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Intervention strategies for emerging viruses: use of antivirals

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Today, small molecule antiviral drugs are available for the treatment of infections with herpesviruses, HIV, HBV and HCV as well as with influenza viruses. Ribavirin, a broad-spectrum (but aspecific) antiviral, has been approved for the treatment of infections with respiratory syncytial virus, HCV and Lassa virus. Yet, for many other viruses that cause life-threatening infections [most of which are considered emerging and/or neglected] there are no drugs available. Ideally, potent and broad-spectrum (i.e., pan-genus or pan-family virus activity) antiviral drugs should be developed whereby one drug could be used for the treatment of a number of such viral infections. We here review recent evolutions in the search for inhibitors of emerging and neglected RNA viruses.

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Introduction

Today, small molecule antiviral drugs are available for the treatment of infections with herpesviruses, HIV, HBV and HCV as well as influenza viruses. More than 25 years after the discovery of HIV, over 25 compounds have been formally approved for the treatment of AIDS and most of these are being used in fixed-dose drug combinations. Potent, highly effective and well-tolerated drugs are also available for the treatment of HBV infections. For HCV two protease inhibitors were recently approved and a number of other direct-acting antivirals (DAAs) is in development, they will ultimately be combined in appropriate drug regimes. Potent nucleos(t)ide analogs (such as acyclovir, ganciclovir and cidofovir), that target the viral polymerase, are available for the treatment of herpesvirus infections, yet novel drugs that target the viral helicase–primase or the CMV terminase are being developed. For influenza virus, novel neuraminidase inhibitors (such as peramivir and laninamivir octanoate) and a polymerase inhibitor (favipiravir) are in development. The broad-spectrum inhibitor ribavirin is approved for the treatment of infections with the respiratory syncytial virus, HCV and Lassa virus. In conclusion, the large number of drugs that are available against HIV (and the many drugs that are in clinical development for the treatment of chronic HCV infections) demonstrates that even for viruses with a short genome, many excellent molecular targets exist for inhibition of viral replication. Yet, for many viruses that cause life-threatening infections in man there are no drugs at hand for treatment. Most of the emerging and/or neglected viral pathogens have an RNA genome, including viruses such as the dengue fever virus (and other flaviviruses), Chikungunya virus, enterovirus 71, rabies virus, HEV, coronaviruses and arenaviruses, bunyaviruses and filoviruses.

Although it should be very well feasible to develop potent inhibitors against each of the (currently known) neglected and/or emerging viruses, this may economically not be a viable option. Therefore, ideally, potent and broad-spectrum drugs should be developed that can be used for the treatment of a variety of such viral infections. Possibly, nucleoside analogs with such characteristics may be designed/discovered. An alternative is to develop drugs that have broad-spectrum antiviral activity within a given genus or family (e.g., broad-spectrum flavivirus or paramyxovirus inhibitors). It is probable that novel, potentially highly pathogenic RNA viruses will emerge in the future; consider for instance the recent fatalities with the novel coronavirus-EMC [1•]. Having broad-spectrum (pan-genus; pan-family or pan-RNA virus) inhibitors at hand may help to contain such future outbreaks. In this review we will provide a nonexhaustive overview of recent developments in the search for small molecule inhibitors of (some) neglected/emerging RNA viruses.

Flaviviruses

About two-fifth of the world's population is now at risk for dengue infection and 50–100 million cases are estimated to occur worldwide every year [2,3]. An estimated 500 000 people with severe dengue require hospitalization each year; a very large proportion of whom are children, resulting in a fatal outcome in about 2.5% of those affected. There is neither vaccine nor a specific antiviral treatment. Likewise, no antivirals are available for the treatment of life-threatening infections with other flaviviruses such as those caused by yellow fever virus [4], Japanese encephalitis virus and West Nile virus. The organization of the genome of flaviviruses resembles — to some extent — that of the related HCV, of which the viral serine protease and the RNA-dependent RNA polymerase have been shown to be excellent targets for inhibition of viral replication (both *in vitro* and in the infected

