Understanding Mycobacterial redox response towards endogenous stress produced by peroxynitrite donor

Thesis submitted towards partial fulfilment of the requirements of BS-MS Dual Degree Programme.

By

Charu Jain

20111033

Indian Institute of Science Education and Research, Pune

Under the Guidance of

Dr. Amit Singh

Assistant Professor

Centre for Infectious Disease and Research

Department of Microbiology and Cell Biology

Indian Institute of Science, Bangalore

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Charu Jain

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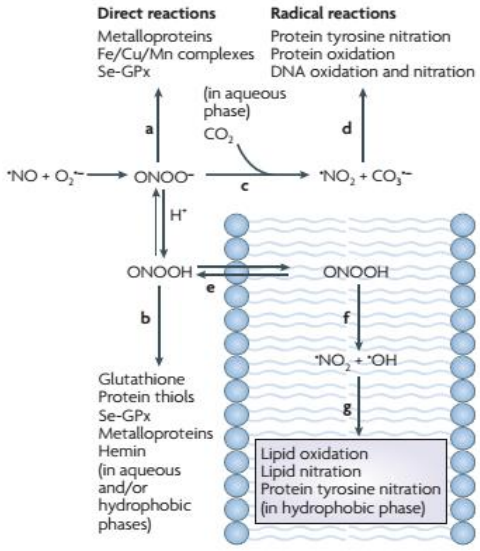
Abstract:

Peroxynitrite is a reactive nitrogen species (RNS), biologically generated from the reaction of superoxide radical with nitric oxide. At elevated levels, $ONOO^-$ causes redox imbalances and disrupt cellular functions which can lead to cell death. Immune cells like macrophages utilises $ONOO^-$ as cytotoxic effector molecule in response to invading bacteria and parasites. It has been shown that IFN-γ activated macrophages releases superoxide and nitric oxide that react to form $ONOO^-$. *Mycobacterium tuberculosis (Mtb)*, the etiologic agent of Tuberculosis, proliferates in human macrophages and also experiences this oxidative and nitrostative stress. It has been recently reported that *Mtb* has higher susceptibility to the endogenously produced superoxide. However, there are not many reports on bactericidal activity of peroxynitrite mainly because of its short half-life. Recently a novel molecule (HyPR-1) has been reported that can enhance intracellular $ONOO^-$ level but it has a low potency. In this study, we have synthesized a peroxynitrite donor (CJ067, analogue of HyPR-1) that generates high fluxes of $ONOO^-$ in buffer as well as inside non-pathogenic *Mycobacterium smegmatis (Msm)*. Using a mycothiol- specific redox biosensor (Mrx1-roGFP2), we have captured the real time oxido-reductive response of mycobacteria in the presence of CJ067 and observed a delayed recovery as compared to other donors. We have also utilised CJ067 to compare the antibacterial activity of $ONOO^-$ with superoxide and nitric oxide donors in *Msm*, and a few pathogenic and drug resistant strains of *Mtb*. We found that a very low concentration (μM) of CJ067 can inhibit the growth of *M. smegmatis* and *Mtb*. In sum, our study constitutes synthesizing a potent peroxynitrite donor, CJ067 and understanding the mycobacterial response to $ONOO^-$.

1. Introduction:

1.1 Peroxynitrite:

Peroxynitrite is one of the reactive species, produced by the coupling reaction of the superoxide ($O_2^{\bullet-}$) and nitric oxide (NO) radicals at the diffusion controlled rate of ~ $1x10^{10}$ $M^{-1}s^{-1}$. NO is small, uncharged, relatively stable and highly diffusible across cellular membranes, while $O_2^{\bullet-}$ is charged short lived and has strictly constrained diffusion. Therefore, it is assumed that the peroxynitrite formation spatially coincides with the sites of superoxide formation i.e. mitochondrial respiratory complexes and phagosomes.[1,2] At physiological pH both peroxynitrite anion ($ONOO^-$, 80%) and peroxynitrous acid (ONOOH; pK_a = 6.8) coexist and both the species are involved in one- and two- electron oxidation reactions with biomolecules containing transition metal centres and thiols. [1-3] Peroxynitrite not only reacts directly with metalloproteins, thiols etc. but also generates a series of radicals ($^{\bullet}OH$, $CO_3^{\bullet-}$, $^{\bullet}NO_2$, $NO_3^{\bullet-}$etc.) either by undergoing homolytic fission or by reacting with carbon dioxide (CO_2) leading to oxidation and nitration of biomolecules like lipids, proteins and DNA [1,3]. In previous studies, peroxynitrite has been reported as a pathogenic mediator in many pathophysiological conditions like cardiovascular, neurodegenerative and inflammatory disorders.[2,4] Peroxynitrite has its biological importance as a signalling molecule at sub-lethal concentrations[5] and also as a cytotoxic effector molecule which is released by the immune cells, notably macrophages in response to invading bacteria and parasites.[2, 6] However, the biological fate of peroxynitrite is determined by the availability of the targets in different biological environments e.g. extracellular, intercellular or intra-mitochondrial, where it is formed.[2] It has been reported that peroxynitrite has better antibacterial ability as compared to its reactant NO [9] and is formed in phagosomes of activated macrophages by reacting with NO.[6]



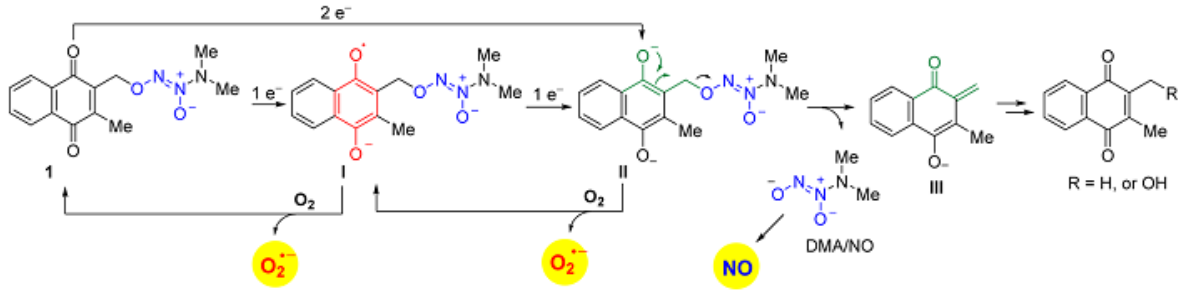
Nat. Rev. Drug Discovery 2007, 6, 662

Figure 1: Reaction of peroxynitrite with Biomolecules.

1.2 Peroxynitrite Donors:

The pathogenic role or the bactericidal activity of peroxynitrite has not been an easy subject of investigation because of its extremely short half-life thereby preventing its direct isolation and detection *in vivo*.[2] Although a number of chemical probes has been developed in recent years for its detection, a very few molecules are available to enhance intracellular $ONOO^-$. In literature most of the cellular studies have been done with two prominent commercially available peroxynitrite donors; (1) sodium salt of peroxynitrite generally referred to as authentic peroxynitrite [7,8] and (2) organic molecule, 3-Morpholinosydnonimine hydrochloride (SIN-1).[4,9] Authentic peroxynitrite has poor stability and bioavailability while SIN-1 releases NO and utilises molecular oxygen to form superoxide in 1:1 stoichiometric ratio to give $ONOO^-$ instantaneously, but does not have spatiotemporal control over $ONOO^-$ generation.[4] However, other probes utilising two component systems to produce $ONOO^-$ have been found useful for the biochemical studies but their use in cellular studies is limited. [4] Recently, a photochemically activated peroxynitrite donor exhibiting spatiotemporal control over peroxynitrite generation has been used in biological application[10] but use of a UV-A light source for activation of molecule is not considered suitable for studying bactericidal activity of $ONOO^-$. Therefore, a peroxynitrite generator which could be triggered by the intracellular enzymes would be a good option for the bactericidal

study. Dr. Harinath Chakrapani's group has recently reported a novel small molecule, HyPR-1 that cogenerates $O_2^{\bullet-}$ as well as NO upon activation with DT-Diaphorase (DT-D) and has biological applications[4]. DT-D is a NADPH-Quinone reductase and belongs to the enzyme family of soluble Quinone oxidoreducatases (QORs) which facilitates NADPH-dependent two-electron reduction of substrates. Several bacterial species are reported to carry the homologues of this class of enzymes for the detoxification of harmful chemical species encountered by them[27]. In *Mtb*, two genes *Rv1454c* and *Rv3141* have been reported to encode homologs of QORs[27]. The compound shows less potency hence cannot be efficient for bactericidal studies. However, an analogue of this scaffold which can generate higher fluxes of $ONOO^-$ or which can serve as a prototype for the further development of the peroxynitrite donors which can generate better fluxes by functionalising the molecule.



