The Riminophenazine Agents Clofazimine and B669 Inhibit the Proliferation of Cancer Cell Lines in Vitro by Phospholipase A2-mediated Oxidative and Nonoxidative Mechanisms

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ABSTRACT

Clofazimine, a riminophenazine antimicrobial agent, and its analogue B669 were investigated for their effects on FaDu cells, a human squamous carcinoma cell line. These agents, at concentrations within the therapeutic range (0.25–2 μg/ml), caused a dose-dependent tumor cell cytotoxicosis which was greatly enhanced in the presence of human neutrophils. The neutrophil-mediated increment in tumoricidal activity, but not the direct antitumor effects of the drugs per se, was inhibited by catalase. The effects of these drugs on three more cell carcinoma lines as well as on two primary cultures and a noncarcinoma cell line were also investigated and compared with the activity of the standard antitumor chemotherapeutic agents bleomycin, cisplatin, and methotrexate. All seven cultures were sensitive to clofazimine and B669 compared to six that were sensitive to cisplatin, three that were sensitive to bleomycin, and one that was sensitive to methotrexate. The treatment of FaDu cells with clofazimine and B669 was associated with enhanced activity of phospholipase A2, as evidenced by increased release of radiolabeled arachidonate and lysophosphatidylethanolamine from membrane phospholipids. Inhibitors of arachidonic acid metabolism, protein kinase C inhibitors, as well as water and lipid soluble antioxidants failed to protect the cells against the cytotoxic activity of clofazimine and B669. However, α-tocopherol, a lysophospholipid-complexing agent, completely blocked the antiproliferative effects of the riminophenazines and also protected the cells against the direct cytotoxic effect of lysophosphatidylethanolamine, while the lysophospholipid-neutralizing enzyme lysophospholipase protected against the riminophenazines. These observations demonstrate that the tumoricidal properties of clofazimine and B669 are probably due to increases in the lysophospholipid content of cell membranes.

INTRODUCTION

The riminophenazine compound clofazimine [B663; 3-(p-chloroanilino)-10-(p-chlorophenyl)-2,10-dihydro-2-(cyclohexylimino)phenazine] is primarily an antileprosy agent (1–3). Apart from its direct antimicrobial activities clofazimine also enhances the phagocytic and antimicrobial activities of human and murine neutrophils (4) by sensitizing the cells to hyperreact to various stimuli leading to increased production of reactive oxidants (5) with antimicrobial and tumoricidal potential (6). Previously published molecular structure/function studies revealed that the prooxidative interactions of the riminophenazines with neutrophils are dependent on the presence and type of alkylimino group at position 2 of the phenazine nucleus (7). One such compound, B669, was identified as being an exceptionally potent stimulant of superoxide generation by activated neutrophils (7). Clofazimine also possesses antiproliferative properties and inhibits the proliferation of phytohemagglutinin-stimulated lymphocytes (8, 9).

In the present study we have investigated the direct effects of clofazimine and B669 on the proliferation of various cancer cell lines in vitro as well as the effects of these agents on neutrophil-mediated killing of tumor cells. Our data demonstrate that clofazimine and B669 per se are potent inhibitors of the proliferation of various cancer cell lines in vitro and also enhance the tumoricidal activity of neutrophils. Moreover, anti-proliferative lysophospholipids generated in the cell membrane of clofazimine/B669-treated cancer cells are the mediators of the direct antitumor activity of these riminophenazine agents. Because of their unique mechanism of action as well as their antimicrobial properties and, in the case of clofazimine, lack of organ toxicity and bone marrow suppression (10), these agents might be useful for inclusion in cytotoxic chemotherapy.

MATERIALS AND METHODS

Agents. The molecular structures of clofazimine and B669 [3-anilino-10-(p-chlorophenyl)-2,10-dihydro-2-(cyclohexylimino)phenazine] are shown in Fig. 1. This material is the subject of a patent program by the University of Pretoria, South Africa (inquiries should be directed to The Head, Research Administration, University of Pretoria). Both agents were synthesized by Dr. J. E. O’Sullivan, Department of Chemistry, University College Dublin, Republic of Ireland. Methotrexate (amethopterin), bleomycin sulfate, and cis-diamine dichloroplatinum (II) (cisplatin) were obtained from Sigma Chemical Co. (St. Louis, MO). All the drugs, except bleomycin sulfate (which is soluble in water), were dissolved in DMSO to a concentration of 2 g/liter and diluted further in MEM supplemented with 10% heat-inactivated FCS directly before use. Control systems contained the corresponding DMSO concentrations only.

