



## International Journal of Current Research and Academic Review

ISSN: 2347-3215 Volume 4 Number 12 (December-2016) pp. 103-117

Journal home page: <http://www.ijcrar.com>

doi: <http://dx.doi.org/10.20546/ijcrar.2016.412.010>



### Determination of the Efficacy of Isoxyl, A Mycolic Acid Inhibitor in Vitro against *M. tuberculosis* Strains in Comparison to Other Mycolic Acid Inhibitors

**Shashikant Vaidya\***, Shreyasi Mulye, Mohan Kulkarni, Geeta Koppikar and Abhay Chowdhary

Clinical Pathology Dept, Haffkine Institute for Training, Research and Testing, Acharya Donde Marg, Parel, Mumbai 400012, India

*\*Corresponding author*

#### KEYWORDS

Multi drug resistant (MDR),  
Tuberculosis (TB),  
Mycolic Acid  
Inhibitor.

#### A B S T R A C T

Multi drug resistant (MDR) tuberculosis (TB) has become more prevalent in recent years and second line drugs are not as effective as the standard therapy and are more toxic and expensive. There is an urgent need for new antitubercular drugs. The components of the cell envelope of Mycobacteria have been the subject of intense research for a number of years because of the fact that enzymes involved in their biosynthetic pathways including mycolic acids, offer attractive and selective targets for the developments of novel antimycobacterial agents. The mechanisms of action of various mycolic acid inhibitors like Isoniazide (INH) and Ethionamide (ETH) have been studied intensively. Recently, mycolic acid inhibitors like Thiolaactomycin (TLM), Triclosan (TRC) and Isoxyl (ISO) have been reported to have potent activity against MDR strains of *M. tuberculosis*. Present study was conducted with the objective of determining the efficacy of ISO in vitro against *M. tuberculosis* strains in comparison to other mycolic acid inhibitors. Minimum Inhibitory Concentration pattern of clinical isolates of *M. tuberculosis* to mycolic acid synthesis inhibitors namely TRC, TLM, ISO, INH And ETH were determined by agar dilution method. Total 10 MDR strains and 10 susceptible strains of *Mycobacterium tuberculosis* (*M. tuberculosis*) along with standard strain of *M. tuberculosis* H37Rv were included in the study. This study shows promising activity of ISO against various strains of *M.tuberculosis* in comparison to other mycolic acid inhibitors. Hence more study on this compound will be beneficial in drug development process.

#### Introduction

Although tuberculosis (TB) is treatable, current treatments have limitations that are contributing to the spread of the disease.

Treatment duration of at least six months is required which leads to patient's non-compliance. The most effective strategy for

treating TB is Directly observed treatment, short-course (DOTS), which is cumbersome, labor intensive, expensive and with unpleasant side effects; particularly for such a long treatment regimen.

MDR TB has become more prevalent in recent years and second line drugs are not as effective as the standard therapy and are more toxic and expensive. It is important to treat latent TB infections (LTBI) and infections due to Mycobacteria other than *M. tuberculosis* (MOTT) in certain risk patients such as the HIV infected ones (Chakrabarth, Sharma Dubey, 1990). There is an urgent need for new antitubercular drugs. New drug that Shortens the duration of treatment or significantly reduce the number of doses needed to be taken under DOTS supervision, improve treatment of Multi drug resistant (MDR) strains, provide a more effective treatment of LTBI to prevent the progression from infection to diseases and have less side effects and toxicity.

A number of approaches are considered to identify targets for novel antimycobacterial agents. They range from biochemical studies of essential pathways to the use of genome scale tools such as transposon mutagenesis, proteomics and transcript mapping on micro arrays (Chopra *et al.*, 2003).

Current chemotherapy for TB relies on Mycobacteria specific drugs that inhibit bacterial metabolism with a heavy emphasis on inhibitors of the cell wall polymer. Mycobacteria have conventionally been considered to be surrounded by a thick waxy coat of lipid. Such a coat around Mycobacterial cells could explain limited permeability, their physical toughness and their rather general insusceptibility to toxic substances. The Mycobacterial cell wall has a unique structure comprised of three

covalently attached macromolecular structure: arabinogalactan, peptidoglycan and mycolic acids (Besra *et al.*, 1995). Mycolic acids (M.A.) are high molecular weight, alfa-alkyl and beta-hydroxy fatty acids. In Mycobacterial cell envelope, they are present in free lipids, they are trehalose dimycolate and trehalose monomycolate and for the most part esterified to the terminal pentaarabinofuranosyl units of arabinogalactan and hence part of the backbone of Mycobacterial cell wall (Minikin, 1992). They are the most characteristic & essential component of the cell walls of Mycobacteria and related genera (Besra *et al.*, 1995). These critical components of the cell envelope have been the subject of intense research for a number of years because of the fact that enzymes involved in their biosynthetic pathways including mycolic acids, offer attractive and selective targets for the developments of novel antimycobacterial agents (Brennan, 2002). In recent years, many excellent reviews on the biosynthesis of mycolic acids have appeared (Asselineau *et al.*, 2002). However, these reviews lack the details on how mycolic acids are synthesized and processed into the final products.

The mechanisms of action of various mycolic acid inhibitors like Isoniazide (INH) and Ethionamide (ETH) have been studied intensively. Early work demonstrated that INH specifically inhibits synthesis of M.A. in *M. tuberculosis* (Takayama, 1974). ETH, a structural analog of INH, is useful second-line antitubercular drug, and the two drugs have almost identical effects in strongly inhibiting the synthesis of mycolic acids (Winder, 1982).

Recently, mycolic acid inhibitors like Thiolactomycin (TLM), Triclosan (TRC) and Isoxyl (ISO) have been reported to have potent activity against MDR strains of *M.*

*tuberculosis* (Kremer *et al.*, 2000 and Slayden *et al.*, 2000).

TLM is a unique thiolactone that has been shown to exhibit antimycobacterial activity by specifically inhibiting fatty acid and mycolic acid biosynthesis (Kremer *et al.*, 2000).

