# FURTHER OBSERVATIONS ON THE ANTIBACTERIAL ACTIVITY TO *MYCOBACTERIUM TUBERCULOSIS* OF A DERIVATIVE OF ISONIAZID, *o*-HYDROXYBENZAL *ISO*NICOTINYLHYDRAZONE (NUPASAL-213)

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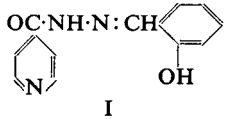
## INTRODUCTION

A previous report[1] described the *in vitro* tuberculostatic activity, and, in some cases, the toxicity and *in vivo* tuberculostatic activity, of a number of new isoniazid derivatives. Of these, compound HP.213 *o*-hydroxybenzal *iso*nicotinylhydrazone (I) by reason of its low toxicity and high activity, merited further examination, the results of which are now reported. The compound has been given the name "Nupasal-213."*




I

## EXPERIMENTAL

### *Methods*

(i) *In vitro* tests were made with the technique previously described[2], using initially a solution of 5 mg. HP.213 in 2 ml. propylene glycol and 18 ml. Dubos Tween albumin medium. The solution, after Seitz filtration, was successively diluted in the same Tween albumin medium. Inoculation of the tubes was carried out using 0·1 ml. of a 10 to 14 day old culture of *Myco. tuberculosis* H37Rv. Growth was finally recorded after 14 days incubation at 37° C.

(ii) *In vitro* tests on the development of resistance to *Myco. tuberculosis* H37Rv were carried out by a technique subsequently described by Steenken[3]. The technique was the same as described above. The tube containing the maximum concentration of the drug in which growth was apparent, for each set of dilutions, was used as the source of inoculum for the second set of dilutions. This represents the first transfer. Subsequent transfers were made in the same way.

(iii) *In vivo* activity was assessed by the corneal test in mice[4] using *Myco. tuberculosis* H37Rv. The intra-corneal injection was made using approximately 1000 organisms in suspension. The drug was administered in the diet immediately after infection. The infected eye was examined frequently for the development of lesions and 28 days after injection final examination of the eyes was made.

* *o*-Hydroxybenzal-*iso*nicotinylhydrazone is available in America under the name "Salizid" and a preliminary account of tests with this material has been given by Barry and Conalty[7].

(*b*) *Toxicity.* Acute toxicity tests were made on male albino mice, weighing between 18 and 25 g. Chronic toxicity tests were made on groups of 10 male rats, of the Wistar strain, weighing from 90 to 110 g., and groups of 4 male and 1 female guinea-pigs weighing 150 to 270 g. The rats were fed on "diet 41" rat cubes, the guinea-pigs on "diet 18" pellets and green food. Doses from 100 mg./kg. to 2·0 g./kg. of HP.213 were given by stomach tube. In the chronic tests, administration was daily, with the exception of Saturdays and Sundays, in the form of a mucilage of acacia suspension. Comparative experiments using isoniazid in doses of 50 mg./kg. and 100 mg./kg. were made and control groups were given mucilage of acacia only.

(*c*) *Estimation in plasma.* Two methods have been used, (i) the cyanogen bromide method of Rubin *et al.*[5] which estimates all *iso*nicotinic acid derivatives present, and (ii) the naphthoquinone method of Short[6] which estimates free isoniazid. HP.213 is not estimated by the latter method, since the terminal amino-group is blocked. In the absence of a specific method for the estimation of HP.213 in plasma, the difference between the results of method (i) and (ii) measures the concentration of HP.213 together with the concentration of metabolites containing an *iso*nicotinic acid group.

## Results

### *Tuberculostatic Activity*

(i) *In vitro*, the minimum inhibitory concentration (M.I.C.) of HP.213 was 0·09 μg./ml. The corresponding figure for isoniazid was 0·03 μg./ml. Against an isoniazid resistant strain, the results were HP.213 15 μg./ml. and isoniazid 7·5 μg./ml.

(ii) Results of experiments on the development of resistance by *Myco. tuberculosis* to HP.213, isoniazid, and mixtures of the two substances are given in the Table I.

