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ScienceDirect
Journal of Biotechnology 128 (2007) 726–734
Journal of BIOTECHNOLOGY
www.elsevier.com/locate/jbiotec

Inositol-1-phosphate synthetase mRNA as a new target for antisense inhibition of *Mycobacterium tuberculosis*

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Received 29 July 2006; received in revised form 5 December 2006; accepted 15 December 2006

Abstract

The need for novel antimicrobial agents to combat the emergence of multi-drug-resistant strains of *Mycobacterium tuberculosis* is a worldwide urgency. This study has investigated the effects on phosphorothioate-modified antisense oligodeoxyribonucleotides (PS-ODNs) against the mRNA of inositol-1-phosphate synthase, the key enzyme in the first step in inositol synthesis. Inositol is utilized by *M. tuberculosis* in the production of its major thiol, which is an antioxidant that helps *M. tuberculosis* to get rid of reactive oxygen species and electrophilic toxins. Real-time RT-PCR analysis revealed that mRNA expression of inositol-1-phosphate (I-1-P) synthase was significantly reduced upon addition of 20 μM PS-ODNs. Treatment with antisense PS-ODNs also reduced the level of mycothiol and the proliferation of *M. tuberculosis* and enhanced susceptibility to antibiotics. The experiments indicated that the antisense PS-ODNs could enter the cytoplasm of *M. tuberculosis* and inhibit the expression of I-1-P synthase. This study demonstrates that the *M. tuberculosis* I-1-P synthase is a target for the development of novel antibiotics and PS-ODN to I-1-P synthase is a promising antimycobaterial candidate.

Keywords: *Mycobacterium tuberculosis*; Antisense oligonucleotides; Inositol; Drug resistance

Abbreviations: mBBr, monobromobimane; ODN, oligonucleotides; PS-ODNs, phosphorothioate-modified oligonucleotides; HPLC, high performance liquid chromatography; TB, tuberculosis; ino1, inositol-1-phosphate synthetase; HIV, human immunodeficiency virus; RT-PCR, real-time PCR; cfu, colony-forming unit; MSH, mycothiol; MIC, minimal inhibitory concentration; I-1-P, inositol-1-phosphate; Ct, threshold cycle; dT, deoxythymine; HEPES, hydroxyethyl piperazine ethanesulfonic acid; Tris, tris (hydroxymethyl)-aminomethane

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0168-1656/$ – see front matter © 2007 Published by Elsevier B.V.
doi:10.1016/j.jbiotec.2006.12.019

1. Introduction

Tuberculosis (TB) is a leading infectious agent causing morbidity and mortality worldwide. The WHO estimates 8 million new cases and 2 million deaths annually (WHO, 2006). One-third of the world population is latently infected by *Mycobacterium tuberculosis*. The increasing emergence of multi-drug-resistant TB strains poses significant threat to the control of the disease in some parts of the world. The HIV coinfection weakens the immune system and could allow latent infection to reactivate or make the individual more susceptible to reinfection. In view of the drug-resistant TB problem there is currently a lot of interest to develop new TB drugs that are active against drug-resistant TB strains and also can potentially shorten the lengthy 6-month therapy (Zhang, 2005).

Inositol, found in *Streptomyces*, ice nucleating bacteria, and mycobacteria (Kozloff et al., 1991; Pittner et al., 1979), is an essential polyol in eukaryotic cells where phosphatidyl inositol plays an important role in membrane construction. Mycobacteria recruit inositol in its major thiol, mycothiol (Fahey, 2001), which is important in maintaining the redox balance in the cell, protecting the cell from oxidative stress, and in storing cysteine. Mycothiol-dependent pathway may play an important role in detoxifying the oxidative radicals produced during normal aerobic metabolism, during infection of activated macrophages, and through the action of the anti-tubercular drug isoniazid (Hayward et al., 2004). Inositol is normally synthesized from glucose-6-phosphate in two steps. The first committed step is the conversion to inositol-1-phosphate (I-1-P) by I-1-P synthase (Ino1; EC 5.5.1.4; *M. tuberculosis ino1*). I-1-P is then dephosphorylated by inositol monophosphate phosphatase to produce inositol.

