CHAPTER III

STUDY OF IN VITRO ANTITUBERCULAR ACTIVITY OF THE SYNTHESISED COMPOUNDS
The effectiveness of antimicrobial agents depends on their capacity to inhibit the multiplication or to kill the invading micro-organisms in vivo. Different lines of approach are available for finding new agents. First, knowledge of the metabolism of micro-organism, so that the new compounds will specifically interfere with metabolism to the point when their multiplication ceases. However, in the case of tubercle bacillus, knowledge of metabolism is not well understood. Second, by determining the ability of a wide variety of substances to inhibit the multiplication of Mycobacterium tuberculosis in vivo. The empirical type of mass screening of compounds is most successful in producing chemotherapeutic agents of clinical usefulness.

In the screening of compounds practical considerations determine the type of test to be employed. Direct in vivo screening has certain limitations, because only a limited number of compounds can be tested, and the amount of sample required is large. In vitro test has proved to be a suitable method for eliminating inactive compounds.

**In vivo studies: General Principles**

There is no standard in vivo screening procedure. Different workers have evolved their own methods and there
is no uniformity regarding the most suitable laboratory animal, the nature of the infecting agents, the size of the infecting dose, the route of infection, the mode of administration of the drug or the manner in which the chemotherapeutic action is measured.

The major purpose of the in vivo screening test is to detect substances which inhibit mycobacterial multiplication and therefore suppressive effect on the acute experimental tuberculous process. It would be quite inappropriate to conduct the test in a manner such that another bacteriostatic mechanism acquired immunity, would enter the picture and possibly obscure or potentiate the action of the drug. Therefore, a screening test for in vivo bacteriostasis should be conducted using animals in which only acute tuberculosis is manifested thus eliminating the factor of acquired immunity. Such in vivo test then becomes an in vivo extension of the in vitro bacteriostatic test. The advantages of such a test are that it can be readily standardized, completed in a short period of time, requires relatively few animals and the result can be obtained which has a quantitative significance.

It is pertinent to point out however, that too great significance should not be attached to the results of any experimental in vivo test, whether the disease is acute or chronic, generalized or local. The fact that a drug is
effective in the suppression of the tuberculosis infections of experimental animal does not mean that this drug will necessarily be effective for the treatment of human disease. No experimental animal is analogous to a human being. Toxic reactions differ markedly from species to species. Furthermore, drugs are not necessarily absorbed, excreted, or metabolized in the same manner by different species. A drug which is highly effective may, for a variety of reasons be ineffective for the treatment of human disease.

For example, the Sulfonamides, the Sulfones and the thiosemicarbazones all of which are effective for the suppression of experimental tuberculosis in laboratory animals but have found little clinical application in men.

Methods used for \textit{in vivo} evaluation are —

1) Tissue culture method$^{2,3}$

2) Microbial Enumeration Technique$^{4}$.

\textbf{In \textit{vivo} test}

For \textit{in vitro} bacteriostatic test several important factors must be considered. These include —

1) Nature of the culture medium

2) Nature of the test micro-organism

3) Amount of inoculum

4) Time and temperature of incubation.
The nature of the medium is of particular importance, since the bacteriostatic activity of a wide variety of substances is markedly influenced by the composition of culture medium. Many substances particularly of organic nature may combine with compound being tested and thereby reduce antimicrobial activity, of certain drugs. Lastly, substances incorporated in a medium may stimulate the growth of mycobacteria and obscure certain growth-inhibiting properties of compounds. For these reasons for absolute antimicrobial activity the simplest possible medium which is chemically defined should be used.

However, substances of biologic origin, such as serum or albumin, may be added to determine their effect on antimicrobial action. In performing test with serum concentration of serum used as an important factor small amounts may not show inhibiting effects of serum on antimicrobial activity, whereas large amounts may inhibit the growth of tubercle bacilli. The optimal growth is 5 to 10% by volume.

Different investigators have used a variety of mycobacterial species for in vitro tests. Wide metabolic differences which exist between different species and varieties of mycobacteria are responsible for their susceptibility to antimicrobial agent. For example, strains of \textit{M. avium} on the whole are more resistant to the bacteriostatic action of streptomycin and \textit{M. tuberculosis} strain No. 607
is far more resistant to the antimicrobial action of 4-aminosalicylic acid\textsuperscript{5,6}. Some strains of *M. bovis* are resistant to it. The \textsuperscript{H 37 Rv} strain of *M. tuberculosis* because of its high degree of virulence and well established metabolic, pathogenic and immunologic characteristics is selected for investigations on bacteriologic aspects of tuberculosis.

**Experimental**

(i) Preparation of the glassware

All glassware should be cleansed thoroughly with soap and water, then rinsed seven to eight times in tap water and three to four times with pure distilled water. The cleansing and rinsing of glassware is very important, since unclean glassware or glassware having a soap film may interfere with the growth of tubercle bacilli.

(ii) Choice of culture medium

A chemically defined medium is most suitable. Because of the high lipid content, the cells of *M. tuberculosis* are quite hydrophobic and therefore tend to adhere firmly and results in the accumulation of non-dispersible clumps or masses of cells. Non-ionic surfactant such as Tween-80 is recommended by Dubos\textsuperscript{7}. Arithmetic linear growth observed is due to relative decrease in the amount of
available oxygen as the number of cells increases in the
culture, aeration restores the exponential growth pattern.

