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Research Article

Encapsulation of Remdesivir in Nanoviricide's Platform Technology Based NV-CoV-2 Polymer Protects the Drug and Improves Its Pharmacokinetics

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Abstract

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), a new member of beta family of human coronaviruses was known to cause highly infectious and fatal respiratory diseases in human Remdesivir (RDV), originally established as an antiviral against Ebola virus is recently approved for COVID-19 therapy by the FDA. However, the efficacy of RDV *in vivo* is limited due to its low stability in presence of plasma. This is the report of analysis of the non-clinical pharmacology study of NV-CoV-2 (Polymer) and NV-CoV-2-R (Polymer encapsulated RDV) in both infected and uninfected rats with h-NL-63 virus, a similar to SARS-CoV-2 but less virulant.

Detection and quantification of RDV, free or encapsulated (NV-CoV-2-R), in plasma samples from rat subjects were done by MS-HPLC chromatography analyses.

- NV-CoV-2-R showed RDV peak in MS-HPLC chromatography, whereas NV-CoV-2 does not, as expected.
- NV-CoV-2 polymer encapsulation protects RDV *in vivo* from plasma-mediated catabolism.
- Body weight measurements of the normal (uninfected) rats after administration of the test materials (NV-CoV-2, and NV-CoV-2-R) show no toxic effects.

Our platform technology based NV-387-encapsulated-RDV (NV-CoV-2-R) drug has a dual effect on coronaviruses. First, NV-CoV-2 itself showed as an antiviral regimen, secondly, protects RDV from plasma-mediated degradation in transit. Therefore, altogether NV-CoV-2-R could be a safest and efficient regimen against COVID-19.

Keywords: *NV-CoV-2; COVID-19; Platform Technology*

Abbreviations

NV: Nanoviricides; PK/PD: Pharmacokinetics/Pharmacodynamics; RDV: Remdesivir; NV-387: Nanoviricides-Polymer 387; NV-387-R: Nanoviricides-Polymer 387-Remdesivir Conjugate; SBECD: Commercial Encapsulating Agent SBECD (GILEAD); SBECD-R: Commercial Remdesivir Conjugated with SBECD; PBS: Phosphate Buffered Saline; DMSO: Dimethyl Sulfoxide; RPL: Rat Plasma; MeOH: Methanol; ADME: Absorption, Distribution, Metabolism and Excretion; SD: Standard Deviation

Introduction

SARS-CoV-2, one of the members of a beta family human coronaviruses causes COVID-19, a severe respiratory illness with a high morbidity and mortality [1]. As of today, some vaccines are available as a protection against this virus infection, but no effective therapeutics identified yet.

SARS-CoV-2 virus can bind with the host cell membrane receptor protein angiotensin-converting enzyme 2 (ACE2) and allows the virus to enter the cell. After entering into the cells, the virus uses the cell replication system RNA dependent RNA polymerase (RdRp) for making their own genome and transcript copies [2].

COVID-19 was first erupted in Wuhan City of China around December 2019. The virus rapidly became a global pandemic with over 100 million people have confirmed infections and more than 3 million deaths [3].

Remdesivir (RDV), a nucleotide analogue, that was originally used as treatment against Ebola virus infection [4]. This drug actually inhibits RNA polymerases (RdRp4), and thus inhibits the viral replication inside the cells. In the cell culture system, RDV has shown a broad antiviral effect against SARS-CoV-2 and other beta-family human coronaviruses such as, Middle East respiratory syndrome coronavirus (MERS-CoV) and severe acute respiratory syndrome coronavirus-1 (SARS-CoV-1) [5-7]. An experiment with rhesus monkeys, RDV was found to be effective against MERS-CoV when given to the animals prior to the infection [8]. Similar outcome was also documented with RDV against *Nipah virus* infection that cause fatal encephalitis to African green monkeys [9]. In a controlled animal study with 12 rhesus monkeys, the SARS-CoV-2 mediated symptoms and any lung damage from the viral infection was reversed when RDV was administered 10-12 hours before the viral infection [10,11].

