Identification and evaluation of antiviral compounds targeting Rift Valley fever virus

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Abstract

Rift Valley fever virus (RVFV), a negative-stranded RNA virus, is the etiological agent of the vector-borne zoonotic disease Rift Valley fever (RVF). RVFV causes significant morbidity and mortality in humans and livestock throughout Africa and the Arabian Peninsula. RVFV is an emerging virus and is capable of infecting a broad range of mosquito species distributed around the world, so it poses a potential threat globally. A wide range of livestock animals (e.g. sheep, goats, cows, and camels) and some wild animals become highly affected by RVFV. In humans, RVFV infection presents as an acute self-limiting febrile illness that may lead to more severe hemorrhagic fever and encephalitis. The severity of the disease is mostly dependent on age and the species of mammal, but other factors are also important.

There are no licensed RVFV vaccines for humans, and there is a lack of effective antiviral drugs. Moreover, due to the severe pathogenicity, higher-level facilities are needed—biosafety level 3 (BSL-3) or more—to work with RVFV, which makes antiviral drug development more challenging. Because RVFV causes severe disease in Africa and the Arabian Peninsula, and has the potential to spread globally, it is essential that safe, efficient antiviral drugs against this virus are developed.

The previously reported antiviral compound benzavir-2 inhibits the replication of several DNA viruses, i.e. human adenoviruses, herpes simplex virus (HSV) type 1, and HSV type 2, indicating a broad-ranging activity. We wanted to evaluate whether benzavir-2 had an effect against the RNA virus RVFV. For these and subsequent studies, we used a recombinant, modified RVFV strain with a deleted NSs gene, which was
replaced by a reporter gene (rRVFVΔNSs::Katushka), enabling the studies to be conducted under BSL-2 conditions. The NSs gene is the main virulence factor for RVFV and without it, RVFV become less pathogenic. The reporter gene made it possible for us to quantify infection with the help of the red fluorescent protein. We found that benzavir-2 effectively inhibited RVFV infection in cell culture at an effective concentration showing 50% inhibition (EC$_{50}$) of 0.6 µM. Benzavir-2 also inhibited the production of progeny virus. When we studied the pharmacokinetic properties, we found that benzavir-2 had good in vitro solubility, permeability, and metabolic stability. When we investigated the oral bioavailability in mice by administering benzavir-2 in peanut butter pellets, high systemic distribution was observed without any adverse toxic effects. Benzavir-2 thus inhibited RVFV infection in cell culture and showed excellent pharmacokinetic properties, suggesting the possibility of evaluating its effectiveness in an animal model. Since benzavir-2 has a broad effect against both RNA and DNA viruses, we speculated that the antiviral mechanism affects cellular targets.

We also wanted to explore a large number of small chemical compounds with unknown properties and identify any anti-RVFV activities. Thus, we developed a whole-cell-based high-throughput reporter-based assay, and screened 28,437 small chemical compounds. The assay was established after optimization of several parameters. After primary and secondary screening, we identified 63 compounds that inhibited RVFV infection by 60% at a concentration of 3.12 µM and showed ≥ 50% cell viability at 25 µM. After a dose-dependent screening of these 63 compounds, several compounds were identified with highly efficient anti-RVFV properties. Finally, N$^1$-(2-(biphenyl-4-
(compound 1) was selected as the lead compound. We performed a structure-activity relationship (SAR) analysis of compound 1 by replacing and changing component after component of the chemical compound to see how this affected the antiviral activity. After the SAR analysis, the antiviral activity did not change, but we could improve the cytotoxicity profile. Our studies suggested that the improved compound, 13a, might be targeting the early phase of the RVFV lifecycle.

In conclusion, we developed an efficient and reliable screening method that creates possibilities for discovering and developing antivirals against RVFV under BSL-2 conditions. We also identified several chemical compounds with anti-RVFV activities, which might lead to development of therapies for RVFV infection.
Enkel sammanfattning på svenska


Frånvaron av licensierade vacciner för människor och brist på effektiva antivirala läkemedel gör behandling och prevention av denna sjukdom utmanande. Dessutom, på grund av att den kan orsaka allvarlig sjukdom och död behövs höga nivåer av biosäkerhet (biosäkerhetsnivå-3, BSL-3) för att arbeta med RVFV vilket försvårar utvecklingen av läkemedel. Eftersom RVFV orsakar allvarlig sjukdom i Afrika och Arabiska halvön och har potential att spridas globalt är det viktigt att utveckla säkra effektiva antivirala medel för detta virus.

Den tidigare rapporterade antivirala föreningen benzavir-2 hämmar flera DNA-virus, dvs humant adenovirus, herpes simplexvirus typ-1 och typ-2 infektion, vilket indikerar en bredverkande aktivitet. Vi ville utvärdera om benzavir-2 också hade en effekt mot RNA-viruset RVFV. För dessa och följdende studier använde vi en rekombinant, modifierad RVFV med en borttagen NSs-gen, ersatt av en reportergen,
som gjorde det möjligt att utföra studierna i BSL-2-förhållanden. NSs-
genen är den huvudsakliga virulensfaktorn för RVFV och med den
borttagen är RVFV mindre sjukdomsframkallande. Reportergenen gjorde
det möjligt att detektera rött fluoroscerande ljus efter virusinfektion och
använda det som ett mått på infektion.

Vi observerade att benzavir-2 effektivt inhiberade RVFV-
infektion i cellkultur med 50% inhibering vid en koncentration av 0,6µM.
Benzavir-2 hämmade också produktion av nya viruspartiklar. När vi
studerede dess farmakokinetiska egenskaper fann vi att benzavir-2 hade
bra läslighet, permeabilitet och metabolisk stabilitet i cellkultur. När vi
undersökte hur bra möss kunde ta upp benzavir-2 genom att äta föreningen
blandad med jordnötssmör (den orala biotillgängligheten) observerades en
hög serumkoncentration av benzavir-2 utan skadliga effekter. Benzavir-2
hämmade alltså RVFV-infektion i cellkultur och visade på utmärkta
farmakokinetiska egenskaper med potential att senare kunna utvärdera
effektiviteten i en djurmodell. Eftersom benzavir-2 har en bredverkande
effekt mot både RNA och DNA-virus, spekulerar vi att den antivirala
mekanismen påverkar cellulära mål.

I den andra studien ville vi undersöka ett stort antal små
kemiska föreningar med okända egenskaper och identifiera anti-RVFV
aktivitet hos dessa. För att genomföra detta utvecklade vi en
helcellsbaserad ”high-throughput-screening” analys då vi använde den
rekombinanta, modifierade RVFV beskriven ovan och screenade 28,437
små kemiska föreningar. Till att börja med optimerades analysmetoden
med avseende på flera olika parametrar. Därefter utfördes primär och
sekundär screening och 63 föreningar som inhiberade RVFV-infektion
identifierades. De inhiberade RVFV-infektion med 60% vid 3,12µM och
uppvisade ≥50% cellöverlevnad vid 25µM. Efter en dosberoende analys av dessa 63 föreningar kunde vi fokusera på några av föreningarna med mycket effektiva anti-RVFV egenskaper. Slutligen valdes N\(^1\)-(2-(biphenyl-4-yloxy)ethyl)propane-1,3-diamine (förening 1) som vår ledande förening. Vi utförde därefter en strukturaktivitetsrelationsanalys (SAR) för förening 1. SAR-analys innebär att man byter ut och förändrar komponent efter komponent i den kemiska föreningen för att se hur det påverkar den antivirala aktiviteten. Efter SAR-analysen förändrades inte den antivirala egenskapen, men vi kunde förbättra cytotoxicitetsprofilen så att föreningen hade en låg toxisk effekt på värdcellen. Den förbättrade föreningen benämns 13a, och vår studie föreslog också att förening 13a kan vara aktiv under den tidiga fasen av RVFV-livscykel.

Sammanfattningsvis har vi identifierat flera kemiska föreningar med anti-RVFV aktiviteter som kan leda till utvecklande av terapi mot RVFV. Denna studie öppnar också möjligheter att upptäcka och utveckla antivirala medel mot RVFV i biosäkerhets-2 nivå.
Included papers

I. Anti-Rift Valley fever virus activity in vitro, pre-clinical pharmacokinetics and oral bioavailability of benzavir-2, a broad-acting antiviral compound.

II. High-throughput screening using a whole-cell virus replication reporter gene assay to identify inhibitory compounds against Rift Valley fever virus infection.

III. Structural modifications and biological evaluations of Rift Valley fever virus inhibitors identified from chemical library screening.

*These authors contributed equally.