patients) [5]. So far, flavivirus NS3 protease inhibitors with a potency comparable to that of the HCV NS3 protease inhibitors have not yet been identified. Particular differences in the characteristics and structure of the different NS3 proteases may be the reason [6]. An exhaustive review on the flavivirus NS3 protease as a target for the design of inhibitors has recently been published [7]. Second, nucleoside as well as non-nucleoside polymerase inhibitors of HCV have been and currently are in clinical development. They exert pan-genotype antiviral activity and have a high barrier to resistance [8]. Nucleoside polymerase inhibitors (that target the enzyme as their 5′-phosphorylated metabolite) have also been shown to exert pan-serotype anti-dengue virus activity *in vitro* and in dengue mouse models [9,10]. Balipiravir, a nucleoside HCV polymerase inhibitor, was evaluated for potential activity in dengue-infected patients. No protective activity was observed [11]. The lack of activity of balipiravir can very probably be ascribed to the very weak *in vitro* potency of this molecule against DENV and should not create any pessimism regarding the potential use of nucleoside analogs for the treatment of dengue. For HCV a large number of non-nucleoside polymerase inhibitors have been reported and at least 4 different allosteric pockets for inhibition of the enzyme activity have been identified on the enzyme [5]. At least one cavity has been found in the dengue polymerase that may potentially serve as a pocket into which inhibitors of the enzyme could be designed [12]. In addition, the flavivirus NS5 gene encodes, besides the viral polymerase, also a methyltransferase (MTase) which is responsible for methylating the viral cap. This MTase is critical for viral replication and therefore represents a valid target for antiviral therapeutics. Efforts are ongoing to develop selective inhibitors of this enzyme [13]. Fourth, the flavivirus NS4b is an essential membrane-associated viral protein (not a homologue of the HCV NS4b) that has been shown to be the target of small molecule dengue inhibitors [14]. Also, an inhibitor of yellow fever virus replication was reported that targets NS4b [15]. We recently identified a class of highly potent and pan-serotype dengue virus inhibitors that target NS4b (our unpublished data). The exact mechanism by which these molecules target NS4b and thereby inhibit viral replication remains to be elucidated. Despite the fact that the precise function of NS4b is not well understood, this protein appears, akin to NS5a of HCV, to be an excellent target for the design of potent inhibitors of viral replication. Finally, inhibition of host factors that are essential for virus replication might be considered. For instance, an inhibitor of dihydroorotate dehydrogenase (DHODH), an enzyme required for pyrimidine biosynthesis, was identified as an inhibitor of *in vitro* dengue virus replication but had no effect in dengue virus-infected mice [16]. Glycosylation inhibitors [N-nonyldeoxynojirimycin and celgosivir] have been shown to inhibit dengue virus replication (by misfolding of NS1). The activity of celgosivir has also been demonstrated in a dengue mouse model [17] and its activity is currently being explored in a clinical study (Celaden Drug Trial; URL: http://www.celaden.sg/). The design of appropriate clinical trials with dengue drugs might be challenging however. Recently, recommendations for the design of such trials have been published [3••].

Enteroviruses

The Enterovirus genus (family Picornaviridae) comprises multiple medically important pathogens of which poliovirus is probably the best known. The Global Polio Eradication Initiative (WHO) aimed at eradicating polio from the globe by 2000, but the virus still remains endemic in Nigeria, Afghanistan and Pakistan. Effective antivirals against poliovirus may be essential for complete eradication of both wild-type and circulating vaccine-derived poliovirus [18]. Enterovirus 71 has emerged as a major pathogen in Southeast Asia during the past 15 years [19]. The virus causes hand-foot-and-mouth disease in young children, but may also result in life-threatening viral encephalitis. Also other enteroviruses may cause serious illness including aseptic meningitis. There are no vaccines (except for polio) or drugs available for the prevention or treatment of enteroviral infections. Such drugs are urgently awaited.

Although multiple compounds have been or are currently in clinical development, none have received marketing authorization thus far. Several of these molecules are capsid binders and interfere with receptor attachment, cell entry and/or virus uncoating [20]. Pleconaril was developed as an oral treatment for the common cold, but was not accepted by the FDA because of safety concerns [21]. Pleconaril, which is also effective against most enteroviruses, has since then been trialed for enteroviral sepsis syndrome or as a nasal spray for common cold symptoms and asthma exacerbations, but no results have been disclosed to date (NCT00031512, NCT00394914; URL: http://www.clinicaltrials.gov/). BTA798 (vapendavir) is another capsid binder and is in clinical development for the treatment of rhinovirus infection in asthmatic patients (NCT01175226; URL: http://www.clinicaltrials.gov/). In a recent press release, Biota reported successful completion of a phase IIb study with vapendavir. Finally, the capsid binder V-073 is being investigated for use in poliovirus eradication [22]. A phase II trial is currently ongoing in Sweden that assesses the effect of V-073 on virus shedding after inoculation with the oral poliovirus vaccine (2011-004804-38; URL: https://www.clinicaltrialsregister.eu/). The main drawback with capsid binders is that they rather easily select for resistance as the enteroviruses tolerate mutations in their capsid proteins quite well [20].

