Exploration of the Potent Toxic Intracellular Effects of the Natural Adenosine Analog Cordycepin against SARS-CoV-2 Replication

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1 MATERIALS AND METHODS

1.1 *In Silico* Toxicological and Stability Properties of Cordycepin

There is an increasing need for such type of *in silico* toxicological and metabolic studies for cordycepin due to its absence in the previous literature. Therefore, a complete simulative toxicological study of cordycepin was implemented in the current work using the ProTox-II "Prediction of Toxicity of Chemicals" web server, which is a speculative web-based laboratory for the prediction of toxicities/adverse effects of small chemical molecules[1]. ProTox-II web server computationally integrates the principles of molecular similarity, fragment propensities, fragment similarity-based cluster cross-validation machine learning, and most frequent features of artificial intelligence (AI) with each other, establishing a diverse collection of more than 33 models for the analysis and prediction of diverse toxicity endpoints, e.g., cytotoxicity, general acute toxicity, organ toxicity "mainly hepatotoxicity", carcinogenicity, immunotoxicity, mutagenicity, adverse outcomes (Tox21) pathways, and several toxicity targets[1,2]. Oral toxic doses in this validated virtual laboratory are given as 50% Lethal Dose (LD_{50}) values in mg/kg of the body weight (BWt)[1-4]. Practically, the LD_{50} can be defined as the experimental dose at which 50% of test subjects die upon exposure to a compound[1-4]. This interesting virtual web server classifies the different chemicals into 6 classes of decreasing order of toxicity (according to the Globally Harmonized System of Classification and Labeling of Chemicals "GHS"); which are Toxicity Class I "fatal if swallowed" (LD_{50}≤5mg/kg BWt), Toxicity Class II "fatal if swallowed" (5mg/kg BWt<LD_{50}≤50mg/kg BWt), Toxicity Class III "toxic if swallowed" (50mg/kg BWt<LD_{50}≤300mg/kg BWt), Toxicity Class IV "harmful if swallowed" (300mg/kg BWt<LD_{50}≤2000mg/kg BWt), Toxicity Class V "may be harmful if swallowed" (2000mg/kg BWtLD_{50}≤5000mg/kg BWt), and Toxicity Class VI "nontoxic" (LD_{50}>5000mg/kg BWt) (see Figures S1 and S2)[1-4]. An additional small complementary simulative/speculative study was also done to determine the expected degree of metabolic stability of cordycepin in its free form, using the PredMS application[5]. This application is a new web server utilized to predict the metabolic stability of a certain chemical compound, where the compound is said to be either stable (if ≥50% was predicted to remain intact after 30min) or unstable (if <50% was predicted to remain intact after 30min) in human liver microsomes[5].

1.2 *In Vitro* Anti-SARS-CoV-2 and Cytotoxic Bioactivities of Cordycepin

This credible and robust *in vitro* anti-COVID-19 test (including the cytotoxicity assay) is based mainly upon the validated procedures of Rabie[6-11]. The complete procedures were carried out in a specialized biosafety level 3 (BSL-3) laboratory. The assayed new strain of SARS-CoV-2 virus, the first Variant of Concern from 2020, December (VOC-202012/01), was isolated from the fresh nasopharynx aspirate and throat swab of a confirmed 50-years-aged COVID-19-infected man using Vero E6 cells (ATCC CRL-1586) on 21 September, 2021. Stock virus ($10^{7.25}$ $TCID_{50}$/mL) was prepared after three serial passages in Vero E6 cells in infection media (DMEM supplemented with 4.5g/L D-glucose, 100mg/L sodium pyruvate, 2% FBS, 100,000U/L Penicillin-Streptomycin, and 25mM HEPES). Cordycepin (3′-deoxyadenosine, CAS Registry Number: 73-03-0) and remdesivir (GS-5734, CAS Registry Number: 1809249-37-3) were purchased from Biosynth Carbosynth (Carbosynth Ltd, Berkshire, U.K.) (for cordycepin, Product Code: ND02930, Purity: ≥98%; for remdesivir, Product Code: AG170167, Purity: ≥98%), while the other reference compound GS-441524 (CAS Registry Number: 1191237-69-0) was purchased from MedChemExpress (MCE®, MedChemExpress LLC, New Jersey, U.S.A.) (Catalog Number: HY-103586, Purity: 99.77%). The ultrapure solvent dimethylsulfoxide (DMSO, CAS Registry Number: 67-68-5) was purchased from a local distributor, El-Gomhouria Company For Drugs (El-Gomhouria Co. For Trading Drugs, Chemicals & Medical Supplies, Mansoura Branch, Mansoura, Egypt) (Purity: ≥99.9% "anhydrous"). Preliminary pilot assays were performed mainly to determine the best concentration of cordycepin, remdesivir, and GS-441524 to start the *in vitro* anti-COVID-19 and cytotoxicity tests with. Accordingly, the stocks of the tested compounds were accurately prepared by dissolving each

