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# Inhibition of mononuclear leukocyte transformation *in vitro* by dihydrophenazines in comparison to clofazimine

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*Summary* To identify the molecular structures of the antileprosy drug clofazimine which mediate its immunosuppressive activity the effects of ten phenazine derivatives on phytohaemagglutinin-stimulated mononuclear leukocyte (MNL) transformations were investigated. It was found that modifications in the substituent at position 2 of the dihydrophenazine moiety decreased the antiproliferative activity. The nature of the chemical group in position 2 also influenced the immunomodulatory effect of halogenation in the paraposition of the phenyl-rings and anilino-rings.

## Introduction

Clofazimine (B663)[1–4] has become an established part of the standard antimycobacterial chemotherapy of leprosy.[5,6] Due to its efficacy in the treatment of erythema nodosum leprosum reactions[5] and various nonmicrobial skin disorders,[7] immunosuppressive effects have been attributed to this agent in addition to its antimycobacterial properties. A variety of techniques have been employed to investigate this immunosuppressive activity *in vitro* and *in vivo*,[8,9] including phytohaemagglutinin (PHA)-stimulated lymphocyte transformations and leukocyte migration assays, which were inhibited by clofazimine at therapeutic concentrations.[10,11]

In an attempt to identify the molecular structure(s) responsible for this clofazimine-mediated immunosuppressive activity we have investigated the effect of ten dihydrophenazine-derivatives on mononuclear leukocyte (MNL) transformation *in vitro* in comparison to clofazimine. The results were correlated with the previously described antimycobacterial properties of the compounds.[3]

## Methods

### AGENTS

#### 1 *Selection*

All agents investigated in this study were synthesized by Dr J F O'Sullivan, Medical Research

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Council of Ireland Laboratories, Dublin. A cross-section of ten dihydrophenzine-derivatives was chosen in order to examine the importance of various substitutions for the inhibition of MNL transformation to PHA in comparison to the antimycobacterial effects. Since the insertion of chlorine in the paraposition of the phenyl-rings and anilino-rings has been found to strongly augment the activity of the agents against murine tuberculosis (TB),[3] all compounds were investigated in their chlorinated and unchlorinated forms. The significance of this halogen was further examined by including the fluorinated form of B663, namely B980, in the study. Since aposafranone derivatives, in which the nitrogen substituent at position 2 of the phenazine core has been replaced by oxygen, were inactive *in vitro* and against murine TB,[3] we also included four representatives of this group of agents in the present study, namely B3722, B433, B685 and B432. The significance of the anilino-group of clofazimine in position 3 of the phenazine molecule was investigated using compounds in which this group had been substituted by a hydroxyl group (B3722, B433). Similarly the importance of the isopropyl-imino-group in position 2 of clofazimine was examined by replacing it with an imino-group resulting in B628 and B283. The chemical precursors of B663 and its unchlorinated analogue B670, which are the imidazophenazines B654 and B621, were also included in this study, since they were virtually without antimycobacterial activity.[3] The chemical structures of the agents are shown in Table 1.

2 *Solubilization*

One milligramme of each compound was solubilized in 0·7 ml dimethyl sulphoxide at 56°C, 0·3 ml hot distilled water was added immediately prior to dilution of the agents in Hepes- (N-2-hydroxyethyl piperazine-N′-2-ethane sulphonic acid, Sigma Chemical Co., St Louis, Mo., USA) buffered tissue culture medium 199 (M199, Grand Island Biological Co., NY, USA) to the concentrations required. All compounds were compared to the appropriate solvent control.

*Cell preparation.* MNL were obtained from heparinized venous blood of healthy volunteers as previously described.[10] The cells were suspended to a concentration of $4 \times 10^6$ MNL/ml in M199.

*Effect of test agents on MNL transformation to phytohaemagglutinin.* This assay was performed according to the method previously described.[10] For dose-response studies the test agents and the solvent control were added to the wells of the micro tissue culture plates at final concentrations of 0·3–10 μg/ml.

*Expression and analysis of results.* Results are expressed as mean values of four different experiments. Statistical analyses were performed by the Student's *t*-test (paired *t*-statistic).