Chemicals, Reagents, and Enzymes. Unless indicated these were obtained from Sigma and radiochemicals were obtained from Du Pont NEN Research Products (Boston, MA) and Amersham International (Aylesbury, England).

Cell Lines and Primary Cultures. The following cell line cells were used: FaDu human pharynx squamous carcinoma (ATCC HTB 43); HeLa human cervix epithelial carcinoma (ATCC CC 42); T24 human transitional cell bladder carcinoma (ATCC HTB 4); Vero African green monkey kidney (ATCC CCL 81); and primary human hepatocellular carcinoma (PLC) kindly supplied by Dr. J. Alexander, National Institute of Virology, Johannesburg, Republic of South Africa. The primary cultures were used as HK and VK cells.

Cytotoxicity Assay. Cytotoxicity assays were performed using round-bottomed 96-well tissue culture plates. Cultured cells were harvested from 75% confluent tissue culture flasks with 0.2% trypsin, washed, and resuspended in MEM (except in the case of T24 where McCoy’s 5A medium was used) supplemented with 10% heat-inactivated FCS. To each well we added 2500 tissue culture cells and the volumes were brought to 200 μl with MEM supplemented with 10% FCS, containing the various solvent controls or concentrations (0.25–4 μg/ml) of clofazimine or B669, the plates were incubated for 1–8 days at 37°C in an atmosphere of 5% CO2. Fewer cells were used (1000/well) for seeding the plates when incubation periods of 5, 7, and 8 days were used. The cytoidal potential of clofazimine and B669 was also investigated using four additional established cell lines and two primary cultures and compared with the activity of bleomycin, cisplatin, and methotrexate. For these studies an incubation period of 3 days was used throughout. At termination,

Received 7/16/92; accepted 11/3/92.

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1 This work was supported by the National Cancer Association of South Africa.

2 To whom requests for reprints should be addressed.

3 The abbreviations used are: DMSO, dimethyl sulfoxide; MEM, minimum Eagle’s medium; FCS, fetal calf serum; PKC, protein kinase C; PLA2, phospholipase A2; HBSS, Hanks’ balanced salt solution; TLC, thin layer chromatography; LPC, lysophosphatidylcholine; AT, α-tocopherol; LDH, lactate dehydrogenase; PLC, phosphatidylcholine; VK, varetic kidney (cells); HK, human fibroblast (cells); ATCC, American Type Culture Collection.
The effects of exposure of FaDu cells to the primary PL A2, hydrolysis products, arachidonic acid (1-30 μg/ml) and lysophosphatidylcholine (1-5 μg/ml), were also investigated. In these experiments the cells were first washed and treated in MEM without serum for 30 min before the addition of 10% FCS.

Neutrophil-mediated Cytotoxic Activity. The effects of the test agents on the tumoricidal properties of neutrophils were also investigated. Neutrophils were isolated from heparinized (5 units preservation-free heparin/ml) venous blood of healthy volunteers and separated on Ficoll-metrizoate cushions by centrifugation at 400 × g for 25 min. Residual erythrocytes in the neutrophil bands were removed by selective lysis with 0.83% (w/v) ammonium chloride. Neutrophils were washed once with MEM and resuspended to a concentration of 2.5 × 10^6/ml. In these experiments neutrophils were added to the test system at a cell ratio of 10 neutrophils/tumor cell and clocalfazine and B669 at final concentrations of 0.125-2 μg/ml. Only the FaDu cells were used in this assay system with a fixed incubation period of 24 h. In some experiments the protective potential of catalase (250 units/well) was investigated. The cells were first preincubated for 5 min with this antioxidative enzyme before the addition of the neutrophils and/or riminophenazines. To investigate the possibility that low levels of neutrophil-mediated oxidants increase the sensitivity of FaDu cells to the riminophenazines, the cells were preincubated with 2.5 and 5 μM reagent H_2O_2 alone for 30 min before the addition of the experimental agents (i.e., in the absence of neutrophils).

Measurement of Cellular Phospholipase A2 Activity. The effects of clofazimine and B669 (final concentrations, 1, 2.5, 5, and 10 μg/ml) on PLA2 activity in FaDu cells were measured according to the extent of release of radiolabeled arachidonate and lysophospholipids by control and riminophenazine-treated FaDu cells.

