

Indian Science Technology and Engineering facilities Map (I-STEM)

Partial financial support for using R&D facilities in other institutions through the I-STEM web portal to Users from Academic Institutions and Start-ups (Format-1)

Format for requesting support for accessing and using facilities through the I-STEM web portal.

(i) Details of the Personal Information

- a. Name of the Researcher/Faculty Member: NAVAMI KRISHNA
- b. Name of the Institution/Start-up: NATIONAL INSTITUTE OF TECHNOLOGY CALICUT
- c. Name of the Department: SCHOOL OF BIOTECHNOLOGY
- d. Degree registered for/Product being developed (for start-up): PhD
- e. Mob. No. 9497333478
- f. Email ID: navami_p180069bt@nitc.ac.in

(ii) Project/Research topic Details:

- a. Project Title/Research Title: **Ischemic preconditioning mimetics as ischemic stroke therapeutics: computational prioritization and experimental validation.**
- b. Sub area: Neuroscience.
- c. Area Description: Ischemic Preconditioning is a well-established, intrinsic, protective phenomenon in which brief episodes of ischemia protect the organs from a subsequent severe ischemic insult. Ischemic preconditioning has numerous clinical correlates such as the treatment of cerebral ischemic stroke, myocardial infarction, kidney ischemic etc. Preconditioning-based therapies, on the other hand, are not commonly used in clinical practise. The need to administer therapy prior to a known ischemic event, as well as the practical difficulty of implementing a preconditioning regimen, are both contributing factors. Preconditioning mimetics (small molecules) that stimulate the biochemical pathways of ischemic preconditioning and protect the organs without inducing ischemia have the potential to be translated into clinical settings due to their ease of administration. The present proposal aims to identify such Preconditioning mimetics and evaluate their neuroprotective efficacy against ischemic injury *in vitro*. The findings are expected to demonstrate that preconditioning mimetics have novel biological activity, with both fundamental and therapeutic implications for the treatment of ischemic stroke.
- d. Aims & Objective (in Bullets): Objectives connected with methodology
 - To identify the top differentially expressed genes by microarray data analysis of precondition experimental samples.
 - To unravel the protective mechanism of preconditioning by systems biology analysis.
 - To identify potential preconditioning mimetics from differentially expressed gene sets.
 - To evaluate the neuroprotective efficacy of preconditioning mimetics against ischemic injury *in vitro*.

e. Duration (MM/YYYY): 06/2022 to 12/2022

f. Project Summary (max. 1000 words):

Ischemic stroke (IS) is a complex, multifactorial and debilitating cerebrovascular disease and the second leading cause of serious long-term disability and mortality worldwide. Thrombolysis by recombinant tissue plasminogen activator (r tPA) is the only FDA-approved therapeutic option, utility of which is limited by short therapeutic window and poor efficacy. Thus, there is an unmet medical need for the discovery of safe and effective neurotherapeutics that may prevent neuronal death and improve neuronal recovery following ischemic stroke.

Preconditioning is a procedure by which a noxious stimulus near to but below the threshold of damage is applied to the tissue. Shortly after preconditioning or after a delay, the organ (and therefore the organism) develops resistance to, or tolerance of the same or different stimuli, thereby protects against subsequent injury. Preconditioning approaches include exposing the brain to mild stress, such as brief episodes of hypoxia/ischemia/seizures, as well as the delivery of bioactive medicines. These methods induce brain tolerance through boosting the process of angiogenesis, apoptosis inhibition and mediation of inflammatory responses thus providing significant neuroprotection against further neuronal injury.

