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The effect of ten phenazine-derivatives in comparison to clofazimine on the production of prostaglandin E_2 by polymorphonuclear leucocytes

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Summary The antileprosy drug clofazimine (B663) apart from its antimycobacterial effects has been shown to stimulate the production of prostaglandin E_2 (PGE_2) by polymorphonuclear leucocytes (PMNL). To separate these two activities on a molecular basis a limited number of ten phenazine-derivatives was investigated for their effects on prostaglandin synthesis. It was found that the *p*-chlorophenyl- and *p*-chloroanilino-groups in position 10 and 3 of the phenazine molecule respectively were indispensable for stimulation of PGE_2 production by PMNL, whereas modifications of the imino-isopropyl-group in position 2 did not affect this activity but as shown previously decreased the antimycobacterial effects of the agents against murine tuberculosis.

Introduction

The Dublin group founded by the late Dr V C Barry has synthesized and described a great variety of phenazine-derivatives to identify agents with antimycobacterial activity.[1–3] The most important of these compounds is B663 (clofazimine), which was first described in 1957.[4] During the last 20 years clofazimine has become one of the standard drugs for the treatment of leprosy.[5,6] Serum concentrations of approximately 1 μg/ml are achieved and tissue levels are considerably higher.[7] Clofazimine is particularly useful in the therapy of erythema nodosum leprosum reactions,[5] indicating anti-inflammatory properties of this drug in addition to its well-documented antimicrobial activity. A variety of mechanisms for the mediation of these anti-inflammatory effects have been proposed[8,9] including stimulation of the synthesis of prostaglandin (PG) E_2 by polymorphonuclear (PMN) and mononuclear (MN) leucocytes (L).[10,11]

The present study was undertaken to investigate the molecular basis of clofazimine-mediated

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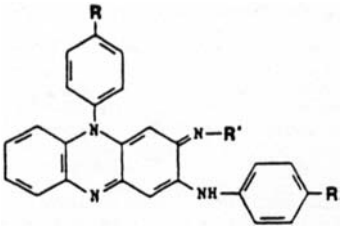
stimulation of PG-synthesis using ten phenazine-derivatives with variable antimycobacterial activity.

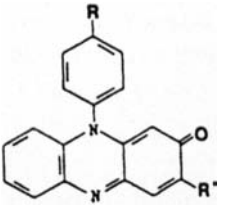
Materials and methods

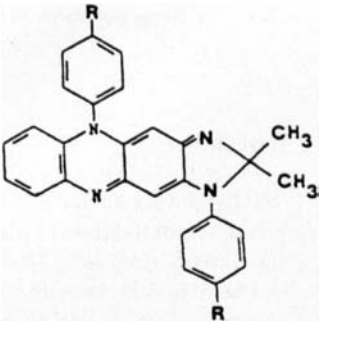
AGENTS

Selection. All agents investigated in this study were synthesized by Dr J F O'Sullivan, Medical Research Council of Ireland Laboratories, Dublin. A cross-section of ten phenazine-derivatives was chosen in order to examine the importance of various substitutions for the stimulation of the synthesis of PGE_2 by PMNL in comparison to the antimycobacterial effects. Since the insertion of chlorine in the para-position of the phenyl-rings and anilino-rings has been found to strongly augment the activity of the agents against murine TB[2], 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 in position 2 of the phenazine core has been replaced by oxygen, were inactive *in vitro* and against murine TB[2], we also included 4 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 imidazo-phenazines B654 and B621, were also included in this study, since they were virtually without antimycobacterial activity.[2] The chemical structures of the agents are shown in Table 1.

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







B663: R = Cl, R′ = $CH(CH_3)_2$
B670: R = H, R′ = $CH(CH_3)_2$
B980: R = F, R′ = $CH(CH_3)_2$
B628: R = Cl, R′ = H
B283: R = H, R′ = H

B3722: R = Cl, R′ = OH
B433: R = H, R′ = OH
B685: R = Cl, R′ = NHC_6H_4Cl
B432: R = H, R′ = NHC_6H_5

B654: R = Cl
B621: R = H

Solubilization. One miligramme 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 Hanks' balanced salt solution (HBSS, Grand Island Biological Co., New York, USA) to the concentrations required. All compounds were compared to the appropriate solvent control.

