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Prooxidative Activities of 10 Phenazine Derivatives Relative to That of Clofazimine

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The objective of this study was to investigate the relationship between the antimycobacterial properties of the antileprosy drug clofazimine and its stimulatory effect on the release of reactive oxidants by polymorphonuclear leukocytes by using a variety of phenazine derivatives. The effects of these compounds on myeloperoxidase-mediated iodination, luminol-enhanced chemiluminescence, and the release of superoxide anion by polymorphonuclear leukocytes were investigated. Dissociation of the antimycobacterial and prooxidative effects of clofazimine was possible by manipulation of the chemical group in position 2 of the phenazine molecule. When nitrogen-containing substituents in this position were replaced by oxygen, the mode of the prooxidative action of the compounds was altered.

Host defense against mycobacterial infection depends largely on the efficacy of a phagocyte myeloperoxidase (MPO)-hydrogen peroxide-halide system (12, 16, 18). The reactive oxidant hydrogen peroxide is produced during the respiratory burst of polymorphonuclear leukocytes (PMN) as a result of superoxide dismutation (11). Various antibiotics have been shown to increase the release of reactive oxidants from PMN (10), including the antileprosy drug clofazimine (B663) (20). Clofazimine is a phenazine derivative (2–5) which apart from its antimycobacterial activity also possesses immunosuppressive properties (15, 21). We have shown recently that the immunosuppressive effects of this drug are partly mediated by reactive oxidants (22).

The present study was undertaken to dissociate on a molecular basis the antimycobacterial activities of clofazimine and its stimulatory effect on the release of reactive oxidants by PMN by using 10 phenazine derivatives.

MATERIALS AND METHODS

Agents. (i) Selection. All agents investigated in this study were synthesized by J. F. O'Sullivan, Laboratories of the Medical Research Council of Ireland, Dublin. Ten phenazine derivatives were chosen to examine the importance of various substitutions on the production of reactive oxidants by PMN in comparison with antimycobacterial effects. Since the insertion of chlorine in the *para* position of the phenyl and anilino rings has been found to strongly augment the activity of the agents against murine tuberculosis (TB) (5), 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, B980, in the study. Since aposafranine derivatives, in which the nitrogen in position 2 of the phenazine core is replaced by oxygen, are inactive in vitro and against murine TB (5), we also included four representatives of this group of agents, B3722, B433, B685, and B432. The significance of the anilino group of clofazimine in position 3 of the phenazine molecule was investigated by using compounds in which this group is replaced by a hydroxyl group (B3722 and B433). Similarly, the importance of the isopropylimino group in position 2 of clofazimine was examined by replacing it with an amino group, resulting in B628 and B283. The chemical precursors of B663 and its unchlorinated analog B670, which are the imidazophenazines B654 and B621, were also included in the study since they are virtually without antimycobacterial activity (5). The chemical structures of the agents are shown in Fig. 1.

(ii) Solubilization. Each compound (4 mg) was solubilized in 0.1 ml of acetic acid–0.3 ml of dimethyl sulfoxide–0.6 ml of distilled deionized water and further diluted in distilled water to a final concentration of 10 μg/ml. The agents were then diluted in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Sigma Chemical Co., St. Louis, Mo.)–buffered Hanks balanced salt solution (HBSS; GIBCO Laboratories, Grand Island, N.Y.) to the concentrations required. All compounds were compared with the appropriate solvent control.

Cell preparation. PMN were obtained from heparinized venous blood (5 U of preservative-free heparin per ml), as previously described (1). The cells were suspended in HBSS and contained >90% viable PMN as determined by trypan blue (0.1%) dye exclusion.

Myeloperoxidase-mediated iodination of PMN. Myeloperoxidase-mediated iodination of PMN was measured by the method of Root and Stossel (14). Reaction mixtures contained 10^6 PMN, 0.05 μCi of ^{125}I (sodium iodide; New England Nuclear Corp., Boston, Mass.; specific activity, 17 Ci/mg), 10% autologous serum, the agent or the drug-free solvent control, and the synthetic chemotactic tripeptide *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP; Miles Laboratories, Inc., Elkhart, Ind.) in a final volume of 1 ml of HBSS. The drugs were used at final concentrations of 1 and 3 μg/ml, and FMLP was used at a final concentration of 1 μM. Results are expressed as femtomoles of ^{125}I per 10^6 PMN in the protein precipitate after 30 min of incubation at 37°C.

