（ 様 式 3 ）

論 文 要 旨

Identification of dihydroorotate dehydrogenase inhibitor, vidofludimus, as a potent and novel inhibitor for influenza virus

（ジヒドロオロト酸デヒドロゲナーゼ阻害剤 **vidofludimus** をインフルエンザウイルスに対する強力かつ新規な阻害剤として同定した）

【序論及び目的】

Influenza viruses have four types, type A, B, C, and D. Most of the outbreaks and epidemics of influenza are caused by influenza A viruses (IAV) and influenza B viruses (IBV). IAV is an enveloped virus belonging to the family Orthomyxoviridae. IAV contains eight negative-sense single-stranded RNA (vRNA) genome segments and four proteins, including RNA dependent RNA polymerase (RdRp) subunits PB1, PB2, PA, and nucleoprotein (NP), which form ribonucleoprotein complexes (RNPs). The RNP of IAV contributes to viral pathogenesis. Two IAV subtypes, subtypes H5 and H7, possess high pathogenicity with the potential to cause severe disease and high mortality in infected animals (chickens, pheasants, etc.). Although the IAV H5N1 is poorly transmissible from avian-human and human-human, an uncommon mutation (T271A) of the viral RdRp subunit PB2 gene was reported in a previous study and recently, infection of avian IAV H5N1 among mammals are reported in mink farm. This is the first report of the avian IAV H5N1 endemic among mammals and means that the warning level of IAV H5N1 endemic among humans might be raised by one rank. Baloxavir marboxil has been approved by the U.S. Food and Drug Administration (FDA) as an antiviral drug for influenza. Whereas, recent clinical studies found that IAV carrying an I38T mutation in the viral RdRp subunit PA showed resistance to baloxavir marboxil. Therefore, development of new compounds for seasonal and highly pathogenic influenza was needed.

【材料及び方法】

Following the previously reported RdRp assay system construction approach in IAV H1N1. We constructed the recombinant plasmids using the cDNA of A/Hong Kong/213/2003 (H5N1). We artificially synthesized the PB1, PB2, PA, and NP genes of H5N1. Then, we cloned the cDNA of the open reading frames (ORFs) of the PB1, PB2, PA and NP genes into a retroviral vector pCX4bsr. To monitor the replication level, we constructed a vRNA containing secreted nanoluciferase (sNLuc). The negative strand of the sNLuc gene was inserted between the 3' and 5' untranslated regions (UTR) of the H5N1 NP gene. By use a QuickChange PCR approach, we constructed the mtPA I38T to test the baloxavir marboxil-resistance, and the mtPB2 T271A to assess its effect on RdRp activity. Using the same approach, we also constructed the recombinant plasmids using the cDNA of A/WSN/1933(H1N1) and B/Yamagata/16/1988 (IBV). Using this RdRp assay system, we examined a Phase II Drop compounds library that is containing 350 compounds cleared Phase I but not Phase II. The antiviral activity and the antiviral mechanism of compounds were evaluated by western blotting analysis, WST-1 cytotoxicity

assay, Cytopathic effect (CPE) assay, and RT-qPCR analysis. We also confirmed if the RdRp assay system could reflect the results of antiviral activities using the RdRp assay and infectious virus of IAV H1N1.

【結　果】

In this study, we focused on the viral replication/transcription stages in the viral life cycle of the IAV H5N1. To monitor the viral specific replication/transcription, we constructed a reporter plasmid for the transcription of anti-sense gene of the sNLuc. The IAV H5N1, PB1, PB2, PA, and NP were cloned into the vector pCX4bsr, which could directly transcribe mRNA in the host cell. Using this RdRp reporter assay system, we screened 350 drugs. Meanwhile, the cytotoxicity was determined by WST-1 analysis. As a result, we found only one drug, vidofludimus as a potent and novel inhibitor for IAV H5N1. Vidofludimus exerted antiviral activity against wild-type and baloxavir marboxil-resistance mutant IAV, with effective concentrations (EC_{50}) of 2.10 and 2.11μM, respectively. Baloxavir marboxil and vidofludimus showed high antiviral activity against RdRp with mtPB2 T271A. Vidofludimus could maintain high antiviral activity even when the RdRp carried both mutations. The anti-IAV activity of vidofludimus was cancelled by the treatment of uridine or cytidine through pyrimidine salvage synthesis pathway, or orotic acid through pyrimidine de novo synthesis pathway. This indicated that the main target of vidofludimus is DHODH in IAV RdRp expressing cells. We also produced recombinant seasonal IAV H1N1 virion and influenza B virus (IBV) RdRp assay system and confirmed vidofludimus also carried highly antiviral activity against seasonal IAV and IBV. Vidofludimus is a candidate drug for the future threat of IAV H5N1 infection among humans as well as seasonal influenza virus infection.

【結論及び考察】

In the present study, we constructed a plasmid-mediated cell-based assay system for IAV H5N1 vRNA replication/transcription inhibitors based on prior studies. Using this RdRp assay system, we found a potential antiviral, vidofludimus, for influenza virus wild-type and mutation-type infection. As the plasmid-mediated assay system expresses each viral polymerase subunit using a single plasmid, the construction of the plasmid with several drug-resistant mutations could be easily developed. Recently unique mutation was reported in PB2 of IAV H5N1 in farmed mink. T271A in PB2 was detected only in mink IAV H5N1 but not in avian IAV H5N1. This mutant was also reported in the pandemic of hog IAV H1N1 in 2009. We constructed a plasmid containing the mutation mtPB2 T271 and evaluated its effects on viral polymerase activity and inhibitors. We found that the mtPB2 T271A mutation enhanced IAV H5N1 RdRp activity. This might be a key point for understanding how the avian HP IAV (H5N1) caused an outbreak among mammal minks. In the case of vidofludimus, the main antiviral mechanism is the inhibition of DHODH. We confirmed that inhibitory activity was through blocking the oxidation of DHO to OA by DHODH in the de novo synthesis of pyrimidines. Furthermore, we examined another DHODH inhibitor, teriflunomide approved by the FDA for the treatment of autoimmune diseases, and brequniar, BAY2402234, ML390, which were published in previous reports. All of these DHODH inhibitors significantly reduced sNluc signals. Previous reports showed inhibitors targeting DHODH had broad-spectrum antiviral activity against RNA viruses. we also evaluate the antiviral activity of vidofludimus against another type of influenza virus B/Yamagata/16/1988 (IBV) for RdRp assay. The result showed vidofludimus also carried highly antiviral activity to IBV. The antiviral activity of vidofludimus shown in this study for IAV H1N1, H5N1 and IBV might also be employed for the other types/subtypes of influenza viruses and other RNA viruses.

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