DATA MANAGEMENT MED-HIGH-TECH GmbH

Administrative Office/Geschäftsstelle: Gaugasse 10, 65203 Wiesbaden·Germany· Phone:+49 (0) 611 18171122

INTERIM REPORT

Evaluation of the antiviral activity of a test item against SARS-CoV-2 in vitro in Vero E6 cells

Implementing Organization

Data Management Med-High-Tech GmbH
Gaugasse 10
65203 Wiesbaden (Germany)

Test Facility

IRTA-CReSA
Campus Universitat Autònoma de Barcelona
08193 Bellaterra (Cerdanyola del Vallès), Barcelona. Spain

IRTA-CReSA study number: SA-10573-20

Version: Interim-01
Date: 22 September 2020

Study Contacts

Implementing Organization's Representative:
Mr. Igor Rechkiman
Data Management Med-High-Tech GmbH
Senior Manager
Gaugasse 10
65203 Wiesbaden (Germany)
Tel. +49 (0) 611 18171122
E-mail: igor.rechkiman@datamanagement-med-high-tech.de

Study director on-site:
Dr. Júlia Vergara-Alert, PhD. Member of WHO expert working group on in vitro and animal models of COVID-19
Dr. Ramon Botet, PhD
IRTA - Centre de Recerca en Sanitat Animal
Campus UAB, Edifici CReSA
08193- Bellaterra (Cerdanyola del Vallès), Barcelona. Spain
Tel.: +34 93 467 40 40
E-mail : julia.vergara@irta.cat ; ramon.botet@irta.cat

Study Location

CReSA BSL3 laboratory
Centre de Recerca en Sanitat Animal (CReSA), IRTA
Campus Universitat Autònoma de Barcelona
08193 Bellaterra (Cerdanyola del Vallès), Barcelona. Spain

INDEX

1. STUDY DATES

START DATE OF EXPERIMENTAL PHASE: June 2020
FINAL DATE OF EXPERIMENTAL PHASE: July 2020

2 . OBJECTIVE

To test the antiviral efficacy of a Test Item by assessing SARS-CoV-2 replication and cytopathic effect (CPE) on Vero E6 cells.

Two tests will be performed: (i) to test the toxicity of a Test Item on cells, and (ii) to test the antiviral activity of this Test Item.

3. MATERIALS AND METHODS

3.1 Test Item

Compound-1 (or Test Item 1): Ethanol extract from *Deschampsia cespitosa* and *Calamagróstis epigéjos* (both Family: *Poaceae*); trading as Proteflazidum® drops

Batch number: 080320
Expiration date: November 2020
Manufacturer: Ecopharm, Ltd. (Ukraine). Contact: Dr. Oleksandr Grynevych PhD
E-mail: grynevych@ecopharm.ua , phone: +380673213191
Storage conditions: between 18 ºC and 25 ºC, in dark place
Appearance: liquid

Remdesivir: compound used as a control

Batch number: not provided (internal batch #202003)
Expiration date: November 2020
Manufacturer: Gilead
Storage conditions: -20ºC for long storage, at 4ºC for few days
Appearance: liquid

3.2 Test System

3.2.1. Virus

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) with ID EPI_ISL_418268 (isolated from a nasopharyngeal swab collected from an 89-year-old male patient) was used. The stock virus was prepared collecting the supernatant culture fluid from Vero E6 cells. The propagation method was specified in the raw data. On the day of use the appropriate number of aliquots were removed, thawed and maintained at a refrigerated temperature until used in the assay.

3.2.2. Cell Culture and Test Culture Media

Vero E6 cells (ATCC CRL-1586) were cultured in Dulbecco's modified Eagle medium (DMEM), supplemented with 5% fetal calf serum (FCS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM glutamine.