Antisense oligonucleotides (ODNs) can be used specially to inhibit gene expression and have been employed as an effective therapeutic strategy (Andratschke et al., 2001) for various human pathogens, including *Plasmodium falciparum*, *Toxoplasma gondii*, and HIV (Barker et al., 1996; Lisziewicz et al., 1992). However, ODNs are not easily taken up by mycobacterial cells. The mycobacterial cell envelope prevents passage of hydrophilic drugs (Rastogi et al., 1981, 1989) into the cell but favors passage of lipophilic drugs, which are easily solubilized within the lipid portion of the outer wall layer (Rastogi et al., 1990). Fortunately phosphorothioate-modified oligonucleotides (PS-ODNs), in which all internucleoside linkages are phosphorothioates, are more easily taken up by mycobacterial cells due to improved lipophilicity (Harth et al., 2002).

In the present work, antisense PS-ODNs have been utilized to study the function of *M. tuberculosis* I-1-P synthase and to evaluate I-1-P synthase as a new drug target using antisense PS-ODNs. It was found that I-1-P synthase-specific antisense PS-ODNs can inhibit the expression of *ino1* mRNA, the production of mycothiol, the proliferation of bacteria and enhance the susceptibility to antibiotics.

2. Materials and methods

2.1. PS-ODNs design and preparation

The coding sequence of *M. tuberculosis* H37Rv *ino1* was amplified by PCR using *M. tuberculosis* H37Ra genomic DNA template with Taq DNA polymerase enzyme (Biostar). Primers for *ino1* were sense 5′-GGAGGTTCGAGTCGCCATTG-3′ and antisense 5′-GGCGGGCTCTTCATCAGGT-3′. PCR products were then sequenced and their secondary structure were analyzed by RNA structure program (Mathews et al., 2004). The fold temperature was fixed at 37 °C and the ionic condition was 1 M NaCl with no divalent ions. Three target sites were chosen from *M. tuberculosis ino1* coding sequences based on their propensities to remain in a linear conformation as determined by RNA structure program. Three subsequent antisense PS-ODNs were then designed from the corresponding sequences. All HPLC-purified PS-ODNs were synthesized and provided by Invitrogen Company.

2.2. Bacterial culture

M. tuberculosis strain H37Ra (ATCC25177) was cultured at 37 °C in 7H9 medium (Difico), supplemented with 2% glucose, 0.05% Tween 80 and ADC (albumin–dextrose–catalase) enrichment (Difico). For analyzing the inhibitory effects of the three PS-ODNs on bacterial growth, *M. tuberculosis* cultures were cultured in 5 ml of 7H9 broth in polystyrene tubes (Fisher)

in the presence of medium alone, or PS-ODNs at final concentrations of 1, 5, 10, 20 and 40 μM, respectively.

2.3. Analysis of the abundance of ino1 mRNA

Aliquots were removed at the end of the sixth week, and the abundance of *ino1* mRNA was assayed by real-time PCR. Trizol (Invitrogen) was used to isolate total RNA from *M. tuberculosis* incubated in the presence of medium alone, or PS-ODNs at final concentrations of 1, 5, 10, 20 and 40 μM. cDNA was generated from 2.5 μg samples of total RNA at 42 °C for 50 min (reaction volume, 20 μl) using oligo dT (Life Technologies) and reverse transcriptase (Superscript II; Life Technologies) according to the manufacturer's protocol.

2.4. Real-time RT-PCR

Real-time (RT)-PCR was performed using SYBR Green (Roche Germany) using an ABI Prism 7900 Sequence Detection System (Applied Biosystems). The PCR was run for 5 min at 95 °C, followed by 40 cycles of 30 s at 94 °C, 60 s at 55 °C, and 30 s at 72 °C. After amplification, a melting curve analysis was performed to verify the specificity of the reactions. The end point used in the real-time RT-PCR quantification, Ct, was defined as the PCR cycle number at which each assay target reached the threshold. The data represent the fold change in mRNA expression relative to *M. tuberculosis* incubated with medium alone, which is arbitrarily defined as 1. Primers for *ino1* were the same as above for PCR amplification of *ino1* gene.

