REGULATION OF MITOCHONDRIAL BIOENERGETICS AND APOPTOSIS INDUCTION IN T CELL LYMPHOMAS

Sanction order no.: UGC-MRP-MAJOR-BIOC-2013-22875.

F. No. 43-44/2014 (SR)

Final report submitted to

The Joint Secretary

University Grants Commission

Submitted by

Dr. Piyali Mukherjee

Assistant Professor and Principal Investigator

Dept. of Life Sciences

Presidency University, Kolkata

UNIVERSITY GRANTS COMMISSION
BAHADUR SHAH ZAFAR MARG
NEW DELHI – 110 002.

Final Report of the work done on the Major Research Project.

1. Project report No. ~~1st~~/~~2nd~~/~~3rd~~/Final
2. UGC Reference No.F. **UGC-MRP-MAJOR-BIOC-2013-22875**
3. Period of report: from: **01.07.2015 to 20.06.2018**
4. Title of research project: "**Regulation of mitochondrial bioenergetics and apoptosis induction in T cell lymphomas"**
5. (a) Name of the Principal Investigator: **Dr. Piyali Mukherjee**
   (b) Deptt.: **Life Sciences**
   (c) University/College where work has progressed: **Presidency University**
6. Effective date of starting of the project: **01.07.2015**
7. Grant approved and expenditure incurred during the period of the report:
   a. Total amount approved Rs. 12,21,000 (Actual received Rs. **1101000**)
   b. Total expenditure Rs.**1049325**
   c. Report of the work done: (Please attach a separate sheet): **Please see attachment 1**
   i. Brief objective of the project
   ii. Work done so far and results achieved and publications, if any, resulting from the work (Give details of the papers and names of the journals in which it has been published or accepted for publication
   iii. Has the progress been according to original plan of work and towards achieving the objective. if not, state reasons:
   iv. Please indicate the difficulties, if any, experienced in implementing the project:

v. If project has not been completed, please indicate the approximate time by which it is likely to be completed. A summary of the work done for the period (Annual basis) may please be sent to the Commission on a separate sheet.

Completed

vi. If the project has been completed, please enclose a summary of the findings of the study. One bound copy of the final report of work done may also be sent to University Grants Commission.

Please see attachment 3

vii. Any other information which would help in evaluation of work done on the project. At the completion of the project, the first report should indicate the output, such as
(a) Manpower trained: No Manpower was requested for this project. However, Master's dissertation student Mr. Avraneel Paul, was trained as part of this project.
(b) Ph. D. awarded: Dr. Malinki Sur who worked on part of this project was awarded PhD degree in 2020.
(c) Publication of results: None so far
(d) other impact, if any

Piyali Mukherjee

SIGNATURE OF THE PRINCIPAL INVESTIGATOR

Dr. Piyali Mukherjee
Asst. Professor, Dept. of Life Sciences
Presidency University, Kolkata

REGISTRAR/PRINCIPAL

23/12/2022

(Seal)

Registrar
Presidency University
Kolkata

Annexure – IX

UNIVERSITY GRANTS COMMISSION
BAHADUR SHAH ZAFAR MARG NEW DELHI – 110 002

PROFORMA FOR SUBMISSION OF INFORMATION AT THE TIME OF SENDING THEFINAL REPORT OF THE WORK DONE ON THE PROJECT

1. Title of the Project: "Regulation of mitochondrial bioenergetics and apoptosis induction in T cell lymphomas"

2. NAME AND ADDRESS OF THE PRINCIPAL INVESTIGATOR:
   Dr. Piyali Mukherjee
   Dept. of Life Sciences
   Presidency University
   86/1 College Street
   Kolkata-700073

3. NAME AND ADDRESS OF THE INSTITUTION:
   Presidency University
   86/1 College Street
   Kolkata-700073

4. UGC APPROVAL LETTER NO. AND DATE:
   F. No. 43-44/2014 (SR) dated: 06.07.2015

5. DATE OF IMPLEMENTATION: 01.07.2015

6. TENURE OF THE PROJECT: 3 years

7. TOTAL GRANT ALLOCATED: Rs. 12,21,000.00

8. TOTAL GRANT RECEIVED: Rs. 11,01,000.00

9. FINAL EXPENDITURE: Rs. 10,49,325.00

10. TITLE OF THE PROJECT:

    "Regulation of mitochondrial bioenergetics and apoptosis induction in T cell lymphomas"

11. OBJECTIVES OF THE PROJECT:
    Aim1: *To examine the role of SARM in cellular apoptosis in nasal NK/T cell lymphoma (NNKTL)*
    Aim2: *To understand the regulation of mitochondrial bioenergetics by SARM leading to cellular apoptosis in NNKTL*
    Aim3*: To identify SARM interacting partners at the mitochondria in NNKTL*

12. WHETHER OBJECTIVES WERE ACHIEVED: Achieved and attached separately

    (GIVE DETAILS)

13. ACHIEVEMENTS FROM THE PROJECT: Attached separately

14. SUMMARY OF THE FINDINGS : Attached separately

(IN 500 WORDS)

15. CONTRIBUTION TO THE SOCIETY .....

(GIVE DETAILS)

16. WHETHER ANY PH.D. ENROLLED/PRODUCED OUT OF THE PROJECT: Nil

17. NO. OF PUBLICATIONS OUT OF THE PROJECT: Nil

(PLEASE ATTACH)

(PRINCIPAL INVESTIGATOR)

Dr. Piyali Mukherjee
Asst. Professor, Dept. of Life Sciences
Presidency University, Kolkata

(REGISTRAR/PRINCIPAL)

Registrar
Presidency University
Kolkata

(Seal)

Annexure – XI

Final Report Assessment / Evaluation Certificate

(Two Members Expert Committee Not Belonging to the Institute of Principal Investigator)

Title of the project: "Regulation of mitochondrial bioenergetics and apoptosis induction in T cell lymphomas"
Project number: UGC-MRP-MAJOR-BIOC-2013-22875
Name of the Principal Investigator: Dr. Piyali Mukherjee

It is certified that the final report of Major Research Project entitled "Regulation of mitochondrial bioenergetics and apoptosis induction in T cell lymphomas" by Dr. Piyali Mukherjee_ Dept. of Life Sciences Presidency University has been assessed by the committee consisting the following members for final submission of the report to the UGC, New Delhi under the scheme of Major Research Project.

