# The Antimycobacterial Activity of B 663

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B 663 is one of a large series of rimino-compounds of structure I synthesized originally in these laboratories as potential antimycobacterial agents. These are orange-red phenazine derivatives whose colour is related to their quinone-imine character. In B 663, R'' represents $\cdot CH(CH_3)_2$ and R' and R''' are $\cdot C_6H_4Cl(p)$ residues. The synthesis and chemistry of these compounds have been described by Barry and co-workers in a series of papers (1956a, 1956b, 1956c, 1956d, 1958a, 1958b, 1959, 1962b).

The forerunner of these compounds, anilino-aposafranine (I, R'' = H, R' = R''' = Ph), had been shown to be highly inhibitory of the growth of tubercle bacilli (H37Rv) *in vitro* (Barry *et al* 1948). It had also shown activity in mouse and guinea-pig tuberculosis (Barry 1951; Conalty 1951; Barry *et al* 1956e) and in human renal disease (Lane 1951). Allday and Barnes (1952) used it with some success in leprosy patients in Nigeria.

I.
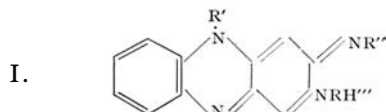
B 663 (I. R'' = $CH(CH_3)_2$ is a crystalline material insoluble in water but highly soluble in fat. Its inhibitory activity as determined by our methods (Conalty 1954) against H37Rv and a number of atypical mycobacteria is shown in Table I.

Mice

In murine tuberculosis B 663 is extraordinarily active (Barry *et al* 1957). At the low dose of 1 mg./kg. bodyweight in the diet for 14 days, beginning on the day of infection with the bovine Ravenel Rv strain, B 663 produces a significant extension of survival time. Again, mice which received 1 mg. each of B 663 intraperitoneally *once* only, on the 11th day of an infection which

TABLE I
Antimycobacterial Activity of B 663
*in vitro**

Mycobacterium	Concentration causing complete inhibition of growth (μg./ml.)
Myco. tuberculosis, H37Rv including wild, isoniazid-resistant, streptomycin-resistant and PAS-resistant strains	1.3–3.3
Myco. bovis, Ravenel Rv, wild and drug-resistant strains. Also BCG	1.3–3.3
Myco. avium, three strains	5.0–10.0
'Unclassified' Mycobacteria	
Myco. kansasii, two strains	3.3–5.0
Scolochromogens, two strains	3.3–5.0
Battey types, 10 strains	1.0–6.6
Runyon Group *IV*	6.6–20.0
*Myco. ulcerans***	2.0

*Proskauer and Beck medium enriched with human serum, 5 per cent. Incubation for 14 days.
**Incubated at 32°C.

caused the death of untreated mice in 19 days, had a median survival time (MST) of 79 days. Perhaps its most striking property is its genuine prophylaxis against tuberculous infection. Thus mice which were fed at the level of 10 mg./kg. in the diet for 14 days and then taken off treatment, were afforded a considerable degree of protection when challenged with the usual lethal intravenous infection with Ravenel Rv four weeks later (Barry and Conalty 1958). In established disease (treatment from the sixth day of infection) B 663 orally (5 mg./kg.) for 14 days effectively reverses the disease process. Further confirmatory reports on the activity of B 663 in murine tuberculosis have been published from

other laboratories (Hirsch 1958; Vischer *et al* 1958; Noufflard and Berteaux 1955a and 1958b; Acharya *et al* 1959; Grumbach 1960; Drabkina and Ginsburg 1962). The drug is also fully active against strains resistant to isoniazid, streptomycin, PAS or thiosemicarbazones.

Syrian hamster
Vischer *et al* (1958) showed that B 663 exerted good curative properties in experimental tuberculosis of the hamster and in this animal also, a striking prophylactic effect could be demonstrated.

