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Review

Discovery of alphavirus inhibitors derived from natural products

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ABSTRACT

Alphaviruses are insect-borne viruses that cause serious and sometimes lethal infections in humans, and are also possible bioterrorism agents with the potential to produce widespread disease and societal disruption. There are currently no approved vaccines or antiviral drugs for humans to prevent or treat infections with alphaviruses. Small molecule libraries are often used to discover new antivirals, but they are limited by factors such as the costs of acquiring or maintaining large drug collections and the boundaries of synthetic and combinatorial chemistry. Thus, there is a pressing need for innovative approaches to antiviral drug discovery that go beyond the limits of synthetic chemistry. One approach takes advantage of the complex biosynthetic pathways of living organisms, which can produce natural products of nearly limitless structural diversity. Although major challenges exist in utilizing natural product resources for antiviral drug discovery, notable progress has been made recently. In this review, we provide an overview of alphavirus epidemiology, clinical disease, pathogenesis, molecular biology, and antiviral drug discovery, with an emphasis on the potential of natural product-derived resources as starting material for the identification of novel compounds to treat these virulent pathogens.

KEYWORDS: alphavirus, natural products, bioterrorism, antivirals, high-throughput screens, RNA virus

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INTRODUCTION

Arboviruses are insect-borne pathogens that cause multiple naturally occurring infectious diseases in humans. Numerous arboviruses are also considered possible bioterrorism agents, with the potential to produce widespread disease and also social and economic disruption. The majority of arboviruses belong to one of three virus families: *Flaviviridae*, *Bunyaviridae*, and *Togaviridae*. Within the last family are found several notable pathogens of the Alphavirus genus. This genus contains approximately 30 viruses, one-third of which cause substantial diseases in humans and animals [1], including Sindbis virus (SINV), Semliki Forest virus (SFV), Chikungunya virus (CHIKV), eastern equine encephalitis virus (EEEV), Venezuelan equine encephalitis virus (VEEV), and western equine encephalitis virus (WEEV) [2]. Alphaviruses are enveloped, single-stranded positive-sense RNA viruses, are transmitted primarily by mosquitoes, and can be classified based on their primary geographic location in the eastern or western hemispheres, also referred to as old world and new world alphaviruses, respectively [3]. SINV and SFV are the prototypic and best-studied alphaviruses experimentally, and are found primarily in the eastern hemisphere, whereas WEEV, EEEV, and VEEV are the primary human pathogenic alphaviruses found in the western hemisphere.

Reemerging disease

Infections caused by arboviruses represent some of the most dramatic examples of disease

reemergence throughout the world [4]. This is due in part to the significant growth in urban centers in the latter half of the 20th century, which has produced societal conditions that can greatly facilitate arbovirus epidemics. One particularly frightening public health scenario that has been observed over the past several decades is the expansion of specific arbovirus diseases outside of their historical geographic boundaries [5-7]. CHIKV is a noteworthy example of an old world alphavirus with a recent dramatic expansion of its geographical distribution. In the early 1950s, CHIKV was identified in remote areas of east central Africa as the causative agent of a large epidemic of viral fever [8]. By the 1970s, CHIKV had spread as far as India, where some epidemics were associated with significant mortality [9]. More recently, a major outbreak of CHIKV infections occurred in the Indian Ocean basin in 2005-2006, which resulted in an estimated two million cases and several hundred deaths [10]. This outbreak serves as a poignant example of the potentially severe and rapid nature of alphavirus epidemics that can quickly devastate populations after years of relatively infrequent infections [7, 11]. Furthermore, clinical and molecular surveillance demonstrated that the CHIKV strain from the Indian Ocean basin outbreak has migrated widely, now including northern India and countries as distant as Cambodia and Italy [12-15]. The appearance of endogenous CHIKV infections in southern Europe was particularly concerning, as it represented adaptation to a new vector thereby allowing wider dissemination. CHIKV is primarily spread by several species of *Aedes* mosquitos, including *A. aegypti*, which have somewhat limited geographic distribution and typically breed in urban environments using water collected in garbage, water containers, and construction sites [1]. However, minor mutations in the CHIKV envelope proteins allowed it to be efficiently transmitted by *A. albopictus*, the Asian tiger mosquito, which has a world-wide distribution [16].

Another example of a reemerging alphavirus is EEEV, although this pathogen is found primarily in the western hemisphere and reemergence has been less dramatic than for CHIKV. EEEV was originally isolated in the mid-Atlantic United States in the 1930s, but it has subsequently spread from southeastern Canada to northern Brazil [17-19]. Similar to other new world alphaviruses, EEEV has an enzootic life cycle described as an avian-mosquito-swamp model, where the virus normally passes among 16 different mosquito species, primarily *Culiseta melanura*, and swamp-dwelling birds. In a recent study surveying *C. melanura* in areas of Massachusetts known to have EEEV activity, these mosquitoes were shown to mainly feed on birds, but 1% of the mosquitoes tested had taken a recent mammalian blood meal. Furthermore, three different arboviruses were detected in *C. melanura* (West Nile virus, Highland J virus, and EEEV), demonstrating the ability of this particular mosquito species to transfer arboviruses between birds and mammals, and potentially serve as a vector for simultaneous transmission of multiple pathogens to humans [20].

Alphavirus clinical disease and pathology

Alphaviruses typically have an incubation period of three to seven days after the infective mosquito bite until the first clinical symptoms appear. Old world alphaviruses cause primarily non-specific flu-like symptoms consisting of fevers, chills, and myalgias, along with a rash and arthralgia syndrome that can last from one to two weeks [7, 21]. However, chronic arthralgias lasting up to three years after initial infection have also been reported [22]. Other uncommon symptoms and potentially lethal complications of old world alphavirus infections include a hemorrhagic syndrome and meningoencephalitis, although these more severe disease outcomes may be more frequent than previously appreciated [1, 23, 24]. For example, CHIKV infections were historically thought to be rarely fatal, but epidemiological data show significant increases in the average monthly mortality rate for affected areas after recent outbreaks, especially among the elderly [24, 25].

In contrast to the old world alphaviruses, the new world alphaviruses, WEEV, EEEV, and VEEV, routinely cause significant morbidity and mortality in both humans and animals. These virulent pathogens can cause severe encephalitis in humans with fatality rates of up to 70%, and

survivors often suffer long-term neurological sequelae [26-31]. The new world alphaviruses directly infect neurons and cause central nervous system (CNS) inflammation with neuronal destruction [32-34]. Initial symptoms of new world alphavirus infections are fever, headache, and nausea or vomiting, followed by the onset of symptoms of meningoencephalitis, which include neck stiffness, seizures, and delirium that may progress to coma and death. Cerebrospinal fluid examination shows a neutrophilic pleocytosis along with increased protein levels and erythrocytes indicating a potent CNS inflammatory response. Magnetic resonance imaging shows focal CNS lesions in the thalamus, basal ganglia, and cerebral cortex [26, 28]. Postmortem examination of CNS tissue support the imaging findings, where histopathology shows cerebral edema and vascular inflammation, and immunohistochemistry shows scattered patches of virus-positive neurons throughout the brain [28].