TRC is a diphenyl ether derivative known as 2, 4, 4-Trichloro-2-hydroxydiphenyl ether is active against wide range of gram positive and gram-negative bacteria (Suller *et al.*, 2000). It inhibits mycolic acid synthesis and is active against *M. tuberculosis* (Slayden *et al.*, 2000). ISO, a thiourea (thiocarlide, 4, 4-diisoamyloxythiocarbanilide) demonstrated potent activity against standard strain of *M. tuberculosis* (Phetsuksisri *et al.*, 1999). It had been noted that it strongly inhibited mycolic acid synthesis in *M. bovis* (Winder, 1982).

There is a need to explore further in vitro activities of these compounds.

As drug development is a long and expensive process, it becomes predominant to reexamine drugs that were formerly deemed effective against TB and increase the permeability of the Mycobacterial cell wall. One such drug is ISO. ISO is an old drug, used for the clinical treatment of TB in 1960's (Winder, 1982). Tischer, 1966 demonstrated modest therapeutic efficacy of ISO monotherapy in cases of untreated pulmonary TB of various degree of difficulty.

Scmid, 1970 concluded that INH and ISO were more effective than monotherapy with either drug. The NCDDG group led by DR Patrick Brennan recently evaluated this drug and found it to be effective against MDR strains of *M. tuberculosis* (Phetsuksisri *et al.*, 1999). Hence there was a thought to do

more work on this compound, as it is an old drug and has proven its efficacy.

Present study was conducted with the objective of determining the efficacy of ISO in vitro against *M. tuberculosis* strains in comparison to other mycolic acid inhibitors.

## **Materials and Methods**

Minimum Inhibitory Concentration pattern of clinical isolates of *M. tuberculosis* to mycolic acid synthesis inhibitors namely TRC, TLM, ISO, INH And ETH were determined by agar dilution method (Hawkins, *et al.*, 1991). Total 10 MDR strains and 10 susceptible strains of *M. tuberculosis* along with standard strain of *M. tuberculosis H37Rv* were included in the study. These strains were provided by Department of Microbiology, P.D. Hinduja Hospital and Medical research centre, Mumbai. Drugs concentrations (mcg/ml) used in the study were:

- i.) ISO: 0.07, 0.15, 0.3, 0.6, 1.2, 2.5, 5, 10, 20 and 40
- ii.) TRC: 0.07, 0.15, 0.3, 0.6, 1.2, 2.5, 5, 10, 20 and 40
- iii.) TLM: 0.4, 0.8, 1.6, 2.5, 3.1, 6.2, 12.5, 25, 50, 100 and 200
- iv.) INH: 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 and 6.4
- v.) ETH: 0.15, 0.3, 0.6, 1.2, 2.5, 5, 10, 20, 40 and 80

Serial two fold dilutions of individual drug namely INH (Lupin Laboratories), ETH (Lupin Laboratories), TLM (Provided by Dr Cliff Barry, NIH), TRC (Balasara Ltd) and ISO (cayman Chemicals) were prepared in sterile water for injection after dissolving it in suitable diluents. 1ml of test drug solution (20X) was mixed with 19 ml of sterile molten Middle brook 7H11 medium with OADC supplements (Hi Media laboratories)

(temperature not more than 45° c) and poured in sterile petri plates. Media was cooled and agar was set.

Test strains were inoculated in sterile Dubos broth with glucose and albumin supplement with 0.05% Tween 80 (Hi Media laboratories) and incubated at 37<sup>0</sup>c for 7 to 10 days.

Cultures were shaken daily on vortex mixture for 30 seconds. The cultures were adjusted to an optical density of 0.1 at 540 nm and then diluted 10 fold in 0.1% Tween 80 containing normal saline. Bacterial suspension in 5-microlit quantities was spotted on agar plates containing various drug concentrations. The control plates, containing no drug was also inoculated along with it. Plates were incubated at 37<sup>0</sup>c for 14 days. The MICS were defined as the minimum concentrations of drugs that completely inhibited the growth of the test organism or allowed growth of not more than five colonies.

### Results and Discussion

Isonicotinic acid hydrazine, INH, was first found to have in vitro activity against Mycobacterial species in 1945. In 1952, INH was reported to be useful agent when two groups independently showed that it

was a potent antiTB drug in animal and human studies. INH remains an inexpensive, well-tolerated and very valuable agent for treatment of TB.

