NUCLEOSIDE ANALOG FA VIR IS A POOR INHIBITOR AGAINST THE SARS-COV-2 VIRUS IN CELL CULTURE, BUT FA VIR IS HIGHLY ACTIVE AGAINST VENEZUELAN EQUINE ENCEPHALITIS VIRUS

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ABSTRACT

The SARS-CoV-2 pandemic has been the largest epidemiological event in the current century, however, it was not the first epidemic with a large tally in the 21st century, nor will it be the last. The majority of pathogens which have caused large epidemics in the 21st century (avian influenza, pandemic influenza, MERS, Ebola, etc.), including the SARS-CoV-2 itself, are RNA-containing viruses. The biological nature of the pathogen which will cause the future epidemic is difficult to predict, but with it highly probable will be an RNA-containing virus. To prepare for future epidemics, drug repurposing is a promising approach. The drugs repurposing against RNA viruses is facilitated by pathogens’ features such that RNA-dependent RNA polymerases have the ability to incorporate modified nucleotides into growing RNA strands; and in the majority RNA viruses, their replicases do not have the editing capacity. In this work, we measured the ability of two registered antiviral drugs with different mechanisms of antiviral action - Favipiravir and Cycloferon - to suppress the replication of two unrelated viruses in Vero E6 cell culture. We measured the antiviral activity against the coronavirus SARS-CoV-2 and Venezuelan equine encephalitis virus (VEEV). Favipiravir was not an effective inhibitor of SARS-CoV-2, because of the high half-maximal effective concentration, EC_{50} > 6.67 mg/ml. But Favipiravir actively suppressed the replication of the VEEV virus in the pharmacological concentration range. Cycloferon appeared to be a prominent inhibitor of the SARS-CoV-2 coronavirus, demonstrating EC_{50} = 0.066 mg/ml. However, Cycloferon was ineffective against VEEV. Such differences in the activity of two drugs against two unrelated RNA-viruses are probably explained by different mechanisms of the antiviral action.

Keywords: Favipiravir; Cycloferon; antiviral activity; SARS-CoV-2.

INTRODUCTION

The SARS-CoV-2 pandemic turned out to be such a large-scale event on the global epidemiological arena that it resembled the “black pox” in medieval Europe, an epidemic that just could not have happened in modern times. However, the SARS-CoV-2 pandemic was not the first epidemiological event of high importance in the 21st century, and it will not be the last. As the SARS-CoV-2 pandemic continues into mid-2022, epidemiologists are sounding the alarm about monkeypox outbreaks around the world.

Actually, various unrelated viruses which genome is represented by RNA molecules, have always been an epidemiological problem of high importance, and have remained so in our times. Before SARS-CoV-2, there were epidemics of severe acute respiratory syndrome caused by the coronavirus SARS in 2002-2004, avian influenza A in 2009 and later (H5N1 and H5Nx, these represent the genus Alphainfluenza-virus), pandemic human influenza H1N1 and N7N9 (also influenzaaviruses), Middle East respiratory syndrome in 2012-2013 caused by coronavirus MERS, Ebola hemorrhagic fever in 2013-2016 (filovirus) and Zika fever in 2015-2016 (flavi-virus) [1]. Add to this list the epidemic of chikungunya fever caused by a member of the genus Alphavirus, that has been ongoing since the 20th century in South America, and the numerous diseases caused by flaviviruses (dengue, West Nile virus, etc.) in Asia and Africa. This is a picture of suffering humanity, struggling to fight diseases caused by RNA viruses.

The biological nature of a pathogen which will cause the next epidemic is difficult to predict, but with a high probability it will also be an RNA-containing virus. For the majority of viruses, etiotropic drugs have not been created, i.e. there are no clinically-approved drugs which work as specific inhibitors of the virus replication process. Accordingly, non-specific treatments and broad activity-spectrum antiviral drugs will be used for a prompt response in case of the future epidemic. Expectedly, attempts will be made to repurpose existing drug, which is when a clinically-approved substance tested against one etiology will be used against a different pathogenic etiology.

