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Efficacy of clofazimine–modified cyclodextrin against *Mycobacterium avium* complex in human macrophages

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Abstract

Clofazimine, a water insoluble substituted iminophenazine derivative with anti-mycobacterial and anti-inflammatory activity, is recommended by the WHO for the treatment of leprosy. It is also active against disseminated *Mycobacterium avium* complex (MAC) disease in HIV-infected patients. Recently, we achieved a 4000-fold increase of clofazimine water solubility using a novel modified clofazimine–cyclodextrin complex synthesized and patented by our group [Wasserlösliche, Iminiophenazinderivate enthaltende pharmazeutische Zusammensetzungen, deren Herstellung und Verwendung, German Patent, DE19814814C2]. In this paper we examine the activity of this complex against MAC in human macrophages, and evaluate its cytotoxicity. MAC-infected macrophages were treated for 24 h with free or complexed clofazimine. The in vitro minimum inhibitory concentrations of both free and complexed clofazimine were 0.1 μg/ml. Free and complexed clofazimine inhibited the growth of MAC inside macrophages to a similar extent, while modified cyclodextrin alone had no observable effects on MAC or macrophages. Complexed clofazimine was not toxic to cells at concentrations effective against MAC. The TD_{50} of the modified cyclodextrin in THP-1 cell line was 297 μg/ml.

Keywords: Clofazimine; Modified cyclodextrin; Solubility; Efficacy; Mycobacterium; Macrophages; Cytotoxicity

1. Introduction

Clofazimine is a substituted iminophenazine derivative with anti-mycobacterial and inflammatory activity (Feng et al., 1981; Anderson et al., 1988). It is active against various Mycobacteria, including *Mycobacterium leprae* (Levy, 1986; Reddy et al., 1996), *Mycobacterium tuberculosis* (Damle et al., 1978; Oliva et al., 1998), *Mycobacterium avium–Mycobacterium intracellulare* (Gangadharam and Candler, 1977) and *Mycobacterium bovis* (Geigy Pharmaceuticals, 1986), among others. Clofazimine also is used in combination with other drugs for the treatment of disseminated *M. avium* complex disease in HIV-infected patients (Masur, 1993; Cohn et al., 1999; Reddy et al., 1999). Clofazimine is known to inhibit the growth of various cancer cell lines, including hepatocellular carcinoma, and recent studies have confirmed that clofazimine could be of value in the treatment of hepatocellular carcinoma in humans (van Rensburg et al., 1993, 2000).

As most iminophenazine derivatives, clofazimine has the disadvantage of being a highly hydrophobic drug (Feng et al., 1981, 1982) with inherent water solubility below 0.3 μg/ml. This restricting property added to the fact that only about 20% of a dose is absorbed

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from the gastrointestinal tract when administered as coarse crystals, knowing that the fraction of the dose absorbed appears to decrease as oral dosage of clofazimine is increased (Mathur et al., 1985), are responsible for the elevated doses administered to patients in order to generate appropriate pharmacokinetic levels and to guarantee therapeutical effects. This restricting property also has limited clofazimine to oral administration, an encapsulated microcrystalline suspension of the substance in oil–wax basis capsules, depriving the advantages of the intravenous and topical administrations.

Various attempts have been made to enhance the solubility of clofazimine and thus its bioavailability. In this context, O'Reilly et al. (1994) investigated in rats the effect of single and mixed systems of sodium cholate and sodium cholate/fatty acid mixtures, respectively, with respect to their solubility and intestinal absorption of clofazimine. A 16- to 63-fold increase of clofazimine solubility was observed, with the 63-fold enhancement being observed with 80 mM sodium cholate. The increase of gastrointestinal absorption found by O'Reilly et al. (1994) was attributed to the enhancement in solubility and increase in membrane permeability. Enhancement of bioavailability was also reported by Peters et al. (2000) following i.v. administration of a nanocrystalline clofazimine suspension to MAC-infected mice, they confirmed that nanocrystalline clofazimine was effective in reducing bacterial loads in the liver, spleen and lung of MAC-infected mice. Other attempts to enhance the solubility of clofazimine, e.g. modifying the clofazimine structure, have not led to better solubility.

Cyclodextrins (CDs), annular water-soluble molecules of glucose units, have been utilized in recent years as host molecules to produce inclusion complexes with a large variety of drugs and other substances (Duchene, 1987). The resulting complexes generally exhibit favorable changes of the characteristics of the guest molecule, such as increased solubility, enhanced stability of the drug to air and light, reduced side-effects, and moreover, a general improvement in the bioavailability (Duchene, 1987; Loftsson and Brewster, 1996; Rajewski and Stella, 1996). Unsubstituted cyclodextrins are generally used for this purpose. Nevertheless, some substituted cyclodextrins, including hydroxypropylated and permethylated cyclodextrins have also been utilized.

