DRUG DISCOVERY AGAINST COVID-19 : EXPLORATION OF NEW CHEMICAL SPACES TO IDENTIFY NEW DRUGS AGAINST SARS-Cov2

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Identification of new chemical hits against COVID19

No recent event in collective human memory has had the breadth and impact of the SARS-Cov2 virus, that has caused the ongoing Covid19 pandemic. The scientific community has put up a heroic response, making SARS-Cov2 one of the most well studied virus within a year's time. Several vaccines have been made in record time and immunisation programs started globally in an effort to combat the virus and contain the disease. Nevertheless, the pandemic continues its relentless march, with infection and death rates at record levels in many parts of the world such as USA and Europe. Although the infection rates seem to have crossed its peak in India, the spectre of a second wave – as seen elsewhere – presents a looming threat. The emergence of new variants such as the UK and South African strains attests to the evolution and robustness of the virus and heavily underscores the need to remain ever vigilant in this war against the virus. New strategies to combat the disease, new methods to enable faster and rapid detection, new drugs to treat the disease and mitigate symptoms are therefore the need of the hour.

Need for new drugs

As the Covid-19 pandemic spread around the globe, efforts to discover vaccine and effective drugs began. In record time, several vaccines working though different technologies have been developed and approved and mass immunization programs started in many countries. However, this does not dampen the need for new drugs for the following reasons. First, immunization programs are layered, with different countries evolving policies on who should be vaccinated first. Thus tremendous logistic – and unprecedented - challenge of vaccinating an entire population in any given region remains to be met. This could provide the virus time to mutate. Second, while the vaccines are highly efficacious in clinical trials, their long term protection ability in populations is not tested. Thus, new anti-viral drugs are absolutely necessary to combat the pandemic. Several groups around the world have screened FDA approved compounds to find anti Covid-19 drugs using a re-purposing strategy. The identification of similar molecules in many of these screens suggest that the chemical spaces represented by this set of compounds is saturated.

In this proposal, we aim to utilise our strength and expertise in cell biology, infection assays and plant biology to develop and perform high-throughput drug screens to identify novel chemical molecules that will be effective against SARS-Cov2 and have the potential for rapid development to clinics.

Preliminary work done in our lab so far

To understand the pathogenesis mechanisms of Covid-19 virus and perform drug screening studies, we need to establish systems to infect cells with Covid-19 virus *in vitro*. The first step towards this is to propagate the virus in vitro culture. Covid-19 virus has been successfully isolated from patient samples and cultured in cell lines elsewhere and detailed protocols available (Harcourt et al, 2020; Kim et al, 2020; Touret et al, 2020). The major challenges involved in successful propagation of the virus and establishment of in vitro culture system is having appropriate biosafety level 3 infrastructure and permissions from statutory committees. We have established a BSL3 suite facility in NCBS with a space exclusively earmarked for the proposed Covid19 related work following appropriate international guidelines (CDC, WHO, DBT). Using this infrastructure, we have now successfully established a SARS-Cov2 infection assay in different cell lines, namely the African Green Monkey kidney derived cell lines Vero and VeroE6, and human cell lines A549 and HEK expressing ACE2, using samples from Covid19 patients (Figure 1).