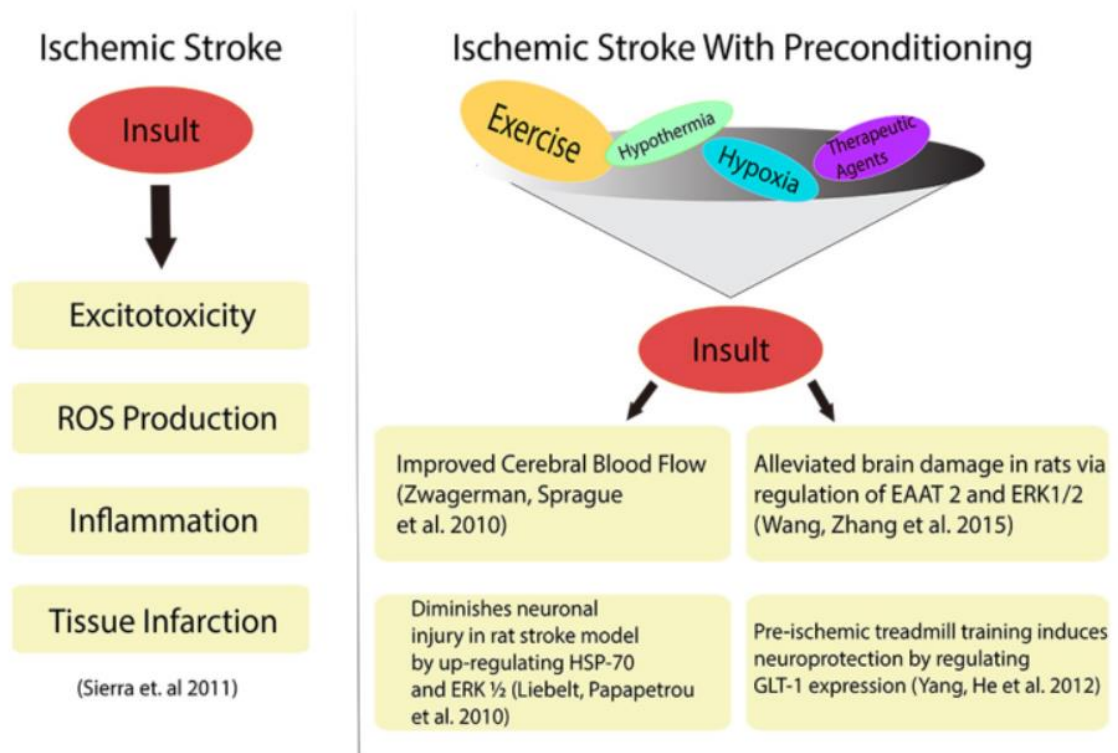


FIGURE 1: - shows the impact of ischemic stroke with or without preconditioning

Research on ischemic preconditioning (IPC) shows the involvement of cellular and molecular elements of the immune system as well as Intrinsic protective factors such as transcriptional factors and endogenous protective proteins. Potent molecular mediators of IPC include Toll-like receptors and type 1 interferons

which are involved in innate immune signaling pathways whereas cellular mediators of IPC include microglia, the resident immune cells of the brain, astrocytes, and neurons. Accumulating evidence indicates that the transcription factor HIF-1 α , a known mediator of ischemic tolerance, plays a key role in neuroprotection against ischemic brain injury, and HIF-1 α mRNA is increased by preconditioning exercise. Studies also suggest that microRNAs (miRNAs) play prominent roles in ischemic tolerance induced by preconditioning.

A set of genes, transcription factors, miRNAs and immune mediators are differentially expressed when the brain is subjected to preconditioning stimulus. Changes caused by these elements are responsible for the neuroprotective effect of the preconditioning technique, as well as its ability to mitigate additional damage. Finding a new therapeutic or pharmaceutical bioactive compound that may alter the expression of corresponding gene or miRNAs and giving the same response as preconditioning strategies is an effective method for increasing the neuroprotection for preventing further damages. Even though hypoxic/ischemic preconditioning gives protection against ischemic stroke, its protective effects are restricted to three days and disappear within a week. Thus, the usage of these small molecule therapeutics, agonists or antagonists that provide the same effect as preconditioning represent a potential opportunity for therapeutic targeting in acute stroke, as well as in prevention of stroke injury in patients at risk of stroke.

