Biochemical Pharmacology, Vol. 46, No. 11, pp. 2029–2038, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

CLOFAZIMINE AND B669 INHIBIT THE PROLIFERATIVE RESPONSES AND Na^+, K^+-ADENOSINE TRIPHOSPHATASE ACTIVITY OF HUMAN LYMPHOCYTES BY A LYSOPHOSPHOLIPID-DEPENDENT MECHANISM

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(*Received* 10 *June* 1993; *accepted* 5 *August* 1993)

Abstract—The relationship between the phospholipase-stimulating and immunosuppressive properties of the riminophenazine anti-mycobacterial agent clofazimine and its experimental analogue, B669, has been investigated *in vitro*. At concentrations of 0.6 μM and upwards, both riminophenazines, particularly B669, caused dose-related inhibition of mitogen- and alloantigen-stimulated uptake of tritiated thymidine by human mononuclear leucocytes (MNL), while in short-term assays both agents increased the release of lysophosphatidylcholine (LPC) and arachidonic acid from these cells. Arachidonate *per se* at a concentration of 20 μM did not affect mitogen-activated lymphocyte proliferation, while cyclooxygenase and 5′-lipoxygenase inhibitors, as well as water- and lipid-soluble oxidant-scavengers and anti-oxidant enzymes, failed to protect the cells against the anti-proliferative effects of clofazimine and B669. However, LPC caused dose-related inhibition of lymphocyte proliferation. Moreover, co-incubation of MNL with α-tocopherol (vitamin E), a lysophospholipid complex-forming agent, or with lysophospholipase, protected the cells against clofazimine and B669, as well as against LPC. Na^+, K^+-adenosine triphosphatase was identified as the primary target of riminophenazine/LPC-mediated inhibition of lymphocyte proliferation. Excessive release of anti-proliferative lysophospholipids during clofazimine or B669 treatment of mitogen- or antigen-activated lymphocytes is the probable biochemical mechanism of the immunosuppressive activity of these agents.

The anti-mycobacterial riminophenazine agent clofazimine, which was first described in 1957 [1], is one of the standard drugs for the treatment of leprosy [2–5] and is now recommended as part of a combination therapy for this disease [6]. It is also useful in the combination antimicrobial chemotherapy of *Mycobacterium avium* infections in AIDS patients [7]. Clofazimine has also been reported to be useful in the treatment of a variety of inflammatory skin disorders of non-microbial origin [8–10] and in suppressing adverse immunological reactions in patients with leprosy [5, 11]. These anti-inflammatory, immunosuppressive properties of clofazimine have been attributed to the inhibitory effects of this agent on neutrophil migration and lymphocyte proliferation [12–14].

The biochemical mechanism of clofazimine-mediated inhibition of lymphocyte proliferation has not been described, but may be related to the stimulatory effects of this agent on the activity of leucocyte phospholipase A_2 (PLA_2†) [15, 16], leading to increased generation of immunomodulatory, anti-proliferative prostaglandins [17, 18]. The drug also sensitises phagocytes to hyper-react to stimuli of membrane-associated oxidative metabolism, leading to increased release of reactive oxidants [15, 19]. Since phagocyte-derived oxidants such as H_2O_2 and HOCl are potent inhibitors of lymphocyte proliferation [20, 21], the pro-oxidative interactions of clofazimine with neutrophils and macrophages may also promote immunosuppression.

In the present study we have investigated the relationship between the phospholipase-activating properties and the immunosuppressive effects of clofazimine and its analogue B669 [22, 23]. The latter agent was included for comparison on the basis of preliminary screening experiments, which indicated that it was a more potent anti-proliferative agent than clofazimine. Our data demonstrate that (1) the primary mediators of clofazimine/B669-induced immunosuppression are lysophospholipids released as a consequence of drug-mediated enhancement of PLA_2 activity and (2) these anti-proliferative effects of the riminophenazines are effectively prevented by the addition of the lysophospholipid-complex-forming agent α-tocopherol (AT, vitamin E) [24] to clofazimine- or B669-treated lymphocytes.

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† Abbreviations: AT, α-tocopherol; [^{3}H]TdR, tritiated thymidine; LECL, lucigenin-enhanced chemiluminescence; LPC, lysophosphatidylcholine; MNL, mononuclear leucocyte; PC, phosphatidylcholine; PHA, phytohaemagglutinin; PLA_2, phospholipase A_2; PMA, phorbol 12-myristate 13-acetate, [^{3}H]AA, [^{3}H]arachidonic acid.

MATERIALS AND METHODS

Chemicals and reagents. The molecular structures of clofazimine [3-(*p*-chloroanilino)-10-(*p*-chlorophenyl)-2,10-dihydro-2-(isopropylimino)-phenazine] and B669 [3-anilino-10-phenyl-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 and inquiries should be directed to The Head, Research Administration, University Pretoria, Brooklyn, Pretoria 0002, South Africa. Both agents were synthesized by Dr J. F. O'Sullivan, Department of Chemistry, University College Dublin, Republic of Ireland. They were dissolved in dimethyl sulphoxide (DMSO) to a concentration of 4 mM and diluted further in distilled water prior to use in the various assay systems and used at final concentrations of 0.6–10 μM (approximately 0.3–5 μg/mL). Serum concentrations achieved during chemotherapy with clofazimine range from 0.7 to 4 μg/mL [25, 26]. Appropriate solvent control systems were included in the various assays described below.

All other chemicals and enzymes were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.) with the exception of *d*L-AT (F. Hoffman-La Roche, Basel, Switzerland) and piroxicam (Pfizer Laboratories, Johannesburg, South Africa). Radiochemicals were obtained from Du Pont NEN Research Products (Boston, MA, U.S.A.) and from Amersham International (Aylesbury, U.K.).