Guinea-pigs
The therapeutic activity of the drug in guinea-pig tuberculosis was much less impressive. Thus although when administered at a dosage level of 140 mg./kg. for 79 days, treatment beginning on the 21st day of infection, the disease index was reduced from 44 to 8.5, the disease being confined to the site of infection and the adjoining lymph nodes (Barry *et al* 1957), at lower dosage levels the results were disappointing. B 663 at 20 mg./kg. in the diet from the 21st day of infection produced a 50 per cent extension in MST and notably less lung disease in the treated animals. Vischer *et al* (1958), Steenken *et al* (1960) and Drabkina and Ginsberg (1962) also reported unimpressive results in this animal species.

Rabbits
In established disease experiments in rabbits, Freerksen observed a good therapeutic effect with oral doses of 50 mg./kg./day.

Monkeys
As with the guinea-pig, results of treatment in the monkey were also disappointing. Schmidt (1959) administered B 663 by stomach tube to infected monkeys. At 10 mg./kg./day there was some effect and at 100 mg./kg./day the effect was marked when treatment was initiated at the time of infection. When, however, treatment was withheld for 30 days after infection, the drug even at the larger dosage level failed completely to hold the disease. Bacilli recovered from the treated monkeys at autopsy were reported to have a reduced susceptibility to B 663.

In vitro resistance
Under our conditions, growth of the human H37Rv and the bovine Ravenel strains of *Myco. tuberculosis* (0.03 mg. inocula) in Proskauer and Beck medium, to which 5 per cent human serum is added, is normally inhibited by 1.3 to 3.3 μg./ml. B 663. To study the development of resistance to B 663 the procedure adopted was to set up serial dilutions of the drug ($1\frac{1}{2}$ fold steps) and inoculate with 0.03 mg. of tubercle bacilli. After two to six weeks incubation, such growth, in the tubes of the highest concentration of drug, as was adequate in amount, was used as inocula for further series. The procedure was repeated at two to six weeks intervals. Under these conditions, 15 successive subcultures produced no change in susceptibility although the total exposure to B 663 was 80 weeks. In other experiments employing 10 fold inocula of the Ravenel strain slight growth was evident at 100 μg./ml. in the fourth serial culture and growth was as heavy as in the control tubes at this concentration of B 663 after the 14th subculture or a total exposure time of 71 weeks. With the H37Rv strain under these same conditions the fifth serial culture showed some growth in the presence of 50 μg./ml.

The Ravenel strain which was now growing freely (large inoculum) at 100 μg./ml. of B 663 also grew freely at this concentration when the usual small (0.03 mg.) inocula were employed. When given to mice in the standard intravenous inocula (0.1 mg.) it proved to be still moderately virulent despite the long period of subculturing – all dead at 66 days with a MST of 28 days. A group of 10 mice also infected in this way but treated for 14 days with B 663 in the diet (20 mg./kg.) had a median survival time of 120 days, only one mouse dying within 95 days. These cannot be considered resistant bacilli.

In vivo resistance
Mme Grumbach (1960) has isolated variants of H37Rv from mice treated for three and four months at a dosage level of 1 mg./mouse/day. These bacilli were much less susceptible to B 663 *in vitro* and it needed five to ten times the dose of drug to control infections produced by them in mice.

Schmidt also isolated bacilli of reduced susceptibility from monkeys treated with B 663 (100 mg./kg./day) for five months. One of these variants which we studied had a very low virulence in mice. It was reisolated after four

months from mice which had been treated for 14 days with B 663 (5 mg./kg.) and still showed reduced susceptibility *in vitro*. Inoculated again into mice it had now fully recovered its virulence but responded to treatment with B 663 (20 mg./kg.). The same variant kept on subculture in Lowenstein slopes for 18 months fully recovered its susceptibility to B 663 *in vitro*, its virulence for mice and also its susceptibility in the mouse to treatment with B 663.

