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Rapid, radiometric *in vitro* assay for the evaluation of the anti-leprosy activity of clofazimine and its analogues

A MITTAL,* P S SESHADRI,† M L CONALTY,‡ J F O'SULLIVAN‡ & INDIRA NATH*§

**Department of Pathology, All India Institute of Medical Sciences, New Delhi-110 029, India; †Central Leprosy Training & Research Institute, Chingleput-603 001, India; and ‡Laboratories, Medical Research Council of Ireland, Trinity College, Dublin 2, Ireland*

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Summary The effect of clofazimine and 6 analogues (B 3691, B 3713, B 3640, B 3648, B 720 and B 749) on the viability of *Mycobacterium leprae* was tested in a rapid, radiometric, *in vitro* assay. Thirteen human and 1 armadillo-derived freshly-extracted *M. leprae*, maintained in peritoneal macrophages, incorporated ^{3}H-thymidine to significant levels as compared to parallel cultures with heat-killed bacilli. Exposure of such cultures to clofazimine for 48 h showed significant inhibition of the radiolabel uptake without any adverse effects on the host macrophages. A sharp linear increase in inhibition was observable at concentrations from 1 to 10 ng/ml, with a plateau up to 40 ng/ml. Further increases of drug concentration up to 100 ng/ml showed marginal increase in the percentage inhibition of ^{3}H-thymidine incorporation. The analogues tested showed levels of inhibition similar to that of clofazimine when left for 72 h and 15 days in *M. leprae* macrophage cultures. However, they were less effective than clofazimine when tested for the shorter duration of 48 h at the lower concentration of 5 ng/ml.

Introduction

In recent years a rapid, radiometric, *in vitro* assay has been developed in the All India Institute of Medical Sciences for the evaluation of *Mycobacterium leprae* viability. Over a 2- to 3-week period, human and armadillo-derived *M. leprae* strains maintained in mouse peritoneal macrophages showed uptake of ^{3}H-thymidine to a significant degree as compared to parallel cultures with heat-killed bacilli.[1] That the incorporation of the radiolabel was in the bacilli and not in the host cell was shown by DNase experiments[2] and autoradiography.[4]

§ Correspondence: Department of Pathology, All India Institute of Medical Sciences, New Delhi-110 029, India.

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This assay was used effectively: (i) to study the effect of dapsone (DDS, 4,4′-diaminodiphenylsulphone); (ii) to identify dapsone-resistant strains,[3] and (iii) to evaluate the antileprosy activity of rifampicin.[4] Moreover, immunologically-mediated inhibition of ^{3}H-thymidine incorporation was observed in *M. leprae* in macrophage cultures treated with antigen-induced lymphokines derived from tuberculoid leprosy patients.[5] Recently, we have been able to miniaturize this technique in microtitre plates (each having 96 flat-bottomed wells) thereby reducing the numbers of bacilli, radiolabel and macrophages required per assay.[6] This rapid *in vitro* assay compared well with the commonly-used mouse foot-pad model.[3] In the present study the inhibitory effect of clofazimine and its analogues has been investigated for 14 human and 1 armadillo-derived *M. leprae* strains maintained in murine macrophage cultures.

Materials and methods

ISOLATION OF *M. LEPRAE*

Skin biopsies from 14 bacilliferous, lepromatous patients, clinically suspected of dapsone resistance, were air freighted on ice from the Central Leprosy Training and Research Institute, Chingleput, India. Briefly, the bacilli were isolated in glass homogenizers with RPMI 1640 (Gibco Bio-Cult, Irvine, Scotland) and were counted by the method of Shepard & McRae.[7] The yield ranged from 3×10^7 to 4×10^8 bacilli per biopsy. The absence of contaminating bacteria and culturable mycobacteria was checked by plating on nutrient agar for 24 h and on Lowenstein–Jensen medium for 8 weeks. A sample from each batch of bacilli was autoclaved at 120°C at a pressure of 15 psi for 15 min.