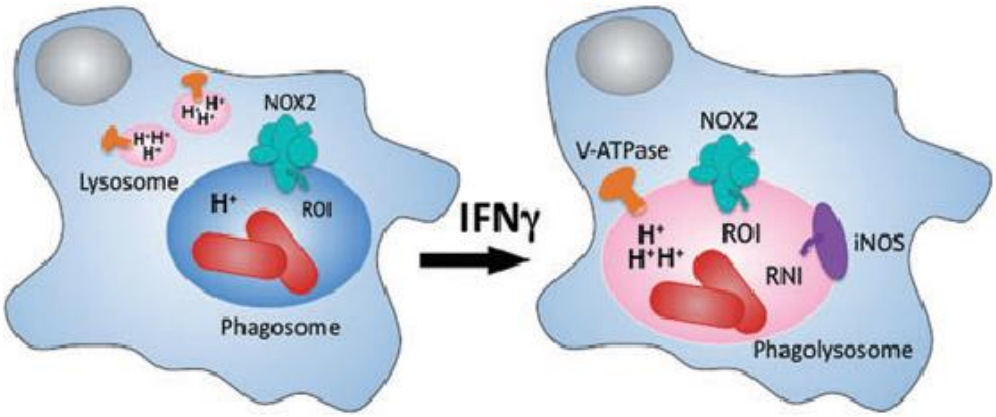
Org. Lett. 2016, 18, 1274−1277

Scheme 1: Peroxynitrite generation by HypR-1 (**1**) in presence of NADPH- quinone reductase.

1.3 Tuberculosis:

Tuberculosis (TB) is one of the deadly bacterial diseases which take over a million lives around the world every year. *Mycobacterium* tuberculosis (*Mtb*), the etiologic agent of Tuberculosis (TB), proliferates in alveolar macrophages. In resting macrophages, *Mtb* is exposed to mildly acidic pH and reactive oxygen intermediates (ROI) produced by phagocytic oxidase (iNOX2) in phagosome[11]. Activation with interferon-γ (IFN-γ) may represent a major anti-mycobacterial mechanism which leads to maturation of *Mtb*-containing phagosomes, wherein phagosomes fuse with

lysosomes to give phagolysosomes[11]. While ROS is generated via pre-existing NOX immediately after phagocytosis (respiratory burst), it is "only" after the activation of macrophage by pro-inflammatory cytokines (such as IFN-γ and tumor necrosis factor-alpha [TNF-α]) or microbial products such as lipopolysaccharide (LPS), that the expression of few genes such as inducible nitric oxide synthase (iNOS$_2$) occurs. Hence, generation of reactive nitrogen intermediate (RNI) is delayed till it gets a "signal".[11,12] Reaction occurs as follows: iNOS, a homodimer oxidizes L-arginine to L-citrulline and NO radical using electrons from NADPH. NO itself is toxic and can harm bacterial moieties, but it also reacts with ROS forming toxic RNI such as $ONOO^-$, dinitrogen trioxide (N_2O_3), nitroxyl (HNO), nitrosothiols and dinitrosyl iron complexes.[12] Additionally, phagolysosomal fusion also activates acid- dependent lysosomal hydrolases, free fatty acids and cationic antimicrobial peptides (CAMPs) which are bactericidal.[26] All of these phagolysosomal arsenals are highly reactive which can directly damage biological components such as lipids, thiols, amino acids, DNA bases and low-molecular weight antioxidants.



Nature Medicine **2008**,*14*, 809 - 810

Figure 2: Phagosome maturation on activation with IFNY.

Reports from experimental murine tuberculosis models using NOS inhibitors have provided evidence that RNI plays critical role in host defence against murine tuberculosis.[7] However biochemical and molecular basis for antimycobacterial function of RNI is not well understood.[7] Over the year's bactericidal activity .of peroxynitrite have been reported in different bacterial spp.[13, 14] including *Mtb*.[7] It has been shown that bacteria like *E.coli* and *Salmonella enterica* takes care of deleterious effect of $ONOO^-$ by peroxiredoxins like KatG and AhpC and by

peroxynitrite scavengers.[28] Transcription factors such QxyR and SoxR coordinates for E.coli antioxidant response towards ROS and NO/$ONOO^-$ respectively. Further comparative transcriptomic study in *E.coli* by microarray has demonstrated that hydrogen peroxide shows milder response as compared to peroxynitrite indicating $ONOO^-$ as a more fatal reactive species.[28] The viability studies in *E.coli* had also pointed out that $ONOO^-$ is more toxic to E.coli as compared to nitric oxide and its by-product.[9] In *Mtb*, redox-sensitive transcription factors such as OxyR and SoxR are absent, therefore we lack a clear understanding of how *Mtb* responds to oxidative or nitrosative stress including peroxynitrite. Dr. Amit Singh's lab has recently demonstrated that *Mtb,* unlike *E.coli*, is highly sensitive towards endogenous superoxide stress[15], suggesting important mechanistic differences underlying redox homeostasis between these two species. A peroxynitrite donor that can be triggered by NAPDH-quinone reductases which has been reported to be encoded by genes like *Rv1454c* and *Rv3141* in *Mtb* will be highly efficient to explore the influence of $OONO^-$ on mycobacterial redox physiology. With all these aspects taken together, this study comprehensively demonstrates the role of peroxynitrite donors to target *Mtb*.

1.4 Objective:

Aim of the thesis is to synthesize an analogue of the novel peroxynitrite donor, HyPR-1 that can generate high fluxes of peroxynitrite and can be later functionalised. The next objective is to explore the generation of peroxynitrite by the donor in the chosen model system, *Mycobacterium spp.*, using different probes and techniques. Following which, the dynamic oxidative stress response of the bacteria towards the donor and susceptibility of different strains of Mycobacterium needs to be probed.

2. Experimental Section

2.1 Synthesis:

All chemicals were purchased from commercial sources and used as received unless stated otherwise. THF and DMF were dried and distilled before use. Column chromatography was performed using Merck silica gel (60-120 mesh) as the solid support.For column chromatography petroleum ether (PE) and ethyl acetate (EtOAc) were distilled before use.^{1}H were recorded on JEOL 400 MHz and ^{13}C spectra on 100 MHz spectrometer using either the residual solvent signal as an internal reference (DMSO-d_6 δH, 2.54 ppm, δC 39.52 ppm) or tetramethylsilane (δH = 0.00, δC =0.0) standard. Chemical shifts (δ) are reported in ppm. The following abbreviations are used: s (singlet), d (doublet), t (triplet) and m (multiplet). Mass spectra were obtained using HRMS-ESI-Q-Time of Flight LC-MS (Synapt G2, Waters) or MALDI TOF/TOF Analyser (Applied Biosystems 4800 Plus). Infrared spectra (IR) were recorded using NICOLET 6700 FT-IR spectrophotometer in DMSO.