Biological weapons

In addition to spontaneous alphavirus epidemics, there is the potential for an intentional release of a virulent alphavirus into the environment or select communities as a biological weapon. The World Health Organization defines biological weapons as:

"...those [agents] that achieve their intended target effects through the infectivity of disease causing microorganisms and other such entities, including viruses, infectious nucleic acids and prions. Such weapons can be used to attack human beings, other animals or plants, but it is with human beings that the report is primarily concerned." [35]

While there are no recorded uses of an alphavirus as a biological weapon, human history contains multiple examples of biological weapons being used during warfare, from the 14th century launching of plague-infested corpses into the city of Kaffa to the use of plague-infected insects by the Japanese in World War II [36, 37]. While the use of biological weapons by nation states has been the primary concern throughout history, there have been sufficient advances in modern molecular biology and microbiology to give even smaller and less sophisticated groups the ability to wield these potentially devastating weapons.

The terrorist attacks of September 11, 2001, heightened concerns that biological weapons may be used in a terrorist attack. However, several such events had already occurred prior to 2001. In 1972, the Order of the Rising Sun was apprehended growing large amounts of typhoid-causing *Salmonella enterica*; in 1980, police raiding the Baader-Meinhof gang found a makeshift laboratory growing *Clostridium botulinum*; in 1986, the Rajneesh cult caused foodborne illnesses by contaminating salad bars with *Salmonella typhi*; and from 1990 to 1994, the Aum Shinrikyo cult carried out multiple attacks with various agents with limited success in Japan [36, 37]. These episodes, in conjunction with the increased awareness of terrorism after 9/11, led the United States Centers for Disease Control and Prevention (CDC) and the National Institute of Allergy and Infectious Diseases (NIAID) to develop criteria for evaluating the threat potential of microorganisms and biological toxins as biological weapons [38]. Threat potential for priority pathogens is designated by category as A (high), B (moderate), or C (low), and is based on the following factors: (i) public health impact; (ii) the ease of production or delivery; (iii) contagiousness to a large population; (iv) public sensitivities and likelihood of societal breakdown after an attack; (v) the ability for surveillance and diagnosis; and (vi) the ability to cache vaccines and pharmaceuticals for treatment if available [39]. The new world alphaviruses, WEEV, EEEV, and VEEV, are category B priority pathogens, whereas the old world alphavirus, CHIKV, is a category C priority pathogen. The neurotropic new world alphavirus pathogens are of special concern due in part to numerous characteristics that make them attractive potential biological weapons: (i) high clinical morbidity and mortality; (ii) no effective treatment; (iii) potential for aerosol transmission; (iv) public anxiety elicited by CNS infections; (v) potential for malicious introduction of foreign genes designed to increase alphavirus virulence; and (vi) ease of production in large quantities [40].

The CDC public health strategy for category B pathogens is to detect shortcomings in the public

health system, increase surveillance programs and knowledge of these diseases by health care workers, and lastly to improve diagnostic testing. Stockpiling of established therapeutics is not emphasized as these organisms are either treatable with drugs effective against category A agents or no known treatment exists [39]. However, this lack of emphasis of therapeutics by the CDC does not diminish the need for the discovery and development of novel anti-infective agents against viruses within this category. Historically, the most noteworthy category B pathogen may be the neurotropic alphavirus VEEV, which was fully weaponized as a biological warfare agent by the United States military, and likely other nations such as the former Soviet Union, until the Biological Weapons Convention of 1972 was signed and enforced. VEEV is a particularly attractive biological weapon due to a low infectious dose and the ability to be readily spread by aerosol transmission [36], and VEEV has the dubious distinction of being one of the most frequent laboratory-acquired viral infections [41]. Aerosol transmission is of particular concern as it presents the possibility of widespread dissemination, and aerosol models of VEEV in mice show direct migration to the CNS via the olfactory bulb, which produces more rapid disease progression compared to infection via the subcutaneous route, as would occur after an insect bite [42]. Similarly, WEEV has also recently been shown to be transmitted by the aerosol route. Cynomolgus macaques exposed to aerosolized WEEV developed encephalitis within six days of exposure and, surprisingly, WEEV was more effective than VEEV at causing clinical and pathological disease in these primates [43]. The observations noted above highlight the potential for neurotropic alphaviruses to cause widespread disease, and justify their inclusion on the list of potential biological weapons.

Molecular biology and pathogenesis of alphaviruses

Alphaviruses infect and replicate in a wide variety of cells in culture, including mammalian, avian, and insect cells [3, 44]. In most cultured mammalian cells, alphaviruses induce rapid cell death that is due in part to specific viral proteins, which may play a central role in alphavirus pathogenesis. For neurotropic alphaviruses, neuronal cell death is either necrotic or apoptotic [45], and the loss of these critical and essentially non-renewable cells has a significant impact on disease outcome. Additionally, *in vivo* studies with SINV, the prototypic alphavirus used to study encephalitis in mice, have revealed that many non-infected neurons are also damaged via "bystander" mechanisms [45, 46]. This secondary neuronal cell death is mediated in part by microglial cell activation and altered glutamate homeostasis, as minocycline and naloxone, which are anti-inflammatory compounds that block microglial cell activation [47-50], and glutamate receptor antagonists protect SINV-infected mice from clinical disease [46, 51-54]. Neuroprotective agents that affect host pathways may provide synergistic protection from encephalitis induced by neurotropic alphaviruses, and may represent an important complimentary approach in combination with direct antiviral agents to treat alphavirus infections.

The genetic organization and replication strategy of alphaviruses have been studied extensively with SINV and SFV [3], and is shown schematically in Figure 1. The alphavirus genome is an 11- to 12-kb single-stranded positive-sense (+) RNA with a 5' terminal cap and 3' polyadenylated tail. Thus, the genome can serve directly as viral mRNA immediately upon entry into the cytosol for the production of the nonstructural proteins (nsPs) needed for viral RNA replication. Viral genomic (+) RNA is translated by host cell ribosomes into one or two polyproteins that undergo regulated autocatalytic processing to eventually form the four non-structural proteins, nsP1 through nsP4. These non-structural proteins form the viral contribution to the membrane-associated replication complex, and represent potential targets for antiviral drug discovery and development [55-57]. Although some alphavirus virulence determinants map to structural genes [44], changes in the 5' untranslated region [58, 59], nsP1 [60, 61], nsP2 [62, 63], and nsP3 [60, 64] have all been correlated with virulence, suggesting that RNA replication complex assembly or function may also impact pathogenesis. During replication through

