ANTITUBERCULOUS ACTIVITY OF ISONIAZID IN COMBINATION WITH SULFISOXAZOLE (GANTRISIN) I.
THE EFFECT OF ISONIAZID-SULFISOXAZOLE *IN VITRO*

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(Received for publication August 10, 1960)

INTRODUCTION

The antituberculous activity of the sulfanilamides has been investigated since the drugs were first found. The discovery of Tibione was probably related to those investigations.

At present, however, sulfanilamides are rarely used in the treatment of tuberculosis.

In 1952 Isoniazid (INH) became available in this laboratory.

Considering the difference in antibacterial spectrum between INH and streptomycin (SM), the present authors administered Sulfisoxazole (SI) in combination with INH to some pulmonary tuberculosis patients who were believed to have mixed infections, and it became apparent that SI was effective not only against the bacteria which caused the mixed infection, but also against *Mycobacterium tuberculosis* when it was used with INH.

Investigations on the antituberculous activity of INH and SI were therefore begun in this laboratory, and the first data obtained were reported at the 29th annual meeting of the Japanese Association for Tuberculosis[1] held in 1954, and the results of the clinical studies[2-7] of the INH-SI regimen for pulmonary tuberculosis in this institute have been successively reported since 1955.

In 1956 Ushiba, Sunahara, Shimamura, Koyama, Takahashi, Tomita and Gomi[8] undertook similar research and reported similar results with satisfaction.

Since then many investigations of this regimen have been carried out in this country.

The present report concerns the *in vitro* effect of this combined use of the drugs which was elucidated in this laboratory.

EXPERIMENTS

I. *In Vitro* Studies on the Tuberculostatic Effects of INH, SM or PAS in Combination with SI and Other Sulfanilamide Derivatives

Methods:

The drugs were evaluated for *in vitro* activity against *M. tuberculosis* var. *hominis* (H37Rv) in 2 ml. of Kirchner's liquid medium[9] containing bovine serum in the concentration of 10 per cent.

The solutions of drugs used were those marketed for injection. Tubes of each drug-combination and appropriate controls were inoculated with approximately 0.04 mg. wet weight of *M. tuberculosis* in fine suspension in the first experiment, 0.8 mg. in the second experiment and 0.0008 to 0.8 mg. in the third experiment.

The suspension was prepared by the following method. A portion of a surface pellicle culture which had grown on Kirchner's medium for ten days was half dried on aseptic filter paper for 30 minutes and weighed. A definite amount of *Mycobacterium* was put into a flask containing glass beads. Then the flask was shaken, while physiological saline solution was added drop by drop. This suspension was diluted to a definite concentration of *Mycobacterium* with physiological saline solution.

Incubation was carried out at 37°C in an incubator. At the end of four weeks the tubes were examined and the bacteriostatic activity of the drug or combination of drugs was recorded as the least amount of the drug or combination which completely prevented the growth of the *Mycobacterium.*

Results:

The results are illustrated in tables 1 to 3.

In the first experiment (table 1) the combined effects of INH, SM or PAS with various concentrations of sulfanilamide derivatives were studied. The minimum inhibitory concentration (MIC) of INH used in combination with various concentrations of sulfanilamide derivatives was half to one-sixth of that of INH used alone. When SM or PAS was combined with sulfanilamide derivatives no additive effect was found. In every experiment it was shown that SI increased the bacteriostatic effect of INH when they were used together. It is well known that SI is one of the sulfanilamide derivatives, which rarely cause gastric trouble, the most frequent side effect of many sulfanilamide derivatives. In the subsequent experiments, therefore, only SI was employed as the sulfanilamide derivative to be used in combination with INH.

In the second experiment (table 2) 2 ml. of the medium was inoculated with 0.8 mg. wet weight of *Mycobacterium.* The MIC of INH used in combination with ten times as much SI was 0.032 γ per ml., while the MIC of INH used alone was 0.125 γ per ml.