Mononuclear leucocytes (MNL) and pure T-lymphocyte suspensions. MNL were prepared as described previously by density centrifugation on Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden) of blood taken from healthy adult human volunteers [14]. The cells were then resuspended to 4×10^6 per mL in HEPES (4.2 mM)-buffered RPMI 1640 supplemented with 1% glutamine, penicillin (100 U/mL) and streptomycin (100 μg/mL).

Suspensions of purified T-lymphocytes were prepared using a standard differential adherence method. Briefly, a 5-mL sterile disposable syringe was loosely filled with 0.15 g of sterile nylon wool (Biotest, Dreieich, Germany) and rinsed with 10 mL prewarmed (37°) RPMI 1640. MNL were resuspended in 1 mL of warm medium, added immediately to the nylon wool column, allowed to equilibrate and the syringe incubated for 30 min at 37° in an atmosphere of 5% CO_2. Thereafter the non-adherent cells were removed and collected by flushing the column with 10 mL of warm RPMI 1640. The resultant, non-adherent T-lymphocyte-enriched cell population was concentrated by centrifugation, adjusted to 2×10^6/mL and the purity determined using flow cytometry to enumerate numbers of B- and T-lymphocytes and monocytes. Lucigenin-enhanced chemiluminescence (LECL) with phorbol 12-myristate 13-acetate (PMA, 20 ng/mL) as the stimulus of superoxide generation, was also used to assess the extent of contamination by phagocytes [27].

Arachidonic acid (AA) release. MNL (2×10^7/mL) were co-incubated with 5 μCi/mL radiolabelled arachidonate [5, 6, 8, 9, 11, 12, 14, 15-^{3}H(N), 79.9 Ci/mmol Du Pont NEN] for 30 min at 37° in Ca^{2+}-free Hank's balanced salt solution (HBSS), containing 10 μM indomethacin, to allow incorporation of radiolabelled arachidonate into membrane phospholipids, then washed twice and resuspended to 1×10^7 per mL in RPMI. The cells (5×10^6 per mL) were then pre-incubated for 5 min at 37° prior to the addition of clofazimine or B669 (1.25, 2.5 and 5 μM). Since riminophenazines cause

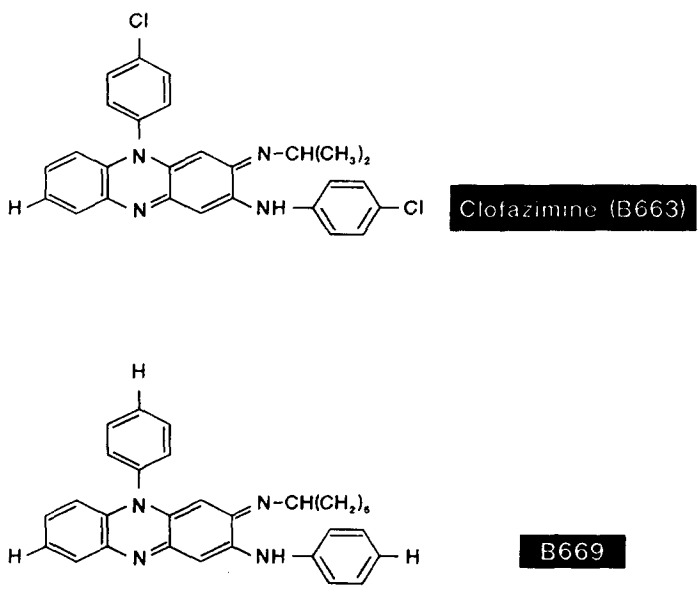

Fig. 1. Molecular structures of clofazimine [3-(*p*-chloroanilino)-10-(*p*-cholorophenyl)-2,10-dihydro-2-(isopropylimino)-phenazine] and B669 [3-anilino-10-phenyl-2,10-dihydro-2-(cyclohexylimino)-phenazine].

rapid activation of PLA_2 in leucocytes [15, 16] the tubes were then incubated for 5 and 10 min at 37°, the final volume in each tube being 5 mL (5×10^6 cells). In some experiments *d*L-AT (50 μM) was added to the MNL during pre-incubation, prior to the addition of the riminophenazines. After incubation the fatty acids were extracted and [^{3}H]AA quantitated by radiometric thin layer chomatography (TLC) as described previously [15, 28].

Lysophosphatidylcholine (LPC) assay. The labelling and incubation procedures were almost identical to those used for measurement of [^{3}H]arachidonate release, except that the cells were treated with 20 μCi/mL of radiolabelled palmitate [9,10-^{3}H(N), 60 Ci/mmol, Du Pont NEN] for 30 min at 37° to allow incorporation of the radiolabelled saturated fatty acid into the C-1 position of the glycerol backbone of membrane phospholipids. After incubation the lysophospholipids were extracted and assayed using a previously described radiometric high-performance TLC method [29].

Assay for the activity of purified PLA_2 and lysophospholipase. The effects of clofazimine and B669 (10 μM) on the activity of purified PLA_2 (from porcine pancreas; final concentration 10 U/mL) or lysophospholipase (2-lysophosphatidylcholine acylhydrolase-phospholipase B, from *Vibrio* species; 200 mU/mL final concentration) was assayed by radiometric TLC [28, 29], using radiolabelled phosphatidylcholine (PC) [L-3-phosphatidylcholine - 1 - stearoyl - 2 - (5,6,8,9,11,12,14,15-^{3}H)-arachidonyl; sp. act. 135 Ci/mmol; Amersham] and LPC (L-lyso-3-phosphatidylcholine-1-[1-^{14}C]-palmitoyl; sp. act. 56 mCi/mmol; Amersham) as the respective substrates.

