Biochemical characterization and novel inhibitor identification of *Mycobacterium tuberculosis* Endonuclease VIII 2 (Rv3297)

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**ABSTRACT**

Nei2 (Rv3297) is a DNA Base Excision Repair (BER) glycosylase that is essential for survival of *Mycobacterium tuberculosis* in prirmtes. We show that MtbNei2 is a bifunctional glycosylase that specifically acts on oxidized pyrimidine-containing single-stranded, double-stranded, S'/S" fork and bubble DNA substrates. MtbNei2 possesses Uracil DNA glycosylase activity unlike *E. coli* Nei. Mutational studies demonstrate that Pro2 and Glu3 located in the active site are essential for glycosylase activity of MtbNei2. Mutational analysis demonstrated that an unstructured C-terminal zinc finger domain that was important for activity in *E. coli* Nei and Fpg, was not required for the glycosylase activity of MtbNei2. Lastly, we screened the NCI natural product compound database and identified three natural product inhibitors with IC50 values ranging between 41.8 μM-92.7 μM against MtbNei2 in in vitro inhibition assays. Surface Plasmon Resonance (SPR) experiments showed that the binding affinity of the best inhibitor, NSC31867, was 74 nM. The present results set the stage for exploiting this important target in developing new therapeutic strategies that target Mycobacterial BER.

**1. Introduction**

Tuberculosis (TB) causes 1.8 million deaths worldwide each year. Cases of drug-resistant (MDR) TB have also risen to more than half a million globally (WHO 2016 Global Tuberculosis report). *Mycobacterium tuberculosis* is an intracellular pathogen that dwells in alveolar macrophages and encounters a diverse range of oxidative stress conditions viz. reactive oxygen and nitrogen intermediates. These free radicals cause DNA base damage. Oxidative DNA damage is mainly repaired by the premier base-excision repair (BER) pathway [1,2]. BER initiates with the recognition of the damaged base by DNA glycosylases. Monofunctional DNA glycosylases cleave the N-glycosyl bond between the damaged base and the sugar creating an abasic (AP) site. However, bifunctional DNA glycosylases also possess an associated AP-lyase activity that cleaves the phosphodiester bond at the AP site by a β- or β-δ elimination mechanism generating either an α, β-unsaturated aldehyde (β-elimination) or a phosphate (β, δ-elimination) group [3].

Based on structural and sequence homology, DNA glycosylases are generally classified into two families viz. the Fpg/Nei superfamily and the Nth superfamily [4,5]. The Fpg/Nei superfamily includes formamidopyrimidine DNA glycosylase or Fpg, also known as MutM, which recognizes both 8-oxoguanine and formamidopyrimidines while Endonuclease VIII (Nei) specifically recognizes oxidized pyrimidines and uracils [6,7]. Fpg is a structural homologue of Nei while Nth is a functional homologue.

Nei proteins consist typically of two domains which are connected by a flexible hinge region. The N-terminal region contains a β-windflank by α-helices. The C-terminal domain consists of α-helices, two of which form a conserved H2TH motif, and a Zn-finger motif formed by two antiparallel β-strands. These motifs are found in both Fpg and Nei subfamilies and additionally the zinc finger and H2TH motifs in Fpg are required for DNA binding [7,8, and 9].

The catalytic mechanism of Nei comprises multiple steps and involves nucleophile attack at the C1′ position of the target nucleotide by an N-terminal proline (Pro2) residue. Glu 3, and a lysine that aligns with position 53 in EcoNei are also important for the activity [10].

Structures of EcoNei with lesion containing duplex DNA are available and have shown that DNA binds to a positively charged groove of the enzyme and also the damaged base is extruded through the major groove. A DNA kink of about 45° occurs at the lesion point upon enzyme binding to enable catalysis. The latter EcoNei -DNA complex is stabilized, among other things, by interactions involving Gin69, Leu70 and...
Tyr71. Nei occurs in actinobacteria and some γ-proteobacteria, and it has been suggested that it is of relatively recent origin [5,6]. The Mtb genome has two paralogs viz. MtbNei1 (Rv2464c) and MtbNei2 (Rv3297). Biochemical characterization of MtbNei1 protein has been reported by two groups. Sidorenko et. al. 2008 showed MtbNei1 (named as MtuNei2 in their study) to remove oxidized pyrimidines and possesses AP site cleavage activity [11]. Guo et. al. reported MtbNei1to be active on uracil containing DNA substrates [7]. Though, MtbNei2 has been shown to complement the spontaneous mutation frequencies in E. coli fpg mutV nei triple and E. coli nei nth double mutants, its biochemical characterization has not been reported yet [7]. Although, nei2(Rv3297) is not essential for in vitro survival of the pathogen, it is required for successful infection and growth of Mtb in primate hosts [12].

