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Pharmacology & Therapeutics 209 (2020) 107512

Contents lists available at ScienceDirect

Pharmacology & Therapeutics

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

Favipiravir, an anti-influenza drug against life-threatening RNA virus infections

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ARTICLE INFO

Available online 22 February 2020

Keywords:
Favipiravir
Influenza
Antiviral agent
Ebola
Chain termination
Resistant virus

ABSTRACT

Favipiravir has been developed as an anti-influenza drug and licensed as an anti-influenza drug in Japan. Additionally, favipiravir is being stockpiled for 2 million people as a countermeasure for novel influenza strains. This drug functions as a chain terminator at the site of incorporation of the viral RNA and reduces the viral load. Favipiravir cures all mice in a lethal influenza infection model, while oseltamivir fails to cure the animals. Thus, favipiravir contributes to curing animals with lethal infection. In addition to influenza, favipiravir has a broad spectrum of anti-RNA virus activities in vitro and efficacies in animal models with lethal RNA viruses and has been used for treatment of human infection with life-threatening Ebola virus, Lassa virus, rabies, and severe fever with thrombocytopenia syndrome. The best feature of favipiravir as an antiviral agent is the apparent lack of generation of favipiravir-resistant viruses. Favipiravir alone maintains its therapeutic efficacy from the first to the last patient in an influenza pandemic or an epidemic lethal RNA virus infection. Favipiravir is expected to be an important therapeutic agent for severe influenza, the next pandemic influenza strain, and other severe RNA virus infections for which standard treatments are not available.

Contents

1. Introduction

Acyclovir enabled the first systemic antiviral therapy, and many antiviral agents have subsequently been developed. Acyclovir targets herpes simplex virus and varicella-zoster infection, and individual antiviral agents target one viral infection caused by one virus or two viruses of the same family (Elion, 1982; Shiraki, 2017, 2018). Among antiviral agents, one of the unique features of favipiravir (T-705) is its broad spectrum activity toward RNA viruses, including influenza virus, rhinovirus, and respiratory syncytial virus, but not DNA viruses, as shown in

Abbreviations: baloxavir, baloxavir marboxil; G-string, guanosine homopolymeric string; IFN, interferon; IL, interleukin; NA, neuraminidase; NAI, NA inhibitor; PEP, postexposure prophylaxis; RdRp, RNA-dependent RNA polymerase; RTP, 4-ribofuranosyl-5′-triphosphate; SFTS, severe fever with thrombocytopenia syndrome.

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https://doi.org/10.1016/j.pharmthera.2020.107512
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Fig. 1 (Furuta et al., 2002; Furuta et al., 2005; Furuta et al., 2009). Favipiravir shows better efficacy in treating influenza infections than oseltamivir (Tamiflu) (Takahashi et al., 2003; Tanaka et al., 2017), and its efficacy in treating pathogenic avian influenza A(H5N1) and oseltamivir-resistant viruses has been confirmed in animals (Kiso et al., 2010; Sidwell et al., 2007).

Clinical trials of treatments for seasonal influenza have been performed in Japan and US, and favipiravir was approved as a treatment for novel or re-emerging influenza viruses in Japan in 2014. Favipiravir is considered for administration to patients only when the government judges that this drug will be used as a countermeasure against novel or re-emerging influenza viruses. The Japanese government and Taiwanese Centers for Disease Control (CDCs) decided to stockpile favipiravir for the people as a countermeasure for severe influenza. Favipiravir has been submitted for additional indications for severe fever with thrombocytopenia syndrome (SFTS) based on clinical trials (Yasukawa, 2016) in addition to influenza in Japan.

Favipiravir was highlighted as a treatment during the lethal Ebola virus epidemic in West Africa in 2014 because a standard treatment for lethal Ebola virus infection is not available. Favipiravir has been reported to be effective for prophylaxis and treating lethal Ebola virus infection in animal models (Oestereich et al., 2014; Smither et al., 2014) and is licensed for influenza treatment based on its confirmed safety and efficacy in clinical trials in humans. Based on this information, favipiravir was successfully used for the post-exposure prophylaxis and treatment of patients with Ebola virus infection (Bai et al., 2016; Jacobs et al., 2015; Sissoko et al., 2016).

Since children died of avian influenza A(H5N1) in Hong Kong in 1997 (Ku & Chan, 1999), concern regarding novel influenza pandemics, such as A(H5N1) and A(H7N9), has been noted because no one is immune to these viruses. Novel influenza strains cause more severe diseases, particularly pneumonia, than seasonal influenza, and the mortality rate is 53.5% (483/903) for influenza A(H5N1) (Lai et al., 2016) and 34% (47/137) for influenza A(H7N9) (Li et al., 2014). A combination therapy with oseltamivir has been used to treat patients with severe influenza in China (Cao, 2018; Wang et al., 2019). Although each novel influenza strain currently occurs sporadically, researchers are concerned about the possibility of a pandemic.

This review describes the specific features of favipiravir, the mechanism of action and the fact that favipiravir alone does not produce resistant viruses among the anti-influenza drugs. These outstanding features of favipiravir among anti-influenza drugs are expected to play a central role in the treatment of lethal influenza pandemic and other severe RNA virus infections for which standard treatments are unavailable and help clinicians, scientists, and policy-makers who are considering preparedness strategies for new influenza pandemics and prevention through vaccine development.

2. Development as an anti-influenza drug

2.1. Discovery of the anti-influenza activity of favipiravir and its development as an anti-influenza drug

In a joint development project with Toyama Chemical Co., Ltd., we initially developed anti-herpes drugs and screened and identified several promising compounds with efficacy, but they did not exceed the efficacy of acyclovir in animals. Approximately 30,000 compounds synthesized in the company were screened for the activity required in each category of drugs, such as antiviral, antibacterial, anti-inflammatory, and nervous system agents. Regarding favipiravir (T-705), one compound showed anti-influenza virus activity and was optimized for activity and efficacy in animal experiments to yield

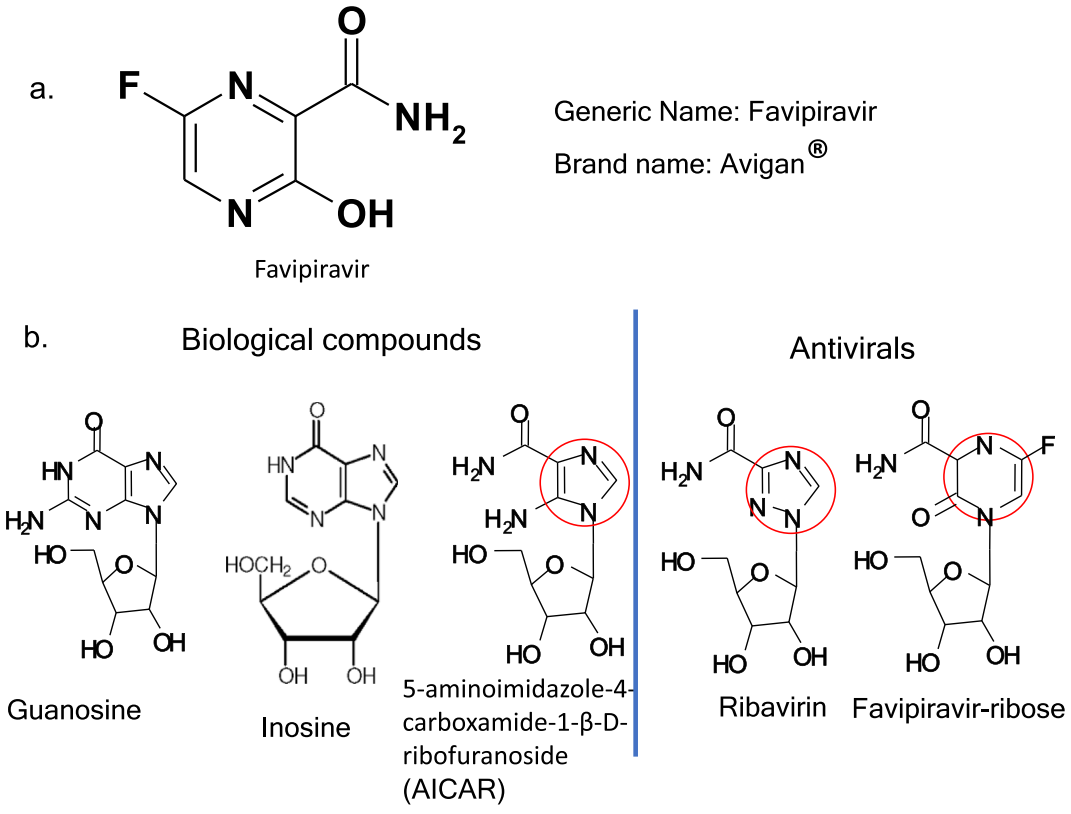

Fig. 1. Chemical structures of favipiravir and its related compounds. A. Favipiravir (6-fluoro-3-hydroxy-2-pyrazinecarboxamide). T-705 is the code number of favipiravir. B. Guanosine, inosine, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), ribavirin, and ribosyl favipiravir. Guanosine, inosine, and AICAR are biosynthesized in the body, and ribavirin and ribosyl favipiravir are synthesized nucleoside analogues. They have similar chemical structures, but favipiravir and the other two compounds differ because they contain pyrazine, triazole, and imidazole that are marked by red circles.

favipiravir. Our laboratory conducted drug efficacy and validation tests in infected mice and ferrets and conducted experiments to compare the efficacy of favipiravir with oseltamivir (Tamiflu). Based on its chemical structure, favipiravir should inhibit RNA synthesis, and our laboratory tried to isolate a resistant virus. Then, the lack of generation of resistant viruses was investigated.