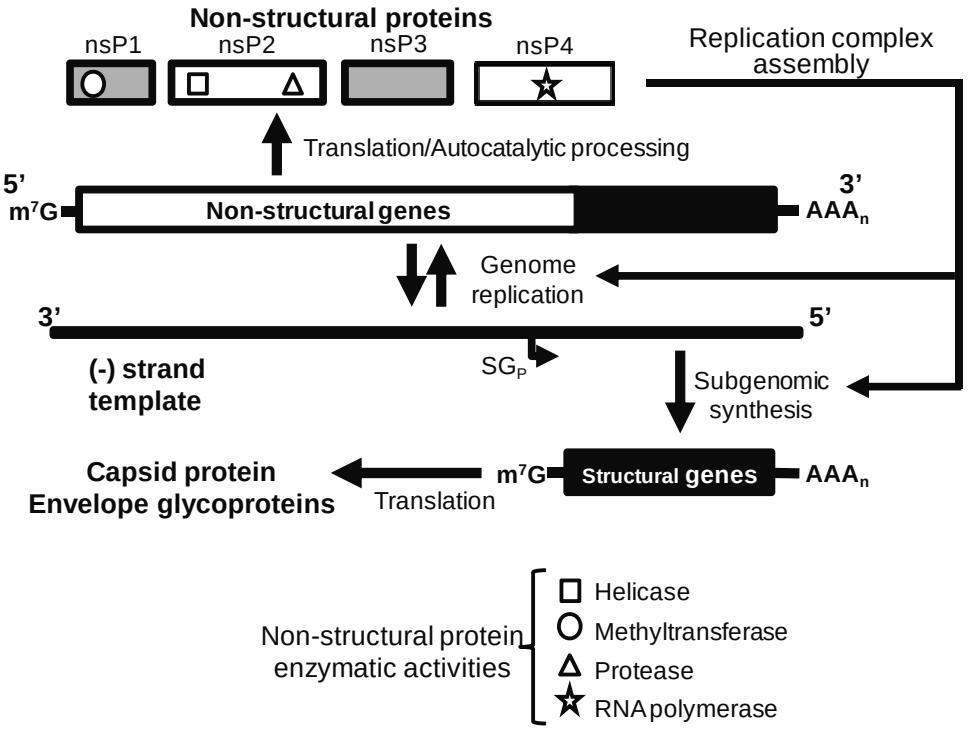

Figure 1. Genetic organization and replication strategy of alphaviruses. The alphavirus genome is a single-stranded positive-sense (+) RNA with a 5' terminal cap and 3' polyadenylated tail. Genomic (+) RNA is directly translated into polyproteins that undergo autocatalytic processing to form the four non-structural proteins nsP1 through nsP4 which together form the viral replication complex. Enzymatic activities of individual alphavirus nsPs needed for replication are indicated by symbols for each nsP. A full-length antigenomic negative-strand (-) RNA functions as an intermediate for the synthesis of progeny genomic (+) RNA by the viral replication complex. Alphaviruses also produce a subgenomic RNA that encodes the structural capsid protein and envelope glycoproteins from an internal subgenomic promoter (SG_P) in the antigenomic RNA.

a full-length antigenomic negative-sense (-) RNA, which serves as an intermediate for the production of genomic (+) RNA, alphaviruses also produce a 4-kb subgenomic RNA that encodes the structural capsid protein and envelope glycoproteins [65]. The alphavirus structural proteins are not essential for genome replication, and can be readily replaced with reporter genes to produce viral replication systems that have improved biosafety characteristics for antiviral drug discovery [66, 67].

All four alphavirus nsPs and their various polyprotein intermediates function in different combinations to regulate the synthesis of the three major viral RNAs that are produced during replication: genomic (+) RNA, antigenomic (-) RNA, and subgenomic RNA. Shortly after full-length nsP1/2/3/4 polyprotein translation, nsP4 is cleaved off and binds the nsP1/2/3 polyprotein to form a complex that recognizes the 3' promoter in genomic (+) RNA for synthesis of antigenomic (-) RNA [68]. Subsequently, nsP1 is cleaved from the nP1/2/3 polyprotein to form a transitional RNA synthesis complex of nsP1-nsP2/3-nsP4, which synthesizes not only antigenomic (-) RNA but also both genomic (+) RNA and subgenomic RNA [69, 70]. Late in the viral replication cycle, nsP2/3 is cleaved and this final RNA synthesis complex, which consists of the 4 separate nsPs, synthesizes primarily genomic (+) RNA [68]. Although the alphavirus nsPs function as a coordinated unit to accomplish the complex task of viral genome replication, each nsP has unique functions that serve as potential targets for antiviral drug discovery and development, and therefore we discuss these essential nsP-specific enzymatic activities and characteristics below. For completeness, we also

briefly discuss the known functions of structural proteins, which are additional possible antiviral targets.

nsP1. Alphaviruses form membrane-associated replication complexes on cytoplasmic vacuoles, which are modified lysosomes and endosomes of infected cells and contain membrane invaginations, called spherules, in which viral RNA synthesis occurs [55-57]. These replication complexes are anchored to the negatively charged phospholipids of cellular membranes by nsP1 through an amphipathic peptide segment located in the center of the protein [71, 72]. The negatively charged membrane phospholipids also activate N-terminus nsP1 guanine-7-methyltransferase domains, whose enzymatic activity is responsible for capping viral RNAs [71, 73, 74]. This alphavirus-encoded activity is necessary as viral replication takes place in the cytosol, whereas host mRNA guanylyltransferases are located predominantly in the nucleus. Furthermore, while many nsP1 methyltransferase characteristics are shared by similar cellular enzymes, the dependence of alphavirus nsP1 on S-adenosylmethionine to form the capping complex is particularly notable. The methyltransferase domain of nsP1 catalyzes the transfer of the methyl group from S-adenosylmethionine to position 7 of GTP, after which the guanylyltransferase domain of nsP1 catalyzes creation of the m^7GMP-nsP1 complex. These nsP1-mediated methyltransferase and guanylyltransferase steps are unique to alphaviruses, and have been proposed as potential targets for novel antiviral inhibitors [73, 74].

nsP2. Amino acid sequence analysis has revealed that the N-terminus of the alphavirus nsP2 contains conserved domains for RNA helicases and nucleotide triphosphatase activity [75]. In addition, the nucleotide triphosphatase domain serves as the RNA 5'-triphosphatase needed to initiate the capping of genomic and subgenomic RNAs [76, 77]. Furthermore, the C-terminus of nsP2 contains a papain-like proteinase domain [76, 78-80]. Thus, nsP2 is a multifunctional protein responsible in part for RNA unwinding and the autocatalytic processing of the viral non-structural polyproteins [81, 82]. Alphavirus polyprotein processing is solely dependent on nsP2, which may act in a cis or trans manner depending on which junction of the polyprotein is being cleaved [82]. It has also been demonstrated through the use of temperature-sensitive mutants that both major nsP2 enzymatic functions, RNA helicase and protease, are required for the expression of subgenomic RNA, although the degree of dependence on the helicase domain varies between alphaviruses and with specific mutations [76, 83]. Finally, the C-terminus of nsP2 from old world alphaviruses, such as SINV and CHIKV, is critical in causing the virus-induced cytopathic effect (CPE) seen in most host cells, primarily through inhibition of host transcription and translation [84, 85]. As noted below in the drug discovery section, this alphavirus nsP2-mediated activity is an intriguing potential target for novel antiviral agents.

nsP3. The function of the alphavirus nsP3 is the least well understood, but it is known to be required for genome replication [70, 86, 87]. The amino acid sequence of nsP3 shows the least homology among alphaviruses, with as little as 52% conservation among viruses. While the N-terminal half of nsP3 is the most conserved region among viruses, the C-terminal half is extremely variable in both sequence and length [88]. Although the C-terminus is highly variable, a conserved proline-rich sequence motif has been identified to which the host proteins amphiphysin-1 and -2 bind. The precise function of this proline-rich conserved domain is unknown, but its disruption leads to reduced genome replication and reduced mortality in SFV-infected mice [89]. Finally, nsP3 is heavily phosphorylated at multiple serine and threonine residues. However, the significance of this post-translational modification is unknown, as it does not appear to impact the functional role of nsP3 in viral genome replication [90, 91].

nsP4. The alphavirus nsP4 serves as the polymerase core for all viral RNA synthesis. The N-terminus of nsP4 contains distinctive domains that resemble RNA-dependent RNA polymerases (RdRps) from other RNA viruses, and it has been shown to interact with other nsPs for the synthesis of viral RNAs [92]. The nsP4 C-terminus also has terminal nucleotide transferase activity for the repair of viral RNA termini [86, 93, 94]. All the enzymatic activities of nsP4 are viable antiviral targets, and in particular the RdRp activity, as

inhibitors of viral polymerases are in clinical use for multiple pathogens, including several herpes viruses, hepatitis B and C viruses, and human immunodeficiency virus (HIV) [95-97].