In the present study, we cloned and purified MtbNei2 (Rv3297). We biochemically characterized MtbNei2 and carried out mutational analysis to identify catalytically important residues. Interestingly, we identified that a C-terminal Zinc-finger domain that was reported to be important for activity in the E. coli Fpg and Nei proteins, is dispensable for glycosylase activity of MtbNei2. Finally, we identified 3 natural product inhibitors of the enzyme following a rational screening experiment. The best of these inhibitors binds to MtbNei2 with a Kd of 74 nM.

2. Materials and methods

2.1. Oligonucleotide substrates and proteins

A 51-mer oligonucleotide containing Uracil at position 26 from the 5’-end was purchased from Integrated DNA Technology, USA (IDT). The sequences of complementary oligonucleotides had Guanine opposite the lesion for generating duplex or contained non-complementary sequences for producing bubble and fork structures as shown in Table S2 [13]. A 51-mer oligonucleotide containing 5-OHU at position 26 from 5’-end and a 35-mer oligonucleotide containing 8-oxo-G at position 14 from 5’-end was purchased from Midland certificate, Texas. For optimal annealing, an equimolar mixture of lesion containing and complementary strands was heated to 94 °C for 2 min in nuclease free water and then slowly cooled to room temperature. All the oligonucleotides were 6-FAM labeled at 5’-end. For DNA binding studies, a 22 mer single stranded oligonucleotide substrate was synthesized from Integrated DNA Technology, USA. EcoFpg protein was purchased from New England Biolabs. EcoUNG protein was purchased from Fermentas (Fig. 1).

2.2. Structure modeling and alignment

A structural model of MtbNei2 was generated using Modeller 9.10 (http://salilab.org/modeller/download_installation.html) with the structure of the MutM (Fpg) from Geobacillus stearothermophilus protein (PDB: 1L1T, 29% identity) [9] as template. The final refined model was validated using RAMPAGE [14] and more than 94% of residues were found to be in the most favorable region of the Ramachandran plot (Fig. S4). MtbNei2 model was superimposed with known Nei and Fpg crystal structures using Rapido [15] in order to locate active site, lesion recognition loop and zinc finger motifs. Visualization of structures was carried out using Chimera [16].

2.3. Cloning, expression and purification of wild-type MtbNei2 and mutants

The genomic DNA of M. tuberculosis H37Rv was kindly provided by Dr. Kishore K. Srivastava (Central Drug Research Institute, India). The gene sequences were retrieved from GenBank for M. tuberculosis H37Rv: NP_217814 Rv3297. The MtbNei2 (Rv3297) and Zinc finger domain deletion gene sequences were PCR amplified using gene specific primers listed in Supplementary Table S1 and cloned between the BamHI and HindIII restriction sites in N-terminal GST tag containing vector pGEX-KG (GE Healthcare). The pGEX-KG-Nei2 (255 amino acids) and C-terminal zinc finger deleted mutant, pGEX-KG-Nei2ZFNF (1–205) constructs were overexpressed in E. coli BL21DE3 cells using 0.5 mM IPTG (Merck). The Nei2WT-GST and Nei2ΔZNF-GST overexpressing cells were re-suspended in lysis buffer A [50 mM Tris-HCl pH 7.5, 250 mM NaCl and 1 mM PMFS] and lysed by sonicator. The lysates were centrifuged at 15,000 rpm for 20 min at 4 °C and the supernatant was allowed to bind for 4 h at 4 °C with glutathione-agarose beads pre-equilibrated with buffer A. The beads were washed with 20 column volumes of buffer A. The Nei2WT-GST and Nei2ΔZNF-GST proteins eluted with buffer A containing 20 mM reduced glutathione and pH adjusted to 7.5. The eluted fractions were analyzed by 12% SDS PAGE. Purified proteins were loaded onto pre-equilibrated Superdex-200 10/300 GL column in buffer containing 50 mM Tris 7.5, 200 mM NaCl Fig. 2(A-B).