MACROPHAGE CULTURES

Macrophage cultures were set up as described earlier.[2] In brief, non-stimulated peritoneal resident cells from Balb/c mice were collected by washing out the peritoneal cavity with 2–3 ml of cold RPMI 1640 containing 10 units per ml of preservative-free heparin (Upjohn Co., Kalamazoo, USA). The cells from individual mice were diluted with an equal amount of RPMI 1640 supplemented with 20% foetal-calf serum (Gibco, Bio-Cult, Irvine, Scotland). One millilitre of cell suspension (containing approximately $0{\cdot}5$–$0{\cdot}75 \times 10^6$ macrophages) was delivered into each Leighton tube incubated at 37°C. Subsequently, nonadherent cells were gently removed and the medium was replaced. The macrophages were maintained at 37°C for 24–48 h, after which 10^6 bacilli were introduced into each Leighton tube.

After the removal of unphagocytosed bacilli at 18 h, the medium was replaced with 1 ml of medium containing 1 μCi of methyl ^{3}H-thymidine (Amersham Corp.,

Arlington Heights, Illinois; specific activity; 42 Ci/mmol and respective concentration of clofazimine (B 663) and its analogues (B 3691, B 3713, B 3648, B3640, B 720 and B 749).

It was not possible to assay all the drug concentrations on each of human-derived *M. leprae* strains. Thus experiments were designed to test low (5 ng/ml); middle (10 ng/ml) and high (100 ng/ml) concentrations of clofazimine on individual *M. leprae* strains exposed for 48 and 72 h and 15 days maintained under similar culture conditions. On 1 armadillo derived *M. leprae* strain full dose response (1, 2·5, 5, 10, 20, 40, 50 and 100 ng/ml) and time kinetics (2, 6, 18, 24, 48, 72 h and 15 days) were done.

In addition 3 human derived *M. leprae* strains were exposed to 5, 10 and 100 ng/ml of clofazimine analogues (B 3691, B 3713, B 3648 and B 3640) along with clofazimine (B 663) for 48 and 72 h and 15 days.

Analogues B 720 and B 749 were tested on 2 human derived *M. leprae* strains at 5, 10 and 100 ng/ml and 15 days exposure.

The drugged medium was then removed and replaced with drug-free medium containing labelled thymidine. This medium was routinely renewed every 4–5 days. Macrophages were harvested by stripping with a rubber policeman after treatment with 100 μl of 2% Xylocaine (Astra, India). Then they were serially washed with saline containing cold thymidine 1 mg/ml, and twice with 5% trichloracetic acid and methanol. The dried discs were counted in a scintillation counter (LKB, 1215 Rackbeta II).

Using the same batch of macrophages, 5 replicates of each of the following were set up: (I) macrophage cultures alone, (II) macrophages with freshly-isolated 'live' *M. leprae,* (III) macrophages and heat-killed *M. leprae* of the same strain, (IV) II plus test concentrations (ng) of clofazimine and its analogues, (V) II plus diluent equivalent, and (VI) III + test concentrations of drug.

Mean counts per minute (cpm) $\pm$ standard error of 3 to 5 cultures were calculated. The percentage inhibition in the presence of clofazimine or its analogues was calculated as follows:

$$\text{Percentage incorporation} = \left[\frac{\text{Mean cpm of IV} - \text{Mean cpm of III}}{\text{Mean cpm of II} - \text{Mean cpm of III}}\right] \times 100$$

Percentage inhibition = 100 − percentage incorporation.

STATISTICAL ANALYSIS

For the assessment of significant values the non-parametric Mann–Whitney *U* test was used.