We have recently synthesized and patented a water-soluble cyclodextrin, which is polysulfated or polyphosphated, and covalently linked to a sterol or sterol derivative (Steffan et al., 2002). The succinic acid monocholesterylester-linked sodium salt of cyclodextrin sulfate, referred to below as "modified cyclodextrin" (mCD), was used to solubilize clofazimine. This mCD could be used also to solubilize other iminophenazine derivatives. Complexes of clofazimine–mCD have been obtained, increasing the water solubility clofazimine by about 4000-fold. Considering that such solutions can be freeze–dried to give solid compositions, which can be dissolved subsequently in smaller amounts of water, an enhancement of solubility in the range of four orders of magnitude or more can be reached easily.

Rather than complex characterization, e.g. complexation ratios, complex confirmation, solubility diagrams, etc. which are objects of a pending paper, the aims of this present study were to determine the minimal inhibitory concentration (MIC) of this novel complex and to investigate the anti-mycobacterial activity of the clofazimine–mCD complex in MAC-infected macrophages in comparison with that of the free, water-insoluble clofazimine. In addition, to evaluate the cytotoxicity of the complex and modified cyclodextrin, using human macrophages and the macrophage-like cell line THP-1.

2. Materials and methods

2.1. Reagents

Clofazimine, bovine serum albumin (BSA), sodium dodecyl sulfate (SDS), NaCl, KCl, sucrose, dimethylsulfoxide (DMSO), ammonium sulfate and HEPES were obtained from Sigma (St. Louis, MO, USA). Other chemicals were purchased from Merck. Solutions were prepared in distilled water further purified in a Barnstead Nanopure filtration apparatus.

2.2. Preparation of modified cyclodextrin

The detailed preparation of the modified cyclodextrin is disclosed in reference (Steffan et al., 2002), basically it was accomplished first by preparing the succinic acid cholesterylester linked

mono-6-deoxy-6-amino-β-cyclodextrin and subsequent sulfatation. The obtained succinic acid monocholesterylester-linked β-cyclodextrin was sulfated using trimethylamine-sulfurtrioxide. The product was then precipitated with a sufficient amount of ethanol, dissolved in a small amount of water and re-precipitated with ethanol. The obtained product was dissolved in an aqueous solution of sodium acetate, laced with activated charcoal, followed by filtration. The crude product was precipitated from this solution with ethanol. The product was finally dissolved in water, sterile filtered and freeze–dried.

2.3. Preparation of clofazimine and the clofazimine–modified cyclodextrin complex

Clofazimine to be used as free drug was dissolved first in DMSO and then diluted in distilled water to at least 40 times the required final concentration (since BACTEC vials contain 4 ml of medium, injections of 0.1 ml results in a 1/40 dilution).

The complex (clofazimine–mCD) was prepared by dissolving the modified cyclodextrin in aqueous solution of ammonia acetate. To this solution appropriate amounts of clofazimine (clofazimine:cyclodextrin at a ratio of about 3:1) were added while stirring. Subsequently, a deep red solution was obtained (Steffan et al., 2002), the solution was filtered through a sterile filter and freeze–dried. Clofazimine–mCD was then dissolved and diluted to appropriate concentrations in distilled water.

The accurate determinations of clofazimine and the clofazimine–mCD samples' concentrations were realized by HPLC using a Rabbit-HP (Rainin, Emeryville, CA) liquid chromatograph and a prepacked 30 cm × 3.9 mm i.d., C_{18} Mirosorb column (Rainin Instruments, Woburn, MA). The assays were performed at room temperature using 0.1 M monobasic sodium phosphate:methanol at a ratio of 20:80 (v/v) as the mobile phase. Flow rates of 0.9 ml/min achieved good resolution. The separation and determinations were monitored at 284 nm.

2.4. Microorganisms

Two different strains of *M. avium–M. intracellulare* complex were used in two different sets of experiments. For the first set of experiments, MAC 11 obtained from Drs. K. Hadley and D. Yajko (San Francisco General Hospital) was used. MAC 101 was provided by Dr. V.M. Reddy (University of Illinois at Chicago). MAC 101 was propagated in beige mice to sustain the virulence of the bacteria. The spleens of the infected mice were homogenized and MAC was cultured on 7H11 plates. Smooth transparent colonies were isolated, grown in 7H9 broth, and stored in 20% (v/v) glycerol in 7H9 in polypropylene vials at −70 °C until use. MAC was thawed and cultured in Middlebrook 7H9 broth and after 7 days of incubation, the bacterial suspension was subcultured 24 h before the infection of the macrophage monolayer, and the bacterial suspension was adjusted to 10^7 ml^{-1} by using a McFarland standard. MAC 101 strain was used to confirm some results obtained using MAC 11.