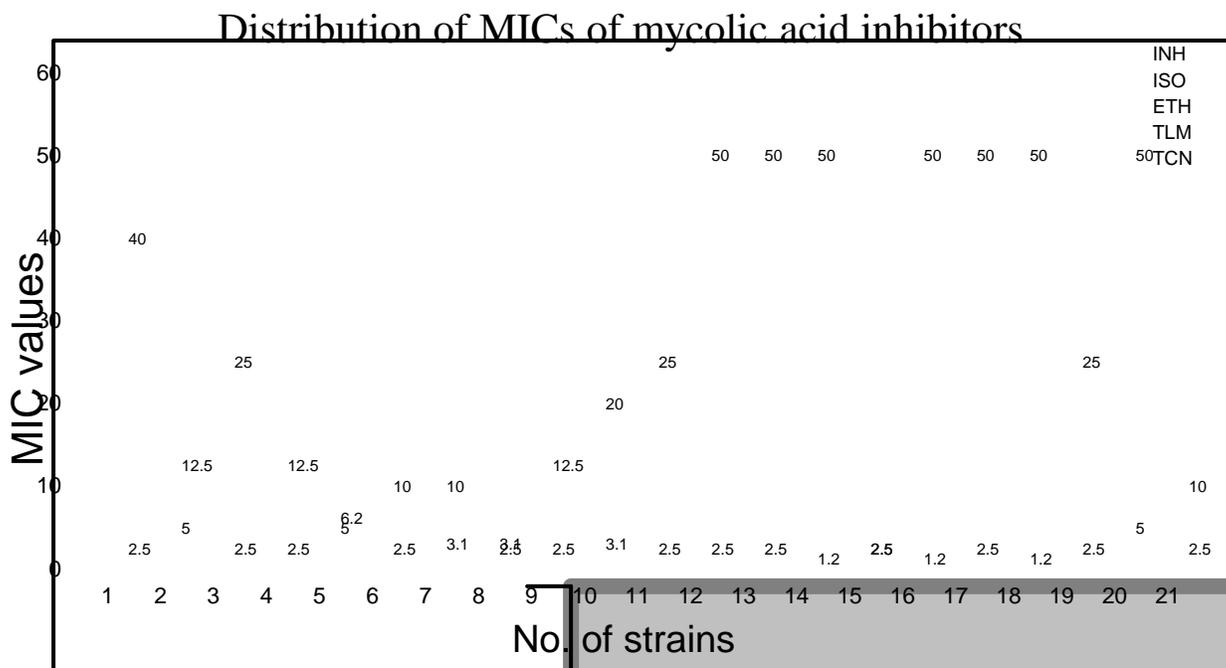
### Mechanism of action and activity

INH is bactericidal for TB. Its mechanism of action, however, remains unclear. One step is the desaturation of the C24 and C 26 long chain fatty acid precursors of mycolic acid, a unique part of Mycobacterial cell wall. This effect may explain the narrow spectrum of INH. The drug also affects, NAD + and pyridoxal phosphate metabolism and produces a cuprous complex toxic to the bacillus. INH appears to be inhibitory to resting bacilli and bactericidal to actively replicating ones. It has been found to be more active than other first line agents in early bactericidal activity against TB. Minimal inhibitory concentration for INH is 0.025 to 0.5 mcg/ml for the tubercle bacillus. Resistance can develop when therapy is inadequate. Recent work has suggested that mechanism of resistance for atleast a subgroup of organisms is deletion or mutation of the catalase peroxidase gene. A second gene involved with mycolic acid synthesis has also been found which encodes the target for both INH and ETH. *M. kansasii* is the most sensitive of the MOTT.

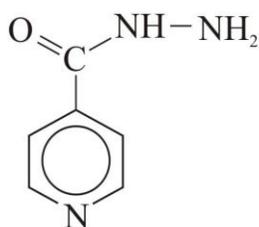
### Susceptibility pattern of *M. tuberculosis* isolates to Mycolic acid inhibitors

N=21

| Number | Mycolic Inhibitor | acid | MIC (mcg/ml) Mean +/- SD |
|--------|-------------------|------|--------------------------|
| 1      | Isoniazide        |      | 0.91 +/- 0.91            |
| 2      | Ethinamide        |      | 6.84 +/- 9.38            |
| 3      | Thiolactomycin    |      | 40.08 +/- 51.83          |
| 4      | Triclosan         |      | 2.43 +/- 2.50            |
| 5      | Isoxyl            |      | 2.78 +/- 2.73            |

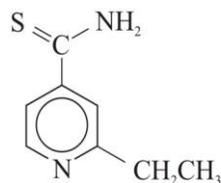


### Isoniazid



Structure of isoniazid

### Ethionamide



Structure of ethionamide

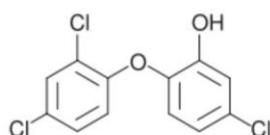
Soon after the discovery of INH, several independent groups discovered the antiTB activity of thioisonicotinamide. The compound's alpha ethyl derivative, ETH, synthesized in France in 1956 was found to be more effective than the parent drug. It is

bright yellow powder with a sulfide odour. ETH is a second line agent that is not recommended for treatment regimens in previously untreated patients, because of its frequent potentially serious toxicities.

## Mechanism of action and activity

While the drug is thought to inhibit peptide synthesis, precise mode of action is unclear. The similarity in structure to INH could offer some answers about ETH's activity, but lack of cross-resistance between INH and ETH suggests that this may not be the case. The report of the gene encoding for the target for INH and ETH, however, suggests that cross-resistance can occur.

## Triclosan



Structure of triclosan

Triclosan (2,4,4'-trichloro-2'-hydroxydiphenyl ether) is a synthetic bisphenol antimicrobial agent, which is active against a wide range of Gram-positive and Gram – negative bacteria. Triclosan, a chemical used for its antibacterial properties, is an ingredient in many detergents, dish-washing liquids, soaps, deodorants, cosmetics, lotions, anti-microbial creams, various toothpastes, and an additive in various plastics and textiles.

The chemical formulation and molecular structure of this compound are similar to some of the most toxic chemicals on earth, relating it to dioxins and PCBs. The Environmental Protection Agency gives triclosan high scores both as a human health risk and as an environmental risk.

In 1998, it was recommended for the control of Methicillin resistant *S. aureus* (MRSA) after being successfully used to control MRSA outbreak in a neonatal nursery and cardiothoracic surgical unit and to promote an alternative to expensive Vancomycin administration.

Active only against some Mycobacteria, ETH inhibits the TB bacillus in vitro at 0.6 to 1.2 mcg/ml. Three quarters of photochromogenic Mycobacteria are inhibited in vitro by 10 mcg/ml or less. ETH is reported to be equally effective in vitro against intracellular and extracellular TB bacilli.

Triclosan has been regarded as a biocide rather than an antibiotic and, as such, has been thought to have numerous intracellular and cytoplasmic target sites. This view has been recently challenged with evidence that triclosan may act on specific target, the enoyl reductase enzyme, which is involved in the synthesis of fatty acids.

However, McDonnell and Pretzer maintain that, although enoyl reductase may be the major target site, triclosan also affects other components. If triclosan does act as a specific inhibitor, it is likely that strains with higher levels of resistance will emerge, as in the case with antibiotic. To date, resistance levels amongst *S. aureus* appear to be low: the clinical importance of this remains to be seen.

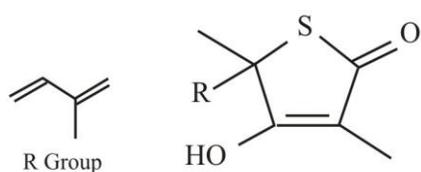
In many instances, triclosan is incorporated into products (e.g. bath products) that have other ingredients, such as surfactants and chelators that promote cell damage. These ingredients may also affect resistance by placing additional stress on the bacteria and so they must be critically evaluated in in

in vitro tests when preparations other than pure compounds are being evaluated.

The efficacy of antimicrobial products may depend on and vary significantly with, the formulation used. It is reported that TRC specifically inhibits InhA, the enoyl reductase from *M. tuberculosis* and a target for the antitubercular drug, INH. The data supports the hypothesis that the Mycobacterial enoyl reductase is a target for TRC and indicates that TRC can stimulate the emergence of InhA resistant Enoyl

Reductase. Also it is indicated that InhA inhibitors targeted at the enoyl substrate-binding site may be effective against existing INH resistant strains of *M. tuberculosis*.<sup>251)</sup> The use of triclosan as a biocide will remain controversial until the mechanism of resistance and the relative importance of low-level resistance in the environment are better understood. Survey of the occurrence of resistance to disinfectants in natural settings is needed to determine whether there is a cause for public health concern.