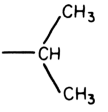
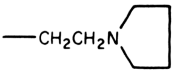
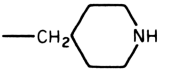
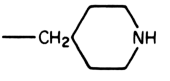
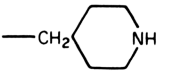
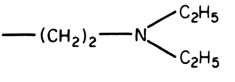
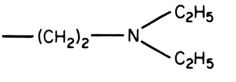
DRUGS

Clofazimine and its analogues B 3691, B 3713, B 3640, B 3648, B 720 and B 749

were prepared in the Laboratories of the Medical Research Council of Ireland. The structures and details of the analogues are given in Table 1.

Of these compounds (B 3691, B 3713, B 3640 and B 3648) were designed as part of a WHO project to develop new analogues of clofazimine which would be active against clofazimine-resistant strains of *M. leprae*. Assessment of activity was carried out *in vitro* using *M. smegmatis strain 607* (sensitive to 1·5 μg/ml) and its clofazimine-resistant variant (resistant to 30 μg/ml). These new analogues have been shown in the Johns Hopkins University, Baltimore, USA, to inhibit the clofazimine-sensitive strain at 0·2–0·4 μg/ml and the clofazimine-resistant variant at 0·4–2·0 μg/ml (N E Morrison, personal communication).

Table 1. Formulae of clofazimine and analogues

Code No.	Structure R′	Structure R	Melting point
B 663	Cl		211° decomp.
B 3691	H		161° decomp.
B 3713	CH_3		168° decomp.
B 3640	Cl		186° decomp.
B 3648	H		156° decomp.
B 720	H		—
B 749	Cl		—

All compounds were chromatographically pure and all analysed satisfactorily.

The compounds were dissolved in ethyl alcohol (1 mg/ml) and further diluted to 1 μg/ml in RPMI 1640, sterilized through 0·22 μm filters (Millipore Corporation, Mass., USA) and kept at 4°C. Working solutions were freshly made in RPMI 1640 just prior to use.

Results

M. leprae strains derived from 14 bacilliferous, lepromatous patients suspected of dapsone resistance, and from 1 infected armadillo liver, were tested in murine macrophage cultures. Of the 14 patients, 12 were proved to have full resistance for DDS in the macrophage culture assay (data not provided). None of these patients was known to have received clofazimine. Thirteen of the 14 human-derived and the 1 armadillo-derived *M. leprae* strains incorporated significant levels of ^{3}H-thymidine in cultures with freshly-isolated 'live' bacilli as compared with control cultures with heat-killed bacilli from the same biopsy ($P < 0{\cdot}005$ to $0{\cdot}001$, Figure 1).

Adherence properties of macrophages were similar in cultures with resident *M. leprae* maintained over a 2 to 3-week period with and without clofazimine or its analogues. After 72-h exposure to drugs many macrophages showed golden