Compounds **A**[4] and **B**[4] have been previously reported in literature and analytical data obtained was consistent with the reported values. **C** was synthesized by following the procedure reported by Khodade *et. al*.[4] was purified and used for the next reaction.

(Z)-1-(4-(tert-butoxycarbonyl)piperazin-1-yl)-2-((3-methyl-1,4-dioxo-1,4-dihydronaphthalen-2-yl)methoxy)diazene oxide (CJ067) : To a solution of **C** (221 mg, 479.89 mmol) in acetonitrile (8 mL) and water (8 mL), ceric ammonium nitrate (1.32 g, 2.40 mmol) was added and the mixture was stirred at RT for 14h. The mixture was diluted with water (20 mL) and extracted with DCM (20 mL x3). The organic layer was dried over anhydrous sodium sulfate, filtered and concentrated. The crude compound was purified using silica gel column chromatography with EtOAc/hexane as eluent to give CJ067 as a pale yellow (150 mg, 72.61%). FTIR (ν, cm^{-1}): 3426, 2998, 2914, 1657, 1436, 1407, 1311, 1018, 952, 898, 698, 669, 509; ^{1}HNMR (400MHz, DMSO-d_6): δ 8.04 (m, 2H), 7.87 (m, 2H), 5.24 (s, 2H), 3.44 (t, *J* = 8 Hz, 4H), 3.26 (t, *J* = 12 Hz, 4H), 2.25 (s, 3H), 1.39 (s, 9H); ^{13}CNMR (100MHz, DMSO-d_6): δ 184.7, 183.1, 153.6, 148.1, 138.1, 134.3, 134.2, 131.8, 131.3, 126.1,

79.4, 65.5, 50.2, 39.7, 39.3, 28.0, 13.1; HRMS (ESI-TOF): for $[C_{21}H_{26}N_4O_6 + Na]^+$ calcd. 453.1799, found 453.1754.

2.2 Preliminary assays in buffer

2.2.1. Stock Solutions: 5-Amino-2,3-dihydro-1,4-phthalazinedione stock solution (Luminol, 4 mM)was prepared in 30 mM aqueous sodium hydroxide and stored under dark conditions in ice. Stock solution of CJ067 (10 mM) and dye **1** (4 mM) was prepared in DMSO. A stock solution of NADH (10 mM) was prepared in pH 7.4 phosphate buffer (PB). The enzyme stock solution was prepared by dissolving 1.5 mg of the lyophilized human DT-Diaphorase (DT-D, Sigma, ≥100 units (U); wherein 1 U is 1.0μmol cytochrome C reduced per min/mg) in 1 mL Phosphate Buffer of pH 7.4 [4]. 20 μL of this enzyme solution was further diluted to 100 μL and was used. Stock solution of catalase (Sigma-Aldrich, catalase from bovine liver 10 kU/mL) was prepared in pH 7.4 phosphate buffer.

2.2.2. Time course of superoxide generation from CJ067: To a 96-well plate, a stock solution of CJ067 (2μL, 10 mM) was added to the phosphate buffer (pH 7.4, 183 μL) followed by NADH solution (8 μL, 10 mM), luminol solution (5 μL, 4mM) and 2 μL of the enzyme DT-D. The time dependent chemiluminescence of the resulting mixture was measured using a microtiter plate reader at 1 min interval for 20 min. Wells containing no DT-D was control.

2.2.3. NO generation from CJ067: In a 96-well plate the reaction mixture was prepared in triplicates by dissolving CJ067 (2 μL, 10 mM), NADH (8 μL, 10 mM) and 2 μL of DT-D in 188 μL of pH 7.4 phosphate buffer. Resulting mixture was incubated at 37 °C for 15 min followed by immediate addition of Greiss reagent (14 μL). The mixture was incubated at 37 °C for another 25 min under dark conditions and the plate was read photometrically at excitation wavelength of 535 nm. Concentration of the nitric oxide was determined by using a calibration curve. For the calibration curve, different concentrations of sodium nitrite (25, 50, 75, 100, 150, 200 μM) were dissolved in phosphate buffer along with greiss reagent and the spectra were recorded.

2.2.4. Peroxynitrite detection using boronate ester probe: The reaction mixture was prepared by dissolving **1** (5 μL, 4 mM), CJ067 (2 μL, 10 mM), NADH solution (8 μL, 10 μL) and DT-D (2 μL) in 183 μL of phosphate buffer pH 7.4. The reaction mixture was incubated at 37 °C for 15 min under dark conditions. The fluorescence spectrum was measured at excitation wavelength 315 nm using a microtiter plate reader.

2.3 Intracellular peroxynitrite detection in *M. smegmatis*

Stock solution of dye **1** (4 mM), Ds-DAB (10 mM), CJ067 (10 mM), JCH (10mM), menadione (10 mM), NO donor(10 mM) and SIN-1 (10 mM) were prepared in filtered DMSO. Stock solution of uric acid (10 mM) was prepared in 0.1N NaOH. The authentic peroxynitrite solution was prepared using reported procedure.[16] Concentration of authentic peroxynitrite was measured after 500 times dilution with 0.1 N NaOH solution by recording absorbance at 302 nm (ε_{302nm} = 1670 $M^{-1}cm^{-1}$) using UV/Vis spectrophotometer.

2.3.1. Time course of peroxynitrite generation using boronic ester probe: *Mycobacterium smegmatis* (MC^2155) was cultured in middle brook 7H9 with 10% albumin-dextrose-saline (ADS) supplement, 0.2% glycerol and 0.05% Tween 80 at 37 °C and grown till exponential phase (OD_{600nm} ~ 0.6). *Msm* grown was diluted to an OD_{600nm} of 0.4 and dye **1** (25 μM) was added. *Msm* only and *Msm*+dye were incubated at 37 °C for 15 min in the dark condition by covering the falcons in an aluminium foil. 200 μL of this incubated culture was dispensed in 96-well flat bottom plate and was treated with 10 μL CJ067, JCH, menadione, NO donor and 100 μM of AD in triplicates. Wells containing *Msm* + dye only was taken as a control. Fluorescence intensity was measured immediately at 1 min intervals for 15 min in a SpectraMax M3 plate reader (Molecular device) in top-reading mode with excitation at 315 nm and emission at 460 nm.

2.3.2. Quenching Experiment Using Uric acid:

Relevant stocks were prepared as mentioned earlier. *Msm* was cultured as mentioned in earlier section and was diluted to anOD_{600nm} of 0.4. *Msm* was co-incubated with 25 μM of dye for 15 min under dark conditions at 37 °C. 200 μL of this incubated culture was dispensed in 96-well flat bottom plate and was treated with

$100\ \mu M$ uric acid along with 10 μM CJ067 and 100 μM of AD in triplicates. Fluorescence intensity was measured immediately at 1 min intervals for 15 min using microplate reader with excitation at 315 nm and emission at 460 nm.

2.3.3. Peroxynitrite detection using Ds-DAB: Relevant stocks were prepared as mentioned before. *Msm* was cultured in 7H9-ADS medium and grown till exponential phase ($O.D_{600nm}$ ~ 0.4). Bacterial culture was co-incubated with 25 μM Ds-DAB for 30 min at 37 $^{\circ}$C in a shaker incubator and then treated with different concentration of CJ067 (10, 25, 50 μM) for 1h. SIN-1 (100 μM) was used as a positive control. Culture was washed 2 times with 1X PBS to remove the extra floating dye and fixed with 4% PFA (paraformaldehyde) for 20 min at room temperature (RT) followed by 1X PBS wash. Culture (~ 10^7 bacilli) was finally resuspended in 200 μL of 1X PBS. For imaging coverslips were mounted using antifade mixture on glass coverslips. Imaging was done on Leica SP5 confocal microscope with 405 laser lines using a 63X objective, NA 1.4 at 4.5X digital zoom.