Luminol-enhanced chemiluminescence of PMN. The effects of the agents, at final concentrations of 1 and 3 μg/ml, on chemiluminescence were investigated by incubating 2×10^6 PMN in a final volume of 1 ml of HBSS containing the agent or the drug-free solvent control for 15 min at 37°C. Luminol

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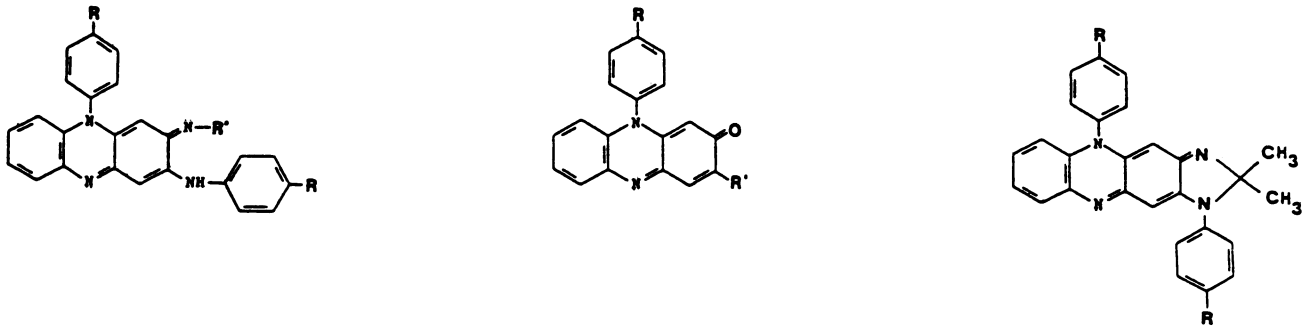

B663: R = Cl, R' = $CH(CH_3)_2$

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

FIG. 1. Chemical structures of 10 phenazine derivatives and clofazimine (B663).

(5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma) at a final concentration of 0.3 μM was added 5 min before counting was performed in a Wallac 1251 luminometer (LKB, Turku, Finland). The results were recorded as millivolt seconds integrated over 5 s.

Superoxide production by PMN. Superoxide production by PMN was measured by a method previously described (17). The reaction mixture contained 2×10^6 PMN, 1 μM FMLP, 1 μg of cytochalasin B per ml, 0.1 mM horse heart ferricytochrome *c* (cyt-*c*; type VI; Sigma), and the agent under investigation or the drug-free solvent control at a final concentration of 1 μg/ml in a final volume of 1 ml of HBSS with or without 200 U of superoxide dismutase (Sigma) per ml. Dose-response studies were performed with clofazimine and B3722 at concentrations of 0.125 to 5 μg/ml. Cells and reagents were prewarmed separately for 15 min at 37°C before the reaction was started by addition of the PMN to the reagent mixture. After incubation of the mixture for 1 min at 37°C in a water bath, the reaction was terminated by addition of 1 ml of ice-cold phosphate-buffered saline (0.15 M, pH 7.1) followed by centrifugation of the tubes at 4°C. The supernatants were assayed for reduced cyt-*c* in an SP 1700 UV spectrophotometer (Unicam, Cambridge, England) at 550 nm. The amount of reduced cyt-*c* was calculated by using an absorbance coefficient of 2.11×10^4 cm^2/mmol (19). Superoxide-dependent reduction of cyt-*c* is expressed as the difference in cyt-*c* reduction between reaction mixtures with and without superoxide dismutase as nanomoles of reduced cyt-*c* per 2×10^6 PMN.

Expression and analysis of results. Results are expressed as mean values with standard errors of the means of four separate experiments in each series using PMN from four different individuals. All experiments were done in duplicate. Statistical analyses were performed by Student's *t* test (paired *t* statistic).