TABLE I

DEVELOPMENT BY *Myco. tuberculosis* OF RESISTANCE TO H.P.213 ALONE AND IN COMBINATION WITH ISONIAZID

| | M.I.C. μg./ml. at start of experiment | M.I.C. μg./ml. after 2½ months (8 transfers) | M.I.C. μg./ml. after 6 months (13 transfers) |
|---|---|---|---|
| HP.213 | 0·06 | 7·81 | not continued |
| Isoniazid | 0·03 | 7·81 | not continued |
| Isoniazid (0·01 μg./ml.) and varying proportions of HP.213 | 0·06 (HP.213) | 0·48 (HP.213) | 15·62 (HP.213) |
| HP.213 (0·01 μg./ml.) and varying proportions of isoniazid | 0·03 (isoniazid) | 0·24 (isoniazid) | 7·81 (isoniazid) |

TABLE II

CORNEAL TEST IN MICE USING *Myco. tuberculosis* H37Rv SENSITIVE TO ISONIAZID

| Compound | Per cent. in diet | No. of mice with lesions | Protection per cent. |
|---|---|---|---|
| Isoniazid | 0·002 | 4/9 | 55 |
| Isoniazid | 0·004 | 0/8 | 100 |
| HP.213 | 0·004 | 5/8 | 38 |
| HP.213 | 0·008 | 0/10 | 100 |
| Untreated controls | — | 6/7 | 14 |

(iii) The results of *in vivo* tests on the tuberculostatic activity of HP.213 are given in Tables II and III.

TABLE III

CORNEAL TEST IN MICE USING A STRAIN OF *Myco. tuberculosis* RESISTANT TO ISONIAZID

| Compound | Per cent. in diet | No. of mice with lesions | Protection per cent. |
|---|---|---|---|
| HP.213 .. .. .. | 0·1 | 4/8 | 50 |
| HP.213 .. .. .. | 0·04 | 7/9 | 22 |
| Isoniazid .. .. .. | 0·02 | 5/6 | 17 |
| Isoniazid .. .. .. | 0·004 | 6/6 | 0 |
| Untreated controls .. | — | 8/8 | 0 |

*Toxicity*

(i) The acute oral LD50 to mice was found to be greater than 10 mg./g. The corresponding dose for isoniazid, determined simultaneously, was 0·14 mg./g.

(ii) The chronic oral toxicity tests were continued for three months. Chronic oral approximate lethal doses were found to be as follows.

| | *Rats* | *Guinea-pigs* |
|---|---|---|
| HP.213 .. .. .. | 250–500 mg./kg. | 500–1000 mg./kg. |
| Isoniazid .. .. | 50–100 mg./kg. | 50–100 mg./kg. |

Weight curves of some typical groups of both rats and guinea-pigs during the chronic toxicity test are shown in Figures 1 (*a*) and (*b*), 2 (*a*) and (*b*). Examination of these curves shows that animals treated with 500 mg./kg. of HP.213 gained weight at approximately the same rate as those treated with 100 mg./kg. of isoniazid. During the course of the experiment, in both species of animals, neither HP.213 nor isoniazid produced any change in the red and white blood cell count or in the blood levels of prothrombin, glucose, urea and amino-acids. Histological specimens taken post-mortem after the termination of the chronic toxicity test showed no significant abnormalities compared with similar specimens taken from control animals.

TABLE IV

PLASMA LEVELS IN RABBITS OF ISONIAZID FOLLOWING ORAL ADMINISTRATION OF HP.213

Dose: 25 mg./kg. of HP.213

| Time after administration, hours | Isoniazid, µg./ml. plasma |
|---|---|
| 1 | 4·7 |
| 2 | 2·0 |
| 4 | 0·6 |
| 6 | 0 |

*Plasma Levels*

Preliminary experiments on rabbits had shown that HP.213 after oral administration was broken down, at least in part, to form isoniazid. The average results from a group of 3 rabbits receiving 25 mg./kg. of HP.213 orally are given in Table IV. Plasma levels of isoniazid in human volunteers receiving a single dose of 600 mg. of HP.213 orally are shown in Figure 3.

To test whether the formation of isoniazid from HP.213 occurs mainly in the gastro-intestinal tract, rabbits were given intraperitoneal injections of HP.213, and plasma levels of isoniazid and HP.213 were determined.