Arachidonic Acid Release. FaDu cells were trypsinized, washed, and resuspended in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered, Ca^{2+}-free HBSS (pH 7.4) to a concentration of 2 × 10^6/ml. The cells were then coincubated with 5 μCi/ml of [5,6,8,9,11,12,14,15-^3H]arachidonic acid (specific activity, 79.9 Ci/mmol; Du Pont NEN) for 10 min at 37°C in Ca^{2+}-free HBSS to allow incorporation of radioarachidonate into the C-2 position of the glycerol backbone of membrane phospholipids and then washed and resuspended to 1 × 10^6/ml in HBSS, containing 5 μg/ml indomethacin to minimize breakdown of [^3H]arachidonate. The cells (1 × 10^6/ml) were then preincubated for 5 min at 37°C prior to the addition of clofazimine/B669 (final concentrations, 1.0, 2.5, 5, and 10 μg/ml) and the tubes were incubated for 15 min at 37°C in an atmosphere of 5% CO_2. The final volume in each tube was 2 ml (2 × 10^6 cells). Appropriate solvent controls were included. The reactions were terminated by the addition of 5 ml of n-hexane/isopropl alcohol/concentrated HCl (final concentration, 0.1 M; 300/200/4, v/v/v) containing 2.7 μg/ml unlabelled arachidonate to facilitate visual detection with iodine vapors. Aliquots of 10 μl were then spotted onto Silica Gel 60-precoated TLC plates (Merck, Darmstadt, Germany). The plates were developed in chloroform/acetone (96/4, v/v). After exposure to iodine vapors the arachidonate spots were localized and the silica removed and assayed for radioactivity.

Lysophosphatidylcholine Assay. A previously described TLC method (12) was used to assay LPC levels in control and riminophenazine-treated tissue culture cells. FaDu cells (2 × 10^6/ml) were coincubated with 50 μCi/ml of [9,10-^3H]palmitic acid (60 Ci/mmol; Du Pont NEN) for 10 min in Ca^{2+}-free HBSS to allow incorporation of palmitic acid into membrane phospholipids and then washed once and resuspended to 1 × 10^6 cells/ml in serum-free MEM. The cells were then preincubated for 5 min at 37°C prior to the addition of clofazimine/B669 (final concentrations, 1.0, 2.5, 5, and 10 μg/ml) and the tubes were incubated for 15 min at 37°C. The reactions were terminated by the addition of 3 ml of chloroform/methanol (2/1, v/v). Water (0.3 ml) was added to cause phase separation. After centrifugation the phospholipid-containing lower phase was removed and evaporated to dryness under a stream of nitrogen and dissolved in 50 μl of chloroform/methanol. Aliquots of 10 μl as well as the appropriate standard (10 μg/ml LPC) were then spotted onto Silica Gel 60-precoated TLC plates (Merck). The plates were developed 2-3 times in chloroform/methanol/isopropl alcohol/0.25% KCl/ethyl acetate (30/25/6/18, v/v/v/v/v). After exposure to iodine vapors the LPC spots were localized, and the silica removed and assayed for radioactivity.

Measurement of the Effects of Clofazimine and B669 on the Activity of Purified Porcine PLA2. The effects of the riminophenazines on the activity of purified PLA2 (from porcine pancreas) were assayed using 1-stearoyl-2-[5,6,8,9,11,12,14,15-^3H]arachidonyl-L-3-phosphatidylcholine (specific activity, 135 Ci/mmol; Amersham) as substrate. The reaction mixtures (1 ml) contained 20 mm Tris-HCl (pH 8.5), 5 mm CaCl_2, 0.625 μCi of radiolabeled PLC, 50 μmol of unlabeled carrier PLC, 200 nmol of unlabeled LPC, and 5 μg/ml of clofazimine or B669. Control systems contained the corresponding concentration of DMSO. LPC was included in the assay system to promote the

**Table 1** Antioxidants and enzyme inhibitors assayed for possible protective activity against the cytotoxic effect of clofazimine and B669 on FaDu cells

