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New Drug/Vaccine RNA-Peptide Named Melody Against SARS-CoV-2: Adapting Antiviral Pathways in Cell Culture WM-266 as Temporal Memory of "In Vitro Cell"

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*CRUZ-RODRIGUEZ L.[1,2,3,4], ZAYAS TAMAYO A.[1,2], DILSIZ N.[5] and *LAMBERT BROWN D.[1,2]

[1]ELIDAN dynamic LLC, Miami, Florida, USA.

[2]ExCELab Co. Ltd., Environmental Biotechnology Department, Kingston, Jamaica.

[3]ELIDAN america LLC, Miami, FL, USA

[4]ELIDAN genome SAS, 1 av du Lycée, 77130 Montereau FY; France

[5]Molecular Biology and Genetics, Faculty of Engineering and Natural Sciences, Istanbul Medeniyet University, Istanbul, Turkey.

*Correspondence authors

Prof. Dr. CRUZ-RODRIGUEZ Luis
ELIDAN america LLC
Miami, FL, USA
Email : luis.cruzrguez@gmail.com
lcr@elidan-genome.com

Dr. LAMBERT BROWN David
CEO,
ExCELab Co. Ltd.
Environmental Biotechnology Department
Kingston, Jamaica.
Email : dalamja1@gmail.com

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Abstract

The new coronavirus formed a clade within the subgenus Orthocoronavirinae, sarbecovirus subfamily. The first time these cases were published, and they were classified as "pneumonia of unknown etiology." The Chinese Center for Disease Control and Prevention (CDC) and local CDCs organized an intensive outbreak investigation program. The etiology of this illness is now attributed to a novel virus belonging to the coronavirus (CoV) family, COVID-19. This disease has inflicted catastrophic damages in public health, economic and social stability-putting life globally on hold in 2020 and presumably a year more. The authorized vaccine against severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) more often are Pfizer-BioNTech, Moderna, Johnson & Johnson and AstraZeneca in order to face the global pandemic COVID-19. Our aim was focused on toxicological evaluation of a new drug/vaccine model against SARS-CoV-2 with therapeutic and prophylactic actions, also useful in post-COVID-19 infection rehabilitation. Our candidate of drug/vaccine RNA-peptide named Melody was tested in cell culture WM-266 as temporal memory of "In vitro cell". We carry out our studies of this RNA target Human Malignant Melanoma cell lines, (WM-266) monitoring dead cell number. The lethal concentration (LC) at 50% and 100% (CL_{50} and CL_{100}) were calculated and reported the toxicological and efficacity findings in each study.

Keywords: SARS-CoV-2, Exosomes, Lethal Concentration (LC), Toxicological Test, Efficacity.

Historical overview and problems

Pandemics are increasingly common. Our project has suggested geological-geogenic-climactic-zoonotic connectivity's. Their elucidation and risk profiling are ancillary goals through nanotechnology research. Combined, viruses such as **SARS-CoV-2,** HIV-1, breast/ovarian and lung cancers incur enormous, suffering, health, and nation costs. Indeed, such cost burdens are so great they inflict national dept, even recessions through social costs, evidenced recently of the likes we have seldom seen in recent history [1-3].

Nowadays, Pfizer and Moderna biopharmaceutical companies have developed two RNA vaccine to face COVID-19 pandemic [4]. A vaccine for severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is needed to control the coronavirus disease 2019 (COVID-19) global pandemic. Structural studies have led to the development of mutations that stabilize *Betacoronavirus* spike proteins in the prefusion state, improving their expression and increasing immunogenicity. Moderna vaccine shows that mRNA induces potent neutralizing antibody responses and protects against SARS-CoV-2 infection in the lungs and noses of mice without evidence of immunopathology [5].

Our aim was focused on toxicological evaluation of a new drug/vaccine model against SARS-CoV-2 with therapeutic and prophylactic actions, also useful in post-COVID-19 infection rehabilitation. Our candidate of drug/vaccine RNA-peptide named Melody was tested in in cell culture WM-266 as temporal memory of "In vitro cell" [6,7], which has undergone clinical investigations.

