be controlled and cured by chemotherapy, treatment usually requires four specific drugs during 6 months, which produces significant adherence problems (2). The consequence of this is disease recurrence and the
arising of multidrug-resistant (MDR) strains (2). In recent years, MDR have increased their frequency, afflicting ~480,000 people worldwide and producing 190,000 deaths in year 2014 (1). Treatment of MDR-TB is resource intensive and requires second-line drugs, which are more expensive, toxic, and less effective than primary drugs (3). These problems have motivated the search for new drugs and treatment strategies. New drug candidates should shorten conventional chemotherapy and be effective against MDR-TB.

The cell wall of \textit{M. tuberculosis} is a significant target for several conventional and new anti-TB agents (4). Ethambutol (EMB) is one of the first-line anti-TB drugs, its mechanism of action is interfering with the integrity of the \textit{M. tuberculosis} cell wall through the inhibition of arabinogalactan biosynthesis (5), although other possible pathways have also been proposed (6). We previously showed that from a group of 2-aminoalkanol derivatives one diamine- and two amino ether analogues of EMB have high in vitro mycobactericidal potency, with a minimal inhibitory concentration (MIC) of 1.25 \(\mu\text{g/mL}\) when evaluated against drug-susceptible strain H37Rv and against several clinical isolates with different patterns of multidrug resistance (7). These encouraging results motivated the continuation of the current research through the structure manipulation and the extension of the series aiming to obtain better anti-\textit{M. tuberculosis} compounds, which could be submitted to efficacy and toxicity in in vivo studies.

We report here the results of in vitro evaluation against H37Rv \textit{M. tuberculosis} and those of selected dihydrosphingosine analogues structurally related to EMB more potent compounds against the CIBIN-99 strain, a MDR clinical isolate resistant to all five first-line anti-TB drugs. We also

<table>
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<td>-</td>
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<td>R': -CO(CH_2)_3COOH</td>
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<td>11d</td>
<td>68.0</td>
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<td></td>
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<td>13</td>
<td>12b</td>
<td>&gt;100</td>
<td>-</td>
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</tbody>
</table>

\(^a\)Anti-\textit{Mt}b potency relative to Ethambutol. \(P_E = \text{MIC}_{\text{EMB}}/\text{MIC}_{\text{COMP}}\). \textbf{Bold-faced} MIC and \(P_E\) values for compounds more potent than EMB.
describe the efficacy and toxicity of selected compounds in in vivo evaluation using a murine model of pulmonary TB infected with drug-susceptible H37Rv or with the MDR CIBIN-99 strain, as well as on their therapeutic efficacy when used in combination with conventional chemotherapy.

Materials and Methods

Compounds and Structures

The structures of the 69 evaluated compounds are represented in Tables 1–3. Through the first group of 2-aminoalkanol derivatives (35 compounds, 1a–12b, Table 1), we analyzed the influence of the side-chain size and lipophilicity on the activity by the preparation and evaluation of compounds with C10–C18 alkyl chains. In the second group (13 compounds, 13a–15, Table 2), the positions of hydroxyl and amino groups were interchanged to that of 1-amino-2-alkanol derivatives, whereas in some members of these series the nitrogen atom was incorporated as part of different heterocyclic moieties as piperidine, piperazine and morpholine, some typical activity-inducing fragments present in many clinically used chemotherapeutic drugs. The third group (22 compounds, 16a–22d, Table 3) included those containing the central ethylenediamine fragment of EMB with the amino groups either free, alkylated or partially carbamoylated, looking to reduce the amphiphilic character of certain compounds. The synthetic procedures applied for the preparation of the new compounds were similar to those applied in our previous work (8), whereas the complete chemical characterization of the new substances evaluated here will be the object of another report.

Mycobacteria and In Vitro Evaluation

The evaluation of the anti-mycobacterial activity of the three series of compounds was initially carried out using the M. tuberculosis reference strain H37Rv (American Type Culture Collection [ATCC 27294, http://www.atcc.org/])
and the clinical strain CIBIN—UMF: 15:99 (CIBIN—99) was used for the evaluation of some selected compounds. H37Rv is susceptible to all five first-line anti-TB drugs: streptomycin (STR), pyrazinamide (PZA), isoniazid (INH), rifampicin (RIF) and EMB. CIBIN-99 is an isolate from a patient with advanced pulmonary TB that is resistant to all the above-mentioned antibiotics and particularly highly resistant to EMB, with a MIC of 32 mg/mL (9) under the in vitro experimental conditions used in this research. CIBIN-99 was previously fully characterized (10).

