REPORT

Report on participation of the ICMR International Fellow (ICMR-IF) in Training/Research abroad.

1. Name and designation of ICMR- IF	:	Dr. Insaf Ahmed Qureshi, Asst. Professor
2. Address	:	Department of Biotechnology & Bioinformatics, School of Life Sciences, University of Hyderabad, Prof. C.R. Rao Road, Hyderabad 500046, India
3. Frontline area of research in which training/research was carried out	:	Structural Biology
4. Name & address of Professor and host institute	:	Prof. John J. Tanner, Department of Biochemistry, University of Missouri, Columbia, MO 65211, USA E-mail: tannerjj@missouri.edu
5. Duration of fellowship	:	Three months (December 4, 2018 to March 3, 2019
6. Highlights of work conducted	:	

i) Technique/expertise acquired: High Throughput Screening of Inhibitors

ii) Research results, including any papers, prepared/submitted for publication:

Project 1: Molecular cloning, Purification and Crystallization of HsALDH9A1:

Aldehyde dehydrogenases (ALDHs) belong to a superfamily of nicotinamide adenine dinucleotide (phosphate) (NAD(P)+)-dependent enzymes that catalyze the oxidation of endogenous and exogenous aldehydes to their corresponding carboxylic acids. In addition to aldehyde metabolizing capacity, ALDHs have additional catalytic (e.g. esterase and reductase) and non-catalytic activities. To date, 24 ALDH gene families have been identified in the eukaryotic genome, whereas human genes are found in ALDH1-9, ALDH16 and ALDH18 families. ALDH9A1 is a cytosolic tetramer and highly expressed in the liver, skeletal muscle and kidney.

1) Cloning and expression of HsALDH9A1:

CDS of aldehyde dehydrogenase (ALDH) enzymes were amplified from cloning plasmid available in lab with primers (containing *BamH* I and *Xho* I restriction sites) by using a thermal cycler. Amplification was checked on 1% agarose gel and amplified product was purified by gel extraction kit (Qiagen). Amplified product and pET-SUMO expression vector were digested with enzymes and were further purified by PCR purification kit (Qiagen). Purified insert was ligated into expression vectors by using T4 DNA Ligase and transformed into *E. coli* DH5 alpha competent cells. Positives clones were identified by colony PCR (Fig. 1) followed by DNA sequencing using T7 forward and reverse primers.

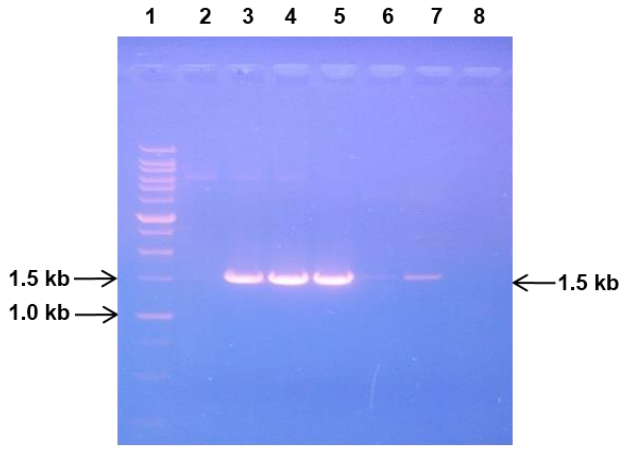

Figure 1: Confirmation of ALDH9A1 into pET-SUMO vector by PCR
Lane 1: DNA Ladder; Lane 2-7: Amplified ALDH9A1 from different clones; Lane 8: Negative control

Plasmid carrying desired insert was further transformed into *E. coli* BL21 (DE3) to over-express the protein. A single colony of *E. coli* BL21 carrying constructs of ALDH clone was inoculated into 5ml of LB medium supplemented with kanamycin. Overnight culture was diluted to 100ml of LB medium and expression was induced by addition of 0.25-1mM of isopropyl thio-β-d-galactoside (IPTG) at OD~0.6. Cells were grown for an additional 4 h at 37°C and then centrifuged. Pellet was checked for the expression of the target proteins on 12% SDS-PAGE gel. ALDH-9A1 was highly expressed with all concentrations of IPTG and its resolving pattern on SDS-PAGE gel indicates that its molecular weight corresponds to 65kDa along with SUMO tag at its N-terminus.

