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***Mycobacterium tuberculosis* is resistant to streptolydigin**

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Summary

Drug resistant strains of *Mycobacterium tuberculosis* (*Mtb*) undermine tuberculosis (TB) control. Streptolydigin is a broadly effective antibiotic which inhibits RNA polymerase, similarly to rifampicin, a key drug in current TB chemotherapeutic regimens. Due to a vastly improved chemical synthesis streptolydigin and derivatives are being promoted as putative TB drugs. The Microplate Alamar Blue Assay revealed that *Streptococcus salivarius* and *Mycobacterium smegmatis* were susceptible to streptolydigin with minimum inhibitory concentrations (MIC) of 1.6 mg/L and 6.25 mg/L, respectively. By contrast, the MICs of streptolydigin and two derivatives, streptolydiginone and dihydrostreptolydigin, against *Mtb* were 100 mg/L demonstrating that *Mtb* is resistant to streptolydigin in contrast to previous reports. Further, a porin mutant of *M. smegmatis* is resistant to streptolydigin indicating that porins mediate uptake of streptolydigin across the outer membrane. Since the RNA polymerase is a validated drug target in *Mtb* and porins are required for susceptibility of *M. smegmatis*, the absence of MspA-like porins probably contributes to the resistance of *Mtb* to streptolydigin. This study shows that streptolydigin is not a suitable drug in TB treatment regimens.

Keywords

drug resistance; RNA polymerase; inhibitor; porins; permeability

Introduction

Mycobacterium tuberculosis (*Mtb*), the causative agent of tuberculosis in humans, kills approximately 1.5 million people each year¹. Infections with drug-resistant strains are increasing world-wide¹. It is, therefore, critical to identify new compounds or new variants of old drugs with activity against drug-resistant strains. Streptolydigin, a broadly effective antibiotic naturally produced by *Streptomyces lydicus*, inhibits RNA polymerase (RNAP) during the nucleotide addition step^{2,3}. Rifampicin is a key first-line drug against *Mtb*, used for almost 50 years in TB chemotherapy; however, resistance to this drug is increasing. Both drugs inhibit RNA polymerase, but bind to different sites and have different mechanisms of action^{4–6}. Rifampicin-resistant bacteria are often susceptible to streptolydigin⁷, which has, therefore, been proposed as an alternative to rifampicin in treating resistant strains of

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Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Mtb^{8, 9}. An additional advantage of streptolydigin is its low toxicity, with an LD50 of 533 mg/kg in rats². To facilitate the development of streptolydigin as an antibiotic in tuberculosis chemotherapy, Pronin and co-workers synthesized streptolydigin using a new strategy with fewer steps (15 compared to 24 in the older method)^{8, 9}. Several derivatives of streptolydigin were also synthesized, with varying activities *in vitro* against *Thermus aquaticus* (*Taq*) RNAP, a well-characterized representative of bacterial RNA polymerases⁹.

Previously, streptolydigin was shown to have a minimal inhibitory concentration (MIC) of 15 and 25 mg/L against *Mtb* and *M. smegmatis* in liquid cultures using turbidity measurements². However, turbidity measurements to determine drug efficacies against *Mtb* are notoriously unreliable¹⁰. The microplate Alamar Blue assay (MABA) is now the standard method of susceptibility measurements in the tuberculosis field and is also utilized in whole cell-based high throughput screens to identify growth inhibitors of *Mtb*^{11, 12}. The Alamar Blue reagent is used as an indicator of cellular growth and viability and has been extensively validated for *Mtb* by comparison to the well-established BACTEC 460 system¹³. Therefore, we used the MABA to determine the MIC of streptolydigin and its derivatives. Our aim was to verify the activity of streptolydigin against *Mtb* using state of the art methods and determine whether the newly synthesized derivatives had increased potency. We determined the *in vivo* efficacy of synthesized streptolydigin and the derivatives streptolydiginone and dihydrostreptolydigin against *Mycobacterium smegmatis* and *Mtb*, using natural streptolydigin as a control.

