Available online at www.sciencedirect.com
SCIENCE DIRECT
Research in Microbiology 156 (2005) 904–910
Research in Microbiology
www.elsevier.com/locate/resmic

Exploring *Mycobacterium avium* inhibition by macrocyclic compounds

Suzana David [a,*], Vanessa Barros [a], Krassimira Passos Guerra [b], Rita Delgado [b,c]

[a] *Mycobacteriology Unit, Instituto de Higiéne e Medicina Tropical/Universidade Nova de Lisboa (UNL), Rua da Junqueira 96, 1349-008 Lisbon, Portugal*
[b] *Instituto de Tecnologia Química e Biológica, UNL, Apartado 127, 2781-901 Oeiras, Portugal*
[c] *Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisbon, Portugal*

Received 3 May 2005; accepted 3 May 2005

Available online 24 June 2005

Abstract

Derivatives of synthetic macrocyclic compounds, MCC, 12- and 14-membered tetraazamacrocycles with *N*-pendant arms, such as *N*-methyl (Mepy14), *N*-acetate (DOTA, TETA and ac_3py14) and *N*-methylphosphonate (DOTP) groups, were investigated in terms of their in vitro activity against *Mycobacterium avium* and for intracellular clearance, using the murine macrophage cell line J-774. Perspective results on a laboratory strain, of opaque morphology, showed in vitro activity with varying inhibitory patterns from one compound to another. The most active compounds, such as TETA, presented *N*-acetate pendant arms. Inhibition levels of 90% and above were obtained at 50 mg/l. Inhibition was confirmed with both the free compound and its iron(III) complex for DOTP, Mepy14, ac_3py14, and TETA. However, with DOTA, no inhibitory effect was observed for the iron(III) complex, suggesting that chelation was at the origin of the inhibitory effect or that the donor atoms of the ligand were strongly involved. Nevertheless, simple experiments indicated that ferric ion might not be responsible for this reversed activity. Intracellular activity using 50 mg/l of TETA confirmed in vitro results with the laboratory strain. Results expressed as relative growth (%), of the drug-containing samples compared to control samples ranged from 2 to 123% (growth promotion) with no apparent relationship between inhibitory activity and the colony morphology of the strains. These studies showed that the evaluation of synthetic macrocycles may be relevant in development of a new family of compounds for use against *M. avium* infections.

Keywords: *Mycobacterium avium*; Chemotherapy; Macrocyclic compounds

1. Introduction

Second to *Mycobacterium tuberculosis*, the most important group of opportunistic pathogens responsible for pulmonary disease of mycobacterial origin are the members of the *M. avium* complex (MAC), also considered emerging pathogens in the advent of the AIDS pandemic [1]. MAC strains are characteristically resistant to antimycobacterial drugs, in part due to a highly stringent cell wall barrier [12,13,25,27], reducing the number of drugs available for treatment. MAC infections are also considered difficult to treat because they are often polyclonal [1,5,32] and subject to variation in drug susceptibility testing due to such external factors as temperature, growth phase, growth rate etc., and to factors for which regulatory mechanisms are still unknown, namely as regards colony morphology [18,31,33].

Macrocyclic compounds (MCCs) seem a promising tool in drug development due to the fact that their properties can be considerably changed by modifying the backbone or by introducing *N*-substituents [9,10,15], with the latter characteristic also being of interest in a drug delivery perspective. MCCs are also promising in that they already have medical applications as contrast-enhancing agents for magnetic resonance imaging (MRI), in nuclear medicine for radioimmunoscintigraphy or radioimmunotherapy and in chelation therapy [3,4,8,17,19–22,24]. Moreover, experimental studies have recently shown that an acetate derivative of a tetraazamacrocycle exerted in vitro activity against *M. tuberculosis* and also induced the host's mycobactericidal activities [14].

* Corresponding author.
E-mail address: suzanadavid@IHMT.UNL.PT (S. David).

0923-2508/$ – see front matter
doi:10.1016/j.resmic.2005.05.003

From these considerations, it is the purpose of this report to study the effect of synthetic MCCs of different macrocyclic cavity sizes and diverse types of *N*-substituents, their in vitro antimycobacterial activity and their ability to obtain the intracellular clearance of *M. avium*.

2. Materials and methods

2.1. Strains

M. avium strains, a laboratory strain of serotype 4 (pigmented opaque morphology) and four clinical isolates 101, 5196, 61596, 94896 (non-pigmented transparent morphologies), were selected for this study. Strains were characterized according to colony morphology in terms of pigmentation (pigmented or not pigmented) and the ratio of opaque to transparent colonies from a total of 100 colonies (expressed as a percentage), as observed after 8–10 days of culture on Middlebrook 7H11 agar plates (Table 1).