2.5. Analysis of mycothiol levels

M. tuberculosis cultures were analyzed for the amount of mycothiol by high performance liquid chromatography (HPLC). The cell extraction procedure was slightly modified from the procedure described by Fahey (Fahey and Newton, 1987) and Anderberg (Anderberg et al., 1998). Briefly, cell pellets from 3 ml culture were resuspended in 0.5 ml warm (60 °C) 50% acetonitrile–water containing 2 μM monobromobimane (mBBr) and 20 μM Tris–HCI, pH 8.0. The cell suspensions were then incubated in the dark at 60 °C for 15 min and 2 μl concentrated 12 M HCl was added to acidify the suspensions. The suspensions were then centrifuged to collect the cell debris and the supernatant was diluted with 10 μM HCl and analyzed by HPLC analysis. Control samples were extracted with 50% aqueous acetonitrile, 5 μM *N*-ethylmaleimide and 20 μM HEPES (pH 8.0). The suspensions were incubated for 15 min at 60 °C. After addition of 2 μM mBBr, the suspensions were incubated again for 15 min at 60 °C. The suspensions were then centrifuged to collect the cell debris and the supernatant was diluted with 10 μM HCl and analyzed by HPLC analysis on a Beckmam Ultrasphere C18 ion-pair HPLC column. The thiols were eluted with 0.25% glacial acetic acid pH 3.6 (buffer A) and 100% methanol (buffer B) using the following gradient: initial conditions, 10% buffer B; at 15 min, 18% buffer B; at 30 min, 27% buffer B; at 33 min, 100% buffer B; at 36 min, 10% buffer B; at 52 min, 10% buffer B. The flow rate was 1 ml/min and the fluorescence was detected as described previously (Newton et al., 2000).

2.6. Inhibition of M. tuberculosis by PS-ODNs

The effect of PS-ODNs on the growth of the bacilli was monitored weekly for 6 weeks by gently sonicating the culture to break up bacterial clumps, removing a small aliquot, washing the bacteria by centrifugation, and plating serial dilutions of washed bacteria on 7H11 agar (Difco) plates. The number of viable bacteria, colony-forming units (cfu), was counted after incubation at 37 °C for 3 weeks.

2.7. Sensitivity assays for antibiotics and oxidative agents

To determine whether the antisense PS-ODNs treated *M. tuberculosis* was more sensitive to oxidative agents, disk assays were performed (Rawat et al., 2002). Briefly, cells were grown to the mid-log phase in the presence of different concentrations of antisense PS-ODNs, and a lawn of cells was plated onto 7H11 agar plates. Various amounts of oxidative agents were added to the disk (sterile filter paper disks, 7.5 mm in diameter) in a 10 μl volume and the disks were allowed to dry. The disks were placed onto the lawn of cells and incubated for 2–3 weeks. To determine the MICs of isoniazid, rifampin and vancomycin E-test strips (AB Biodisk) were used (Rawat et al., 2002). All assays were performed in triplicate at least three times.

2.8. Statistical methods

Comparisons were made between groups by using the *t*-test for comparing experimental groups and control groups at the $P = 0.05$ level.

3. Results

3.1. Selection of PS-ODN targets in M. tuberculosis ino1 gene

The secondary structure of *ino1* RNA was predicted by RNAstructure (Mathews et al., 2004), which is a program for the prediction and analysis of RNA secondary structure based on free energy minimization. Three favorable targets for antisense PS-ODNs were selected if the favorable structural elements were conversed among a set of minimum free energy (Patzel et al., 1999) and its secondary structure motifs were assumed to contain accessible sequences (Patzel et al., 1997). According to the target sites three antisense PS-ODNs were designed: PS-ODN1, PS-ODN2 and PS-ODN3. These three antisense PS-ODNs were complementary in sequence to nt 366–386, 544–564 and 737–757 of *ino1*, respectively. The sequences of the antisense PS-ODN are: PS-ODN1, 5′-TCGACGGGCTCCGCGTCGGAG-3′; PS-ODN2, 5′-GTCGGTGAACTTCTTGGCCCA-3′; PS-ODN3, 5′-TCTTGGAGATCTTCTTGGACT-3′. The secondary structures and energies of the three targets are shown in Fig. A in supporting information. The propensity of PS-ODN to remain linear is: PS-ODN 1 > PS-ODN 3 > PS-ODN 2. All three regions were devoid of any stable secondary structures, and thus, they are theoretically readily accessible to complementary PS-ODNs. Besides these three *ino1* specific PS-ODNs, we included two control oligonucleotides. Control 1 is mismatched at 4 nt positions with *ino1*: 5′-TCGTCGGGATCCGTGTCGCAG-3′; and Control 2 was designed to contain a pronounced secondary structure, 5′-CCGTCCGACCACGTCGGATGG-3′. All PS-ODNs were 21 bases in length.