Publications not included in the thesis


<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>ADME</td>
<td>absorption, distribution, metabolism, and excretion</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole</td>
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<tr>
<td>CC50</td>
<td>50% cytotoxic concentration</td>
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<tr>
<td>EC50</td>
<td>half maximum effective concentration</td>
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<tr>
<td>EMA</td>
<td>European Medicines Agency</td>
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<td>FDA</td>
<td>US Food and Drug Administration</td>
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<tr>
<td>cRNA</td>
<td>complementary RNA</td>
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<td>HAdV</td>
<td>human adenovirus</td>
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<td>HEK293t</td>
<td>human embryonic kidney 293 cells</td>
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<td>HIV</td>
<td>human immunodeficiency virus</td>
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<td>high-throughput screening</td>
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<td>human papillomavirus</td>
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<td>HSV</td>
<td>herpes simplex virus</td>
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<tr>
<td>ICTV</td>
<td>International Committee on Taxonomy of Viruses</td>
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<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
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<tr>
<td>N</td>
<td>nucleocapsid</td>
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<tr>
<td>NS3</td>
<td>non-structural protein 3</td>
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<tr>
<td>NSm</td>
<td>non-structural protein M</td>
</tr>
<tr>
<td>NSs</td>
<td>non-structural protein S</td>
</tr>
<tr>
<td>PB</td>
<td>peanut butter</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
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<tr>
<td>PK</td>
<td>pharmacokinetic</td>
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<td>PKR</td>
<td>protein kinase R</td>
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<td>PRNT</td>
<td>plaque reduction neutralization test</td>
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<td>Abbreviation</td>
<td>Description</td>
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<td>--------------------------------------------------</td>
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<tr>
<td>qRT-PCR</td>
<td>quantitative real-time polymerase chain reaction</td>
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<tr>
<td>RdRp</td>
<td>RNA-dependent RNA polymerase</td>
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<tr>
<td>RNAP II</td>
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<tr>
<td>RNPs</td>
<td>ribonucleoproteins</td>
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<td>RSV</td>
<td>respiratory syncytial virus</td>
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<td>RVFV</td>
<td>Rift Valley fever virus</td>
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<tr>
<td>RVF</td>
<td>Rift Valley fever</td>
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<tr>
<td>SAR</td>
<td>structure-activity relationship</td>
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<td>VLPs</td>
<td>virus-like particles</td>
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<td>vRNA</td>
<td>viral RNA</td>
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1. Introduction and background

Rift Valley fever virus (RVFV) is the etiological agent of acute Rift Valley fever (RVF) infection. RVFV is mainly transmitted by mosquitoes and causes mild to fatal febrile illness in a broad range of hosts, from humans to domestic and wild ruminants (1-4). RVF was first identified in the Rift Valley region of Kenya and characterized by Daubney et al., following sudden death and abortions in lambs and ewes during an outbreak in 1930 (5). Since then, small and major epizootics and epidemics have been recorded occasionally throughout sub-Saharan Africa. RVF was mainly considered to be a major veterinary disease in East Africa, but the 1977 outbreak in Egypt in humans, with severe clinical manifestations and massive mortality, made the disease a severe health concern for humans (6-8). Lately, the virus has spread all over the African continent and the occurrence of a RVFV outbreak outside Africa was first observed in Saudi Arabia and Yemen in 2000 (9). The mosquito genera (Aedes, Anopheles, Culex, Eretmapoites, Mansonia) that play a significant role in transmitting this virus are abundant all over the world and are capable of initiating epidemics in livestock and humans (10, 11). Thus, despite being previously endemic in sub-Saharan Africa alone, RVFV has crossed significant natural geographic barriers such as the Indian Ocean (Madagascar), the Sahara Desert (Egypt), and the Red Sea (Saudi Arabia, Yemen) (12-14). Recently, RVFV seropositivity in animals has been reported in Iran and Turkey, although further work is needed to confirm the presence of RVFV in these countries (15, 16). For decades, RVFV remained neglected by major global donors and disease control programs,
even though it had a high potential to spread to non-endemic regions. Recently, this view has changed, after its classification as a potential bio-terror agent by the “Centre for Disease Control and Prevention”. Consequently, RVFV is considered to be one of the most important emerging pathogens that can pose a serious public health threat to non-endemic countries. Moreover, the lack of efficient vaccines and drugs to prevent and treat RVF infection has meant that authorities have had to classify RVFV as a biosafety level 3 (BSL-3) agent in Europe and a BSL-4 agent in the USA (17, 18).

1.1 Classification of Rift Valley fever virus
RVFV is a single-strand negative-sense RNA virus, which was previously classified as belonging to the Bunyaviridae family. In 2017, the International Committee on Taxonomy of Viruses (ICTV) re-classified the negative-sense RNA viruses. They created a new order by elevating the previously existing family Bunyaviridae to the order “Bunyavirales”, which comprises nine families and a total of thirteen genera. The order Bunyavirales consists of 160 species that cause diseases in animals, insects, and plants. RVFV is now assigned to a new family called Phenuiviridae containing four genera, Goukovirus, Phasivirus, Phlebovirus, and Tenuivirus. RVFV belongs to the genus Phlebovirus, together with nine other species (19). Viruses in the Phenuiviridae family are distributed all over the world and are transmitted by mosquitoes, sandflies, and ticks (20-23).
RVFV taxonomy:

Order: Bunyavirales

Family: Phenuiviridae

Genus: Phlebovirus

Species: Rift Valley fever phlebovirus

1.2 The virus particle

RVF virions are spherical and are between 80 and 120 nm in diameter. The outermost enveloped layer is embedded in a lipid bilayer, and contains the RVFV-specific glycoproteins Gc and Gn heterodimers (24, 25). Each virus particle has three segments of single-stranded RNA, which are surrounded by nucleocapsid protein and the viral RNA-dependent RNA polymerase (RdRp), forming viral ribonucleoprotein (RNP) (Figure 1). RNPs are filamentous and strongly connected with the cytoplasmic tail of glycoproteins, and in this way enable the packaging of the genome into the virus particles (26-29).

Figure 1: Three-dimensional reconstruction of the RVFV particle, made from data gathered from cryo-electron microscopy and single-particle averaging. Reprinted from (26) with permission.
1.3 Genome coding strategy and functions of RVFV proteins

The single-stranded RNA genome of RVFV has three segments of negative or ambisense polarity, designated large (L), medium (M), and small (S) based on their relative nucleotide length. No viral proteins are synthesized directly from viral genomic RNA, due to the lack of a cap-structure at the 5’ end (30).

Figure 2: Schematic diagram of the RVFV genome.

The L segment

The L segment is 6,404 nucleotides long and encodes an RNA-dependent RNA polymerase (Figure 2), which synthesizes both viral RNA (vRNA), complementary RNA (cRNA) and mRNA (31) and is therefore essential for viral transcription, replication, and maturation. In addition to polymerase activity, the viral L protein also has exonuclease activity (32-34).

The M segment

The M segment is 3,885 nucleotides long. After the M segment is transcribed to the M mRNA, it will be translated to a precursor polyprotein, which is co-translationally cleaved into four proteins: two structural proteins (Gn and Gc) and two non-structural proteins (NSm and a 78-kDa protein) (Figure 2) (35-37). The glycoproteins Gn and Gc facilitate the
attachment and entry of the virion into the host cells. Gn and Gc are also required for virus assembly in infected cells and for maturation of progeny particles (38-40). The NSm protein integrates and targets the mitochondrial outer membrane protein and exerts its anti-apoptotic function. It has been reported that mutant RVFV lacking the NSm gene shows reduced virulence in the rat, relative to wild-type RVFV, suggesting that the anti-apoptotic function of RVFV plays a role in viral pathogenesis (41-43). Thus, NSm may function as a virulence factor by suppressing the host cell apoptotic pathway after infection. There is also evidence that NSm has an important role in viral replication and spread in mosquitoes. The function of 78-kDa protein is currently unknown, but there is evidence that it is not required for virus infection and replication in either mammalian or insect cell cultures, although it may be important for virus dissemination in mosquitoes (36, 44-46).