Comments/Suggestions of the Expert Committee:-

The committee members felt that the proposed objective was satisfactorily met by the PI of the project.

Name & Signatures of Experts with Date:-

Name of Expert	University/College name	Signature with Date
1. Prof. Anita Mukherjee	University of Calcutta	Anita Mukherjee 15.01.2019
2. Prof. Sampa Das	Bose Institute, Kolkata	Sampa Das. 15.01.2019

It is certified that the final report has been uploaded on UGC-MRP portal on .....
It is also certified that final report, Executive summary of the report, Research documents, monograph academic papers provided under Major Research Project have been posted on the website of the University/College.

(Registrar/Principal)
Seal

Registrar
Presidency University
Kolkata

Attachment-1

Report of the work done

i. Brief objective of the project

Tumor cells have evolved strategies to down regulate proteins primarily involved in the induction of apoptosis. Since mitochondria play a key role in the regulation of apoptosis identification of novel mitochondrial proteins that programs the tumor cells to endorse the apoptotic pathway is urgently required to develop new anti-cancer therapies and better prognosis of more aggressive forms of cancer like T cell lymphomas. SARM holds such a promise and a detailed mechanistic insight of SARM regulation at the mitochondria is the major goal of this study. How this single-molecule SARM regulates the mitochondrial proteome that leads to excess ROS generation? How SARM regulates mitochondrial bioenergetics that skews the cells to the apoptotic pathway and how this regulation is deranged in cancer. *The goal of this proposal is to robustly investigate this novel regulation of mitochondrial energy metabolism by SARM and how this pathway is altered in cancer with emphasis on T cell lymphomas.*

The primary objectives of this project were:

Aim1: *To examine the role of SARM in cellular apoptosis in nasal NK/T cell lymphoma (NNKTL)*
Aim2: *To understand the regulation of mitochondrial bioenergetics by SARM leading to cellular apoptosis in NNKTL*
Aim3*: To identify SARM interacting partners at the mitochondria in NNKTL*

ii. Work done so far and results achieved and publications, if any, resulting from the work (Give details of the papers and names of the journals in which it has been published or accepted for publication:

Introduction

Several diseases including cancers have mitochondrial origin which is considered as the major site of cellular reactive oxygen species (ROS) generation. A variety of cancers have elevated ROS levels which may lead to oxidative stress that subsequently leads to cell death. Inhibition of this pathway and its upstream regulators offers added advantage to tumor cell survival. Thus it is important to identify the trigger behind this oxidative stress and how it contributes to the development, progression and maintenance of cancers. Identifying a master regulator that orchestrates excess ROS generation through modulation of the mitochondrial bioenergetic pathway may lead to the identification of novel targets in tumor cells and delineate them from non-malignant cells.

One protein of interest is the sterile alpha and heat armadillo-motif containing protein (SARM), a purported TLR adaptor, which has recently been shown to induce the intrinsic apoptotic pathways in activated T cells. Quite interestingly, the level of

SARM was shown to be decreased in NK/T cell lymphoma tissues as compared to healthy cells and it is quite possible that down regulation of the SARM apoptotic machinery is targeted in this cancer. SARM is known to induce mitochondrial ROS in neurons, a contributing factor in neuronal apoptosis. *Whether SARM regulates ROS generation and subsequent apoptosis in T cells and how this pathway is deranged in T cell lymphomas is the key question of this proposal.*

Methodology

1. **Reagents:-** Tissue culture reagents like DMEM were purchased from Gibco/Life Technologies and RPMI 1640 from HiMedia Laboratories. FBS was purchased from Gibco/Life Technologies.

