Evaluation of Minor Groove Binders (MGBs) as novel anti-mycobacterial agents, and the effect of using non-ionic surfactant vesicles as a delivery system to improve their efficacy

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Running title: Non-ionic surfactant vesicles to deliver Minor Groove Binders intracellularly for *M. tuberculosis* sterilization

ABSTRACT

Objectives: The slow development of major advances in drug discovery for the treatment of *Mycobacterium tuberculosis* (*Mtb*) infection have led to a compelling need for evaluation of more effective drug therapies against tuberculosis. New classes of drugs are constantly being evaluated for anti-mycobacterial activity with currently a very limited number of new drugs approved for TB treatment. Minor Groove Binders (MGBs) have previously revealed promising anti-microbial activity against various infectious agents; however have not yet been screened against *Mtb*.

Methods: Mycobactericidal activity of 96 MGB compounds against *Mtb* was determined using H37Rv-GFP microplate assay. MGB hits were screened for their intracellular mycobactericidal efficacy against clinical Beijing *Mtb* strain HN878 in bone marrow-derived macrophages using standard colony-forming unit counting. Cell viability was assessed by CellTiter-Blue assays. Selected MGB were encapsulated into non-ionic surfactant vesicles (NIVs) for drug delivery system evaluation.

Results: H37Rv-GFP screening yielded a hitlist of 7 compounds at an MIC_{99} between 0.39 and 1.56 μM. MGB-362 and MGB-364 displayed intracellular mycobactericidal activity against *Mtb* HN878 at MIC_{50} of 4.09 μM and 4.19 μM respectively, whilst being non-toxic. Subsequent encapsulation into NIVs demonstrated a 1.6 and 2.1-fold increased intracellular mycobacterial activity, similar to that of rifampicin when compared to MGB alone formulation.

Conclusions: MGBs anti-mycobacterial activities together with non-toxic properties indicate that MGB compounds constitute an important new class of drug/chemical entity, which holds promise in future anti-TB therapy. Furthermore, NIVs ability to better deliver entrapped MGB compounds to an intracellular *Mtb* infection has provided merit for further preclinical evaluation.

Introduction

Mycobacterium tuberculosis (*Mtb*), the causative agent of tuberculosis (TB), has become the top infectious killer worldwide. According to the 2016 World Health Organization (WHO) Global Tuberculosis Report,[1] TB killed approximately 1.8 million people in 2015, up from 1.5 million deaths in 2014.[2] The current six-month treatment regimen for drug-susceptible *Mtb*, although still effective in most cases, is gradually becoming ineffective due to increasing resistance against the drugs used to treat TB.[3] Several advances have been made in the field of TB drug discovery, spearheaded by global partnerships. For example, the Global Alliance for TB Drug Development currently manages the largest array of novel anti-TB drug compounds and novel regimens for MDR and XDR TB.[4] Other initiatives to eradicate TB include the STOP TB partnership that includes an international working group to develop new TB drugs.[5] Furthermore, several large consortia of pharmaceutical companies (TB Drug Accelerator) and academia (MM4TB) drive the discovery of new TB drugs.[6] Despite the progress in the pipeline for new diagnostics, drugs, regimens, and vaccines, research remains relentlessly underfunded. As a consequence, only a few new drugs have been approved for clinical use, i.e. delamanid, bedaquiline and pretomanid, and only ten new drugs are in advanced phases of clinical trials as of 2016.[7, 8] With the slow development of major advances in anti-mycobacterial drug discovery and the emergence of multi-drug-and extremely drug-resistant TB, there is an urgent need for the development of more effective therapies and formulations of existing drugs for the treatment of TB.[8, 9] In the area of novel therapeutics discovery, progress has been made in developing new drug classes such as benzothiazinones, which inhibit cell wall arabinan synthesis, and imidazopyridines, which inhibit respiratory chain ATP synthesis.[10, 11] Minor Groove Binder compounds (MGBs) have revealed promising antibacterial properties, but have not yet been investigated for their anti-mycobacterial activity against *Mtb in vitro.*