Combination therapy must consider many factors to determine the efficacy of any combination. The combination effect of two agents/methods should be significantly higher than the individual agent/method. Also, they must improve the treatment window and the ceiling effect between base and combination treatments. The present study is aimed at evaluating the post-ischemic neuroprotective efficacy and mechanisms of these two drugs alone or in combination in vitro. Also, it defines a new possibility of treating cerebral ischemia with a refined model of combination therapy with drug repurposing perspective

References

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g. Technical Details

a. TRL

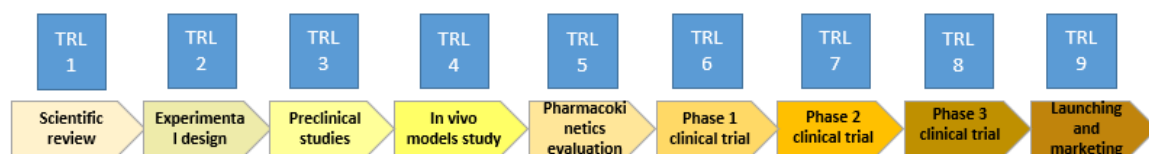


Figure 2. Technology readiness level

The technology readiness level (TRL) stages for drug development are shown in Figure 2. The proposed project estimates the technical development ranked in TLR 3.

Methodology

The general methodology for the proposed project is to be executed in two stages. The first stage involves the application of systems biology tools to gain a better understanding of the preconditioning mechanism and to identify preconditioning mimetics. The second step is related with the in vitro validation of the neuroprotective efficacy of individual and combination medicines. The detailed experimental approach followed in each phase is described as follows:

Stage I (completed): Systems biology analysis to identify preconditioning mimetics/ drugs

Stage 1 starts with the identification and analysis of preconditioning dataset from GEO database. Microarray Data quality checking and normalization done by R/Bioconductor software. DEGs (Differentially Expressed Genes) were screened using the R/Bioconductor limma tools (adj. p-value 0.05). Conversion of mouse genes into human orthologous genes were done by HGNC multisymbol checker. Up and down regulated genes were identified based on Fold change (FC) Functional enrichment analysis of DEGs involves identification of biological processes by GO analysis and Pathway analysis by KEGG and Reactome database. Enrichr was used to identify drugs/small molecules that mimicked the action of preconditioning genes.

Pharmacokinetics evaluation and Drug perturbation signature analysis were carried out for the identified small molecules. The top genes governing preconditioning were identified using network biology analysis of differentially expressed genes, providing greater insight into the mechanism of preconditioning mediated neuroprotection.