Capsid. The alphavirus subgenomic RNA is translated into a polyprotein that is processed into the three main structural proteins: capsid, envelope glycoprotein 1 (E1), and E2 [98, 99]. The capsid protein, which is located at the N-terminus of the structural polyprotein, is a multifunctional protein with autocatalytic protease activity [100]. The capsid protein also serves as a primary structural component to encapsidate the viral genomic RNA during packaging and, at least for the new world alphaviruses, has been demonstrated to inhibit host transcription and translation [101, 102]. Thus, the capsid protein from new world alphaviruses is responsible in part for virus-induced CPE, similar to the function of nsP2 in old world alphaviruses [84, 85]. Consequently, drug discovery and development strategies that target specific virus-encoded functions may differ between new world and old world alphaviruses. Some new world alphavirus capsid proteins also inhibit nuclear export [103, 104], suggesting a potential mechanism whereby this structural protein disrupts innate host cell defenses such as pattern recognition receptors and interferon-dependent signaling pathways [105, 106]. Disrupting this capsid-mediated virus countermeasure represents an additional potential target for drug discovery.

Envelope glycoproteins. The alphavirus envelope consists primarily of cellular membrane phospholipids and the glycoproteins E1 and E2 which, along with the capsid protein, are encoded on the subgenomic RNA [98]. Glycoprotein E1 is primarily responsible for virus penetration into the host cell by facilitating viral membrane fusion with cellular membranes [107, 108]. Glycoprotein E2 is responsible for host cell receptor binding, and plays a key role in the neurovirulence of new world alphaviruses [109]. Furthermore, E2 is responsible for eliciting both protective and therapeutic immune responses [110], and several groups have shown promising results *in vitro* and with preclinical animal models for the development of viral glycoprotein-specific antibodies as therapeutics against alphavirus infections [111-113]. There is also ample clinical precedence for targeting viral envelope glycoprotein activities in the development of antiviral agents, as two commonly used drugs for the treatment of HIV infection, maraviroc and enfuvirtide, inhibit viral glycoprotein-mediated co-receptor binding and membrane fusion, respectively [114, 115].

Antiviral drug discovery

There are currently no approved vaccines or antiviral drugs for humans to prevent or treat infections with alphaviruses, or in fact, most arboviruses. This leaves symptomatic management, such as fluid resuscitation, analgesia, and seizure control, as the mainstay of treatment until the infection resolves by itself. However, there are indispensable enzymatic activities associated with non-structural and structural viral proteins that are common to many, if not all, arboviruses, including RNA polymerase, protease, helicase, and methyltransferase activities. Furthermore, many enveloped viruses share common receptor binding and membrane fusion mechanisms [116], and these shared characteristics are ideal potential targets for the development of broad spectrum antivirals. For example, carbocyclic cytidine is a nucleoside analog that has promise as a broad spectrum antiviral with activity in cell culture against numerous DNA and RNA viruses, including SINV and VEEV [117, 118]. Another nucleoside analog, ribavirin, inhibits alphavirus [119], flavivirus [120], and bunyavirus [121] replication in cultured cells, and has been used clinically in the settings of severe La Crosse virus encephalitis [122] and CHIKV arthralgia [123]. In addition to viral targets, there is an increasing emphasis in antiviral drug discovery on targeting host processes, an approach that has the potential to maintain broad spectrum activity and increase the barrier to the development of resistance [124, 125]. The limited examples noted above indicate that the development of broad spectrum antivirals is achievable, which is an important directive in the NIAID Strategic Plan for Biodefense Research [126]. Nevertheless, there remains a pressing need to discover additional broad spectrum antiviral drugs as part of an effective medical countermeasure strategy to prevent or mitigate illness, suffering, and death resulting from alphavirus infections and other reemerging viruses.

Chemical libraries containing small molecule compounds with known structures provide a rich source of starting material for the identification of antiviral agents. A number of small molecule compounds have been reported to inhibit alphavirus replication, including the nucleoside analogs ribavirin [127, 128], carbocyclic cytidine [117], and triaryl pyrazoline [129]. Our laboratory has also utilized small molecule libraries to identify novel thienopyrrole- and indole-based compounds effective against WEEV and related neurotropic alphaviruses [67, 130, 131]. Although small molecule libraries can be vast in size and scope, they are constrained by factors such as the costs of acquiring or maintaining large drug collections and limitations of synthetic and combinatorial chemistry [132]. Even the largest small molecule libraries, which often contain 10^6 compounds or more, represent an exceedingly small fraction of the number of chemically feasible drug-like molecules, which is projected to be on the order of 10^{60} to 10^{100} compounds [133, 134]. The nearly complete lack of effective drugs to treat alphavirus infections highlights the need for innovative approaches that go beyond the limitations of chemical libraries to identify and develop antiviral agents for these potentially devastating pathogens.

An alternative approach to defined small molecule-based drug discovery is to take advantage of the complex biosynthetic pathways of living organisms, which can produce natural products of almost unlimited structural diversity [135]. Natural product structures are the endpoint of a corresponding complex biosynthetic system that comprises a metabolic pathway, which can include structural, regulatory, resistance, and transport genes [136]. Many anti-infective agents, including the antibiotics penicillin, erythromycin, and streptomycin, as well as other pharmaceuticals such as immunosuppressants, cholesterol-reducing agents, and cancer chemotherapeutics, are produced by plants and microbes as the products of secondary metabolism. Historically, natural products have been utilized quite effectively in the identification and development of antimicrobial agents, as a substantial portion of the currently available drugs used clinically to treat bacterial and fungal infections were derived originally from microbial sources [137]. In fact, from 1983-1994, natural products formed the majority of newly approved chemotherapy agents (78%) and antibiotics (61%), clearly demonstrating that even with advances in chemistry, natural materials remain a vital source for drug discovery [138]. However, major challenges exist in utilizing natural product extracts that differ from approaches using synthetic chemical libraries, as extracts are typically heterogeneous mixtures of complex compounds that are not structurally optimized for high-throughput screening (HTS). Furthermore, natural products require multiple rounds of bioassay-guided fractionation to identify the purified compound or compounds responsible for the bioactivity in the original extract. There are also risks of low yield or isolation of structurally related molecules that may not be easily separated by standard biochemical techniques [139]. For these reasons, natural product-based drug discovery has somewhat fallen out of favor in recent years in the pharmaceutical industry, despite continued progress in HTS and analytical chemistry techniques that have improved the potential utility of natural products [140]. Nevertheless, the increased emphasis on drug discovery within academia and the development of robust assays based on the alphavirus life cycle amenable to HTS have led to the identification of several natural product-derived candidate antivirals. In the following sections, we will discuss the primary alphavirus assays used for antiviral HTS and review select natural product-derived compounds that have already been identified as candidate antivirals against alphaviruses and related arboviruses. Table 1 lists several candidate natural product-derived antiviral compounds, their initial source, potency, proposed mechanism of action, target alphaviruses, and corresponding references.