2.4. Site directed mutagenesis

Based on sequence analysis, two point mutations of residues lying in the catalytic site of MtbNei2 i.e P2A and E3A were generated using pGEX-KG-Nei2 construct as template and appropriate overlapping primers (Table S1). After initial denaturation step at 94 °C for 4 min, PCR was conducted for 25 cycles with denaturation at 94 °C for 60 s, primer annealing at 60.3 °C (for pGEX-KG-Nei2(P2A)) and 62.3 °C (for pGEX-KG-Nei2(E3A)) for 45 s and DNA synthesis at 72 °C for 60 s for each 1 kb of plasmid sequence, e. g. 6 min for a plasmid + gene sequence of 5.768 kb. The high fidelity Platinum Pfx DNA polymerase was used (Thermo Fisher Scientific). The products were digested overnight by DpnI (Fermentas) at 37 °C to remove the template DNA and transformed into E. coli DH5α. Plasmids were purified. Double digestion and
sequencing was done (Chromous Biotech., India) to verify the mutations. *E. coli* BL21 DE3 cells were used to express and purify the verified mutant constructs pGEX-KG-Nei2(P2A) and pGEX-KG-Nei2 (E3A) using above described purification method Fig. 2(A-B).

2.5. DNA Glycosylase activity

In a 20 µl reaction, 100 nM labeled substrate (G1(Uracil)/G2(double stranded)/G3(5’fork)/G4(3’fork), G5(bubble)/G6(5-OHU) or G7(8-oxo-G)) was incubated with increasing concentration of *Mtb*Nei2 enzyme (5 nM, 10 nM, 20 nM, 40 nM or 80 nM) in glycosylase buffer G (10 mM Tris-HCl pH8.0, 75 mM NaCl and 1 mM EDTA) supplemented with 0.1 mg/ml of BSA. The reactions were carried out at 37 °C for 30 min. All the reactions were ended with 20 µl of formamide stop loading buffer F (98% formamide, 5 mM EDTA, 0.1% xylene cyanol and 0.1% bromophenol blue). The cleaved products and uncleaved substrates were separated on a 12% denaturing urea polyacrylamide gel and quantified using ImageQuant LAS4000 and ImageQuant TL 8.1 software (GE Healthcare) [7], (Table S2).

2.6. AP site incision activity

AP site cleavage activity was performed as described above for glycosylase assay but with an AP site containing substrate (A1) generated by annealing a 75-mer oligonucleotide containing Tetrahydrofuran, an AP site analogue to its complementary...
2.7. DNA binding using tryptophan fluorescence quenching assay

MtbNei2 contains 7 tryptophan residues, Trp72 and Trp221 surround the active site pocket whose intrinsic fluorescence can be monitored for DNA binding events of MtbNei2. Variation in the intrinsic tryptophan fluorescence was checked in a solution containing 200 nM MtbNei2, 50 mM Tris-HCl pH7.5 and 50 mM NaCl. The concentration of single stranded DNA was varied (0–180 nM). Excitation wavelength used was 280 nm, while emission spectra were obtained by scanning from 300 to 400 nm using a Cary Eclipse Fluorescence Spectrophotometer (Agilent Tech. USA).

2.8. Inhibition studies

2.8.1. Virtual screening

National Cancer Institute’s Natural Product database was selected for screening against MtbNei2. The reference protein coordinates for screening were obtained from the model of MtbNei2 (template PDB ID: 1L1T) [9] after being shorn off the water molecules and addition of hydrogen and required charges. The N-terminal 1-MPEGDT-5 residues were selected as the docking/screening site. All the compounds were screened in a grid surrounding the docking site using Surflex-Dock of SYBYLX2.0 [22]. A maximum of 5 poses were evaluated for each compound. Top 10 hits were selected based on the highest docking score (Chem score + G score + D score + PMF score). The selected compounds were obtained from National Cancer Institute, USA and...
were further evaluated against in vitro Uracil DNA glycosylase activity of MtbNei2.