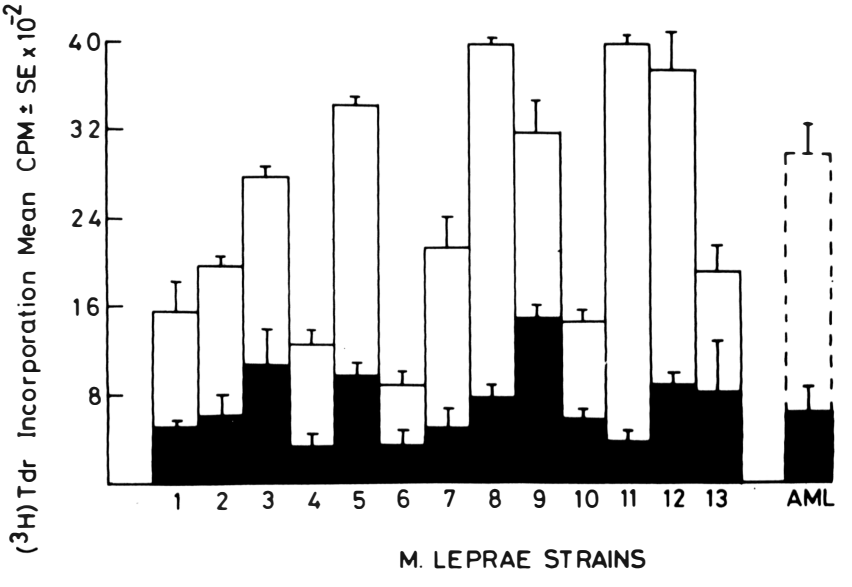


Figure 1. Incorporation of ^{3}H-thymidine by 13 human-derived *M. leprae* strains and 1 armadillo-derived *M. leprae* strain (AML) maintained in murine macrophages (mean cpm ± se of 5 replicate cultures). Numbers along the abscissa refer to *M. leprae* strains. All the strains showed significant incorporation of ^{3}H-thymidine ($P < 0{\cdot}05$) in cultures of live bacilli (□) as compared with heat-killed bacilli of the same strain (■).

brown inclusions in the cytoplasm. The effect of various concentrations of clofazimine and its analogues on individual *M. leprae* strains is expressed as the percentage inhibition of ^{3}H-thymidine uptake in cultures containing *M. leprae* and drug as compared with parallel cultures without drug. In general, it was observed that $\geqslant 50\%$ inhibition gave a P value of $<0{\cdot}05$ to $0{\cdot}01$ by the Mann–Whitney U test.

Table 2 shows representative control experiments where no significant differences in ^{3}H-thymidine incorporation were observed between (a) cultures of live bacilli exposed to ethyl alcohol diluent in concentrations equivalent to those used for dissolving clofazimine analogues, and (b) cultures of heat-killed bacilli in the presence and absence of the drug.

Table 2. Representative experiment showing negligible effects of diluent and drug on control macrophage cultures

(a)

	^{3}H-thymidine incorporation (mean cpm ± se)* Killed = 406 ± 106 Live = 4234 ± 296			
Cultures 'live'	5 ng drug	Equivalent diluent	100 ng drug	Equivalent diluent
+ B 663	1234 ± 236	4011 ± 168	1114 ± 126	4198 ± 185
+ B 3691	1186 ± 265	4210 ± 289	1268 ± 191	4111 ± 273
+ B 3648	1150 ± 256	4027 ± 135	1086 ± 160	4139 ± 225
+ B 3640	1864 ± 315	4269 ± 333	1279 ± 340	4222 ± 373
+ B 3713	1986 ± 230	4085 ± 216	1645 ± 235	4115 ± 207

(b)

Cultures	Drug conc. (ng/ml)	^{3}H-thymidine incorporation (mean cpm ± se)*
Killed + B 663	0	406 ± 106
	1	410 ± 17
	2·5	417 ± 29
	5	331 ± 36
	10	342 ± 122
	20	336 ± 29
	40	398 ± 125
	50	350 ± 20
	100	316 ± 33

* Five replicate cultures

EFFECT OF CLOFAZIMINE (B 663)

(a) *Time kinetics*

Figure 2 shows that at 10 and 100 ng/ml of B 663, inhibition of the ^{3}H-thymidine uptake was discernible at 24 h and reached significant and maximal levels at 48 h. Further incubation of cultures with the drug for 72 and 360 h (15 days) did not increase the inhibitory effects to a significant degree.

(b) *Dose response*

Results obtained on 1 armadillo-derived *M. leprae* strain maintained in macrophage cultures in the presence of 1 to 100 ng/ml of clofazimine are shown in Figure 3. A sharp, linear increase in percentage inhibition of ^{3}H-thymidine incorporation was observed from 1 to 10 ng/ml, followed by a plateau from 20 to 40 ng/ml. Increasing the concentration of B 663 from 50 to 100 ng/ml showed a marginal increment in inhibition of the radiolabel uptake.