Materials and Methods

In order to test the toxicity level of RNA-peptide (Melody) we carry out our studies of this RNA target human malignant metastatic melanoma cell line, (WM-266 purchased from American Type Culture Collection, USA) monitoring dead cell number. The cells were cultured in a 75 cm^2 culture flask in minimal essential medium (MEM) supplemented with 10 % of heat-inactivated fetal bovine serum (Gibco, Life Technologies, Germany) and 5 ml of penicillin/streptomycin (10.000 units/ml). Cells were cultivated in an incubator (5% CO_2, 37°C, >95% humidity). The cells were split when they were 90% confluent. Compounds were added to the cells in MEM supplemented with fetal bovine serum (FBS) in 384-well plates. The Lethal Concentration (LC) at 50 % and 100 % (LC_{50} and LC_{100}) were calculated in each study [8-10].

Study the toxicological action of control assay control assays

- Phorbol 12-myristate 13-acetate (PMA; phorbol ester) activates protein kinase C, which then activates a wide range of signaling pathways, including ERK via effects on the upstream kinase Raf. ERK then activates a wide range of downstream targets, including S6 ribosomal protein. PMA, through its activation of PKC, can activate T-cells and stimulate low-level production of IL-2 [11-13].
- Hydrogen peroxide solution (H_2O_2); Oxygen free radicals generated by H_2O_2 are involved in the multistage carcinogenic process; mechanisms include carcinogen activation, oxidative DNA/RNA damage, and tumor promotion. In this study, we have evaluated another potential mechanism of H_2O_2 in carcinogenesis--modulation of DNA repair activities [14].
- Phosphate-buffered saline (PBS) is a buffer solution commonly used in biological solutions. PBS has many uses because it is isotonic and non-toxic to most cells [15].
- Exosomes are a membrane bound small extracellular vesicles (sEVs), that contain cellular composite cargo of nucleic acids mainly microRNAs (miRNAs), proteins, lipid and metabolites [16-18].
- RNA "target [19-21].

Toxicological studies

Target: RNA polyA-CUCCUAGAACUAGCAUUA-CAGAUG---- [22]

In order to test the toxicity level of control assay we made our studies in Human malignant melanoma cell lines (WM-266) culture cell. We monitored dead cell number using flow cytometry according to the manufacturer's recommendations. The percentage of the dead cells was calculated according to the formula:

$$\% \text{ dead cell} = \frac{\text{Dead cell number}}{\text{Total cell number}} \text{ X } 100\%$$

We calculated LC_{50} and LC_{100}, LC_{50} values are frequently used as a general indicator of a target's acute toxicity.

Flow cytometry of cell surface receptor staining (FACS): Protocol FACS

After confluency stage of the cell lines, wash the cell lines (single cell suspension) and adjust cell number to a concentration of 1-5x10^6 cells/ml in ice cold FACS Buffer (PBS, 0.5-1% BSA or 5-10% FBS, 0.1% NaN_3 sodium azide).

The cell culture is usually stained in polystyrene round-bottom 12 x 75 mm BD polystyrene tube (cat # Z376787). It is convenient to check the viability of the cells which should be around 95% but not less than 90%.

For each cell line suspension:

- 100 µL of each cell line suspension was transferred to each tube.
- 0.1-10 µg/ml of the primary labeled antibody was added to each tube.
- Dilutions, if necessary, was made in FACS buffer.
- Cells were incubated for at least 30 min at 4°C in the dark.
- The cells were centrifuged at 1500 rpm for 5 minutes and resuspended in 200 µL of ice cold FACS buffer.
- The cells were incubated for at least 20-30 minutes at 4°C in the dark. The cells centrifuged at 1500 rpm for 5 minutes and resuspended in 200 µL to of ice cold FACS buffer. The cells were stored in the dark at 4°C in a fridge until analysis.
- The cells were then analyzed on the flow cytometer as soon as possible.
- We made the analysis on the same day. For extended storage (16 hours), we resuspended the five cell lines in 1-4% paraformaldehyde to prevent deterioration.

PMA Toxicological test: % DEAD CELLS (Flow Cytometry)

In order to test the toxicity level of **PMA** we made our studies in WM-266 culture cell. We monitored dead cell number using flow cytometry. We calculated LC_{50} and LC_{100}, LC_{50} values are frequently used as a general indicator of a target's acute toxicity.