High-throughput screening assays

There are three general categories of HTS assays that have been developed to identify candidate antivirals against alphaviruses. The first are assays that specifically target a viral enzymatic activity, such as the methyltransferase, protease, helicase, or RNA polymerase activities of individual alphavirus nsPs or structural proteins described above.

Table 1. Candidate antiviral agents derived from natural products that are active against alphaviruses.

Compound	Source	Species	Potency (EC_{50} or IC_{50})	Mechanism of Action	Viruses tested	Ref.
Seco-pregnane steroid analogs	Plant	*Strobilanthes cusia; Cynanchum paniculatum*	1-1.5 nM	Viral subgenomic RNA synthesis	SNIV, EEEV	[162]
Trigocherrins A - F Trigocherriolides A	Plant	*Trigonostemon cherrieri*	1-50 μM	Viral RNA synthesis	CHIKV, SINV, SFV	[163, 164]
Harringtonine	Plant	*Cephalotaxus harringtonia*	0.24 μM	Viral proteins synthesis	CHIKV, SINV	[153]
Mycophenolic acid	Fungi	*Penicillium stoloniferum; Penicillium echinulatum*	0.26-7.8 μM	Host cell enzyme inosine-5'-monophosphate dehydrogenase	SINV, CHIKV, WEEV	[119, 156, 174, 175]
Tunicamycin	Bacteria	*Streptomyces lysosuperficus*	<0.03 μg/ml	Host cell N-linked glycoprotein synthesis	SFV, SINV	[151, 177, 178]
Antimycin A	Bacteria	*Streptomyces kaviengensis*	3-4 nM	Host cell pyrimidine biosynthesis	WEEV	[156]

In general, although biochemical assays to measure many of these enzymatic activities have been developed [74, 82, 141-146], the majority have not yet been adapted to an HTS format. However, it has recently been demonstrated that host transcriptional inhibition can be modeled by transiently expressing old world alphavirus nsP2 with a luciferase reporter plasmid [147]. Lucas-Hourani *et al.* took advantage of this assay to develop an HTS to identify CHIKV nsP2 inhibitors from a library of >3,000 known compounds, with which they identified one biologically active compound as a candidate antiviral [148]. Further development of alphavirus enzymatic activity-specific assays will undoubtedly lead to additional candidate antivirals targeting these essential viral proteins.

The second category of HTS assays for alphavirus inhibitors relies on protection from virus-induced CPE in host cells. The earliest versions of these CPE-reduction assays were developed decades ago and represent some of the earliest attempts at antiviral HTS [149]. Although this assay provides a relatively crude evaluation of alphavirus replication, it was used successfully to identify the antiviral activity of tunicamycin [150, 151]. Significant advances in molecular biology techniques and instrument development have led to decreased labor intensity and miniaturized scales, which has increased the throughput for CPE-reduction assays. For example, measuring ATP metabolism using readily available commercial reagents results in highly reproducible HTS antiviral assays with excellent signal-to-noise ratios [152].

The third category of HTS assays for alphavirus inhibitors uses sensitive techniques to measure the expression of either an endogenous viral protein or an engineered reporter gene as a surrogate marker for viral replication. Recently developed HTS assays that detect endogenous viral proteins include an immunofluorescence-based assay for CHIKV envelope glycoprotein [153] and an ELISA-based assay for VEEV envelope glycoprotein [154]. The genetic flexibility of alphaviruses also allows the insertion of reporter genes into several genomic regions, whereby reporter gene expression correlates with viral genome replication. Pohjala *et al.* developed an HTS assay with infectious SFV by inserting the luciferase gene between nsP3 and nsP4, thereby creating a reporter virus in which polyprotein processing leads to luciferase expression and activity [155]. Similarly, we have developed an

HTS assay using a WEEV-based replicon, where the subgenome-encoded structural proteins were replaced with the luciferase reporter gene [67]. We have used this system extensively to identify a series of novel small molecule alphavirus inhibitors [130, 131], and also to identify the microbial-derived natural product antimycin A, as noted below [156].

Plant-derived antivirals

The use of plant material likely represents the earliest attempts to derive therapeutics from the surrounding environment for the treatment of illnesses. It has been postulated that traces of medicinal plants in Neanderthal graves provide evidence of such primitive therapeutic uses [157]. There are an estimated 250,000 different plant species known, of which only approximately 5-6% have been screened for any type of therapeutic indication [158]. Perhaps the most successful use of a plant-derived natural product as an antiviral is oseltamivir, a neuraminidase inhibitor that is clinically effective for influenza virus infections. Oseltamivir was originally synthesized from the precursor shikimic acid, which is derived from the Chinese star anise *Illicium anisatum* [159, 160]. Advantages of plants as source material for drug discovery include significant diversity in specific biochemical pathways, such as terpenoid and phenylpropanoid synthesis, compared to microorganisms. Plants also offer a relative ease of obtaining large amounts of starting material [158]. Finally, plants are multicellular organisms that actively defend themselves against viruses, indicating that plants may contain specific metabolites with intrinsic antiviral properties [161]. Thus, plants provide source material for antiviral discovery that can offer novel or complex structures not found in other natural product sources or synthetic libraries. Below, we describe several recent examples of natural products isolated from plants that have antiviral activity against alphaviruses, yet function through different mechanisms.

Chinese herbal medicines have been used for centuries to treat many illnesses, including infectious diseases, and they represent an attractive source of starting material for potential antiviral agents. Li *et al.* screened extracts from *Strobilanthes cusia* and *Cynanchum paniculatum*, two plants commonly used in traditional Chinese medicine, for antiviral activity using an assay based on tobacco mosaic virus, an alphavirus-like plant pathogen [162]. This approach led to the isolation of several *seco*-pregnane steroids with antiviral activity, including glaucogenin C, cynatratoside A, and paniculatumoside C, D, and E. These purified compounds have potent activity against SINV and EEEV, but not other RNA or DNA viruses. Mechanism of action studies revealed that these plant-derived steroids inhibit subgenomic RNA synthesis, which potentially explains their restricted antiviral activity for alphaviruses and related plant pathogens. Furthermore, paniculatumoside C improved survival in mice challenged with a lethal SINV inoculum, but only when administered in advance or simultaneously with infection, suggesting limited applicability in the setting of established infections [162].

In 2012, Allard *et al.* published two papers describing the isolation and characterization of antiviral compounds derived from the bark and wood of *Trigonostemon cherrieri*, a rare plant native to the sclerophyllous forest of New Caledonia [163, 164]. They isolated a family of highly oxygenated daphnane diterpenoid orthoesters with an unusual chlorinated moiety, which were termed trigocherrins A through F and trigocherriolides A through D. These compounds were active in CPE reduction assays using CHIKV, SINV or SFV, and in contrast to the plant-derived *seco*-pregnane steroids noted above, trigocherrin A and trigocherriolides A and B were also active against dengue virus, a flavivirus unrelated to alphaviruses. Mechanism of action studies showed suppression of radiolabeled nucleotide incorporation into newly synthesized viral RNA [163, 164], suggesting the potential for broad spectrum antiviral activity amongst viruses with RdRp activity.