EFFECT OF CLOFAZIMINE ANALOGUES

B 3691, B 3713, B 3640 and B 3648 were tested in parallel with clofazimine on human-derived *M. leprae* strains. Representative results obtained on 3 strains tested with 5, 10 and 100 ng/ml of drugs for 48 and 72 h and 15 days are given in Figure 4. These analogues showed significantly less inhibition than the parent compound at 48 h exposure at the lower concentration of 5 ng/ml ($P < 0{\cdot}005$ to

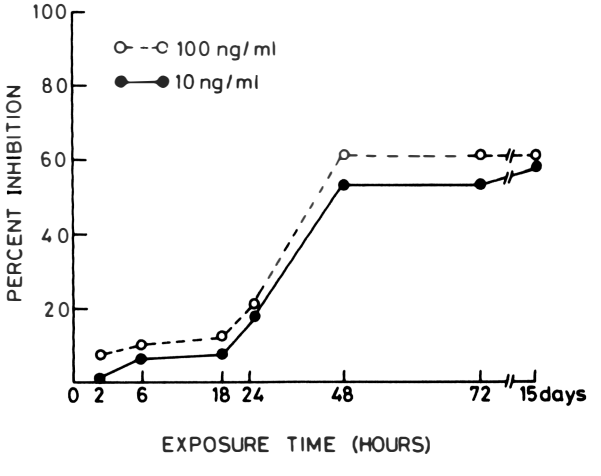


Figure 2. The response of an armadillo-derived *M. leprae* strain to a different exposure time (2 h to 360 h) of B 663 at 10 ng (●——●) and 100 ng/ml (○——○) on ^{3}H-thymidine incorporation expressed as percent inhibition.

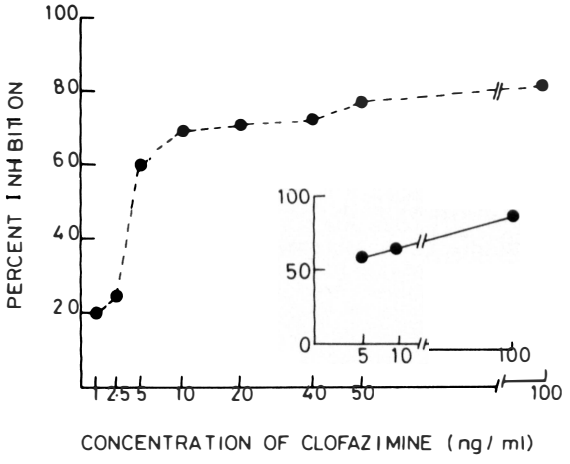


Figure 3. The effect of 1 to 100 ng of clofazimine (B 663) per ml on ^{3}H-thymidine incorporation in 1 armadillo-derived *M. leprae* strain (●– – –●) expressed as percent inhibition. The inset shows mean percent inhibition obtained with 3 human-derived *M. leprae* strains (●——●) each of which were tested at 5 ng, 10 ng and 100 ng/ml of B 663.

