In-Vitro Fluorescence Microscopy Studies Show Retention of Spike-Protein (SARS-Cov-2) on Cell Membrane in the Presence of Amodiaquin Dihydrochloride Dihydrate Drug

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The ability of S-glycoprotein (S-protein) in SARS-Cov-2 to bind to the host cell receptor protein (angiotensinconverting enzyme 2 (ACE2)) leading to its entry in cellular system determines its contagious index and global spread. Three available drugs (Riboflavin, Amodiaquin dihydrochloride dihydrate (ADD) and Remidesivir) were investigated to understand the kinetics of S-protein and its entry inside a cellular environment. Optical microscopy and fluorescence-based assays on 293T cells (transfected with ACE2 plasmid) were used as the preamble for assessing the behaviour of S-protein in the presence of these drugs for the first 12 hours post S-protein - ACE2 binding. Preliminary results suggest relatively long retention of S-protein on the cell membrane in the presence of ADD drug. Evident from the %-overlap and colocalization of S-protein with endosome studies, a large fraction of S-protein entering the cell escape endosomal degradation process, suggesting S-protein takes non-endocytic mediated entry in the presence of ADD, whereas in the presence of Riboflavin, S-protein carry out normal endocytic pathway, comparable to control (no drug) group. Therefore, present study indicates ADD potentially affects S-protein's entry mechanism (endocytic pathway) in addition to its reported target action mechanism. Hence, ADD substantially interfere with S-protein cellular entrance mechanism. However, further detailed studies at molecular scale will clarify our understanding of exact intermediate molecular processes. The present study (based on limited data) reveal ADD could be potential candidate to manage Covid-19 functions through yet unknown molecular mechanism.

The rapidity with which SARS-Cov-2 is transmitted is unprecedented and the rapid mutation rate resulting in genetic changes, particularly in the spike protein [1], requires special attention to the binding affinity and action mechanism of Spike protein (S-protein) with cell receptor [3]. The ongoing COVID-19 pandemic and many such infections are likely to attract such pandemic emergencies in future with close human-to-human contacts. With rise in global temperature, the situation has become favourable for viruses and bacteria to flourish and emerge in new climate zones where it was never known to exist [4] [5]. Covid-19 may just be the beginning of such pandemic emergencies and frequency of such pandemics are more likely to occur in future.

In the midst of pandemic, we are possibly well-placed to understand the rapidity of transmission, and prevention but not eradication. At the core of this crisis lies the basic understanding of many biological aspects that are currently unknown and the true picture is more likely to emerge in near future. However, the virus SARS-Cov-2 has striking similarities with SARS coronavirus [6]. The host-virus interaction has been well understood , primarily the spike (S) glycoprotein of SARS-Cov-2 that binds with angiotensin converting enzyme 2 (ACE2) on host cell-surface with high affinity primarily by its surface S-glycoprotein [7] [8] [9]. The structural basis of receptor recognition by receptor-binding domain (RBD) of the S-protein of SARS-CoV-2 with the ACE2 reported in details by Shang et al. [10], reveals the interaction of ACE2 with RBD of S-protein result in conformational rearrangements of S-protein that could be triggered due to receptor binding with the RBD at cell surface or a change in pH within endosomal environment or both [11].

Present report evaluates S-protein fate upon binding with ACE-2 receptor and estimates how re-purposed drugs may affect S-protein action mechanism. The three re-purposed drugs chosen were Riboflavin [12] [21], Amodiaquin Dihydrochloride Dihydrate (ADD) [15], and Remidesivir [13]. Recent study on bulk whole blood have shown that Riboflavin + UV study significantly could reduce the SARS-CoV-2 titre both in plasma and platelet. However, mechanism of action is still unknown. The ADD second test drug, an FDA-approved drug for anti-malarial, anti-Ebola activity [14] [15], is also in the list of re-purposed drug that could inhibit SARS-Cov-2 [16]. The third drug Remidesivir, an established antiviral drug could also inhibit SARS-CoV-2 viral titre compared to control [13]. Further, in-vitro and in-vivo study report suggested Remdesivir could be suitable for viral prophylaxis [17] reportedly by inhibition of SARS-CoV-2 virus RNA-dependent RNA polymerase [18], however possibility other mechanism has not been explored. Therefore, lack of understanding about the action of these re-purposed drugs may be the leading cause of evaluating exact potential of these drugs. However, such studies are extremely useful in the middle of a pandemic. Understanding individual proteins rather than complete virus may provide keys to understand virus/host cell receptor binding and its kinetics in the presence of external agent (drugs). As a first preliminary study, present report utilizes above-mentioned three drug candidates to understand kinetics of S-protein upon binding with ACE2-receptor.