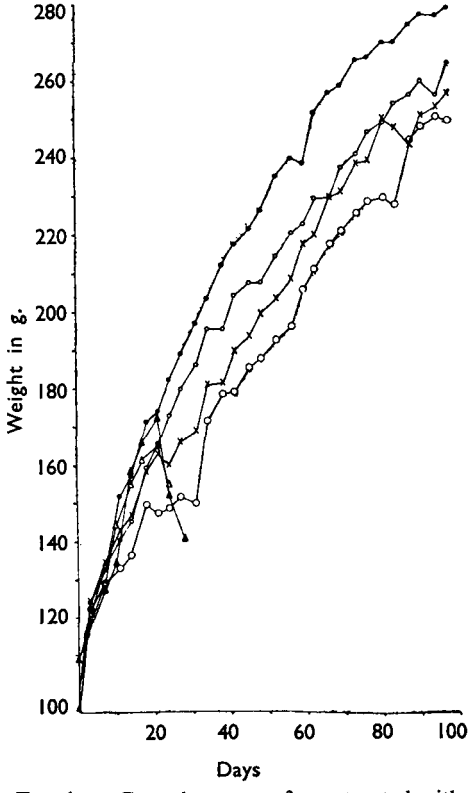



FIG. 1A. Growth curves of rats treated with HP.213.

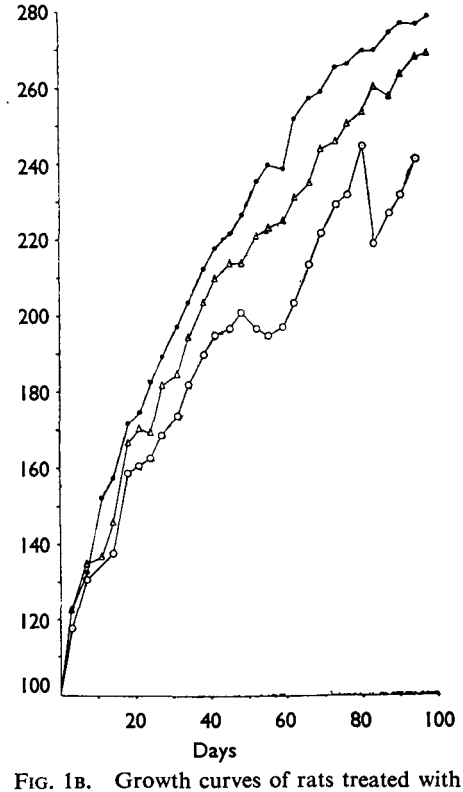



FIG. 1B. Growth curves of rats treated with isoniazid.

●——● Controls. Saline.
○——○ 100 mg./kg. HP.213.
×——× 250 mg./kg. HP.213.
⊙——⊙ 500 mg./kg. HP.213.
▲——▲ 1·0 g./kg. HP.213.
△——△ 2·0 g./kg. HP.213.

●——● Controls. Saline.
△——△ 50 mg./kg. isoniazid.
⊙——⊙ 100 mg./kg. isoniazid.

The average results from a group of six rabbits receiving 20 mg./kg. of HP.213 intraperitoneally are given in Table V.

## DISCUSSION

The foregoing results show that HP.213 has a tuberculostatic activity of the same order as that of isoniazid, with only one-fifth to one-tenth of the toxicity of the latter drug. Cross resistance has been observed between the two

TABLE V

PLASMA LEVELS IN RABBITS OF ISONIAZID AND HP.213 FOLLOWING INTRAPERITONEAL ADMINISTRATION OF HP.213

Dose: 20 mg./kg. of HP.213

| Time after injection, hours | Isoniazid, µg./ml. plasma | HP.213, µg./ml. plasma |
|---|---|---|
| 1 | 0 | 5·8 |
| 2 | 0 | 3·8 |
| 4 | 0 | 2·8 |
| 6 | 0 | 2·0 |