<table>
<thead>
<tr>
<th>Agent</th>
<th>Mode of action</th>
<th>Final concentration tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>dl-α-Tocopherol</td>
<td>Lipid-soluble antioxidant</td>
<td>25 μg/ml</td>
</tr>
<tr>
<td>dl-α-Tocopherol acetate</td>
<td>Lipid-soluble antioxidant</td>
<td>50 μg/ml</td>
</tr>
<tr>
<td>Retinol acetate</td>
<td>Lipid-soluble antioxidant</td>
<td>31.2 μg/ml</td>
</tr>
<tr>
<td>Butylated hydroxytoluene</td>
<td>Lipid-soluble antioxidant</td>
<td>50 μg/ml</td>
</tr>
<tr>
<td>Butylated hydroxyanisole</td>
<td>Lipid-soluble antioxidant</td>
<td>12.5 μg/ml</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>Reducing agent</td>
<td>60 μg/ml</td>
</tr>
<tr>
<td>Cystine</td>
<td>Water-soluble antioxidant</td>
<td>500 μg/ml</td>
</tr>
<tr>
<td>Catalase</td>
<td>Antioxidant enzyme</td>
<td>500 units/ml</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>Prostaglandin synthetase inhibitor</td>
<td>2.5 μg/ml</td>
</tr>
<tr>
<td>Piroxicam</td>
<td>Prostaglandin synthetase inhibitor</td>
<td>10 μg/ml</td>
</tr>
<tr>
<td>Nordihydroguaiaretic acid</td>
<td>5’-Lipoxygenase inhibitor</td>
<td>2 μg/ml</td>
</tr>
<tr>
<td>H-7</td>
<td>PKC inhibitor</td>
<td>100 μM</td>
</tr>
<tr>
<td>Lysophospholipase</td>
<td>Lysophospholipid inactivator</td>
<td>250 milligrams/ml</td>
</tr>
</tbody>
</table>

Fig. 1. Molecular structures of clofazimine and B669.
formation of phospholipid micelles (13). Reactions were initiated by the addition of PLA₂ (final concentration, 10 units/ml) and the tubes were incubated for 15 min at 37°C. The 15-min incubation period and the fixed concentration of the enzyme were determined in a series of preliminary experiments. The reactions were then terminated by the addition of n-hexane/isopropyl alcohol, and \([^{3}H]\)arachidonate in the reconstituted lipid evaporates assayed by radiometric TLC as described above. PLA₂ activity was directly related to the amount of \([^{3}H]\)arachidonate released from radiolabeled PLC.

Measurement of the Effects of Clofazimine and B669 on the Activity of Purified Lysophospholipase. The effects of the riminophenazines on the activity of purified lysophospholipase (2-lysophosphatidylcholine acylhydro-lase-phospholipase B from Vibrio species) were assayed using 1-[1-\(^{14}C\)]palmitoyl-\(\alpha\)-lysophosphatidylcholine (specific activity, 56 mCi/mmol; American) as substrate. The reaction mixtures (1 ml) contained 20 nm Tris/HCl (pH 7.0), 0.25 \(\mu\)Ci of radiolabeled LPC, 200 nmol of unlabeled LPC, and 5 \(\mu\)g/ml of clofazimine or B669. Control systems contained the corresponding concentration of DMSO. Reactions were initiated by the addition of 200 milliunits (final concentration) of lysophospholipase and the tubes were incubated for 15 min at 37°C. The reactions were then terminated by the addition of 1.5 ml chloroform/methanol (2:1, \(v/v\)) and the remaining intact \([^{14}C]\)LPC was assayed by radiometric TLC as described above.

Measurement of Possible Complexing of AT with Clofazimine and B669. The UV absorption spectra of mixtures of AT (up to 100 \(\mu\)g/ml) and clofazimine or B669 in ethanol (up to 10 \(\mu\)g/ml) relative to identical concentrations of the individual agents were measured as described previously (14), using a Pye Unicam SP 1700 double beam UV spectrophotometer.

LDH Release Assay. The cytotoxic potentials of clofazimine and B669 for FaDu cells after a 60-min treatment were assayed using a standard spectrophotometric assay for the measurement of LDH (15). Briefly, \(5 \times 10^5\) cells were coincubated at 37°C with 5 and 10 \(\mu\)g of the experimental drugs in 1 ml of Ca²⁺-free HBSS without serum for 60 min. After treatment the cells were removed by centrifugation and the supernatants were assayed for cytosolic LDH.

RESULTS

Effects of Clofazimine and B669 on the Survival of FaDu Cells after Exposure for Up to 8 Days. Clofazimine and B669 caused a dose-dependent inhibition of the proliferation of FaDu cells (Figs. 2 and 3) which was significant (P < 0.05) at concentrations as low as 0.25 \(\mu\)g/ml and detectable after 24 h. B669 was considerably more active (approximately 3-fold) than clofazimine.

