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THE INTERNATIONAL ARABIC JOURNAL OF ANTIMICROBIAL AGENTS

2013
Vol. 3 No. 2:1
doi: 10.3823/728

Evaluation of Structurally Related 3-Substituted 4-Amino-2-arylquinolines and 2-Aryl-4-methoxyquinolines for Potential Antimycobacterial Activity

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Abstract

Background: A series of structurally related 2-aryl-4-(amino/methoxy)quinoline derivatives were evaluated for potential antimycobacterial activity against *Mycobacterium tuberculosis* strain H37Rv.

Methods: The chemical compounds were tested against a drug sensitive and drug-resistant strains of *M. tuberculosis* using rapid radiometric techniques. The selected derivatives were tested for their intracellular activity against TB- infected macrophages. Two 4-amino-2,3-diarylquinoline derivatives were investigated for their immune modulatory effect with regard to to Th1 and Th2-subset cytokines

Results: A complete inhibition of a drug sensitive strain of *M. tuberculosis* was observed at 20.0 µg/mL for 4-amino-2-(4-chlorophenyl)quinoline 3b, 4-amino-3-iodo-2-(4-methoxyphenyl)quinoline 5d, 4-amino-2,3-diphenylquinoline 6a, 4-amino-2-(4-fluorophenyl)-3-phenylquinoline 6b and 4-amino-2-(4-methoxyphenyl)-3-phenylquinoline 6d. These derivatives were further evaluated for activity against a multidrug resistant strain of *M. tuberculosis*. The minimum inhibitory concentration (MIC) against a two drug-resistant strain was found to be ≥5.0≤20.0 µg/mL. Systems 6a and 6b were, in turn, subjected to cytotoxicity assay using U937 human macrophages and their subsequent intracellular antimycobacterial activity was determined. Intracellular *M. tuberculosis* growth was inhibited with 64 and 61% by compounds 6a and 6b, at concentrations of 18.00 and 14.00 µg/mL, respectively. Moreover, these two 4-amino-2,3-diarylquinoline derivatives were also investigated for their immune modulatory effect according to Th1 and Th2-subset cytokines. System 6b indicated activity that stimulated multi-effector macrophages with a mixed Th1/Th2 cytokine profile.

Conclusions: The presence of a primary amino group at C-4 and phenyl ring at C-3 and the accompanying increased basicity of the quinoline ring as well as electronic effect and lipophilicity of the substituent on the *para* position of the 2-phenyl ring seem to be critical for the antimycobacterial activity of the 2,3-diarylquinoline derivatives. Overall, the production of cytokines from this specific experiment gives an idea of the amount of individual cytokines produced daily instead of a cumulative response to the test samples during infection. Compound 6a holds potential to modulate the functionality of *M. tuberculosis* infected macrophages.

Keywords: antimycobacterial activity, *Mycobacterium tuberculosis*, immunomodulation, 2-aryl-4-methoxyquinolines, 4-amino-2-arylquinolines.

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Introduction

Tuberculosis (TB) is a contagious disease caused by respiratory infection from the Gram positive bacteria, *Mycobacterium tuberculosis*, and this disease remains one of the major world health problem with an estimated annual death toll of about 2 million [1]. The situation is further complicated by the association of TB with HIV infections [2] and the emergence of multidrug resistant (MDR) strains of *M. tuberculosis* [3]. The multidrug resistant strains of *M. tuberculosis* have become insensitive to one or more of the first-line drugs currently in the market, *viz*., streptomycin (STR), isoniazid (INH), ethambutol (ETH), pyrazinamide (PA) and rifampicin (RIF) [3]. Moreover, it is well known that *M. tuberculosis* survive in macrophages by various mechanisms and that anti-TB drugs that are effective in the acidic vacuoles of macrophages are needed for the treatment of TB [4-6]. Several fluoroquinoline antibacterial drugs have previously been examined as potential chemotherapeutics for *M. tuberculosis* infection [7]. The naturally occurring 4-methoxy-2-phenylquinoline and its 2-(methylenedioxyphenyl) analogue have recently been found to show inhibitory activity against *M. tuberculosis* $H_{37}Rv$ [8]. The analogous 4-aminoquinolines are known to exhibit antimalarial, anti-inflammatory, antibacterial, and antihypertensive activities [9] and some derivatives have also been found to serve as immunostimulants [10] and non-nucleoside HIV-1 inhibitors [11].

In the absence of an obvious immunosuppressive disorder such as HIV infection, the tubercle bacillus is non-pathogenic in 90% of those individuals infected [12, 13]. There are two types of T cell mediated immune responses against tuberculosis infection [14]. T cells are divided into mainly two subsets depending on the different cytokines produced, type 1 helper T cells (Th1; characterized by the production of IL-2, IL-12, IFN-γ and TNF-α) and type 2 helper T cells (Th2; characterized by the production of IL-4, IL-5 and IL-10). A predominant Th2-type cell response causes tissue damage and may, under some circumstances, have a minor protective role by isolating bacteria in necrotic foci. However, the immune response associated with a Th2 cell profile does not actively destroy tubercle bacilli and thus does not lead to sterilisation of the tissues. The second type of response is associated with Th1-type cells that induce a state of protective immunity (14). It has been proposed that a balanced stimulation of both responses is necessary for protective responses accompanied by minimal immunopathology [15].