An observation of great interest which we have made and which has been confirmed by Grumbach (1961) is that tubercle bacilli which have emerged from *in vitro* studies or in the mouse and monkey and which exhibit a reduced susceptibility to B 663 are still susceptible to other rimino-compounds, *e.g.* B 749 (I, $R' = R'''$ = *para*chlorophenyl; $R'' = N \cdot CH_2 \cdot CH_2NEt_2$). This phenomenon is being studied further.

Bacilli resistant to isoniazid or ethionamide can be developed readily in a few subcultures in a tween-free medium enriched by serum. We have shown that if sub-inhibitory concentrations (*e.g.* 0.5 μg./ml.) of B 663 are present, no change in susceptibility of H37Rv to isoniazid could be demonstrated after ten successive titrations extending over a period of 17 months. In a series of experiments in mice infected with the H37Rv strain of *Myco. tuberculosis*, Grumbach (1960) has shown that, under treatment with B 663 (16 to 20 mg./kg.) or isoniazid (4 to 5 mg./kg.) or ethionamide (40 to 50 mg./kg.), the bacillary count in the lungs falls rapidly until about the 65th day and then in each case begins to rise rapidly again. This is the well-known 'fall and rise' phenomenon associated with the development of drug-resistant bacilli. When, however, B 663 was combined with isoniazid or ethionamide, at the dosage levels mentioned, the bacillary count continued to fall, reaching almost zero in 150 days, and no resistant bacilli appeared. Vischer and Roulet (1963) also found that B 663 delayed the emergence of isoniazid resistant strains in mice.

Absorption, distribution and retention
The genuine chemoprophylaxis of B 663 against experimental tuberculosis referred to earlier makes the pharmacology of this substance of great interest (Barry *et al* 1959b, 1960). As ordinarily presented in powder form, B 663 on ingestion is absorbed in amounts adequate to exert high antituberculosis activity in the mouse, hamster and rabbit. It is also well absorbed in the rat. Blood levels attainable in the guinea-pig are very low and in man 90 per cent of the drug presented in this state is excreted unchanged in the faeces. To counter this poor absorption in man the drug has been prepared in micronized form by Dr E. Hodel, Geigy Pharma, Basle.

In mice B 663, whether given as a powder or in micronized form, is well absorbed. At the end of 28 days treatment with B 663 (25 mg./kg.) in the diet, the lungs contained approximately 800 μg./g., the spleen 4,000 μg./g., the fat 800 μg./g. and the plasma 3.0 μg./ml. Twenty-one days off treatment reduced these values to 60 (lungs), 85 (spleen), 350 (fat) and 1.1 (plasma). Higher dosage or extended periods of treatment had little effect on the plasma level.

In rats treated at the high level of 100 mg./kg./day in the diet, plasma levels reached 8 μg./ml. and lung levels, 1,000 μg./g.

Guinea-pigs on a dose of 20 mg./kg. continued for 30 days proved to have 140 μg./g. of drug in the lungs but even at the end of this period plasma levels never reached even 1 μg./ml. At the end of 326 days treatment (50 mg./kg. in the diet) the plasma level was still little more than 1 μg./ml. although the lung tissue contained 18,500 μg./g.

In man when the drug is given orally in the micronized form 70 to 90 per cent is absorbed and serum levels on a dosage of 600 mg. per day reach 3 to 4 μg./ml.; skin and lymph node may contain 40 μg./g. (Knight 1964). Excretion is slow and some colour may be seen in the urine many weeks after cessation of treatment.

Reticulo-endothelial system
The low plasma and high tissue levels of B 663 which can be reached in different animal species are due to the active take up of the drug by the cells of the reticulo-endothelial system (Conalty and Jackson 1962a, 1962b). This phenomenon has so far been most studied in mice. When mice are treated at the rate of 50 mg./kg. (in the diet), in three to four days orange-red rounded bodies, 1 to 2μ diameter, appear in the cytoplasm of macrophages. On continuing treatment bright red crystals are seen accompanying the round inclusions (seven

to ten days) and at the end of a few weeks most of the cells contain crystals only. The above sequence of events is particularly well seen in the mesenteric lymph nodes and in the spleen. The appearance of crystals in the lung macrophages is somewhat delayed. Macrophages already loaded with crystals continue to take up carbon particles and tubercle bacilli. B 749, the analogue of B 663 referred to earlier, does not colour the fat in mice and appears even on prolonged treatment as a diffuse colouring of the cytoplasm and is never seen in crystalline form.

