REPORT

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

1. Name and designation of ICMR- IF : **Dr. M. Janaki Ramaiah**

2. Address : **Professor, Biotechnology, KLEF**
KL deemed University.
Vaddeswaram
Guntur, ANDHRA PRADESH,
INDIA

3. Frontline area of research in which training/research was carried out : **Cancer Biology and epigenetics.**

4. Name & address of Professor and host institute : Dr. Muralidhar L. Hegde
Professor of Neurosurgery
Everett E. and Randee K. Bernal Centennial Endowed Director of DNA Repair
HOUSTON METHODIST RESEARCH INSTITUTE (HMRI),
HOUSTON, TEXAS, USA

5. Duration of fellowship with exact date : 3 months. Starts on 24th March2023.
and ENDS on 24th June 2023

6. Highlights of work conducted

Aim 1: Molecular Understanding of Chemo-resistance in GBM
Aim 2: Identification of gene expression, and proteomic changes in the chemo-sensitization in GBM cancer cell

Introduction:

Tubastatin, a known HDAC-6 inhibitor, was found successful in neuroprotection. Tubastatin A overcomes radio-resistance by inhibiting GPX4 and induces ferroptosis **(Shan Liu et al., 2023)** Tubastatin A directly interacts with GPX4. In GBM (grade IV) astrocytoma chemo-resistant, and radioresistant **(Alejandro Urdiciain et al., 2019)**. Tubastatin A inhibits HDAC-6 and causes apoptosis in GBM cancer. Also, tubastatin A causes deacetylation of alpha-tubulin and inhibits clonogenicity.

Novelty:

1) We identified the differential expression of certain genes involved in DNA damage and repair was involved in the sensitization of GBM.
2) Various protein biomarkers were identified through RPPA assay (conducted in MD Anderson cancer centre, Houston, USA.)

Results

In this study we found the cytotoxic behaviour of tubastatin A in sensitive as well as resistant GBM cancer cells. Treatment of cells with tubastatin A has induced substantial change in the gene expression pattern of DNA damage and DNA repair proteins such as ATM, pATM, γ H2AX , DNA ligase IV, and several other factors involved in cell-cycle such as E2F1, Aurora A, BRCA1, BIRC5 in both chemo-sensitive as well as chemo-resistant cell lines. This clearly indicates DNA damage nature of the drug. Interestingly, tubastatin A, enhanced the cell death preferentially in resistant cells at lower concentrations. The response of GBM cancer cells towards chemo-sensitive and resistant cells was different. Also, RPPA analysis to understand several proteomic biomarkers was found to be confined to DNA damage and Repair proteins. Thus the study has identified several factors that can be used as a biomarker for drug-sensitization in resistant cancer cells as well as role of tubastatn A on DNA damage and repair. The effect of tubastatin A on several microRNAs was under investigation at KL University.

Achieved Targets

1. Biomarkers for effective DNA damage in resistant GBM cancer cells
2. Proteomic changes in chemo resistant GBM cancer cells as Biomarkers.

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i) Technique/expertise acquired : Protein translational modification study, gene expression study by RT2 profiling, Proteomics profiling, Western blotting

RT2 Profiing: The U87MG (TMZ sensitivity 60.4%) and T98 cell lines (TMZ sensitivity 26.4%) will be grown at confluence of $1x10^6$ **(Miichae T.C. Pooni et al., 2021)**. The RNA was isolated using trizol method. The PAHS-020ZA (Cell-cycle), PAHS-042ZA (Human DNA Repair) plates were used in RT2 profiling and the SYBR green dye 1ml of 2X dye. Gene expression profiling changes will be monitored. The tubastatin stock use was 50 mg and was dissolved in 100 µl DMSO (i.e .1.28M). During cDNA preparation Turbo DNase buffer was used to activate TurboDNase in an RNA sample and was incubated at 37^0C for 20 minutes.

A) **MTT band cell viability**: MTT Assay was conducted in U87 (GBM sensitive cancer cells) as well as T98 (GBM resistant cancer cells). Here cells were seeded at a density of 5 x 10^5/well and the treatment was conducted and effective dose of tubastatin A was monitored.