Mitogen-activated MNL transformation. Fifty microlitres of MNL or purified T-lymphocyte suspension (2×10^5) cells were added to 100 μL of RPMI 1640 followed by 20 μL of the riminophenazines (0.6–5 μM) or drug-free solvent control system. Appropriate volume adjustments were made when clofazimine or B669 were used in combination with the other agents described below. After an incubation period of 30 min at 37°, 20 μL of 50% autologous, heat-inactivated serum (5% final concentration) was added to each well followed immediately by 20 μL of RPMI 1640 or the mitogen, phytohaemagglutinin (PHA; 5 μg/mL final concentration) in control and stimulated systems, respectively. After 24–48 hr of culture at 37° in an atmosphere of 5% CO_2, 0.5 μCi of tritiated thymidine ([^{3}H]TdR, Du Pont NEN) was added to each well and the plates re-incubated for 18 hr and the cells then assayed for [^{3}H]TdR uptake. In some experiments the potential of various lipid- and water-soluble anti-oxidant chemicals and enzymes, the LPC-hydrolysing enzyme lysophospholipase, as well as inhibitors of cyclooxygenase, 5-lipoxygenase and protein kinase C (PKC) to protect against riminophenazine- (1.25, 2.5 and 5 μM) mediated inhibition of lymphocyte proliferation was investigated. These agents and the final concentrations at which they were used in the assays of lymphocyte proliferation are shown in Table 1. At the predetermined concentrations shown, none of the test agents *per se*, with the exception of cysteine, either inhibited or enhanced the uptake of [^{3}H]TdR by PHA-activated lymphocytes. Cysteine (500 μM) *per se* increased the PHA-activated uptake of [^{3}H]TdR by MNL. However, when analysing the data, the effects of cysteine on riminophenazine-mediated inhibition of MNL proliferation were always compared with the corresponding cysteine-treated, riminophenazine-free control system. In other experiments the effects of the following on PHA-activated MNL transformation were investigated: (a) pre-treatment of MNL with AT (50 μM) for 60 min at 37° followed by washing and exposure to the riminophenazines (2.5 and 5 μM) (b) co-incubation of MNL with arachidonate or PC (1–20 μM) *per se* and (c) co-incubation of MNL with LPC (5–20 μM) in the presence or absence of AT (10 and 50 μM).

Mixed lymphocyte cultures. Fifty microlitres of responder MNL were co-cultured with an equal number of mitomycin C (150 μM) pre-treated MNL from an unrelated donor in a final volume of 200 μL

Table 1. Anti-oxidants, enzymes and enzyme inhibitors assayed for possible protective activity against riminophenazine-mediated inhibition of mitogen-induced lymphocyte proliferation

Agent	Mode of action	Final concentration
dL-AT	Lipid-soluble anti-oxidant	10 and 50 μM
dL-AT acetate	Lipid-soluble anti-oxidant	10 and 50 μM
Retinol	Lipid-soluble anti-oxidant	10 μM
Butylated hydroxytoluene	Lipid-soluble anti-oxidant	25 μM
Butylated hydroxyanisole	Lipid-soluble anti-oxidant	15 μM
Cysteine	Water-soluble anti-oxidant	500 μM
Catalase	Anti-oxidant enzyme	500 U/mL
Indomethacin	PG synthetase inhibitor	15 μM
Piroxicam	PG synthetase inhibitor	15 μM
NDGA	5′-Lipoxygenase inhibitor	10 μM
Staurosporine	PKC inhibitor	0.025 and 0.05 μM
H-7	PKC inhibitor	25 μM
Lysophospholipase	Hydrolysis of LPC	25–200 mU//mL

of RPMI 1640 containing 5% heat-inactivated AB serum with and without the riminophenazines (0.6–5 μM). After 5 days of incubation at 37° in an atmosphere of 5% CO_2, 0.5 μCi of [^{3}H]TdR were added to each well and the plates re-incubated for 18 hr after which the cells were assayed for [^{3}H]TdR.

Assay of transmembrane fluxes of K^+ *in lymphocytes.* ^{86}Rb was used as tracer for measuring K^+ uptake [30, 31]. Briefly, MNL or purified T-lymphocytes (10^6/mL) were suspended in isotonic Tris buffer (122 mM NaCl, 4 mM KCl, 1 mM $MgSO_4$, 1 mM KH_2PO_4, 20 mM Tris, 5 mM glucose) pH 7.4 and pre-incubated for 30 min at 37° followed by the serial addition of clofazimine or B669 (1, 2.5 and 5 μM), foetal calf serum (FCS) (10%) and PHA (5 μg/mL). After 60 min of incubation at 37° 2 μCi of ^{86}Rb (Rubidium-86 chloride, 37 MBq, Amersham) were added to each tube (final volume 2 mL). The final volume in each tube was 2 mL. After 30 min the reactions were terminated by the addition of ice-cold Tris buffer. The cells were then washed twice and the pellets finally dissolved in 0.4 mL of 1% Triton X-100/0.1 M NaOH and the radioactivity assayed in a liquid scintillation spectrometer. Na^+, K^+-ATPase activity was taken as the difference in ^{86}Rb uptake in the presence and absence of 2 mM ouabain. AT (50 μM) or lysophospholipase (200 mU/mL) was added to some systems.

The activity of Na^+, K^+-ATPase in purified lymphocyte membranes was measured using a previously described spectrophotometric method [32]. Incubation procedures were similar to those described above for ^{86}Rb uptake. Briefly, after a 60 min incubation at 37° in the presence and absence of the riminophenazines (2.5 μM), the cells (10^6 per mL) were concentrated by centrifugation and the pellets, which contained 2×10^7 cells, resuspended in 3 mL of 0.34 M sucrose containing 0.1 mM EDTA and the cells disrupted by sonication. Cellular debris was then removed by centrifugation at 1400 rpm/10 min/4° and the membrane fractions in the supernatants concentrated by centrifugation at 25,000 rpm/30 min. The membrane pellets were dispersed in 2 mL sucrose and assayed for ouabain-inhibitable Na^+, K^+-ATPase activity. These data are expressed as nmoles NADH oxidized at a wavelength of 340 nm [32]. This method was also used to investigate the effects of clofazimine and B669 (5 and 10 μM), as well as those of reagent LPC (5–40 μM) on the activity of purified Na^+, K^+-ATPase (from dog kidney, Sigma) at a final concentration of 100 mU/assay.