However, the clinical outcome of using RDV in humans does not match the efficacy of RDV *in vitro* or *in vivo* [12]. Further, RDV during the Ebola trial has shown some side effects like, liver damage due to increase in liver enzymes in blood. Similar findings were also documented in three U.S. COVID-19 patients treated with RDV [13,14].

The efficacy of RDV *in vivo* is limited due to the low stability in the plasma. We have tested the stability of RDV encapsulated with our platform technology-based polymer NV-CoV-2-R, in presence of plasma *in vitro*. The result is very supportive on the polymer protecting RDV from plasma-mediated catabolism [15].

In this paper, we wanted to test our *in vitro* results in an *in vivo* rat model of systemic exposure of NV-CoV-2 and NV-CoV-2-R. The drugs were administered once per day for 5 days (0, 1, 3, 5, and 7) over a 7-day time period. The results were compared with commercially available Gilead RDV and used DMSO as a negative control. Here we report our results.

Materials and Methods

Test articles

- NV-CoV-2 used in Batch ID NV1067-387: Polymer
- NV-CoV-2-R used in Batch ID NV1067-387-R: Polymer encapsulated RDV
- NV1067-376: RDV in SBECD (Gilead)

In vivo treatment

Thirty-six Sprague Dawley female rats (Taconic Biosciences, USA) (three/-treatment groups) were administered with NV-CoV-2 or NV-CoV-2-R, once a day for 5 days (0, 1, 3, 5, and 7) over a 7-day time period. Injection on day 0 was considered the 1st injection and on day 7 is the 5th injection. NV-376 (RDV-in-SBECD; Gilead) was given in two doses on day 1 followed by once a day until day 7. Each compound was delivered via slow-push IV injection.

Blood sample for systemic exposure assay were collected at 0, 0.08, 0.5, 1, 2, 4, 8 and 24 hours after 1st and 5th injection of the drugs.

At each time point, the blood from one animal in each test article treated group was collected. The same animal was used throughout the entire study from "day 0" to "day 7" injections.

The procedures for *in vivo* experiment done by Dr. Krishna Menon from AR Biosystems, (17633 Gunn Highway, Odessa, FL 33556), based on the protocol #IACUC No. 14/17ARB. The study design was shown in table 1.

Group	Drug	Dose (mg/Kg)	Dose Volume (mL/Kg)	Concentration (mg/mL) NV-CoV-2/ Remdesivir
1	NV-1067-387-High	320	10	32/0
2	NV-1067-387-Medium	160	10	16/0
3	NV-1067-387-R-Low	80 of NV-CoV-2, plus 8 of Remdesivir	10	8/0.8
4	NV-1067-387-R-Medium	160 of NV-CoV-2, plus 16 of Remdesivir	10	16/1.6
5	NV-1067-376	0 of NV-CoV-2, plus 16 of Remdesivir	10	0/1.0
6	NV-1067-377	0	10	0/0

Table 1: *Study Design.*

Assay for RDV in plasma by LC-MS spectroscopy

Preparation of standard curve of pure RDV (Purchased from Sigma-Aldrich Co. USA). Reagents and their source were shown in (Table2).

Item(s)	Vendor	Cat#	Lot#	Description
PBS 1X, pH 7.4	Life Technologies			pH 7.2
Low-binding tubes	Eppendorf	20431081		
Na-Acetate Buffer 1M, pH 5.2	Sigma	S7899	SLBS5549	
Acetonitrile for precipitation				100%
Rat- Plasma-Sprague Dawley	Innovative Research	IGRTNaEDTA 22443		Anticoagulant-NaEDTA
Remdesivir (RDV)	Medkoo Biosciences, Inc.	329511	E20004S01	
ISTD ($^{13}C_6$-isotope) for RDV	AlsaCHIM	8845	MJ-ALS-20-037-P1	
Isopropanol	Sigma-Adrich			75%

Table 2: *Sources of Reagents.*

Standard curve preparation of RDV was determined by using LC-MS. Different concentrations of the standard solutions in DMSO + MeOH (1:9) ranging from 0-5 ng/uL final concentrations was prepared. As an internal standard (ISTD), $^{13}C_6$ –RDV was used in the mixture. The final concentration of the ISTDs in the reaction mixture became 0.125 ng/uL.