The selected strains were subcultured in Bactec 12B vials prior to the experiments involving radiometric technology. For macrophage infection, bacterial suspensions were prepared from Lowenstein–Jensen slants. Otherwise, all strains were stored as frozen suspensions at −70 °C.

2.2. Drugs

The compounds used in this study were based on different MCCs: cyclen (1,4,7,10-tetraazacyclododecane), cyclam (1,4,8,11-tetraazacyclotetradecane), py14 [3,7,11,17-tetraazabicyclo[11.3.1]heptadeca-1(17),13,15-triene] and its 7-methyl derivative (Mepy14). From these MCCs were synthesized the following derivatives: 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetramethylenephosphonic acid (DOTP), 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (TETA), and 3,7,11,17-tetraazabicyclo[11.3.1]-heptadeca-1(17),13,15-triene-3,7,11-triacetic acid (ac3py14). The molecular weight of each compound is: 347.42 for DOTA, 548.14 for DOTP, 432.35 for TETA, 248.33 for Mepy14 and 408.35 for ac_3py14. Cyclam was obtained commercially from Strem Chemicals (Bischheim, France) and the other parent macrocycles for synthesis were synthesized and characterized in our laboratory according to procedures previously described: cyclen [15] and py14 or Mepy14 [11]. The final derivatives were also synthesized and characterized by us following described procedures, DOTA and TETA [15], DOTP [16] and ac_3py14 [9].

Their ferric complexes K[Fe(DOTA)], K_5[Fe(DOTP)], [Fe(MePy14)]$(NO_3)_3$, [Fe(ac_3py14)] and K[Fe(TETA)] were prepared by the addition of $Fe(NO_3)_3$ to the MCC in a 1:1 ratio, followed by the increase of the pH of the mixture with a solution of KOH until complete formation of the complex, as indicated by the speciation diagram [2].

Ethylenediamine-di-*o*-hydroxyphenylacetic acid (EDDHA), used for the verification of mycobactin production, was purchased from Sigma–Aldrich Química (Madrid, Spain). EDDHA was freed from contaminating iron using the method suggested by Rogers [29]. The iron-free compound was then solubilized using the method proposed by Ong [23].

To prepare the ferric complexes and to study the effect of free iron(III) on the biological effect of DOTA, $Fe(NO_3)_3{\cdot}9H_2O$ p.a. was used (Merck/VWR International, Lisbon, Portugal).

2.3. Drug sensitivity studies

For examining the antimycobacterial activity of the MCC, stock solutions of 5 mg/ml were prepared in sterile distilled water, sterilized by filtration using 0.2 µm Filtropur filters (Sarstedt, Nümbrecht, Germany) and frozen at −20 °C until use. Volumes of 0.1 ml of the appropriate dilutions were injected into 12B vials to obtain concentrations ranging from 10 to 300 mg/l.

In vitro activities were evaluated using radiometric methodologies applied to the Bactec 460TB instrument, as previously described [14,30]. Since the growth index (GI) during exponential growth is directly proportional to the number of colonies in the vials [26], it was used in this simple relationship to express inhibition. Thus, relative growth was calculated in relation to a control vial having received the same initial inoculum but no added compound, representing 100% growth or 0% inhibition. Bacterial inhibition was also confirmed by bacterial counts carried out at the end of the experiment by plating serial 10-fold dilutions of the bacterial suspensions from the individual Bactec vials onto Middlebrook 7H11 agar plates; results were expressed in CFU/ml. Viability was determined by comparison of CFU/ml from individual Bactec vials at the beginning and end of the experiments. The experiments were completed within 6 days.

Table 1
Intracellular activity of 50 mg/l of TETA used against *M. avium* growing in J-774 macrophages

Strains	Colony morphology[a]		Relative growth (%)[b]
	Pigmentation	Ratio of opaque to transparent (%)	
Laboratory strain	+	100	55
Isolate 61596	–	50	24
Isolate 94896	–	50	2
Isolate 101	–	<1	123
Isolate 5196	–	<1	32

[a] Strains were characterized according to colony morphology as regards pigmentation (pigmented or not pigmented) and the ratio of opaque to transparent colonies from a total of 100 colonies, as observed after 8–10 days of culture on Middlebrook 7H11 agar plates.

[b] The results shown were obtained after two days of active growth in the macrophage. They are expressed as relative growth (%), representing the averages of three independent experiments for viable counts in the drug containing samples compared to those obtained in the control samples. Standard errors for viable counts ranged from 0.3 to 5.0 for the control samples and from 0.2 to 0.9 for the drug-containing samples.

Results represent the average of three independent experiments.