The S segment
The length of the S segment is 1,690 nucleotides. The structural nucleocapsid protein (N) derives from the negative-sense portion of the S segment, whereas the non-structural NSs protein derives from the positive-sense portion (Figure 2). The N protein, the viral genomic RNA, and the L protein are packaged into an RNP complex, which is important for transcription and RNA replication (47-49). The NSs protein is a major virulence factor of RVFV. This protein blocks host cell RNA polymerase II (RNAP II) by interacting with the p44 subunit of the essential transcription factor TFIIH (50), and acts as an interferon antagonist by inhibiting the production of interferon-β (51). NSs promotes the degradation of double-stranded viral RNA sensing and the IFN effector
protein kinase R (PKR) (52-54), and it also induces the degradation of TFI IH p62 (55)

1.4 RVFV replication cycle

The replication cycle of RVFV takes place entirely in the cytoplasm (Figure 3). It begins by attachment of the virus to the plasma membrane of the host cell, which is mediated by the viral glycoproteins (Gn and Gc) and cell-surface receptor molecules. DC-Sign and heparan sulfate have been described as cellular binding partners or receptors for RVFV (56-58). After attachment, bunyaviruses are endocytosed through either a clathrin-dependent (59-61) process or a clathrin-independent process (62). One study also suggested that RVFV enters the host cell by caveola-mediated endocytosis (63). Once internalized in the endosome, the low pH there causes conformational changes in the glycoproteins and therefore initiates the last event of the entry process—fusion of the viral envelope with the endosomal membrane (62, 64, 65). This allows the release of the RNPs into the cytoplasm of the host cell.
After uncoating, primary transcription of the negative-sense viral RNA to mRNA occurs via viral RNA-dependent RNA polymerase (Figure 4: Step 1). Only ribonucleocapsids can serve as template for primary transcription. Free RNA cannot be transcribed into mRNA as it is unable to act as template (48). The exonuclease activity of the viral polymerase scavenged the capped 5’ oligos from host mRNAs and uses them as primers to start the viral mRNA transcription. L and S segment-mRNA are translated by free cytoplasmic ribosomes, whereas proteins that originate from the M segment are translated using membrane-bound ribosomes (Figure 4: Step 2) (67). After primary transcription of viral mRNA, the genome replication takes place. Viral RNPs also act as templates to replicate the full-length cRNA i.e. the antigenomes (cRNAs). These antigenomes serve as templates for the replication of full-length
viral RNA (vRNA) (Figure 4: Step 3) (68-70). Transcription of NSs mRNA occurs from full-length cRNA (Figure 4: Step 4) (1, 71). Assembly and budding of RVFV particles take place in the Golgi apparatus. Glycoproteins are transported through the endoplasmic reticulum (ER), are glycosylated, and accumulate in the Golgi; other viral proteins and RNPs are also transported to this location. It is believed that glycoproteins are responsible for recruiting the core viral proteins to the site of budding. Genomes are packaged into virus particles, which bud into the Golgi apparatus, and leave the cell via secretory pathway (72-75).

**Figure 4:** Different steps of RVFV replication. In the first step, primary transcription of negative-sense vRNA to mRNA occurs. In the second step, L and S segment-capped mRNAs are translated using free ribosomes and the M segment is translated using membrane-bound ribosomes. In step 3, the full-length cRNA templates are synthesized, followed by production of full-length vRNA. In step 4, as the S segment is ambisense in nature, the full-length complementary RNA (cRNA) acts as template for secondary transcription of NSs mRNA.
1.5 Transmission

The bite from mosquitoes carrying RVFV is the main means of transmission to animals (76, 77). Other than mosquitoes, numerous blood-feeding arthropods (ticks and sandfly species) have been suggested to be vectors of the virus (11, 78). RVFV has been isolated from more than 53 species in eight genera of the family Culicidae, and vector competence studies have revealed that RVFV-competent mosquitoes exist all over the world (79). Aedes species especially have a major role in maintaining RVFV in nature during inter-epizootic periods, as the virus is capable of being transmitted from the female host vector to the offspring through the ovum (transovarian transmission) (Figure 5) (80-82). In endemic regions, RVFV-infected Aedes eggs can survive and remain dormant during the dry season. Then seasonal rainfall or floods cause the eggs to hatch, resulting in numerous Aedes offspring that carry virus. The virus remains viable in the offspring until they become adults, at which time they transmit the virus to susceptible domestic and wild animals. This leads to viremia in vertebrates. Uninfected Aedes and other mosquito species (such as Culex) feed on viremic vertebrates, which results in an explosion of mosquito numbers infected with RFVF. Humans can become infected through bites from infected mosquitoes, but another important route of transmission is handling of infected animals, leading to contact with infected tissues such as aborted fetuses, blood, or body fluids, and ingestion of raw milk from infected animals. People working as abattoir workers, herdsmen, farm workers, or as veterinarians are therefore at high risk of RVFV infection (78, 83-88). Infected animals’ body parts, aborted fetal material, and placentas contain large numbers of virus particles, which can either contaminate the local environment directly or infect other animals that are
in close contact (89). Human-to-human horizontal transmission is uncommon, and has only been documented in one fatal case (90). Another important route of transmission for RVFV is exposure to aerosols. Virus has been isolated from human nasal discharge (91), and studies have shown that viral invasion of brain tissues occurred much earlier and was more severe in mice exposed to aerosols than in peripherally exposed mice (92), which increases the risk of using RVFV as bio-terror agent (93). Humans are known to be a dead-end host for RVFV, and the possibility of acting as amplifying host is very low (94). It has also been shown that vertical transmission of RVFV from mother to fetus leads to miscarriage (95).
Figure 5: The RVFV transmission cycle, adapted from (96) with permission. Three routes of transmission have been described for RVFV: vectoral, direct, and vertical.

1.6 Pathogenesis

After a mosquito bite, incoming virus first encounters dermal dendritic cells and replicates at the site of infection, and from there it reaches the local lymph nodes (56). Multiplication of RVFV in the local lymph nodes leads to a systemic viremia. As a result, the virus reaches the primary replication sites i.e. liver, spleen, and brain. Rapid viral replication in these
organs leads to a high viral titer, in turn resulting in hepatic necrosis, lesions in the spleen and lymph nodes. The overall incubation period varies from 3 to 7 days (97-99). RVFV has a broad range of cell tropism, and can be found in almost all body parts of infected animals (100, 101). Susceptibility to RVFV infection is extensive and exhibition of RVF disease symptoms is high in domestic ruminants (e.g. sheep, goats, camels, cows, and buffaloes), and to some extent also in wild ruminants (3).

Disease in domestic ruminants is often associated with symptoms such as anorexia, bloody diarrhea, and nasal discharge. The infection may progress and more severe symptoms occur, such as hepatitis and hemorrhagic fever (97, 102). Infected animals remain highly viremic for between 2 and 7 days after infection, and act as amplifying hosts (6). There is a clear divergence in mortality rates in RVFV-infected animals of different ages. Young animals are more susceptible to the infection, and the mortality rates can reach 100% in newborn sheep, goats and cattle. Adult animals are less susceptible, and have lower mortality rates (between 10% and 30%). A sudden increase in the rate of abortions in sheep and goats often indicates the start of a new outbreak. Thus, the classical hallmark of RVFV outbreaks is termed an “abortion storm” due to the large number of simultaneous abortions in infected pregnant ruminants in herds. Abortion rates are almost 100% in pregnant livestock infected with the virus. The most severe disease is often seen in young animals of exotic sheep and cattle breeds (89, 97-99, 103, 104). As a result, RVF outbreaks cause devastating economic damage to the livestock industry (for both producers and consumers) in affected countries, regarding national and international livestock trade (105, 106).
Human infections are often asymptomatic or entail mild influenza-like symptoms such as fever, weakness, myalgia, dizziness, headache, anorexia, and weight loss (107). The fever is biphasic: the body temperature rises for 2 days, decreases, and then rises again. These symptoms are mostly self-limited and usually resolve within 7 days (99). However, 1–2% of affected individuals suffer from more severe disease, associated with retinitis, renal impairment, neurological manifestations such as meningoencephalitis, and hemorrhagic fever (107, 108). Retinitis and retinal hemorrhage have been seen in infected patients during outbreaks. The retinitis is usually reversible, with complete recovery; however, a more severe outcome can be impaired eyesight or permanent loss of vision (109). Neurological complications occur in less than 1–2% of patients within 1–3 weeks after the onset of disease. Development of severe headache, confusion, or drowsiness would indicate possible encephalitis (99, 110, 111). Most patients recover, but some may live with residual neurological damage (108). The deadly outcome of infection is hemorrhagic fever. Patients develop hemorrhagic features generally as a result of complications arising from acute hepatic injury, multiple organ failure including renal failure, and disseminated intravascular coagulations, which finally leads to death (112-114). In severely affected individuals who are hospitalized, the case fatality rate is approximately 10–20% (115). However, the overall mortality rate in humans is around 2%, but during recent outbreaks the case fatality rate has reached 29% (116, 117).
1.7 Prevention and control of RVFV

Countries affected by RVFV often face significant public health threats due to the high morbidity and mortality in both humans and animals. Extensive loss of economically important livestock species such as cattle and sheep put these countries in financial jeopardy. Most of the RVFV endemic countries are poorly developed and do not have the infrastructure to deal with emergency situations. Moreover, the lack of licensed or commercially available vaccine for humans and the lack of fully licensed vaccines for livestock outside endemic areas create additional burdens for the whole of society.