Extraction of RDV for LC-MS assay was done by using an Acetonitrile cocktail solution (Acetonitrile: ISTDs: 75% Isopropanol at 10:1:1), added at the ratio of 1:4 by volume. The mixture was vortexed and centrifuged at 10,000 RPM for 5 minutes to separate the supernatants from the precipitated solids. The ratio of concentrations of RDV vs. ISTDs, ($^{13}C_6$-RDV) was used to generate a standard curve for RDV.

Assay of remdesivir in rat plasma after the drugs injection

To test the *in vivo* stability of our in-house made anti-COVID-19 product, NV-CoV-2 polymer-encapsulated RDV was injected along with the polymer as a negative control at two different doses. As a positive control, we included other market available Gilead RDV compounds in our *in vivo* experiments with female rats. After the 1st and 5th injection (day 1 and day 7), blood samples were collected at different time points for 24 hrs. The received samples from the test site were pre-diluted (1:2) and diluted further to (1:20) with plasma:3M Na-acetate, pH 5.0 (1:1). In those samples, reaction mix including ISTDs, 75% Isopropanol and Acetonitrile (1: 1:10) was added at the ratio of 1:4 by volume for the extraction of RDV from the test materials. The samples were vortexed and spun at 10,000 RPM for about 5 minutes to separate the precipitated solids from the supernatants.

Detection of RDV by LC-MS spectroscopy

LC/MS analysis was performed based on the conditions mentioned in table 3. The multiple reaction monitoring (MRM) data are shown in table 4. Shim-Pack Sceptor™ C18-120 (50 mm x 2.1 mm I.D., 1.9 uM) analytical column was used for the assay.

LC Analysis Conditions	
HPLC Method	RDVmetIS-LC-NoSlit-0.4mL-ExtRinse-202010231219.1cm
Column	Shimadzu, Shim Pack, Sceptor, C18-120, 2.1 x 50 mm, 1.9 um, Cat#227-31120-01
Pre-Column	Guard Column, Shimadzu, Shim-pack, C18-120 EXP (G), 1.9 um, 2.1 x 5 mm, or Restek prefilter: Ultrafield UHPLC, o.2 um frit, Catalog# 25810.
Mobile Phase	A: Water containing 0.05% formic acid B: Acetonitrile containing 0.05% formic acid
Gradient	0 - 0.3 min: 5% B, 0.3 - 0.35 min: 30% B, 0.35 - 1.5 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)
Injection Volume	1 uL
Auto-sampler was used in external rinsing mode, with R0 = 95/5 Water/Acetonitrile and R3 = 1/1 IPA/Methanol.	

Table 3: *Chromatographic conditions.*

Compounds	Ion	Precursor Ion (m/z)	Product Ion (m/z)
RDV (target, drug):	Quantitation ion	603.2	200.0 (@41)
	Quantitation ion	603.2	229.0 (@21)
$^{13}C_6$-RDV (Internal standard for RDV)	Quantitation ion	609.2	206.0 (@25)
	Quantitation ion	609.2	229.0 (@26)

Table 4: *MS MRM Transitions Observed (for quantitation).*

Calculation

The ratio of RDV and its isotope, $^{13}C_6$-ISTD (as an internal standard) was calculated from the chromatogram. The amount of RDV was determined using the linear equation derived from their respective standard curve.