2.4. Effect of free iron on inhibition by DOTA

To determine whether free iron affected inhibition by DOTA, ferric nitrate, $Fe(NO_3)_3{\cdot}9H_2O$, was added at various concentrations, (0, 30, 60, 100 mg/l), as an exogenous source of iron to Bactec vials of 12B medium containing 50 mg/l of the MCCs. These concentrations included values high above the equimolar concentration of iron to DOTA. The vials were left to stand for 24 h prior to inoculation. As an additional control, ferric nitrate at the above concentrations was also tested in vials without the presence of MCCs.

2.5. Mycobactin production

Bacterial growth and mycobactin production using a modified EDDHA two-step method were carried out as previously described [7]. For this, mycobacteria were cultured in 10 ml of liquid 7H9 medium. After an initial growth phase, the bacteria were transferred, following centrifugation, to fresh 7H9 medium complemented with 1 mg/ml of deferrated EDDHA. The chelation of iron from the growth medium by the EDDHA does not permit bacterial growth, but during this production phase it induces optimal production of mycobactins between the 10th and 20th day of incubation.

2.6. Macrophage monolayer culture

The J-774 mouse BALB/c monocyte macrophage cell line, purchased from The European Collection of Cell Cultures (ECACC), was maintained in Dulbecco's Modified Eagle's Medium (DMEM), containing 4500 mg/l of D-glucose, 25 mM Hepes, 1 mM sodium pyruvate, and 10% (vol/vol) heat-inactivated fetal calf serum (FCS). The culture reagents were GIBCO™ from the Invitrogene Corporation (Invitrogene/ALFAGENE, Carcavelos, Portugal). Cell stocks were prepared in 80 cm^2 Nunc™ tissue culture flasks (OXVITAL, Lisbon, Portugal). Confluent cells were obtained after 5–7 passages from the frozen culture, or after 3 days of incubation from a preculture. Cells were incubated in 10 ml of medium at 37 °C in the presence of 5% CO_2. Cells were recovered from the flasks by scraping. Viable cells were monitored using the trypan blue dye (Gibco™, Invitrogene Corporation, Barcelona, Spain).

2.7. Macrophage infection by M. avium

For studying the action of the drugs, cell cultures were prepared in 8.8 cm^2 Nunc tissue culture dishes containing 2 ml DMEM medium with 10% FCS (vol/vol). Each culture dish was seeded with approximately 5×10^4 cells, to obtain, after 3 days of incubation, 10^6 cells. At that time the medium was replaced by DMEM medium containing 1% FCS (vol/vol) and the appropriate concentration of bacterial cells, approximately 10 times more than macrophage cells. For infection, bacterial cell suspensions were obtained from fresh Lowenstein–Jensen culture. The FCS concentration of 1% (vol/vol) was adequate to stop macrophage division but maintain cellular viability. Phagocytosis was allowed to proceed for 4 h at 37 °C. Then the cells were washed twice with phosphate buffer saline pH 7, 0.01 M, in order to eliminate any extracellular bacteria. Ziehl–Neelsen staining of the mycobacteria confirmed that extracellular bacteria had been eliminated and revealed that about two thirds of the macrophages were infected with 1–20 bacteria per cell.

2.8. Drug action against intracellularly growing mycobacteria

The procedure used to assess drug action against intracellularly growing mycobacteria was adapted from [28]. For this, infected macrophages were incubated for 48 h in order to allow for intracellular growth of phagocytized mycobacteria. After this time the desired concentrations of the drugs to be tested were added to the cultured macrophages. Mycobacterial cell counts were then measured at two and 5 days after the addition of the drugs. For this the macrophages were lysed by the removal of the growth medium followed by the addition of 0.5 ml of 1% (vol/vol) Igepal (Sigma–Aldrich Química, Madrid, Spain). At that concentration Igepal did not affect mycobacterial viability. A total of 0.5 ml of the lysate was diluted in 4.5 ml of sterile distilled water to obtain a 10^{-1} dilution, followed by subsequent tenfold dilutions which were appropriately plated on 7H11 agar medium and incubated at 37 °C the amount of time required for colony formation and counting of CFU. The results were expressed in relative growth (%), corresponding to the averages of three independent experiments, for viable counts in the drug-containing samples compared to those obtained in the control samples. The drugs were used at concentrations non-toxic to the macrophages as assessed by the trypan blue method.

3. Results

3.1. Inhibition of M. avium

These studies on the *M. avium* laboratory strain revealed in vitro activity by tetraaza-MCCs with methyl, acetate and methylphosphonate pendant arms (Fig. 1).