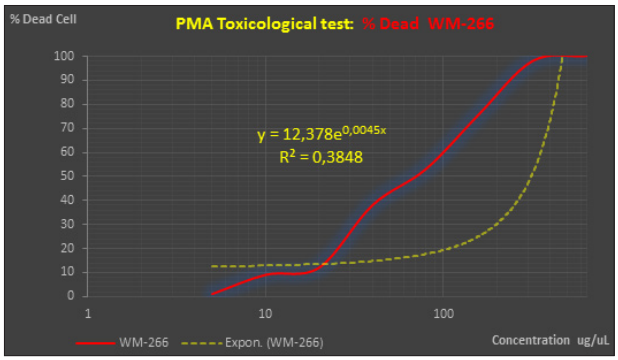

Figure 1: We are showing the PMA toxicological test in WM-266 cell lines. The yellow curve represents the exponential dead cell according to serial concentration of PMA (5 µg/µL, 10 µg/µL, 20 µg/µL, 40 µg/µL, 80 µg/µL, 160 µg/µL, 320 µg/µL and 640 µg/µL.

H_2O_2 Toxicological test: % DEAD CELLS (Flow Cytometry)

In order to test the toxicity level of H_2O_2 we made our studies in WM-266 culture cell. We monitored dead cell number using flow cytometry. We calculated LC_{50} and LC_{100}, LC_{50} values are frequently used as a general indicator of a target's acute toxicity.

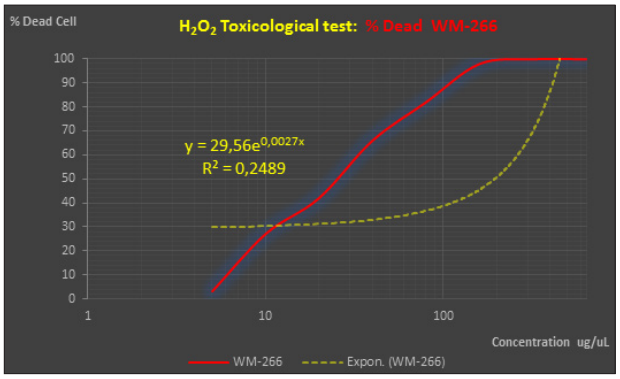

Figure 2: We are showing the H_2O_2 toxicological test in WM-266 cell lines. The yellow curve represents the exponential dead cell according to serial concentration of H_2O_2 (5 µg/µL, 10 µg/µL, 20 µg/µL, 40 µg/µL, 80 µg/µL, 160 µg/µL, 320 µg/µL and 640 µg/µL.

$CuSO_4$ Toxicological test: % DEAD CELLS (Flow Cytometry)

In order to test the toxicity level of $CuSO_4$ we made our studies in WM-266 culture cell. We monitored dead cell number using flow cytometry. We calculated LC_{50} and LC_{100}, LC_{50} values are frequently used as a general indicator of a target's acute toxicity.

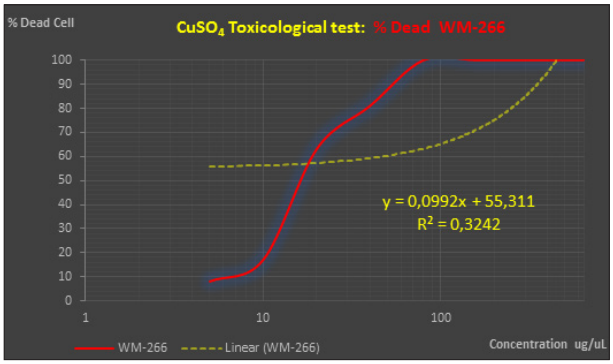

Figure 3: We are showing the $CuSO_4$ toxicological test in WM-266 cell lines. The yellow curve represents the exponential dead cell according to serial concentration of $CuSO_4$ (5 µg/µL, 10 µg/µL, 20 µg/µL, 40 µg/µL, 80 µg/µL, 160 µg/µL, 320 µg/µL and 640 µg/µL.

PBS Toxicological test: % DEAD CELLS (Flow Cytometry)

In order to test the toxicity level of PBS we made our studies in WM-266 culture cell. We monitored dead cell number using flow cytometry. We calculated LC_{50} and LC_{100}, LC_{50} values are frequently used as a general indicator of a target's acute toxicity.