Recent studies indicate the efficiency of a specific protein complex (Spike-protein) that binds to the cell ACE2 receptor leading to its entry in target cells [3] [25] [26] [27]. To tackle and manage the virus, several potential neutralizing agents have been proposed but the virus has managed to escape by mutation [28] [29] [30] [31] [32] [34]. Peptide derivatives of ACE2 has shown promise as a potent inhibitor [35]. Our aim is to understand the drug action mechanism at the S-protein to ACE2 receptor level inside a cellular system in the presence of these drugs.

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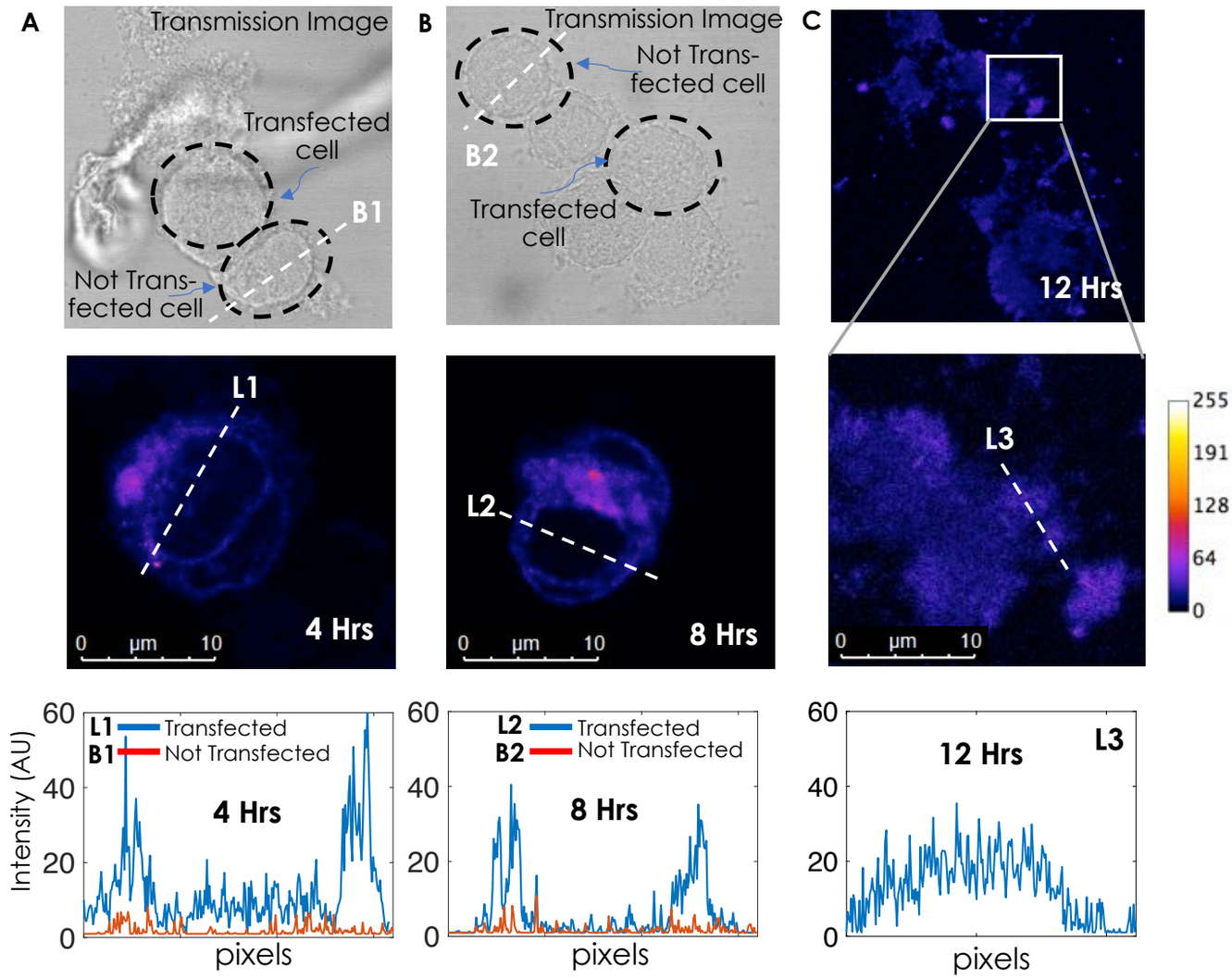

FIG. 1: S-protein (GFP-S) binding with ACE2 transfected 293T cells over time. A) 4 hrs and B) 8 hrs GFP-S treated cells. Column represents: Top- Cells under transmission light; Middle- GFP-S/ACE2 positive cell under fluorescent light (excitation: 488 nm; Emission:520 nm); Bottom: Intensity vs pixel plots, L1 and L2 line represent the distribution of S-protein in transfected cell, whereas, B1 and B2 line represent background cell intensity (non-transfected cell). C) 12 hrs GFP-S/ACE2 positive cells under fluorescent light (top), single cell area magnified image (middle), and the intensity vs pixel plots (bottom), where L3 represent the distribution of S-protein in transfected cell.