Understanding of how the immune response and functioning is controlled by delicate balances between these two distinct subsets, based on cell cytokine profile, has increased dramatically in recent years [16]. Immune functions are mediated by the secretion of cytokines in a supposedly polarized fashion, classified as either Th1- or Th2-type cytokines [17]. Th1-type cytokines include mainly IFN-γ, IL-2 and IL-12 secretions [18, 19]. Th1 cells also secrete TNF, thereby enhancing B-cell responses that elicit IgG2a, the antibody subclass most efficient in binding the serum complement proteins that enhance antigen-antibody reactions [18]. Th2 cells express IL-4, IL-5, IL-10 and IL-13 [19, 20] that activate the production of high levels of IgG1, IgA, and IgE by B cells [18]. This cytokine heterogeneity is not restricted to CD4+ T cells, as other cell types also contribute to the secretion of regulatory cytokines. Thus, the terms Th1-type and Th2-type cytokines or cells are used to characterize the cytokine profile of different cell types. Immunological findings indicate that a therapeutic measure able to switch from a Th2 to a Th1 response would be of great benefit in treating TB [17, 18, 21]. In this context, compounds that can induce the Th1 response and subsequently suppress the Th2 response by increasing or decreasing the related cytokines can be valuable for anti-TB treatments.

The alarming increase in bacterial infections that have become resistant to most commonly used antibiotics prompted us to probe the known 4-methoxy-2-arylquinoline and 4-amino-2-arylquinoline derivatives [22-24] for potential antibacterial activity on *M. tuberculosis*. The cytotoxicity of the systems found to exhibit direct inhibitory antimycobacterial activity was determined using the U937 monocytic cell line, which maintains potential to differentiate macrophages upon stimulation. Moreover, their intracellular antimycobacterial activity was also determined. In light of previous results for 4-aminoquinolines serving as immunostimulants [10], the possibility that the present analogues can increase or decrease specific cytokine levels intracellularly in naive and *M. tuberculosis* infected U937 macrophages was also investigated.

Materials and Methods

General

A drug-susceptible strain of *M. tuberculosis* (sensitive to the first-line antituberculous drugs; INH, RIF, ETH and STR), H37Rv (ATCC 27294) was obtained from the American Type Culture Collection (Rockville, MD, USA). The two drug-resistant strain of *M. tuberculosis* (AW 4/2), resistant to both INH and ETH, was obtained from a clinical specimen (Medical Research Council, Pretoria [MRC], South Africa). All procedures involving manipulation of mycobacteria were carried out in a biological safety cabinet (Level 3, MRC). 4-Amino-2-arylquinolines 3a–c, 4-amino-2-aryl-3-bromoquinolines 4a–d, 4-amino-2-aryl-3-iodoquinolines 5a–d, 4-amino-2,3-diarylquinolines 6a–d, 2-aryl-3-iodo-4-methoxyquinolines 7a–d and 2,3-diaryl-4-methoxyquinolines 8a–d used in this investigation (Figure. 1.) were synthesized as described in our

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2013
Vol. 3 No. 2:1
doi: 10.3823/728

previous communications [22-24]. The test compounds were prepared in DMSO at concentrations of 5–20 μg/mL.

Preparation of micro-organisms

The micro-organisms, H37Rv drug-sensitive strain and the clinical drug-resistant strain (AW 4/2) of *Mycobacterium tuberculosis*, were plated onto slants of Löwenstein-Jensen (LJ) medium and allowed to grow for 3-4 weeks at 37 °C. The H37Rv reference strain of *M. tuberculosis* was used in the screening procedure and the AW4/2 strain was used in the subsequent activity assays at the MRC, Pretoria, South Africa. Both the strains were maintained and prepared in the same way. A representative amount of growth was taken from the LJ cultures by using a sterile applicator stick. This sample was transferred to a sterile 16×125 mm screw capped round tube containing six to eight glass beads (1-2 mm) and 3.0-4.0 mL of the diluting fluid (0.1% Tween 80/saline solution). The culture mixture was homogenised by mixing the tube on a Vortex mixer for 5-10 minutes. A homogenous suspension was obtained by placing the tube on the Vortex mixer for five minutes and then left for 15 minutes to allow the particles to settle. After the large particles had settled, the supernatant was transferred into a separate sterile test tube and more diluting fluid was added and adjusted to McFarland no. 1 turbidity standard (1×10^4 to 1×10^5 colony-forming units per millilitre [CFU/mL]). A hundred microlitres of the adjusted growth suspension was streaked onto two blood agar plates to determine any contamination of non-mycobacterial organisms.

Screening samples for antimycobacterial activity (*M. tuberculosis H37Rv*) using automated radiometric respiratory technique

The radiometric respiratory technique using the BACTEC 460 instrument was used for susceptibility testing against *M. tuberculosis* as described previously [25-27]. Solutions of all the compounds were prepared in DMSO to yield a final test concentration of 20.0 μg/mL in the BACTEC 12B vials containing 4 mL of 7H12 medium broth. Every vial was supplemented with antimicrobial PANTA solution (Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim and Azlocillin; Becton Dickinson and Company, Ferndale, South Africa). Control experiments showed that a final concentration of DMSO (1%) in the medium had no adverse effect on the growth of *M. tuberculosis*. Isoniazid (Sigma Chemical Co., South Africa), was used as the positive drug control. A homogenous culture (0.1 mL) of *M. tuberculosis*, yielding 1×10^4 to 1×10^5 CFU/mL, was inoculated in the vials containing the compounds as well as in the control vials. Three sample-free vials were used as controls (medium+1% DMSO): two vials (V1) were inoculated in the same way as the vials containing the samples, and one (V2) was inoculated with a 1:100 dilution of the inoculum (1:100 control) to produce an initial concentration representing 1% of the bacterial population (1×10^2 to 1×10^3 CFU/mL). Mycobacterium growing in 7H12 medium containing ^{14}C- labelled substrate (palmitic acid) use the substrate and produced $^{14}CO_2$. The amount of $^{14}CO_2$ detected (reflecting the rate and amount of growth occurring in the sealed vial) is expressed in terms of the growth index (GI) [28]. Inoculated bottles were incubated at 37 °C and each bottle was assayed at 24 h intervals at approximately the same hour (±2 h) until cumulative results were interpretable to measure the GI or until the GI of the V2 control reached a reading ≥30. The difference in the GI values of the last two days is designated as ΔGI. The ΔGI readings of the vials containing the test samples were compared with the V2 control vial. If the ΔGI values of the vials containing the test compounds were less than that of V2, the population was reported to be susceptible to the compound. Each test was replicated three times. Whenever results suggested contamination (e.g., large, rapid increase in GI), the bottles were inspected, Ziehl-Neelsen (ZN) staining was carried out and a 100.0 μL of the suspected vial medium was streaked on blood agar plates to determine whether the visible microbial growth was organisms other than *Mycobacterium tuberculosis*.

