ANTITUBERCULOSIS ACTIVITY IN THE PHENAZINE SERIES. ISOMERIC PIGMENTS OBTAINED BY OXIDATION OF *O*-PHENYLENEDIAMINE DERIVATIVES

By Vincent C. Barry, Michael L. Conalty and Ethna E. Gaffney

From the Laboratories of the Medical Research Council of Ireland, Trinity College, Dublin

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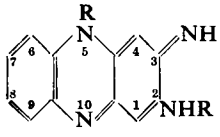
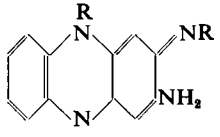
Introduction

The products obtained on oxidation of derivatives of *o*-phenylenediamine with ferric chloride or *p*-benzoquinone have been under investigation in these laboratories for some years. The main products formed are derivatives of anilino*apo*safranine (2-anilino-3 : 5-dihydro-3-imino-5-phenylphenazine), I (R = Ph), and its isomer, 2-amino-3 : 5-dihydro-3-phenylimino-5-phenylphenazine, II (R = Ph).



I



II

Anilino*apo*safranine is strongly inhibitory of the growth of *Mycobacterium tuberculosis* in serum enriched media and it has been shown to exert a suppressive effect in experimental tuberculosis in mice and guinea-pigs[1–4]. It is also claimed to show a curative effect in lepromatous leprosy[5]. The present paper describes the evaluation in mouse and guinea-pig tuberculosis of a number of new derivatives of structure II and a comparison of their activity with that of the corresponding isomers of structure I where these were available. The chemistry of these compounds has been described recently[6–8].

Experimental Methods

The experimental methods have been described previously[9]. In brief, groups of 10 albino mice (Schofield or Parkes) of average weight 25–30 g. were infected intravenously with 0·1 mg. (moist weight) of the bovine Ravenel Rv strain of *Myco. tuberculosis* grown for 9 days on a Dubos type liquid medium.

The drugs were administered in a ground biscuit diet for 14 days, beginning on the day of infection. The measured daily intake of drug, used in the Tables, is the mean of the daily intakes during the five days, second to the sixth inclusive.

Every mouse which died was examined to ascertain the cause of death. The criterion of antituberculosis activity was a significant increase (19/20 probability, or better) in the median survival time (MST) of the treated mice over the controls. The MST of untreated control mice varied in different experiments from 13 to 23 days.

For the investigations in guinea-pigs, animals of average weight 625 g. were used. Each animal was infected subcutaneously in the left flank with 0·01 mg. of the Ravenel Rv strain grown as above.

Drugs were incorporated in the ordinary diet of crushed oats. Liberal quantities of greenstuffs, mainly cabbage, were also provided. From the daily intake of the drug-oats mixture the mean daily intake of drug over the period of treatment was calculated.

In determining the Disease Index[9] extensive disease in the lungs, liver, spleen, site of inoculation and adjacent lymph nodes combined, is awarded 35, 30, 25 and 10 points, respectively; a total maximum of 100 points. Moderate disease is awarded, 25, 20, 15 and 10 points; slight disease 15, 10, 5 and 5 points.

Results and Discussion

The investigation has shown (Table I) that the isomer (II, R = Ph) of anilino*apo*safranine and certain derivatives of both isomers are more active in suppressing experimental tuberculosis in mice than anilino*apo*-safranine (283). Of the 11 derivatives of structure I, Nos. 283, 628, 616, 623, 658, 637 and 662 show significant activity and of these only No. 628, I, (R = $C_6H_4Cl(p)$) had outstanding activity. High activity in this group is associated with the presence of a methyl or higher alkoxy group in the *p*-position of the phenyl substituents on the 2:5-nitrogens. A similar pattern of behaviour is evident in the compounds of structure II. Here again the outstanding compound is the chloro-derivative, 629, (R = $C_6H_4Cl(p)$) which is isomeric with compound 628, although the *p*-ethoxyphenyl derivative 630, is little behind it in activity. Included in this group of compounds are a series of alkyl and *cyclo*alkyl derivatives, and one aralkyl derivative of type II, the corresponding I isomers of which could not be prepared. On the whole they showed low activity with the exception of the *cyclo*hexyl derivative, 430.

Apart from the type I compound, 628, which is the most active of all the compounds examined, it appears to be true that on the whole the type II compounds show higher activity than the group I isomers. This is clear from the comparison of the following 7 pairs:—283, 595; 616, 619; 422, 607; 658, 659; 637, 636; 656, 657; 662, 661. The last pair are exceptional in that both isomers show identical activity.

