Virus Research 317 (2022) 198826

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Short communication

Antiviral activity of brequinar against African swine fever virus infection in vitro

Rafayela Grigoryan [a,#], Erik Arabyan [a,#], Roza Izmailyan [a], Zaven Karalyan [b], Nuno Jordão [c,d], Fernando Ferreira [c,d], Hovakim Zakaryan [a,e,*]

[a] Laboratory of Antiviral Drug Discovery, Institute of Molecular Biology of NAS, E. Hasratyan 7, 0014, Yerevan, Armenia
[b] Laboratory of Cell Biology and Virology, Institute of Molecular Biology of NAS, E. Hasratyan 7, 0014, Yerevan, Armenia
[c] Centre for Interdisciplinary Research in Animal Health, Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal
[d] Associate Laboratory for Animal and Veterinary Sciences (AL4AnimalS), Portugal
[e] Denovo Sciences CJSC, H. Hakobyan 3, 0033, Yerevan, Armenia

ARTICLE INFO

Keywords:
African swine fever virus
Antiviral
Brequinar
Pyrimidine

ABSTRACT

African swine fever virus (ASFV) is a double-stranded DNA virus that causes an acute and hemorrhagic disease in domestic swine, resulting in significant economic losses to the global porcine industry. The lack of vaccines and antiviral drugs highlights the urgent need for antiviral studies against ASFV. Here, we report that brequinar (BQR), which is a specific inhibitor of dihydroorotate dehydrogenase, robustly inhibits ASFV replication in Vero cells, as well as in porcine macrophages. We demonstrate that BQR exerts its antiviral activity in a dose-dependent manner through the depletion of pyrimidine pool. Although BQR does not affect the synthesis of an early viral protein, pI215L, the synthesis of late viral proteins, p17 and p72, is suppressed in the presence of BQR. We also show that BQR is able to induce cellular antiviral response in ASFV-infected macrophages by enhancing the expression of interferon-stimulated genes. Taken together, our study reveals that targeting nucleotide biosynthesis represents a promising strategy for developing antiviral agents against ASFV.

Text

African swine fever virus (ASFV) is the causative agent of a highly contagious animal disease, African swine fever (ASF), affecting both wild and domestic swine of all breeds. It is a large double-stranded DNA virus which belongs to the genus Asfivirus within the Asfarviridae family (Alonso et al., 2018), and the only known DNA arbovirus. Highly virulent ASFV strains like those that are currently circulating in Europe (apart from Sardinia) and Asia cause acute disease with up to 100% lethality within 2 weeks. The clinical signs include hemorrhagic lesions, high fever, anorexia, cyanosis, and ataxia (Pikalo et al., 2019).

Due to the lack of effective vaccines against ASFV (Rock, 2021; Liu et al., 2021), it represents a global threat to agriculture, causing huge economic losses in affected countries. For instance, the outbreak of ASF caused a considerable loss of pig capacity in China, where it entered in August 2018 and continues to spread across the entire country. In the first quarter of 2020, because of the persistent impact of ASF, the number of pigs in China was 131.29 million, which was 57.14 million less than the same period last year (Wu et al., 2020). Therefore, there is a pressing need to develop antiviral strategies against ASFV for reducing the spread of virus.

Nucleotides, composed of purines and pyrimidines, play an important role in the constitution of nucleic acids. *De novo* nucleotide biosynthesis and salvage pathway are the two mechanisms for the recovery of intracellular nucleotide pool. Since viruses largely depend on the nucleotide supply, the pharmacological inhibition of nucleotide biosynthesis may disrupt viral lifecycle. Dihydroorotate dehydrogenase (DHODH) and inosine monophosphate dehydrogenase (IMPDH) are key enzymes in *de novo* pyrimidine and purine synthesis, respectively. Several independent studies revealed the antiviral potential of DHODH and IMPDH inhibitors against different human pathogenic viruses such as Zika virus, hepatitis C and E viruses, dengue virus, HIV, cytomegalovirus and SARS-CoV-2 (Borroto-Esoda et al., 2004; Hoffmann et al., 2011; Marschall et al., 2013; Wang et al., 2016; Tong et al., 2018; Hahn et al., 2020; Xiong et al., 2020; Luban et al., 2021), thereby confirming that nucleotide biosynthesis enzymes may represent promising target in

* Corresponding author at: Institute of Molecular Biology, Armenia.
E-mail address: h_zakaryan@mb.sci.am (H. Zakaryan).
# Authors have equal contribution

https://doi.org/10.1016/j.virusres.2022.198826
Received 2 May 2022; Received in revised form 20 May 2022; Accepted 22 May 2022
Available online 23 May 2022
0168-1702/© 2022 Elsevier B.V. All rights reserved.

antiviral drug discovery.