Fig. 2 shows results of radiometric growth in the presence of 50 mg/l of different derivatives of the three MCC families cyclen, cyclam and py14, respectively, DOTA, DOTP, TETA, Mepy14, and ac_3py14. These were used in the form of the free ligand and as the iron(III) complex. Inhibition patterns varied significantly from one compound to another. While Mepy14 and DOTP molecules were poor inhibitors, the *N*-tris-acetate derivative of py14, ac_3py14, resulted in

up to 90% inhibition both with the free ligand and with the iron(III) complex. Likewise, TETA, the *N*-tetraacetate derivative of the 14-membered macrocycle cyclam, proved to be one of the most active compounds, showing up to 90% inhibition with both the free ligand and the iron(III) complex. On the other hand, DOTA, the tetraacetate derivative of cyclen, the 12-membered tetraazamacrocycle, showed a high degree of inhibition with the free ligand, over 99%, but contrary to what was observed with the *N*-acetate derivatives of both the other 14-membered macrocycles ac_3py14 and TETA, lost this effect with the iron(III) complex.

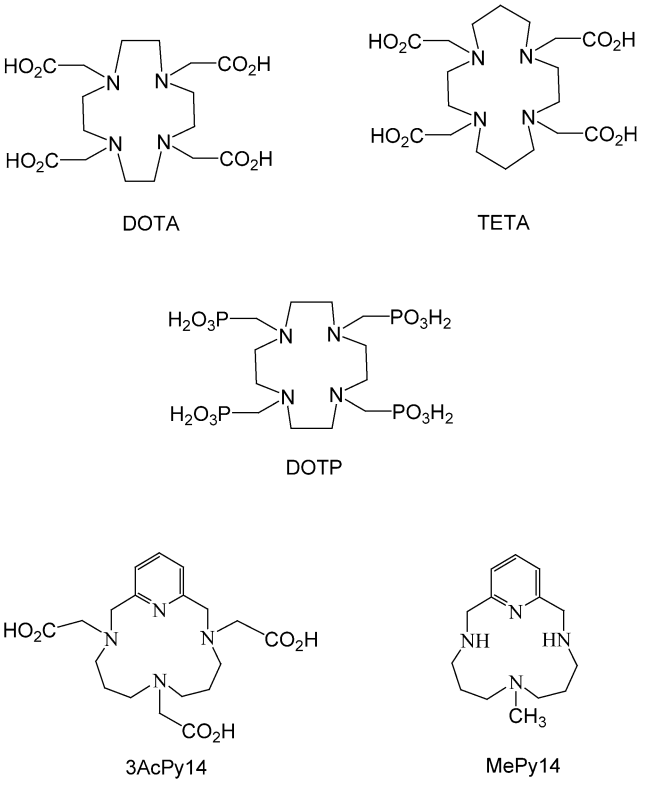

Fig. 1. Macrocyclic compounds used in this study.

These results suggest that the chelating properties of DOTA may be at the origin of the antimycobacterial effect or that the donor atoms of the macrocyclic ligand are strongly involved in this mechanism. However, in experiments in which free iron(III) was added as an exogenous source in the form of $Fe(NO_3)_3$, growth was not restored in Bactec vials of 12B medium containing 50 mg/l of DOTA; relative growth remained <0.2% compared to the control vial with no added compound. Free iron(III) did not in itself inhibit growth, showing 100% growth for all the concentrations tested (0, 30, 60, 100 mg/l) (data not shown).

Likewise, at a concentration of 200 μg/ml, which is sufficient to stop mycobacterial growth, DOTA failed to induce mycobactin production, as would be expected if iron was being removed from the growth medium by DOTA. In this experiment, mycobactin production was confirmed with an EDDHA control (data not shown).

A slight degree of growth promotion was observed early in growth with the iron complexes of TETA, DOTA and DOTP.

As shown in Fig. 3, radiometric bacterial inhibition was also confirmed at the end of the experiment by plating bacterial suspensions from the individual BACTEC vials on Middlebrook 7H11 agar plates, followed by counts of CFU/ml. Conformity of radiometric data with bacterial counts indicated reductions in growth inhibition (GI) were not deter-

