Two specific publications/research papers of the applicant relevant to the research work mentioned above in support of Prof. Ravishankar Ramachandran

The following are attached as examples of the work carried out by Prof. Ravishankar Ramachandran

I. Disease biology and molecular mechanism

a) *M. tuberculosis* class II apurinic/apyrimidinic-endonuclease/3′-5′ exonuclease (XthA) engages with NAD+ -dependent DNA ligase A (LigA) to counter futile cleavage and ligation cycles in Base Excision Repair
Taran Khanam, Mohammad Afsar, Ankita Shukla, Faiyaz Alam, Sanjay Kumar, Horam Soyar, Kunzes Dolma, Ashish, Mukesh Pasupuleti, Kishore Kumar Srivastava, Ravi Sankar Ampapathi & **Ravishankar Ramachandran***
Nucleic Acids Research. **48**, 4325-4343, 2020
https://pubmed.ncbi.nlm.nih.gov/32232338/

b) Mechanistic insights from the crystal structures of a feast/famine regulatory protein from *Mycobacterium tuberculosis H37Rv*
Tripti, S & **Ravishankar Ramachandran**
Nucleic Acids Res. **35***,* 7324-7335, 2007
https://pubmed.ncbi.nlm.nih.gov/17962306/

II. Translational application

a) **Ramachandran, Ravishankar** and Bhosale, Vivek and Reddy, Himanshu and Atam, Virendra and Faridi, MMA and Fatima, Jalees and Shukla, Vaibhav and Singh, Vikram and Singh Negi, Mahendra Pal and Srivastava, Mukesh and Srivastava, Ajay Kumar and Tripathi, Chandra Bhushan and Ghosh, Nayan and Majumdar, Nilanjana and Tripathi, Raj Kamal and Rath, Srikanta Kumar and Mishra, Prabhat Ranjan and Sharma, Sharad and Kundu, Tapas K., Phase III, Randomized, Double-Blind, Placebo Controlled Trial of Efficacy, Safety and Tolerability of Antiviral Drug Umifenovir vs Standard Care of Therapy in Non-Severe Covid-19 Patients (September 7, 2021).
http://dx.doi.org/10.2139/ssrn.3919585

Note: *The above are all open access publications. Due to limitation of upload size as per the application format, the first pages of each of these are attached herewith*.

Published online 30 March 2020

Nucleic Acids Research, 2020, Vol. 48, No. 8 ***4325–4343***
doi: 10.1093/nar/gkaa188

M. tuberculosis class II apurinic/apyrimidinic-endonuclease/3′-5′ exonuclease (XthA) engages with NAD$^+$-dependent DNA ligase A (LigA) to counter futile cleavage and ligation cycles in base excision repair

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Received October 04, 2019; Revised February 24, 2020; Editorial Decision March 10, 2020; Accepted March 27, 2020

ABSTRACT

Class-II AP-endonuclease (XthA) and NAD$^+$-dependent DNA ligase (LigA) are involved in initial and terminal stages of bacterial DNA base excision repair (BER), respectively. XthA acts on abasic sites of damaged DNA to create nicks with 3′OH and 5′-deoxyribose phosphate (5′-dRP) moieties. Co-immunoprecipitation using mycobacterial cell-lysate, identified MtbLigA-MtbXthA complex formation. Pull-down experiments using purified wild-type, and domain-deleted MtbLigA mutants show that LigA-XthA interactions are mediated by the BRCT-domain of LigA. Small-Angle-X-ray scattering, ^{15}N/^{1}H-HSQC chemical shift perturbation experiments and mutational analysis identified the BRCT-domain region that interacts with a novel $_{104}$DGQPSWSGKP$_{113}$ motif on XthA for complex-formation. Isothermal-titration calorimetry experiments show that a synthetic peptide with this sequence interacts with MtbLigA and disrupts XthA–LigA interactions. *In vitro* assays involving DNA substrate and product analogs show that LigA can efficiently reseal 3′OH and 5′dRP DNA termini created by XthA at abasic sites. Assays and SAXS experiments performed in the presence and absence of DNA, show that XthA inhibits LigA by specifically engaging with the latter's BRCT-domain to prevent it from encircling substrate DNA. Overall, the study suggests a coordinating function for XthA whereby it engages initially with LigA to prevent the undesirable consequences of futile cleavage and ligation cycles that might derail bacterial BER.

INTRODUCTION

Mycobacterium tuberculosis is a deadly human pathogen that can survive for years in the human host. This is partly attributed to its ability to repair DNA damage caused by exposure to the hostile oxidative environment present within the host cell (1–3). In the absence of a mismatch repair (MMR) pathway in the mycobacterium, the non-canonical MMR by EndoMS/NucS (4,5) or DNA base excision repair (BER) pathway assumes importance for countering host-inflicted oxidative DNA damaging responses (6,7). BER is a multi-step process initiated by the recognition and removal of damaged DNA bases by DNA glycosylases (8–10), to generate non-coding, abasic sites which can block DNA replication and transcription if left unprocessed (11,12). Subsequently, Class II AP-endonucleases cleave the phosphodiester backbone of DNA at the abasic sites (13,14). The repair process is then completed by the

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7324–7335 *Nucleic Acids Research, 2007, Vol. 35, No. 21* *Published online 25 October 2007*
doi:10.1093/nar/gkm850

Mechanistic insights from the crystal structures of a feast/famine regulatory protein from *Mycobacterium tuberculosis H37Rv*

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Received August 9, 2007; Revised September 25, 2007; Accepted September 26, 2007

ABSTRACT

***Rv3291c* gene from *Mycobacterium tuberculosis* codes for a transcriptional regulator belonging to the (leucine responsive regulatory protein/regulator of asparigine synthase C gene product) Lrp/AsnC-family. We have identified a novel effector-binding site from crystal structures of the *apo* protein, complexes with a variety of amino acid effectors, X-ray based ligand screening and qualitative fluorescence spectroscopy experiments. The new effector site is in addition to the structural characterization of another distinct site in the protein conserved in the related AsnC-family of regulators. The structures reveal that the ligand-binding loops of two crystallographically independent subunits adopt different conformations to generate two distinct effector-binding sites. A change in the conformation of the binding site loop 100–106 in the B subunit is apparently necessary for octameric association and also allows the loop to interact with a bound ligand in the newly identified effector-binding site. There are four sites of each kind in the octamer and the protein preferentially binds to aromatic amino acids. While amino acids like *Phe*, *Tyr* and *Trp* exhibit binding to only one site, *His* exhibits binding to both sites. Binding of *Phe* is accompanied by a conformational change of 3.7 Å in the 75–83 loop, which is advantageously positioned to control formation of higher oligomers. Taken together, the present studies suggest an elegant control mechanism for global transcription regulation involving binding of ligands to the two sites, individually or collectively.**