With this regard, it is important that most RNA viruses have common features which make it possible to consider drug repurposing as an efficient strategy to promptly identify candidate drugs. There are two such features in RNA viruses, which is a broad biochemical class of evolutionarily unrelated viruses. One feature which distinguishes RNA-viruses from DNA-containing viruses is a low ability of RNA-replicase to discriminate modified nucleotides (i.e. select unmodified natural ribonucleotides in the presence of modified bases) [2]. The other feature is that in the majority of RNA viruses, their replicases have no proof-reading capacity, i.e. these RNA-polymerases cannot edit growing RNA chains to delete wrongly incorporated nucleotides [3].

RNA-dependent RNA polymerases do not have the intrinsic editing (3’->5’ exonuclease) activity [4]. This means that, in principle, it is possible to use a limited number of chemical substances which are nucleoside- or ribonucleotide-analogues, to find among them a substance which will inhibit the replication of nearly any RNA-virus. The inhibition will be pos-
sible if found nucleoside-analog is not discriminated against by cellular enzymes of nucleotide biosynthesis and the resulting ribonucleotide is not discriminated against by the viral RNA polymerase, i.e. efficiently incorporated into growing RNA strands.

One hassle in this way is that some RNA-viruses having a very large RNA genome actually acquired the proof-reading function which removes erroneously included bases from 3'-termini of growing RNA strands [5]. Such molecular editing is present in a minority of genera of RNA viruses, but it is present, for example, in coronaviruses, including the pandemic pathogen SARS-CoV-2 [5].

The editing function is not a feature of a RNA-dependent RNA polymerase itself, but pertains to a separate protein which associates with the polymerase within the replicase complex. Thus, in coronaviruses including SARS-CoV-2, the nsp14 protein has the activity of 3'-5' exonuclease. The actual RNA-dependent RNA polymerase in coronaviruses is the nsp12 protein, which works in a complex with nsp7, nsp8 [6], and nsp14 also somehow participates in the replication process. The ability of the coronavirus replicase to remove erroneously incorporated bases during the replication is probably the most serious hassle on the way of developing potential drugs - inhibitors of coronavirus replication - from the nucleoside-analogs class.

In countries with top-level virology and synthetic chemistry, such as the USA, only one nucleotide-mimicking inhibitor, Remdesivir, has received clinical approval against SARS-CoV-2 [7]. Remdesivir was originally developed to fight infection caused by the hepatitis C virus (HCV, also a RNA-virus, member in the Hepacivirus genus). But upon drug repurposing, Remdesivir appeared active against SARS-CoV-2. Unfortunately, Remdesivir’s availability in our part of Eurasia is limited to nearly the inexistence of the drug.

In Kazakhstan, at some time during 2021, the recommended clinical protocols for SARS-CoV-2 included a different nucleoside-analog drug, Favipiravir. By chemical classification, Favipiravir is a pyrazinecarboxamide derivative, it was initially developed by the Japanese company Fujifilm Toyama Chemical as a drug to treat influenza caused by influenza A virus, within the pharmacologically achievable concentration range.

Favipiravir efficiently suppressed the replication of the VEEV virus, low EC₅₀ = 0.066 mg/ml. However, cycloferon appeared ineffective against VEEV.

Such differences in the activities of two drugs against two unrelated RNA-viruses are probably due to different mechanisms of the antiviral action of these drugs.

MATERIALS AND METHODS

Cell culture and virus strain

Vero E6 (ATCC CRL-1586) are from collection at the National Center for Biotechnology (Nur-Sultan, Kazakhstan). Vero E6 cells were grown in DMEM with high glucose (Lonza BEI-604 F/U1) with addition of 10% FBS (Gibco Cat# 16000-044), 2 mM L-glutamine, 1% MEM vitamin solution (ThermoScientific Cat# 11120052), 1% non-essential amino acids (ThermoScientific Cat# 11140050), penicillin (100 U/mL), streptomycin (100 µg/mL).