Table 1. Minimum Inhibitory Concentrations of Antituberculous Agents (INH, SM and PAS) in Combination with Various Concentrations of Sulfanilamide Derivatives (SA) (*in vitro*)

Drug used in combination	Ratio of antituberculous agent and sulfanilamide									
	INH : SA						SM : SA		PAS : SA	
	1 : 25			1 : 10	1 : 3		1 : 5	1 : 3	1 : 1/2	1 : 1/3
None	0.08	0.06	0.06	0.06	0.06	0.12	1.56	1.25	3.13	1.56
Sulfisoxazole	0.03	0.02	0.03	0.03	0.03	0.02	1.0	4.0	2.5	1.56
Sulfapyridine		0.02		0.03	0.03	0.04	2.0	4.0	2.5	1.56
Homosulfamine		0.03		0.06	0.03	0.08	2.0	4.0	2.5	1.56
Sulfamerazine		0.12		0.03	0.06	0.16	0.5	2.0	2.5	1.56
Sulfathiazole		0.02		0.03	0.06	0.04	2.0	2.0	2.5	1.56
Sulfadiazine		0.12		0.06	0.12	0.12	1.0	2.0	1.56	1.56
Sulfapyrimidine				0.03	0.12	0.12		2.0		1.56

* Figures indicate the minimum inhibitory concentration of INH, SM or PAS in γ per ml.

Table 2. MIC of INH in Combination with SI *in vitro*

Drug	MIC of INH (γ/ml.)
INH	0.125
INH+SI (1 : 10)	0.032

In the third experiment (table 3) the combined effect was studied with various amounts of inoculum, and found to be present when the concentration of inoculum was higher than 0.04 mg. per ml. of medium. But a combined effect was not apparent when the concentration of inoculum was less than 0.004 mg. per ml. of medium.

Table 3. Effect of Inoculum Size on Tuberculostatic Activity of INH and SI

Drug	Concentration of *Mycobacteria* in the medium			
	0.4 mg./ml.	0.04 mg./ml.	0.004 mg./ml.	0.004 mg./ml.
INH	*1.0	0.1	0.005	0.005
INH+SI (1 : 10)	0.1	0.02	0.005	0.005

* Figures indicate the MIC of INH (γ per ml.).

II. *In Vitro* Studies on the Tuberculostatic Effect of SI

Methods:

Three different experiments were carried out as follows:

(1) An inoculum of 0.01 mg. of H37Rv per ml. of Kirchner's liquid medium[9]

with 10 per cent serum was used. The suspension of the inoculum was prepared as previously described.

(2) Dubos liquid medium with the same inoculum.

(3) Inoculum of 0.02 mg. in each tube of 5 ml. of Oka-Katakura's egg medium[10].

Cultures employing Kirchner's and Oka-Katakura's medium were macroscopically read after a four-week incubation at 37°C and culture employing Dubos medium after a two-week incubation.

Results:

As stated in table 4, in every medium the MIC of SI after a two-week-incubation was one-half or one-fourth of the MIC after a four-week-incubation.

As stated in table 5, after a four-week-incubation the MIC of SI in acid media was less than in neutral or alkaline (Kirchner's Medium). In summary, SI has a weak but definite tuberculostatic action *in vitro*.

Table 4. MIC of SI in Different Media and Different Periods of Incubation

Media	Period (days)	MIC of SI (γ/ml.)
Kirchner's M.	14	31.3
	28	125.0
Dubos M.	14	7.8
	28	15.6
Oka-Katakura's egg M.	14	15.6
	28	31.3

Table 5. Effect of pH of Media on MIC of SI

pH	MIC of SI (γ/ml.)
5.5	31.3
6.0	62.5
6.5	250.0
7.0	500.0
7.5	500.0
8.0	500.0

III. Effect of pH on Tuberculostatic Activity of INH alone and in Combination with SI

Methods:

The pH of Kirchner's medium containing 10 per cent serum was adjusted to 5.5, 6.5 and 7.5 with NaOH or HCl. The concentration of the inoculum was 0.04 mg. per ml. of the medium.