B) **FACS**: Make 1x binding buffer. Harvest cells. Resuspend the 1x 50 µl binding buffer from 5x dye. Add 5µl of Annexin-FITC to 100 µl cell suspension. Incubate at 10-15 min at RT. Cover with aluminium foil. Add 500µl 1x binding buffer and centrifuge at 400-600g for 5 min at Room temperature. Discard the supernatant. Resuspend the 200 µl of 1x binding

buffer. Add 5 µl of propidium iodide (PI) solution and incubate for 5-15 min at RT. Excitation is 499/521 and emission of 635/617nm. Analyze the samples for cell-cycle arrest (BD Bioscience)

C) **RNA Isolation**: Take cell pellet. Add 1ml Trizol and mix up/down for 15'. Incubate 55°C-60°C. Vortex for 3minutes. Add 0.2ml chloroform: Isoamyl alcohol (24:1). Vortex for 2-3 min at RT. Centrifuge at 13,000 rpm. Take only supernatant. Add glycogen 2µl and incubate at RT and add 0.5 mL 2-propanol. Incubate for 10 min @ 4°C. Centrifuge for 13k for 60 minutes. Wash pellet with 75% alcohol. Centrifuge 7500g for 5 minutes. Dry the Pellet. Add RNAse-free 30µl water.

D) **Senescence**: U87 and T98 cells were cultured. Cells were pelleted at 10000rpm for 2 mins. Add 1x PBS. Add 1ml of 4% formaldehyde for 10-15' RT. Rinse twice in 1xx PBS overnight at 4°C. Add 1 ml of β-gal solution and keep incubated 37°C overnight.

E) **Immunofluorescence**: Add 100 µl of 10x paraformaldehyde into 900 µl PBS and make as 1x solution. Incubate the cells 37°C for 15 min. Wash with PBS for 5 min. Add 0.5% Triton-X-100 and incubate at RT for 15min. Wash 3 times. Each wash for 5 min. Add 200 µl of 5% BSA and incubate for1 h. Add primary antibody with 1% BSA (1:500) for 3 hr Wash 3 times with PBS. Add 200µl of secondary antibody with 1% BSA for 1hr. 3 times PBS wash. Finally add Mounting media 1h and keep at 20°c.

F) **Colonogenic Assay**: 5000 cells/60mm dish. Different dilutions of the drug will be used. Once colonies are formed for 1-3 weeks. Fix in 6% v/v of glutaraldehyde for 10 minutes Add crystal violet 0.5% w/v. **(Nicolas AP Franker et al., 2006; Vijayalakshmi Rajendran & Mayur Vilas Jain 2018)**.

G) **RT2- Profiling**: cDNA up to 50µl. SYBR green 1ml 2x master mix. Make the total volume up to 950 µl. The gene expression profile was conducted using (PAHS-020ZA: Human cycle- Qiagen) and (PAHS-042ZA: Human cycle- Qiagen)

H) **Western Blotting**: The cell lysate was obtained using 10x RIPA buffer (cat 20-188, millipore). Then 12% SDS PAGE was run and allowed to run for 1.5 hr. The gel was transferred using transfer buffer (50 ml of trans buffer 20x; 200 ml of methanol (certified ACS A-412-4), 750 ml Millipore water (M1DSC1 800-227-9997). The sample loading dye (Novex). Antibodies used pATM, ligase III, γH_2AX, $p^{53}BP$, (S1778), $p^{53}BP_1$, MSH_1, MSH_3, Aurora A, Aurora B, GAPDH, $E2F_1$, GADD45, $BRCA_1$, $BRCA_2$

I) **Proteomic Analysis**: The treated and untreated cells from sensitive and resistant cells were subject to RPPA-based proteomic assay to understand the protein modifications **(Haibo Wang et al., 2020).**

References

- Liu, S., Zhang, H. L., Li, J., Ye, Z. P., Du, T., Li, L. C., ... & Zhu, X. F. (2023). Tubastatin A potently inhibits GPX4 activity to potentiate cancer radiotherapy through boosting ferroptosis. *Redox Biology*, *62*, 102677.
- Rajendran, V., & Jain, M. V. (2018). In vitro tumorigenic assay: colony forming assay for cancer stem cells. *Cancer Stem Cells: Methods and Protocols*, 89-95.
- Urdiciain, A., Erausquin, E., Meléndez, B., Rey, J. A., Idoate, M. A., & Castresana, J. S. (2019). Tubastatin A, an inhibitor of HDAC6, enhances temozolomide-induced

apoptosis and reverses the malignant phenotype of glioblastoma cells. *International journal of oncology*, *54*(5), 1797-1808.

- Wang, H., Rangaswamy, S., Kodavati, M. *et al.* RT^2 PCR array screening reveals distinct perturbations in DNA damage response signaling in FUS-associated motor neuron disease. *Mol Brain* **12**, 103 (2019). https://doi.org/10.1186/s13041-019-0526-4

ii)	Research results, including any papers,	: Data Acquired and submitting to Scientific Reports or cell Death Differentiation
	prepared/submitted for publication	
iii)	Proposed utilization of the experience in India	: Initiating the Brain cancer center at KLUniversity

M. Faneki Ramaiah

Signature of ICMR-IF

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