博士論文（要約）

Identification and characterization of antiviral compounds

in vitro and *in vivo* against Chandipura virus

（チャンディプラウイルス感染症に対する抗ウイルス薬の

探索および *in vitro*/*in vivo* 系での検討）

北浦　慧

Chandipura virus (CHPV) is a highly lethal pathogen that causes acute encephalitis outbreaks in the pediatric population across the Indian subcontinent. CHPV is a single-stranded, negative-sense RNA virus, which belongs to the genus *Vesiculovirus*, family *Rhabdoviridae*, order *Mononegalovirales*. CHPV was first isolated from adults presenting with febrile illness during a large dengue fever and chikungunya fever epidemic in 1964 and 1965. While its pathogenic significance was not well known, several fatal outbreaks of encephalitic syndrome have been reported. The pediatric population is especially vulnerable to disease with a high case fatality rate of 44–75%. Currently, there is no standardized treatment for the Chandipura virus. The aim of this study was to develop a platform for evaluating drug efficacy *in vivo* and identifying potential candidate drugs *in vitro*.

Several regions within India have been investigated for the presence of human CHPV infection. Endemic areas with human CHPV infection include Andra Pradesh, Gujarat, Odisha, and Nagpur state. While no human patients have been identified outside of India, CHPV may be endemic over a wide geographic area. For instance, serological surveys of domestic animals in Andra Pradesh have shown a high proportion of positive neutralizing antibody titers among pigs, buffalos, cattle, goats, and sheep, ranging from 8–31%. In addition, the presence of CHPV has been detected by virus isolation from *Atelerix albiventris* and Phlebotomine sandflies in West Africa and by anti-CHPV antibodies from macaques in Sri Lanka.

CHPV is an arthropod-borne virus (arbovirus) transmitted mainly by sandflies. Past studies detected CHPV RNA in sandfly pools collected from houses and the surrounding environment during outbreaks. Other studies have detected positive CHPV RNA in *Sergentomyia* species collected in Nagpur, Maharashtra, and Gujarat regions. In addition, *Sergentomyia* species in the Gujarat region showed positive CHPV RNA in male sandflies, suggesting vertical transmission within sandfly colonies. Laboratory-setting studies revealed venereal transmission in *Phlebotomus papatasi* and transmission capability from *P. papatasi* and *P. argentipes* to mice. In the laboratory setting, vectorial capacities of mosquitos have also been indicated in *Culex* and *Aedes* species, suggesting a wider vectorial range than expected.

CHPV infection is known to present with neurological symptoms, such as altered sensorium, seizures, and quadri-/hemi-paresis. The clinical progression is aggressively acute, where deaths usually occur within 48 hours of admission and symptom onset. Most cases have been diagnosed using either reverse-transcriptase polymerase chain reaction (RT-PCR) or CHPV IgM antibody detection

methods, with occasional use of virus isolation. Diagnosis usually requires the exclusion of other infectious etiologies such as the Japanese encephalitis virus, dengue virus, and enteroviruses. Due to rapid clinical deterioration, scarce data on pathology is available. As a result, the exact pathophysiology remains elusive. While clinical manifestations were concordant with an encephalitic syndrome, most cerebrospinal fluid (CSF) examination results were unremarkable, except for heightened CSF pressure in some cases. Imaging modalities have shown signs of ischemia in the middle cerebral artery region, brain edema, and midline shift. These laboratory and imaging studies led to another speculation that CHPV infection may be causing an "epidemic brain attack," which suggests a possible vascular pathology. Nevertheless, the clinical presentation suggests a neurological manifestation, and further in-depth investigation into CHPV pathogenesis is warranted.

As there is no specific standardized treatment for patients with CHPV infection, the overall goal of this study was to identify antiviral agents against CHPV infection utilizing both *in vitro* and *in vivo* platforms. To achieve this goal, I conducted a two-part study with different objectives. The first part of the study was aimed at developing a platform for *in vivo* antiviral efficacy testing. The second part of the study aimed to identify potential drugs effective against CHPV infection *in vitro* from a drug library. I presumed that the results from the two studies would then contribute to further advancements in the search for an optimal treatment strategy.

In the first part of this study, I developed a new lethal animal model and investigated the efficacy of broad-spectrum antivirals against Chandipura virus *in vivo*. While 10–14 days old mouse is a regularly used mouse model in past studies, some of the potential drawbacks include technical difficulties arising from small size and cannibalism confounding survival analysis. In addition, adult BALB/c mice are known to be non-lethal to CHPV except for intracranial administration. Hence, I aimed to investigate immunocompromised mice as a potential lethal model for assessing drug efficacy. I specifically focused on a mouse with defective acquired immunity to provide a platform that enables exclusive antiviral evaluation isolated from the host immune response.

