รายงานวิจัยฉบับสมบูรณ์

โครงการ กลไกการออกฤทธิ์ของสารต้านเชื้อวัณโรค thiourea และอนุพันธ์ใหม่ และผลต่อการแสดงออกของยีน *desA3* ในเชื้อวัณโรค
(Thiourea and new derivatives Mode of action and effects on the expression of the *Mycobacterium tuberculosis desA3* gene)

โดย ดร. เบญจวรรณ เพชรสุขศิริ
สังกัดเดิม กรมควบคุมโรคติดต่อ กระทรวงสาธารณสุข
สังกัดปัจจุบัน สถาบันวิจัยวิทยาศาสตร์สาธารณสุข กรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข

วันที่ 30 มิถุนายน 2546

รายงานวิจัยฉบับสมบูรณ์

โครงการ กลไกการออกฤทธิ์ของสารต้านเชื้อวัณโรค thiourea และอนุพันธ์ใหม่ และผลต่อการแสดงออกของยีน *desA3* ในเชื้อวัณโรค

(Thiourea and new derivatives: Mode of action and effects on the expression of the *Mycobacterium tuberculosis desA3* gene)

โดย ดร. เมญจวรรณ เพชรสุขศิริ

สังกัดเดิม กรมควบคุมโรคติดต่อ กระทรวงสาธารณสุข

สังกัดปัจจุบัน สถาบันวิจัยวิทยาศาสตร์สาธารณสุข กรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข

วันที่ 30 มิถุนายน 2546

รายงานวิจัยฉบับสมบูรณ์

โครงการ กลไกการออกฤทธิ์ของสารต้านเชื้อวัณโรค thiourea และอนุพันธ์ใหม่ และผลต่อการแสดงออกของยีน *desA3* ในเชื้อวัณโรค

(Thiourea and new derivatives: Mode of action and effects on the expression of the *Mycobacterium tuberculosis desA3* gene)

ผู้วิจัย พ.ต. เมธาวรรณ เพชรสุขดิรี

สังกัดเดิม กรมควบคุมโรคติดต่อ กระทรวงสาธารณสุข

สังกัดปัจจุบัน สถาบันวิจัยวิทยาศาสตร์สาธารณสุข กรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข

สนับสนุนโดย สำนักงานกองทุนสนับสนุนการวิจัยและกรมควบคุมโรคติดต่อ

(ความเห็นในรายงานนี้เป็นของผู้วิจัย ไม่จำเป็นต้องเห็นด้วยเสมอไป)

กิตติกรรมประกาศ

การศึกษาวิจัยนี้ผู้วิจัยและคณะ ขอขอบคุณผู้สนับสนุนงานวิจัย ดังต่อไปนี้

1. สำนักงานกองทุนสนับสนุนการวิจัย สนับสนุนทุนดำเนินงานวิจัย
2. กรมควบคุมโรคติดต่อ กระทรวงสาธารณสุข หน่วยงานต้นสังกัดเดิม
   สนับสนุนการปฏิบัติงานและทุนสมทบการดำเนินงานวิจัย
3. สถาบันวิจัยวิทยาศาสตร์สาธารณสุข กรมวิทยาศาสตร์การแพทย์
   หน่วยงานต้นสังกัดปัจจุบัน สนับสนุนการปฏิบัติงาน
4. รศ.นพ ประสิทธิ์ ผลิตผลการพิมพ์
   ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล
   อาจารย์ที่ปรึกษา
5. Professor, Dr. Patrick J. Brennan,
   Department of Microbiology, Immunology and Pathology,
   Colorado State University, USA
   ที่ปรึกษาโครงการวิจัย
6. นายแพทย์ จรูญ ปิยะวราภรณ์
   ผู้ทรงคุณวุฒิ กรมควบคุมโรคติดต่อ กระทรวงสาธารณสุข
   สนับสนุนการปฏิบัติงาน
7. นายแพทย์ ปฐม สวรรค์ปิญญานิค
   ผู้อำนวยการสถาบันวิจัยวิทยาศาสตร์สาธารณสุข
   ที่ปรึกษาโครงการ
8. รศ.ดร อังคณา ฉายประเสริฐ
   ภาควิชาชีววิทยา คณะแพทยศาสตร์ศิริราชพยาบาล
   สนับสนุนวัสดุวิจัย (เชื้อวัณโรค และเชื้อมัยโคแบคทีเรียต่างๆ)
9. นางสาวจนิพรา อุดิลเนกรินทร์
   ฝ่ายโรคเรื้อนและมัยโคแบคทีเรียอื่น สถาบันวิจัยวิทยาศาสตร์สาธารณสุข
   ช่วยเหลือเทคนิค
10. นางสาวไสยา ศรีสังข์งาม
    ฝ่ายโรคเรื้อนและมัยโคแบคทีเรียอื่น สถาบันวิจัยวิทยาศาสตร์สาธารณสุข
    ช่วยเหลือเทคนิค
11. นางสาวมิ่งขวัญ วรศันสิ
    ฝ่ายโรคเรื้อนและมัยโคแบคทีเรียอื่น สถาบันวิจัยวิทยาศาสตร์สาธารณสุข
    ช่วยเหลือเตรียมต้นฉบับ

12. นางศิริรัตน์ ลิกานนท์สกุล
กลุ่มงานอิมมูโนวิทยา สถาบันบำราศนราดูร
อนุเคราะห์เครื่อง LightCycler PCR
13. ดร. สุภาพรรณ ธีรชนลักษณ์
ภาควิชาทรัพยากรและเทคโนโลยี สถาบันเทคโนโลยีพระจอมเกล้าธนบุรี
อนุเคราะห์สารมาตรฐานกรดไขมัน
14. เจ้าหน้าที่ นักศึกษาห้องปฏิบัติการ เชื้อมัยโคแบคทีเรีย
ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล
อำนวยความสะดวกเครื่องมือ สถานที่ในการปฏิบัติงาน

ในนามของผู้รับผิดชอบโครงการวิจัยขอขอบพระคุณผู้มีส่วนสนับสนุนงานวิจัยนี้เป็นอย่างยิ่ง

ดร. เบญจวรรณ เพชรสุขศิริ

Project Code	PDF/ 60/ 2544
Project Title	Thiourea and new derivatives: Mode of action and effects on the expression of the *Mycobacterium tuberculosis* desA3 gene
Investigation	Dr. Benjawan Phetsuksiri Department of Communicable Disease Control Ministry of Public Health Current Address Thai National Institute of Health Department of Medical Sciences Ministry of Public Health, Thailand.
E-mail Address	benjapsk@health.moph.go.th
Project Period	1 July, 2544 - 30 June, 2546

PDF/60/2544. Thiourea and new derivatives: Mode of action and effects on the expression of the *Mycobacterium tuberculosis desA3* gene.

Benjawan Phetsuksiri. Department of Communicable Disease Control, Ministry of Public Health. Current Address: Thai National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Thailand. benjapsk@health.moph.go.th

1 July, 2544 - 30 June, 2546

ABSTRACT

Isoxyl, a thiourea (4, 4' diisoamyloxydiphenylthiourea) and new synthetic derivatives were investigated in vitro for their antimycobacterial activity against *Mycobacterium tuberculosis*. By employing the rapid microplate alamar blue assay, isoxyl was shown to be highly effective against Thai clinical isolate of *Mycobacterium tuberculosis* with MIC of 0.30-1.25 µg/ml. Four new derivatives also exhibited potent activity against *M. tuberculosis* with MIC in the range of 0.30-10.0 µg/ml. The selective mode of action of thiourea was demonstrated by whole cell labeling of *M. tuberculosis* with (1, 2 -C^{14}) acetate. Analysis of labeled fatty acids by thin layer chromatography (TLC) demonstrated the inhibitory effects on the synthesis of oleic acid, indicating a unique mechanism of action. The specificity of the inhibition on oleic acid synthesis pointed to a $\Delta 9$ stearoyl-desaturase as a drug target. The transcripts of the *M. tuberculosis desA3*, which encodes $\Delta 9$ stearoyl-desaturase, were detected by reverse transcription polymerase chain reaction (RT-PCR) demonstrating the expression and importance of enzymatic function. A new LightCycler real-time RT-PCR was developed for quantitative detection of mRNA of *M. tuberculosis desA3*. *M. tuberculosis* RNA could be extracted by the use of a modified commercially ready-to use guanidine-phenol extraction method. The single tube reaction of real-time RT-PCR was performed in glass capillary containing the SYBR Green I dye as a detection signal. The amplification of 341 nucleotide region of the *desA3* gene specific for *M. tuberculosis* by real-time RT-PCR demonstrated that *desA3* transcripts were diminished in cells exposed to thiourea. These results confirmed that thiourea is a novel antituberculosis agent, which had specific mechanism by inhibiting the synthesis of oleic acid and affected the expression of *desA3* in *M. tuberculosis*. We propose here that thioureas serve as a promising compound for future antituberculosis drug development and DesA3 is a new therapeutic target worthy for further study.

Keyword : Thiourea, oleic acid, tuberculosis, *desA3*

Project Code	PDF/ 60/ 2544
Project Title	Thioureа and new derivatives: Mode of action and effects on the expression of the *Mycobacterium tuberculosis desA3 gene*
Investigation	Dr. Benjawan Phetsuksiri Department of Communicable Disease Control Ministry of Public Health Current Address Thai National Institute of Health Department of Medical Sciences Ministry of Public Health, Thailand.
E-mail Address	benjapsk@health.moph.go.th
Project Period	1 July, 2544 - 30 June, 2546

PDF/60/2544 กลไกการออกฤทธิ์ของสารต้านเชื้อวัณโรค thiourea และอนุพันธ์ใหม่ และผลต่อการแสดงออกของยีน *desA3* ในเชื้อวัณโรค

เบญจวรรณ เพชรสุขศิริ กรมควบคุมโรคติดต่อ กระทรวงสาธารณสุข สังกัดปัจจุบัน สถาบันวิจัยวิทยาศาสตร์สาธารณสุข กรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข
benjapsk@health.moph.go.th 1 กรกฎาคม 2544 - 30 มิถุนายน 2546

บทคัดย่อ

Isoxyl (4, 4 diisoamyloxydiphenylthiourea) และสารประกอบอนุพันธุ์ใหม่ของ thiourea ที่ได้จากการสังเคราะห์ มีฤทธิ์ต้านเชื้อวัณโรคโดยการทดสอบที่ให้ผลเร็วด้วย microplate alamar blue assay พบว่า isoxyl สามารถทำลายเชื้อวัณโรคที่แยกได้จากผู้ป่วยด้วยความเข้มข้นต่ำสุด 0.30-1.25 µg/ml และสารอนุพันธุ์ของ thiourea มีฤทธิ์ต้านเชื้อวัณโรคด้วยค่าความเข้มข้นต่ำสุด 0.30-10.0 µg/ml การศึกษากลไกการออกฤทธิ์ที่จำเพาะของสาร thiourea ทำโดยการติดฉลากสารเมตาโบไลท์ในขบวนการสังเคราะห์กรดไขมันในเชื้อวัณโรคด้วย (1, 2 C^{14})acetate และวิเคราะห์กรดไขมันที่ถูกติดฉลากด้วย thin layer chromatography สาร thiourea และอนุพันธ์ใหม่มีฤทธิ์ยับยั้งการสังเคราะห์ oleic acid ปกติว่าเอนไซม์ Δ9 stearoyl-desaturase ซึ่งสังเคราะห์ oleic acid เป็นเป้าหมายที่สารออกฤทธิ์ การตรวจพบ transcript ของยีน *desA3* ที่ถอดรหัสให้เอนไซม์ Δ9 stearoyl-desaturase แสดงความสำคัญของหน้าที่เอนไซม์โดยยีนมีการแสดงออก ผลของสาร thiourea ต่อการแสดงออกของยีน *desA3* ศึกษาโดยการสกัด RNA ด้วยน้ำยา Trizol วิเคราะห์หาปริมาณของ *desA3* transcript ด้วย real-time reverse transcription PCR แบบปฏิกิริยาเดียวโดยใช้ primer ที่จำเพาะต่อเชื้อวัณโรค ตรวจพบผลผลิตปฏิกิริยาที่ใช้สาร SYBR green I ติดฉลากขนาด 341 base pair ของยีน *desA3* สาร thiourea มีผลทำให้ transcript ของยีน *desA3* ในเชื้อวัณโรคลดลง ผลการศึกษานี้ แสดงให้เห็นว่า สาร thiourea มีฤทธิ์ต้านเชื้อวัณโรคโดยมีกลไกการออกฤทธิ์ยับยั้งการสังเคราะห์ oleic acid ที่เฉพาะ สารมีผลทำให้การแสดงออกของยีน *desA3* ลดลง และเอนไซม์ DesA3 ในเชื้อวัณโรคมีคุณสมบัติเป็นเป้าหมายของยาต้านวัณโรค

คำหลัก : สาร thiourea, กรดไขมัน oleic, วัณโรค, ยีน *desA3*

INTRODUCTION

Identifying and understanding the functions of specific genes is a fundamental and essential step to find and validate new targets for drug design and development. Elucidation of such genes can be approached by using molecular genetics, biochemical analysis and enzyme inhibitors that affect the function of gene products. Such compounds or drugs may selectively inhibit specific targeted enzymes and thereby cause an accumulation of precursor(s) and depletion of product(s) leading to an identification of the mode of action of drugs and the specific gene that encodes the targeted enzyme.

A thiourea, Isoxyl (thiocarlide; 4, 4' diisoamyloxy diphenylthiourea), is known to be an effective anti-tuberculosis drug, active against a range of multidrug-resistant strains of *Mycobacterium tuberculosis*, and has been used clinically (Titscher, 1966; Urbancik, 1966; Urbancik, 1970). Little was known of its mode of action. Recently, it has been shown that exposure of *M. tuberculosis* to an inhibitory level of ISO caused an accumulation of stearic acid concomitant with the depletion of oleic acid. Synthesis of mycolic acid is also affected. The anti-bacterial effect of ISO was reversed by supplementing growth medium with oleic acid. The specificity of oleic acid inhibition pointed to a $\Delta 9$ stearoyl-desaturase, an enzyme that introduces one double bond at carbon 9 of stearic acid to form oleic acid as the drug target (Phetsuksiri, et al., 2003). Development of a cell-free assay for $\Delta 9$ desaturase activity allowed direct demonstration of the inhibition of oleic acid synthesis by ISO. The three putative fatty acid desaturases in the *M. tuberculosis* genome, *desA1*, *desA2*, and *desA3*, were cloned and expressed in *Mycobacterium bovis* BCG. Whole cell labeling demonstrated increased synthesis of oleic acid only in the *desA3* overexpressing strain and an increase in the minimal inhibitory concentration for ISO (Phetsuksiri, et al., 2003). The results validated $\Delta 9$ desaturase, DesA3, as a target of thiourea. In this study, antimycobacterial activities of thioures and new derivatives against clinical isolate of *M. tuberculosis* were evaluated and the effects of thioureas on the synthesis of oleic acid and on the expression of the *desA3* gene, which is known to encode $\Delta 9$ desaturase in *M. tuberculosis* were explored. This is the extent of published work conducted on the new drug target discovery and evaluation of efficiency of the thioureas as anti-tuberculosis drugs worthy of further development.

MATERIALS AND METHODS

Mycobacterial strain and growth conditions.

M. tuberculosis H37Ra (TMCC 25711), *M. tuberculosis* H37Rv and Thai clinical isolate of *M. tuberculosis* were grown in 250-ml tissue culture flasks containing 50 ml of liquid Sauton's medium, and were incubated without agitation. Cells were grown to early exponential phase (~21 days), and harvested, and sterile glycerol was added to a final concentration of 10%. Cell suspension was dispensed into tubes and stored at -70°C until required. For further studies, thawed suspensions were added to 50 ml of Sauton's medium containing 5% tyroxapol (Sigma) and incubated at 37° C to mid-exponential phase.

