CLOFAZIMINE: CURRENT STATUS AND FUTURE PROSPECTS

M.C. Cholo,[1] H.C. Steel,[1] P.B. Fourie,[2] A. Germishuizen,[2] R. Anderson.[1]

[1]Medical Research Council Unit for Inflammation and Immunity, Department of Immunology, Faculty of Health Sciences, University of Pretoria, and Tshwane Academic Division of the National Health Laboratory Service, Pretoria, South Africa;

[2]Medicine in Need, Pretoria, South Africa.

Correspondence: Dr M.C. Cholo
Department of Immunology
P.O. Box 2024
Pretoria
South Africa
0001

Telephone: +27-12-319-2162
Telefax: +27-12-319-0732
E-mail: moloko.cholo@mrc.up.ac.za

Abstract

Clofazimine, a lipophilic riminophenazine antibiotic, possesses both antimycobacterial and anti-inflammatory activities. However, its efficacy has been demonstrated only in the treatment of leprosy, not in human tuberculosis, despite the fact that this agent is impressively active *in vitro* against multidrug-resistant strains of *Mycobacterium tuberculosis*. Recent insights into novel targets and mechanisms of antimicrobial and anti-inflammatory activity coupled with the acquisition of innovative drug delivery technologies have, however, rekindled interest in clofazimine as a potential therapy for multidrug- and extensively multidrug-resistant tuberculosis in particular, as well as several autoimmune diseases. The primary objective of this review is to critically evaluate these recent developments and to assess their potential impact on improving the therapeutic efficacy and versatility of clofazimine.

Keywords: anti-inflammatory activity, leprosy, lysophospholipids, membrane destabilisation, mycobacteria, potassium transporters, reactive oxygen species, redox cycling, spray-drying, tuberculosis, T lymphocytes.

1. Introduction

Clofazimine, originally described in 1957, is the prototype riminophenazine antibiotic. The primary clinical application of clofazimine since 1962 has been in the treatment of multibacillary leprosy as a component of the WHO-recommended triple drug regimen.[2] Notwithstanding its antimicrobial activity, the efficacy of clofazimine in the treatment of leprosy is attributable to both the lipophilicity and anti-inflammatory properties of this agent. Lipophilicity enables clofazimine to accumulate in skin and nerves, while its anti-

inflammatory activities are potentially useful in controlling harmful erythema nodosum leprosum and reversal immunity reactions, which may complicate antimicrobial chemotherapy. [3-6].

Although impressively active against *Mycobacterium tuberculosis* (MTB) *in vitro*, including multidrug-resistant (MDR) strains of this microbial pathogen,[7,8] clofazimine is generally considered to be ineffective in the treatment of pulmonary tuberculosis. This contention arose from early studies that demonstrated inconsistent therapeutic activity of clofazimine in various animal models (hamsters, guinea pigs, rabbits, non-human primates) of experimental tuberculosis (TB).[9] Enthusiasm for the development of clofazimine as an anti-TB agent was also blunted by the unusual pharmacokinetic properties and side effects profile of this agent (see below),[10] further complicated by the fact that its discovery coincided with the emergence of the more potent anti-TB agents isoniazid and pyrazinamide in the early 1950s and rifampicin and ethambutol in the early 1960s. Although of low priority for several decades, the emergence of MDR and extensively drug-resistant (XDR) TB, together with advances in technology for the delivery of lipophilic drugs to target organs, has triggered renewed interest in clofazimine as an anti-TB agent. In addition, novel insights into the targets and molecular mechanisms of both clofazimine-mediated antimicrobial and anti-inflammatory activity have provided the impetus for the design and development of novel riminophenazines with improved antimicrobial efficacy.[11,12]

The primary objective of the current review is to evaluate recent insights into: i) putative mechanisms of antimicrobial activity of clofazimine; ii) mechanisms of anti-inflammatory immunosuppressive activity of this agent as these may modulate therapeutic efficacy in mycobacterial infections; and iii) innovations which may promote the efficient delivery of clofazimine to target organs. Of necessity, these will be preceded by a brief consideration of the pharmacokinetic and current clinical applications of clofazimine.

2. Clinical experiences and recommendations

Clofazimine is mainly indicated in the treatment of lepromatous leprosy, including dapsone-resistant lepromatous leprosy and lepromatous leprosy complicated by erythema nodosum leprosum, and has been included as an anti-leprosy medicine in the current WHO Model Lists of Essential Medicines for adults and children.

The literature on clinical experience with clofazimine in the treatment of especially pulmonary TB is sparse. Earlier reviews largely demonstrate uncertainty as to the efficacy of the drug in humans, and emphasize a fair degree of intolerance to long-term use. The drug is mainly recommended for use in combination with other drugs in the second-line treatment of drug-resistant TB.[15,16] Treatment of a small group of five TB patients with clofazimine in combination with linezolid and other drugs has also been described.[17] Although some degree of efficacy with these combination regimens was observed, adverse events were significant.

Even though clofazimine has no official indications for the treatment of drug-resistant TB, it is recommended by WHO as a Group 5 medicine, i.e. an agent with unclear efficacy, for use in patients with XDR TB.[18]

Studies in TB patients with HIV co-infection are largely lacking. Coyne *et al.*[19] observed that drug–drug interactions in such patients might be a potential concern, because clofazimine is a weak inhibitor of CYP3A4, and so protease inhibitor and etravirine concentrations may be increased, but no interaction studies have been undertaken. In earlier work, Chaisson *et al.*[20] found that the addition of clofazimine to a regimen of clarithromycin and ethambutol for *Mycobacterium avium* complex (MAC) bacteraemia in AIDS patients does not contribute to the clinical response and is associated with higher mortality. The safety and effectiveness in children has not been established.