CHPV infection in C.B-17 severe combined immunodeficiency (SCID) mouse were initially characterized. All mice inoculated with a viral load equivalent to or more than 10^3 $TCID_{50}$/mouse died from infection. Apart from non-specific clinical signs, neurological signs including hemi-paralysis, ataxia, and seizures were observed. In addition, viral load dynamics revealed that after an incubation period of approximately 5 days, CHPV viral loads increased in whole blood and solid organs in a parallel fashion. Kidney/Adrenal glands and brain tissue revealed especially higher viral loads. The

pathological evaluation also showed a high antigen burden in the brain, spinal cord, and adrenal medulla, which suggested tropism for neuronal structures. However, the meninges, choroid plexus, and central canal of the spinal cord were more evidently positive in viral antigens than the parenchyma. As a result, I suspected a meningeal pathology as the predominant mechanism in these animal models. In addition, strong antigen staining in adrenal glands suggested potential adrenal failure as part of the pathophysiology in these mice. Despite a different clinical phenotype compared to suckling mice, this model appeared to be a feasible animal model for assessing drug efficacy *in vivo*.

As a proof-of-concept experiment to show the utility of the C.B-17 SCID mouse model, I investigated the *in vitro* efficacy of favipiravir and ribavirin to select a candidate. *In vitro* experiments were conducted to determine the 50% inhibitory concentrations (IC_{50}) and 50% cytotoxicity concentrations (CC_{50}) for both drugs in Vero and N2a cells. The results from these experiments suggested higher viral suppressive effects and lower cytotoxicity of favipiravir in comparison to ribavirin. As a result, I selected favipiravir as a model drug for *in vivo* antiviral efficacy testing. I next investigated survival benefits associated with pre-symptomatic treatment (from day 5 to 14) and post-symptomatic treatment (from day 9 to 18) in comparison to a control (vehicle only from day 5 to 14). I found significantly improved median survival in both groups, while earlier treatment resulted in better survival. Viral dynamics upon pre-symptomatic treatment and post-symptomatic treatment were compared against control. A significant decrease in viral load was observed in the whole blood, kidney/adrenal glands, and brain. However, a stronger viral suppression was observed in whole blood and kidney/adrenal glands compared to the brain. I speculate that this difference is attributable to difficulty in penetration of the blood-brain barrier. Regardless, favipiravir appeared to exhibit effective anti-CHPV response *in vivo* utilizing newly developed C.B-17 SCID mouse model.

In the second part of this study, I conducted a high-throughput screening of a drug library to identify antiviral drugs *in vitro*. Primary screening (cell viability assay) and secondary screening (virus yield reduction assay) identified four compounds: digitoxin, nelfinavir, niclosamide, and ponatinib. Further *in vitro* characterizations revealed that niclosamide and nelfinavir exhibited reproducible efficacy among different cell lines: Vero, BHK, and SK-N-SH. Digitoxin and ponatinib did not show dose-dependent virus yield reduction in the BHK cell. The IC_{50} for niclosamide and nelfinavir were within ranges achievable in human plasma or serum. In addition, only niclosamide and nelfinavir exhibited significant instantaneous inhibitory potential within achievable concentrations. These data suggested that both niclosamide and nelfinavir may be potentially effective antiviral agents. Time-of-

addition experiments revealed that all four drugs affected the post-entry step of viral infection, except for ponatinib, which had mild pre-entry and during entry virus yield reduction effect. One limitation of this study was accumulating cytotoxicity over longer incubation periods *in vitro* and sub-optimal selectivity indices. Therefore, I further evaluated the combined effects of niclosamide and nelfinavir as a dose-sparing strategy since these agents were the most promising in achieving antiviral efficacy against CHPV.

Assuming that each drug exerts its antiviral effects independently, I utilized the Bliss model and the dose model to define synergism. Based on the Bliss model, I identified considerable synergy across a variety of concentrations. In addition, the interaction parameters determined from the dose model revealed that niclosamide interacts synergistically with nelfinavir and vice versa. Oral formulations of niclosamide and nelfinavir enable prophylaxis in the setting of laboratory exposure or outbreaks. Although further *in vivo* evaluation and pharmacokinetic/pharmacodynamic (PK/PD) data for pediatric dosing is required, these findings expand the possibility of strategies that can be taken against CHPV.

Overall, the findings in this study lay the foundation for the development of treatment strategies against CHPV. The first part of the study built an *in vivo* platform for assessing drug efficacy. The second part of the study identified a repertoire of promising drugs, which have potential as single or dual therapy against CHPV infection. Together, the finding in this study lay the foundation for potential anti-CHPV strategies in preparation for future outbreaks.