The enteroviral 3C protease ($3C^{pro}$) may be another interesting drug target. The peptidomimetic $3C^{pro}$

inhibitor rupintrivir elicits pan-entero/pan-rhinovirus activity. The compound was effective in volunteers with experimental rhinovirus infections but lacked efficacy in naturally infected patients [23]. An orally available analog was designed (compound 1, AG7404), but clinical development was halted despite successful phase I studies [24]. Using a structure-based drug design approach, a class of broad-spectrum enteroviral $3C^{pro}$ inhibitors was recently developed [25].

The 2C helicase-NTPase constitutes another promising target [20]. A number of molecules that target this viral protein has been reported (MRL-1327, 2-(α-hydroxybenzyl)-benzimidazole (HBB) and TBZE-029) [26,27]. We recently identified a class of highly potent pan-entero/pan-rhinovirus compounds that target 2C, have a high barrier to resistance and that are highly effective in enterovirus infection models in mice (unpublished results). Despite the fact that the precise mechanism by which the compounds interact with 2C remains to be discovered, this protein can be viewed as an excellent target for the development of potent and pan-enterovirus inhibitors.

The multifunctional 3A protein is involved in replication complex formation [28•]. Several enterovirus inhibitors were found to select for drug-resistance mutations in 3A, for example, enviroxime, TTP-8307 and T-00127-HEV1 [28–30]. Enviroxime was evaluated in clinical trials, but caused gastro-intestinal side effects and failed to protect from natural rhinovirus infection [31]. The molecule was recently shown to block enterovirus replication through direct inhibition of the host enzyme phosphatidylinositol-4 kinase IIIβ (PI4KIIIβ) that is recruited by 3A to the replication complex [28•]. The resistance mutations in 3A render the virus independent of PI4KIIIβ for replication. Whereas the viral polymerase has proven to be an excellent target for inhibition of viral replication of a number of viruses (including for HIV and HCV), selective inhibitors of the enterovirus polymerase were so far not reported. We recently discovered a class of non-nucleosides with broad-spectrum anti-entero/anti-rhinovirus activity that target the viral RNA-dependent RNA polymerase by competing with the template primer (van der Linden *et al.*, submitted for publication).

Alphaviruses

Chikungunya virus (CHIKV) belongs to the genus Alphavirus, family Togaviridae. Infection is associated with an acute pathology characterized by fever, rash and arthralgia. Care is still limited to supportive treatment aiming at alleviating the infection-induced symptoms [32]. Limited *in vitro* antiviral activity of chloroquine, alpha-interferon (IFNα) and ribavirin was demonstrated but no benefit of these drugs was observed in the clinical setting [33]. A number of natural products such as terpenoid compounds [34], 5,7-dihydroxyflavones [35], prostratin and 12-O-tetradecanoylphorbol 13-acetate [36] and approved drugs such as phenothiazinyls [35] and arbidol [37] were shown to inhibit *in vitro* CHIKV replication. Computer-aided design resulted in the identification of inhibitors of the nsP2 protease [38]. We recently demonstrated that the anti-influenza drug favipiravir inhibits the *in vitro* replication of CHIKV (including clinical isolates) and results in protective activity in an animal model (unpublished data).

Coronaviruses

Four coronaviruses (CoV) are known to be endemic in the human population and are associated with mild to severe respiratory symptoms. In 2003 a novel CoV, Severe Acquired Respiratory Syndrome (SARS)-CoV, caused an epidemic of respiratory disease in almost 8000 people with a ~10% case fatality rate. SARS-CoV is most probably the result of a zoonotic event: a bat virus jumping into the human population (directly or via another animal). In 2012 a novel CoV was discovered in a patient in Saudi Arabia. To this day the virus has been diagnosed in 16 patients (connected with different regions of the Middle-East), of which 9 have died. For one case there is now also evidence for person-to-person transmission [1•] (Health Protection Agency, UK; URL: http://www.hpa.org.uk/NewsCentre/NationalPressReleases/2013PressReleases/130213statementonlatestcoronavirus-patient/). Because of the association with severe respiratory illness and the potential of outbreaks of new strains with a high transmission and fatality rate, there is an urgent need for inhibitors of CoV replication. So far no small molecule antivirals have yet shown activity in experimental infection models or in the clinical setting.