3. Molecular structure and antimicrobial spectrum of clofazimine

The key structural features of riminophenazines are the phenazine nucleus with an alkylimino (R-imino) group at position 2 and phenyl substituents at positions 3 and 10 of the phenazine nucleus. The alkylimino group is critical for antimicrobial activity, with varying, albeit secondary, contributions according to the number and type of halogen atoms on the phenyl substituents.[21,22] The molecular structure of clofazimine, which has an isopropylimino group at position 2 of the phenazine nucleus, is shown in Figure 1.

Clofazimine and other riminophenazines are active against mycobacteria, both slowly and rapidly growing, as well as most other Gram-positive bacteria *in vitro*, including those that are multidrug resistant, with MICs of 0.5–2 mg/L in most cases.[7,8,23–29] Gram-negative bacteria, on the other hand, are uniformly resistant to clofazimine.[23] As mentioned earlier, the clinical applications of clofazimine as an antimicrobial agent are extremely limited, despite impressive antimycobacterial activity *in vitro*. Notwithstanding its primary use as an anti-leprosy agent, clofazimine has been used with limited success in the treatment of MDR and XDR TB as a category 5 agent,[30] as well as in the multidrug therapy of infections caused by MAC, also with limited efficacy.[31,32]

Nonetheless, there has been a recent revival of interest in clofazimine, which can be attributed to the following: (i) impressive *in vitro* activity against MDR/XDR clinical isolates of MTB *in vitro*;[7,9,23] (ii) the extremely low frequency of drug resistance in MTB,[2] with possible emergence among rapidly growing mycobacteria;[33] and (iii) synergistic interactions with *trans*-cinnamic acid[34] and amikacin[24] against MDR isolates of MTB and rapidly growing mycobacteria *in vitro*, respectively, as well as with isoniazid, preventing the development of resistance to this agent.[7,35] Clearly, the identification of other anti-TB agents with which clofazimine interacts optimally may enable a more discerning and efficacious strategy on which to base clofazimine-containing drug regimens. Although speculative, this contention appears to be supported by the recent study by van Deun *et al*.,[36] who reported on the

efficacy (87% relapse-free use) of a treatment regimen for MDR TB based on a minimum of 9 months of treatment with the combination of gatifloxacin, clofazimine (50–100 mg/daily), ethambutol and pyrazinamide throughout the treatment period supplemented by prothionamide, kanamycin and high-dose isoniazid for a minimum of 4 months.

4. Mechanisms of antimicrobial action

Although the exact mechanism(s) of clofazimine-mediated antimicrobial activity remains to be established, the outer membrane appears to be the primary site of action of this agent.[12,24,28,29,37-39] Putative targets include the bacterial respiratory chain and ion transporters.

4.1 Redox cycling

Because of its highly lipophilic nature and redox potential (-0.18V at pH 7), Barry *et al*, [1] in their original description of clofazimine, proposed that intracellular redox cycling was likely to contribute to the antimicrobial activity of this agent by a mechanism involving oxidation of reduced clofazimine, leading to generation of the antimicrobial reactive oxygen species (ROS), superoxide and hydrogen peroxide.[1] This putative mechanism was, however, challenged by Van Rensburg *et al*.[25] Using a large series of Gram-positive bacteria, which, with 2 exceptions, had clofazimine MICs ranging from 0.25-4 mg/L, these authors observed the following: i) there was no clear association of catalase positivity/negativity with the degree of susceptibility to clofazimine; ii) sensitivity of 3 different species of Gram-positive facultative anaerobes to clofazimine was undiminished, and actually increased, during exposure to the riminophenazine under strictly anaerobic conditions; and iii) inclusion of either

water-soluble or lipid-soluble scavengers of ROS, as well as anti-oxidative enzymes in the bacteriological culture medium did not attenuate the inhibitory effects of clofazimine on the growth of Gram-positive bacteria. The exception was α-tocopherol (AT), which, at a ratio of 25 mg/L: 0.5 mg/L (AT: clofazimine), almost completely attenuated the inhibitory effects of clofazimine on the growth of *S. aureus*.[25] As mentioned below, however, AT, in addition to ROS scavenging activity, also possesses membrane-stabilizing properties, which may underpin its antagonistic effects on clofazimine. In addition to these observations, 11 different species of Gram-negative bacteria, which are apparently sensitive to the antimicrobial actions of redox cycling agents,[40,41] were found to be uniformly resistant to clofazimine with MIC values of >32 mg/L.[25]

Very recently, however, the concept of intracellular redox cycling as a mechanism of clofazimine-mediated antimicrobial activity has been revived by Yano *et al*.[12] These authors reported that addition of clofazimine, at MIC concentrations, to isolated membrane fractions from *Mycobacterium smegmatis*, in the presence of the terminal cytochrome respiratory chain inhibitor potassium cyanide (KCN) and the oxidizable cofactor, reduced nicotinamide adenine dinucleotide (NADH), resulted in serial oxidation of NADH, reduction and oxidation of clofazimine, and generation of superoxide and H_2O_2.[12] Substitution of membrane fractions with recombinant NDH-2 (from MTB), the primary respiratory chain NADH: quinoneoxidoreductase, which functions early in the respiratory chain and is insensitive to KCN, was equally effective in promoting formation of ROS. The authors proposed that clofazimine competes with the NDH-2 substrate, menaquinone, for electrons donated by NADH, an initial event in the mycobacterial respiratory chain, to generate reduced clofazimine, which is in turn oxidized by molecular oxygen with resultant formation of

superoxide and H_2O_2. This contention was supported by observations that inclusion of either water-soluble (N-acetylcysteine) or lipid-soluble [AT, or 4-hydroxy-TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl)] anti-oxidants in the bacteriological culture medium neutralized the antimicrobial action of clofazimine,[12] in contrast to earlier findings with *S. aureus* and *S. epidermidis*.[25]