In this study, we evaluated the antiviral activity of DHODH and IMPDH inhibitors against ASFV. For this purpose, we studied brequinar (BQR; TRC, Canada), teriflunomide (TFM; Sigma-Aldrich, Germany), mycophenolic acid (MPA; Sigma-Aldrich, Germany) and AVN-944 (AVN; Cayman Chemical, USA). BQR and TFM inhibit pyrimidine *de novo* synthesis by blocking the DHODH enzyme (Fig. 1A) (Vyas and Ghate, 2011). MPA and AVN-944 are selective, non-competitive inhibitors of IMPDH enzyme (Fig. 1A) (Chen and Pankiewicz, 2007). First, we tested different concentrations at which these compounds may display cytotoxic effects on Vero cells. Confluent Vero cells in 96-well cell culture plates (seeding density: 2×10^4 cell/well) were treated with increasing concentrations of compound ranging from 12.5 to 100 μM. Treated cells were incubated for 24 h at 37 °C in 5% CO_2. Then, cell viability was studied by MTT assay as described (Sirakanyan et al., 2021). The percentage of viable cells were calculated for each concentration as $[(OD_T/OD_C) \times 100]$, where OD_T and OD_C correspond to the absorbance of treated and control cells, respectively. As shown in Fig. 1B, all compounds did not affect cell viability at concentrations up to 100 μM, indicating that these concentrations could be used in antiviral assays. Next, we examined whether these inhibitors may disrupt the ASFV infection in Vero cells. Vero cells grown in 24-well cell culture plate (seeding density: 2×10^5 cell/well) were infected with the ASFV Ba71V strain at an MOI of 0.2 $TCID_{50}$/cell and treated with compounds at 50 μM (BQR and AVN) and 100 μM (TFM and MPA) concentrations. The infection was allowed to proceed for 24 h, after which the supernatant was collected and titrated by the cytopathic effect (CPE)-based serial dilution assay (Carrascosa et al., 2011). All tested compounds exerted antiviral effects on ASFV replication in Vero cells. TFM, AVN and MPA reduced the viral yield from 4.2 ± 0.13 log $TCID_{50}$/ml to 3.14 ± 0.36 log $TCID_{50}$/ml ($P < 0.05$), 3.2 ± 0.24 log $TCID_{50}$/ml ($P < 0.05$), and 3.25 ± 0.09 log $TCID_{50}$/ml ($P < 0.02$), respectively (Fig. 1C). The most potent compound, BQR, reduced the viral yield by 4 log ($P < 0.02$), thereby inhibiting the ASFV infection by 99.99%. Although BQR and TFM have overlapping binding sites on DHODH enzyme, McLean et al. (2001) demonstrated that TFM is much less potent DHODH inhibitor than BQR. To further validate whether the potent anti-ASFV activity of BQR was achieved through the depletion of pyrimidine pool, we conducted antiviral experiments in the presence of exogenous uridine in the cell culture medium. Here we used 2 mM uridine to recover the pyrimidine nucleotide pool. Although the physiological concentration of uridine is significantly less than 2 mM, uridine alone did not affect the ASFV replication in Vero cells even at this high concentration (Fig. 1D). The anti-ASFV activity of 50 μM BQR was significantly reversed by the presence of uridine at 2 mM concertation (Fig. 1D), suggesting that the anti-ASFV mechanism of BQR relied on the depletion of pyrimidine nucleotides.