Lastly, Kaur *et al.* screened a library of 502 purified natural products using a CHIKV immunofluorescence assay, and identified 44 compounds with antiviral activity. Four were chosen for further validation, including daunorubicin, harringtonine, hypocrellin A, and rottlerin, where harringtonine, an alkaloid derived from the plant

Cephalotaxus harringtonia, showed the most potent activity against both CHIKV and SINV [153]. Harringtonine is a known inhibitor of peptide elongation during eukaryotic translation [165], and mechanism of action studies showed that harringtonine blocked an early step in the alphavirus replication cycle before the synthesis of (+) and (-) viral RNA, suggesting inhibition of initial viral protein translation [153].

Microbe-derived antivirals

The use of microorganism (e.g., bacteria and fungi) in natural product drug discovery has a long and fruitful history dating back to the discovery of penicillin in the 1940s [166]. Microorganisms have been obtained from a wide range of geographical regions, terrestrial and marine habitats, and plant or animal source materials to produce antibiotics. Their secondary metabolites have changed the face of human and veterinary medicine over the past 60 years, and continue to provide new drug leads for pharmaceutical development [136]. In recent years, there have been significant advances in the identification, cultivation, and analysis of novel microorganisms from the marine environment, which has sparked much speculation and research into using these marine microbes for drug discovery [167, 168]. Marine sediments from shallow and deep-water habitats are proving to be a particularly rich source of actinomycetes, whose metabolic products are providing entirely new structural diversity with potential broad clinical applicability [167-172], including possible novel antiviral compounds [173]. With their track record of success against bacterial and fungal infections, and the continued discovery of new marine microorganisms and improvements in HTS and analytical chemistry, the possibility of finding novel antiviral agents derived from microorganisms has never been more promising. Below, we describe several examples of natural products isolated from microorganisms that have antiviral activity against alphaviruses, yet similar to the plant-derived compounds described above, functioning through different mechanisms.

Mycophenolic acid is one of the most studied and widely employed inhibitors used experimentally with broad spectrum antiviral activity, including against alphaviruses, and is derived from a metabolite of the fungi *Penicillium stoloniferum* or *P. echinulatum*. Mycophenolic acid inhibits the host cell enzyme inosine-5′-monophosphate dehydrogenase and was shown more than 50 years ago to have antiviral activity [149]. Mycophenolic acid depletes the pool of cellular GTP, which inhibits GTP-dependent viral enzyme functions, such as alphavirus capping enzyme (nsP1) or RdRp (nsP4) activities, and is active against multiple alphaviruses, including SINV [119, 174], CHIKV [175], and WEEV [156].

As noted above, Takatsuki *et al.* used an early version of the CPE-reduction assay to screen 4,000 terrestrial actinomycetes for antiviral activity, and discovered that tunicamycin, a nucleoside antibiotic derived from *Streptomyces lysosuperficus*, had potent and broad spectrum antiviral activity [150]. Tunicamycin has a complex chemical structure and blocks cellular glycosylation, thereby inhibiting N-linked glycoprotein synthesis [176]. This cellular process is essential for viral glycoprotein production, and its disruption potently inhibits infectious particle assembly of enveloped viruses, such as SFV and SINV [151, 177, 178]. Unfortunately, preclinical studies of tunicamycin in animal models were disappointing, where it was shown to enhance alphavirus pathogenesis [179, 180], potentially through decreased glycosylation of essential host antiviral response proteins, such as type I interferons [181]. Thus, tunicamycin vividly demonstrates both the potential and challenges of developing antivirals that target host pathways.

We recently completed an HTS of over 2,200 pre-fractionated extracts derived from marine actinomycetes using a WEEV replicon-based assay, and subsequently used a bioassay-guided fractionation process to isolate a purified compound from *Streptomyces kaviengensis* that displayed potent and broad spectrum antiviral activity in cultured cells [156]. We identified this compound as antimycin A, a secondary bacterial metabolite initially identified in the 1940s [182] and a known inhibitor of host mitochondrial electron transport [183]. We demonstrated that antimycin A antiviral activity against WEEV is mediated in part through suppression of pyrimidine biosynthesis, which is linked to the mitochondrial

electron transport chain via the enzyme dihydroorotate dehydrogenase. Furthermore, in contrast to many candidate antiviral compounds that have potent *in vitro* activity yet fail during *in vivo* animal model efficacy studies, we demonstrated that antimycin A improves clinical disease, prolongs survival, and decreases CNS virus titers in mice given a lethal inocula of WEEV, albeit with a narrow therapeutic window [156].

CONCLUSION

Alphaviruses continue to pose a threat to human health around the world both as reemerging infectious diseases and as potential biological weapons. There are currently no antivirals or vaccines approved for these virulent pathogens, and thus there is an urgent need to develop viable treatment options that can be distributed quickly in the event of a large scale outbreak or intentional exposure. One significant bottleneck to antiviral discovery is acquiring sufficient source material to explore a larger expanse of chemical space. Natural products have a proven track record of being fruitful sources for drug discovery, primarily due to the extensive and complex metabolic pathways living organisms possess to produce a nearly limitless supply of structural diversity. The recent development, optimization, and utilization of alphavirus assays with HTS capabilities, coupled with improvements in analytical biochemistry techniques and microorganism cultivation, has reopened the door for further discovery and development of antiviral compounds derived from natural product resources.

CONFLICT OF INTEREST STATEMENT

Authors report no conflict of interest.

ABBREVIATIONS

CDC, Centers for Disease Control and Prevention; CHIKV, Chikungunya virus; CNS, central nervous system; CPE, cytopathic effect; E1, envelope glycoprotein 1; E2, envelope glycoprotein 2; EEEV, eastern equine encephalitis virus; HIV, human immunodeficiency virus; HTS, high-throughput screen; NIAID, National Institute of Allergy and Infectious Diseases; nsP, non-structural protein; RdRp, RNA-dependent RNA polymerase; SFV, Semliki Forest virus; SINV, Sindbis virus; VEEV, Venezuelan equine encephalitis virus; WEEV, western equine encephalitis virus