Normalization of the value using the dilution factor from the original plasma sample

Samples	Received as	Further Diluted with the Vehicle	Diluted in the reaction Mix	Final Dilution Factor
Plasma Samples	1:2 (x 2) pre-diluted	1:20 (x 20)	1:4 (x 4)	2 x 20 x 4 (x160)

Table 5: *Sample dilution calculation.*

Results and analysis of the data

- A standard curve for RDV from a representative experiment were shown in table-6 and (Figure 1).
- RDV values in rat plasma obtained (mg/mL) after 1st and 5th injections of the drugs were normalized by dividing with the amount of RDV administered (mg/kg of rat body weight) and shown in (Figure 2)anfd (Figure 3), respectively.
- Comparative analysis of RDV level in rat plasma after 1st and 5th injections of the drugs were shown in (Figure 4) **Values** were normalized as a ratio of RDV found in blood (mg/mL) and the amount of RDV that was administered (mg/Kg of rat body weight).
- Toxicity/Tolerability study of NV-CoV-2-R: Loss of body weight analysis of rats after drug administration i.v. (Figure 5) (Table 6).

RDV (ng/mL)	RDV MRM Response	$^{13}C_6$ RDV MRM Response (ISTD)	Ratio (RDV MRM Resp./ ISTD MRM resp.)	Ratio: Minus Blank
0.00	1490	1817785	0.0008	0.0000
0.05	8621	1584501	0.0054	0.0046
0.10	22660	1505391	0.0151	0.0143
0.20	54772	1364275	0.0401	0.0393
0.50	1600401	1510440	1.0596	1.0588
1.00	3027001	1708963	1.7713	1.7705
2.50	5219885	1528426	3.4152	3.4144
5.00	9943162	1331972	7.4650	7.4642

Table 6: *A representative standard curve of RDV.*

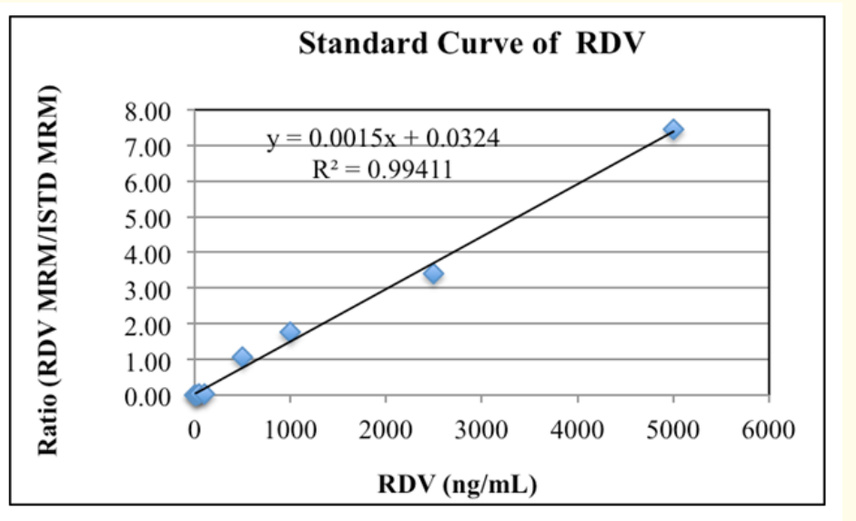

Figure 1: *Standard curve of RDV was determined by using LC-MS, using different concentration of the standards solution in DMSO + MeOH (1:9). Final concentrations of RDV ranged from 0-5 ug/mL, which is described in the method section. Values (Mean ± SD) are from a representative experiment done in duplicate.*