RESULTS

Myeloperoxidase-mediated iodination of PMN. Myeloperoxidase-mediated iodination of PMN was investigated to measure the effects of the phenazines on the MPO-H_2O_2-halide system of phagocytes in comparison with those of clofazimine. At a concentration of 1 μg/ml, B663 (clofazimine), B980, B628, B3722, B433, and B432 caused statistically significant stimulation of iodination (P between <0.05 and <0.005). However, the stimulatory effects of B980 and

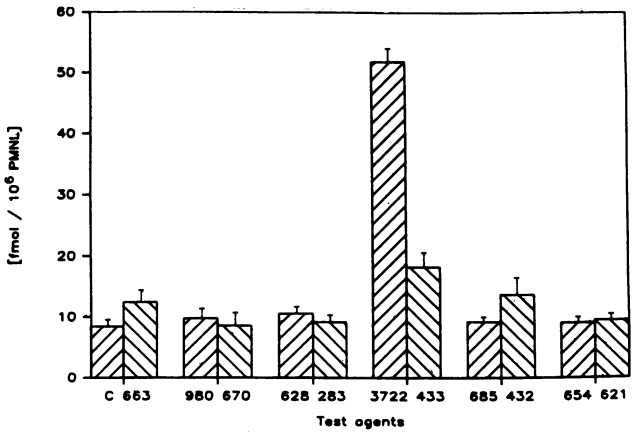

FIG. 2. Effects of clofazimine (B663) and the phenazine derivatives on MPO-mediated iodination of 10^6 PMN per ml in comparison with those of a drug-free solvent control (C) at a final concentration of 1 μg/ml. Results are shown as means with standard errors of the means (bars) in femtomoles of ^{125}I per 10^6 PMN for four different experiments. Statistically significant stimulation was found in the presence of clofazimine (B663; $P < 0.025$), B980 ($P < 0.05$), B628 ($P < 0.05$), B3722 ($P < 0.005$), B433 ($P < 0.025$), and B432 ($P < 0.05$).