2.9. In vitro inhibition assay

In a reaction of 20 μl, 100 nM labeled single stranded DNA substrate containing Uracil/THF and 80 nM MtbNei2 was incubated with increasing concentration of inhibitors (50 μM, 100 μM, 200 μM, 400 μM and 800 μM) in buffer G. The reactions were carried out at 37 °C for 30 min. All the reactions were stopped with 20 μl of formamide stop buffer F. Cleaved and uncleaved substrates were resolved on 12% denaturing urea polyacrylamide gel and quantified using ImageQuant LAS 4000 and ImageQuant TL 8.1 software (GE Healthcare). Potency of compounds was measured by determining IC50 values. IC50 values were determined by plotting the % MtbNei2 activity versus inhibitor concentration using GraphPad Prism software and fitting to the equation:

\[ \frac{V_i}{V_0} = \frac{IC_{50}}{IC_{50} + [I]} \]

where, \( V_0 \) and \( V_i \) are the rates of MtbNei2 glycosylase/AP lyase activity in the absence and presence of the inhibitor respectively, and [I] represent the inhibitor concentration.

To gain further insights into the mode of inhibitor-protein interactions, inhibitors were docked against the MtbNei2 model using Autodock4.2 [17].

2.10. Surface plasmon resonance (SPR) binding assay

The anti-GST antibody (Santa Cruz Biotechnology) was immobilized up to 8000 response units (RU) onto a CM5 sensorchip (BIAcore3000 system, GE Healthcare) using standard amine coupling chemistry. Binding using capturing molecule methodology was employed where 7 nM MtbNei2 dialyzed in HBS buffer was injected as capturing molecule. Concentration series of NSC31867 (0, 25, 50, 100 and 200 nM) were injected in HBS (10 mM HEPES pH 7.4, 150 mM KCl).
**NaCl, 3 mM EDTA and 0.8% DMSO) at a flow rate of 30 µl/min. The binding response was measured for 150 s after the end of injection. The interactions between MtbNei2 and inhibitors were analyzed and steady-state binding was determined at each concentration. Following each injection cycle, chip surfaces were regenerated with an injection of 10 mM Glycine pH 2.0. Sensorgrams were processed by BIAevaluation software (GE Healthcare) to obtain kinetic parameters of protein–inhibitor interaction. The steady-state binding constants were determined by fitting the data to a 1:1 Langmuir isotherm as per the Biacore manual [18].

![Figure 7](image)

**Table 1**

Inhibition studies against *in vitro* Uracil DNA glycosylase activity of MtbNei2.

| S. No. | Compound Identifier | Inhibitor scaffold 2D | IC50 (µM) UDG activity | IC50 (µM) AP site cleavage activity | Dissociation constant Kd (nM) | H-bond forming residues |
|--------|---------------------|-----------------------|------------------------|-----------------------------------|-------------------------------|-------------------------|
| 01     | NSC31867 Norlobaric acid | 41.80                  | 64.84                  | 74nM                              | PRO2, ASN76, TYR166          |
| 03     | NSC345647 Chaetochromin | 92.71                  | 84.68                  | ND                                | SER61, ASN76, TYR166         |
| 04     | NSC250430 Gavetin      | 76.25                  | 43.65                  | ND                                | ASN76, ARG77, TYR166         |

*Fig. 7. A. Effect of compounds on Uracil DNA glycosylase activity of MtbNei2 (i) NSC31867, (ii) NSC345647 and (iii) NSC250430; 100 nM labeled substrate was incubated with 80 nM MtbNei2 and either no inhibitor or increasing concentrations (50 µM, 100 µM, 200 µM, 400 µM and 800 µM) of compounds; B. Inhibition curves obtained after plotting % DNA cleaved against the inhibitor concentrations; C. SPR analysis. NSC31867 (0, 25, 50, 100 and 200 nM concentrations) (solid lines; double referenced) were injected on the Nei2WT-GST captured on CMS chip immobilized with anti-GST antibody. The sensograms were fitted to the 1:1 reaction model to calculate Kd as per Biacore manual.*
3. Results

