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Activity against *Mycobacterium tuberculosis* with concomitant induction of cellular immune responses by a tetraaza-macrocycle with acetate pendant arms

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Abstract – The novel tetraaza-macrocyclic compound 3,7,11-tris(carboxymethyl)-3,7,11,17-tetraaza-bicyclo[11.3.1]heptadeca-1(17),13,15-triene, abbreviated as ac_3py14, was investigated for its activity against *Mycobacterium tuberculosis* and for induction of protective cellular immune responses. Perspective results show that ac_3py14 and its Fe^{3+} 1:1 complex, [Fe(ac_3py14)], inhibited radiometric growth of several strains of *M. tuberculosis*. Inhibition with 25 µg/mL varied from 99% for H37Rv to 80% and above for multiple drug-resistant clinical isolates. The capacity of ac_3py14 to elicit a beneficial immune response without cellular apoptosis was assessed and compared to the effects of virulent *M. tuberculosis*. The present study produces evidence that after stimulation with ac_3py14 there was significant production of interferon gamma (IFN-γ), whereas the production of interleukin-5 (IL-5) remained low, and there was development of a memory population (CD45RO). The level of binding of Annexin V, a marker of apoptosis, was not sufficient to result in toxic effects toward $\alpha\beta$ and $\gamma\delta$ T cells and $CD14^+$ macrophages. This preliminary study is the first report of a compound that simultaneously exerts an inhibitory effect against *M. tuberculosis* and induces factors associated with protective immune responses. © 2001 Éditions scientifiques et médicales Elsevier SAS

***Mycobacterium tuberculosis* / chemotherapy / immunotherapy / macrocyclic compounds**

1. Introduction

Tuberculosis, caused by *Mycobacterium tuberculosis*, constitutes a major problem for public health worldwide. The annual rate of new cases is calculated at 8 million and mortality at nearly 3 million [16, 27].

Multiple drug-resistant tuberculosis is a threat in many settings where tuberculosis control has become difficult, notably as a result of the emerging AIDS pandemic [1, 4, 21, 24, 28]. In Portugal data from the WHO reports an alarming rate of 1.7% primary and 18.8% acquired multiple drug resistance along with the highest incidence in Europe, 56.77/100 000 [28]. Thus, there is an urgent need to discover effective new drugs and strategies to circumvent multiple drug resistance.

Because of their potential drug delivery capabilities, we examined the activity of a family of macrocyclic compounds (MCCs) both against *M. tuberculosis* and upon Th1 stimulation. MCCs are of particular interest as they can be easily derivatized by introducing N-substituents [6, 7]. Our objective in studying these compounds was to contribute to their evaluation as potential candidates for the preparation of antibiotic conjugates. The purpose of the present report was to show that one of the macrocyclic compounds

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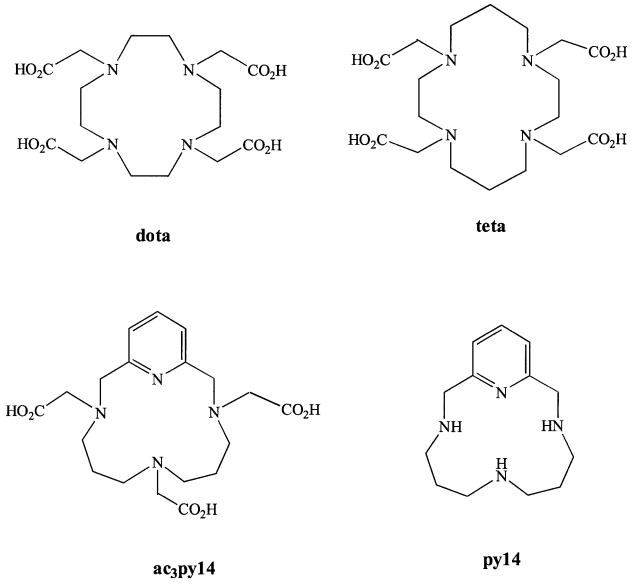

Figure 1. Macrocyclic compounds dota, teta and their derivatives, py14 and ac_3py14.

tested, compound ac_3py14 (*figure 1*), exerted a significant antituberculous activity in vitro and induced beneficial cellular immune responses.