Derived from the natural product distamycin, MGBs are a class of compounds that selectively bind to the minor groove of bacterial DNA with their helical structure matching

that of DNA.[12] Most often, proteins binding to bacterial DNA bind to the major groove, leaving the minor groove exposed and thus, a vacant target for MGBs. Natural forms of MGBs are currently used in clinical treatment of disease. For example, aromatic diamidines, such as pentamidine,[13, 14] and berenil,[15] known to bind to the minor groove at adenosine-thymine tracts, have been administered clinically against human African trypanosomiasis and *Pneumocystis carinii* pneumonia.[16-18] MGBs display a wide variety of activity profiles against many infectious organisms evaluated, including Gram-positive bacteria,[19] *Mycobacterium aurum,*[20] chloroquine sensitive and resistant *Plasmodium falciparum,*[21] and *Trypanosoma brucei brucei*.[17] In partnership with MGB-Biopharma, one MGB compound has successfully completed phase I clinical trials for the treatment of *Clostridium difficile* infections.[22] We recently screened a limited number of MGBs for their anti-mycobacterial activity against the laboratory *Mtb* H37Rv strain with MIC_{99} reaching 3.1 uM.[23] We have now further extended this work by producing more active MGBs with higher MIC_{99} values against *Mtb* H37Rv. In addition we examined the anti-mycobacterial activity of MGBs against intracellular clinical HN878 Beijing strain of *Mtb* and evaluated the effect of MGBs exposure on cell viability in macrophages.

Oral drug administration has various limitations such as drug inefficiency resulting from drug insolubility caused by gastric low pH or poor absorbance in the gastrointestinal tract. However, an effective drug delivery system can improve drug retention at the site of infection. Therefore, an ability to deliver the drug to the site of infection may provide a sustained drug concentration enabling increased effectiveness of a drug against its target. In the case of pulmonary TB treatment, oral drug administration leads to high systemic concentrations of the drugs with associated side effects such as liver toxicity and cytotoxicity, amongst others.[24] Ultimately, the drawbacks associated with the oral administration of antibiotics laid the foundation for the development of innovative drug delivery approaches. The use of liposomes as a drug delivery system has been previously reported to reduce microbial drug resistance through faster drug delivery and increasing the antimicrobial drug concentration thereby preventing microbial drug efflux pump activity.[25]

Liposome encapsulated drugs kill microbes faster before microbial mutations can develop. For example the incorporation of the antibiotic levofloxacin into liposomes improved the anti-mycobacterial activity to kill *Mtb* strain resistant to levofloxacin.[26] Other drug delivery systems such as non-ionic surfactant vesicles (NIV) have the ability to encapsulate both hydrophobic and hydrophilic drugs for direct delivery to the site of infection.[27] NIVs are small colloidal particles made of a non-aqueous, non-ionic surfactant bilayer that surrounds a central aqueous compartment. They are thermodynamically stable, easily manufactured and do not require special storage conditions. One of the major advantages of NIVs is that they are able to entrap different types of drug substances and can have their size altered. Their capacity to improve the delivery of small molecules is an important trait that allows for precise targeting of deposition of particles within the respiratory tract. Previous studies have shown NIVs to be a promising inhalable drug delivery system against pulmonary aspergillosis with aerosolized amphotericin B (AMB)-NIV administration reducing fungal lung burden when compared to AMB solution only.[28] More recent studies are showing antibacterial action of moxiflacin[29] and cefixime[30] and antiviral action of nevirapine[31] in NIV formulations. Although many different drug delivery systems have been utilised to entrap first-line TB drugs,[32] only a few have systematically explored their anti-mycobacterial activity against *Mtb* and against intracellular *Mtb* in infected primary macrophages. Thus, we have investigated the use of NIVs as a drug delivery system on the improvement of delivery and efficacy of novel MGB compounds to *Mtb*-infected macrophages.

Materials and methods

Minor Groove Binder compounds

MGB compounds were synthesized using distamycin template, a natural product with known infective properties as previously reported.[17, 23, 33] Alterations of the head, tail, side chains and body resulted in a number of diverse compounds with later synthesis driven by acquired screening data (Table S1). MGBs were re-suspended in DMSO to a concentration of 1.25 mM and were stored at -80˚C.