The SARS-CoV-2 virus strain in this work has been produced from a clinical sample by the authors themselves and registered in the GISAID database (accession number EPI_ISL_454501). This strain was published [10].

Venezuelan equine encephalitis virus (VEEV) was rescued from a molecular infectious clone (MIC). The MIC named cTC-83/TrD is published in [11].

Virus stocks

Vero E6 cells (2 × 10⁶ cells) were seeded in 100 dishes. The cultures were grown to 90% confluence. A medium with reduced amount of serum (2% heat-inactivated FBS) was used to infect the cultures and produce stocks of viruses. Infectious inocula (SARS-CoV-2 or VEEV) were prepared to achieve multiplicity of infection (MOI) 0.01. Cultures infected with viruses were incubated in a CO₂ incubator. Virus-containing media were collected 72 h after infection. The media were clarified by centrifugation, aliquoted and stored at −80°C.

Virus titers

The limiting dilution (Reed-Muench) method in a 96-well-plate format was used. Vero E6 cells were seeded in 96-well plates (37,500 cells per well). Serial dilutions of virus stocks were made using DMEM+2% heat-inactivated FBS as a diluent. Eight tenfold dilutions were prepared (1:10 to 1:10⁹). The dilutions were distributed in plate’s long rows. In each plate, vertical row 12 was filled with medium without virus and served as the normal cells control. The plates were incubated for 3–4 days until the virus-induced cytopathic effect (CPE) is visible. The wells with CPE per row of the plate were counted and used to obtain the virus titer employing the clas-
Favipiravir (6-fluoro-3-hydroxypyrazine-2-carboxamide) and Cycloferon (10-carboxymethyl-9-acridanone methylguanamine salt) are registered drugs in Kazakhstan. The drugs were purchased in a form of tablets containing the following amounts of active substances, 200-mg Favipiravir (Glenmark Pharmaceuticals Ltd., Mumbai, India), and 150-mg for Cycloferon (LLC “Polisan NTFF”, St.-Peterburg, Russia).

Drugs stock solutions were prepared by dissolving tablets. One tablet was thoroughly crushed in a mortar; the resulting powder was transferred in a tube. Then, 10-ml of infection medium (DMEM+1% heat-inactivated FBS) were added to Cycloferon to obtain the drug’s stock at 15 mg/ml. Similarly, 20-ml of the infection medium were added to Favipiravir to obtain 10 mg/ml. Resulting mixtures were placed on a rotating platform for 15 min, then the solutions were clarified by centrifugation and sterilized by filtering through 0.22-um filters. The substances are water-soluble at the indicated concentrations.

Cytotoxicity test

Cytotoxicity of Cycloferon and Favipiravir was measured by determining the half-maximal inhibitory concentration $IC_{50}$, i.e. the concentration at which the compound present in culture medium reduces amounts of live cells by 50% [12].

Vero E6 cells were seeded in wells of a 96-well plate at 20,000 cells per well. The plates were incubated overnight. Next day, media were changed to fresh medium (DMEM+1% FBS, 100 ul per well). Existing media were removed from the wells of row H (wells 1-10). The wells 1-10 of row H were filled with 150 ul-aliquots of the drugs stock solutions (Cycloferon at 15 mg/ml; Favipiravir at 10 mg/ml). Aliquots of 50 ul were picked from wells of row H and transferred to row G with accurate mixing. This procedure continued until rows A-H (vertical rows 1-10) were filled with drugs’ stock dilutions with a concentration step of 3. Vertical rows 11 and 12 were left without the addition of drugs to serve as unaffected controls.

The plates were incubated for at least 3 days with daily microscopy to record signs of the drug’s cytopathic effect on cell monolayer. At the end of the experiment, 100 ul-aliquots of fresh media containing 0.011% of neutral red (Cat# N4638 Sigma) were added to all wells of a plate. Upon 2-hour incubation in a CO$_2$-incubator, the media were completely removed, the wells were gently rinsed with PBS and dried. Acetic acid (1% solution in water) was added to wells to allow the dye to re-dissolve. The optical absorbance was measured using plate reader at 540 nm.