Measurement of the cytotoxic potential of the riminophenazines and LPC. This was determined by measurement of intracellular ATP levels using a sensitive luciferin/luciferase chemiluminescence method [33] and by a fluorescence microscopic dye exclusion method based on the differential uptake of ethidium bromide by dead and viable cells. The lymphocytes (10^6 per mL) were incubated with clofazimine or B669 (1.25, 2.5 and 5 μM) or LPC (5–20 μM) for 2, 24 and 66 hr at 37° then assayed for viability.

Measurement of the possible complexing of clofazimine and B669 with AT. The UV absorption spectra of mixtures of AT, tocopherol acetate (both at 400 μM), butylated hydroxyanisole or butylated hydroxytoluene (both at 1000 μM) with clofazimine or B669 (20 μM) were measured as described previously [34] using a Pye Unicam SP1700 double-beam UV spectrophotometer.

Expression and statistical analysis of results. The results are expressed as the mean value ± SEM for each series of experiments. Levels of statistical significance were calculated using the Student's *t*-test (paired *t* statistic).

RESULTS

Effects of clofazimine and B669 on the release of [^{3}H]*AA and* [^{3}H]*LPC by MNL*

These data for the 10 min incubation period are shown in Table 2. Treatment of MNL with either clofazimine or B669 (1.25, 2.5 and 5 μM) caused dose-related enhancement of the release of both [^{3}H]AA and [^{3}H]LPC. All values shown in Table 2 are statistically significant ($P < 0.05$–$P < 0.005$), with the exception of those for 1.25 μM clofazimine.

Inclusion of AT (50 μM) during exposure of MNL to 5 μM clofazimine or B669 did not affect the release of [^{3}H]AA by these cells. MNL treated with 5 μM clofazimine in the absence and presence of AT released 303 ± 67 and 432 ± 87%, respectively, of the amount of [^{3}H]AA released by the corresponding control systems. The respective values for MNL treated with 5 μM B669 with and without AT were 500 ± 145 and 637 ± 165% (data from three separate experiments).

Measurement of the effects of clofazimine and B669 on the activities of purified PLA_2 *and lysophospholipase*

Neither clofazimine nor B669 at a fixed concentration of 10 μM affected the activities of purified PLA_2 or lysophospholipase. The amount of AA released from radiolabelled PLC in the presence of PLA_2 was 19 ± 1, 20 ± 1 and 21 ± 1 nmol for the control system and systems containing 10 μM clofazimine and B669, respectively (data from four

Table 2. Generation of radiolabelled AA and LPC by riminophenazine-treated MNL

System	Production of [^{3}H]AA (% of control)	Production of [^{3}H]LPC (% of control)
Clofazimine 1.25 μM	124 ± 10	128 ± 17
2.5 μM	228 ± 26	174 ± 10
5 μM	315 ± 57	261 ± 27
B669 1.25 μM	205 ± 36	208 ± 17
2.5 μM	428 ± 136	308 ± 118
5 μM	647 ± 176	605 ± 144

Data from four experiments are presented as the mean values ± SEMs. The absolute values for the drug-free control systems were 5195 ± 1100 and 9520 ± 2125 cpm for generation of [^{3}H]AA and [^{3}H]LPC, respectively. With the exception of those for 1.25 μM clofazimine, all values shown are statistically significant ($P < 0.05$–$P < 0.005$).

Table 3. Effects of clofazimine and B669 on PHA- and alloantigen-activated MNL transformation

System	Uptake of [^{3}H]TdR by MNL activated with	
	PHA (5 μg/mL)	Alloantigens
Control	45502 ± 6984	17488 ± 4936
1.25 μM Clofazimine	40702 ± 5862	10884 ± 5560
2.5 μM Clofazimine	33667 ± 5078	9902 ± 4667
5 μM Clofazimine	25034 ± 4441	ND
1.25 μM B669	32863 ± 5367	8303 ± 5289
2.5 μM B669	19259 ± 5334	3029 ± 2113
5 μM B669	6396 ± 3386	ND

The results are presented as the mean cpm ± SEMs of eight and four different experiments for PHA- and alloantigen-activated systems, respectively. The unstimulated background values were 1005 ± 295 and 761 ± 251 cpm for the PHA- and alloantigen-activated control systems, respectively. All the values shown for the riminophenazine-containing systems are significantly less ($P < 0.01$–$P < 0.005$) than those of the corresponding drug-free control systems. ND = not done.

Table 4. Effects of cysteine and catalase on riminophenazine-mediated inhibition of PHA-activated MNL transformation

System	PHA-activated uptake of [^{3}H]TdR by MNL
Control	50963 ± 7556
Cysteine (500 μM)	57129 ± 7152
Catalase (500 U/mL)	48298 ± 5728
Clofazimine (2.5 μM)	38491 ± 4421 (24)*
B669 (1.25 μM)	38222 ± 6083 (25)
Clofazimine + cysteine	45577 ± 7194 (20)
Clofazimine + catalase	41280 ± 4640 (19)
B669 + cysteine	53960 ± 7222 (6)
B669 + catalase	44847 ± 5041 (12)

Data from three separate experiments are presented as the mean cpm ± SEMs. The mean value for the control, unstimulated background system was 495 ± 21 cpm.

* Per cent inhibition.

different experiments). The amount of LPC hydrolysed by lysophospholipase was 103 ± 16, 100 ± 2 and 91 ± 7 nmol for the control system and systems containing 10 μM clofazimine and B669, respectively (data from two different experiments).