Determination of MICs of ISO and new derivatives.

ISO, a derivative of thiourea was a gift from M.J. Colston and P. Draper, National Institute of Medical Research, London, United Kingdom. New derivatives of thioureas were included as shown Table 1; the synthesis of ISO and new derivatives will be documented separately. The MICs of ISO and new derivatives in liquid medium were determined by microplate alamar blue assay which was modified from the method of from Yajko *et al.* (1995). Briefly, inocula were prepared from *M. tuberculosis* clinical isolate. Cells were grown in Sauton's medium to a turbidity equal to that of a No. 1 McFarland standard (~2.0×10^7 CFU/ml). Serial dilutions of ISO were prepared from DMSO stock in Sauton's medium. From each dilution 5 µl was then inoculated into 85 µl of cultures that had been dispensed into clear-bottomed, 96-well microplates prior to adding thiourea. Duplicate treatments with different concentrations of each thiourea were performed. Four controls were included which contained medium only, culture only, medium plus DMSO, and culture plus DMSO. At the outset, 10 x Alamar Blue solution (Accumed; OH) was added to the controls. After overnight incubation at 37°C in a moisture-controlled chamber, Alamar Blue was added to the all treated wells. Plates were further incubated, and the color in each treated culture was recorded until the color in the culture control turned pink. The MIC was determined as the lowest concentration of ISO in which the blue dye in the treated culture did not turn pink. Antimycobacterial activity of thioureas against *M. tuberculosis* was evaluated based on the MIC values.

Determination of effects of ISO and new derivatives on the synthesis of oleic acid and mycolic acids. *M. tuberculosis* clinical isolate were grown in 5 ml of Sauton's medium in a

thioureas included compound of C06, C26, JDDD18, JDDD38, JDDD45, JDDD46) and INH) was added. The cells were then incubated with gentle shaking for 6 h prior to adding [1,2-^{14}C]acetate (110 mCi/mmol) (Dupont NEN; Boston, Mass.) at 0.5 µCi/ml to both control and drug-treated cultures which were further incubated at 37°C with gentle agitation for an additional 18 h. The resulting [^{14}C]-labeled cells were harvested by centrifugation at 2500 x g, and washed twice with saline and once with sterile water (Slayden, *et al.*, 1996). In parallel experiments, *M. tuberculosis* H37Ra was grown in Sauton's medium to early exponential phase, pre-incubated with ISO for 6 h and exposed to [1,2-^{14}C]acetate for 12 h. After labeled, cells were harvested by centrifuge at 3,000 g. Fatty acids as well as mycolic acids were derivatized to be methyl esters as followed. The [^{14}C]-labeled control and drug-treated cells were resuspended in 2 ml of 15% tetrabutylammonium hydroxide (Sigma; St. Louis, MO) and saponified at 100°C overnight. After cooling, 2 ml of water, 3 ml of dichloromethane and 300 µl of iodomethane (Aldrich Chemical Co.; Milwaukee, Wis.) were added to the entire reaction mixture which was then shaken on a rolling shaker for 1 h. After centrifugation, the upper layer was discarded and the lower organic phase was washed three times with 3 ml of water. The washed lower phase was dried by a nitrogen flow, extracted with 4 ml of diethylether, sonicated for 5 min, and centrifuged at 2500 × g (Desktop Centrifuge). The ethereal extract was transferred into new 13 x 100-ml glass tubes, dried, and resuspended in 200 µl of dichloromethane for counting of radioactivity. Finally, an aliquot of the extracts containing fatty acid methyl esters (FAMEs) and mycolic acid methyl esters (MAMEs) (20 µl) from control and thioureas-treated cells were subjected to thin layer chromatography (TLC) on silica gel plates (silica gel 60_{254} Merck; Darmstaadt, Germany) with had been impregnated with 5% silver nitarte and activated (Morris, 1966). After the plates were developed four times in petroleum ether-acetone (90:10), autoradiograms were produced by overnight exposure at -70°C to Kodak X-Omat AR film. Separate bands of FAMEs and MAMEs were marked, cut from the TLC plates, and placed directly in 10 ml of EcoLume™ (Ecolume™; Costa Mesa, CA), and radioactivity was counted to estimate the degree of inhibition of the synthesis of oleic acid as well as mycolic acids. Oleic acid methyl ester standard was visualized by spraying with 10% sulfuric acid and anisaldehyde (Dunphy et al., 1967).

RNA isolation *M. tuberculosis* was grown in Sauton's medium for RNA isolation and subsequent reverse transcription PCR (RT-PCR). Total RNA was isolated from *M.*

tuberculosis as follows. Cell pellets were resuspended in 94 µl of RNase free water containing RNase inhibitor. The suspension was then sonicated at 4 °C for 10 min followed by the addition of 6 µl of 3 mg/ml lysozyme. After incubation at 25 °C for 10 min, the 900 µl of phenol and guanidine thiocyanate was added. The homogenate mixture was stored for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes. The following step is phase separation, which performed by adding 100 µl of chloroform to homogenate mixture. Resulting samples were then shaken vigorously in seconds and allowed to stand at room temperature for subsequent 15 min. Sample tubes were then centrifuged at 12,000 x g at 4 °C for 15 min. After centrifugation, the mixtures was separated into three phases; aqueous, interphase and organic phase. The aqueous phase, which contains RNA was transferred with caution to leave the interphase and organic layers. RNA pellet was further precipitated by the addition of absolute cold ethanol, 0.3 M sodium acetate and 1 µl of 20 mg/ml glycogen. The resulting pellet was washed in 75% ethanol, dried at room temperature, and solubilized in RNase free water. The purified RNA was subsequently used in RT-PCR for determination of the expression of *desA3*.

RT-PCR for detection of the expression of *desA3*. A reverse transcription and PCR were carried out sequentially in a single tube using RT-PCR beads. Each room-stable bead contains *M*-MuLV reverse transcriptase, RNase inhibitor, buffer, nucleotides and *Taq* DNA polymerase. The only additional reagents needed to compose the reactions are 1 µl of 20 pmol of each primer, and 30 ul of RNA template in RNase free water. The RT-PCR reaction was conducted in a programmable master-gradient thermal machine using specific *desA3* primers, GGT GAC CGA CTT AGC CAT ACG CTT, upper primer; AGC ATC ACC TCT ATC CGG AC TGC C, lower primer. The thermal program involved reverse transcription at 55 °C for 30 min, followed by the initial denaturation at 94 °C for 5 min. The amplification was performed for 35 cycles of denaturation at 94 °C for 1 min, annealing and extension at 72 °C for 1 min. The final extension was set at 72 °C for 10 min. Negative controls, which contained all reaction components except RNA template, was included to detect carry over contamination.

For experiment to determine whether transcribed RNA or contaminating DNA was amplified by this protocol, purified nucleic acids were subjected to DNase and RNase treatment prior to cDNA synthesis and PCR. In brief, DNase reaction was performed in a total volume of 30 µl containing 1 unit of RNase free DNase and RNA template. The

reaction mixture was incubated at 37 °C for 10 min. Subsequently, the enzyme was inactivated at 80 °C for 10 min. The resulting DNase-treated RNA was subjected to RT-PCR. To ensure that the DNase reaction used was an optimal condition, the DNase control reaction, which contains RNase free DNase and DNA template was included. After incubation for DNAse activity, the latter reaction mixture was subjected to PCR.

Again, to assure that amplicons derived from RT-PCR was originated from RNA, the control containing RNase was performed. Briefly, RNA obtained from extraction procedure was treated with 1 µl of 10 mg/ml RNase. After incubation at 37 C°, the RNase reaction was terminated by heat inactivation at 80 °C for 10 min. The resulting RNase treated RNA was used as a control template in parallel with RNase untreated RNA, which then were added directly to the mixture of RT-PCR reaction. The control RT-PCR was completed by our simple protocol as described above.

The amplicons obtained from RT-PCR were analyzed for 341-bp fragment of *M. tuberculosis* by 2.0% agarose gel electrophoresis in Tris-borate-EDTA (TBE) buffer (0.089 M Tris-HCl, pH 8.0), 0.044 M Boric acid, and 0.001 M EDTA). Electrophoresis was conducted at 100 V for 1 h. Gels were stained with ethidium bromide and visualized by UV transilluminator.

LightCycler Real-time PCR for quantitative *desA3* transcripts

The optimized conditions in conventional RT-PCR and LightCycler real-time PCR (LC-PCR) was applied to develop LightCycler real time RT-PCR (LC-RT-PCR) targeting 341 bp of the *desA3* transcripts. In LC-PCR, the *desA3* amplicons were amplified in the reaction sealed capillary tube using a Roche LightCycler instrument. Firstly, the "HotStart" reaction mix was prepared by pippetting the total volume 60 µl LightCycler-FastStart reaction Mix SYBR Green I into the LightCycler-FastStart enzyme (LightCycler-DNA master mix, Roche Diagnostics). After gentle mixing, the resulting "HotStart" reaction mixture is ready for a final volume of 20 µl each reaction. The component of LC-PCR in a final volume of 20 µl included 2 µl of ready to use reaction mix which contains *Taq* DNA polymerase, reaction buffer, deoxynucleoside triphosphate mix, and $MgCl_2$. An aliquot of10 pmol of each primer corresponding to 341 base pairs of *M. tuberculosis desA3* gene as used in conventional RT-PCR was applied again. To determine the optimal concentration of $MgCl_2$, the concentration of $MgCl_2$ was varied from 1 to 5 mM. Prior to PCR a pre-incubation step (95° for 7 min) was performed to activated the Faststart enzyme. The PCR consisted of 40 cycles with the

following thermal sequences: 95 C for 10 s, 70 C for 8 S, and 72 C for 20 s. the reaction was performed in capillary tubes in LightCycler instrument.

For LC-RT-PCR), the reaction mixture of the real-time RT-PCR was composed of reaction mixture containing 10 pmol of each of the primers, 4 mM $MgCl_2$ and ready to use real-time RT-PCR reaction mixture. The RT-PCR consists of the reverse transcription at 55° C for 60 S and PCR cycles. Initial denaturation step in PCR was performed at 95° C for 10 S followed by 40 cycles of two-step thermal sequences: (95° C for 10 S and 69° C for 10 S). Amplicons were detected by fluorometer equipped with the LightCycler machine and data acquisition was facilitated through the detection of the fluorescent dsDNA binding dye, SYBR® Green. The melting temperature (T_m) was used to characterize the amplified product. When the amplification was completed, a melting program of from 80 to 93 C at 0.2 C /s with continuous monitoring of the fluorescence. A negative control, which is a reaction that template RNA, was replaced by the PCR-grade water was run with samples.

Determination of effects of thiourea on the expression of *desA3*

Recently developed LC-RT-PCR targeting 341 bp-of *desA3* transcripts was applied to determine the effects of thioureas on the expression of the *desA3* in *M. tuberculosis*. Briefly, *M. tuberculosis* was grown in 5 ml of Sauton's medium in a set of culture tubes to early exponential phase, at which point ISO and INH was added to a final concentration of 2.0 μg/ml. The cells were then incubated with gentle shaking for 6 h prior to centrifugation at 3,000 g for harvesting. *M. tuberculosis* RNA was extracted as described previously. The resulting RNA was subjected to quantifcation of *desA3* transcripts by LC-RT-PCR.

RESULTS

MICs of ISO and new derivatives

It is impossible to find accord in the literature on a standardized means of measuring MICs applicable to the different physical properties of drugs and different mycobacteria. Accordingly, MICs were evaluated under the defined conditions described in Materials and Methods, with species of *M. tuberculosis* selected to reflect clinical application. Isoxyl, a thiourea, and new derivatives were subjected to a evaluation of their *in vitro* activity against species of *M. tuberculosis* by applying our defined quantitative microplate alamar blue test involving the use of Suaton's liquid medium containing 0.5% tyroxapol. The Alamar Blue oxidation-reduction dye was applied as an indicator to determine the MIC of isoxyl and new derivatives in *M. tuberculosis*. In this assay, the blue, oxidized form becomes red due to the normal redox reactions within mycobacterial cells and thus the red color represents cell viability.

The MIC of ISO for *M. tuberculosis* as analyzed by microplate alamar blue assay was in the range of 0.30-1.25 µg/ml compared to published values of 0.02 to 0.2 µg/ml for INH and 5 to 10 µg/ml for ETH (Bernstein, 1952). The MICs of the newly synthesized derivatives of thiourea (Fig. 1) (Derivatives of thiourea included compound of C06, C26, JDDD18, JDDD38, JDDD45, JDDD46) against *M. tuberculosis* were in the range of 0.30-10.0 µg/ml. Under the test conditions, isoxyl and new derivatives exhibited potent antimycobacterial activity against clinical isolate of *M. tuberculosis*.

Selective effects of ISO and new derivative on inhibition of oleic acid and mycolic acid synthesis.

Previous studies with [1,2-^{14}C]acetate as a precursor of fatty acid synthesis showed that ISO inhibited the synthesis of both mycolic acids and shorter chain-fatty acids in *M. tuberculosis* (Phetsuksiri, et al., 2003). In this latter respect, ISO differed from INH and ETH, suggesting a different mode of action of ISO. In this study, effects of ISO and thioureas on the synthesis of oleic acid, a major unsaturated fatty acid, and mycolic acids were determined. *M. tuberculosis* was grown in the presence and absence of ISO and new derivatives, following which cultures were labeled with [1,2-^{14}C]acetate. Extracts containing [^{14}C]labeled fatty acids and mycolic acid methyl esters were prepared from untreated and thioureas-treated *M. tuberculosis* cultures and analyzed by argentation-TLC. Combined MAMEs and FAMEs were resolved and fractionated on TLC plates. The results demonstrated a decrease in the incorporation of radioactivity into FAMEs and MAMEs in the

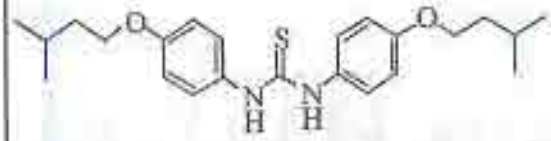
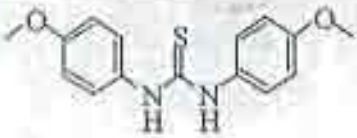
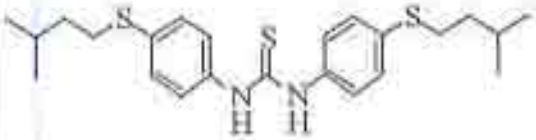
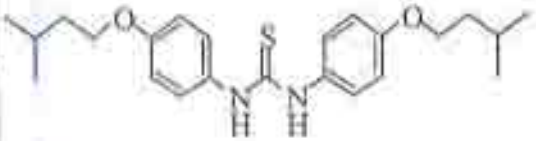
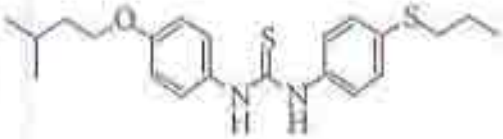
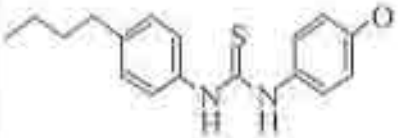
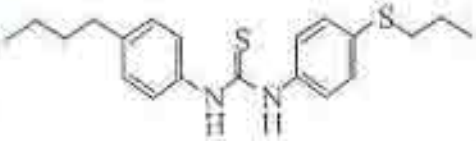
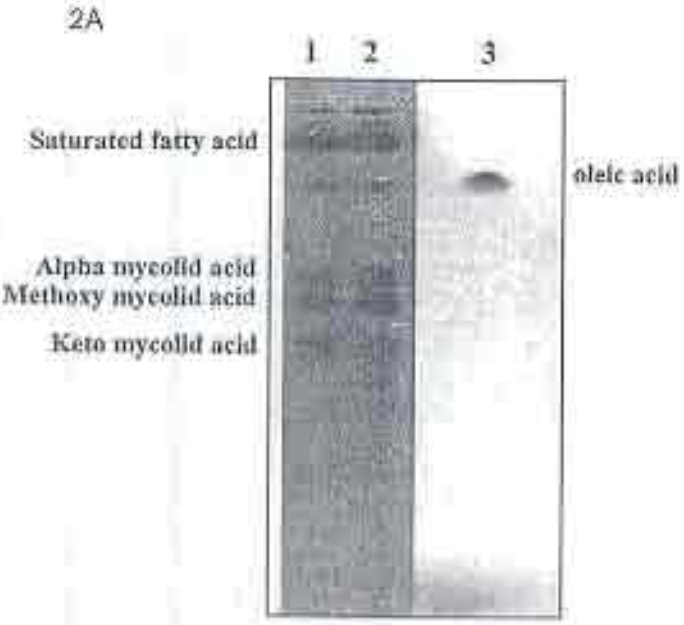
ISO derivative			MIC (ug/ml) *M. tuberculosis*
No	**Code**	**Structure**	
1	B27		0.3
2	C06		5.0
3	C26		10.0
4	JDDD18		5.0
5	JDDD38		0.3
6	JDDD45		N.D
7	JDDD46		N.D