### Thiolactomycin



Thiolactomycin ((4R) (2E,5E)-2,4,6-trimethyl-3-hydroxy-2,5,7-octatriene-4-thiolide) is a unique thiolactone antibiotic isolated initially from soil *Nocardia* spp., which exhibits potent in vitro activity against many pathogenic bacteria.<sup>252)</sup> It is of relevance in the present context that TLM inhibits bacterial and plant type II fatty acid synthase (FAS II), but not mammalian or yeast type I fatty acid synthase (FAS-I), for instance, in *E. coli*, TLM inhibits both  $\beta$ -ketoacyl-ACP synthase I to III and acetyl coenzyme A (Co A. ACP transacylase activities in in vivo and in vitro.

Consequently, an understanding of the mode of action of TLM is important in the development of more effective antibiotics that exhibit selective action against bacterial FAS –II. The effects of TLM on Mycobacterial multifunctional Fas-I, mono functional Mycobacterial polypeptide FAS-II, and the largely undefined mycolate synthase were investigated in the search for new antituberculosis drug targets and treatments of drug resistant *M. tuberculosis*.

Genetic and biochemical evidence has implicated two different target enzymes for INH, within unique type II fatty acid synthase (FAS) system involved in the production of mycolic acid. These two components are an enoyl acyl carrier protein (ACP) reductase, of *M. tuberculosis*, with two inhibitors having well defined targets: TRC, which inhibits InhA, TLM, which inhibits KasA.

TLM resistant mutants of *M. tuberculosis* were more cross resistant to INH than TRC resistant mutants. Over expression of Kas A conferred more resistance to TRC than to INH and TLM. Co-over expression of both InhA and KasA resulted in strongly enhanced levels of INH resistance, in addition to cross-resistance to both TLM and TRC.

These results suggest that these components of the FAS II complex are not independently regulated and that alterations in the expression level of InhA affect expression levels of KasA. Nevertheless, INH appeared

to resemble TLM more closely in overall mode of action, and Kas A levels appeared to be tightly correlated with INH sensitivity. Kremer *et al.*, study presented evidence that TLM targets two  $\beta$ -ketoacyl-acyl-carrier protein synthases, KasA and KasB, consistent with the fact that both the enzymes belong to the fatty-acid synthase type II system involved in fatty acid and mycolic acid biosynthesis. Overexpression of KasA, KasB, and KasAB in *Mycobacterium bovis* BCG increased *in vivo* and *in vitro* resistance against TLM. In addition, a multidrug-resistant clinical isolate was also found to be highly sensitive

to TLM, indicating promise in counteracting multidrug-resistant strains of *M. tuberculosis*.

The design and synthesis of several TLM derivatives have led to compounds more potent both *in vitro* against fatty acid and mycolic acid biosynthesis and *in vivo* against *M. tuberculosis*. Finally, a three-dimensional structural model of KasA has also been generated to improve understanding of the catalytic site of mycobacterial Kas proteins and to provide a more rational approach to the design of new drugs.

## ISOXYL



Structure of thiocarlide (isoxyl)

At present only a few alternative chemotherapeutic regimens are available, resulting in poor therapeutic outcomes and high mortality rates among multi-drug-resistant tuberculosis patients. 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), thiocarlide is a thiourea derivative that was successfully used in the 1960s to treat tuberculosis. Recently, ISO was shown to have considerable antimycobacterial activity *in vitro* and to be effective against various clinical isolates of multidrug-resistant strains of *M. tuberculosis* in the range of 1-10  $\mu\text{g/ml}$ . An early note reported that ISO, like isoniazid (INH) and ethionamide (ETH), strongly inhibits the synthesis of mycolic acids (a result since confirmed with the

demonstration that all types of mycolic acids are affected. In addition it was noted that ISO also inhibited shorter chain fatty acid synthesis, suggesting inhibitory effects different from those of INH and ETH and raising the prospects of novel fatty acid biosynthetic targets.

ISO is an old drug used for the clinical treatment of TB in the 1960s. It is 4,4'-diisoamyloxydiphenylthiourea. 4,4'-diisoamyloxythiocarbonylurea, thiocarlide). It was suggested in 1953 by Buu-Hoi and Dat-Xuong, ISO, a potential anti-tuberculosis and anti-leprosy drug. Its activity against *M. tuberculosis* was subsequently confirmed by Tacquet and others in 1958.<sup>(263)</sup>

Many authors have since shown it to have a powerful inhibitory action on human species of tubercle bacilli *in vitro*. *In vitro* activity has also been confirmed in mice and in guinea pigs. After oral administration in animals experiment, ISO is generally

considerably less effective than INH, only slightly less effective than SM, superior to PAS and similar to or better than most second-line tuberculostatic drugs.

It is insoluble in water, and is a highly specific antibacterial agent only having an effect on certain mycobacterial species. Metabolism studies in animals and man have been made particularly difficult due to the lack of a suitable assay technique in body fluids at old time.<sup>266)</sup>

A reliable method for determining the in vitro effect of ISO against *M. tuberculosis* using Tarshis media was evolved by Virtanen in 1963. This technique was adapted to determine human blood levels of ISO by B.W. Lacey in 1965, who found blood levels of 6 to 16 mcg/ml in subjects taking 6 g ISO daily.

It was pointed out by Schmid *et al.*, in 1970, that requirements from any combination drug were

- 1) It must have a tuberculostatic activity
- 2) It must be able to delay the sensitivity against INH and SM
- 3) It has to be atoxic
- 4) It must be of good compatibility. These 4 demands were fulfilled by the new tuberculostaticum, Thiocarbanilide = ISOXYL.

Its tuberculostatic effect had been confirmed in vivo and in vitro as well as in monotherapy in man. The ability to delay the sensitivity against INH and SM had also been tested. Toxic sensations were never detected in adults. There was a great deal of information about the efficacy and good compatibility in adults, concerning the experience with children in hospitals had reported until now.

Since 1964, ISO was used as a combining drug in cases of primary TB in children together with INH and had up to now treated about 600 children from the age of 6 months to 6 years.