Apart from fat cells, B 663 is only noticeable in macrophages; its retention for long periods in these cells probably favours its therapeutic effect but its rapid withdrawal from the blood makes it difficult to establish or maintain therapeutic levels in some animal species. We had thought for this reason that another rimino-compound more soluble in aqueous media, e.g. B 749 also highly effective in mice, might produce higher blood-levels and thus show a better effect in, for example, the guinea-pig. Unexpectedly, however, we found that although B 749 does not accumulate to any extent in fat, it builds up very quickly in the solid tissues. So effective is the uptake of this drug by the macrophages that it has not been possible to obtain blood levels even as high as those obtained with B 663 treatment. Accordingly it is much less active than B 663 in guinea-pig tuberculosis.

The distribution of B 663 in the animal after ingestion, resulting in high concentrations in certain organs and low levels in the blood, throws some light on its therapeutic effect in different animal species. Thus it is very effective in the mouse and hamster where the bacilli tend to be intracellular and poor in the guinea-pig where the bacilli are for the most part extracellular. This is not the whole story, however; we know that B 663 is present at a very low level in the plasma in mice when an impressive prophylaxis can be demonstrated. It has been suggested that the binding and transport of the drug by serum proteins may be different in the mouse and guinea-pig. Binding to lipoprotein in the serum of mice is, however, complete and yet the therapeutic effect of the drug is excellent in this animal (Buggle). The nature of the lesions in experimental tuberculosis in different species is obviously of great importance.

Murine leprosy
In view of the intracellular location of B 663 in macrophages its examination in leprosy was clearly indicated. Chang (1962) states that 'B 663 has emerged as the most active drug yet tested in the treatment of murine leprosy'. It is the first drug to hold the infection in check for 816 days although the infection was not eradicated. Of various combinations of several antituberculosis drugs, the combination of B 663 and isoniazid was apparently the only one which caused an actual reduction of the established infection. There was evidence that in mice treated with this combination, Myco. lepraemurium acquires some degree of resistance to isoniazid but not to B 663. Shepard (1963), emplying his mouse foot-pad technique, has shown that 0.1 per cent B 663 in the diet causes complete suppression of growth of human leprosy bacilli.

Experience in man
The first trial of B 663 in human leprosy was carried out by Browne and Hogerzeil (1962a, 1962b, 1962c) who reported that B 663 had a definite action in lepromatous leprosy, inducing clinical and bacteriological improvement. No signs of toxicity were noticed over a 12 month period. A preliminary small scale trial has also been completed in Kuala Lumpur with encouraging results (Rees 1964). Knight and his associates at the National Institutes of Health, Bethesda are conducting a very thorough examination of the behaviour of B 663 in a small number of leprosy patients (Knight 1964). Much more extensive clinical investigation of B 663 in leprosy has now been planned or is already under way in Nigeria, Malaya and elsewhere (Rees 1964) and when the resulting reports appear it will be possible to assess more completely the status of B 663 as an antileprosy drug.

Other mycobacterial infections
The report (Lunn and Rees 1964) of the successful treatment of mycobacterial skin ulcers, due to atypical mycobacteria resembling Myco. ulcerans, in Uganda with B 663 has aroused considerable interest. These authors also reported that B 663 showed a very high activity against three strains of Uganda mycobacteria, three strains of Myco. ulcerans and two freshly isolated strains from Australia. Standardized infections

in the foot-pads of mice produced by these strains of mycobacteria were completely suppressed by 0.006 per cent of B 663 in the diet. This report has already led others to consider the possibility of exploiting B 663 in a variety of mycobacterial infections in man.

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