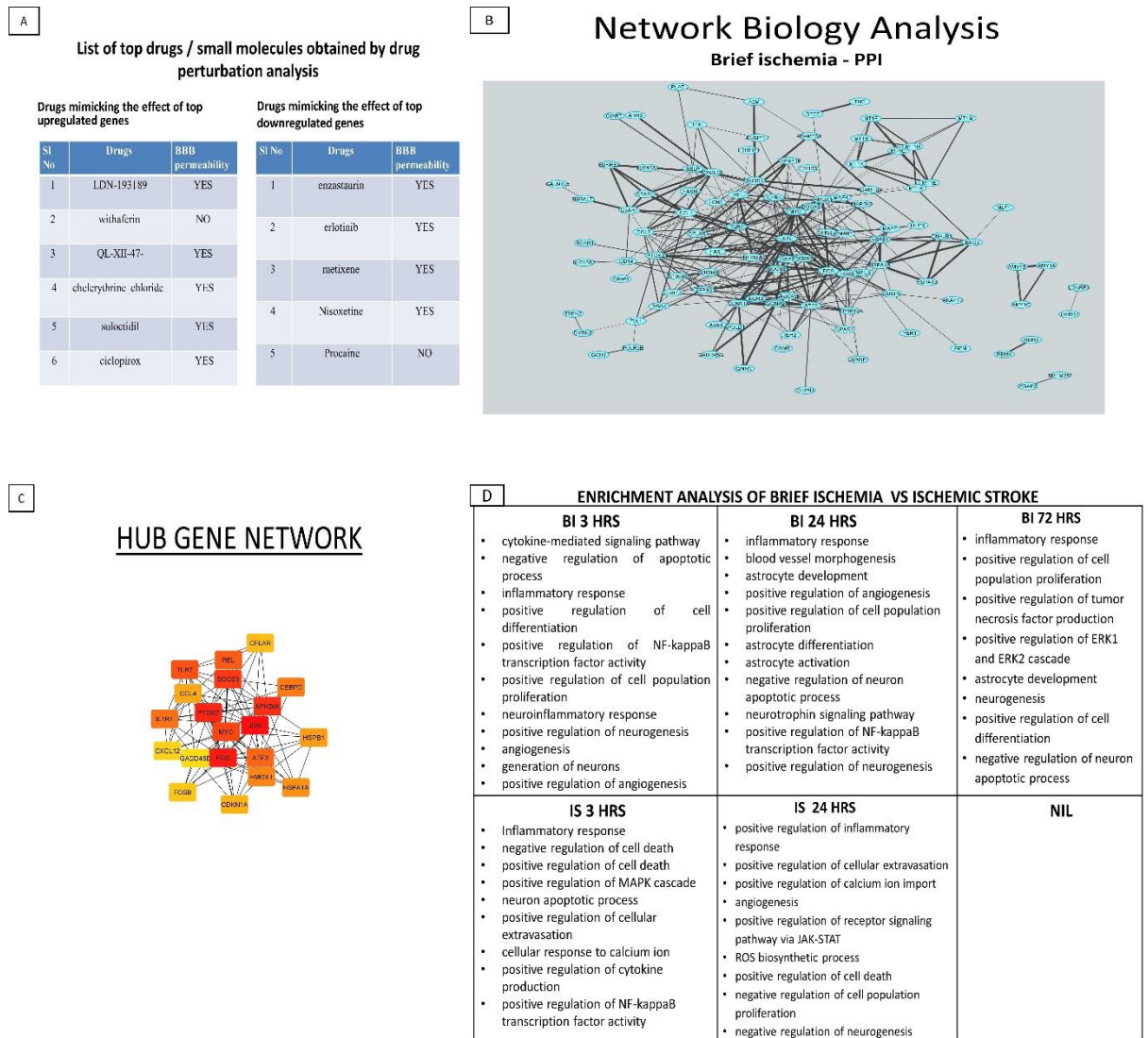


Figure 2. A. TOP DRUGS OBTAINED BY DRUG PERTURBATION ANALYSIS B. NETWORK BIOLOGY ANALYSIS SHOWING PROTEIN-PROTEIN INTERACTION NETWORK C. TOP HUB GENES IN PRECONDITIONING D. FUNCTIONAL ENRICHMENT ANALYSIS OF PRECONDITIONING AT DIFFERENT TIME POINTS VERSUS ISCHEMIC STROKE

Stage II (on-going): In vitro experimental validation for neuroprotective efficacy

Oxygen glucose deprivation (OGD)/re-oxygenation (OGD/R) is a simple yet highly useful techniques an in vitro model for stroke, not only for the elucidation of the role of key cellular and molecular mechanisms underlying brain ischemia, but also for the development of novel neuroprotective strategies. It induces severe injury to neuronal cells, showing similarities with the in vivo models of brain ischemia. In order to perform OGD/R, cell or tissue cultures, such as primary neurons or organotypic slices, and acutely prepared tissue slices are usually incubated in a glucose-free medium under a deoxygenated atmosphere, for example in a hypoxic chamber.

The therapeutic drugs identified by network biology approach will be evaluated for their neuroprotective efficacy in vitro, using the classical OGD/R model, established in our laboratory. Sh-Sy5y cells (NCCS, Pune) will be subjected to OGD/R injury by replacing the culture medium with Earle's balanced salt solution (EBSS) (Thermo Fisher Scientific, MA, USA), and placing them in hypoxic chamber (Billups-Rothenberg, Del Mar, USA), flushed with a mixture of 95% N₂ and 5% CO₂ for two hours. OGD/R will be terminated by replacing the EBSS with DMEM/F12 medium containing 10% FBS. The culture will be then returned to the incubator for reoxygenation. The control cells will be untreated and maintained under normoxic condition (5% CO₂ and 95% air) with DMEM/F12 containing 10% FBS. Cells will be treated with increasing concentration of drugs to be repurposed one hour after OGD/R. The cell viability will be measured using MTT and ATP assays 24 hours post-OGD/R.

Combination Drug Treatment The cells were divided into control group (normoxic), OGD group, and treatment groups for different combinatory-concentrations of Drug 1 and Drug 2. The cells were plated in a 96 well plate with cell density of 2 x 10⁴ cells per well. The combination doses were determined based on the optimal neuroprotective assay result of the individual treatment groups. The OGD, reoxygenation and combination treatment durations were followed similar to the individual treatment set. The neuroprotective efficacy was evaluated from the MTT and ATP assay results. The cell viability result for combination treatment was given as input into Cohen's d and the effect-size correlation data. The effect-size correlation data was further applied into multiple combination analysis models to retrieve the optimal combination dose.