Molecular formula of ISO is C<sub>23</sub>H<sub>32</sub>N<sub>2</sub>O<sub>2</sub>S. For long storage, it is suggested that ISO be stored at -20°C temperature. It is a crystalline solid. It is soluble in organic solvents like ethanol, Dimethyl sulfoxide; Dimethyl Formamide. Solubility of ISO is 1 mg/ml in ethanol, 30 mg/ml in DMSO and DMF. ISO is sparingly soluble in aqueous buffers, therefore further dilutions of organic solvent solutions into aqueous buffers or isotonic saline should be made prior to performing biological experiments

#### **Methods for determining antimicrobial activity of anti-tuberculosis drugs in vitro**

Methods used for evaluation of the antimicrobial activity of conventional and experimental agents may include some of the techniques, used for drug susceptibility testing of the clinical isolates. The results obtained by these techniques can provide information of the inhibitory (bacteriostatic) activity of an agent if the test is performed in a quantitative manner and if inactivation of a drug in the medium is minimal, allowing the obtained MIC values to be reasonably compared with the pharmacokinetic data.

In addition to the evaluation of the bacteriostatic activity of drugs present in relatively constant concentrations in the medium during the specific period of incubation, at least two more in vitro methods are essential for evaluation of the potentials of an antimicrobial agent: determination of its killing (bactericidal) activity, most often expressed quantitatively

as the minimal bactericidal concentration (MBC); and its ability to affect the bacterial growth by a timed pulse exposure, or the so-called post antibiotic effect (PAE). Generally, there are two types of drug susceptibility tests available in the fields of clinical Microbiology, qualitative & quantitative.

Qualitative tests are designed to report the minimum inhibitory concentration (MICs), the lowest drug concentration that produces complete inhibition of the bacterial growth in vitro, usually more than 99% of the bacterial population. Such a report can also suggest interpretation as very susceptible, moderately susceptible, moderately resistant and very resistant.

A qualitative test provides only suggested interpretation without actual values. The

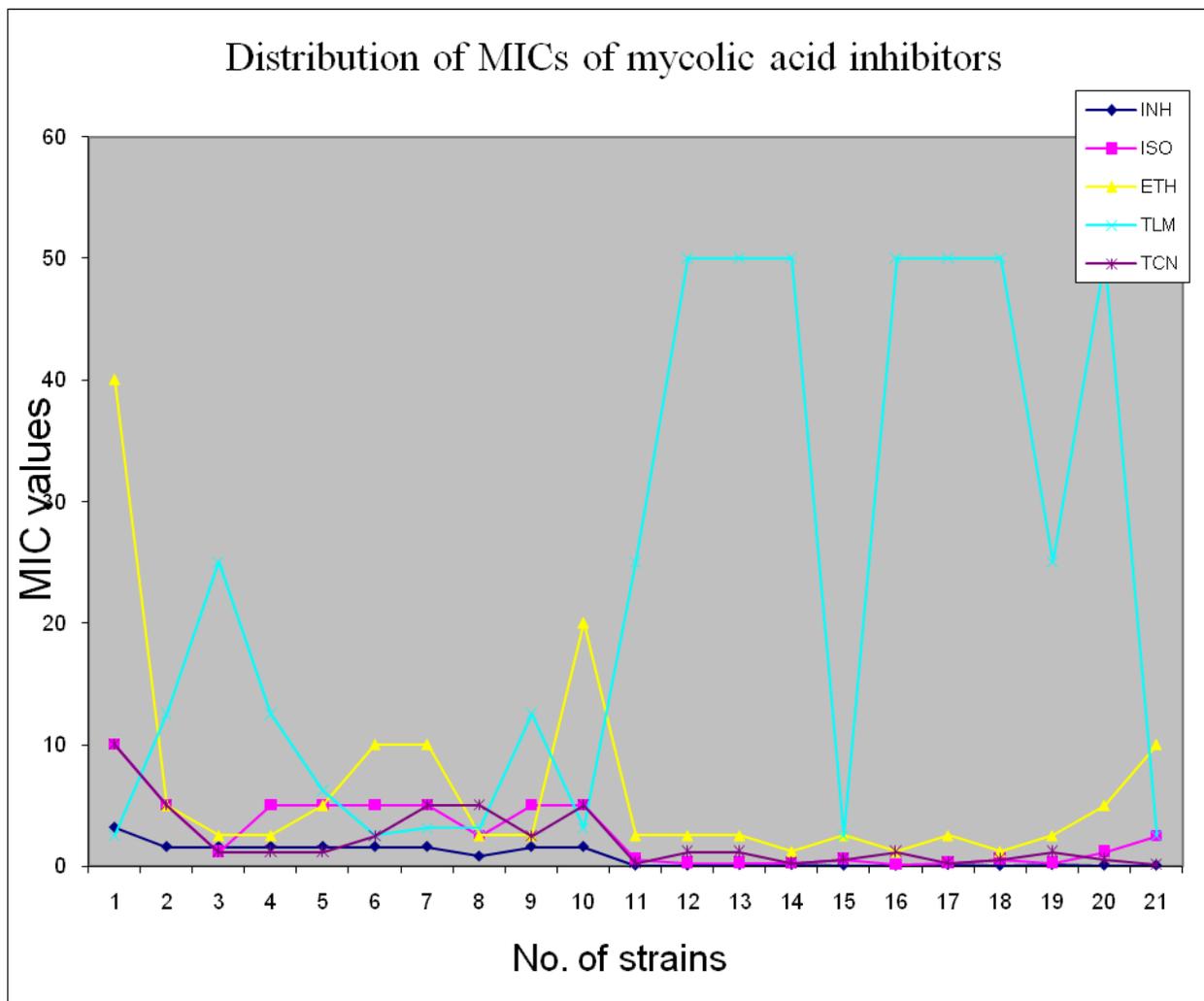
purpose of drug susceptibility tests of *M tuberculosis* clinical isolates have three goals (1) To guide the choice of drugs for the initial therapy, (2) To confirm the emergence of drug resistance and to guide the choice of drugs for further treatment, (3) To estimate the prevalence of drug resistance in the community.