The first vaccine, which is still in use in livestock, is the live attenuated Smithburn strain (118). It is able to protect non-pregnant adult animals (119) but it causes miscarriage and/or birth defects in fetuses (120-122). This limits its use to non-pregnant female animals in endemic areas (84, 123). Formalin-inactivated RVFV vaccine has also been tested, but it gives a low level of protection, and these kinds of vaccines require multiple inoculations and annual boosters (124, 125). Several different laboratories and research groups have prioritized research in developing RVFV vaccines both for humans and for livestock, but so far none have come up with a safe and efficacious candidate. Efforts have been made to develop vaccines using different approaches such as subunit vaccines, DNA vaccines, virus-like particles (VLPs), virus replicon particle vaccines, virus-vector vaccines, modified live vaccines, and live attenuated vaccines (126-138). Despite these substantial efforts, a safe, fully protective, cost effective RVFV vaccine is yet to be developed.

Currently, there is no approved specific treatment for RVFV infection in humans and animals, other than supportive care. Ribavirin, a
nucleoside analog, is an approved drug for the treatment of selected viral hemorrhagic fevers (139). It inhibits RNA virus replication and has been used to treat hemorrhagic patients during the RVFV outbreak in Saudi Arabia (140, 141). However, the use of ribavirin remains limited as it has shown teratogenic effects in some animal species and can cause undesirable side effects such as hemolytic anemia in humans (142, 143). Ribavirin has also failed to protect mice from RVFV infection when they were exposed through the aerosol route. It has a limited ability to cross the blood-brain barrier, resulting in failure to prevent delayed-onset neurological disease (92).

Favipiravir (T-705) is a non-nucleoside viral RNA-dependent RNA polymerase inhibitor, which was recently approved for use against influenza virus infection in Japan, and the phase-III clinical trial has been completed in the USA (144-147). It has broad-spectrum antiviral activity and efficiently inhibits a wide variety of RNA viruses, such as influenza A virus, Ebola virus, human pneumovirus, paramyxoviruses, Arena viruses, and Phenuiviruses including RVFV in animal models. One study also suggested that favipiravir could protect rats from RVFV infection by aerosol exposure (139, 140, 148-151). However, as yet, no clinical trials using favipiravir for RVFV have been performed. Several other compounds have been reported to efficiently inhibit RVFV infection in vitro and in vivo (139, 152-155), but potent antiviral compounds with improved safety features are urgently needed.
2. Antiviral drugs

The first antiviral drug, idoxuridine, was developed by William H. Prusoff and was approved by the US Food and Drug Administration (FDA) in 1963 for treatment of HSV. Over the last five decades (up to 2016), 90 antiviral drugs were formally approved, but only for nine human viruses (156-158). Many viruses still remain without any antivirals being available. Development of antiviral drugs is not an easy task and have many challenges. It requires a combined effort from chemists, biologists, and pharmacologists. Viral proliferation is strongly linked to the metabolic processes of host cells, and systemic toxicity of the candidate antiviral agent is always a major concern. Moreover, the emergence of antiviral drug-resistant viruses and drug-related side effects make antiviral drug development more challenging, and necessitates further refinement of antiviral drug design and development. An antiviral drug should have some specific features: it should have the ability to reach the virus target organ; it should be chemically and metabolically stable; it should be readily absorbed; it should have the ability to kill/inactivate the virus without affecting the cellular functions; and it should not be toxic, carcinogenic, allergenic, or mutagenic. Many compounds exhibit potent antiviral activity in vitro, but only a limited number of them show activity in animal systems. Of those that have good potency and acceptable toxicity in animals, only a few become antiviral drugs for use in humans. It is believed that it might take 10–15 years for a compound to be approved as a drug from its first proven activity in vitro (159, 160). This explains why,
after five decades of effort, only a few virus infections have treatments available.

Of the antiviral drugs that are available today, half of them are for human immunodeficiency virus (126); approximately one-third are for treatment of herpes simplex virus (HSV), human cytomegalovirus, varicella-zoster virus, and influenza virus infection; and the rest of them are for hepatitis B virus, hepatitis C virus, respiratory syncytial virus (RSV), and human papillomavirus (HPV) infections (157, 158). These 90 antiviral drugs have been categorized into 13 functional groups based on their mode of action and/or targets. These antiviral drugs acts using several mechanisms of action that include inhibition of viral penetration, uncoating, nucleic acid synthesis, integration of viral DNA into the host genome, maturation, and release from host cells. Another group of antiviral drugs are interferons and immunostimulators to treat hepatitis B virus, hepatitis C virus, and HPV. This group of drugs shows specific antiviral activity without directly targeting viral proteins (158).
2.1 General aspects of antiviral drug mechanisms
Viruses have different ways of infecting cells to maintain the continuation of progeny production. However, all the human viruses have a common set of steps or stages in their life cycle (Figure 6). All of these stages of the virus life cycle can be targeted by antivirals. Nowadays, antiviral drugs are available that target most of the stages of the virus life cycle.

Figure 6: General steps and antiviral drug targets in the virus life cycle. Reprinted from (161) with permission.

2.1.1 Inhibitors of viral attachment and entry
From a therapeutic point of view, aiming at virus-cell attachment or virus entry is an attractive strategy, as this is the first opportunity to inhibit the virus life cycle. Prevention of virus binding to the cell surface and entry into the cell also prevents all the subsequent steps in virus infection, which
allows the immune system to counteract and clear the virus and thus inhibit spread of virus within the tissue. Antiviral drugs that inhibit viral attachment and entry have been developed using two approaches. One approach is creation of drugs that bind to the virion and block fusion with cellular receptors. Enfuvirtide is one such drug; it is a synthetic peptide, and is also called HIV fusion inhibitor (162). Another approach is for the compound to target the cellular receptor, thereby preventing virion attachment. An example is the anti-HIV compound TAK-779, which binds to the cellular receptor (CCR5) and inhibits binding of virus to the cells (163, 164).

2.1.2 Inhibitors of penetration and uncoating
One of the major steps in the virus life cycle is penetration of the virus into the cells, and uncoating and release of the viral genome into the cytoplasm. Uncoating procedures are highly variable and depend on the nature of the virus and the cells, although the stepwise process is similar for most viruses. Successful completion of this process enables the release of genome to the cellular machinery, and so leads to transcription and replication. Development of antivirals that target these events can restrict virus infection. One efficient uncoating inhibitor is amantadine. This compound binds and blocks the hydrogen ion (H+) channel formed by the influenza A virus M2 protein. Lack of H+ prevents the dissociation of M1 matrix proteins from the nucleocapsid (uncoating), so the nucleocapsid cannot be released to the replication machinery (165-167).
2.1.3 Inhibitors of genome replication

Inhibition of viral genome replication is one of the most successful drug development strategies. Most antiviral drugs are nucleoside analogs and inhibit viral genome replication, and almost all of them inhibit viral DNA polymerase. The two main categories of nucleoside analogs are anti-herpesvirus agents and anti-HIV agents. Some of these drugs are actually nucleotide analogs, as they mimic the nucleoside monophosphates.

Nucleoside or nucleotide analogs enter cells through specific nucleoside transporters. Nucleoside transporters are membrane pumps that allow the uptake and/or the efflux of nucleosides by cells. Upon entering into the virus-infected cell, these drugs must be activated by phosphorylation mediated by viral or cellular kinases. Phosphorylated nucleosides act by inhibiting viral polymerases, and by being incorporated into newly synthesized viral DNA or RNA. Incorporation into growing DNA leads to the termination of chain elongation, or altered base recognition and base pairing cause base modification, leading to the accumulation of inactivating mutations in virus progeny (168). The anti-herpesvirus drug acyclovir is one of the archetypal antiviral nucleoside analogs that inhibit viral nucleic acid chain elongation. Another analog is ribavirin; it has been used clinically for hepatitis C virus, RSV, and hemorrhagic fever virus infections (169-171). Ribavirin causes lethal mutagenesis of viral RNA genomes, potentiates the action of interferons, and inhibits viral transcription (172-174).