3.2. Effect of PS-ODNs on the abundance of ino1 mRNA

M. tuberculosis was first cultured in 5 ml broth medium for 2 weeks to provide a sufficient number of bacteria for reliable measurements of the abundance of *ino1* mRNA. Different concentrations of antisense PS-ODNs (1, 5, 10, 20 and 40 μM) were then inoculated with the cultures. The cultures were assayed for *ino1* mRNA at the end of sixth week. Fig. 1 shows the fold change of *ino1* mRNA in *M. tuberculosis* in the presence of PS-ODNs relative to *M. tuberculosis* incubated with medium alone. The results revealed that among the three concentrations of PS-ODNs, 20 μM was the minimum effective concentration. At concentrations of 20 and 40 μM, all three antisense PS-ODNs exerted a substantial inhibitory effect on the *ino1* mRNA expression ($P < 0.01$). By the end of the 6-week observation period, the fold change of *ino1* mRNA in *M. tuberculosis* treated with 40 μM PS-ODN 1 relative to *M. tuberculosis* incubated with medium alone was 0.45 (±0.21; S.D.), and that of PS-ODN 2 was 0.67 (±0.17;

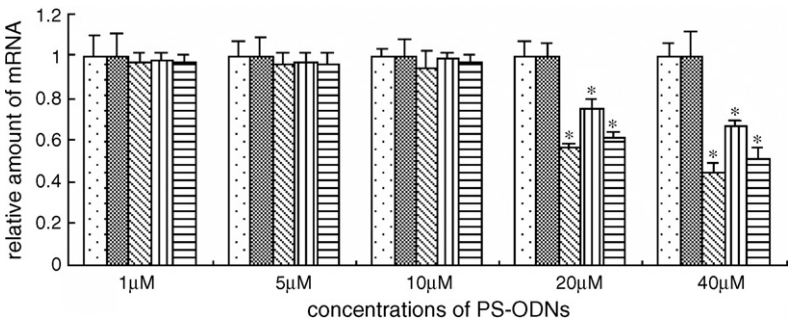

Fig. 1. Relative amounts of mRNAs in *M. tuberculosis* in the presence of PS-ODNs or controls. Each value represents the amount of mRNA relative to *M. tuberculosis* incubated with medium alone, which is designed as 1. Each experiment was performed in triplicate. $^{*}P < 0.01$. (▫) Control 1; (▩) control 2; (▨) PS-ODN1; (▥) PS-ODN2; (▤) PS-ODN-3.

S.D.). As the concentration of PS-ODN 1 increased, the inhibitory effect became more apparent. It is interesting to note that the inhibitory capacity of a PS-ODN was directly related to its propensity to remain in a linear conformation, as assessed by the RNA structure program. The inhibitory capacity of the PS-ODNs could be ranked in the following order: PS-ODN 1 > PS-ODN 3 > PS-ODN 2. At all concentrations tested, the control PS-ODNs did not affect *ino1* mRNA expression.

3.3. Effect of PS-ODNs on the amount of mycothiol

Inositol is utilized by *M. tuberculosis* in the production of its major thiol, which suggests the possibility that inhibition of enzymes involved in inositol synthesis may reduce the endogenous mycothiol. This was investigated by determining the amount of this cell wall structure in *M. tuberculosis* grown in the presence of PS-ODN 1 and control PS-ODNs at concentrations of 1, 5, 10, 20 and 40 μM. Cells were harvested weekly at the end of sixth week, and analyzed for mycothiol content by HPLC.

Mycothiol levels declined over the 6-week period in the presence of 20 and 40 μM of antisense PS-ODN 1. As the concentration of PS-ODN 1 increased, the mycothiol levels decreased. PS-ODN 1 at 1, 5 and 10 μM did not interfere with the mycothiol synthesis (Fig. 2). Control PS-ODNs failed to affect the mycothiol levels (Fig. B in supporting information).

