



# **Novel Antimicrobial Molecules for Treatment of *Mycobacterium tuberculosis***

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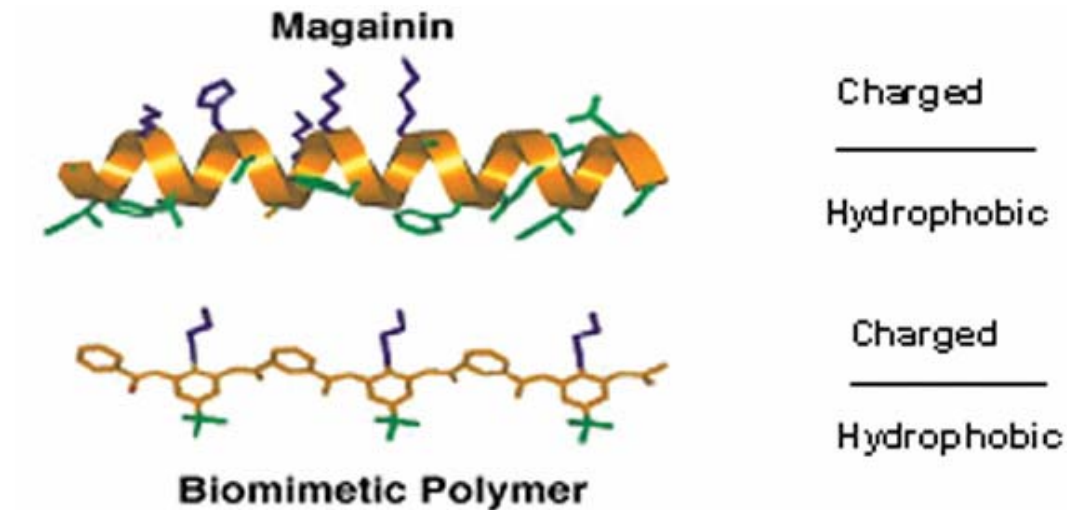
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## ABSTRACT

There is a dire need for development of new antimicrobial agents that attack new targets to evade resistance issues which limit the usefulness of many antibiotics. Pandemic levels of multi-drug resistant tuberculosis (MDR-TB) and extensively drug resistant tuberculosis (XDR-TB), with 90% mortality rate among HIV/MDR-/XDR-TB co-infected, are becoming a worldwide medical crisis as record levels of infection occurred in 2007 with 45 countries reporting at least one case of XDR-TB. A series of small, stable, synthetically-produced arylamide antimicrobials has been developed which mimic the activity of the host defense proteins and represent a novel class of therapeutics for the clinical treatment of TB. These small non-peptidic analogues (MW < 1,000) have many advantages over other antibiotic drugs because of their mechanism of action which is associated with rapid killing times and a lower risk for the development of resistance. One of the lead compounds, PMX30063, is being developed as an IV antibiotic to treat pan-Staphylococcal infections and is now in Phase 1 human clinical trials. A number of analogues spanning several structural series were screened by TAACF (Tuberculosis Antimicrobial Acquisition Facility, NIAID) in in vitro assays to measure susceptibilities against the H37Rv strain of *M. tuberculosis* and for cytotoxicity against monkey VERO cells. Several compounds were highly active (IC<sub>90</sub> < 5 μM) and greater than 30 to 120 fold selective. Mechanism of action studies in model systems investigating interactions with lipid membranes and bacterial membrane permeabilization indicate that the compounds interact preferentially with bacterial membranes but do not induce widespread permeabilization and leakage of cellular contents (which could have detrimental consequences in therapeutic settings). Future efforts will be aimed at developing this promising series of compounds as effective ant-tubucular agents that meet the goals for future TB drugs. Furthermore, because of their mechanism of action, there is the added benefit for a lower risk of resistance development.

## ABSTRACT (Cont'd)

The biological activities of the Host Defense Proteins (HDPs) are dependent on amphiphilicity.