Although of interest, there are two possible caveats in respect of the mechanism of clofazimine-mediated antimicrobial activity described by Yano *et al*.[12] Firstly, the magnitude of generation of ROS by isolated membranes exposed to clofazimine *in the absence of KCN* was considerably less than in the presence of the respiratory chain inhibitor. Secondly, isolated membrane fractions from *S. aureus* behaved somewhat differently to those of *M. smegmatis*, with inhibition of NADH oxidation observed at clofazimine concentrations >2 mg/L, compatible with the existence of additional mechanisms of antimicrobial activity.[12]

4.2 Membrane destabilization and dysfunction

Van Rensburg *et al.,*[25] who originally reported on the selectivity of clofazimine for Gram-positive bacteria, including mycobacteria, proposed a mechanism of antimicrobial activity caused by disruption of membrane structure and function.[24] This contention was based on the following observations: i) as mentioned above, these authors found no compelling evidence to implicate intracellular redox cycling in the antimicrobial activity of clofazimine; ii) exposure of Gram-positive bacteria, as well as MTB, at MIC concentrations of clofazimine, was accompanied by accumulation of lysophospholipids, detergent-like agents with membrane-disruptive properties, in the bacterial cells;[25,38] iii) the inhibitory effects of clofazimine on the

growth of Gram-positive, but not Gram-negative bacteria, were mimicked by low micromolar concentrations of lysophospholipds;[25,37] iv) the earliest indicator of clofazimine- or lysophospholipid-mediated membrane dysfunction was almost complete inhibition of uptake of K^+, which preceded, and was proposed to be the probable cause of the subsequent decrease in microbial ATP levels due to interference with the membrane potential in Gram-positive bacteria and MTB, but not Gram-negative bacteria;[37-39,42,43] and v) the inhibitory effects of both clofazimine and lysophospholipids were effectively attenuated by pre-treatment of the bacteria with AT, which, in addition to its anti-oxidative activity, also possesses well-documented membrane-stabilizing properties.[44]

Several mechanisms exist by which AT may protect the bacterial membrane against the disruptive effects of clofazimine. These include possible interference with the binding of this cationic amphiphile to its target anionic/zwitterionic phospholipids in the bacterial membrane, neutralization of lysophospholipids, and/or inhibition of bacterial phospholipases. In the case of lysophospholipids, AT neutralizes the membrane-disruptive activity of these agents by two types of interaction, specifically, formation of a hydrogen bond between the chromanol nucleus hydroxyl group of AT and the C-O group of the lysophospholipid, as well as interactions of the acyl chains of the lysophospholipids with the chromanol nucleus methyl groups of AT.[25,44]

We originally proposed that clofazimine-mediated enhancement of microbial phospholipases, resulting in increased generation of anti-proliferative lysophospholipids, was likely to underpin the anti-bacterial activity of this agent.[25,37-39] However, this proposed mechanism can be discounted on the basis of two lines of evidence. Firstly, no genes encoding conventional A-type phospholipases have been documented in the MTB genome, in contrast to several putative C-type

phospholipases (*plc* A, B, C, D).[42] Secondly, a mutant of MTB deficient in all four *plc* genes displayed a level of sensitivity to clofazimine equivalent to that of the wild-type strain.[43] While these findings appear to eliminate microbial phospholipases as putative mediators of the antimicrobial activity of clofazimine, they certainly do not exclude membrane destabilization, or even the involvement of lysophospholipids. This contention is based on the study by Baciu *et al*,[45] who reported that cationic amphiphiles such as clofazimine partition rapidly to the polar-apolar region of the membrane, where, at physiological pH, the protonated groups on the drug catalyse the acid hydrolysis of the ester linkage of the phospholipid chains.[44] The consequence is production of a fatty acid and a lysophospholipid, both of which possess antimicrobial activity.[46]

As mentioned above, interference with uptake of K^+ is the earliest detectable indicator of membrane dysfunction, occurring within minutes of exposure of MTB to clofazimine at MIC concentrations.[38,39] Notwithstanding non-specific membrane potential-driven uptake at high extracellular concentrations of the cation in the bacteriological culture media, the high intracellular concentrations of K^+ necessary to sustain diverse, essential cellular processes in MTB are achieved by two major, structurally distinct K^+-transporters, *viz.* the constitutively operative Trk A/B system, and the inducible Kdp system.[47] Because both systems are strongly inhibited following exposure of MTB to clofazimine, it seems unlikely that this agent functions as a primary, selective inhibitor of these K^+-transporters. Alternatively, and more realistically, clofazimine is simply a membrane-destabilizing agent, dismantling membrane architecture both directly and via lysophospholipids, with consequent dysfunction of vulnerable K^+-transporters.[25,38,39]

Notwithstanding possible interference with RNA polymerase, which seems to have been largely discounted,[28] intracellular redox cycling[1,12] and membrane disruption[9,28,29,37-39] are clearly the two favoured membrane-directed mechanisms of clofazimine-mediated antimicrobial activity. Although these are not mutually exclusive and possibly co-exist, it is likely that sensitive bacterial pathogens are differentially affected by these mechanisms. On the other hand, the insensitivity of Gram-negative bacteria to clofazimine is likely to reflect poor penetration of the outer membrane by this agent, and/or differences in the inner membrane phospholipid compositions of Gram-negative bacteria and Gram-positive bacteria.