Drugs activity

Activity of the drugs was measured in a variant of the 96-well-plate-based test. The plates were prepared and filled with drugs dilutions (horizontal rows A-H, vertical rows 1-10) similarly to the procedure described above in the section “Cytotoxicity test”. Then infectious inocula containing a virus (SARS-CoV-2 or VEEV) were distributed to the wells. Universally, multiplicity of infection used was MOI=0.01.

After 3 days of incubation, media were collected from the wells with the infected cultures. Viral titers in the collected media samples were determined using the Reed-Muench method as described above.

Data processing

Data processing was done in GraphPad Prism (GraphPad Inc, CA, USA). Four-parameter non-linear regression was used to compute half-maximal effective concentration $EC_{50}$ (the measure of drug’s efficiency) and inhibitory concentration $IC_{50}$ (the measure of drug’s cytotoxicity).

**RESULTS**

Results of the cytotoxicity test are presented in Figure 1 and show that both drugs are not toxic for Vero E6 cells when applied in pharmacologically-relevant concentrations. The half-maximal inhibitory concentrations ($IC_{50}$) appeared 676 ug/ml for Cycloferon, and 3393 ug/ml for Favipiravir, which values are well above the physiologically relevant concentrations range.

The two drugs Favipiravir and Cycloferon showed very different patterns of activity against the two viruses in this study - SARS-CoV-2 and VEEV (Figure 2). Favipiravir appeared to be a surprisingly poor inhibitor of the SARS-CoV-2 replication, the half-maximal effective concentration ($EC_{50}$) above the drug’s own cytotoxicity limit ($EC_{50} > 6.67$ mg/ml). Favipiravir actively suppresses the VEEV replication, $EC_{50} = 49$ ug/ml.

A different drug, Cycloferon shows a pattern of the antiviral activity which is in a sharp contrast to that of Favipiravir (Figure 3). With a huge relevance to the current epidemiological situation, Cycloferon is an efficient inhibitor of the SARS-CoV-2 replication in Vero E6 cells, $EC_{50} = 66$ ug/ml. However, with a kind of surprise, Cycloferon does not show activity against the VEEV replication in Vero E6 cells, $EC_{50} = 5$ mg/ml.

**DISCUSSION**

The SARS-CoV-2 pandemic continues in the world and in Kazakhstan, despite the perceived success of vaccination. A list of drugs with antiviral activity against SARS-CoV-2 in the clinic is very limited. In Kazakhstan, for some time at the height of the epidemic, the only one drug belonging to a group of direct-acting antivirals (DAA) had been listed as recommended against SARS-CoV-2. This is Favipiravir. But currently it Favipiravir is not mentioned in the clinical protocol for management of patients with SARS-CoV-2.

Curing the coronavirus infection in Kazakhstan is largely supportive if not mention usage of such understudied drugs as Ingavirin (imidazolyl ethanamide pentandioic acid), and Arbidol (Umifenovir, a complex substituted indole-derivative). Therefore, there is an obvious need for new drugs for the treatment and possibly prophylaxis of the coronavirus infection.

In developed countries, among small-molecular-weight inhibitors (DAA), only Remdesivir has an unlimited approval for clinical use [13]. An Emergency Use Authorization had been issued to Paxlovid [14]. But this authorization was recently revoked because Paxlovid was found to be ineffective against the SARS-CoV-2 strain omicron. Monoclonal antibodies with virus-neutralizing ability against SARS-CoV-2 are also effectively used as drugs; five brand names have the full authorization. However, this type of anti-COVID drugs...
is practically unavailable in the CIS countries. On their turn, many Eurasian countries approved Favipiravir to treat SARS-CoV-2 [15]. Favipiravir is highly active against the pathogen for which it was developed— influenza viruses of types A, B and C (EC_{50} values 0.014-0.55 μg/ml) [16]. But with regard to the actual efficacy of Favipiravir against SARS-CoV-2, the results published in literature are controversial. One study reports for Favipiravir quite low values of the effective concentration EC_{50} = 61.88 μM [17]. However, different papers report much higher values, such as 118.3 μM for inhibition of the virus-induced cytopathicity, or 207.1 μM for inhibition of replication [18], or as high as EC_{50} > 500 μM [19].