Bacilli were inoculated in 13 × 100 mm screw-capped tubes containing 4 mL sterile Middlebrook 7H9 broth (Difco, Detroit MI) supplemented with 0.2% glycerol, 10% oleic acid, albumin, dextrose and catalase (OADC) (Difco) incubated at 37°C in 5% CO₂ atmosphere. Anti-mycobacterial activity was determined by the Microplate Alamar Blue Assay (MABA) as previously described (11). The concentration range of each evaluated compound was 100⁻⁰.⁸⁸ mg/mL. Those compounds having MICs of one digit were considered active. In each microplate 2.0⁻⁰.⁶⁰ μg/mL RIF/mL, 16.0⁻⁰.⁵⁰ μg ofloxacin/mL, and 1⁻³₂ μg EMB/mL were used as positive controls. In addition, OADC enriched Middlebrook 7H9 broth and 5% DMSO dissolved in enriched Middlebrook broth were included as negative and solvent controls, respectively. All evaluations were carried out in triplicate.

The relative anti-mycobacterial potency (PE) of each compound was calculated with respect to the anti-M. tuberculosis potency of EMB by means of the following equation:

\[
P_E = \frac{\text{MIC}_{\text{EMB}}}{\text{MIC}_{\text{COMP}}}\]

where MIC_{EMB} is the MIC of EMB and MIC_{COMP} is the MIC of each experimental compound.

### Murine Model of Progressive Pulmonary Tuberculosis

Strains of M. tuberculosis H37Rv and CIBIN-99 were cultured in Middlebrook 7H9 broth. After 1 month, mycobacteria were harvested, adjusted to 2.5 × 10⁷ cells in 100 μL of PBS and stored at −70°C until used (12). Pathogen-free male BALB/c mice, 6–8 weeks old, were anesthetized (sevoflurane; Abbott Laboratories, Abbott Park, IL) and infected by endotracheal cannulation administering 2.5 × 10⁷ viable bacteria suspended in 100 μL of PBS. Infected mice were kept in groups of five in cages fitted with micro-isolators connected to negative pressure. All procedures were performed in a biological security cabinet in a biosafety level III facility. Animal work was performed in accordance with Mexican National Regulations on Animal Care and Experimentation (NOM 062-ZOO-1999) following the guidelines and approval of the Ethical Committee for Experimentation in Animals of the INCMNSZ, permit number 224.

### Table 3. Structures, MIC (mmol) values and relative potency (PE) values for 1,2-alkanediamine derivatives against H37Rv M. tuberculosis

<table>
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<th>Base structure</th>
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<th>n</th>
<th>Comp. no.</th>
<th>MIC</th>
<th>PE</th>
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<tr>
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<td>R²: Ethyl</td>
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<td>R¹: H; R²: Boc</td>
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<td>20c</td>
<td>&gt;100</td>
<td>-</td>
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<td>R¹: H; R²: Boc</td>
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<td>21a</td>
<td>&gt;100</td>
<td>-</td>
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**Bold-faced** MIC and PE values for compounds more potent than EMB.

*Anti-Mtb potency, relative to Ethambutol (EMB). P_E = MIC_{EMB}/MIC_{COMP}.*
Drug Administration

The animals surviving 60 days after infection were randomly allocated to the required treatment groups. Treatment was started 60 days after infection and euthanized after 2 months of treatment. Due to the fairly lipophilic character of the selected compounds, their expected high intestinal absorption and aiming to obtain more realistic pre-clinical efficacy and toxicity results, the oral route of administration was selected. Thus, aqueous solutions/suspensions of the compounds were used to carry out the in vivo assays in mice. Indeed, log $p$ values for the compounds ranged from 4.64 (2b) to 8.31 (9b) according to the results provided by commercial ChemDraw software (13). Other pharmacokinetic-ADME parameters as the human intestinal absorption (HIA) rate ranged from 92.0 (5b) to 100% (9b) according to web-based online pre-ADMET predictions (14).

