Non-toxic metal-cyclam complexes, a new class of compounds with potency against drug-resistant *Mycobacterium tuberculosis*

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ABSTRACT: Tuberculosis (TB) accounted for 1.5 million deaths in 2014 and new classes of anti-TB drugs are required. We report a class of functionalized 1,8-disubstituted cyclam derivatives that display low micromolar activity against pathogenic mycobacteria. These compounds inhibit intracellular growth of *Mycobacterium tuberculosis*, are non-toxic to human cell lines and are active against multidrug-resistant *M. tuberculosis* strains, indicating a distinct mode of action. These compounds warrant further appraisal as novel agents to control TB in humans.

INTRODUCTION

The increasing prevalence of bacterial resistance to antibiotics is well documented.[1-3] There is scientific consensus, and a growing awareness in society, that antimicrobial resistance constitutes an acute threat to the expected standard of medical care. The speed and efficiency of microbial evolution means the need for new antibiotics with novel modes of action grows ever more urgent.

Tuberculosis (TB) remains a major cause of mortality and morbidity worldwide: one third of the world's population is currently infected with *Mycobacterium tuberculosis* and annually there are almost 9 million new cases of clinical TB and 1.3 million deaths.[4] Current drugs and vaccines have had no significant impact on TB control, with TB incidence rates now higher than they were 25 years ago.[4] The emergence of drug resistant TB is considered a public health crisis, with some strains now resistant to all available drugs.[5] Thus there is a particularly urgent need to develop new, effective anti-TB drugs with efficacy against drug resistant *M. tuberculosis* strains.[6]

Metal-based drugs have gained increasing attention in recent years. Well-established medicinal agents like platinum- and ruthenium-based anti-cancer agents[7] have been joined by an increasing range of metal centered therapeutics.[8-10] In antibiosis, the antimicrobial potential of metals has long been known and exploited,[11,12] and the entire field of chemotherapy originated from arsenic-based antimicrobials.[13,14] Yet inspection of the compounds in the antitubercular development pipeline reveals only a handful of examples in the early discovery phase based on metal complexes of existing organic drug candidates, with no metal-containing compounds in the development or clinical phases.[15-21] More specifically, studies with *M. tuberculosis* have demonstrated that metal complexes of known drugs (isoniazid) may act against drug-resistant strains[19,22] and the search for novel anti-TB metal complexes is becoming more important.[23] Over the past decade metal complexes of ruthenium have shown promise in developing new anti-TB medicines.[15,24] It is essential that we develop as broad a range of compounds as possible to maximize the diversity of *in vivo* targets and thereby minimize the chances of resistance developing.

RESULTS AND DISCUSSION

Metal complexes of cyclam are known for their anti-HIV activity and ease of synthesis[25] but the antimicrobial activity of cyclam complexes is far less explored. We have previously prepared diverse functionalized azamacrocycles and their metal complexes (Figure 1) for ion sensing and other potential biological applications.[26-32] Many of these compounds possessed good levels of solubility and the ability to enter cells. Given their contrast to existing or pipeline antibiotics, and the paucity of metal-containing compounds with reported activity against mycobacteria, we sought to evaluate their activity through phenotypic screening against pathogenic mycobacteria, Gram-positive and Gram-negative medically-important bacteria.

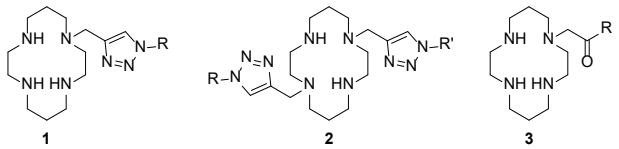

Figure 1. General structures of the macrocyclic compounds investigated; compounds of interest include ligands themselves, copper(II) and zinc(II) complexes of these ligands. R and R′ include amino acids and a short peptide,[26] fluorescent naphthalimide dyes,[27,29] biotinylated sidechains,[29] marimastat derivatives,[28] and amyloid β recognition peptides.[30]

Table 1. Antibacterial activity of select cyclam–metal complexes.

Compound	MIC (μM)[a]			
	M. avium	M. bovis BCG	MRSA	Pseudomonas aeruginosa
36	6.25	6.25	>50	>50
37	6.25	6.25	>50	>50
38	6.25	12.5	>50	>50
39	50	25	>50	>50
43	3.13	3.13	50	>50
44	1.56	1.56	50	>50
45	1.56	3.13	>50	>50
46	>50	>50	>50	>50
47	>50	>50	>50	>50
48	>50	>50	>50	>50
49	>50	>50	>50	>50
INH	0.31	0.31	N.D.	N.D.
PMB	N.D.	N.D.	N.D.	1.56
VAN	N.D.	N.D.	3.13	N.D.