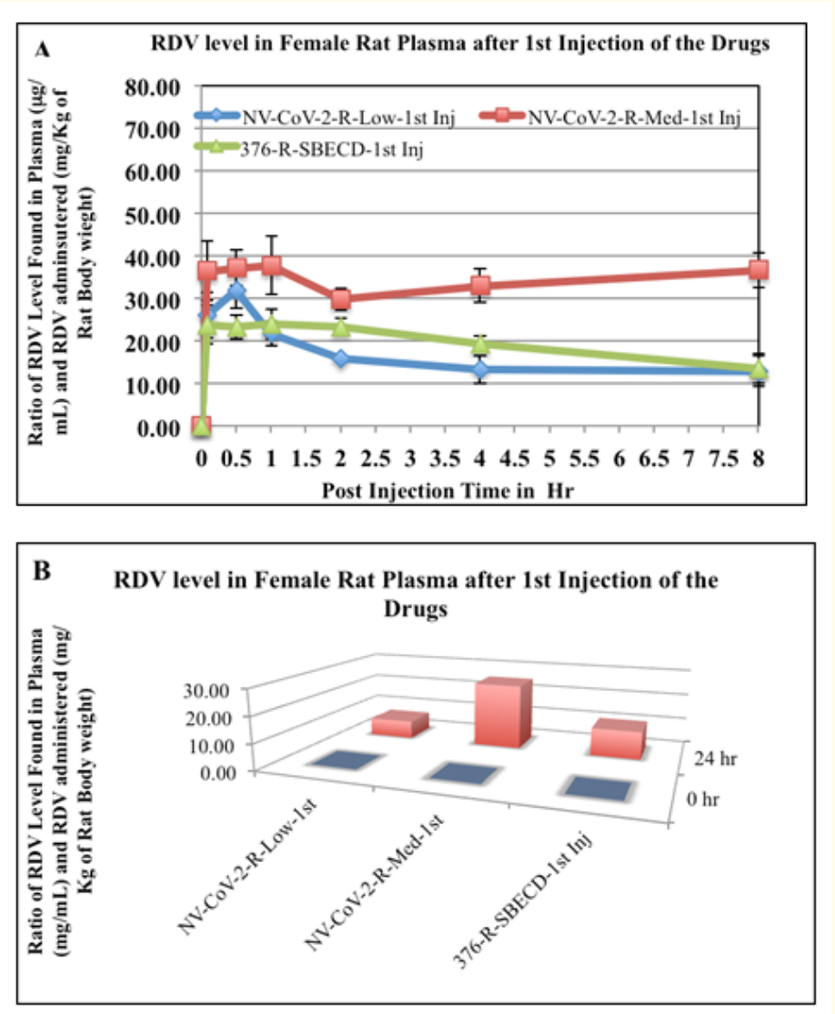

Figure 2: *RDV values in female rat plasma after 1st injection of the drugs.*

The blood samples collected at different time points after drug administration i.v. to the animals, were collected and measured for RDV level as described in the method section. The values obtained as mg/mL were normalized by dividing with the RDV amount administered (mg/kg of rat body weight). Each data point is the mean (±SD) of 3 values and the experiment was repeated three times with similar results.