2.4 Redox Stress studies using Mrx1-roGFP2 by flow cytometry

Msm expressing Mrx1-roGFP2 was cultured in Middlebrook7H9 medium (Difco) supplemented with 10% ADS, 0.2% glycerol, 0.05% Tween 80 and 0.05% (0.5 mg/mL) Hygromycin (100 mg/ml stock). The culture was grown till exponential phase (OD_{600nm} ~ 0.4-0.6). The culture was treated with 10 μM CJ067, AD, Menadione, JCH and NO donor and the sensor response was recorded using BD FACS Verse Flow cytometer (BD Sciences) at 3 min time interval for half an hour. The biosensor response was analysed by taking the ratio at a fixed emission (510 nm) after excitation at 405 nm and 488 nm.

2.5 MIC *assays*:

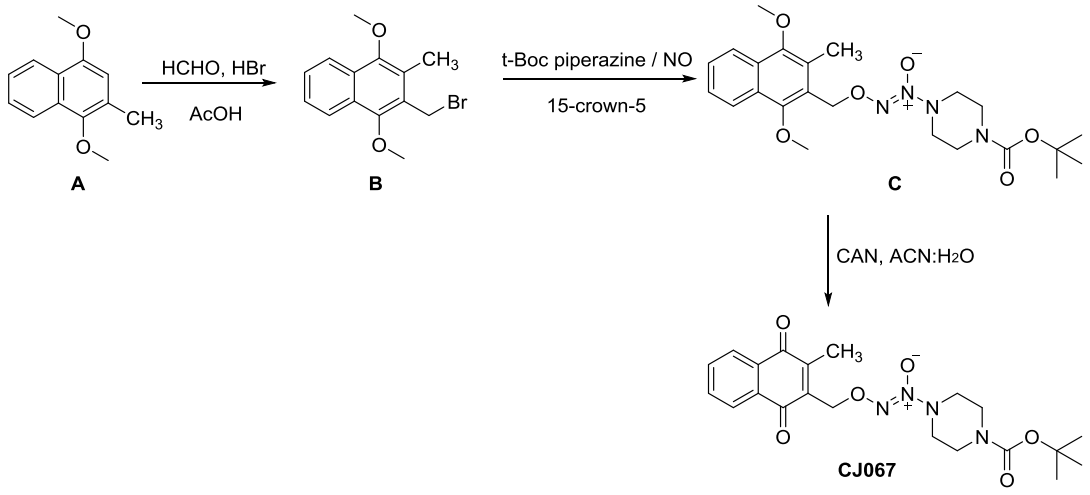
MIC of all the strains was determined by microplate Alamar blue assay (MABA). The assay was performed in a 96-well flat bottom plate.100 μL of 7H9-ADS medium without tween was dispensed in each well of the plate and serial two fold dilutions of each compound were prepared directly in the plate. Mycobacterial strains were cultured in 7H9-ADS medium and grown till exponential phase (OD_{600nm} ~ 0.6-0.8). Approximately $1x10^5$ ($1x10^4$ for *Msm*) bacteria were added per well in a total volume

of 200 μL of 7H9-ADS. Wells containing cells and medium only were growth control and wells containing no bacterial culture were the autofluorescence control. After 5 days (1 day) of incubation at 37 °C, 20 μL (10% of total volume) of 10X Alamar blue was added and reincubated for 24 h (12 h for *Msm*). Fluorescence intensity was measured in a Spectra Max M3 plate reader (Molecular device) in top-reading mode with excitation at 530nm and emission at 590nm. Percentage of growth inhibition was calculated with relative fluorescence unit obtained. The minimum concentration at resulted in 90% inhibition was identified as MIC[15].

3. Results and Discussion

3.1 Synthesis of the Peroxynitrite Donor Analogue:

(Z)-1-(4-(tert-butoxycarbonyl)piperazin-1-yl)-2-((3-methyl-1,4-dioxo-1,4-dihydronaphthalen-2-yl)methoxy)diazene oxide (**CJ067**) was synthesized by following the procedure reported by Khodade *et. al* [4] with some changes in reaction time. **B** was obtained from the one-pot formylation and bromination of the 1,4-dimethoxy-2-methylnapthalene (**A**) followed by the reaction with t-Boc piperazine/NO to give **C.** Reaction of **C** by Ceric ammonium nitrate gave oxidised product CJ067.



Scheme 2: Synthesis of CJ067

3.2 Preliminary Assays in Buffer:

For confirming the peroxynitrite generation from CJ067, preliminary experiments were performed in phosphate buffer pH 7.4 under ambient aerobic conditions in the presence of DT-Diaphorase (DT-D) enzyme and cofactor-NADH. To demonstrate that CJ067 dissociates only in the presence of DT-D and is stable in buffer, CJ067 without DT-D was taken as a control. Firstly, the ability of CJ067 to generate $O_2^{\bullet-}$ was examined using a reported luminol-based chemiluminescence assay.[4,15,17] In the time dependent measurement, ~15-fold increase in the chemiluminescence was observed within 10 min as compared to the control followed by a gradual decrease (Fig.3).

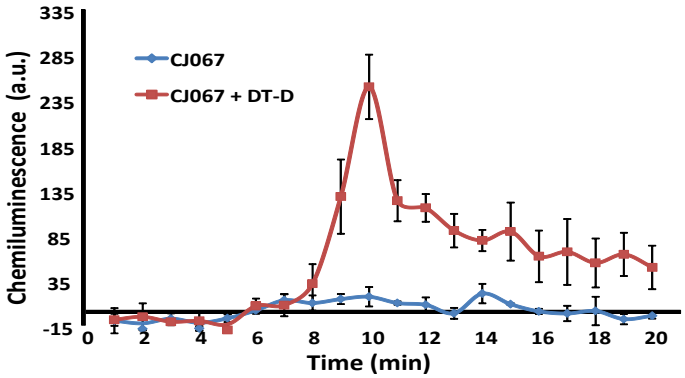


Figure 3: Superoxide generation during incubation of CJ067 (100 μM) with DT-D in phosphate buffer pH 7.4 using a luminol assay.

Next, the ability of CJ067 to generate NO was examined using colorimetric Greiss assay.[18] Nitric oxide readily gets oxidised by oxygen to give nitrite or nitrate which undergoes diazotization reaction with the Greiss reagent to give colorimetric change. Within 25 min of incubation of CJ067 (100 μM) with the greiss reagent in presence of DT-D produced 45 μM of NO. No significant evidence of NO generation was detected in control.

Next, $ONOO^-$ formation was examined using a "turn on" fluorescent dye, an arylboronate ester. 3-methyl-coumarin-7-(pinacolboronate ester) **1** was used for the study.[4] This boronate ester undergoes hydrolysis in buffer to produce a weakly fluorescent boronic acid. The boronic acid reacts rapidly with $ONOO^-$ and produces corresponding alcohol which shows strong fluorescence emission at 460 nm on excitation at 315 nm (Fig. 4). Upon 15 min incubation of CJ067 + DT-D with **1**, an enormous increase in fluorescence was observed (Fig. 5) as compared to the control signifying that $ONOO^-$ was being formed.