2.1.4 Inhibitors of virus assembly and maturation

Virus assembly and subsequent events are attractive targets for antiviral drug discovery, since they are indispensable for formation of an infectious
virion. Virus-encoded proteases are essential for viral maturation, because they catalyse the processing of viral polyproteins and this catalytic activity is required for the production of infectious virions. Drugs that target this protease activity have been developed for HIV and hepatitis C virus, and have been approved for clinical practice. Most of these protease inhibitors were designed as peptidomimetics of the viral peptide substrates; thus, they bind to the cleavage site and prevent the enzyme from releasing the individual core proteins so that new virus particles cannot mature and become infectious. Examples of these anti-HIV drugs are saquinavir, ritonavir, and tipranavir (175-178). Two other drugs that selectively target non-structural protein 3 (NS3) and NS3/4A protease of hepatitis C virus are boceprevir and telaprevir (179, 180).

2.1.5 Inhibitors of virion release

Release of new progeny virus from the host cell is the last step in the virus life cycle. Drugs that inhibit virus release from the host cell have been developed for influenzavirus infection and approved for human use. The influenzavirus neuraminidase inhibitors zanamivir and oseltamivir prevent the release of newly produced virus from the host cell by inhibiting the virus-encoded neuraminidase. At the end of the infectious cycle, hemagglutinin on nascent influenzavirus particles binds to cell-surface sialic acid, preventing virus from being released from the host cell. To overcome this problem, virus-encoded neuraminidase cleaves sialic acid from the cell surface. Neuraminidase inhibitors are designed to limit influenzavirus release by blocking this enzymatic activity (181-183).
2.2 Approaches used for antiviral drug discovery

During the early stage of drug development history, the general belief was that due to the close relationship between the virus and its host cell, it would be difficult for any antivirals to inhibit infection without also affecting the host. Despite this consideration, various strategies have been adopted to develop antivirals, and some licensed antiviral drugs have been established. However, antiviral drug discovery and development is still a long, complex, and multi-stage process with lots of challenges, where the probability of success is relatively low. Challenges regarding the low success rate are that most of the candidate drugs are inactive in animal models or in patients, show undesirable side effects or toxicity upon in vivo administration, and have poor pharmacological properties. To overcome these challenges and improve the efficiency and success rate, it is crucial for basic scientists and technology developers to determine areas that require improvement and fruitful innovation (158, 184).

Basic research in the academic institutions and industry is the mainstay of the drug discovery process. Drug discovery is mostly represented as a linear, consecutive process that begins with target and lead discovery, followed by lead optimization and pre-clinical studies in vitro and in vivo. These studies determine whether the compounds fulfill a number of pre-set criteria that are required for initiation of clinical development. In general, each phase of the drug discovery process must be completed before proceeding to the next phase (Figure 7).
The remaining parts of this section will mainly focus on describing the early phases of the antiviral drug discovery procedure, as the purpose of this thesis is to assay development and identification of chemical compounds with potential antiviral activity.
2.2.1 Target identification

Target selection plays a crucial role in the successful identification of hit and lead compounds. In most cases, the critical parts of the virus life cycle, i.e. genes and proteins that are essential for virus replication, are suitable targets, since it is relatively easy to modulate them. Before selecting the target, one must consider several criteria. The target should be crucial for the viral life cycle, and it should be easily accessible to the candidate drug (i.e. the target should be druggable). The targets selected can be either the virus or the host cell, and both have advantages and disadvantages. Development of drugs that target viral components will have high specificity and fewer side effects, as it should inhibit only viral replication without affecting the host cell machinery. However, some antiviral drugs have strong side effects, e.g. viral protease inhibitors. A limitation of this approach would be that the resulting drug may not have broad antiviral activity; sometimes it may not be active against different serotypes of the same virus. Another limitation of this approach is that due to high genetic adaptability, the virus may develop drug-resistant mutants (185, 186).

To avoid these limitations, identification of targets derived from the host can sometimes be a better alternative. Using this approach, a drug with a broad spectrum of activity might be developed—as many viruses use common host cell machinery for progeny particle production, but this approach can lead to a drug that has adverse side effects on the host. However, regardless of targets, it is desirable to develop antivirals with a broad spectrum of activity and few side effects—or none at all. This goal is difficult to achieve and requires collaborative work between academics, biotechnology firms, and the pharmaceutical industry (187).
2.2.2 Assay development and high-throughput screening (HTS)

This is entry point of the chemist in the drug discovery process. HTS is currently the most widely used approach to identify compounds of interest from a library containing thousands of chemical compounds with known or unknown characteristics. The possibility of using fully automated robots for liquid handling and pipetting makes HTS even more rapid and easy. HTS assays can be performed in several ways, based on the number of compounds screened, the assay protocol, and the format. However, target- and cell-based assays are often the only practical and available tools for drug discovery programs. In a target-based assay, the target of interest (a protein, enzyme etc.) is first produced, isolated, and purified and then an assay is developed to monitor and measure the interaction between the target and other compounds. Target-based assays often provide information about the mode of action of the hit compounds, or at least give an understanding of the kinetics of the process involved. Identification of hits through this assay does not always confirm the true activity, as real cell culture or physiological conditions may differ from the assay conditions. Follow-up evaluation is therefore needed to determine the off-target effects (184, 186).

In the phenotypic or cell-based assay, the effect of a compound on virus infection of a host is measured. A cell-based infection assay with the full infection cycle allows discovery of putative antiviral agents that can effectively act on any stage of the virus life cycle and provide the opportunity to discover new drug targets. Here the readout can be cell viability, virus titer, or a fluorescence-based assay to monitor the viral infection. The readout does not always confirm that the compound of interest inhibits virus replication, as it can also inhibit host cell functions...
that can lead to virus inhibition and/or cell toxicity. Therefore, a follow-up assay must be performed to identify and exclude cytotoxic compounds. Before starting the screening campaign, the assay has to be optimized and validated. It is very important to confirm the uniformity and reproducibility of the results and determine the positive and negative signals in order to identify true hit compounds. The Z factor can be used to determine the assay performance and quality (184, 186, 188).

One of the crucial steps in HTS is to select the library of compounds. A desirable library can be diverse, so that it covers a large chemical space. While designing the assay, it is also important to select those compounds that would be expected to have antiviral activity. Compounds that have been approved in humans for other reasons are also interesting, as the mechanism may already have been studied and it could shorten the development procedure. Currently, it is very common to screen approved drugs that are used for other diseases, for their antiviral properties (189).

A new alternative antiviral screening approach that is gaining more and more attention is virtual screening or computer-aided drug design. Here, the crystallographic structure of the target is used to identify the ligand compound by using computational methods to perform \textit{in silico} screening of hypothetical structures and compounds. Hit compounds that are identified through this process still need to be confirmed by biological assays (186).
2.2.3 Hit confirmation and lead identification
After all the criteria are assured and assay validation is complete, the primary HTS campaign is performed. In most cases, to minimize the cost, all the compounds are initially screened at a single concentration in one experiment. Based on the control, selection criteria are set to determine the hit compounds. Hit compounds identified from primary screening must be validated in a secondary screening campaign, possibly at several concentrations to ensure their activity, potency, and efficacy with dose-response curves. The efficacy of identified hits should then be further confirmed with orthogonal assays. The cytotoxic effects of the compounds also need to be determined. Based on the both antiviral and cytotoxicity profiles, the compound(s) is/are selected for molecular optimization by medicinal chemistry to improve the potency, safety, and efficacy. Structure-activity relationship (SAR) analysis assesses the chemical space around a compound with the goal of finding structural analogs with improved activity. In order to perform the SAR analysis, analogs can be purchased or synthesized. In most studies, analogs are synthesized in generations and the activity of each generation is measured and considered before synthesizing the next. The SAR analysis allows narrowing down of the number of hits to a few compounds with improved activity and reduced toxicity. These optimized hits are considered as leads.

After lead identification, it is desirable to perform experiments to understand the mechanism of action of the lead compound, although it is not necessary for the advancement to pre-clinical development (186).
2.2.4 Lead optimization

Lead optimization can be performed with a variety of parameters using both *in vitro* and *in vivo* assays. A compound may be highly potent in cell culture, but this does not necessarily mean that it would have same potency in an animal model. It is therefore crucial to analyze the pharmacokinetic (PK) properties of the compound, both in cell culture and in animal models. This analysis determines the potential of the compound to be used as a drug in humans. PK analysis includes absorption, distribution, metabolism, and excretion (ADME) properties of the compound in cell culture. If the compound has desirable properties, then PK analysis is performed in animals using different routes of administration. If any undesirable results are obtained, then the reasons should be found out and solved. For instance, the pharmaceutical formulation of the compound can be optimized to increase the oral bioavailability, or the medicinal chemistry of the compound can be optimized and modified. Finally, the PK properties of the compound could allow investigation of the antiviral efficacy of the compound in an animal model, as a proof-of-principle antiviral effect, and thus accelerate its further development as a drug.