Figure 1 Structue of isoxyl and new thiourea derivatives

presence of ISO or new derivatives (Fig. 2). With the use of oleic acid standard (Fig. 2), this simple means of analysis demonstrated that isoxyl and new derivatives inhibited the synthesis of oleic acid. Methyl ester form of oleic acid was applied on the impregnated TLC plate. The oleic acid standard could be visualized by staining with anisaldehyde and 10% sulfuric acid as described previously (Fig. 2). The decrease in the incorporation of [1,2-^{14}C]acetate into oleic acid was quantitated by liquid scintillation counting. The inhibition of isoxyl and new derivatives on the synthesis of mycolic acids could be seen (Fig. 2). According to one-dimensional TLC plates, Isoxyl at its respective MICs inhibited the synthesis of fatty acids and mycolic acids defined as to α-mycolates, methoxy mycolates and ketomycolates by in *M. tuberculosis* H37Rv (Fig. 2).



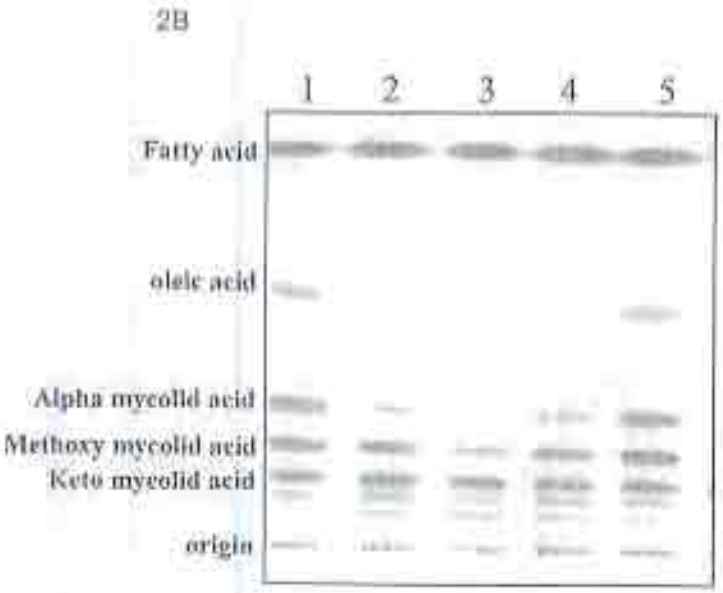


Figure 2 : 2A: Identification of separated *Mycobacterium tuberculosis* oleic acid using oleic acid standard in form of methyl ether; Lane 1-2, fatty acid from *M. tuberculosis*; Lane 3, standard of oleic acid; 2B: Lane 1, control; Lane 2, JDD38 2 µg/ml; Lane 3, JDD45 2 µg/ml; Lane 4, JDDD 46 2 µg/ml; Lane 5, control with DMSO

The general effects of isoxyl were in accordance with those reported by Winder et al. (1971), i.e., a generalized inhibition of fatty acid and mycolic acid synthesis. The approach extended to an examination of the effects of isoxyl and new derivatives on oleic acid synthesis confirmed a selective action of thioureas. In the presence of isoxyl or new derivatives at concentration as low as 2 µg/ml, there was an apparent decrease in the synthesis of oleic acid concomitant with a partial increase in the synthesis of saturated fatty acid (Fig. 2). Measurement of the radioactive counts in the relevant bands established that ISO at 2 µg/ml inhibited oleic acid by 60%. Clearly, new derivatives were also inhibited the incorporation of [1, 2-^{14}C]acetate into oleic acid and resolved mycolic acid (Fig. 2).

Comparisons of the effects of isoxyl and new derivatives of thiourea with those of INH on the synthesis of oleic acid.

Comparison of the effects of ISO and the derivatives of thiourea to those of INH on oleic acid acid biosynthesis was studied through cell labeling with [1,2-^{14}C]acetate. The effects of all these drugs were similar in that the synthesis of mycolic acid acids was inhibited. More importantly, ISO inhibited the synthesis of oleic acid but this effect had not been observed with INH in *M. tuberculosis*, suggesting that target of drug might be different. Isoxl and new derivatives were all had inhibitory effects on the synthesis of oleic acid as shown in Fig. 2

Detection of the expression of *desA3* by RT-PCR.

In the amplification presented in this study, primers specific for *M. tuberculosis desA3* was designed resulting in the single band of PCR product at the size of 341 bp as analyzed by agarose gel electrophoresis. Specificity of primers for *M. tuberculosis* were further analyzed with various mycobacterial genomic DNA as well as those of other organisms. PCR product dose not exist with other DNA indicating specific of the primers. Figure 3 showed that the primers designed and used were specific for *M. tuberculosis* including *M. bovis*, which classified as to a member of *M. tuberculosis* complex. RT-PCR targeting the 341-bp region of *desA3* specific for *M. tuberculosis* was further developed.

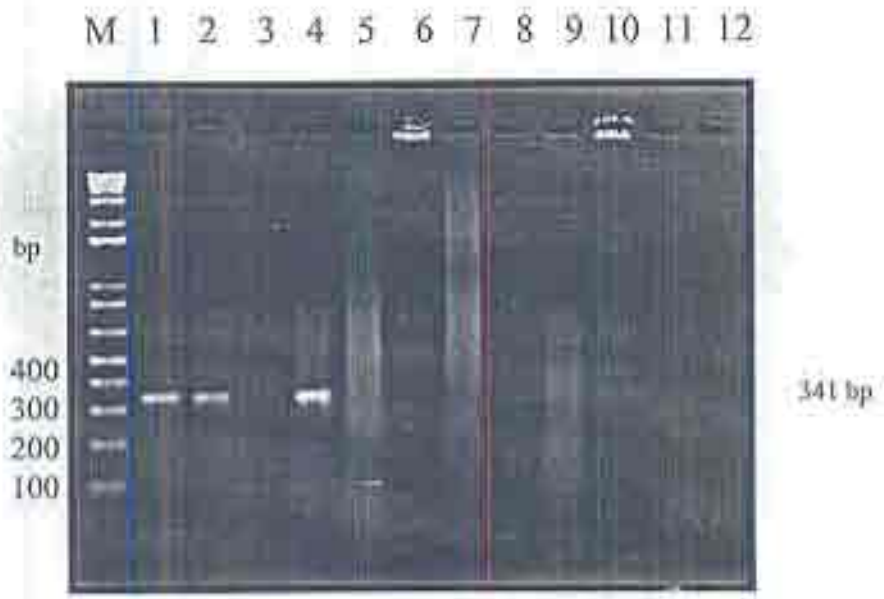


Figure 3 Specificity of *des3A* primer: Lane M, DNA marker; Lane 1, *M. tuberculosis* (H37Rv); Lane 2, *M. tuberculosis* (clinical isolate); Lane 3, *M. smegmatis*, Lane 4, *M.bovis*; Lane 5, *M. kansasii* ATCC 12478; Lane 6, *M. avium* ATCC 2529; Lane 7, *M. gordonae* 330; Lane 8, *M. intracellulare* ATCC 18950; Lane 9, *M. fortuitum* ATCC 23048; Lane 10, *M.marinum* ATCC 927; Lane 11, *M.serofulaceum*; Lane 12, Negative control.

Subsequently, the conditions of conventional PCR were further applied to set up a system of conventional RT-PCR and real-time RT-PCR. It proved that the RT-PCR product was corresponding to the presence of RNA template in the reaction by the use of DNase and RNase (Fig. 4).

Using specific primers resulting in a single band product of RT-PCR as showed in Fig 4. The *desA3* transcript was detected by RT-PCR during exponential growth emphasizing the importance of enzymatic product and function (Fig. 4).

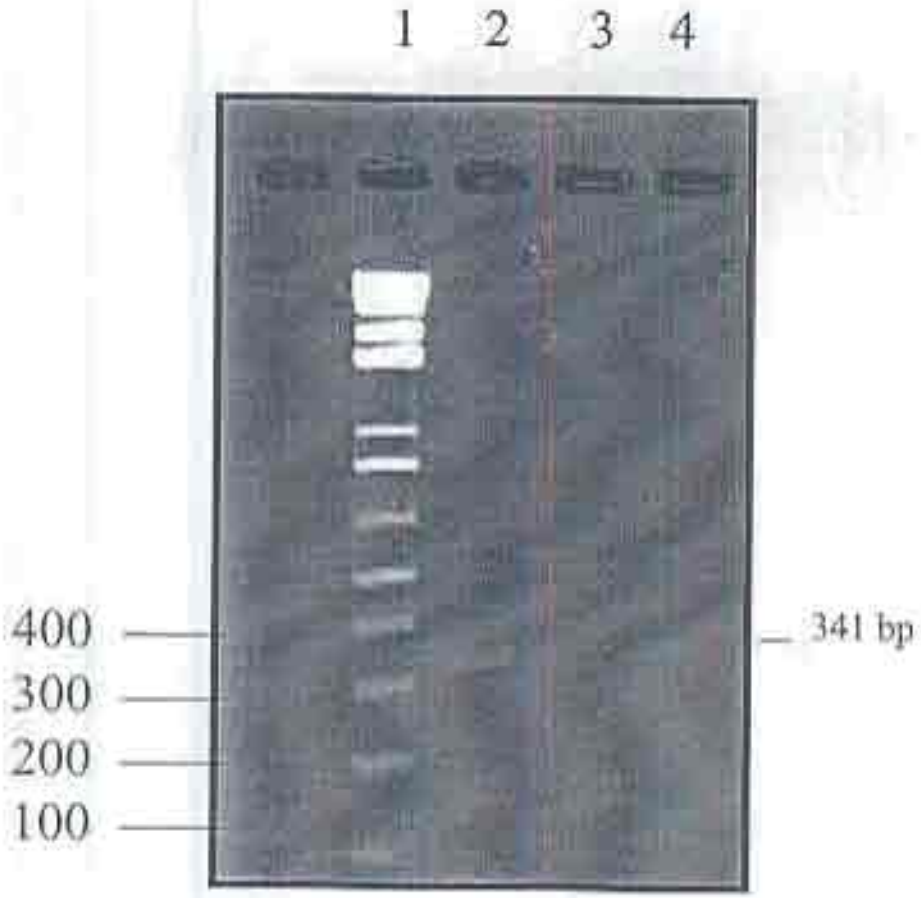


Figure 4 Detection of mRNA transcript of *M. tuberculosis desA3* by RT-PCR. RNA isolated from *M. tuberculosis* H37Rv was subjected to cDNA synthesis, PCR, agarose gel electrophoresis. Replicate RNA samples were treated with DNase prior to cDNA synthesis. Lane 1, DNA marker; Lane 2, DNAse; Lane 3, DNase and RNase; Lane 4, Negative control.

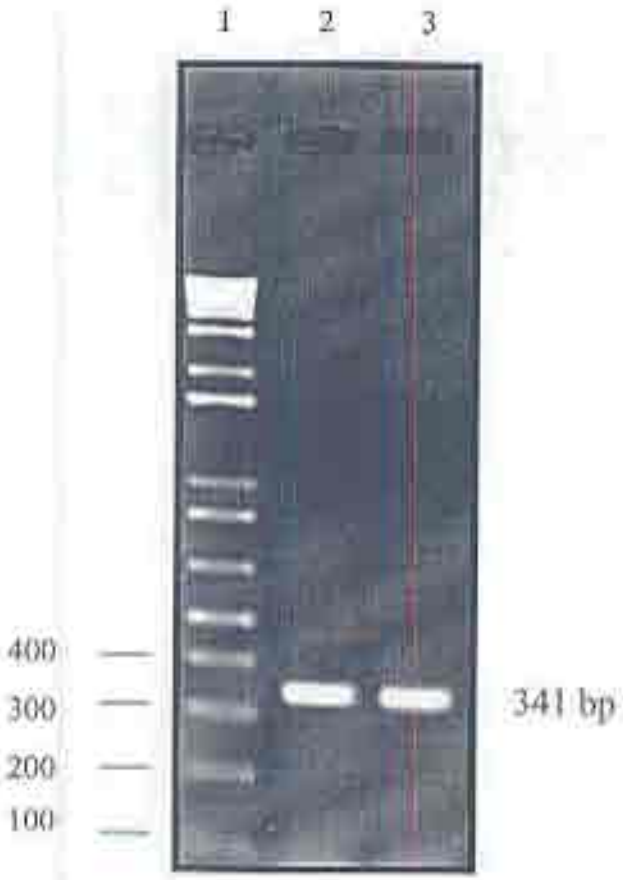


Figure 5 Determination of effect of thiourea on the expression of *M.t uberculosis desA3* by conventional RT-PCR. Lane 1, DNA Marker; Lane 2, *M. tuberculosis* without isoxyl treatment; Lane 3, *M. tuberculosis* with isoxyl treatment at 2 ug/ml for 18 h exposure.

Quantitative analysis of *M. tuberculosis desA3* transcripts by conventional RT-PCR.

RT-PCR targeting the mRNA of *desA3* was successfully developed and could be detect des3 transcripts. However, quantitative analysis of the expression of *desA3* transcripts could not be achieved by the conventional RT-PCR. This application could not be able to determine the difference in small amount of mRNA of *desA3*. Figure 5 showed that the difference in amount of *desA3* transcripts could not be distinguished at the end of multiple cycles of conventional RT-PCR (Fig. 5)

Quantitative analysis of the effect of ISO on the expression of *desA3* by real-time RT-PCR.

We designed a real-time RT-PCR approach using the SYBR Green I fluorescent dye and the LightCycler. Primers designed to amplify a 341 bp of *desA3* of *M. tuberculosis* in conventional RT-PCR was applied in real-time RT-PCR. We used the same primers for conventional PCR and LightCycler, because the target was small enough that this rapid cycling worked well. The establishment of real-time RT-PCR was successfully performed to quantitate transcripts of *M. tuberculosis desA3*. The LightCycler real-time RT-PCR was simple to perform and interpret and was able to quantify *M. tuberculosis desA3*transcripts. Very fast amplification of DNA in small volumes can be continuously monitored with a rapid cycler that incorporates fluorimetric detection. The 341-bp cDNA template was amplified, and the RT-PCR product was quantitatively detected using SYBR Green I chemistry. The accumulation of PCR product was monitored by measuring the level of fluorescence. The use of SYBR Green I in a real-time PCR assay permitted the quantitative detection of RT-PCR product. Based on the principle that PCR products are labeled by fluorescent dye in each round of real-time PCR cycle, PCR products could then be monitored and quantitated by measuring the level of fluorescence (Fig. 6).

