ABSTRACT

As of today, seven coronaviruses were identified to infect humans, out of which only 4 of them belong to the beta family of coronavirus, like HCoV-HKU1, SARS-CoV-2, MERS-CoV and SARS-CoV. SARS family of viruses were known to cause severe respiratory disease in humans. SARS-CoV-2 infection causes pandemic COVID-19 disease with high morbidity and mortality. Remdesivir (RDV) is the only antiviral drug so far approved for COVID-19 therapy by FDA. However, it's efficacy is limited in vivo due to its low stability in presence of Plasma.

Here we show the stability of RDV encapsulated with our platform technology based polymer NV-387 (NV-CoV-2-R), in presence of Plasma in vitro in comparison to naked RDV when incubated in plasma. The potential use of this polymer in vivo will be discussed, here.

Keywords
Remdesivir, COVID-19, SARS-CoV-2, Therapy, Vaccine, Antiviral, NV-CoV-2 (387-Polymer), Nanoviricides.

Introduction
A new threat to human life, in recent days, is the outbreak of SARS-CoV-2 human corona virus in China, at Wuhan City in late December 2019 [1]. SARS-CoV-2 is a beta family of human coronavirus and stands for “Severe Acute Respiratory Syndrome Coronavirus 2” that causes the severe lower tract infection called COVID-19 [2-4]. As of Sept 30, 2021, the world-wide SARS-CoV-2 infected cases are more than 219M and death number is about 4.55M [5]. In USA, there have been over 44.5 million confirmed cases with 715K deaths (approx.) [6].

The infection capabilities of SARS-CoV-2 are believed to be primarily due to its increased affinity for the angiotensin-converting enzyme 2 (ACE2) receptor on the body’s cell surface [1,2]. We have recently written an article on referring the zoonosis, susceptibility and different strategies to develop therapeutics [3].

COVID-19 was declared as a global pandemic on 11 March 2020, by the WHO, however, the evidence for therapies against this virus is as yet inadequate. Various medical teams are prescribing drugs for this collection of ailments based on some mechanistic data but with limited clinical findings in support of their activity. The clinical importance of pre-clinically validated regimens relies on the available pharmacokinetic (PK) data for the particular drug [1,3].
SARS-CoV-2 virus enters a host cell by binding to and fusing with cell membrane receptor, ACE-2, followed by membrane fusion. Once inside, the virus uses the host cell’s machinery to replicate, using the virus’s RNA dependent RNA polymerase (RdRp) for making genome and transcript copies. Among the different strains of the corona virus, this non-structural protein is unique in structure, and thus making it a potentially useful drug target. *Sofosbuvir*, a synthetic analogue of nucleosides and nucleotides which inhibits RdRp has led to a successful treatment for hepatitis C infection [7]. Based on these facts, *remdesivir* was approved by FDA in various clinical trials for the treatment of COVID-19 infected people [8].

*Remdesivir*, formerly known as GS-5734, is a nucleotide analogue that is claimed to have been originally developed as a treatment against Ebola [3]. This drug can also inhibit corona virus replication by inhibiting RNA polymerases (RdRp4). This compound has shown broad antiviral activity *in vitro* against Middle East respiratory syndrome coronavirus (MERS-CoV), severe acute respiratory syndrome coronavirus 1 (SARS-CoV-1), and SARS-CoV-2 [9-11].

In animal studies, *remdesivir* has been found effective in protecting rhesus monkeys from MERS-CoV infection, when given prior to infection [12]. It also protected African green monkeys from Nipah virus, a cause of fatal encephalitis; and also, rhesus monkeys from Ebola virus [13,14]. A randomized, well-marked, controlled animal study with 12 rhesus monkeys infected with SARS-CoV-2 reported that an attenuation of respiratory symptoms and reduction in lung damage with *remdesivir* administered 12 hours after virus infection [15].

However, there are a lot of limitation of using *remdesivir* in vivo against COVID-19. (i) Efficacy of *remdesivir* *in vitro* does not match with the clinical outcomes in humans. (ii) Further, *remdesivir* has some side effects. In the Ebola trial, the side effects of *remdesivir* (RDV) were possible liver damage [16] and also affects kidney and mitochondria [17,18].

Besides, *remdesivir* is not stable in presence of Plasma. Metabolism of RDV decreases the effective concentration of the drug, *in vivo*, and the time of exposure with the drug that may be needed to eliminate the virus.

We searched for the methodologies to protect *remdesivir* from plasma-mediated metabolism. NanoViricides, Inc. (Shelton, CT, USA) is probably the only company that has developed a PEG-based polymer comprised of a polyethylene glycol (PEG-1000) and C16-alkyl pendants in the monomer unit. PEG forms the hydrophilic shell; while the alkyl chains float together to make a flexible core, like an immobilized oil droplet. This polymer can (a) directly attack the virus and disable them from infecting human cells, and simultaneously, and (b) block the reproduction of the virus that has already gone inside a cell (Figure 1). Together, this double-whammy would result in a cure [3].