Cell preparation. PMNL were obtained from heparinized venous blood as previously described.[10] They were suspended in HBSS and contained >90% viable cells as determined by Trypan blue (0·1%) dye exclusion.

PGE_2 release by PMNL. This assay was performed according to the method previously described.[10] Drug effects were investigated at the fixed final concentration of 5 μg/ml in the presence and absence of the synthetic tripeptide N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, Miles Laboratories, Elkhart, Indiana, USA) at the final concentration of 10^{-6} M. Additionally dose-response studies were performed for B621 for a concentration range of 0·3–10 μg/ml. Interactions of the agents with the assay system were excluded using cell-free drug preparations. Results are expressed as pg PGE_2 per 10^6 PMNL.

To obtain a measure for the interactions of the phenzine-derivatives with the stimulus FMLP with respect to PGE_2 release by PMNL, a stimulation index (SI) was calculated according to the formula: SI = pg PGE_2 (FMLP + phen)/pg PGE_2 (phen) + pg PGE_2 (FMLP) with pg PGE_2 (FMLP + phen) = results in the presence of FMLP and the test agents or the solvent control, pg PGE_2 (phen) = PGE_2 release in the presence of the phenazines (or solvent control) only and pg PGE_2 (FMLP) = FMLP-stimulated PGE_2 release in the presence of the solvent control.

Expression and analysis of results. Results are expressed as mean values with standard error (SEM) for each series of experiments. Statistical analyses were performed by the Student's *t*-test (paired *t*-statistic).

Results

PGE_2 release by PMNL. The effects of ten phenazine-derivatives on FMLP-activated generation of PGE_2 by PMNL are shown in Figure 1. Statistically significant stimulation of PG production was observed for the compounds B663, B628, B654 and B621 (*P*-values between $<0{\cdot}025$ and $<0{\cdot}01$). The effects of the agents on spontaneous PGE_2 production by PMNL were similar (Figure 2, *P*-values between $<0{\cdot}05$ and $<0{\cdot}01$). The very strong stimulator B621 was the only unchlorinated compound with a more potent activating effect in comparison to its chlorinated counterpart. Dose-response studies with this agent showed dose-dependent stimulation of PGE_2 synthesis by PMNL at the concentration range of 0·3–10 μg/ml (results not shown).

The stimulation indices indicate the effect of the drugs on the response of PMNL to stimulation by FMLP. An SI > 1 indicates a synergistic effect of the phenazine-derivatives with the stimulus FMLP. Synergism was therefore observed with clofazimine (SI = 1·53 +/− 0·36), B980 (SI = 1·52 +/− 0·52), B628 (SI = 1·36 +/− 0·31) and B654 (SI = 1·57 +/− 0·31). In the presence of B621 an SI of 1·07 +/− 0·15 was calculated and for all other agents the SIs were < 1. With the exception of B621 the compounds that significantly stimulated PGE_2 release by PMNL therefore also primed the cell response to FMLP-stimulation.

Discussion

The phenazine-derivative clofazimine (B663) is used as part of the standard regimen for the treatment of leprosy.[5] Apart from its antimycobacterial effects, this drug also possesses

