Mycobacterium tuberculosis arylamine N-acetyltransferase acetylates and thus inactivates *para*-aminosalicylic acid

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Running title: NAT from *M. tuberculosis* can inactivate PAS

***Mycobacterium tuberculosis* Arylamine N-acetyltransferase (TBNAT) is able to acetylate *para*-aminosalicylic acid (PAS) both *in vitro* and *in vivo* as determined by HPLC and ESI/MS techniques. Anti-tuberculosis activity of the acetylated PAS is significantly reduced. As a result, over-expression of TBNAT in *M. tuberculosis* results in PAS resistance, as determined by MIC tests and drug exposure experiments. Taken together, our results suggest that TBNAT from *M. tuberculosis* is able to inactivate PAS by acetylating the compound.**

Tuberculosis (TB) remains a major threat to global public health. Increasing emergence of multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) calls for urgent development of new drugs to combat drug-resistant TB. Understanding molecular mechanisms of action and

resistance of current drugs will provide new perspectives and approaches for drug discovery. *Para*-aminosalicylic acid (PAS) used to be one of the first-line, and is now still used as second line anti-tuberculosis agents (1). The emergence of MDR and XDR *M. tuberculosis* prompted the reintroduction of PAS to protect companion anti-tuberculosis drugs from additional acquired resistance.

Arylamine N-acetyltransferases (NATs, EC. 2.3.1.5) are cytosolic enzymes that catalyze the transfer of the acetyl group from acetyl coenzyme A (AcCoA) to the free amino group of arylamines, hydrazines and N-hydroxyarylamines (2-5). NATs participate in detoxification and metabolic activation of xenobiotics and are found in a wide variety of prokaryotic and eukaryotic species (6). The NAT enzyme was first identified in humans in the 1960s due to its ability to inactivate the anti-tubercular drug isoniazid (INH) (7). The human genome contains two polymorphic NAT genes, NAT1 andNAT2 (8, 9), which play important pharmacogenetics roles in cancer susceptibility and have the potential to contribute to personalized medicine (10, 11). The corresponding enzymes possess distinct substrate profiles: NAT1 preferentially N-acetylates the arylamines *p*-aminobenzoic acid (pABA), *p*-aminobenzoyl-glutamate (pABGlu) and PAS, whereas NAT2 has higher activity towards the hydrazines isoniazid (INH), hydralazine (HDZ) and the arylamines sulfamethazine (SMZ) (12, 13). NATs from prokaryotes had also been studied, which display unique substrate specificity for various arylamine, hydrazide, and hydrazine substrates (14-21).

Previous studies showed that, NAT from *M. tuberculosis* (TBNAT) and *Mycobacterium smegmatis* (MSNAT) can inactivate INH by transfer an acetyl group

from AcCoA onto the terminal nitrogen of the drug (17, 22). Since PAS is a drug specific for tuberculosis, and it has been shown by a colorimetric assay that PAS is a substrate for TBNAT (23), we are wondering if NAT from *M. tuberculosis* could acetylate and thus inactivate PAS. To test this hypothesis, NAT from *M. tuberculosis* H37Rv (MYCTU NAT) was over-expressed in *Escherichia coli* BL21 (DE3) using pCA24N vector (24) and soluble recombinant protein was obtained through Ni-NTA column purification. Then enzymatic activity of purified recombinant TBNAT for PAS was determined by using high-pressure liquid chromatography-electrospray ionization mass spectrometry (HPLC-ESI/MS). The standard reaction consists of TBNAT, AcCoA and substrates in 20 mM Tris-HCl buffer (pH 7.5). After overnight reaction at 37°C, the TBNAT in reaction solutions was removed by passing through a 10 kDa Microcon (Millipore) centrifugal filter, and the filtered mixture was subsequently injected into a C18 liquid chromatography column (Thermo Fisher, Hypersil Gold aQ, 150×4.6 mm, 3 μm) in an UltiMate 3000 HPLC system (Thermo Fisher Scientific). Samples were eluted with a gradient from 95% buffer A (H_2O plus 0.1% acetic acid) and 5% buffer B (methanol plus 0.1% acetic acid) to 5% buffer A and 95% buffer B for 30 min, at a flow rate of 0.3 ml/min. Retention times of PAS (substrate) and acetylated PAS (4-acetamidosalicylic acid, AcPAS) (product) were determined to be 16.53 min and 21.15 min according to the pure standard (Fig.1A). We observed that PAS can be almost completely converted into AcPAS after overnight reaction, resulting a peak of m/z at 196.05 ($[M+H]^+$) representing the AcPAS detected by ESI/MS (Thermo Fisher Scientific, LCQ Fleet) in the positive

ionization mode (Fig.1B). This result showed that, TBNAT can transfer the acetyl group from AcCoA to the N4 position (the amino group) of PAS and yield AcPAS *in vitro*.