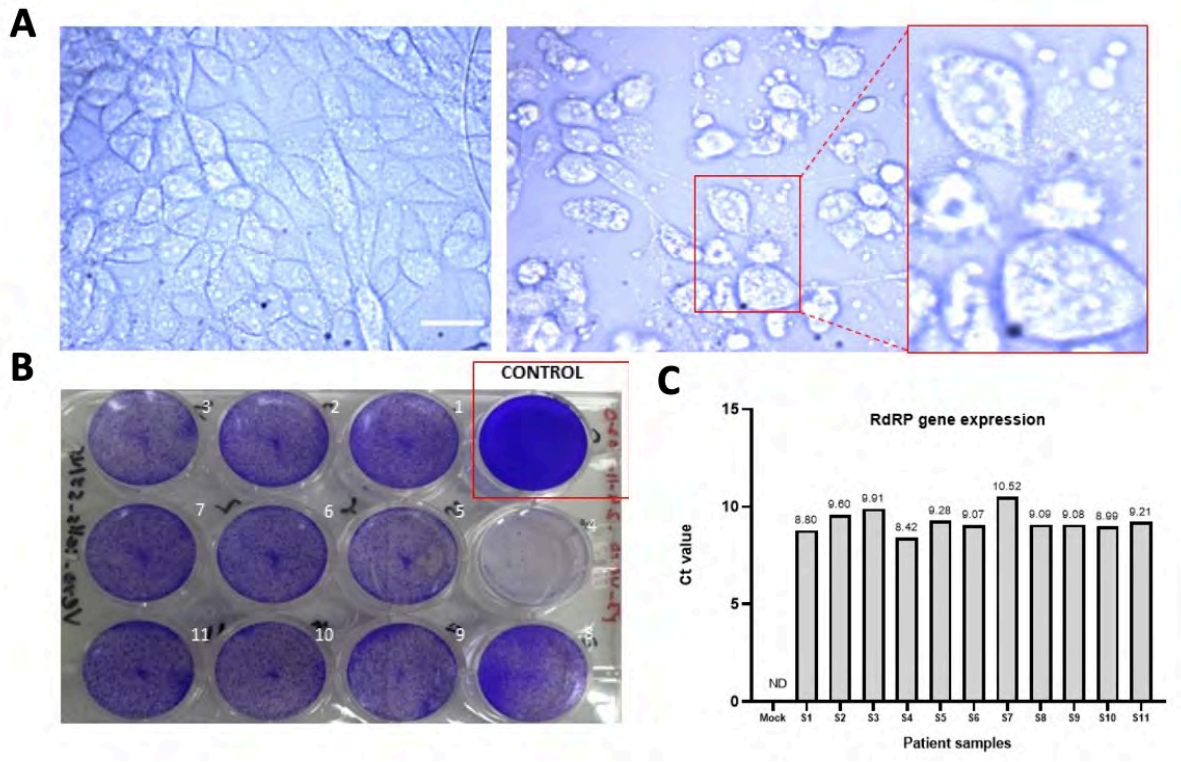

Figure 1. Isolation and propagation of SARS-Cov2 virus. A. Vero cells are infected with SARS-Cov2 for 72 hours and images taken under bright field microscope. Clear signs of cytotoxicity and cell lysis are seen in virus treated cells (right) compared to untreated cells (left). B. Vero cells infected with 11 different clinical isolates of SARS-Cov2 for 72 hours and stained with crystal violet. Control cells that are not lysed are fully blue indicating cell viability, while cells incubated with SARS-Cov2 undergo cytolysis. C. RNA was extracted from the supernatant from the cells infected with the 11 strains and subjected to RT-PCR with SARS-Cov2 specific primer. All 11 strains show amplification with low Ct values indicating high viral load.

With these infections, we have optimised work-flows to characterize the virus using a variety of approaches including cytotoxicity assays, RT-PCR, plaque formation assays and immune-fluorescence assays (Figure 2). These assays serve as a robust platform to launch our campaign for drug discovery against SARS-Cov2.