Clinical trials of treatments for seasonal influenza have been performed in Japan and the US, and favipiravir was approved as a treatment for novel or re-emerging influenza viruses in Japan in 2014. However, favipiravir (Avigan®) is only considered for administration to patients when the government judges that this drug will be used as a countermeasure for novel or re-emerging influenza viruses. In addition, favipiravir is contraindicated for use in pregnant women because it exerts teratogenic and embryotoxic effects on animals. The Taiwanese CDC decided to stockpile favipiravir for people who became infected with new strains of influenza, including avian and swine influenzas, in 2015. The Japanese government decided to stockpile favipiravir (Avigan®) as a novel influenza countermeasure for 2 million people in 2017. Recent clinical trials have been performed to assess its efficacy in treating SFTS in Japan (Yasukawa, 2016) and as a combination therapy with oseltamivir to treat patients with severe influenza in China (Cao, 2018; Wang et al., 2019). In addition to influenza, favipiravir has been submitted for additional indications for SFTS in Japan, based on clinical trials, indicating its outstanding feature as a broad spectrum anti-RNA virus drug.

2.2. Chemical structure of the anti-influenza drug favipiravir and its broad anti-RNA virus activity

A compound that first showed anti-influenza activity was obtained in the screen of antiviral drugs targeting influenza, and favipiravir was optimized in terms of activity and stability in animals. Dr. Shiraki, K. suggested that the chemical structures of the active compound and its derivatives, including favipiravir, resembled a nucleoside analogue, and the presumed mechanism of action was to inhibit RNA synthesis (Fig. 1). Three compounds, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), which was biosynthesized de novo from amino acids, ribosyl-favipiravir, and ribavirin, have similar chemical structures, suggesting that favipiravir functions as a purine homologue and inhibits viral RNA synthesis. Therefore, Dr. Shiraki, K. suggested the synthesis of the ribosyl form of favipiravir for its better efficacy, but the ribosylation of favipiravir did not improve its antiviral activity. Addition of 10 × 50% effective concentration for plaque reduction (EC_{50} = 63.7 μM) of adenine, guanine, adenosine, guanosine, and inosine in the assay medium abolishes the anti-influenza virus activity of favipiravir (Furuta et al., 2005). Based on these findings, favipiravir is mainly incorporated in the salvage pathways for purine nucleotides through the purine phosphoribosyltransferases (Craig 3rd & Eakin, 2000) and is further phosphorylated to favipiravir-triphosphate as the substrate for viral RNA-dependent RNA polymerase (RdRp). The phosphorylation of favipiravir by hypoxanthine guanine phosphoribosyltransferase was confirmed in a subsequent study (Naesens et al., 2013). Favipiravir showed anti-influenza virus activity toward all subtypes of seasonal influenza virus strains, including types A, B and C of laboratory strains and clinical isolates, as well as an oseltamivir-resistant virus, with an EC_{50} ranging from 0.014 to 0.55 μg/mL (Furuta et al., 2002). Although the effectiveness against influenza viruses at the cell culture level was confirmed, the use of favipiravir as an anti-influenza drug was confirmed by analyzing its efficacy in animal models of influenza infection, as described below, which led to drug development.

Favipiravir was effective against other RNA viruses, poliovirus, rhinovirus, and respiratory syncytial virus but not effective against DNA viruses, herpes simplex virus-1, cytomegalovirus, and adenovirus (Furuta et al., 2002). Based on the antiviral activity toward four RNA viruses, we expected that favipiravir should be active against a broad range of RNA viruses. Favipiravir has been evaluated and developed as a broad spectrum anti-RNA virus drug, including lethal RNA virus infections. The anti-RNA virus activity of favipiravir was analyzed at the cellular level, and efficacy studies were performed in animal models of human lethal RNA virus infections, as shown in Table 1. Patients with an Ebola virus infection in West Africa in 2014 and patients with other RNA virus infections have been treated with favipiravir (Avigan®) based on the efficacy in human lethal RNA virus infections, clinical experience, efficacy in patients with seasonal influenza, and its licensure for human use.

2.3. Influenza infection model

We have identified two antipyretic steps in influenza infections, as shown in Fig. 2. Influenza infection and its replication in the epithelium of the upper respiratory tract induce the production of interferon (IFN) and cytokines that induce cyclooxygenase expression and prostaglandin E_2 production and cause fever. The effects of interferon and interleukin (IL)-1α were determined by neutralizing their actions through the intravenous injection of their respective antibodies in influenza-infected mice (Kurokawa, Imakita, Kumeda, & Shiraki, 1996; Kurokawa, Watanabe, Shimizu, Sawamura, & Shiraki, 2010). The effects of aspirin, NSAIDs, and cinnamyl compounds derived from herbal extracts on influenza-infected mice or mouse macrophage-derived P388D1 cells were determined, and 48 cinnamyl compounds showed compound-specific responses to fever and increased IL-1 levels in influenza-infected mice. Cinnamyl compounds regulate cytokine levels by modulating the amount of NF-κB (Kurokawa et al., 1998; Kurokawa, Brown, Kagawa, & Shiraki, 2003). Cinnamyl compounds and clarithromycin increase the levels of IL-12 on day 2 and IFN-γ on day 3 in the bronchoalveolar fluids of mice and decrease the area of pneumonia throughout the lungs. The role of IL-12 on day 2 was confirmed through its direct nasal application, and nasal administration of IL-12 reduced the virus yield in the bronchoalveolar fluids from influenza-infected mice (Hama et al., 2009; Kurokawa, Tsurita, Brown, Fukuda, & Shiraki, 2002; Tsurita et al., 2001). Cinnamyl compounds that are mainly derived from medicinal herbs prevent the induction of fever in influenza-infected mice by decreasing the serum IL-1 level. In contrast, aspirin suppresses fever by inhibiting hypothalamic cyclooxygenase activity and prostaglandin E_2 production without affecting the high level of IL-1 (Kurokawa, Imakita, Kumeda, & Shiraki, 1996; Kurokawa, Imakita, Kumeda, Yukawa, & Shiraki, 1996; Kurokawa, Kumeda, Yamamura, Kamiyama, & Shiraki, 1998). We have determined two steps of antipyretic action by cinnamyl compounds and NSAIDs in the fever cascade in influenza infection.

Studies of the efficacy of favipiravir on influenza in animals have been performed in our biosafety level 3 laboratory, and sterile and pyrogen-free distilled water for injections was used for drinking to ensure traceability. As we have experience in conducting a pharmacological study for a famciclovir approval application in Japan, the efficacy and validation studies of favipiravir were conducted in mice and ferrets using the influenza infection system described above in our laboratory.

2.4. Efficacy of favipiravir in influenza-infected animals

Since our laboratory had established an influenza infection model to investigate the cytokine cascade that induces fever in influenza virus-infected animals, we performed a virological analysis by examining the effects of crude drugs on the pathogenesis and cytokine levels, as well as the antiviral activity (Kurokawa et al., 2002; Kurokawa, Kumeda, Yamamura, Kamiyama, & Shiraki, 1998; Tsurita et al., 2001). Our animal model for influenza was used to study the efficacy of favipiravir in the influenza-infected animals.

As favipiravir was effective in cell culture, its effectiveness in animals infected with influenza virus must be confirmed. We first observed the prevention of a lethal influenza infection. Oral administration of favipiravir was significantly effective in alleviating influenza infection

Table 1
Therapeutic activity of favipiravir in human RNA virus infections or in animal models of human RNA virus infections. (Abdelnabi et al., 2018; Arias et al., 2014; Bixler et al., 2018; Caroline et al., 2014; Escribano-Romero et al., 1994; Gowen et al., 2013; Gowen, Westover, Miao, et al., 2017; Gowen, Westover, Sefing, et al., 2017; Gowen et al., 2007; Gowen et al., 2010; Hawman et al., 2018; Jochmans et al., 2016; Julander, Shafer et al., 2009; Julander, Smee et al., 2009; Mendenhall et al., 2011; Morrey et al., 2008; Oestereich et al., 2016; Oestereich, Rieger, et al., 2014; Safronetz et al., 2013; Safronetz et al., 2015; Scharton et al., 2014; Tani et al., 2016; Westover et al., 2016; Yamada et al., 2015; Zhu et al., 2018).

Efficacy shown in animal infection models and human use	Mortality (%)	References
Ebola virus infection	50% [a], 25 to 90% [a]	(Bai, et al., 2016; Bixler, et al., 2018; Jacobs, et al., 2015; Oestereich, Ludtke, et al., 2014; Sissoko, et al., 2016; Smither, et al., 2014)
Severe fever with thrombocytopenia syndrome (SFTS) caused by a virus infection	12–30%[b]	(Gowen, Westover, Miao, et al., 2017a; Tani, et al., 2016; Yasukawa, 2016)
Severe influenza		(Cao, 2018; Wang et al., 2019)
Lassa virus infection	1%[a], severe cases 15% [a]	(Gowen, Westover, Sefing, et al., 2017b; Mendenhall, et al., 2011; Oestereich, et al., 2016; Raabe, et al., 2017; Safronetz, et al., 2015)
Rabies	Approximately 100% [a, b]	(Baker, 2017; Yamada, Noguchi, Komeno, Furuta, & Nishizono, 2015)
Norovirus infection	50,000 deaths/year [b]	(Arias, Thorne, & Goodfellow, 2014; Ruis, et al., 2018)
Avian influenza A(H5N1)	52.8% (Sep 9, 2019) [a]	Favipiravir is effective against influenza in animals and humans and has been approved as an anti-influenza drug in Japan. Its indications are for novel influenza strains. Favipiravir has not been used in human avian influenza cases.
Avian influenza A(H7N9)	39.2% [a]	

Therapeutic efficacy of favipiravir in animal models of human RNA virus infections

Efficacy shown in animal infection models	Mortality (%)	References
Crimean-Congo hemorrhagic fever	3-30% [a]	(Hawman, et al., 2018; Oestereich, Rieger, et al., 2014)
Western equine encephalitis	3-4, 8-15%[d]	(Julander, Smee, Morrey, & Furuta, 2009b)
Marburg virus infection	50% [a], 24% to 88% [a]	(Bixler, et al., 2018; Zhu, et al., 2018)
Argentine hemorrhagic fever virus infection	15-30%[e]	(Gowen, et al., 2013; Gowen, Westover, Sefing, et al., 2017b; Mendenhall, et al., 2011; Westover, et al., 2016)
Rift Valley fever virus infection	1%[a], hemorrhagic form 50% [a]	(Caroline, et al., 2014; Gowen, et al., 2007; Scharton, et al., 2014)
Yellow fever virus infection	7 to 65.2%[c] 30,000 deaths/year [c]	(Gowen, et al., 2010; Julander, Shafer, Smee, Morrey, & Furuta, 2009a)
Hemorrhagic fever with renal syndrome	5-15%[b]	(Safronetz, et al., 2013)
Hantavirus pulmonary syndrome	18.6% to 40%[a], 38%[b]	
Chikungunya virus infection	0.012 to 4.9%[f]	(Abdelnabi, Jochmans, Verbeken, Neyts, & Delang, 2018)
West Nile fever	13%[g]	(Morrey, et al., 2008; Escribano-Romero, Jimenez de Oya, Domingo, & Saiz, 2017)
Metapneumovirus		(Jochmans, et al., 2016)

References where favipiravir has been used in humans are underlined.
The mortality rate is derived from [a]the World Health Organization (WHO), [b]CDC, [c]Pan American Health Organization (PAHO) & WHO, [d]Western equine encephalitis (Spickler, 2017), [e]Argentine hemorrhagic fever virus infection (Enria & Maiztegui, 1994), [f]Chikungunya (Cardona-Ospina, Henao-SanMartin, Paniz-Mondolfi, & Rodriguez-Morales, 2015; Renault, Josseran, & Pierre, 2008), and [g]West Nile fever (Philpott et al., 2019).

in mice (Furuta et al., 2002), and its efficacy was characterized under various conditions. We are convinced that favipiravir will be developed as an influenza drug for humans after the confirmation of its therapeutic activity in two species of animals, mice and ferrets, infected with influenza virus.