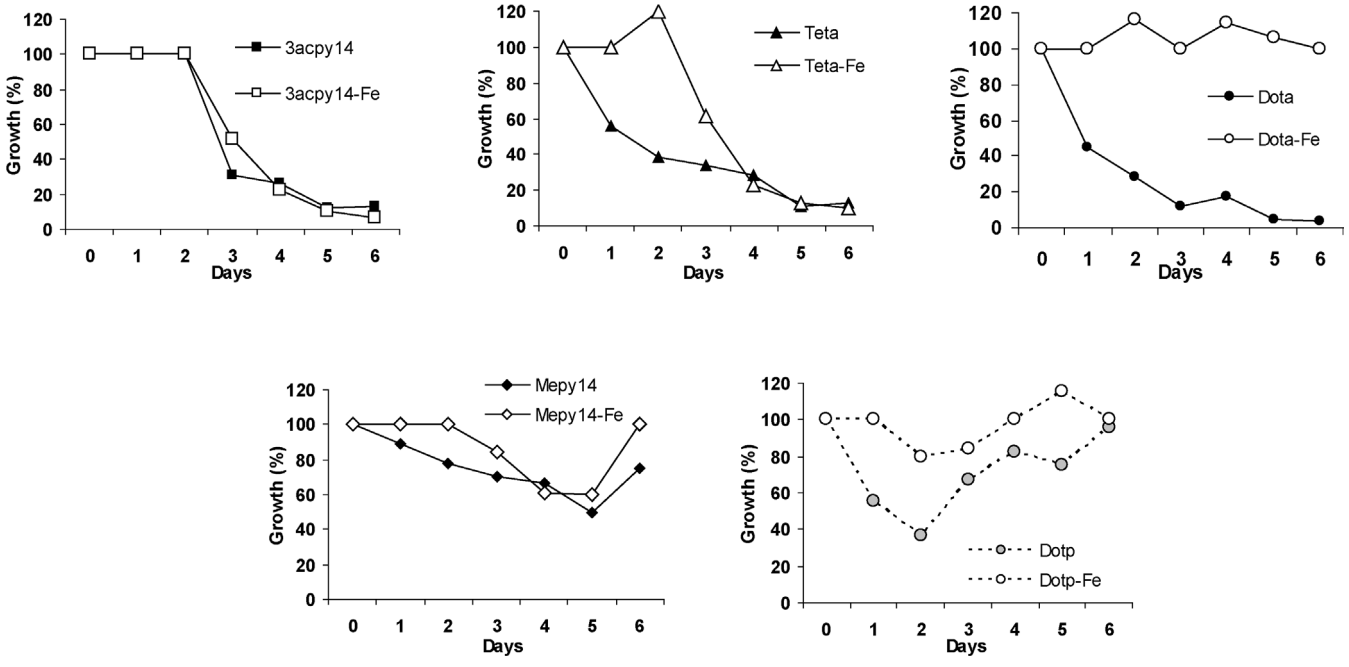

Fig. 2. Growth of *M. avium* laboratory strain in the presence of 50 mg/l of various MCCs, in the form of their iron(III) complex and as the free compound. Radiometric data, expressed as the percentage of growth in relation to the control vial, with no added compound, is plotted as a function of time.

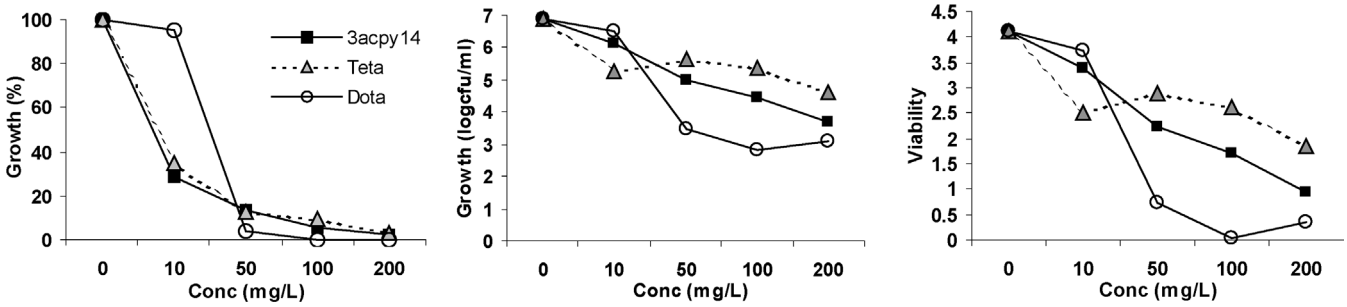

Fig. 3. Growth inhibition of the *M. avium* laboratory strain in the presence of increasing concentrations of compounds ac_3py14, TETA, and DOTA evaluated from (A) radiometric data and expressed as the percentage of growth in relation to the control vial, with no added compound, and corresponding (B) bacterial counts on 7H11 medium expressed as CFU/ml of culture in BACTEC vials. Viability (C) was expressed as log[(CFU/ml)t6] − log[(CFU/ml)t0] and was determined by plating the bacterial suspensions from individual Bactec vials at the beginning (t0) and at the end of the experiments (t6).

mined by direct inhibition of respiratory activity by these chelating compounds. Also in Fig. 3, viability results indicated bacteriostatic activity, as seen when comparing the bacterial population by plating suspensions from individual Bactec vials at the beginning and end of the experiments.