INTRODUCTION

Mycobacterium tuberculosis is a successful pathogen mainly because of its ability to persist in the human host for several years while evading the immune system (1). The *rv3291c* gene codes for an Lrp/AsnC (leucine responsive regulatory protein/regulator of asparigine synthase C gene product) type global transcriptional regulator (MtbLrp) (2) and belongs to the large family of DNA-binding transcriptional regulators. It has been suggested that Lrp/AsnC type proteins should more appropriately be called feast/famine regulatory proteins because of their involvement in regulation of metabolic pathways in response to the availability of amino acids and nitrogen bases in the external environment (3,4). These proteins are also involved in DNA bending, condensation of DNA into globular nucleo-protein structures, chromosome structure and organization, among other roles (3,5).

Individual subunits of these proteins have a molecular weight of ~17 kDa and are divided into two domains namely DNA and effector-binding domains which occur at the N- and C-terminal regions, respectively. The DNA-binding domain contains the helix-turn-helix motif while the effector-binding domain is also involved in oligomeric interactions. Higher order assemblies usually exist as multiples of dimers and include formation of tetramers, octamers, dodecamers and chromatin-like cylinders (5–9). How these proteins translate the effector-binding event to the protein–DNA-binding site is unclear. Some global regulators like the *Escherichia coli* Lrp, which reportedly controls over 10% of all genes, are known to bind to a variety of amino acids including leucine, alanine, valine, proline and lysine, whose binding can effect a positive/negative regulation of the target genes (5,10). On the other hand, local regulation involving proteins like AsnC, is controlled by the binding of a

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The Lancet Infectious Diseases

Phase III, Randomized, Double-blind, Placebo controlled trial of Efficacy, Safety and Tolerability of Antiviral drug Umifenovir vs Standard care of therapy in non-severe COVID-19 patients.

--Manuscript Draft--

Manuscript Number:	
Article Type:	Article (Clinical Trials)
Keywords:	Phase III clinical trials; Umifenovir; Arbidol; COVID-19; CSIR-Central drug research institute
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Manuscript Region of Origin:	INDIA
Abstract:	Summary Background SARS-Cov2 has caused millions of deaths worldwide and effective antivirals are not yet available. We evaluated the efficacy, tolerability and safety of Umifenovir, a drug used originally against influenza in Russia and China, in non-severe COVID19 adult patients from India. Method

A double-blind placebo controlled Phase III trial spread over 3 hospitals in Lucknow, India viz. King George's Medical University, ERAs Lucknow Medical College and Ram Manohar Lohia Institute of Medical Sciences was carried out. RT-PCR confirmed COVID19 patients in the age group from 18-75 yrs. were recruited and further stratified into Mild-asymptomatic and Moderate cases. Exclusions included, pregnant or lactating women, severe patients, those suffering from Acute respiratory distress syndrome (ARDS), sepsis shock, requiring invasive ventilator support, ECMO or shock requiring vasopressor support, severe liver disease, severe renal impairment, comorbid conditions like asthma, diabetes with second-third line medicines as defined in the WHO guidance document. Patients were randomized 1:1 on placebo with standard care of therapy or Umifenovir (800 mg BID, maximum 14 days) with standard care of therapy respectively. Computerized randomization was carried out for each group separately through sequentially numbered, opaque, sealed envelopes (SNOSE) in block sizes of six and was administered independently through clinical-site coordinators and to have sufficient number of Mild-asymptomatic and moderate patients. The primary endpoint for Asymptotic-mild patients was time to nasopharyngeal swab negativity by two RT-PCR tests for SARS COV2 antigens taken 24 hrs apart from the date of randomization. For Moderate patients, the average change in the ordinal scale from the baseline scores from randomization on the eight-point ordinal scale as defined by WHO was calculated as the primary endpoint. This trial is registered with the Clinical Trial Registry of India (CTRI) Number: CTRI/2020/09/027535.

Findings

A total of 132 patients were recruited in the trial after screening between 3rd October 2020 to 28th April 2021. Of these, 9 patients withdrew consent/ stopped medication on their own. The remaining 123 patients were almost equally distributed into Asymptomatic (35%), Mild (32%) and Moderate (33%) symptoms groups respectively. No Serious adverse events were noted in any of the patients. Only few minor events like headache, stomach ache and nausea were reported and this also was observed almost equally between the Umifenovir and Standard-of-care arms respectively. In the Primary endpoint corresponding to the Mild-asymptomatic patient group (n=82), we found that 73% patients in the Umifenovir arm were RT-PCR negative on the 5th day (P=0.004), while only 40% patients in the placebo arm were negative. In the moderate group, the WHO scores for the Umifenovir arm corresponded to faster clinical improvement as compared to the Placebo arm (P=0.125 on day 3). In the Mild-asymptomatic group, the clinical improvement assessed by the WHO score on day 5 was statistically significant (P=0.019) in the Umifenovir arm compared to the placebo arm and agrees well with the primary endpoint results.

Interpretation

Umifenovir is efficacious for Mild-asymptomatic patients and meets the primary and secondary endpoint criteria of the trial. The drug is safe and well tolerated at a dosage of 800 mg BID, maximum 14 days, in adult patients. It exhibits faster time to cure with median 5 days (95%CI, 5-14) in Umifenovir group and median 7 days (95%CI, 5-14) for the placebo group. In moderate patients, administration of Umifenovir co-relates with faster clinical recovery, albeit with less statistical significance. In future studies, the drug should be evaluated as a prophylactic in high-risk adults, and for efficacy in children and pregnant/lactating women.

Phase III, Randomized, Double-blind, Placebo controlled trial of Efficacy, Safety and Tolerability of Antiviral drug Umifenovir *vs* Standard care of therapy in non-severe COVID-19 patients.