**Approach: Design small non-peptidic mimics of the HDPs using torsionally-constrained chemical backbones**

# 1. Primary Screen of Selected PMX Antimicrobial Compounds in Susceptibility Assays versus *M. tuberculosis* (H37Rv strain) and Cytotoxicity Assays versus Monkey VERO Cells

Compound	IC <sub>90</sub> (µg/mL)	EC <sub>50</sub> (µg/mL)	SI (EC <sub>50</sub> /IC <sub>90</sub> )
PMX10070	2.2	>300	>136.4
PMX10072	4.5	>300	>66.7
PMX30053	3.6	>100	>27.8
PMX10138	18.4	>300	>16.3
PMX30024	57.6	129.8	2.25
PMX30006	60.4	39.7	0.66
PMX70004	40	12.4	0.31
PMX10129	100	251.7	2.52
PMX30016	100	55	0.55
PMX30063	100	120.4	1.2

Methods: *M. tuberculosis* (H37Rv strain, ATCC 27294) was screened using the Microplate Alamar Blue Assay (MABA) in BACTEC 12B medium by the Tuberculosis Antimicrobial Acquisition Facility, NIAID (TAACF). Compounds were tested in ten 2-fold dilutions to determine IC<sub>90</sub> values (reduction in fluorescence of 90% relative to controls). Viability in the VERO cell cytotoxicity assay was measured after a 72 hour exposure using a luminescent cell viability assay that measures ATP levels. Cytotoxicity was determined using a curve fitting program to calculate EC<sub>50</sub> values. The EC<sub>50</sub> is divided by the IC<sub>90</sub> to derive an SI (Selectivity Index) value.

**Results: Three arylamide compounds met criteria for further testing PMX10070, PMX10072 and PMX30053. Several compounds that are broadly active against Gram-positive and Gram-negative bacteria (PMX70004, PMX30016 and PMX30063) demonstrate poor activity against *M. tuberculosis*.**

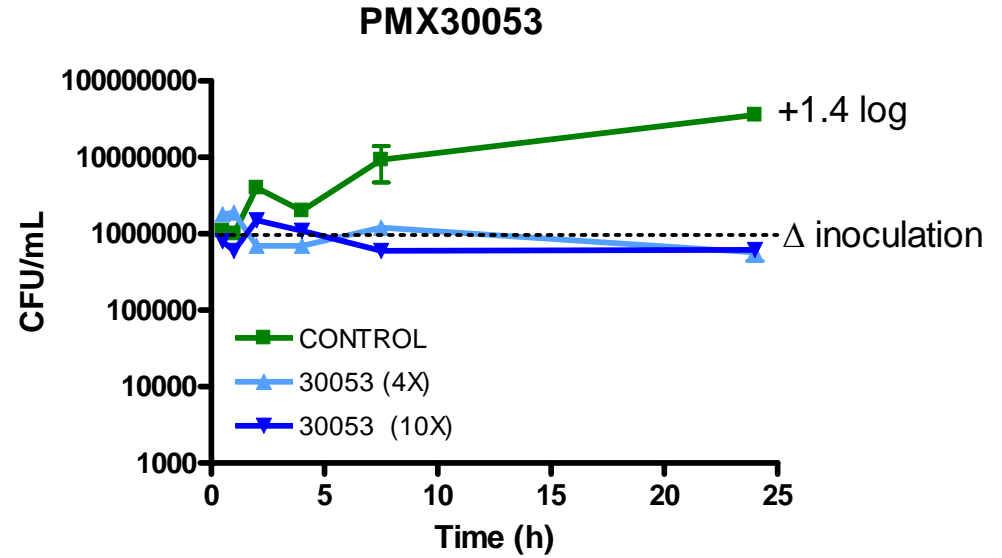
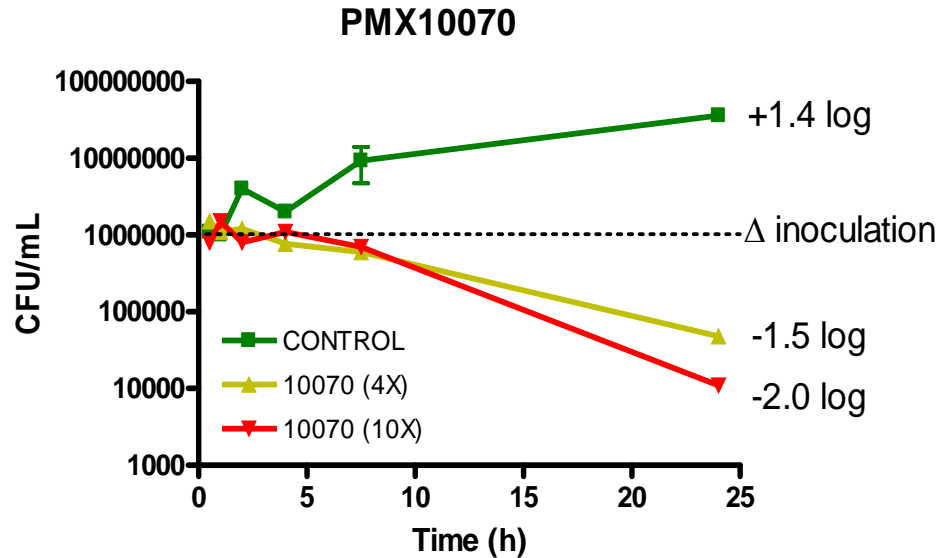
## 2. Antimicrobial and Cytotoxic Activities of *M. tuberculosis*-Active Compounds