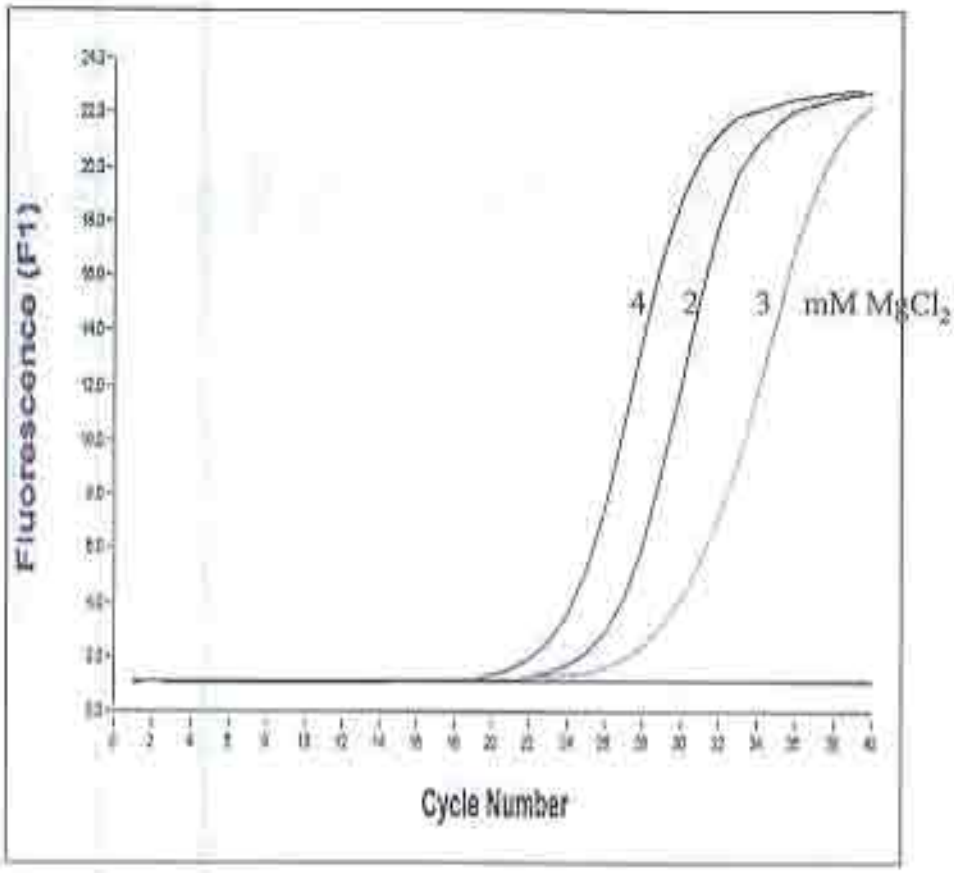


Figure 6 Accumulation of the fluorescence during PCR using different concentration of $MgCl_2$: 2, 3 and 4 mM $MgCl_2$. NC: Negative control. F2 is the channel used by the LightCycler to detect the fluorescence.

For efficient amplification by real-time RT-PCR, it is essential to optimize the target-specific $MgCl_2$ concentration. The result of effects of $MgCl_2$ concentration on the amplification of *desA3* was shown in (Fig. 6). The PCR product was observed to accumulate in an exponential manner. The concentrations of Mgl2 also affected the efficiency of amplification. It can be seen that the signal started to rise at different times depending on the efficiency of amplification and the DNA concentration. The concentrations of DNA dose not affect the interpretation of the Tm. When the amplification was completed, a melting step was performed, in which the temperature of the tube was slowly increase to analyze the melting pattern of PCR product. Melting pattern was monitored directly from the LC instrument. Within 45 min, the LightCycler (LC) method amplify target sequence without the need of any post-PCR sample manipulation. The approach described here is rapid, reliable and simple and can be used to estimate relatively levels of transcripts of *desA3* in *M. tuberculosis* cells. As expected, the real-time RT-PCR provide a single peak of the RT-PCR product which had a melting curve was equivalent to 90^{o} C approximately corresponding to 341-fragment of *desA3* RT-PCR product (Fig. 7).

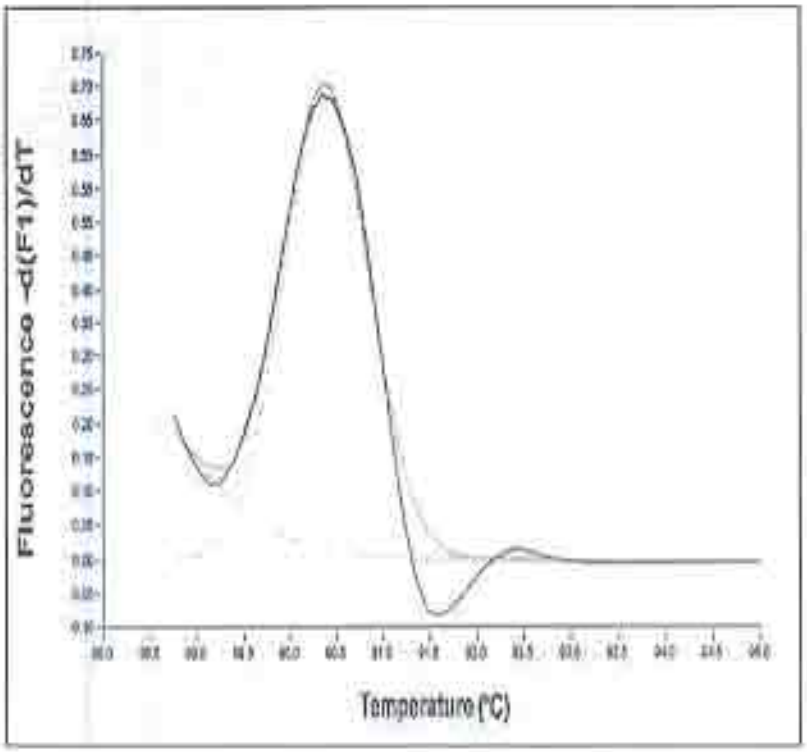


Figure 7. Representative experimental melting pattern for 341-bp fragment of *M. tuberculosis desA3*. The temperature of the tube was slowly increased to analyze the melting temperature of RT-PCR product. Melting pattern was monitored directly from the LC instrument.

Agarose gel electrophoresis of real-time RT-PCR product indicated the single product of the reaction, which has a melting point at approximately 90° C is equivalent to 341-bp specific to *desA3* sequence (Fig. 8).

Demonstration of effects of thiourea on the expression of *M. tuberculosis desA3*

The use of real-time RT-PCR demonstrated that the transcripts of *M. tuberculosis desA3* decreased when *M. tuberculosis* was treated with isoxyl at 2 µg/ml for 18 h (Fig 9). Using this technology we found that *desA3* transcripts of *M. tuberculosis* decreased when cells exposed to thiourea.

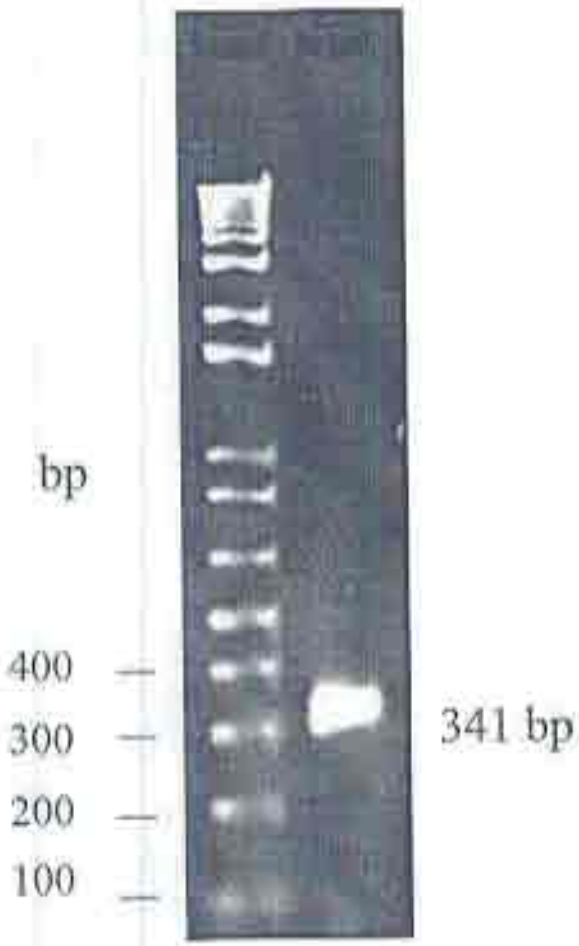


Figure 8 Agarose gel electrophoresis for verification of real-time RT-PCR product. Real-time RT-PCR product was verified for 341-bp fragment specific to the portion of *M. tuberculosis desA3* gene by 1 % agarose gel electrophoresis.

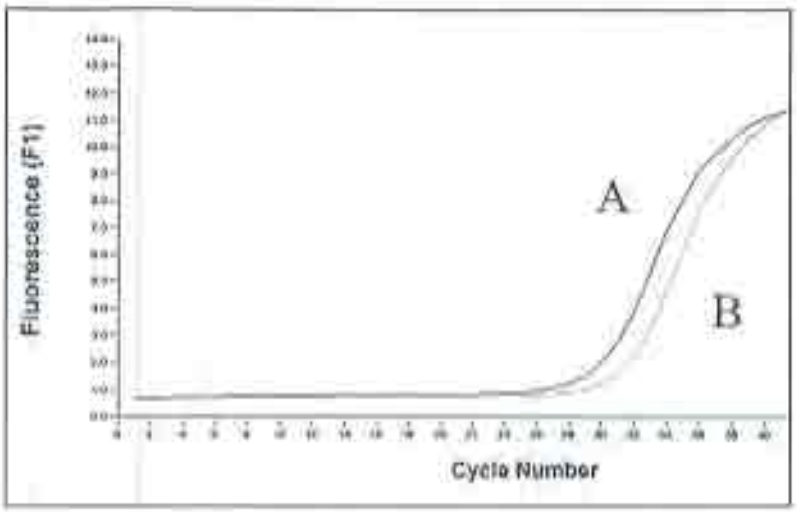


Figure 9 Analysis of the effect of thiourea, isoxyl, on the expression of *desA3* in *M.t uberculosis* by real-time RT-PCR. A; Fluorescence signal detected in *M.t uberculosis* without treatment of thiourea, isoxyl. B; Fluorescence signal detected in *M.t uberculosis* treated with isoxyl at 2 ug/ml for 18 h.

DISCUSSION

The development of new antituberculosis drugs is essential in combating drug resistant *M. tuberculosis*. Isoxyl (ISO), a 4,4'-diisoamyloxydiphenylthiourea (4,4' diisoamyloxythiocarbanilide; thiocarlide) (Winder, 1982) is an old drug used for the clinical treatment of tuberculosis in the 1960s. Titscher (1966) and Urbancik (1966; 1970) demonstrated modest therapeutic efficacy of thiourea monotherapy in cases of untreated pulmonary tuberculosis of various degrees of difficulty. The drug was able to convert about 25% of bacteriologically chronically positive cases to negative after 6 to 8 weeks of 6 g of ISO daily. However, when the treatment was extended to 10 to 18 weeks, about 50% of the patient population had their sputa converted to negative (Kampelmann, 1970). Schmid (1970) concluded that combined INH and ISO was more effective than monotherapy with either drug. It had been noted in the early 1950s that ISO exhibited strong antimycobacterial activity in vitro (Winder, 1982). A note from Winder, et al. (1971) showed that like INH and ETH, ISO strongly inhibited mycolic acid synthesis in *Mycobacterium bovis* during 6 h of exposure to 10 µg/ml. ISO also partially inhibited the synthesis of the fatty acids of free lipids, which were stimulated by INH and ETH. The examination of effects of thiourea in *M. tuberculosis* may enable us to define its mode of action.

The results of the MIC studies show that isoxyl is capable of inhibiting the growth of clinical isolates of *M. tuberculosis*. The organism exhibited susceptibility to isoxyl when exposed to isoxyl in the range of 0.3 to 1.25 µg/ml. Isoxyl is a substituted diacyl thiourea, and previous studies had demonstrated that the thiourea nucleus is required for antimycobacterial activity. In the hope of generating more-effective variants, random substitutions were made in the side chains attached to the key structure. This strategy resulted in an array of new derivatives of thiourea with variations in the symmetry and asymmetry of the side chains attached to the key structure. Some derivatives of thioureas, for instance, the butyl derivative of thiourea (Phetsuksiri et al., 1999) the first synthesized thiourea derivative, possessed low MICs (0.1 to 0.5 µg/ml) and was chosen for further evaluation of its effects on mycobacteria. It was demonstrated in this study that thiourea derivatives with different side chains were effective against *M. tuberculosis* clinical isolate.

Potential new targets for antimycobacterial drug development may exist among the synthetic enzymes needed to make the unique lipids produce by mycobacteria, such as

fatty acids and mycolic acids (Parrish, et al., 2001). Recently, it was reported that isoxyl inhibited the synthesis of oleic acids and caused accumulation of stearic acid. Base on the specific inhibition it was proposed that mode of action of isoxyl is through the inhibition of 9Δ desaturase. Oleic acid is the most abundant unsaturated fatty acids in *Mycobacterium spp* (Ratledge, 1982) and is a vital constituent of mycobacterial membrane phospholipids (Okuyama, et al., 1967; Walker, et al., 1970), where it apparently plays an essential role in membrane physiology (Los and Murata, 1998). At physical temperatures, phospholipid containing only saturated fatty acid cannot form a lipid bilayer, but the introduction of appropriate fatty acids decrease s the transition from gel to the liquid crystaline phases and provide membranes with necessary fluidity for physiological function (Stubbs and Smith, 1984; Russel, 1984; Hazel, 1995; Houslay and Gordon, 1984). This report showed that isoxyl and new derivatives are effective against *M. tuberculosis* and inhibited the synthesis of oleic acid which is an important molecule to cells. The vital function of oleic acid lead to the conclusion that the inhibition of its synthesis leads to cell death and the enzymes involved in oleic acid synthesis are probably lethal target for drugs effective against *M. tuberculosis*.

A key technique for analyzing purified RNA is expression analysis through quantitative real-time PCR. RT-PCR can be performed in either one or two steps, depending on the enzymes, primers and buffers used. The one-step real-time RT-PCR approach presented in this study was able to quantify *M. tuberculosis desA3* transcripts. The primers designed conferred species specificity in PCR assays. Based on the notion that mRNA is present in viable bacteria and vulnerable to degradation upon cell death (Albert, et al., 1989; Gabriella et al., 1995), the presence of mRNA may indicate cell viability (Bej, 1991; Patel, 1993). This RT-PCR assay, which is able to detect mRNA may be useful in detection of viable *M. tuberculosis* in a number of clinical situations. Comparing to conventional RT-PCR, which detect PCR product at the end point of PCR cycles, the LC-RT-PCR coupled to fluorogenic detection would provide superior advantages especially in term of relative quantitative detection particularly when used to determine the difference in amount of mRNA in small scales.