Macrocyclic compounds, especially dota, teta and some of their derivatives (*figure 1*), are currently used in medicine as contrast-enhancing agents for magnetic resonance imaging (MRI), in nuclear medicine for radioimmunoscintigraphy and radioimmunotherapy and in chelation therapy [2, 3, 5, 9, 13, 15, 20].

2. Materials and methods

2.1. Subjects

This study included 4 Portuguese individuals (2 female and 2 male); these healthy controls were seronegative for HIV-1 and -2. Individuals enrolled in this study ranged in age from 20 to 34 years (mean age, 32.1 years), and all (100%) had been vaccinated with bacillus Calmette-Guerin (BCG) and were Mantoux-positive (> 5-mm enduration).

2.2. Organisms

M. tuberculosis H37Rv (Mtb H37Rv) and 4 clinical isolates were selected for this study. Their susceptibility to first-line antibiotics was determined using the Bactec 460TB radiometric criteria, SIRE (Becton Dickinson, Sparks, MD, USA). The clinical isolates included one strain fully susceptible to four primary drugs (streptomycin, isoniazid, rifampicin and ethambutol) and three multiple drug-resistant strains, which were resistant to the same four primary drugs.

2.3. Drugs

Streptomycin, isoniazid, rifampicin, and ethambutol were purchased from Becton-Dickinson for use in the Bactec 460TB procedure (SIRE) (Becton-Dickinson, Microbiology Systems, Cockeysville, MD, USA).

The macrocycle ac_3py14 was synthesized and characterized in our laboratory according to the procedure previously described [7, 8].

[Fe(ac_3py14)] corresponds to the 1:1 complex of ac_3py14 with Fe^{3+}, prepared by addition of a standard solution of $Fe(NO_3)_3$ to an equivalent amount of a solution of ac_3py14 and the pH adjusted at 7.4 by addition of a standard solution of KOH. The prepared solution of the complex was left to equilibrate one day before use. The thermodynamic stability constant of the complex in aqueous solution is 1.622×10^{19}, 19.21 in log units [7]. At physiological pH the conditional stability constant is equal to 5.3×10^8, the main species in solution is $[Fe(ac_3py14)(OH)]^-$, which exists at 98.9% of the total metal concentration, and the $pFe = -\log [Fe^{3+}]$ at the same pH is 17.66 (value calculated for 100% excess of free ac_3py14, pH = 7.4, with the total concentration of Fe^{3+} being 1.0×10^{-5} M, and that of the ligand 2.0×10^{-5} M).

2.4. Drug sensitivity studies

For examining the antimycobacterial activity of ac_3py14, a 1 mg/mL aqueous solution of the compound was prepared and then filter-sterilized using 0.2 µm Filtropur filters (Sarstedt, Nümbrecht, Germany). Volumes of 0.1 mL of the appropriate dilutions were injected into 12B vials to obtain serial concentrations of 0.5, 1.0, 5.0, 7.5, 10.0, 25.0 µg/mL. Drug sensitivity testing of Mtb H37Rv and the clinical isolates was established using the Bactec 460TB instrument. Briefly, a primary culture from a Bactec 12B vial having reached a growth index (GI) of 500 was used as the inoculum. For drug sensitivity testing, 0.1 mL of this inoculum was injected directly into the drug-containing vials and the control vial with no added compound. This inoculum contained approximately 10^4–10^5 colony-forming units

(CFU)/mL. Readings of the GI were recorded daily except weekends. As the growth GI during exponential growth is directly proportional to the number of colonies in the vials [23], 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% of growth, or 0% inhibition.

At the end of an experiment, bacterial inhibition by the compounds was also determined by plating serial 10-fold dilutions of the bacterial suspensions from the individual Bactec vials onto Middlebrook 7H11 agar plates [22]. Results were expressed as the logarithm of the CFU/mL.