Automated radiometric respiratory detection of resistant *M. tuberculosis* susceptibility AW4/2 clinical strain

For preliminary studies, the derivatives were tested against a drug-sensitive strain of *M. tuberculosis* only at 20.0 μg/mL conentration. Five derivatives completely inhibited the growth of the mycobacteria and these were further selected for testing at two concentrations, namely, 20.0 and 5.0 μg/mL against a two drug-resistant strain of *M. tuberculosis* (AW 4/2). The latter was obtained from a clinical specimen (MRC, Pretoria, South Africa) and prepared in the same manner as the H37Rv strain, mentioned before (see 2.2). The samples were dissolved in 100% DMSO to obtain stock concentrations of 2.0 mg/mL to yield a final test concentration of 20.0 μg/mL and 5.00 μg/mL in the BACTEC 12B vials. The assay was carried out as mentioned for the H37Rv screening, test samples were prepared in triplicate and the assay performed on two separate occasions. The two drug-resistant test organism (resistant to both INH and ETH) was used as the drug-control for these bioassays at a final concentration of 0.2 μg/mL.

Cytotoxicity on differentiated U937 cells

The human promonocytic cell line U937 (ATCC available from Highveld Biological (Pty) (Ltd), (Sandringham, South Africa) was maintained in complete medium (RPMI 1640 medium

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[pH 7.2], with 10% heat inactivated foetal calf serum (FCS), 2 mM L-glutamine and a 0.1% antimicrobial solution consisting of pennicilin, streptomycin and fungizone). Reagents were procured from Highveld Biological (Pty) (Ltd) (Sandringham, South Africa). The U937 cell concentration was adjusted to $1{\times}10^5$ cells/mL, seeded into the inner wells of a 96-well tissue culture plate and treated with 0.10 µg/mL phorbol 12-myristate 13-acetate (PMA; Sigma) for 24 h at 37 °C in an atmosphere of 5% CO_2 to induce differentiation into macrophage-like cells [29, 30]. After the incubation period, the differentiated U937 cells were washed three times with phosphate-buffered saline (PBS), and adherent monolayers were replenished with complete medium without antibiotics. Cytotoxicity was measured by the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2-*H*-tetrazolium hydroxide (XTT) method using the Cell Proliferation Kit II (Roche Diagnostics GmbH) as described by the method of Mahapatra *et al.* (2007) [31]. The pure compounds 6a and 6b were made up to a stock concentration of 20.00 mg/mL and serially diluted to start with a concentration of 200.0 to 1.562 µg/mL from the first wells to the last in the microtitre plates. The anti-tubercular drug, INH was diluted in the same way. These dilutions were added to the inner wells of the microtiter plate and incubated for 72 h. After 72 h, 50.0 µL of XTT reagent (0.3 mg/mL) was added to the wells and the plates were then incubated for 1-2 h. The positive drug, (Actinomycin D; Sigma), at a final concentration range of $5.0{\times}10^{-2}$ to $3.9{\times}10^{-4}$ µg/mL, was included. DMSO (0.04%) was added to serve as the control for cell survival. The assay was carried out in triplicate. After incubation, the absorbance of the colour complex was spectrophotometrically quantified using an ELISA plate reader (PowerWave XS, Bio-Tek), which measured the OD at 450 nm with a reference wavelength of 690 nm. GraphPad Prism 4.03 software was used to statistically analyse the fifty percent inhibitory concentration (IC_{50}) values. The IC_{50} was defined as the concentration of the compounds at which absorbance, and thus cell viability, was reduced by 50%.

Parallel intracellular MIC determination and cell supernatant harvesting for immune modulation: U937 cell line.

The cells were infected with *M. tuberculosis* by diluting a concentration of actively growing bacilli into the cell culture medium and incubating with the cells over sixteen hours. The samples were then added to verify antimycobacterial activity intracellularly and the resultant cell supernatants were analyzed to determine possible immunomodulation by these compounds.

Preparation of cells and mycobacteria

U937 cells were cultured, plated in 96-well plates at $1{\times}10^5$ cells/mL and differentiated as described in section 2.5. *Mycobacterium tuberculosis* H37Rv was maintained as previously described (section 2.2). Three week-old colonies were scraped from LJ slants and a concentration of $6{\times}10^5$ CFU/mL was prepared in 10% heat inactivated FCS RPMI medium supplemented with a final concentration of 2.5% PANTA prevent contamination by non-mycobacterial organisms.

In vitro *Mycobacterium tuberculosis* infection of U937 cells.