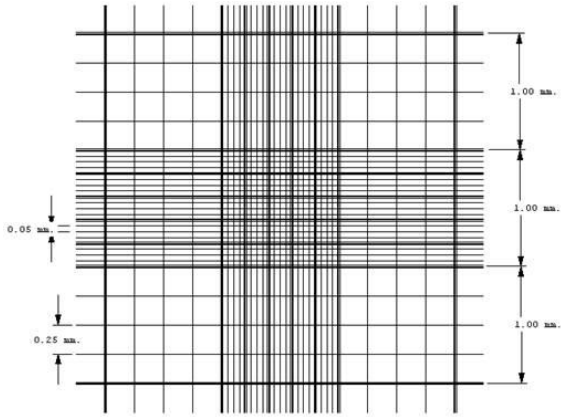


***Fig 3:-** Petroff Hauser Counting Chamber from electron microscopy sciences*

2. **Culture and maintenance of SHSY5Y, JURKAT and K562 cells:-**
   (All the cells were purchased from NCCS, Pune) **SHSY5Y;** Homo sapiens bone marrow neuroblastoma cell lines, **Jurkat**; Homo sapiens acute T cell leukemic peripheral T-lymphocyte cell line, **K562**; Homo sapiens bone marrow chronic myelogenous leukemia (CML) cell lines were maintained in 1:1 mixture of Ham's F12 and (DMEM) Dulbecco's Modified Eagle's Medium (for SHSY5Y cells) and RPMI 1640 Medium (for Jurkat and K526 cell lines) with 10% fetal bovine serum as supplement with 0.1% Pen-Strep and grown in presence of 5% CO2 in an incubator at 37°C. Within short intervals, the cell medium was replaced depending upon their confluence, and were sub-cultured when confluence was reached. Differences in morphology between proliferative and differentiated cells were evaluated by phase contrast light microscopy.
   After counting by Petroff Hauser chamber haemocytometer from EMS, $0.25X10^6$ cells were seeded into 25 cm^2 flasks (for SHSY5Y) or 33mm plates (for Jurkat and K5265 cells).So that at every chemical treatment, 0.5 $X10^{6\ cells}$ were treated accordingly.
3. **Cell treatment:-** Chemicals used to target different mitochondrial complexes for Electron Transport Chain were brought from Sigma Aldrich, in powder form and were dissolved in Dimethyl Sulfoxide (DMSO). Chemicals were, FCCP (an uncoupler of ETC), Rotenone (ETC complex I inhibitor), and Oligomycin (F0 /F1

ATPase inhibitor), each with the stock concentration of 2mM. The treated concentration or the working concentration was made primarily 100 µM and then 5µM and 10 µM while treatment, for each chemical, determined by literature studies. As these inhibitors were insoluble in water, for dilution purpose, they all were solubilized in DMSO which is also harmful for cells. That's why for each treatment a vehicle control of DMSO was used in a same concentration as the concentration of DMSO used in Rotenone, FCCP or Oligomycin dilution. From ach 2mM stock of chemicals,5,2.5 uM working concentration was made.

4. **DAPI and MitotrackerRed staining:-** DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) was used to stain nucleus and MitotrackerRed dye was used to stain mitochondria. Cells from 24 well plates were pipetted out from 24 well plates for each sample and 1X DAPI (BIO-RAD) stain was added from 100X stock (500 µl water mixed with lyophilized DAPI dye to make a stock conc of 100X). Kept for 5 minutes at 37^{o} C and then was twice washed with 1X PBS. Finally was observed under phase contrast microscope.
   In case of MitotrackerRed dye, the lyophilized sample was dissolved in DMSO to achieve the stock concentration of 1mM. The working concentration was maintained at 150nm and treated to the cells and observed in a same manner.
5. **Cell viability quantification by MTT assay:-** Viability of SHSY5Y cells was measured by the MTT (Sigma Aldrich) assay. In 24 well culture plates, SHSY5Ycells were cultured and at the end of the experiments, the MTT solution was added to fresh culture at 10% of the total culture volume according to the manufacturer's instructions. After addition one hour and three hours in CO2 incubator incubation at 37°C, the absorbance at 590 nm of solubilized MTT formazan products was measured by Synergy, H1 micro plate reader.
6. **Determination of reactive oxygen species generation:-** The Reactive Oxygen Species production of SHSY5Y cells was measured by the ROS generation assay with DCFDH2 treatment. SHSY5Ycells were cultured and 50 µM of DCFDAH2 was added from the stock of 5mM. After 45mins in CO2 incubator incubation at 37°C, the fluorescence at 480nm excitation and 520 emission spectra of products was measured by Synergy, H1 micro plate reader.
7. **RNA isolation, cDNA preparation and Real time PCR:-** RNA was isolated SHSY5Y cells using the Purozol reagents. cDNA was prepared from RNA with primers, which were designed using primer3 website with a Tm of 58°C. SYBR green dye (Bio-Rad) was used for measurement of real-time PCR amplification. Data for each sample was calculated as the percent difference in CT value (ΔCT = CT Gapdh - CT gene of interest). The data was plotted as mean percent Gapdh values for each gene of interest for each sample. Data were calculated as fold differences between control and treated cells. Followings are the primer sequences of the genes targeted for quantitative PCR analysis.

Gene	Primer	Tm
NDUFS2 F	3'ACCCTGAACTTTGGGCCCC5'	60.5
NDUFS2 R	3'TGCAGGAGCCCGATGTGA5'	59.3

COX5B F	3'GGCTGCATCTGTGAAGAGGACAA5'	59.4
COX5B R	3'TGGGGCACCAGCTTGTAATGG 5'	60.2
ATP12A F	3'AGAGCAGCTGGTGGTGGG5'	60.3
ATP12A R	3'CTGGGGCTCAGACTCCCC5'	60.2
TNF F	3'GAGTGACAAGCCTGTAGCCCA5'	59.2
TNF R	3'GGTGTGGGTGAGGAGCAC5'	58.5
SARM1 F	3'GAGCAGATCCTGGTGGCTGAGAA 5'	60.8
SARM1 R	3'GCTTGAACATGTGCTCCAAGATGCC5'	60.8
GAPDH F	3'CCCATCACCATCTTCCAGGAGC5'	59.8
GAPDH R	3'GCCTTCTCCATGGTGGTGAAGAC5'	59.9

Table 1: Primer sequences of the genes targeted for quantitative PCR analysis

8. **Mutational cloning of different domains of SARM1:-**Mutation of different domains of SARM1 was acheived by mutational cloning with help of pCMV6 vector and the primers mentioned in table 2. The cloning was first done by mutation PCR and transforming them in a bacterial system using Kanamycin as a primary selection marker. The plasmids containing the domain mutation from selected colonies were isolated and maxi-prep was done followed by the preservation at -20^{o} C.