Comparison of the Cytotoxic Effects of B669, Clofazimine, Cisplatin, Bleomycin, and Methotrexate for Five Established Tissue Culture Cell Lines and Two Primary Cultures. All the cultures tested were sensitive to clofazimine and B669 (Fig. 4). The order of

![Fig. 2](image-url) - Effects of clofazimine on the growth of FaDu cells after a 1-day, 2-day, 5-day, 7-day, and 8-day treatment. Data are expressed as the mean percentage of the corresponding control systems of 4 different experiments.

![Fig. 3](image-url) - Effects of B669 on the growth of FaDu cells after a 1-day, 2-day, 5-day, 7-day, and 8-day treatment. Results of 4 different experiments are expressed as the mean percentage (OD₆₂₀, absorbance at 620 nm) of the corresponding control systems.

![Fig. 4](image-url) - Comparison of the cytotoxicity of B669, clofazimine, cisplatin, bleomycin, and methotrexate for HF, VK, human pharynx squamous carcinoma (FaDu), primary human hepatocellular carcinoma (PLC). Vero African green monkey kidney (Vero), human transitional cell bladder carcinoma (T24), and human cervix epithelioid carcinoma (HeLa) cell cultures after a 3-day treatment. Results of three different experiments are expressed as the mean percentage (OD₆₂₀, absorbance at 620 nm) of the corresponding control systems.
Fig. 5. Effect of catalase (250 units/well) on the cytotoxic effects of neutrophils on FaDu cells treated with clofazimine and B669 after a 24-h treatment. Results are expressed as the mean percentage (OD at 620 nm) inhibition ± SEM.

Table 2 Effects of clofazimine and B669 in the presence and absence of AT (25 μg/ml) on the growth of FaDu cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AT, 25 μg/ml (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absent</td>
</tr>
<tr>
<td>0.5 μg/ml B669</td>
<td>7.0 ± 0.6 a</td>
</tr>
<tr>
<td>1 μg/ml B669</td>
<td>15.0 ± 4.4</td>
</tr>
<tr>
<td>2 μg/ml B669</td>
<td>16.3 ± 4.8</td>
</tr>
<tr>
<td>1 μg/ml clofazimine</td>
<td>62.0 ± 12.7</td>
</tr>
<tr>
<td>2 μg/ml clofazimine</td>
<td>47.3 ± 12.3</td>
</tr>
<tr>
<td>4 μg/ml clofazimine</td>
<td>29.0 ± 4.4</td>
</tr>
</tbody>
</table>

a Results from 3-4 separate experiments are presented as the mean percentage ± SEM of the relevant control systems. The absolute values for the AT/riminophenazine-free and the AT-containing riminophenazine-free control systems were 0.385 ± 0.029 and 0.401 ± 0.031 at A620, respectively.

b All the values in this column are statistically significant (P < 0.005) compared to the corresponding riminophenazine-treated, AT-free systems.

susceptibility for clofazimine was HeLa > T24 > FaDu > HF > VK > PLC > Vero and for B669 the order was HeLa > T24 > FaDu > HF > PLC > Vero > VK. B669 was again more potent than clofazimine and caused complete cell death at concentrations 2 to 3 times less than those of clofazimine. All the cultures tested, except PLC, were sensitive to cisplatin (Fig. 5). The order of susceptibility was T24 > Vero > FaDu > VK > FA > T24 and to a much lesser extent, HeLa, VK, and Vero cells were sensitive to bleomycin. Vero and HeLa cells, but not the other cell lines, were slightly sensitive to mettretexate at the concentrations and incubation conditions used in this assay. Results obtained with cultures not sensitive to one or more of the test agents are not shown.

Effects of Various Antioxidants as well as Cyclooxygenase, 5'-Lipoxygenase, and PKC Inhibitors on Clofazimine and B669-Mediated Inhibition of FaDu Cell Growth. Of the various agents listed in Table 1, only AT and lysophospholipase protected FaDu cells against the anti-proliferative effects of both clofazimine and B669. The effects of AT (25 μg/ml) on the growth of FaDu cells coincubated with clofazimine (1, 2, and 4 μg/ml) and B669 (0.5, 1, and 2 μg/ml) are shown in Table 2. However, preincubation of the cells with AT (25 μg/ml) for 60 min, followed by 2 washings and exposure to clofazimine and B669 (0.5, 1, and 2 μg/ml) did not protect the cells against the cytotoxic effect of the drugs. Coincubation of the cells with lysophospholipase (250 milliunits/ml) caused partial but statistically significant protection against the anti-proliferative effects of the riminophenazines. The growth of cells treated with 2.5 μg/ml clofazimine in the absence and presence of lysophospholipase was 61 ± 12 and 72 ± 11% (P < 0.005) of the untreated control value. The corresponding values for B669 (1 μg/ml) were 57 ± 5 and 74 ± 6% (P < 0.01; data from 2 experiments). Lesser protection was observed with 30 milliunits/ml of lysophospholipase, while heat inactivation (80°C/10 min) of the enzyme totally abolished the protective activity (data not shown).