In this brief report, we provide preliminary comparative study of Riboflavin, ADD and Remidesivir drugs effect on the kinetics of S-protein. Precisely, we studied effect of these drugs on the S-protein entry pathway. The study is concentrated on an isolated S-protein of SARS-Cov-2 virus and does not involve the entire virus. So, the results described in this report may be considered limiting in nature.

I. RESULTS

Fluorescence imaging based investigation has emerged as one of the conclusive evidences for investigating diseases and therapy. The studies involve following up the biological processes using high resolution confocal microscopy.

A. S-protein binding study with ACE2-transfected cells

It is well studied that the RBD of the SARS-CoV2 S-protein efficiently binds with human ACE2 expressed on the surface of 293T cells [3] [33]. In the same direction, to study the S-protein binding with ACE2 on cell surface, ACE2 transfected 293T cells were treated with GFP tagged S-protein (GFP-S) and the binding was evaluated by fluorescent microscopy over time. Figure 1, shows GFP-S protein with the ACE2 transfected 293T cells in absence of treatment. The intensity plots were carried out alongside (Fig. 1 bottom row) shows only those 293T cells that were transfected expresses ACE2 receptors. Upon 4 h treatment, S-protein (GFP-S) binds only to those cells that expresses ACE2 receptors (Fig. 1A), whereas low transfected 293T cells don't show S-protein binding and have only background intensity (see B1, B2 intensity plots in Fig. 1). This is evident from the corresponding intensity plots at 4 hrs and 8 hrs (Fig. 1A and 1B). However, S-protein seems to uniformly disperse in the cell after 12 hrs. This is evident from overall reduced intensity throughout 293T cells (see, L1, L2, L3 in Fig. 1). Therefore, the study illustrates S-protein's specific binding affinity with ACE2 receptors on cell surface. This is in-line with the studies reported recently by Procko's group [2] [3].

To comparatively evaluate drug effect on S-protein binding,

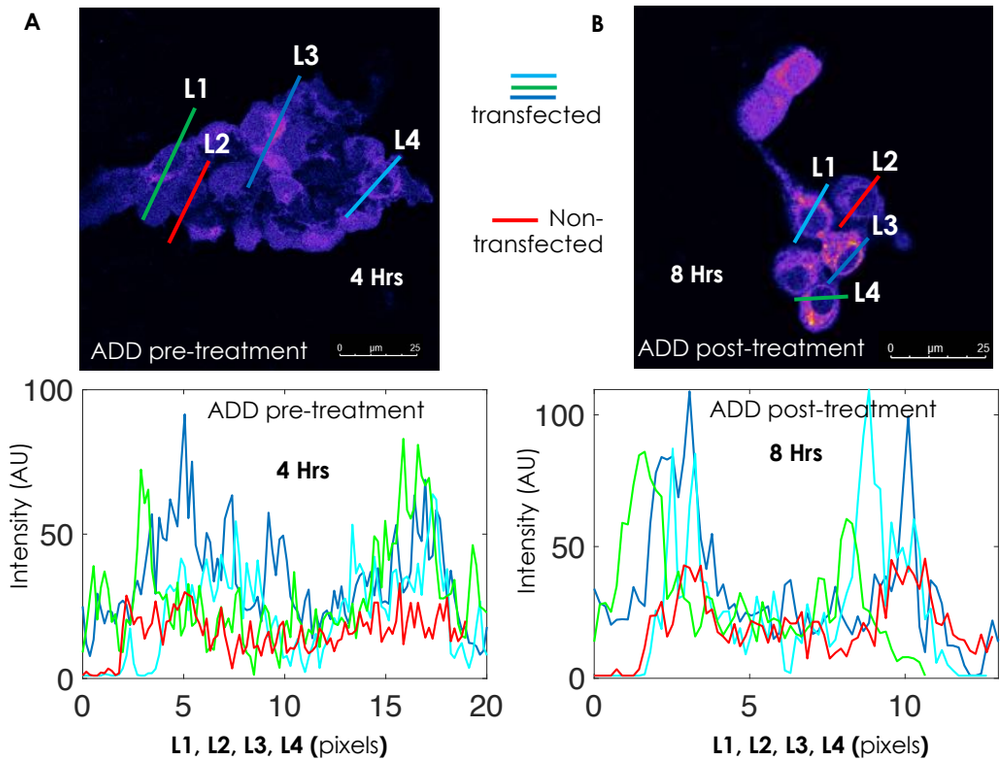

FIG. 2: Comparative S-protein (GFP-S) binding study. A) ADD pre-treated (15 mins ADD treatment prior to 4 hrs GFP-S incubation). B) 8 hrs post-treatment: 4 hrs GFP-S incubated ADD pre-treated cells were washed-off and followed 4 hrs in fresh media (with no GFP-S and ADD). Graphs (below) represents fluorescent intensity through the line L1, L3, and L4 (sky-blue, green and red lines) of GFP/ACE2 bound cells vs line L2 (red) for background cell intensity.