These insights into the mechanisms of antimicrobial activity of clofazimine, which are summarised in Figure 2, have therefore identified novel targets/strategies for future development of antibiotics. These include firstly the respiratory chain of MTB.[12] Secondly, the major K^+-transporters of MTB, which are admittedly, diverse and complex targets.[42,43,48] Thirdly, and as recently proposed by others, the differences in phospholipid composition between the outer membranes of eukaryotic and prokaryotic cells may be exploited in the design of membrane disruptive agents which selectively target prokaryotes.[49,50]

5. Pharmacokinetic properties

Because of its lipophilicity, clofazimine is administered as a microcrystalline suspension in an oil-wax base in order to improve absorption.[51] In humans, the absorption of orally administered drug varies considerably (45%–62%) depending on whether the drug is taken with or without food. Ingestion of a 200 mg tablet taken with food resulted in a peak plasma concentration of 0.41 mg/L, with a time to C_{max} of 8 h; without food the corresponding peak plasma concentration was 30% less, while the time to C_{max} was 12 h.[52] Administration by the

oral route of 100, 300 or 400 mg clofazimine daily to leprosy patients resulted in average plasma levels of 0.7, 1.0 and 1.41 mg/L, respectively,[51] while peak serum levels of 4 mg/L were reported following daily intake of 600 mg of this agent.[52] As mentioned above, the limited activity of clofazimine against MTB in humans may be due to inadequate peak drug concentrations or an insufficient total dose as a result of low oral bioavailability and gastric intolerance.[53]

Because of its lipophilicity, clofazimine distributes primarily into fatty tissues, as well as cells of the mononuclear phagocyte system,[9] and following administration of clofazimine to mice at a dose of 25 mg/kg body weight for 28 days, the average concentrations of this agent in the lungs, spleen, fat and plasma were ~800, 4000 and 80 mg/kg and 3 mg/L, respectively.[9] In man, the concentration of clofazimine in the fat of leprosy patients has been reported to be as high as 5.3 mg/g, while concentrations of >1 mg/g were found in bile, gall bladder, kidney, pancreas, skin, liver, spleen, lymph nodes, eyes and lung.[54].

6. Adverse effects

The adverse effects of clofazimine have been reported in detail elsewhere,[10,55] and are generally dose related, primarily affecting the skin, eyes and gastrointestinal tract.[10] Though tolerable, but not necessarily acceptable, reddish-brown discolouration of the skin and conjunctiva are gradually reversible on cessation of therapy. Gastrointestinal tract side effects may be mild to moderate (abdominal/epigastric pain, nausea, diarrhoea, vomiting, gastrointestinal intolerance), or, less frequently, severe (splenic infarction, bowel obstruction, bleeding), and occasionally fatal.[10] Clofazimine crosses the placenta[56] and, albeit in very small amounts, the blood–brain barrier.[54] Importantly, clofazimine is not teratogenic/mutagenic[55,57] and does not possess myelosuppressive properties.[55]

7. Analogues of clofazimine

Two major strategies are being used in an attempt to increase the antimycobacterial potency of clofazimine in the setting of improved safety, namely, the development of: (i) analogues of clofazimine; and (ii) drug delivery systems.

In the case of analogue development, the late Dr J. F. O'Sullivan, a medicinal chemist and member of the Trinity College, Dublin team that originally developed clofazimine, designed and synthesized hundreds of analogues of clofazimine based primarily on varying the alkylimino substituents at position 2 of the phenazine nucleus, as well as the numbers, types and positions of halogen atoms on the phenyl substituents. The most promising of these were the tetramethylpiperidyl (TMP) derivatives, first described in the late 1980s, in which the isopropyl alkylimino group of clofazimine was replaced with the TMP group.[21] Apart from being equivalent to, or slightly superior to clofazimine with respect to antimycobacterial activity,[21,22,58,59] these agents were reported to have the following advantages over clofazimine: (i) they cause less skin discolouration because they are not taken up by body fat; (ii) they do not crystallize within macrophages, potentially decreasing their half-life in the body; and (iii) they accumulate in tissues at higher levels than clofazimine.[58,59] Unfortunately, however, these novel TMP-phenazines were described at a time when the global impact of the HIV pandemic and its ominous implications for the resurgence of TB, particularly in developing countries, were not fully appreciated. Consequently, there was little or no impetus for the clinical development of these agents.

Very recently, however, the Global TB Alliance, in partnership with several academic institutions, has embarked on a riminophenazine-based drug development programme in the belief that these agents show promise for treatment shortening in TB.[11] This is based on the premise that riminophenazines inhibit mycobacterial energy metabolism, an important target in slow-growing persisters.[11] Ideally, this research will identify a novel derivative with improved antimycobacterial activity, pharmacokinetics and safety profile, which can move quickly from preclinical to clinical development.[11] Several potential drug candidates

originating from Global TB Alliance-sponsored research were recently described by Yano *et al*.[12] and Lu *et al*.[60] The first of these, known as K56, is a more water-soluble form of clofazimine with comparable redox properties, in which the isopropyl group bonded to the imino nitrogen at position 2 on the phenazine nucleus is replaced by an aminoethyl-ethoxyethoxy group.[12] This agent is currently under investigation with respect to antimycobacterial activity *in vitro* and in animal models of experimental TB.[12] Lu *et al*.[60] have described a series of 12 analogues with improved solubility, as well as pharmacokinetic and therapeutic activity in a murine model of experimental infection. Interestingly, these agents, most of which possess heterocyclic groups on the imino nitrogen at position 2 on the phenazine nucleus, are structurally similar to B669.[25,38] This latter agent, as shown in Figure 1, possesses a cyclohexyl group on the imino nitrogen and was found to be more potent than clofazimine against Gram-positive bacteria.[25]

8. Alternative formulations of clofazimine

Low drug solubility is the primary rate-limiting step in the absorption of clofazimine,[51,61] improvement on which offers several advantages:[62]

- Increased rate and extent of drug absorption;
- Reduction of inter-subject variability in bioavailability;
- Reduction of dietary effects and other gastro-intestinal variables on drug absorption;
- Reduction of the drug dose, thus reducing the cost of therapy and dose-related side-effects; and
- Increased application of clofazimine in the treatment of MDR-TB and other mycobacterial diseases.