3.2. Intracellular clearance of M. avium *infections*

TETA, one of the most active compounds, as assessed by the in vitro methods used, was selected for intracellular studies. The intracellular activity of 50 mg/l of TETA was carried out using a J-774 murine macrophage cell line on laboratory strains (opaque morphology) and clinical strains (transparent morphologies). Results in Table 1 express relative growth (%) of the drug-containing samples compared to control samples. Values ranged from 2 to 123% (growth promotion), with no apparent relationship between inhibitory activity and colony morphology of the strain. Results with the laboratory strain, 55% relative growth, confirmed the inhibition shown by in vitro methods. There was no significant toxicity toward macrophages in the presence of any of the MCCs tested up to 100 mg/l, as assessed by the trypan blue method (data not shown).

4. Discussion

This study reports in vitro activity and effective intracellular clearance of *M. avium* infections by derivatives of the synthetic macrocyclic compounds MCC, 12- and 14-membered tetraazamacrocycles with *N*-pendant arms, such as *N*-methyl (Mepy14), *N*-acetate (DOTA, TETA and ac_3py14) and *N*-methylphosphonate (DOTP) groups.

In *M. avium*, the inhibitory concentrations of the MCCs were higher than those previously observed in *M. tuberculosis* (strain 38396), with inhibition levels of 90% and above obtained with 50 mg/l of the compounds ac_3py14 and [Fe(ac_3py14)] in the former, compared to 7.5 mg/l in the latter [14]. Nevertheless, observations were similar to those previously obtained with *M. tuberculosis*, namely in showing that reductions in the Bactec GI were not determined by direct inhibition of respiratory activity and that MCCs have an apparent bacteriostatic effect on mycobacterial growth.

While ac_3py14 and TETA appear to exert intrinsic activity toward *M. avium*, this did not seem to be the case with DOTA. With this MCC, the inhibitory effect observed with the free compound was reversed with the iron(III)-saturated complex, suggesting that chelation equilibrium was at the origin of the inhibitory effect or that the donor atoms of the macrocyclic ligand were needed in this process and could not be previously blocked by coordination with a metal. Nevertheless, preliminary investigations do not point to the loss of iron(III) as the source of inhibition, as experiments in which free iron(III) was added to the growth medium containing DOTA failed to restore growth. Also, at a concentration of 200 µg/ml, which is sufficient to stop mycobacterial growth, DOTA failed to induce mycobactin production, as would be expected if this GI were related to iron-deficient conditions created by DOTA (data not shown).

In these studies, the greater activity of the MCCs seemed less related to the size of the macrocyclic cavity than to the type of *N*-substituents. Indeed, acetate pendant arms seemed related to activity, since DOTA, ac_3py14 and TETA with acetate pendant arms, although derived from three different kinds of macrocycles (presenting different macrocyclic cavity sizes), showed in vitro activity, whereas other derivatives of the same macrocycles showed little activity. In fact, DOTP and Mepy14 exhibit slight activity; however, the former ligand is the phosphonate analogous of DOTA, and Mepy14 is the methyl derivative of py14, such that $ac_3$14py is the acetate derivative of the same macrocycle. Several chemical reasons can be advanced, emphasizing the differences in the ligands, especially the type of pendant arms of the macrocycles, but the real role of these ligands in in vitro inhibition is unknown for the moment, as we cannot predict the mechanism of action or the type of interaction. In fact, DOTP and Mepy14 are, in general, poorer ligands for metal chelation when compared with the related DOTA or $ac_3$14py, respectively [9,11,15,16], but it is also known that DOTP is a powerful chelator, on the order of, or stronger than, ac_3py14 and TETA. The basicity of the ligands and the structural behavior of the ligands in chelation may also contribute to dif-

ferences observed in in vitro activity. On another hand, each phosphonate group of DOTP exhibits, at physiological pH, two negative charges and therefore the electrostatic effects are much stronger when compared with the mononegative charge of each carboxylate group of DOTA, with this having strong repercussion on all interactions of these groups, including metal coordination.

Intracellular clearance by 50 mg/l of TETA using a J-774 murine macrophage cell line revealed marked activity in a number of infections by clinical strains of *M. avium*. However, variability was considerable, with values for relative growth (%) of the drug-containing samples compared to the control samples ranging from 2 to 123% (growth promotion). These observations showed no apparent relationship between the inhibitory activity and colony morphology. Clinical strains on primary culture are predominantly of transparent morphology with the opaque population increasing with subsequent cultures. Colony phenotype is known to strongly influence susceptibility to antibiotics and other chemical agents, with the transparent type being more resistant [6,12,18,31]. However, in this study, the selected strains presented both transparent and opaque variants. The ratio of opaque to transparent colonies varied from 100 to <1% (Table 1), with only one of the opaque strains also being pigmented. Another possible clue to explaining observed differences between the strains may be related to their serotypes, corresponding to variations in the cell wall's peptidoglycolipids. However, this aspect was not explored.