Primers for Human SARM1 Mutational Cloning:-

Mutant	Primer
OnlyTIR domain F	5' GGCTAAGAATTCATGCTACACTCCCCGCTG 3'
OnlyTIR domain R	5'TAATTAACGCGTCTCAGGCCACTCGAAGCC 3'
Only SAM1 domain F	5'GGAATAGAATTCATGGTAGAGAAGCGCGCA3'
Only SAM1 domain R	5'TAATTAACGCGTATAGTTGGCGAAGGTCTT 3'
Only SAM1+SAM2 domain F	5'GGAATAGAATTCATGGTAGAGAAGCGCGCA3'
Only SAM1+SAM2 domain R	**:** 5'TAATTAACGCGTCATTTCTCTGGCCGCCGT 3'
N term deletion F	5' TAATTAACGCGTGGTTGGACCCATGGGTGC 3'
N term deletion R	5'GGAATAGAATTCATGGTAGAGAAGCGCGCA3'

Table 2: Primer sequences used for Human SARM1 Mutational Cloning

9. **Protein isolation and Western blot analysis:-** Cells were scrapped from well and dissolved in lysis buffer (MPER) and the protein was isolated in presence of 1X protease inhibitor and consecutive centrifugation. After running a SDS gel, it was transferred on NCM and blotted against primary antibody and secondary antibody (HRP tagged).
10. **Transient transfection by Lipofectamine 2000:-** Cells were plated until it reaches 70% confluence at the time of transfection. Plasmid DNA lipid complex were prepared by adding 3µg of DNA of SARM full length and its mutants (SAM, only SAM1, only TIR and ∆N27 in FLAG tagged pCNV6) and 5-12 µl of lipofectamine 2000. This was added to the cells in 6 well plates and cellular morphology and cell survival rate was observed.

Results and Discussion

Rotenone treatment induces morphological changes in T-cell lymphoma cell line

In our laboratory we have used the pesticide rotenone to study environmental toxin induced neuronal death. We have observed that rotenone treatment induced neuronal death and was associated with the increased expression of the neurodegenerative molecule SARM1 (Fig. 1A and B). The molecule SARM1 is predominantly expressed in the neuronal cells in the brain and also in the T lymphocytes. Hence induction of the pro-apoptotic molecule SARM1 may help control T cell lymphoma. Since rotenone served as a natural inducer of SARM1, we treated the T cell lymphoma cell line Jurkat with increasing concentration of rotenone at varying time points.

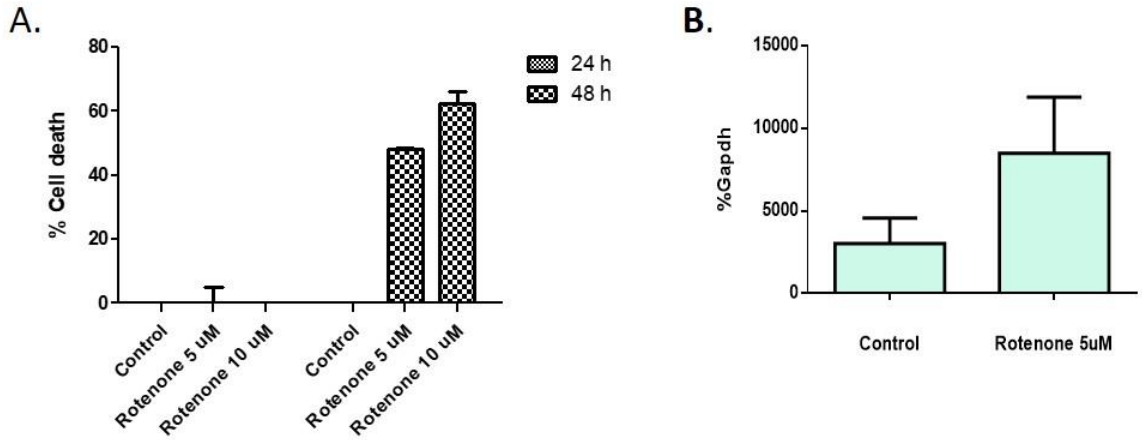


***Fig. 1**: MTT assay and real-time PCR analysis of Sarm1 in Jurkat cells in the presence or absence of rotenone*

Rotenone treatment induces morphological charges in T cell lymphoma cell line

In our laboratory we have used the pesticide rotenone to study environmental toxin induced neuronal death. We have observed that rotenone treatment induced neuronal death and was associated with the increased expression of the neurodegenerative molecule SARM1(Data not shown). The molecule SARM1 is predominantly expressed in the neuronal cells in the brain and also in the T lymphocytes. Hence induction of the pro-apoptotic molecule SARM1 may help control the progression of T cell lymphoma. Since Rotenone served as a natural inducer of SARM1, we treated the T cell lymphoma cell line Jurkat with increasing concentration of rotenone at varying time points. When cells were treated with 5 or 10uM of Rotenone they were resistant to rotenone induced cell death but cells started dying at 48h post treatment (Fig 1). Interestingly, cells that were resistant to rotenone mediated cell death at 24h post treatment assumed an elongated morphology and appeared swollen (Fig 2A) which was not present in the chronic myeloid leukemic cell line K562 (Fig 2B).).