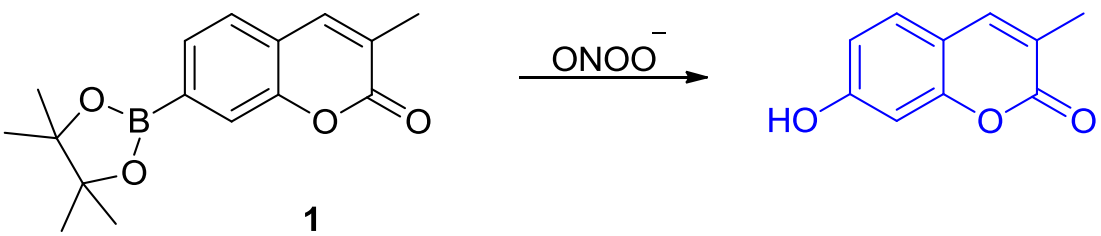


Figure 4: Conversion of probe **1** on reaction with peroxynitrite.

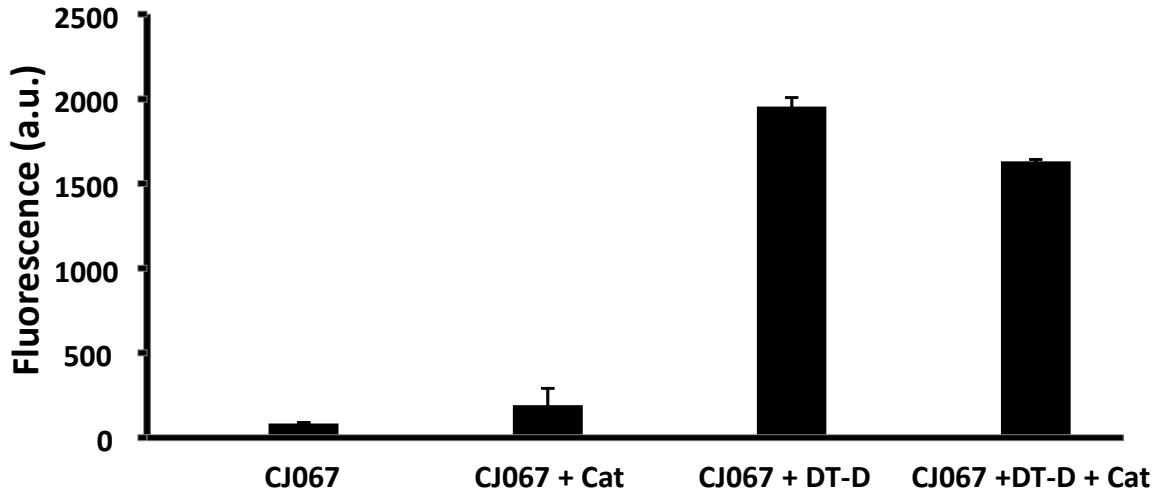


Figure 5: Fluorescence intensity of 1 on incubation with CJ067 (100 µM) + DT-D in pH 7.4 buffer**.**

Arylboronic acids have been reported to react with hydrogen peroxide at low rates[19, 20] and it is also known that superoxide dismutates to form hydrogen peroxide (H_2O_2) and oxygen. To exclude the fluorescence produced by H_2O_2, emission profiles were also measured in the presence of catalase (Cat), an enzyme that detoxifies H_2O_2 effect by converting it to water and oxygen. No distinct change in the fluorescence in the presence of catalase (CJ067 + DT-D + Cat) was observed as compared to CJ067 + DT-D indicating that major reactive species formed is $ONOO^-$.

The fluorescent intensity obtained for CJ067 was higher compared to HypR-1 indicating CJ067 can generate better fluxes of $ONOO^-$. The efficiency of peroxynitrite generation would depend on the kinetics of superoxide and nitric oxide generation. For both the compounds till the formation of diol intermediate the reaction kinetics is going to be same, but upon release of dizeniumdiolate, the half-life of dizeniumdiolate is going to determine the yield of peroxynitrite. The half-life of DMA/NO is only few second in pH 7.4 buffer on the other hand tBOC-PI/NO takes little longer to release NO. Therefore, the kinetics of NO and superoxide generation might be overlapping well for CJ067 hence giving better yield of peroxynitrite.

Together, these experiments demonstrated that the compound (CJ067) is stable in buffer and generates superoxide and nitric oxide, only in the presence of a NAPDH

Quinone reductase thereby establishing $ONOO^-$ is the major reactive species formed by CJ067.

3.3 Intracellular peroxynitrite detection in *M.smegmatis*:

Next, it was checked if the donor undergoes similar dissociation inside the bacteria (*Mycobacterium smegmatis mc²155, Msm*) and $ONOO^-$ is the major reactive species getting generated. Sodium salt of peroxynitrite (Authentic Donor, AD) was used as a positive control for this experiment. ROS donors were incorporated in studies to rule out the possibility that fluorescent signal obtained was due to H_2O_2. JCH [21] and menadione were used as ROS donors. Menadione is a commercially available ROS donor which bioreductively generates superoxide, while JCH generates superoxide exogenously. Along with the ROS donors, an esterase activated nitric oxide generator [4] (NO Donor) was also used to exclude any possibility of side reaction in bacteria.

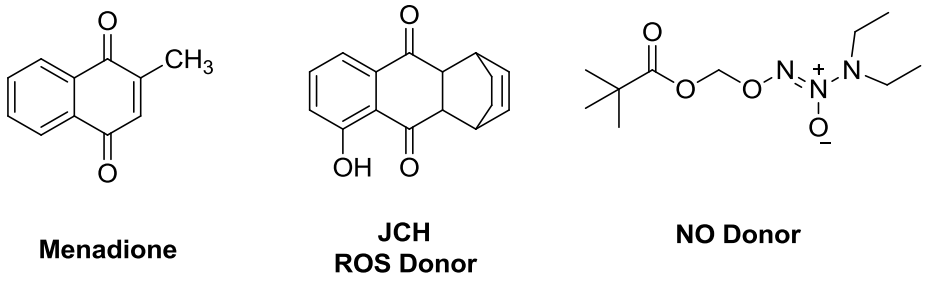


Figure 6: Structures of ROS donors Menadione, JCH and Nitric oxide donor (NO).

Next, the time course of peroxynitrite generation by CJ067 inside *Msm* was examined. Co-incubation of *Msm* with **1**(25 μM) for 15 min followed by the treatment with CJ067 (10 μM) and immediate fluorescence measurement at an interval of 1 min by microplate reader showed an overwhelming increase in fluorescence (Fig. 7). Saturation of the fluorescence within 15 min was observed which was consistent with the result obtained earlier that $ONOO^-$ formation occurs within 15 min of incubation. A higher concentration of AD (100 μM) was used due to its short half-life and a significant increase in the fluorescence was detected but lesser than CJ067 (~15-fold). A minor fluorescence was observed for menadione while no significant fluorescence was detected for JCH. The small fluorescence obtained in case of menadione can be attributed to the slow side reaction of the dye with H_2O_2.

However, a distinct difference in the fluorescent intensity of CJ067 and menadione was observed indicating that the prominent reactive species formed is $ONOO^-$. The difference in the fluorescence profile can be due to the fact that CJ067, menadione produce reactive species intracellularly while AD, JCH dissociate extracellularly to generate respective reactive species. Finally, no significant fluorescent intensity was detected for NO donor that was consistent with the reports that the dye does not react with NO.