Fig. 3 shows one of the comparisons of efficacy between favipiravir and oseltamivir in the influenza virus infection model established with a high titer of virus in a series of experiments, and the better efficacy of favipiravir compared with oseltamivir was observed in an animal with a severe lethal influenza infection with a high viral load (Takahashi et al., 2003). Favipiravir and oseltamivir show similar efficacy in low-dose infections, but the efficacy of favipiravir as an influenza drug is clearly increased compared with oseltamivir in high-titer virus infections (Fig. 3). Mice in the control group die on day 3 after a high-dose infection, and an oseltamivir treatment prolongs the survival period for three days but does not prevent death. The favipiravir treatment cures lethal infection, and all mice survive. Favipiravir exhibits better efficacy than oseltamivir after a delayed administration beginning 1, 24, 48, or 72 h after infection with seasonal influenza A(H1N1) (Takahashi et al., 2003).

Mice infected with a low titer represent nonlethal infection models and are considered to correspond to human seasonal infection models. In this model, oseltamivir and favipiravir are equally effective and do not differ in efficacy despite the difference in their mechanisms of action. A high-titer infection represents a lethal infection model and is considered to correspond to a novel influenza infection in humans. Although oseltamivir is ineffective in this model, favipiravir effectively improves survival in all cases, indicating the importance of administering a drug with a mechanism of action that reduces viral load.

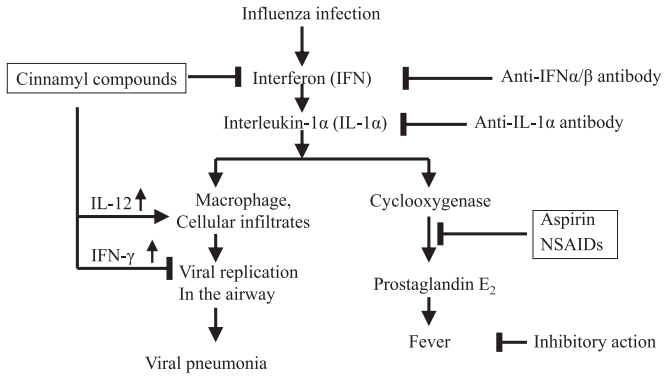

Fig. 2. Cascade of fever induction by influenza and antipyretic action of cinnamyl compounds and NSAIDs. Influenza infection induces interferon (IFN) production that subsequently induces interleukin (IL)-1 production to act on the hypothalamus. Next, cyclooxygenase is expressed to induce prostaglandin (PG)E_2 production and generate fever. Cinnamyl compounds from medicinal herbs, anti-IFN antibody, anti-IL-1 antibody, and nonsteroidal anti-inflammatory drugs (NSAIDs) (aspirin) act at each step of this process. Cinnamyl compounds and NSAIDs inhibit the induction of IFN and cyclooxygenase activity, respectively, in the fever cascade to work as antipyretics in influenza. Regarding pneumonia, IL-12 and IFN-γ production are induced in the bronchoalveolar fluid (BALF) on the second and third day of infection, respectively, and an increase in IL-12 levels reduces the viral load in the BALF and the area of pneumonia throughout the lungs.

TNF-α production is induced through recognition of the single-strand RNA or double-strand RNA of the influenza virus genome by Toll-like receptor-7/8 (Yang & Chen, 2012) or 3 (Diebold et al., 2003; Guillot et al., 2005; Poux et al., 2019; Wong et al., 2009), respectively, in infected cells. Influenza infection induces TNF-α production in mouse macrophage-derived P388D1 cells, and the suppressive effects of favipiravir and oseltamivir were compared in this P388D1 cell-based system. Favipiravir significantly suppresses the production of TNF-α in influenza virus-infected P388D1 cells compared with the active form of oseltamivir (Tanaka et al., 2017). TNF-α appears first and disappears first in P338D1 cells among TNF-α, IL-1, and IL-6 (Kurokawa et al., 2003), and we observed a significant reduction in the levels of TNF-α in P388D1 cells and influenza-infected mice treated with favipiravir. The antiviral activity of favipiravir has been attributed to a decrease in the pulmonary viral load and TNF-α level in the airways of influenza virus-infected mice compared with oseltamivir, and the reduction in the viral RNA load induced by favipiravir might have resulted in a reduction in TNF-α production and alleviation of lung pathogenesis (Damjanovic et al., 2011; Tanaka et al., 2017). Based on these findings, the intracellular viral RNA is less recognized by intracellular Toll-like receptors and results in a decrease in the production of the inflammatory cytokine TNF-α. This mechanism may also contribute to the enhanced effects of favipiravir treatment on alleviating influenza infection compared with oseltamivir treatment.

Favipiravir displays superior properties to oseltamivir in mouse models, and we confirmed its efficacy in influenza-infected mice and ferrets. The confirmation of the efficacy of this drug in two species of animals convinced us of the efficacy in humans and its possible use as the standard treatment of choice for influenza in the future.

3. Mechanisms of action of current anti-influenza drugs and their resistant viruses

3.1. Mechanisms of action of current anti-influenza drugs

Fig. 4 shows the influenza virus growth cycle and the sites of action of anti-influenza drugs. The viral hemagglutinin binds to sialic acid on the cell surface, and the viral particle is incorporated into the endosome. When the endosome is acidified and the pH decreases to a value of 5, which is characteristic of the late endosome, the structure of the hemagglutinin changes through a process mediated by the Matrix-2 (M2) ion channel. Then, the endosomal membrane and the viral envelope fuse, and the viral genome in the viral particle is released into the cytoplasm (uncoating). The viral genome and RdRp complex are transported to the nucleus where the transcription (replication) of viral RNA synthesis begins. The synthesized RNA does not contain the Cap structure required for mRNA, and the Cap structure from the host mRNA is excised and transferred to the viral RNA by the Cap-dependent endonuclease of viral RdRp complex, resulting in the formation of the viral mRNA (Cap-snatching). After the viral mRNA is produced, the viral protein is translated. The viral proteins and RdRp-RNA complex form viral particles that subsequently bud from the cell membrane. Hemagglutinin on viral particles budded from the cell surface binds to the sialic acid on the surface of the infected cell, and the viral particles are released from the infected cell through the cleavage of sialic acid by viral neuraminidase (NA) for the next round of infection.

Amantadine inhibits the uncoating of influenza A in late endosomes, and viral RNA does not replicate in the infected cells. Amantadine inhibits only influenza A and not B. However, influenza A virus becomes

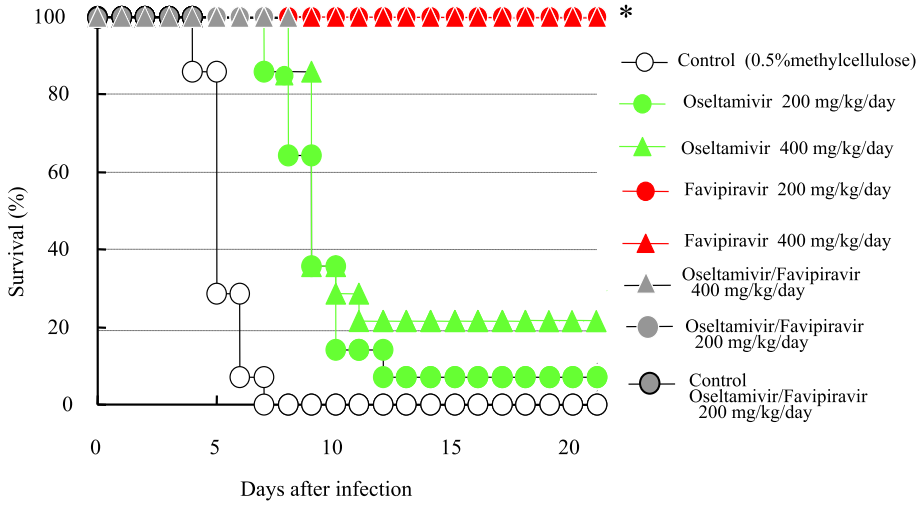

Fig. 3. Comparison of the efficacy of favipiravir and oseltamivir in lethal influenza virus infection. Mice were infected with 3×10^4 plaque forming units of influenza A/PR/8/34 virus and were orally administered favipiravir and oseltamivir at doses of 200 and 400 mg/kg/day for 5 days beginning at 1 h post-infection ($n = 14$). The results presented in this figure were obtained from a representative experiment. *$P < .01$ compared to 0.5% methylcellulose solution-treated controls and oseltamivir-treated groups (log-rank test). The authors obtained permission from the Antiviral Chemistry and Chemotherapy to reuse this figure (Takahashi et al., 2003).

resistant, even in an epidemic occurring in a closed facility, and the current influenza A viruses are not susceptible to amantadine.