Combination Therapy: Evaluation for Synergistic Neuroprotection The combination-treatment cell viability results were statistically transformed into Cohen's d and the effect-size correlation data, from the following equation;

$$Cohen's\ d = M_1 - M_2 / s_{pooled}$$

$$where\ s_{pooled} = \sqrt{[(s_1^2 + s_2^2) / 2]}$$

$$r_{Y1} = d / \sqrt{(d^2 + 4)}$$

Results were further compared and studied using combination index analysis (Chou Talalay), Loewe additivity reference model, Bliss independence model, and Highest Single Agent (HSA) model (references for all models). Cohens effect size co-relation compares the cell-viability difference between the OGD group and the treatment-combination group. The value of Cohen's d = 0.2 is considered to be a small effect size, 0.5 represents a medium effect size and values greater than 0.8 shows significant effect difference in the real world. Combination index analysis for determining the additive, synergistic, and antagonist effects of various drug 1 - drug 2 duo-

combinations was done based on the combination index equation of the CompuSyn software.

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2}$$

Where (D_x)₁ and (D_x)₂ indicate the mono protection doses of drug 1 and drug 2, and (D)₁ and (D)₂ are the doses of drug 1 and drug 2 that can cause the similar protection effect in combination. Drug 1- drug 2 duo-combinations were further tested with Loewe additivity reference model, Bliss independence model, and HSA model from <https://synergyfinder.fimm.fi>. The Loewe additivity model depends on the dose equivalence principle and can be expressed as:

$$Effect(a + b) = E_A(a + a_b) = E_B(b_a + b) = E_{AB}$$

Where E_A is the dose-effect value for drug A, (a + ab) corresponds to the dose A giving the effect E_{AB} and, respectively, for drug B. The Bliss independence model evaluates the additivity as the independent activity of drug without ever interacting with their targets and contributing to the same result. Whereas the HSA model approach is based on the statistical testing of the combination effects against the maximum individual effect.

$$CI = \frac{\max(E_A, E_B; \dots; E_N)}{E_{AB}; \dots; E_N}, \text{ for HSA model};$$

$$CI = E_A + E_B + \dots + E_N - E_A E_B - E_A E_N - E_B E_N - \dots - E_A E_B \dots E_N,$$

for the Bliss independence Model

$$CI = \frac{a}{A} + \frac{b}{B} + \dots + \frac{n}{N}, \text{ for the Loewe additivity model}$$

b. Design & development

- The therapeutics available for the treatment of cerebral ischemia is very limited. Insufficient blood flow to the brain following cerebral occlusion reduces the metabolic functions. The involvement of multiple biomolecules and biological pathways limit the discovery of a complete neuroprotective agent at the moment. The ultimate objective can be attained by the use of multiple drugs for targeting the multifactorial molecular cascade responsible for the ischemic injury. Repurposing the already agency-approved drugs for nonrelated diseases could save immense time and resources to aid human health and lifestyle diseases.

- Preconditioning is a process in which a noxious stimulus is administered to the tissue near but below the threshold of damage. Shortly after preconditioning or after a delay, the organ (and thus the organism) develops resistance to, or tolerance for, the same or different stimuli, protecting against future harm. But most of the preconditioning strategies cannot be clinically translated in humans and thus the use of small molecules or already approved drugs to mimic the preconditioning effect could be a valuable discovery in the stroke management. The present study is a stepping stone towards the use of existing drugs for ischemic neuroprotection with the effective application of combination therapy and drug repurposing strategies.

c. Facilities and Instrumentation required

The successful completion of the project depends on the availability of consumables and detection kits for the successful evaluation of different drug testing strategies in invitro models. In vitro cell culture Laboratory facilities are available in the department. However, a vast number of consumables for invitro cell culture and test detection kits are required to evaluate the neuroprotective efficacy of medicines.

