Ivermectin Derivatives: A Pivotal Anti COVID-19 Drug Molecules Blocking the Manifestation of Coronavirus

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In early December 2019, an outbreak of coronavirus disease 2019 (COVID-19), caused by a novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), occurred in Wuhan City, Hubei Province, China. Notably, on January 30, 2020, the World Health Organization declared the outbreak as a pandemic and is of International Concern. Perceived risk of acquiring disease has led many governments to issue control measures. Recently, numerous studies demonstrated the drug molecules controlling the COVID-19 are ongoing and some have reached to the clinical trial phase III. However, none of them are worthy in controlling the corona virus. We hypothesized that derivatives of FDA approved drugs Ivermectin may block the manifestation of Coronavirus. Here, we used MTT assay to check damage levels in the lung's cells affected with coronavirus. In conclusion, we showed that derivatives of Ivermectin, an FDA approved drugs possess a powerful efficacy as an anti-COVID-19 drugs.

Keywords: - COVID-19, Ivermectin, qRT-PCR, MTT
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Over the past two decades, outbreaks of SARS (severe acute respiratory syndrome) and MERS (Middle East respiratory syndrome) have resulted in coronavirus (CoV) public health emergencies in 2002 and 2012, respectively. In Wuhan, the most populous city in central China with more than 11 million inhabitants, an outbreak of pneumonia with unknown etiology was identified at the end of 2019 [1]. SARS-CoV-2, formerly known as 2019-nCoV, is a newly emerging virus belonging to the family of Coronaviridae, likely derived from a bat-like SARS coronavirus and transmitted to humans after spike glycoprotein (protein S) and nucleocapside N [2] mutations have appeared. The World Health Organization's zoonotic pathogen, dubbed Coronavirus Disease 2019.

Coronaviruses (CoVs) belong to the Nidovirales order, Coronaviridae family, which comprises of two subfamilies, namely Orthocoronavirinae and Letovirinae (International Committee on Taxonomy of Viruses) (Carlos et al., 2020; Lei et al., 2018). CoVs are genotypically classified into four genera: Alpha coronaviruses (a), Beta coronaviruses (b), Gamma coronaviruses (g), and Delta

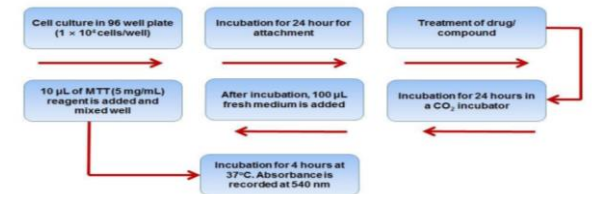
coronaviruses (d), according to their phylogenetic and genomic data. Further, β-coronavirus is subdivided into four viral lineages of A to D (Woo et al., 2012; Li, 2016). Coronavirus is an enveloped and non-segmented virus, which has a large positive-sense single-stranded RNA virus genome (27–32 kb), capped and polyadenylated (Song et al., 2019). Coronavirus also has crown-shape spikes projecting from its surface (80–160 nM in size), from which its name derived (Woo et al., 2010). The CoV Spike (S) glycoprotein attaches to cellular receptors on the host cell and mediates viral entry resulting in interspecies transmission and pathogenesis (Li, 2016; Zhu et al., 2018). A virion consists of two basic components: genomic RNA and a protein capsid packaged forming a nucleocapsid. The nucleocapsid is surrounded by a phospholipid bilayer, composed of the spike glycoprotein trimmer (S) and the hemagglutinin-esterase (HE). All viruses have Nucleocapsid (N), Spike (S), Envelope (E) and Membrane (M) structural proteins. Besides this, they also have several non-structural and accessory proteins. The membrane (M) protein and the envelope (E) protein are placed amongst the S proteins in the virus capsid (Luk et al., 2019; Li, 2016; Fahimi et al., 2018). Novel coronavirus-2019 (nCoV-2019) RNA genome, has 29891 nucleotides in length, which encode 9860 amino acids (Luk et al., 2019). nCoV-2019 genome contains following components: two flanking untranslated regions (UTRs), a single long open reading frame (ORF1ab) (7096-aa), a non-structural polyprotein (7096-aa), four structural proteins – Spike (S) (1273-aa), Envelope (E) (75-aa), Membrane (M) (222-aa), Nucleocapsid (N) (419-aa), and five accessory proteins (ORF3a, ORF6, ORF7a, ORF7b, ORF8 and ORF10) (Phan, 2020; Chan et al., 2020b). (Fig. 1).