Compound	MW	MIC ( $\mu\text{g/ml}$ )				EC <sub>50</sub> ( $\mu\text{g/ml}$ )	
		<i>E. coli</i>	<i>S. aureus</i>	<i>M. smegmatis</i>	BCG	Human RBCs	Mouse 3T3
PMX10070	794	12.5	0.2	3.13	12.5	214	18
PMX10072	895	12.5	0.2	12.5	25	486	58
PMX30053	781	12.5	1.5	12.5	12.5	>1,000	697

Methods: MICs were determined in standard microbroth dilution assays according to CLSI guidelines (*M. smegmatis* and BCG) or CLSI guidelines with Hancock modifications for testing cationic agents (*S. aureus* and *E. coli*). Mammalian cell cytotoxicities were tested in mouse 3T3 and human HepG2 cells in the absence of serum using an MTS viability assay. EC<sub>50</sub> values represent compound concentrations that cause 50% lethality. EC<sub>50</sub> values are corroborated in trypan blue exclusion assays. Hemolysis (HC<sub>50</sub>) was measured following incubation of human red blood cells in the presence of compound for 1 hour. Melittin, a lytic antimicrobial peptide, was used as a positive control agent. [*S. aureus* + serum] represents MICs done in the presence of 40% mouse serum.

**Results:** PMX10070, PMX10072 and PMX30053 are highly active against *S. aureus* ATCC 27760. PMX10070 demonstrates the greatest potency against the avirulent surrogates *M. smegmatis* ATCC 14468 and BCG ATCC 19015. PMX10070 and PMX10072 possess low hemolytic activity with EC<sub>50</sub> values that exceed the highest MIC values by factors of 17 and 39 fold, respectively. Their selectivity against mouse fibroblasts is less pronounced, with selectivity for PMX10072 being approximately 5 fold. PMX30053 is significantly less cytotoxic than either PMX10070 or PMX10072 and has selectivity indices (EC<sub>50</sub>/highest MIC) of 56 to >80 fold.