To verify if TBNAT could also acetylate PAS *in vivo*, the vector pMV261-NAT_{TB} was constructed and electroporated into *M. tuberculosis* H37Ra to over-express TBNAT, then levels of AcPAS in both the wild type strain and the TBNAT over-expressed strain were analyzed by HPLC after PAS treatment. 5 μM PAS was added into the culture media when *M. tuberculosis* cells were grown to mid-log phase (OD600 of 0.5–1.0) and incubated under 37°C for 12 hours. Then, cells were collected and disrupted by glass beads, and proteins in cell extracts were removed by using a 3 kDa Microcon centrifugal filter, and the level of AcPAS in the extract was analysis by HPLC as described above. As shown in Figure 2, an obvious accumulation of AcPAS could be observed (Fig.2A, indicated by square frame) in the TBNAT over-expressed strain after PAS treatment, which was not observed in the wild type strain (Fig.2B). Meanwhile, when the TBNAT over-expressed and wild type strain was not treated with PAS, AcPAS could not be detected (Fig.2C and D). These results showed that, TBNAT is also able to acetylate PAS *in vivo*.

To further investigate acetylation of PAS could affect the anti-tuberculosis activity of the compound, MICs of PAS and AcPAS for *M. tuberculosis* H37Ra were tested. The MIC of PAS was determined to be 0.02 μg/ml (0.1 μM), whereas that for AcPAS (From Santa Cruz Biotechnology, Catalog: sc-207963) was determined to be 10 μg/ml (50 μM). Thus, the anti-tuberculosis activity of acetylated PAS was

significantly reduced. Meanwhile, in order to investigate whether TBNAT could affect the anti-tuberculosis activity of PAS *in vivo*, PAS susceptibilities were determined for both the wild type strain and the TBNAT over-expressed strain. We found that, over-expression of TBNAT leads to a two times increase of PAS MIC. Besides, drug exposure experiments were also performed, and the results were shown in Figure3. For the TBNAT over-expressed strain, the killing effect of PAS peaked after 7 days of treatment, and then bacteria started to grow again. However, for the wild type strain, continuous killing by PAS could be observed even after 21 days of treatment. In addition, after 7days of PAS treatment, the survival rate of the TBNAT over-expressed strain is much higher than that of the wild type strain. These results demonstrated that, over-expression of TBNAT in *M. tuberculosis* greatly enhances the tolerance to PAS treatment.

We found that, though acetylation greatly reduces the anti-tuberculosis activity of PAS, AcPAS still has weak anti-tuberculosis activity. Previous studies showed that, PAS acts as a prodrug, it needs to be incorporated into the folate pathway through dihydropteroate synthase (DHPS) and dihydrofolate synthase (DHFS) and thus forms a hydroxyl dihydrofolate antimetabolite, which in turn inhibits dihydrofolate reductase (DHFR) (25, 26). We speculated that the AcPAS might also be incorporated into the folate biosynthesis pathway, though with much lower efficiency or could compete with pABA as they are structural analogues, thus affect folate biosynthesis (25). In conclusion, our results presented here demonstrate that NAT from *M. tuberculosis* can acetylate PAS and thus inactivate the anti-tuberculosis drug both *in*

vitro and *in vivo*.

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Figure Legends

Figure 1. HPLC-ESI/MS analysis of TBNAT activity for PAS. TBNAT reaction mixture was separated by HPLC. PAS (retention time 16.53 min) (A) could be acetylated by TBNAT, yielding acetylated PAS (retention time 21.15 min) (A). Acetylated products were then detected by ESI/MS (m/z =196.05) ($[M+H]^+$) which were indicated by arrows and chemical structures were shown alongside (B). MS data was acquired in the positive mode.

Figure 2. HPLC analysis of AcPAS accumulation in *M. tuberculosis*. The TBNAT over-expressed (A and B) and wild type (C and D) *M. tuberculosis* were grown to mid-log phase (OD600 of 0.5–1.0), and treated with (A and C) or without (B and D) 5 μM PAS for 12 hours, then cell extracts were prepared and filtered to remove protein before HPLC analysis.

Figure 3. Killing curve of PAS against *M. tuberculosis*. *M. tuberculosis* was grown to mid-log phase (OD600 of 0.5–1.0) and diluted to about 10^7 CFU/ml in fresh medium. PAS was then added and aliquots were taken at regular intervals. Serial dilutions were performed before plating. *M. tuberculosis* H37Ra (■) and *M. tuberculosis* H37Ra over-expressing TBNAT (▲) with no drug added; *M. tuberculosis* H37Ra (□) and *M. tuberculosis* H37Ra over-expressing TBNAT (△) with 0.5 μg/ml PAS added. The data represent the mean ± SD (standard deviations) of three independent experiments.