METHODS

qRT-PCR (Real Time Polymerase Chain Reaction)

The COVID-19 RT-PCR Test is a real-time reverse transcription polymerase chain reaction (rRT -PCR) test. We used SYBR green, cDNA and SARS-COV2 specific primers. The test can be run in a singleplex format (three individual assays) or multiplexed into a single reaction and amplification set up. In a singleplex format, the test uses three primer and probe sets to detect three regions in the SARS-CoV-2 nucleocapsid (N) gene and one primer and probe set to detect human RNase P (RP) in a clinical sample. When multiplexed into a single reaction, the test uses two primer and probe sets to detect two regions in the SARS-CoV-2 N gene and one primer and probe set to detect RP. RNA isolated from upper and lower respiratory specimens (such as nasal, nasopharyngeal or oropharyngeal swabs, sputum, lower respiratory tract aspirates, bronchoalveolar lavage, and nasopharyngeal wash/aspirate or nasal aspirate) is reverse transcribed to cDNA and subsequently amplified using Applied Biosystems QuantStudio7 Flex (QS7) instrument with software version 1.3. During the amplification process, the probe anneals to a specific target sequence located between the forward and reverse primers. During the extension phase of the PCR cycle, the 5' nuclease activity of Taq polymerase degrades the bound probe, causing the reporter dye to separate from the quencher dye, generating a fluorescent signal. Fluorescence intensity is monitored at each PCR cycle by QS7.

Figure 1.
workflow of MTT assay



MTT assay

We performed MTT assay to check the manifestation/proliferation of SARS-COV2 through ivermectin derivatives compared to control cells. We prepared MTT stock solution by dissolving 500 mg MTT powder in 10 mL phosphate buffer solution. Stir the solution with a magnetic stirrer for about 1 hour in the dark. Filter the sterilized solution with a 0.22 mm filter (Millipore, Ireland) and then store it in 10-mL aliquots (50 mg/mL) at -20°C (van Meerloo et al.,

2011). The working solution (5 mg/mL) will be prepared on the day of experiment by dilution.

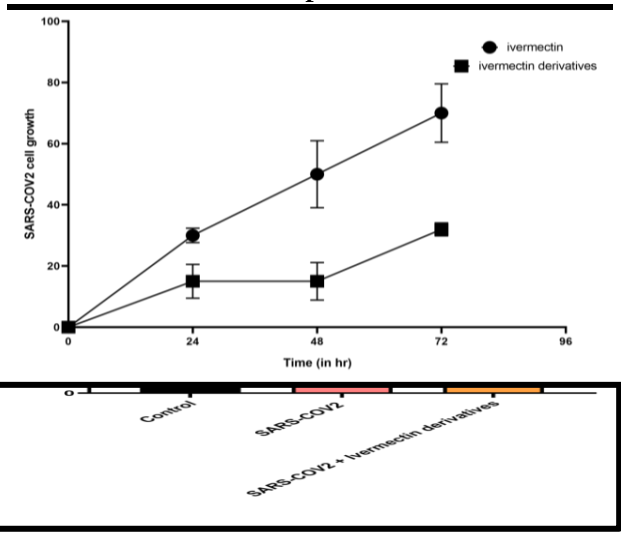
RESULTS

Expression of SARS-COV2 in COVID-19 patients

The COVID-19 RT-PCR test is a real-time reverse transcription polymerase chain reaction (rRT-PCR) test for the qualitative detection of nucleic acid from SARS-CoV-2 in upper and lower respiratory specimens (such as nasopharyngeal or oropharyngeal swabs, sputum, lower respiratory tract aspirates, bronchoalveolar lavage, and nasopharyngeal wash/aspirate) collected from individuals suspected of COVID-19 by their healthcare provider (HCP), as well as upper respiratory specimens (such as nasopharyngeal or oropharyngeal swabs, nasal swabs, or mid-turbinate swabs) collected from any individual, including for testing of individuals without symptoms or other reasons to suspect COVID-19 infection. This test is also for use with individual nasal swab specimens that are self-collected by individuals using the LabCorp At Home COVID-19 test home collection kit when directly ordered by a HCP. The COVID-19 RT-PCR test is also for the qualitative detection of nucleic acid from the SARS-CoV-2 in pooled samples, using a matrix pooling strategy (i.e., group pooling strategy), containing up to five individual upper respiratory swab specimens (nasopharyngeal, mid-turbinate, nasal or oropharyngeal swabs collected using individual vials containing transport media) per pool and 25 specimens per matrix. Nasal swab specimens are collected in individual vials containing transport media either under observation by a HCP or self-collected using a home collection kit authorized for use with this test. 1 Negative results from pooled testing should not be treated as definitive. If a patient's clinical signs and symptoms are inconsistent with a negative result or results are necessary for patient management, then the patient should be considered for individual testing. Specimens included in pools where the positive sample cannot be identified using the matrix must be tested individually prior to reporting a result. Specimens with low viral loads may not be detected in sample pools due to the decreased sensitivity of pooled testing.

Results are for the identification of SARS-CoV-2 RNA (Figure 1). The SARS-CoV-2 RNA is generally detectable in respiratory specimens during the acute phase of infection. Positive results are indicative of the presence of SARS-CoV-2 RNA; clinical correlation with patient history and other diagnostic information is necessary to determine patient infection status. Positive results do not rule out bacterial infection or co-infection with other viruses.