MIC is the method used for evaluation of the antimicrobial activity of conventional, experimental agents. The results obtained by these techniques can provide information on the inhibitory (bacteriostatic) activity of an agent if the test is performed in a qualitative manner and if inactivation of a drug in the medium is minimal, allowing the obtained MIC values to be reasonably compared with the pharmacokinetic data.<sup>171)</sup>

**Susceptibility pattern of *M. tuberculosis* isolates to M.A. inhibitors.**

|                             |            | No.        | Mean              | SD          | T-value                          | p-value |
|-----------------------------|------------|------------|-------------------|-------------|----------------------------------|---------|
| <b>Pair 1</b>               | <b>INH</b> | 21         | 0.91              | 0.91        | -4.555                           | 0.00019 |
|                             | <b>ISO</b> | 21         | 2.78              | 2.73        | <b>Difference is significant</b> |         |
| <b>Pair 2</b>               | <b>ISO</b> | 21         | 2.78              | 2.73        | -2.496                           | 0.021   |
|                             | <b>ETH</b> | 21         | 6.84              | 9.38        | <b>Difference is significant</b> |         |
| <b>Pair 3</b>               | <b>INH</b> | 21         | 0.91              | 0.91        | -3.111                           | 0.006   |
|                             | <b>ETH</b> | 21         | 6.84              | 9.38        | <b>Difference is significant</b> |         |
| <b>Pair 4</b>               | <b>INH</b> | 21         | 0.91              | 0.91        | -3.427                           | 0.003   |
|                             | <b>TLM</b> | 21         | 40.08             | 51.83       | <b>Difference is significant</b> |         |
| <b>Pair 5</b>               | <b>INH</b> | 21         | 0.91              | 0.91        | -3.748                           | 0.001   |
|                             | <b>TRC</b> | 21         | 2.43              | 2.50        | <b>Difference is significant</b> |         |
| <b>Pair 6</b>               | <b>ISO</b> | 21         | 2.78              | 2.73        | -3.198                           | 0.005   |
|                             | <b>TLM</b> | 21         | 40.08             | 51.83       | <b>Difference is significant</b> |         |
| <b>Pair 7</b>               | <b>ISO</b> | 21         | 2.78              | 2.73        | 1.010                            | 0.325   |
|                             | <b>TRC</b> | 21         | 2.43              | 2.50        | Difference is not significant    |         |
| <b>Pair 8</b>               | <b>ETH</b> | 21         | 6.84              | 9.38        | -2.725                           | 0.013   |
|                             | <b>TLM</b> | 21         | 40.08             | 51.83       | <b>Difference is significant</b> |         |
| <b>Pair 9</b>               | <b>ETH</b> | 21         | 6.84              | 9.38        | 2.738                            | 0.013   |
|                             | <b>TRC</b> | 21         | 2.43              | 2.50        | <b>Difference is significant</b> |         |
| <b>Pair 10</b>              | <b>TLM</b> | 21         | 40.08             | 51.83       | 3.247                            | 0.004   |
|                             | <b>TRC</b> | 21         | 2.43              | 2.50        | <b>Difference is significant</b> |         |
| <b>Correlation between-</b> |            | <b>No.</b> | <b>Correlatio</b> | <b>Sig.</b> |                                  |         |

|         |           | n  |        |          |
|---------|-----------|----|--------|----------|
| Pair 1  | INH & ISO | 21 | 0.949  | 5.57E-11 |
| Pair 2  | ISO & ETH | 21 | 0.779  | 0.00003  |
| Pair 3  | INH & ETH | 21 | 0.731  | 0.00017  |
| Pair 4  | INH & TLM | 21 | -0.596 | 0.00400  |
| Pair 5  | INH & TCN | 21 | 0.787  | 0.00002  |
| Pair 6  | ISO & TLM | 21 | -0.579 | 0.00600  |
| Pair 7  | ISO & TCN | 21 | 0.823  | 4.54E-06 |
| Pair 8  | ETH & TLM | 21 | -0.361 | 0.10800  |
| Pair 9  | ETH & TCN | 21 | 0.852  | 9.66E-07 |
| Pair 10 | TLM & TCN | 21 | -0.504 | 0.02000  |



Mycolic acids are  $\beta$ -hydroxy acids substituted at the  $\alpha$  position with a moderately long aliphatic chain. The acids

are formed either by step by step elongation of the carbon chains or by the head to tail fusion of shorter performed chain (Claisen

Condensation Backsdale and Kim (1977). As defined by Asselineau *et al.*, 1976 mycolic acids are  $\beta$ -hydroxy fatty acids with a long  $\alpha$ -alkyl side chain. They exist as homologous series of fatty acids differing by 28 atomic mass units (a two-carbon unit), and in *M. tuberculosis* they are characterized by very hydrophobic C<sub>54</sub> to C<sub>63</sub> fatty acids with C<sub>22</sub> to C<sub>24</sub>  $\alpha$ side chains. Three distinct structural classes of mycolic acids are found in *M. tuberculosis* and they are  $\alpha$ -, methoxy- and keto-mycolic acids. The  $\alpha$ -mycolic acid is the most abundant form (>70%), whereas methoxy and keto-mycolic acids are the minor components (10 to 15%) (Qureshi, N., 1978)

INH is bactericidal for TB. Its mechanism of action, however, remains unclear. One step is the de saturation of the C24 and C 26 long chain fatty acid precursors of mycolic acid, a unique part of Mycobacterial cell wall (Hawkins *et al.*, 1991). This effect may explain the narrow spectrum of INH.

While the drug is thought to inhibit peptide synthesis, precise mode of action is unclear. The similarity in structure to INH could offer some answers about ETH's activity, but lack of cross-resistance between INH and ETH suggests that this may not be the case. The report of the gene encoding for the target for INH and ETH, however, suggests that cross-resistance can occur (Altamirano *et al.*, 1994). It is reported that TRC specifically inhibits InhA, the enoyl reductase from *M. tuberculosis* and a target for the antitubercular drug, INH. The data supports the hypothesis that the Mycobacterial enoyl reductase is a target for TRC and indicates that TRC can stimulate the emergence of InhA resistant Enoyl Reductase. Also it is indicated that InhA inhibitors targeted at the enoyl substrate-binding site may be effective against existing INH resistant strains of *M.*

*tuberculosis* (Parikh *et al.*, 2000). The use of triclosan as a biocide will remain controversial until the mechanism of resistance and the relative importance of low-level resistance in the environment are better understood. Survey of the occurrence of resistance to disinfectants in natural settings is needed to determine whether there is a cause for public health concern.