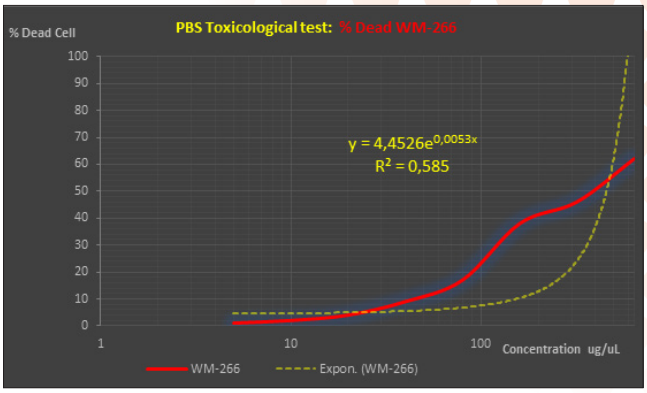

Figure 4: We are showing the PBS toxicological test in WM-266 cell lines. The yellow curve represents the exponential dead cell according to serial concentration of PBS (5 µg/µL, 10 µg/µL, 20 µg/µL, 40 µg/µL, 80 µg/µL, 160 µg/µL, 320 µg/µL and 640 µg/µL.

Exosome Toxicological test: % DEAD CELLS (Flow Cytometry)

In order to test the toxicity level of exosome we made our studies in WM-266 culture cells. We monitored dead cell number using flow cytometry. We calculated LC_{50} and LC_{100}, LC_{50} values are frequently used as a general indicator of a target's acute toxicity.

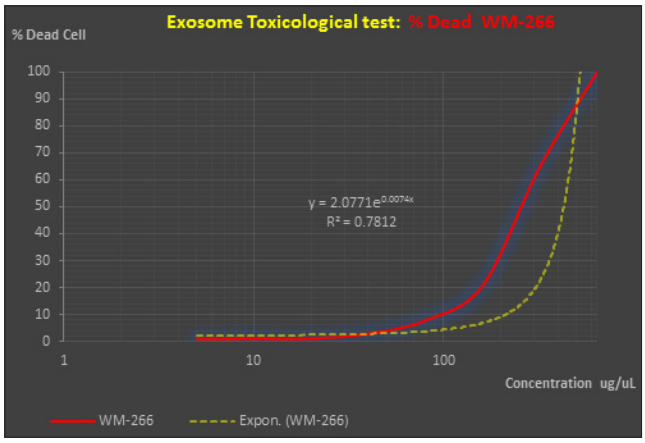

Figure 5: We are showing the exosome toxicological test in WM-266 cell lines. The yellow curve represents the exponential dead cell according to serial concentration of exosome (5 µg/µL, 10 µg/µL, 20 µg/µL, 40 µg/µL, 80 µg/µL, 160 µg/µL, 320 µg/µL and 640 µg/µL.

RNA Toxicological test: % DEAD CELLS (Flow Cytometry)

In order to test the toxicity level of **RNA** we made our studies in WM-266 culture cell [23]. We monitored dead cell number using flow cytometry. We calculated LC_{50} and LC_{100}, LC_{50} values are frequently used as a general indicator of the target's acute toxicity

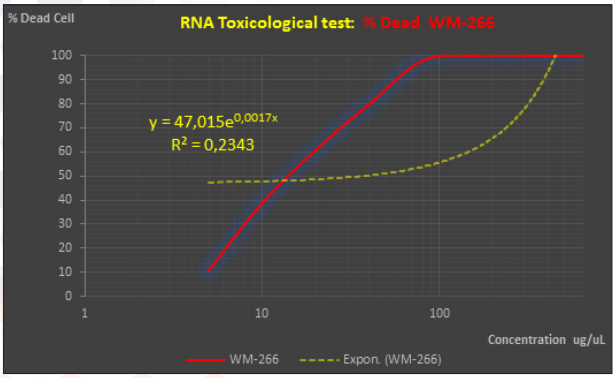

Figure 6: We are showing the RNA toxicological test in WM-266 cell lines. The yellow curve represents the exponential dead cell according to serial concentration of RNA (5 µg/µL, 10 µg/µL, 20 µg/µL, 40 µg/µL, 80 µg/µL, 160 µg/µL, 320 µg/µL and 640 µg/µL.

Results and discussion of toxicological test at concentrations lethal of 50%

The studies of cell proliferation action of this invention were carried out at concentrations lethal of 50% (CL50) in *WM-266* cell lines. We obtained as a result that: The present studies do not present a statistical difference in toxicity values with PMA (positive inductor of cell proliferation). Meanwhile, the invention melody presents a statistical difference with the negative controls of proliferation such as: H_2O_2 and $CuSO_4$. Being a statistically significant difference with sulfate of copper (II).

