



ISSN Print: 2394-7500
ISSN Online: 2394-5869
Impact Factor: 5.2
IJAR 2016; 2(12): 27-32
www.allresearchjournal.com
Received: 06-10-2016
Accepted: 07-11-2016

Shashikant Vaidya
Clinical Pathology
Department, Haffkine
Institute, Mumbai, India

Shreyasi Muley
Haffkine Bio-Pharmaceutical
Corporation Ltd. Mumbai,
India

Rashmi Shimpi
Haffkine Bio-Pharmaceutical
Corporation Ltd. Mumbai,
India

Mohan Kulkarni
T.N. Medical College and
B.Y.L. Nair Charitable
Hospital, Mumbai, India

Geeta Koppikar
Breach Candy Hospital Trust,
Mumbai, India

Abhay Chowdhary
Grant Medical College and Sir
J.J. Group of Hospitals,
Mumbai, India

Correspondence
D Shashikant Vaidya
Clinical Pathology
Department, Haffkine
Institute, Mumbai, India

To study intracellular bactericidal activity of Isoniazid & Rifampicin, alone and in combination with Isoxyl against standard and multi drug resistant strain of *Mycobacterium tuberculosis* in macrophage cell line

Shashikant Vaidya, Shreyasi Muley, Rashmi Shimpi, Mohan Kulkarni, Geeta Koppikar and Abhay Chowdhary

Abstract

Tuberculosis (TB) remains a major health problem in developing nations. 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 Isoxyl (ISO). Interest in the use of combination of antibiotics was started many years ago, almost as soon as two antibiotics were available. To minimize the probability of emergence of drug resistance, to increase the activity, particularly the bactericidal activity of the agents. Current use of drug combinations in the therapy of TB is aimed at designing the most efficient short-course regimens. It is reported the use of a murine macrophage continuous cell line J774 as a model for testing the susceptibility of mycobacteria to antimicrobial agents. Present study was conducted with the objective of determining the efficacy of ISO. To assess cytotoxic reaction to macrophage cell line by various concentrations of ISO. To study intracellular bactericidal activity of ISO against standard strain of *Mycobacterium tuberculosis* H37Rv in macrophage cell line. To study intracellular bactericidal activity of INH and RF, alone and in combination with ISO against standard and MDR strain of *M. tuberculosis* in macrophage cell line. It is observed that, this thiourea is apparently not acutely toxic for macrophage cell line J 774A.1. It showed some bactericidal activity in intracellular bacteria. There is an intracellular synergistic interaction, though little, between ISO & INH and ISO & RF against standard strain and MDR strain of *M. tuberculosis*

Keywords: Tuberculosis, Isoxyl, cytotoxicity, rifampicin, isoniazid

1. Introduction

TB remains a major health problem in developing nations. Here TB infections are responsible for one in four avoidable adult deaths^[1]. *Mycobacterium tuberculosis* (MTB) has been shown to be not only the greatest scourge of mankind but also adaptable to changing conditions. Only a few years after the introduction of effective chemotherapy, drug resistance began to be reported^[2].

With the widespread introduction of control programmes, drug resistance began to increase, at first only in non-compliant cases. Thereafter, as resistant organisms retain their virulence and infectivity, the resistant strains have been gradually increasing in the community^[3]. The most significant emergence has been that of the multi-drug resistant (MDR) strains, which is resistant to Isoniazid (INH) and Rifampicin (RF) with or without being resistant to other drugs^[4].

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 *Mycobacterium tuberculosis* (MOTT) in certain risk patients such as the HIV infected ones. There is an urgent need for new antitubercular drugs (5). Developing new antitubercular drugs is an expensive exercise and TB is not a disease of rich nations. Some development projects are underway, but more are needed. TB still remains a neglected disease in relation to drug development^[6].