Ravishankar Ramachandran[1*], Vivek Bhosale[1], Himanshu Reddy[2], Virendra Atam[2], MMA Faridi[3], Jalees Fatima[3], Vaibhav Shukla[3], Vikram Singh[4], Mahendra Pal Singh Negi[1], Mukesh Srivastava[1], Ajay Kumar Srivastava[1], Chandra Bhushan Tripathi[1], Nayan Ghosh[1], Nilanjana Majumdar[1], Raj Kamal Tripathi[1], Srikanta Kumar Rath[1], Prabhat Ranjan Mishra[1], Sharad Sharma[1] and Tapas K Kundu[1]*

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Summary

Background SARS-Cov2 has caused millions of deaths worldwide and effective antivirals are not yet available. We evaluated the efficacy, tolerability and safety of Umifenovir, a drug used originally against influenza in Russia and China, in non-severe COVID19 adult patients from India.

Method A double-blind placebo controlled Phase III trial spread over 3 hospitals in Lucknow, India *viz.* King George's Medical University, ERAs Lucknow Medical College and Ram Manohar Lohia Institute of Medical Sciences was carried out. RT-PCR confirmed COVID19 patients in the age group from 18-75 yrs. were recruited and further stratified into Mild-asymptomatic and Moderate cases. Exclusions included, pregnant or lactating women, severe patients, those suffering from Acute respiratory distress syndrome (ARDS), sepsis shock, requiring invasive ventilator support, ECMO or shock requiring vasopressor support, severe liver disease, severe renal impairment, comorbid conditions like asthma, diabetes with second-third line medicines as defined in the WHO guidance document. Patients were randomized 1:1 on placebo with standard care of therapy or Umifenovir (800 mg BID, maximum 14 days) with standard care of therapy respectively. Computerized randomization was carried out for each group separately through sequentially numbered, opaque, sealed envelopes (SNOSE) in block sizes of six and was administered independently through clinical-site coordinators and to have sufficient number of Mild-asymptomatic and moderate patients. The primary endpoint for Asymptotic-mild patients was time to nasopharyngeal swab negativity by two RT-PCR tests for SARS COV2 antigens taken 24 hrs apart from the date of randomization. For Moderate patients, the average change in the ordinal scale from the baseline scores from randomization on the eight-point ordinal scale as defined by WHO was calculated as the primary endpoint. This trial is registered with the Clinical Trial Registry of India (CTRI) Number: CTRI/2020/09/027535.

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Funding *Council of Scientific and Industrial Research*, Ministry of Science and Technology, Government of India.

Research in context

Evidence before this study

PubMed was searched for published clinical trials involving Umifenovir till July 2021 with search terms 'Covid-19', 'Umifenovir', 'Arbidol' and 'clinical trial'. Umifenovir has been suggested as a candidate drug for repurposing against COVID-19 based on its known broad spectrum antiviral activity and cell-culture inhibition assays involving Sars-Cov2. Published clinical trials involving Umifenovir have reported mixed results regarding the efficacy of the drug against COVID-19. E.g. a trial comparing KALETRA with Umifenovir concluded that Umifenovir 'significantly contributes to clinical and laboratory improvements, including peripheral oxygen saturation, requiring ICU admissions, duration of hospitalization, chest CT involvements, WBC, and ESR'. Another trial involving moderate and severe patients, concluded that there was no statistically significant improvement in time to clinical improvement or in the mortality rate between the Umifenovir and standard-of-care arms. In a limited randomized study, involving mild and moderate patients it was concluded that 'Umifenovir presents advantages for ameliorating mild and moderate COVID-19 in patients who were symptomatic in combination with supportive treatment'. None of the earlier reported trials were double-blind placebo controlled or were tested at the dosage used in the present study.

Added value of this study

To our knowledge, the present report is the first for a double-blind placebo controlled trial to evaluate Umifenovir against COVID-19. In view of the earlier reported mixed results, we stratified patients into Mild-asymptomatic and moderate groups to probe the efficacy of the drug. We also defined distinct primary endpoints for Mild-asymptomatic and moderate patient groups respectively. Importantly, the dosage of 800 mg BID was designed to achieve predicted C_{max} for anti-Sars-Cov2 activity. Earlier trials have reported a maximum of 200 mg TID. We found that Umifenovir is efficacious in Mild-asymptomatic patients and exhibits statistically significant faster virus clearance as assessed by RT-PCR assays and faster clinical recovery as assessed by WHO scores. In moderate patients, there was a tendency for faster clinical recovery of patients in the Umifenovir arm, but it was not statistically significant. The study supports the safety of the drug in adult patients at the dosage used. Administration of the drug in the tested dosage is shown to be beneficial for Mild-asymptomatic patients and suggests that it may also be useful as a prophylactic for persons at high-risk of contracting COVID19.

Implications of all the available evidence

Overall, our present results advocate the efficacy and use of Umifenovir in Mild-asymptomatic adult COVID-19 patients in the dosage tested here.

We recommend future studies for evaluation of Umifenovir as a prophylactic and a larger trial for moderate patients in view of the tendency for faster clinical improvement reported here. Additionally, in view of the safety profile and earlier approved use in children and pregnant women, we suggest studies to evaluate efficacy in children and pregnant/ breast-feeding women too.

Introduction

The COVID-19 pandemic caused by the Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-Cov2) has ravaged almost every nation across the globe (1). In India alone, over 30 million persons have been infected by the virus and about 0.4 million people have been officially declared dead due to the disease and its complications (https://www.mygov.in/covid-19). Vaccination strategies are obviously vital to control the pandemic (https://www.who.int/emergencies/diseases/novel-coronavirus-2019/covid-19-vaccines/advice) and at the same time it is critical to have evidence-based therapeutics that can mitigate the disease that can occur in, both vaccinated, and unvaccinated persons.

Umifenovir (Arbidol) is known to have broad spectrum anti-viral activity and has earlier been approved in China and Russia for treating influenza, SARS, and Lassa viruses (2-6). It has been suggested and tested in multiple studies as a candidate for use as an anti-COVID19 therapeutic and has been suggested to act at the entry stage and at the post-entry stages by preventing viral attachment and inhibiting the release of virus particles from intracellular vesicles respectively (7-9). Earlier clinical trials have reported mixed results about its efficacy (10-18). The EC_{50}, 50% maximal effective concentration has been reported to be 4.11 µM while the 50% cytotoxic concentration, CC_{50}, has been reported to be 31.79 (7,19). Our hypothesis, based on the evaluation of multiple *in vitro* and clinical studies, was that Umifenovir is a drug with a good safety profile (LD_{50} ~4g/kg), and with the capacity of achieving the required EC_{50} with a dose of 800mg. Earlier relevant human studies had identified a C_{max} ~4.1 µM upon administration of 800 mg of Umifenovir and a half-life of about 16 hrs. (19). On the other hand, other reported clinical trials involving Umifenovir have all used a maximum of 600 mg/day as the dosage.