The minimum inhibitory concentrations (MIC) of clofazimine for these strains were determined by the BACTEC method in Middlebrook 7H12 broth.

2.5. MIC determination by BACTEC

The applied quantitation method has been described previously in detail (Salem and Düzgünes, 2003). Briefly, growth of MAC was monitored radiometrically by means of a BACTEC 460-TB instrument (Becton Dickinson). Growth was measured as function of the release of ^{14}C-labeled CO_2 resulting from the metabolism of ^{14}C-palmitate in Middlebrook 7H12 broth (Siddiqi et al., 1981). Growth was expressed as the growth index (GI). The data are presented in each case as the mean of three determinations.

2.6. Infection and treatment of human monocyte-derived macrophages

Human peripheral blood was obtained from a healthy donor and mononuclear cells were isolated by Histopaque-1077 (Sigma) density gradient centrifugation. The cells were then cultured at a density of 2×10^6 per well in 48-well tissue culture plates (Falcon) at 37 °C and 5% CO_2 in a cell culture incubator, details are in reference (Salem and Düzgünes, 2003).

MAC was cultured in Middlebrook 7H9 broth. After 7 days of incubation, the bacterial suspension was subcultured just 24 h before the infection of the macrophage monolayer, the bacterial suspension was adjusted to 10^7 ml^{-1} by using a McFarland standard.

The macrophage layer was inoculated with the MAC suspension at a ratio of 5:1 (Salem and Düzgünes, 2003). After the first day of infection the cell number and the viability of the cells were ascertained by the counting solution technique (naphthol blue black stain) and by Trypan Blue exclusion, respectively.

Infected monolayers were treated with 0.1, 0.25, 0.5 and 1 μg/ml of either free clofazimine, or clofazimine–mCD complex for 24 h. These concentrations of clofazimine are in a concentration range achievable in the serum of treated patients. The medium was then removed, and the cells were washed three times with HBSS and incubated for 7 days in DME-HG-L-glu + 20% FBS. Seven days after the treatment, the growth index of MAC was determined after lysis of the macrophages with 0.25% SDS, which was subsequently neutralized with 10% BSA. The lysate was diluted serially to 10^3 and aliquots of the diluted lysates (200 μl) were injected into the BACTEC vials, which were incubated at 37 °C and assayed every 24 h. In all the macrophage experiments, triplicate wells of macrophages were utilized for each condition. The growth index of MAC obtained from each of the wells was determined in duplicate.

2.7. Toxicity tests in THP-1 cells

The toxicity of the modified cyclodextrin was determined both in monocyte-derived macrophages culture (which do not proliferate) and in the THP-1 cell line. The latter cells were used to ascertain the lack of toxicity in a cellular system where continuous cell division is taking place, to provide further support for the potential use of the cyclodextrin formulation in therapy.

The Alamar Blue Assay was used to establish relative cytotoxicity of the modified cyclodextrin used in this study. This assay incorporates a colorimetric growth indicator based on detection of metabolic activity of the cells (Konopka et al., 1996). THP-1 cells were plated and differentiated into adherent, macrophage-like cells by treatment with 160 nM phorbol 12-myristate 13-acetate (Sigma) for 24 h in 24-well plates at a cell density of 10^6 per well. They were incubated for 1 week in RPMI + 10% FBS + 1% S/P + 1% L-glu, after which the culture medium was removed and the cells were washed twice. Different amounts of mCD dissolved in water, 0.9, 4.5, 9.1, 91 and 455 μg/ml, were added to the wells. The cells were then incubated for 24 h at 37 °C. The medium was removed, the wells were washed twice, 2 ml of culture medium with 10% Alamar Blue was added to each well and then incubated for 2 h. The untreated control wells were submitted to the same procedure.

2.8. Toxicity tests in human monocyte-derived macrophages

Macrophages isolated for experiments on the intracellular activity of clofazimine were also used to evaluate the cytotoxicity of the modified cyclodextrin. They were incubated with 1.5, 3.75, 7.5, 15.0, 22.5 and 45 μg/ml of mCD for 24 h at 37 °C in 5% CO_2. After this period of time the medium (with the mCD) was removed and the wells were washed twice. One milliliter of culture medium with 10% Alamar Blue was added to each well and then incubated for 2 h. The untreated control wells were submitted to the same procedure. Alamar Blue was removed from all these wells and the cells were incubated in fresh media for 24 h more. The experiment was realized in the same way in order to assess the toxicity of the mCD at 48 h of incubation.