There is an urgent need to develop new effective antitubercular compounds, compounds that increase the permeability of the Mycobacterial cell wall by inhibiting the synthesis of cell wall components and enhance the activity of conventional drugs as a result of increased penetration of these latter agents to susceptible internal targets [7]. This enhancement of antimicrobial activity theoretically affords the use of lower concentration of antibiotics associated with toxicity [8]. 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 Isoxyl (ISO). ISO is an old drug, used for the clinical treatment of TB in 1960's [9]. Studies demonstrated modest therapeutic efficacy of ISO monotherapy in cases of untreated pulmonary TB of various degree of difficulty [10, 11].

INH and ISO were more effective than monotherapy with either drug [12]. The NCDDG group led by DR Patrick Brennan recently evaluated this drug and found it to be effective against MDR strains of MTB [13]. Hence there was a thought to do more work on this compound, as it is an old drug and have proven its efficacy. Interest in the use of combination of antibiotics was started many years ago, almost as soon as two antibiotics were available. This corresponded to a belief among physicians that antimicrobial agents are widely effective and fairly harmless and in their search for security in treatment they prescribe antibiotic combinations because of a general feeling that if one antibiotic is good, two should be better and three should cure almost everybody of almost every ailment [14].

The use of antimicrobial in combination has been known since the discovery of the first antibiotics. A combination of Penicillin with Streptomycin was effective in the therapy of enterococcal endocarditis, whereas Penicillin alone was not effective [14]. At the very beginning of the era of antibiotic, it was also observed that combinations of antimicrobial agents were not always more effective than single drugs. Some combinations were even found to be harmful or antagonistic. Combinations of antimicrobial agents are most often used for the following reasons [14, 15].

To minimize the probability of emergence of drug resistance, to increase the activity (particularly the bactericidal activity) of the agents which have bactericidal effect, to reduce a potentially toxic effect by employing lower dosages of each drug, to provide broad coverage of infections caused by unidentified organisms and for treatment of polymicrobial infections.

The rationale for combination chemotherapy of Mycobacterial infections varies, depending on the causative agent.

Cell culture has become a powerful technique in research and technical field as well as industry, since it was first described in the early 1900s. Recently, reported the use of a murine macrophage continuous cell line J774 as a model for testing the susceptibility of mycobacteria to antimicrobial agents. Macrophage J774.1A cells possess many of the biological and biochemical properties of both resident and activated murine peritoneal macrophages [16]. The use of a continuous cell line for antimicrobial susceptibility tests reduces the variability associated with the use of different batches of experimental animals. The immunobiologic role of the macrophage as a key defense cell against

mycobacterial infections has been thoroughly established [17, 18].

With the increasing prevalence of Mycobacterial infections, the development of new antimycobacterial agents and strategies for treating Mycobacterial infections is of paramount importance, while *in vitro* methods such as radiometric and calorimetric assays are important to determine the MICs of antimicrobials and effective doses of antimycobacterial drugs and should also be evaluated in a macrophage model to ensure intracellular drug effectiveness [19]. In this regard, several macrophage models have been used. A variety of murine macrophage models have been described, however, the one that appears to be most commonly used is the J774.1A macrophage cell line [16].

Present study was conducted with the objective of determining the efficacy of ISO to assess cytotoxic reaction to macrophage cell line by various concentrations of ISO. To study intracellular bactericidal activity of ISO against standard strain of *Mycobacterium tuberculosis* H37Rv in macrophage cell line. To study intracellular bactericidal activity of INH and RF, alone and in combination with ISO against standard and MDR strain of *M. tuberculosis* in macrophage cell line.

2. Material and methods

2.1 Assessment of cytotoxic reaction to macrophage cell line by various concentrations of ISO [13].

Splitting of cells, J774A.1 Macrophage cell line, which was procured from National Centre for cell sciences, Pune (N.C.C.S.) was carried out and cell suspension containing 10^6 cells/ml was prepared. Twenty-four wells tissue culture plates (Nunc) were seeded with 10^6 macrophage cells/ml per well and incubated at 37 °C in 5% CO₂ atmosphere (Forma Scientific). On day 6, after seeding the plates with cells, Dulbaco's minimum essential medium. (DMEM) (Himedia Laboratories) with 5% FBS was changed and fresh 2 ml of DMEM without antibiotics was added to each well.