We therefore aimed to evaluate the Efficacy, Safety and Tolerability of Umifenovir vs Standard care of therapy through a randomized Phase III double-blinded placebo controlled trial in non-severe COVID-19 adult patients in the age group of 18-75 yrs using a dosage of 800mg BID. An entry inhibitor is expected to have more efficacy in the earlier stages of the COVID19 disease, while moderate/severe disease is supported by other host-directed clinical measures for alleviation of symptoms. Accordingly, separate endpoints were devised for Mild-asymptomatic and moderate patients respectively based on the known disease progress and nationally adopted standard-of-care treatment strategies. To our knowledge, this report is the first for a double-blind placebo controlled

Phase III trial for Umifenovir against COVID-19 and furthermore no other trial has involved the dosage of 800 mg BID that has been used here.

Methods

Study design, randomization, and inclusion/exclusion of participants

A double-blind placebo controlled Phase III trial was designed to be carried out in three clinical trial centres based in Lucknow, India, *viz.* King George's Medical University, Ram Manohar Lohia Institute of Higher Medical Sciences and Era's Lucknow Medical College and Hospital for a total of 132 patients. All National regulatory and respective ethical committees' permissions/ approvals were secured before the commencement of the trial. Patients were referred to the respective hospitals by a central command center under the Directorate of Medical & Health Services, State government of Uttar Pradesh (http://dgmhup.gov.in/en/default) based on positive RT-PCR results of persons with symptoms or through contact tracing of already identified COVID-19 positive patients (https://lucknow.nic.in/noval-corona-virus-covid-19/). Dosage of Umifenovir used in the study was 800mg twice daily for 14 days plus standard care of therapy. Each patient enrolled in the study gave written consent and was observed for a total of 28 days normally. Case categories according to severity was defined as per Ministry of Health & Family Welfare, Govt of India guidelines. As per the earlier reported pharmacokinetic studies, a dosage of 800mg achieves sufficient concentration to inhibit the pathogen. The drug has a half-life of about 16 hours and it was therefore decided to be administered twice daily. The standard care of therapy used was as per the Ministry of Health, Govt. of India COVID-19 treatment guidelines (https://www.mohfw.gov.in/pdf/UpdatedDetailedClinicalManagementProtocolforCOVID19adultsdated24052021.pdf). Patients were randomized using Computerised randomization (Sequentially numbered opaque, sealed envelopes –SNOSE).

The inclusion criteria involved chiefly the following: *Asymptomatic persons*: aged 18-75 years, at the time of signing the Informed Consent Form (ICF), with Nasopharyngeal swab positivity in RT-PCR tests for SARS-Cov-2 antigens detected during screening of contacts or sentinel surveillance. Mild patients were those with uncomplicated upper respiratory tract viral infection and who may have non-specific symptoms such as fever, cough, expectoration, shortness of breath, myalgia, fatigue, sore throat, nasal congestion, diarrhea, loss of taste with Nasopharyngeal swab positivity in RT-PCR tests for SARS-Cov-2 antigens. Moderate disease was considered as Pneumonia with

no signs of severe disease. Adults with presence of clinical features of dyspnea and or hypoxia, fever, cough, including SpO2 <94% (range 90-94%) on room air, respiratory rate more or equal to 24 per minute were included in the moderate patient category.

The main exclusion criteria were: patients with severe covid and with respiratory rate >30 breaths/min, severe respiratory distress, SpO2 <90% on room air, Cases of Acute respiratory distress syndrome (ARDS), sepsis/ septic shock, pregnant/ lactating women, patients with severe lever disease, severe renal impairment, or other comorbidities like asthma, diabetes with second and third line medicines as defined in the WHO guidance document (20). The clinical trial protocol is attached as Supplementary information.

Randomization and masking

Patients who were eligible as per the inclusion criteria were asked to give their consent to participate in the trial. Randomization and recruitment was administered by an independent clinical trial coordinator for true double-blinding. Patients were almost equally stratified into the Mild-asymptomatic and Moderate arms. All laboratory staff and doctors were also masked to treatment allocation and samples were identified by serial numbers.

Study population and criteria

Calculation of sample size for the overall study

The patients were assigned to the three hospitals by a Central COVID-19 command center of the State government of Uttar Pradesh, India. A total of 132 patients were to be recruited with 66 patients in each arm of the trial. The sample size of the present study was chosen based on formal statistical power calculation for the primary outcome measure *i.e.* nasopharyngeal swab negativity by RT-PCR test. Sample size estimation was based on assumption that the average time (duration) of discharge of patient in Standard-of-care (SOC) group is 13± 2.5 days. For any patient to be discharged in lesser time than 11.7 days we require the sample size to be calculated as:

$\eta = 2(Z\ \alpha/2 + Z\ \beta\ )2\ \sigma2\ /\ (x1 - x2\ )2$

Where $Z\ \alpha/2 = 1.96$ level of significance, $Z\ \beta = 0.842$ power of test= 80%, x1 = 11.7 days, x2 = 13 days, $(x1 - x2) = 1.3$, $\sigma = 2.5$ days, $x1 - x2$ the minimum time difference which can be significant.

$\eta = 2\times (1.96 + 0.842)2 \times 2.52 /1.32 = 58$

With 10% margin of dropouts and also taking into account randomization block size of 6, the required sample size was calculated to be 66 in each arm. Ultimately, 9 patients withdrew from the trial by not appearing for subsequent tests or stopped taking the medication (either Umifenovir/ placebo) leading to a total of 123 patients divided into placebo (n=63) and Umifenovir (n=60) arms respectively.

Outcomes and safety assessments

The primary endpoints for the Mild-asymptomatic patients was different from Moderate patients. For the Mild-asymptomatic patients, the primary endpoint was Time from randomization to nasopharyngeal swab negativity by two RT-PCR tests, for SARS-Cov-2 antigens, taken 24 hours apart. For moderate patients, the end point was time to improvement by one category from randomisation on the eight-category ordinal scale defined by WHO (21) (**Table S1**) & average change in the ordinal scale from baseline. The secondary outcome was Time from randomization to clinical recovery or deterioration, assessed at 0, 7, 14, 21 and 28 days, on eight-category ordinal scale defined by WHO (21). Also assessed was the proportion of patients to clinical recovery or deterioration, at 0, 7, 14, 21 and 28 days respectively, on the WHO defined eight-category ordinal scale consisting of the following categories: (a) Proportion of patients hospitalized with Severe Covid-19 pneumonia (with respiratory rate $\geq$30/minute and/or SpO2 < 90% in room air) or ARDS or Septic shock as per Government of India guidelines. (b) Adverse events in the two groups.