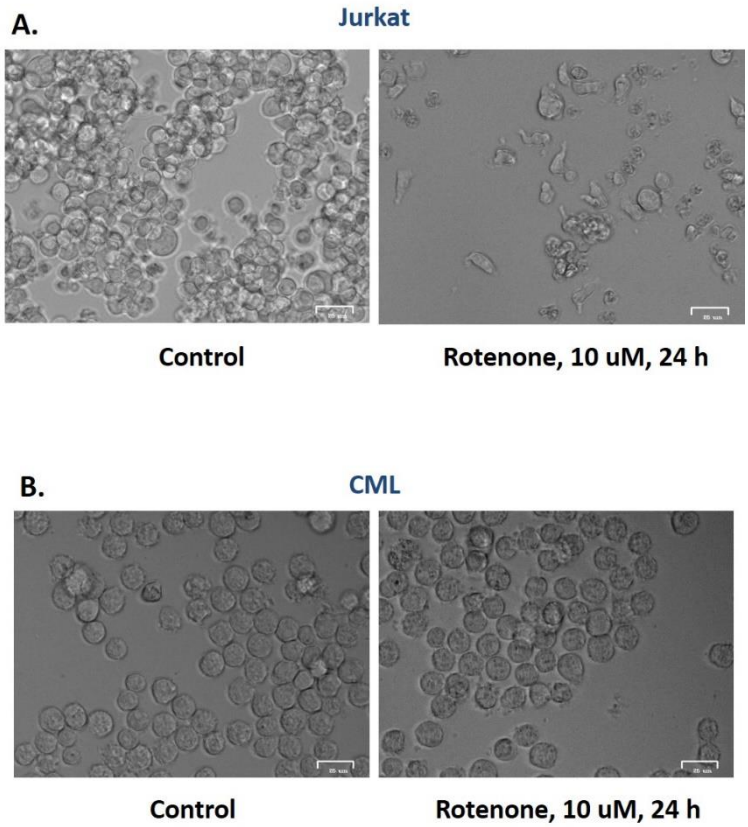


***Fig. 2**: Morphology of the T cell lymphoma cell line Jurkat and the CML cell line K562 in the presence of the mitochondrial stressor rotenone*

This elongated morphology as observed in the Jurkat cells following rotenone treatment was not due to cell fusion as indicated by DAPI staining (Fig.3)

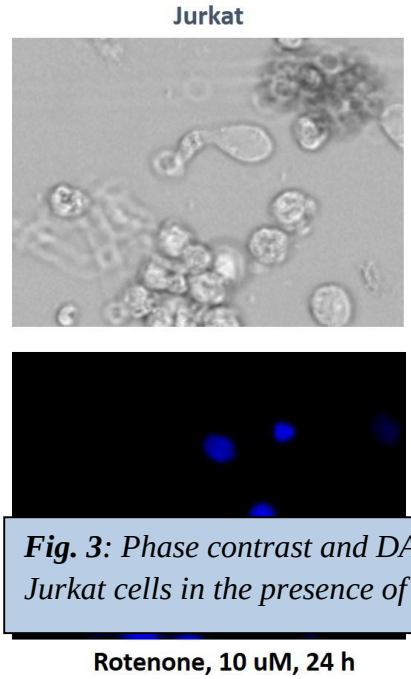
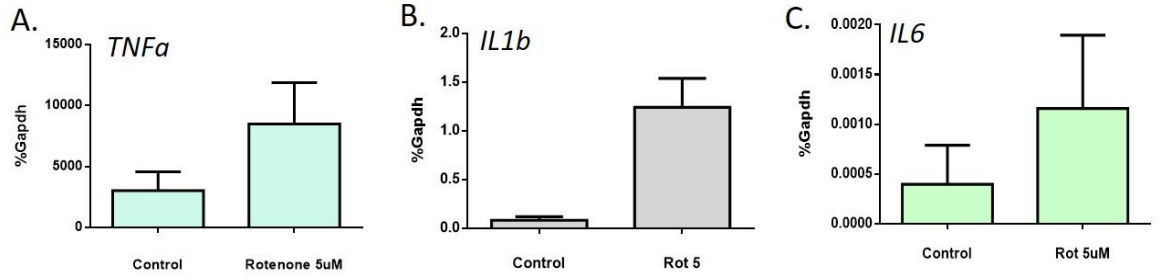


***Fig. 3**: Phase contrast and DAPI-staining images of Jurkat cells in the presence of rotenone*

Rotenone induced cell death at 48h was associated with enhanced SARM1 expression and increased inflammatory response

SARM1 has been shown to induce T cell apoptosis and the increased cell death of the cells following 10uM of rotenone treatment was associated with increased levels of SARM1(Fig. 2B). The molecule SARM1 has been linked to modulate TNFα production to restrict viral infection. Since rotenone treated Jurkat cells showed increased SARM1 expression, we also analysed the expression of TNFα and other pro-inflammatory cytokines in these cells. Significant increase in TNFα expression alongwith IL1b and

Fig. 4. Realtime PCR analysis of the inflammatory genes in the T cell lymphoma cell line in the presence and absence of rotenone



Interestingly, TNFα expression followed a similar increase as observed with Sarm1 in these cells. Further, treatment with the uncoupler FCCP did not result in the induction of either SARM1 or any inflammatory response (Fig. 5).

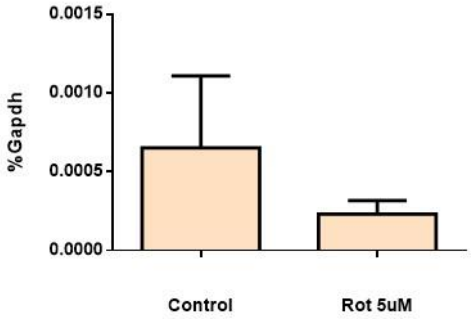


Fig. 5. Realtime PCR analysis of TNFα in the T cell lymphoma cell line in the presence and absence of FCCP

Creation of deletion mutants of SARM1 and studying their role in cell death

The structure od SARM1 contains a common SAM (Sterile Alpha Motif) domain shared by all the homologous, flanked by Armadillo motif (ARM) at N terminal and a TIR domain at C terminal (Fig. 6). SARM1 also contains a 27 amino acids long mitochondrial localization signal(N27).