3.3. Test Method

3.3.1. Testing Schedule

- Compound-1 + Vero E6 cells + Virus
- Remdesivir + Vero E6 cells + Virus

- Compound-1 + Vero E6 cells
- Remdesivir + Vero E6 cells

3.3.2. Assay description

A detailed explanation of the methods used for this assay can be found in our preprint published in biorxiv *(Rodon et al., 2020).*

- *Cell preparation in plate:*

  In a 96-well plate 15,000 Vero E6 cells per well were added in 100 µl c-DMEM ($1{,}5x10^6$ cells per plate will be needed) and incubated at 37°C, 5% CO_2 overnight.
  Two 96-well plates were prepared.

- *Preparation of the Test Item:*

  Trial was performed with serial 1:5 dilutions starting at 160µg/ml (final concentration).
  To perform serial dilutions, a working stock at 320µg/ml of Test Item-1 was used.
  As positive inhibiting control Remdesivir was used at concentrations ranging from 100µM to 0.0512nM, as previously described (Rodon *et al*., biorxiv 2020). The Compound was added once together with the virus on top of the Vero E6 cells monolayer.

- *Virus addition:*
  SARS-CoV-2 at approximately $10^{1.8}$ $TCID_{50}$/mL* (2x concentrated) in c-DMEM media + 5% FBS were added to each well (including the C+ wells) from the antiviral activity test rows.

  **A $TCID_{50}$ concentration that achieved a 50% of cytotoxicity 3 days after inoculation with the number of Vero E6 cells used in the titration experiment was used (approximately $10^{1.8}$ $TCID_{50}$/mL).*

  Plates were incubated for 3 days until CPE was observed at 37°C and 5% CO_2.

- Results:

  Viral infectivity was analyzed by microscopy assessing cellular cytopathic effect.

Cytopathic effect was confirmed using the cell Titer-Glo Assay based on luciferase to measure ATP released by living cells.

4. MODIFICATIONS and DEVIATIONS

not applicable

5. ARCHIVE

5.1 Test Substance Retention

Unused test substance will be kept for "umpire assay", returned to the Sponsor or destroyed if approved by the Sponsor.

5.2. Records Retention

All the original raw data developed exclusively for this study will be archived at CReSA for five years following the study completion date. After this time, the Sponsor (or the Sponsor Representative, if applicable) will be contacted to keep such data and to determine its final disposition. These original data include, but are not limited to, the following:

All handwritten raw data for control and test substances including, but not limited to, notebooks, data forms and calculations.

Any study plan amendments/deviation notifications.

All measured data used in formulating the final report.

Memoranda, specifications, and other study specific correspondence relating to interpretation and evaluation of data, other than those documents contained in the final study report.

Original signed study plan.

Certified copy of the final study report.

6. REFERENCES

A detailed explanation of the methods used for this assay can be found in our preprint published in biorxiv (Rodon *et al*., 2020): https://www.biorxiv.org/content/10.1101/2020.04.23.055756v1?rss=1.

7. Interim RESULTS and CONCLUSIONS

Antiviral and cytotoxic effect on Vero E6 cells exposed to a fixed concentration of SARS-CoV-2 in the presence of decreasing concentrations of the test Item-1 is shown in Table 1 and Figure 1. Test Item was used at the concentrations mentioned below. Non-linear fit to a variable response curve from one representative experiment with two replicates is shown (red lines). Cytotoxic effect on Vero E6 cells exposed to decreasing concentrations of the drug in the absence of virus is also shown (grey lines). CC50 value is indicated. Remdesivir was used as positive control (see Figure 2).