Thiolactomycin ((4R) (2E,5E)-2,4,6-trimethyl-3-hydroxy-2,5,7-octatriene-4-thiolide) is a unique thiolactone antibiotic isolated initially from soil *Nocardia* spp. , which exhibits potent in vitro activity against many pathogenic bacteria.<sup>252)</sup> an understanding of the mode of action of TLM is important in the development of more effective antibiotics that exhibit selective action against bacterial FAS –II. The effects of TLM on Mycobacterial multifunctional Fas-I, monofunctional Mycobacterial polypeptide FAS-II, and the largely undefined mycolate synthase were investigated in the search for new antituberculosis drug targets and treatments of drug resistant *M. tuberculosis* (Sladen *et al.*, 1996). Genetic and biochemical evidence has implicated two different target enzymes for INH, within unique type II fatty acid synthase (FAS) system involved in the production of mycolic acid. These two components are an enoyl acyl carrier protein (ACP) reductase, of *M. tuberculosis*, with two inhibitors having well defined targets: TRC, which inhibits InhA, TLM, which inhibits KasA.

Isoxyl (ISO), thiocarlide is a thiourea derivative that was successfully used in the 1960s to treat tuberculosis (Urbanck, 1966, Urbancik, 1970 and Titscher, 1966). Recently, ISO was shown to have considerable antimycobacterial activity *in vitro* and to be effective against various clinical isolates of multidrug-resistant strains

of *M. tuberculosis* in the range of 1-10 µg/ml (Phetsuksisri *et al.*, 1999). An early note reported that ISO, like isoniazid (INH) and ethionamide (ETH), strongly inhibits the synthesis of mycolic acids (a result since confirmed with the demonstration that all types of mycolic acids are affected. In addition it was noted that ISO also inhibited shorter chain fatty acid synthesis suggesting inhibitory effects different from those of INH and ETH and raising the prospects of novel fatty acid biosynthetic targets (Winder *et al.*, 1971 and Winder, 1982).

In the present study, tested five mycolic acid inhibitors against 10 MDR strains and 10 susceptible strains of MTB along with standard strain of MTB H37Rv. We found lowest MIC of INH (0.91mcg/ml), while highest MIC was shown by TLM (40.08 mcg/ml). MIC of TRC and ISO was similar. Difference between mean MIC values of all the compounds was statistically significant except between TRC and ISO. ISO is an old drug used for the clinical treatment of TB in the 1960s. It is 4,4'-diisoamyloxy-diphenylthiourea. 4,4'-diisoamyloxy-thiocarbanilide, thiocarlide).

It was suggested in 1953 by Buu-Hoi and Dat-Xuong, 1953. ISO, a potential anti-tuberculosis and anti-leprosy drug. Its activity against *M. tuberculosis* was subsequently confirmed by Tacquet and others in 1958.

Many authors have since shown it to have a powerful inhibitory action on human species of tubercle bacilli in vitro. In vitro activity has also been confirmed in mice and in guinea pigs (Crowle *et al.*, 1963). After oral administration in animals experiment, ISO is generally considerably less effective than INH, only slightly less effective than SM, superior to PAS and similar to or better than most second-line tuberculostatic drugs.

It is insoluble in water, and is a highly specific antibacterial agent only having an effect on certain mycobacterial species. Metabolism studies in animals and man have been made particularly difficult due to the lack of a suitable assay technique in body fluids at old time.

A reliable method for determining the in vitro effect of ISO against *M. tuberculosis* using Tarshis media was evolved by Virtanen in 1963. This technique was adapted to determine human blood levels of ISO by B.W. Lacey in 1965, who found blood levels of 6 to 16 mcg/ml in subjects taking 6 g ISO daily (Emerson and Nicholson, 1965).

### **Conclusion**

Tuberculostatic effect of ISO had been confirmed in vivo and in vitro as well as in monotherapy in man. The ability to delay the sensitivity against INH and SM had also been tested. Toxic sensations were never detected in adults. There was a great deal of information about the efficacy and good compatibility in adults, concerning the experience with children in hospitals had reported until now. Since 1964, ISO was used as a combining drug in cases of primary TB in children together with INH and had up to now treated about 600 children from the age of 6 months to 6 years.

This study also shows promising activity of ISO against various strains of *M. tuberculosis*. Hence more study on this compound will be beneficial in drug development process.

### **References**

- Alshamaony, L., Goodfellow, M. and Minnikin, D.E. 1976. Free Mycolic Acids as criteria in the classification of