Results:

The MIC of INH was 0.125 γ per ml. at pH 6.0 to 7.0 and 0.0625 γ per ml. at pH 5.5. The combined effect of the two drugs was more marked in an acid than in a neutral or alkaline medium (table 6).

Table 6. Effect of pH on the Combined Use of INH and SI

pH	INH alone	INH+SI (1:10)
5.5	*0.0625	0.015
6.0	0.125	0.031
6.5	0.125	0.062
7.0	0.125	0.125
7.5	0.0625	0.125

* Figures indicate the MIC of INH (γ per ml.).

IV. The Bacteriostatic Effect of INH and SI *In Vitro* on *M. Tuberculosis* Resistant to SM or PAS

Methods:

The methods were those of the previous experiment (I), but *M. tuberculosis*, strain H37Rv resistant to 100 γ of SM per ml. and that resistant to 50 γ of PAS per ml. were employed as inocula. The concentration of the inoculum was 0.02 mg. per ml. of the medium.

Results:

As may be seen in table 7, the combined effect of INH and SI was recognized on both SM and PAS resistant strains.

Table 7. The Combined Effect of INH and SI on *M. Tuberculosis* Resistant to SM or to PAS

Drug	*M.* Resistant to SM	*M.* Resistant to PAS
INH alone	*0.025	0.025
INH +SI (1:10)	0.0062	0.0062

* Figures indicate the MIC of INH (γ/ml.).

V. The Bactericidal Effect of INH and SI *In Vitro*

Methods:

1) Physiological saline solution was placed in 2 ml. amounts in centrifuge tubes, the drugs were added to the first tube and serial dilutions were made through the tubes. Each tube was then inoculated with 0.05 mg. of *M. tuberculosis* and incubated for 24 hours at 37°C.

2) Kirchner's medium containing 10 per cent serum was placed in 2 ml. amounts in the centrifuge tubes, the drugs were added to the first tube and serial dilutions were made through the tubes. Inoculation was the same as above and incubation was for 4 weeks at 37°C.

After incubation each tube was centrifuged for 15 minutes at 3,000 r.p.m., the supernatant was discarded, physiological saline solution was added to the precipitate and the tubes were centrifuged as above. The washing process was repeated three times, and after the final washing the precipitate was dispersed in approximately 0.1 ml. of physiological saline solution.

One loop-full of the suspension was then smeared on 2 egg media (Oka-Katakura's), and incubated for 4 to 6 weeks.

Results:

The bactericidal effects of INH and SI for *M. tuberculosis* in physiological saline solution at pH 5.5, 6.5 and 7.5 with a 24-hour-incubation are shown in table 8. The minimum bactericidal concentration of INH alone was 12.5 γ per ml. at pH 5.5, 25.0 γ per ml. at pH 6.5, and 25.0 γ per ml. at pH 7.5. The minimum bactericidal concentration of INH in combination with three or ten times as much SI was approximately 1.56 γ per ml.

Table 8. Bactericidal Effects of INH Combined with SI in Physiological Saline Solution with a 24-Hour Incubation

pH of medium	5.5	6.5	7.5
INH alone	*12.5(−)	25.0(−)	25.0(−)
	6.25(+)	12.5(+)	12.5(+)
INH+SI (1:3)	1.56(−)	1.56(−)	3.13(−)
	0.78(+)	0.78(+)	1.56(+)
INH+SI (1:10)	1.56(−)	1.56(−)	0.39(−)
	0.78(+)	0.78(+)	0.19(+)

* Figures indicate the concentration of INH (γ/ml.).
(−): Bactericidal (+): Not bactericidal

The bactericidal effects of INH and SI for *M. tuberculosis* in Kirchner's medium at various pH values with a four-week incubation are shown in table 9. The minimum bactericidal concentration of INH alone-was 6.25 γ per ml. at pH 5.5, 12.5 γ per ml. at pH 6.5 and 7.5, and that of INH in combination with three or ten times as much SI was approximately 1.56 γ per ml.