Next, we evaluated the inhibitory activity of BQR at different concentrations. For this purpose, Vero cells grown in 24-well cell culture plate (seeding density: 2×10^5 cell/well) were infected with the virus at an MOI of 0.2 $TCID_{50}$/cell and treated with BQR at decreasing concentrations up to 3.125 μM. After 24 h post-infection, the supernatant was collected and titrated. BQR inhibited the ASFV infection in Vero cells at all tested concentrations in a dose-dependent manner (Fig. 2A). Even at the lowest concentration (3.125 μM), it decreased the viral yield by 0.9 log (more than 80% inhibition). The IC_{50} (the half maximal inhibitory concentration) value was 2.83 μM, while the CC_{50} (50% cytotoxic concentration) value was more than 100 μM, indicating a high specificity and therapeutic activity of BQR against ASFV. Indeed, BQR successfully inhibited the ASFV-induced CPE in Vero cells at both high and low concentrations (Fig. 2A). To determine whether this potent antiviral effect depends on viral MOI, confluent Vero cells grown in 24-well cell

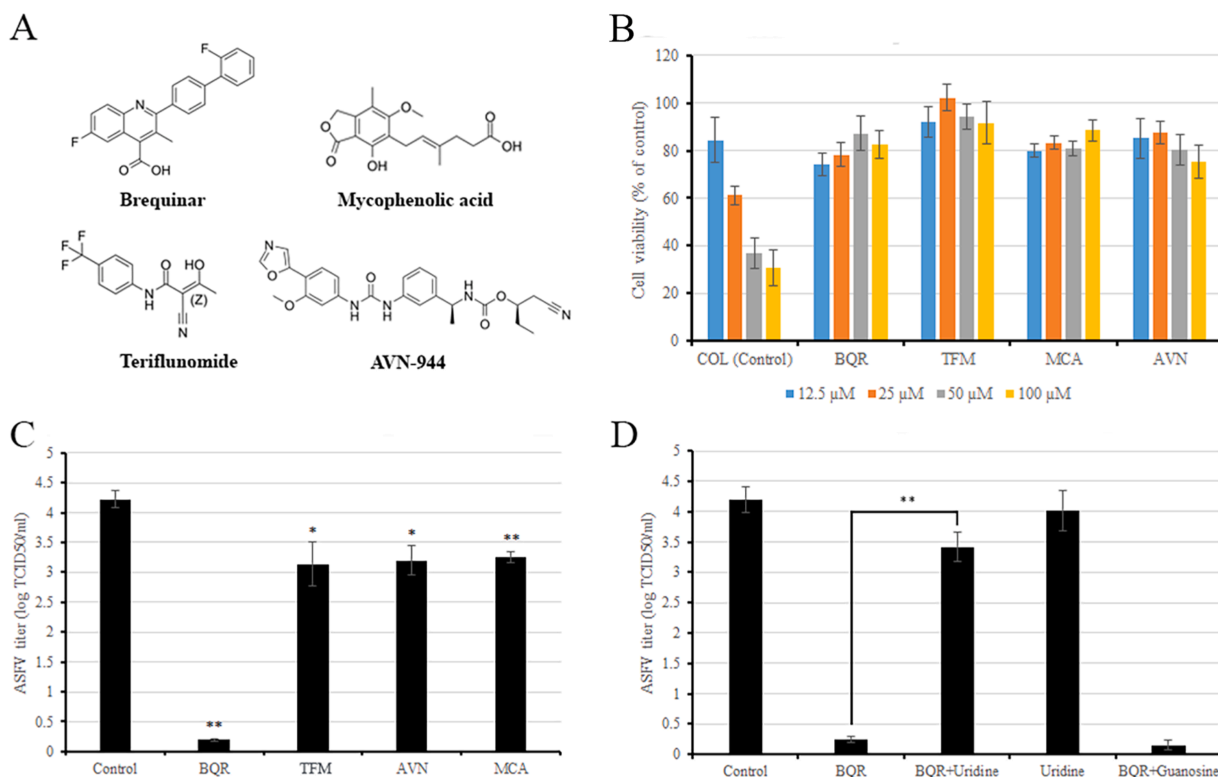

Fig. 1. Antiviral activity of DHODH and IMPDH inhibitors against ASFV. (A) Chemical structures of inhibitors selected for this study. (B) Cytotoxicity of inhibitors on Vero cells evaluated by MTT method. Colchicine (COL) was used as a cytotoxicity control. Control cells were treated with DMSO (<1% v/v). Viability of control cells was considered as 100%. (C) ASFV titer in Vero cells upon treatment with inhibitors at 50 μM (BQR and AVN) and 100 μM (TFM and MCA) concentrations. (D) Effect of BQR on ASFV replication in the presence of uridine at 2 mM concentration. Results represent the mean (± SD) of three independent experiments ($n = 3$). Data were analyzed by Student's *t* test. Significant differences compared to control are denoted by $^*P < 0.05$ and $^{**}P < 0.01$.