We have subsequently confirmed the high antituberculosis potency of compound 630 in a mouse infection which had been allowed to progress for 5 days before treatment was initiated. The increase in MST in this case was 56 days. (Dosage = 130 mg./kg. daily for 14 days.)

The chlorinated derivatives are of especial interest. No. 639, the 8-chloro-derivative of No. 283, showed a reduced MST. Despite this the disease in the lungs of the mice was small and their early death suggests a toxic effect. No. 640 the only other compound with a chlorine substituent in the phenazine structure proper, showed no activity. Apparently the substitution of this chlorine atom has destroyed the activity of the parent 595. The only pair of I, II, type isomers available for comparison in this chlorinated group was 628 and 629. In both of these compounds

TABLE I

ACTIVITY IN MOUSE TUBERCULOSIS

No.	Compound	Measured daily intake of drug (mg./kg.)	Change in MST (days)
293	II, R = methyl	161	−2
510	II, R = allyl	191	+0·5
550	II, R = *n*-propyl	111	+2·5
617	II, R = *iso*propyl	252	0
449	II, R = *n*-heptyl	110	+1·5
415	II, R = benzyl	119	+3·5 S*
653	II, R = *cyclo*pentyl	155	+2·5
430	II, R = *cyclo*hexyl	268 193 140 62	+27 S +24 S +19 S +1·5
283	I, R = phenyl	70 41	+ 7·5 S + 1·5
595	II, R = phenyl	350 125 66 41	+14 S +12·5 S +10·5 S 0
628	I, R = *p*-chlorophenyl	120 92·5	+117 S +159 S
629	II, R = *p*-chlorophenyl	145 127	+108 S + 57 S
622	I, R = *o*-tolyl	158 58	− 1·5 + 4 S
616	I, R = *p*-tolyl	144	+11 S
619	II, R = *p*-tolyl	213	+26 S
623	I, R = *o*-methoxyphenyl	172 90	+ 9 S + 8·5 S
627	II, R = *o*-methoxyphenyl	156 82	0 + 6 S
422	I, R = *p*-methoxyphenyl	200	+ 2·5
607	II, R = *p*-methoxyphenyl	295 200 111 71	+28 S +30 S +31 S +14 S
630	II, R = *p*-ethoxyphenyl	117 65	+91 S +30 S
658	I, R = *p*-*n*-propoxyphenyl	70	+18 S
659	II, R = *p*-*n*-propoxyphenyl	131	+28 S
637	I, R = *p*-*iso*propoxyphenyl	168	+12·5 S
636	II, R = *p*-*iso*propoxyphenyl	162	+44 S
656	I, R = *p*-*iso*butoxyphenyl	75	+ 3
657	II, R = *p*-*iso*butoxyphenyl	131	+20 S
662	I, R = *p*-*sec*.butoxyphenyl	115	+30 S
661	II, R = *p*-*sec*.butoxyphenyl	115	+30 S
640	II, R = phenyl (Also Cl in the 7-position)	135	− 1
639	I, R = phenyl (Also Cl in the 8-position)	120	− 4 S

*S = significant at 19/20 level or better.

the chlorine is situated on the periphery of the molecule and its introduction has had a remarkable enhancing effect on the antituberculosis activity (see Table I).

The activity in mice of compounds 628, 629 and 630 is of a higher order than we have obtained with PAS, the thiosemicarbazones or streptomycin, and when compared on a molecular basis is only equalled by certain isoniazid derivatives.

TABLE II

RESULTS IN GUINEA-PIGS INFECTED WITH *Myco. tuberculosis* RAVENEL RV (0·01 MG. S.C.) AND TREATED FOR 21 DAYS COMMENCING ON THE DAY OF INFECTION. THE ANIMALS WERE THEN KILLED

No. of drug all given 0·5 per cent. in diet	Disease Index maximum = 100	Lungs showing macroscopic evidence of tuberculosis	Lungs giving positive cultures for *Myco. tuberculosis* on Lowenstein-Jensen medium
628 (260 mg./kg.)	7	0/5	0/5
629 (230 mg./kg.)	11	0/5	0/5
671 (50:50 mixture of 628 and 629) (243 mg./kg.)	17	0/5	0/5
630 (197 mg./kg.)	29	0/5	4/5
Control	45	4/5	5/5

The above three compounds were further investigated in experimental tuberculosis of guinea-pigs and the results are set out in Tables II, III and IV. In the first experiment (Table II) the drugs were administered in the diet for 21 days from the day of infection and the animals were then killed. As was found in mice the protective effect of the drugs was impressive. No. 671 which was a 50:50 mixture of compounds, 628 and 629, showed no better results than those obtained with either constituent.