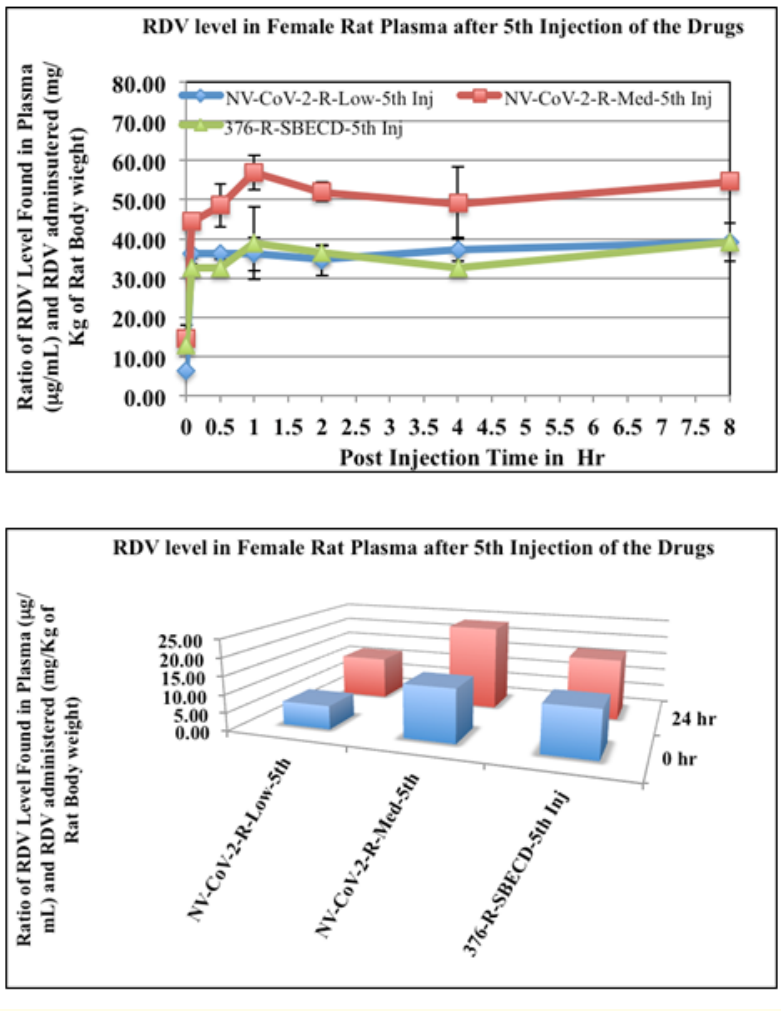

Figure 3: *RDV level in Female Rat Plasma after 5th Injection of the Drugs*

The blood samples collected at different time points after 5th i.v injection of drug to the animals, were collected and measured for RDV level as described in the method section (A: During time points upto 8hs; and B: Values at 24 hrs. of Post injection). The values obtained as µg/mL were normalized by dividing with the RDV amount administered (mg/kg of rat body weight). Each data point is the mean (±SD) of 3 values and the experiment was repeated three times with similar results.

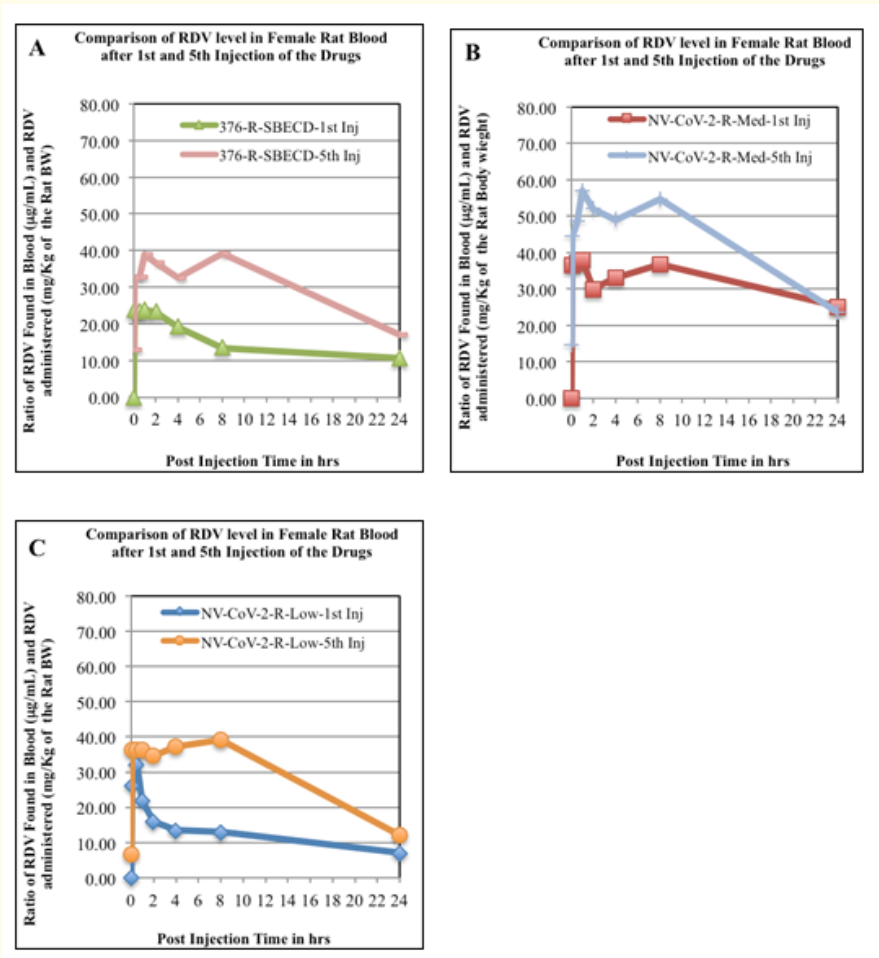

Figure 4: *Comparison of RDV level in female rat plasma after 1st and 5th injections of the drugs.*