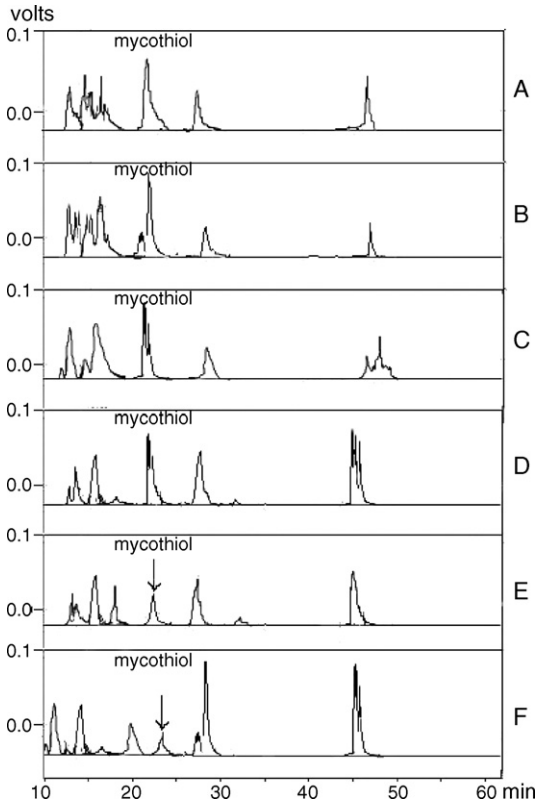

Fig. 2. Mycothiol levels of *M. tuberculosis* in the presence of antisense PS-ODN 1 determined by HPLC. Panel A: no PS-ODN 1; panel B: 1 μM of PS-ODN 1; panel C: 5 μM of PS-ODN 1; panel D: 10 μM of PS-ODN 1; panel E: 20 μM of PS-ODN 1; panel F: 40 μM of PS-ODN 1.

3.4. Inhibition of M. tuberculosis by antisense PS-ODNs

Besides evaluating the effect of PS-ODNs on mycobacterium *ino1* mRNA and the amount of mycothiol, we also determined the ability of antisense PS-ODNs to inhibit bacterial growth. PS-ODNs were added to *M. tuberculosis* cultures and cfu's were quantified weekly for 6 weeks. The results showed that 20 μM is still the minimum effective concentration (Fig. 3). Only 20 and 40 μM of PS-ODNs had the ability to inhibit the bacterial growth significantly ($P < 0.005$). At concentration of 1, 5, and 10 μM, the proliferation of *M. tuberculosis* did not change. At concentration of 20 μM, PS-ODN 1 decreased growth by 1.3 log units (±0.2 log units; S.D.), and that of 40 μM was 1.7 log units (±0.1 log units; S.D.) (Fig. 3). Under the same condition, the cfu's for controls did not decrease significantly (Fig. C in supporting information).

3.5. Effect of antisense PS-ODNs on the susceptibility of M. tuberculosis

Since the mycothiol is important for maintaining the redox balance, in protecting the cell from oxidative stress and antibiotic in *M. tuberculosis* (Rawat et al., 2002), we hypothesized that the antisense PS-ODNs might enhance the susceptibility of *M. tuberculosis* to antibiotics, and oxidative agents. Thus, we examined the sensitivity of antisense PS-ODN 1 treated *M. tuberculosis* to oxidative stress and antibiotic. As seen in Fig. 4, *M. tuberculosis* treated with 40 μM antisense PS-ODN 1 was about six times more sensitive to

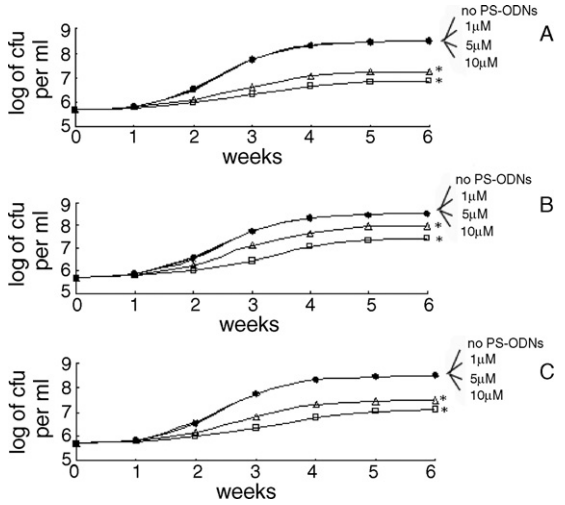

Fig. 3. Inhibition of cell proliferation of *M. tuberculosis* by antisense PS-ODNs. Panel A: antisense PS-ODN 1; panel B: antisense PS-ODN 2; panel C: antisense PS-ODN 3; (△) 20 μM of antisense PS-ODNs; (□) 40 μM of antisense PS-ODNs. $^{*}P < 0.005$, comparison with no PS-ODNs at the end of sixth week.