Differentiated U937 cells were washed three times with phosphate-buffered saline (PBS), and adherent monolayers were replenished with complete medium without antibiotics. U937 cells ($1{\times}10^5$ cells/mL) were infected with mycobacteria ($3{\times}10^5$ CFU/mL) using bacteria-to-cell ratios of 3:1. The macrophages were allowed to phagocytose the bacteria for 16 h at 37 °C in a humidified atmosphere of 5% CO_2. In preliminary experiments phagocytosis of *M. tuberculosis* was evaluated by light microscopy using ZN staining. To evaluate the effect of *M. tuberculosis* phagocytosis on cell mortality, cells were periodically counted using the trypan blue dye (0.4%, Sigma) exclusion method. *M. tuberculosis* causes lysis and cell death in 10-14 days. In preliminary experiments, it was observed that a 10:1 ratio between *M. tuberculosis* and cells has a high cytopathic effect, a 5:1 ratio is better in terms of cell viability (25% in cultures with *M. tuberculosis* versus 45% in control cultures), while the 3:1 ratio gave the best cell viability (38% in cultures with *M. tuberculosis* versus 45% in control cultures). Therefore 3 bacilli/cell was the *M. tuberculosis*/cell concentration used in all the experiments.

Intracellular MIC assay and parallel collection of cell supernatants for immune modulation: U937 cell line

Preparation of sample solutions

Samples tested for determining the intracellular antimycobacterial and immunomodulatory activity included sample 6a and 6b at their IC_{50}, and half MIC concentrations as their MIC concentrations would lead to loss of cell viability. The anti-tubercular drug INH was included at half MIC, MIC and IC_{50} concentrations of 0.10, 0.20 and 200 µg/mL. The highest concentration of DMSO present in the assay (0.25%) was included as the vector control to measure cell viability. To ensure for enough volume of sample (two sets of samples, for

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infected and uninfected macrophages, each sample tested in triplicate and samples replaced at 24 and 72 h after the first sample addition) a volume of 4 mL for each sample concentration was prepared in RPMI medium (no supplementation) with a DMSO concentration of no higher than 0.25%. Sample solutions were prepared fresh on the first day of the experiment (designated as 0 h) and the remaining sample solutions were stored at 4 °C until used again at 24 and 72 h after initial sample addition. Cell viability was not affected as the highest concentrations of samples tested never exceeded the IC_{50} concentrations of the individual samples.

Confirming the infective dose

After the infection period the cells were washed with PBS an additional three times to remove any unphagocytosed, extracellular bacilli. To confirm the infection dose for all the samples, designated wells containing untreated, infected macrophages were lysed with 0.25% (w/v) sodium dodecyl sulfate (SDS, Sigma), performing 10-fold serial dilutions (10^0, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) and plating the lysate on Middlebrook 7H11 agar (Fluka Analytical, Sigma) for viable count determinations of intracellular organisms.

Intracellular MIC assay and supernatant collection

After the 16 h phagocytosis, 200.0 µL of fresh medium containing the desired test samples was transferred to each macrophage containing well (replicates of three per treatment). The cell supernatants were collected at 24, 72 and 120 h, transferred to sterile Eppendorf tubes and frozen at -70 °C for subsequent cytokine analysis. The sample-medium mixtures were replaced at 24 and 72 h. The culture was enumerated after lysing the macrophages with 0.25% SDS on day 5 (120 h). The lysed contents of each well were resuspended by multiple passages through a 25-gauge needle, and 100.0 µL was transfered to 7H11 agar medium for viable count determinations, serial 10-fold dilutions of the lysate (10^0, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) was included. An additional set of uninfected macrophages were handled and treated in identical conditions to the infected set of macrophages, to investigate the immunomodulatory effects of the samples on the macrophages alone. Methods used for intracellular infection were adapted from Passmore *et al.* (2001) [29], Rastogi *et al.* (1996) [32] and Lall *et al.* (2005) [33].

CFU enumeration

The conventional method for viable count determinations of intracellular *M. tuberculosis* involved plating of 10-fold serial dilutions (10^0, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) of sample lysate directly on 7H11 agar plates in triplicate for each concentration of samples and controls [32, 34]. The plates were incubated at 37 °C in 5% CO_2 atmosphere for 3-4 weeks followed by CFU enumeration via the colony counting system ColorQCount® (Spiral Biotech, Model 530). Appropriate positive and negative controls were included. A compound was considered bactericidal if it effectively reduced the bacteria viable counts in the test sample compared with the initial inoculum added at the time of compound addition [32, 34].

Cytokine detection via Cytometric Bead Array (CBA) analysis

Cell culture supernatants were thawed once and examined for IL-2, IL-4, IL-5, IL-10, IFN-γ and TNF-α concentrations by multiplex cytokine array analysis performed by using the Cytometric Bead Array (CBA) method using the Human Th1/Th2 Kit (BD-Biosciences). Using the same lots of samples, IL-12 levels were acquired with a commercial Human IL-12p70 Flex Set (BD-Biosciences). The IL-12 assay was run separately but in parallel with the Th1/Th2 Kit. The methods used were based on literature procedures [12-35, 36]. Assay procedures were performed as specified by the manufacturer. The methods for both the Th1/Th2 Kit and the Flex Set are similar, except that with the Th1/Th2 Kit, six cytokines were measured simultaneously, six standard curves (each ranging from 0 to 5000 pg/mL) and six cytokine results were acquired from one set of mixed calibrators and one test sample (cell supernatant) whereas with the Flex Set only the one cytokine (IL-12) was measured from a single test sample, and only one standard curve (0 to 2500 pg/mL) from the one calibrator was obtained. Briefly, the appropriate Capture Beads were mixed on a Vortex mixer before adding 50.0 µL to each of the assay tubes. Fifty microlitres of the relevant phycoerythrin (PE) detection reagent was then added to the assay tubes. For each of the test samples, 50.0 µL of cell supernatant was added to the test assay tubes and 50.0 µL of the cytokine standard dilutions were added to the control assay tubes. The assay tubes were then incubated for 3 h at room temperature away from direct exposure to light. During the incubation, the Cytometer Setup procedure was performed. After the incubation time, 1.00 mL of wash buffer was added to each assay tube and centrifuged at 200×g for 5 minutes. The supernatant from each assay tube was carefully aspirated (not to disturb the bead pellet) and discarded. To resuspend the bead pellet, 300 µL of wash buffer was added to each assay tube and mixed on a Vortex for 3-5 seconds before plating the samples out in 96-well microtiter plates. Acquisition was performed with flow cytometry utilising the BD FACSArray bioanalyzer and the data was analyzed with the FCAP Array software. The sensitivity for each cytokine using the BD CBA Human Th1/Th2 Cytokine Kit is as follows: IL-2, 2.6 pg/mL; IL-4, 2.6 pg/mL; IL-5, 2.4 pg/mL; IL-10, 2.8 pg/mL; TNF-α, 2.8 pg/mL; IFN-γ, 7.1 pg/mL and for the Human IL-12p70 Flex Set the limit of detection is 0.6 pg/mL These theoretical limits of