3.1. Purification of wild-type Nei2WT-GST, Nei2ΔZNF-GST, catalytic site mutants Nei2(P2A)-GST and Nei2(E3A)-GST

MtbNei2 wild type/mutants were cloned and overexpressed (Fig. S1). Proteins Nei2WT-GST (26 kDa GST + 29 kDa Nei2WT = 55 kDa), Nei2ΔZNF-GST (26 kDa GST + 23 kDa Nei2ΔZNF = 49 kDa), Nei2(P2A)-GST (26 kDa GST + 29 kDa Nei2WT = 55 kDa) and Nei2(E3A)-GST (26 kDa GST + 29 kDa Nei2WT = 55 kDa) were purified to homogeneity using GST affinity purification followed by SEC. SEC profile shows Nei2WT-GST wild type and mutant proteins to be octamer with elution volume of 10.9 ml (Fig. 2).

3.2. Biochemical characterization of MtbNei2

3.2.1. Undamaged DNA binding

Presence of tryptophan residues in various domains of MtbNei2 which are involved in catalysis led us to investigate the effect of DNA binding on the overall conformation of the protein by monitoring intrinsic tryptophan (Trp) fluorescence. A first increase (0–40 nM) followed by decrease in the intrinsic Trp fluorescence was observed with increasing DNA concentration from 0 to 180 nM. Change in Trp fluorescence with increase in DNA concentration suggests the protein undergoes conformational change upon binding to DNA and this probably modulates the activity (Fig. 3).

3.2.2. MtbNei2 specifically recognizes oxidized pyrimidines

We tested MtbNei2 activity on single-stranded oligodeoxynucleotide containing Uracil, 5-Hydroxy-Uracil (5-OHU) and 8-oxo-Guanine. MtbNei2 successfully recognized and cleaved the Uracil and 5-OHU containing substrates (Fig. 4A, B). At the same time, MtbNei2 failed to cleave 8-oxo-G containing substrate whereas control enzyme EcoFpg could cleave it (Fig. 4D). The results suggest MtbNei2 specifically recognizes oxidized pyrimidines. Furthermore, MtbNei2 found to cleave the AP site substrate as well via β and β-δ eliminations as depicted by the two bands formed after the cleavage (Fig. 4C). Like other Nei and MtbNei1 proteins, MtbNei2 is a bifunctional glycosylase. In control experiments, GST alone didn’t exhibit any of the above activities (Fig. S3).

3.2.3. Effect of DNA structures on MtbNei2 activity

To investigate MtbNei2’s activity on various types of DNA structures, we tested its activity on Uracil containing duplex DNA, 5′fork, 3′fork and bubble DNA substrates. MtbNei2 could recognize and remove base lesions on double stranded, 3′fork and bubble DNA substrates. This suggests MtbNei2 has active roles in pre-replicative and pre-transcriptive associated BER (Fig. 5).

3.3. Pro2 and Glu3 are essential active site residues of MtbNei2

Activity of C-terminal Zinc finger domain deleted mutant, Nei2ΔZNF-GST was tested on Uracil containing DNA substrate. Nei2ΔZNF-GST was found to be fairly active and it successfully cleaved...
the uracil containing substrate (Fig. 6C). This result suggests that the Zinc-finger domain is not important for the glycosylase activity of MtbNei2 in contrast to EcoNei and Fpg where it was found to severely compromise the activity of the respective enzymes.

3.5. Identification of inhibitors against MtbNei2

Ten best scoring compounds were selected after virtual screening of the NCI natural product database against MtbNei2. These were then assayed against Uracil DNA glycosylase and AP site cleavage activities of MtbNei2. NSC31867 (Norlobaric acid), NSC345647 (Chaetochromin) and NSC250430 (Geterin) showed good inhibition of both Uracil DNA glycosylase (Fig. 7A, B; Table 1) and AP site cleavage activity (Fig. 8A, B; Table 1). Binding affinity of the inhibitor NSC31867 for MtbNei2 was measured using SPR (Fig. 7C and Table 1). All three compounds were predicted to interact with the inter-domain catalytic-DNA binding cleft (Fig. 9A). NSC31867 forms H-bonds to the active site Pro2 and Ile62, Asn76 and Tyr166 (Fig. 9B i) and inhibits glycosylase activity. Moreover, the other two inhibitors NSC345647 and NSC250430 were predicted to form H-bonds with Ser61, Asn76, Arg77 and Tyr166. The latter residues are hypothesized to be involved in lesion recognition and void filling after base extrusion activities and inhibition of the glycosylase activity (Fig. 9B ii and iii).