An understanding of the specific mode of action of isoxyl involved the defining of drug effects in *M. tuberculosis* is important in the search for new antimycobacterial drug targets and for the development of more effective chemotherapy. It is prudent to re-examine drugs

that were formerly deemed effective against tuberculosis. We conclude that isoxyl and new derivatives have antimycobacterial activity. The unique mechanism of isoxyl and new thioureas derivatives on the inhibition of oleic acid was observed. The reduction of oleic acid in cells was due to the inhibition of enzyme involved in oleic acid synthesis, the 9Δ desaturase. The decrease in the expression of *desA3* may be related to the antimycobacterial activity of thiourea, which resulted in the decrease of cellular metabolic rate. Isoxyl and new thiourea derivatives inhibited the growth of *M. tuberculosis* and hence metabolic rate was retard and may cause a decrease of *desA3* gene expression in *M. tuberculosis*. Further study to develop new generation of inhibitors of oleic acid synthesis and other approaches to show that *desA3* is an essential gene for *M. tuberculosis* should be pursued.

CONCLUSION

Isoxyl and new derivatives of thioureas possess antimycobacterial activity and are effective against *Mycobacterium tuberculosis* clinical isolate with MIC of 0.30-10.0 µg/ml. The selective action of thiourea, isoxyl and new derivatives is on the inhibition of oleic acid synthesis. The expression of *desA3*, a gene that encodes the 9Δ desaturase involved in the synthesis of oleic acid existing in *M. tuberculosis* indicated the importance of gene function. The use of real-time RT-PCR demonstrated that the transcripts of *M. tuberculosis desA3* decreased when *M. tuberculosis* was treated with isoxyl at 2 µg/ml for 18 h. Dissection of effects of thiourea on *M. tuberculosis* therefore, revealed a promise of new antituberculosis agents and a new therapeutic new drug target.

REFERENCES

Albert, B., D. Bray, J. Lewis, M. Raff, K. Robert, and J. D. Watson. 1989. Control of gene expression, p.595. Molecular biology of the cell, 2nd ed. Garland Publishing, Inc., New York.

Bej, A.K., M. H. Mahbubani, and R.M. Atlas. 1991. Detection of viable *Legionella pneumophilla* in water by polymerase chain reaction and gene probe methods. Appl. Environ. Microbiol. **57:** 597-600.

Bernstein, J., W.A. Lott, B.A. Steinberg, and H.L. Yale. 1952.Chemotherapy of experimental tuberculosis: Isonicotinic acid hydrazide (Nydrazid) and related compounds. Ann. Rev. Tuberc. **65:**357-364.

Fulco, A.J., and K. Bloch. 1964. Cofactor requirements for the formation of 9-unsturated fatty acids in *Mycobacterium phlei*. J. Biol.Chem. **239:** 993-997.

Dunphy, P.J., J.D Kerr, J.F. Pennock, K.L. Whittle, and J. Feeney. 1967. Biochim. Biophys. Acta. **136:** 136-147.

Gabrielle, M. E., V. D. Vliet, P. Schepers, R. A. F. Schukkink, B. V. Gemen, and P. R. Klaster. 1994. Assessment of mycobacterial viability by RNA amplification. Antimicrob. Agents Chemother. **38:**1959-1965.

Hazel, J.R. 1995. Thermal adaptation in biological membrane is homoviscous adaptation the explanation? Annu. Rev. Physiol. **57:** 19-22.

Housley, M.D., and L.M. Gordon. 1984.The activity of adenylate cyclase is regurated by the nature of its lipid environment. Curr. Top. Membr. Transp. **18:** 179-231.

Los, D.A., and N. Murata. 1998. 1998. Review: Structure and expression of fattya cid desaturase. Biochim Biophys. Acta. **1394:** 3-15.

Kampelmann, F. 1970. Results of monotherapy with Isoxyl in untrated pulmonary tuberculosis cases of difficulties and isoxyl in routine therapy. Antibiot. Chemther. 26: 96-

104.

Kashiwabara, Y., and R.Sato.1973. Electron transfer mechanism involved in stearoyl-coenzyme A desaturase activity. Tokyo. **74:** 803-810.

Kashiwabara, Y. H. Nakagawa, G. Matsuki, and R. Sato. 1975. Effect of metal ions in the culture medium on the stearoyl-coenzyme A desaturase activity of *Mycobacterium phlei*. J. Biochem. Toyko. **74:** 803-810.

Lennarz, W.J. G. Scheuerbrandt, and K. Bloch. 1962. The biosynthesis of oleic acid and 10-methylstearic acids in *Mycobacterium phlei*. J. Biol. Chem. **237:** 664-671.

Morris, L.J. 1966. Separation of lipids by silver ion chromatography. J. Lipid Res. **7:** 717-730.

Parris, N.M., T. Houston, P.B. Jones, G. Townsend, and J.D. Dick. 2001. In vitro activity od a novel antimycobacterial compound, N-Octanesulfonylacetamide, and its effects on lipid and mycolic acid synthesis. **45:** 1143-1150.

Patel, B. K. R., D. K. Banerjee, and P.D. Butcher. 1993. Determination of *Mycobacterium leprae* viability by polymerase chain reaction amplification of 71-kDa heat-shock protein mRNA. J. Infect. Dis. **168:** 799-800.

Okuyama, H., T. Kankura, and S. Nojima. 1967. Position distribution of fatty acids in phospholipids from mycobacteria. J. Biochem, Tokyo. **61:** 732-737.

Phetsuksiri B., A.R. Baulard, A.M. Cooper, D.E. Minnikin, J.D. Douglas, G.S. Besra, and P.J. Brennan. 1999. Antimycobacterial activities of isoxyl and new derivatives through the inhibition of mycolic acid synthesis. Antimicrob. Agents Chomother. **43:** 1042-1051.

Phetsuksiri B., M. Jackson , H. Scherman, M. McNeil, G.S. Besra, A.R. Baulard, R.A. Slayden, A.E. DeBarber, C.E. Barry 3rd, M.S. Baird , D.C. Crick, and P.J. Brennan. 2003. Unique mechanism of action of the thiourea drug isoxyl on *Mycobacterium tuberculosis*. J. Biol. Chem. **278:** 53123-53130.

Ratledge, C. 1982. Lipids: cell composition, fatty acid biosyntheses, p.53-94. *In* C. Ratledge and J. Standford (ed.), The biology of mycobacteria, vol. 1. Academic Press, Inc., San Diego, California.

Russel, N.J. 1984. Mechanisms of thermal adaptation in bacteria: blueprint for survival. Trands Bichem. Sci. **9:** 108-112.

Slayden, R.A., R.E. Lee, J.W. Armour, A.M. Cooper, I.M. Orme, P.J. Brennan, and G.S. Besra. 1996. Anti-mycobacterial action of thiolactomycin: an inhibitor of fatty acid and mycolic acid synthesis in mycobacteria. Antimicrob. Agents Chemother. **40:**2813-2819.

Stubbs, C.D. and A.D. Smith. 1984. 1984. The modification of mammalian membrane polysaturated fattya cid composition in relation to membrane fluidity and function. Biochim. Biophy. Acta. **779:** 89-137.

Titscher, R. 1966. Monotherapie mit isoxyl/DAT bei tuberculose-asylierungsfallen. Prax. Pneumol. **20:** 202.

Urbancik, B. 1966. A clinical trial of thiocarlide (isoxyl). Tubercle. Edinb., **47:** 283-288.

Urbancik, B. 1970. Clinical experiences with thiocarlide (isoxyl). Antibiot. Chemother. **16:** 117-123.

Walker, R.W., H. Barakat, and J.G.C. Hung. 1970. The positional istribution of fattya cids in phospholipids and tri-glycerides of Mycobacterium smegmatis and M. bovis, Lipids. 5: 684-691.

Winder, F.G., and Collins. 1970. Inhibition by isoniazid of synthesis of mycolic acids in *Mycobacterium tuberculosis* BCG. J. Gen. Microbiol. **66:** 379-380.

Winder, F.G., P.B. Collins, and D. Whelan. 1971. Effect of ethionamide and isoxyl on mycolic acid synthesis in *Mycobacterium tuberculosis*. Antimicrob. Agent Chemother. **40:** 426-428.

Ratledge, C. 1982. Lipids: cell composition, fatty acid biosyntheses, p.53-94. *In* C. Ratledge and J. Standford (ed.), The biology of mycobacteria, vol. 1. Academic Press, Inc., San Diego, California.

Russel, N.J. 1984. Mechanisms of thermal adaptation in bacteria: blueprint for survival. Trands Bichem. Sci. **9:** 108-112.

Schimid, P.C.H. 1970. Clinical experience in cases of primary tuberculosis with tuberculostaticum isoxyl. Antibiot. Chemther. **16:** 108-116.

Slayden, R.A., R.E. Lee, J.W. Armour, A.M. Cooper, I.M. Orme, P.J. Brennan, and G.S. Besra. 1996. Anti-mycobacterial action of thiolactomycin: an inhibitor of fatty acid and mycolic acid synthesis in mycobacteria. Antimicrob. Agents Chemothor. **40:**2813-2819.

Stubbs, C.D. and A.D. Smith. 1984. 1984. The modification of mammalian membrane polysaturated fattya cid composition in relation to membrane fluidity and function. Biochim. Biophy. Acta. **779:** 89-137.

Titscher, R. 1966. Monotherapie mit isoxyl/DAT bei tuberculose-asylierungsfallen. Prax. Pneumol. **20:** 202.

Urbancik, B. 1966. A clinical trial of thiocarlide (isoxyl). Tubercle. Edinb., **47:** 283-288.

Urbancik, B. 1970. Clinical experiences with thiocarlide (isoxyl). Antibiot. Chemother. **16:** 117-123.

Walker, R.W., H. Barakat, and J.G.C. Hung. 1970. The positional istribution of fattya cids in phospholipids and tri-glycerides of Mycobacterium smegmatis and M. bovis, Lipids. 5: 684-691.

Winder, F.G., and Collins. 1970. Inhibition by isoniazid of synthesis of mycolic acids in *Mycobacterium tuberculosis* BCG. J. Gen. Microbiol. **66:** 379-380.

Winder, F.G., P.B. Collins, and D. Whelan. 1971. Effect of ethionamide and isoxyl on mycolic acid synthesis in *Mycobacterium tuberculosis*. Antimicrob. Agent Chemother. **40**: 426-428.

Winder, F.G. 1982. Mode of action of the antimycobacterial agents and associated aspects of the molecular biology of mycobacteria. P 353-438. In C. Ratledge and J. Standford (ed.) The biology of mycobacteria. Vol. 1. Academic Press, Inc., New York. N.Y.

Yajko, D.M., J.J. Madez, M.V. Lancaster, C.A. Sanders, V.L. Cawthon, B. gee, A. Babst, and W.K. Hadley. 1995. Colorimetric method for determining MICs of antimicrobial agents for *Mycobacterium tuberculosis*. J. Clin. Microbiol. **33**: 2324-2327.

Out put จากโครงการวิจัย

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ ดังนี้

   เรื่อง Unique mechanism of action of the thiourea drug, Isoxyl, on *Mycobacterium tuberculosis.*

   ผู้แต่ง Benjawan Phetsuksiri *et al.*

   วารสาร Journal of Biological Chemistry (JBC). 2003. 278: 53123-53130.

   เรื่อง New LightCycler real-time reverse transcription-PCR for quantitative detection of mRNA of *desA3* in *Mycobacterium tuberculosis.*

   ผู้แต่ง Benjawan Phetsuksiri *et al.*

   วารสาร Journal of Molecular Cell Probe (Mol & Cell Probes)

   เรื่อง Investigation of effects of isoxyl and new thiourea derivatives involved oleic acid synthesis in *Mycobacterium tuberculosis.*

   ผู้แต่ง Benjawan Phetsuksiri *et al.*

   วารสาร Antimicrobial Agents and Chemotherapy

2. การนำผลงานวิจัยไปใช้ประโยชน์

ผลงานวิจัย ทำให้ทราบว่า สารประกอบ thiourea และอนุพันธ์ใหม่ มีคุณสมบัติในการต้านเชื้อวัณโรค มีฤทธิ์ทำลายเชื้อวัณโรคที่ความเข้มข้นต่ำ ด้วยความเข้มข้น 0.35-10.0 μg/ml และยอกฤทธิ์ยับยั้งการสังเคราะห์ oleic acid ซึ่งไม่พบผลนี้ในเชื้อวัณโรคที่เกิดจากการใช้ยา isoniazid และ ethionomide ดังนั้นกลไกการออกฤทธิ์ของสาร thiourea จึงแตกต่างจากยา isoniazid และ ethionomide ที่เป็นยาสำคัญในการรักษาวัณโรคใช้สารต้นแบบ

- มีการสร้างเครือข่ายความร่วมมือวิจัย
- มีการอ้างอิง จากผลงานวิจัยที่เผยแพร่ในวารสาร
- มีการนำไปศึกษาต่อ โดยผลจากการศึกษาวิจัยพบว่า สาร thiourea และอนุพันธุ์ใหม่ มีฤทธิ์ทำลายเชื้อวัณโรคที่ความเข้มข้นต่ำในช่วง 0.35-10.0 μg/ml ฤทธิ์ทำลายเชื้อวัณโรคที่ศึกษาแล้ว พบว่า เกิดจากการยับยั้งการสังเคราะห์กรดไขมัน ได้แก่ กรดไขมันไม่อิ่มตัว (unsaturated fatty acid) ที่สำคัญ คือ oleic acid และยับยั้งการสังเคราะห์ mycolic acids โดยฤทธิ์ยับยั้งการสังเคราะห์ oleic acid ไม่ปรากฏในการใช้ยา isoniazid และ rifampicin ซึ่งเป็นยาหลักในการรักษาวัณโรค ดังนั้น กลไกการออกฤทธิ์ของสาร thiourea และอนุพันธุ์ใหม่จึงน่าจะแตกต่างจากกลไกการออกฤทธิ์ของยา isoniazid และ rifampicin

การสังเคราะห์ oleic acid ในเชื้อวัณโรคอาศัยเอนไซม์ desaturase พบว่า overexpression ของยีน *desA3* ในเชื้อวัณโรค มีผลทำให้เซลล์มีการสังเคราะห์ oleic acid มากขึ้นและค่า Minimal Inhibitory Concentration (MIC) ของ isoxyl ในเชื้อวัณโรคเพิ่มสูงขึ้นด้วย ดังนั้น ยีน *desA3* จึงเป็นยีนสังเคราะห์ desatrirase ที่เป็นเอนไซม์ที่เป็นเป้าหมายที่สาร thiourea ใช้ออกฤทธิ์ การศึกษาต่อไป จึงเป็นที่น่าสนใจ เนื่องจาก ได้แสดงให้เห็นแล้วว่า desaturase ที่เป็นผลผลิตของยีน *desA3* มีคุณสมบัติในการเป็นเป้าหมายใหม่ของยาต้านวัณโรคและสารในกลุ่ม thiourea มีคุณสมบัติในการต้านเชื้อวัณโรค การศึกษาโครงสร้างของเอนไซม์ จะนำไปสู่การค้นพบยาใหม่ และการศึกษาปฏิกิริยา deasaturation ในเชื้อวัณโรค จะทำให้เข้าใจชีววิทยาของเชื้อที่เป็นประโยชน์ต่อการพัฒนายาต้านวัณโรคใหม่ด้วย

- มีการนำเทคโนโลยีหรือวิธีการตรวจวิเคราะห์ไปประยุกต์ใช้ในการศึกษาวิจัยยีนหรือใช้เพื่อการตรวจวิเคราะห์เชื้อวัณโรค

Out put จากโครงการวิจัย

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ ดังนี้

   เรื่อง Unique mechanism of action of the thiourea drug, Isoxyl, on *Mycobacterium tuberculosis*.