On day 8, the old medium was removed and 1.8 ml of DMEM with antibiotics containing ISO (Cayman Chemicals), at concentrations of (mcg/ml) 5.0, 2.5, 2.0, 1.5, 1.0, 0.5, and 0.25 was added with 200 microlitre of sterile Alamar Blue reagent. (Accumed International, Westlake, OH, USA)

Drug concentrations were added in triplicate in wells, while 3 wells were kept as control, in which drug solution was not added. Plates were incubated at 37 °C in 5% CO₂ atmosphere (Forma Scientific). Colour of the Alamar Blue dye mixed with DMEM, without antibiotics and the cell morphology were observed periodically within 3 days. Red colour of the DMEM, without antibiotic mixed with Alamar Blue dye, represents cell viability.

Table 1: Arrangement of concentrations of ISO (mcg/ml) in 24 wells tissue culture plate

5.0	5.0	5.0	2.5	2.5	2.5
2.0	2.0	2.0	1.0	1.0	1.0
0.5	0.5	0.5	0.25	0.25	0.25
0.125	0.125	0.125	Control	Control	Control

2.2 Study of intracellular bactericidal activity of RF, INH and ISO alone and in combination in J774A. 1 macrophage cell line [13].

Twenty-four well tissue culture plates (Nunc) were seeded with 10⁶-macrophage cells/ml/well (N.C.C.S.) and incubated at 37 °C in 5% CO₂ atmosphere. (Forma Scientific)

After 4 hours the macrophage monolayer were washed with sterile Hanks balanced salt solution (Haffkine Bio-pharmaceuticals Co. Ltd) to remove non-adherent cells. Macrophage monolayer was infected with 1.0 ml of test culture (MDR and standard strain of MTB which were procured from P.D. Hinduja Hospital and Medical Research centre, Mumbai)) containing 10⁶ bacteria per ml. Test culture suspension was prepared as follows. Test organisms were grown in sterile Dubos broth with glucose and albumin supplements with tween 80 (0.05%), (Himedia Laboratories) containing sterile glass beads, for 2 weeks. Growth was vortexed everyday for 30 seconds for breaking the bacterial clumps. On 14th day culture was kept in upright position in tube to settle the clumps in growth. Supernatant was transferred into another sterile test tube and the growth was washed thrice with sterile saline. Culture suspension was adjusted to McFarland number 1 turbidity. The culture was diluted in Dulbecco’s modified Eagles medium with 2% FBS. (Himedia Laboratories). This culture is ready for infecting the macrophage monolayer. After 3 hours of incubation non-phagocytosed organisms were removed by washing the wells three times with warm sterile Hanks balanced salt solution. The cells were replaced with fresh DMEM with 5% FBS containing different concentrations of drugs alone and in combination. Drug concentrations (mcg/ml) tested as follows. Values in bracket indicates the drug concentrations used for *M. tuberculosis* H37Rv strain while other concentrations are used for MDR TB strain.