MTT/ATP assays with various medication doses will be used to test cell survival. The optical density of the samples will be measured using a plate reader to determine cell survival.

d. Consumables (Scientific materials)

Cell culture experiments are carried out using the SH-SY5Y cell line. The SH-SY5Y cell line is a comparatively homogeneous neuroblast-like cell line, which exhibits neuronal marker enzyme activity (tyrosine and dopamine- β -hydroxylases), specific uptake of norepinephrine (NA), and expresses one or more neurofilament proteins. SH SY5Y cells also express opioid, muscarinic, and nerve growth factor receptors. In addition, SH-SY5Y cells are able to proliferate in culture for long periods without contamination, which is a prerequisite for in vitro cell model developing. Thus, SH-SY5Y cells widely used in neurobiology research. Cell culture research necessitates the use of numerous agents and consumables. Drugs/small molecules and cell culture mediums such as Dulbecco's modified eagle medium (DMEM), Dimethyl sulphoxide (DMSO), Fetal bovine serum (FBS), antibiotics, and trypsin are examples. The research will also require culture well plates, tips, and culture flasks. MTT test kits, ATP assay kits, and neurite outgrowth assay kits are examples of assay kits for analysis.

h. **Expected output and outcome**

The present study is conceptualized in designing a novel integrated multi-omics network biology approach to prioritize gene candidates in ischemic stroke preconditioning strategies and repurpose FDA approved drugs that target these genes to confer neuroprotection. The relative ease of administration, low cost, and potential wide generalizability of remote ischemic preconditioning using therapeutics (RIPC) make the concept a particularly attractive clinical strategy for select populations of

patients that are at high risk for cerebral ischemic events in the immediate (hours to days) future. Further, the research methodologies outlined in this proposal will provide experimental validation data on the neuroprotective mechanisms of repurposed drugs. Combinatorial studies will provide insights into the development of drug combination which will have the maximum therapeutic effects.

Overall, it is expected that the findings will demonstrate the neuroprotective effects and mechanisms of repurposed drugs against ischemic stroke with both fundamental and potential therapeutic implications. Further, the repurposed drug(s) may be covered by intellectual property rights for its novel therapeutic effect. Drug repurposing strategy could dramatically reduce the cost and time of drug discovery by narrowing down the field to testing and validation. This research will also result in research paper publications and the possibility of a patent.

i. **Industry/Institute Collaboration: NO**

- (i) Name of the Collaborating Industry/Institute
- (ii) Collaboration Details
- (iii) Additional Details if any

j. **Funding required from I-STEM (with justification): (Most important section- better if you can add more points in this part)**

The funding required is mostly for the testing of the drugs in invitro cell cultures to assess its neuroprotective efficacy. From the list of drugs obtained from systems biology analysis of preconditioning dataset, we selected the top ones (Drug 1 and Drug 2) based on the pharmacokinetics evaluation and BBB permeability. The effect of these two drugs in the cell cultures were assessed individually as well as in combinations. Drug 1 is expected to mimic the upregulated expression of genes in preconditioning, whereas Drug 2 is expected to mimic the downregulated expression of genes, thus conferring maximum neuroprotection.

The cells were divided into control group (normoxic), OGD group, and treatment groups for different individual concentrations of drug 1 and drug 2. The cells were plated in a 96 well plate with cell density of 2×10^4 cells per well. Cells were subjected to OGD injury for 5-hours and were brought back to the normoxic condition (reoxygenation) thereafter. Drugs were added (Table 3) 1-hour after reoxygenation (post-OGD) and continued till 18-hour. Thereafter, the cells were harvested for cell viability and optimal neuroprotective doses were calculated from MTT and LDH assay results.

Detailed testing and thorough validation are required to identify such drug candidates and to achieve the impossible task of neuroprotection following cerebral ischemia. Drug repurposing strategy could dramatically reduce the cost and time of drug discovery by narrowing down the field to testing and validation. Several neuroprotective agents have failed in the clinical trials despite their therapeutic potential to act as calcium channel

antagonists, glutamate receptor antagonists, antioxidants, immunosuppressants, nitric oxide synthase (NOS) inhibitors, and exogenous growth factors. In this scenario, there arises a hope on the combination therapy strategy to act on multiple degenerative pathways by increasing survival rate and plasticity.

The proposed work study will be a proof of concept on the neuroprotective efficacy of preconditioning and here we are employing individual and combinatorial drug treatment studies to employ maximum neuroprotection targeting multiple pathways and biological processes. In vitro drug testing will be done by MTT/ATP cell survival assay.