Table 9. Bactericidal Effects of INH Combined with SI in Kirchner's Media with a Four-Week Incubation

pH of medium	5.5	6.5	7.5
INH alone	*6.25(−)	12.5(−)	12.5(−)
	3.13(+)	6.25(+)	6.25(+)
INH+SI (1:3)	1.56(−)	1.56(−)	1.56(−)
	0.78(+)	0.78(+)	0.78(+)
INH+SI (1:10)	0.78(−)	3.13(−)	1.56(−)
	0.39(+)	1.56(+)	0.78(+)

* Figures indicate the concentration of INH (γ/ml.).
(−): Bactericidal (+): Not bactericidal

VI. Development of Resistance of *M. Tuberculosis* to INH through Successive Transfers in Media Containing Increasing Concentrations of INH alone and of INH plus SI

Methods:

Tubes with 2 ml. of Kirchner's media with 10 per cent serum containing various dilutions of INH alone or INH plus SI were inoculated with 0.04 mg. of *M. tuberculosis*, prepared as described above. Monthly, after the MIC of INH was estimated, the *Mycobacterium* growing in the test medium containing the highest concentration of drugs was transferred into the second, newly prepared test media containing the drugs in higher concentrations. The test media were adjusted at pH between 5.4 and 5.6 (acid), 6.6 and 6.8 (neutral) and 7.2 and 7.4 (alkaline).

Table 10. Development of Resistance of *M. tuberculosis* to INH on Successive Transfers in the Media Containing Increasing Concentration of the Drugs

pH		Acid			Neutral			Alkaline		
Generation		I	II	III	I	II	III	I	II	III
INH alone	Not inhibited	*0.005	0.1	1.0	0.1	10.0	25.0	0.25	0.02	10.0
	Inhibited	0.01	1.0	10.0	1.0	100.0	100.0	0.1	0.1	100.0
INH+SI (1:3)	Not inhibited	0.005	0.01	0.1	0.02	0.02	1.0	0.02	0.02	1.0
	Inhibited	0.01	0.02	1.0	0.1	0.1	10.0	0.1	0.1	10.0
INH+SI (1:25)	Not inhibited	0.01	0.1	0.1	0.02	0.02	1.0	0.02	0.02	1.0
	Inhibited	0.02	1.0	1.0	0.1	0.1	10.0	0.1	0.1	10.0

* Figures indicate the concentration of INH (γ/ml.).

Results:

Development of resistance of *M. tuberculosis* to INH on successive transfers in media containing increasing concentrations of INH and of INH plus SI at various pH values is illustrated in table 10. It may be seen that emergence of resistance of *Mycobacterium tuberculosis* to INH was delayed by adding three or ten times as much SI, especially in neutral media.

VII. Development of Resistance of *M. Tuberculosis* to INH in Media Containing Fixed Concentrations of INH Alone and of INH Plus SI

Methods:

Tubes with 2 ml. of Kirchner's serum medium containing various concentrations of INH or INH plus SI were inoculated with 0.04 mg. of *M. tuberculosis*.

At intervals of one month the supernatants were discarded aseptically, and the precipitates were re-suspended in newly prepared media containing the same concentrations of the drugs, and each time the resistance of *Mycobacterium* to INH was estimated in egg media.

Results:

The results are shown in table 11, in which it may be seen again that the emergence of resistance of *Mycobacterium* to INH was delayed in media containing INH plus SI.

Table 11. Development of Resistance of *M. tuberculosis* to INH by Culturing in Media Containing a Fixed Concentration of the Drugs

pH		Acid			Neutral			Alkaline		
Generation		I	II	III	I	II	III	I	II	III
INH alone	Not inhibited	0.0125	100.0	100.0	0.0125	4.0		0.25	4.0	4.0
	Inhibited	0.025	200.0	200.0	0.025	8.0		0.5	8.0	8.0
INH+SI (1:3)	Not inhibited	0.0031	1.0	4.0	0.0062	0.0062	0.1	0.0031	0.0062	0.0062
	Inhibited	0.0062	2.0	8.0	0.0125	0.0125	0.2	0.0062	0.0125	0.0125
INH+SI (1:10)	Not inhibited	0.0031	0.05	0.25	0.0031	0.0062	0.25	0.0031	0.0062	0.0062
	Inhibited	0.0062	0.1	0.5	0.0062	0.0125	0.5	0.0062	0.0125	0.0125

DISCUSSION

The combined effect of INH, SM or PAS and various sulfanilamide derivatives, especially SI, on *M. tuberculosis* var. *hominis*, strain H37Rv *in vitro* was studied.