2.5. Napthol blue-black method

Peripheral blood mononuclear cells (PBMCs) were isolated from heparized blood by density sedimentation [25] and resuspended at 1×10^6 cells/mL in growth medium; 180 µL of this suspension were incubated with serial dilutions from 100, 50, 25, 10 to 1 µg/mL of the macrocyclic compound added in 20-µL aliquots to 96-well tissue culture plates (Nalge Nunc Int., Naperville, USA). Cells were incubated at 37°C in the presence of 5% CO_2. Adherent cells were washed with Hank's balanced salt solution (Gibco BRL Life Technologies, CA, USA). On day two adherent macrophages were assayed using the naphthol blue-black method [19], for number of nuclei present, representing the viable population after stimulation with various concentrations of ac_3py14. Briefly, after removal of excess liquid, 50 µL of the napthol blue-black stock solution containing 1% Triton X-100 (w/v), 0.1 M citric acid and 0.05% (w/v) napthol blue black (Sigma-Aldrich Co. Ltd., UK), final pH adjusted to 2.0, was added per well and the mixture was left to stand at room temperature for 15 min while repeatedly mixing cells with a pipette tip. Cells stained by the dye were counted in a hemocytometer. Optimal concentration for the viability of cells was 25 µg/mL of ac_3py14 (data not shown). This concentration was selected for all other experiments in immunology.

2.6. Lymphocyte proliferation assay

PBMCs were isolated from heparized blood by density sedimentation. Assays were performed as described by Silveira and collaborators [25]. PBMC were stimulated with Mtb H37 Rv and ac_3py14. Incorporated radioactivity was measured on an LS 6500 scintillation counter (Beckman, Fullerton, CA, USA) and expressed as counts per minute (cpm). Lymphocyte proliferation, after 6 days of stimulation, was expressed as mean cpm of triplicates minus the mean cpm of cells without the compound.

2.7. Bacterial infection of monocyte-derived macrophages

The infection ratio of monocyte derived macrophages with Mtb H37Rv was based on 1 CFU/1 macrophage, admitting that approximately 10% of the PBMCs are monocytes adhering to the plate. Assays were performed as described by Silveira et al. [25].

2.8. Cytokine assay

The amount of IFN-γ and IL-5 present in culture was analyzed by commercial ELISA (Endogen, Woburn, MA, USA). Supernatants were collected in parallel plates on day 6, stored at -20°C and analyzed later according to the instructions of the manufacturer (Endogen, Woburn, MA, USA).

2.9. Apoptosis assay

Isolated PBMCs were resuspended at 1×10^6 cells/mL in growth medium and 2 mL of this suspension incubated with or without ac_3py14 in 24-well tissue culture plates (Nalge Nunc Int., Naperville, USA). Cells were incubated at 37°C in the presence of 5% CO_2. At 0, 1, 2 and 6 days the cells were processed according to the instructions in the AnnexinV-FITC kit (R & D systems, MN, USA). Briefly, cells were resuspended using a sterile pastette and transferred to a 15-mL sterile conical tube (Greiner Labortechnik, Germany). The cell suspension was centrifuged at 410 g for 10 min and the cells were resuspended in RPMI at 1×10^6 cells/mL. Aliquots of 100 µL of this suspension were incubated with 10 µL of fluorescent labeled antibody for surface receptors ($\alpha\beta$ TCR, $\gamma\delta$ TCR, CD45RA) (Becton Dickinson, Oxford, UK) and 100 µL of fluorescent labeled antibody for Annexin V and propidium iodide in 6-mL tubes (Greiner Labortechnik, Germany) for 30 min at 4°C in the dark. Cells were washed once in cold binding buffer (410 g for 10 min) and resuspended in 1.5 mL of 2% paraformaldehyde. The tubes were stored at 4°C overnight and in the dark until analyzed.