2) Purification and crystallization of HsALDH-9A1:

To purify SUMO tagged ALDH9A1, fusion protein was over-expressed into *E. coli* BL21 (DE3) with 0.25mM IPTG at 18°C for 20 hrs. Cells were collected by centrifugation and resuspended in buffer A (50 mM Tris pH 8.0, 300 mM NaCl, 10 mM imidazole and 5% glycerol) containing 0.1% Triton X-

100. Cells were lysed by sonication and then centrifuged at 20000 rpm for 30 min at 4ºC to remove the remaining insoluble material. The supernatant was subjected to immobilized metal-affinity chromatography (IMAC) on a HisTrap HP column (GE Healthcare) pre-equilibrated with buffer A. Unbound proteins were washed sequentially with ten column volumes of buffer A with imidazole concentrations of 40, 100 and 150 mM. The His-tagged proteins were eluted with buffer A containing 200 mM imidazole. To excise the N-terminal SUMO-tag, SUMO protease (purified separately by affinity chromatography) was added to the pure protein in 1:100 ratio and subjected to dialysis for 12–16h at 4°C in 25mM Tris-HCl pH 8.0, 100mM NaCl, 5% glycerol and 0.5% TCEP. The digested protein was again passed through Ni-NTA column to cause the entrapment of SUMO tag and SUMO protease as both carry an N-terminal 6×His-tag. While as, the pure protein was collected in the flow through and then protein quality analyzed with SDS-PAGE (Fig. 2). Consequently, ALDH9A1 was concentrated with a 50kDa Millipore concentrator and applied for Gel filtration chromatography on HiLoad 16/60 Superdex 200 column with a buffer containing 50mM Tris-HCl pH 8.0, 600mM NaCl, 5% glycerol and 0.5% TCEP. The purity of the recombinant ALDH9A1 was verified by SDS-PAGE and the protein concentration was determined on Nanodrop using molar absorption coefficient and molecular weight.

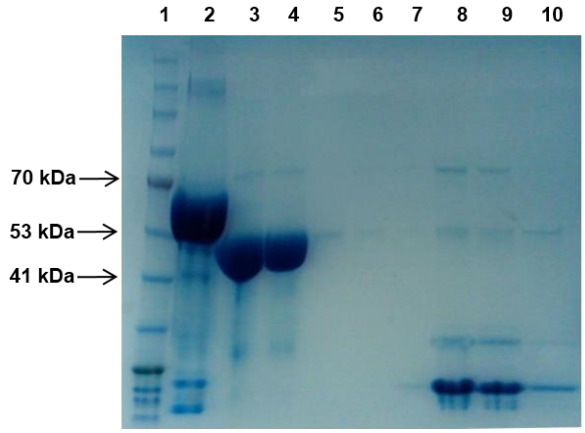

Figure 2: Purification of ALDH9A1 using affinity chromatography
Lane 1: Protein marker; Lane 2: Purified SUMO tagged ALDH9A1; Lane 3-4: SUMO protease cleaved ALDH 9A1; Lane 5-10: Different fractions from Ni-Sepharose column

Crystallization screening was initially carried out by the sitting-drop vapor-diffusion method at 296 K using an Oryx8 protein crystallization robot (Douglas Instruments) in MRC 96-well plates by mixing ALDH9A1 with precipitant solution. Commercially available crystallization kits from Hampton Research (Index, Crystal Screen and Crystal Screen 2) were used to screen for suitable conditions for ALDH9A1 in apo and complexed form with nicotinamide adenine dinucleotide (NAD). Crystals were

appeared after 1 d in a condition consisting of 200mM NaCl, 25% PEG 3350, and 0.1 M Bis-Tris pH 6.5 (Fig. 3). To obtain crystals suitable for diffraction experiments, further optimization is on the way by varying the concentration of protein, pH of the buffer and the ionic strength.

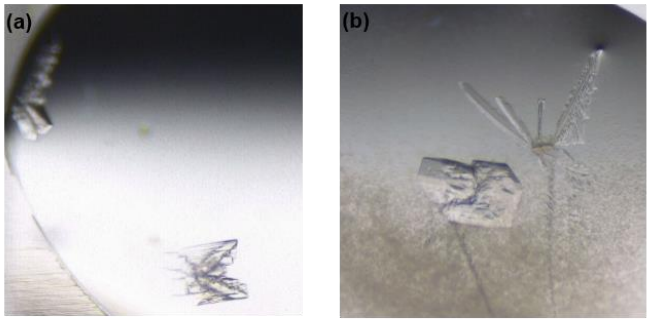

Figure 3: Crystallization of ALDH9A1 with and without NAD.
ALDH9A1 crystals in reservoir buffer containing 200mM NaCl, 25% PEG 3350, and 0.1 M Bis-Tris at pH 6.5 with NAD (a) and without NAD (b).