In this communication our objective is to determine the efficacy of the polymer whether can protect RDV from plasma-mediated catabolism. Time dependent RDV level were measured by LC-MS analysis after incubation of the naked RDV vs polymer encapsulated RDV with rat plasma *in vitro*. RDV-metabolite, GS-441524 was also measured to justify the RDV breakdown. For comparison we used commercially available Gilead Remdesivir, and as a negative control we used vehicle (DMSO:MeOH, 1:9). Here we report our results.

### Materials and Methods

#### Test Articles (Table 1)

<table>
<thead>
<tr>
<th>SL#</th>
<th>Test Article</th>
<th>Test Article description</th>
<th>Concentration (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NV-1067-387-Medium</td>
<td>NV-CoV-2 (Polymer)</td>
<td>16 / 0</td>
</tr>
<tr>
<td>2</td>
<td>NV-1067-387-High</td>
<td>NV-CoV-2 (Polymer)</td>
<td>32 / 0</td>
</tr>
<tr>
<td>3</td>
<td>NV-1067-387-R-Low</td>
<td>NV-CoV-2+Remdesivir</td>
<td>8 / 0.8</td>
</tr>
<tr>
<td>4</td>
<td>NV-1067-387-R-Medium</td>
<td>NV-CoV-2+Remdesivir</td>
<td>16 / 1.6</td>
</tr>
<tr>
<td>5</td>
<td>NV-1067-376</td>
<td>SBECDF+Remdesivir</td>
<td>0 / 1.0</td>
</tr>
<tr>
<td>6</td>
<td>NV1067-377</td>
<td>Dulbecco’s buffer (PBS)</td>
<td>0 / 0</td>
</tr>
</tbody>
</table>

#### Sources of Reagents (Table 2)

<table>
<thead>
<tr>
<th>Item(s)</th>
<th>Vendor</th>
<th>Cat#</th>
<th>Lot#</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS 1X, pH 7.4</td>
<td>Life Technologies</td>
<td></td>
<td></td>
<td>pH 7.2</td>
</tr>
<tr>
<td>Low-binding tubes</td>
<td>Eppendorf</td>
<td>20431081</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na-Acetate Buffer 1M, pH 5.2</td>
<td>Sigma</td>
<td>S7899</td>
<td>SLBS5549</td>
<td></td>
</tr>
<tr>
<td>Acetonitrile for precipitation</td>
<td></td>
<td></td>
<td></td>
<td>100%</td>
</tr>
<tr>
<td>Rat- Plasma-Sprague Dawley</td>
<td>Innovative Research</td>
<td>IGRT-Na-EDTA 22443</td>
<td></td>
<td>Anticoagulant-Na-EDTA</td>
</tr>
<tr>
<td>Remdesivir (RDV)</td>
<td>Medkoo Biosciences, Inc.</td>
<td>329511</td>
<td>E20004S01</td>
<td></td>
</tr>
<tr>
<td>GS-441524</td>
<td>Medkoo Biosciences,</td>
<td>555299</td>
<td>C20R07B09</td>
<td></td>
</tr>
<tr>
<td>ISTD (13C5-isotope) for RDV</td>
<td>AlsoCHIM</td>
<td>8845</td>
<td>MJ-ALS-20-037-P1</td>
<td></td>
</tr>
<tr>
<td>ISTD (13C6-isotope) for GS</td>
<td>AlsoCHIM</td>
<td>8855</td>
<td>MJ-ALS-20-050-P1</td>
<td></td>
</tr>
<tr>
<td>Isopropanol</td>
<td>Sigma-Adrich</td>
<td></td>
<td></td>
<td>75%</td>
</tr>
</tbody>
</table>
Standard Curve Preparation of Remdesivir and GS-441524 were determined by using LC-MS, using different concentration of the standards solution in DMSO + MeOH (1:9) ranging from 0-5 ng/mL final concentrations. Plasma and/or PBS were used in the reaction mixture, as when needed. As an internal standard (ISTD) \(^{13}C_5-RDV\) and \(^{13}C_6-GS441524\), 2.5 ng/mL each, were used, respectively for RDV and GS assay, and or in mixture. The final concentration of the internal standards in the reaction mixture becomes 0.125 ng/mL. Extraction of RDV and its metabolite GS-441524, for LC-MS assay were done by using Acetonitrile.