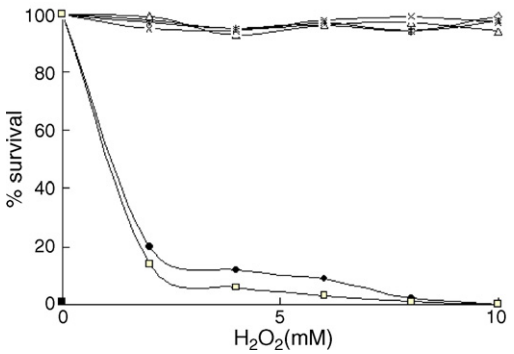

Fig. 4. Sensitivities of antisense PS-ODN 1 treated *M. tuberculosis* vs. normal *M. tuberculosis* to hydrogen peroxide. (△) No PS-ODN 1; (◇) 1 μM of PS-ODN 1; (*) 5 μM of PS-ODN 1; (×) 10 μM of PS-ODN 1; (♦) 20 μM of PS-ODN 1; (□) 40 μM of PS-ODN 1.

hydrogen peroxide than normal *M. tuberculosis*. This was proved by that *M. tuberculosis* treated with 40 μM antisense PS-ODN 1 was unable to tolerate as little as 1.5 μM H_2O_2, but the untreated *M. tuberculosis* can survive in the presence of 10 μM H_2O_2. *M. tuberculosis* treated with 20 μM antisense PS-ODN 1 was intermediate between them (Fig. 4). As the concentration of antisense PS-ODN 1 increased, *M. tuberculosis* became more sensitive. Similar results were obtained in the antibiotics sensitivity assay (Table 1). *M. tuberculosis* treated with 20 μM antisense PS-ODN 1 was three- to five-fold more sensitive than the control *M. tuberculosis* for a variety of antibiotics, including isoniazid, rifamcin and vancomycin. *M. tuberculosis* treated with 40 μM antisense PS-ODN 1 was seven- to nine-fold more sensitive than the normal *M. tuberculosis*.

4. Discussion

M. tuberculosis infection is a leading cause of death worldwide. The emergence of multi-drug-resistant TB strains has given new urgency to the need of developing new antimicrobials to shorten treatment course, improve patient compliance and provide more effective treatment. Comparison with traditional antituberculosis drugs, antisense ODNs as therapeutic strategies for *M. tuberculosis* are lagging and we propose that antisense PS-ODNs against *M. tuberculosis* I-1-P synthase could be a new strategy against *M. tuberculosis*.

Mycobacterial cell wall synthesis and assembly is the target of many antituberculosis agents. Inositol is utilized by *M. tuberculosis* in the production of its major thiol and of essential cell wall lipoglycans (Movahedzadeh et al., 2004). We tested the potential for antisense PS-ODNs to inhibit *M. tuberculosis*. RT-PCR shows that in the presence of antisense PS-ODNs, the

Table 1
Antibiotic response of *M. tuberculosis* treated with or without PS-ODN1 *in vitro*

Drugs	MIC (μg/ml)			
	No PS-ODN1	PS-ODN1 (1, 5, and 10 μM)	PS-ODN1 (20 μM)	PS-ODN1 (40 μM)
Isoniazid	2	2	0.4*	0.2*
Vancomycin	6	6	2*	0.85*
Rifampin	32	32	7.8*	3.7*

PS-ODN1 at concentrations 1, 5 and 10 μM has no effects. $^{*}P < 0.05$ comparison with no PS-ODN.