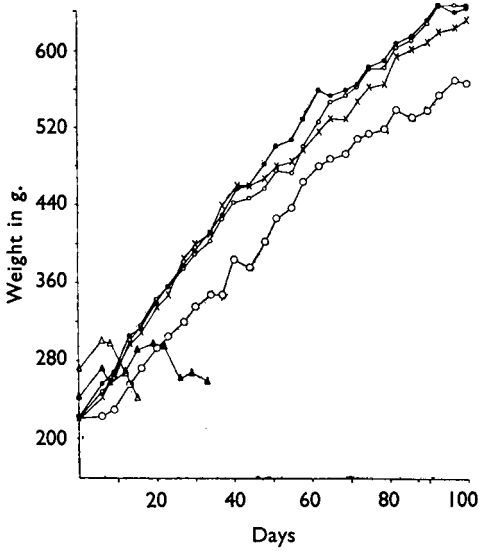



FIG. 2A. Growth curves of guinea-pigs treated with HP.213.

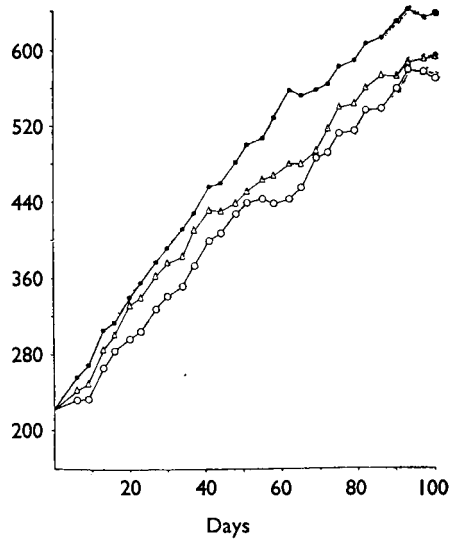



FIG. 2B. Growth curves of guinea-pigs treated with isoniazid.

| | |
|---|---|
| ●——● | Controls. Saline. |
| ○——○ | 100 mg./kg. HP.213. |
| ×——× | 250 mg./kg. HP.213. |
| ⊙——⊙ | 500 mg./kg. HP.213. |
| ▲——▲ | 1·0 g./kg. HP.213. |
| △——△ | 2·0 g./kg. HP.213. |

| | |
|---|---|
| ●——● | Controls. Saline. |
| △——△ | 50 mg./kg. isoniazid. |
| ⊙——⊙ | 100 mg./kg. isoniazid. |

compounds in the sense that strains of *Myco. tuberculosis* highly resistant to isoniazid were also resistant to HP.213. However, the low toxicity of the latter substance enables sufficiently high doses to be used *in vivo* to produce some effect against isoniazid-resistant organisms. Moreover, isoniazid and HP.213 appear to possess a mutual action on the emergence of resistance, inasmuch as each substance delays the development of resistance by *Myco. tuberculosis* to the other compound. While this work was in progress, similar results were reported by Steenken, Wolinsky and Montalbine[3]. Further work is now being carried out to determine whether a similar mutual effect can be reproduced *in vivo*.

Absorption experiments in rabbits show that, after oral administration of moderate doses of HP.213, isoniazid is present in the plasma, whereas no isoniazid was detected after the intraperitoneal injection of a similar dose of HP.213.

In a group of 12 mice receiving daily subcutaneous injections of 60 mg./kg. of HP.213 for 5 days, 2·7 μg./ml. of isoniazid was found in the plasma. These results suggest that HP.213 is much more easily decomposed to isoniazid by the gastro-intestinal tract than by tissue enzymes. This result is not unexpected since it is known that HP.213 is easily decomposed by dilute acids to form isoniazid and salicylaldehyde. Plasma levels of

isoniazid in human volunteers after a clinical dose by mouth of HP.213 were of the same order as those produced by an equivalent dose of isoniazid. Further work on the estimation of HP.213 in plasma and its metabolic products is in hand.

The question whether HP.213 owes all its tuberculostatic action to the formation of isoniazid, or whether it has an action *per se*, cannot be answered categorically at present. The facts that HP.213 has a marked tuberculostatic action *in vitro*, that HP.213 and isoniazid act mutually in the delay of resistance emergence, and that HP.213 is considerably less toxic than isoniazid strongly suggest that the properties of HP.213 are not solely due to isoniazid formed as a breakdown product.