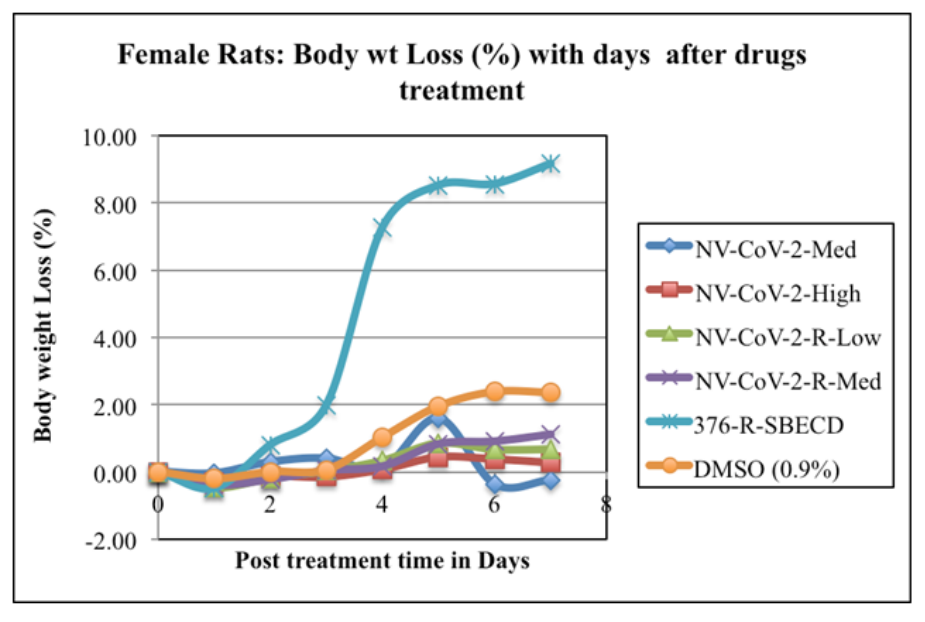

Figure 5: *Toxicity/Tolerability study of NV-CoV-2-R: Loss of Body weight analysis of female rats after drug administration.*

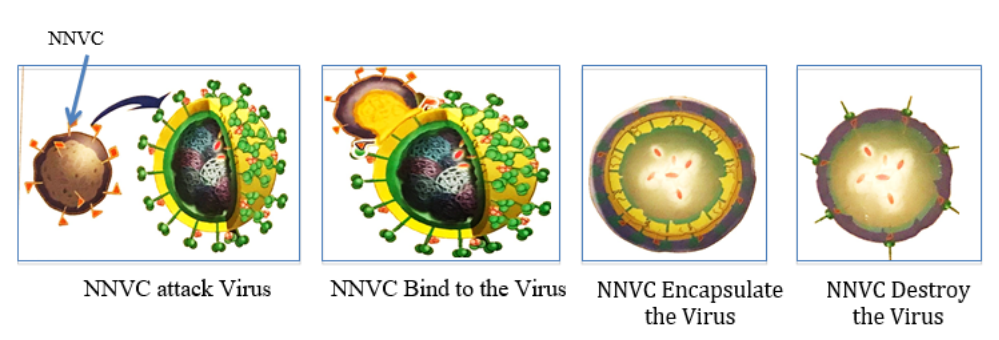

Figure 6: *Mechanism of Nanoviricide action.*

From all the above figures it appears that

- After the 5th injection of NV-CoV-2-Med, the accumulation of RDV was better than the 1st injection, and also from all the other drugs injected.
- For all the doses of NV-CoV-2-R, RDV were detected in rat plasma producing an initial increase that peaked between 4-8 hours and decreases significantly between 24 to 48 hrs. Similar pattern of pharmacokinetics have been noticed with 376-R-Gilead.