Materials and Methods

Cell growth experiments and MABA were performed as previously described^{14, 15}. *M. smegmatis* cultures were grown to mid-log phase in standard liquid medium (Middlebrook 7H9 (Difco) with 0.2% glycerol) containing 0.02% tyloxapol to promote dispersed growth. *Mtb* cultures were supplemented with 10% (vol./vol.) OADC (oleic acid, albumin, dextrose and catalase) (BD). *Streptococcus salivarius* was cultured in Bacto Tryptic Soy Broth (BD). Cells were transferred to a microplate containing twofold serial dilutions of the compound to be tested. After appropriate drug exposure times (*S. salivarius*, 4 hours; *M. smegmatis*, 16 hours; *Mtb*, 5 days), Alamar Blue (Invitrogen) was added according to manufacturer instructions and allowed to react until a visible color change was observed compared to negative controls. Dye conversion was measured fluorometrically (excitation 530nm, emission 590nm) using a plate reader (Biotek Synergy HT). Streptolydigin is most stable in ethanol out of the solvents which yielded the highest activity². The concentration of ethanol alone which does not inhibit mycobacterial growth was determined to be 1.25% by MABA. Hence, we used a 1% final concentration of ethanol to determine the MIC of streptolydigin. The maximum concentration of streptolydigin or its derivatives tested in this study was 200 mg/L.

Results and Discussion

Validation of activity of streptolydigin and its derivatives against model bacteria

To assess the activity of the natural streptolydigin sample, we tested the efficacy against *Streptococcus salivarius* strain MN8248 (from Dr. Moon Nahm) and found a MIC of 1.6 mg/L, which is within the range of activities reported for other *Streptococcus* species². We determined the MIC of both natural and synthetic streptolydigin against *M. smegmatis* to be 6.25 mg/L, about four-fold lower than previously reported (Table 1, Figure 1)².

Although streptolydiginone and dihydrostreptolydigin had lower activities against *Taq* RNAP compared to streptolydigin⁹, structural and chemical changes may have improved permeability across the outer membrane, a key resistance determinant in mycobacteria¹⁶.

The partition coefficients of compounds correlate with hydrophobicity and indicated that dihydrostreptolydigin may be able to cross membranes more efficiently than streptolydigin (Table 1). Further, streptolydiginone and dihydrostreptolydigin had similar efficacies against *S. salivarius*, but different *in vitro* activities against RNAP, suggesting there may be secondary targets⁹. Using the MABA we determined the MICs of streptolydiginone and dihydrostreptolydigin for *M. smegmatis* to be 100 mg/L and 50 mg/L, respectively (Table 1) demonstrating that these modifications rendered streptolydigin less effective against *M. smegmatis*.

Activity of streptolydigin and its derivatives against *M. tuberculosis*

We used the MABA to determine the MIC of natural streptolydigin against *Mtb* grown in standard medium to be 200 mg/L (Table 1). The same MIC was obtained using the Nitrate Reductase Assay (NRA)¹⁷ (data not shown). Because the albumin in the OADC supplement is capable of binding and sequestering a wide variety of compounds including antibiotics¹⁸, we repeated the MABA using the minimal, albumin-free Sauton's Medium¹⁹. However, even in the absence of albumin, the MIC of streptolydigin for *Mtb* was 200 mg/L, nearly 14-fold higher compared to previous results². It should be noted that streptolydigin in medium alone did not show any autofluorescence which might interfere with the MABA. Since mycobacteria are known to form clumps in liquid media even in the presence of detergents, rendering turbidity measurements inaccurate¹⁰, we believe the discrepancy between our results and those previously published have been caused by unreliable turbidometric measurements in the earlier experiments². Additionally, we determined the MIC of synthetic streptolydigin, streptolydiginone and dihydrostreptolydigin against *Mtb* to be 100 mg/L, 200 mg/L and greater than 200 mg/L, respectively (Table 1). These measurements showed that the modifications of these two derivatives did not improve the efficacy of streptolydigin against *Mtb*, similar to the results obtained for *M. smegmatis*. The NIH Tuberculosis Antimicrobial Acquisition and Coordinating Facility (TAACF) defined primary hits as having a MIC of less than 10 mg/L¹¹. The MICs of streptolydigin and its derivatives for *Mtb* are well above this limit indicating that these compounds would not be considered as viable leads for TB drugs.