Concentrations of RDV and GS-441524 vs. their ratio with the internal standards, ISTDs \(^{13}C_5-RDV\), \(^{13}C_6-GS\), respectively were used to generate a standard curve for RDV and GS-441524, respectively, as shown in the table 2 and figure 2.

**Incubation of test materials with rat plasma (RPL) in vitro, and isolation of the RDV/GS-441524 by Acetonitrile**

Ten microliter of the test materials (20 ng/mL) were incubated with 30 uL rat plasma for different time points (as indicated in the Figures), and then extracted with Acetonitrile (100 uL). The mixture is centrifuged for 10 mins at 10,000 x g to separate the supernatants from the precipitates. The supernatants containing either RDV and/or its metabolites, GS-441524 were determined by LC-MS chromatography (as described below).

**Detection of RDV and GS-441524 by LC-MS spectroscopy**

Analysis was performed using the LC and MS analysis conditions shown in Table 3, and the multiple reaction monitoring (MRM) data acquisition parameters shown in Table 4. Shim-Pack Sceptor™ C18-120 (50 mm x 22.1 mm I.D., 1.9 uM) was used as the analytical column.

### Table 3: Chromatographic Conditions.

<table>
<thead>
<tr>
<th>LC Analysis Conditions</th>
<th>HPLC Method:</th>
<th>Shimadzu, Shim Pack, Sceptor, C18-120, 2.1x50 mm, 1.9 um, Cat#227-31120-01</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-Column:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mobile Phase:</td>
<td>A: Water containing 0.05% formic acid B: Acetonitrile containing 0.05% formic acid</td>
<td></td>
</tr>
<tr>
<td>Gradient:</td>
<td>0-0.3 min: 5% B, 0.3-0.35 min: 30% B, 0.35-1.1 min: 70% B, 1.5-1.8 min: 90% B, 1.8-2.8 min: 90% B, 2.8-2.9 min: 5% B, 2.9-4.5 min: 5% B, (A: Solvent A; B: Solvent B)</td>
<td></td>
</tr>
<tr>
<td>Injection Volume:</td>
<td>1 uL</td>
<td></td>
</tr>
<tr>
<td>Auto-sampler was used in external rinsing mode, with R0 = 95/5 Water / Acetonitrile and R3 = 1/1 IPA / Methanol.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 4: MS MRM Transitions observed (for quantitation).

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Ion</th>
<th>Precursor Ion (m/z)</th>
<th>Product Ion (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RDV (target, drug):</td>
<td>Quantitation ion</td>
<td>603.2</td>
<td>200.0 (@41)</td>
</tr>
<tr>
<td>(^{13}C_5-RDV) (Internal standard for RDV)</td>
<td>Quantitation ion</td>
<td>603.2</td>
<td>229.0 (@21)</td>
</tr>
<tr>
<td>GS-441524 (target, metabolite):</td>
<td>Quantitation ion</td>
<td>292.1</td>
<td>163.0 (@25)</td>
</tr>
<tr>
<td>(^{13}C_6-GS-441524) (Internal standard for metabolite)</td>
<td>Quantitation ion</td>
<td>292.1</td>
<td>136.0 (@26)</td>
</tr>
</tbody>
</table>

**Calculation of RDV and GS-441524 from the chromatogram**

The ratio of RDV and its isotope, \(^{13}C_5\)-ISTD (as an internal standard) was calculated. Similarly, the ratio of GS and its internal standard, \(^{13}C_6\)-ISTD was also calculated. From that ratio, the amount of RDV and GS were determined using the linear equation derived from their respective standard curve.

The values were normalized using the Dilution factor (2 x 20 x 4=160) used for Original Plasma sample.

### Results and Discussion

**Standard Curve of RDV and GS-441524 (Table 2, Figure 2)**

A representative standard curve for RDV and GS-441524, both were shown in Figure 2 with R² value (>0.99). The experiment was repeated 3 times with similar results.

**Stability of different RDV test materials in presence of Rat Plasma in vitro were shown in Figures 3-5**

Test materials are:

1. RDV in DMSO: MeOH (1:9)
2. RDV-NV387 (RDV encapsulated in NV-387 polymer)
3. RDV-in SBEDC (NV1104-376A, Gilead)

Other test materials used in these experiments are the different vehicles used as a negative controls, such as, DMSO:MeOH (1:9), NV-387 polymer itself, and PBS buffer.

In brief, NV-387 Polymer can encapsulate Remdesivir efficiently, as we see RDV-387 show RDV peak in LC-MS chromatography, whereas only the polymer NV-387 does not show any RDV peak (data not shown). We have also tried to measure RDV and GS-441524 level in the respective vehicles, like DMSO, Polymer itself NV-387, and PBS, but the results are below the detection level (data Not shown).