Statistical analysis:

Discrete (categorical) nasopharyngeal swab/RTPCR output (negative/positive) of two groups (*placebo, n=63* and *umifenovir, n=60*) over the periods (day 5, 7, 9, 11, 13, 15, 17, 19, 21 and 28) were summarised in number (n) and percentage (%) and compared by chi-square ($\chi 2$) test. The WHO score of two groups over the periods (day 3, 5, 7, 14, 21 and 28) were summarised in Mean ± SE (standard error of the mean) and compared by repeated measures two factor (groups and periods) analysis of variance (ANOVA) and the significance of mean difference within (intra) and between (inter) the groups was done by Newman-Keuls post hoc test. A two-tailed (α=2) $P < 0.05$ was considered statistically significant.

This study is registered with the Clinical Trial Registry of India (CTRI) with Number: CTRI/2020/09/027535 and was conducted between 3rd October 2020 – 28th April 2021.

Role of the funding source

The funder had no role in the study design, conduct of the trial or the writing of the report

Results

Patients were recruited into the trial and randomized into the Umifenovir arm + standard of care or Placebo + standard of care respectively. They were stratified into Asymptomatic, Mild and Moderate categories almost uniformly. Out of 132 patients who were recruited, 9 withdrew consent or stopped taking medication on their own and were discontinued from the trial. The remaining 123 patients were found to be divided as placebo group (n=63) and umifenovir group, (n=60) respectively (**Figure 1**). The baseline characteristics of recruited participants was assessed and is quite similar in both groups of patients and also within stratified Mild-asymptomatic and moderate patients (**Table 1**).

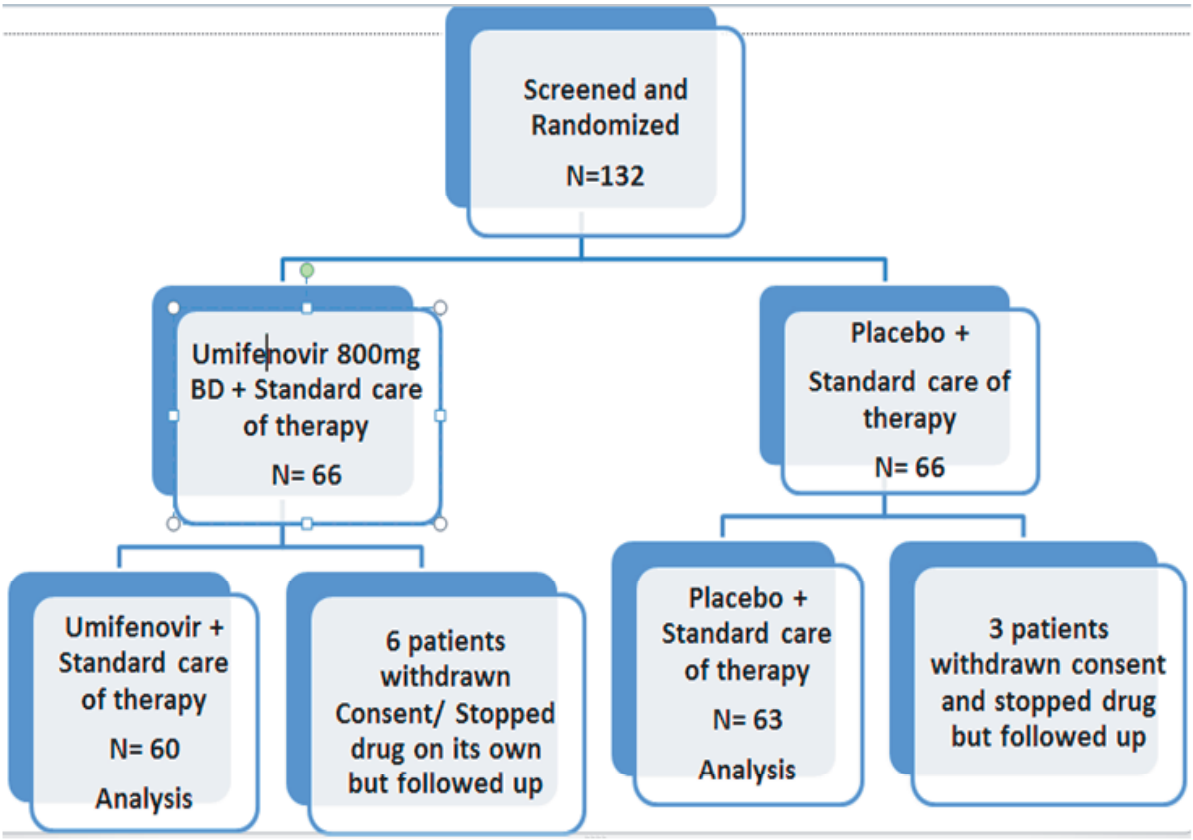

Figure 1. Patient randomization and distribution shown as a CONSORT diagram. The Umifenovir and placebo groups contained 60 and 63 patients respectively in the analysis.

Table 1. Comparison of baseline demographic characteristics of all recruited patients between two drug groups. Age, height and weight of two groups were summarised in Mean ± SE and compared by Student's t test whereas sex were summarised in number (n) and percentage (%) and compared by χ^2 test

(A) Overall patients (n=123)

Variable	**Placebo (n=63) (%)**	**Umifenovir (n=60) (%)**	**t/χ2 value**	***P* value**
Age (yrs)	47.35 ± 1.96	46.08 ± 1.93	0.46	0.646
Sex:				
Female	19 (30.2)	12 (20.0)	1.68	0.195
Male	44 (69.8)	48 (80.0)		
Height (cm)	164.86 ± 0.88	165.60 ± 0.81	0.62	0.537
Weight (kg)	69.51 ± 1.02	69.03 ± 1.09	0.32	0.751

(B) Mild-asymptomatic patients (n=82)

Variable	**Placebo (n=42) (%)**	**Umifenovir (n=40) (%)**	**t/χ2 value**	***P* value**
Age (yrs)	45.50 ± 2.45	42.35 ± 2.38	0.92	0.360
Sex:				
Female	14 (33)	9 (23)	1.19	0.275
Male	28 (67)	31 (78)		
Height (cm)	164.50 ± 1.06	164.25 ± 1.05	0.17	0.867
Weight (kg)	69.19 ± 1.43	68.40 ± 1.43	0.39	0.697

(C) Moderate patients (n=41)

Variable	**Placebo (n=21) (%)**	**Umifenovir (n=20) (%)**	**t/χ2 value**	***P* value**
Age (yrs)	51.05 ± 3.17	53.55 ± 2.61	0.61	0.548
Sex:				
Female	5 (24)	3 (15)	0.51	0.477
Male	16 (76)	17 (85)		
Height (cm)	165.57 ± 1.61	168.30 ± 1.00	1.42	0.163
Weight (kg)	70.14 ± 1.14	70.30 ± 1.58	0.08	0.936

When we examined the symptom category of patients, we found that the recruited patients were similarly distributed with Asymptomatic (35%), Mild (32%) and Moderate (33%) respectively.

