Summary of case study lecture at Biophysics and Structural Biology at Synchrotrons Workshop Triumph over Adversity: structure of arylamine N-acetyltransferase from *M. tuberculosis*

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Mycobacterium tuberculosis (TB) remains the worldwide leading cause of death by an infectious agent. It is contagious and very widespread: in 2017, there were 10 million new cases worldwide and 1.6 million deaths from TB, and 0.3 million of these were associated with HIV infection. TB can lie latent for years in the body until the host becomes immunocompromised, and then the disease takes hold. Treatment is complicated and lengthy, involving 4 different antibiotics taken concurrently for 6 months. The bacterium has become multidrug, extensively or totally drug resistant over the last 50 years, so new treatments for TB are urgently required. To aid the search for new alternatives to classical antibiotics, which ideally would have a low ability to induce resistance and represent a large potential range of molecular moieties, the 3-D structures of more proteins from TB are needed.

One possible drug target is arylamine N-acetyltransferase (TBNAT), a 31-kDa, 273 amino acid cytosolic enzyme. The *nat* gene in mycobacteria is in a gene cluster which is essential for cholesterol catabolism and intracellular survival of mycobacteria inside the macrophage. The NAT enzymes are known to catalyse the transfer of an acetyl group from acetyl-CoA (Ac-CoA) to an arylamine substrate. Studies suggested that the TBNAT is involved in biosynthesis of the mycolic acid component of the protective coating of the bacterium that it grows to avoid detection by the body's immune system when within a human cell. Deletion of the gene coding for this protein in *M. bovis* (bovine TB) has been found to deplete the amount of mycolic acid and to render the bacterium sensitive to the antibiotic gentamicin. Starting in 1995, extensive efforts to

characterise TBNAT by Professor Edith Sim and her group at the Oxford University Pharmacology Department had found the protein to be very hard to express at the purity and in the quantities required for crystallisation trials, and for 15 years, successive generations of graduate students had worked to solve the challenges involved. As a model for TBNAT, they used a homologous enzyme from *M. marinum* (MMNAT), a bacterium affecting fish. MMNAT has 74% sequence identity with TBNAT and had been thought to be a suitable model for drug discovery, but both high-throughput screening experiments for inhibitors and in silico modelling showed the two proteins to have different inhibitor profiles, TBNAT giving IC₅₀ values for putative inhibitors that were between 5 and 60 times larger than those for MMNAT. The team had already determined the structure (see Fig. 1a) of MMNAT which readily gave crystals (see Fig. 1b), but knowledge of the TBNAT structure was clearly required to understand the different inhibitor profiles. Thus, renewed efforts were made by a new and very dedicated, skilled and determined graduate student from Jordan, Areej Abuhammad (see Fig. 1c), to obtain enough pure TBNAT protein for crystallisation trials.

The first major challenge was to find a new vector and better recombinant expression system (*E. coli*, pVLT31, NEB express strain), and after 2 years of hard work, Areej managed to increase the expression efficiency from 2 mg/l to 8 mg/l. Additionally, by optimising the purification protocol (using a cobalt gradient IMAC column for His-tagged cleaved protein and a nickel column for His-tagged protein), she pushed this up to 16 mg/l and the purity from < 90% to > 95%, good enough for crystallisation trials to commence.

Screening for crystallisation conditions is still rather unscientific and is largely a process of setting up many screens at different temperatures and a range of protein concentrations with different precipitants and additives. In the case of TBNAT, over 7000 (100 nl protein+100 nl precipitant) conditions were tested, but with no crystals at all being obtained. In a final attempt to grow TBNAT crystals, we tried cross-

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Fig. 1 **a** The 5 key steps of structure solution, each one of which gave significant challenges on this project. **b** The crystal of MMNAT used for cross-seeding. **c** The only TBNAT crystal ever obtained after it had been irradiated in a Gaussian profile X-ray beam (note blackened centre due to radiation damage effects). **d** The structure and **e** the heroine of the story, Dr. Areej Abuhammad, whose determination and perseverance resulted in ultimate success

seeding using a suspension of MMNAT microcrystals for nucleation and a Douglas Instruments Oryx 8 crystallisation robot, which was being demonstrated for 1 day in our laboratory. Some crystals appeared in the following few weeks and were tested on an in-house X-ray generator. All of them turned out to be salt except for one of 23 µm (see Fig. 1c) in size. Diffraction data were collected from this crystal at the Diamond Light Source (DLS) to 2.1-Å resolution. Space group $C222_1$ ($a = 135.1$ Å, $b = 137.9$ Å, $c = 139.1$ Å) was detected by the automatic analysis software on the beamline, so the 120° wedge collected should have been more than enough for a complete dataset. The structure of TBNAT was then solved by molecular replacement (MR) using MMNAT ($P4_12_12$). The four protein chains in the asymmetric unit (ASU) were refined using BUSTER, and the electron density map looked good. However, the refinement statistics were poor: $R_{\text{cryst}} = 0.26$ and $R_{\text{free}} = 0.31$ with disappointing geometry (6.4% rotamer outliers) and many missing residues (~4%) and side chains. Something was wrong, and the structure was not good enough to explain the observed inhibitor profile!

On closer inspection of the reflection intensity distributions for the dataset, our X-ray Facilities Manager, Dr. Ed Lowe, thought something was very odd about them. He thus collected 180° of data at DLS the next week. Due to radiation

damage from the first collection, the crystal now only diffracted to 2.9 Å, but as it turned out, these data saved the project. Lesson learned is as follows: if you only have one crystal, never throw it out until the structure is solved!

At this point we called in a known code breaker, Dr. Edward Snell from Hauptmann Woodward Medical Research Institute in Buffalo, USA. He looked at our problem and said ‘you may have some translational pseudosymmetry with a peak at 0.25 0.25 0.0 at a height of 44.192 and also looks like you actually have two peaks associated with non-crystallographic symmetry at 41.0% of the origin at 0.244, 0.256, 0.00 and 23.2% of the origin at 0.0, 0.5, 0.0. The refinement (only the second cycle so I may be premature) suggests you may have a space group problem which would be caused by this NCS.’ Following this suggestion, we indexed and processed the 180° of 2.9-Å data in P1. For these data, MR found that (a) there were 16 chains in the P1 ASU, (b) the correct space group was $P2_1$ (not $C222_1$) with 8 molecules in the ASU, (c) the unit cell was $a = 96.50$ Å, $b = 139.20$ Å, $c = 96.51$ Å, and $\beta = 91.2^\circ$ cell (note the close values of a and c and the nearness of β to 90°), and (d) the refinement was further complicated by the presence of pseudo-merohedral twinning with a twinning fraction of 0.43.

With all these ingredients, the final $R_{\text{cryst}} = 0.16$ and $R_{\text{free}} = 0.19$ are much more respectable values. The electron density and geometry were both much improved, with only 1.9% rotamer outliers and very few missing residues and side chains.

The final structure (Fig. 1d) allowed us to see small but significant differences between the active sites of MMNAT and TBNAT and beautifully explained their different inhibitor profiles, so we published our results (Abuhammad et al. 2013). After the challenges of the project, we definitely felt it was Triumph over Adversity!

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Reference

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