two sets of experiments were carried out (Fig. 2). In ADD pre-treated set (4 hrs S-protein incubated ADD pre-treated cells, Fig. 2A), the S-protein binding in presence of drug were found predominantly on cellular periphery in presence of ADD (Fig. 2A, sky-blue, green and red lines), indicating ADD does not interrupt S-protein binding with ACE2 and S-protein remains adherence on cellular surface. Whereas post-treated cells (8 hrs, Fig. 2B, sky-blue, green and red lines), ADD pre-treated cells (15 minutes) were incubated with GFP-S for 4 hrs and after free GFP-S wash-off followed over another 4 hrs making it a total of 8 hrs study. The results show S-protein still remained adhered to the cell surface. In both conditions, the S-protein binding to ACE2 transfected 293T cells were not affected, demonstrating tested drugs (ADD) doesn't affects the S-protein binding with ACE2 receptors. However, over-time the S-protein shows clustering effect (GFP-S intensity increased, Fig. 2B).

B. Drug effect on S-protein intracellular fate

The S-protein (GFP-S) binding and its intracellular pathway mechanism was evaluated following early and late endosomal signal using LAMP-1 antigen/IgG-AlexaFluor 594 lysosome labelled ACE2 transfected 293T cells. Figure 3, demonstrated comparative S-protein (GFP-S, green) binding with ACE2 and its endosomal (LAMP-1, red) fate study of three different re-purposed drug candidates (Riboflavin, ADD and Remidesivir) compared to the control group (untreated). As expected, in case of control (untreated cells), S-protein (green) were endocytosed and enters endocytic pathway (late endosome, red) (Fig. 3, left column) [24]. Similar result was observed in case of Riboflavin and Remidesivir treated cells (Fig. 3, second and fourth column). This demonstrates that the drug treatment doesn't interferes with the S-protein binding and intracellular uptake mechanism / endocytic pathway (as evident from %- overlap analysis, Fig. 3B). However, ADD shows negligible colocalization illustrating that even after 12 hrs of treatment S-protein doesn't enters endosomes (S-protein not present in endosomes, see Fig. 3, third column). Colocalization studies (Fig. 3, fourth row) indicate strong proximity of S-protein and endosomes in 293T cells when treated with Riboflavin (Pearson's correlation coefficient, $r = 0.51$), whereas the least colocalization is reported in the presence of ADD ($r = 0.19$), and Remidesivir (r=0.32, similar to untreated control (r=0.33)). In addition, associated intensity plots (L1-L4, Fig. 3, third row) provides a direct evidence. As reflected from 3D cross-section analysis of a cell treated with ADD (Figure 4), it appears that S-proteins are mainly located on the cell membrane post 12 hrs of S-protein - ACE2 binding. Therefore, present study indicated ADD treatment interferes with S-protein cellular endosomal entrance pathway. In addition, ADD predictively prolongs S-protein retention on the cell surface.

II. CONCLUSION AND DISCUSSION

The high infection and death rate of SARS-CoV-2 virus has necessitated quick response to current pandemic. In this study, three re-purposed drugs (Riboflavin, Remidesivir and ADD) were investigated to understand their effect on S-protein (SARS-CoV-2 surface protein) kinetics inside and at the surface of 293T cells. The ADD, Remidesivir and Riboflavin drugs were chosen due to their reported potency against Ebola and Covid-19 [15] [21] [17]. Preliminary findings and investigations were carried out that suggest distinct effect of these drugs on mediated SARS-CoA-2 entry mechanism.