Figure 3.
Dot plot represents the proliferation pattern of SARS-COV2 with ivermectin and its derivatives treatment at 24, 48 and 72 hours time point.



Cytotoxic levels of SARS-COV2 in COVID-19 patients

In the present study, the systematic experimental steps in order to determine the potential cytotoxicity of drug at different concentrations by MTT assay are presented in video form. It is shown that a decreasing absorbance at 540 nm in the cells treated with increasing concentration of the drug in comparison to the control cells without any treatment. A decreased absorbance in the cells treated with drug suggesting cytotoxicity. MTT assay significantly helps the researchers to determine whether any of the test compounds has cell toxicity or proliferative activity (Alley et al., 1988, Mosmann et al., 1983). In this study, attached cells are used in

the microtiter plate. That is why, flat-bottomed 96-well microtiter plate is preferred. But in case of suspension cells, either round bottom wells or flat bottom wells are used. The number of cells in the microplate is not unique for different cell lines and primary cells. The number of cells in the microtiter plate must be optimum to get good result. The number of cells is influenced the level of mitochondrial activity and the rate of proliferation. To get optimum result, several concentrations of cells should be plated in 5-7 plates. Then measure the optical density using colorimeter daily to determine the growth curve of the cell line to prevent overgrowth, which will influence the experiment.

DISCUSSION

Early diagnosis is the most important step to manage and treat COVID-19. The diagnostic tools are generally molecular methods, serology and viral culture. Initial laboratory investigations of hospitalized patients consist of a complete blood count, coagulation testing and serum biochemical test such as creatine kinase (CK), lactate dehydrogenase, procalcitonin, and electrolytes. Based on laboratory tests, most patients showed a significant decrease in total number of lymphocytes, suggesting that lymphocytes (particularly T lymphocytes) are likely target of SARS-CoV-2. In the COVID infection, virus particles begin to spread through the respiratory tract and infect the surrounding uninfected cells. This leads to initiate a cytokine storm and consequently trigger a series of sever immune responses. This process results in some changes in immune cells, particularly lymphocytes, and then leads to immune system dysfunction. Hence, the decreased number of the circulating lymphocytes could be considered as a diagnostic marker for SARS-CoV-2 infection and its severity (Chen et al., 2020b). Previous studies reported that there is a correlation between elevated level of pro-inflammatory cytokines like IL1B, IL6, IL12, IFNγ, IP10, and MCP1, and cytokines such as IFNγ, TNFα, IL15, and IL17, in SARS-CoV and MERS-CoV infection respectively, with pulmonary inflammation and lung injury. Notably, the high value of cytokines like IL1B, IFNγ, IP10, and MCP1, may activate T helper 1 cells (Th1) response. This cytokine storm is probably associated with disease severity. However, in SARS-CoV2 infection, enhanced secretion of T helper 2 (Th2) and cytokines like IL4 and IL10, reduce peripheral white blood cells and immune cells such as lymphocytes, probably leading to suppression of the inflammatory response and immune system function followed by serious lung damage, which differs from SARS-CoV infection. These findings suggest that sever and uncontrolled inflammatory response have a more damaging effect on COVID-19 induced lung injury than viral pathogenicity. Therefore, in SARS-CoV-2 pneumonia, it is vital to control cytokines or chemokines to detect the impact of 2019 coronavirus on their production in the critical phase of the disease. RT-PCR (reverse-transcription polymerase chain reaction) or Real-Time PCR and genome sequencing for respiratory or blood specimens are the next methods to confirm COVID-19 infection.

Currently, RT-PCR method is opted for the SARS-CoV-2 Viral RNA detection in samples collected from infected patients, which has been facilitated by availability of full genome sequence of the 2019-nCoV in Gene Bank. WHO approved real-time RT-PCR assays for detecting viral nucleic acid (RNA) of common viruses, such as SARS-CoV and MERS-CoV, influenza, avian influenza, respiratory syncytial virus, adenovirus, para-influenza virus present in respiratory samples, including oropharyngeal and nasopharyngeal swabs, sputum or bronchial aspirates, bronchoalveolar lavage fluids. Sputum is a noninvasive lower respiratory tract specimen, but only 28% of patients with 2019-nCoV in 1 case series could produce sputum for diagnostic evaluation. Notably, lower respiratory tract specimens compared with upper respiratory tract samples can provide significantly more viral load and genome fraction for 2019-nCoV test. In addition, stool or blood samples are also used for gene sequencing of the viral nucleic acid. Viral culture is more time-consuming method as compared to the other methods, and it was much more useful in the first stage of outbreaks, before other diagnostic methods became clinically

available. Besides, viral cultures can be used in the in-vitro and in-vivo antiviral treatment and vaccine evaluation trials.

ACKNOWLEDGEMENTS

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