Several strategies have been developed to improve the dissolution and absorption properties of clofazimine, mainly by formulation into solid dispersions with non-toxic polymers/polymers such as polyethylene glycol, polyvinyl pyrrolidone, polyvinylmethyl ether/maleic anhydride, cyclodextrins, and biodegradable polylactic-co-glycolic acid.[61-64] These, as well as liposomal and nanosuspension formulations of clofazimine,[65-67] have demonstrated efficacy equivalent or superior to that of native clofazimine in the setting of decreased toxicity in models of experimental chemotherapy in mice infected with MTB or MAC. However, none of these has progressed to pre-clinical development, probably underscoring the low priority attributed to clofazimine as an anti-TB agent, as well as lack of financial incentive on the part of pharmaceutical companies.

Spray-drying is an alternative strategy that can be applied to improve solubility and enhance the dissolution rate and oral availability of poorly soluble drugs.[68,69] This process dries the droplets of their volatile substance and leaves non-volatile components in the form of dry particles, with particle size, morphology, density and volatile content controlled by the drying process parameters. Moreover, spray-drying crystalline drugs typically yields drug particles in the amorphous state, usually with increased water solubility, because little additional energy is required to break up the crystal lattice during the dissolution process.[70,71] Additional benefits of spray-drying include relatively low cost and energy-efficiency of the equipment, the possibility of continuous operation, and good control of the resulting physical properties of the dried material which can allow for the possibility of formulating for multiple delivery routes.[72] For example, spray-drying has been applied to formulate various drugs and biopharmaceuticals for inhalation. The lung has a large surface of absorption and a thin alveolar epithelium, allowing for rapid absorption, enabling a reduction in dose,

while maintaining an efficacious systemic concentration. By spray-drying using appropriate conditions and excipients, large porous particles are formed with geometric diameter d_{geo}> 5 μm, density ρ< 0.1 g/cm^3, with ideal aerodynamic properties such as aerodynamic diameter d_a of 1-5 μm for delivery to the alveolar region of the lungs.[73]

Several anti-TB drugs have been formulated as dry powder microparticles for pulmonary delivery. These include capreomycin,[74,75] para-aminosalicyclic acid,[76] PA-824 [77,78] and rifampicin,[79-81] studies in animal models of experimental TB, have demonstrated that direct delivery to the lungs results in high local concentrations and reduced bacterial burden compared to the same treatments delivered via other routes.

Although not yet tested in animal models of experimental TB chemotherapy, spray-dried amorphous, microparticle formulations of clofazimine, optimised with respect to physical properties and dissolution rates for oral and inhaled administration, have the potential to increase bioavailability and efficacy.[82]

9. Anti-inflammatory/immunosuppressive properties of clofazimine

In addition to its primary antimicrobial activity, clofazimine also possesses anti-inflammatory/immunosuppressive properties which underpin the reported therapeutic efficacy of clofazimine in various non-microbial, chronic inflammatory disorders, predominantly cutaneous in origin. These have been reviewed by Van Rensburg *et al*.[25] and include discoid lupus erythematosus, pustular psoriasis, Melkersson–Rosenthal syndrome, necrobiosis lipoidica and granuloma annulare, as well as cutaneous lesions in systemic lupus erythematosus.[83] More recently, coincident with the identification of novel targets/mechanisms of immunosuppressive activity, it has been proposed that clofazimine

holds broader therapeutic promise, encompassing non-cutaneous autoimmune disorders, such as multiple sclerosis and type I diabetes mellitus.[84] T lymphocytes in particular are sensitive to the immunomodulatory actions of clofazimine.

9.1 T-lymphocytes

Clofazimine, at concentrations comparable with peak serum concentrations attained during the chemotherapy of leprosy, has been reported to cause significant suppression of the mitogen– and antigen-driven proliferative responses of isolated T lymphocytes *in vitro*.[84,85] Two major mechanisms, both targeting plasma membrane K^+ transport, appear to underpin the inhibitory effects of clofazimine on the activation and proliferation of T lymphocytes. One of these targets is the sodium-potassium exchanger, Na^+, K^+-ATPase,[86] and the other is the Kv1.3 potassium channel,[84] both of which are electrogenic.

9.2 Na^+, K^+-ATPase

Na^+, K^+-ATPase exchanges 3 Na^+ for 2 K^+ ions, enabling eukaryotic cells to accumulate K^+intracellularly, which is essential for sustaining multiple activities, including the resting membrane potential, active transport of nutrients, various enzyme activities involved in cellular metabolism, and biosynthesis of macromolecules. The requirement for Na^+, K^+-ATPase in the mitogen-activated proliferation of T lymphocytes is well-recognised, being rapidly up-regulated by several fold, a critical event in cell proliferation.[86,87] Treatment of T-lymphocytes with clofazimine (at low micromolar concentrations) for 60 min was found to cause considerable inhibition of the mitogen-activated increase in Na^+, K^+-ATPase activity,