Preparation of compounds and non-ionic surfactant vesicles

MGB compounds (Stock: 1.25 mM) and rifampicin (Stock: 20 mM) were diluted to a starting concentration of 50 µM followed by 2-fold dilutions in 7H9 broth medium or DMEM to yield required screening range. Freeze dried NIVs were prepared as previously described[28] and rehydrated in DMEM + 10% FCS (Gibco, Thermofisher Scientific, USA) to a NIV concentration range of 23-5000 µM (empty NIV) and subsequently added to bone marrow-derived macrophages (BMDMs) in order to assess cell viability through CellTiter-Blue (Promega, Wisconsin, USA) assay with fluorescence detection at (544_{ex}/590_{em} nm). Subsequently, drug-NIV solutions were prepared in DMEM + 10% FCS at 2:5 molar ratio (MGB: NIV) at compound two-fold serial dilution range from 1.56 to 12.5 µM (3.91-31.25 µM NIV) to assess cell viability and intracellular anti-mycobacterial activity. Two-fold serial drug dilution was performed as previously reported in other drug screening studies.[34]

H37Rv-GFP microplate screening assay

MGB compounds were screened for their anti-mycobacterial activity using 96-well, black clear flat-bottom microplates (Greiner Bio-One, Germany) as previously reported.[35, 36] Single cell suspension of H37Rv-GFP from frozen stock with working concentration of $1x10^6$ cfu/mL, was prepared in Middlebrook 7H9 supplemented with 25 mg/l kanamycin, 10% Middlebrook OADC (v/v) and 0.05% tween 80 (w/v). 100 µL of H37Rv-GFP at a

concentration of $1x10^5$ cfu/well was added to each experimental well. 100 µL of drug compounds prepared in 7H9 broth supplemented with 25 mg/L kanamycin to generate 0.195-50 µM screening range, was added to well containing H37Rv-GFP for final screening range of 0.0977-25 µM. Wells containing compound only at the highest screening concentration were used to detect autofluorescence of compounds and broth (vehicle control). Fluorescence (485_{ex} /520_{em} nm) was measured at designated time points; days 0, 4, 8, 10 and 12 with BMG Labtech Omega Plate Reader (Germany). The addition of sterile water to the outer wells of each plate served to minimize the evaporation. Time intervals were selected as previously reported in other drug screening studies.[36]

Bone marrow-derived macrophages generation and Mtb infection

BMDMs were generated from 8-12 week old C57BL/6 mice as previously reported.[37] After differentiation, BMDMs were plated into 96-well plates (Nunc, Denmark) at 2 x 10^5 cells per well. Following overnight adherence, BMDMs were then infected with *Mtb* HN878 (MOI=5) and cultured at 37˚C under 5% CO_2 for 4 hours. BMDMs were washed once with pre-warmed culture media to remove extracellular bacteria or lysed and lysates plated on 7H10 agar plates supplemented with 10% OADC and 0.5% glycerol for cfu counting to determine bacilli uptake. Drug compounds prepared in DMEM media supplemented with 10% FCS at defined concentrations were added to infected BMDMs to determine anti-mycobacterial activity and cell viability. After 5 days of culture, cells were lysed for cfu plating or assessed for cell viability by CellTiter-Blue assay.

Statistical analysis

All data were analysed using R, a student t-test (two-tailed with equal variance) or unless otherwise stated in figure legends. A *p value of less 0.05 was considered significant, with **$p < 0.01$ and ***$p < 0.001$.

Results

Minimum inhibitory concentration (MIC_{99}) of MGB compounds against H37Rv-GFP

We screened 96 MGBs for their anti-mycobacterial activity against GFP-labelled H37Rv *Mtb* in liquid broth culture using a 96-well plate assay (Table 1). Relative fluorescence was measured at 0, 4, 8, 10 and 12 days in broth culture of MGBs (serially diluted from 25 μM to 0.19 μM) to determine the minimum inhibitory concentration (MIC_{99}) of MGBs required to eradicate 99% of *Mtb* (Figure 1). Hit compounds, defined as previously reported,[38] were identified as drugs that were active at or below the threshold concentration of 3.12 μM. A hitlist of 7 compounds were identified with an MIC_{99} of 1.56 μM or less (Figure 1 and Table 1). Rifampicin, which had an MIC of 0.0977 μM, was used as the positive control. The selected hit compounds were MGBs 362, 368, 361, 365, 359, 364 and 367 with MIC_{99} range (0.391-1.56 μM) and therefore were identified for subsequent intracellular anti-mycobactericidal activity screening.