Effects of clofazimine and B669 on PHA- and alloantigen-activated uptake of [^{3}H]TdR by MNL

These results are shown in Table 3. Both riminophenazines caused dose-related inhibition of PHA-activated MNL transformation which achieved statistical significance at 1.25 μM of both clofazimine ($P < 0.01$) and B669 ($P < 0.005$). B669 was a more potent inhibitor of mitogen-activated MNL proliferation than clofazimine. At concentrations of 1.25, 2.5 and 5 μM clofazimine the mean percentage inhibition of PHA-activated MNL transformation was 11% ($P < 0.01$), 26% ($P < 0.005$) and 45% ($P < 0.005$), respectively. The corresponding values for B669 were 28, 58 and 86% (all P values < 0.005). At all concentrations tested the inhibition observed with B669 was significantly greater than that observed with clofazimine ($P < 0.01$–$P < 0.005$).

Both riminophenazines at concentrations of 1.25 μM and upwards caused significant dose-related inhibition of the uptake of [^{3}H]TdR by responder lymphocytes in the MLR (Table 3). As observed with MNL transformation, B669 was a more potent inhibitor than clofazimine.

Measurement of the effects of cyclooxygenase, 5'-lipoxygenase, PKC inhibitors, anti-oxidants and lysophospholipase on riminophenazine-mediated inhibition of PHA-stimulated MNL transformation

Indomethacin, piroxicam, NDGA, H-7, staurosporine, cysteine, catalase, retinol, butylated hydroxytoluene, butylated hydroxyanisole and tocopherol acetate did not protect MNL against the anti-proliferative effects of 5 μM clofazimine or B669 (data not shown). However, cysteine, and to a lesser extent catalase, protected the PHA-induced proliferative responses of MNL against lower concentrations of both agents (1.25 and 2.5 μM clofazimine and 1.25 μM B669). The data for 2.5 μM clofazimine and 1.25 μM B669 are shown in Table 4. However, both AT and lysophospholipase protected the MNL against the anti-proliferative effects of higher concentrations of clofazimine and B669. The effects of AT on clofazimine and B669-mediated inhibition of PHA-activated MNL transformation are shown in Fig. 2. Co-incubation of MNL with 10 μM AT partially protected the cells ($P < 0.005$) against the anti-proliferative effects of 5 μM clofazimine or B669, while complete protection was observed with lower concentrations (1.25 and 2.5 μM; $P < 0.005$) of the immunosuppressive agents. At the higher concentration (50 μM), AT completely protected the MNL against the anti-proliferative effects of all three concentrations of the rim-

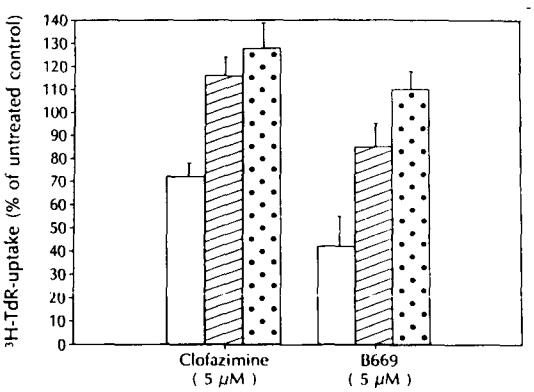

Fig. 2. Effects of clofazimine and B669 without (□) and with 10 μM (▨) or 50 μM (▩) AT on the PHA-activated proliferative responses of MNL. Data from six separate experiments are expressed as the mean percentage ± SEMs of the corresponding drug-free control systems. The absolute values for the unstimulated and PHA-stimulated control systems were 987 ± 67 and 31,875 ± 4107 cpm, respectively.

Table 5. Effects of AT on riminophenazine-mediated inhibition of alloantigen-activated MNL proliferation

System	Uptake of [^{3}H]TdR by alloantigen-activated MNL
Control	31058 ± 2071
50 μM AT	33152 ± 2135
2.5 μM Clofazimine	23915 ± 4671
2.5 μM Clofazimine + 50 μM AT	35133 ± 5712
2.5 μM B669	18945 ± 3875
2.5 μM B669 + 50 μM AT	37928 ± 3712

The results of four experiments are expressed as the mean cpm ± SEMs. The background value for unstimulated MNL was 835 ± 108 cpm.

inophenazines. The level of protection remained the same if the AT was added 60 min before or 60 min after clofazimine or B669. However, pre-incubation of the MNL with AT (50 μM) for 60 min, followed by washing (×2) and exposure to clofazimine or B669 (1.25–5 μM) did not protect the proliferative responses of the cells, demonstrating a requirement for the continuous presence of AT in order to neutralize the anti-proliferative effects of the riminophenazines (data not shown).

AT at both concentrations tested, also protected the alloantigen-activated responses of MNL against riminophenazine-mediated inhibition. These results are shown in Table 5.

The protective effects of lysophospholipase (200 mU/mL) on clofazimine (5 μM), or B669 (2.5 μM) mediated inhibition of PHA-activated MNL proliferation are shown in Table 6. Lysophospholipase was added to MNL 30 min after the riminophenazines, followed 30 min later by the addition of serum and PHA. Inclusion of the enzyme almost completely abolished ($P < 0.005$) the inhibitory effects of the riminophenazines on MNL proliferation. Heat inactivation of lysophospholipase was associated with loss of protection, while lesser protection was observed with lower concentrations of the enzyme (not shown).

Effects of clofazimine and B669 ± AT on the proliferative responses of purified T-lymphocytes

The mean percentages of monocytes, B-lymphocytes and T-lymphocytes in the unfractionated MNL preparations were 2 ± 1, 17 ± 2 and 77 ± 9, respectively. The corresponding values for the same MNL preparations after purification of T-lymphocytes were 0, 2 ± 1 and 95 ± 6. The PMA-stimulated LECL responses of unfractionated and fractionated MNL were 814 ± 251 and 56 ± 15 mV/sec, respectively, with corresponding unstimulated values of 40 ± 1 and 16 ± 1 mV/sec.