2.2.5 Drug development

This is the second phase of the antiviral drug discovery process. At this stage, pre-clinical studies of candidate drugs are performed. Several key properties must be addressed and passed before the candidate drug can go to clinical trials. This includes (i) the pharmacological profile of the drug, (ii) acute toxicity of drug in at least two species of animal, (iii) short-term toxicity results, depending on the proposed duration of use of the drug. Full ADME has to be analyzed to understand the absorbance rate in blood,
the distribution throughout the body, the toxicity of the drug metabolites, and its rate of excretion. After fulfilling all the criteria, clinical trials can be performed after a proper approval from a national authority or the European Medicines Agency (EMA). Briefly, the clinical trials are performed in three phases. In the first phase, the candidate drug is tested in a small group of healthy volunteers to evaluate its safety without apparent side effects in humans. In the second phase, the drug is tested in a relatively larger group of patients (100 to 500) who actually have the disease. Phase-III trials include possibly thousands of patients with the disease. These patients are closely monitored clinically to understand the safety, efficacy, and an overall benefit/risk factor. If the drug is passed in clinical trials, then it can be marketed for patient use with approval from medical agencies (160).

The discovery and development of drugs is no longer a single-institution endeavor. Close collaboration, communication, and exchange of knowledge between academics and the pharmaceutical industry are a key factor for a successful drug development program.
3. Aims

The overall aim of the work in this thesis has been to identify and characterize chemical compounds with antiviral activity against RVFV.

The specific aims were:

I. To evaluate the anti-RVFV activity of benzavir-2, and investigate its pre-clinical pharmacokinetics and oral bioavailability.

II. To develop a whole-cell-based screening assay, screen a library of small chemical compounds for their anti-RVFV properties, and select potential hits.

III. To perform SAR analysis of selected hit compounds identified from screening for structural optimization and biological evaluation.
4. Methodology

4.1 Cell-based high-throughput screening assay

Fluorescence-based assays are being used widely for antiviral drug screening procedures. We used a recombinant replication-competent (NSs-deleted) RVFV (rRVFVΔNSs::Katushka) throughout our studies, unless stated otherwise. The NSs gene was replaced with a red fluorescent marker, Katushka, thus allowing us to quantify the efficacy of viral infection in cell culture by measuring fluorescence in the red spectrum. By using this modified RVFV, we developed an HTS assay and screened a library of chemical compounds to identify their anti-RVFV activity in BSL-2 conditions. For the primary screening campaign of 28,437 compounds, 5000 A549 cells (a human lung epithelial cell line) were seeded/well of 384 well plate using a Matrix WellMate dispenser (Thermo Fisher Scientific, Langenselbold, Germany). Next day, compounds were diluted from the stock plate to a dilution plate and added to the cells with the Biomek NXp automated multichannel workstation (Beckman Coulter, USA) with the final concentration is 50 µM. Next the rRVFVΔNSs::Katushka (MOI-3) was added to the cells using the Matrix WellMate dispenser (Thermo Fisher Scientific, Langenselbold, Germany). The fluorescence intensity (infection efficacy) was measured with a Synergy microplate reader after 16 h of infection (Figure 8), as we found that at 16 h the fluorescence expression was high and had reached a plateau and katushka signal can easily be observed and quantified with a fluorescence microscope or a plate reader. After measuring the antiviral activity, we also measured the cytotoxic effects of the compounds.
Figure 8: Primary and secondary screening of chemical compounds.

The cytotoxicity assay was performed using the resazurin cell viability assay (Sigma-Aldrich). Resazurin is a colorless dye; upon contact with living cells with active metabolism, it is reduced to the pink fluorescent dye resorufin. To determine the toxicity of screened chemical compounds, after measuring the katushka expression, we added resazurin (at a final concentration of 40 µM) to the wells, which was followed by incubation for 3‒4 h at 37°C in an atmosphere of 5% CO₂, and the fluorescence intensity of resorufin was measured with a Synergy microplate reader (Figure 8). After primary screening, we performed a secondary screening of the selected 641 hits at four different concentrations (25 µM, 12.5 µM, 6.25 µM and 3.12 µM) following the same protocol as primary screening.
4.2 Translational inhibition analysis

The translational inhibition assay was performed to ensure that inhibition of Katushka expression by chemical compounds was due to antiviral activity. To perform the assay, HEK293t (human embryonic kidney 293) cells were transfected with an SV40 expression plasmid encoding the Renilla luciferase gene. The transfection reagent and plasmid were removed after 2.5 h of incubation, and compounds were added to the cells. After 24 h of incubation at 37°C in an atmosphere of 5% CO₂, luciferase expression was measured with the Renilla luciferase assay system (Promega) with a Tecan plate reader.

4.3 Plaque reduction neutralization test (PRNT)

PRNT was used as an orthogonal assay to detect the antiviral activity of some of the compounds selected. PRNT allowed us to evaluate the ability of the compounds to reduce the number of plaques, formed from cytopathic effect of the virus. Briefly, pre-seeded Vero B4 cells were infected with RVFV clone 13 (40 PFU/well) for 1.5 h at 37°C. Unbound viruses were removed and the cells were covered with growth medium, containing compounds, and Avicel (1:1), and incubated for 72 h. The overlay was then washed away and the cells were fixed with 4% paraformaldehyde (PFA) and stained with crystal violet (1%) for plaque counting.
4.4 Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Real-time qRT-PCR was used to assess the antiviral activity of the compounds. This is very a sensitive assay, which is capable of distinguishing between small variations in genomic numbers. The assay was used to quantify newly synthesized viral RNA in response to chemical compound treatment, using specific primers and probe corresponding to the L segment, which encodes the RNA-dependent RNA polymerase of RVFV. In parallel, we also detected the cellular actin mRNA and the Ct values of RVFV RNA were normalized to that of actin mRNA; RVFV RNA was quantified by relative quantification (190).

4.5 Production of progeny virus

We determined the production of RVFV progeny particles in response to benzavir-2 treatment. Briefly, A549 cells were infected with virus for 1 h in the presence of different concentrations of benzavir-2. Then the virus inoculum and benzavir-2 mixture was removed and replaced with fresh medium containing benzavir-2, and incubated for another 15 h at 37°C. After incubation, the supernatants were collected and used to infect new A549 cells for 16 h at 37°C. Finally, numbers of infected cells were counted using a Trophos Plate Runner HD.

4.6 Time-of-addition assay

Understanding the mechanism of action of active compound is an important step in antiviral drug development. A time-of-addition assay is a good start to get an idea of the possible mode of action of an antiviral compound. The time based assay determines how long the addition of
compound can be postponed before losing its activity in cell culture. The standard way of performing this assay is to first infect the cells with virus for 1 h; followed by wash away the unbound virus and adding the test compound at different time points (0 h, 1 h, 2 h, 3 h, ....) after virus infection. Then the plate is incubated for certain time period based on the type of virus (DNA or RNA virus) used. Thereafter the virus infection is monitored.

We performed time-of-addition assays for both benzavir-2 (paper I) and compound 13a (paper III) by using the fluorescent cell foci assay. In the case of benzavir-2, A549 cells were treated with benzavir-2 (at a final concentration of 20 µM) for a 2-h period before or after infection for up to 8 h. After 8 h of infection, the cells were fixed with 3% paraformaldehyde (PFA) for 1 h and the number of infected cells in each well was quantified using a Trophos Plate Runner HD. In the case of 13a, A549 cells were infected with RVFV and the compound (at a final concentration of 50 µM) was added 1 h before infection, at the time of infection (0 h), and 2, 4, 6, and 8 h after infection. After 13 h of infection, the cells were fixed with 3% PFA and cellular nucleoli were stained with 0.1% DAPI (4’,6-diamidino-2-phenylindole), and the number of infected cells was determined using a Trophos Plate Runner HD.
5. Results and discussion

Paper I

Anti-Rift Valley fever virus activity in vitro, pre-clinical pharmacokinetics and oral bioavailability of benzavir-2, a broad-acting antiviral compound.


Benzavir-2 is an antiviral compound that is known to be active against human adenovirus (HAdV), HSV-1, and HSV-2. It was previously developed by identifying benzavir-1 as an inhibitor of HAdV infection through a cell-based screening assay. Later, the substance was modified and developed as benzavir-2 using SAR analysis, with improved potency and low cytotoxicity (191). Benzavir-2 was also found to be a potent inhibitor of acyclovir-resistant clinical isolates of HSV-1 and HSV-2 (192). HAdV and HSVs are DNA viruses, but they have differences in their infectious cycle, indicating that this was a broad-acting antiviral mechanism. Hence, the idea was to determine whether benzavir-2 is also active against RNA viruses. For this reason, we chose to determine the antiviral activity of benzavir-2 against RVFV. We also wanted to assess its pre-clinical PK parameters, which would allow evaluation of the antiviral efficacy of benzavir-2 in vivo.