Intracellular drug activity against clinical Mtb and macrophage cell viability

The ability of anti-TB drug compounds to penetrate macrophages and induce mycobactericidal activity, while being non-toxic to the macrophages, is a salient property sought after in TB drug development. Hence, BMDMs were exposed to serial MGB drug concentrations from 1.56 to 12.5 μM to evaluate their anti-mycobacterial activity against the clinical *Mtb* strain HN878, after 5 days of infection. Compounds were screened for the concentration which eradicated 50% of bacilli (MIC_{50}, Figure 2A). Two of the 7 hit compounds identified from screening studies against *Mtb* in Figure 1 had good intracellular mycobacterial killing efficacy against *Mtb*-infected macrophages, with MIC_{50} values of 4.09 μM (MGB 362) and 4.19 μM (MGB 364). Rifampicin, selected as a positive control, had a MIC_{50} of 1.7 μM. CellTiter-Blue cell viability assay was performed to assess for macrophage

cell viability in MGBs-treated BMDMs after 5 days of exposure (Figure 2B). MGB 362 and 364 and rifampicin had no significant effect on macrophage viability at the respective intracellular drug activity MIC_{50} concentrations (Figure 2B). These data suggests that MGB 362 and 364 have an efficient intracellular anti-mycobacterial activity against *Mtb* while being non-toxic to the host cells.

MBGs-NIV encapsulation increased intracellular drug activity against clinical strain of Mtb

We next investigated whether encapsulating our hit MGB compounds into NIVs, a drug delivery system that was previously reported to improve drug delivery of amphotericin B to macrophages,[28] would improve MGBs drug efficacy against the intracellular clinical HN878 *Mtb* strain. We demonstrated that encapsulating MGBs into NIVs improved the intracellular anti-mycobacterial abilities by 2.1-fold for MGB 362, and 1.6-fold for MGB 364 in *Mtb* HN878-infected BMDMs, displaying a significant cfu reduction ($P < 0.01$) compared to controls (Figure 3A). The anti-mycobacterial killing activity of MGB 362-NIV and MGB 364-NIV were similar to that of rifampicin. MGB-NIV 364 displayed a significant decreased cfu counts ($P < 0.033$) when compared to MGB alone. Furthermore, *Mtb*-infected macrophages were viable following MGB-NIV treatment (Figure 3B). Treatment with NIV-alone also had no significant effect on macrophage viability (data not shown). These results demonstrate that NIVs can act as a suitable delivery system by transporting MGB inside macrophages, the target cells for *Mtb*.

Discussion

MGB compounds have shown great potential for their use as antibacterial therapeutic agents.[33] However, their activity against *Mtb* remains unknown. Here, we demonstrated the anti-mycobacterial (MIC_{99}) properties of MGBs against *Mtb* (H37Rv-GFP) with a reliable screening method that enables the detection of most active compounds,[39] using rifampicin as a positive control. All of the active MGB compounds belong to the well-established alkene-linked minor groove binder family discovered at the University of Strathclyde with high killing activities against different pathogens as previously reported.[17, 19-21, 23, 33] Since the primary binding sites of all of these MGBs in the DNA minor groove are AT rich regions it is unlikely that target sequence specificity is responsible for the selectivity observed. This is true also for the active compounds against *Mtb* described here. However, it is more likely that activity and selectivity against a particular pathogen is caused by differential access to cells caused by differing cell wall and cell membrane structures in a way that with the current state of knowledge is idiosyncratic and unpredictable.[33] What can be reliably stated is that the alkene-linked compounds are significantly the most biologically active of the Strathclyde MGB family. In general, MGBs with the most significant antibacterial activity possess a range of different tail groups, all of which are exemplified within the set in our screen. However, all of the most active MGBs identified in this study possess an amidine-containing tail group, which perhaps suggests an important role of tail group pKa for targeting mycobacteria.

Screening of MGB compounds in the context of their cell viability and anti-mycobacterial activity against intracellular clinical *Mtb* strain HN878 have identified two compounds with promising results, giving a hit rate of 2.1% (2/96). In most studies the hit rate for hit compounds is in the order of 1%, in-line with previous studies.[40] These findings however warrant *in vivo* testing which aims to allow for better clinical therapeutic translation of the findings. The use of non-ionic surfactant vesicles (NIVs) has been demonstrated repeatedly in literature before and constitutes a prominent focus within current *Mtb* research in order to combat the infection.[27, 41] NIVs given by nebulisation delivered amphotericin B to

253 the lungs and liver with significantly improved treatment outcome when compared to AMB
254 solution against pulmonary aspergillosis and visceral leishmaniasis.[28] Our investigation of
255 NIVs as a delivery device indeed demonstrate that NIVs can be used to enhance the efficacy
256 of MGB compounds against HN878 in infected BMDMs whilst not increasing the toxicity of
257 the drug to BMDMs. MGB contain hydrophobic head groups[12] which allows for encapsulation
258 into NIV. Liposomes have previously been reported to encapsulate an alkyl derivative of
259 distamycin A[42] which are naturally occurring backbones for MGB compound synthesis.