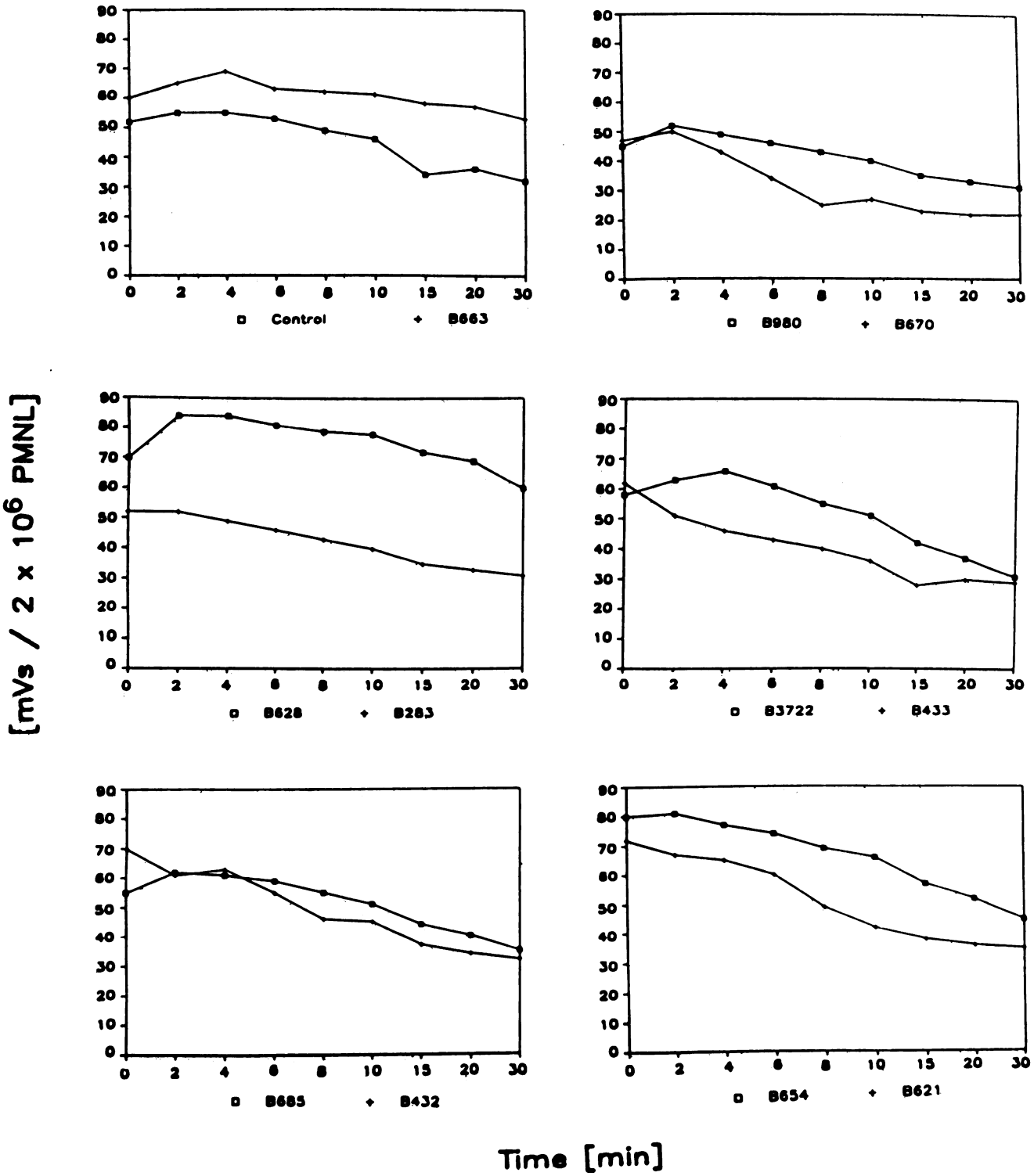

FIG. 3. Effects of clofazimine (B663) and the phenazine derivatives on luminol-enhanced chemiluminescence of 2×10^6 PMN in comparison with those of a drug-free solvent control at a final concentration of 1 μg/ml. Results are shown as means (millivolt seconds per 2×10^6 PMN) of four different experiments. Statistically significant stimulation was caused by clofazimine (B663; $P < 0.05$) and B628 and B654 (P between <0.05 and <0.025 for the different time intervals).

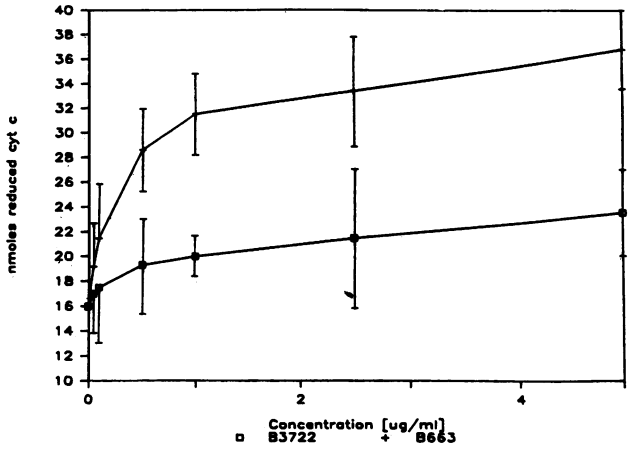

FIG. 4. Effects of clofazimine (B663) and B3722 at concentrations of 0.05 to 5 μg/ml on superoxide generation by PMN. Unstimulated background values were subtracted, and the results are expressed as nanomoles of reduced cyt-*c* per 2×10^6 PMN after 1 min of incubation (means ± standard errors of means of four different experiments). At concentrations of 0.5 to 5 μg/ml, clofazimine (B663) significantly ($P < 0.025$ to $P < 0.005$) increased superoxide generation by FMLP-activated PMN. No statistically significant effects were observed with B3722.

B628, although statistically significant, were minimal. The other agents had no effect. The results are shown in Fig. 2.

Similar results were obtained at 3 μg/ml except that B654 and B621 also significantly stimulated iodination of PMN ($P < 0.025$; data not shown).

Luminol-enhanced chemiluminescence of PMN. Luminol-enhanced chemiluminescence of PMN was used to investigate the effects of the phenazines on the release of the reactive oxidants hydrogen peroxide, hydroxyl free radical, and superoxide anion by PMN relative to those of clofazimine (6). At a concentration of 1 μg/ml, B663 (clofazimine), B628, and B654 significantly stimulated the chemiluminescence of PMN at all time intervals (*P* between <0.05 and <0.025). The other compounds had no effect. The results are shown in Fig. 3.

The findings at 3 μg/ml were similar except that B283 significantly inhibited PMN chemiluminescence at this concentration (*P* between <0.05 and <0.005 for the different time intervals; data not shown).