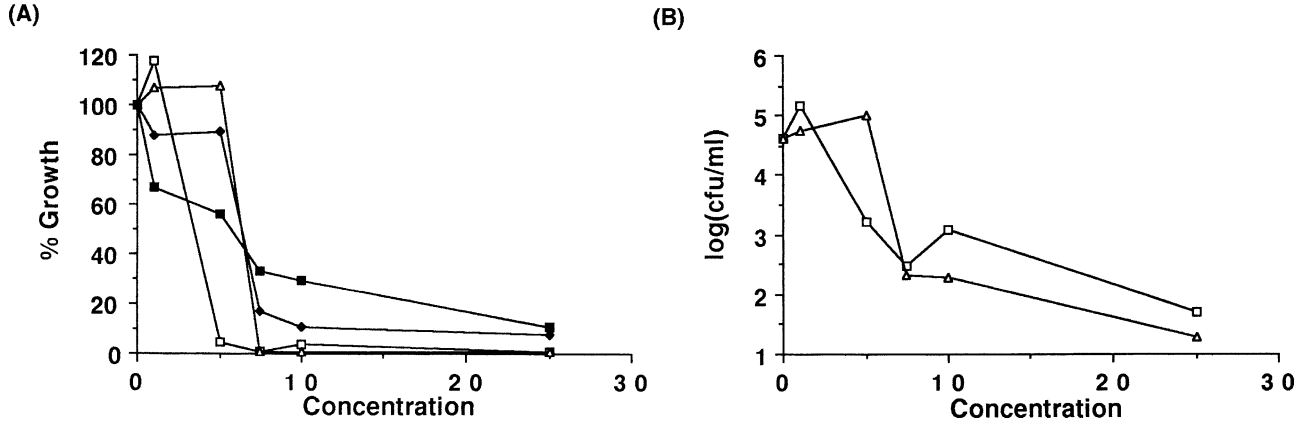

Figure 2. Growth inhibition of *M. tuberculosis* H37Rv in the presence of increasing concentrations of compounds ac_3py14, □, and [Fe(ac_3py14)], △, and that of a clinical isolate (38396), also in the presence of ac_3py14, ■, and [Fe(ac_3py14)], ◆, evaluated from (A) radiometric data and expressed as the percentage of growth in relation to the control vial, with no added compound, and corresponding (B) bacterial counts for the H37Rv strain on 7H11 medium, expressed as CFU/mL of culture in the Bactec vials, indicating that low GI values were not caused by direct inhibition of respiratory activity.

Analysis was carried out using an Ortho Cytoron Absolute Cytometer (Ortho Diagnostics, Johnson & Johnson, NJ, USA). Parameters were set to measure lymphocytes by forward scatter and side scatter. Fluorescence was analyzed by gating on all mononuclear cells and measuring fluorescein isothiocyanate (FITC), phycoerythrin (PE) Cy5-phycoerythrin tandem (CyP) and allophycocyeain (APC) profiles.

3. Results

3.1. Inhibition of *M. tuberculosis*

Figure 2A illustrates radiometric data showing significant growth inhibition by low concentrations of compounds ac_3py14 and [Fe(ac_3py14)] on Mtb H37Rv and on a fully sensitive clinical isolate. Inhibition reached 99% and 90% levels for Mtb H37Rv and the clinical isolate, respectively, at concentrations above 7.5 µg/mL. Comparative levels of inhibition were obtained with either the iron-free compound or its iron saturated complex. Bacterial inhibition was also confirmed at the end of the experiment by plating on Middlebrook 7H11 agar plates bacterial suspensions from the individual Bactec vials (*figure 2B*). Both of these observations indicate that the reductions in the GI were not determined by direct inhibition of the respiratory activity by these quelating compounds. Growth inhibition of *M. tuberculosis* multiple drug-resistant clinical isolates was within an 80–90% bracket at 25 µg/mL of compound ac_3py14 (data not shown).

3.2. IFN-γ and IL-5 production

Comparisons were made between the capacity of T cells to proliferate and to produce IFN-γ and IL-5 after stimulation with live Mtb H37Rv, ac_3py14 or no stimulation (no Ag) (*figure 3*). Healthy donors ($n = 4$) were evaluated for IFN-γ (*figure 3A*) and IL-5 (*figure 3B*) production after 6 days of stimulation. These studies demonstrated that the strongest mean proliferative (*figure 4*) and IFN-γ responses were towards Mtb H37Rv. The capacity to induce IFN-γ was slightly lower in response to ac_3py14, although these levels could still be considered relatively high. Mtb H37Rv induced increased IL-5 production in comparison to ac_3py14, showing moderate to low levels of IL-5 secretion.