260 NIVs ability to trap the drug within its hydrophilic/-phobic compartment allows the
261 drug to be taken up by phagocytosis by the infected macrophage, thereby transporting the
262 drug to the site of infection. Using NIV drug formulations resulted in higher drug levels
263 compared to similar treatment with drug solution at the site of infection after treatment by the
264 pulmonary or intravenous routes for water soluble[43, 44] and lipid soluble drugs[28]. Studies in
265 dogs treated by the intravenous route with a sodium stibogluconate-dextran (SSG)-NIV
266 formulation increased the elimination half-life and the volume of distribution at steady state
267 compared to SSG-dextran solution.[45] Therefore NIV-MGB formulation can be a feasible
268 pulmonary treatment for *Mtb*.

269 In conclusion, this study showed that MGBs constitute an important new class of
270 drug/chemical entity with favourable anti-mycobacterial activity and holds promise in future
271 anti-TB therapy. Furthermore, we demonstrate that NIVs contribute to better delivery of
272 drugs to an intracellular infection and secondly act as a delivery device for entrapped MGB
273 compounds and lastly serve as the initial step into future research of targeted delivery of
274 entrapped drug to *Mtb*-infected cells.

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Transparency Declarations

None to declare.

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Table 1. MIC_{99} of all screened MGBs against H37Rv-GFP. 7 hits were identified out of 96 MGBs screened. MGBs marked with asterisk symbols were previously screened as reported.[23]

	Compound	MIC_{99}	Compound	MIC_{99}	Compound	MIC_{99}
	Rifampicin	0.0977	**371**	25	**235**	>25
Hits	**362**	0.391	**372**	25	**245**	>25
Hits	**368**	0.391	**373**	25	**246**	>25
Hits	**361**	0.781	**374**	25	**247**	>25
Hits	**365**	0.781	**381**	25	**248**	>25
Hits	**359**	1.56	**1**	>25	**270**	>25
Hits	**364**	1.56	**2**	>25	**271**	>25
Hits	**367**	1.56	**9**	>25	**283**	>25
	353*	3.12	**12**	>25	**286**	>25
	354*	3.12	**74***	>25	**287**	>25
	391	3.12	**85**	>25	**288**	>25
	263	6.25	**92**	>25	**289**	>25
	343	6.25	**114**	>25	**300**	>25
	385	6.25	**121**	>25	**303**	>25
	386	6.25	**122**	>25	**304**	>25
	351*	12.5	**123**	>25	**305**	>25
	352*	12.5	**124**	>25	**306**	>25
	376	12.5	**131**	>25	**322**	>25
	377	12.5	**134**	>25	**323**	>25
	378	12.5	**147**	>25	**324***	>25
	379	12.5	**154**	>25	**325**	>25
	380	12.5	**176**	>25	**329***	>25
	383	12.5	**185**	>25	**331***	>25
	387	12.5	**187**	>25	**332***	>25
	390	12.5	**188**	>25	**333***	>25
	282	12.5 - 25	**192**	>25	**334***	>25
	4*	25	**210**	>25	**335***	>25
	116	25	**212**	>25	**336***	>25
	164	25	**213**	>25	**338***	>25
	292	25	**214**	>25	**356**	>25
	317*	25	**222**	>25	**357**	>25
	330*	25	**234**	>25	**358**	>25
	337	25				