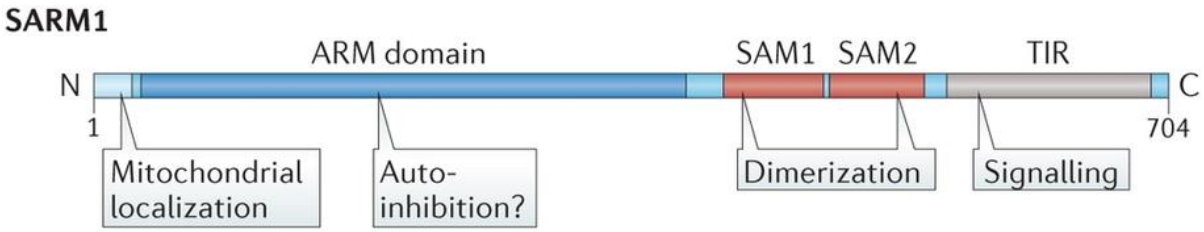


***Fig. 6**: Different domains of Sarm1*

Other than N27 domain, the only function known to us is of TIR domain, whose dimerization is important for activation of SARM1 and SARM1 mediated cell death, but the exact pathway is still unknown. To understand the role of these domains of SARM1 in the induction of cell death in Jurkat cell lines, several deletion mutants of SARM1 were created using standard PCR based technique (Table 1 and Fig. 7).

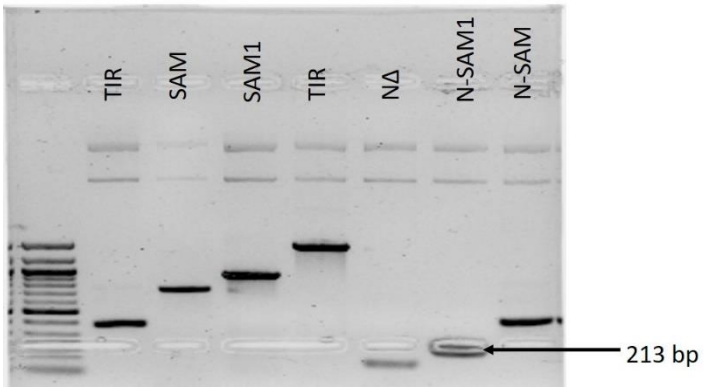


***Fig. 7**: Creation of deletion mutants of Sarm1*

Jurkat cell lines were transfected with 10uM of Full Length (FL) SARM1, TIR only and N terminal deletion mutant of SARM1. Following 24h post-transfection cells there was significant cell death associated with FL-SARM1 and moderate amount with SARM1-TIR. However, the cells with no N terminal domain showed the elongated or swollen morphology as observed with 24h Rotenone (Fig. 8). These results indicate that mitochondrial localization is important for SARM1 mediated cell death in Jurkat cells.

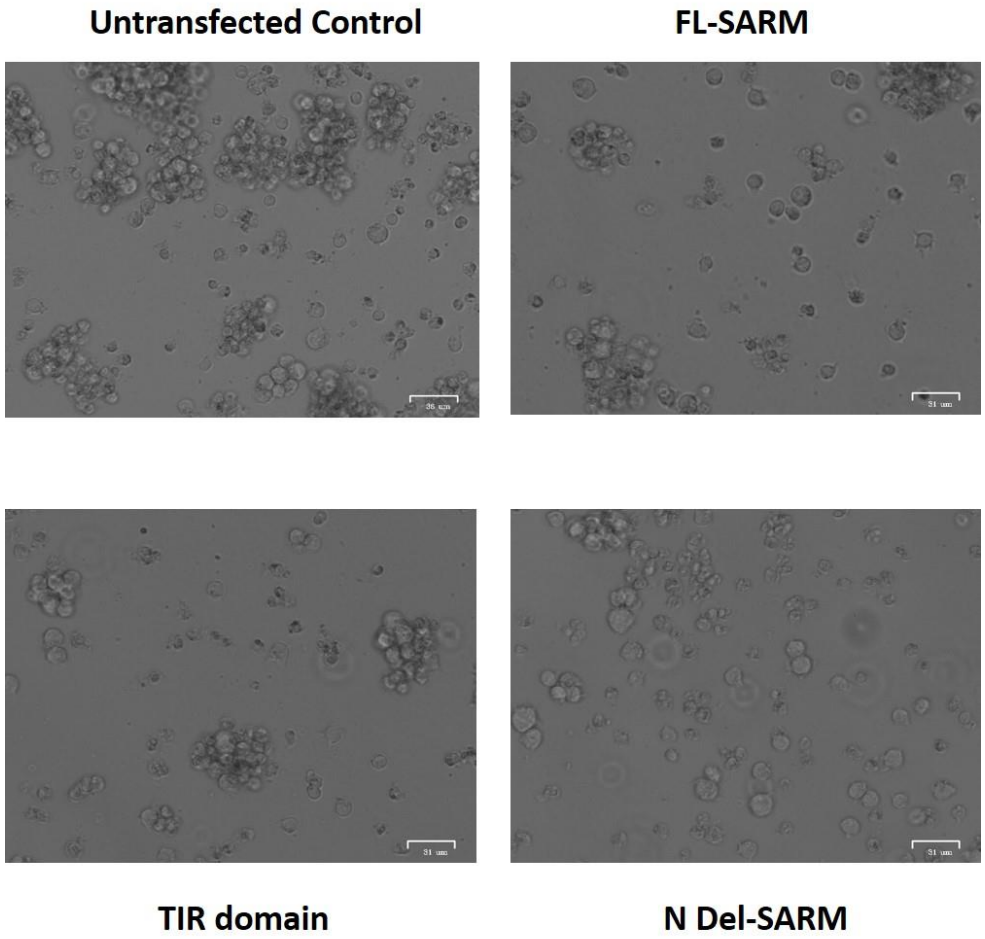
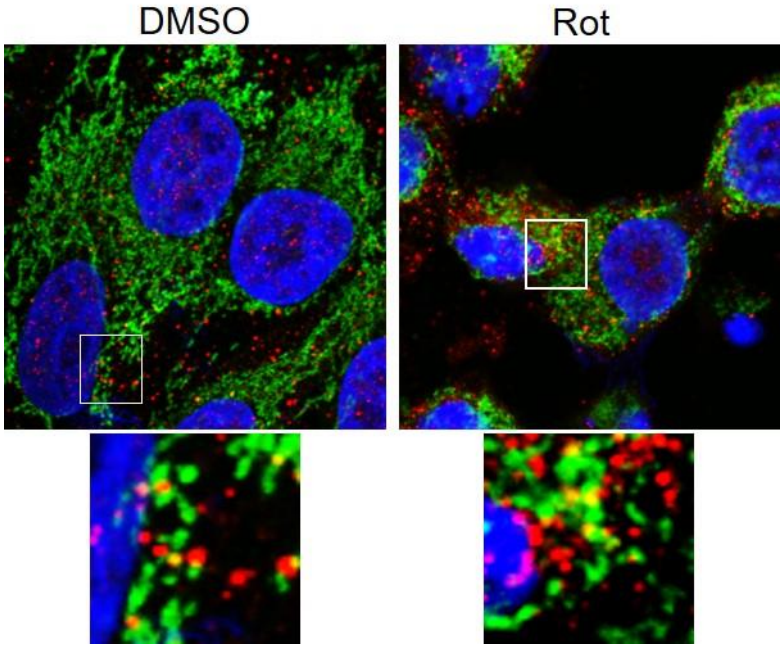