## Results

*Effect of test agents on MNL transformation to phytohaemagglutinin.* Apart from the compounds B670, B628, B685 and B432 all agents investigated caused statistically significant inhibition of MNL transformation at concentrations $\geqslant 0{\cdot}6$ μg/ml with *P*-values between $< 0{\cdot}05$ and $< 0{\cdot}005$. At a concentration of 0·3 μg/ml only the compounds B663, B980, B3722 and B654 significantly inhibited PHA-stimulated blastogenesis (*P*-values between $< 0{\cdot}05$ and $< 0{\cdot}025$). Results are shown in Figure 1.

## Discussion

The dihydrophenazine-derivative clofazimine (B663) is one of the standard drugs in the treatment of leprosy.[5,6] Serum concentrations of approximately 1 μg/ml are achieved with this agent,[6] but since it is concentrated in macrophages[12–14] and in fat, tissue levels are significantly higher.[15] Apart from its antimycobacterial properties clofazimine also possesses anti-inflammatory activity.[8] *In vitro* it

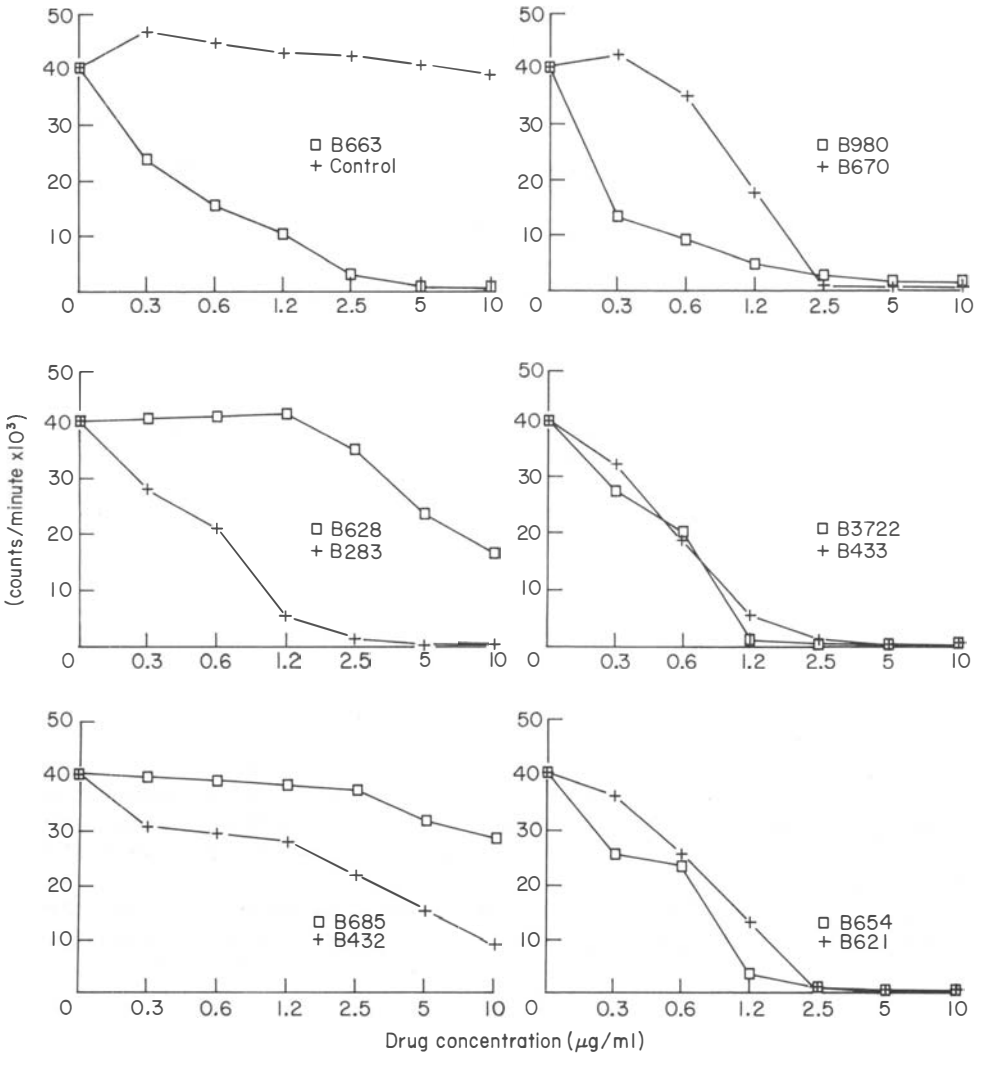
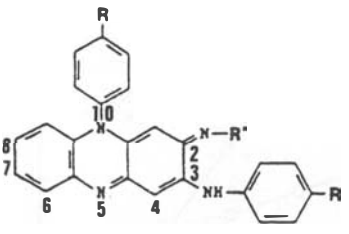