REFERENCES

1. Griffin, D. E. 2001, Fields Virology, 4th Edition, D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman and S. E. Strauss (Eds.), Lippincott Williams & Wilkins, Philadephia, 917.
2. Porterfield, J. S., Casals, J., Chumakov, M. P., Gaidamovich, S. Y., Hannoun, C., Holmes, I. H., Horzinek, M. C., Mussgay, M., Oker-Blom, N., Russell, P. K. and Trent, D. W. 1978, Intervirology, 9, 129.
3. Schlesinger, S. and Schlesinger, M. J. 2001, Fields Virology, 4th Edition, D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman and S. E. Strauss (Eds.), Lippincott Williams & Wilkins, Philadephia, 895.
4. Gubler, D. J. 2002, Arch. Med. Res., 33, 330.
5. Nash, D., Mostashari, F., Fine, A., Miller, J., O'Leary, D., Murray, K., Huang, A., Rosenberg, A., Greenberg, A., Sherman, M., Wong, S. and Layton, M. 2001, N. Engl. J. Med., 344, 1807.
6. Soldan, S. S. and Gonzalez-Scarano, F. 2005, J. Neurovirol., 11, 412.
7. Enserink, M. 2007, Science, 318, 1860.
8. Ross, R. W. 1956, J. Hyg., 54, 177.
9. Padbidri, V. S. and Gnaneswar, T. T. 1979, J. Hyg. Epidemiol. Microbiol. Immunol., 23, 445.
10. Charrel, R. N., de Lamballerie, X. and Raoult, D. 2007, N. Engl. J. Med., 356, 769.
11. Bessaud, M., Peyrefitte, C. N., Pastorino, B. A., Tock, F., Merle, O., Colpart, J. J., Dehecq, J. S., Girod, R., Jaffar-Bandjee, M. C., Glass, P. J., Parker, M., Tolou, H. J. and Grandadam, M. 2006, Emerg. Infect. Dis., 12, 1604.
12. Singh, P., Mittal, V., Rizvi, M. A., Bhattacharya, D., Chhabra, M., Rawat, D. S., Ichhpujani, R. L., Chauhan, L. S. and Rai, A. 2012, J. Infect. Dev. Ctries., 6, 563.
13. Duong, V., Andries, A. C., Ngan, C., Sok, T., Richner, B., Asgari-Jirhandeh, N., Bjorge, S., Huy, R., Ly, S., Laurent, D., Hok, B.,

Roces, M. C., Ong, S., Char, M. C., Deubel, V., Tarantola, A. and Buchy, P. 2012, Emerg. Infect. Dis., 18, 2066.
14. Wangchuk, S., Chinnawirotpisan, P., Dorji, T., Tobgay, T., Yoon, I. K. and Fernandez, S. 2013, Emerg. Infect. Dis., 19, 1681.
15. Napoli, C., Salcuni, P., Pompa, M. G., Declich, S. and Rizzo, C. 2012, J. Travel. Med., 19, 294.
16. Tsetsarkin, K. A. and Weaver, S. C. 2011, PLoS Pathog., 7, e1002412.
17. Giltner, L. T. and Shahan, M. S. 1933, Science, 78, 587.
18. Armstrong, P. M. and Andreadis, T. G. 2013, N. Engl. J. Med., 368, 1670.
19. Silva, M. L., Galiza, G. J., Dantas, A. F., Oliveira, R. N., Iamamoto, K., Achkar, S. M. and Riet-Correa, F. 2011, J. Vet. Diagn. Invest., 23, 570.
20. Molaei, G., Andreadis, T. G., Armstrong, P. M., Thomas, M. C., Deschamps, T., Cuebas-Incle, E., Montgomery, W., Osborne, M., Smole, S., Matton, P., Andrews, W., Best, C., Cornine, F. 3rd., Bidlack, E. and Texeira, T. 2013, Vector Borne Zoonotic Dis., 13, 312.
21. Caglioti, C., Lalle, E., Castilletti, C., Carletti, F., Capobianchi, M. R. and Bordi, L. 2013, New Microbiol., 36, 211.
22. Brighton, S. W., Prozesky, O. W. and de la Harpe, A. L. 1983, S. Afr. Med. J., 63, 313.
23. Hochedez, P., Jaureguiberry, S., Debruyne, M., Bossi, P., Hausfater, P., Brucker, G., Bricaire, F. and Caumes, E. 2006, Emerg. Infect. Dis., 12, 1565.
24. Josseran, L., Paquet, C., Zehgnoun, A., Caillere, N., Le Tertre, A., Solet, J. L. and Ledrans, M. 2006, Emerg. Infect. Dis., 12, 1994.
25. Mavalankar, D., Shastri, P., Bandyopadhyay, T., Parmar, J. and Ramani, K. V. 2008, Emerg. Infect. Dis., 14, 412.
26. Deresiewicz, R. L., Thaler, S. J., Hsu, L. and Zamani, A. A. 1997, N. Engl. J. Med., 336, 1867.
27. Carrera, J. P., Forrester, N., Wang, E., Vittor, A. Y., Haddow, A. D., Lopez-Verges, S., Abadia, I., Castano, E., Sosa, N., Baez, C., Estripeaut, D., Diaz, Y., Beltran, D., Cisneros, J., Cedeno, H. G., Travassos da Rosa, A. P., Hernandez, H., Martinez-Torres, A. O., Tesh, R. B. and Weaver, S. C. 2013, N. Engl. J. Med., 369, 732.
28. Silverman, M. A., Misasi, J., Smole, S., Feldman, H. A., Cohen, A. B., Santagata, S., McManus, M. and Ahmed, A. A. 2013, Emerg. Infect. Dis., 19, 194.
29. Przelomski, M. M., O'Rourke, E., Grady, G. F., Berardi, V. P. and Markley, H. G. 1988, Neurology, 38, 736.
30. MMWR Morbidity and Mortality Weekly Report. 2006, 55, 697.
31. Feemster, R. F. 1957, N. Engl. J. Med., 257, 701.
32. Aguilar, M. J. 1970, Infect. Immun., 2, 533.
33. Zlotnik, I., Peacock, S., Grant, D. P. and Batter-Hatton, D. 1972, Br. J. Exp. Pathol., 53, 59.
34. Liu, C., Voth, D. W., Rodina, P., Shauf, L. R. and Gonzalez, G. 1970, J. Infect. Dis., 122, 53.
35. World Health Organization. 2005, Biosecur. Bioterror., 3, 268.
36. Medical Management of Biological Casualties Handbook. 2011, Z. F. Dembek (Ed.), Fort Detrick, MD: U.S. Army Medical Research Institute of Infectious Diseases.
37. Venkatesh, S. and Memish, Z. A. 2003, Int. J. Antimicrob. Agents, 21, 200.
38. NIH/NIAID Category A, B, & C Priority Pathogens. 2003, available at http://www.niaid.gov/biodefense/PDF/cat.pdf.
39. Rotz, L. D., Khan, A. S., Lillibridge, S. R., Ostroff, S. M. and Hughes, J. M. 2002, Emerg. Infect. Dis., 8, 225.
40. Sidwell, R. W. and Smee, D. F. 2003, Antiviral. Res., 57, 101.
41. Rusnak, J. M., Kortepeter, M. G., Hawley, R. J., Anderson, A. O., Boudreau, E. and Eitzen, E. 2004, Biosecur. Bioterror., 2, 281.
42. Ryzhikov, A. B., Ryabchikova, E. I., Sergeev, A. N. and Tkacheva, N. V. 1995, Arch. Virol., 140, 2243.
43. Reed, D. S., Larsen, T., Sullivan, L. J., Lind, C. M., Lackemeyer, M. G., Pratt, W. D. and Parker, M. D. 2005, J. Infect. Dis., 192, 1173.
44. Strauss, J. H. and Strauss, E. G. 1994, Microbiol. Rev., 58, 491.