Primary endpoint analysis for Mild-asymptomatic patients

As mentioned earlier, the primary endpoint for this category of patients was time to RT-PCR nasopharyngeal swab negativity by two RT-PCR tests for SARS COV2 antigens taken 24 hrs apart from the date of randomization. In the Mild-Asymptomatic group (n=82), we found that: 73% patients on the Umifenovir arm were RT-PCR negative on the 5th day (P=0.004) as compared to only 40% patients on the placebo arm (**Figure 2, Table 2**).

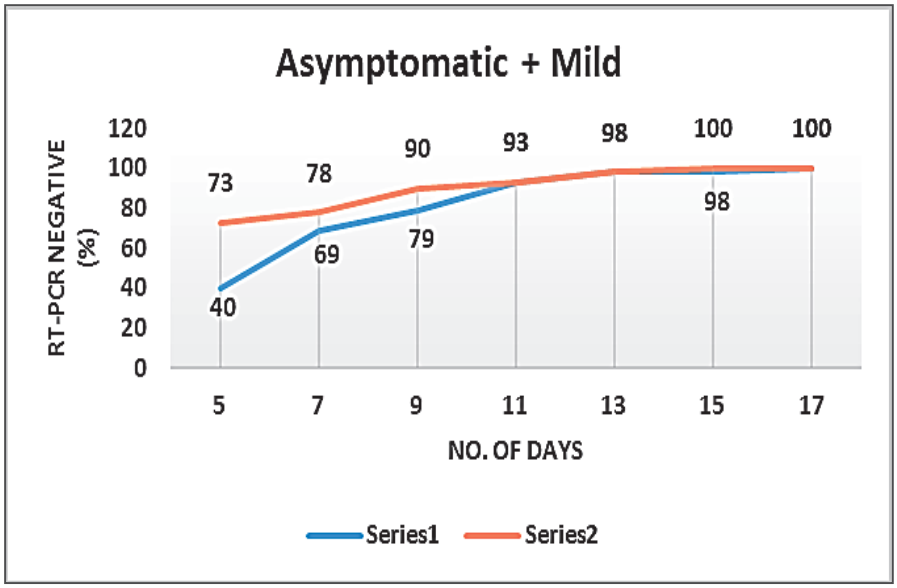

Figure 2. Time to RT-PCR-negativity in the two groups of Mild-asymptomatic patients. Orange line corresponds to Umifenovir arm while the blue curve corresponds to the placebo arm.

Table 2. Statistical and RT-PCR negativity summary of Mild-Asymptomatic patients recruited in the clinical trial (n=82)

RT-PCR test Day (negative)	Placebo (n=42) (%)	Umifenovir (n=40) (%)	Diff (%)	*P value*
5	17 (40)	29 (73)	32	0.002
7	29 (69)	31 (78)	8	0.194
9	33 (79)	36 (90)	11	0.078
11	39 (93)	37 (93)	0	0.475
13	41 (98)	39 (98)	0	0.486

15	41 (98)	40 (100)	2	0.163
17	41 (98)	40 (100)	2	0.163
19	42 (100)	40 (100)	0	-

Secondary endpoint analysis for the Mild-asymptomatic patients' category

The secondary endpoint was the average change in the ordinal scale by at least one category from the baseline scores from randomization on the eight-point ordinal scale as defined by WHO. This would assess the clinical recovery of the patients on both arms of the trial in the Mild-asymptomatic patients. In this analysis we found that the WHO score on day 5 was 48.9% lower in the Umifenovir group (P=0.019) compared to the placebo group (**Figure 3, Table 3**).

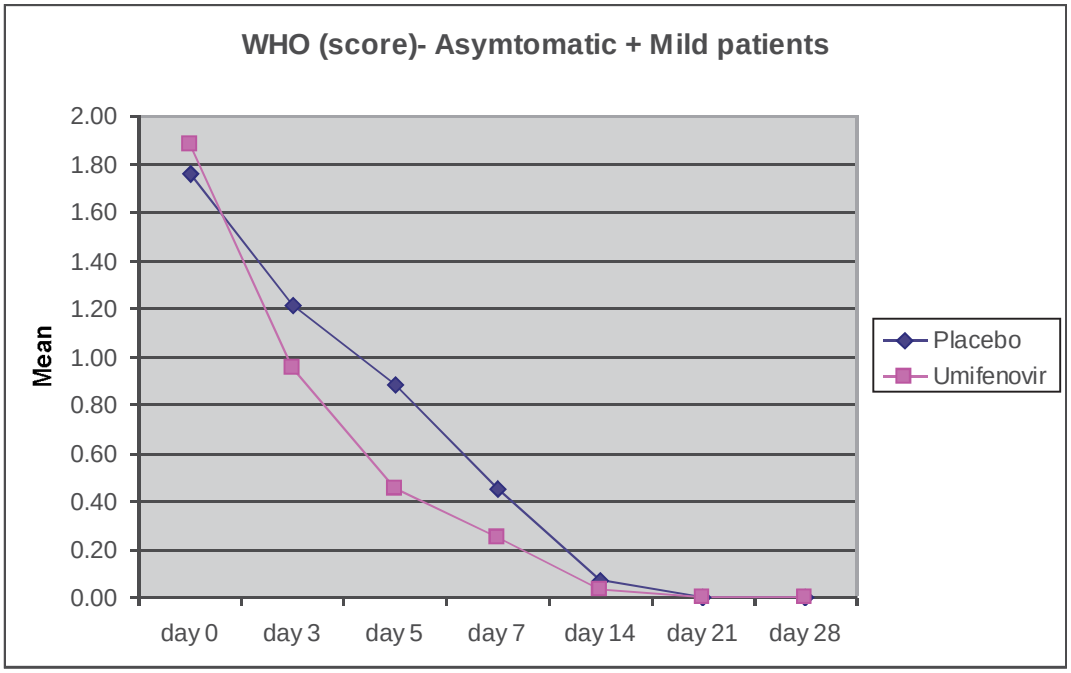

Figure 3. Reduction in the mean WHO scores plotted in Asymptomatic and Mild patients (n=82). Pink curves represent the reduction in the mean WHO scores on days 0,3,5,7,14,21 and 28 respectively while blue curves depict the reduction in the average WHO scores on the respective days plotted on the X-axis. Significant difference in the reduction in the mean WHO score was observed on day 5 in the Mild-Asymptomatic patients (P=0.019).