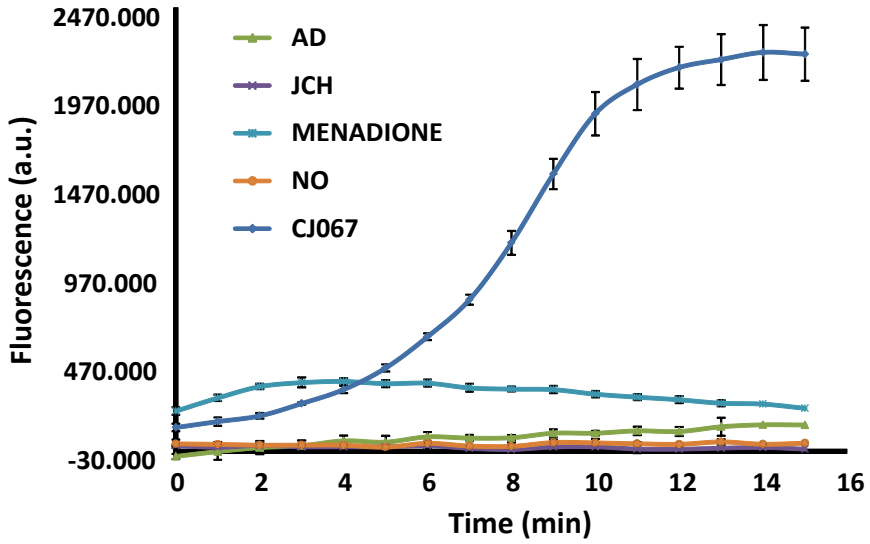


Figure 7: Time course of intramycobacterial peroxynitrite generation during incubation of 10μM CJ067, AD, JCH, Menadione and NO donor using probe **1.**

In addition, fluorescence was measured in presence of $ONOO^-$ specific quencher, uric acid. Uric acid is known as a biological scavenger of $ONOO^-$ and is reported as a therapeutic agent for the $ONOO^-$ mediated disease[22]. When uric acid (100 μM) was added to the co-incubated bacterial culture with dye (**1**) along with CJ067, a significant decrease in fluorescence of the dye was observed (Fig. 8). This observation indicated that the fluorescence was due to $ONOO^-$ which resulted in quenching upon addition of uric acid.

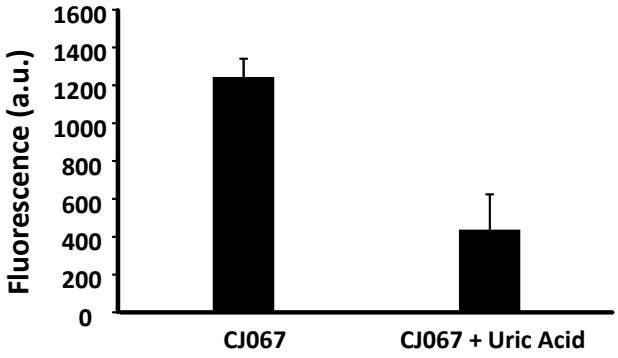
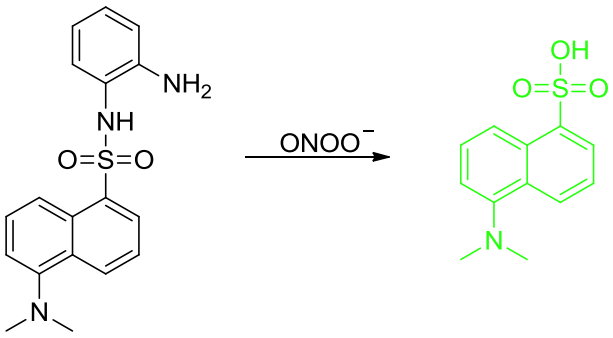


Figure 8: Decrease in the fluorescence of probe **1** on addition of uric acid (100 μM) to *Msm* + CJ067 (10 μM).

Next, the intracellular generation of $ONOO^-$ was validated by cell imaging using N-(2-aminophenyl)-5-(dimethylamino)-1-naphthalene sulfonic amide (Ds-DAB).[4, 23] Ds-DAB is an $ONOO^-$ sensitive probe which converts into dansylsulfonic acid to give a strong fluorescence emission at 505 nm (Fig. 9).[23] When *Msm* co-incubated with Ds-DAB (25 μM) was treated with CJ067 (10, 25 μM) a dose dependent enhancement in the fluorescence signal was observed (Fig. 10). SIN-1, taken as positive control also showed a significant fluorescent signal but at a concentration of 100 μM.



Ds-DAB

Figure 9: Peroxynitrite probe Ds-DAB

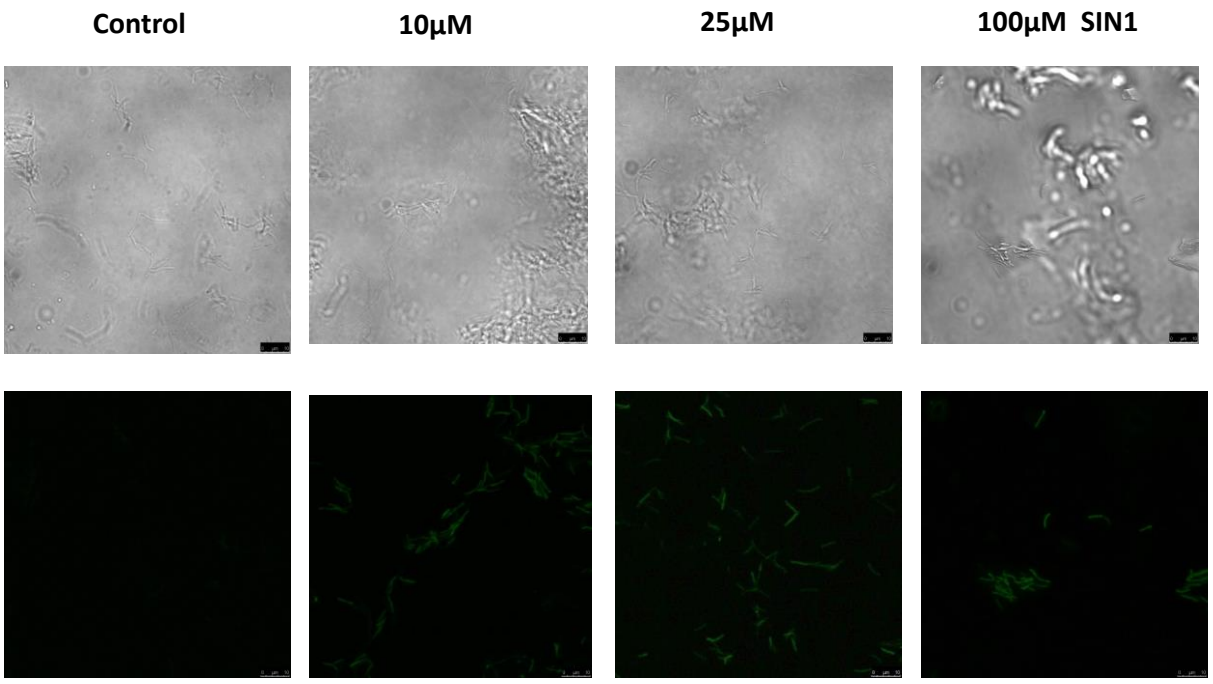


Figure 10: Fluorescence images of *Msm* on 1h incubation with CJ067 in presence of Ds-DAB.

Together, these experiments established that CJ067 is permeable and enhances specifically peroxynitrite levels inside *M. smegmatis*.

3.4 Redox Stress studies using Bio-sensor in *M.smegmatis*:

Next, $ONOO^-$ fluxes generated by CJ067 is significant enough to disrupt the redox balance of mycobacteria was examined. To measure the dynamic response of mycobacteria towards the redox perturbation induced by CJ067, a non-invasive redox biosensor, Mrx1-roGFP2[24] was used. The available fluorescent dye based probes were not used because these probes can introduce oxidation artifacts which are not suitable for dynamic measurements in real time.