[a]Minimal inhibitory concentration of compound as determined by the resazurin viability assay. INH: Isoniazid; PMB: Polymyxin B; VAN: Vancomycin; N.D.: not determined.

Ligands and metal complexes (Figure 1 and Table S1, Supporting Information) were prepared as reported previously: amino acid and simple peptide derivatives,[26] naphthalimide derivatives,[27,29] biotinylated sidechains,[29] marimastat derivatives,[28] and amyloid β related peptides.[30] Briefly, suitably protected doubly-propargylated cyclam was coupled with pendant motifs through the copper-catalyzed azide-alkyne cycloaddition reaction, with metal complexation being performed, where applicable, following deprotection.

The antibacterial activity of compounds **4**–**49** against selected medically-relevant bacteria was determined using a modified version of the resazurin viability assay.[33] The structures of the most promising candidates and key controls are shown in Figure 2 and bioactivity data in Table 1; structures of all compounds tested are shown in Table S1 and bioactivity data in Table S2 (Supporting Information).

In initial screening, compounds were tested against *Mycobacterium bovis* BCG and *Mycobacterium avium*, the latter an opportunistic pathogen that is a major cause of infection in immuno-compromized individuals and which typically displays a generalized pattern of resistance to antibiotics.[34] A subset of the compounds tested displayed potent activity against mycobacteria, with MIC values in the low micromolar range (Table 1). The antimycobacterial activity of these compounds appeared to be selective, as the compounds showed no significant activity against Gram positive methicillin-resistant *Staphylococcus aureus* (MRSA) or Gram negative *Pseudomonas aeruginosa* (clinical strain CJ2009) (Table 1).

The most active compounds are 1,8-naphthalimide derivatives bearing two pendant groups (Figure 2). Most of these leads (**36-38, 43-45**) contain a metal ion (Cu(II) or Zn(II)) coordinated to the macrocycle, although the uncomplexed macrocycle **43** displays comparable activity to its Zn(II)-complex **45** (in contrast to trends observed in anti-HIV cyclam complexes, the Zn(II) complexes are not more active than Cu(II)).[25] Importantly, studies on cyclam have demonstrated rapid Zn(II) binding under physiological conditions,[35] therefore it is possible that **43** binds a metal ion prior to exerting its antibiotic effect under the assay conditions. The mono-pendant derivative **39** exhibits low bioactivity, while free cyclam **46** and the pendant groups alone (naphthalimides **47** and **48** and biotin **49**) show no activity, indicating the composite structure is required for the antimycobacterial effect. These data suggest future exploration of modified side chains in these structures would be productive.

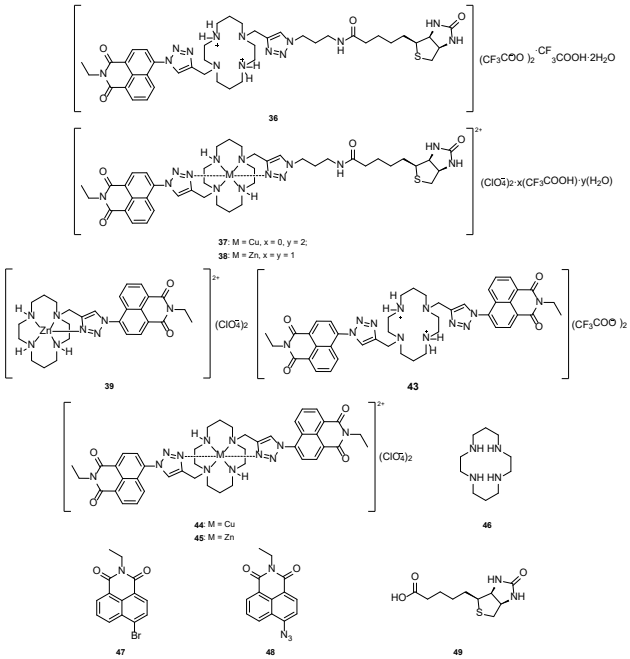

Figure 2. Structures of the most potent of the derivatives tested (36-38, 43-45) and key control compounds (39, 46-49); for details of bioactivity see Tables 1 and 2 and Table S2 (Supporting Information).

Table 2. Antibacterial activity of 43, 44 and 45 against *M. tuberculosis* H37Rv and toxicity against mammalian THP-1 cells.