As benzavir-2 was developed from benzavir-1 and both of them efficiently inhibited HAdV and HSV infections in vitro, we first examined the anti-RVFV (rRVFVΔNSs::Katushka) efficacy of benzavir-
2 and four of its structural analogs (including benzavir-1) with a cell-based fluorescence assay. Briefly, A549 cells were infected with virus—with and without compounds—and incubated for 16 h. After incubation, the antiviral activity of benzavir-2 and its analogs was evaluated by counting the number of infected cells (i.e. fluorescing cells) in each well. We found that benzavir-2 was the most potent inhibitor of RVFV infection among the five compounds tested, with an EC$_{50}$ (half maximum effective concentration) value of 0.6 µM. Interestingly, the trend of potency observed was similar to previous studies using HAdV (191), and the sigmoidal dose-response curve of benzavir-2 for RVFV was similar to those from testing it against HAdV and HSV in earlier studies (192). As benzavir-2 was the most potent antiviral, we evaluated its potency in inhibiting RVFV RNA expression further using a qRT-PCR assay. It inhibited RNA expression in a dose-dependent manner with an EC$_{50}$ value of 1.7 µM. However, this potency did not always confer the highest level of inhibition. Thus, apart from potency determination, maximum reduction of virus replication should be assessed in a virus-yield or titer-reduction assay, to give an idea of the compound’s efficacy (186). We then determined whether benzavir-2 would affect the production of progeny RVF virus, and found that it efficiently inhibited the production of infectious progeny particles.

The mechanism of action of benzavir-2 is presently unknown. It inhibits both DNA and RNA viruses with similar potency, even though these different viruses use different cellular machinery (HAdV and HSV replicate in the nucleus and RVFV replicates in the cytoplasm). Thus, it is likely that benzavir-2 has one or more host cell targets that are shared by HAdV, HSV, and RVFV during their life cycles.
In this study, we performed a simple time-of-addition assay to better understand the timing of the antiviral effect and to obtain a hint of where in the virus replication cycle benzavir-2 exerts its antiviral effect. Our data indicated that benzavir-2 was most active when added during the early phase of the virus life cycle. Preliminary studies in our group have suggested that benzavir-2 does not inhibit the attachment and entry of RVFV to the cell (unpublished data), indicating that it acts on an intracellular target that is essential in the early phase of the RVFV replication cycle (e.g. transcription and/or translation).

As we found that benzavir-2 was a possible inhibitor of RVFV, we determined its PK properties by performing in vitro ADME and in vivo PK studies in mice. The ADME studies were performed with both benzavir-1 and 2, and both compounds had similar properties. These compounds showed good solubility and permeability in both human and mouse cells. Clearance of benzavir-2 was also comparable for human hepatocytes (4.1 ± 0.4 µl/mg/min), liver microsomes (3.8 ± 0.4 µl/mg/min), and mouse liver microsomes (3.8 ± 0.9 µl/mg/min). Both compounds were highly bound to plasma protein, but this did not affect the tissue distribution of the benzavir-2 that we later found in the in vivo studies. In order to perform in vivo PK studies, we first assessed several excipients so that the solubility of benzavir-2 could be increased and an acceptable concentration could be reached. Finally, two formulations were selected for use in the whole experiment, and the choice of formulation did not influence the PK properties of benzavir-2 in any way. The formulations were administered to mice by different routes (either intraperitoneally, intravenously, or orally) and with different dosages. We found that benzavir-2 had good oral bioavailability properties. Our data also indicated
that it had very fast extravascular absorption and relatively fast disposition and elimination, irrespective of the route of administration. This is sometimes a drawback in an *in vivo* efficacy situation, as it requires repetitive administration to maintain a continuous presence in the system.

The using of liquid formulation for the *in vivo* PK assessments of benzavir-2 restricted us to use the maximum administered dose in mice. Furthermore, we wanted to understand the possible negative clinical signs that can be associated with a high dose of benzavir-2 in mice and to possibly obtain a high systemic concentration *in vivo*, without repeated injections. Mice also become more stressed with repetitive drug administration, which sometimes have effects on mice response to drug treatment and on overall experiment results. To address this issue, we used a peanut butter (PB) pellet-based formulation containing benzavir-2 (60 mg/kg), administered to mice orally. This allows repetitive oral dosing in mice without being stressed. PB formulation has been used successfully in other studies (193, 194). Benzavir-2 was mixed with PB to high concentrations, and the mice were fed PB pellets. The benzavir-2 concentration in plasma was measured, and we observed a satisfactory level of benzavir-2 in plasma 6 h after administration (15 to 20 µM). The benzavir-2 concentration in plasma was even higher when measured 3 h after administration of PB pellets. Interestingly, no adverse effects (weight loss or negative behavioral signs) were observed with high doses of benzavir-2 in PB over the period of the study. Thus, benzavir-2 was well tolerated in mice, and the concentration in plasma was high enough to possibly give full antiviral effectiveness *in vivo* when correlating with the EC$_{50}$ value (0.6 µM) observed in cell culture.
In summary, in this study we found that benzavir-2 was a potent inhibitor of rRVFVΔNSs::Katushka \textit{in vitro}. This substance also showed a promising pre-clinical profile \textit{in vivo}, and oral administration in PB pellets gave high systemic exposure. Moreover, benzavir-2 was well tolerated in mice at high concentrations without having any negative effects. These results suggest that studies on the antiviral efficacy of benzavir-2 in \textit{in vivo} models would be valuable.
Paper II
High-Throughput Screening Using a Whole-Cell Virus Replication Reporter Gene Assay to Identify Inhibitory Compounds against Rift Valley fever virus Infection.
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Searching for new antiviral candidates is always a challenge, and requires collaborative efforts by people from different backgrounds, with expertise in different subjects. In this study, we wanted to develop a screening platform that would allow us to identify small chemical compounds with antiviral activity against RVFV. There are mainly two approaches for development of new antivirals: the target-based assay, targeting specific proteins or pathways of the virus lifecycle, and the phenotypic or cell-based screening assay. In most cases, with the target-based assay one has a preconceived idea of the mechanism of action, with limitations such as non-target interaction, which can lead to toxicity. Furthermore, permeability issues may arise at later stages of the study, when the compounds identified are analyzed in cell culture assays. To overcome this issue, a cell-based assay can be used instead. The main drawback of the cell-based assay is not having any knowledge of the targets or mechanisms of action of compounds identified. Bearing this in mind, we developed a whole-cell-based HTS assay to screen a large library of chemical compounds. We used the recombinant, replication-competent (NSs-deleted) RVFV virus containing red fluorescent reporter gene Katushka (rRVFVΔNSs::Katushka), which allowed us to quantify the virus infection
by measuring the expression of red fluorescent protein. This construct was originally generated by our collaborators, and was similar to their construct of a GFP expressing virus rRVFVΔNSs::GFP (136).

Before starting the screening of the compound library, several conditions were optimized and fixed to ensure the accuracy, uniformity, and robustness of the assay. We optimized cell culture plates, cell density, virus MOI (multiplicity of infection), and incubation time, and also performed a dimethyl sulfoxide (DMSO) tolerability test. After evaluating all the different parameters, we used 5,000 A549 cells per well of a 384-well plate, infected the cells with rRVFVΔNSs::Katushka at a MOI of 3, and incubated for 16 h. The compounds were added to the cells at the same time as the virus to ensure identification of compounds that were active at any stage of the virus infection cycle. The final working concentration of the compounds was 50 µM. At the same time, we also performed a cytotoxicity assay to exclude all the compounds that had adverse effects on the cells. We used an automated dispensing robot to ensure the accuracy of dispensing amounts of compounds and also of dispensing other liquids. After optimization and validation, we screened 28,437 compounds. After primary screening, we identified 641 compounds as primary hits that showed >80% inhibition of virus infection (i.e. reduction of fluorescence) and >50% cell viability at a concentration of 50 µM.

The Z factor is used to quantify the accuracy of an assay to be used in full-scale high-throughput screening (188). Statistically, the ideal Z factor is 1.0. Experiments having a Z factor of between 0.5 and 1.0 are considered excellent, and those between 0 and 0.5 are considered to be marginal. If the Z factor is below zero, then the assay cannot be used in
HTS. After performing the primary screening, we calculated the Z values for all the screened compounds and found that the average Z value was 0.54, which confirmed that the HTS assay developed was reliable and that the compounds identified from primary screening data were true hits. After identifying the primary hits, we performed a secondary screening of the 641 compounds at four different concentrations, following the same protocol as primary screening. From the secondary screening, we identified 63 compounds as hits (with a cut-off of > 60% reduction of fluorescence at 3.12 µM).