Role of porins in mycobacterial susceptibility to streptolydigin

We wondered whether the low permeability of the outer membrane and its low content of porins¹⁶ might contribute to the resistance of *Mtb* to streptolydigin compared to *M. smegmatis*. It has been shown that general porins mediate antibiotic uptake in *M. smegmatis*^{14, 20}, but no general porins are known in *Mtb*²¹. A molecular model of the major *M. smegmatis* porin MspA indicates that streptolydigin is small enough to pass through the constriction zone of MspA. To examine whether the absence of MspA-like porins is correlated with resistance to streptolydigin, we tested the activity of streptolydigin against an *M. smegmatis* triple porin mutant, lacking the general porins MspA, MspC and MspD (ML16, $\Delta mspA \Delta mspC \Delta mspD$). As a control, we determined the MIC for ampicillin against the porin mutant ML16, which has previously been shown to be more resistant to ampicillin than wild-type *M. smegmatis*¹⁴. In preliminary dose-response curves, we determined the activity of ampicillin against mycobacteria and *S. salivarius*. The MICs of ampicillin were 0.06 mg/L, 62.5 mg/L and 160 mg/L for *S. salivarius*, *M. smegmatis* and *Mtb*, respectively (Table 1). These results are consistent with previous measurements^{14, 22}. The MICs for streptolydigin and ampicillin against ML16 were both greater than 200 mg/L (Table 1). Expression of *mspA* in *Mtb* did not increase streptolydigin susceptibility (data not shown), likely due to very low MspA levels in *Mtb*²³. We conclude that porins are required for susceptibility of *M. smegmatis* to streptolydigin, probably by mediating its uptake across the outer membrane.

Conclusions

In this study, we showed that *Mtb* is resistant to streptolydigin, while *M. smegmatis* is susceptible. The porin MspA and its paralogs are a major susceptibility factor, promoting streptolydigin uptake in *M. smegmatis*. Hence, the absence of MspA-like porins in *Mtb* may contribute to its resistance to streptolydigin. However, reduced uptake across the outer membrane is only efficient in mediating drug resistance in combination with other mechanisms such as drug efflux²⁴. It is unknown whether streptolydigin is a substrate of drug efflux pumps in *Mtb*. In conclusion, these results show that neither streptolydigin nor its derivatives are useful antibiotics to treat tuberculosis.

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Abbreviations

wt	wild-type
MABA	microplate alamar blue assay
MIC	minimum inhibitory concentration

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Figure 1.
Structures of streptolydigin and the derivatives dihydrostreptolydigin and streptolydiginone.

TABLE 1

Efficacies of antibiotics against mycobacteria and *S. salivarius* (MIC₉₀, in mg/L)

Drug	MW (g/mol)	Partition coefficient	<i>M. tuberculosis</i>	<i>M. smegmatis</i>	<i>M. smegmatis</i>	<i>S. salivarius</i>
			H37Rv wt	SMR5 wt	ML16 Δ mspACD	MN8248 wt
Streptolydigin natural	600.7	1.41	200	6.25	>200	1.6
Streptolydigin synthetic	600.7	1.41	100	6.25	n.d.	n.d.
Streptolydiginone	486.6	0.99	200	100	n.d.	n.d.
Dihydrostreptolydigin	602.7	1.7	>200	50	n.d.	n.d.
Ampicillin	349.4	0.43	160	62.5	>200	0.06

Partition coefficients were calculated using the XlogP2 server ²⁵. MW: molecular weight; n.d.: not determined