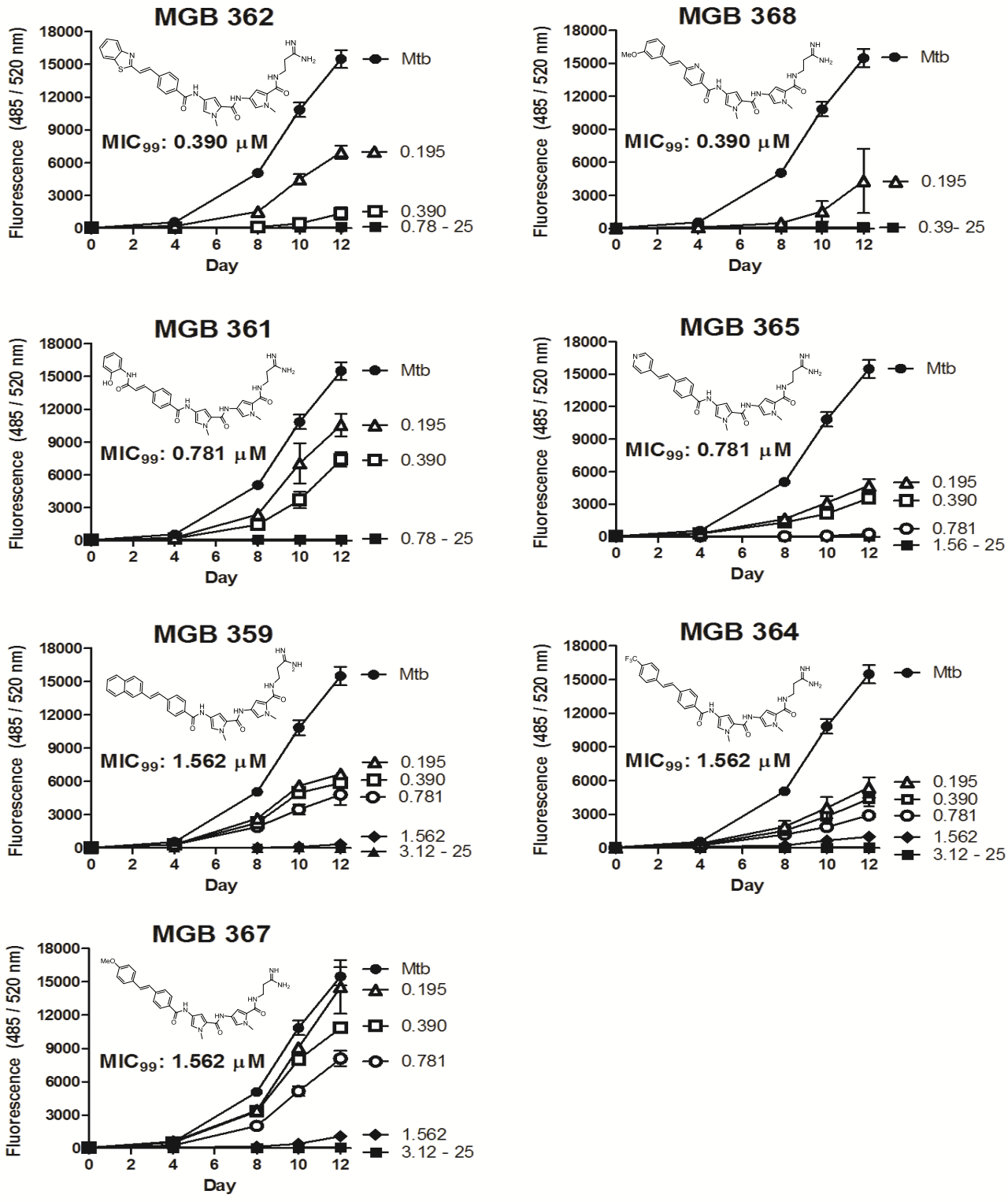

Figure 1. Screening of anti-mycobacterial activity of MGB compounds against H37Rv-GFP. Direct antimicrobial activity of MGB compounds at the drug concentration range of 0.195 - 25 µM was tested against H37Rv-GFP ($1x10^5$ cfu/well) in 7H9 liquid broth culture using microplate assay. The anti-mycobacterial activity of MGB treatment on H37Rv-GFP was determined at a concentration-dependent manner by measuring fluorescence ($485_{ex}/520_{em}$ nm) on days 0, 4, 8, 10 and 12. Data was corrected for background 7H9 fluorescence. Data show mean ± SEM of duplicates.