with smaller, but nevertheless detectable inhibition of pump activity in unstimulated cells.[85] At the same concentrations, clofazimine caused significant inhibition of the proliferative responses of T lymphocytes, the probable consequence of impaired Na^+, K^+-ATPase activity.[85] From a mechanistic perspective, the clofazimine-mediated decrease in T lymphocyte Na^+, K^+-ATPase activity was not preceded by detectable alterations in cellular ATP levels, but was associated with increased generation of arachidonic acid, compatible with hydrolysis of membrane phospholipids.[85] As mentioned above, this latter event may result from the increased activity of PLA_2, and/or direct acid hydrolysis of the ester linkage at the C2 position of the glycerol backbone of membrane phospholipids.[45] In either case, the result is formation of lysophospholipids. The involvement of lysophospholipids in clofazimine-mediated inhibition of mitogen- and antigen-activated up-regulation of Na^+, K^+-ATPase and proliferation of T lymphocytes was supported by three lines of evidence: i) the effects of clofazimine on cellular Na^+, K^+-ATPase activity and proliferation were mimicked by lysophosphatidylcholine; ii) inclusion of lysophospholipase or AT effectively abrogated the inhibitory actions of clofazimine and lysophosphatidylcholine; and iii) the activity of Na^+, K^+-ATPase in isolated membrane fractions was inhibited by lysophosphatidylcholine, but not by clofazimine.[85]

9.3 Kv 1.3 potassium channels

More recently, Ren *et al.*,[84] using a cell-based screen strategy, identified clofazimine as a pharmacological inhibitor of T cell receptor for antigen (TCR)-mediated intracellular signalling mechanisms involved in transcriptional activation of the gene encoding interleukin 2 (IL-2). More specifically, these authors observed that clofazimine interfered with Ca^{2+} signalling in T cells activated with the potent, non-physiological stimulus phorbol myristate

acetate (PMA)/ionomycin, by antagonism of the Kv 1.3 potassium channel.[84] This is a voltage-gated, delayed rectifier K^+ ion channel that regulates membrane potential and Ca^{2+} signalling in effector memory T cells.[88] The consequence of aberrant function of this electrogenic ion channel is interference with K^+ efflux and failure of the efficient membrane repolarization response necessary to drive Ca^{2+} influx via calcium release-activated Ca^{2+} (CRAC) channels, and possibly other types of Ca^{2+} channels. The consequence is impaired Ca^{2+} influx, leading to suppression of the cytosolic Ca^{2+} oscillation frequency necessary for activation of the calcineurin-nuclear factor of activated T cells (NFAT) signalling pathway, which initiates transcriptional activation of the IL-2 gene.[84]

9.4 Reactive oxygen species (ROS) and prostaglandin E_2

In mixed leucocyte suspensions, clofazimine may indirectly interfere with the proliferation of T cells by promoting the release of ROS and E-series prostaglandins (PGs), especially PGE_2, from bystander neutrophils and monocytes.[89]Arachidonic acid released from membrane phospholipids is the substrate for cyclooxygenase, resulting in production of PGE_2. Following its interaction with adenylyl cyclase-coupled EP2 receptors on T cells, PGE_2 initiates the production of anti-proliferative 3'-5'-cyclic adenosine monophosphate (cAMP).Arachidonic acid is also a direct activator of the ROS-generating system of phagocytes, NADPH oxidase.[90]These phagocyte-derived ROS are indiscriminate and interfere with the mitogen/antigen-driven proliferation of T cells.[89]

These three mechanisms of clofazimine-mediated T lymphocyte activation and proliferation are summarized in Figure 3.

In the setting of mycobacterial infection, the secondary immunosuppressive properties of clofazimine may be detrimental or potentially beneficial. In TB patients with severe disease

associated with advanced immunosuppression due to primary or acquired immunodeficiency, clofazimine-mediated interference with cell-mediated immunity may restrict the efficacy of other antimycobacterial agents. Taken together with the contention that clofazimine may be particularly effective in targeting slow-growing persisters,[11] this agent may be most efficacious if administered later, rather than earlier, in the course of antimycobacterial therapy. On the other hand, the immunosuppressive activity of clofazimine may be useful in controlling the adverse effects of therapy-associated recovery of cell-mediated immunity, as previously reported in leprosy patients.[3–6] This is of potential benefit in HIV-infected patients on dual highly active antiretroviral therapy (HAART)/anti-TB therapy who are vulnerable to the development of immune reconstitution inflammatory syndrome (IRIS).

10. Summary

The emergence of MDR and XDR TB has rekindled interest in the riminophenazine group of antibiotics. This has resulted in novel insights into putative mechanisms of antimicrobial activity, as well as a potential pipeline of analogues of clofazimine with improved pharmacokinetic profiles and therapeutic efficacies, possibly potentiated by the development of drug delivery systems. Although in the pre-clinical stages of development, the acquisition of novel analogues and/or formulations of clofazimine, together with a reassessment of the optimum timing of administration, especially in immunosuppressed individuals, may lead to a more discerning and efficacious clinical utilisation of these agents in the treatment of TB.

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Transparency Declaration

None of the authors has any conflicts of interest to declare.