The effects of the riminophenazines (5 μM), in the presence and absence of 50 μM of AT, on the PHA-activated proliferative responses of purified T-lymphocyte suspensions are shown in Table 7. As with unfractionated MNL, both riminophenazines inhibited the PHA-stimulated proliferative responses of purified T-lymphocytes, which was prevented by the inclusion of AT.

Effects of LPC, PC and AA on PHA-stimulated MNL transformation

The effects of LPC (5–20 μM) on PHA-induced lymphocyte proliferation are shown in Fig. 3. This agent caused a dose-related inhibition of MNL

Table 7. Effects of clofazimine and B669 with and without AT on the PHA-activated proliferative responses of purified T-lymphocytes

Systems	Uptake of [^{3}H]TdR by PHA (5 μg/mL)-activated T-lymphocytes
Control	39412 ± 3175
50 μM AT	41233 ± 2578
5 μM Clofazimine	26618 ± 4671
5 μM Clofazimine + 50 μM AT	40979 ± 5321
5 μM B669	7239 ± 175
5 μM B669 + 50 μM AT	38975 ± 5471

Data from two experiments in triplicate are presented as the mean cpm + SEMs. The background values for unstimulated MNL were 3782 ± 193 cpm.

Table 6. Effects of added lysophospholipase on clofazimine- and B669-mediated inhibition of PHA-activated MNL transformation

System	Uptake of [^{3}H]TdR by MNL
a) Control	898 ± 162
b) Lysophospholipase (200 mU/mL)	770 ± 242
c) PHA	26532 ± 4168
d) PHA + lysophospholipase	27416 ± 4542
e) PHA + 5 μM clofazimine	12974 ± 5548
f) PHA + clofazimine + lyusophospholipase	24056 ± 6104*
g) PHA + 2.5 μM B669	11914 ± 2476
h) PHA + B669 + lysophospholipase	22888 ± 4230*

The results are presented as the mean cpm ± SEMs of five separate experiments.
* $P < 0.005$ for comparison of system e with f and for comparison of system g with h.

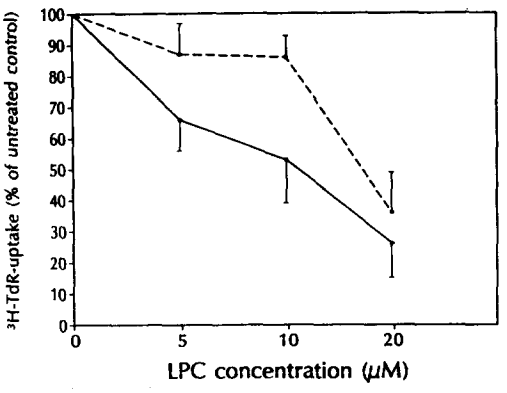

Fig. 3. Effects of LPC only (●——●) and in the presence of 50 μM AT (●---●) on the PHA-activated uptake of [^{3}H]TdR by MNL. The results of six experiments are presented as the mean percentage ± SEMs of the corresponding LPC-free control systems. The absolute values for the unstimulated and PHA-stimulated control systems were 1482 ± 171 and 40667 ± 3721 cpm, respectively.

Table 8. Effects of clofazimine and B669 in the presence or absence of PHA on the uptake of ^{86}Rb by MNL

Treatment	Uptake of ^{86}Rb by MNL (% of control)	
	Without PHA	With PHA
1.25 μM Clofazimine	95 ± 5	90 ± 3
2.5 μM Clofazimine	90 ± 6	77 ± 5
5 μM Clofazimine	90 ± 5	60 ± 3
1.25 μM B669	90 ± 5	73 ± 7
2.5 μM B669	82 ± 9	53 ± 5
5 μM B669	69 ± 4	35 ± 7

Data from four separate experiments are expressed as the mean percentages of the corresponding riminophenazine-free control systems ± SEMs. The absolute values for uptake of ^{86}Rb by the control MNL with and without PHA were 1067 ± 101 and 2172 ± 21 cpm, respectively. All the values shown are statistically significant ($P < 0.05$–$P < 0.005$), with the exception of those for 1.25 and 2.5 μM clofazimine in the absence of PHA.

transformation which was statistically significant at 5 μM ($P < 0.05$) and upwards. Treatment of MNL with 50 μM AT prior to exposure to LPC protected the proliferative responses of cells exposed to 5 μM ($P < 0.005$) and 10 μM ($P < 0.05$). However, AT added to MNL 30 min after LPC was unable to reverse the inhibitory effect of the lysophospholipid (not shown). On the other hand, AA at concentrations of up to 20 μM did not detectably alter the uptake of [^{3}H]TdR by PHA-activated MNL. The respective values for PHA-treated control MNL and cells co-incubated with 20 μM AA were 48,064 ± 1531 and 44,413 ± 1832 cpm (means ± SEM of three experiments). The corresponding value for unstimulated control MNL was 412 ± 87 cpm. Likewise PC at concentrations of up to 20 μM did not affect PHA-activated MNL transformation. The respective values for unstimulated MNL and PHA-activated MNL without and with 20 μM PC were 1222 ± 177, 56808 ± 1579 and 53942 ± 3551 cpm.