Compound	Antibacterial activity (MIC, μM)					THP1 cells IC_{50}
	M. tb INH[Ra]	M. tb RIF[Rb]	M. tb INH^R RIF^R	M. tb INH^R RIF^R ETH^{Rc}	M. tb H37Rv	
43	6.25	3.13	6.25	6.25	6.25	>100
44	6.25	3.13	6.25	6.25	6.25	>100
45	6.25	3.13	6.25	6.25	6.25	>100
INH	>10	N.D.	5.0	5.0	0.16	N.D.
RIF	N.D.	0.33	1.0	0.33	0.01	N.D.
ETH	N.D.	N.D.	N.D.	25	3.13	N.D.

[a]*M. tb* INH^R: isoniazid-resistant strain; [b]*M. tb* RIF^R: rifampicin-resistant strain; [c]*M. tb* ETH^R: ethambutol-resistant strain. N.D.: not determined.

Bis-naphthalimide analogues **43-45**, which demonstrated the most promising activity, were selected as leads for further evaluation. These compounds were tested for their ability to inhibit the growth of virulent *M. tuberculosis* strains, including clinical isolates resistant to first-line antimycobacterial drugs (Table 2). All three compounds were active in the low micromolar range (6.25 μM) against drug-sensitive *M. tuberculosis* H37Rv, with activities well below (5.06-5.44 μg/mL) the 6.25 μg/mL and 10 μg/mL benchmarks for compound progression.[36,37]

Importantly, this inhibitory effect was maintained against clinical isolates of *M. tuberculosis* resistant to single or multiple antimycobacterial drugs, most notably strains resistant to isoniazid, rifampicin and ethambutol (Table 2). Activity against multi-drug resistant *M. tuberculosis* strains is a property shared by anti-TB candidate compounds in late-stage clinical trials.[38] In addition, these data suggest that cyclam–metal complexes possess a mode of action distinct from three major front-line drugs used for TB treatment. Furthermore, these compounds were not active against differentiated THP1 cells, a macrophage-like human cell line,[39] demonstrating specific activity for mycobacteria without toxicity for mammalian cells (Table 2) with selectivity indices over 16 (SI = IC_{50}/MIC) (SI of 10 or over are typically used to assess progression to preclinical stages).[37] Work towards elucidation of the mechanism of action is underway, facilitated by the reasonable potency of biotinylated compound **36-38** (Table 1).

Table 3. Solubility and *in vitro* metabolic stability of lead compounds (43-45).

	43	**44**	**45**
Solubility at pH 2.0 (μM)	62-124	62-124	31-62
Solubility at pH 6.5 (μM)	31-62	31-62	7.8-15
Degradation half-life (min)	84	>247	49
In vitro CL_{int} (μL/min/mg protein)	21	<7	36
Microsome-Predicted E_H	0.45	<0.22	0.59

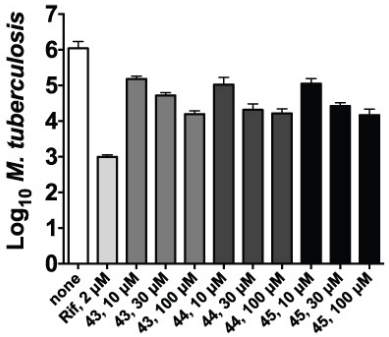

Figure 3. Novel inhibitors 43, 44 and 45 reduce mycobacterial load within host cells. THP-1 cells (2×10^5) were infected with *M. tuberculosis* H37Rv at a MOI of 1:1, treated with inhibitors and bacterial numbers determined 7 days post infection. Rif: rifampicin. Data are mean bacterial load ± SEM and represent two independent experiments.

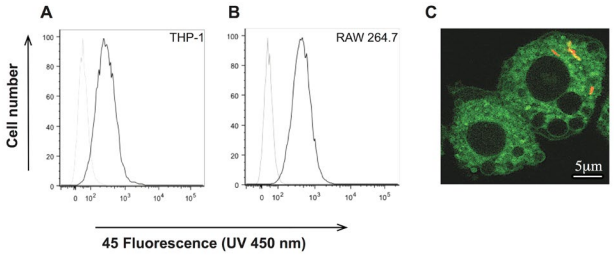

Figure 4. Visualization of metal cyclam derivatives within host cells. 2 x 10^5 THP-1 (A) or RAW 264.7 cells (B) were left untreated or treated for 1 hour with 100 μM of 45. The percentage of fluorescent cells in non-treated (grey line) and treated cells (black line) was determined by flow cytometry. RAW 264.7 cells (C) infected with BCG:mCherry at a MOI of 10:1 (red) and then treated with 100 μM of 45 (green) were examined by confocal microscopy with a ×63 oil immersion objective.