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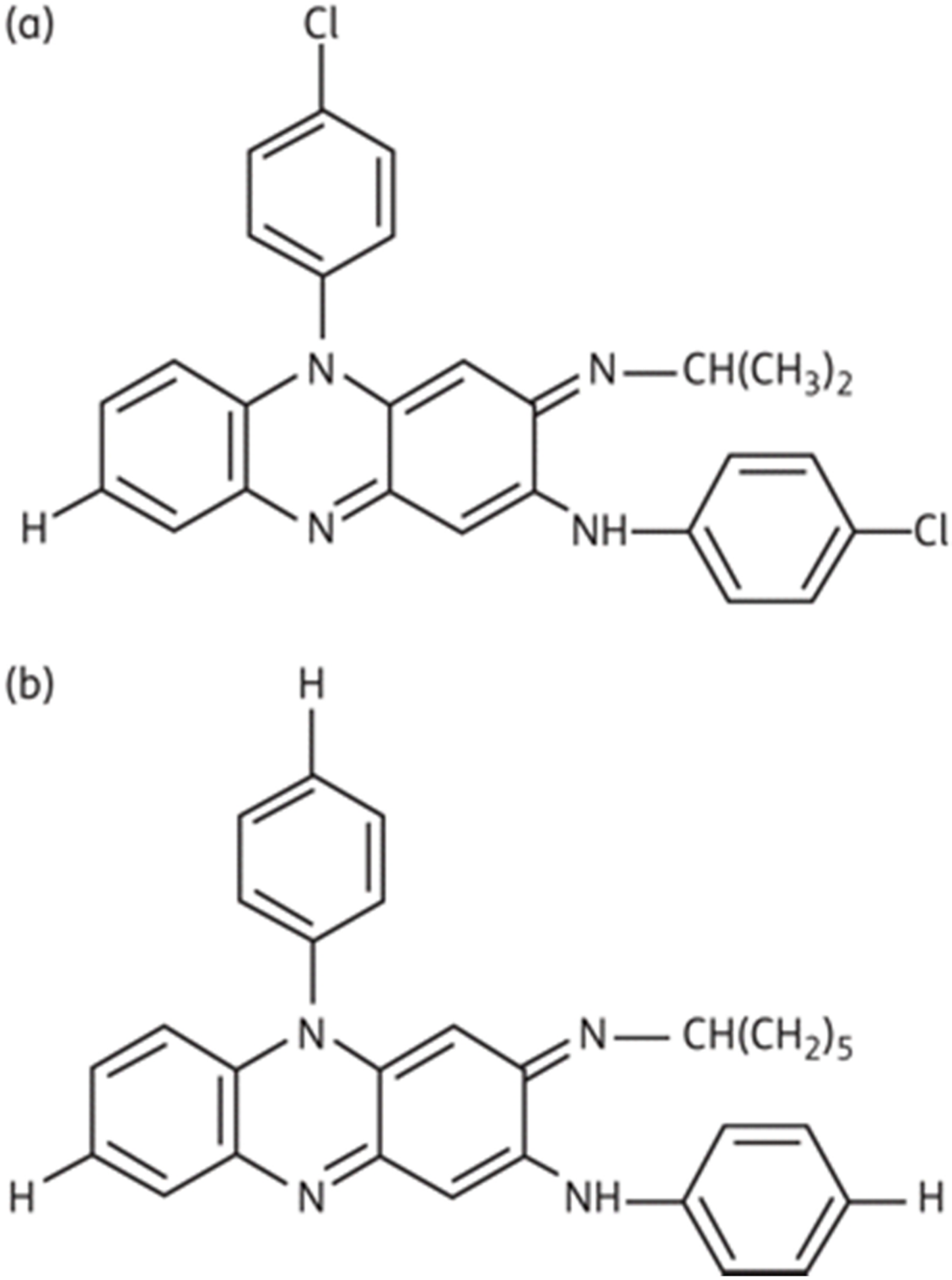

Figure 1: Molecular structures of (a) clofazimine [3-(*p*-chloroanilino-10-(*p*-chlorophenyl))-2,10-dihydro-2-(isopropylimino)-phenazine], molecular weight 473.4, and (b) B669 [3-anilino-10-phenyl-2,10-dihydro-2-(cyclohexylimino)phenazine], molecular weight 445.4.