The dose of 5 mg/kg of body weight was tested for each of the four selected compounds. Each compound was administered using an intragastric cannula daily as a suspension, dissolving the drug in 100 μL of acidified water (0.1% HCl, pH 5). To evaluate the therapeutic effect of each compound, two different experiments were done. In the first one, animals surviving 60 days after H37Rv or CIBIN-99 strains infection were randomly allocated into two treatment groups: mice treated with each compound and infected mice that only received the vehicle as a control group. Groups of six animals were killed at day 60 after treatment. In the second experiment, mice surviving 60 days after infection with drug-sensible strain H37Rv were randomly allocated to three experimental groups. The first group of four infected mice was treated with conventional chemotherapy: a combination of 10 mg RIF/kg, 10 mg INH/kg, and 30 mg PZA/kg (RIP therapy), administered daily by intragastric cannula plus 5 mg/kg of either compound administered daily by the same route. The second group of four infected mice exclusively received the RIP chemotherapy, and the control group was solely treated with the diluent. Mice were sacrificed by exsanguination under terminal anesthesia after 60 days of treatment. To determine the effect of treatments, we quantified lung bacillary loads by colony-forming units ($CFU$). To accomplish this, lungs were removed and frozen by immersion in liquid nitrogen immediately after the animals were euthanized. Frozen lungs were disrupted in a Polytron homogenizer (Kinematica, Lucerne, Switzerland) in sterile 50 mL tubes containing 3 mL of isotonic saline solution. Four dilutions of each homogenate were spread onto duplicate plates containing Middlebrook 7H10 agar (Difco) enriched with OADC. The incubation time was 21 days. Data points are the means of four animals. Two independent experiments were performed.

Toxicity

Short-term toxicity of compound 2b was determined in BALB/c male and female adult mice. Both determination of LD$\text{50}$ and observation of animals were done from the first day of the drug administration and for 2 weeks until they were euthanized. Two groups (male and female) of five animals for drug testing and one for control were used. The initial drug concentration was 175 mg/kg of body weight (considered to be a sublethal dose). This dose was progressively increased by a factor of 3.2 (progressive sequence of 175, 550 and 2000 mg/kg of body weight), prepared by dissolving compound 2b after vigorous shaking in corn oil and adjusting to a volume of 0.2 mL. Animals were kept in a fasting state condition for 4 h before and 2 h after the drug administration. At the end of the initial 4 h of fasting, animals were weighed. The substance was administered by intragastric cannula using water as vehicle; control animals received only the same amount of vehicle. Animals were observed during the first 24 h and 48 h (short term) and daily for a period of 14 days (long term), registering any toxic sign (gait, feeding habit alteration, diuresis, convulsions, diarrhea, changes in skin, eyes, mucosal epithelia). Animals were euthanized under pentobarbital anesthesia and the liver, kidney, lungs, heart, spleen, intestine and brain were weighted, fixed in 10% formalin solution and embedded in paraffin for histopathological examination.

Statistics

One-way analysis of variance (ANOVA) and Student $t$-test were used to compare CFU determinations in infected mice treated with each compound and non-treated control animals; $p < 0.05$ was considered significant.

Results

In vitro Anti-mycobacterial Activity

MIC values corresponding to each experimental compound determined against the H37Rv and CIBIN-99 strains are shown in Tables 1—4. Bold-faced numbers in the MIC/$P_e$ columns, respectively, represent values lower/higher than those found for EMB, the reference drug structurally close to those evaluated compounds.

For the group of 2-aminoalkan-1-ols (Table 1), carbamoylation (Boc-protection) or acylation of the amino group led to practically inactive compounds (3, 4, 11 and 12), whereas monoalkylation (compounds 5, 6, 8 and 10) or dialkylation (7 and 9) of such group of substances led to potent anti-$M$. $tuberculosis$ compounds, with preference for the benzyl-etherification of the primary hydroxyl group. The $n$-$C_{14}$H$_{29}$ chain size (corresponding to letter b attached to the number of any compound) could significantly be associated to the most potent anti-$M$. $tuberculosis$ compounds of the series (Table 1).

The exchange of positions between the amino and the hydroxyl groups 1-aminoalkan-2-ols, (Table 2) resulted unsuccessful and led to lesser potent compounds, whereas
other structure-activity relationship comparisons would be similar as those made for the series of 2-aminoalkan-1-ols. Related to the alkane-1,2-diamine (1,2-ethylenediamine) derivatives (Table 3), more closely related to EMB, dialkylation of the amino group at position C-1 led to an increased anti-\textit{M. tuberculosis} activity (18b—19b < 22b), whereas the carbamoylation of the amino group at position C-2 fairly reduced the antibacterial potency (17b > 22b).

These \textit{in vitro} results showed that the aminoethers 2b and 9b (2-ethylaminopalmitol benzyl ether and 2-diethylaminopalmitol benzyl ether, respectively), the aminoalcohol derivative 5b (2-ethylaminopalmitol), and the ethylene-1,2-diamine 17b (N\textsubscript{1},N\textsubscript{1}-diethylhexadecane-1,2-diamine) were the most potent mycobactericidal compounds against the drug-susceptible strain H37Rv.