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2013
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doi: 10.3823/728

detection is defined as the corresponding concentration at two standard deviations above the median fluorescence of 20 replicates of the negative control (0 pg/ml).

Results and Discussion

Antimycobacterial activity

Antimycobacterial activity was assayed using the BACTEC radiometric assay, which is a well-documented technique for testing susceptibility of *M. Tuberculosis* [37-39]. Preliminary studies were performed on a drug-susceptible strain of *M. tuberculosis* H37Rv (sensitive to the first-line antituberculosis drugs; INH, RIF, ETH and STR). The antituberculosis positive drug, isoniazid (INH), was used as a control and it inhibited the growth of *M. tuberculosis* at 0.2 μg/mL during the screening assay. A compound is considered to be active if the difference in GI (ΔGI) of the test compounds is less than that of the control vial. If the ΔGI values of the vials containing the test samples were less than the control vials, the population was reported to be susceptible to the compound and if it was equal to or greater than that in the control vials, the test organisms were considered to be resistant to the drugs. The vast majority of quinoline derivatives tested, namely, 4-amino-2-arylquinolines, 4-amino-2-aryl-3-bromo/iodoquinolines and 4-amino-2, 3-diarylquinolines contain a primary 4-amino group (Figure1). The 2-aryl-3-iodo-4-methoxyquinolines and 2, 3-diaryl-4-methoxyquinolines were included for structure activity relationship analysis. Although the naturally occurring 4-methoxy-2-phenylquinoline and its 2-(methylenedioxyphenyl) analogue have recently been found to show inhibitory activity against *Mycobacterium tuberculosis* $H_{37}Rv$ [11] results from preliminary assays (**Table 1**) using drug susceptible strain of *M. tuberculosis* showed no activity for all the 4-methoxy-2-aryl-3-iodoquinolines 7a–d (MPOMe16–MPOMe19) and 2,3-diaryl-4-methoxyquinoline derivatives 8a–d (MPOMe20–MPOMe23). Most of the 4-amino-2-aryl-3-halogenoquinolines 3–5 (MPHJA01, MPHJA03–MPHJA10) displayed reduced or lack of activity against the drug-sensitive strain (results of inactive compounds not shown) with the exceptions of 3b (MPHJA02) and 4-amino-3-iodo-2-(4-methoxypheyl)quin-

Figure 1. List of the 4-heteroatom substituted 2-arylquniline derivatives evaluated for antimicobacterial activity.

Compound structure	NQ	X	4-R
3–6 (4-NH₂ quinoline, 3-X, 2-(4-R-phenyl))	3a (MPHJA01)	H	H
	3b (MPHJA02)	H	Cl
	3c (MPHJA03)	H	OMe
	4a (MPHJA04)	Br	H
	4b (MPHJA05)	Br	F
	4c (MPHJA06)	Br	Cl
	4d (MPHJA07)	Br	OMe
	5a (MPHJA08)	I	H
	5b (MPHJA09)	I	F
	5c (MPHJA10)	I	Cl
	5d (MPHJA11)	I	OMe
	6a (MPHJA12)	$-C_6H_5$	H
	6b (MPHJA13)	$-C_6H_5$	F
	6c (MPHJA14)	$-C_6H_5$	Cl
	6d (MPHJA15)	$-C_6H_5$	OMe
7–8 (4-OCH_3 quinoline, 3-X, 2-(4-R-phenyl))	7a (MPOMe16)	I	H
	7b (MPOMe17)	I	F
	7c (MPOMe 8)	I	Cl
	7d (MPOMe19)	I	OMe
	8a (MPOMe20)	$-C_6H_5$	H
	8b (MPOMe21)	$-C_6H_5$	F
	8c (MPOMe22)	$-C_6H_5$	Cl
	8d (MPOMe23)	$-C_6H_5$	OMe

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Table 1. Cytotoxicity and *in vitro* growth inhibition of *Mycobacterium tuberculosis.*

Compounds	GI[a] (20.0 µg/mL)	ΔGI[b]		MIC[c] (µg/mL)	nIC_{50}[d] (µg/mL)
		5.00 (µg/mL)	20.0 (µg/mL)		
3b (MPHJA02)	1.00	59.5 ± 4.95	5.00 ± 4.24	> 5.00 < 20.0	8.445± 0.048
5d (MPHJA11)	0.00	86.0 ± 14.1	41.0 ± 7.07	> 20.0	6.752±0.034
6a (MPHJA12)	8.00	76.00 ± ns	1.00 ± 0.00	> 5.00 < 20.0	18.43±0.421
6b (MPHJA13)	8.00	85.0 ± 18.4	3.00 ± 1.41	> 5.00 < 20.0	14.37±0.323
6d (MPHJA15)	0.00	87.5 ± 10.6	12.5 ± 3.54	> 5.00 < 20.0	7.968±0.178
V2[e]	23.0	36.0 ± 1.41		-	-
INH[f]	-3.00	40.1 ± 3.75		≥ 0.20	> 200.0
Actinomycin D	-	-		-	0.0038±0.0005