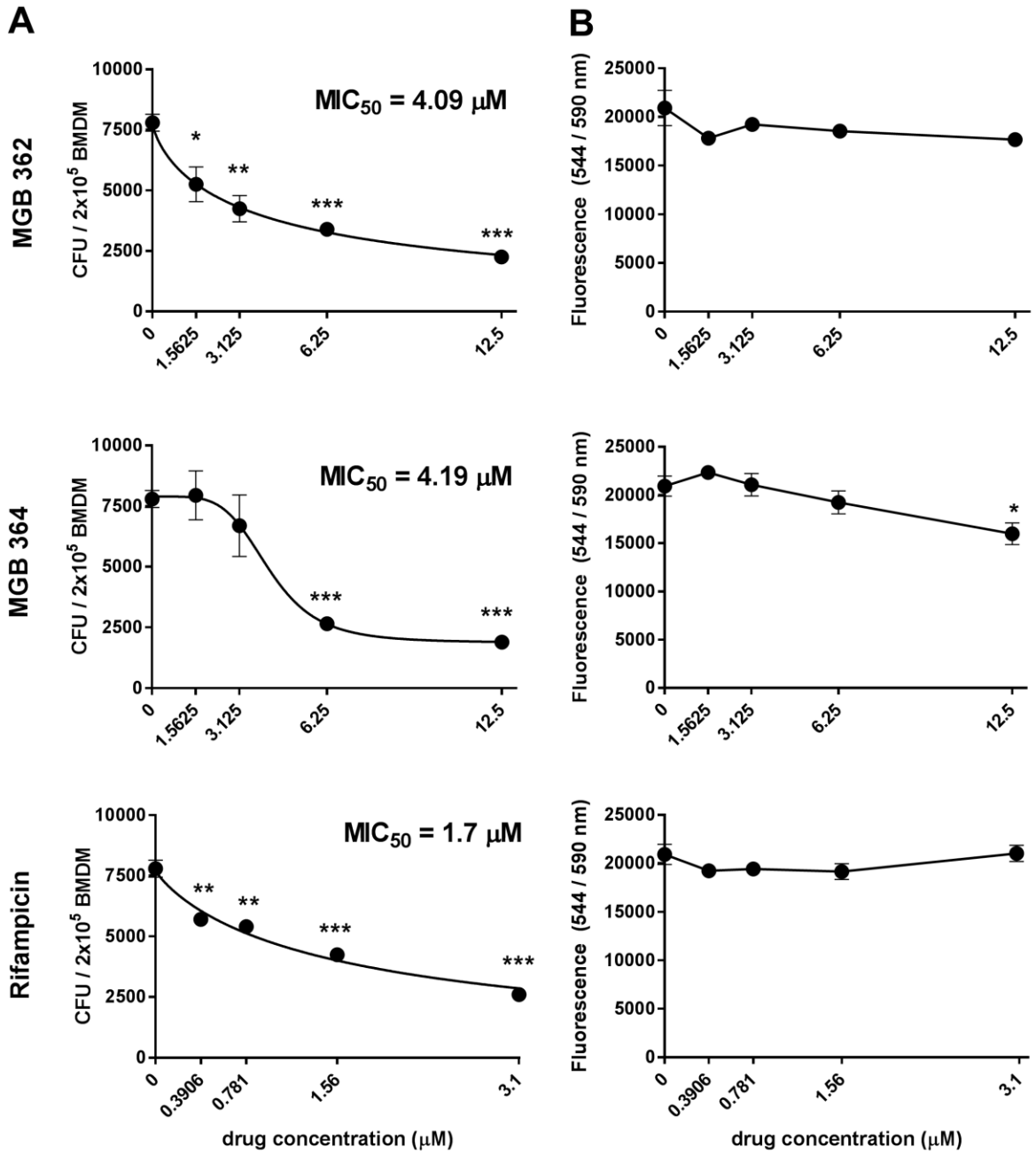

Figure 2. MIC_{50} of MGB compounds in HN878 *Mtb*-infected BMDMs and cell viability**.** A) The intracellular anti-mycobacterial activities of MGBs (1.5625-12.5 μM) and rifampicin (0.3906-3.125 μM) were assessed by counting cfu at the respective concentration at 5 days post Mtb HN878 infection. MIC_{50} values of each drug compound were identified in GraphPad Prism by non-linear regression analysis. B) Macrophage cell viability was determined at 5 days of MGB compound exposure and measured by CellTiter-Blue assay with fluorescence detection at ($544_{ex}/590_{em}$ nm). Data were corrected for background culture medium fluorescence and are shown mean ± SEM, representative of triplicates. Two-tailed Student's t-test, *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$ compared to control.