The florescence microscopy based study indicates ADD drug prolongs S-protein surface retention (Fig. 2) and blocks endosomal entry of S-protein (Figure 3 and 4). The present data reveals that ADD drug have efficacy in Covid-19 treatment predictively may be by blocking SARS-CoV-2 endosomal entry (preventing pH dependent S-protein conformational change for needed viral entry [11]) as well

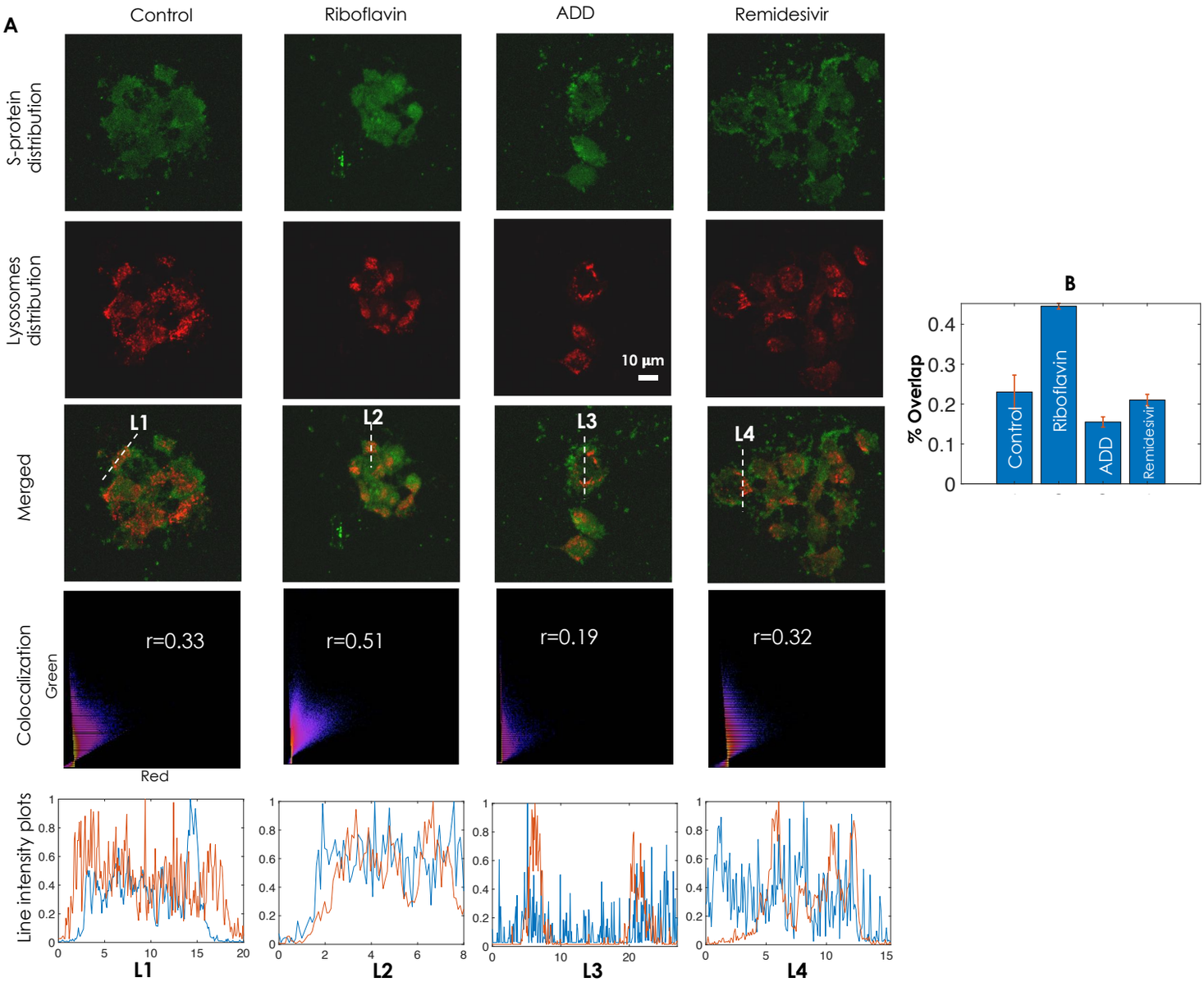

FIG. 3: Effect of drugs (Riboflavin, ADD and Remidesivir) on S-protein (GFP-S) distribution and endosomal colocalization study after 12 hours treatment, in comparison to drug untreated control. A) Fluorescent images showing GFP-S (green) distribution and endosomal (red, LAMP1-AlexaFluor 594) colocalization. The colocalization efficiency was evaluated based Pearson's correlation coefficient, r analysis (4th row) and the line intensity plot (5th row) represent respective line's fluorescent intensity (GFP-S-blue and Endosome-red) curve. B) The % overlap represent percentage GFP-S protein localization in endosomes in present or absence (control) of treatment.

as prolong surface retention of virus on cell surface. This is important since it gives longer time for immune system to act and facilitate medical diagnosis.

In view of the present work being preliminary with limited data, our findings require more investigation and trials for conclusive evidence of the proposed mechanism. We also plan to carry out studies at different time points and with different concentrations of the proposed drug candidates. In addition, detailed investigation may bring out a clear picture and possible modifications for the mechanisms involved. However, from present study, it can be speculated that ADD may help moderate Covid-19 symptoms at the time of Covid-19 crises.