**Comparative analysis of RDV breakdown and GS-441524 production by the above test materials in presence of Rat Plasma, in vitro (Table 3, Figure 6):**

NV-387-polymer encapsulation protects RDV in vitro from plasma-mediated catabolism, thus could protect RDV in vivo for longer period of anti-viral effect even more than that of Gilead-RDV (Figure 6).
Figure 1A: Novel Platform technology: Nanoviricide is a Cell Mimic: A Virus particle binding to a Cell via CD4 and a co-Receptor. A nanoviricide “looks like” a human cell to the virus. Nanoviricide is large enough for a virus particle to latch onto it, however, it is yet small enough to circulate readily in the body. Rather than a virus particle entering into a nanoviricide, a nanoviricide wraps around the virus particle and encapsulate it, by using the virus particle’s very same ability to enter a cell. Viral resistance to the Nanoviricide drug is unlikely because even as the virus mutates, it still binds to the same cell surface receptor(s), in the same fashion. 

Figure 1B: A schematic presentation of nanoviricide action on virus particle.

Figure 2: Standard Curve of Remdesivir and GS-441524 were determined by using LC-MS, using different concentration of the standards solution in DMSO + MeOH (1:9) ranging from 0-5 ug/mL final concentrations. Plasma and/or PBS were used in the reaction mixture, as when needed. As an internal standard (ISTD) 13C6-Remdesivir and 13C5-GS, 2.5 ug/mL each, were used, respectively for RDV and GS assay, and or in mixture. Final extraction of RDV and its metabolite GS, were dome by using acetonitrile.
Nonetheless our Platform technology based NV-387-encapsulated-RDV drug has a dual effect on coronavirus, as it being itself possess an antiviral activity [19,20], and also by protecting RDV more than that of Gilead-RDV, renders RDV effectiveness against virus. Further, potential mutations in the virus are unlikely to enable it to escape these drug candidates.

Further, we have already scaled up production of key portions to multi-kilogram scales. We are now initiating an animal model study to finalize two to three best candidates for further testing. We intend to perform certain animal model safety studies, in order to further advance the final candidate for limited human clinical (compassionate) use scenario. This is the fastest timeframe that a drug candidate truly directed at the SARS-CoV-2 has been developed by any Company to date (https://www.marketwatch.com/press-release/nanoviricides-develops-highly-effective-broad-spectrum-drug-candidates-against-coronaviruses-2020-05-12). Nanoviricides Develops Highly Effective Broad-Spectrum Drug Candidates Against Coronaviruses. Published: May 12, 2020 at 7:15 a.m. ET)

Conclusions
It is needless to say that in the present days, rapid design & construction, as well as synthesis and manufacture of anti SARS-CoV-2 has a crying need. Although our approach is very potential for the COVID-19 therapy, but we have been limited to studying effectiveness against available BSL2 level strains of coronaviruses, as they do not cause the severe pathology in humans. No other
Figure 5: Stability of RDV in SBEC (NV1104-376A; Gilead) in RPL, in vitro.
The samples RDV in SBEC (Gilead) were tested for their stability in presence of Rat Plasma in vitro as described in Methodologies section. GS-441524, the RDV metabolite formations are representative of RDV breakdown, and supportive to each other data. Each data points are the Mean ± SD of three experiments done in duplicate.
Figure 6: Comparison of Stability of RDV in DMSO, in polymer 387 encapsulated, and in SBECDE-\textit{In vitro} in presence of Rat Plasma.

Stability in RPL: The samples, RDV, RDV-encapsulated in MM6 Polymer, RDV in SBECDE (Gilead) were tested for their stability in presence of Rat Plasma in \textit{vitro}. Methodologies of incubation in reaction mixture, extractions, are same as we did for standard curve determinations of RDV/GS. The results show that RDV alone has a very short life in presence of Plasma, but encapsulation in NV-387 polymer make them very stable even after overnight incubation, even compared to Gilead RDV product. GS-441524 metabolite formations are representative of RDV breakdown, and supportive to each other data.
drug currently available in the development for SARS-CoV-2 virus in the USA, be it antibody, small chemical, or otherwise, has been tested against any coronaviruses at present, to the best of our knowledge.

We are in contact with USAMRIID (United States Army Medical Research Institute of Infectious Diseases) and Robert Davey at NIEDL in Boston, for testing against SARS-CoV-2 itself. We have not received back a Materials Transfer Agreement to enable initiating these studies. Meanwhile its report to scientific community as well as to the common people for their attention and assistance in furthering this endeavor, to contribute to the fight against the SARS-CoV-2 pandemic and position to win the fight.

Conflict of Interests
Author, Anil Diwan, was employed by the company Nanoviricides, Inc. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Fundings
Nanoviricide's Inc own fund.

References
5. COVID.cdc.gov/ COVID-data-tracker/