   ผู้แต่ง Benjawan Phetsuksiri *et al.*

   วารสาร Journal of Biological Chemistry (JBC). 2003. 278: 53123-53130.

   เรื่อง New LightCycler real-time reverse transcription-PCR for quantitative detection of mRNA of *desA3* in *Mycobacterium tuberculosis*.

   ผู้แต่ง Benjawan Phetsuksiri *et al.*

   วารสาร Journal of Molecular and Cellular Probes (Mol Cell Probes)

   เรื่อง Investigation of effects of isoxyl and new thiourea derivatives involved oleic acid synthesis in *Mycobacterium tuberculosis*.

   ผู้แต่ง Benjawan Phetsuksiri *et al.*

   วารสาร Antimicrobial Agents and Chemotherapy (Antimicrob. Agents Chemother.)

2. การนำผลงานวิจัยไปใช้ประโยชน์

ผลงานวิจัย ทำให้ทราบว่า สารประกอบ thiourea และอนุพันธ์ใหม่ มีคุณสมบัติในการต้านเชื้อวัณโรค มีฤทธิ์ทำลายเชื้อวัณโรคที่ความเข้มข้นต่ำ ด้วยความเข้มข้น 0.35-10.0 µg/ml และยกฤทธิ์ยับยั้งการสังเคราะห์ oleic acid ซึ่งไม่พบผลนี้ในเชื้อวัณโรคที่เกิดจากการใช้ยา isoniazid และ ethionomide ดังนั้นกลไกการออกฤทธิ์ของสาร thiourea คงแตกต่างจากยา isoniazid และ ethionomide ที่เป็นยาสำคัญในการรักษาวัณโรคใช้สารต้นแบบ

- มีการสร้างเครือข่ายความร่วมมือวิจัย
- มีการอ้างถึง จากผลงานวิจัยที่เผยแพร่ในวารสาร
- มีการนำไปศึกษาต่อ โดยผลจากการศึกษาวิจัยพบว่า สาร thiourea และอนุพันธุ์ใหม่ มีฤทธิ์ทำลายเชื้อวัณโรคที่ความเข้มข้นต่ำในช่วง 0.35-10.0 μ g/ml ฤทธิ์ทำลายเชื้อวัณโรคที่ศึกษาแล้ว พบว่า เกิดจากการยับยั้งการสังเคราะห์กรดไขมัน ได้แก่ กรดไขมันไม่อิ่มตัว (unsaturated fatty acid) ที่สำคัญ คือ oleic acid และยับยั้งการสังเคราะห์ mycolic acids โดยฤทธิ์ยับยั้งการสังเคราะห์ oleic acid ไม่ปรากฏในการใช้ยา isoniazid และ rifampicin ซึ่งเป็นยาหลักในการรักษาวัณโรค ดังนั้น กลไกการออกฤทธิ์ของสาร thiourea

และอนุพันธุ์ใหม่จึงน่าจะแตกต่างจากกลไกการออกฤทธิ์ของยา isoniazid และ rifampicin

การสังเคราะห์ oleic acid ในเชื้อวัณโรคอาศัยเอนไซม์ desaturase พบว่า overexpression ของยีน *desA3* ในเชื้อวัณโรค มีผลทำให้เซลล์มีการสังเคราะห์ oleic acid มากขึ้นและค่า Minimal Inhibitory Concentration (MIC) ของ isoxyl ในเชื้อวัณโรคเพิ่มสูงขึ้นด้วย ดังนั้น ยีน *desA3* จึงเป็นยีนสังเคราะห์ desatrirase ที่เป็นเอนไซม์ที่เป็นเป้าหมายที่สาร thiourea ใช้ออกฤทธิ์ การศึกษาต่อไป จึงเป็นที่น่าสนใจ เนื่องจาก ได้แสดงให้เห็นแล้วว่า desaturase ที่เป็นผลผลิตของยีน *desA3* มีคุณสมบัติในการเป็นเป้าหมายใหม่ของยาต้านวัณโรคและสารในกลุ่ม thiourea มีคุณสมบัติในการต้านเชื้อวัณโรค การศึกษาโครงสร้างของเอนไซม์ จะนำไปสู่การค้นพบยาใหม่ และการศึกษาปฏิกิริยา desaturation ในเชื้อวัณโรค จะทำให้เข้าใจชีววิทยาของเชื้อที่เป็นประโยชน์ต่อการพัฒนายาต้านวัณโรคใหม่ด้วย

- มีการนำเทคโนโลยีหรือวิธีการตรวจวิเคราะห์ไปประยุกต์ใช้ในการศึกษาวิจัยอื่นหรือใช้เพื่อการตรวจวิเคราะห์เชื้อวัณโรค

บทความเผยแพร่ผลงานวิจัย

โครงการ กลไกการออกฤทธิ์ของสารต้านเชื้อวัณโรค thiourea และอนุพันธ์ใหม่ และผลต่อการแสดงออกของยีน *desA3* ในเชื้อวัณโรค
(Thiourea and new derivatives: Mode of action and effects on the expression of the *Mycobacterium tuberculosis desA3* gene)

โดย ดร. เบญจวรรณ เพชรสุขศิริ
สังกัดเดิม กรมควบคุมโรคติดต่อ กระทรวงสาธารณสุข
สังกัดปัจจุบัน สถาบันวิจัยวิทยาศาสตร์สาธารณสุข กรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข

สัญญาเลขที่ PDF/60/2544

บทความที่ 1 เรื่อง Unique Mechanism of Action of the Thiourea Drug, Isoxyl, on *Mycobacterium tuberculosis*.
ส่ง Journal of Biological Chemistry

Investigation of effects of isoxyl/thiourea and new derivatives involved oleic acid synthesis in *Mycobacterium tuberculosis*.

Benjawan Phetsuksiri§, Janisara Rudeeaneksin§, Dean C. Crick‡**, Gurdyal S. Besra∫, James D. Douglas∫, Prasit PalittapongarnpimΦ, and Patrick J. Brennan‡**

*From § the Department of Medical Sciences, ‡**Department of Microbiology, Pathology and Immunology, Colorado State University, Fort Collins, Colorado 80523-1682, USA, USA, Φ the Department of Microbiology, Mahidol University, Bangkok, Thailand and ∫Department of Chemistry, University of Wales, Bangor, Wales,*

§Present Address: *The Sasakawa Research Building, Thai National Institute of Health , Department of Medical Sciences, Ministry of Public Health, Nonthaburi Province, Thailand 11000.*

∫Present Address: *School of Biosciences, University of Birmingham, Birmingham, United Kingdom*

*This work was funded by the Thailand Research Fund (TRF). BP was a recipient of a grant PDF/60/2544 from TRF.

**Corresponding author. Mailing address: Thai National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Thailand. Phone: (66 2) 589-1567. Fax: (66 2) 965-9700. E-mail: beniapsk@health.moph.go.th

Running title: Effect of the Thiourea, Isoxyl, on *Mycobacterium tuberculosis*

ABSTRACT

Isoxyl, a thiourea (4, 4' diisoamyloxydiphenylthiourea) and new synthetic derivatives were investigated in vitro for their antimycobacterial activity against *Mycobacterium tuberculosis*. By employing the rapid microplate alamar blue assay, isoxyl was shown to be highly effective against clinical isolate of *Mycobacterium tuberculosis* with MIC of 0.30-1.25 µg/ml. New derivatives also exhibited potent activity against *M. tuberculosis* with MIC in the range of 0.30-10.0 µg/ml. The selective mode of action of thiourea was demonstrated by whole cell labeling of *M. tuberculosis* with [1, 2 -C^{14}] acetate. Analysis of labeled fatty acids by thin layer chromatography (TLC) demonstrated the inhibitory effects on the synthesis of oleic acid, indicating a unique mechanism of action. The specificity of the inhibition on oleic acid synthesis pointed to a $\Delta 9$ stearoyl-desaturase as a drug target. The transcripts of the *M. tuberculosis desA3*, which encodes $\Delta 9$ stearoyl-desaturase, were detected by reverse transcription polymerase chain reaction (RT-PCR) demonstrating the expression and importance of enzymatic function. A new LightCycler real-time RT-PCR was developed to monitor the expression of *desA3* in response to thiourea. *M. tuberculosis* RNA could be extracted by the use of a modified commercially ready-to-use guanidine-phenol extraction method . The single tube reaction of real-time RT-PCR was performed in glass capillary containing the SYBR Green I dye as a detection signal. The amplification of 341 nucleotide region of the *desA3* gene specific for *M. tuberculosis* by real-time RT-PCR demonstrated that *desA3* transcripts were diminished in cells exposed to thiourea. These results confirmed that thiourea is a novel antituberculosis agent which had specific mechanism by inhibiting the synthesis of oleic acid and affected the expression of *desA3* in *M. tuberculosis*. We propose here that thioureas serve as a promising compound for future antituberculosis drug development and DesA3 is a new therapeutic target worthy for

further study.

Tuberculosis continues to be the leading cause of death caused by infectious agent worldwide (Bloom and Murray, 1992). The prevalence of tuberculosis, particularly in concert with HIV infection and AIDS, has been well documented (WHO, 2002, Williams and Dye. 2003). In this regard, it is estimated that over 8 million people contract tuberculosis each year, and approximately 2 to 3 million people die of this disease (Murray and Salomon, 1998; Dye et al., 1999). In addition, it is thought that as many as 2 billion people have been exposed to the tuberculosis bacillus and are therefore at risk of developing active disease. This problem is further compounded by a dramatic increase in multidrug-resistant strains of *M. tuberculosis* (Dye et al., 2002). At present, there is a limit of alternative chemotherapeutic regimens, resulting in poor therapeutic outcomes and high mortality rates among MDR-TB patients (Espinal, 2003). The development of new effective antituberculosis drugs with bactericidal mechanisms different from those of the presently available agents is an urgent need to provide more effective treatment and to shorten treatment course to improve patient compliance. It is hoped that new drug target could be identified and new compounds would be effective against the growing number of drug-resistant strains and against bacilli that may be in a state of latency (Orme, 2001).

The thiourea isoxyl (thiocarlide; 4, 4' diisoamyloxy diphenylthiourea), is an old drug and was used clinically to treat tuberculosis during 1960s (Titschcr, 1966; Urbancik, 1966; Urbancik, 1970). Little was known of its mode of action. Previously, isoxyl was shown to have considerable antimycobacterial activity *in vitro* and to be effective against various clinical isolates

of multidrug-resistant strains of *Mycobacterium tuberculosis* in the sensitive range of 1-10 µg/ml (Phetsuksiri, et al., 1999). An early note reported that ISO, like isoniazid (INH) and ethionamide (ETH), isoxyl exhibited strong inhibition on the synthesis of mycolic acids (Winder, 1982), a result since confirmed with the demonstration that all types of mycolic acids are affected (Phetsuksiri, et al., 1999). In addition, it was noted that isoxyl partially inhibited the synthesis of shorter chain fatty acids of free lipids (Phetsuksiri, et al., 1999; Winder and Collins, 1970; Winder, et al., 1971; Winder, 1982), which was stimulated by INH and ETH, suggesting inhibitory effects different from those of INH and ETH. Recently, we documented that isoxyl inhibited the synthesis of unsaturated fatty acids identified as to oleic acid. In turn, esterified oleic acid is a direct precursor of tuberculostearic acid (Lennarz, et al., 1962), and, therefore, the consequent effect of ISO is the inhibition of tuberculostearic acid synthesis. Inhibitory effects of isoxyl, on the synthesis of oleic acid and tuberculostearic acid in *M. tuberculosis* was in dose-response manner with complete inhibition at 3 µg/ml. As a result, isoxyl caused accumulation of stearic acid in *M. tuberculosis* and *M. bovis* BCG (Phetsuksiri, et al., 2003). This evidence suggested that ISO acts by inhibiting $\Delta 9$ desaturase, an enzyme that introduces one double bond at carbon 9 of stearic acid to form oleic acid (Fulco and Bloch, 1964; Kashiwabara and Sato, 1973; Kashiwabara, et al., 1975).

It is a fundamental and essential step to find and validate new targets for drug design and development. Elucidation of such gene encode enzymatic target was approached by using molecular genetics, biochemical analysis and enzyme inhibitors that affect the function of gene products. Isoxyl was used as a tool to identify a specific gene that encodes $\Delta 9$ desaturase. The purified DesA3 enzyme from the *M. tuberculosis* was partially characterized and shown to

increase the synthesis of oleic acid and was susceptible to isoxyl. These results validated membrane-bound $\Delta 9$ desaturase, DesA3, as a new therapeutic target, and the thioureas as anti-tuberculosis drugs worthy of further development (Phetsuksiri, et al., 2003).

The aims of this study were to assess the in vitro activity of new derivatives of thioureas against *M. tuberculosis* and examine potential effects of thiourea compounds on the expression of the *desA3* gene, known to encoded $\Delta 9$ desaturase in *M tuberculosis*. In this study, three new derivatives of thioureas were tested for capacity to kill *M. tuberculosis* in vitro by a rapid test, a microplate alamar blue assay. Effects of new derivatives on the inhibition of oleic acid synthesis was determined by whole cell labeling using [1, 2-C^{14}] sodium acetate, a precursor for fatty acid synthesis. Monitoring of change in the expression of the *desA3* gene to Isoxyl was performed by lightcycler real-time reverse transcription PCR. In general, the results were promising and the apparent antimycobacterial effects of new derivatives of thiourea could be seen.

The microplate alamar blue assay modified from Yajko et al. (1995), was used to determine the MIC of isoxyl and new derivatives. Inocula were prepared from *M. tuberculosis*. Cells were grown in Sauton's medium to a turbidity equal to that of a No. 1 McFarland standard (~2.0 x 10^7 CFU/ml). Serial dilutions of isoxyl and new derivatives were prepared from dimethylsulfoxide (DMSO) stock in Sauton's medium. From each dilution 5 µl was then inoculated into 85 µl of cultures that had been dispensed into clear-bottomed, 96-well microplates prior to adding the drug. Duplicate treatments with different concentrations of ISO were performed. Four controls were included which contained medium only, culture only, medium plus DMSO, and culture plus DMSO. At the outset, 10 x Alamar Blue solution

(Accumed; OH) was added to the controls. After overnight incubation at 37°C in a moisture-controlled chamber, Alamar Blue was added to the all treated wells. Plates were further incubated; and the color in each treated culture was recorded until the color in the culture control turned pink. The MICs was determined as the lowest concentration of isoxyl and new thiourea derivatives in which the blue dye in the treated culture did not turn pink.

To determine inhibitory effect of thioureas on the synthesis of oleic acid, whole cell labeling with [1,2-^{14}C]acetate, of *M. tuberculosis* fatty acids and mycolic acids were performed. Briefly, *M. tuberculosis* H37Rv were grown in 5 ml of Sauton's medium in a set of culture tubes to early exponential phase, at which point different concentrations of each thiourea was added, followed by further incubation gentle shaking at 37°C for 6 h prior to the addition of [1,2-^{14}C] acetate (Dupont NEN; Boston, Mass.) at 0.5 µCi/ml. Cells were labeled for 18 h, harvested by centrifugation at 2,500 x g, and washed twice with saline and once with sterile water (Slayden, et al., 1996). Saponification was conducted with 15% tetrabutylammonium hydroxide at 100° C overnight. After cooling, dichloromethane and iodomethane were added to generate fatty acid methyl esters (FAMEs) and mycolic acid methyl esters (MAMEs) which then were extracted by diethylether (Phetsuksiri, et al., 1999). Finally, equal volumes of this extract, which contained FAMEs and MAMEs, were applied to preparative silica gel TLC plates (silica gel 60 F_{254}, Merck; Darmstadt, Germany) impregnated with 5% silver nitrate (Morris, 1966). After the plates were developed two times in petroleum ether-acetone (90:10), autoradiograms were produced by overnight exposure of the impregnated TLC plates to Kodax X-Omat AR film at -70°C. Separated bands of oleic acid, saturated fatty acid and individual mycolic acids were marked, cut from the TLC plates, placed directly in 5 ml of scintillation fluid (EcoLume®). Radioactivity

was counted to estimate the degree of inhibition of the synthesis of oleic acid and individual populations of MAMEs by liquid scintillation counter.