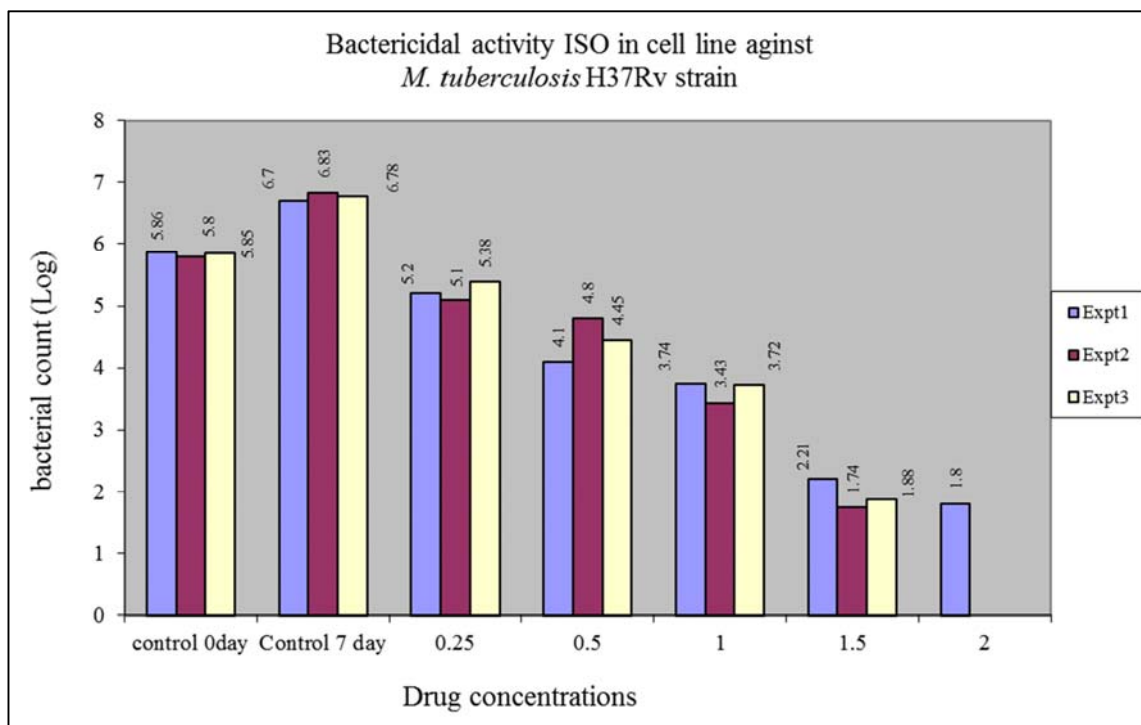
1. ISO(Cayman Chemical Co., U.S. A.): 5.0,2.5, 2.0,1.5, 1.0 and 0.5 (2.5, 2.0,1.5, 1.0, 0.5 and 0.25)
2. RF (Lupin Laboratories): 5.0, 2.5, 1.25, 0.6, 0.3 and 0.15 (2.5, 1.25, 0.6, 0.3, 0.15 and 0.07)
3. INH(Lupin Laboratories): : 5.0,2.5, 1.25, 0.6, 0.3 and 0.15 (2.5, 1.25, 0.6, 0.3, 0.15 and 0.07)
4. ISO + RF :5.0+1.5, 2.5+ 1.5, 1.25+1.5, 0.6+1.5, 0.3+1.5 and 0.15+1.5 ((2.5+ 1.5), (1.25+1.5), (0.6+1.5), (0.3+1.5), (0.15+1.5) and (0.07+ 1.5))
5. ISO + INH: 5.0+ 1.5, 2.5+1.5, 1.25+1.5, 0.6+1.5, 0.3+1.5 and 0.15+1.5 ((2.5+1.5), (1.25+1.5), (0.6+1.5), (0.3+1.5), (0.15+1.5) and (0.07+ 1.5))

Study was carried out in triplicate. Culture medium controls and cell control was included in the study. The tissue culture plates were incubated at 37 °C in 5% CO₂ atmosphere. Infected macrophages were continuously exposed to the drugs for 6 days.