Favipiravir inhibits viral RNA synthesis, and therefore viral RNA is not produced in the infected cells.

The NA inhibitors (NAIs) zanamivir, oseltamivir, peramivir, and laminamivir inhibit the NA activity of viral particles on the surface of the infected cell and the release of viral particles from the cell surface to other cells for the next round of infection. NAIs result in the accumulation of viral particles on the cell surface and inhibit the spread of virus infection. NAIs are currently the main choice of anti-influenza drugs.

Baloxavir marboxil (baloxavir, Xofluza®) is a selective inhibitor of influenza Cap-dependent endonuclease of the viral RdRp complex. Baloxavir inhibits mRNA synthesis and subsequent viral protein synthesis, but genomic RNA without the Cap structure is synthesized by the incorporated RdRp-viral RNA complex in the presence of baloxavir.

3.2. A reservoir of resistant virus is generated in influenza virus-infected cells treated with anti-influenza drugs other than favipiravir

Favipiravir inhibits viral RNA synthesis as a chain terminator. This inhibition of genomic RNA synthesis is the most important difference from other anti-influenza drugs, which substantially forms the pool of genomic RNA containing drug-resistant mutants synthesized by viral RdRp with low fidelity from the RdRp-viral RNA complex. The anti-influenza drugs NAIs and baloxavir inhibit the spread of infection but allow the synthesis of genomic RNA, and the genomic RNA pool is important from the perspective of the generation of resistant viruses as a source of drug-resistant viruses.

The spontaneous mutation rate of the influenza virus RdRp complex is approximately 1×10^4. Titers of influenza virus in culture reach approximately 10^8 plaque forming units/mL in 5 mL in a 25 cm^2 flask, and their genomes contain approximately 13,600 bases, indicating a probability of 10^4 alterations per nucleotide in the genome. Therefore, all types of mutated genomes should be synthesized and present as genomic RNA, even in cells treated with anti-influenza drugs.

There is a high probability that the resistant virus is already present in the virus that grew rapidly and in large quantities before the start of treatment. In addition, the viral RNA formed in the cells in patients treated with anti-influenza drugs serves as a source of drug-resistant viruses. Subsequently, drug-resistant influenza viruses should appear more readily from the mutant genomic RNA pool during drug treatment.

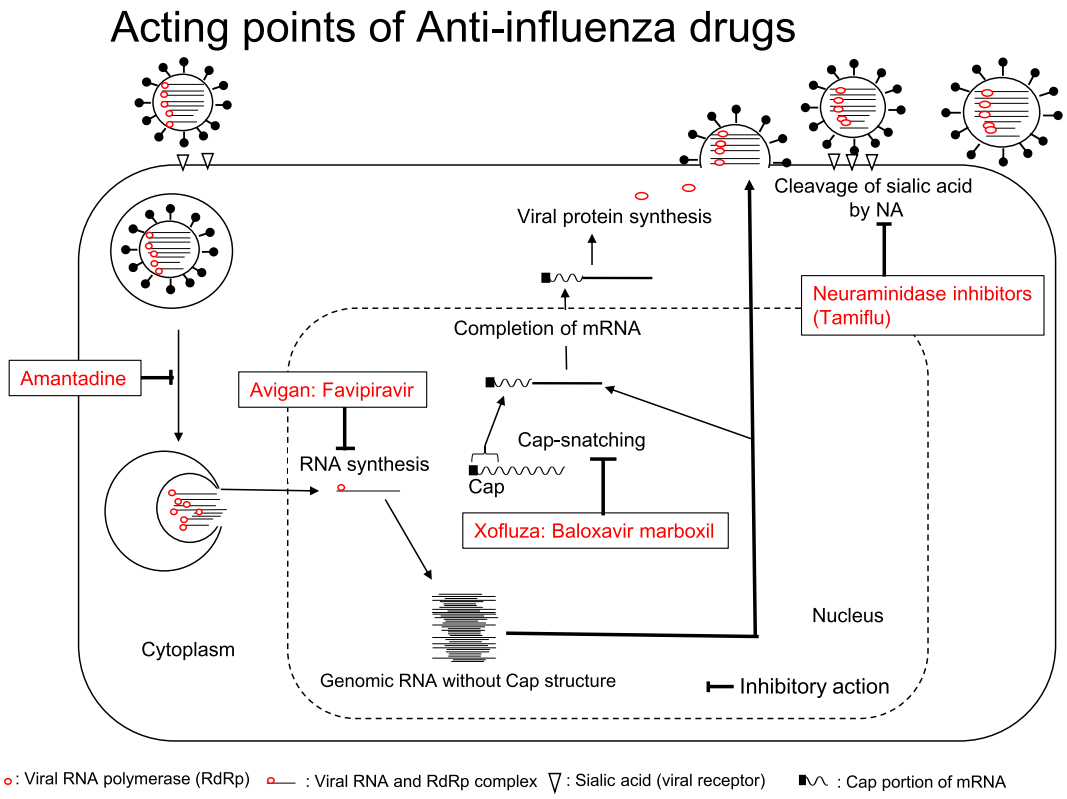

Fig. 4. Replication cycle of influenza and sites of action of anti-influenza drugs. Influenza virus hemagglutinin (HA) binds to its receptor, sialic acid on the cell surface and is taken up into endosomes by endocytosis. Endosomes are gradually acidified to produce late endosomes, viral matrix-2 (M2) ion channels acidify viral particles, and the structure of trimeric HA molecules changes and shows membrane fusion activity. The endosomal membrane and envelope are then fused, and the genomic RNA and RdRp complex in the virus particle are released into the cytoplasm (uncoating) and transported to the nucleus through the cytoplasm. The transcription (replication) of genomic RNA by the RdRp complex occurs in the nucleus, and the genomic RNA is abundantly produced. Genomic RNA lacks the Cap structure required for mRNA function, and the Cap portion of the host mRNA is removed by a Cap-dependent endonuclease. Then, the Cap portion is coupled to genomic RNA, and viral mRNA synthesis is complete (Cap-snatching). Viral proteins are synthesized from the mRNA, and viral proteins and genomic RNA are transported to the cell surface and bud from the membrane to form viral particles. Since the budding virus is bound to the sialic acid on the infected cell surface via the HA protein of virus particles, it is unable to leave the infected cell and infect new cells. For this reason, the sialic acid-HA bond is cleaved by neuraminidase (NA) on the surface of the virus particle, and the virus particle is released from the surface of the infected cell and proceeds to the next round of infection. Amantadine blocks uncoating by inhibiting acidification mediated by the M2 protein in the virus particle in late endosome, and thus the infection is unable to be completed. Favipiravir inhibits viral RNA synthesis by terminating chain elongation at its incorporated site, and no new RNA is generated in the cell. Baloxavir marboxil prevents Cap-snatching, and the viral mRNA is not produced, resulting in a failure to produce viral proteins and infectious viruses. Genomic RNA is synthesized and remains in the cell. NA inhibitors block the cleavage of the sialic acid-HA bond in the virus on the surface of infected cells and prevent the spread of the viral infection. NA inhibitors allow genomic RNA synthesis. Favipiravir inhibits viral RNA synthesis, while baloxavir and NAIs allow viral RNA synthesis. Although viral spread is inhibited by baloxavir and NAIs, viral RNA is synthesized, and the pool of genomic RNA serves as a rich source of resistant viruses.

4. Seasonal influenza and pandemic influenza

4.1. Seasonal influenza

The replication cycle of influenza viruses is approximately 6 h from entry to the production of new virus, and robust growth begins after infection at a rate of four replication cycles per day. The course of influenza virus infection was analyzed in 56 different studies with 1280 healthy participants after an experimental influenza virus infection (Carrat et al., 2008). The A(H1N1) and A(H3N2) infections resulted in a substantial increase in viral shedding on the first day after experimental influenza virus infection, and they reached their maximum values on the second day. Fever was reported in 34.9% of infected individuals. Systemic symptoms (fever, muscle aches, fatigue, and headache) peaked earlier, by day 2 after inoculation, and resolved faster than respiratory or nasal symptoms. The presence of a preexisting antibody modified the mean duration of illness of 4.4 days in participants with pre-hemagglutinin inhibition titers of ≤1/8 compared with 1.0 day in participants with pre-hemagglutinin inhibition titers of >1/8 after the inoculation of a wild-type A(H1N1) virus (Doyle et al., 1994). Influenza causes dry cough (90%), fever (83.8%), and headache (82.5%), and immunocompromised patients exhibit a significantly longer length of illness with delayed virus clearance (Memoli et al., 2014). The mean durations of viral shedding in immunocompromised and non-immunocompromised patients are 19.4 and 6.38 days with median values of 8.0 and 5.0 days, respectively, indicating that viral replication persists for a week after the disappearance of major symptoms. Preexisting immunity and immunocompetence are important in modifying the severity of symptoms of influenza.

4.2. Difference in influenza pathogenesis between seasonal and novel influenza

Seasonal influenza strains that are adapted to humans include A (H1N1), A(H3N2), and B influenza and cause seasonal epidemics of influenza among humans every year. Seasonal influenza viruses mainly infect the epithelium of the upper respiratory tract because their hemagglutinins bind to their receptor, sialic acid linked to galactose by an alpha-2,6 linkage that is distributed in the upper respiratory tract. Although these strains cause epidemics every year, the susceptibility and severity of illness are modified by the degree of immunity of the host as described above.

Novel influenza is derived from avian influenza A and is a source of concern as a cause of an influenza pandemic. Avian influenza is divided into highly pathogenic avian influenza and low-pathogenic avian influenza based on the molecular characteristics of the virus and their abilities to cause disease and mortality in chickens. Both low-pathogenic avian influenza and highly pathogenic avian influenza viruses have caused severe and lethal infections in humans (CDC, 2017).