These studies show that the evaluation of synthetic macrocycles may be relevant in the development of a new family of compounds active against *M. avium* infections. Results suggest that the preparation of derivatives with greater in vitro activity against *M. avium* may further contribute to the evaluation of MCCs in a chemotherapeutic perspective. Further studies should also include the evaluation and improvement of their intracellular uptake. Testing a large number of strains of clinical origin and studies in an animal model are needed to determine whether in vivo antimycobacterial activity exists, and whether these compounds are indeed stimulatory to the immune response, as had been previously suggested.

Acknowledgements

We would like to thank Professor Hugo L. David and Professor Leonard Amaral, Unit of Mycobacteriology, Instituto de Higiéne e Medicina Tropical, Lisbon Portugal, for fruitful discussion and help in preparing the manuscript. We also thank Elsa Anes, Faculdade de Farmácia de Lisboa, UL for her helpful suggestions for work with the J-774 mouse BALB/c monocyte macrophage. This investigation received financial support from the Fundação para a Ciência e Tecnologia (FCT), Portugal (grant reference: POCTI/35877/ESP/2000), with co-participation by the European Community fund FEDER.

References

[1] W.W. Barrow, N. Rastogi, Laboratory and clinical aspects of the *Mycobacterium avium* epidemic: Contributing factors associated with variability of drug susceptibility and immune responsiveness, and the multifaceted nature of pathogenicity, Res. Microbiol. 145 (1994) 167–261.

[2] L. Alderighi, P. Gans, A. Ienco, D. Peters, A. Sabatini, A. Vacca, Hyperquad simulation and speciation (HySS): A utility program for the investigation of equilibria involving soluble and partially soluble species, Coord. Chem. Rev. 184 (1999) 311–318.

[3] O. Andersen, Principles and recent developments in chelation treatment of metal intoxication, Chem. Rev. 99 (1999) 2683–2710.

[4] C.J. Anderson, M.J. Welch, Radiometal-labeled agents (non-technetium) for diagnostic imaging, Chem. Rev. 99 (1999) 2219–2234.

[5] R.D. Arbeit, A. Slutsky, T.W. Barber, J.N. Maslow, S. Niemczyk, J.O. Falkinham III, G.T. O'Connor, C.F. Reyn, Genetic diversity among strains of *Mycobacterium avium* causing monoclonal and polyclonal bacteremia in patients with AIDS, J. Infect. Dis. 167 (1993) 1384–1390.

[6] L.E. Bermudez, C.B. Inderlied, P. Kolonoski, M. Wu, L. Barbara-Burnham, L.S. Young, Activities of Bay Y 3118, levofloxacin, and ofloxacin alone or in combination with ethambutol against *Mycobacterium avium* complex in vitro, in human macrophages, and in beige mice, Antimicrobiol. Agents Chemother. 40 (1996) 546–551.

[7] S. Bosne, F. Papa, S. Clavel-Sérès, N. Rastogi, A simple and reliable EDDA method for mycobactin production in mycobacteria: Optimal conditions and use in mycobacterial speciation, Curr. Microbiol. 26 (1993) 353–358.

[8] P. Caravan, J.J. Ellison, T.J. McMurry, R.B. Lauffer, Gadolinium(III) chelates as MRI contrast agents: Structure, dynamics, and applications, Chem. Rev. 99 (1999) 2293–2352.

[9] J. Costa, R. Delgado, M.G.B. Drew, V. Felix, Design of selective macrocyclic ligands for the divalent first-row transition metal ions, J. Chem. Soc. Dalton Trans. (1998) 1063–1071.

[10] J. Costa, R. Delgado, M.G.B. Drew, V. Felix, Methyl pyridine derivatives of 14-membered tetraazamacrocycles: A new host with high selectivity for cadmium, J. Chem. Soc. Dalton Trans. (1999) 4331–4339.

[11] J. Costa, R. Delgado, Metal complexes of macrocyclic ligands containing pyridine, Inorg. Chem. 32 (1992) 5257–5265.

[12] H.L. David, Basis for the lack of drug susceptibility of atypical mycobacteria, Rev. Infect. Dis. 3 (1981) 878–884.

[13] H.L. David, S. Clavel-Sérès, F. Clément, K.S. Goh, Uptake of selected antibacterial drugs in *Mycobacterium avium*, Int. J. Microbiol. Hyg. A 265 (1987) 385–392.

[14] S. David, D. Ordway, M.-J. Arroz, J. Costa, R. Delgado, Activity against *Mycobacterium tuberculosis* with concomitant induction of cellular immune responses by tetraazamacrocycle with acetate pendant arms, Res. Microbiol. 152 (2001) 569–576.

[15] R. Delgado, J.J.R. Fraústo da Silva, Metal complexes of cyclic tetraazatetraacetic acids, Talanta 29 (1982) 815–822.