On the other hand, we have verified that the melody invention *WM-266* cell line a statistical difference between the toxicity values CL_{50} with the controls assay such as: PBS and exosomes. Finally, the melody vaccine shows in *WM-266* cell lines a statistical difference between the toxicity values at CL50 with the test RNA target.

Conclusion

We can conclude that this invention at CL_{100}, presents equivalents mechanisms of toxicity comparing with the inductor of cell proliferation (PMA) and $CuSO_4$. And melody, not presents equivalents mechanisms of toxicity comparing with H_2O_2. The melody invention does not present a significant difference between the toxicity values at CL_{100} with the assay controls and targets [18-20].

Results and discussion of toxicological test at concentrations lethal of 100%

The studies of cell proliferation action of this invention were carried out at concentrations lethal of 100% (CL100) in *WM-266* cell line. We obtained as a result that: The present invention does not present a statistical difference in toxicity values with PMA and $CuSO_4$. Meanwhile, the invention melody presents a statistical difference with the H_2O_2.

On the other hand, we have verified that the melody invention shows in *WM-266* a significative statistical difference in the toxicity values CL_{100} with the control assay PBS and not presents significant difference in the toxicity values with the exosomes.Finally, the melody invention shows in *WM-266* a not statistical difference between the toxicity values at CL_{100} with target RNA.

Conclusion

We can conclude that this invention at CL_{100}, presents equivalents mechanisms of toxicity comparing with the inductor of cell proliferation (PMA) and $CuSO_4$. And melody, not presents equivalents mechanisms of toxicity comparing with H_2O_2. The melody invention does not present a significant difference between the toxicity values at CL_{100} with the assay controls and targets [18-20].

Efficacity Studies of Melody

Efficacity test one melody incubation: % DEAD CELLS (Flow Cytometry)

In order to test the toxicity level of RNA after melody incubation 16 hours, we made our studies in the WM-266 culture cell. We monitored dead cell number using flow cytometry. We calculated LC_{50} and LC_{100}, LC_{50} values are frequently used as a general indicator of a target's acute toxicity.

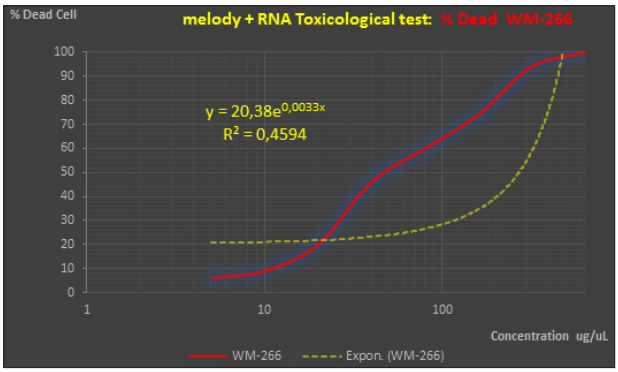

Figure 7: We are showing the RNA toxicological test in WM-266 cell lines after melody incubation of 16 hours. The yellow curve represents the exponential dead cell according to serial concentration of RNA (5 µg/µL, 10 µg/µL, 20 µg/µL, 40 µg/µL, 80 µg/µL, 160 µg/µL, 320 µg/µL and 640 µg/µL.

Efficacity test two melody incubations: % DEAD CELLS (Flow Cytometry)

In order to test the toxicity level of RNA after melody incubation two times of 16 hours each incubation, we made our studies in the *WM-266* culture cell. We monitored dead cell number using flow cytometry. We calculated LC_{50} and LC_{100}, LC_{50} values are frequently used as a general indicator of a target's acute toxicity.

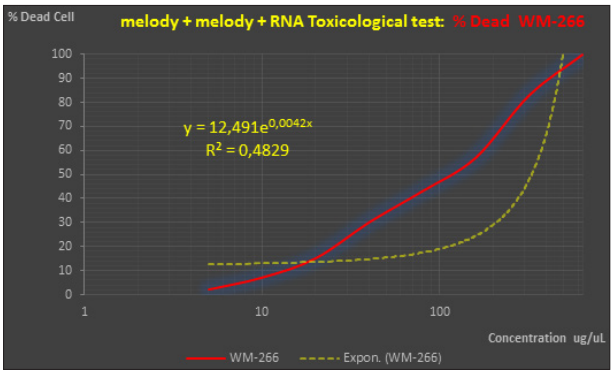

Figure 8: We are showing the RNA toxicological test in WM-266 cell lines after melody incubation of 16 hours two times. The yellow curve represents the exponential dead cell according to serial concentration of RNA (5 µg/µL, 10 µg/µL, 20 µg/µL, 40 µg/µL, 80 µg/µL, 160 µg/µL, 320 µg/µL and 640 µg/µL.