3.3. Apoptosis

Annexin V binding (*figure 5A*) and propidium iodide uptake assessed the effect of ac_3py14 and Mtb H37Rv on the viability of $\alpha\beta$, $\gamma\delta$ T cells and CD14 positive macrophages. The ability of Annexin V to bind to the exposed phospholipid monomers and the exclusion of propidium iodide, indicative of early cellular death, were evaluated in healthy donors ($n = 3$)

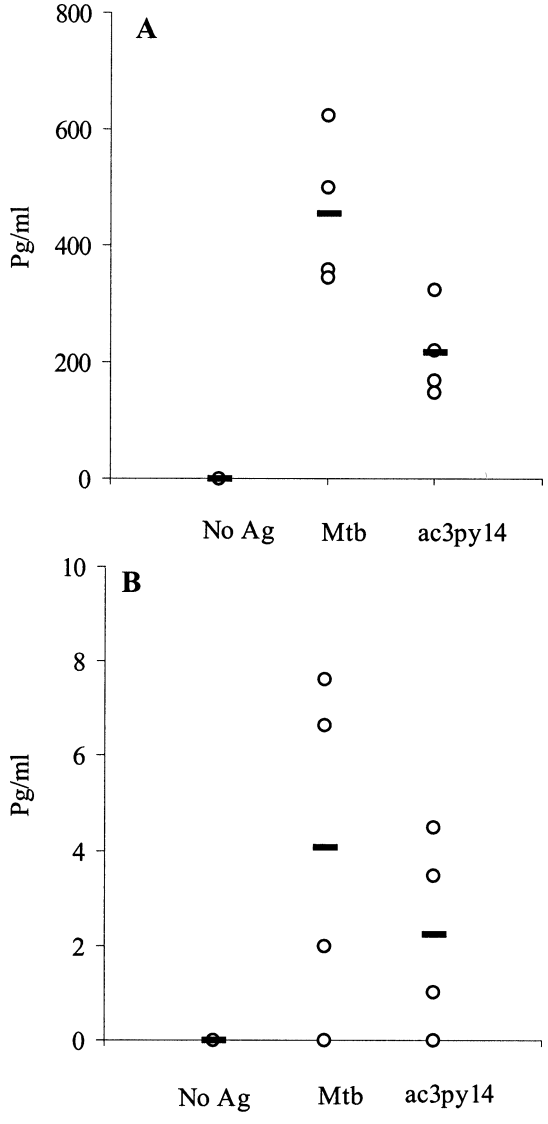

Figure 3. IFN-γ and IL-5 secretion by PBMC induced by live *M. tuberculosis* H37Rv (Mtb H37Rv), or ac_3py14, or the nonstimulated cell control (No Ag). PBMCs from healthy donors (n = 4) were stimulated for 6 days and supernatants were then removed for measurement of IFN-γ production (A) and IL-5 (B) by ELISA and expressed as pg/mL.

after 6 days of stimulation. After stimulation with ac_3py14, the highest mean % of Annexin V binding was found in cells of the $\alpha\beta$ T-cell phenotype (*figure 5A*) followed by $\gamma\delta$ T cells and CD14 macrophages in comparison to Mtb H37Rv. The percent of Annexin V binding induced by ac_3py14 was below 10% for all cell phenotypes.

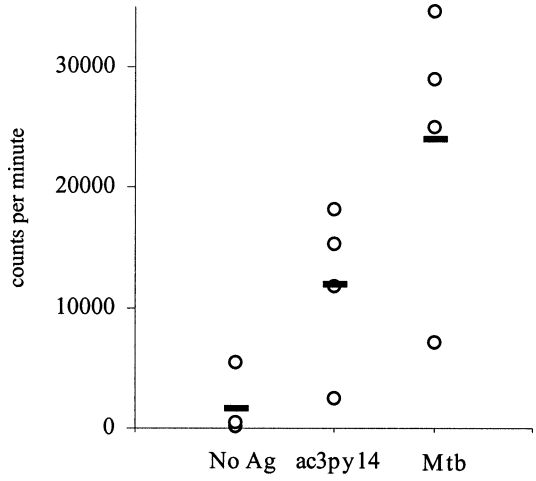

Figure 4. Comparison of individual proliferative responses by PBMC to Mtb H37Rv, ac_3py14, and the nonstimulated cell control (no Ag). PBMC (n = 4) were incubated with antigens for 6 days. Incorporation of ^{3}H-thymidine into the transforming cell DNA was measured on a beta particle counter and expressed as counts per minute.