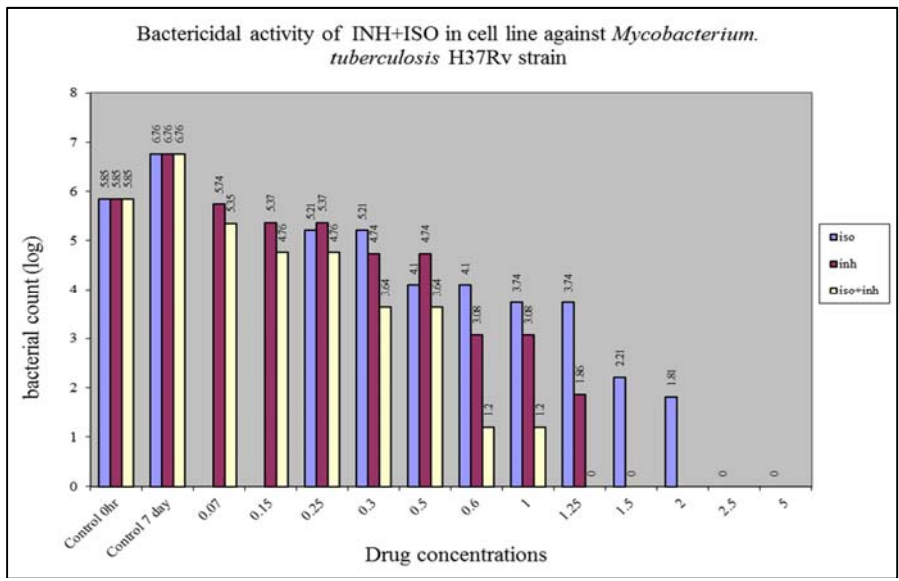
On 6th day; macrophage cells were lysed with 1ml of sterile water for injection containing 0.25% Sodium dodecyl sulfate (Qualigen).

Three, 10 fold dilutions of lysate were made and 0.1 ml of each dilution was plated on sterile Lowenstein Jensen medium (LJM) slants in duplicate. LJM slants were incubated at 37 °C for 21 days. Number of viable bacteria in each well was scored by counting the number of colonies resulting from each dilution on LJM. As a control, cells in 3 control wells were lysed immediately after initial infection to determine the number of bacteria phagocytosed and to assess the extent of growth over time. Number of CFUs obtained on LJM, were compared with the drug concentrations to determine the drug dose response. Also number of CFUs obtained on LJM were compared with the exposure of cells with combination of drugs.