Also, there are no properly organized double-blind placebo-controlled clinical trials which show significant clinical benefits from Favipiravir during treatment of SARS-CoV-2 patients [20]. Favipiravir shows teratogenic effects, it is forbidden for use in pregnant women. It is supposedly the failure Favipiravir in registered clinical trials is the reason why Favipiravir was terminated from the standard clinical protocol in Kazakhstan. Illustratively, the currently effective protocol (June 2022) from the Ministry of Health does not recommend Favipiravir as a drug in a standard therapeutic scheme.
Original articles

Fig. 2. Favipiravir does not possess the property of inhibiting the SARS-CoV-2 virus but the drug efficiently suppress the replication of the Venezuelan equine encephalitis virus (VEEV). Results of the yield reduction assay are shown. Favipiravir was added to Vero E6 cell cultures to make concentrations 0 - 10 mg/ml, indicated in the X-axis in the Log10 scale. The cultures were infected with SARS-CoV-2 or VEEV, as described in the Materials and Methods. The Y-axis is viral titers determined in samples collected at 72 hours post-infection (hpi). The experiment was repeated in triplicates. Data points are geometric mean titers (GMT) with 95% confidence intervals (CI). The sigmoid line is a nonlinear regression curve. The dotted horizontal line indicates a virus titer in control cultures without addition of Favipiravir. Panel A, inhibition of the SARS-CoV-2 virus. The half-maximal effective concentration for Favipiravir is high, EC$_{50} > 6.67$ mg/ml (higher concentrations are not informative for EC$_{50}$ because they are toxic). Panel B, inhibition of VEEV, EC$_{50} = 0.049$ mg/ml.

Fig. 3. Cycloferon efficiently inhibits the replication of the SARS-CoV-2 virus in cell cultures, however Cycloferon is unable to suppress the Venezuelan equine encephalitis virus (VEEV). Results of the yield reduction assay are shown. Cycloferon was added to Vero E6 cell cultures to make concentrations 0 - 15 mg/ml, indicated in the X-axis in the Log10 scale. The cultures were infected with SARS-CoV-2 or VEEV, as described in the Materials and Methods. The Y-axis is viral titers determined in samples collected at 72 hpi. The experiment was repeated in triplicates. Data points are geometric mean titers (GMT) with 95% confidence intervals (CI). The sigmoid line is a nonlinear regression curve. The dotted horizontal line indicates a virus titer in control cultures without addition of Cycloferon. Panel A, inhibition of the SARS-CoV-2 virus. The half-maximal effective concentration for Cycloferon is EC$_{50} = 0.066$ mg/ml. Panel B, inhibition of VEEV, EC$_{50} = 5$ mg/ml.

a model of viral infection to test antivirals potentially active against SARS-CoV-2.

Also, to control different effects in virus-infection models, we applied Cycloferon as a control substance in our assays. Cycloferon (10-carboxymethyl-9-acridanone, CMA) is a low-molecular organic compound, which is a registered drug in a group of “Antiviral and immunomodulatory drugs, interferon synthesis inducers” in the Kazakhstan’s pharmacopoeia.

Actually, the authors’ own studies have shown that Cycloferon does not induce interferon-alpha or interferon-beta in animals evolutionary distant from mice, at least at dosages equivalent to used in humans [11]. Also, other groups had published research on that CMA [active substance of Cycloferon] does not induce interferon in humans. Despite the posed to differ among viruses with different RNA-dependent RNA-polymerases (RdRp) [23-25]. At least two models of the Favipiravir’s antiviral action have been proposed, which are the models of lethal mutagenesis [23-25] or chain termination [24]. For both models it is important that the drug’s active form Favipiravir-RTP enters the RdRp active center mimicking natural GTP (guanosine ribosyl triphosphate) or ATP (adenosine ribosyl triphosphate). Figure 5 illustrates the molecular basis of the Favipiravir’s capacity to mimic both natural purine nucleotides.