Effects of Clofazimine and B669 Treatment in the Presence of Neutrophils on the Survival of FaDu Cells after a 24-h Incubation Period. Coincubation of neutrophils with FaDu cells (10:1) caused an 8 ± 4% decrease in cell survival (Fig. 5), while the corresponding decreases observed with clofazimine (0.5 μg/ml) or B669 (0.5 μg/ml) alone (in the absence of neutrophils) were 11 ± 6 and 27 ± 10%, respectively. However, exposure of FaDu cells to combinations of neutrophils and clofazimine or B669 caused a significant (P < 0.05 and P < 0.005, respectively) synergistic increase in tumor cell lysis. Preincubation with catalase (250 units/ml) prevented neutrophil-mediated enhancement of the cytotoxic activity (Fig. 5) but had no effect on the direct tumoricidal properties of the drugs per se (results not shown). Similar results were observed with 1 and 2 μg/ml clofazimine and 0.125 and 0.25 μg/ml B669 (results not shown).

The treatment of FaDu cells with 2.5 and 5 μg H2O2 caused a 92 ± 8 and 35 ± 8% inhibition of growth, respectively. However, exposure of the cells to combinations of H2O2 and either clofazimine (0.5–2 μg/ml) or B669 (0.125–1 μg/ml) did not cause any synergistic increases in tumor cell death.

Effects of Clofazimine and B669 on Phospholipase A2 Activity. The effects of the riminophenazines (1–10 μg/ml) on the activity of PLAr in FaDu cells were measured according to the release of the two primary hydrolysis products, unsaturated fatty acid and LPC from phosphatidylycholine pre-labeled in the C-2 and C-1 positions of the glycerol backbone with [3H]arachidonate and [3H]palmitate, respectively. Data for the release of [3H]arachidonate and [3H]LPC are shown in Table 3. The riminophenazines, especially B669, caused dose-related enhancement of the release of these two primary PLA2 hydrolysis products, indicating that exposure of FaDu cells to the riminophenazines increases the activity of PLA2. The addition of AT (25 μg/ml) to the experimental system had no effect on the riminophenazine-mediated increase in [3H]arachidonic acid release (results not shown).

Effects of Lysophospholipids and Arachidonic Acid on the Growth of FaDu Cells. The effects of LPC (1–5 μg/ml) on FaDu cell growth are shown in Table 4. This agent caused a dose-related inhibition of cell growth which was statistically significant at all concentrations tested (P < 0.05–P < 0.005). Treatment of FaDu cells with 25 μg/ml AT prior to exposure to LPC protected against the inhibitory effect of LPC on cell growth (Table 4). On the other hand, arachidonic acid at concentrations of up to 30 μg/ml did not affect cell growth.

Table 3 Release of [3H]arachidonate and [3H]LPC from FaDu cells after a 15-min treatment with clofazimine or B669

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[3H]Arachidonate (%)</th>
<th>[3H]LPC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 μg/ml clofazimine</td>
<td>ND a</td>
<td>131 ± 14 b</td>
</tr>
<tr>
<td>2.5 μg/ml clofazimine</td>
<td>155 ± 14</td>
<td>205 ± 27</td>
</tr>
<tr>
<td>5 μg/ml clofazimine</td>
<td>127 ± 8</td>
<td>158 ± 18</td>
</tr>
<tr>
<td>10 μg/ml clofazimine</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>1 μg/ml B669</td>
<td>194 ± 8</td>
<td>148 ± 14</td>
</tr>
<tr>
<td>2.5 μg/ml B669</td>
<td>ND a</td>
<td>167 ± 6</td>
</tr>
<tr>
<td>5 μg/ml B669</td>
<td>198 ± 26</td>
<td>321 ± 31</td>
</tr>
<tr>
<td>10 μg/ml B669</td>
<td>240 ± 33</td>
<td>ND</td>
</tr>
</tbody>
</table>

a ND, not done.
b All the values shown in this column are statistically significant (P < 0.005–P < 0.005) by comparison with the relevant control systems.