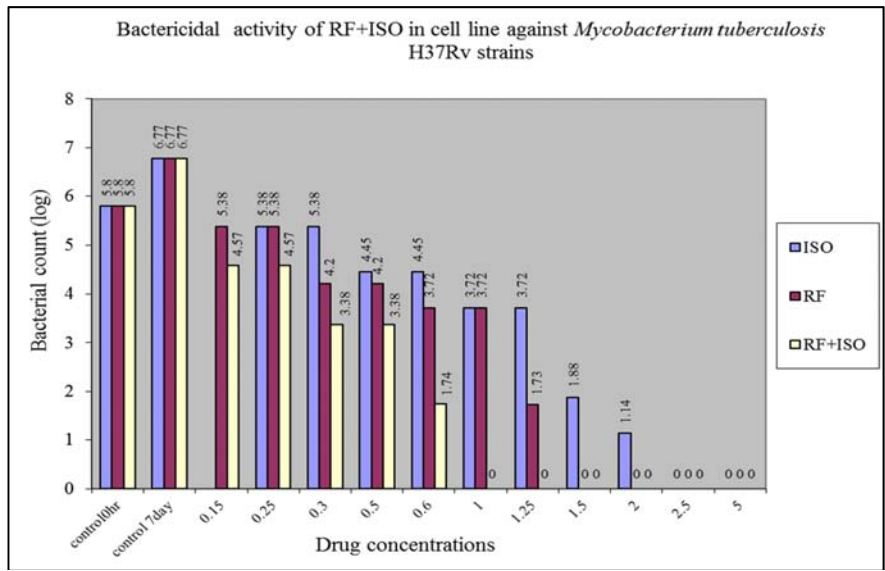
3. Results



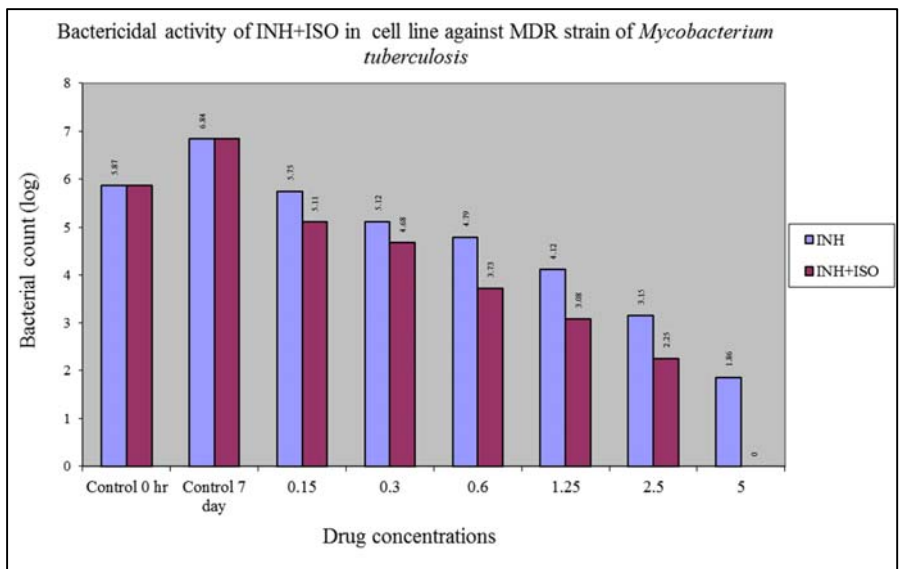
Graph 1



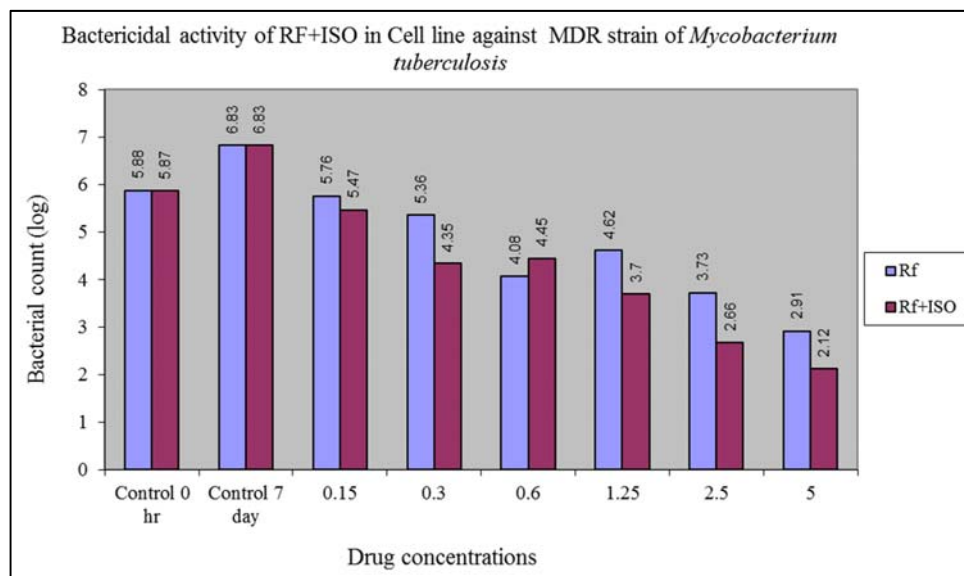
Graph 2



Graph 3



Graph 4



Graph 5

4. Discussion

The use of J 774 A.1 macrophage cell line, to study the activity of drugs to the MTB is one step close to the *in vivo* condition than the use of both susceptibility methods.

The alamar blue oxidation-reduction dye was applied as an indicator to ascertain the effects of ISO in macrophage cell viability. In this assay, the blue oxidized form becomes red due to the normal redox reactions within macrophage cells. Thus the red colour represents cell viability. Macrophage cells were grown in tissue microplates and treated with various concentrations of ISO. When the alamar blue dye was mixed with incomplete DMEM, the colour was purple. All the dilutions that contained ISO up to highest tested concentrations (5.0 mcg/ml) exhibited; the red colour. Similar results were observed in previous studies [20]. Accordingly, this thiourea is apparently not acutely toxic for macrophage cell line J774A.1.

