Annotation

for the dissertation for the Doctor of Philosophy (PhD) degree

on the educational program “8D05110 – Virology” of Khaidarov Saken

on the theme « Study of the antiviral activity of drugs against the SARS-CoV-2 virus in vitro»

General description of the research: The dissertation is devoted to the study of the biological and molecular genetic properties of current SARS-CoV2 strains isolated on the territory of the Republic of Kazakhstan using RT-PCR, cell-viability, viral titter production and COVID-19 express test techniques and appropriate primers to detect NSP12 gene product. An in vitro study of the antiviral activity of drugs against the SARS-COV-2 virus was carried out on the Vero E6 cell culture - *green monkey kidney tissue.*

The relevance of the research: The COVID-19 pandemic in 2020 showed the extreme vulnerability of healthcare systems worldwide. First and foremost, the speed and intensity of spreading viral infection was a profound issue due to high viral load rates that most of the population could not withstand. Secondly, hospitalization issues, neither bed nor drug capacities were sufficient for effective containment. Thirdly, the severe cases of immune response caused significant health damage, predominantly to the lungs and due to this feature, it was called a severe acute respiratory syndrome. Lastly, the primary objective was to achieve fast and reliable viral infection diagnostics in Kazakhstan and the world.

One of the most significant issues of SARS-COV 2 infection is relatively fast mutation rates and human host adaptation mechanisms through spike protein variations, and the most effective way to hold the population infection intensity is to implement mass vaccination and find effective therapies against it. And, of course, the fundamental understanding of this viral infection's nature will help us minimize the adverse outcomes in combating novel viruses in the future.

The purpose of the research: Study of the antiviral activity of drugs against the SARS-CoV-2 virus in vitro

The main tasks of the research to accomplish the purpose are as follows:

1. Sequencing genome Kazakhstan variant of the SARS-CoV-2 virus and characterizing significant virus genes. Comparative and phylogenetic analysis of the nucleotide sequence of viral genes;
2. To identify the mutation of two SARS-CoV2 strains, the Alpha variant, which was to be isolated in Kazakhstan by comparing it with the original Wuhan strain;
3. To find the cytotoxicity - safe concentration of four antiviral drugs;
4. To identify the most effective and potent antiviral drug among three candidates: Ribavirin, Favipiravir (Fabiflu), and Tenofovir (Tenvir), as well

as a corticosteroid with a safe concentration that minimises cell toxicity while maintaining high cell viability.

5. To determine the inhibition coefficient $IC_{10} \rightarrow IC_{50} \rightarrow IC_{100}$ - Range antiviral drug-Tenofovir (TAF) with acceptable SI (selectivity index);
6. To perform and confirm the preclinical test on wild-type (WT) mice to determine the antiviral efficacy of the potent drug at a safe concentration.

The research objects and materials: The SARS-CoV-2/human/KAZ/B1.1/2021 strain: the intact virus from viral titter, its ORF1ab genome, particularly the NSP12-*gene product* and Vero E6 cell line

Research methods: The study employed biomolecular, genetic, cellular-based biotechnological, microbiological, and pharmaceutical methods.

The scientific novelty of the research:

A three-stage dissertation study examining the effectiveness of the antiviral activity of the tableted drug against Kazakhstan's SARS-CoV-2/human/KAZ/B1.1/2021, the Alpha variant strain isolated and characterised, and an MTT assay on the Wuhan strain using original Tenvir (TAF) stock from China. Antiviral drug cytotoxicity and cell viability assays – Determining the optimal antiviral drug concentration (CC50) using two colourimetric methods, CCK8 and MTT, in vitro in Kazakhstan. Three tableted forms of drugs demonstrated further inhibitory activities: Tenofovir (TDF and TAF), with IC10 = 0.174 μM, IC50 = 1.74 μM, and IC100 = 174 μM at a concentration of 50 μg/ml. Ribavirin: IC10 = 2 μM, IC50 = 7 μM, and IC80-90 = 205 μM of 50 μg/ml., Favipiravir: with IC10 = 1.65 μM, IC37=318μM, of 50 μg/ml. Dexamethasone showed no inhibitory properties at any concentration or volume. Comparison of the efficacy and cytotoxicity (CC50/IC50) of three tableted antiviral drugs, identifying an antiviral drug with a positive selectivity index (SI value), and analysis of the CCK8 assay test. Antiviral activity of three tableted prodrugs (active agents): Ribavirin, Tenofovir, and Favipiravir on Vero E6 cells line that is both susceptible and permissive for SARS-CoV2 virus – RdRP-inhibition, causing the lethal mutagenesis for viral replication with a significantly higher viral load MOI:2 or TID50=10, whereas MOI of 0.01is enough to cause cytopathic effect within 24 hours (200 times increase virus load decrease potential). The molecular and genetic characterisation of the RdRP (RNA dependent RNA polymerase) gene (NSP12- none structural proteins) and its 'genetical conservatism' of SARS-CoV-2/human/KAZ/B1.1/2021, Alpha variant strain in comparison with the original Wuhan strain; The assumption of antiviral activity was confirmed using Tenvir TAF (pure Aldrich stock concentration) with 10μg/ml solution from original concentration 25mg/ml). All three antiviral drugs target the RdRP activity, making viral replication more challenging.

The theoretical and practical significance of the research:

To understand the effectiveness of three disputed antivirals and one hormonal (steroid) drug in vitro. The work's theoretical and practical significance is clearly

understanding the efficacy of three disputed antivirals and one hormonal (steroid) drug in vitro. To understand which drug demonstrates a sensible, i.e., pre-clinical, effect on wild-type mice in China and to establish a safe dosage for viral load, the author aims to enhance strategies for combating SARS-CoV-2 viral infection. During the pandemic, Kazakhstan faced multiple and numerous different cases of COVID-19 progressions and complication stages among infected patients with devastating post-corona effects and even lethal outcomes due to a poor understanding of the biological nature of the SARS-COV 2 virus. The primary objective of this dissertation is to provide a deeper understanding of not only COVID-19 treatment but also similar viral infection cases in the future. To evaluate the vitro effectiveness of three antiviral agents and one hormonal (steroid) drug. To understand which of these drugs demonstrate not only practical, i.e., clinical, effects but also to establish the safe dosage in treatment strategies for COVID-19. The value of this work lies in several additional aspects: 1. Tenvir, available in its two isoforms – TDF and TAF, has two origins: one is tableted (TDF), and the second is a laboratory standard stock (TAF). Both these forms showed similar effectiveness; furthermore, the last in vitro test on Tenvir efficacy was performed in the 2000s. The antiviral effects were demonstrated on the Vero E6 cell model. Furthermore, the cytotoxic profiles of all four drugs were evaluated and confirmed. The non-structural protein sites on the viral genome were identified and quantified, and their biological 'conservative' nature was verified in the SARS-CoV-2/human/KAZ/B1.1/2021 Alpha variant strain.

The main provisions for the defense:

1) The SARS-CoV-2/human/KAZ/B1.1/2021, Alpha variant strain – is an object of antiviral study, aiming at the entire - ORF1ab, where ORF1a NSP1-11(Protease section) and ORF1b NSP12-16 (viral RNA replication site) with the strain-specific mutation that is responsible for the replication of viral genome RNA as well as for sub-genomic RNA that regulate the final assembly of virions.

2) The potent inhibition of the RdRP (RNA-dependent RNA Polymerase is detectable through an MOI count of 2 or lower (two viral particles for each host cell) in a 12 wells-sampling by Tenofovir (tableted TDF against The SARS-CoV-2/human/KAZ/B1.1/2021, Alpha variant) and TAF (lab stock) against original Wuhan strain) The tablet safe concentrations of Tenofovir, Favipiravir, Ribavirin, and Dexamethasone must support cell viability with a noncytotoxic viral load value of— MOI 2 (one host cell for two intact viral particle or plaque forming unit (PFU/ml)) with 200µl of virus volume) per 10.000 cells.

3) The cell counting techniques CCK8 and MTT demonstrate true cytotoxicity and antiviral assays on reliable Vero E6 cells with a high proliferation growth profile for four selected drugs: Tenofovir, Favipiravir, Ribavirin, and Dexamethasone.