3.4. CD45RA and CD45RO populations

After stimulation with ac_3py14 and Mtb H37Rv, $\alpha\beta$ and $\gamma\delta$ T-cell phenotypes were analyzed for their capacity to develop naïve CD45RA, and memory CD45RO T-cell populations (*figure 5B*). It was evident that ac_3py14 stimulated a higher percent of CD45RA and CD45RO T cells in both $\alpha\beta$ and $\gamma\delta$ subsets in comparison to Mtb H37Rv. It should also be noted that ac_3py14 seemed to induce $\gamma\delta$ T-cell expansion. The ac_3py14 compound elicited a larger quantity of memory cells than naïve cells, as did Mtb H37Rv. Assessment of both populations also revealed that stimulation with both ac_3py14 and Mtb H37Rv resulted in more Annexin V binding in the CD45RO$^+$ memory T-cell subsets (data not shown).

4. Discussion

In the treatment of any disease, including tuberculosis, the individual's capacity to construct protective immunity is at stake in determining the outcome of chemotherapeutic treatment. With this in mind, this study sought to evaluate, in healthy donors, both the antituberculosis effect of and the immune response to two compounds from the macrocyclic family, ac_3py14 and [Fe(ac_3py14)]. Perspective data show that ac_3py14 and [Fe(ac_3py14)] are inhibitory toward

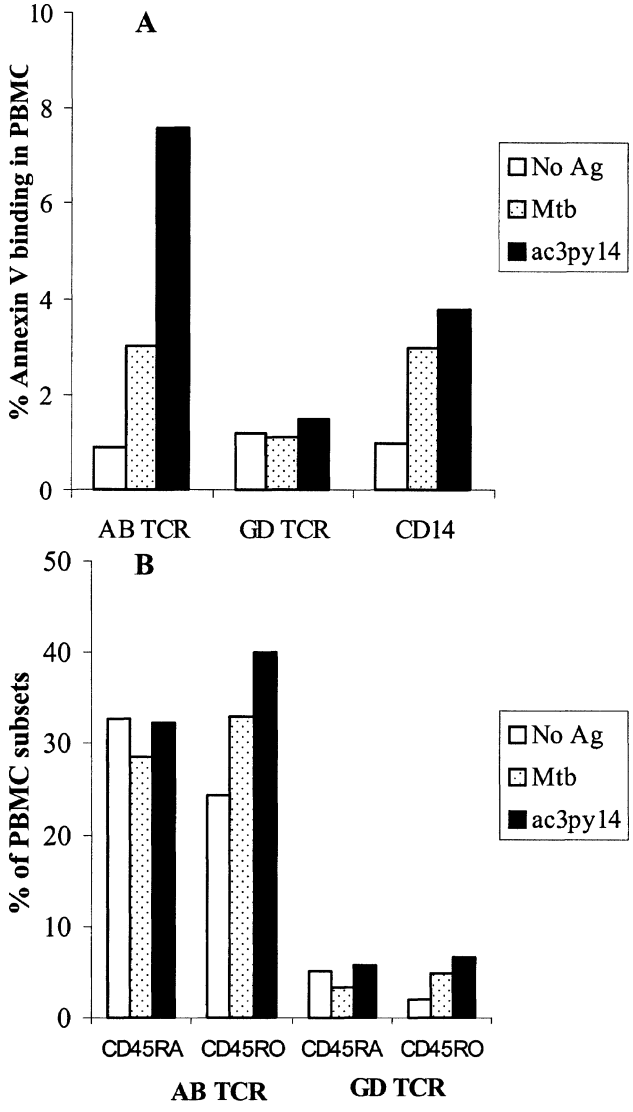

Figure 5. PBMC populations from healthy donors (n = 3) stimulated for seven days with *M. tuberculosis* (Mtb H37Rv) or ac_3py14, or nonstimulated cells (no Ag). On day seven cells were harvested and stained with fluorescent antibodies against $\alpha\beta$ TCR (T-cell receptor), $\gamma\delta$ TCR and $CD14^+$ macrophages in combination with Annexin V (A) or CD45 RA (B). Cells were analyzed on an Ortho Absolute Flow cytometer. Results are expressed as mean % Annexin V binding in PBMCs or mean % in PBMC.