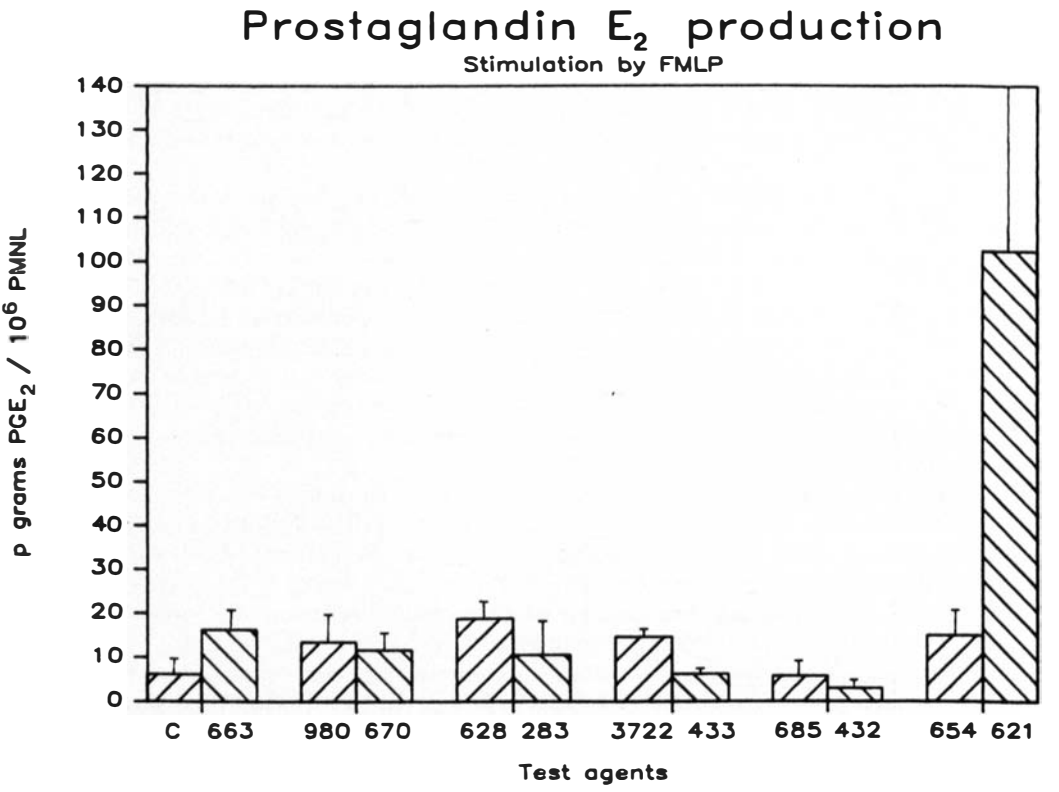


Figure 1. The effect of ten phenazine-derivatives at a concentration of 5 μg/ml in comparison to clofazimine (B663) and the solvent control (C) on the production of PGE_2 by human PMNL in the presence of 10^{-6} M FMLP. Results are expressed as the mean value +/− SEM in pg $PGE_2/10^6$ PMNL of three different experiments. ($P<0{\cdot}01$ for B663 and B628, $P<0{\cdot}025$ for B654 and B621).

immunosuppressive properties which may be related to its stimulatory effect on the synthesis of PGE_2 by PMNL and MNL.[10,11] In an attempt to distinguish between antimycobacterial and anti-inflammatory properties on a molecular basis we have investigated the effect of ten phenazine-derivatives in comparison to B663 on the PGE_2 production of PMNL.

It was found that the *p*-chlorophenyl-group in position 10 and the *p*-chloroanilino-group in position 3 of the phenazine-molecule were indispensable for the stimulatory effect of the compounds on the release of PGE_2 by PMNL. However, substitution of the imino-isopropyl-group in position 2 by an imino-group resulting in the agent B628 did not affect this activity, though it diminished the antimicrobial effect of the agent against murine TB[2]. Formation of an imidazole-ring between the nitrogens in position 2 and 3 similarly did not interfere with the stimulatory activity on PGE_2 generation, though the resulting imidazophenazine B654 possessed virtually no antimycobacterial activity.[2] The unchlorinated analogue of B654—B621—also lacked antimycobacterial properties[2] but was an extremely potent stimulator of PGE_2 production by PMNL. However, this agent may have a different mode of action relative to clofazimine. The lack of priming activity of B621 is possibly due to its very strong stimulatory effect on spontaneous PGE_2 release by PMNL, so that in the presence of a stimulus the upper limit of the cells for the production of this mediator could be approached.

It is concluded that the antimycobacterial properties of phenazine-derivatives and their stimulatory effects on PG synthesis by PMNL can be related to specific substitutions of the