- Nocordia and the Rhodochrous complex. *J. Gen. Microbiol.*, 92: 188.
- Altamirano, M., Marostenmaki, J., Wong, A. *et al.* 1994. Mutations in the catalase-peroxidase gene from isoniazid-resistant *Mycobacterium tuberculosis* isolates. *J. Infect. Dis.*, 165: 1162-5.
- Asselineau, C., J. Asselineau, G. Laneelle, and M. A. Laneelle. 2002. The biosynthesis of mycolic acids by mycobacteria. Current and alternative hypotheses. *Prog. Lipid Res.*, 41: 501-523.,
- Backsdale, L. and Kim, K.S. 1977. Mycobacterium. *Bacterial Rev.*, 41: 212-272.
- Besra, C.S., Khoo, K.H., McMeil, M.R., Dell, A., Morris, H.R. and Brennan, P.J. 1995. A new interpretation of the structure of the mycolyl-arabinogalactan complex of *Mycobacterium tuberculosis* as revealed through the characterisation of oligoglycosyl alditol fragments by fast atom bombardment mass spectrometry and <sup>1</sup>H nuclear magnetic resonance spectroscopy, *Biochem.*, 34: 4257 – 4266.
- Brennan, P.J. 2002. Plenary lecture, Current developments in drug Discovery for Tuberculosis. Targeting Cell wall Biogenesis. *Introduction symposium on current developments in drug discovery for tuberculosis*, 5-6.
- Buu-Hoi, N.P., and Dat-Xuong, N. 1953. Sur les composés tuberculostatiques du groupe de la Thiouree' et leur mecanisme d'action. *Cr.hebd. jeanc. Acad. Sci. Paris*, 237: 498.
- Chakrabarthy, A., Sharma, Dubey, M.L. 1990. Isolation rates of different mycobacterial species from Chandigarh. *Ind. J. Med. Res.*, 91: 111.
- Chopra, P., Meena, L.S. and Singh, Y. 2003. New drug targets for *Mycobacterium tuberculosis*. *Ind. J. Med. Res.*, 117: 1-9.
- Crowle, A.J., Mitchell, R.S. and Petty, T.L. 1963. The effectiveness of a thiocarbanilide (Isoxyl) as as therapeutic drug in mouse Tuberculosis. *Am. Rev. Resp. Dis.*, 88, 69 B: 716.
- Emerson, P. and Nicholson, J.P. 1965. The absorption of ISOXYL using radioactive material (S.35 tagged). In *Trans.24<sup>th</sup> Res. Conf. Palm. Dis. Veterans administration-Armed Forces*, Washington: 44.
- Hawkins, J.E., Wallace, R.J., and Brown, B.A. 1991. Antibacterial susceptibility tests: mycobacteria, In *Manual of Clinical Microbiology*, 5th Edn, Balows A, Hausler WJ, Herrmann KL, Isenberg HD and Shadomy HJ, Eds. Am Society For Microbiology, Washington DC. 1138-52.
- Kremar, L, Douglas, J.D., Baulard, A, R., Morehour, C., Guy, M.R., Alland, D., Dover, L.G., Lakey, J.H., Jacob, W.R. Jr, Brennan, P.J., Minnikin, D.E., Besra, G.S. 2000. Thiolactomycin and related analogues as novel anti-mycobacterial agents targeting Kas A and Kas B condensing enzymes in *Mycobacterium tuberculosis*. *J. Bio. Chem.*, 275, 22:16857-64.
- Minikin, D.E. 1992. Lipids: Complex lipids, their chemistry, biosynthesis and roles; in Rattedge C and Stanford J (ed), *The biology of the Mycobacteria*, Vol 1. Academic Press; London: 95-184.
- Parikh, S.L., Xiao, G. and Tonge, P.J. 2000. Inhibition of InhA, the enoyl reductase from *M. tuberculosis*, by TRC and INH. *Biochem.*, 39(26): 7645-50.
- Phetsuksisri, B., Baulard, A., Cooper, R., Minnikin, D.E, Douglas, D.J., Besra, G.S. and Brennan, P.J. 1999. Antimycobacterial activities of Isoxyl and new derivatives through the inhibition of mycolic acid synthesis. *Antimicrob. Agents and Chemoth.*, 43, 5: 1042-1051.
- Phetsuksisri, B., Baulard, A., Cooper, R., Minnikin, D.E., Douglas, D.J., Besra, G.S. and Brennan, P.J. 1999. Antimycobacterial activities of Isoxyl and new derivatives through the inhibition of mycolic acid synthesis. *Antimicrob. Agents and Chemoth.*, 43, 5: 1042-1051.

- Qureshi, N., K. Takayama, H. C. Jordi, and H. K. Schnoes. 1978. Characterization of the purified components of a homologous series of  $\alpha$ -mycolic acids from *Mycobacterium tuberculosis* H37Ra. *J. Biol. Chem.*, 253: 5411-5417.
- Schmid, C.H. 1970. Clinical experience in cases of primary tuberculosis with tuberculostaticum isoxyl. *Antibiot chemother.*, 16: 108-116.
- Sladen, R.A., Lee, R.E, Armour, J.W., Cooper, A.M., Orme, I.M., Brennan, P.J. and Besra, G. 1996. *Antimicrob. Agents and Chemoth.*, 40(12): 2813-2819.
- Slayden, R.A., Lee, R.E., Barry, C.E. III. 2000. Isoniazide affects multiple components of the type 11 fatty acid synthase system of *Mycobacterium tuberculosis*. *Mol. Microbiol.*, 38, 3: 514-25.
- Suller, M.T.E. and Russel, A.D. 2000. Triclosan and antibiotics resistance in *Staphylococcus aureus*. *J. Antimicrob. Chemoth.*, 46: 11-18.
- Takayama, K. 1974. Selective action of isoniazid on the synthesis of cell wall mycolates in mycobacteria, *Ann. Y. Acad. Sci.* 235: 426-438.
- Tischer, R. 1966. Monotherapie mit isoxyl/DAT bei tuberculose-asylierungsfällen. *Prax Pneumoniol.*, 202.
- Tischer, R. 1966. Monotherapie mit Isoxyl/DAT bei Tuberkulose-Asylierungsfällen. *Prax. Pneumol.*, 20: 202.
- Urbancik, B. 1970. *Antibiot. Chemother.*, 16: 117-123.
- Urbanck, B. 1966. A clinical trial of Thiocarlide (Isoxyl). *Tubercle. Edinb*, 47: 283.
- Virtanen, S. 1963. Determination of the sensitivity of mycobacteria to Isoxyl in vitro. *Annl. Med. Exp. Bio. Fenn.*, 41: 430.
- Winder, F. G., Collins, P.B. and Whelan, D. 1971. Effects of ethionamide and isoxyl on mycolic acid synthesis in *Mycobacterium tuberculosis* BCG. *J. Gen. Microbiol.*, 66: 379-380.
- Winder, F.G. 1982. Mode of action of the antimycobacterial agents and associated aspects of the molecular biology of Mycobacteria.. In (Ratledge and J Standford 9Ed) *The biology of Mycobacteria*. Vol 1, academic Press Inc. New York. N.Y.: 353-438 .
- Winder, F.G. 1982. Mode of action of the antimycobacterial agents and associated aspects of the molecular biology of mycobacteria,. In C. Ratledge, and J. Standford (ed.), *The biology of mycobacteria*, vol. 1. Academic Press, Inc., New York, and N.Y: 353-438.

**How to cite this article:**

Shashikant Vaidya, Shreyasi Mulye, Mohan Kulkarni, Geeta Koppikar, Abhay Chowdhary. 2016. Determination of the Efficacy of Isoxyl, A Mycolic Acid Inhibitor in Vitro against *M. tuberculosis* Strains in Comparison to Other Mycolic Acid Inhibitors. *Int.J.Curr.Res.Aca.Rev.*4(12). 103-117. doi: <http://dx.doi.org/10.20546/ijcrar.2016.412.010>