3. Results

3.1. Solubility and stability of clofazimine in modified cyclodextrin

The accurate determination of clofazimine and the clofazimine–mCD concentrations and dosing were ascertained using a highly specific and reproducible HPLC method of analysis. The HPLC analysis was ran over 2 days in order to achieve the correlation curves. A straight-line calibration plot was obtained for the range of concentrations between 0.1 and 100 μg/ml with the corresponding correlation coefficient of $r = 0.9941$ ($r^2 = 98.82\%$). Concentrations above the higher limit of linearity were properly diluted for appropriate determination. The study of the precision of the HPLC assay was performed over a 2-day period, calculating the variation coefficient for five determinations per day of two different solutions of the clofazimine standard. The resulting coefficient of variation (CV) was 0.87%. The limit of

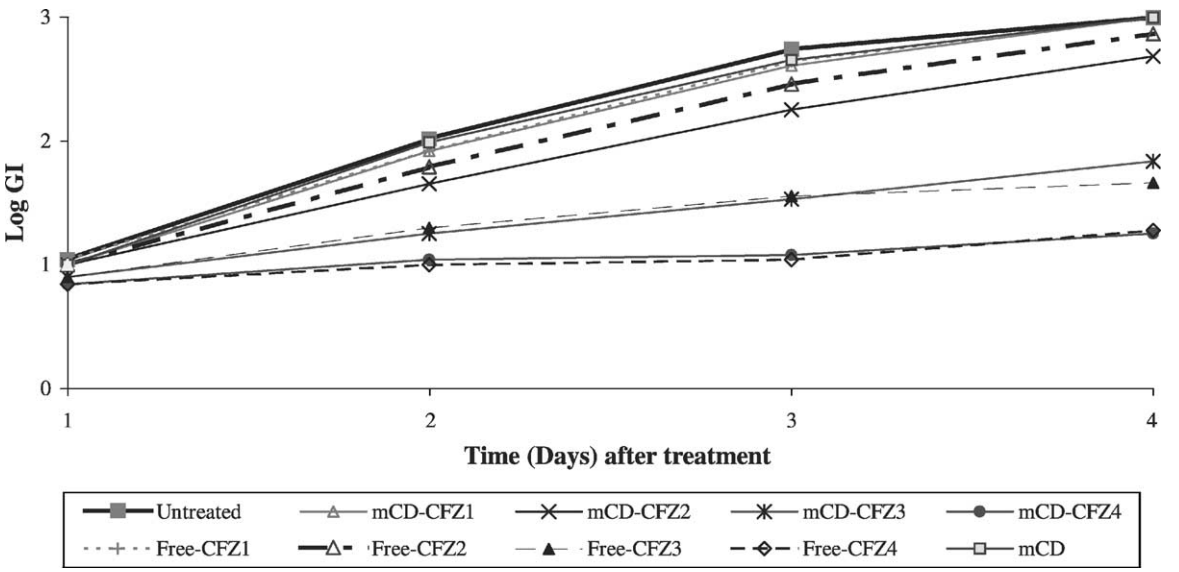

Fig. 1. Representative radiometric data showing the effects of free or a complex containing clofazimine on the growth of *M. avium* complex. These data were used to establish the MICs for the clofazimine (free-CFZ; 1, 2, 3 and 4 corresponding to 0.01, 0.05, 0.1 and 0.2 μg/ml of drug, respectively), or complex containing clofazimine (mCD–CFZ; 1, 2, 3 and 4 corresponding to 0.01, 0.05, 0.1 and 0.2 μg/ml of drug, respectively). Growth is represented as log GI values obtained with BACTEC instrument for 4 days following the exposure to drug as described in Section 2.

detection was defined as the lowest detectable concentration, with a signal-to-noise ratio > 3:1. A value of 0.02 μg/ml was found for clofazimine.

Complexation effects on clofazimine water solubility were assessed using the aforementioned method of analysis. Solubility studies showed that water solubility of free clofazimine was 0.210 μg/ml, while water solubility of clofazimine–mCD was 0.830 mg/ml (about 4000-fold enhancement of clofazimine water solubility). The stability study proved no degradation products after 20 days of storage of clofazimine–mCD solutions at 37 and 48 °C. The solutions retained their initial content of clofazimine and no other chromatographic peaks were observed.

3.2. MIC of clofazimine–modified cyclodextrin

The BACTEC vials were read at the same time every day till 1/100 control showed a GI > 30 with an increase in GI > 10 for three consecutive days (Salem and Düzgünes, 2003). In this study, the MIC was considered the lowest concentration of clofazimine or clofazimine–mCD complex that inhibited growth by 90%. The in vitro determinations of MICs for clofazimine and the clofazimine–mCD complex were 0.1 μg/ml in both cases (Fig. 1), indicating that the host molecule (mCD) has had no negative effect on the activity of the guest molecule (clofazimine).