**Figure 1.** The effect of ten phenazine-derivatives on MNL transformation to PHA relative to clofazimine (B663) and a drug-free solvent control. Results are shown as man counts/minute $\times 10^3$ of four different experiments.

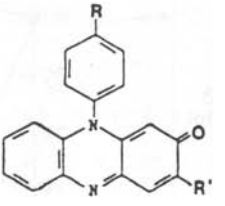
significantly inhibits PHA-stimulated MNL transformation and leukocyte migration at therapeutic concentrations[10,11] and similar effects have been observed after oral administration of the drug.[10]

In the present study we have used 10 dihydrophenazine-derivatives to correlate the chemical substitutions with the inhibitory activity of clofazimine on MNL transformation. Our findings indicate that this effect was dependent on the chemical groups in position 2 of phenazine. When the isopropyl-imino-group in position 2 of clofazimine was replaced by an imino-group (B628) or an oxygen (B685/B432), the antiproliferative potency of the compounds was decreased. The nature of the chemical group in position 2 of these agents also influenced the immunomodulatory effect of halogenation in the paraposition of the phenyl-rings and anilino-rings: the halogen-free analogues

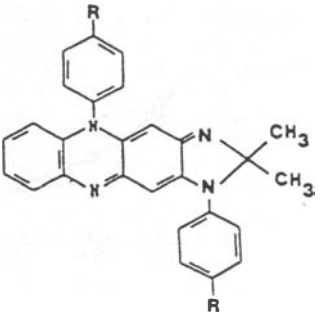
**Table 1.** Chemical structures of ten phenazine derivatives in comparison to clofazimine (B663)



B663: R = Cl, R′ = $CH(CH_3)_2$



B3722: R = Cl, R′ = OH



B654: R = Cl

B670: R = H, R′ = $CH(CH_3)_2$

B433: R = H, R′ = OH

B621: R = H

B980: R = F, R′ = $CH(CH_3)_2$

B685: R = Cl, R′ = $NHC_6H_4Cl$

B628: R = Cl, R′ = H

B432: R = H, R′ = $NHC_6H_5$

B283: R = H, R′ = H

of B628 and B685—B283 and B432—were more potent inhibitors of MNL transformation than their chlorinated counterparts, whereas the antiproliferative potency of the other compounds tested was increased by the presence of chlorine. Interestingly B685 and B283 as well as B670, B654 and B621 were all virtually inactive against murine TB, whereas B628 increased the median survival time of *Mycobacterium tuberculosis*-infected mice, though not to the same extent as clofazimine.[3] Therefore most of the agents investigated apparently possessed little or no antimycobacterial activity in murine TB, but had nevertheless retained antiproliferative effects equivalent to clofazimine.

It is concluded that the suppressive activity of dihydrophenazine-derivatives on MNL transformation to PHA depends on the nature of the chemical group in position 2 of the compounds tested. Furthermore the antiproliferative effects of the agents are influenced by interrelations of the group in position 2 with halogen-substitution of the paraposition of the phenyl-rings and anilino-rings. Our results also indicate that the antimycobacterial and immunosuppressive properties of clofazimine are probably unrelated.

**Acknowledgment**

The authors wish to thank Dr M K Felten for the production of the figures.

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## *NEWS AND NOTES*

### Robert Cochrane Fund for Leprosy

The fund, in memory of the contribution of the great leprologist Robert Cochrane, is administered by the Royal Society of Tropical Medicine and Hygiene. It is to be used to finance up to 3 travel fellowships each year to a maximum value of £1200 each.

The intention is to enable leprosy workers to travel for practical training in field work, or in research, or to enable experienced leprologists to travel in order to provide practical clinical training in a developing country. There is no restriction on the country of origin or destination providing the above requirements are fulfilled.