In order to obtain a further insight on the potentiality of these groups of aminoalkanols and alkanediamines against MDR strains of \textit{M. tuberculosis}, a dozen representative compounds selected within those most potent against the drug-susceptible strain were submitted to further \textit{in vitro} evaluation against the SIREP-resistant CIBIN-99 strain with the results shown in Table 4.

In fact, many compounds retained those MIC values found against the susceptible strain and, consequently, with a fair enhancement of their \(P_E\) relative potency indexes. As expected, due to the high resistance of the CIBIN-99 strain against the reference drug EMB, all the compounds assayed on this strain resulted significantly more potent than EMB. The aminoalkanol derivatives 9b, 5b and 2b displayed anti-\textit{M. tuberculosis} activity (18b—19b < 22b), whereas the carbamoylation of the amino group at position C-2 fairly reduced the antibacterial potency (17b > 22b).

\begin{table}[h]
\centering
\caption{MIC values (mmol) for selected aminoalkanol and diamine derivatives against the CIBIN-99 MDR-strain of \textit{M. tuberculosis}}
\begin{tabular}{lll}
\hline
\textbf{Comp. no.} & \textbf{CIBIN-99} & \textbf{PE}\textsuperscript{a} \\
\hline
1b & 49.0 & > 3.2 \\
2b & 3.60 & > 44 \\
3b & 84.2 & > 1.8 \\
5b & 3.12 & > 50 \\
7b & 39.8 & > 3.9 \\
8b & 16.6 & > 9.5 \\
9b & 3.10 & > 51 \\
16a & 12.6 & > 12 \\
17b & 7.80 & > 20 \\
18b & 30.9 & > 5.1 \\
19b & 32.0 & > 4.9 \\
22b & 16.3 & > 9.7 \\
EMB & > 158 & 1.0 \\
\hline
\end{tabular}
\textsuperscript{a}Anti-\textit{MtB} potency relative to ethambutol. \(P_E = \text{MIC}_{\text{EMB}}/\text{MIC}_{\text{COMP.}}\)
\end{table}

\textbf{Figure 1.} Effect of compounds 2b, 5b, 9b and 17b on bacterial loads in lungs from mice infected with the \textit{M. tuberculosis} drug-susceptible H37Rv strain. Five mg per kg of the indicated compound was administrated intragastrically for 60 days (white bars) starting at 60 days after infection. All compounds decreased bacterial loads when compared with control mice (black bars). Asterisks represent statistical significance (*\(p < 0.05\)).
$P_E$ values $>40$ and the diamine 17b a value $>20$ (Table 4). Thus, these four compounds were studied in vivo.

**Therapeutic Effects on Mice**

In comparison with non-treated control mice, animals infected with H37Rv and treated only with each of the selected compound showed a significant decrease of live bacilli in the lungs after 2 months of treatment: 60% less in animals treated with compound 5b or 17b, 70% lower in mice that received compound 9b, and 96% fewer bacilli loads in animals treated with compound 2b (Figure 1).

In the study of a possible additive effect between these dihydrosphingosine analogues and conventional chemotherapy, mice treated with compounds 2b or 5b and RIP showed significantly lower bacilli burdens than those treated with RIP chemotherapy alone, whereas animals treated with compounds 9b or 17b did not show this effect (Figure 2).

In animals infected with CIB-99, the clinical isolate resistant to all first-line anti-TB drugs, mice treated with compounds 2b, 5b or 17b showed significantly lower lung bacillary loads than the control non-treated mice (Figure 3).

**Toxicity**

Because the in vitro and the in vivo anti-TB assays showed that the best compound against H37Rv strain was 2b, its acute toxicity was tested in female and male mice (Table 5). LD$_{50}$ values were $>2000$ mg/kg for both genders, without any significant weight changes and histological alterations in the examined organs from treated animals.

**Discussion**

As shown in Tables 1 and 3, the aminoalcohol derivatives 2b, 5b and 9b and the diamine 17b showed high anti-mycobacterial in-vitro activity, with similar MIC values against the susceptible and MDR strains. Moreover,
mice infected with drug-susceptible strain H37Rv and treated with either analogue showed a significant decrease of pulmonary bacilli loads in comparison to control non-treated animals, compound 2b being the most efficient. In fact, treatment with this compound alone produced a similar CFU reduction as the treatment with conventional chemotherapy. Similarly, in animals infected with the MDR strain the treatment for 2 months with compounds 2b, 5b or 17b significantly decreased pulmonary bacilli loads when compared with the control animals.

These dihydrosphingosine analogues are structurally related to EMB, an ethylenediamine derivative discovered in 1961 (15) that affects the cell wall biosynthesis by specifically targeting the polymerization of arabinogalactan and lipoarabinomannan (16). The fact that our dihydrosphingosine-EMB analogues efficiently eliminate MDR strains resistant to EMB suggest that another mechanism, which should be established, would be involved in the bacilli killing.