DMSO, solvent used to solubilize the free clofazimine, has been proved not to affect MAC growth at the highest concentration normally used to dissolve the drug.

The MICs found for clofazimine and for the complex were within the published range for MAC, which ranges for clofazimine between 0.1 and 2 μg/ml (Lindholm-Levy and Heifets, 1988; Siddiqi et al., 1993). Once MICs were established, higher concentrations of both the free drug and clofazimine–mCD complex were tested in MAC-infected macrophages.

3.3. Intracellular activity of clofazimine and the clofazimine–modified cyclodextrin complex

The effect of the clofazimine–mCD complex against MAC inside human macrophages was compared with that of the free clofazimine in a concentration range achievable in the serum of treated patients. As shown

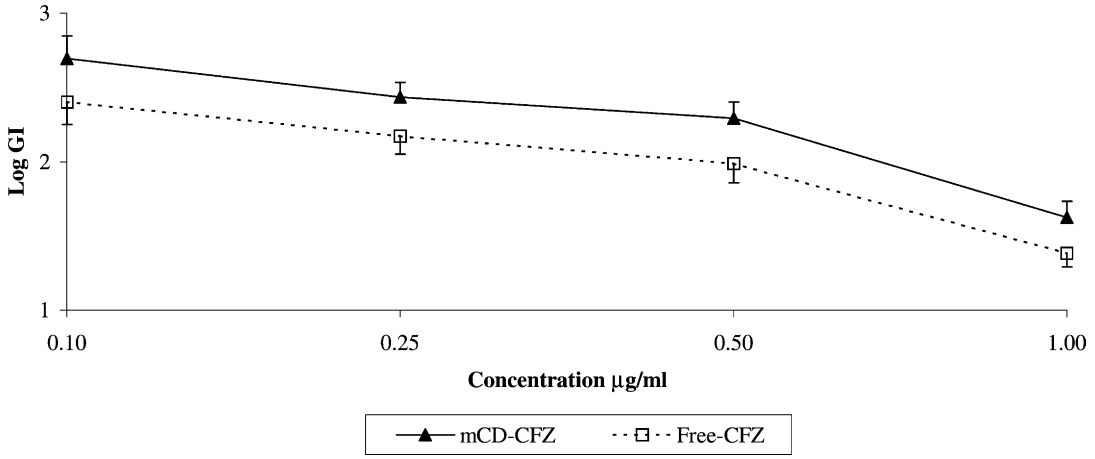

Fig. 2. Effects of free and complex containing clofazimine against MAC growth inside monocyte-derived macrophages. Infected cells were incubated with free (free-CFZ) or modified cyclodextrin clofazimine complex (mCD–CFZ) for 24 h. After 7 days, cells were lysed to determine MAC growth by BACTEC. The untreated controls are represented by 0 μg/ml on the abscissa.

in Fig. 2, the growth index of MAC decreased as the clofazimine or clofazimine–mCD complex concentration increased, in comparison with the untreated controls at the end of the third day of incubation following the initial treatment. Both treatments, at 0.5 μg/ml, were effective in reducing the growth index by more than 80% compared to the untreated. Similar results were obtained with the MAC 101 strain (Fig. 3). At 1 μg/ml, free or the clofazimine–mCD complex reduced the growth index to 95 and 91% of the untreated control, respectively.

Although there were slight differences in the GI readings between the clofazimine–mCD complex and the free drug (Figs. 4 and 5), the ANOVA test indicated that these differences were not statistically significant. These differences in activity found between free drug and the clofazimine–mCD complex cannot be attributed to the inaccuracy in sample concentrations,