In Kirchner's liquid medium containing 10 per cent serum, the sulfanilamide derivatives, especially SI, increased the bacteriostatic effect of INH. Although the combined effect of INH and SI was clearly noted when the concentration of

the inoculum was higher than 0.04 mg. wet weight per ml., it was not obvious when the concentration was lower than 0.004 mg. wet weight per ml.

Sulfanilamide derivatives did not increase the bacteriostatic effect of SM or PAS.

The MIC of SI was 125.0 γ per ml. in Kirchner's medium, 31.3 γ per ml. in egg medium and 15.6 γ per ml. in Dubos medium. The MIC was lower in acid than in neutral or alkaline media. Thus, SI has a weak but definite tuberculostatic effect *in vitro*.

The combined effect of INH and SI was also more marked in acid than in neutral or alkaline media.

It is worth emphasizing that this combination is effective even on *Mycobacteria* resistant to SM or to PAS.

The tuberculocidal effect of INH has been well known to be relatively higher than that of other tuberculostatic drugs. In the present studies it was found that SI increased the tuberculocidal effect of INH markedly. The minimum bactericidal concentration of INH used with SI was approximately 1.56 γ per ml. This concentration can probably be achieved in human body fluids.

The aim of combined use of antituberculous agents is, of course, not only the increase of bacteriostatic effect, but also the delay of the development of drug resistance. Therefore the *in vitro* effect of SI on the development of resistance of *Mycobacterium* to INH was studied. Both in the experiment with successive transfers in media containing increasing concentrations of INH and in that with media containing a fixed concentration of INH, SI was noted to delay the emergence of resistance of *Mycobacterium* to INH.

Thus the combined effect of INH and SI *in vitro* was demonstrated clearly. The mode of this synergic action, results of animal experiments and clinical studies will be successively reported.

SUMMARY

1) A synergic bacteriostatic effect was demonstrated in the combined use of INH and Sulfisoxazole (SI) *in vitro*.
2) SI was found to have a weak but definite tuberculostatic effect *in vitro*.
3) This combined effect was recognized even with SM and PAS resistant strains.
4) SI also increased the tuberculocidal effect of INH.
5) The emergence of resistance of *Mycobacterium* to INH *in vitro* was markedly delayed in the presence of SI.

REFERENCES

1) Naito, M., Watanabe, R., Maekawa, N., Hineno, Y., Nakamura, A., and Iba, K.: Kekkaku, 29: 248, 1954.
2) Naito, M.: Kekkaku no Rinsho, 3: 458, 1955.
3) Naito, M.: Jap. Arch. Internal Med. 2: 639, 1955.
4) Naito, M.: Japan. J. Clin. Tuberc. 15: 674, 1956.
5) Naito, M.: Naika, 2: 540, 1958.
6) Yoshida, T.: Rep. Tuberc. Res. Inst., Kyoto University, 6: 259, 1958.
7) Naito, M.: V International Congress on Diseases of the Chest, 315, 1958.
8) Ushiba, D., Sunahara, S., Shimamura, K., Koyama, Y., Takahashi, K., Tomita, Y. and Gomi, J.: Japan. J. Clin. Tuberc. 15: 664, 1956.
9) Kirchner, O.: Zbl. Bakt. I, Org., 124: 403, 1932.
10) Oka, S., Katakura, T. and Ishikawa, Y.: Tohoku Igaku Zasshi, 21: 692, 1937.