Application forms are available from the Society and must be received by the Society at least 6 months ahead of the proposed trip. All applications must be sponsored by a suitable representative of the applicant's employer or study centre, and agreed by the host organization. A 2 page report on the travel/study should be submitted to the Society within 1 month of the recipient's return. Apply: The Administrator, Royal Society of Tropical Medicine and Hygiene, Manson House, 26 Portland Place, London W1N 4EY.

### Posters and other Visual Material for Health Education in Developing Countries

Mr Bob Linney, Holly Tree Farm, Walpole Lane, Walpole, Halesworth, Suffolk, UK, continues to develop his interest, previously reported in this Journal, on the production of posters and other visual material for health education in Third World countries. From his commercial poster studio in London, Mr Linney has branched out into the production of coloured posters on health issues in developing countries, giving emphasis to the involvement of local artists and technicians and to the importance of getting to grips with local customs and culture. He has already organized workshops in India and Nepal. Pretesting and further testing of any poster developed are considered essential, in view of the evidence he has documented for widespread misinterpretation of many, perhaps the majority of posters produced in the health field. He is currently working towards the foundation of a charity called 'Health Images' which will concentrate on Third World needs, using local ideas and expertise whenever possible. Bob Linney (address above) would like to hear from anyone who would be interested in cooperation or who would like to learn more about his work.

# *NEWS AND NOTES*

## Ciba-Geigy Leprosy Fund

In 1986 the Pharmaceutical Division of Ciba-Geigy Ltd established the Ciba-Geigy Leprosy Fund with a budget of Sfr. 3 million (US $1·7 m) for an initial period of 3 years.

Purpose of the Fund

The Ciba-Geigy Leprosy Fund has been established to support leprosy control programmes which implement or intend to implement multidrug therapy (MDT) as recommended by the World Health Organization (WHO).

The primary emphasis of the Fund is to help create the preconditions for the correct implementation of MDT and thus to increase the total number of patients on MDT.

Conditions for funding

The proposed field activities should be undertaken in a programme which currently uses or wishes to introduce the WHO recommended MDT regimens to treat leprosy patients.

Only one project proposal may be submitted per year by each institution or agency. The Ciba-Geigy Leprosy Fund reserves the right to visit project sites. The use of the Fund's resources may be audited. Research dimensions can be added to projects (e.g. costs involved in MDT implementation, acceptance of MDT, effectiveness of measures taken to prevent disabilities). All applicants must agree to supply a bi-annual progress report on the specific activities supported by the Fund. Field projects must use the Ciba-Geigy form to give the project specific details.

Application procedure

Proposals must be submitted to the: Ciba-Geigy Leprosy Fund, PO Box, K-24.2.09, CH-4002 Basle, Switzerland before 15 February or 15 August.

All proposals must be accompanied by the following documentation: the objective of the proposed activities; detailed plan of action which includes methods, targets, timetable, staff; detailed budgetary request; details of other support reveived and requested.

In the case of field projects the following additional information is required: a brief description of the project area (e.g. size, population, patients by type, attendance rates) and the leprosy control efforts in the country; current extent of MDT implementation; a copy of the most recent annual report.

Selection criteria

Given a limited budget and the fact that most projects will require financing during the 3 year life span of the Fund, priorities must be set for the types of projects that would be favoured.

Only project proposals submitted by institutions (e.g. governments, non-governmental organizations and voluntary organizations) will be considered.

Any application should receive the approval of the government of the country where the project will be executed.

Field projects will be given top priority. Eighty per cent of the budget will be allocated to such projects.

Preference will be given to an intensification of ongoing work but projects which start from scratch are not excluded.

The contribution of the Ciba-Geigy Leprosy Fund, either in a professional or a financial sense, should be a significant one in the context of the total project.

The number of patients treated in the course of the project is of great importance.

The existence of the preconditions for the correct use of MDT (e.g. trained personnel, laboratory facilities, basic infrastructure, health education, or their creation as part of the project is essential.

Specific activities within the context of a project (e.g. health education, training) will be financed rather than contributing to the total project costs.

Ciba-Geigy Leprosy Fund, PO Box, K-24.2.09, CH-4002 Basle. *Telephone* (061) 36 26 55; *Telex* 962 355; *Telefax* (061) 36 22 39.