[a] Growth index for samples active against the drug sensitive strain (H37Rv) at 20.0 *µg*/mL.
[b] Difference in growth index for the drug resistant strain (AW 4/2) as the mean ± standard deviation.
[c] Minimum inhibitory concentration against drug resistant strain.
[d] Fifty percent inhibitory concentration of differentiated U937 macrophage cell-growth as the mean ± standard deviation.
[e] 1:100 dilution of the inoculums (control vial), representing 1% of the mycobacterial population ($1{\times}10^2$-$1{\times}10^3$ CFU/mL).
[f] INH tested at the known MIC of 0.20 µg/ml.

oline 5d (MPHJA11). Only the five compounds 3b, 5d, 6a, 6b and 6d bearing primary 4-amino group exhibited significant inhibition of drug sensitive *M. tuberculosis*. The strong conjugative effect of the 4-amino group due to compatible C_{2P}-N_{2P} pi bond interaction has been found to render the quinoline ring sufficiently basic so that it is protonated at physiological pH (pK>8.4) [40, 41]. The moderate conjugative effect of oxygen atom of the 4-methoxy group, on the other hand, would lead to reduced basicity of the quinoline ring and therefore less or no activity for the 4-methoxyquinoline derivatives. Despite the presence of a primary 4-amino group, which is expected to increase electron density at N1 and C3 atoms to led to increased basicity of the quinoline ring, all the 3-halogeno derivatives with the exception of 4-amino-3-iodo-2-(4-methoxyphenyl)quinoline 5d showed no activity against the drug sensitive strain. Increased activity displayed by 5d and the 4-amino-2, 3-diarylquinoline derivatives 6a, b and d against drug sensitive strain, may point to electronic effect of the 3-substituent on the electron density of the heterocyclic ring and also the basicity of N1. In our view, the electron withdrawing inductive effect of bromine or iodine reduces the electron density at C-3 leading to reduced electron density of the heterocyclic ring and therefore the basicity of N1. The increased propensity of the 4-methoxy group for electron donation to the phenyl ring of 5d, on the other hand, would lead to increased electron density on C-2 and in turn counteract the relatively weak electron withdrawing inductive effect of the 3-iodo group (**Figure 1**).

Based on these preliminary results, the five 4-aminoquinolone derivatives 3b, 5d, 6a, 6b and 6d were evaluated against drug resistant strain, *M. tuberculosis* H37Rv, and for minimum inhibitory concentrations (MIC) determination (**Table 1**). Increased activity against drug-resistant strains was observed for the 4-amino-2-(4-chlorophenyl)quinoline 3b (MPHJA02) and the 4-amino-2,3-diarylquinoline derivatives 6a (MPHJA12), 6b (MPHJA13) and 6d (MPHJA15). Compound 5d however showed no activity against the drug-resistant strain at any of the concentrations employed. Although 4-amino-2,3-diphenylquinoline 6a was not the most active compound during the preliminary screening against the drug sensitive strain, it showed increased activity against of *M. tuberculosis* H37Rv at 20.0 µg/mL than the other analogues (**Table 1**). Reduced activity displayed by 3b against the drug resistant strain points to the importance of the 3-phenyl substituent on activity (**Table 1**).

The observed trend in activity for the 4-amino-2, 3-diarylquinolines, on the other hand, is presumably the result of a combination of electronic and steric effects of the 2-aryl group. Accordingly, activity seems to decrease ($-C_6H_5$>$4-FC_6H_4-$>$4-MeOC_6H_4-$) with increasing size of the substituent ($H<F<OCH_3$) at the *para* position of the 2-phenyl ring.

Intracellular antimycobacterial activity

The enumerated CFU/mL of mycobacteria treated with test samples at 120 h were compared with the initial inoculum

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Table 2. Intracellular growth inhibition of *Mycobacterium tuberculosis* 120 h post-treatment.

Treatment	Viable count (CFU/mL)[a]
H37Rv[b]	(7.00 ± 1.62) × 10^5
INH	
0.10 µg/mL[c]	(5.80 ± 2.10) × 10^4
0.20 µg/mL[d]	(1.20 ± 0.18) × 10^4
6a	
10.0 µg/mL[c]	(6.30 ± 0.42) × 10^5
18.0 µg/mL[e]	(2.50 ± 0.68) × 10^5
6b	
10.0 µg/mL[c]	(3.60 ± 0.44) × 10^5
14.0 µg/mL[e]	(2.70 ± 0.12) × 10^5