 https://www.doi.org/10.53964/jmbdd.2022001

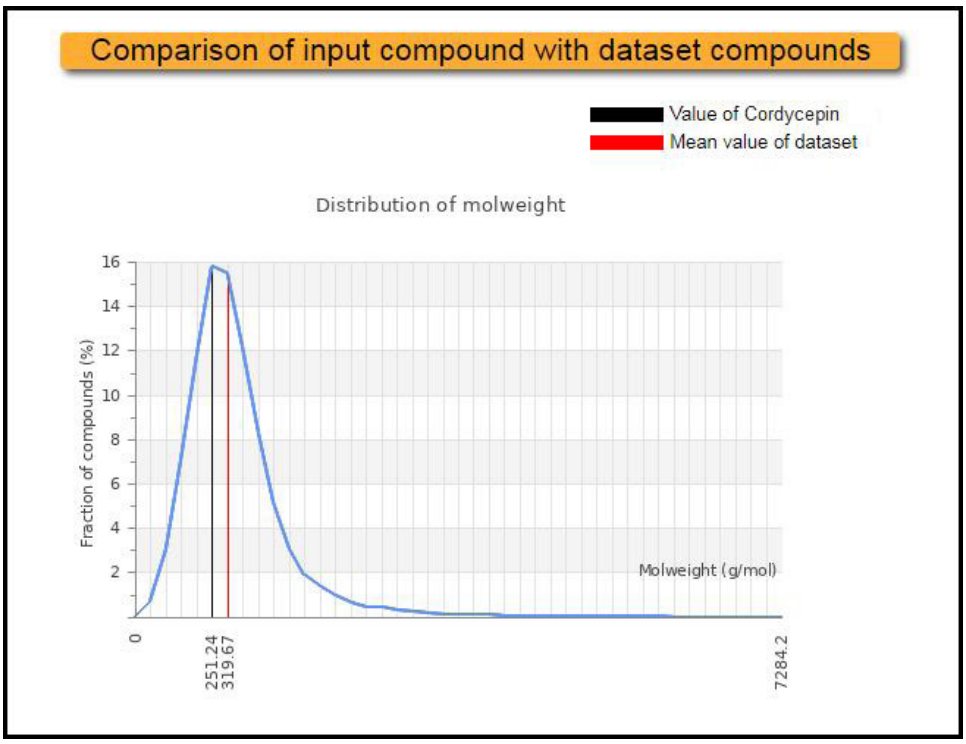

Figure S1. Graphic comparison of the molecular weight (molweight) of cordycepin with the mean value of molecular weights of all the dataset compounds (using ProTox-II Virtual Laboratory methodology).