Data produced in this study are more compatible with that Favipiravir cannot cause either chain termination or extensive lethal mutagenesis in SARS-CoV-2. This is actually an expected consequence of the proof-reading ability of the SARS-
CoV-2 RdRp complex (including nsp14), which manifests itself that the virus replication complex detects and removes erroneously incorporated bases. In contrast, the unrelated virus VEEV which RdRp has no editing capacity, is sensitive to Favipiravir at very low concentrations (EC$_{50}$, 49 ug/ml).

Future studies must concentrate on finding inhibitors of the coronavirus nsp14 enzyme. If such inhibitors are found and prove to be bioavailable and non-toxic, their use in combination with Favipiravir, will convert the drug Favipiravir into an efficient cure against the pandemic coronavirus.

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**CONCLUSION**

Favipiravir is ineffective against SARS-CoV-2 in _in vitro_ tests. On the contrary, Cycloferon has shown capacity to suppress the SARS-CoV-2 replication at concentrations achievable during human treatment. This signifies that Cycloferon is a perspective and currently overlooked drug with a poten-
tial to cure the SARS-CoV-2 infection.

**LITERATURE**


REFERENCES


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ФАВИПИРАВИРДІҢ НУКЛЕОЗИДТІК АНАЛОГЫ-БҰЛ ЖАСУША КУЛЬТУРАСЫНДАҒЫ ВИРУСА ВЕНЕСУЭЛЬСКОГО КОНСКОГО ЭНЦЕФАЛИТА РЕПЛИКАЦИЯСЫН БАСУ ҚАРСЫ ДЕРІ-ДЕРМЕКТЕРДІҢ БАЙЛАНЫСЫ

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АБСТРАКТ

Пандемия SARS-CoV-2 стала крупнейшим эпидемиологическим событием в текущем столетии, однако это была не первая пандемия с большим числом случаев в 21 веке и не последняя. Большинство возбудителей, вызвавших крупные эпидемии в 21 веке (птичий грипп, пандемический грипп, MERS, Эбола и др.), включая сам SARS-CoV-2, являются РНК-содержащими вирусами. Биологическую природу возбудителя, который вызовет будущую эпидемию, предсказать сложно, но при этом весьма вероятно, что это будет РНК-содержащий вирус. Для подготовки к будущим эпидемиям многообещающим подходом является перепрофилирование лекарств. Перепрофилированию лекарств против РНК-вирусов способствуют такие особенности патогенов, как РНК-зависимые РНК-полимеразы, обладающие способностью включать модифицированные нуклеотиды в репликацию вирусов с РНК-содержащим нуклеотидным диапазоном.

Ключевые слова: Фавипиравир; Циклоферон; противовирусная активность; SARS-CoV-2

ТУЙІН

SARS-CoV-2 пандемиясы қазіргі гасырдағы ең үлкен эпидемиологиялық оқиға болды, бірақ бул 21 гасырда көптеген жағдайлар бар алғашқы індет емес жасаны індет емес. 21 гасырдан ірі індеттер тұдарын қоқырмашырының көпшілігі (құс тұмауы, пандемия тұмауы, MERS, Эбола және т.б.), соның ішінде SARS-CoV-2-нің олар РНК бар вирустар. Білімді жағдайлардың біріншінде, патогениң биологиялық табиғаты болуы мүмкін, бірақ оның күрісінде РНК бар вирустар болуы мүмкін. Жағдайлар жаңа өзгерер және соңғысы емес.

SARS-CoV-2 вирусының жаңа қасиеттері арқылы, бірақ фавипиравир вирусқа қарсы екі препараттың репликациясын басу қабілетін өлшейді. Біз фавипиравир мен циклофероннан дәрі-дәрмектердің қосу мүмкіндігі бар РНҚ-ға тәуелді РНҚ полимеразалары сияқты патогендердің болып табылады.

Түйінді сөз: Фавипиравир; Циклоферон; противовирусная активность; SARS-CoV-2