- In all the cases, our encapsulated polymer was nontoxic to the animals, based on their steady level of body weight.

Discussion

RDV originally developed as an anti-Ebola drug [4] but found also to inhibit H-CoV (SARS-CoV-1, MERS-CoV). RDV actually inhibits RNA-dependent-RNA polymerases (RdRp4) which hCoV viruses generally uses for replication inside the cells [5,6].

The efficacy of RDV alone and in combination with chloroquine was reported in cell culture system [7]. In animal studies with rhesus monkeys, RDV was found to be active when administered at least 10-12 hours before the MERS-CoV infection [8]. Similarly, RDV was also found to be effective in protecting African green monkeys from *Nipah virus* infection and rhesus monkeys from the Ebola virus infection [9,10]. SARS-CoV-2 infection modalities in rhesus monkey were found to be reverse with RDV treatment [11]. Therefore, RDV is the only drug that has been considered by the FDA for using against COVID-19 [16]. However, the clinical outcome of using RDV in humans with COVID-19 does not match with the efficacies of RDV *in vitro* or in animal studies with SARS-CoV-2.

One particular reason for this is the low stability of RDV in presence of plasma [15]. In addition, re-dissolving and filtration of RDV (Veklury, Gilead) removes undissolved RDV and thus reduces the dose applied. Cyclodextrins that form colloids at high concentrations hold insoluble RDV in the colloid, dilutes out into the bloodstream quickly and lead to falling out of the API of RDV. Protecting RDV from metabolism and keeping it in an encapsulated form is essential if its full potential is to be realized clinically.

We searched for other regimen that could be used in conjunction with RDV to minimize the breakdown of RDV in the system and can also potentiate its effect against SARS-CoV-2. Recently, it was shown that our lab-made nano-polymer (NV-CoV-2) can encapsulate RDV and protects it from degradation in the blood stream [17-21]. This bio-mimetic polymer (Nanoviricide®) can bind and engulf a virus particle into its polymeric shell, and therefore acts like a "Venus-fly-trap". Once engulfed, the virus particle gets dismantled (Figure 6).

This Nanoviricide is a broad-spectrum antiviral biopolymer and can be manipulated the ligand part of it to be fitted for various receptors of the host cells. Several drug candidates were already tested for their effectiveness in cell culture studies. One of the non-virulent human coronavirus strains (h-CoV-NL63) that uses the same cell surface receptor ACE2 (angiotensin converting enzyme-2) like SARS-CoV-2 and SARS-CoV-1, was used as a right surrogate virus for SARS-CoV-2 studies in a BSL-2 lab like ours [22]. Out of several test drug candidates, Nanoviricides lab-made biopolymer (NV-CoV-2) showed 15-times more potentiality than favipiravir (a known antiviral drug, [23] against two different coronaviruses (h-CoV-NL63 and HCoV-229E) [19-21].

Safety and tolerability of NV-CoV-2 and NV-CoV-2-R was studied in a rat model. Body weights remained constant with no clinical signs of any allergic reactions such as itching, biting, twitching, rough coat, etc., or any immunity issues. Furthermore, on postmortem and in gross histology, no observable changes in any organs including large intestine or colon were found. This non-GLP safety/tolerability study was conducted under GLP-like conditions by AR BioSystems, Inc., Odessa, Tampa, FL. Microscopic analyses for histology and blood works are in progress [19,20].

Conclusion

Detection and quantification of NV-CoV-2 in rat plasma samples from toxicology study was done using a validated LC-MS spectrometry. For all doses, NV-CoV-2 was detected in the rat plasma producing an initial increase that peaked between 4-8 hours. The results show that the plasma concentrations decreased to below detection level between 24 to 48 hrs.

Conflict of Interests

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

Authors' Contribution

All the authors contributed equally to preparing this article, read and approved the final manuscript.

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Ethical Statement

The procedures for *in vivo* experiments done by Dr. Krishna Menon from AR Biosystems, (17633 Gunn Highway, Odessa, FL 33556), based on the protocol #IACUC No. 14/17ARB.

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