Toxicity Model Report

Classification	Target	Shorthand	Prediction	Probability
Organ toxicity	Hepatotoxicity	dili	Inactive	0.63
Toxicity end points	Carcinogenicity	carcino	Inactive	0.68
Toxicity end points	Immunotoxicity	immuno	Inactive	0.99
Toxicity end points	Mutagenicity	mutagen	Active	0.69
Toxicity end points	Cytotoxicity	cyto	Inactive	0.58
Tox21-Nuclear receptor signalling pathways	Aryl hydrocarbon Receptor (AhR)	nr_ahr	Inactive	0.89
Tox21-Nuclear receptor signalling pathways	Androgen Receptor (AR)	nr_ar	Inactive	0.99
Tox21-Nuclear receptor signalling pathways	Androgen Receptor Ligand Binding Domain (AR-LBD)	nr_ar_lbd	Inactive	1.0
Tox21-Nuclear receptor signalling pathways	Aromatase	nr_aromatase	Inactive	0.91
Tox21-Nuclear receptor signalling pathways	Estrogen Receptor Alpha (ER)	nr_er	Inactive	0.98
Tox21-Nuclear receptor signalling pathways	Estrogen Receptor Ligand Binding Domain (ER-LBD)	nr_er_lbd	Inactive	1.0
Tox21-Nuclear receptor signalling pathways	Peroxisome Proliferator Activated Receptor Gamma (PPAR-Gamma)	nr_ppar_gamma	Inactive	0.80
Tox21-Stress response pathways	Nuclear factor (erythroid-derived 2)-like 2/antioxidant responsive element (nrf2/ARE)	sr_are	Inactive	0.99
Tox21-Stress response pathways	Heat shock factor response element (HSE)	sr_hse	Inactive	0.99
Tox21-Stress response pathways	Mitochondrial Membrane Potential (MMP)	sr_mmp	Inactive	0.97
Tox21-Stress response pathways	Phosphoprotein (Tumor Supressor) p53	sr_p53	Inactive	0.81
Tox21-Stress response pathways	ATPase family AAA domain-containing protein 5 (ATAD5)	sr_atad5	Inactive	0.71

Figure S2. A screenshot of the output table for the ProTox-II Toxicity Model Report which demonstrates the expected probabilities of the main toxicities computationally analyzed for cordycepin molecule inside the human body (using ProTox-II Virtual Laboratory methodology).

of the three compounds in DMSO to obtain a 100μM concentration of each compound. Additionally, DMSO was used for the purpose of a negative control comparison to make the study placebo-controlled. To evaluate the *in vitro* anti-SARS-CoV-2 activity of the target compound, cordycepin, in comparison to that of each of the two positive control drugs, remdesivir and GS-441524, along with that of the negative control solvent, DMSO, Vero E6 cells were pretreated with the four compounds diluted in infection media for 1h prior to infection by the new variant of the SARS-CoV-2 virus at MOI = 0.02. The four tested compounds were maintained with the virus inoculum during

the 2-h incubation period. The inoculum was removed after incubation, and the cells were overlaid with infection media containing the diluted test compounds. After 48 h of incubation at 37 °C, supernatants were immediately collected to quantify viral loads by $TCID_{50}$ assay or quantitative real-time RT-PCR "qRT-PCR" (TaqMan™ Fast Virus 1-Step Master Mix). Viral loads in this assay were fitted in logarithm scale (log_{10} $TCID_{50}$/mL and log_{10} viral RNA copies/mL), not in linear scale, under increasing concentrations of the tested compounds. Four-parameter logistic regression (GraphPad Prism) was used to fit the dose-response curves and determine the EC_{50} of the tested compounds that inhibit SARS-CoV-2 viral replication ($CPEIC_{100}$ was also determined for each compound). Cytotoxicity of each of the four tested compounds was also evaluated in Vero E6 cells using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega). Final results (see Table 1 in the Main Paper) were represented as the mean (μ) ± the standard deviation (*SD*) from the triplicate biological experiments. Statistical analysis was performed using SkanIt 4.0 Research Edition software (Thermo Fisher Scientific) and Prism V5 software (GraphPad). All reported data were significant at $P<0.05$.

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