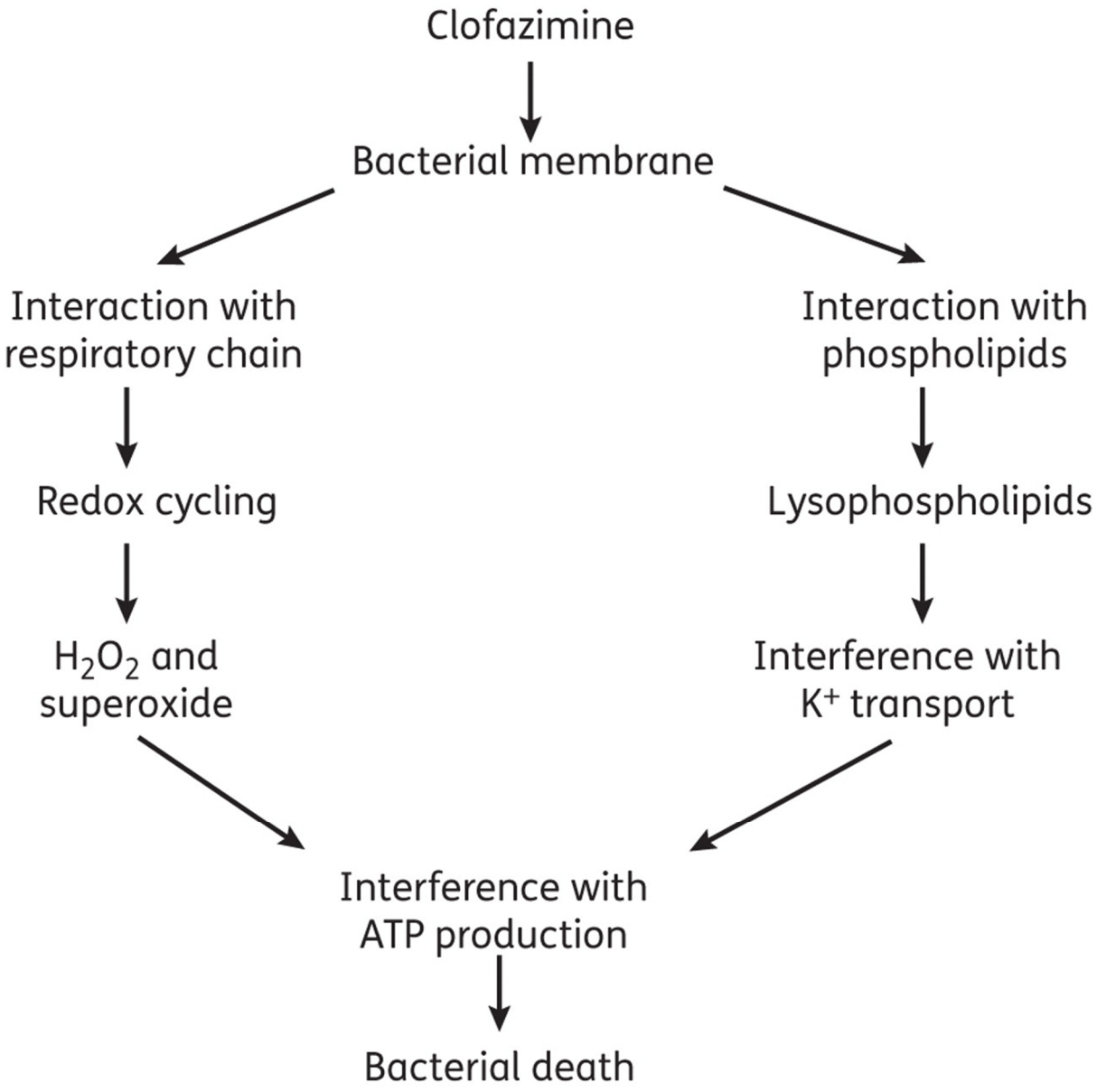

Figure 2 Putative membrane-directed mechanisms of the antimicrobial activity of clofazimine include the respiratory chain and ion transporters. Intracellular redox cycling, involving oxidation of reduced clofazimine, leads to the generation of antimicrobial ROS, superoxide and H_2O_2. Secondly, interaction of clofazimine with membrane phospholipids results in the generation of antimicrobial lysophospholipids, which promote membrane dysfunction, resulting in interference with uptake of K^+. Both mechanisms result in interference with cellular energy metabolism.

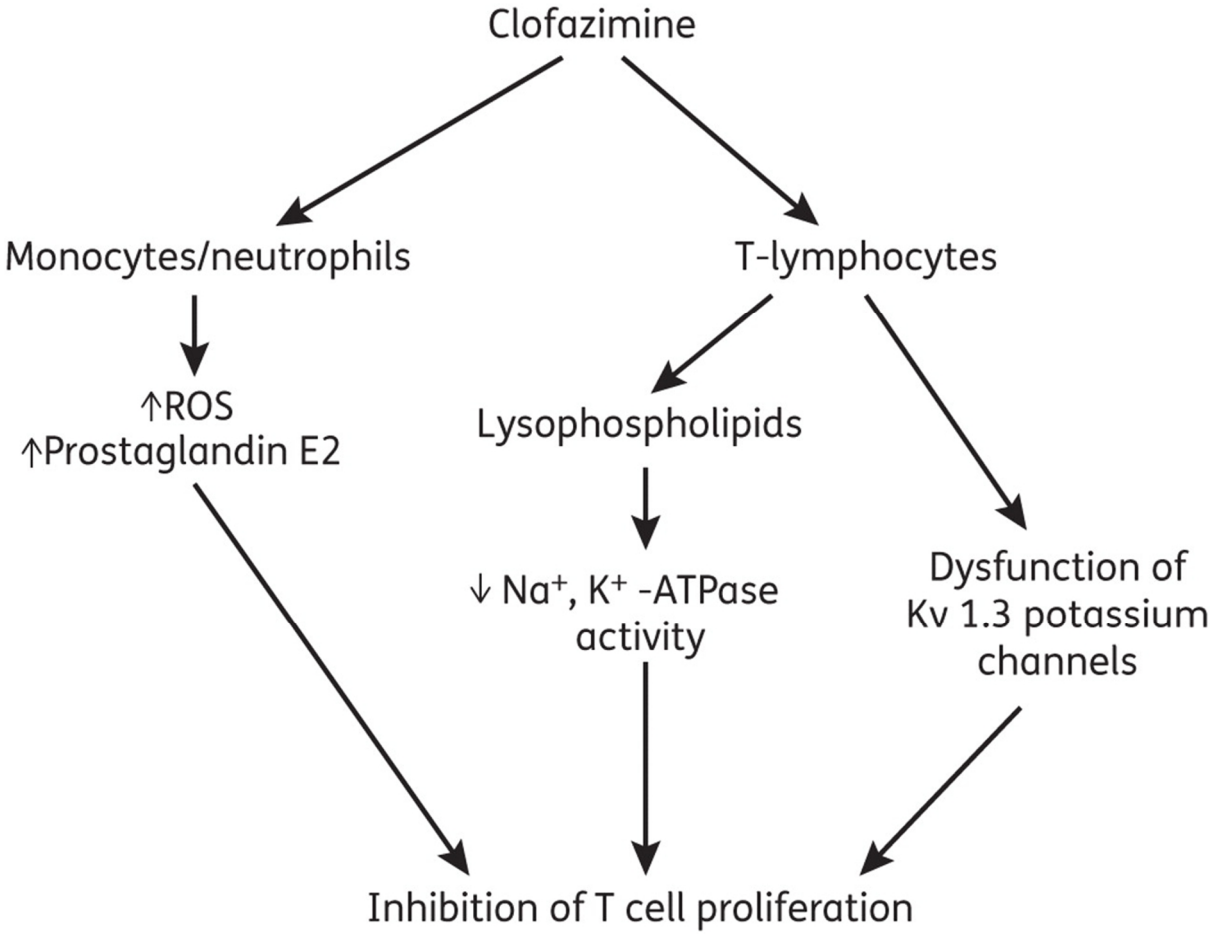

Figure 3: Putative membrane-directed mechanisms of the immunosuppressive activity of clofazimine