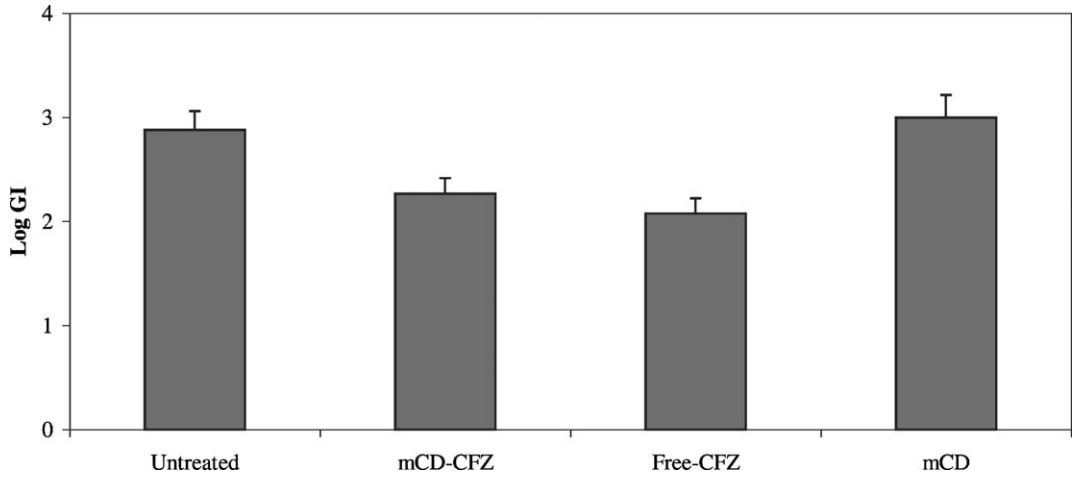

Fig. 3. Effects of free (free-CFZ) or complex containing clofazimine (mCD–CFZ) and modified cyclodextrin alone against MAC 101 growth inside monocyte-derived macrophages. The macrophages were lysed to determine the GI of MAC following the procedure described in text.

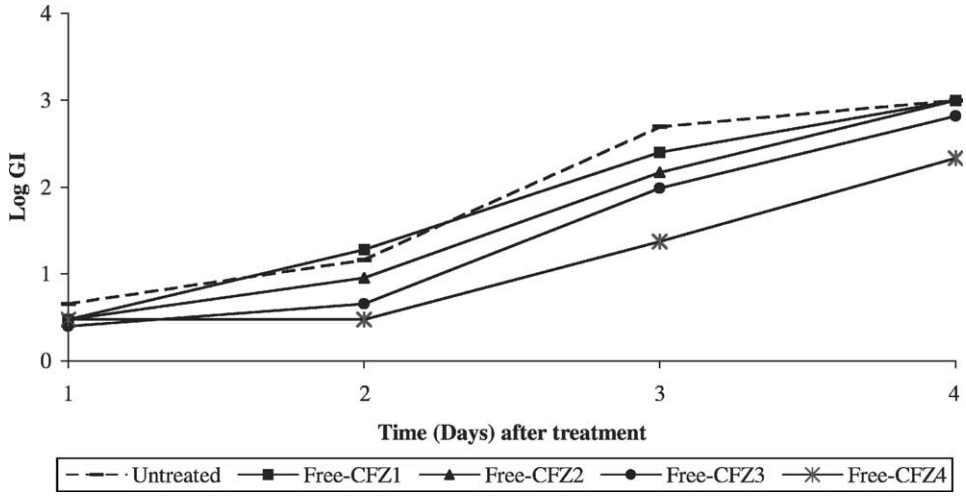

Fig. 4. Treatment of MAC-infected monocyte-derived macrophages with free clofazimine. Following infection, the cells were treated for 24 h with free clofazimine (free-CFZ; 1, 2, 3 and 4 corresponding to 0.1, 0.25, 0.5 and 1.0 μg/ml of drug, respectively). The macrophages were lysed on day 7 and the growth index was determined as described in Section 2.

yet HPLC method was applied to ensure that drug dosing was accurate.

The overestimation of the drug activity, due to possible removal of macrophages and MAC during washing procedures, was also excluded. To confirm that, macrophages viability was established by counting viable cells in infected but untreated controls, as well as MAC-infected cells treated with clofazimine–mCD, free drug or incubated alone with mCD. Macrophages viability results, compared to the untreated controls after 7 days of infection, were 114, 107 and 98% for clofazimine–mCD, free clofazimine and modified cyclodextrin, respectively. These results helped to confirm that the exhibited activity is integrally intrinsic to