Chemicals and consumables:

The consumables required for the experiments mainly includes drugs/small molecules, cell line, culture plates, culture flasks, medium, antibiotics and trypsin.

Maintenance of cell culture: - The SH-SY5Y cell line is used for cell culture experiments. DMEM medium with 10% FBS with 1% antibiotics is used to culture the cell line. Aspirate medium under sterile conditions. Rinse adherent cells once with sterile $1 \times$ PBS pre-warmed to 37°C or room temperature. To prevent detaching cells, add the PBS to the inner wall of the petri dish that doesn't have cells attached. Do not add PBS directly onto cell monolayer. Gently tip the flask so that the PBS washes over the cell monolayer. Aspirate PBS. Add trypsin to adherent cells for approximately 2 min or until cells visibly detach from petri dish. Neutralize trypsin by adding an equal volume of DMEM medium containing 10 % FBS. Collect detached cell suspension and centrifuge at 1,000 rpm for 5 min at room temperature to concentrate cell pellet. Aspirate supernatant carefully without disturbing the cell pellet. Gently suspend pellet in DMEM medium containing 10 % FBS. To separate cells from clumps, pipette up and down gently until the suspension appears homogenous. Count cells using a hemocytometer and plate at approximately 3×10^3 to 1×10^5 cells/cm². Culture cells at 37°C , 5 % CO₂. Growth medium should be refreshed every 3–5 days. Monitor cells for confluence. When cells reach 80–90 % confluence, subculture as described below.

The cell survival assay will be conducted in triplicates. The HiMedia brand is chosen to procure the consumables as it is also available with the local suppliers. Primary testing of drugs requires addition of drugs at different concentration and at different timepoints to understand the concentration at which it gives maximum neuroprotection. Combinatorial drug studies will also require multiple experiments to assess maximum amount of protection offered by the drug combinations. Hence, a good number of tissue culture well-plates, medium and culture flasks are required for carrying out the experiments.

Table 1. Chemicals and consumables for drug studies

Sl. No.	Chemicals/Consumables	Price (INR)	Quantity	Total price (INR)
1.	cells	15000	1	15000.00
2.	96 well plates/ Culture flasks/tips	30000	1	30000.00
3.	MTT/ATP assay kits	35000	1	35000.00
4.	Medium/ trypsin/ antibiotics	20000	1	20000.00
5.	Drugs/small molecules	30000	1	30000.00
Total (INR)			1,30000.00	

References:

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Details of Requirements & Mode of the Engagement: (Attach the quotation for the consumables)

(i) Short term Engagement:

a. Usage of testing/characterization facilities across the country (Characterization Mode)

i. Consumable charges

Sl. No.	Consumables	Expected charges
1.	cells	15000.00
2.	96 well plates/ Culture flasks/tips	30000.00
3.	MTT/ATP assay kits	35000.00
4.	Medium/ trypsin/ antibiotics	20000.00
5.	Drugs/small molecules	30000.00
Total		130,000.00 INR

ii. Travel Cost (Boarding and Lodging, if any)

The total expense is estimated to be:

Sl. No.	Category	Amount (INR)
1	Consumables	1,30,000.00
Total expenses		1,30,000.00

Details of User's Bank Account:

Beneficiary Name (User's name): NAVAMI KRISHNA

PAN: DQQPK0986J

Bank Account No: - 20488605047

Name of the Branch: SBI NIT Calicut branch

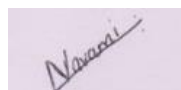
IFSC Code: SBIN0002207

Note: Proposed proposal must be executed within stipulated duration (max. 6 month) to avoid fund availability issues. Travel form will be provided by the I-STEM office. The users will submit the technical report, original invoice (duly signed by the Head of the Institution) raised by the Host Institution through I-STEM portal only along with a copy of the travel forms and original tickets through post to the Nodal Officer, I-STEM, Indian Institute of Science, Bangalore. A scan copy of all the documents may be sent in advance.

Declaration:

We have read the terms and conditions for the I-STEM scheme under “*Partial financial support for using R&D facilities through the I-STEM web portal to Users from Academic Institutions and Start-ups (Proposal Format-1)*”

a) Researcher/ PhD scholar: NAVAMI KRISHNA



b) Faculty member/ Guide: Dr. RAJANIKANT G.K



12-05-2022

NIT Calicut