4. Discussion

DNA repair mechanisms contribute to the survival and persistent infection of mycobacterium. *M. tuberculosis* genome is highly G/C rich which renders it to be more susceptible to oxidative DNA damage. This is the reason that *Mtb*’s DNA repair mechanism is highly evolved and it harbors at least three fpg/nei members and one Nth glycosylases as well as other BER factors to deal with this major problem. Occurrence of multiple DNA glycosylases each specific for a particular type of base lesion enables pathogen to rectify a large spectrum of base lesions. The DNA Base excision repair is a premier pathway in *M. tuberculosis*, given the absence of a known mismatch repair pathway that is present in *E. coli*. Targeting this pathway can be a self-limiting strategy from the point of view of development of resistance as suggested earlier [23]. MtbNei2 (Rv3297) is a previously uncharacterized member of the DNA glycosylase family. MtbNei2 was found to bind undamaged and damaged DNA substrates. MtbNei2 specifically recognizes oxidized pyrimidines similar to its paralog MtbNei1. MtbNei2 removes base lesions not only on duplex DNA but also from single stranded, 5′ fork, 3′ fork and bubble DNA substrates like its prokaryotic and eukaryotic homologues. On the other hand, MtbNei2 possesses Uracil DNA glycosylase activity unlike the *E. coli* homologue pointing to the significance of uracil repair in *Mtb*.

Another significant difference highlighted by the present study is that the Zn-finger motif in MtbNei2 is not essential for the glycosylase activity. Indeed the zinc-finger domain-deleted mutant MtbNei2ΔZF was fairly active on the uracil containing DNA substrate (Fig. 6C). In our modeling experiments, despite having four cysteines in the extreme C-terminus, MtbNei2 exhibited an unstructured C-terminal zinc finger domain (Fig. 6Ai, iii). For instance, the human Neil1 contains a C-terminal锌less finger domain which interacts with many repair and replication factors [19–21]. Based on the activity of MtbNei2ΔZF and modeling experiments, we hypothesize that the zinc-finger domain of MtbNei2 might similarly function as a protein-protein interaction domain.

Structural alignment of various members of fpg/nei superfamily indicated the conservation of active site residues (Pro2 and Glu3), helix two turn helix and zinc finger motif (4 cysteines). Complete loss of glycosylase activity observed in the active site mutants Nei2(P2A)-GST and Nei2(E3A)-GST of MtbNei2 strongly suggests that Pro2 and Glu3
are essential (Fig. 6).

The mutational analysis was helpful in selecting the target site sequence for the protein for the virtual screening experiments against the NCI database. We identified three natural products, Norobaric acid (NSC31867), Chaetochromin (NSC345647) and Gengetin (NSC250430) as inhibitors of both uracil DNA glycosylase activity as well as AP site cleavage activity of MtbNei2. Based on docking results, these inhibitors predictably interact with the active site of MtbNei2. The best of them, Norobaric acid, binds to MtbNei2 with an affinity of 74 nM and has a relatively small core scaffold (Table 1). Residues (Pro2, Arg77 and Tyr166) involved in inhibitor binding as predicted by docking are well conserved in MtbNei1. Therefore, we hypothesize that the inhibitors identified in this study may inhibit MtbNei1 as well. These compounds represent the very first inhibitors against Nei and can form the basis of an inhibitor optimization program against mycobacterial BER.

Despite low sequence identity, both MtbNei1 and MtbNei2 have similar activities, conserved catalytic residues and DNA binding motifs (Fig. S5). Besides, both MtbNei1 and MtbNei2 could complement the similar activities, conserved catalytic residues and DNA binding motifs of the SPLenDID grant [BSC0104]. This manuscript bears the CSIR-CDRI first inhibitors against Nei and can form the basis of an inhibitor optimization program against mycobacterial BER.

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Appendix A. Transparency document

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2017.07.010.

Appendix B. Supplementary material

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