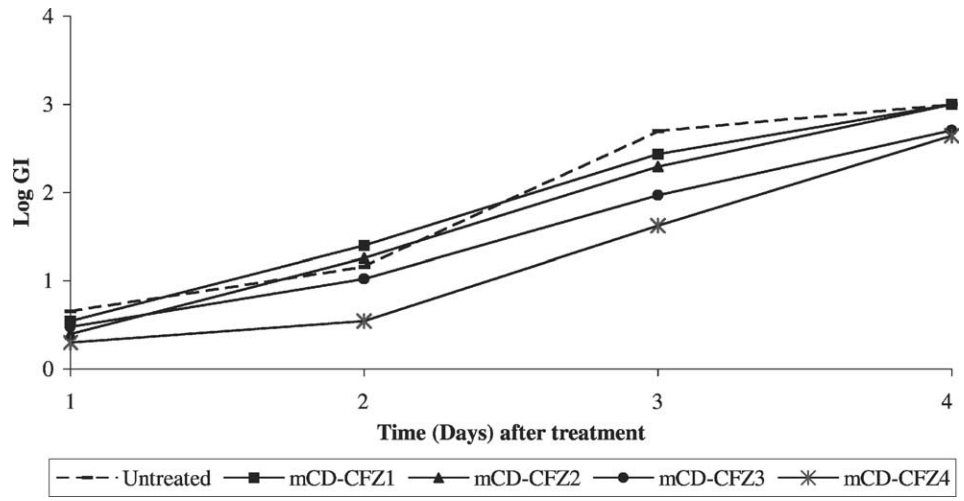

Fig. 5. Treatment of MAC-infected monocyte-derived macrophages with modified cyclodextrin clofazimine complex. Following infection, the cells were treated for 24 h with complex containing clofazimine (mCD–CFZ; 1, 2, 3 and 4 corresponding to 0.1, 0.25, 0.5 and 1.0 μg/ml of drug, respectively). The macrophages were lysed on day 7 and the growth index was determined as described in Section 2.

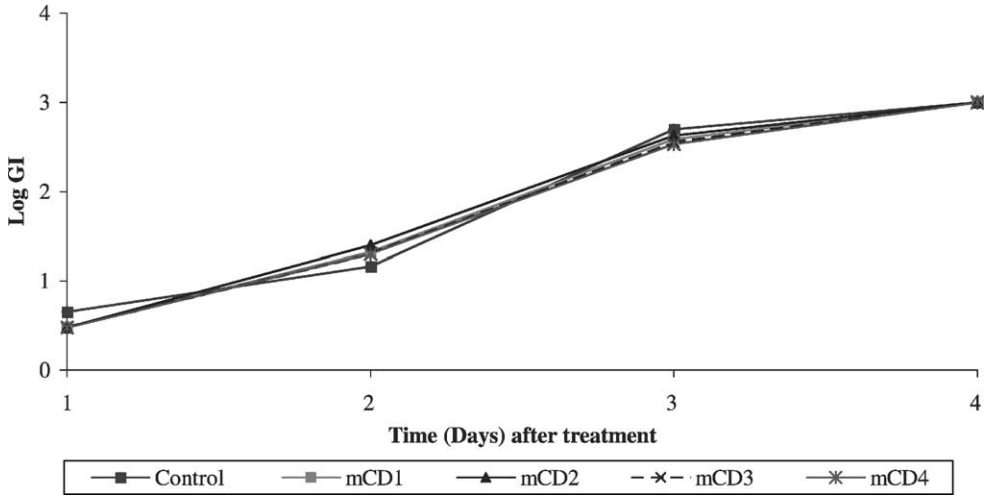

Fig. 6. Effects of modified cyclodextrin alone on MAC growth. Following infection, the cells were incubated for 24 h with modified cyclodextrin (mCD; 1, 2, 3 and 4 corresponding to 1.5, 3.75, 7.5 and 15 μg/ml of modified cyclodextrin, respectively). The monocyte-derived macrophages were lysed on day 7 and the growth index was determined as described in Section 2.

drug mechanism of action and not due to differences in MAC numbers due to removal procedures.

The possible inhibitory effect of DMSO on the growth of MAC, or on macrophage viability was also investigated. For these experiments, MAC or MAC-infected macrophages were incubated with DMSO at the highest concentration normally used to prepare the free clofazimine. No effects of DMSO were detected in either case. Modified cyclodextrin alone caused no effect on MAC growth compared to untreated controls in the range of concentrations present in the clofazimine–mCD complex (Fig. 6).