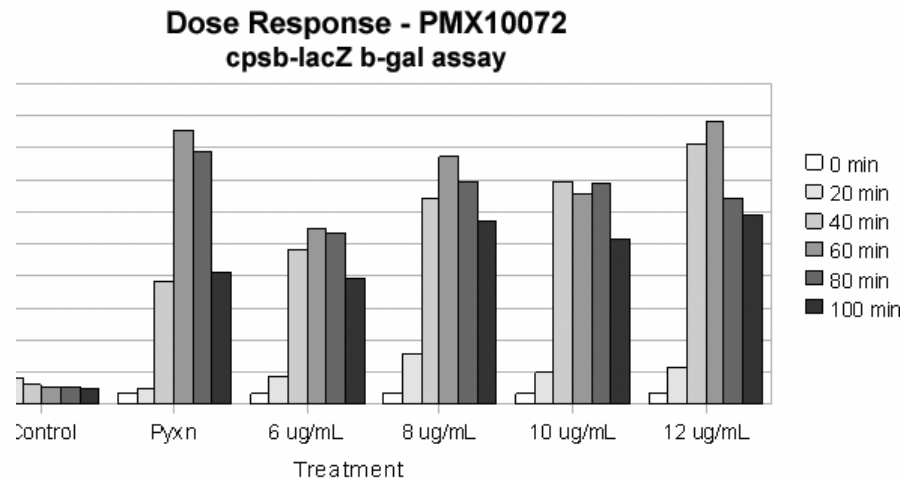
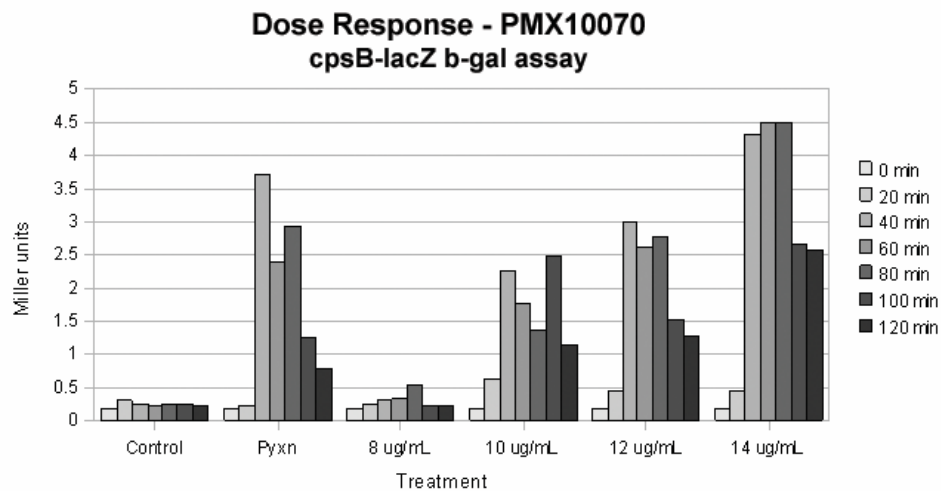
### 3. Time-Kill vs *Mycobacterium Smegmatis*



Methods: Time-kill studies were done by adding compound dilutions (4X and 10X MIC) to a suspension of *M. smegmatis* ATCC 14468 (~10<sup>6</sup> cfu/ml) in Middlebrook 7H9 medium with ADC supplement and 0.05% Tween 80. Incubations were done at 37°C and viable bacteria were counted at the indicated time points by serial dilution on 7H11 (with 0.5% glycerol) agar plates after an 48 hour incubation.

**Results:** Control (untreated) cultures show a 1.4 log increase in cfus/ml over 24 hours. Bacterial densities in the presence of PMX30053 at 4x and 10x MIC concentrations show no change over 24 hours indicating static activity. With PMX10070 at 24 hours, bacterial densities are reduced 1.5 and 2.0 logs at 4x and 10x MIC concentrations, respectively, indicating cidal activity.