An important requirement of antimycobacterial compounds is that they restrict bacterial growth within host cells. Compounds **43-45** gave a dose-dependent inhibition of *M. tuberculosis* bacterial load at day 7 post-infection of THP-1 cells (Figure 3). All three compounds displayed similar activity profiles although **44** and **45** resulted in a greater reduction in *M. tuberculosis* growth at the intermediate concentration (30 μM) compared to **43**. The fluorescent properties of **45**[40] were used to examine the uptake and distribution of cyclam–metal complexes within host cells. As demonstrated by flow cytometry, **45** was strongly associated with both THP-1 and RAW264.7 cell lines (murine macrophage cell line),[41] with all cells within the population becoming highly fluorescent when incubated with 100 μM of compound (Figures 4A and 4B). After infection of RAW264.7 cells with fluorescent *M. bovis* BCG, treatment with **45** and analysis by confocal microscopy, **45** was seen to be distributed throughout RAW264.7 cells and appeared to colocalize with fluorescent *M. bovis* BCG (Figure 4C). Therefore cyclam–metal complexes were able to efficiently enter host cells and exert their inhibitory action against intracellular mycobacteria.

Finally, the solubility and stability of **43-45** were examined (Table 3). Both **43** and **44** displayed moderate to good solu-

bility, particularly at pH 2.0 that is representative of gastric pH. Compound **44** displayed excellent metabolic stability in human liver microsomes, with a long-half degradation life, a very low intrinsic clearance value (CL_{int}) and a low predicted hepatic extraction ratio (E_H). These data predict that **44** would be subjected to low hepatic clearance *in vivo*, while **43** and **45** are predicted to display moderate clearance.

CONCLUSIONS

We have identified a new class of compounds with micromolar antimycobacterial activity, efficacy against both intracellular and drug-resistant *M. tuberculosis*, and with suitable safety (SI>16), solubility and metabolic stability. These are exciting leads in the search for drug candidates with novel modes of action against drug-resistant *M. tuberculosis.* These data clearly mark this series out for further evaluation by the community. Their ease of synthesis, coupled with their lack of patent protection, will make this task particularly feasible.

EXPERIMENTAL SECTION

Bacterial growth conditions. All mycobacterial strains (*M. avium* 104, *M. bovis* BCG Pasteur, *M. tuberculosis* H37Rv and *M. tuberculosis* clinical isolates) were grown at 37˚C in complete Middlebrook 7H9 media (Bacto, Australia) containing albumin, dextrose and catalase (ADC), 20% Tween 80 and 50% glycerol (Sigma-Aldrich, Australia). Fluorescent *M. bovis* BCG expressing mCherry was constructed by electroporation of BCG with the pSMT3-mCherry plasmid and transformants selected on Middlebrook 7H11 plates supplemented with 25 μg/mL hygromycin (Sigma-Aldrich).[42] *M. tuberculosis* EAI genotype isoniazid mono-resistant (*katG* Ser315Thr), Delhi/CAS genotype rifampicin mono-resistant (*rpoB* His445Asn), Beijing genotype isoniazid/rifampicin multi-resistant (*katG* Ser315Thr, *rpoB* His445Asn) or Euro-American Superlineage genotype isoniazid/rifampicin/ethambutol multi-resistant strains (*katG* Ser315Thr, *rpoB* Ser441Leu, *embB* Met306Val) were sourced from the NSW Mycobacterium Reference Laboratory, Centre for Infectious Diseases and Microbiology Laboratory Services Strain Collection, Westmead Hospital, Sydney. Different genotypes were selected to reduce potential lineage bias.

Resazurin assay of growth inhibition. The minimal inhibitory concentration (MIC) of compounds was determined using a modified version of the resazurin viability assay.[33] All compounds were initially prepared as 100 mM stocks in 100% DMSO and then adjusted to the required concentration in diluent (0.1% DMSO). Compounds (0.2-100 μM) were added to wells in 2-fold dilutions and incubated with bacteria previously diluted to an $OD_{600\ nm}$ of approximately 0.001 (determined by spectrophotometry or comparison with McFarland standard). Compounds and bacteria were incubated in complete 7H9 media for 5 or 7 days for *M. avium* or BCG/*M. tuberculosis*, respectively. Resazurin (10 μL; 0.05% w/v; Sigma-Aldrich, Australia) was then added and plates incubated for 24 h at 37 ˚C. The MIC was calculated either by visual determination of colour change within wells or detection of fluorescence at 590 nm using a FLUOstar Omega microplate reader (BMG Labtech, Germany).