M. tuberculosis, including clinical isolates of differing susceptibility status to primary antibiotics. Under the experimental conditions tested, growth in the Bactec vials was strongly inhibited. Inhibition of *M. tuberculosis* was obtained regardless of whether the compound was iron-free, ac_3py14, or in the form of the saturated complex [Fe(ac_3py14)]. Bacterial inhibition was also confirmed by plating bacterial suspensions from individual Bactec vials at the end of the experiments (*figure 2B*). Both observations indicate that the reduction in GI was not determined by direct inhibition of the respiratory activity by these chelating compounds.

There was no apparent drop in bacterial viability, as seen when comparing the bacterial population, by plating the suspensions from individual Bactec vials at the beginning and end of the experiments (data not shown). The compounds were thus apparently bacteriostatic.

The present study also produced evidence that stimulation with ac_3py14 induced proliferation of PBMCs and elicited IFN-γ production. On the other hand, IL-5 was produced at low levels. The action of cytokines of the Th1 type may be beneficial to the host via activation of macrophages, resulting in intracellular killing of mycobacteria.

Immunity against tuberculosis is generally assumed to be T-cell-dependent through the effect of cytokines, for example IFN-γ and TNF-α , resulting in activation of infected macrophages, or through lysis of infected cells by $CD4^+$, $CD8^+$ ($\alpha\beta$ T cells) and $\gamma\delta$ T cells. Although it has been difficult to prove, by in vitro experimentation with human macrophages, that IFN-γ induced bacterial killing, it has been shown to mediate a 1.9-fold reduction in bacterial growth [26], and it has been reported that T-cell-derived IFN-γ can inhibit intracellular growth of Mtb H37Rv [12].

In tuberculosis patients, IL-2 and IFN$-\gamma$ production (Th1) by PBMCs stimulated with *M. tuberculosis* H37Rv or Erdman strains was reduced compared to healthy BCG-vaccinated individuals [11]. IL-4 (Th2) has the capacity to inhibit the immune response by deactivating macrophages [10] and through downregulation of IL-2 receptor expression, thus reducing T-cell proliferation [14]. These results suggest that Th-1-like cells play an important role in antimycobacterial defenses.

However, the dichotomy of type 1 and type 2 cytokine production has been described in several infections, with one type of response conferring protection and the other being associated with disease [18]. Th1 cells are producers of IL-2, IFN-γ and lymphotoxin as well as other cytokines, while Th2 cells produce IL-4, IL-5, IL-6, and other cytokines but not IL-2 or IFN-γ [17].

Our results further indicate that there was no significant toxicity to $\alpha\beta$ and $\gamma\delta$ T cell and $CD14^+$ macrophages in the presence of ac_3py14, which could lead to T-cell functional abnormalities or a quantitative reduction in numbers leading to a deficiency of T cells. Although the level of Annexin V binding was increased compared to *M. tuberculosis* it was not of quantities which resulted in toxic effects toward T cells of the $\alpha\beta$ and $\gamma\delta$ phenotype. Macrophages which were $CD14^+$ resulted in increased binding of Annexin V in comparison to *M. tuberculosis*, but those values were not enough to result in high levels of cellular death or toxicity toward viable cells. The capacity of ac_3py14 to stimulate the differentiation of a large population of CD45RO T cells in both $\alpha\beta$ and $\gamma\delta$ T-cell phenotypes may indicate the ability to enhance a beneficial response. Further studies are needed in order to define specificity, effector function and induction of immunological memory in healthy donors and tuberculosis patients.

This is the first report showing that a compound may be both inhibitory to *M. tuberculosis* and stimulatory to the immune response against this organism. Studies on this family of compounds are currently in progress, in the hope that further investigations may clarify many of these aspects.

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