Since the novel influenza strain is transmitted in the original host bird, the avian receptor is sialic acid linked to galactose by an alpha-2,3 linkage, and this receptor is distributed in respiratory bronchioles and alveolar epithelial cells in humans (Shinya et al., 2006). Although the name of the influenza virus is common to both seasonal influenza and novel influenza, a new subtype of influenza causes severe influenza in humans because it is a completely new subtype of virus, and humans have neither a history of infection nor immunity. Seasonal influenza mainly infects the upper respiratory tract, while novel influenza causes pneumonia, mainly due to an infection of the pulmonary epithelium. Furthermore, unlike the presence of a certain level of immunity to seasonal influenza, immunity does not exist for novel influenza. Therefore, the severity of the infection appears to be caused by a prolonged virus growth period and the affinity for the pulmonary epithelium (Shinya et al., 2006). As described above, novel influenza causes severe diseases, particularly pneumonia, compared to seasonal influenza, and the mortality rate is 53.5% (483/903) for A(H5N1) (Lai et al., 2016) and 34% (47/137) for A(H7N9) (Li et al., 2014).

4.3. Oseltamivir-resistant influenza in seasonal influenza

Oseltamivir, an NAI, has been the treatment of choice for influenza infection. The emergence of a resistant virus (after day 1) was detected in 43/1207 (3.56%) oseltamivir-treated influenza A-infected patients, with a higher frequency observed in 1- to 5-year-olds (11.8%) than in children aged >5 years (1.4%), and viral clearance occurred in 8–10 days (Lina et al., 2018). The overall incidence of an oseltamivir-resistant virus was 10 of 182 (5.5%) (Whitley et al., 2001) and 9 of 50 (18%) (Kiso et al., 2004) oseltamivir-treated children. Oseltamivir-resistant influenza appears and becomes dominant during treatment.

Another concern is the prevalence of oseltamivir-resistant strains that was observed during the 2008 to 2009 season. Oseltamivir-resistant seasonal A(H1N1) viruses possessing an NA H275Y substitution spread globally, 12.3% (142/1155) in the US (Dharan et al., 2009), 64% in South Africa, Oceania and SE Asia (Hurt et al., 2009), and 20.1% in Europe. In particular, a prevalence of 67% (184/272) was observed in Norway, with a gradual increase observed in Europe from approximately 0% in week 19 to 56% in week 40 (Meijer et al., 2009). Thus, once the oseltamivir-resistant virus has adapted to humans, it will become or replace an epidemic virus worldwide.

Single-dose baloxavir is superior to the placebo in alleviating influenza symptoms and to both oseltamivir and placebo in reducing the viral load 1 day after initiation in patients with uncomplicated influenza (Hayden et al., 2018). The emergence of baloxavir-resistant mutants with PA/I38X substitutions occurred in 2.2% and 9.7% of baloxavir recipients in the phase 2 and phase 3 trials, respectively. Patients with a substitution at position I38 in the viral polymerase acidic protein (PA/I38X) of the baloxavir-resistant virus exhibited sustained alleviation and virus clearance, and baloxavir-resistant viruses were not cross-resistant to favipiravir and oseltamivir (Omoto et al., 2018). Baloxavir-resistant viruses were identified after 3–9 days in 9.7% (36/370) of baloxavir-treated immunocompetent adults and adolescents (Uehara et al., 2020). Baloxavir-resistant viruses emerged in 18 of 77 (23.4%) patients. Emergence was associated with a prolonged detection of the infectious virus (median time, 180.0 h) and time to illness (median, 79.6 vs 42.8 h in patients without PA/I38T/M-substituted viruses) (Hirotsu et al., 2019). Baloxavir-resistant influenza viruses cause new human-to-human infections and have the ability to spread infections (Takashita et al., 2019).

Oseltamivir- and baloxavir-resistant mutants emerge and become dominant viruses during their treatment, and the resistant viruses are transmitted to other hosts. Thus, these resistant viruses may become dominant pandemic viruses. In contrast, favipiravir treatment does not change the susceptibility of 57 pairs of viruses to favipiravir before and after treatment (Takashita et al., 2016). Although the number of cases is limited, 57 pairs might be sufficient to detect a virus resistant to the current anti-influenza drug, suggesting that favipiravir-resistant mutants will not appear. Favipiravir alone may maintain its efficacy from the beginning to the end of an influenza pandemic without replacement by resistant strains for which the effectiveness of drugs is reduced during treatment or during the pandemic.

4.4. Effectiveness of favipiravir among anti-influenza virus drugs used to treat pandemics of novel influenza

An influenza pandemic is a global outbreak of a novel influenza A virus. Pandemics occur when novel influenza A viruses emerge that are able to infect people easily and spread from person to person in an efficient and sustained manner. Two decades have elapsed since an

avian influenza case was reported in Hong Kong in 1997, and a decade has elapsed since the most recent influenza pandemic that occurred in 2009. Sporadic cases of A(H5N1) and A(H7N9) influenza infections have been accumulating, and we must prepare for these strains or other novel influenza strains that might progress to an influenza pandemic and spread globally, such as A(H1N1)pdm09. Influenza pandemics can cause severe pneumonia, generate a drug-resistant virus, and render antiviral drugs ineffective during prolonged viral growth in patients treated with oseltamivir or baloxavir. Although drug-resistant viruses may have a reduced ability to grow, the use of drugs in many patients continues the selection pressure to ensure that resistant viruses become dominant. Drug selection continues, and the resistant strain becomes dominant during pandemics because drug-resistant strains are selected in many patients treated with the same anti-influenza drug. Regarding a resistant virus with good replication capability, oseltamivir-resistant A(H1N1) viruses possessing the NA H275Y substitution spread globally during the 2008 to 2009 season. Thus, we have experience with a global pandemic of an oseltamivir-resistant strain. Favipiravir maintains the same efficacy from the first to the last patient of a pandemic because its resistant strain does not appear or does not replace the original strain.

Favipiravir is significantly more effective in treating mice with severe influenza infections characterized by a high viral load than oseltamivir (Takahashi et al., 2003), and a favipiravir-resistant virus does not emerge during treatment. This outstanding feature of favipiravir as an anti-influenza drug has been exploited in treating patients with severe influenza in combination with oseltamivir in China (Cao, 2018; Wang et al., 2019). Favipiravir and oseltamivir combination therapy accelerated clinical recovery compared to oseltamivir monotherapy in severe influenza. The dose of favipiravir used in the study mentioned above was 1600 mg twice a day on day 1 followed by 600 mg twice a day for 9 days, and the approved favipiravir dose in Japan is 1600 mg twice a day on day 1 followed by 600 mg twice a day for 4 days. The viral replication period is 6 days or longer for seasonal influenza (Lina et al., 2018; Memoli et al., 2014). When drug administration is stopped during the virus replication period or when resistant strains appear, virus replication and fever relapse. Thus, 10 days of administration may be required for severe influenza or novel influenza.

4.5. Pandemics of the 20th century

According to the WHO (EuroWHO, 2019), "Three influenza pandemics occurred at intervals of several decades during the 20th century, the most severe of which was the so-called 'Spanish Flu' (caused by an A (H1N1) virus), estimated to have caused 20–50 million deaths in 1918–1919. Milder pandemics occurred subsequently in 1957–1958 (the 'Asian Flu' caused by an A(H2N2) virus) and in 1968 (the 'Hong Kong Flu' caused by an A(H3N2) virus), which were estimated to have caused 1–4 million deaths each. While most cases of pandemic H1N1 were mild, globally it is estimated that the 2009 pandemic caused between 100,000–400,000 deaths in the first year alone." Elderly individuals had immunity to pandemic A(H1N1)pdm09, and although people developed a milder form of influenza, it spread quickly throughout the world. Once an avian influenza outbreak occurs, more people are infected than in the pandemic of 2009, with similar or faster global spread. Predictions of whether the next pandemic will occur are difficult to determine, but researchers are currently concerned about the A(H5N1) and A(H7N9) strains with high lethality. When these strains adapt to humans and become pandemic, their pathogenicity may be milder, similar to previous pandemics. If an influenza pandemic causes severe disease, it may cause substantial damage to human health and social dysfunction. The need for pandemic countermeasures is an important consideration.

5. A broad spectrum of anti-RNA virus drugs: efficacy in animal models and human infection

5.1. The broad spectrum of the anti-RNA virus activity of favipiravir

Favipiravir has a broad spectrum of activity toward RNA viruses, including life-threatening RNA viruses, and exhibits efficacy in animal models of these infections. Table 1 summarizes the efficacy of favipiravir in animal models of human infections. Based on the efficacy in animal models, it has been used to treat humans with diseases such as Ebola virus infection (Bai et al., 2016; Jacobs et al., 2015; Sissoko et al., 2016), Lassa fever (Raabe et al., 2017), norovirus (Ruis et al., 2018), and rabies (Baker, 2017). Notably, as a broad spectrum anti-RNA virus drug, favipiravir has been submitted for additional indications for SFTS in Japan, based on clinical trials (Yasukawa, 2016).

The broad spectrum of activity of favipiravir toward RNA viruses has been reviewed, including anti-RNA virus activity in vitro and in vivo in animal models (Delang, Abdelnabi, & Neyts, 2018; Furuta et al., 2009; Furuta et al., 2013; Furuta, Komeno, & Nakamura, 2017).

5.2. Ebola virus infection

Concerning Ebola virus infection, the efficacy of favipiravir in post-exposure prophylaxis (PEP) was shown in a mouse model (Smither et al., 2014) and a therapeutic mouse model of Ebola virus disease (Oestereich, Ludtke, et al., 2014). Treatment with favipiravir from 6 to 13 days after lethal infection with Ebola virus cured all mice when the treatment was started at the initiation of liver damage (elevation of AST and ALT) and virus detection in blood. However, the administration of favipiravir from 8 to 14 days prolonged survival, but four of five mice died when the liver damage and viremia advanced. Early treatment with favipiravir was effective, but when the disease is advanced, including liver damage, the efficacy in prolonging survival is limited to one of five mice, indicating that treatment should be started before liver damage progresses to irreversible levels. Thus, favipiravir may be able to cure an Ebola virus infection in the early phase of infection, but the curative activity of favipiravir may be limited in patients with an advanced infection.