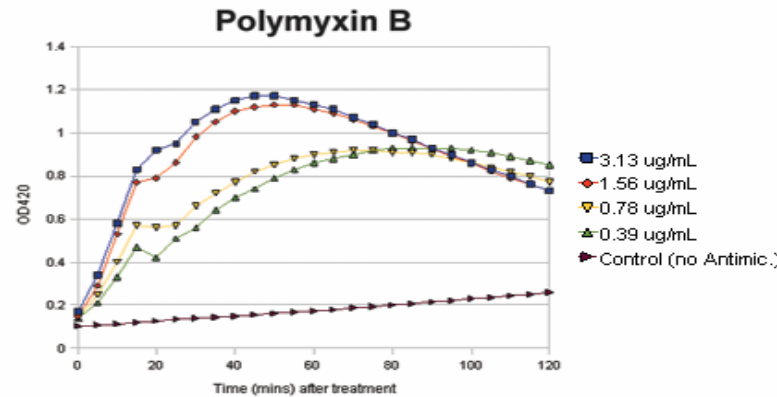
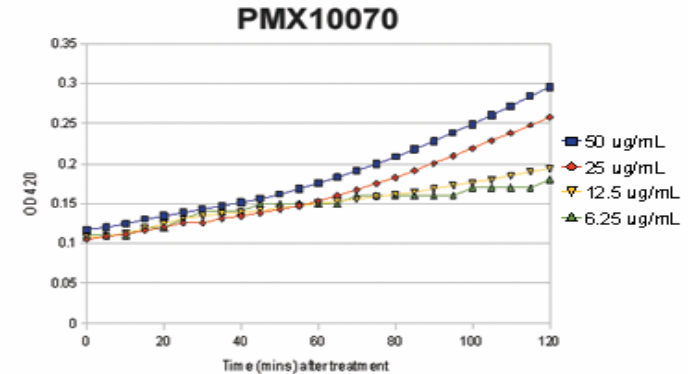
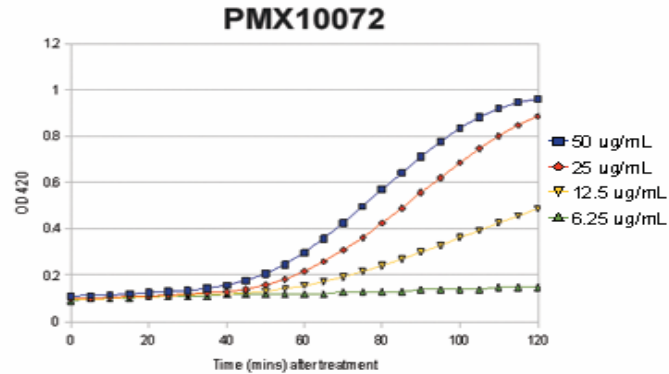
## 4. Induction of the Membrane Perturbation Marker, *rcs*, Following Exposure to the PMX Mimics



**Methods:** *E. coli* D31 containing a chromosomal copy of the *rcs* reporter gene *cpsb-lacZ-b-gal* was grown to OD600 0.4 in LB media and diluted 2X into fresh LB containing antimicrobial. Growth was monitored by measuring OD600 at each timepoint. 250 uL aliquots were removed every 20 minutes and diluted 4X into Z-buffer and lysed with 40 uL chloroform and 20 uL 0.1% SDS. 200 uL of 4 mg/mL ONPG was added to lysate and incubated at 37°C for 16 hours. The reaction was stopped with 0.5 volume of 1M Na<sub>2</sub>CO<sub>3</sub> and OD420 was measured in 96-well plates in a spectramax plate reader.

**Results:** *rcs* phosphorelay, a sensor for membrane disturbance, is upregulated by PMX10072 at MIC concentrations and by PMX10070 at concentrations slightly higher than its MIC. Upregulation of *rcs* by Polymyxin also occurs at MIC concentrations but to a greater magnitude than the PMX mimics. The magnitude of *rcs* induction is proportional to the degree of growth inhibition caused by the compound (not shown).

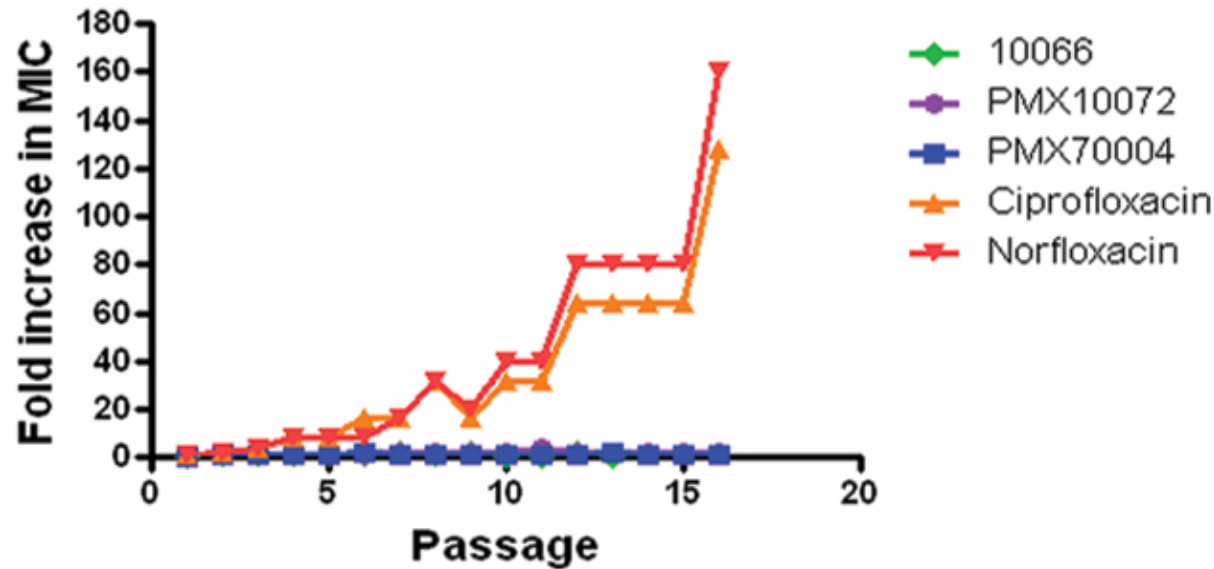
# 5. Concentration-Dependence of Membrane Leakage