4) Tenvir (Tenofovir-TDF and TAF) is an effective antiviral drug among the four selected for inhibiting viral accumulation in infected Vero cells. It achieves a maximum log2 value of 100% with high cell viability rates at a relatively high viral load MOI of 2 or lower. In vivo, preclinical studies in China have shown decreased MOI4 → MOI2 viral load.

Key research findings and conclusion:

- ✓ 1)The whole genome of SARS-CoV-2/human/KAZ/B1.1/2021, Alpha variant strain was sequenced. The SARS-CoV-2/human/KAZ/B1.1/2021, also known as the Alpha variant strain, originated in Europe in 2021 and serves as the starting point of the phylogenetic tree. This branch has close relationships with European strains (Appendix A-C).
- ✓ 2) Mutations of SARS-CoV-2/human/KAZ/Britain/2021 and SARS-CoV-2/human/KAZ/B1.1/2021 strains compared to the reference sequence Wuhan-Hu-1 SARS-CoV-2. In my dissertation, only the ORF1b segment was relevant. It showed no significant mutations that could cause antiviral resistance in SARS-CoV-2/human/KAZ/B1.1/2021, the Alpha variant strain (Appendix D).
- ✓ 3) The cytotoxicity - safe concentration of Tenofovir was found to be - 50μg/ml or 174μM both for TDF and TAF (10nM is much safer than TDF's) – for IC_{100}. Furthermore, the evaluation of Tenofovir, Favipiravir, Ribavirin, and Dexamethasone using MTT and CCK8 cell viability assays in the context of SARS-CoV-2 infection provides critical insights into their potential antiviral efficacy and cytotoxicity. These assays, which measure mitochondrial activity (MTT) and cellular dehydrogenases (CCK8), provide a robust framework for quantifying the effects of drugs on cell viability and antiviral activity in vitro, particularly in models such as Vero E6 or other susceptible cell lines.
- ✓ 4)Tenvir (Tenofovir) is the most effective and potent antiviral drug among the three selected ones; however, it is not the safest. Accurate and proper usage can easily coupe the COVID-19 progression into mild and moderate illness stages. Tenvir (Tenofovir) is a potent antiviral drug among the four selected ones against SARS-CoV-2/human/KAZ/B1.1/2021, Alpha variant strain. Its cytopathic optimal viral load was found at MOI 2. The EC_{50} in Tenvir's exposure (TDF) was not detected due to high cytotoxicity in renal Vero E6 cells; we obtained only an ECmax/ICmax at 50 μg/ml. The approximate estimation is 0.5 μg/ml or 1.74 μM of the EC_{50} of tableted Tenvir (TDF).
- ✓ 5) The Inhibition coefficient $IC_{10} \rightarrow IC_{50} \rightarrow IC_{100}$ -Range was determined only in China, where the stock and pure TAF had an acceptable SI (selectivity index).
- ✓ 6) The preclinical test on WT mice confirmed TAF's Antiviral efficacy at a concentration of 50μg/ml.

The author's contribution to the results described in the dissertation: The author carried out independently the analysis of literature data on the researched problem, the setting of research goals and objectives, the conduct of experimental research, the analysis of the obtained results, statistical processing, and the writing of the dissertation.

Research approbation: The research results and the main principles of the dissertation were presented and discussed at the following international scientific conferences and symposiums:

- Modern scientific technology» (February 9-10, 2023). Stockholm, Sweden, 2023

- 3rd International Conference on Virology, infectious disease COVID-19, October 24-25, 2022, Dubai, UAE

- Proceedings of the 1st International Scientific Conference, 26-27 January 2023, Warsaw, Poland

- II International Forum "Asfen Forum, new generation-2024" on June 6-7, 2024, in Almaty, Kazakhstan

Publications: The main result of the dissertation consists of 9 published works, including two articles in peer-reviewed international scientific journals indexed in the Web of Science or Scopus databases, two articles in the list of the Committee for Control in the Sphere of Education and Science of the Republic of Kazakhstan, and five theses published at international conferences.

Dissertation structure: The dissertation comprises 138 pages of computer-generated text, symbols, and abbreviations, an introduction, a literature review, research materials and methods, research results and their discussion, a conclusion, and a list of used literature, totalling 115 entries. The work has seven tables, six mathematical formulas, 50 figures, five appendixes, and one monograph.