***Fig. 8**: Phase contrast images of Jurkat cells overexpressed with different mutants of Sarm1*

To further understand the importance of mitochondrial localization of Sarm1 we exposed the cells to mitochondrial stress and observed increased translocation of Sarm to the mitochondria (Fig. 9).



We also verified this enhanced mitochondrial l a

***Fig. 9**: Confocal microscopic imaging of CoX-IV and Sarm1 in the presence or absence of rotenone*

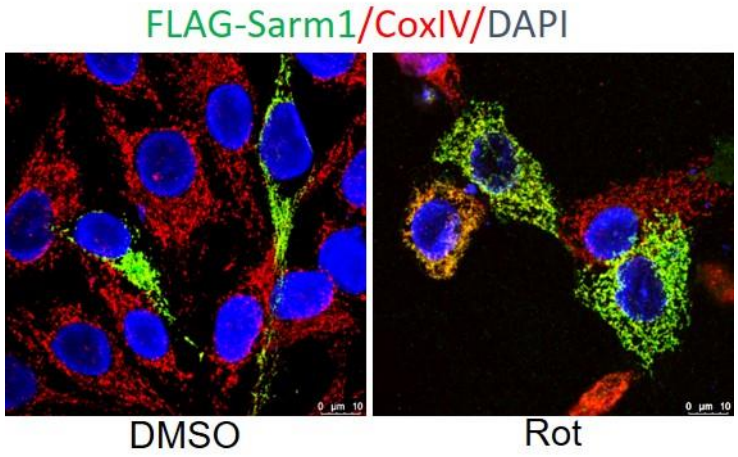


***Fig. 10**: Confocal microscopic imaging of CoX-IV and Sarm1 in HEK293 cells overexpressing FL-Sarm1*

Rotenone treatment in T cell lymphoma cells is associated with increased ROS generation

SARM1 translocate to the mitochondria and induces cell death. Rotenone is known to mediate cell death through the generation of Reactive oxygen species (ROS). ROS has been shown to be induced early upon rotenone treatment. Hence, we measured the level of ROS in these cells 3h, 4h and 6h post treatment with 5uM of Rotenone. A significant increase in the ROS production was observed at 6h post treatment (Fig. 11).

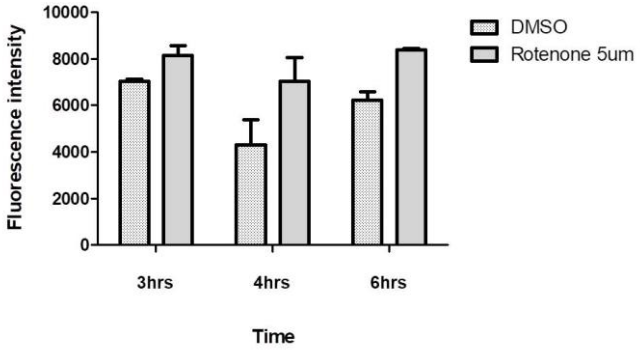


***Fig. 11**: Measurement of ROS generation by DCFDA assay in Jurkat cells in the presence or absence of rotenone*

However, a closure look at the mitochondrial morphology showed no disruption of mitochondrial integrity (Fig. 12).