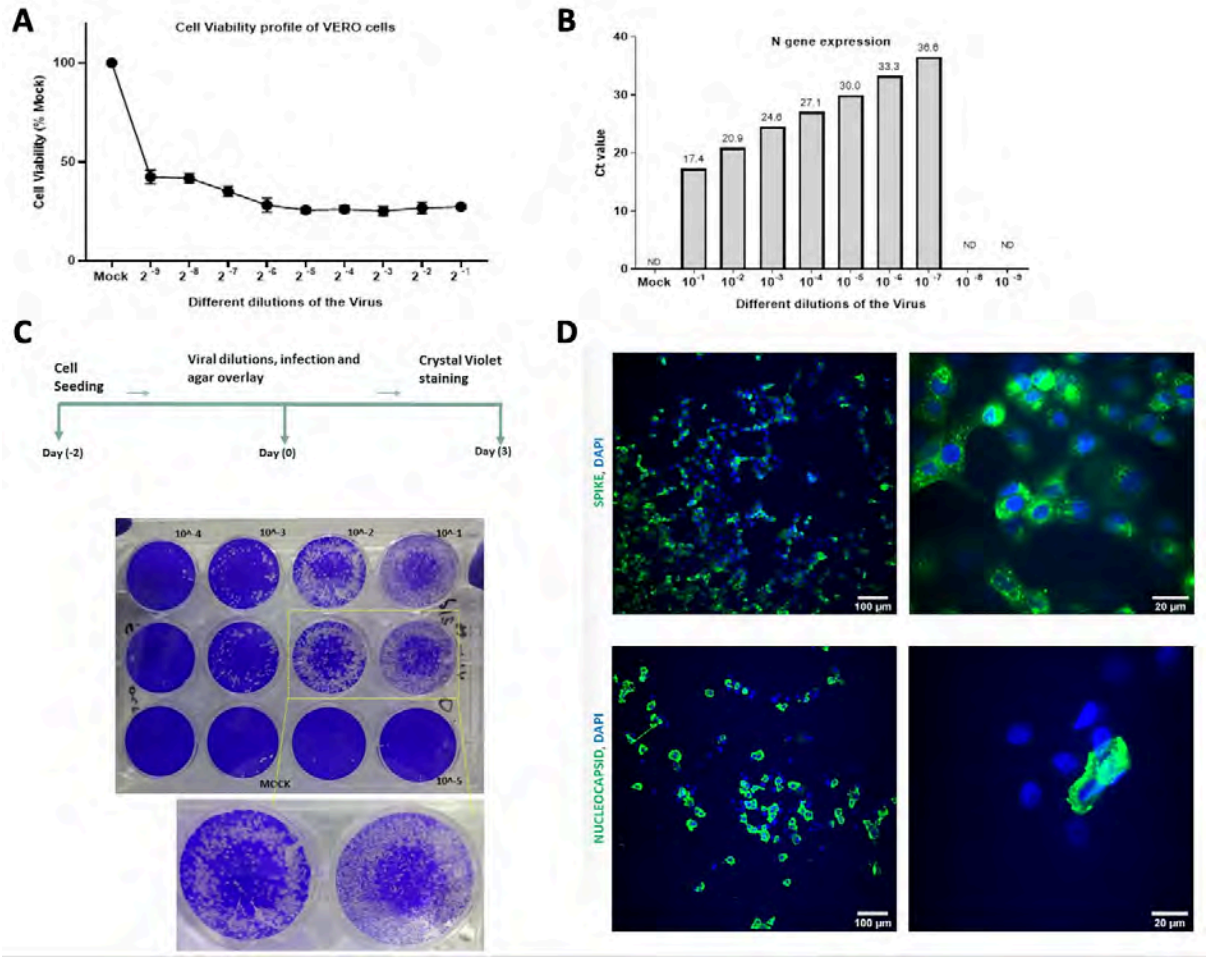

Figure 2. Characterization of SARS-Cov2 virus. A. Vero cells are infected with different dilutions of SARS-Cov2 for 72 hours. Cytotoxicity assessed using CellTitreGlow reagent. Graph shows the dose dependent cytotoxicity of cells. B. RT-PCR analysis of supernatant from cells infected with different doses of SARS-Cov2 virus showing dose dependent response. C. Plaque forming unit (PFU) assays are performed following the scheme given. Plates with plaques show clear dose dependent patterns. D. SARS-Cov2 infected cells are immunostained with different antibodies against spike (top) or nucleocapsid (bottom) proteins to detect infected cells (green), and imaged with confocal microscope. Host cell nuclei is stained with DAPI (blue)

Using the defined viral titres, we have set up a drug testing and screening platform to screen drugs active against SARS-Cov2 virus.

In this proposal, we aim to utilise this platform with the following two objectives.