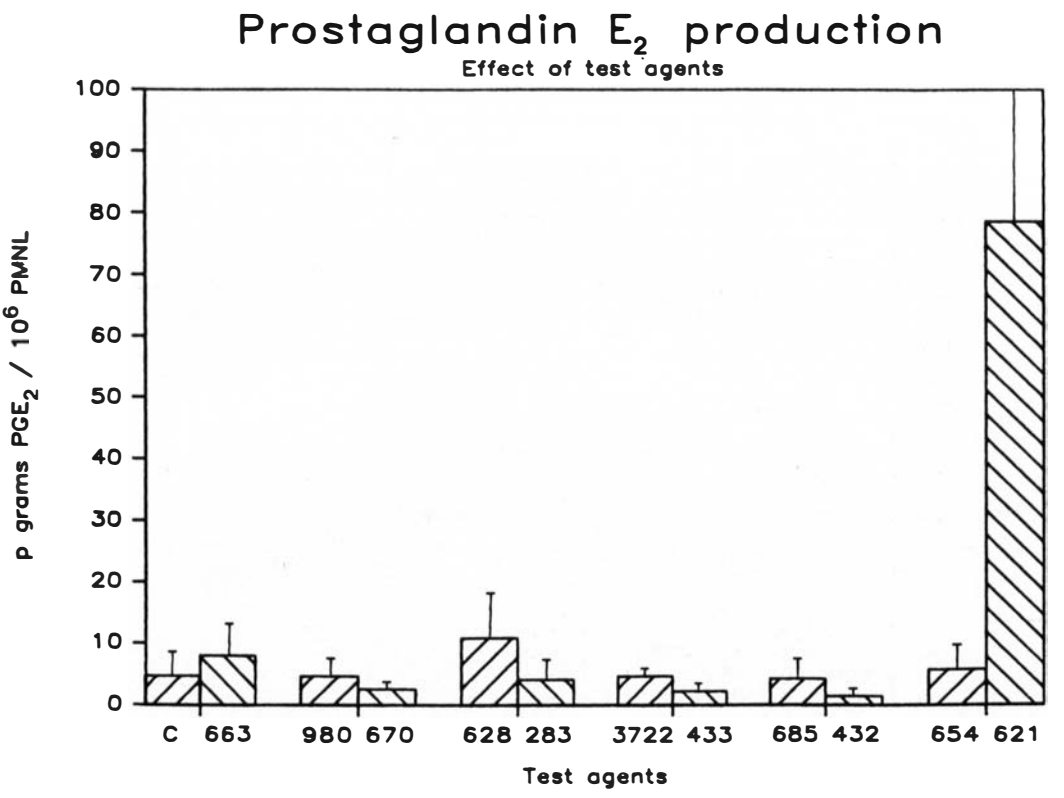


Figure 2. The effect of ten phenazine-derivatives at a concentration of 5 μg/ml in comparison to clofazimine (B663) and the solvent control (C) on the spontaneous release of PGE_2 by human PMNL. Results are expressed as the mean value +/− SEM in pg $PGE_2/10^6$ PMNL of three different experiments. ($P<0{\cdot}05$ for B663 and B628, $P<0{\cdot}01$ for B654, $P<0{\cdot}025$ for B621).

phenazine-molecule and that modifications of the imino-isopropyl-group in position 2 of this molecule are particularly important in this context.

Acknowledgment

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

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NEWS AND NOTES

Robert White Fellowships in Immunology

The closing date for this year, 1987, has past, but this Memorial Fund invites applications each year for its fellowships. The details are as follows:

A number of fellowships named in the memory of Professor Robert White will be awarded to individuals from developing countries in order to aid or further their education or scientific experience in immunology. The Society interprets these aims in the broadest possible terms and consideration will be given (for example) to support travel to study in a University or to gain experimental or technical and scientific expertise or for the purchase of books, journals or equipment.

All members of the Society, and particularly those resident overseas, are urged to bring the Robert White Fellowships to the attention of their colleagues. For further details of the scheme, and any enquiries about it, please contact the Secretary, The British Society for Immunology, 11 Hobart Place, London SW1N 0HL.

Leprosy Relief Organization, Munich; new office in Pune, India

We were delighted to hear from the Chairman of AHM; Leprosy Relief Organization, Munich, that they have recently opened an India office at Pune. The following is extracted from the announcement:

Dr Kalpara Mutatkar, who is trained in leprosy, and who has worked in a leprosy control project as a medical officer, is the Regional Director for India. He has the overall responsibility for promoting and co-ordinating AHM activities in India.

We are fortunate that Mr R D Sathe, who has been the Foreign Secretary of the Government of India and Ambassador to the Federal Republic of Germany, has agreed to be the chairman of the committee. Professor Mutatkar has agreed to be the Advisor to AHM office in India. Professor Mutatkar is the first social scientist who has worked closely with World Health Organization on social aspects of leprosy. Of 12 scientists invited to the 1984 Workshop of the Pontifical Academy of Sciences Vatican, Professor Mutatkar was the only one who represented a developing country. On this occasion he had an audience with his Holiness, Pope John Paul II.

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