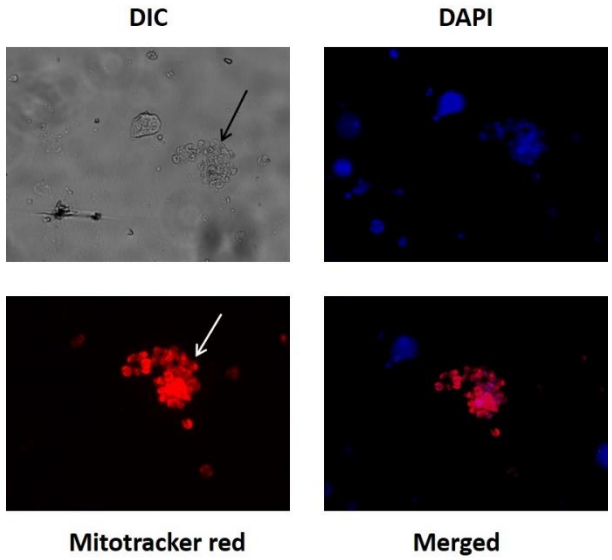
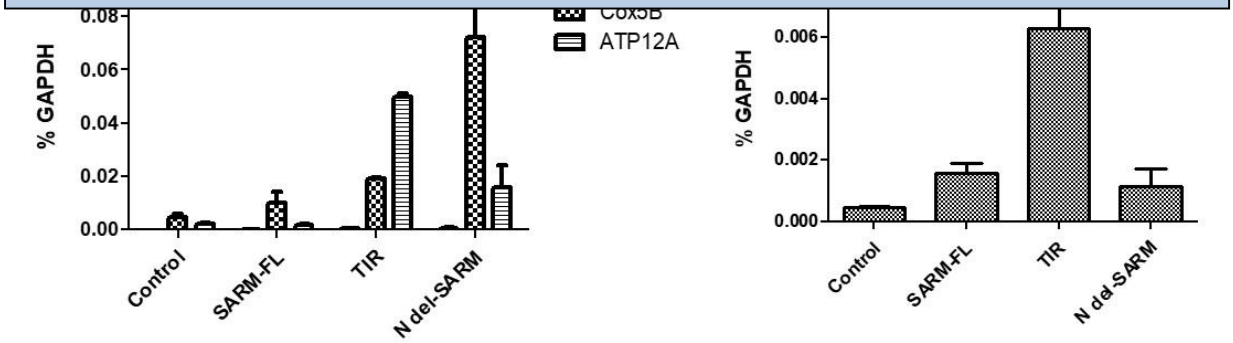


***Fig. 12**: Mitotracker staining of Jurkat cells in the presence of rotenone*

As observed with rotenone treatment, overexpression of Jurkat cells with either FL-SARM1 or its mutants did not induce any significant changes in the ETC complex genes (Fig 13A). However, one of the genes Cox5B showed significant increase following SARM1 overexpression in these cells. This was also accompanied by an increase in the inflammatory response with a similar increase in TNFα expression (Fig 13B) as observed previously with rotenone treatment. Whether SARM1 regulates the transcription of both Cox5B and TNFα and how they may regulate cellular death in T cell lymphoma remains to be seen.

***Fig. 13**: (A-B) Real time PCR analysis of mitochondrial complex genes and the inflammatory gene TNFα in Jurkat cell lines overexpressing FL-Sarm1 and its different mutants*



The distinct morphology of T cell lymphoma cell line did not arise due to cell fusion

The structure of Jurkat cells generated by the rotenone treatment might possibly be the result of nuclear fusion. Interestingly, DAPI staining showed the presence of a single nucleus were that confirmed that there was no cell fusion in the Jurkat cells following rotenone treatment (Fig. 14).

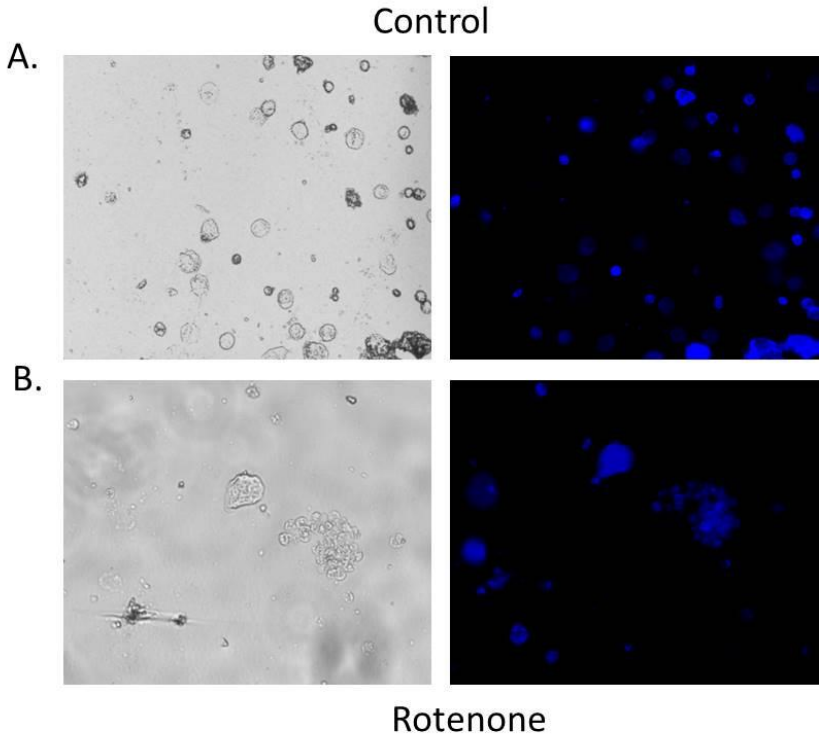


***Fig. 14**: Phase contrast and DAPI stained images of Jurkat cells in the presence of rotenone*

ii. Has the progress been according to original plan of work and towards achievingthe objective. if not, state reasons: Yes

iii. Please indicate the difficulties, if any, experienced in implementing theproject: NA