Superoxide production by PMN. Superoxide production by PMN was used to investigate the effects of the agents on the respiratory burst of phagocytes after stimulation with FMLP. At a concentration of 1 μg/ml, clofazimine was the only compound that significantly stimulated the generation of superoxide anion by PMN, with the release of 25.0 ± 2 nmol of reduced cyt-*c* per 2×10^6 PMN, in comparison with 15.6 ± 1 nmol in the presence of the drug-free solvent control ($P < 0.01$). Significant inhibition was observed only with B432 (9.7 ± 1 nmol per 2×10^6 PMN [$P < 0.025$]); the other agents had no effect (data not shown).

Dose-response studies with clofazimine and B3722 at concentrations of 0.125 to 5 μg/ml yielded dose-dependent stimulation of superoxide release by clofazimine, whereas B3722 had no effect. The results are shown in Fig. 4.

DISCUSSION

The phenazine derivative clofazimine is a well-established part of the standard regimen for the treatment of leprosy (15, 21). Concentrations of approximately 1 μg/ml are achieved in serum (21), but since the drug is concentrated in macrophages (7–9) and in fat, concentrations in tissue are considerably higher (13).

The previously reported stimulatory effect of clofazimine on the release of reactive oxidants by PMN (20) is probably partly responsible for both its antimycobacterial and immunosuppressive properties (22). In the present study we investigated the effects of 10 phenazine derivatives on the release of reactive oxidants by PMN to dissociate the antimycobacterial and prooxidative effects of clofazimine on a molecular basis. Except for B3722, B433, and B432, all agents examined have previously been investigated for their efficacy against murine TB (5), and other than B663 (clofazimine), B628, and B980, they are all virtually inactive (5). The antimycobacterial activities of the agents correlated well with the results of the superoxide anion assay in this study, since clofazimine is the most potent of the test drugs against murine TB (5) and also stimulated the release of superoxide anion by PMN. The other two compounds which also possess some antimycobacterial activity against murine TB, B628 and B980, were among the five phenazine derivatives that significantly increased MPO-mediated iodination at a concentration of 1 μg/ml, although their stimulatory effects in absolute terms were minimal. Data on the antimycobacterial properties of the other three agents were not available. However, we investigated the effect of the most potent stimulator in this assay, B3722, relative to that of clofazimine on PMN-mediated killing of *Staphylococcus aureus* and found that B3722 was inactive, whereas clofazimine caused increased killing of this microorganism (data not shown). This finding, together with the lack of activity of B3722 and its unchlorinated analog B433 in the chemiluminescence and superoxide anion assays, indicates that this agent works by a mode of action different from that of clofazimine. The aposafranines B3722 and B433 possibly participate in an NADPH-oxidase-independent redox cycle which leads to the generation of hydrogen peroxide, whereas clofazimine, in addition to this mechanism, seems to interact with the membrane-associated respiratory burst of PMN, since it significantly enhanced superoxide generation by phagocytes.

Furthermore, the finding that the phenazines B628 and B654 significantly stimulated MPO-mediated iodination and luminol-enhanced chemiluminescence of PMN at concentrations of 3 and 1 μg/ml, respectively, indicates that modifications in position 2 of the phenazine molecule do not interfere with the effect of the agents on the release of reactive oxidants by PMN as long as nitrogen is present, although the antimycobacterial properties of the compounds against murine TB are diminished by substitutions other than the isopropylimino group of clofazimine (5).

It is concluded that dissociation of the antimycobacterial and prooxidative effects of clofazimine is possible based on the chemical group in position 2 of the molecule.

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LITERATURE CITED

1. **Anderson, R., and A. J. van Rensburg.** 1979. The *in vitro* effects of propanolol and atenolol on neutrophil motility and post-phagocytic metabolic activity. Immunology 37:15–24.
2. **Barry, V. C., J. G. Belton, M. L. Conalty, J. M. Denneny, D. W.**