III. METHODS AND PROTOCOLS

A. Study Protocol

In order to investigate the effect of these potential anti-malarial / anti-viral drug candidates on the binding efficiency (of spike protein and cell receptor) and its translation in the cellular system, we developed a simple study protocol as shown in Fig. 5. The central idea is to study both short and moderate time effect of these drugs on 293T cells with S-protein attached to its surface. Step 1 involved the extraction of S-protein primarily based on protocols developed by Procko's group [3]. The study is broadly categorized into three steps: (1) S-protein extraction, (2) ACE2 receptor transfection of 293 cells, and (3) Drug interaction on S-protein kinetics over time.

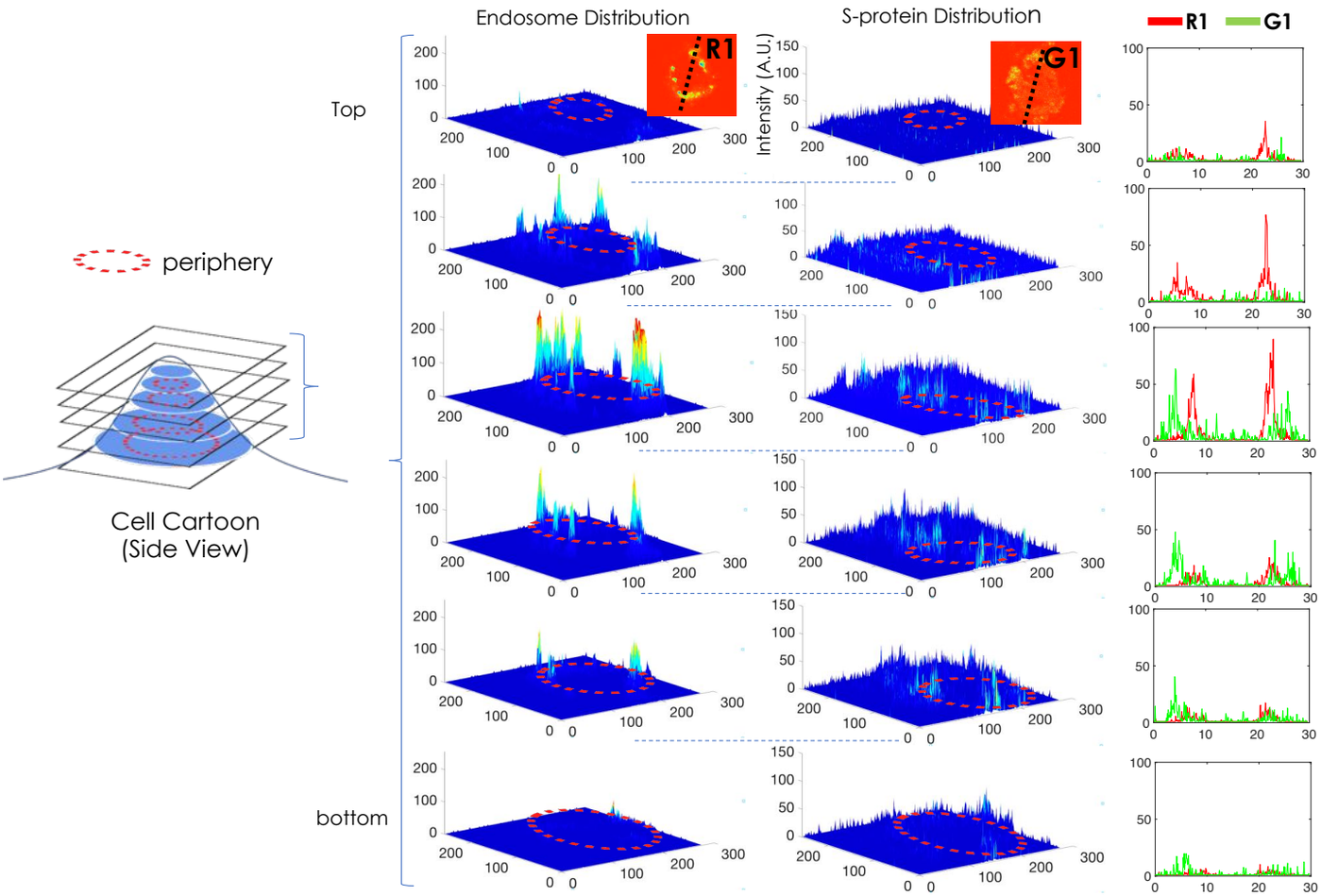

FIG. 4: Fluorescent intensity over 3D plane of a 12 hrs ADD treated and GFP-S bound ACE2 transfected 293T cell. A) Side view of a cell cartoon with slices from different planes (top to bottom) is shown. B) 3D surface plots show fluorescent intensity at the respective z-axis surface (all the planes), illustrating the distribution of Endosomes (left column) and S-protein (right column). C) The graphical presentation of fluorescent line intensity of endosome (LAMP1, R1) and S-protein (GFP-S, G1) across the cell in each plane (inset image shows the line across the cell). The line intensity graph shows S-protein (green line) are at the periphery and doesn't overlap with endosomes (LAMP1, red line).