TABLE III

RESULTS IN GUINEA-PIGS INFECTED WITH *Myco. tuberculosis* RAVENEL RV (0·01 MG. S.C.) AND TREATED FOR 21 DAYS COMMENCING ON THE DAY OF INFECTION

Drug, all given 0·5 per cent. in diet	Mean survival time in days	Disease Index maximum = 100	Disease index in lungs maximum = 35
628 (260 mg./kg.)	127	77·0	15
629 (230 mg./kg.)	97	77·5	17·5
671* (243 mg./kg.)	118	80·0	20·0
630 (197 mg./kg.)	87	86·0	35·0
Control*	59	82·5	35·0

* One animal from each of these groups of 5 animals was lost through intercurrent infection and was not included in the analysis.

In the second guinea-pig experiment (Table III) the animals received similar infection and treatment to that described for the first experiment. In this case, however, when the administration of the drug was discontinued after 21 days, the disease was allowed to run its course. Final assessment of the activity was based on mean survival time and on the extent of the

TABLE IV

RESULTS IN GUINEA-PIGS INFECTED WITH *Myco. tuberculosis*, RAVENEL RV (0·01 MG. S.C.). TREATMENT* COMMENCING ON THE 22ND DAY AFTER INFECTION AND CONTINUED UNTIL THE 83RD DAY

Drug	Median survival time time (days)	Disease Index in lungs maximum = 35
628* (241 mg./kg.)	70	9·4
629 (191 mg./kg.)	63	13·5
671 (238 mg./kg.)	76	15
630 (182 mg./kg.)	79	19
Pre-controls	—	12
Controls*	51	30·5

* One animal from each of these groups of 10 animals was lost through intercurrent infection and was not included in the analysis.

disease. For an experiment of this kind the extension of the mean survival time is impressive particularly with Nos. 628, 629 and 671.

Table IV shows the results obtained with an established infection in guinea-pigs. At start of treatment, i.e., 21 days after infection, 5 animals (precontrols) were killed. These had a disease index of 45/100. As far as increase in MST is concerned, the results embodied in this Table are not striking. It will be noticed, however, here as well as in Tables II and III, that the amount of infection in the lungs is markedly less than that in the control animals. It is characteristic of these coloured substances that they accumulate in many organs and persist there long after treatment has been discontinued. This phenomenon is less noticeable with No. 630. It may be that the compounds remain in lung tissue to a large extent without extensive decomposition.

The disparity between the activity exhibited by these pigments in mouse and guinea-pig tuberculosis is interesting and is reminiscent of the different effect obtained by streptomycin treatment in established disease in the same animal species, except that with this drug the disparity noted was in the opposite direction. An obvious explanation in our case would be that the guinea-pig metabolises the phenazine derivatives more rapidly than does the mouse. Alternatively, we have noted that guinea-pigs receiving these compounds in the diet, eat much less well than the control animals. The lessened food intake may be in itself enough to explain the species difference in results.

We have already pointed out[2] the formal resemblance in structure between these phenazines and riboflavine and suggested that their activity might be related to this fact. Since riboflavine does not antagonise their growth-inhibitory properties for *Myco. tuberculosis*, it may be that this was too simple an approach to the problem of their mode of action. All these compounds contain a *p*-quinonoid system which is readily reduced, and reoxidised in the air. It is not improbable that they become involved in the hydrogen transfer system of the bacillus, accepting hydrogen from reduced diphosphopyridine nucleotide and transferring it to oxygen with the formation of hydrogen peroxide in the cell.

Winder[10] in these laboratories has already postulated that the anti-mycobacterial action of isoniazid is due to the intracellular formation of peroxide. In support of our hypothesis we have shown that hydrogen peroxide is produced when these phenazine pigments are exposed to air after catalytic reduction.

If this hypothesis is correct, high activity in this series should be related to the ease of reduction and re-oxidation of the compound and also to its capability of undergoing this reversible transformation repeatedly without decomposition. Some evidence has already been obtained which shows that the most active compounds are those which form the most permanent redox systems, and investigation is continuing in this field.