The dose of favipiravir used to treat a human with an Ebola virus infection is 6000 mg on the first day and 2400 mg/day on days 1–9 for a total of 27,600 mg when administered for both PEP and treatment. Four of eight health-care workers, including two with maximum risk exposures from penetrating injuries with freshly used hollow-bore needles, were administered PEP with favipiravir alone or favipiravir with other anti-Ebola agents and did not develop Ebola virus disease (Jacobs et al., 2015). Although the needle stick had not been confirmed to result in infection, the probability of infection was high based on previous observations, and PEP was considered effective, as observed in PEP using a mouse model (Smither et al., 2014).

An Ebola study conducted in Guinea included 126 patients, and 111 were analyzed and compared with 540 patients as a historical control group. Favipiravir treatment reduced the mortality rate in the low viral load group to 33% compared with the historical control group that was not treated with favipiravir, but this reduction in the mortality rate was not statistically significant (Sissoko et al., 2016). An Ebola study conducted in Sierra Leone included 39 favipiravir-treated patients and 85 historical control patients. The overall survival rate in the favipiravir treatment group was higher than the control group (56.4% [22/39] vs 35.3% [30/85]; $P = .027$). (Bai et al., 2016). During the 2014 epidemic of Ebola virus infection in West Africa, patients who were treated without favipiravir in the period before preparation for favipiravir treatment were classified as historical controls, and the therapeutic efficacy of favipiravir was compared between the patient group treated with favipiravir and the historical patient group in the two clinical trials described above. Randomized placebo-controlled trials are desirable for confirming therapeutic effects in clinical trials, but the placebo group

has an ethical problem of not being able to receive an effective drug that can recover fatal infections in animal models. The studies were conducted to compare the effectiveness of favipiravir treatment between patients who were not treated in the period prior to the start of drug administration as the historical control patient group and those who were treated with favipiravir after the clinical study was ready.

5.3. Effectiveness of favipiravir in other human RNA infections

The number of patients is limited, and favipiravir has been used as an emergency or compassionate treatment for Lassa fever, norovirus, and rabies cases. This review focuses on human administration in terms of the broad spectrum of RNA virus activity. Therefore, favipiravir has been positioned as a valuable anti-influenza drug and a broad spectrum anti-RNA drug, as listed in Table 1. Favipiravir has been used in human therapy for Ebola hemorrhagic fever (Bai et al., 2016; Jacobs et al., 2015; Sissoko et al., 2016), Lassa fever (Raabe et al., 2017), norovirus (Ruis et al., 2018), rabies (Baker, 2017), and SFTS (Yasukawa, 2016). Some treatments were used in emergencies, and some were used in the setting of a clinical trial. In addition, combination therapy of favipiravir with the other existing therapies is also an option and favipiravir and oseltamivir combination therapy showed accelerated clinical recovery compared to oseltamivir monotherapy in severe influenza in China (Cao, 2018; Wang et al., 2019).

5.4. The dose of favipiravir for influenza and Ebola virus infection

The antiviral activity (EC_{50}) of favipiravir against influenza and Ebola viruses is different from the range of 0.014–0.55 μg/mL in medium without adenosine and guanosine and 10 μg/mL, respectively (Furuta et al., 2002; Oestereich et al., 2014). Addition of 10 x EC_{50} (63.7 μM) of adenine, guanine, adenosine, guanosine, and inosine in the assay medium abolishes the anti-influenza virus activity of favipiravir (Furuta et al., 2005). The intracellular concentration of ATP is 1–9 mM in various tissues (Beis & Newsholme, 1975), suggesting favipiravir may need a higher concentration in vivo. Pharmacokinetic values of favipiravir for the maximum drug concentration (*C*max), the area under the curve (*AUC*), the maximum drug concentration time (*T*max), and the half-life period ($t1/2$) are 65 μg/mL, 450–550 μg·hr/mL, 1 h, and 4.8–5.6 h, respectively (Avigan Tablets 200 mg package insert).

The approved favipiravir dose for influenza in Japan is 1600 mg twice a day on day 1 followed by 600 mg twice a day for 4 days, and the dose for Ebloa virus infection is 6000 mg on the first day and 2400 mg/day on days 1–9. The antiviral concentration of favipiravir in influenza is achieved in the lung epithelium by diffusion from the blood, and in the case of Ebola virus infection, direct perfusion of blood reaches target tissues, vascular endothelial cells, hematopoietic cells, and hepatocytes. Differences in the two factors between antiviral concentrations and target cells do not appear to be significantly reflected in dosage. Intravenous preparations are being prepared to improve oral administration of favipiravir.

6. Mechanism of action of favipiravir as a chain terminator

6.1. Inhibition of elongation of RNA synthesis as a chain terminator

Favipiravir inhibits viral RdRp by terminating elongation at the incorporation site as a chain terminator (Jin, Smith, Rajwanshi, Kim, & Deval, 2013; Sangawa et al., 2013). Favipiravir functions as a purine analogue, as expected from the chemical structure, and it is incorporated instead of guanosine and adenosine. Favipiravir terminates elongation after the incorporation of a single favipiravir molecule (Sangawa et al., 2013) and after the incorporation of two consecutive favipiravir molecules (Jin et al., 2013), and the synthesis of this complementary viral RNA strand cannot be completed. In contrast, the anti-RNA virus drug ribavirin is incorporated into the replicating strand, which further elongates and accumulates mismatched nucleotides at the incorporated sites. Base pairing with ribavirin in the complementary strand during replication, transcription, and translation of the RNA strand causes mismatched base pairing, the production of nonfunctional proteins, and a loss of viral infectivity. Accumulated mutations (mismatched nucleotides) cause the replicated viruses to lose their replicative capability, which is known as “lethal mutagenesis” (Vignuzzi, Stone, & Andino, 2005), as shown in Fig. 5. When the number of mutations is limited and infectious viruses with mutations in viral RdRp that affect ribavirin incorporation are produced, drug-resistant viruses can be selected in the presence of ribavirin.

6.2. Increased number of mutations in viral genomes in favipiravir-treated cultures

Favipiravir treatment increases the frequency of transition (Delang et al., 2014; Goldhill et al., 2019; Vanderlinden et al., 2016) and transversion (Baranovich et al., 2013) in viral genomes, and these mutations are hypothesized to be caused by favipiravir, resulting in lethal mutagenesis (Baranovich et al., 2013; Delang et al., 2014; Goldhill et al., 2019; Vanderlinden et al., 2016). We observed a transition of influenza virus and poliovirus in cultures treated with favipiravir but did not assess the frequency because we were unable to compare the transition rate with the proper antiviral agents with similar mechanisms as a control (Daikoku et al., 2017; Daikoku, Yoshida, Okuda, & Shiraki, 2014).

Ribavirin is known to cause lethal mutagenesis, as shown in Fig. 5. Ribavirin is incorporated into the elongating RNA strand, resulting in the production of multiple mismatches that lead to lethal mutagenesis (Vignuzzi et al., 2005). Favipiravir inhibits RNA synthesis through chain termination, and this inhibitory mechanism more consistently explains the observations reported than a mechanism similar to ribavirin, as described below.

Favipiravir-4-ribofuranosyl-5′-triphosphate (RTP) has a higher affinity for the RdRp of influenza virus than GTP and functions as a chain terminator (Jin et al., 2013; Sangawa et al., 2013). Favipiravir-RTP and ribavirin TP inhibit the RdRp activity in a dose-dependent manner, with 50% inhibitory concentrations (IC_{50}s) of 0.14 and 2.4 μM, respectively (Furuta et al., 2005). The RdRp activity was determined by measuring the incorporation of labeled GTP in the elongating RNA strand. Favipiravir-RTP has a higher affinity for RdRp than GTP, and when incorporated, favipiravir-RTP stops the elongation of the RNA strand in which it is incorporated. This termination prevents the incorporation of radioactivity, and thus favipiravir-RTP exhibits high inhibitory activity and a low IC_{50} (0.14 μM). On the other hand, ribavirin induces competitive inhibition with GTP, and incorporation results in mismatch mutations; the strand continues to elongate and incorporate labeled GTP without stopping at the incorporation site. Thus, ribavirin has a high IC_{50} value (2.4 μM) because its RNA strand is further elongated by incorporating radiolabeled GTP. Therefore, its ability to inhibit enzyme activity becomes weaker, and it displays a higher IC_{50} value than favipiravir. Furthermore, a marked decrease in the amount of the viral genome has been observed in favipiravir-treated cultures compared with ribavirin-treated cultures (Vanderlinden et al., 2016). Favipiravir is incorporated into the viral genome and terminates elongation, resulting in shorter genome sizes and the marked loss of the viral genome in favipiravir-treated cultures (Rocha-Pereira et al., 2012; Vanderlinden et al., 2016). These observations are consistently explained by the chain termination induced by favipiravir.

6.3. Mutation due to chain termination and proofreading activity by acyclovir

Proofreading activity mediated by enzymes such as the 3′-5′ exonuclease of herpesvirus DNA polymerase removes the terminal mismatched base and corrects the base pairing during the elongation