Mycothiol (MSH) is an antioxidant maintained in millimolar concentrations in mycobacterial which functions as a redox buffer in the cytoplasm to protect against oxidative stress encountered during infection.[11,25] The biosensor Mrx1-roGFP2 consists of roGFP2, an oxidation-reduction sensitive GFP covalently fused to mycothiol specific oxidoreductase (mycoredoxin-1, Mrx1)[24]. Electrons are reversibly transferred between the mycothiol redox buffer and the thiol groups of roGFP2[24]. Continuous formation and release of roGFP2 disulphide bridges are associated with

the actual redox potential of the mycothiol buffer (E_{MSH}) inside mycobacteria (Fig. 11). Redox stress leads to the formation of oxidized mycothiol (MSSM) which is selectively monitored by roGFP2 once coupled with mycoredoxin[24]. Mrx1-roGFP2 exhibits two distinct excitation peaks, 390 (oxidised) and 490 (reduced) at fixed emission wavelength of 510nm.[24]

The redox response of cells is detected by measuring fluorescence intensity at 405 and 488 using Fluorescence activated cell sorting (FACS). Increase or decrease in 405/488 ratio corresponds to the oxidative or reductive shift in E_{MSH} of *Msm.*[24]

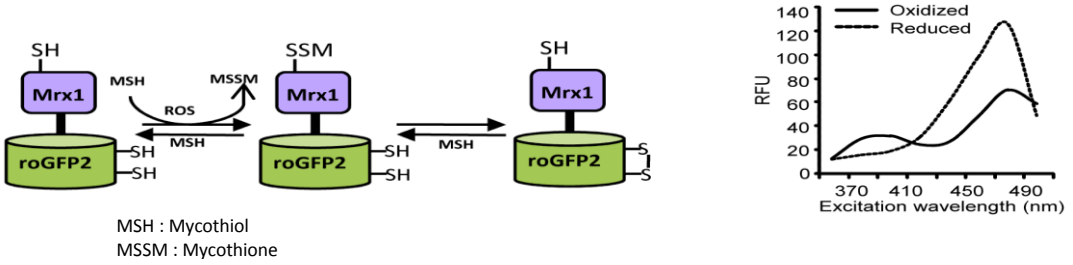


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Figure 11: Schematic representation of the working principle of Mrx1-roGFP2.

Treatment of *Msm* expressing Mrx1-roGFP2 with 10 μM CJ067 resulted in a gradual increase in the 405/488 ratio which was retained till 12 min followed by a gradual recovery to the basal level after 50 min (Fig. 12). In contrast, 10 μM addition of AD showed a transient increase in the biosensor ratio as full recovery from the oxidative stress was evident within 15 min. JCH produced a very marginal oxidative shift in E_{MSH} of *Msm* which normalizes to basal level immediately within few minutes while menadione produces a modest but persistent (for ~ 10min) oxidative shift. However, no shift was observed in case of NO donor. Since, the biosensor response corresponds to the oxidative stress experienced by the bacteria due the treatment by various donors; the above observations indicate that CJ067 can induce huge oxidative burst while NO donor has the least ability to induce the oxidative stress among all the donors. Our data indicate that NO is relatively ineffective in inducing oxidative changes in the E_{MSH} of *Mtb*. This could be due to multiple NO detoxification mechanisms such as haemoglobins and α-ketoglutarate (α-KG) dehydrogenase complex (KDHC) in *mycobacteria*.[29] For higher concentrations of CJ067, instantaneous and long lasting increase in oxidative shift in E_{MSH} of *Msm* was

observed as biosensor remain oxidized till 2 hours post-treatment. But *Msm* was able to recover from higher concentration of AD within 15min.

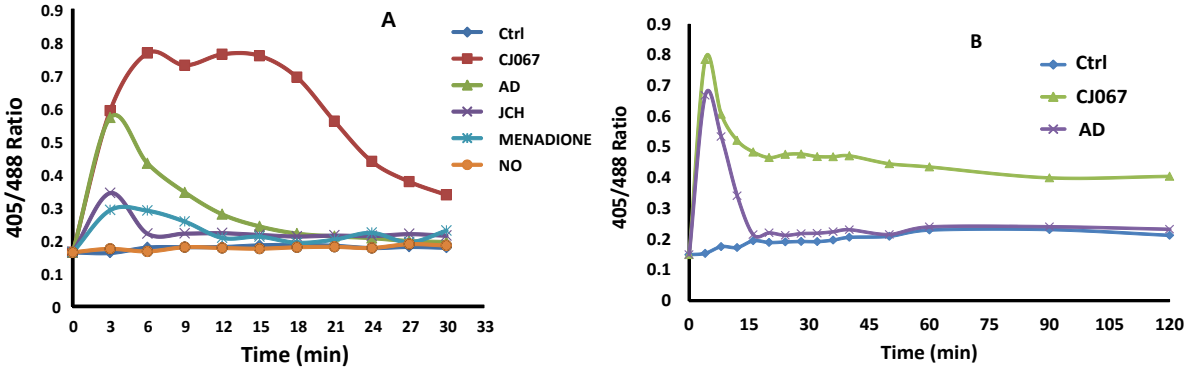

Figure 12: (A) Shift in the 405/488 ratio on treatment of *Msm* expressing Mrx1-roGFP2 with 10 μM concentration of CJ067, AD, JCH, Menadione and NO donor (B) 50 μM concentration of CJ067 and AD.

3.5 MIC studies:

Next, a correlation between the oxidative stress induced by the $ONOO^-$ donor and growth inhibition was examined. Micro Plate Alamar Blue Assay (MABA) was used for determining MICs (Minimum Inhibitory Concentration) of anti-TB drugs in mycobacteria. MIC in mycobacterial strains is defined as the lowest concentration of the drug that gives 90% growth inhibition. Alamar blue is a dye that incorporates a redox indicator which shows fluorometric and colorimetric changes depending on the oxidized or reduced environment within the cells. Growing cells produce reduced equivalents due to their metabolic activity while inhibition of growth results in oxidised environment which forms the working principle of this dye. Therefore, dye will remain in oxidised state and will be blue (non-fluorescent) in case of growth inhibition while during cell growth, it will get reduced and will appear pink (fluorescent).

Most of the donors were incapable of inhibiting growth of *Msm* at low concentration and showed MIC greater than equal to 400 μM. However, 100 μM of CJ067 was sufficient to inhibit bacterial growth (Table 1). The $ONOO^-$ donor, CJ067, which resulted in the highest and persistent oxidative shift in the E_{MSH} of *Msm* has

consistently demonstrated the lowest MIC, while NO donor which showed no change in 405/488 ratio has highest MIC (>800μM). Altogether, MIC measurements obtained were in agreement with the Mrx1-roGFP2 data. This direct correlation between the MIC and the level of oxidative stress induced by $ONOO^-$ donor indicates the role of $ONOO^-$ in disrupting mycothiol redox homeostasis in mycobacteria, which may be the underlying mechanism by which CJ067 is showing antibacterial effect.[15]

Strains	CJ067	AD	JCH	Menadione	NO Donor
WT msm	100	400	>400	400	>800
Δ Mtr msm	25	200 – 400	N/D	N/D	N/D
Δ MshA msm	50-100	400	400	200	>800
H37Rv	6.25	400	6.25	50	200
XDR (Myc-431)	12.5	400	N/D	N/D	N/D
MDR (Jal 2287)	25	400	3.125	50	200
MDR (Jal 2261)	6.25	400	3.125	50	400
MDR (BND320)	25	400	12.5	100	400

Table 1: Minimum inhibitory concentrations (MICs) of donors against different Mycobacterial *spp*. Concentrations in μM.