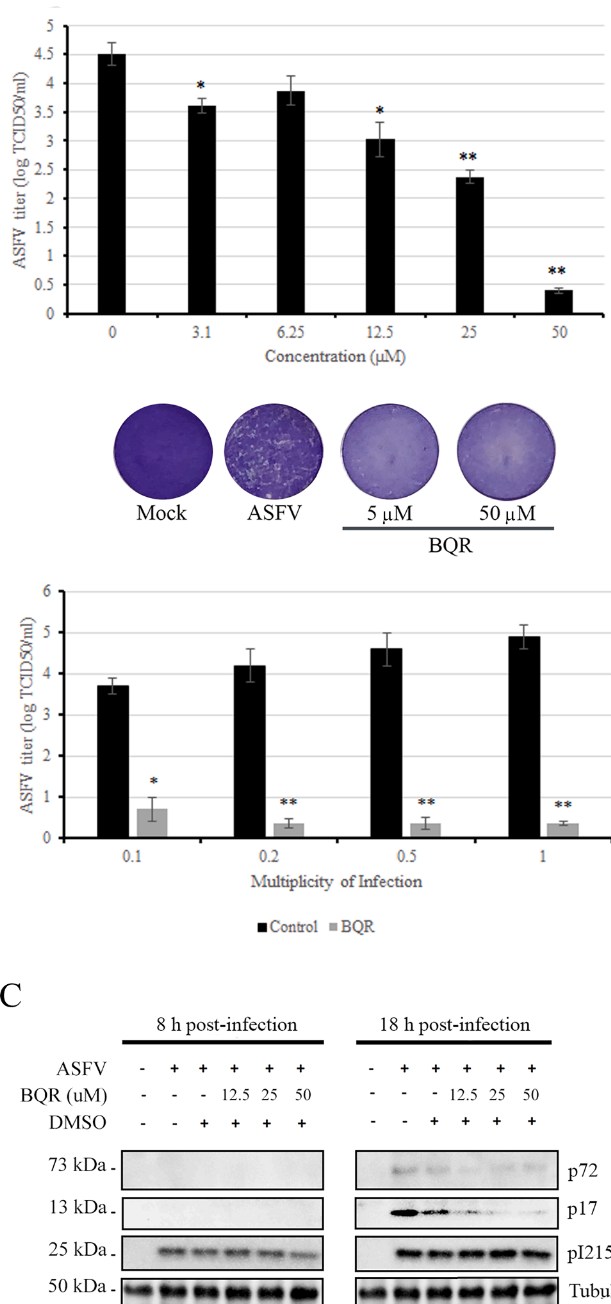

Fig. 2. Antiviral effect of BQR on ASFV replication in Vero cells. (A) ASFV titer and CPE in cells exposed to BQR at increasing concentrations. ASFV-inducted CPE was visualized by crystal violet staining method. (B) Effect of BQR on cells infected with ASFV at different MOIs. (C) Analysis of ASFV early (pI215L) and late (p73 and p17) proteins synthesis in ASFV-infected cells exposed to BQR. Alpha-tubulin was used as a loading control. Results represent the mean (± SD) of three independent experiments ($n = 3$). Data were analyzed by Student's *t* test. Significant differences compared to control are denoted by $^{*}P < 0.05$ and $^{**}P < 0.01$.

culture plate were treated with a fresh medium containing BQR at concentration of 50 μM and subsequently infected with ASFV at four different MOIs of 0.1, 0.2, 0.5 and 1 $TCID_{50}$/cell. After 24 h post-infection, viral titers were determined from collected supernatants. As shown in Fig. 2C, treatment of ASFV-infected Vero cells with BQR resulted in ASFV inhibition at all tested MOIs by a similar extent.