[16] R. Delgado, L.C. Siegfried, T.A. Kaden, Protonation studies and complexation properties of tetraazamacrocyclic methylenephosphonates with earth-alkali ions, Hev. Chim. Acta 73 (1990) 140–148.

[17] K.-P. Eisenwiener, M.I.M. Prata, I. Buschmann, H.-W. Zhang, A.C. Santos, S. Wenger, J.-C. Reubi, H.R. Mäcke, NODAGATOC, a new chelator-coupled somatostatin analogue labeled with $^{67/68}$Ga and ^{111}In for SPECT, PET, and targeted therapeutic applications of somatostatin receptor (hsst2) expressing tumors, Bioconj. Chem. 13 (2002) 530–541.

[18] J.O. Falkingham III, Factors influencing chlorine susceptibility of *Mycobacterium avium*, *Mycobacterium intracellulare*, and *Mycobacterium scrofulaceum*, Appl. Environ. Microbiol. 69 (2003) 5685–5689.

[19] W.R. Harris, K.N. Raymond, F.L. Weitl, Ferric ion sequestering agents: The spectrophotometric and potentiometric evaluation of sulfonated tricatecholate ligands, J. Amer. Chem. Soc. 103 (1981) 2667–2675.

[20] M. Li, C.F. Meares, G.-R. Zhong, L. Miers, C.-Y. Xiong, S.J. DeNardo, Labeling monoclonal antibodies with ^{90}yttrium- and ^{111}indium-DOTA chelates: A simple and efficient method, Bioconj. Chem. 5 (1994) 101–104.

[21] S. Liu, D.S. Edwards, Bifunctional chelators for therapeutic lanthanide radiopharmaceuticals, Bioconj. Chem. 12 (2001) 7–34.

[22] C.F. Meares, Chelating agents for the binding of metal ions to antibodies, Nucl. Med. Biol. 13 (1986) 311–318.

[23] S.A. Ong, T. Peterson, J.B. Neilands, Agrobactin, a siderophore from *Agrobacterium tumefaciens*, J. Biol. Chem. 254 (1979) 1860–1865.

[24] D. Parker, Tumor targeting with radiolabeled macrocycle-antibody conjugates, Chem. Soc. Rev. 19 (1990) 271–291.

[25] N. Rastogi, C. Fréhel, A. Ryter, H. Ohayon, M. Lesourd, H.L. David, Multiple drug resistance in *Mycobacterium avium*: Is the wall architecture responsible for the exclusion of antimicrobial agents?, Antimicrob. Agents Chemother. 20 (1981) 666–677.

[26] N. Rastogi, K.S. Goh, H.L. David, Drug susceptibility testing in tuberculosis: A comparison of the proportion methods using Lowenstein–Jensen, Middlebrook 7H10 and 7H11 agar media and a radiometric method, Res. Microbiol. 140 (1989) 405–417.

[27] N. Rastogi, H. Hellio, H.L. David, A new insight into the mycobacterial cell envelope architecture by the localization of antigens in ultrathin sections, Int. J. Med. Microbiol., Virol., Parasitol., Infect. Dis. 275 (1991) 287–302.

[28] N. Rastogi, M.-C. Potar, H.L. David, Intracellular growth of pathogenic mycobacteria in the continuous murine macrophage cell line J-774: Ultrastructure and drug-susceptibility studies, Curr. Microbiol. 16 (1987) 79–92.

[29] H.J. Rogers, Iron-binding catechols and virulence in *Escherichia coli*, Infect. Immun. 7 (1973) 445–456.

[30] S.H. Siddiqi, L.B. Heifets, M.H. Cynamon, N.M. Hooper, A. Laszlo, J.P. Libonati, P.J. Lindholm-Levy, N. Pearson, Rapid broth macrodilution method for determination of MICs for *Mycobacterium avium* isolates, J. Clin. Microbiol. 31 (1993) 2332–2338.

[31] R.S. Stormer, J.O. Falkinham III, Differences in antimicrobial susceptibility of pigmented and unpigmented colonial variants of *Mycobacterium avium*, J. Clin. Microbiol. 27 (1989) 2459–2465.

[32] C.F. Von Reyn, N.J. Jacobs, R.D. Arbeit, J.N. Maslow, S. Niemczyk, Polyclonal *Mycobacterium avium* infections in patients with AIDS: Variations in antimicrobial susceptibilities of different strains of *M. avium* isolated from the same patient, J. Clin. Microbiol. 33 (1995) 1008–1010.

[33] C.L. Woodley, H.L. David, Effect of temperature on the rate of transparent to opaque colony type transition in *Mycobacterium avium*, Antimicrob. Agents Chemother. 9 (1976) 113–119.