Effects of clofazimine and B669 on lymphocyte Na^+, K^+-*ATPase activity*

These results are shown in Table 8. Inclusion of PHA almost doubled the extent of ^{86}Rb uptake by MNL ($P < 0.005$). Treatment of these cells with the riminophenazines caused dose-related inhibition of uptake of ^{86}Rb by both resting and PHA-activated MNL, with the latter being particularly sensitive. Similar results were obtained using purified T-lymphocytes with 60 ± 4% ($P < 0.05$) and 22 ± 10% ($P < 0.005$) of the control values observed for uptake of ^{86}Rb by PHA-activated cells treated with 5 μM clofazimine and B669, respectively (data from four experiments). Inclusion of AT or lysophospholipase protected Na^+, K^+-ATPase in PHA-activated cells against the inhibitory effects of the riminophenazines. In the presence of 5 μM clofazimine or 2.5 μM B669 the respective uptakes of ^{86}Rb by PHA-activated MNL were 69 ± 4% ($P < 0.005$) and 53 ± 5% of the drug-free control systems. The corresponding values in the presence of AT (50 μM) were 92 ± 4 and 92 ± 5%, demonstrating significant protection ($P < 0.005$ for both systems), and 95 ± 2 and 89 ± 5% ($P < 0.005$ and $P < 0.01$, respectively) in the presence of 200 mU/mL lysophospholipase (data from five experiments). Neither AT nor lysophospholipase *per se* affected the uptake of ^{86}Rb by MNL (not shown).

The activities of Na^+, K^+-ATPase in the membrane fractions of PHA-activated MNL in the absence of the drugs and in the presence of 2.5 μM clofazimine or B669 were 4.3 ± 0.4, 3.1 ± 0.3 ($P < 0.05$) and 1.3 ± 0.1 ($P < 0.05$) nmol NAD/min/2 × 10^7 MNL, respectively (data from two experiments).

Neither clofazimine nor B669 affected the activity of purified Na^+, K^+-ATPase (activities of 6.2 ± 0.2, 6.3 ± 0.2 and 6.2 ± 0.2 nmol NAD/min in the absence and presence of 5 μM clofazimine or 2.5 μM B669, respectively). However, LPC caused a dose-dependent inhibition of this enzyme; the mean percentages of the control system observed in the presence of 5, 10, 20 and 40 μM LPC were 98 ± 1, 93 ± 2, 80 ± 6 and 49 ± 13, respectively (data from three separate experiments).

Effects of clofazimine and B669 on MNL ATP levels and viability

LPC caused a dose-dependent decrease in MNL viability measured by dye exclusion. Using a 2 hr incubation period the numbers of non-viable cells in control systems and in those treated with 5, 10 and 20 μM LPC were 2 ± 0.2, 45 ± 1, 68 ± 2 and 77 ± 6%, respectively (data from three experiments). The corresponding values for cellular ATP levels were 9.9 ± 1.4, 4.7 ± 0.2, 3.2 ± 0.2 and 1.9 ± 0.1 nmol ATP/10^7 MNL. The effects of clofazimine and B669 on the viability and ATP levels of MNL after 2, 24 and 66 hr of incubation at 37° are shown in Table 9. Clofazimine at concentrations of up to 5 μM did not affect either the viability or ATP levels of MNL exposed to the drug for either 2 or 24 hr, although

Table 9. Effects of clofazimine and B669 on the viability and intracellular ATP levels of MNL

System	Percentage non-viable cells			Intracellular ATP levels (nmol/10^7 MNL) after		
	2 hr	24 hr	66 hr	2 hr	24 hr	66 hr
Control	2 ± 1	3 ± 1	4 ± 1	9 ± 1	8 ± 1	4 ± 0.1
Clofazimine: 1.25 μM	2 ± 1	3 ± 1	9 ± 4	9 ± 1	7 ± 1	3.9 ± 0.2
2.5 μM	4 ± 1	4 ± 1	23 ± 4	9 ± 1	8 ± 1	3.2 ± 0.2
5 μM	5 ± 1	5 ± 1	41 ± 4	8 ± 1	8 ± 1	2.3 ± 0.4
B669: 1.25 μM	3 ± 1	4 ± 1	27 ± 5	9 ± 1	9 ± 1	2.7 ± 0.3
2.5 μM	7 ± 1	20 ± 3	77 ± 4	9 ± 1	5 ± 1	0.7 ± 0.1
5 μM	5 ± 1	54 ± 2	83 ± 2	5 ± 1	3 ± 1	0.6 ± 0.1

Data from three to four experiments are expressed as the mean values ± SEMs.

a 66 hr exposure of MNL to 2.5 and 5 μM clofazimine caused decreased viability and ATP levels. B669 also caused time and concentration-dependent decreases in cellular viability and ATP levels (Table 9).

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.

DISCUSSION

In this study we have investigated the effects of clofazimine and its experimental analogue B669 on the activity of lymphocyte PLA_2, as well as the potential involvement of PLA_2 breakdown products in the mediation of clofazimine/B669-induced immunosuppression. Co-incubation of MNL with therapeutically relevant concentrations of the riminophenazines (1.25, 2.5 and 5 μM) for 5–10 min was associated with increased activity of PLA_2 as measured by the release of [^{3}H]AA and [^{3}H]LPC. This is in agreement with previous reports in which clofazimine was found to increase the release of AA and prostaglandin E_2 by neutrophils [10–12] and MNL [13, 17] *in vitro*. Riminophenazine-mediated stimulation of PLA_2 was dose-related, with B669 being the more potent of the two test agents. The activities of purified PLA_2 and lysophospholipase were unaffected by either clofazimine or B669 (10 μM), demonstrating that the riminophenazines do not act directly on either enzyme. In the lymphocyte cell-membrane these highly lipophilic riminophenazines may disrupt membrane structure making the integral phospholipids more susceptible to attack by PLA_2.