c Results from 4–9 experiments are presented as the mean percentage ± SEM of the riminophenazine control systems. The absolute values for the drug-free control systems were 10158 ± 1628 and 7575 ± 82 cpm for the release of [3H]arachidonate and [3H]LPC, respectively.
Effects of Clofazimine and B669 on the Activities of Purified Phospholipase A2 and Lysophospholipase. Neither clofazimine nor B669 at a fixed concentration of 5 µg/ml affected the activities of purified PLA2 or lysophospholipase. The amount of arachidonate released from radiolabeled PLC in the presence of PLA2 was 19 ± 1, 20 ± 1, and 21 ± 1 nmol for the control system and systems containing 5 µg/ml clofazimine and B669, respectively (data from 4 different experiments). The amount of LPC hydrolyzed by lysophospholipase was 103 ± 16, 100 ± 2, and 91 ± 7 nmol for the control system and systems containing 5 µg/ml clofazimine and B669, respectively (data from 2 different experiments).

Spectrophotometric Analysis of Mixtures of AT and Clofazimine or B669. The UV spectra of ethanol solutions of clofazimine or B669 before and after the addition of AT were unchanged, demonstrating the absence of interactions between the test agents.

Effects of Clofazimine and B669 on LDH Release. Clofazimine and B669 at 5 and 10 µg/ml had no effect on LDH release by FaDu cells after a 60-min treatment at 37°C in Ca²⁺-free HBSS.

DISCUSSION

The cytotoxic effects of clofazimine described here were observed at physiologically relevant concentrations. Ingestion of 200 or 600 mg clofazimine daily gives peak serum levels of 0.7 to 1 µg/ml and 3 to 4 µg/ml of the drug, respectively (2, 3). Tissue levels are considerably higher (16). Although the antiproliferative effect of clofazimine on phytohemagglutinin-stimulated lymphocytes is well documented (8, 9), this is, to our knowledge, the first report on the in vitro antitumor potential of this agent.

In contrast to the other standard chemotherapeutic agents investigated, clofazimine and especially B669 were active against all the cultures tested, although they were somewhat less active against slower-growing primary cultures and Vero cells. The carcinoma cell lines, especially PLC and to a lesser extent FaDu and HeLa cultures, were mostly resistant to methotrexate and bleomycin, while cisplatin was effective against all the cultures except PLC. Many cancer patients treated with anticancer drugs die of progressive disease, which acquires resistance to different chemotherapeutic agents (17). The lack of resistance to the rimonophenazine compounds tested suggests that these agents may have a novel mechanism of action. Most of the standard chemotherapeutic drugs act directly or indirectly on DNA, with the possible restriction that protective repair mechanisms may circumvent the cytotoxic effect of chemotherapy (17).

Clofazimine and B669 also increase the production of reactive oxidants by phagocytes (5, 7). These agents (oxidants) are both antimicrobial and tumoricidal (6). The addition of neutrophils to clofazimine- or B669-treated FaDu cells greatly enhanced the cytotoxic effect of the rimonophenazines. It is unlikely that this enhancement is due to increased sensitivity of oxidant-exposed FaDu cells to clofazimine or B669 since reagent H₂O₂ did not increase the susceptibility of FaDu cells to the rimonophenazines. However, catalase protected the cells against the increase in cell death mediated by rimonophenazine-treated neutrophils, demonstrating that the enhanced cytotoxic effect is due to increased production of oxidants. Clofazimine- and B669-mediated enhancement of the generation of reactive oxidants by stimulated neutrophils is associated with increased activity of phospholipase A₂ and is blocked by pretreatment of the phagocytes with an inhibitor of this enzyme (5, 7).

Since clofazimine activates PLA₂ in lymphocytes and phagocytes (5, 8), we investigated the relationship between enhanced activity of this enzyme and the antiproliferative properties of the rimonophenazines. Increased release of [³H]LPC and [³H]arachidonic acid was observed during exposure of FaDu cells to clofazimine or B669. Neither agent, at the concentrations tested, affected the viability of FaDu cells during a 1-h incubation period. The activities of purified PLA₂ and lysophospholipase were unaffected by either clofazimine or B669 (5 µg/ml), demonstrating that the rimonophenazines do not appear to act directly on either enzyme. In the cell membrane these highly lipophilic agents may disrupt membrane structure making the integral phospholipids more susceptible to attack by PLA₂.