The antitubercular screening was carried out in
Lowenstein Jensen medium.

Composition of the media:

<table>
<thead>
<tr>
<th>I</th>
<th>Dihydrogen potassium phosphate</th>
<th>2.5 gm</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Magnesium sulphate</td>
<td>0.25 gm</td>
</tr>
<tr>
<td></td>
<td>Magnesium citrate</td>
<td>0.60 gm</td>
</tr>
<tr>
<td></td>
<td>Aspergine</td>
<td>3.60 gm</td>
</tr>
<tr>
<td></td>
<td>glycerol</td>
<td>12.00 ml</td>
</tr>
<tr>
<td></td>
<td>Distilled water</td>
<td>600 ml</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>II</th>
<th>Malachite green solution</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2% solution of malachite green in sterile distilled water.</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>III</th>
<th>Egg solution</th>
<th>1000 ml</th>
</tr>
</thead>
</table>

All the three solutions were prepared separately. 600 ml of mineral salt solution was mixed with 20 ml of malachite green solution. To this 1000 ml of egg solution was added with constant stirring.

Slopes of Lowenstein-Jensen medium were prepared in 10 ml capacity screw capped bottles. 10 ml of medium with and without standard drug or compound to be tested
was inspissated in these bottles at 90° for three consecutive days for one hour each in the inspissatorin slanting portion. The bottles were kept in the incubator at 37°C for 12 hours to check the sterility.

Test micro-organism

A virulent strain of Mycobacterium tuberculosis H 37 Rv strain was employed for screening the antitubercular compounds.

Preparation of the inoculum

Inoculum was prepared from the well grown granular growth of Mycobacterium tuberculosis on the slopes of flat inoculum bottles. 20 ml of sterile saline was added into these bottles and growth was lightly scraped. This suspension was collected in sterile flask and was kept on shaker for one hour to homogenize the cells for uniformal growth.

0.1 ml of this suspension was used to inoculate each bottle.

Preparation of drug dilution

The solvent is selected depending on the solubility of compound. Commonly used solvents are - ethyl alcohol, ether, propylene glycol, 0.1 sod. hydroxide, 0.1 M hydrochloric acid, dimethyl formamide. Heat labile substances
should be filtered through sintered glass. Heat stable compounds can be diluted and then sterilised in an autoclave. The concentration used are in the range of 5.0 and 50 ug/ml.

Preparation of compound solution

50 mg of compound was weighed and dissolved in 10 ml of dimethylformamide in sterile bottles. 0.1 ml of this solution was added into 10 ml of medium to give 50 mcg/ml concentration.

Incubation

Bottles were incubated at 37°C in the incubator for 20 days. On every 5th day observations were made for the growth and contaminants.

Observations on 10th, 15th and 20th day were recorded. All the compounds were tested at 50 ug/ml in duplicate. Active compounds at 50 ug/ml were tested at lower concentration to determine the exact minimum inhibitory concentration value. No growth at the lowest concentration was taken as MIC value for the compound. Proper blanks, drug and solvent controls were run with each batch. Results are tabulated in Table - II.
### Table II

**Antitubercular activity**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Name of compound</th>
<th>MIC value in mcg/ml against <em>M. tuberculosis</em> H 37 RV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Para-aminosalicyl phenyl hydrazide</td>
<td>More than 50</td>
</tr>
<tr>
<td>2</td>
<td>N-glucosyl nicotinamide</td>
<td>More than 50</td>
</tr>
<tr>
<td>3</td>
<td>Isorucotinyl anilide</td>
<td>More than 12.5</td>
</tr>
<tr>
<td>4</td>
<td>Nicotinyl 4-aminosalicylic acid</td>
<td>More than 50</td>
</tr>
<tr>
<td>5</td>
<td>Isonicotinyl 4-aminosalicylic acid</td>
<td>More than 50</td>
</tr>
<tr>
<td>6</td>
<td>Fructosyl isonicotinic acid</td>
<td>More than 50</td>
</tr>
<tr>
<td>7</td>
<td>Glucosyl isonicotinic acid</td>
<td>More than 50</td>
</tr>
<tr>
<td>8</td>
<td>N-Glucosyl isonicotinamide</td>
<td>More than 50</td>
</tr>
<tr>
<td>9</td>
<td>Isonicotinyl phenyl hydrazide</td>
<td>More than 50</td>
</tr>
<tr>
<td>10</td>
<td>Para-aminosalicylic acid (Standard)</td>
<td>More than 0.78</td>
</tr>
<tr>
<td>11</td>
<td>Isonicotinic acid (Standard)</td>
<td>More than 1.56</td>
</tr>
</tbody>
</table>
Maintenance of the culture

Culture was maintained on Lowenstein Jensen medium slopes in big rectangular bottles, granular growth free from contamination on these slopes was used as inoculum.

The purity of the culture was checked periodically by acid fast differential stains and microscopy.

Result

Compound isonicotinyl anilide has moderate anti-tubercular activity. Other compounds tested showed activity at a conc. more than 50 mcg/ml.
REFERENCES

1 Schnitzer, R.J. and Hawking, F., Experimental Chemotherapy, 2, 403, (1964).