The first step towards S-protein study involves the extraction of S-protein through mamallian cell culture. 293T cells were transfected using standard Lipofectamine based protocol. This involves mixing equal proportion of RBD-plasmid (Mammalian expression plasmid for RBD of SARS-CoV-2 protein S (spike) fused with sfGFP) and Lipofectamine to prepare complexes that were added to healthy 293T cells. The protein is secreted. Subsequently, the media from 293T cells secreting the SARS-Cov-2 RBD fused to sfGFP was collected after 72 hrs. The expression medium is then centrifuged to remove cell debris and used for measuring binding. The media containing the protein is directly used as such without purification [2] [3]. No separate protein isolation is carried out.

The next logical step is preparation of 293T cells for drug interaction study. In step 2, a new set of healthy 293T cells were transfected with ACE2 plasmid in a 12-well plate (both with and without coverslip). Coverslip based cell culture and preparation are useful for Confocal microscopy studies. Lipofectamine based protocol with ACE2 plasmid were used to prepare the complexes and added to cells cultured in 12-well plate. In about 20 hrs, about 20 percent of cells were found to be transfected (as seen in fluorescence microscope with blue light excitation) and are ready for next step.

The final step (step 3) is the drug interaction study where these transfected cells were incubated with extracted S-protein medium. The binding efficiency of S-protein (SARS-Cov-2 RBD) labelled with sfGFP was visualized using a blue light (470-490 nm) using a standard inverted fluorescence microscope. Subsequently, the drug is added and these cells (GFP labelled on the cell membrane) were visualized. Finally the cells were fixed at specific time points (12 hrs) and prepared for confocal microscopy studies.

B. Cell Culture

293T cells were cultured using the standard protocol. Cells were cultured in in a 35 mm and 12-well plate supplemented with medium (DMEM and FBS along with antibiotic (Ampicillin)). The cells were plated at 100, 000 counts and incubated in CO2 incubator. After each 24 hrs, the cell media is removed, cells were washed with PBS to remove dead cells and fresh media was added.

C. Cell Transfection with ACE2

HEK293T cells were used as host for the present study. Since, HEK293 cells do not express any appreciable level of

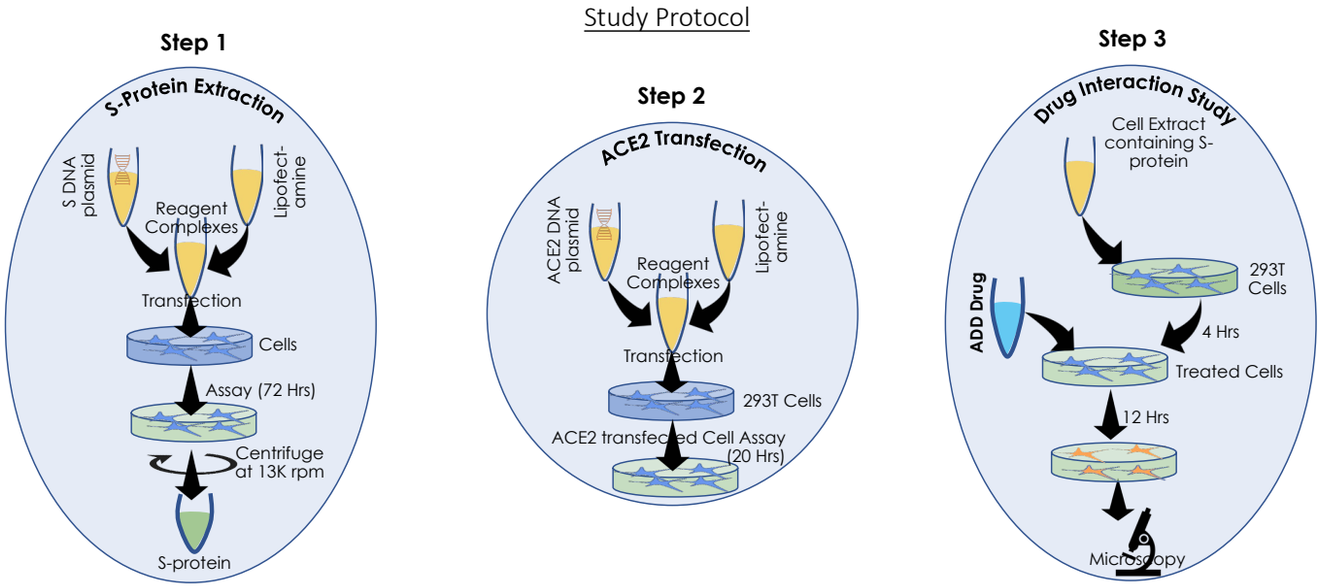

FIG. 5: The study protocol for Spike protein extraction, ACE2 transfection and drug interaction study.