SQ 109 is another very efficient ethylene-1,2-diamine EMB analogue, which is currently included in clinical trials. This compound was discovered after an extensive combinatorial study on synthetic ethylenediamines (17) and was active against EMB-resistant M. tuberculosis strains; therefore, as probably happens with our compounds, it should act in a different manner than EMB (18). The MIC of SQ109 reported in the literature ranged from 0.63 to 1.56 mmol against the H37Rv drug-susceptible strain and

Table 5. Toxicity results from female (fm)/male (mm) BALB/c mice receiving compound 2b

<table>
<thead>
<tr>
<th>Test sequence</th>
<th>Animal ID</th>
<th>Dose (mg/kg)</th>
<th>Short-term results</th>
<th>Long-term results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>fm1/mm1</td>
<td>175</td>
<td>O/O</td>
<td>O/O</td>
</tr>
<tr>
<td>2</td>
<td>fm2/mm2</td>
<td>550</td>
<td>O/O</td>
<td>O/O</td>
</tr>
<tr>
<td>3</td>
<td>fm3/mm3</td>
<td>2000</td>
<td>O/O</td>
<td>O/O</td>
</tr>
<tr>
<td>4</td>
<td>fm4/mm4</td>
<td>2000</td>
<td>O/O</td>
<td>O/O</td>
</tr>
<tr>
<td>5</td>
<td>fm5/mm5</td>
<td>2000</td>
<td>O/O</td>
<td>O/O</td>
</tr>
</tbody>
</table>

Summary of long-term (fm + mm) results

<table>
<thead>
<tr>
<th>Dose</th>
<th>O</th>
<th>X</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>175</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>550</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>2000</td>
<td>6</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>All doses</td>
<td>10</td>
<td>0</td>
<td>10</td>
</tr>
</tbody>
</table>

(X = Died, O = Survived). Statistical estimate based on long-term outcomes: LD50 is >2000 mg/kg.
from 0.7–1.4 mmol against single-drug (EMB, INH or RIF) resistant (18).

A detailed comparison of the anti-\textit{M. tuberculosis} activity between SQ109 and our compounds will be not consid-
ered here because the efficacy assays were performed on
animals infected through different routes, treated after
different periods of time and only compound 17b contains
the ethylenediamine fragment present in SQ109 and
EMB. Overall, our dihydrosisphingosine analogues resulted in
2.8–3.2 times more potent than EMB against the
drug-susceptible strain, and although they showed lesser anti-
ymycobacterial potency than SQ109 against \textit{H37Rv} strain,
they have displayed more interesting \textit{in vitro} and \textit{in vivo}
results against the MDR strain (7).

It was demonstrated that SQ109 had synergistic activity
with INH and RIF (19). In a chronic TB mouse model,
SQ109 at doses of 10 mg/kg and 25 mg/kg was able to
significantly reduce the number of bacilli loads in lung and
spleen after 30 days of treatment, and studies in the
same \textit{in vivo} model showed that substitution of SQ109
(10 mg/kg) for ethambutol (100 mg/kg) improved efficacy
of first-line drug therapy combination after 4 or 8 weeks
of treatment (20). Although it is not strictly comparable
because our murine model is different, we obtained similar
results with compounds 5b and 2b but with a lower dose. In
this regard it is also important to consider that our toxic-
ology study showed that compound 2b is almost innoc-
uous, with a LD50 of >2000 mg/kg. Thus, it would be
possible that a better additive or synergistic effect could
be obtained with conventional chemotherapy using a higher
dose of these compounds. Moreover, MDR infected mice
-treated with the same low dose of compounds 2b, 5b or
17b for 2 months also showed a significant decrease of pul-
monary bacilli loads. Perhaps higher doses of either of
these compounds could produce a better bacterial elimina-
tion as well as their combinations with new efficient anti-
TB drugs as was demonstrated in a study of the combined
effect of SQ109 and bedaquiline (20).

In conclusion, dihydrosisphingosine analogues showed a
significant mycobactericidal effect \textit{in vitro} and good anti-
TB efficacy \textit{in vivo} against drug- susceptible and highly
MDR mycobacterial strains. Although currently there are
several new drugs in clinical trials, these types of studies
looking for new classes of anti-TB compounds should
continue with the aim to discover new drugs in a constant
effort to fight this ancient and significant re-emerging infec-
tious disease.

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SQ109, a new ethylene diamine, with front-line antitubercular drugs

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