Edward, J. F. O'Sullivan, D. Twomey, and F. Winder. 1957. A new series of phenazines (rimino-compounds) with high antituberculosis activity. Nature (London) **179**:1013–1015.
3. **Barry, V. C., J. G. Belton, M. L. Conalty, and M. McInerney.** 1970. Antitubercular substances—XXII. Rimino-phenazines—the effect of further substitution. Proc. R. Ir. Acad. **70**:179–194.
4. **Barry, V. C., J. G. Belton, M. L. Conalty, and D. Twomey.** 1948. Anti-tubercular activity of oxidation products of substituted o-phenylene diamines. Nature (London) **162**:622–624.
5. **Barry, V. C., and M. L. Conalty.** 1958. Antituberculosis activity in the phenazine series. II. N3-substituted anilinoaposafranines (rimino-compounds) and some derivatives. Am. Rev. Tuberc. Pulm. Dis. **78**:62–73.
6. **Bird, J., and J.-P. Giroud.** 1985. An appraisal of the technique of polymorphonuclear leukocyte chemiluminescence as a means to detect compounds with antiinflammatory activity. J. Pharmacol. Methods **14**:305–312.
7. **Conalty, M. L.** 1966. Rimino-phenazines and the reticuloendothelial system. Ir. J. Med. Sci. **491**:497–501.
8. **Conalty, M. L., V. C. Barry, and A. Jina.** 1971. The antileprosy agent B.663 (clofazimine) and the reticuloendothelial system. Int. J. Lepr. **39**:479–492.
9. **Conalty, M. L., and R. D. Jackson.** 1962. Uptake by reticuloendothelial cells of the rimino-phenazine B.663 (2-p-chloroanilino-5-p-chlorophenyl-3:5-dihydro-3-isopropyliminophenazine). Br. J. Exp. Pathol. **43**:650–654.
10. **Duncker, D., and U. Ullmann.** 1986. Influence of various antimicrobial agents on the chemiluminescence of phagocytosing human granulocytes. Chemotherapy (Basel) **32**:18–24.
11. **Hertz, F., and A. Cloarec.** 1984. Pharmacology of free radicals; recent views on their relation to inflammatory mechanisms. Life Sci. **34**:713–720.
12. **Klebanoff, S. J., and C. C. Shepard.** 1984. Toxic effect of the peroxidase-hydrogen peroxide-halide antimicrobial system on *Mycobacterium leprae*. Infect. Immun. **44**:534–536.
13. **Mansfield, R. E.** 1974. Tissue concentrations of clofazimine (B663) in man. Am. J. Trop. Med. Hyg. **23**:1116–1119.
14. **Root, R. K., and T. P. Stossel.** 1974. Myeloperoxidase-mediated iodination by granulocytes. Intracellular site of operation and some regulating factors. J. Clin. Invest. **53**:1207–1215.
15. **Schulz, E. J.** 1972. Forty-four months' experience in the treatment of leprosy with clofazimine (Lamprene (Geigy)). Lepr. Rev. **42**:178–187.
16. **Sharp, A. K., M. J. Colston, and D. K. Banerjee.** 1985. Susceptibility of *Mycobacterium leprae* to the bactericidal activity of mouse peritoneal macrophages and to hydrogen peroxide. J. Med. Microbiol. **19**:77–84.
17. **Theron, A., and R. Anderson.** 1985. Investigation of the protective effects of the antioxidants ascorbate, cysteine, and dapsone on the phagocyte-mediated oxidative inactivation of human alpha-1-protease inhibitor *in vitro*. Am. Rev. Respir. Dis. **132**:1049–1054.
18. **Urbanczik, R., and K. F. Petersen.** 1985. Ueberlegungen zur Entwicklung der Bakteriologie und der experimentellen Chemotherapie von Mykobakteriosen. Prax. Klin. Pneumol. **39**:421–430.
19. **van Gelder, B. F., and E. C. Slater.** 1962. The extinction coefficient of cytochrome c. Biochim. Biophys. Acta **58**: 593–595.
20. **van Rensburg, C. E. J., E. M. S. Gatner, F. M. J. H. Imkamp, and R. Anderson.** 1982. Effects of clofazimine alone or combined with dapsone on neutrophil and lymphocyte functions in normal individuals and patients with lepromatous leprosy. Antimicrob. Agents Chemother. **21**:693–697.
21. **Yawalkar, S. J., and W. Vischer.** 1979. Lamprene (clofazimine) in leprosy. Lepr. Rev. **50**:135–144.
22. **Zeis, B. M., and R. Anderson.** 1986. Clofazimine-mediated stimulation of prostaglandin synthesis and free radical production as novel mechanisms of drug-induced immunosuppression. Int. J. Immunopharmacol. **8**:731–739.