The experimental results obtained, so far, with HP.213 indicate its possible value in the treatment of human tuberculosis, and clinical trials of the substance are in progress.

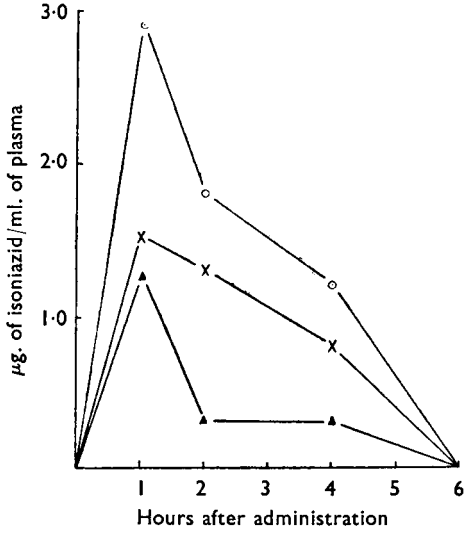



FIG. 3. Human plasma levels of isoniazid following administration of HP.213. Dose: 600 mg. orally of HP.213.

×——× Subject J.H.
▲——▲ Subject H.Y.
⊙——⊙ Subject S.T.

## SUMMARY

1. *o*-Hydroxybenzal *iso*nicotinylhydrazone ("Nupasal-213") possesses tuberculostatic activity of the same order as that of isoniazid.

2. Its chronic toxicity towards rats and guinea-pigs is about 1/5 to 1/10 of that of isoniazid.

3. Isoniazid and *o*-hydroxybenzal *iso*nicotinylhydrazone act mutually to delay the development of resistance by *Myco. tuberculosis* H37Rv to either substance.

4. *o*-Hydroxybenzal *iso*nicotinylhydrazone is decomposed, at least partially, in the gastro-intestinal tract to form isoniazid. This decomposition occurs to a much smaller extent when the substance is administered parenterally.

## REFERENCES

1. Bavin, Drain, Seiler and Seymour, *J. Pharm. Pharmacol.*, 1952, **4**, 844.
2. Goodacre, Mitchell and Seymour, *Quart. J. Pharm.*, 1948, **21**, 301.
3. Steenken, Wolinsky and Montalbine, *Proc. Soc. exp. Biol. N.Y.*, 1954, **87**, 245.
4. Rees and Robson, *Brit. J. Pharmacol.*, 1950, **5**, 77.
5. Rubin, Drekter, Scheiner and de Ritter, *Dis. Chest.*, 1952, **21**, 439.
6. Short, *Lancet*, 1954, **266**, 656.
7. Barry and Conalty, *Lancet*, 1954, **2**, 494.

## DISCUSSION

The paper was presented by MR. E. M. BAVIN.

MR. H. GRAINGER (London) said that no information was given about the possible route of administration. It appeared that if any great advantage was to be derived clinically from the use of the compounds it would probably be by parenteral administration, because given orally the effect was largely that of the breakdown compound.

MR. G. SYKES (Nottingham) said it was apparent that even traces of one compound in the presence of the other delayed the development of resistance of the organism, and he wondered whether the authors had any idea of the mechanism of the combined effect. It seemed that the administration of Nupasal *in vivo* would be advantageous, because a mixture of the two compounds would be present and therefore, presumably, optimum conditions for delaying resistance to the organism.

DR. G. BROWNLEE (London) said that the capacity of the new substance to deal with strains resistant to isoniazid was of interest, and he suggested that it was necessary to have information about a number of strains shown to be resistant. The acid test would be an animal experiment in which strains resistant to isoniazid were shown to produce generalised infection which was adequately suppressed by the new substance.

MR. E. M. BAVIN, in reply, said that the compound was not intended for parenteral use, but orally it was much less toxic than isoniazid. It had been shown that it was possible to give up to 1·5 g. per day clinically without any trace of side reaction. Some breakdown to isoniazid did occur, but no method was available for identifying Nupasal specifically in the presence of isoniazid. It was not possible to give a detailed reply on the question of the mechanism of resistance. One could only hazard a guess that substances like isoniazid and Nupasal, which were similar chemically, did interfere mutually with each other's metabolic pathway in the tubercle organism.