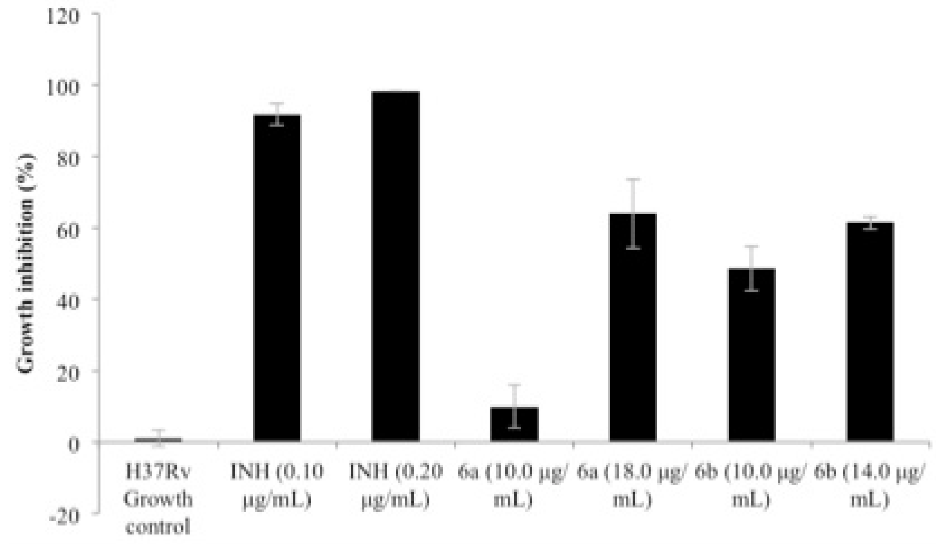
[a] Viable counts were performed by plating serially diluted cultures from cell lysate onto 7H11 agar medium. Expressed as the mean ± standard deviation.
[b] H37Rv inoculum containing 0.25% DMSO (initial infective dose determined as (2.20 ± 0.41) × 10^5 CFU/mL).
[c] At ½ MIC concentration.
[d] At MIC concentration.
[e] At IC_{50} concentration.

added at the time of compound addition (0 h), and a sample was considered bactericidal if it effectively reduced the bacteria viable counts (a one log decrease or 90% inhibition). The number of enumerated CFU's for the growth control (2.20×10^5 CFU/mL) corresponded well with the amount of bacilli used to infect the cells (3.00×10^5 CFU/mL). As expected, the positive drug INH inhibited the mycobacteria by a one log reduction in viable bacterial counts, from 10^5 to 10^4 CFU/mL at both concentrations tested (**Table 2**/**Figure 2**). The 4-amino-2, 3-diarylquinolines 6a and 6b, on the other hand, did not exhibit any significant inhibition of intracellular *M. tuberculosis* growth at the concentrations tested (**Table 2**/**Figure 2**). The direct MIC values of 6a and 6b are closer to 20.00 *µg*/mL and had to be tested intracellulary at sub-MIC concentrations, below or equal to their IC_{50} concentrations (**Table 2**) to avoid loss of U937 cell viability. Still, the general inactivity of 6a and 6b at their IC_{50} concentrations is surprising as the IC_{50} concentrations are not far from the proposed MIC values (**Table 2**) and these compounds are fairly fat soluble and would readily cross the cell membrane. Another reason for sample inactivity could be physicochemical, especially considering a pH change inside of the macrophages where the lysosomes containing the *M. tuberculosis* has a much lower pH as the rest of the cell, which could have a drastic effect on the structure and activity of the compounds. This acidic environment affects the action of anti-TB drugs such as streptomycin (activity reduced) and pyrazinamide (activity increased) (7) (**Table 2** and **Figure 2**).

Immunomodulation

In response to infection with mycobacteria, macrophages produce specific cytokines including the Th1-type cytokine, IL-12. These cytokines synergistically stimulate production of IFN-γ

Figure 2. Intracellular growth inhibition of *Mycobacterium tuberculosis* after 120 h of sample treatment.



All sample treatment results statistically significant comparisons for CFU/mL: P>0.05 when compared to the 120 h H37Rv inoculum containing 0.25% DMSO. Viable counts were performed by plating serially diluted cultures from cell lysate onto 7H11 agar medium. Viable counts have been expressed as percentage growth.

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doi: 10.3823/728

Table 3.1. Cytokine profiles (pg/mL) of sample treated, uninfected macrophages (72 h treatment).

Test Sample	Th1 Cytokines				Th2 Cytokines		
	IL-2	IL-12	IFN-γ	TNF	IL-4	IL-5	IL-10
Control	0.00	0.00	0.00	0.00	0.00	1.31	0.00
DMSO (0.25%)	0.00	0.00	0.00	0.00	0.00	0.00	0.00
INH:							
½ MIC (0.01 µg/mL)	0.00	0.00	0.00	0.00	0.00	0.00	0.00
MIC (0.02 µg/mL)	0.00	1.12	0.00	0.00	1.85	0.00	1.39
IC_{50} (200.0 µg/mL)	0.00	0.00	0.00	0.00	0.00	0.00	2.17
6a							
½ MIC (10.0 µg/mL)	0.00	0.00	0.00	0.00	0.00	0.00	0.00
IC_{50} (18.0 µg/mL)	0.00	1.23	0.00	0.00	0.00	1.22	2.17
6b							
½ MIC (10.0 µg/mL)	0.00	1.70	0.00	0.00	0.00	0.00	0.00
IC_{50} (14.0 µg/mL)	0.00	1.41	0.00	0.00	0.00	0.00	1.68

Table 3.2. Cytokine profiles of *M. tuberculosis* infected sample treated macrophages (72 h treatment).

Test Sample	Th1 Cytokines				Th2 Cytokines		
	IL-2	IL-12	IFN-γ	TNF	IL-4	IL-5	IL-10
Control	0.00	0.00	0.00	0.00	0.00	0.00	1.88
DMSO (0.25%)	0.00	1.30	0.00	0.00	0.00	0.00	0.00
INH:							
½ MIC (0.01 µg/mL)	0.00	1.14	0.00	0.00	1.11	0.00	2.03
MIC (0.02 µg/mL)	0.00	0.00	0.00	0.00	1.85	1.11	2.03
IC_{50} (200.0 µg/mL)	0.00	0.00	0.00	0.00	0.00	0.00	1.39
6a							
½ MIC (10.0 µg/mL)	0.00	1.47	3.49	1.76	3.69	1.70	2.55
IC_{50} (18.0 µg/mL)	6.30	1.23	22.31	8.21	17.19	3.91	8.81
6b							
½ MIC (10.0 µg/mL)	0.00	0.00	0.00	0.00	0.00	0.00	0.00
IC_{50} (14.0 µg/mL)	0.00	1.51	0.00	0.00	2.92	0.00	1.78