1. **High throughput screen of 18,000 compound diversity library from Dundee University**
2. **Screen of a home made natural product library from Rubiaceae family**

Both the libraries proposed to be screened here are enriched for bioactive compounds that have high potential to be drug-like. While the Dundee library is hand-picked for such compounds, Rubiaceae family is well known to contain bioactive compounds with known anti-

viral properties (see below). Identification of such hits will hugely accelerate drug discovery and enrich the known chemical scaffolds with anti SARS-Cov2 activity.

Objectives of the proposal:
In this proposal, we aim to utilise two different approaches for drug discovery against SARS-Cov2.

a. <u>High throughput screen of drug-like molecules in SARS-Cov2 infection assays</u>

We propose to screen a set of 18,000 compounds from the Diversity library from University of Dundee, Drug Discovery Center. This set of compounds is carefully chosen to represent different chemical scaffolds and are selected to serve as excellent starting points for medicinal chemistry optimisations. Several compounds from this collection have been screened in other phenotypic screens for infectious diseases, with a good hit rate (Abraham et al, 2020; Love et al, 2017), indicating bioavailability and bioactivity. This library is available in the Screening facility in NCBS.

High throughput screening
Screening will be performed in 384 well plates. Briefly, compounds will be pre-added to 384 well plates at 10 μM concentration and Vero cells infected with SARS-Cov2 virus at a low MOI (0.1) will be added to wells. Cells will be incubated with compounds for 72 hours, followed by addition of CellTitreGlow reagent to measure cell viability using a luminescence in a multi-mode plate reader. Cells uninfected with the virus will be used as a normalizing control, while infected cells treated with the vehicle (0.1% DMSO) will be treated as negative control. Compounds known to kill the virus, and therefore prevent virus induced cell death, such as Remdesvir will be used as positive control. The screen will be performed in duplicate.

Hit selection and validation assays
Virus induced cell death is the primary read out of the assay. Compounds that overcome the virus induced cell death will be considered as hits. We will identify the hits based on their reproducibility in the two screens and the distance from the negative control. Since the library is chosen for bioactive compounds, the hit rate is expected to be higher than the conventional 1%. We will choose the top 25 hit compounds, and source it independently from Molport. Verifying activity of the same compound from an independent source is essential to ensure that the hits obtained are robust. We will perform dose response using cytotoxicity and RT-PCR assays using atleast 7 different dilutions of the compounds to generate the IC50 values. Cytotoxocity assays will be performed in uninfected cells at concentrations higher than the IC50 to ensure that the compounds are not toxic.

b. <u>Screen a set of natural products for anti-Covid19 effects.</u>

We will screen library of plant-based extracts with putative antiviral properties, utilized in traditional medicine. Of particular interest will be plant-based extracts with putative antiviral properties sourced from the Rubiaceae family. A wide variety of active phytochemicals derived from Rubiaceae members such as quinines, quinidines, flavonoids, terpenoids, lignans, sulphides, polyphenolics, coumarins, saponins, furyl compounds, alkaloids, polyines and thiophenes have been identified as novel antiviral agents (Jassim and Naji, 2003; Perez, 2003; Kapoor et al., 2017; Benelli et al., 2017, Savarino et al., 2003). Several of these phytochemicals have complementary and overlapping mechanisms of action, including inhibition of entry, replication of viral nucleic acids or suppression of the activities of viral proteins.

The most common pathway that generates strong bioactive compounds in this family is the acetate-polymalonate pathway. From a literature search of precursors and pathway genes of this pathway and the presence of phytochemicals, we find that members of Rubiaceae with similar phytochemical properties available locally are Ixora spp., *Pentas lanceolata*, *Mussaenda frondosa*, *Coffea arabica*, members of the order Gentianales (in which Rubiaceae belongs to) such as *Strychnos* spp., *Mitragyna speciosa*, members of Lamiaceae such as *Ocimum sanctum*, *Plectranthus* spp. (Bekut et al., 2018; Benelli et al., 2017; Cui et al., 2010; Drevinskas et a., 2018). Among these Ixora, Mussaenda, Strychnos, Plectranthus, Mitragyna, Pentas have been referred richly in folk medicine against viral diseases, and also backed with scientific literature (Jassim and Naji, 2003; Kapoor et al., 2017; Nolkemper, 2006).Using the commonly used extraction and available traditional methods, 8 variants of the concoctions will be prepared in the lab from the above mentioned plants.