In order to further compare the inhibiting ability of CJ067 and other donors, MICs in pathogenic strains like *Mycobacterium tuberculosis* (H37Rv), multidrug resistant strain (MDR) and extensively drug resistant (XDR) strains were examined. Surprisingly, H37Rv, MDR, and XDR strains displayed lower MICs of CJ067 as compared to the non-pathogenic *Msm* strain. This result indicates fundamental difference between how different species of mycobacteria tolerate oxidative stress. These results are in agreement with our findings indicating an exceptional sensitivity of *Mtb* strains to endogenous ROS/RNS generated by various redox cycling drugs.[14] On the other hand, *Msm* is better equipped to tolerate endogenous ROS/RNS, in parts, due to activation of a protective oxidative response similar to OxyR-mediated induction of antioxidant in other bacteria.[14] Additionally, we earlier reported higher

antioxidant capacity of *Msm* owing to lower intrabacterial E_{MSH} (-300 mV) as compared *Mtb* (-275 mV).[24,14]

Earlier report proposed that *Mtb* is resistant to peroxynitrite as compared to non-pathogenic strains like *Msm* and BCG.[7] This study first time showed that ability to resist peroxynitrite is compromised in *Mtb* if it is generated endogenously as compared to *Msm*. This finding can be exploited to target *Mtb* by developing molecules which can generate $ONOO^-$ endogenously.

However no difference in MIC was observed in case of AD may be because of its less bioavailability and due to its exogenous mode of peroxynitrite generation. For NO donor higher MICs were obtained in each strain as compared to CJ067 and AD which is consistent with the studies that peroxynitrite is more toxic as compared to nitric oxide.[7,9]

Surprisingly, we also observed that the MICs obtained against CJ067 for MDR and XDR strains is higher compared to that of H37Rv while other donors showed similar or lower MICs in these strains as compared to H37Rv. The lesser susceptibility of these strains to CJ067 can be due to the less permeability of CJ067 in MDR and XDR as these have different membrane properties. These strains are resistant to isoniazid which is usually encountered due to the mutation in KatG, which has also been reported to function as a peroxynitratase in *Mtb*. The lesser activity of CJ067 can also be attributed to the hypermorphic mutation of the peroxynitratase activity of the KatG enzyme which is leading to the detoxification of $ONOO^-$ generated by CJ067 and hence giving resistance in MDRs and XDR.

4. Conclusions:

The study encompasses synthesis of a peroxynitrite donor which can generate significantly high fluxes of $ONOO^-$. Kinetic and confocal study with fluorescent dyes demonstrated that the synthesized compound is cell permeable and generates peroxynitrite as major reactive species in *Msm*. Next, the $ONOO^-$ fluxes produced by the compound is significant enough to perturb the redox balance of *Msm* was positively determined using redox biosensor Mrx1-roGFP2. Finally, MIC studies showed that *mycobacterium spp.* are more susceptible to endogenous $ONOO^-$ as compared to the one produced exogenously and $ONOO^-$ shows better cytotoxic activity compared to nitric oxide. Our study provides new information into mechanism of how mycobacteria respond to a physiologically relevant oxidant, $ONOO^-$, which can be explored to develop better strategies to control TB infection.

NMR Spectra

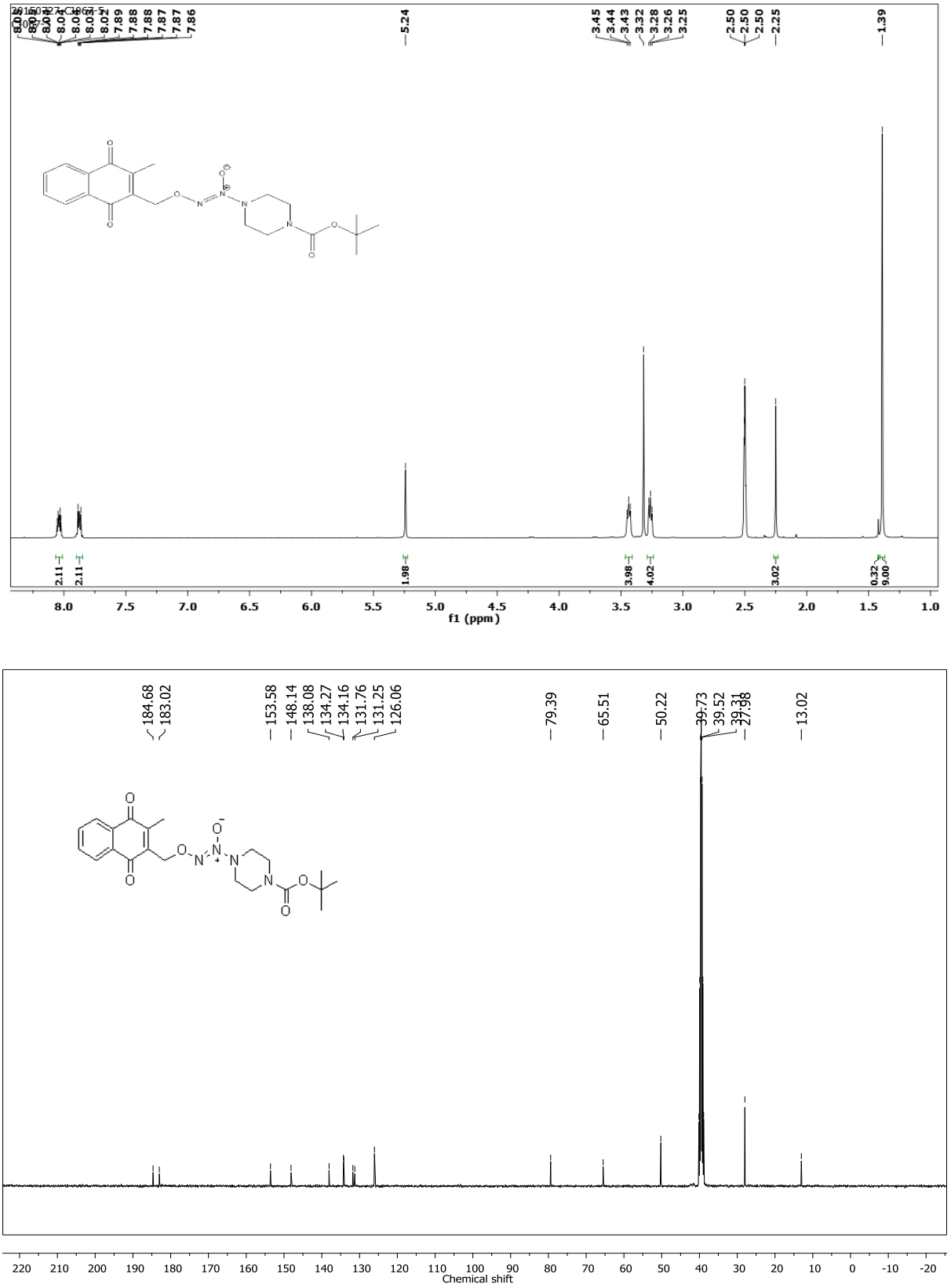

Figure 13: ^{1}H and ^{13}C NMR of CJ067

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Certificate

This is to certify that this dissertation entitled **Understanding Mycobacterial redox response towards endogenous stress produced by peroxynitrite donor** towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents the research carried out by **Charu Jain** at Indian Institute of Science (IISc.), Bangalore under the supervision of **Dr. Amit Singh**, Assistant Professor, Centre for Infectious Disease and Research (CIDR), Department of Microbiology and Cell Biology, IISc. Bangalore during the academic year 2015-2016.

Date: 27-04-2016

Place: Bangalore

Dr. Amit Singh

IISc. Bangalore

Dr. Amit Singh
Assistant Professor
Dept. of Microbiology & Cell Biology
Centre for Infectious Disease Research
Indian Institute of Science, Bangalore - 560 012

Declaration

I hereby declare that the matter embodied in the report entitled **Understanding Mycobacterial redox response towards endogenous stress produced by peroxynitrite donor** are the results of the investigations carried out by me at the Department of Microbiology and Cell Biology, IISc. Bangalore, under the supervision Dr. Amit Singh and the same has not been submitted elsewhere for any other degree.

Date: 27/04/16

Place: Bangalore

Charu Jain

IISER Pune