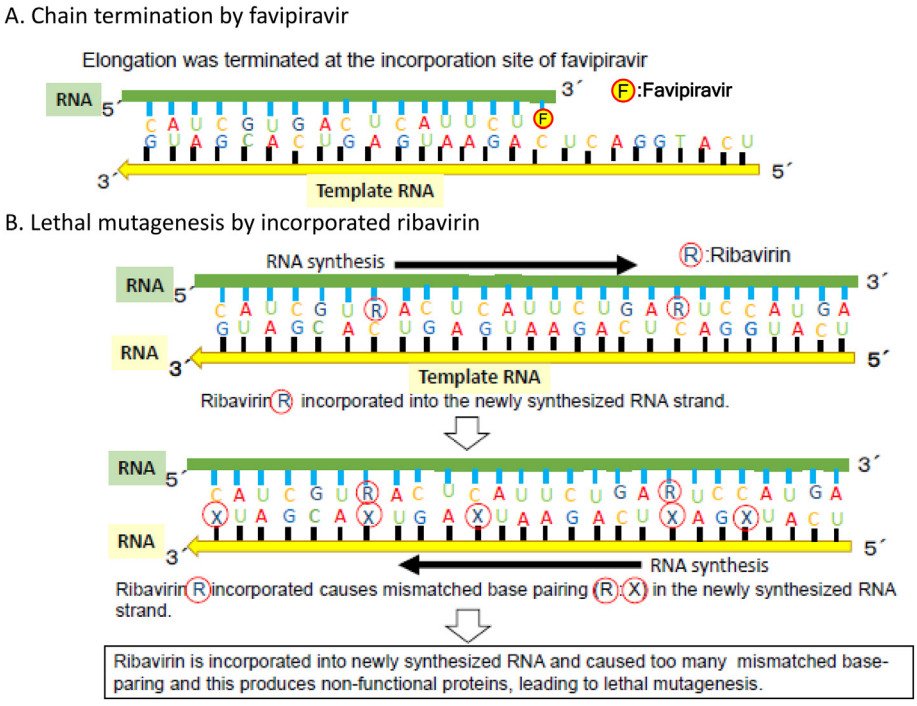

Fig. 5. Chain termination and lethal mutagenesis. A. Chain termination Favipiravir is converted to favipiravir-RTP and incorporated into the elongating RNA strand. Then, chain elongation stops at the site of favipiravir incorporation, and elongation does not proceed because favipiravir functions as a chain terminator. This RNA-favipiravir(−RdRp) complex will not be repaired by the proofreading enzyme and would be disposed of as unnecessary RNA, resulting in the extinction of the viral genome (Rocha-Pereira et al., 2012; Vanderlinden et al., 2016). B. Lethal mutagenesis Ribavirin is incorporated into the elongating RNA strand, and viral RdRp continues to elongate until completion. Next, the RNA strand with multiple ribavirin incorporation sites further incorporates ribavirin or serves as mRNA for viral protein synthesis. The incorporation of ribavirin in the viral RNA causes the mismatch of base pairs (transition mutation), and translation of this RNA causes mutations in the amino acid sequence of the protein, resulting in impaired function. Viral proteins with impaired function fail to replicate or produce infectious viral particles, and viral infection ceases. Thus, lethal mutagenesis terminates viral infection through a different mechanism than chain termination.

process. Viral RdRp lacks proofreading activity and is unable to complete the elongation step when favipiravir-RTP is incorporated as a chain terminator. Coronavirus has been reported to expresses a 3′-to-5′exoribonuclease and its proofreading function among RNA viruses (Smith, Blanc, Surdel, Vignuzzi, & Denison, 2013).

Acyclovir causes chain termination at the incorporated site during the elongation of herpes simplex virus and varicella-zoster virus DNA and prevents viral DNA synthesis. However, the incorporated acyclovir is removed by the proofreading activity of viral DNA polymerase, and viral DNA elongation continues. Therefore, the sequence containing the guanosine homopolymeric string (G-string) in the genome is the target of the incorporation and removal of acyclovir, and the incorporation of guanosine followed by repeated corrections by the proofreading machinery create a hot spot of mutations in G-strings of our laboratory and clinical acyclovir-resistant isolates (Akahoshi et al., 2017; Daikoku et al., 2016; Ida et al., 1999; Shimada et al., 2007; Talarico, Phelps, & Biron, 1993). Penciclovir (famciclovir) and ganciclovir (valganciclovir), which are guanosine analogues, function as an anti-herpes simplex virus and varicella-zoster virus agent and anti-cytomegalovirus agent, respectively, with mechanisms similar to acyclovir, but they do not induce mutations or have hot spots in the G-string. These compounds do not prevent elongation at the incorporation site but are incorporated, and elongation pauses at normal nucleotides after the incorporated site. Therefore, mutations associated with proofreading do not occur in the G-string, and this difference in the mode of chain termination and proofreading activity causes a lower mutation frequency in subjects treated with penciclovir and ganciclovir than in subjects treated with acyclovir.

If favipiravir induces mismatches as a mutagen, favipiravir should allow elongation after its incorporation into the elongating RNA strand and induce mismatches at the favipiravir-incorporated sites in the new complementary strand. This possibility is inconsistent with the mechanism of favipiravir as a chain terminator. Thus, the increased mutation rates observed in response to favipiravir treatment are unlikely to be due to the incorporation of favipiravir into viral RNA followed by elongation, similar to ribavirin or acyclovir (Fig. 5). Favipiravir is unlikely to induce mismatches upon its incorporation into the RNA strand and transitions in the influenza virus genome. The increased rate of transition mutations observed after favipiravir treatment is presumed to be due to the biased nucleotide pool induced by the increase in the level of favipiravir-RTP and the properties of viral RdRp.

6.4. RNA-dependent RNA polymerase (RdRp) of influenza virus causes mutations

A biased nucleotide pool is a major factor that promotes polymerase-induced mutation synthesis (Wheeler, Rajagopal, & Mathews, 2005). Influenza RdRp complex is composed of PB1, PB2, and PA and requires four nucleotides, ATP, GTP, CTP, and UTP, as substrates for RNA synthesis. Influenza RdRp incorporates mismatched nucleotides in a primer-extension-based misincorporation assay in the presence of completely biased nucleotide pools consisting of only three nucleotides instead of all four nucleotides (Aggarwal, Bradel-Tretheway, Takimoto, Dewhurst, & Kim, 2010). Even if one of the four nucleotides is missing, its absence is compensated by other nucleotides, and the RNA strand continues to elongate, causing mismatches. Therefore, even in the absence of a mutagen, mismatches should occur during RNA synthesis under biased nucleotide pool conditions as if the mutagen is present.

Influenza RdRp displays a significantly higher fidelity than human immunodeficiency virus-1 reverse transcriptase and T7 RNA polymerase and an equivalent or higher fidelity than murine leukemia virus reverse transcriptase (Aggarwal et al., 2010). The mutation frequency of influenza RdRp is 7.06×10^{-4} nucleotides in wild-type H3N2 virus

(Cheung et al., 2014) and >7.26×10^{-5} nucleotides deduced from 108 sequenced clones of an average of 849 bases from the database (Drake, 1993), and these values are much larger than those of herpesvirus DNA polymerases with proofreading activity at the levels of 1.38×10^{-7} per nucleotide (Lee et al., 2015) to 3×10^{-9} substitutions per site per year (McGeoch, Dolan, & Ralph, 2000). These results indicate the low fidelity of the RdRp activity of influenza lacking proofreading activity.

Jurkat cells exposed to 500 μM guanosine for 24 h show an increase in GTP pools to 600% of the control and a decrease in ATP to 40% of the control (Batiuk, Schnizlein-Bick, Plotkin, & Dagher, 2001). Thus, the intracellular condition of biased nucleotide pools alone is sufficient to increase the ratios of mismatched transition mutations without treatment with mutagens such as ribavirin. Ribavirin reduces GTP levels by inhibiting inosine monophosphate dehydrogenase, but favipiravir has little effect on GTP levels (Furuta et al., 2005; Vanderlinden et al., 2016).

As a guanosine analogue, extracellular favipiravir may bias the nucleotide pools, and favipiravir-RTP competes with GTP or ATP, resulting in an increase in the transition mutations, although data are not available to support this speculation. An observed increase in the transition frequency is presumed to be due to the low fidelity of RdRp of influenza virus and misincorporation of nucleotides by the biased nucleotide pools in favipiravir-treated cells. Favipiravir is a chain terminator without direct mutagenic activity but increases the number of mismatches in the genome due to the induction of biased nucleotide pools in the favipiravir-treated cultures.

7. Favipiravir-resistant mutants

7.1. Comparison of the generation of resistant herpesvirus and influenza virus

We have isolated a herpes simplex virus resistant to acyclovir, phosphonoacetic acid, and foscarnet, a varicella-zoster virus resistant to acyclovir, penciclovir, foscarnet, and vidarabine, and a cytomegalovirus resistant to ganciclovir, foscarnet, and mizoribine by culturing the virus in the presence of these antiviral agents (Ida et al., 1999; Ida et al., 2000; Kamiyama, Kurokawa, & Shiraki, 2001; Kuramoto et al., 2010; Kurosaki et al., 2004; Miwa et al., 2005; Mori et al., 1988; Shiraki, Ogino, Yamanishi, & Takahashi, 1983, 1985). These resistant viruses replace the virus population in the presence of drug in vitro and in patients. The emergence and replacement of resistant strains occurs in the herpetic lesions in immunocompromised individuals when a lesion with viral growth is treated for at least one or two weeks.

An oseltamivir- or baloxavir marboxil (baloxavir)-resistant virus has emerged in patients with seasonal influenza during treatment, and the isolated virus has been replaced by a resistant virus. A subsequent new infection by the resistant virus has been confirmed. Therefore, we investigated the possibility that a favipiravir-resistant virus appeared in cultured cells while influenza virus was growing in the presence of favipiravir and that the proliferating virus was replaced by a resistant virus.

7.2. The lack of generation of favipiravir-resistant mutants in cultures treated with favipiravir

We tried to isolate favipiravir-resistant influenza virus and poliovirus from 28 and 10 25 cm^2 flasks, respectively, after independent cultures in the presence of increasing concentrations of favipiravir for a month, but no resistant virus was isolated (Daikoku et al., 2014; Daikoku et al., 2017). Then, we decided to deny the possibility of replacement with the resistant virus. Spontaneous mutation rates of the influenza virus RdRp complex and poliovirus 3D RdRp are both approximately 1×10^{-4} (Cheung et al., 2014; Drake, 1993; Gubareva & Fry, 2019). Titers of influenza virus and poliovirus in the culture reach approximately 10^8 infectious viral particles/mL in 5 mL of media in a 25 cm^2 flask, and their genomes contain approximately 13,600 and 7500 base pairs, respectively, stochastically indicating 10^4 alterations per nucleotide in the genome. Therefore, every type of mutant should be generated during replication, and continuous cultivation for a month might increase the favipiravir-resistant virus population. If favipiravir induces mutations more frequently than natural processes, mutants resistant to favipiravir should be isolated easily, and the culture should be replaced by the favipiravir-resistant mutants. We isolated susceptibility variants of influenza virus and poliovirus in the cultures treated with favipiravir for a month and identified nucleotide substitutions (mutations) in their RdRp genes, but these mutations were not related to resistance or mutations common to favipiravir (Daikoku et al., 2014; Daikoku et al., 2017). Therefore, theoretical resistant mutants should be generated but are unable to replicate or replicate with reduced fidelity to replace the entire virus population. The favipiravir-resistant mutant replicates as an artificially generated clone but does not become dominant among the entire virus population that grows in the presence of favipiravir (Abdelnabi et al., 2017; Delang et al., 2014; Goldhill et al., 2019). Our results are consistent with the absence of a resistant virus in the entire virus population treated with favipiravir.