Compound intracellular efficacy and toxicity. THP-1 cells (TIB-202R), a human monocyte cell line, were grown and differentiated in complete Dulbecco's Modified Eagle Media (DMEM; LifeTechnologies, Australia) as described previously.[33] The determination of inhibition of *M. tuberculosis* load within THP-1 cells was performed using 2×10^5 THP-1 cells, a multiplicity of infection (MOI) of 1 bacteria per cell and compound concentrations of 10, 30 and 100 μM for **43**, **44**, **45**, or 2 μM of the rifampicin control.[33] After 7 days cells were lysed with cells with H_2O and bacterial numbers determined by serial dilution of the suspension onto 7H11 Middlebrook agar (Bacto). To examine the toxicity of selected compounds, 2×10^5 THP-1 cells/well were added to a 96-well plate and left for 48 h at 37°C to adhere. Compounds (0.2-100 μM) were added to the wells in 2-fold dilutions and incubated for 7 days at 37°C. Then 0.05% w/v resazurin was added for 4 hours and fluorescence measured. Cell viability was calculated as percentage fluorescence in comparison to untreated cells.

Flow cytometry and confocal microscopy. 2×10^5 THP-1 or RAW 264.7 cells, were incubated for 1 hour with 100 μM of compound. Cells were washed twice with FACS wash and fixed with 10% neutral buffered formalin and acquired using LSR Fortessa. The percentage of fluorescent cells in treated and non-treated cells was determined using Flowjo software and gated on the UV450 channel.[41]

For direct visualization of compound within host cells, RAW 264.7 cells were infected with *M. bovis* BCG:mCherry for 1 hour at a MOI of 10:1. Cells were washed three times with DMEM to remove excess bacteria. Infected cells were then incubated for 1 hour with 100 μM of compound, cells washed and incubated with fresh media for a further 40 mins. These cells were washed again with PBS and fixed with 10% neutral buffered formalin and imaged using a SP5 confocal microscope (Leica Microsystems) with × 63 oil immersion objective. The images were analysed using ImageJ software.

Kinetic Solubility Assays. Compound (**43**, **44** or **45**) in DMSO was spiked into either pH 6.5 phosphate buffer or 0.01 M HCl (approx pH 2.0) with the final DMSO concentration being 1%. Samples were then analysed with nephelometry to determine a solubility range.[43]

Metabolic Stability Assays. Compound (**43**, **44** or **45**, 1 μM) was incubated with human liver microsomes (Xenotech, Lot #1210057) at 37°C and 0.4 mg/mL protein concentration. The metabolic reaction was initiated by the addition of an NADPH-regenerating system (i.e. NADPH is the cofactor required for CYP450-mediated metabolism) and quenched at various time points over the 60 minute incubation period by the addition of acetonitrile to determine the rate order of the first-order kinetics and the clearance calculated.[44] Control samples (containing no NADPH) were quenched at 2, 30 and 60 minutes, to monitor potential degradation in the absence of cofactor. Samples were analysed by UPLC-MS (Waters/Micromass Xevo G2 QTOF) under positive electrospray ionisation and MS spectral data acquired in a mass range of 80 to 1200 Daltons.

ASSOCIATED CONTENT

Supporting Information.
Bioactivity data, metal complexation, solubility assays, metabolic stability assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

BCG, Bacillus Calmette-Guérin; CL_{int}, intrinsic clearance; ETH, ethambutol; IC_{50}, half-maximal inhibitory concentration; INH, isoniazid; MIC, minimum inhibitory concentration; MOI, multiplicity of infection; MRSA, methicillin-resistant *Staphylococcus aureus*; *M. tb.*, *Mycobacterium tuberculosis*; RIF, rifampicin; SEM, scanning electron microscope; SI, selectivity index; TB, Tuberculosis.

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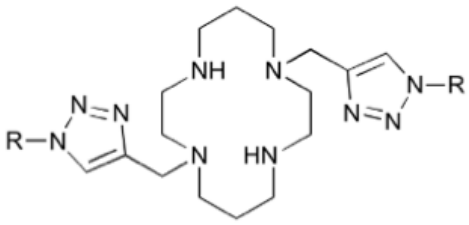
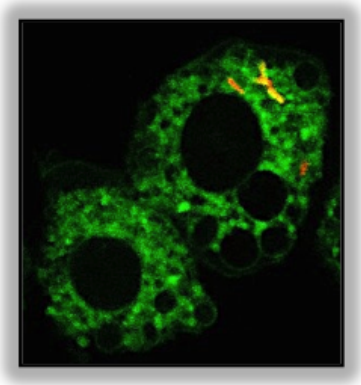


MICs down to 3.13 μM

Free Ligands and Metal Complexes Potent vs. Virulent and Drug-Resistant Mycobacterium Tuberculosis



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