Methods: *E. coli* D31 was grown to early log phase (OD600 = 0.2) in LB media and induced with 1 mM IPTG for 1 hour to induce endogenous beta-galactosidase. 70  $\mu$ L of culture was combined with 20  $\mu$ L of 4 mg/mL ONPG/Z-buffer solution and 10  $\mu$ L of the appropriate 10x concentration of antimicrobial in a 96 well plate. Hydrolysis of ONPG to o-nitrophenol indicates entry of substrate into the bacterial cytoplasm and was monitored by measuring OD420 every 5 minutes for 2 hours at 37°C on a Spectramax plate reader. Pyxn = Polymyxin B, a membrane-lytic host defense protein. MICs: Pyxn = 0.39  $\mu$ g/ml; PMX10070 = 12.5  $\mu$ g/ml; PMX10072 = 12.5  $\mu$ g/ml.

**Results: Inner membrane leakage is evident for PMX10070 and PMX10072 at 2x MIC concentrations and PMX10072 demonstrates a greater magnitude of effect within the 2 hr. timeframe. The highly lytic antimicrobial peptide, Polymyxin B, displays membrane leakage at its MIC with more rapid kinetics than the PMX mimics.**

## 6. Serial Passage Resistance Assays: *S. aureus* ATCC 29213



Methods: Serial passage and MICs were performed in microtiter panels in cation-adjusted Mueller-Hinton broth containing antimicrobials, each over a range of doubling dilution concentrations. After a 20 hour incubation period, the entire content of the well (100  $\mu$ L) with the highest concentration of compound permitting visible growth was taken, diluted to the correct inoculum ( $5 \times 10^5$  CFU/mL), and re-inoculated in a fresh panel with compound dilutions.

**Results:** Passage of *S. aureus* with two fluoroquinolones was associated with a significant rise in MIC values by passage 3 (4 doubling dilutions) that reached 128-fold and 64-fold increases, respectively, by passage 15. Conversely, there was no change in the MICs for PMX10070 and the other PMX compounds over the entire 17 passage time course. Identical results have been obtained with these and additional PMX compounds against MSSA ATCC 29213 and MRSA ATCC 33591, and *P. aeruginosa* ATCC 27853 (not shown).

**In collaboration with TAACF (Tuberculosis Antimicrobial Acquisition Facility, NIAID), we have screened select members of the PMX antimicrobial library in in vitro assays to measure susceptibilities against the H37Rv strain of *M. tuberculosis* (ATCC 27294). Three compounds exhibited high antimicrobial activity (IC<sub>90</sub> < 5 µg/ml) against H37Rv, with selectivity greater than 30-120 fold for TB versus mammalian cells. The compounds are active against *M. smegmatis* and BCG and PMX10070 demonstrates cidal activity against *M. smegmatis* within a 24 hour timeframe of exposure. Mechanism of action studies with the TB-active compounds in model systems argue that they act at the membrane and, like the HDPs, the incidence of resistance development is low. These preliminary results support further investigation of the PMX mimics as novel agents to treat TB.**



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