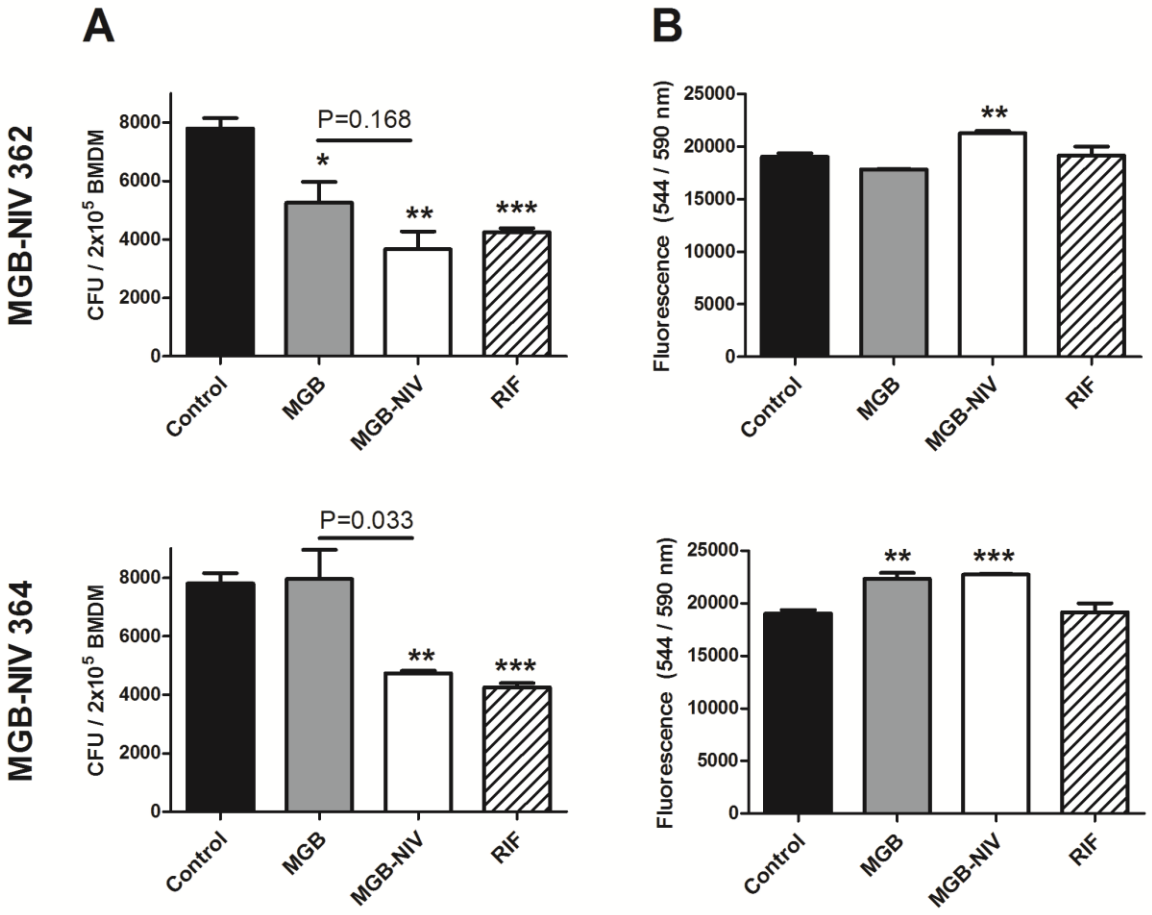

Figure 3. MGBs-NIV formulation intracellular mycobacterial activity in HN878 Mtb-infected BMDMs and cell viability. A) The intracellular anti-mycobacterial activity of MGBs only, MGBs-NIV formulation and rifampicin was determined in comparison to control (no drug treatment). Cfu was determined at 5 days post Mtb HN878 infection. B) Macrophage cell viability was determined at 5 days post *Mtb* HN878 infection and measured by CellTiter-Blue assay with fluorescence detection at (544_{ex}/590_{em} nm). Data were corrected for background culture media fluorescence and are shown as show mean ± SD, representative of triplicates. Two-tailed Student's t-test, *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control.