Real-time RT-PCR was developed and applied to monitor the change of the expression of *desA3* in response to isoxyl in *M. tuberculosis*. Cells were grown in Sauton medium to early exponential phase, at which time isoxyl was added to final concentrations of 2.0 µg/ml. To control cell numbers in both control and isoxyl treated culture, cells were harvested after 6 h exposure to the drug. Total RNA was then extracted by the use of Trizol reagent according to manufacturer's instruction with modification. Briefly, cells were lysed by sonication at 4° C for 10 min followed by the addition of lysozyme (Amersham Pharmacia) prior to the addition of Trizol reagent. At the final step, RNA pellet was recovered by precipitating with absolute cold ethanol, 0.3 M sodium acetate and 1 µl of 20 mg/ml glycogen. After washed and dried, the purified RNA was resuspended in RNase free water for subsequent analysis to quantify *M. tuberculosis desA3* transcript by in real-time RT-PCR.

A single-tube real-time RT-PCR reaction in a total volume of 20 µl was carried out in a programmable lightcycler machine (Roche) using specific *desA3* primers, GGT GAC CGA CTT AGC CAT ACG CTT, upper primer; AGC ATC ACC TCT ATC CGG AC TGC C, lower primer, which were designed by the Oligo software (Version 6.0; National Bioscience Inc; Plymouth, Minn), and were synthesized at BIOTEC, Thailand. The component of LC-PCR in a final volume of 20 µl included 2 µl of ready to use reaction mix, which contains reaction buffer, reverse transcriptase and *Taq* polymerase enzymesdNTP, 4 mM $MgCl_2$, 10 pmol of the primer pairs and SYBR Green I dye. The thermal program of RT-PCR consists of the reverse transcription at 55°

C for 60 S and PCR cycles. Initial denaturation step was performed at 95° C for 30 S followed by 40 cycles of two-step thermal sequences; 95° C for 10 S and 69° C for 10 S. Amplicons were detected by fluorometer equipped with the LightCycler machine and data acquisition was facilitated through the detection of the fluorescent dsDNA binding dye, SYBR Green. The melting temperature (T_m) of RT-PCR product was used to characterize the amplified product. When the amplification was completed, a melting program was conducted from 60° C to 99° C at 20 ° C/S with continuous monitoring of the fluorescense. Direct sequencing of RT-PCR product was performed with automated sequencer using forward and reverse primers. Data analysis was carried out with system software of LightCycler (Roche). The amplicons obtained from real-time RT-PCR were also analyzed for 341-bp fragment of *M. tuberculosis* by 1.0% agarose gel electrophoresis A negative control, which is a reaction that template RNA, was replaced by the PCR-grade water was included to detect carry over contamination.

Figure 1 shows the structures of isoxyl and new thiourea derivatives. The rapid microplate alamar blue assay was employed to investigate the inhibitory activity of these thioureas against *M. tuberculosis*. Results indicate that thioureas, were effective against *M. tuberculosis*. Thioureas inhibited the growth of the *M. tuberculosis* clinical isolate in a dose-dependent manner. The minimal inhibitory concentration of isoxyl is 0.30 mg/L and four new derivatives were 0.30, 5.0 and 10.0, respectively. Studies confirmed the in vitro activity of thioureas against 2.86×10^5 CFU of drug-susceptible strain of *M. tuberculosis* clinical isolate.

Mechanism-of-action studies were conducted by examining the effect of thiourea on the incorporation of radiolabelled precursors of fatty acids into respective mycobacterial oleic acids.

The inhibitory effect of isoxyl and thioureas on the synthesis of oleic acid is indicated in Fig. 2. ISO inhibited the incorporation of [^{14}C] from [1,2-^{14}C]acetate into oleic acid and MAMEs of *M. tuberculosis*.

Genomic technologies have the potential to greatly increase the efficiency of the drug and drug target discovery. By conventional RT-PCR, the fragment of 341-bp specific to the portion of *desA3* sequence could be detected. The presence of mRNA of *desA3* was demonstrated in *M. tuberculosis* by conventional RT-PCR indicating the essential of gene function. Fig 3 showed that the effects of isoxyl on the expression of *desA3* in *M. tuberculosis* could not be observed by means of conventional RT-PCR (Fig 3). In attempt to define effect of thioures on the expression of *desA3* in *M. tuberculosis*, we have developed a real-time RT-PCR targeting 341-bp fragment of *M. tuberculosis desA3* transcripts. The RT-PCR product could be identified by melting curve analysis. The single product of RT-PCR as illustarted in Fig 3 has a melting temperature pattern as shown in Fig 4. This real-time RT-PCR is able to demonstrate the change of *desA3* gene expression in thiourea-treated *M. tuberculosis*. This approach provided a means to rapidly confirm the mode of action of thioureas involved oleic acid synthesis.

It is prudent to re-examine drugs that were formerly deemed effective against tuberculosis. Isoxyl is a thiourea derivative that was successfully used in the 1960s to treat tuberculosis (Titscher, 1966; Urbancik, 1966; Urbancik, 1970). We conclude that isoxyl and new derivatives have antimycobacterial activity. The unique mechanism of new derivatives on the inhibition of oleic acid was observed. Isoxyl inhibited the growth of *M. tuberculosis* and hence metabolic rate was retard and may cause a decrease of *desA3* gene expression in *M. tuberculosis*.

ACKNOWLEDGEMENT

This work was supported by the grant from the Thailand Research Fund and the supplementation from Department of Disease Control, Ministry of Public Health, Thailand. We are grateful to Sirirat at Bamrasnradura Institute, Department of Disease Control for providing facilities of LC machine, Culture Collection Section of Thai NIH, Department of Medical Sciences, and Dr. Aniksana Chalprasert, Siriraj Hospital, for providing mycobacterial cultures. We thank Sopa Srisangngam for excellent technical assistance.

REFERENCE

Bloom, B., and C. Murray. 1992. Tuberculosis: commentary on a reemergent killer. Science. **257:** 1055-1064.

Dye, C., S. Scheele, P. Dolin, V. Pathania, and M.C. Raviglione. 1999. Consensus statement . Global Burden of tuberculosis: estimated incidence, prevalence, and mortality by country. WHO global surveillance and monitoring project. JAMA. **282:** 677-686.

Dye, C., M.A. Espinal , C.J. Watt , C. Mbiaga, andB.G. Williams. 2002. Worldwide incidence of multidrug-resistant tuberculosis. J Infect Dis. **185:** 1197-202.

Espinal, M.A. 2003. Tuberculosis (Edinb). The global situation of MDR-TB. **8:** 44-51.

Fulco, A.J., and K. Bloch. 1964. Cofactor requirements for the formation of 9-unsturated fatty acids in *Mycobacterium phlei*. J. Biol.Chem. **239:** 993-997.

Kashiwabara, Y., and R.Sato.1973. Electron transfer mechanism involved in stearoyl-coenzyme A desaturase activity. J. Biochem. Tokyo. **74:** 803-810.

Kashiwabara, Y. H. Nakagawa, G. Matsuki, and R. Sato. 1975. Effect of metal ions in the culture medium on the stearoyl-coenzyme A desaturase activity of *Mycobacterium phlei*. J. Biochem. Toyko. **74:** 803-810.

Lennarz, W.J., G. Scheuerbrandt, and K. Bloch. 1962. The biosynthesis of oleic acid and 10-methylstearic acids in *Mycobacterium phlei*. J. Biol. Chem. **237:** 664-671.

Morris, L.J. 1966. Separation of lipids by silver ion chromatography. J. Lipid Res. **7:** 717-730.

Murray, C.J., and J.A. Salomon. 1998. Modeling the impact of global tuberculosis control strategies. Proc. Natl. Acad. Sci. USA. **95:** 13881-13886.

Orme, I.A. 2001. Serach for new drug for treatment of tuberculosis. Antimicrob. Agents Chemother. **45:** 1943-1946.

Phetsuksiri B., A.R. Baulard, A.M. Cooper, D.E. Minnikin, J.D. Douglas, G.S. Besra, and P.J. Brennan. 1999. Antimycobacterial activities of isoxyl and new derivatives through the inhibition of mycolic acid synthesis. Antimicrob. Agents Chemother. **43:** 1042-1051.

Phetsuksiri B., M. Jackson , H. Scherman, M. McNeil, G.S. Besra, A.R. Baulard, R.A. Slayden, A.E. DeBarber, C.E. Barry 3rd, M.S. Baird , D.C. Crick, and P.J. Brennan. 2003. Unique mechanism of action of the thiourea drug isoxyl on *Mycobacterium tuberculosis*. J. Biol. Chem. **278:** 53123-53130.

Slayden, R.A., R.E. Lee, J.W. Armour, A.M.. Cooper, I.M. Orme, P.J. Brennan, and G.S. Besra. 1996. Anti-mycobacterial action of thiolactomycin: an inhibitor of fatty acid and mycolic acid synthesis in mycobacteria. Antimicrob. Agents Chemother. **40:** 2813-2819.

Titscher, R. 1966. Monotherapie unit isoxyl/DAT bei tuberculose-asylierungsfallen. Prax. Pneumol. **20:** 202.

Urbancik, B. 1966. A clinical trial of thiocarlide (isoxyl). Tubercle. **47:** 283-288.

Urbancik, B. 1970. Clinical experience with thiocarlide (isoxyl). Antibiot. Chemother. **16:** 117-123.

Williams, B.G., and C. Dye. 2003. Antiretroviral drugs for tuberculosis control in the era of HIV/AIDS. **12:** 1535-7.

Winder, F.G. 1982. Mode of action of the antimycobacterial agents and associated aspects of the molecular biology of mycobacteria. P 353-438. In C. Ratledge and J. Standford (ed.) The biology of mycobacteria. Vol. 1. Academic Press. Inc., New York. N.Y.

Winder, F.G., and Collins. 1970. Inhibition by isoniazid of synthesis of mycolic acids in *Mycobacterium tuberculosis* BCG. J. Gen. Microbiol. **66:** 379-380.

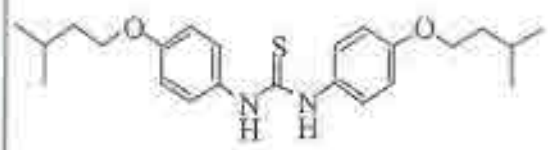
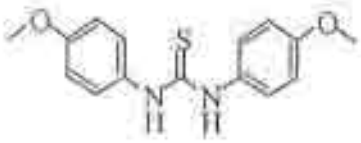
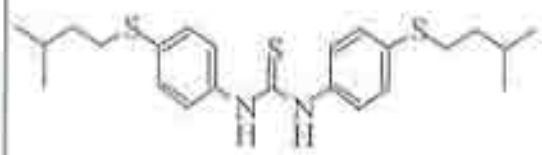
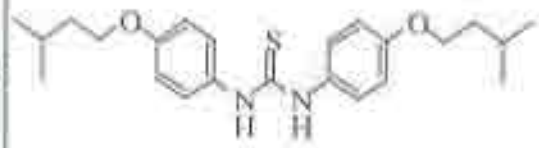
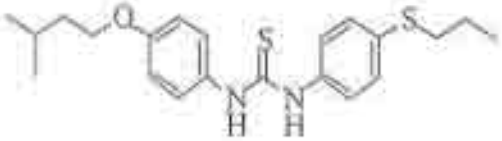
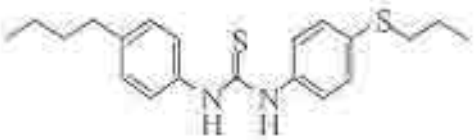
Winder, F.G., P.B. Collins, and D. Whelan. 1971. Effect of ethionamide and isoxyl on *mycolic* acid synthesis in *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. **40:** 426-428.

Winder, F.G. 1982. Mode of action of the antimycobacterial agents and associated aspects of the molecular biology of mycobacteria. P 353-438. *In* C. Ratledge and J. Standford (ed.) The biology of mycobacteria. Vol. 1. Academic Press, Inc., New York, N.Y.

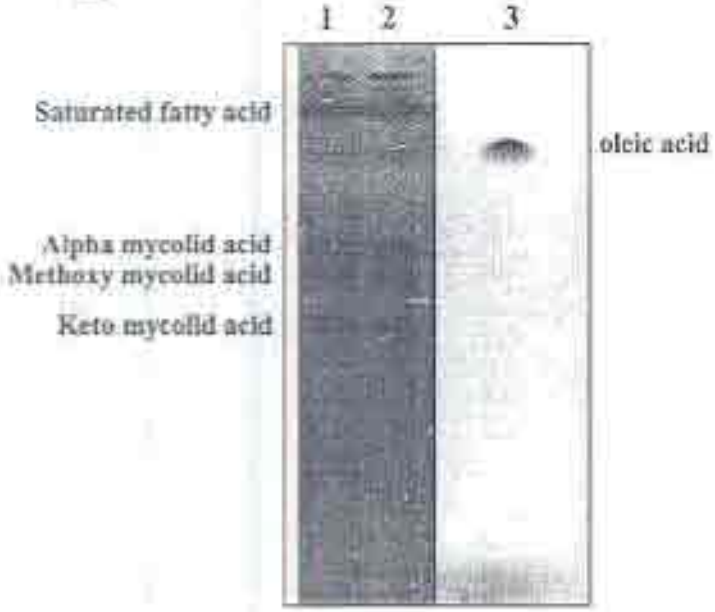
World Health Organization. 2002. Strategic framework to decrease the burden of TB/HIV. Document WHO/CDS/TB2002.296, WHO/HIV_AIDS/2002.2. World Health Organization, Geneva, Switzerland, 2002.

Yajko, D.M., J.J. Madez, M.V. Lancaster, C.A. Sanders, V.L. Cawthon, B. Gee, A. Babst, and W.K. Hadley. 1995. Colorimetric method for determining MICs of antimicrobial agents for *Mycobacterium tuberculosis*. J. Clin. Microbiol. **33**: 2324-2327.