Knowing that BQR interferes with ASFV progeny production, we also investigated whether the viral protein synthesis was affected by this compound. We compared the expression of an early (pI215L) and two late (p73 and p17) viral proteins by western blot analysis. For this purpose, Vero cells grown in 6-well cell culture plates (2×10^5 cells/well) were infected with the ASFV Ba71V at MOI of 1. Following this step and before protein extraction, mock-infected and ASFV-infected cells either exposed or not to the compound were washed twice with cold PBS and processed as previously reported (Freitas et al., 2018). As shown in Fig. 2B, from the early (6 h post-infection) to the late phase of infection (18 h post-infection), the expression of ASFV-pI215L was similar in ASFV-infected cells exposed to different BQR concentrations and in positive control. In contrast, the synthesis of two late ASFV proteins (p17 and p72) was reduced in ASFV-infected cells exposed to BQR.

In pigs, the primary replication of ASFV takes place in the cells of the mononuclear-phagocytic system (Gómez-Villamandos et al., 2013). Therefore, we conducted additional experiments to define the effect of BQR on the replication of highly virulent ASFV isolate (Arm/07) in porcine macrophages. Preparation of porcine macrophages were done as previously described (Carrascosa et al., 2011). As no cytotoxicity was observed at concentrations up to 100 μM (Fig. 3A), porcine alveolar macrophages grown in 24-well cell culture plate (seeding density: 4×10^4 cell/well) were infected with ASFV Arm/07 isolate at MOI of 0.5 $HADU_{50}$/cell and subsequently treated with 50 μM BQR in the presence or absence of 2mM uridine. The infection was allowed to proceed for 24 or 48 h, then the supernatant was collected and titrated by hemadsorption (HAD) assay (Carrascosa et al., 2011). After 24 h post-infection, BQR reduced the virus titer from 5.5 ± 0.4 $HADU_{50}$/ml to 3.4 ± 0.5 $HADU_{50}$/ml ($P < 0.05$) (Fig. 3B). At 48 h post-infection, the ASFV yield decreased from 6.3 ± 0.25 $HADU_{50}$/ml to 4.1 ± 0.3 $HADU_{50}$/ml, indicating that ASFV did not overcome the inhibitory effect over time. It has been previously shown that pyrimidine depletion promotes the enhanced expression of interferon-stimulated genes (ISGs), resulting in cellular antiviral response against West Nile virus, Ebola virus, and vesicular stomatitis Indiana virus (Lucas-Hourani et al., 2013; Chung et al., 2016; Luthra 2018). Thus, we hypothesized that porcine ISGs could be involved in the antiviral effect of BQR against ASFV. We examined expression of ISGs in ASFV-infected macrophages by qRT-PCR. The following primers were used: 1) pCXCL10: TGCCCACATGTTGAGATCAT (forward) and CGGCCCATCCTTATCAGTAG (reverse); 2) pIFIT1: CTGACTCACAGCAACCATG (forward) and CTTTCAGGTGTTTCACATAGG (reverse); 3) pIRF1: GCAACAGATGAGGACGAG (forward) and GCTTTCAACTTCTGGCTC (reverse); 4) pISG15: GACTGCATGATGGCATCGGA (forward) and TGCACCATCAACAGGACCAT (reverse); 5) pISG56: TTAGAAAACAGGGTCTTGGAGGAG (forward) and CGTAAGGTAATACAGCCAGGCATA (reverse), 6) pMx1: TACGACATCGAATACCAGATCAA (forward) and ATGGTCCTGTCTCCTTCGG (reverse). The qRT-PCR assay was performed as described previously (Fan et al., 2020). Our results showed that when ASFV-infected macrophages were treated with BQR, it robustly induced the expression of a panel of antiviral ISGs (Fig. 3C). The most significant induction, a five-fold increase, was observed for ISG56 ($P < 0.01$). As exogenous uridine completely abrogated the transcription of ISGs (Fig. 3C), we could conclude that overexpression of ISGs was associated with nucleotide depletion. However, the precise mechanism remains unclear.