by CD4+ T cells and NK cells (lymphocytes). IFN-γ ligand is known to bind to its receptor on the macrophage cell surface and to activate the macrophage resulting in enhanced TNF-α production, production of other cytokines and chemokines, upregulation of MHC class II expression, enhanced antigen processing, and production of reactive oxygen species [42]. An infection is likely to induce a complex cytokine profile, including both stimulatory and inhibitory cytokines. Since multiple cytokine effects on macrophages are exerted simultaneously, the net result may be ultimately determined by the nature of cytokines present, the timing of their production and their relative concentration levels. In this investigation, the level of seven cytokines (Interleukin [IL] -2,-4,-5,-10,-12, Interferon gamma [IFN-γ] and Tumour necrosis factor alpha [TNF-α]) were measured via Cytometric Bead Array (CBA), a particle based immunoassay combined with flow cytometry. The levels of cytokine production was very low (**Table 3.1** and 3.2) mainly due to the experimental circumstances where the supernatant was initially collected and stored at 24 h after incubation and replaced with fresh sample-containing medium and only collected and stored again after another 48 h. Due to technical difficulties, only the supernatants collected

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72 h after infection are reported herein. Cytotoxicity was measured 72 h after sample addition and can thus be correlated with the cytokine results. Considering the experimental outcomes for the naive (uninfected) macrophages (**Table 3.1**), none of the test samples produced any toxic effects as most samples exhibited minor cytokine production similar to that of the untreated differentiated cells and the DMSO (0.25 %) control (**Table 3.1.** and **Table 3.2**).

At first glance it seems as though the data generated from the cytokines profiles in the context of this study has limited value. However, relative to the naive cells, the degree of cytokine production in the infected cells treated with samples 6a and 6b was greater with respect to the range and concentration of cytokines (Table 3.1). Cells with concomitant production of different cytokines harbour multiple effect or functions and are functionally superior to their single-cytokine counterparts [43]. Therefore the functionality of the infected cells is increased when treated with 6a and 6b especially upon treatment with 6a. This effect is not attributed to intracellular antimycobacterial activity of 6a as no significant inhibition was observed (Table 2/Figure 2). Infected cells treated with compound 6a represent the only sample that induced production of all seven cytokines measured during this study and at significantly higher levels than any of the other test samples. This is interesting since $CD4^{+}$ lymphocytes rather than macrophages are the primary cells for IFN-γ and IL-2 production during *M. tuberculosis* infection and therefore low levels of these two Th1 cytokines were expected. System 6a induced production of both these Th1 cytokines whereas the other samples or controls did not induce the production of IFN-y or IL-2. Untreated cells (control cells) infected with *M. tuberculosis* did not express any cytokines save for the Th2 cytokine IL-5 at a negligible mean concentration of 1.31 pg/mL. Similar results were observed for TNF where only infected cells treated with 6a produced this important cytokine. TNF plays a critical role in the control of mycobacteria, which drives granuloma development and also concentrates antimycobacterial drugs within macrophages [44]. However, TNF-α does not play a strictly beneficial role in tuberculosis pathogenesis and has been referred to as a "double-edged-sword". Increased plasma TNF-α levels have been associated with clinical deterioration early in the treatment of severe tuberculosis. TNF may have deleterious roles such as overt tissue damage in the setting of overproduction, as TNF-α is the cytokine widely thought to be responsible for the effects of tuberculosis including fever, weight loss, tissue necrosis and the Koch phenomenon [45]. Treatment with samples 6a and 6b slightly altered the production of both type 1 and 2 cytokines during infection, but did not induce a marked polarized type 1 to type 2 state but rather a mixed Th1/Th2 response. Very low levels of Th2 cytokines (especially IL-4 and IL-10) are needed to negate the effects of Th1 cytokines even if the latter are at higher concentrations [46]. In addition, the Th1-type cytokine IFN-γ is synthesized earlier than the type 2 cytokine, IL-4 [17]. According to a previous report, when carried out gene expression analyses using the reverse transcription polymerase chain reaction for the iNOS and the cytokines IL-1, IL-12, IL-18, TNF-α, IFN-α, and IFN-γ in non-infected and in *Leishmania major*-infected RAW 264.7 cells was carried out, low mRNA levels in non-infected cells, but considerably up-regulated transcript expressions in infected cells. Production of IFN-γ mRNA was also stimulated for the methanol-insoluble fraction (MIF) and gallic acid. The methanol-soluble fraction and umckalin did not show any significant gene-inducing capabilities.

Conclusion

The presence of a primary amino group at C-4 and phenyl ring at C-3 and the accompanying increased basicity of the quinoline ring as well as electronic effect and lipophilicity of the substituent on the *para* position of the 2-phenyl ring seem to be critical for the activity of the 2, 3-diarylquinoline derivatives. Overall, the production of cytokines from this specific experiment gives an idea of the amount of individual cytokines produced daily instead of a cumulative response to the test samples during infection. Compound 6a holds potential to modulate the functionality of *M. tuberculosis* infected macrophages. Research is currently underway in our laboratories to prepare series of analogues substituted with various aryl or heteroaryl rings at the 3-position for further *in vitro* immunoassays with macrophages from PBMC's and co-cultures with lymphocyte cells.

Acknowledgements

The authors are grateful to the staff at the Medical Research Council (Pretoria, South Africa) for kindly allowing us the use of their facility and for donating the Mycobacterial strains, Karlien Le Roux and Elizabeth Mogapi (University of Pretoria) for maintaining the U937 cells. Financial assistance from the University of South Africa and the National Research Foundation is gratefully acknowledged.

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