Project 2: High Throughput Screening of inhibitors against PYCR1:

Pyrroline-5-carboxylate reductase (PYCR) is the final enzyme in proline biosynthesis and catalyzes the NAD(P)H-dependent reduction of 1-pyrroline-5-carboxylate (P5C) to proline. Recent studies revealed overexpression of PYCR1 in multiple cancers, and its knock-out suppresses tumorigenic growth, suggesting that PYCR1 is a potential cancer target. However, inhibitor development has been stymied by limited mechanistic details for the enzyme.

1) Purification and crystallization of HsPYCR1:

E. coli cells carrying human PYCR1:pET-24b construct was grown in Luria broth medium overnight at 18°C and expression was induced by addition of 0.5mM of IPTG. Induced cells were resuspended in buffer A (50mM HEPES, pH 7.8, 300 mM NaCl, 10 mM imidazole and 5% glycerol) along with mixture of DNase I, lysozyme and PMSF. After lysing cells via sonication, cell debris was removed by centrifugation and then supernatant was subjected to immobilized metal-affinity chromatography (IMAC) on a Ni-NTA agarose. Unbound proteins were washed with buffer A supplemented with 30mM imidazole and eluted with 300mM imidazole. Fractions containing desired protein were pooled and and dialyzed at 4°C overnight into 50 mM HEPES pH 7.5, 300 mM NaCl and 5% glycerol. After dialysis, the protein was concentrated to 5ml and then applied for gel filtration chromatography on a HiLoad 16/600 Superdex 200 column using a column buffer with the same composition as the dialysis buffer.

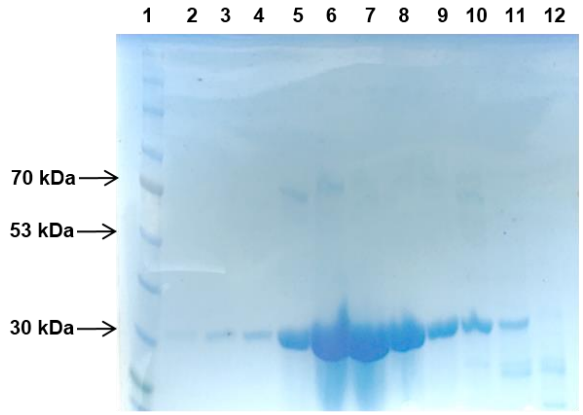

Figure 4: Purification of HsPYCR1 using affinity and gel filtration chromatography
Lane 1: Protein marker; Lane 2-11: Different fractions containing hsPYCR1 from Superdex 200

Purified fractions were pooled (Fig. 4) and concentrated to 8 mg ml^{-1} and subjected for crystallization with 500µl reservoir volumes and drops containing 1.5µl of protein and 1.5µl of the reservoir solution. Several small size crystals were grown using reservoir solution containing 250mM LiSO4, 19% (w/v) polyethylene glycol (PEG) 3350, and 0.1 M HEPES at pH 7.5, while a single crystal was grown from reservoir solution containing 3 M NaCl and 0.1 M HEPES at pH 7.5 (Fig. 5).

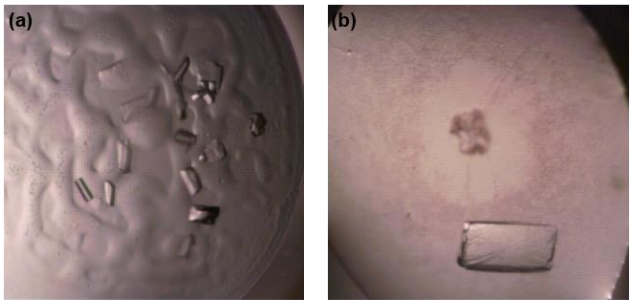

Figure 5: Crystallization of HsPYCR1.
Recombinant HsPYCR1 crystals with reservoir buffer containing (a) 250mM $LiSO_4$, 19% PEG 3350, and 0.1 M HEPES at pH 7.5, (b) 3M NaCl, and 0.1 M HEPES at pH 7.5.