The addition of ISO to macrophage cell cultures containing *M. tuberculosis* H37Rv strain resulted in bacterial killing in a dose-dependent manner. In the absence of ISO, viable bacteria grew to log 6 within 7 days, while in its presence, not only was growth inhibited but there was a reduction in the initial inoculum, indicating some bactericidal activity. There was significant difference in bacterial count in 7 day control and each ISO concentrations. Similar results were obtained by a study [20].

The use of an *in vitro* macrophage model allowed an assessment of the ability of ISO to cross membranes and target viable bacteria within the confines of the macrophage and phagosome. The drugs also demonstrated strong intracellular bactericidal activity by reducing the initial inoculum of virulent MTB strains suggesting cidal rather than static action.

When INH & ISO and RF & ISO were used in combination against *Mycobacterium tuberculosis* H37Rv strain and MDR strain of MTB, enhancement of activity was observed. The MICs of INH and RF dropped. Though there was no significant difference in lowering of MICs of INH and RF with combination of ISO in cell line.

The explanation for this apparent little synergistic interaction between ISO & INH and ISO & RF in the face of resistance to all is unclear. The mechanism of action of ISO

against Mycobacteria is inhibition of mycolic acid synthesis. As ISO affects cell wall in MTB, this disruption allows INH & RF to gain access to the drug resistant and susceptible cells. This impermeability plays an important role in the resistance to drugs, a view held by some investigators [21]. Alternatively, enough minor cell wall disruption may be caused by INH, despite *in vitro* resistance to allow for the penetration of ISO into cell, when would normally be excluded. Indeed RF has been observed to enhance ISO activity. The hypothesis presupposes that impermeability is the mechanism of resistance to ISO in MTB. This may be the case, since ISO apparently penetrates the envelope of other Mycobacteria to degree sufficient to its inhibition.

Some of the *in vitro* studies and clinical trial experiences of ISO in combination with INH discussed earlier shows synergistic activity of ISO along with INH [12, 22, 23]. It is not yet possible, however to predict with certainty that drug combinations that kill MTB inside J774 macrophages will be of therapeutic value in patient. Following conclusions can be drawn from these studies. This thiourea is apparently not acutely toxic for macrophage cell line J 774A.1. ISO showed some bactericidal activity in intracellular bacteria. There is an intracellular synergistic interaction, though little between ISO & INH and ISO & RF against standard strain and MDR strain of MTB.

5. Conclusion

The remarkable propensity of members of the pathogenic mycobacteria to survive within the intracellular environment of the macrophages poses particular problems for an antimicrobial agent. To exert an effect, the antibiotic must be able to penetrate the mammalian cell membrane and remain stable within the hostile cellular environment and then achieve appreciable concentrations at the intracellular site in which pathogen resides. The burden of organisms sequestered within macrophages during the course of illness represents a sizable source of viable organisms, so that the ability to ascertain whether an antibiotic can affect these organisms should be useful criteria to predict therapeutic efficacy [19]. This thiourea is apparently not acutely toxic for macrophage cell line J 774A.1, showed some bactericidal activity in intracellular bacteria. There is an intracellular

synergistic interaction, though little between ISO & INH and ISO & RF against standard strain and MDR strain of MTB.