In the next step, we performed a dose-response validation assay of these 63 compounds. Here, we quantified the fluorescence intensity of individual infected cells by identifying single cells expressing Katushka protein. From this assay, we selected six compounds that inhibited rRVFVΔNSs::Katushka infection in a dose-dependent manner and exhibited high inhibitory activity with IC₅₀ values ranging from 2.1 µM to 7.0 µM. The fluorescence-based assay is based on correct transcription (by the viral RNA polymerase) and translation (by the cellular machinery) of the Katushka reporter gene. We therefore performed a translational inhibition assay of three selected compounds to confirm that inhibition of Katushka expression was due to inhibition of virus, and not because the compounds inhibited the translation of Katushka mRNA. None of the compounds tested inhibited the translation, confirming that they were inhibiting some step(s) in RVFV infection. We also confirmed the anti-RVFV activity of these three compounds by performing an orthogonal PRNT assay.

In summary, we developed a sensitive and robust whole-cell virus replication reporter HTS assay and demonstrated that it is an
effective method for identification of possible antiviral candidates against highly pathogenic RVFV under BSL-2 conditions. Moreover, we identified several candidate compounds with anti-RVFV properties and low toxicity.
Paper III

Structural modifications and biological evaluations of Rift Valley fever virus inhibitor identified from chemical library screening.

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In this study, we selected N\textsuperscript{1}-(2-(biphenyl-4-yloxy)ethyl)propane-1,3-diamine (designated compound 1) as our parent compound; this was originally identified from our screening in previous studies described in paper II. Compound 1 has two parts: one is a biphenyl group, which is connected by an ether bond to the other part, a 2-(3-aminopropylamino)ethyl chain. We wanted to assess which part of compound 1 was chemically more important for its antiviral activity, and to possibly discover analogs with improved potency and reduced toxicity. We therefore performed SAR analysis of compound 1.

SAR is a useful way of molecular optimization of any potential compound using medicinal chemistry, to improve the safety, potency, and efficacy. One of the key goals of SAR analysis is to quantify the potency of identified HTS hits by measuring the EC\textsubscript{50} and/or the EC\textsubscript{90}. In addition to potency and maximum efficacy, the CC\textsubscript{50} values of hits and their analogs should also be evaluated (186).

First, we synthesized the first-generation analogs and evaluated their biological activities, which led to the synthesis of the second-generation analogs. A total of 25 compounds were synthesized. For biological evaluation of the compounds, we used rRVFVΔNSs::Katushka and two different assays (fluorescence assay and qRT-PCR) to evaluate
the antiviral activity of synthesized analogs. The resazurin cytotoxicity assay was used to evaluate the cytotoxicity of the compounds.

For the first-generation compounds, we determined the importance of the 2-(3-aminopropylamino) ethyl chain of compound 1. Eleven analogs were synthesized by modifying the length and adding a cyclic ring at the end of the chain. The general conclusion from the experiments with the first-generation compounds was that the length of the chain was important in order to retain the antiviral activity. Adding a butyl piperazine to the para-position of the biphenyl group (compound 9a, EC$_{50}$ = 12.8 ± 0.2 µM, CC$_{50}$ = 74.8 ± 1.2 µM) gave a similar antiviral and cytotoxicity activity to that of compound 1 (EC$_{50}$ = 12 µM, CC$_{50}$ = 86 ± 9 µM). Therefore, we moved to the second-generation compounds by keeping the butyl piperazine ring linked to the biphenyl group. Here, fourteen analogs were synthesized and after performing several modifications at the biphenyl group, we could not detect any pronounced improvement in antiviral activity. We observed a slight improvement in antiviral activity when an isopropyl group was added to the different positions of the outer ring of the biphenyl group. On the other hand, when we determined the CC$_{50}$ values, we observed that these analogs were highly toxic to the cells. When we studied the stereo confirmation of the biphenyl part, we found that moving the piperazine-phenyl linker to the ortho-position of the biphenyl group (compound 13b) had an adverse effect on the compound’s antiviral activity. The antiviral activity improved, to a level similar to that of compound 1 and compound 9a, when the piperazine-phenyl linker was added to the meta-position (compound 13a, EC$_{50}$ = 13.8 ± 5.3 µM). We also observed an improved cytotoxicity
profile ($CC_{50} = 144.8 \pm 5.5 \mu M$) when the phenyl was located in the meta-position.

Taking into account both the antiviral activity and the cytotoxicity of all the compounds, compound 13a was the best compound among all the synthesized analogs, though it had a low selectivity ratio ($CC_{50}/EC_{50}$). Theoretically, a drug candidate is expected to have a very low $EC_{50}$ value. The higher the value, the higher the dose needed to achieve the inhibitory effect and the higher the possibility of toxicity. However, compounds with high $EC_{50}$ values can also be potent antiviral drugs. For instance, the broad-spectrum antiviral compound favipiravir is active against RVFV with an IC$_{50}$ value of 31 µM (145, 146, 150). Another promising broad-spectrum antiviral compound, galidesivir (BCX4430), which is an adenosine nucleoside analog, has a wide range of $EC_{50}$ values that vary from ~3 to ~68 µM; and a phase I clinical trial has been performed for this compound (195). Galidesivir has been shown to be able to limit RVFV infection in Syrian golden hamsters, despite having a very narrow selectivity ratio in vitro (196).

We wanted to understand at which stage of the RVFV infection cycle the compound 13a was active. We therefore performed time-of-addition assay to better understand the mode of action of compound 13a. This study suggested that compound 13a was active at the early stages of the virus replication cycle, most likely attachment and/or post-entry events. However, more studies should be performed to investigate this further.

In conclusion, we performed SAR analysis of novel anti-RVFV compound 1 and modified it to compound 13a, which had a better cytotoxicity profile. Based on the selectivity index (SI), it was the best of
all the analogs synthesized that might target the early and/or post-entry stages of the RVFV life cycle.

**Figure 9:** Overall summary of paper II and III.
Concluding remarks and future prospects

RVF has a significant socioeconomic impact on affected countries, as it causes recurrent outbreaks in endemic regions, with the latest being reported in Kenya in June 2018, by the World Health Organization (197). The lack of protective vaccines and treatments for humans and animals make this disease very difficult to combat.

In the first study described in this thesis, we evaluated the anti-RVFV activity of the previously reported antiviral compound benzavir-2. We found that benzavir-2 efficiently inhibited RVFV infection in cell culture, and had similar antiviral activity to that reported in previous studies using other viruses. In addition, PK studies revealed that benzavir-2 has good solubility and permeability \textit{in vitro}, and good tissue distribution \textit{in vivo}. The oral bioavailability of benzavir-2 was also good, but it showed very fast systemic elimination. We overcame this issue by administering benzavir-2 orally in a PB formulation. Overall, benzavir-2 is a promising antiviral compound \textit{in vitro} and has the potential to be active against RVFV in \textit{in vivo} models.

The mode-of-action studies of benzavir-2 are ongoing, and preliminary results (unpublished data) suggest that it might target replication and/or translation in the RVFV life cycle, but more studies are required to confirm this issue. Interestingly, it has a broad-acting effect against both DNA and RNA viruses, which suggests that it targets a common cellular pathway. Most drugs selectively target a single virus, and viral pathogens rapidly develop resistance to conventional drugs. Most of the antiviral drugs in use today inactivate viral target molecules. In contrast, when targeting cellular pro-viral factors, broad-spectrum
antivirals can cover several viruses and genotypes and still avoid resistance. Thus, the development of broad-spectrum antivirals that target cellular factors is a novel and interesting concept in antiviral drug development (198).

In the second study (papers II and III), we developed an HTS assay and screened a large number of chemical compounds with unknown characteristics. This assay proved to be efficient and useful, and it could be used as tool to screen more compounds for their anti-RVFV properties, possibly already known compounds or FDA-approved drugs that are used for other purposes. Our HTS assay allowed us to identify small molecules that were active against RVFV as primary hits. We then performed SAR analysis on one of the selected hits (compound 1) and improved its cytotoxic profile (compound 13a). The overall findings of the second study are summarized in Figure 9. However, from a chemistry point of view there is still room for additional characterization and optimization of compound 13a, to improve its antiviral potency even further. Our time-of-addition study also suggested that compound 13a might be active at the early and/or post-entry stages of the RVFV life cycle. Even so, more comprehensive studies are needed to gain in-depth knowledge of its mechanism of action.
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