Efficacity test three melody incubations: % DEAD CELLS (Flow Cytometry)

In order to test the toxicity level of RNA after melody incubation three times of 16 hours each incubation, we made our studies in the WM-266 culture cell. We monitored dead cell number using flow cytometry. We calculated LC_{50} and LC_{100}, LC_{50} values are frequently used as a general indicator of a target's acute toxicity.

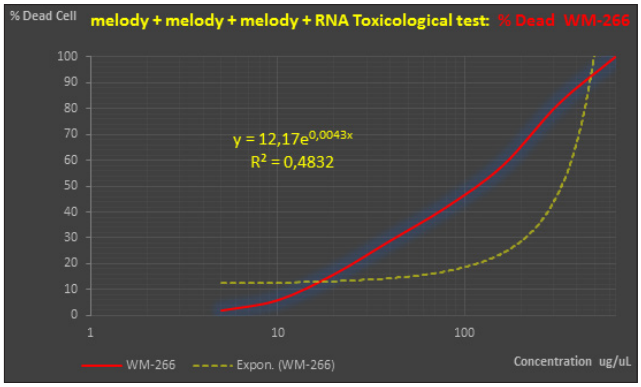

Figure 9: We are showing the RNA toxicological test in WM-266 cell lines after melody incubation of 16 hours three times. The yellow curve represents the exponential dead cell according to serial concentration of RNA (5 µg/µL, 10 µg/µL, 20 µg/µL, 40 µg/µL, 80 µg/µL, 160 µg/µL, 320 µg/µL and 640 µg/µL.

Results and discussion of efficacity test after melody incubations at concentrations lethal of 50%

The studies of melody efficacity were monitored by flux cytometry (cell viability) at concentrations lethal of 50% (CL_{50}) in *WM-266* cell line. We obtained as a result that: The cell line after melody incubation of 16 hours before treatment with RNA induced metabolic adaptation and keep this behavior in new adapting cellular pathways as "temporal memory in cell culture".

Conclusion

We can conclude that this invention at CL_{50} shows not equivalents mechanisms of toxicity comparing the *WM-266* cell line incubated with melody and the same cell lines with placebo assay. We suggested that use three doses of melody can induce temporal memory in adapting pathway against RNA infection [18-20].

Results and discussion of efficacity test after melody incubations at concentrations lethal of 100%

The studies of melody efficacity were monitored by flux cytometry (cell viability) at concentrations lethal of 100% (CL100) in *WM-266* cell line. We obtained as a result that: The line cell after melody incubation of 16 hours before treatment with RNA induced metabolic adaptation and keep this behavior in new adapting cellular pathways as "temporal memory". The present invention shows statistical difference in the toxicity values between *WM-266* cell line incubated with melody vaccine one or two doses and the placebo groups of cells (without melody incubation). With three doses of melody the *WM-266* cell line and placebo not shows statistical difference in the toxicity values [24].

Conclusion

We can conclude that this invention at CL_{100}, presents equivalents mechanisms of toxicity comparing the cell lines *WM-266* incubated with melody and placebo assay. We suggested using two doses of melody to induce temporal memory in adapting pathway *in vitro* cell against RNA infection.

Perspectives

To control the coronavirus disease 2019 (COVID-19) pandemic and aid the return to pre-pandemic normalcy, vaccines are urgently required. A large number of vaccine candidates are being developed, with many of them having completed late-stage clinical trials with promising results. We explore which viral components are used in COVID-19 vaccine candidates in this progress article, as well as why they may be used. In this summary, according to our results we are describing here supports the further evaluation of melody as a vaccine candidate for COVID-19. It will be key to determine this *in vitro* activity in the Covid-19 preclinical and clinical studies. Further studies are required to evaluate the long-term stability and immune response in animal models and the effectiveness of melody in humans [25].

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