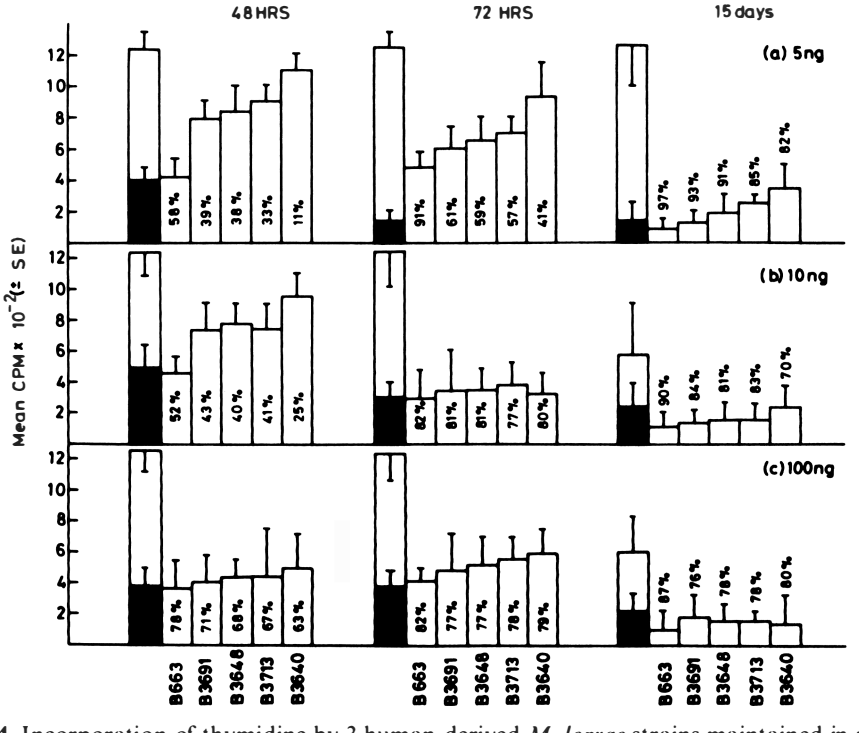


Figure 4. Incorporation of thymidine by 3 human-derived *M. leprae* strains maintained in murine macrophage (mean cpm ± se) in the presence of clofazimine (B 663) and clofazimine analogues (B 3691, B 3648, B 3713, B 3640) at different concentrations of drug: (a) 5 ng/ml, (b) 10 ng/ml, and (c) 100 ng/ml exposed for 48 h, 72 h and 15 days. The values in the bar indicate the percent inhibition of the uptake of the radiolabel in the presence of drug. □, mean cpm ± se with live bacilli ■, mean cpm ± se with heat-killed bacilli.

Table 3. Effect of clofazimine analogues (B 720 and B 749) on ^{3}H-thymidine incorporation in 2 human-derived *M. leprae* strains maintained within macrophages

		Mean cpm of 'live' *M. leprae*			
			Drug ng/ml		
Strain No.	Drugs	Without drug	5	10	100
I	B 720	872	384 (56)*	346 (60)	280 (67)
	B 749	872	427 (51)	261 (70)	273 (71)
II	B 720	5108	1700 (67)	1440 (72)	764 (85)
	B 749	5108	1381 (73)	1205 (77)	784 (82)

* Percent inhibition of ^{3}H-thymidine is shown within parenthesis.

Mean cpm $\pm$ se of cultures with killed *M. leprae* of strain I and II was respectively 338 ± 34 and 944 ± 18.

P value $< 0{\cdot}05$ to $0{\cdot}01$ by the Mann–Whitney *U* test.

0·001). However, they showed similar levels of inhibition as clofazimine when left for 72 h and 15 days.

Analogues B 720 and B749 were also tested on 2 human *M. leprae* strains in a 15-day assay. Significant inhibition of ^{3}H-thymidine incorporation was observed at 5, 10, 100 ng/ml (Table 3).

Discussion

In general, it may be observed that the clofazimine analogues exerted levels of inhibition similar to those of the parent compound. In experiments with lower concentrations of drugs and a short exposure of 48 h, clofazimine was more effective than the analogues. However, at higher concentrations of 10 ng/ml and 100 ng/ml similar levels of inhibition were observed with all drugs. As expected, percentage inhibition for each strain varied for the same analogue. Amongst the analogues B 3691 appeared to be the most inhibitory.

It is of interest that of the 4 analogues examined B 3691 is the one most closely related structurally to 2 others of our compounds, B 720 and B 749, which have been shown[8] to be active in the mouse foot-pad assay although they were of only very moderate activity in experimental tuberculosis of mice.[9] Not all clofazimine analogues are well absorbed from the mouse gut on oral administration. Of the above 6 analogues, only B 720, B 749 and, to a lesser extent, B 3691 are satisfactory in this respect.

Acknowledgments

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