In conclusion, we showed that both DHODH and IMPDH inhibitors have antiviral activities against ASFV. The anti-ASFV activity of BQR is mediated by pyrimidine depletion, which negatively affects the virus replication in Vero and porcine macrophages, indicating that nucleotide biosynthesis plays an important role in ASFV infection. Furthermore, we also revealed that BQR induces the expression of some ISGs in ASFV-infected macrophages. Although we did not show that ISGs responses are required for the antiviral activity of BQR, some ISGs could nonetheless contribute to anti-ASFV effects by evading ASFV defenses against host innate immunity. Additional research into *in vivo* antiviral uses for BQR is warranted to evaluate its potential as a promising anti-ASFV drug candidate.

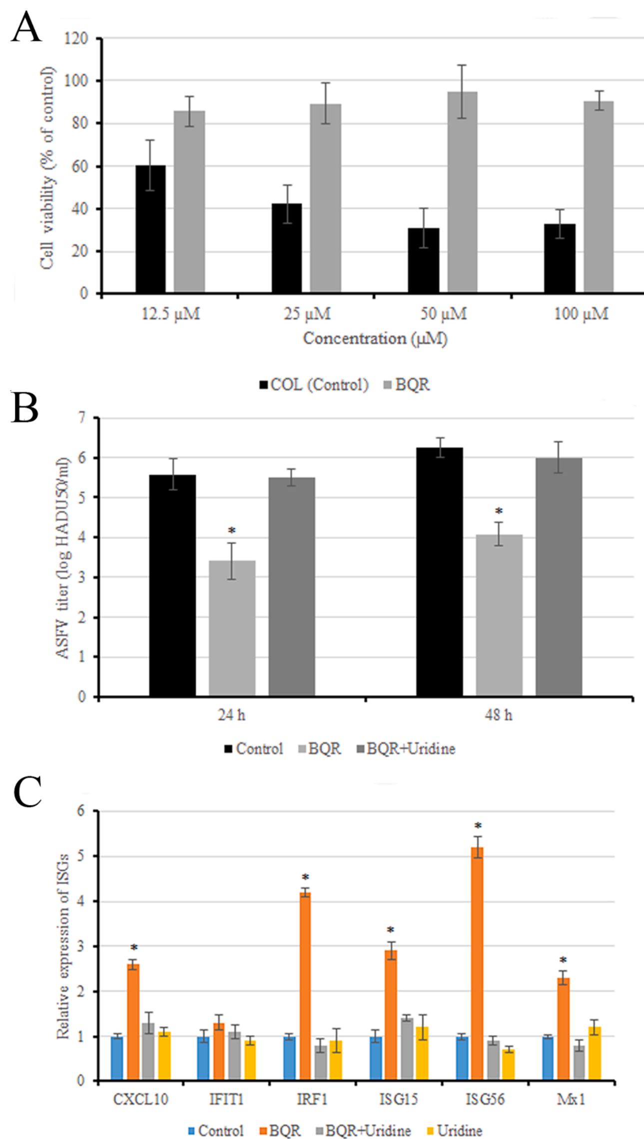

Fig. 3. Effect of BQR on ASFV infection in porcine macrophages. (A) Cytotoxicity of BQR on porcine macrophages evaluated by MTT method. Colchicine (COL) was used as a cytotoxicity control. Control cells were treated with DMSO (<1% v/v). Viability of control cells was considered as 100%. (B) ASFV titer in macrophages treated with BQR for 24 and 48 h. (C) Expression of ISGs in ASFV-infected macrophages determined by qRT-PCR. Data were normalized to basal ISG expression without treatment in ASFV-infected cells. Results represent the mean (± SD) of three independent experiments ($n = 3$). Data were analyzed by Student's t test. Significant differences compared to control are denoted by $^{*}P < 0.02$.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

Founding sources

This work was supported by the Science Committee of the Ministry of Education, Science, Culture and Sport RA (grant number: 20TTSG-1F007) and by Fundação para a Ciência e a Tecnologia (Portugal) through the projects UIDB/00276/2020 and LA/P/0059/2020 - AL4AnimalS.