We also confirmed the previously described inhibitory effects of clofazimine on PHA-activated lymphocyte transformation [8, 9, 13, 16], which were about 2-fold less than those observed with B669. Both agents also inhibited alloantigen-stimulated uptake of [^{3}H]TdR by lymphocytes. Using both unfractionated MNL suspensions and purified T-lymphocytes, inhibition was observed at concentrations of 1.25 μM and upwards of both riminophenazines. To identify the products of phospholipase activation with anti-proliferative activity we investigated the effects of the primary hydrolysis products LPC and AA, generated during cleavage of PLC by PLA_2, on PHA-activated lymphocyte proliferation. LPC at concentrations of 5 μM and upwards inhibited MNL proliferation, while AA at concentrations of up to 20 μM had no effect. Likewise PC (20 μM), the major phospholipid substrate for PLA_2, did not affect MNL proliferation. While implicating LPC, which has detergent and membrane-destabilizing properties [24, 33], these data did not exclude other metabolites and reactive oxidants generated during the metabolism of AA by cyclooxygenase and 5-lipoxygenase, or by riminophenazine-mediated enhancement of the NADPH-oxidase activity of contaminating phagocytes [15, 16]. However, inclusion of a water-soluble anti-oxidant (cysteine) or enzyme (catalase), lipid-soluble anti-oxidants (retinol, butylated hydroxytoluene or butylated hydroxyanisole), prostaglandin synthetase inhibitors (indomethacin or piroxicam) or an inhibitor of 5′-lipoxygenase (NDGA) all failed to protect the cells against the anti-proliferative effects of 5 μM clofazimine and 2.5 and 5 μM B669. At lower concentrations of the riminophenazines (<5 μM clofazimine and <2.5 μM B669) however, cysteine and catalase partially protected the PHA-activated proliferative responses of MNL. These observations, as well as the failure of catalase and cysteine to protect purified T-lymphocytes, confirm the involvement of oxidants originating from riminophenazine-stimulated neutrophils and monocytes in the anti-proliferative activity of low concentrations of clofazimine and B669 [18], while prostaglandins or 5′-lipoxygenase products are not primarily involved in clofazimine/B669-mediated inhibition of lymphocyte proliferation. Although tocopherol-acetate did not protect MNL against the anti-proliferative effects of the riminophenazines, AT, originally included as a lipid-soluble anti-oxidant, 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 5 μM clofazimine and B669 on the proliferative responses of both unfractionated MNL and purified T-lymphocytes. 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 clofazimine of B669 since (a) spectrophotometric analysis of mixtures of AT and clofazimine or B669 revealed no interactions, (b) the protective effects of AT were still evident when this agent was added to the cells 1 hr after clofazimine or B669; and (c) AT did not inhibit the effects of the riminophenazines on the activity of PLA_2. It also seemed unlikely that these protective effects of AT were related to the anti-oxidant properties of the molecule since other lipid-soluble anti-oxidants were ineffective. Notwithstanding its well-documented anti-oxidant properties, AT possesses other biological activities which could account for the observed protection against the anti-proliferative effects of clofazimine and B669. AT has been reported to inhibit both the activation and translocation of cytosolic PKC [35, 36], and to interfere with the activity of 5'-lipoxygenase [37] and PLA_2 in some experimental systems [38], but not in others [39]. The failure of staurosporine, H-7 and NDGA to protect the mitogen-activated proliferative responses of lymphocytes appears to discount PKC or 5'-lipoxygenase inhibition, respectively, as potential mechanisms of prevention of riminophenazine-induced immunosuppression. Likewise, we were unable to demonstrate any inhibitory effects of AT on lymphocyte PLA_2. AT also complexes with, and neutralises lysophospholipids through two types of interaction, namely formation of a hydrogen bond between the AT chromanol nucleus hydroxyl group and the C-O group of the lysophospholipid; and interaction of the acyl chains of the lysophospholipids with the chromanol nucleus methyl groups of AT [24]. In the present study we observed that pretreatment of lymphocytes with AT effectively protected these cells against the anti-proliferative activity of LPC. The most compelling evidence in favour of the involvement of LPC in riminophenazine-mediated immunosuppression however, was derived from experiments with the LPC-hydrolysing enzyme, lysophospholipase. The anti-proliferative effects of both clofazimine and B669 were neutralised by co-incubation of mitogen-activated MNL with lysophospholipase.

These data implicate LPC as a potential mediator of riminophenazine-induced immunosuppression. Although cellular lysophospholipase may confer partial protection, the lymphocytes are apparently ill-equipped to neutralise excessive amounts of LPC. LPC-mediated damage to the plasma membrane and consequent cytotoxicity seemed a likely mechanism of anti-proliferative activity and was supported by observations of loss of viability in cells treated for short periods (2 hr) with either reagent LPC or high concentrations (5 μM) of B669. Non-specific cytotoxicity due to abrupt exposure of lymphocytes to high concentrations of LPC (added exogenously or generated endogenously during exposure to high concentrations of B669) cannot however explain the non-lethal, anti-proliferative effects of clofazimine (up to 2.5 μM) and lower concentrations of B669. Intra-membrane accumulation of non-lethal concentrations of endogenously generated LPC during exposure of lymphocytes to these concentrations of the riminophenazines was associated with decreased activity of the membrane-associated enzyme Na^+, K^+-ATPase. The activity of this enzyme is up-regulated during activation of T-lymphocytes by mitogens and is indispensable for the differentiation and proliferation of these cells [30, 40, 41]. Clofazimine and B669, at concentrations which did not affect cellular ATP levels, inhibited the activity of Na^+, K^+-ATPase in both intact cells and purified membranes, demonstrating a direct inhibitory effect on the enzyme. Importantly, both AT and lysophospholipase protected the enzyme against riminophenazine-mediated inactivation, implicating LPC as the probable mediator. The inhibitory effects of LPC on Na^+, K^+-ATPase activity in various cell types have been described previously, and it has been proposed that the lysophospholipid may interfere with essential interactions between the enzyme and boundary phospholipids in the inner membrane [31, 42]. Although LPC is the major lysophospholipid generated in mammalian cells, the involvement of other types of lysophospholipid in riminophenazine-mediated inhibition of lymphocytes Na^+, K^+-ATPase cannot be excluded.

Finally, the data presented here are compatible with enhancement of phospholipid hydrolysis in mitogen- or antigen-activated T-lymphocytes, leading to increased generation of the anti-proliferative agent LPC, as being the probable primary mechanism of riminophenazine-mediated immunosuppression.

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