Table 3. Average WHO scores tabulated for the Mild-asymptomatic group.

Time (days)	**Mild-asymptomatic (n=82)**		
	Placebo (n=42)	**Umifenovir (n=40)**	***P* value**
day 0	1.76 ± 0.14	1.88 ± 0.15	0.479
day 3	1.21 ± 0.13	0.95 ± 0.12	0.098
day 5	0.88 ± 0.13	0.45 ± 0.11	0.019
day 7	0.45 ± 0.12	0.25 ± 0.09	0.414
day 14	0.07 ± 0.05	0.03 ± 0.02	0.771

Overall, the primary and secondary endpoints are met for the Mild-asymptomatic category of patients.

Calculation of sample size and power of test for Mild-asymptomatic patient category.

We carried out calculations to determine the *post hoc* power of the above results.

Assuming a difference of 20% to be significant between Placebo and Umifenovir arms in the Mild-asymptomatic category and with α level of significance and with 80% power of the test the sample size per group is:

$n = \{2*(Z\alpha/2 + Z\beta)2 *P*Q\}/\Delta 2$

where; $Z\alpha/2 = 1.96$, $Z\beta = 0.842$, $P = 0.9$, $Q = 0.1$ and $\Delta = 0.2$.

This gives n=35.3, *i.e* n=36.

Hence the minimum sample size per group in this study was determined to be n=36.

[P = Pooled rate of response; Q = 1-P; Zα/2 = Desired level of significance (0.05)

Zβ = Value of Z when power is 80%; Δ = minimum difference in rate of response of placebo and treatment group to be significant].

Based on this, the *post hoc* power of the results was estimated to be 84.5%. Since the estimated power is more than the expected power of test, it can be concluded that the sample size studied is sufficient to justify the significant effect of the Umifenovir group over the placebo group in the Mild-asymptomatic patients too.

Analysis of trial endpoints for Moderate category patients.

As mentioned earlier, for Moderate patients, the average change in the ordinal scale from the baseline scores from randomization on the eight-point ordinal scale as defined by WHO was calculated as the primary endpoint. The distribution of WHO score, Mean ± SE, of the two treatment groups in Moderate patients (n=41) is given in **Figure 4, Table4**.

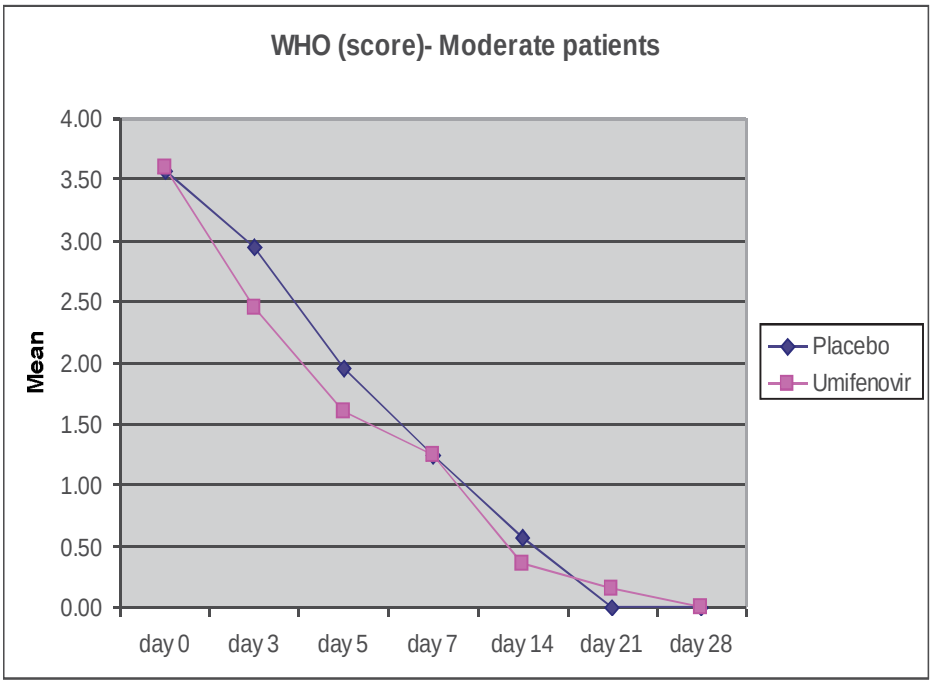

Figure 4. Pink lines corresponds to Umifenovir patients in the Moderate category, while blue represents the placebo category. Both sets of patients received the standard-of-care.

We found that in the Moderate patients group also the reduction in the mean WHO score was faster in the Umifenovir group as compared to the patients on Placebo, although the reduction was not statistically significant (P=0.125 & 0.281 on days 3 and 5 respectively).

Table 4. Average WHO scores (Mean ± SE) tabulated for the Moderate group (n=41)

Time (days)	**Moderate (n=41)**		
	Placebo (n=21)	**Umifenovir (n=20)**	***P* value**
day 0	3.57 ± 0.11	3.60 ± 0.11	0.930

day 3	2.95 ± 0.19	2.45 ± 0.22	0.125
day 5	1.95 ± 0.32	1.60 ± 0.32	0.281
day 7	1.24 ± 0.32	1.25 ± 0.32	0.971
day 14	0.57 ± 0.24	0.35 ± 0.20	0.497
day 21	0.00 ± 0.00	0.15 ± 0.15	0.646

Adverse Events (AE)

We found that Umifenovir was well tolerated. No serious adverse events were noted in the patients and additionally no deaths were seen in any of the groups. A total of 14 patients with minor adverse events were noted (**Table 5**) with symptoms ranging from headache, stomach ache, nausea and vomiting. The patients who exhibited minor AEs were almost equally divided between the Umifenovir and Placebo groups respectively. Further our assessment of all patients on 0,7,14,21 and 28 days on eight-category ordinal scale defined by WHO supported no deterioration of the clinical status. Additionally, the analysis of laboratory parameters also showed that clinically significant changes were not found in both patient groups. This is as expected, as Umifenovir has been safely used for over 25 years as an over the counter medicine and is in line with other reported trials.