References

Alonso, C., Borca, M., Dixon, L., Revilla, Y., Rodriguez, F., Escribano, J.M., 2018. Ictv Report, C ICTV virus taxonomy profile: asfarviridae. J. Gen. Virol. 99, 613–614.
Borroto-Esoda, K., Myrick, F., Feng, J., Jeffrey, J., Furman, P., 2004. In vitro combination of amdoxovir and the inosine monophosphate dehydrogenase inhibitors mycophenolic acid and ribavirin demonstrates potent activity against wild-type and drug-resistant variants of human immunodeficiency virus type 1. Antimicrob. Agents Chemother. 48 (11), 4387–4394.
Carrascosa, A.L., Bustos, M.J., de Leon, P., 2011. Methods for growing and titrating African swine fever virus: field and laboratory samples. Curr. Protoc. Cell Biol. 26, 14, 26.
Chen, L., Pankiewicz, K.W., 2007. Recent development of IMP dehydrogenase inhibitors for the treatment of cancer. Curr. Opin. Drug Discov. Devel. 10 (4), 403–412.
Chung, D.H., Golden, J.E., Adcock, R.S., Schroeder, C.E., Chu, Y.K., Sotsky, J.B., Cramer, D.E., Chilton, P.M., Song, C., Anantpadma, M., et al., 2016. Discovery of a broad-spectrum antiviral compound that inhibits pyrimidine biosynthesis and establishes a type 1 interferon-independent antiviral state. Antimicrob. Agents Chemother. 60, 4552–4562.
Fan, W., Jiao, P., Zhang, H., Chen, T., Zhou, X., Qi, Y., Sun, L., Shang, Y., Zhu, H., Hu, R., Liu, W., Li, J., 2020. Inhibition of African Swine Fever Virus Replication by Porcine Type I and Type II Interferons. Front. Microbiol. 11, 1203.
Freitas, F., Frouco, G., Martins, C., Ferreira, F., 2018. African swine fever virus encodes for an E2-ubiquitin conjugating enzyme that is mono- and di-ubiquitinated and required for viral replication cycle. Sci. Rep. 8, 3471.
Gómez-Villamandos, J.C., Bautista, M.J., Sánchez-Cordón, P.J., Carrasco, L., 2013. Pathology of African swine fever: the role of monocyte-macrophage. Virus Res. 173 (1), 140–149.
Hahn, F., Wangen, C., Häge, S., Peter, A.S., Dobler, G., Hurst, B., Julander, J., Fuchs, J., Ruzsics, Z., Überla, K., Jäck, H.M., Ptak, R., Muehler, A., Gröppel, M., Vitt, D., Peelen, E., Kohlhof, H., Marschall, M., 2020. IMU-838, a Developmental DHODH Inhibitor in Phase II for Autoimmune Disease, Shows Anti-SARS-CoV-2 and Broad-Spectrum Antiviral Efficacy In Vitro. Viruses 12 (12), 1394.
Hoffmann, H.H., Kunz, A., Simon, V.A., Palese, P., Shaw, M.L., 2011. Broad-spectrum antiviral that interferes with de novo pyrimidine biosynthesis. Proc. Natl. Acad. Sci. U S A. 108 (14), 5777–5782.
Liu, L., Wang, X., Mao, R., Zhou, Y., Yin, J., Sun, Y., Yin, X., 2021. Research progress on live attenuated vaccine against African swine fever virus. Microb. Pathog. 158, 105024.
Luban, J., Sattler, R.A., Mühlberger, E., Graci, J.D., Cao, L., Weetall, M., Trotta, C., Colacino, J.M., Bavari, S., Strambio-De-Castillia, C., Suder, E.L., Wang, Y., Soloveva, V., Cintron-Lue, K., Naryshkin, N.A., Pykett, M., Welch, E.M., O'Keefe, K., Kong, R., Goodwin, E., Jacobson, A., Paessler, S., Peltz, S.W., 2021. The DHODH inhibitor PTC299 arrests SARS-CoV-2 replication and suppresses induction of inflammatory cytokines. Virus Res. 292, 198246.