abundance of *ino1* mRNA decreased markedly and the proliferation of bacteria was inhibited. These findings suggest that the antisense PS-ODNs could enter the *M. tuberculosis* cell, bind to its cognate targets and specifically inhibit gene expression and alter the metabolism. There is a concentration threshold that PS-ODNs could show inhibitory effect on mRNA expression, below which PS-ODNs did not exert inhibitory effect. The effect of the antisense PS-ODNs on *M. tuberculosis* was highly specific. In our experiment, control 1, which is mismatched at 4 nt positions with *ino1*, had no apparent inhibition on *M. tuberculosis* growth or I-1-P synthase expression. The effect of a non-specific control was the same as that of control 1. The activity of the antisense PS-ONDs was inversely correlated with the potential for secondary structure formation. The inhibitory capacity of PS-ODNs was PS-ODN 1 > PS-ODN 3 > PS-ODN 2, which was in accordance with previously published antisense studies on *M. tuberculosis* (Harth et al., 2000). The inverse correlation could be explained by the mechanism of antisense proposed by Aboul-Fadl and Milner: the sequence-specific binding of an antisense oligonucleotide to target mRNA, resulting in the prevention of gene translation. The essential step of the antisense process is the hybridization between the antisense oligo and its target mRNA; this can be simply viewed as a two-step process of nucleation at an accessible (single-stranded, unstructured) site and elongation by a 'zippering' process. The likelihood of successful hybridization is greatly influenced by secondary structural features of the target RNA (Aboul-Fadl, 2005; Milner et al., 1997).

The antisense PS-ODNs exhibit four simultaneous effects on *M. tuberculosis*: inhibition of *ino1* mRNA expression, reduction in the formation of mycothiol, inhibition of bacterial growth, and enhancement of susceptibility to antibiotics. The direct consequence of antisense PS-ODNs is a decrease in I-1-P synthase mRNA expression, leading to a decrease in I-1-P synthase activity, which will in turn cause the decrease in inositol and also mycothiol levels. Mycothiol is an antioxidant that helps *M. tuberculosis* to cope with reactive oxygen species and electrophilic toxins (Newton et al., 1999, 2000). We showed that antisense PS-ODN-treated *M. tuberculosis* have increased sensitivities to several antibiotics and oxidative stress. Actinomycetes and especially *M. tuberculosis* have the natural ability to resist a wide range of antibiotics. Drug inactivation by mechanisms involving low-molecular-weight thiols has been reported before (Arca et al., 1990). A plasmid-encoded glutathione *S*-transferase is able to confer resistance to the antibiotic fosfomycin (Arca et al., 1990). It seems that in *M. tuberculosis* MSH serves a role analogous to that of glutathione in eubacteria and eukaryotes, which may account for the correlation between antibiotic susceptibility and mycothiol decrease in our study. In *Streptomyces* MSH appears to detoxify and protect the cell from a variety of endogenously generated antibiotics reactive intermediates, as evidenced by the isolation of mercapturic acids from fermentation broths (Aoyama et al., 1993; Newton et al., 2000). It is proposed that mycobacteria retained this capability and can similarly detoxify exogenously supplied antibiotics using MSH and MSH S-conjugate amidase (Rawat et al., 2002). The antibiotic response of PS-ODN 1 treated *M. tuberculosis* was a reduction in the MICs, which may be explained by this proposal. Moreover, the mechanism of action of antisense PS-ODNs differs substantially from conventional antibiotics. Even if this kind of response may explained as synergistic action, the *M. tuberculosis* I-1-P synthase involved in synthesis of mycothiol may be developed as a target for antimicrobial drug and also as small-drug inhibitors, which could be used to "sensitize" antibiotics to which bacteria have developed resistance.

Pharmacokinetic studies by Agrawal and his colleagues indicate that therapeutic concentrations of ODNs are achievable in blood and tissues at nontoxic doses (Agrawal and Zhang, 1997; Agrawal and Zhao, 1998); tissue concentrations remain relatively high for several days. With respect to tuberculosis, it is noteworthy that when FITC-labeled ODNs are administered i.v. to mice, the ODNs are preferentially taken up by mononuclear phagocytes (Zhao et al., 1998). Thus, our study and the pharmacologic work of Agrawal and his colleagues (Agrawal and Zhang, 1997; Agrawal and Zhao, 1998; Zhao et al., 1998) suggest the feasibility of employing modified nuclease-resistant ODNs as candidates (Rawat et al., 2002) of antituberculous drugs. Further studies are needed to determine whether antisense PS-ODNs against *M. tuberculosis* I-1-P synthase have any antituberculosis activity and exert a synergistic action with current TB drugs in animal models of tuberculosis.

Acknowledgements

This study was supported by National Natural Science Foundation of China (30170051 and 30328031). We thank Dr. Yi Ren and Dr. Zhijun Fu at Wuhan Tuberculosis Hospital for technical assistance.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jbiotec.2006.12.019.

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