Two cysteine proteases reside within the SARS-CoV polyprotein, a papain-like protease and a 3C-like protease. For both proteases different inhibitors have been discovered and optimized. Despite the efforts in structural biology and chemical optimization the best inhibitors are currently only effective in the 1–10 μM range [39–43]. The SARS-CoV virus uses different host cell proteases for cellular entry. Inhibition of these proteases was shown to block entry of the virus in *in vitro* assays [44]. Interestingly one of the host cell proteases being targeted using this approach (TMPRSS2) is also pivotal in the replication of other respiratory viruses like influenza A and human metapneumovirus (hMPV). Inhibitors of the SARS-CoV helicase were reported that also inhibit the *in vitro* replication of the virus [45,46]. More recently new inhibitors of the viral helicase were discovered in a target-based screen that inhibit the *in vitro* replication of the virus. It is hoped that optimization of this class may deliver potent compounds [47].

Hepatitis E virus

Hepatitis E virus (HEV) is a positive-sense single-stranded RNA virus classified into the Hepevirus genus

in the Hepeviridae family and is transmitted faeco-orally. Infections can be asymptomatic, especially in young children, but usually present as a mild acute hepatitis. Nevertheless, fulminant hepatitis requiring liver transplantation can occur, particularly in pregnant women [48]. HEV genotype 1 and 2 are endemic in developing countries and their host range is restricted to humans. Genotypes 3 and 4 are zoonoses with a major reservoir in domestic pigs; they cause sporadic infections worldwide which have been linked to the consumption of undercooked pig and deer meat [49]. Even though a recombinant vaccine (HEV 239) has been developed [50], it has only been approved in China yet. HEV causes 20 million infections, 70 000 deaths and 3000 stillbirths annually [51]. Experience in hepatitis E treatment is limited and largely restricted to chronic infections. For transplant patients, the first consideration is lowering immunosuppressive therapy which clears the infection in over 30% of patients [52]. Ribavirin monotherapy was reported successful in 10 out of 13 chronic hepatitis E patients [53•,54–56]. Anemia was the most frequent side effect and necessitated dose reductions in a number of cases. Patients were treated for 3 months or longer and ribavirin is contraindicated during pregnancy. PEG-IFNα treatment is another option [57]. Successful combination therapy of PEG-IFNα and ribavirin was reported for a chronically HEV-infected HIV-1 patient [58] and ribavirin also substantially decreased HEV RNA levels in a case of severe acute hepatitis E [59]. Nevertheless, there is an urgent need for efficacious and nontoxic treatments for HEV infections that can be used to contain outbreaks and that are safe in pregnant woman. The first efficient cell culture systems for HEV have only been described recently [60,61] and will be instrumental in developing more potent and selective inhibitors.

Paramyxoviruses

Several paramyxoviruses cause disease in man; human respiratory syncytial virus (RSV), hMPV and parainfluenza virus are broadly prevalent respiratory pathogens. Nipah and Hendra viruses are highly pathogenic paramyxoviruses that have emerged from bats within the past two decades. Both are capable of causing fatal disease in humans. It would seem prudent, when developing drugs for the treatment of RSV (and hMPV) infections, to aim at generating drugs that elicit broad-spectrum antiparamyxovirus activity and that could thus be deployed to treat infections with Nipah and Hendra (or with highly pathogenic paramyxoviruses if they would emerge). Ribavirin is currently the only drug licensed to treat RSV infection, but its clinical efficacy remains unclear. The majority of reported small-molecule antivirals target the RSV entry step by blocking the fusion protein. MDT-637 (MicroDose Therapeutics) is currently (one of) the most advanced RSV fusion inhibitor (currently entering phase IIa). TMC353121 (Johnson & Johnson), another fusion inhibitor, causes a local disturbance of the F-protein post-fusion conformation [62]. The molecule reduces RSV replication and consequential lung inflammation in mice [63]. Phase I studies with yet another fusion inhibitor, BTA-C286 (Biota), are anticipated to start in 2013 (Biota; URL: http://www.biotapharma.com/?page=1021001&subpage=1021017/). Efficacy with this compound has been shown in two nonclinical models of human RSV infection. Gilead Sciences recently initiated a phase II safety and efficacy RSV challenge study of a novel molecule with an undefined mechanism (GS-5806) in healthy volunteers (NCT01756482; URL: http://www.clinicaltrials.gov/). In addition, a non-nucleoside inhibitor of RSV replication was reported to inhibit guanylylation of the cap of viral transcripts [64]. Finally, Alios Biopharma reported the discovery of nucleoside analogs that potently inhibit RSV replication by targeting the viral polymerase. The company plans to bring ALS-8176, an orally bioavailable analog, into clinical trials in 2013 (Alois Biopharma; URL: http://www.aliosbiopharma.com/).