Value Tables

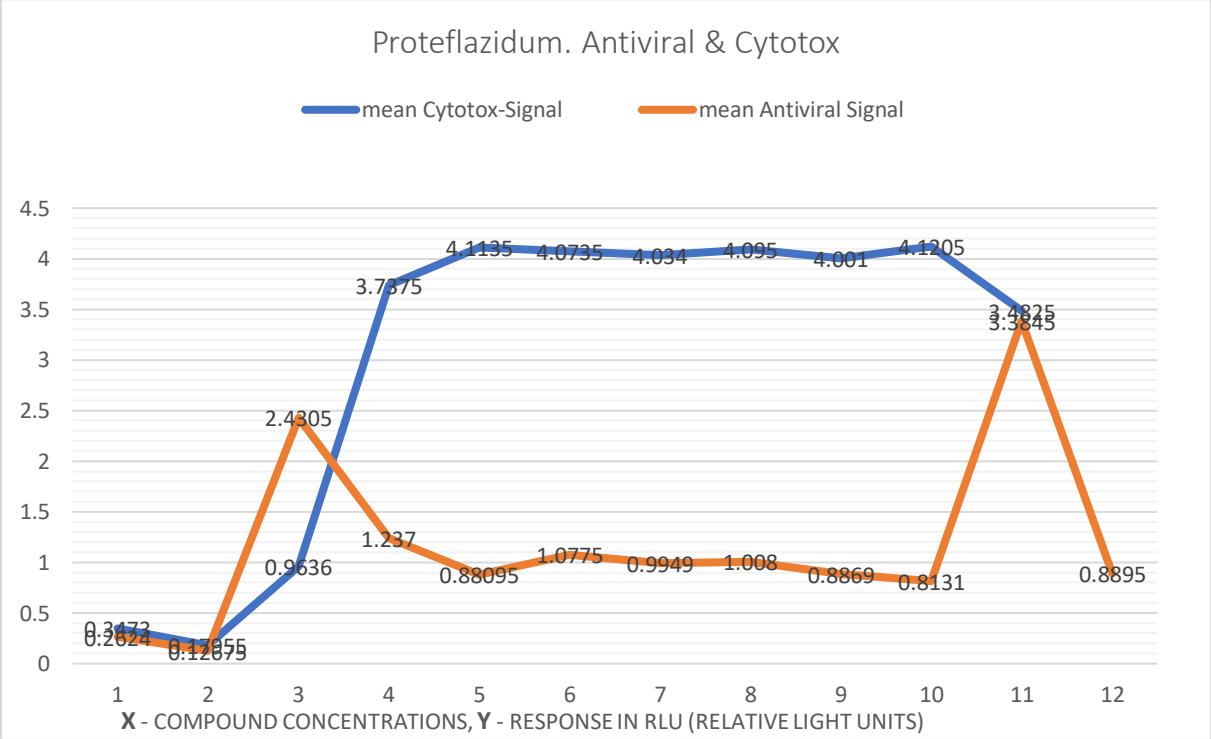
*By using Promega® (luciferin-luciferase) cell viability and cytotoxicity assay kit. At that, light production is proportional to the number of live cells in culture (light intensity is linearly related to ATP concentration).

Table 1. **Proteflazidum**

Concentration, ug/ml	Quantitative Indicator of *Antiviral* Effect. Units of measure	Quantitative Indicator of *Antiviral* Effect. Units of measure	Quantitative Indicator of *Cytotoxicity*. Units of measure RLU.	Quantitative Indicator of *Cytotoxicity*. Units of measure RLU.	Comments (if applicable)

	Relative Light Units (RLU). REPLICATE 1	RLU. REPLICATE 2	REPLICATE 1	REPLICATE 2	
Control without compound and virus	2,968	3,801	3,524	3,441	
160	0,2603	0,2645	0,3519	0,3427	
32	0,1329	0,1206	0,1837	0,1754	
6,4	2,017	2,844	0,9786	0,9486	
1,28	1,382	1,092	3,707	3,768	
0,256	0,8349	0,927	4,087	4,14	
0,0512	1,061	1,094	4,027	4,12	
0,01024	0,8958	1,094	4,095	3,973	
0,002048	1,049	0,967	4,119	4,071	
0,0004096	0,9207	0,8531	4,002	4	
0,0000819	0,7573	0,8689	4,18	4,061	
Control only virus (without compound)	0,8892	0,8898	-	-	

Figure 1.