6. References

1. Cornwall J. Tuberculosis: a clinical problem of international importance. *Lancet*. 1997; 349:660-661.
2. Paramasivan CN. An overview on drug resistant tuberculosis in India. *Lung India*. 1998; 15:21.
3. Chandrasekaran S, Jagota P, Chaudhari K. Initial drug resistance to antituberculosis drugs in urban and rural district tuberculosis programme. *Ind J. Tub.* 1992; 39:171.
4. Rosha D, Kataria VK. Impact of initial drug resistance pattern as the maintenance phase of short course chemotherapy with reference to the emergence of multi drug resistance *Ind. J Tub.* 2001; 48:205.
5. Bates JN. Tuberculosis Chemotherapy: The need for new antituberculosis drugs is urgent. *Am J Respir. Crit. Care Med.* 1995; 151:942.
6. Chopra P, Meena LS, Singh Y. New drug targets for *Mycobacterium tuberculosis*. *Ind. J Med.Res.* 2003; 117:1-9.
7. David HL, Rastogi N, Clavel-Seres S, Clement F. Alterations on the outer wall architecture caused by the inhibition of mycoside C biosynthesis in *Mycobacterium avium*. *Current microbiology*. 1988; 17:61-8.
8. Matlola NM, Steel HC, Anderson R. Antimycobacterial action of B4128, a novel tetramethylpiperidyl-substituted phenazine *J. of Antimicrob. Chemoth.* 2001; 47:199-202.
9. Winder FG. Mode of action of the antimycobacterial agents and associated aspects of the molecular biology of *Mycobacteria*. In (Ratledge and J Stanford 9Ed) *The biology of Mycobacteria*. Academic Press Inc. New York. N.Y. 1982; 1:353-438.
10. Urbancik B. Clinical experiences with thiocarlide (Isoxyl). *Antibiot. Chemother.* 1970; 16:117-123.
11. Tischer R. Monotherapie mit isoxyl/DAT bei tuberculose-asylierungfallen. *Prax Pneumoniol*: 1966, 202.
12. Schmid CH. Clinical experience in cases of primary tuberculosis with tuberculostaticum isixyl. *Antibiot chemother.* 1970; 16:108-116.
13. Phetsuksisri B, Baulard A, Cooper R, minnikin DE, Douglas DJ, Besra GS, Brennan PJ. Antimycobacterial activities of Isoxyl and new derivatives through the inhibition of mycolic acid synthesis. *Antimicrob. Agents and Chemoth.* 1999; 43(5):1042-1051.
14. Jawetz E. The use of combinations of antimicrobial drugs. *Ann. Rev. Pharmacol.* 1968; 8:151.
15. Young LS. Antimicrobial synergism and combination therapy. *The Antimicrobial Newsletter*. 1984; 1:1.
16. Rastogi N, Labrousse V. Extracellular and intracellular activities of clarithromycin used alone and in association with ethambutol and rifampicin against *Mycobacterium avium* complex. *Antimicrob. Agents Chemother.* 1991; 35:462-470.
17. Lowri DB. The macrophage and mycobacterial infections. *Trans. R. Soc. Trop. Med. Hyg.* 1983; 77:646-55.
18. Hibbs JB, Chapman Jr, HA, Weinberg JB. The macrophage as an antineoplastic surveillance cell: Biological perspectives. *J. Reticuloendothel. Soc.* 1978; 24:549.
19. Wright EL, Quenelle DC, Suling WJ, Barrow WW. Use of Human Monocytic cell Line and J774 Murine macrophage cell Line in parallel antimycobacterial Drug Studies. *Antimicrob. Agents and Chemotherapy* 1996; 40:2206-2208.
20. Phetsuksisri B, Baulard A, Cooper R, minnikin DE, Douglas DJ, Besra GS *et al.* Antimycobacterial activities of Isoxyl and new derivatives through the inhibition of mycolic acid synthesis. *Antimicrob. Agents and Chemoth.* 1999; 43(5):1042-1051.
21. Winder FG, Collins PB, Whelan D. Effects of ethionamide and isoxyl on mycolic acid synthesis in *Mycobacterium tuberculosis* BCG. *J Gen. Microbiol.* 1971; 66:379-380.
22. Rink H. Uber den derzeitigen stand der Chemotherapie bei ambulanter Anwendung. *Z. Tuberk.* 1967; 126:1.
23. Gallen CS. Isoxyl: A review of the results of its use over a five year period in the Tuberculosis field service of large urban area. *Antibiotica et Chemotherapia.* 1970; 16:139-148.