Figure 1 The structures of isoxyl and new thoures derivatives

ISO derivative		
No	**Code**	**Structure**
1	B27	
2	C06	
3	C26	
4	JDDD18	
5	JDDD38	
6	JDDD45	
7	JDDD46	

2A



2B

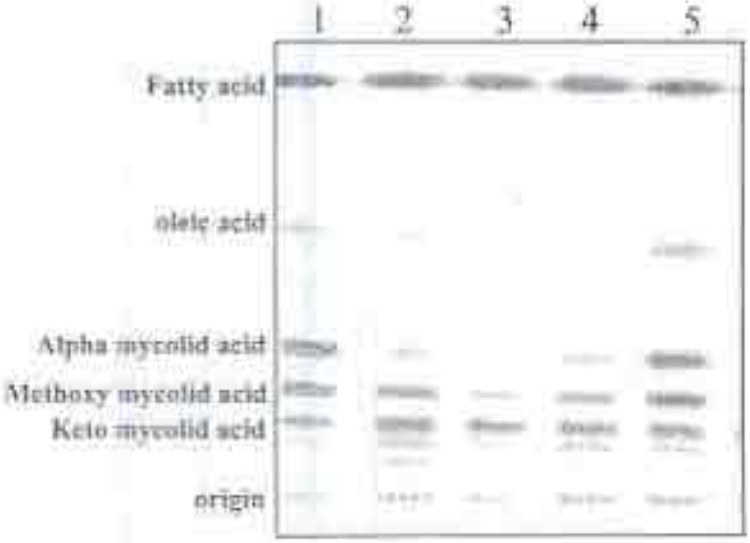


Figure 2: 2A and 2B

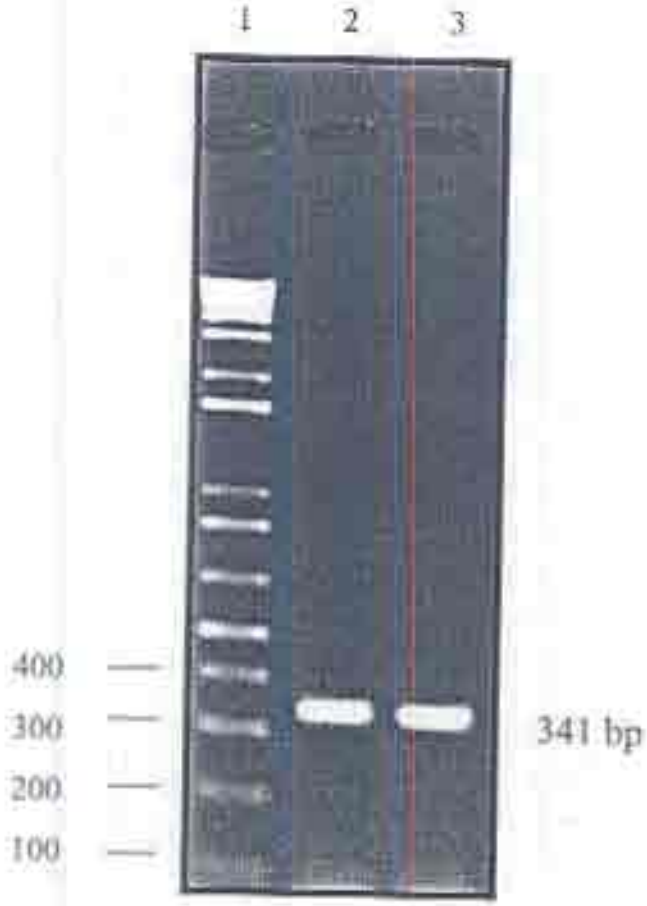


Figure 3

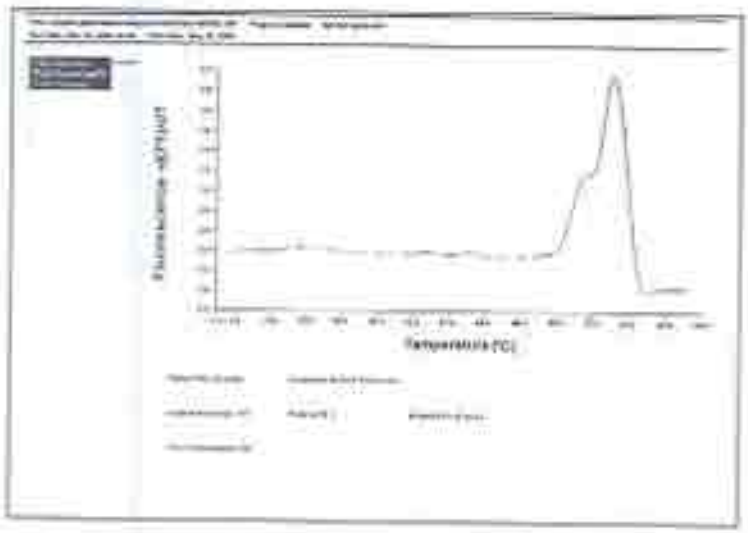


Figure 4

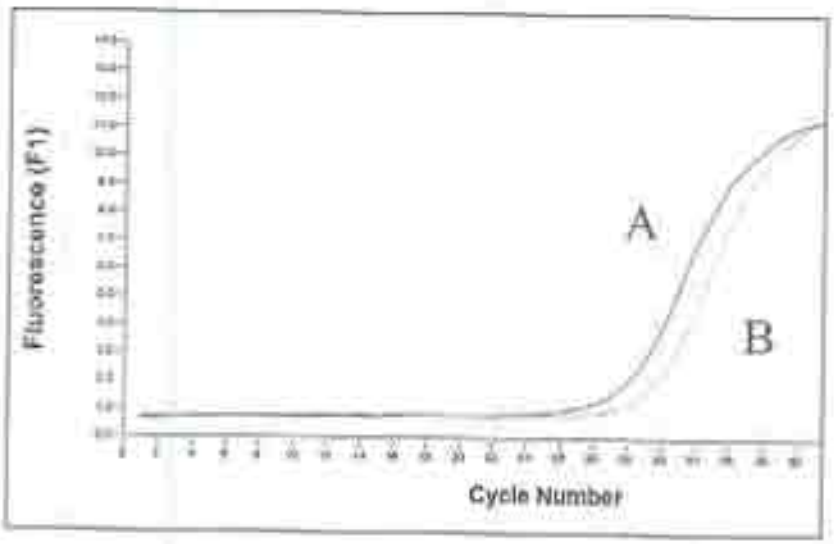


Figure 5

LEGEND

Figure 1 The structures of isoxyl and new thourea derivatives

Figure 2 : 2A: Identification of separated *Mycobacterium tuberculosis* oleic acid using oleic acid standard in form of methyl ether; lane 1-2, fatty acid from *M. tuberculosis*; lane 3, standard of oleic acid; 2B: Lane 1, control; Lane 2, JDD38 2 µg/ml; Lane 3, JDD45 2 µg/ml; Lane 4, JDDD 46 2 µg/ml; Lane 5, control+DMSO

Figure 3 Determination of effect of thiourea on the expression of *M. tuberculosis desA3* by conventional RT-PCR. Lane 1, DNA Marker; Lane 2, *M. tuberculosis* without isoxyl treatment; Lane 3, *M. tuberculosis* with isoxyl treatment at 2 µg/ml for 18 h exposure.

Figure 4 Melting curve analysis pattern. Melting point analysis of amplicons was performed at the end of the amplification run to identify the 341-bp fragment of the *desA3* sequence of *M. tuberculosis*.

Figure 5 Analysis of the effect of thiourea, isoxyl, on the expression of *desA3* in *M. tuberculosis* by real-time RT-PCR. A; Fluorescence signal detected in *M. tuberculosis* without treatment of thiourea, isoxyl. B; Fluorescence signal detected in *M. tuberculosis* treated with isoxyl at 2 µg/ml for 18 h.

Unique Mechanism of Action of the Thiourea Drug, Isoxyl, on *Mycobacterium tuberculosis**

Benjawan Phetsuksiri‡§, Mary Jackson‡¶, Hatairhanok Scherman‡, Michael McNeil‡, Gundyal S. Besra‡Φ, Alain R. Baulard‡⊥, Richard A. Slayden‖, Andrea E. DeBarber‖, Clifton E. Barry, 3rd‖, Mark S. Baird‖, Dean C. Crick‡, and Patrick J. Brennan‡**

From the ‡Department of Microbiology, Pathology and Immunology, Colorado State University, Fort Collins, Colorado 80523-1682, USA, the ‖National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, Maryland 20852, USA, and ‖Department of Chemistry, University of Wales, Bangor, Wales

§Present Address: *The Sasakawa Research Center, Leprosy Division, Department of Communicable Disease Control, Ministry of Public Health, Nonthaburi Province, Thailand 11000*

Present Address: ¶*Institut Pasteur, Unite de Genetique Mycobacterienne, 75724 Paris Cedex 15, France*

ΦPresent Address: *School of Biosciences, University of Birmingham, Birmingham, United Kingdom*

⊥Present Address: *INSERM U447, Institut Pasteur de Lille, 59019 Lille, France*

*This work was funded by grant AI-18357 and grant AI-38087 (a Research Project Agreement from the National Cooperative Drug Discovery Groups for the Treatment of Opportunistic Infections (NCDDG-OI) in AIDS), from the National Institute of Allergy and Infectious Diseases (NIAID), NIH. The work was also supported in part by Thailand Research Fund (TRF). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

BP was a recipient of a Thai Government Scholarship and a grant PDF/60/2544 from TRF. MJ was a recipient of a Post-Doctoral Fellowship from the Heiser Program for Research in Leprosy and Tuberculosis.

**Corresponding author. Mailing address: Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, CO 80523-1682. Phone: (970) 491-6700. Fax: (970) 491-1815. E-mail: Patrick.Brennan@ColoState.edu.

Running title: Effect of the Thiourea, Isoxyl, on *Mycobacterium tuberculosis*

SUMMARY

The thiourea isoxyl (thiocarlide; 4, 4' diisoamyloxy diphenylthiourea), is known to be an effective anti-tuberculosis drug, active against a range of multidrug-resistant strains of *Mycobacterium tuberculosis*, and has been used clinically. Little was known of its mode of action. We now demonstrate that isoxyl results in a dose-dependent decrease in the synthesis of oleic and, consequently, tuberculostearic acid in *M. tuberculosis* with complete inhibition at 3 µg /ml. Synthesis of mycolic acid was also affected. The anti-bacterial effect of isoxyl was partially reversed by supplementing growth medium with oleic acid. The specificity of this inhibition pointed to a Δ9 stearoyl-desaturase as the drug target. Development of a cell-free assay for Δ9 desaturase activity allowed direct demonstration of the inhibition of oleic acid synthesis by isoxyl. Interestingly, sterculic acid, a known inhibitor of Δ9 desaturases, emulated the effect of isoxyl on oleic acid synthesis but did not affect mycolic acid synthesis, demonstrating the lack of a relationship between the two effects of the drug. The three putative fatty acid desaturases in the *M. tuberculosis* genome, *desA1*, *desA2*, and *desA3*, were cloned and expressed in *Mycobacterium bovis* BCG. Cell-free assays and whole cell labeling demonstrated increased Δ 9 desaturase activity and oleic acid synthesis only in the *desA3* overexpressing strain and an increase in the minimal inhibitory concentration for isoxyl, indicating that Des A3 is the target of the drug. These results validate membrane-bound Δ9 desaturase, DesΔ3, as a new therapeutic target, and the thioureas as anti-tuberculosis drugs worthy of further development.

INTRODUCTION

The prevalence of tuberculosis, particularly in concert with HIV infection and AIDS, has been well documented (1). An equally serious public health problem is increasing multi-drug-resistant tuberculosis (MDR-TB) (2). At present, only a few alternative chemotherapeutic regimens are available, resulting in poor therapeutic outcomes and high mortality rates among MDR-TB patients (3). There is an urgent need to develop new effective antituberculosis drugs with bactericidal mechanisms different from those of the presently available agents.

It is prudent to re-examine drugs that were formerly deemed effective against tuberculosis. Isoxyl (ISO)[1] (thiocarlide) (Fig. 1) is a thiourea derivative that was successfully used in the 1960s to treat tuberculosis (4, 5, 6, 7). Recently, ISO was shown to have considerable antimycobacterial activity *in vitro* and to be effective against various clinical isolates of multidrug-resistant strains of *Mycobacterium tuberculosis* in the sensitive range of 1-10 μg/ml (8). An early note reported that ISO, like isoniazid (INH) and ethionamide (ETH), strongly inhibits the synthesis of mycolic acids (9), a result since confirmed with the demonstration that all types of mycolic acids are affected (8). In addition, it was noted that ISO also inhibited shorter chain fatty acid synthesis (8, 9, 10, 11), suggesting inhibitory effects different from those of INH and ETH and raising the prospects of novel fatty acid biosynthesis therapeutic targets exploitable for new drug development against MDR-TB.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions--*Escherichia coli* strain XL-1 Blue (Stratagene, La Jolla, CA), cultured on Luria-Bertani broth or agar medium (Gibco BRL, Rockville, MD) and containing kanamycin at a concentration of 25 μg/ml, was used for generating recombinant clones. *M. bovis* BCG strain 1173P2 was used for whole-cell labeling, MIC determinations, cell-free reactions, and expression of *M. tuberculosis* H37Rv *desA1*, *desA2* and *desA3* genes. Liquid cultures of *M. bovis* BCG and *M. tuberculosis* H37Rv (ATCC 25711) were grown in Sauton medium containing 0.025% tyloxapol (12). Recombinant *M. bovis* BCG clones were selected on Middlebrook 7H11 agar supplemented with oleic acid-albumin-dextrose-catalase (OADC) enrichment (Difco, Detroit, MI) (13) or on Sauton medium containing 20 μg/ml kanamycin. To determine whether the addition of this oleic acid supplement could override the effects of ISO, a series of 10-fold dilutions of *M. tuberculosis* was prepared from stock culture in a glycerol-alanine-salts medium with PBS as diluent. An aliquot (5 μl) of each dilution was spotted on two types of agar plates, those with 7H11 agar medium supplemented with OADC or those with non-oleic acid-containing ADC (albumin-dextrose-catalase), prepared with the incorporation of ISO to a final concentrations of 0.1, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 μg/ml. After inoculation, the plates were incubated at 37°C for 21 days. The growth rates and bactericidal effects were scored by comparing size and number of colonies on plates.

Whole-cell Radiolabeling and Analysis of the In vivo Effects of ISO and Sterculic Acid on Fatty Acid and Mycolic Acid Synthesis--Mycobacteria were grown in Sauton medium at 37°C to A_{650nm} 0.250. ISO (a gift from Dr. P. Draper, National Institute of Medical Research, London, U.K.) was added, followed by further incubation for 8 h prior to the addition of 1,2-[^{14}C]acetate

(110 mCi/mmole; Dupont NEN, Boston, MA) to a final concentration of 1 μCi/ml. Cells were labeled for 24 h, harvested, washed, saponified with 15% tetrabutylammonium hydroxide at 100°C overnight, methylated, and extracted (8). Extracts containing equal amounts of radiolabeled fatty acid methyl esters and mycolic acid methyl esters from control and ISO-treated cells were subjected to TLC on silica gel plates (silica gel $60F_{254}$, Merck, Darmstadt, Germany) which had been impregnated with 10% silver nitrate and activated (14). Plates were developed twice in petroleum ether-acetone (90:10), radioactive bands were located by autoradiography, and the oleic acid methyl ester standard was visualized by spraying with 10% sulfuric acid and anisaldehyde (15).

To analyze the effects of ISO on the synthesis of individual fatty acids, extracted methyl esters were treated with Trisil® (Pierce, Rockford, IL) to silate any free hydroxyl groups, and products were dissolved in hexane and injected onto a capillary HP-1 column (5 m x 0.53 mm i.d.) (Supelco Inc., Bellefonte, PA) coupled to a Hewlett Packard HP 5890 Series II GC with a thermal conductivity detector attached to a "GC-RAM™ radio-detector" (Inus Systems, Tampa, FL). The initial column temperature was 80°C, which was increased to 185°C at a rate of 30°C/min, followed by an increase at the rate of 5°C/min to a final temperature of 345°C. The eluted peaks of labeled FAMEs were identified by comparison of their retention time with those of available fatty acid methyl ester standards. Analysis of the effects of sterculic acid, the Δ9 desaturase inhibitor, on oleic acid synthesis was performed by whole-cell labeling with [1,2-^{14}C]acetate, followed by argentation-TLC and GC analysis, as described above. The effects of sterculic acid and ISO on mycolic acid synthesis were compared by argentation-TLC.

Confirmation of Position of Double Band in Mono-Unsaturated Fatty Acid--Fatty acids were derived from harvests of *M. tuberculosis* H37Rv wild-type, *M. bovis* BCG wild-type, and