Further support of this hypothesis is provided by the fact that compound 595 which exerts a moderate suppressive effect in mouse tuberculosis (increase in MST, 12·5 days at 125 mg./kg. dosage) has been found by us to be highly active against a mouse infection induced by an isoniazid-resistant variant of the same strain as was used in the other experiments. Thus in the latter experiment compound 595 at a dose of 153 mg./kg. caused an increase in MST of 43 days. It is well established that isoniazid-resistant strains of *Myco. tuberculosis* are much more susceptible than isoniazid-sensitive strains to the action of hydrogen peroxide[11].

Other factors which will determine activity in these series will be those physico-chemical properties of the molecules which govern absorption from the gastrointestinal tract and which allow access to the bacilli present in the tuberculous lesions and then to diffuse into and be held in a suitable orientation in the bacillary cell. Included in these will be lipoid/water solubility, the planar area of the molecule and the disposition of its active groupings.

Summary

1. A number of phenazine compounds obtained by oxidation of derivatives of *o*-phenylenediamine have been investigated for antituberculosis activity in mice and guinea-pigs. Considerable activity was found in mice in more than half of 30 derivatives examined.

2. The activity was outstanding, however, and of the order obtainable with isoniazid, with 2-*p*-chloroanilino-5-*p*-chlorophenyl-3 : 5-dihydro-3-iminophenazine (No. 628), its isomer, 2-amino-5-*p*-chlorophenyl-3-*p*-chlorophenylimino-3 : 5-dihydrophenazine (No. 629), and the analogue of the latter in which the chlorine is replaced by ethoxyl (No. 630).

3. The results in guinea-pigs were clear cut but less impressive.

4. The mode of action of these compounds may depend on their ability to produce hydrogen peroxide within the mycobacterial cell.

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DISCUSSION

The paper was presented by DR. VINCENT C. BARRY.

DR. G. BROWNLEE (London) said he welcomed this painstaking structure-action study. Like Dr. Barry he found the peroxide hypothesis stimulating but not quite acceptable as a theory. The equal antibacterial actions against both susceptible and resistant isoniazid strains, and the observed non-inhibitory actions of serum were both stumbling-blocks to accepting, in its simplest form at least, the peroxide hypothesis. The discovery of a reversing agent, perhaps to be sought for among the ribose-nucleic acids, would help to test the theory.

MISS A. E. ROBINSON (London) said that it seemed on consideration of the shape, size and chemical characteristics of the phenazine molecules that by analogy with the acridine type of compounds, penetration of the bacterial cell was precluded. The authors, however, seemed to suggest that penetration of the bacterial cell was necessary for their activity, although a hydrogen transfer mechanism was postulated. Was it not possible that the site of metabolic interference was in fact at the cell surface? It would be interesting to know whether the authors had undertaken any bacterial uptake or metabolism studies using those compounds. The authors had come out strongly in favour of one hypothesis concerning the mode of action of isoniazid, namely, peroxide formation, and they suggested that the phenazine compounds acted by a similar mechanism. Could they give a little more information concerning that choice to the exclusion of other currently postulated mechanisms; for example, chelation of trace metals, formation of diphosphopyridine nucleotide analogue, and direct inhibition of various enzyme systems?

DR. R. F. TIMONEY (Dublin) asked whether the authors had considered the possibility of testing sulphur analogues of these compounds. A number of sulphur compounds had been shown to be effective in the treatment of leprosy.

DR. V. C. BARRY, in reply, said that sulphur derivatives had not been considered because he did not like mixing his ideas! He had expected a violent reaction to his hypothesis. The hydrogen peroxide hypothesis was not being put forward very strongly. It was being submitted because there was some evidence which seemed to support it. A number of isoniazid derivatives were quite active, but they could not chelate. Evidence had been produced that isoniazid decomposed in solution liberating hydrogen peroxide. It was known that isoniazid gets into the

cell, and it was not asking much to expect that under proper conditions it would decompose, in that way liberating peroxide. Isoniazid did not accumulate. The planar area of the molecule and the disposition of the polar groupings would depend on how they orientated. The stability of the molecule was also all important. Reduction and reoxidisation were carried out a number of times without decomposition. It was well known that isoniazid-resistant bacilli were more resistant to hydrogen peroxide than sensitive strains. It was possible that the hydrogen peroxide was formed within the bacillus cells as well as outside it, and that that gave the increased curative effectiveness. It might also be that the small amount of catalase present in the tubercle bacilli was near the outer membrane, and its function might be to remove peroxide from the environment.