Extraction methods to be used are already well-described in Nolkemper et al., 2006; El-Awady et al., 2014; Donalisio et al., 2013. These extracts will be tested in SARS-Cov2 infection assays. Extracts that show activity will be analyzed in house. In further studies, we will fractionate the active extracts and test the fractions for anti-Covid19 effect. This will help us identify the active ingredient in the fractions.

Deliverables

The major deliverable of this aim is

a) Generation of hit list from Diversity library against SARS-Cov2
b) IC_{50} values for most prominent hits
c) Identification of natural products active against SARS-Cov2

Expected Outcome

We expect to generate a set of strong hits from the diversity library that can be used as starting points for hit-to-lead discovery. We also expect to assemble, test and characterize a natural product library and identify active ingredients from this.

The expected outcome of this proposal is this to generate a set of hits from screening a bioactive drug-like library and natural product library. Once the hits and fractions are identified, we will partner with interested drug discovery companies to develop the hits into leads for Covid19 drug discovery.

Deployment plan and timelines

SARS-Cov2 infection assays are established. They will be miniaturized to 384 well format for drug screening in 1-3 months. Dundee library will be aliquoted and ready for screening in 1-3 months. High throughput screening will be performed in duplicate in 3-4 months. Screen will be analysed and hits selected by 3-4 months, validation assays performed in 4-6 months.

Plant extracts will be prepared fresh, protocols for extraction are optimized. They will be tested in uninfected cells for toxicity in 1-2 months. Extracts will be tested in SARS-Cov2 infection assays in 2-4 months. Selected hit mixtures will be fractionated and infection assays performed on purified fractions to identify active ingredient in 4-6 months.

Expertise of the groups involved:

Dr. Sundaramurthy's laboratory works on the interface between host and intracellular pathogens. Of particular interest is the trafficking pathways of the host cell, primarily endocytic and autophagic pathways, which eventually fuse with lysosomes. Ongoing work is investigating the relationship of these pathways in the context of intracellular M. tuberculosis infection and modulation of these pathways towards interfering with pathogenesis mechanisms. His expertise inlcude high content quantitative imaging, automated image analysis and chemical perturbation of the endo-lysosomal pathways. Of particular interest for

this proposal is their expertise in BSL3 infections and phenotypic screens in cellular infection models with compound libraries.

Dr. P.V. Shivaprasad is a plant virologist with expertise in biochemistry, genetics and small RNA mediated epigenetics. He has used RNAi based immunity to cure DNA viral disease in mungbean, first such attempt against any DNA virus. He identified how host factors (miRNAs, resistance genes) and DNA/RNA viral pathogenicity factors (that act against host defence) counteract in diverse model systems in both DNA and RNA viruses. He has developed efficient strategies for virus resistance in crops and model systems. Currently his lab focuses on a plant geminivirus, looking at various aspects of host-pathogen interactions. Of particular interest to this proposal, Shivaprasad lab has used various methods to look for methylgloxalase and glyoxalase activities in a variety of medicinal plants, and similar approach will be used in the plants mentioned above.

Proposed budget:

Items	**Cost (INR)**	**Justification**
Cell culture reagents, Consumables, plastic ware	7,50,000	
Detection kits, qRT-PCR reagents	5,00,000	Includes mycoplasma detection, cell viability measurement kits
BSL3 and screening facility access costs	7,50,000	To cover the facility access charges
Cell culture microscope	7,50,000	For use in BSL3
Multimode reader	30,00,000	For plate measurements in BSL3 for cytotoxicity assays
CO2 incubator	5,00,000	Spare incubator in BSL3
Post-doc level scientist	5,40,000	Responsible for planning and execution of the proposed experiments
Tecan repair cost	7,50,000	Robotic arm of existing Tecan liquid handler needs part replacement
Library aliquoting, plant extraction, fractionation	15,00,000	Costs include tips and plates for library aliquoting and daughter plates; for production of plant extracts
Total	87,90,000	

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