Summary of dissertation

Development and Implementation of a Compound Screening Assay to Identify Antivirals against Rabies Virus

(狂犬病ウイルスに対する抑制作用を有する化合物の
スクリーニングアッセイ法の開発と化合物の探索)

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Rabies is an acute and invariably fatal encephalomyelitis, resulting from infection by any of the viruses in the genus *Lyssavirus*, family *Rhabdoviridae* and the principal lyssavirus that causes this devastating neurological disease is rabies virus (RABV). Rabies is mainly transmitted through the bite of rabid animals even though evidence for airborne routes and organ transplantation has been reported for rabies transmission in a limited number of cases. Rabies can infect a wide range of susceptible mammalian hosts; however, rabies cases in humans are predominantly related to transmission from rabid dogs and the number of stray dogs present in the endemic areas since most of the cases arise from dog bites. Rural areas in Asian and African countries, with a large number of free-roaming dogs, suffer the highest disease burden from rabies. World Health Organization (WHO) estimates that 60,000 people die from rabies with more than 15 million people receiving post-exposure prophylaxis (PEP) worldwide annually (WHO Rabies Fact Sheet 2016, accessed June 10, 2017, from http://www.who.int/mediacentre/factsheets/fs099/en/).

RABV is an enveloped virus containing a single-stranded, nonsegmented, negative sense RNA genome of ~12 kb composed of five open reading frames (ORFs) encoding: nucleoprotein, N;

phosphoprotein, P; matrix protein, M; glycoprotein, G and the RNA-dependent RNA polymerase, L. Each RABV gene is flanked by short regulatory sequences, referred to as start and stop signals, that regulate transcription of the contiguous open ORF. Between each RABV gene, there is an intergenic region (IGR) with differing nucleotide (nt) lengths: 2 nts between N and P (N/P) IGR, 5 nts between P and M (P/M) IGR, 5 nts between M and G (M/G) IGR and 24-29 nts between G and L (G/L) IGR. These nucleotide sequences are neither transcribed into mRNA nor translated into protein. The length of the nucleotide sequence in the RABV IGR has been reported to contribute to transcriptional attenuation of the downstream viral gene and to be involved in regulating viral protein expression.

Once RABV is transmitted to a susceptible individual, about 80% will develop an encephalitic rabies, a classical form of rabies which is characterized by the occurrence of general arousal and hyperexcitability that can be accompanied with hydrophobia, aerophobia and sensitivity towards bright lights or loud sounds; whereas the remainder of individuals (~20%) will develop a paralytic rabies which is characterized by general muscle weakness. Both forms of rabies have similar clinical stages that include: incubation period which length depends on the bite site, prodromal period, acute neurological signs, coma and, finally, death.

Rabies vaccine is available both as a preventive measure and included in a PEP regimen given immediately after a person is bitten by a rabid animal, together with wound cleaning and administration of rabies immunoglobulin. A number of drugs, such as, ribavirin, amantadine, interferon alpha and ketamine have been employed to treat symptomatic rabies patients; however, clinical efficacy has not been demonstrated, with few survival cases recorded. Thus, novel antiviral drugs to treat symptomatic rabies patients are urgently needed to mitigate the devastating neurological sequelae associated with this high-consequence pathogen.

A limited number of studies involving screening of small chemical compounds have been performed on RABV to find novel antivirals. The assays employed in the screening depended on the target of the small compounds in the RABV life cycle. A cell-free protein synthesis system has been developed to identify small compounds that inhibit the assembly of the RABV ribonucleoprotein capsid. Cell-based screening assays have also been successfully utilized to identify compounds which target viral replication and protein synthesis steps of the viral life cycle.

In chapter I, recombinant RABVs (rRABVs) encoding NanoLuc luciferase (NanoLuc) were generated to facilitate the screening of compound libraries for viral inhibitors. The infectivity of rRABVs was determined by measurement of viral titres and quantification of NanoLuc expression after inoculation of rRABV into cells. Detailed characterization of the rRABV showed that the luciferase activities could be maintained over ten serial passages of the rRABV and correlated with the viral inputs. In addition, the rRABV could be used to demonstrate a dose-dependent antiviral activity of ribavirin against RABV *in vitro*, reflected by the decrease of NanoLuc signal.

In chapter II, the rRABV encoding non-secreted type NanoLuc was employed to examine the antiviral activity of ribavirin-related nucleoside analogs *in vitro*. It was found that several nucleoside analogs could more effectively decrease the NanoLuc expression in the rRABV-infected human neuroblastoma cells compared to ribavirin in a dose-dependent manner. Subsequently, a time-of-addition assay was done and the result suggested that these compounds inhibit the viral transcription and genome replication stages of the RABV life cycle. This finding was further confirmed by use of a RABV minigenome assay and qRT-PCR quantification of viral gene expression and genome copy.

Altogether, the presented studies provide insights into screening methodologies and chemical compounds that could feasibly be employed to generate antivirals to inhibit RABV infection in exposed individuals in order to decrease the morbidity and mortality associated with rabies.