Table 5 Tabulation of adverse events.

Umifenovir group			
Category	**Symptom**	**Number of patients**	**Resolved (Y/N)**
Asymptomatic	Stomach ache	1	Y
Mild	Nausea	2	Y
Mild	Headache	1	Y
Asymptomatic	Nausea with Vomiting	2	Y
Asymptomatic	Headache/ Nausea	1	Y
Placebo group			
Asymptomatic	Stomach ache	1	Y
Mild	Nausea	1	Y
Asymptomatic	Vomiting	1	Y
Moderate	Nausea with Vomiting	1	Y
Moderate	Headache/ Nausea	1	Y
Asymptomatic	Stomach ache/ headache	1	Y
Mild	Stomach ache / Nausea/ Vomiting	1	Y

Discussion

Umifenovir is a safe drug used for over 25 years in Russia and China against Influenza. It has been approved for use in children and pregnant women from the second trimester onwards in these countries. It was used as a standard of care/ trialled in the latter countries in the earlier stages of the COVID19 pandemic and the earlier trials suggested better benefits as compared to drugs like Lopinavir/Ritonavir. However, retrospective studies involving hospitalization or severe cases were not clear in their conclusion and the reports suggested that additional studies are needed.

Our own hypothesis, based on earlier reports, suggested that early administration of the drug should be useful for COVID-19 patients and also that the dosage of Umifenovir was much less than that needed to achieve the C_{max} suggested for use against SARS-Cov2. This was also suggested by other studies (7). We therefore designed separate primary endpoints for Mild-asymptomatic and moderate patients respectively.

To the best of our knowledge, the present trial is the first one involving Umifenovir against SARS-Cov2 that is double-blinded, placebo controlled one. The earlier clinical trials involving Umifenovir against SARS-Cov2 did not involve placebo control. Further, the dosage in the earlier reported trials did not take into account the earlier suggested Cmax of 4.1 µM needed for efficacy of Umifenovir against SARS-Cov2. A single dose of 800 mg of Umifenovir in healthy patients were reported to have a Cmax of about 4.1 µM and this corresponds to the IC50 of ~4.1 µM reported against SARS-Cov2 for Umifenovir. The reported half-life of ~14-16 hrs and the good safety profile of the drug led us to rationally propose a dosage of 800mg twice a day for the repurposing strategy involving Umifenovir against SARS-Cov2.

In the trials, we found that Umifenovir was safe and well tolerated and only few minor events like headache, stomach ache and nausea were reported and this also was distributed almost equally between the Umifenovir and standard of care arms respectively. No negative disease progression was noted in both arms and the patients steadily improved. No deaths were also reported in either arm. This is similar to the reports of minor adverse events in other trials involving Umifenovir.

In the present trial the primary endpoint involving asymptotic and mild patients was time to nasopharyngeal swab negativity by two RT-PCR tests for SARS COV2 antigens taken 24 hrs apart from the date of randomization. While the secondary endpoint was the average change in the ordinal scale from the baseline scores from randomization on the eight-point ordinal scale as defined by WHO.

In the Mild-asymptomatic patients group (n=82), we found that 73% patients on the Umifenovir arm were RT-PCR negative on the 5th day as compared to only 40% patients on the placebo arm (P=0.004). Hence the trial meets the primary endpoint criteria for this patient category. Our confidence in the result for the Mild-asymptomatic patients is further bolstered by the *post hoc* statistical analysis that was estimated to be 84.5% as compared to the originally calculated 80%. Statistically significant clinical recovery (P = 0.002) was also observed for the Mild-asymptomatic patients on the 5^{th} day as assessed by the WHO score analysis (secondary endpoint) for Umifenovir vs Placebo groups. The WHO score is a measure of how the patients in the cohort are becoming clinically better and was captured on days 0 (date of randomization), 3, 5,7,14, 21, and 28 respectively.

For Moderate patients, the average change in the ordinal scale from the baseline scores from randomization on the eight-point ordinal scale as defined by WHO was the primary endpoint. The baseline scores were similar between the respective placebo and Umifenovir arms on day 0. We found that the WHO scores for the Umifenovir arm corresponded to faster improvement as compared to the Placebo arm (P=0.125 on day3) in the moderate patients too. However, a limitation of the trial was the smaller number of patients in the moderate patients group, and we therefore suggest a larger trial for moderate patients to take these results further.

In view of the safety profile we suggest studies to evaluate efficacy in children and pregnant/ breast-feeding women too, especially as no other therapeutic is available for this population segments. We also recommend future studies for evaluation of Umifenovir as a prophylactic as this would be useful for high-risk contacts. Both the latter suggestions are supported by the fact that Umifenovir is used as a prophylactic against influenza and also approved for use in children and pregnant women.

Overall, there is an urgent need for effective and safe treatments for COVID-19 patients and our results demonstrate the efficacy and use of Umifenovir in Mild-asymptomatic adult COVID-19 patients in the dosage tested here.

Contributors

TKK, VB, RR, SS, SKR and HR contributed to the study concept and protocol design. HR, VA, MMAF, JF, VaS and VS contributed to protocol implementation and verified the clinical data integrity. AS, CBT, NG and NM contributed to the chemistry inputs for the study. TKK, RR, VB,

SS, PRM, SKR and RKT coordinated the collation of the data. Integrity of the data were independently audited by a third party and all the authors had access to the data. MPSN and MS conducted statistical analysis coordinated by RR and VB. Study was supervised by RR, VB, SS, PRM, SKR and TKK.

Declaration of interests

The authors declare no competing interests

Data sharing

The study protocol is attached as a supplementary file.

Individual patient data used in the study is not available.

Acknowledgements

M/s Medizest Pharmaceutical Pvt Ltd, Goa, India, is acknowledged for providing the Umifenovir and placebo tablets used in the study. We acknowledge *Council of Scientific and Research*, Ministry of Science and Technology, Government of India for funding the study. This manuscript is communicated by CSIR-Central Drug Research Institute.

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