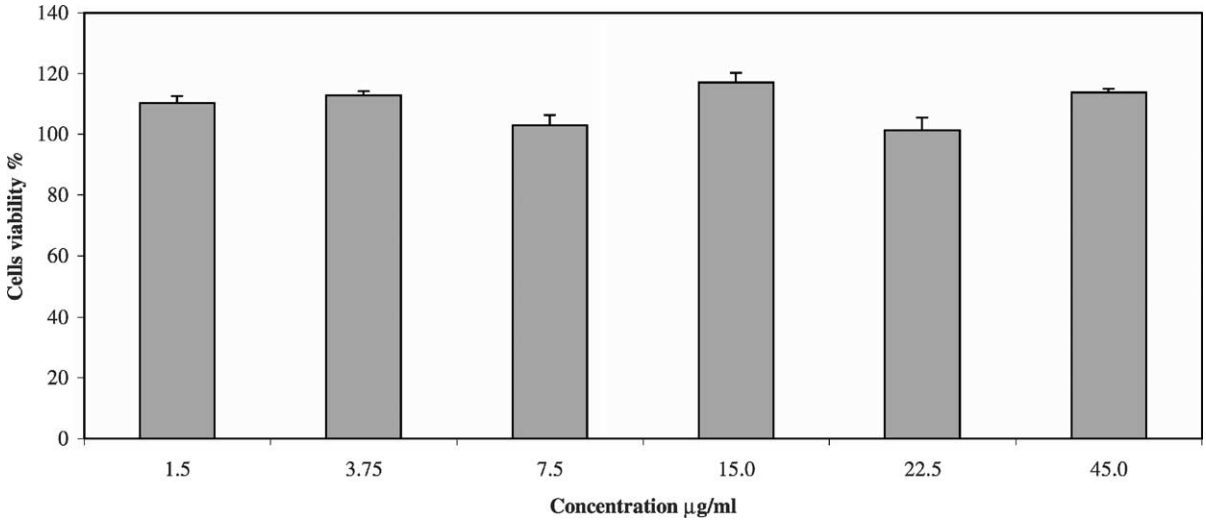

Fig. 7. Cytotoxicity of the modified cyclodextrin in monocyte-derived macrophages applying the Alamar Blue technique. Readings were obtained in comparison against the o.d. results of untreated controls (macrophages with no added cyclodextrin).

3.4. Cytotoxicity

The characteristic value for cytotoxicity has been obtained from the cytotoxicity curves by representing four different concentrations of mCD against THP-1 cell viability percentage, measured by Alamar Blue method against untreated controls. In order to predict the mCD cytotoxicity in THP-1, calculations were made on the basis of TD_{50} defined as the cyclodextrin concentration that produces 50% cytotoxic effect. This was obtained by extrapolating in the graph where the 50% point intercepts the dose response curve to the concentration along the *x*-axis. TD_{50} was found to be 297 μg/ml.

When the Alamar Blue technique was applied to determine the cytotoxicity of the modified cyclodextrin in monocyte-derived macrophages, the results of cell viability were about 100% as can be observed in Fig. 7. The unexpected results above 100% were obtained in comparison against the o.d. results of untreated macrophages (no added cyclodextrin) used as controls. The possible interference of mCD with the Alamar Blue method was discarded after verifying that mCD incubated alone with the reagent in cell-free wells gave zero optical readings after 24 and 48 h at the highest concentration of mCD.

4. Discussion

In this study we have found that clofazimine water solubility could be greatly enhanced by complexation with a modified cyclodextrin in comparison with the ineffective use of β or hydroxypropyl-β-cyclodextrins (data not shown). The approximately 4000-fold enhancement of the solubility is by far the highest among reported attempts to solubilize this antimicrobial agent. For example, the study by O'Reilly et al. (1994) reported an increase of 16- to 63-fold in clofazimine water solubility.

Cyclodextrins do not normally interfere with microbial cells, and it was of interest to us to investigate whether our modified cyclodextrin could cause any extra inhibition in MAC growth, or whether it could reduce the activity of the free drug. The results obtained in the MIC determination proved clearly that both the complex and the free drug have the same MIC, which was in accordance with the published data for clofazimine (Lindholm-Levy and Heifets, 1988; Siddiqi et al., 1993). The inhibition of MAC inside macrophages was similar, confirming that the complexed clofazimine was available inside macrophages. The slight differences found in activity between the free drug and the modified cyclodextrin complex could be related to the association–dissociation process in the aqueous substrate–cyclodextrin system, since it is a very dynamic process and is a function of drug and cyclodextrin concentrations, binding constant, and dilution (Uekama et al., 1994). Further studies are necessary to ascertain whether the complex is taken up by macrophages, or whether the drug is transferred from the complex to the macrophage.

One of the major requirements for drug carriers, enhancers or excipients is that they should have either no or acceptably low levels of intrinsic cytotoxicity. Cytotoxicity studies concluded with the determination of the TD_{50} for THP-1 cells, showing a value of 297 μg/ml. On the other hand, the human monocyte-derived macrophages experiment showed clearly that in the range of concentrations of mCD applied to cells the viability was alike. It was expected that the behavior of both cell lines would be different due to the biological differences between them, thus results were accepted as an indicator of low toxicity and as a first approach to evaluate this novel modified cyclodextrin.

Improving the solubility of poorly soluble drugs is essential to achieve better bioavailability and to reduce side-effects. In this context, our studies indicate that this modified cyclodextrin can solve the solubility problem of clofazimine and, potentially, other iminophenazine derivatives. It is expected that this clofazimine complex would provide the means for an orally administered formulation that could contribute to higher gastrointestinal absorption. This formulation may also be applicable to other routes of administration, such as intravenous, with improved therapeutic efficacy for the treatment of disseminated MAC disease. The clofazimine–mCD formulation may also be incorporated in liposomes, which has been shown to be active against MAC in infected human macrophages (unpublished data).

The excellent results obtained enhancing clofazimine water solubility, and the similarity of efficacy when compared to the free drug, justify obviously further research with this clofazimine–mCD complex.

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