ACE2 and thus are not susceptible to SARS-CoV-2 infection. Hence, the cells were first transfected with an ACE2 plasmid (name, "pcDNA3.1-hACE2" from AddGene) in order to see binding of the RBD at the surface. Lipofectamine based protocol as described in step 2 of Fig. 5 was used to transfect the cells. For trensfection, antibiotic free medium was used. The cells were incubated for 12 hrs and subsequently the drug interaction study was carried out. This has enabled high efficiency binding of S-protein on host cell surface. These cells are used as a preamble to understand the effect of S-protein-ACE2 binding and its entry in cellular system.

D. Drug Preparation

The drugs, Amodiaquin dihydrochloride dihydrate and Ribosome were purchased from Sigma Aldritch. DMEM was used to prepare a 250 nM solution of Ribosome, whereas, DMSO is used to prepared 1mM solution of ADD and finally diluted with DMEM to obtain 250 nM solution. In the present study, we have used the above mentioned concentrations although we have seen similar effects at lower concentration as well for which the studies are not reported here. For each study, we have used 200 μL of respective drug solution.

E. S-Protein Extraction using 293T cells as Host

Healthy 293 cells were cultured for extracting S-protein. The cells were thawn and plated in a 35 mm disc with cell medium (95%DMEM + 4.5%FBS + 0.5%Ampicillin). After 4 hrs, the cells were washed with PBS and fresh media was added. The cells were incubated for 48 hrs in a CO2 incubator. A confluency of $>$ 70% is ensured. In the next possage (passage #22) the cells were plated in a fresh 35 mm plate. Following transfection protocol as mentioned in Fig. 1 (step 1), the cells were transfected with SARS-Cov-2 RBD plasmid and left for 72 hrs. The transfected cells were visualization using blue light in a standard inverted fluorescence microscope (Olympus, IX73). The protein is secreted, and the expression medium is centrifuged to remove cells and the medium is used for binding studies. A weak light green color is noted in the cell medium.

F. S-protein binding and drug treatment method

For the S-protein binding study, the ACE2 transfected HEK293T (293T) cells (Figure 5) were washed and incubated with expression medium containing GFP-S (i.e., GFP expressing S-protein) for 4 and 8 hrs at 37 degrees and 5% CO2. At respective time-point, the cells were washed, fixed by standard protocol (i.e., 30 min incubation in 3.7% formaldehyde at room temperature, treatment wash-off and mounting with FluoroSave, Sigma-Aldrich) and were observed in fluorescence microscope fitted with 20X objective lens and blue light (470-490 nm). For the ADD treatment study, ACE2 transfected 293T were first treated with ADD (250 nM) for 15 mins followed incubated with GFP-S (i.e., GFP expressing S-protein) for 4 and 8 hrs as describe above. At respective time-point, the cells were washed, fixed and were observed in fluorescence microscope as described in pervious paragraph.

G. Endosome/Lysosome Labelling

For the long term (12 hrs) drug-treatment study, finally, the ACE2 transfected 293T were treated with respective drugs, i.e., Riboflavin, ADD and Remidesivir (250 nM) along with GFP-S containing medium for 12 hrs in the same condition mentioned above. To visualize Lysosomes / endosomes, LAMP-1 antigen present in late endosomal compartment were targeted. Briefly, the drug treated cells were fixed. The cells were then incubated with the primary mouse anti-LAMP1 antibody (Sigma-Aldrich), at a 50 nM concentration in blocking for 30 min at room temperature. After washing-off primary antibody treatment, the cells were treated with the secondary antibody, Alexa Fluor 594 (emission at 620 nm) tagged donkey anti-mouse IgG antibody (Thermofisher) at 50 nM concentration in blocking buffer for 30 min at room temperature. The cells were then washed 2 times and mounted with FluoroSave before carrying out Confocal studies

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V. DECLARATION

This is to declare that the experimental work was performed at Nanobioimaging Lab, Instrumentation and Applied Physics, Indian Institute of Science, Bangalore, India. We have performed few sets of experiments to report the findings and further investigation need be performed for conclusive evidence.

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