To identify the primary products of phospholipase activation with anti-proliferative activity, we investigated the effects of the major degradation products of PLA₂, LPC, and arachidonic acid on the proliferation of FaDu cells. LPC, at concentrations of 1 µg/ml and higher, caused dose-related inhibition of cell growth, while arachidonic acid at concentrations of up to 30 µg/ml had no effect. While implicating LPC, which possesses both detergent and membrane-stabilizing properties (19), these data did not exclude other metabolites and reactive oxidants generated during the metabolism of arachidonic acid by cyclooxygenase and lipoxygenase enzymes. It is also possible that, because of the extremely lipophilic properties and redox potential (~0.18 V at pH 7) of clofazimine (1), intracellular generation of H₂O₂ by redox cycling mechanisms may contribute to the cytotoxic activity of this agent. However, inclusion of a water-soluble antioxidant (cysteine) or enzyme (catalase), lipid-soluble antioxidant (retinol, retinol acetate, butylated hydroxytoluene, or butylated hydroxyanisole), prostaglandin synthase inhibitors (indomethacin or piroxicam), or an inhibitor of 5'-lipoxygenase (nordihydroguaiaretic acid) all failed to protect the cells against the cytotoxic effects of the rimonophenazines. These observations demonstrate that oxidants generated intracellularly by the drugs per se due to possible redox cycling mechanisms, as well as products generated during the metabolism of arachidonic acid, are not primarily involved in rimonophenazine-mediated inhibition of cell growth. Although tocopherol acetate did not protect the cells against the toxic effects of clofazimine and B669, AT, originally included as a lipid-soluble antioxidant, proved to be a striking exception, indicating a critical requirement for the hydroxyl group on the chromanol nucleus of AT. This agent (AT) almost completely blocked the inhibitory effects of the rimonophenazines on cell growth. The protective effects of AT were eliminated by washing the cells, demonstrating a continuous requirement for this agent throughout the incubation period. It is improbable that the protective effects of AT are due to interaction of this agent with the rimonophenazines since (a) no complex formation could be demonstrated by spectrophotometric analysis of mixtures of the two compounds and (b) AT did not inhibit the increase in PLA₂ activity mediated by clofazimine and B669. It also seemed unlikely that these protective effects of AT were related to the antioxidant properties of the molecule since other lipid-soluble antioxidants were ineffective. Notwithstanding its antioxidant properties, AT also possesses other biological activities which could account for the observed protection against the cytotoxic effects of the rimonophenazines. It has been reported that AT inhibits both the activation and translocation of cytosolic PKC (20, 21) and may interfere with the activity of
5’-lipoxygenase (22) and PLA2 in some experimental systems (23) but not in others (24). The failure of H-7 and nordihydroguaiaretic acid to protect FaDu cells from the cytotoxic effect of clofazimine and B669 appears to discount PKC or 5’-lipoxygenase inhibition, respectively, as potential mechanisms of riminophenazine-induced cytotoxicity.

We were also unable to demonstrate any inhibitory effects of AT on PLA2 activity in FaDu cells. AT also possesses membrane-stabilizing properties due to its ability to complex with, and neutralize, lysophospholipids through two types of interaction, namely, the formation of a hydrogen bond between the AT chromanol nucleus hydroxyl group and the C-O group of the phospholipid and the interaction of the acyl chains of the lysophospholipids with the chromanol nucleus methyl groups of AT (19). In the present study we observed that pretreatment of FaDu cells with AT effectively protected these cells against the antiproliferative activity of LPC. The most compelling evidence in favor of the involvement of lysophospholipids as mediators of the antiproliferative activity of the riminophenazines was derived from experiments with the LPC-hydrolyzing enzyme, lysophospholipase. The antiproliferative effects of clofazimine and B669 were partly, but significantly, neutralized by coincubation of FaDu cells with this enzyme. These data strongly support enhancement of phospholipid hydrolysis in FaDu cells, leading to increased release of the membrane destabilizing antiproliferative agent LPC, as being the primary biochemical mechanism of the cytotoxic action of the riminophenazines.

Finally we have observed that clofazimine, primarily an antimycobacterial agent, which is also active against gram-positive bacteria, and its analogue B669 possess direct antitumor activity in vitro. Moreover, these agents also potentiate the oxidant-mediated tumoricidal mechanisms of neutrophils. This apparently unique dual mechanism of antitumor activity, together with their antimicrobial properties and apparent low toxicity, makes these drugs attractive contenders for evaluation in clinical and experimental cancer chemotherapy.

REFERENCES

The Riminophenazine Agents Clofazimine and B669 Inhibit the Proliferation of Cancer Cell Lines \textit{in Vitro} by Phospholipase A$_2$-mediated Oxidative and Nonoxidative Mechanisms

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