7.3. Characteristics of favipiravir-resistant mutants

Many laboratories have attempted to isolate favipiravir-resistant influenza viruses but have not been successful. Recently, mutants of influenza virus (Goldhill et al., 2018) and chikungunya virus (Abdelnabi et al., 2017; Delang et al., 2014) that are less susceptible to favipiravir have been reported. Mutated sequences of these viruses have been detected in cultures treated with favipiravir, and reverse-engineered viruses showed favipiravir resistance with altered RdRp activity related to the fidelity and reduced growth property.

The K229R mutation in motif F of the PB1 gene was observed in the virus population cultivated in the presence of favipiravir, and a virus with the K229R mutation was created by reverse engineering and confirmed as a favipiravir-resistant virus. However, the virus was artificially produced and grown as a clone and is not considered a dominant virus in the culture. A K229R mutation in P1 shows reduced polymerase activity and acquired P653L in the PA during replication. The acquisition of the additional PA P653L mutation restores the polymerase activity and favipiravir resistance (Goldhill et al., 2018).

The K291R mutation in the F1 motif of the RdRp (nsP4) in chikungunya virus is less susceptible to favipiravir, displays a reduced growth property and acquired an additional Y543C mutation in the helicase-protease (nsP2) during passages in the absence of favipiravir (Delang et al., 2014). The corresponding K-to-R substitution (K159R) of the chikungunya virus K291R mutation was introduced in the coxsackievirus B3 RdRp, but the engineered virus with the K159R mutation in RdRp was a nonviable virus (Abdelnabi et al., 2017). The replication competence of the K159R variant is restored by the additional acquisition of an A239G substitution in the RdRp. The variant virus with the K159R and K291R mutations is more susceptible to favipiravir and exhibited lower fidelity than the wild-type virus.

A common feature of less susceptible viruses is high fidelity of RdRp that may distinguish favipiravir-RTP and GTP and replicate in the presence of favipiravir by avoiding the incorporation of favipiravir-RTP. Since their proliferative ability is not high, clones that are less susceptible to favipiravir grow well alone or are not viable, but an additional alteration that modulates RdRp activity restores the replication capability and the susceptibility to favipiravir (Abdelnabi et al., 2017; Delang et al., 2014; Goldhill et al., 2019). Favipiravir-resistant mutants of influenza virus have been created by reverse-engineering and replicate as a clone with a reduced growth property. Therefore, this virus is unlikely to replace the entire growing virus population when it is produced during favipiravir treatment, as has been observed in many laboratories.

Two highly pathogenic A(H5N1) influenza viruses from chicken and Muscovy duck and one H3 influenza virus from ruddy turnstone and two swine (H1N1) origin influenza viruses possess the PB1-V43I

mutation that may result in a high-fidelity RdRp but has not been confirmed (Cheung et al., 2014). Thus, researchers should consider the possibility of changes in the susceptibility to favipiravir when these types of influenza viruses cause a pandemic.

7.4. A favipiravir-resistant virus has not appeared in clinical trials

Antiviral susceptibility was examined in 57 pairs of influenza viruses isolated from patients before and after the administration of favipiravir in phase 3 clinical trials. No viruses displayed reduced susceptibility to favipiravir, although two of 20 paired A(H1N1)pdm09 isolates, one of 17 paired A(H3N2) isolates and one of 20 paired B viruses possessed amino acid substitutions in the RdRp subunits PB1, PB2 and PA after favipiravir administration (Takashita et al., 2016). These amino acid substitutions in the RdRp had nothing to do with the susceptibility of favipiravir and seemed to have occurred regardless of favipiravir treatment. This trial was a limited clinical study to assess the emergence of favipiravir-resistant virus, but the appearance of oseltamivir or baloxavir resistance might be observed in clinical trials of influenza treatment with oseltamivir and baloxavir with a similar number of patients, as described in Section 4.3. The lack of the emergence and replacement of resistant viruses during favipiravir treatment in vitro and in humans indicates that the same effectiveness of favipiravir is expected to be maintained from the beginning to the end of the influenza pandemic and that all patients could be treated effectively.

8. Organ function and lethal virus infection

Causes of death from severe infections may be liver failure, renal failure, respiratory failure and encephalitis. Ebola virus infection seemed to be difficult for patients when they had the chance of infection or fever after infection, and the amount of virus was used as an indicator of the time of infection (Sissoko et al., 2016). Renal function represented by creatinine levels was found to be an important factor for prognosis. The factor that determines survival and death rate with favipiravir treatment seems to be residual organ function at the start of treatment in fatal infections caused by cytotoxic RNA viruses.

The residual function necessary for the recovery of each organ can be estimated from the indication criteria for organ transplantation. One of the criteria for lung function for lung transplant patients is a forced expiratory volume in 1 s (FEV1) less than 25–30% (Maurer, Frost, Estenne, Higenbottam, & Glanville, 1998). Concerning renal function, hemodialysis starts when renal function falls to 10–15%, and death occurs within one to several weeks when dialysis is stopped (National Kidney Foundation, 2019). Concerning liver function, the donor's liver is left as a residual liver volume of at least 30% of the total liver volume to ensure hepatic graft with excellent results and low donor morbidity (Pomfret, Pomposelli, & Jenkins, 2001). It is important to begin treatment before losing the recoverable function of the target organ.

Since pneumonia is the main cause of death by influenza, it may progress to some extent even after the start of treatment. Time from illness onset to oseltamivir treatment in avian influenza A(H7N9) virus infection is 6 days (5–9 days), and time from illness onset to the development of acute respiratory distress syndrome (ARDS) is 7 days (5–9 days) (Li et al., 2014), indicating the importance of early diagnosis and treatment.

In Ebola virus infection, other than the direct cause of death related to bleeding such as hemorrhagic shock, the degree of liver dysfunction and renal dysfunction seems to be related to survival and death rate as seen in an animal model treated with favipiravir (Oestereich, et al., 2014). SFTS is mainly caused by tick bites and develops with fever. Liver and renal dysfunction in SFTS may be the cause of death. In Yamaguchi Prefecture, where the first patient was found in Japan, dermatologists who have examined tick bites monitor the development of SFTS to enable early treatment.

9. Conclusions and future perspectives

Favipiravir has been developed as an anti-influenza drug with efficacy against severe infections caused by a high viral load and was approved as an anti-influenza drug in Japan. Favipiravir has a similar mechanism of action to the antiherpesvirus drug acyclovir and has the property of not producing a resistant virus. Favipiravir is contraindicated in pregnant women due to its teratogenicity and embryotoxicity in animals. Subsequent stockpiling of doses for 2 million people has been performed as a countermeasure against novel influenza in Japan. Favipiravir has been used to treat lethal infections in humans because its efficacy has been confirmed in a wide range of animal models of lethal RNA infections. Severe human RNA infections are sporadic, and the number of cases is limited. In patients with these infections, favipiravir has been used for urgent and life-saving purposes because there is no standard effective treatment, rather than to show efficacy. Clinical trials have been performed to compare historical control patients and patients treated with favipiravir for Ebola virus infection (Bai et al., 2016; Jacobs et al., 2015; Sissoko et al., 2016) and SFTS (Yasukawa, 2016). Subsequently, favipiravir has been submitted for additional indications based on clinical trials for SFTS in Japan. The establishment of a placebo group that does not receive an effective drug in a lethal animal model is a challenging problem to confirm the efficacy of favipiravir by a randomized placebo-controlled trial. As an example of this situation, PEP of human immunodeficiency virus has been performed to protect against needle sticks or occupational exposures without placebo and has become a standard procedure in the guideline due to its prophylactic effect as a result of many years of implementation (Kuhar et al., 2013).

Since children died of avian influenza A(H5N1) in Hong Kong in 1997 (Ku & Chan, 1999), concern has been expressed about novel influenza pandemics, such as A(H5N1) and A(H7N9). An influenza pandemic consisting of a global outbreak of influenza A(H1N1)pdm09 virus occurred in 2009, and although the pathogenicity of this virus was milder than A(H5N1) and A(H7N9), it caused a pandemic and health problems. Although there was immunity against influenza A (H1N1)pdm09 among the elderly, it caused health problems for young people and a substantial social impact. As a next candidate for a pandemic influenza, avian influenza, such as A(H5N1) or A(H7N9), causes a severe infection and pneumonia due to the prolonged viral replication caused by the lack of immunity and its tropism to the pulmonary epithelium with a high mortality rate (Lai et al., 2016; Li et al., 2014; Shinya et al., 2006). The ability to predict whether a pandemic will occur remains challenging, but a necessary strategy appears to be to stockpile vaccines and anti-influenza drugs for novel influenza strains to cope with pandemics.

The specific features and mechanism of action of favipiravir and the fact that favipiravir alone does not produce resistant viruses among anti-influenza drugs suggests that it is expected to play a central role among anti-influenza drugs in the treatment of a lethal influenza pandemic. This review helps clinicians, scientists, and policy-makers considering preparedness strategies for an avian influenza pandemic, such as the use of the anti-influenza drug favipiravir for prophylaxis and treatment, as well as prevention through vaccination.

Declaration of Competing Interest

K.S. reports the receipt of consulting fees from Maruho Co., Ltd., lecture fees from Maruho Co., Ltd., MSD, and Novartis, and research funding from Maruho Co., Ltd., MSD, and Japan Blood Products Organization; all payments were sent to the institution.

Acknowledgments

We appreciate Mr. K. Yamada from FUJIFILM Toyama Chemical Co., Ltd. for supplying the information on the international supply of

favipiravir and Elsevier Language Editing for editing the English language of the manuscript. This study was supported in part by JSPS KAKENHI Grant Number 16K09929, 19K07597 and 19K08940 from the Ministry of Education, Culture, Sports, Science and Technology of Japan and joint research project with Toyama Chemical Co., Ltd.

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