Lucas-Hourani, M., Dauzonne, D., Jorda, P., Cousin, G., Lupan, A., Helynck, O., Caignard, G., Janvier, G., André-Leroux, G., Khiar, S., Escriou, N., Desprès, P., Jacob, Y., Munier-Lehmann, H., Tangy, F., Vidalain, P.O., 2013. Inhibition of pyrimidine biosynthesis pathway suppresses viral growth through innate immunity. PLoS Pathog. 9, e1003678.
Luthra, P., Naidoo, J., Pietzsch, C.A., De, S., Khadka, S., Anantpadma, M., Williams, C.G., Edwards, M.R., Davey, R.A., Bukreyev, A., Ready, J.M., Basler, C.F., 2018. Inhibiting pyrimidine biosynthesis impairs Ebola virus replication through depletion of nucleoside pools and activation of innate immune responses. Antiviral Res. 158, 288–302.
Marschall, M., Niemann, I., Kosulin, K., Bootz, A., Wagner, S., Dobner, T., Herz, T., Kramer, B., Leban, J., Vitt, D., Stamminger, T., Hutterer, C., Strobl, S., 2013. Assessment of drug candidates for broad-spectrum antiviral therapy targeting cellular pyrimidine biosynthesis. Antiviral Res. 100 (3), 640–648.
McLean, J.E., Neidhardt, E.A., Grossman, T.H., Hedstrom, L., 2001. Multiple inhibitor analysis of the brequinar and leflunomide binding sites on human dihydroorotate dehydrogenase. Biochemistry 40 (7), 2194–2200.
Pikalo, J., Zani, L., Hühr, J., Beer, M., Blome, S., 2019. Pathogenesis of African swine fever in domestic pigs and European wild boar - Lessons learned from recent animal trials. Virus Res. 271, 197614.
Rock, D.L., 2021. Thoughts on African Swine Fever Vaccines. Viruses 13 (5), 943.
Sirakanyan, S., Arabyan, E., Hakobyan, A., Hakobyan, T., Chilingaryan, G., Sahakyan, H., Sargsyan, A., Arakelov, G., Nazaryan, K., Izmailyan, R., Abroyan, L., Karalyan, Z., Arakelova, E., Hakobyan, E., Hovakimyan, A., Serobian, A., Neves, M., Ferreira, J., Ferreira, F., Zakaryan, H., 2021. A new microtubule-stabilizing agent shows potent antiviral effects against African swine fever virus with no cytotoxicity. Emerg. Microbes Infect. 10 (1), 783–796.
Tong, X., Smith, J., Bukreyeva, N., Koma, T., Manning, J.T., Kalkeri, R., Kwong, A.D., Paessler, S., 2018. Merimepodib, an IMPDH inhibitor, suppresses replication of Zika virus and other emerging viral pathogens. Antiviral. Res. 149, 34–40.
Vyas, V.K., Ghate, M., 2011. Recent developments in the medicinal chemistry and therapeutic potential of dihydroorotate dehydrogenase (DHODH) inhibitors. Mini Rev. Med. Chem. 11 (12), 1039–1055.
Wang, Y., Wang, W., Xu, L., Zhou, X., Shokrollahi, E., Felczak, K., van der Laan, L.J., Pankiewicz, K.W., Sprengers, D., Raat, N.J., Metselaar, H.J., Peppelenbosch, M.P., Pan, Q., 2016. Cross Talk between Nucleotide Synthesis Pathways with Cellular

Immunity in Constraining Hepatitis E Virus Replication. Antimicrob. Agents Chemother. 60 (5), 2834–2848.

Wu, K., Liu, J., Wang, L., Fan, S., Li, Z., Li, Y., Yi, L., Ding, H., Zhao, M., Chen, J., 2020. Current State of Global African Swine Fever Vaccine Development under the Prevalence and Transmission of ASF in China. Vaccines (Basel) 8 (3), 531.

Xiong, R., Zhang, L., Li, S., Sun, Y., Ding, M., Wang, Y., Zhao, Y., Wu, Y., Shang, W., Jiang, X., Shan, J., Shen, Z., Tong, Y., Xu, L., Chen, Y., Liu, Y., Zou, G., Lavillete, D., Zhao, Z., Wang, R., Zhu, L., Xiao, G., Lan, K., Li, H., Xu, K., 2020. Novel and potent inhibitors targeting DHODH are broad-spectrum antivirals against RNA viruses including newly-emerged coronavirus SARS-CoV-2. Protein Cell 11 (10), 723–739.