Rhabdoviruses

Rabies causes a nearly 100% fatal encephalomyelitis in humans. An effective vaccine is available, but many people at risk remain unvaccinated. Current strategies of post-exposure prophylaxis involve infiltration of the wound with human rabies immunoglobulin and vaccination. Each year an estimated 50 000 people (of which many children) die because of a rabies infection [65]. In recent years, a treatment protocol, known as the Milwaukee protocol, was set up that involved induction of coma and treatment with ketamine (a neuroprotective anesthetic with putative anti-rabies activity [66]) as well as amantadine and ribavirin [67]. Although both amantadine and ribavirin inhibit the *in vitro* replication of the virus to some extent [68], activity has never been demonstrated in experimental infection models or in the clinical setting [69–71]. Despite the fact that the Milwaukee protocol has been used several times, the effectiveness remains controversial [72•]. Potent inhibitors of rabies replication that penetrate into the brain are urgently needed. Such drugs might help patient to survive and recover, even after neurological complications have already developed.

Arenaviruses, bunyaviruses and filoviruses

A limited number of phylogenetically distinct viruses that belong to the Arenaviridae, Bunyaviridae, Filoviridae families can cause a severe, often lethal hemorrhagic fever syndrome. Lassa virus (Arenaviridae) is responsible for a large number of deaths in West Africa. Ribavirin is the only licensed antiviral with reported activity against Lassa virus and is also effective against arenaviruses of the New World (Junin and Machupo virus). Favipiravir (T-705) which is being developed for the treatment of influenza virus infections has also shown efficacy in experimental mouse and hamster models of arenavirus [73]. Such broad-spectrum anti-RNA virus drug could

thus potentially be used off-label (once approved for the treatment of influenza virus infections) for the treatment of life-threatening arenavirus infections. In a large screenings effort using lentiviral pseudotypes with the Lassa virus envelope glycoprotein, a class of compounds was identified that inhibit Lassa (and other arenavirus) entry. The lead compound ST-193 is efficacious in a lethal LASV guinea pig model with superior activity compared to ribavirin [74–76]. The latest developments in the search for inhibitors of arenavirus have recently been reviewed [77].

Rift Valley fever (RVF) is caused by a bunyavirus; it not only affects ungulates but can also cause disease in humans. There are currently no FDA-approved antivirals for the treatment of RVF. Ribavirin has shown some efficacy in animal model systems of RVF but is only indicated under compassionate use guidelines in the event of emergency [78]. Favipiravir (T-705) and its analog T-1106 has shown efficacy against RVF in cell culture and in a surrogate animal model [79,80]. Crimean-Congo hemorrhagic fever (CCHF) is caused by CCHF virus, another bunyavirus. The virus causes a fatal infection and is transmitted by the bites of ticks, by contact with a patient with CCHF during the acute phase of infection or by contact with blood or tissues from viremic livestock. The reported case fatality rate ranges from 10% to 50%. A number of observational studies have indicated that treatment with the antiviral drug ribavirin is beneficial for CCHF, but no placebo-controlled trials have been performed [81,82,83•].

The Filoviruses Ebolavirus (EboV) and Marburg cause severe hemorrhagic fever with high case fatality rates [84]. Neither vaccine nor antiviral treatment is currently available. Although a vaccine would be greatly appreciated, a potent and fast-acting antiviral drug will be required as well considering the sporadic nature of the infections and the rapid disease progression. Some of the earliest reported inhibitors of filovirus replication are carbocyclic 3-deazaadenosine and 3-deazaneplanocin A, they function through blocking of the host enzyme S-adenosyl-L-homocysteine hydrolase thereby preventing methylation of the cap [85,86]. Both molecules protect mice from experimental EboV infection. Another class of filovirus inhibitors targets virus entry [87••,88,89]. For instance, compounds 3.0 and 3.47 block binding of EboV glycoprotein (GP) to its receptor Niemann–Pick C1 [87••] and a benzodiazepine-derivative inhibits entry through binding a pocket in the GP [88]. For other molecules, details regarding the mechanism of action are missing, for example, for the broad-spectrum antiviral compounds FGI-103, FGI-104 and FGI-106 [90–92]. All three inhibitors are protective in a lethal EboV mouse model. A last antiviral molecule that was reported recently is NSC62914, an antioxidant that resulted in activity in an infection model as well [93].

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