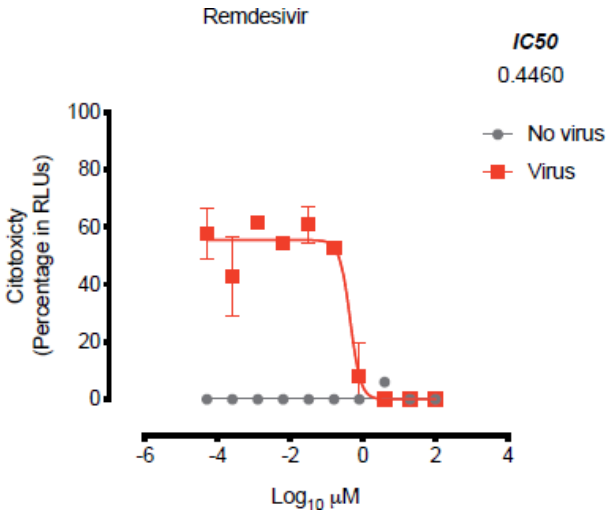
Point number	1	2	3	4	5	6	7	8	9	10	11	12
Concentrations, ug/ml	160	32	6,4	1,28	0,256	0,0512	0,01024	0,002048	0,0004096	0,0000819	Cells only	Virus only
Accompanying ethanol concentration,		9,6	1,92	0,384	0,0768							

%												

Table **2**. Remdesivir as positive control

Concentration, ug/ml	Quantitative Indicator of Antiviral Effect (where applicable, i.e. if such a measurement was performed). Units of measure RLU. ASSAY 1	Quantitative Indicator of Antiviral Effect (where applicable, i.e. if such a measurement was performed) Units of measure RLU. ASSAY 2	Quantitative Indicator of Cytotoxicity (where applicable, i.e. if such a measurement was performed). Units of measure RLU. ASSAY 1	Quantitative Indicator of Cytotoxicity (where applicable, i.e. if such a measurement was performed). Units of measure RLU. ASSAY 2	Comments (if applicable)
Control without compound and virus	*2,597*	*3,202*	*2,304*	*2,941*	
2000	*2,302*	*2,212*	*2,298*	*2,559*	
400	*2,783*	*3,121*	*3,706*	*3,728*	
80	*2,784*	*2,944*	*3,479*	*3,75*	
16	*2,683*	*2,207*	*3,684*	*3,864*	
3,2	*0,9339*	*1,288*	*3,004*	*3,846*	
0,64	*0,8832*	*1,579*	*3,879*	*3,877*	
0,128	*1,008*	*1,617*	*2,377*	*3,07*	
0,0256	*1,163*	*1,491*	*2,424*	*3,407*	
0,00512	*1,029*	*1,365*	*2,844*	*3,62*	
0,001024	*0,8344*	*1,222*	*2,299*	*3,161*	
Control only virus (without compound)	*0,7542*	*0,9336*	-	-	

Figure ***2.*** *As positive control Remdesivir was included.*



Conclusion: The Item-1 (Proteflazidum® Drops) displays protective activity against SARS-CoV-2 at concentrations between 1.28 ug/ml and 6.4 ug/ml in the performed in vitro assay. However, quite a high content of accompanying ethanol seems to complicate evaluation of Proteflazidum's antiviral effect to the right degree. Therefore, with the aim of further proper clarification and verification, we feel quite confident in recommending for supplementary examination of the Item at, at least, such additional concentration as e.g. 4,0 (or 4.5 µg/ml) and 8.0 µg/ml (or 8.5 or even up to 9.0-9.5 µg/ml).

T. Karamuska

DATA Management
Med-High-Tech GmbH
Wiesbaden Germany

Managing director
Data Management Med-High-Tech GmbH