2) Inhibition studies of HsPYCR1 with compounds:

PYCR1 enzyme activity assay was performed by measuring the P5C-dependent oxidation of NADH at 340 nm as described by Christensen and co-authors. Inhibitory compounds used in this experiment were obtained from Atomwise, Inc., USA at 10mM concentration in 100% DMSO. Before proceeding for inhibition assay, effect of DMSO was observed on PYCR1 activity varying conc. from 0-10%. DMSO tolerance assay suggested that there was not any significant effect observed on PYCR1 activity, but further increase in DMSO concentration affects it (Fig. 6).

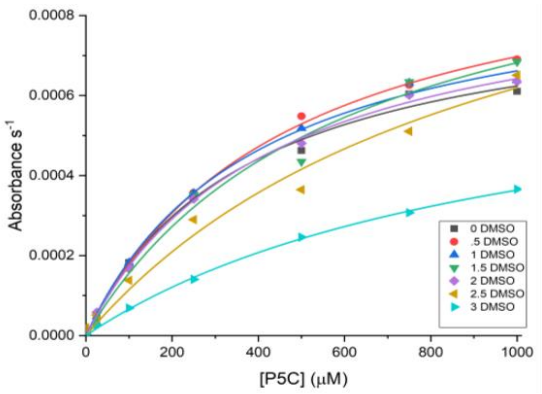

Figure 6: Effect of different concentrations of DMSO on HsPYCR1 activity

Therefore, 2% DMSO which contains 200 µM inhibitory compounds was used to analyze the inhibition of PYCR1 activity. Screening of compounds was performed using N-Formyl-*L*-proline, a known inhibitor at 1mM concentration, as a control. Out of 84 compounds, it was observed that B4 showed significant inhibition at 200 µM in activity of PYCR1 (Fig. 7).

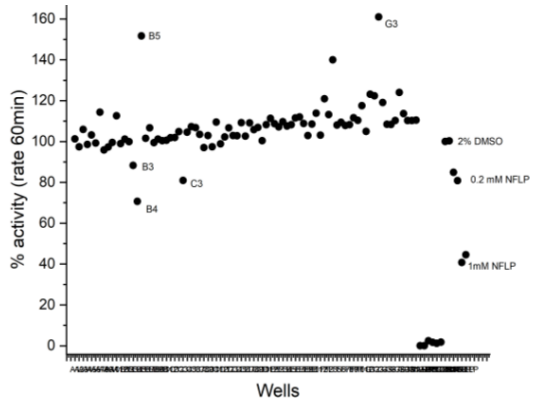

Figure 7: Screening of compounds against HsPYCR1.
Eighty four compounds (A1-G12) from Atomwise were screened against activity of PYCR1, while DMSO (2%) and NFLP (0.2 and 1 mM) were used as control.

Furthermore, different concentrations (0 to 200 µM) of B4 were used to calculate IC50 and it was found to be 80 µM (Fig. 8). Further validation of compound B4, which in progress, will help us to identify first inhibitor against PYCR1 that is a potential cancer target.

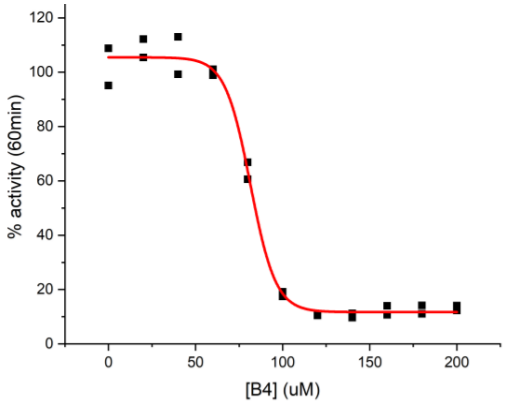

Figure 8: Dose-dependent response of B4 against HsPYCR1 activity.

iii) Proposed utilization of the experience in India:

Presently, my laboratory is focusing upon the crystallographic studies of various proteins from human pathogens and determined the structural and functional attributes of several significant proteins from human parasites including *Leishmania* and *M. tuberculosis*. During my visit, I have learnt a new technique i.e. High Throughput Screening of inhibitors that will help me to screen available compounds library against validated targets of human parasites. Additionally, my visit to Prof. Tanner's laboratory has strengthen our collaboration which would be helpful to explore novel targets of human diseases like tuberculosis, malaria etc. and designing of inhibitors to improve the health of Indian population.

Signature of ICMR-IF

ICMR Sanction No.: INDO/FRC/452/Y-69/2018-19-IHD