45. Havert, M. B., Schofield, B., Griffin, D. E. and Irani, D. N. 2000, J. Virol., 74, 5352.
46. Irani, D. N. and Prow, N. A. 2007, J. Neuropathol. Exp. Neurol., 66, 533.
47. Wu, D. C., Jackson-Lewis, V., Vila, M., Tieu, K., Teismann, P., Vadseth, C., Choi, D. K., Ischiropoulos, H. and Przedborski, S. 2002, J. Neurosci., 22, 1763.
48. Tikka, T., Fiebich, B. L., Goldsteins, G., Keinanen, R. and Koistinaho, J. 2001, J. Neurosci., 21, 2580.
49. Das, K. P., McMillian, M. K., Bing, G. and Hong, J. S. 1995, J. Neuroimmunol., 62, 9.
50. Kong, L. Y., McMillian, M. K., Hudson, P. M., Jin, L. and Hong, J. S. 1997, J. Pharmacol. Exp. Ther., 280, 61.
51. Prow, N. A. and Irani, D. N. 2007, Exp. Neurol., 205, 461.
52. Nargi-Aizenman, J. L., Havert, M. B., Zhang, M., Irani, D. N., Rothstein, J. D. and Griffin, D. E. 2004, Ann. Neurol., 55, 541.
53. Darman, J., Backovic, S., Dike, S., Maragakis, N. J., Krishnan, C., Rothstein, J. D., Irani, D. N. and Kerr, D. A. 2004, J. Neurosci., 24, 7566.
54. Greene, I. P., Lee, E. Y., Prow, N., Ngwang, B. and Griffin, D. E. 2008, Proc. Natl. Acad. Sci. USA, 105, 3575.
55. Peranen, J. and Kaariainen, L. 1991, J. Virol., 65, 1623.
56. Ahola, T., Kujala, P., Tuittila, M., Blom, T., Laakkonen, P., Hinkkanen, A. and Auvinen, P. 2000, J. Virol., 74, 6725.
57. Kujala, P., Ikaheimonen, A., Ehsani, N., Vihinen, H., Auvinen, P. and Kaariainen, L. 2001, J. Virol., 75, 3873.
58. Kinney, R. M., Chang, G. J., Tsuchiya, K. R., Sneider, J. M., Roehrig, J. T., Woodward, T. M. and Trent, D. W. 1993, J. Virol., 67, 1269.
59. Kobiler, D., Rice, C. M., Brodie, C., Shahar, A., Dubuisson, J., Halevy, M. and Lustig, S. 1999, J. Virol., 73, 10440.
60. Suthar, M. S., Shabman, R., Madric, K., Lambeth, C. and Heise, M. T. 2005, J. Virol., 79, 4219.
61. Heise, M. T., Simpson, D. A. and Johnston, R. E. 2000, J. Virol., 74, 4207.
62. Fazakerley, J. K., Boyd, A., Mikkola, M. L. and Kaariainen, L. 2002, J. Virol., 76, 392.
63. Rikkonen, M. 1996, Virology, 218, 352.
64. Tuittila, M. T., Santagati, M. G., Roytta, M., Maatta, J. A. and Hinkkanen, A. E. 2000, J. Virol., 74, 4579.
65. Kaariainen, L., Keranen, S., Lachmi, B., Soderlund, H., Tuomi, K. and Ulmanen, I. 1975, Med. Biol., 53, 342.
66. Frolov, I., Hoffman, T. A., Pragai, B. M., Dryga, S. A., Huang, H. V., Schlesinger, S. and Rice, C. M. 1996, Proc. Natl. Acad. Sci. USA, 93, 11371.
67. Peng, W., Peltier, D. C., Larsen, M. J., Kirchhoff, P. D., Larsen, S. D., Neubig, R. R. and Miller, D. J. 2009, J. Infect. Dis., 199, 950.
68. Shirako, Y. and Strauss, J. H. 1994, J. Virol., 68, 1874.
69. Li, M. L. and Stollar, V. 2004, Proc. Natl. Acad. Sci. USA, 101, 9429.
70. Wang, Y. F., Sawicki, S. G. and Sawicki, D. L. 1994, J. Virol., 68, 6466.
71. Ahola, T., Lampio, A., Auvinen, P. and Kaariainen, L. 1999, EMBO J., 18, 3164.
72. Spuul, P., Salonen, A., Merits, A., Jokitalo, E., Kaariainen, L. and Ahola, T. 2007, J. Virol., 81, 872.
73. Ahola, T. and Kääriäinen, L. 1995, Proc. Natl. Acad. Sci. USA, 92, 507.
74. Ahola, T., Laakkonen, P., Vihinen, H. and Kaariainen, L. 1997, J. Virol., 71, 392.
75. Gorbalenya, A. E., and Koonin, E. V. 1993, Curr. Opin. Struct. Biol., 3, 419.
76. Balistreri, G., Caldentey, J., Kaariainen, L. and Ahola, T. 2007, J. Virol., 81, 2849.
77. Vasiljeva, L., Merits, A., Auvinen, P. and Kaariainen, L. 2000, J. Biol. Chem., 275, 17281.
78. Barrett, A. J. and Rawlings, N. D. 2001, Biol. Chem., 382, 727.
79. Russo, A. T., White, M. A. and Watowich, S. J. 2006, Structure, 14, 1449.
80. Gorbalenya, A. E., Koonin, E. V. and Lai, M. M. 1991, FEBS Lett., 288, 201.
81. Hardy, W. R. and Strauss, J. H. 1989, J. Virol., 63, 4653.
82. Merits, A., Vasiljeva, L., Ahola, T., Kaariainen, L. and Auvinen, P. 2001, J. Gen. Virol., 82, 765.

83. Lulla, V., Merits, A., Sarin, P., Kaariainen, L., Keranen, S. and Ahola, T. 2006, J. Virol., 80, 3108.
84. Garmashova, N., Gorchakov, R., Frolova, E. and Frolov, I. 2006, J. Virol., 80, 5686.
85. Fros, J. J., van der Maten, E., Vlak, J. M. and Pijlman, G. P. 2013, J. Virol., 87, 10394.
86. Hahn, Y. S., Grakoui, A., Rice, C. M., Strauss, E. G. and Strauss, J. H. 1989, J. Virol., 63, 1194.
87. Lastarza, M. W., Lemm, J. A. and Rice, C. M. 1994, J. Virol., 68, 5781.
88. Strauss, E. G., Levinson, R., Rice, C. M., Dalrymple, J. and Strauss, J. H. 1988, Virology, 164, 265.
89. Neuvonen, M., Kazlauskas, A., Martikainen, M., Hinkkanen, A., Ahola, T. and Saksela, K. 2011, PLoS Pathog., 7, e1002383.
90. Li, G. P., La Starza, M. W., Hardy, W. R., Strauss, J. H. and Rice, C. M. 1990, Virology, 179, 416.
91. Peranen, J., Takkinen, K., Kalkkinen, N. and Kaariainen, L. 1988, J. Gen. Virol., 69 (Pt 9), 2165.
92. Rupp, J. C., Jundt, N. and Hardy, R. W. 2011, J. Virol., 85, 3449.
93. Poch, O., Sauvaget, I., Delarue, M. and Tordo, N. 1989, EMBO J., 8, 3867.
94. Tomar, S., Hardy, R. W., Smith, J. L. and Kuhn, R. J. 2006, J. Virol., 80, 9962.
95. Ferrer-Orta, C., Arias, A., Escarmis, C. and Verdaguer, N. 2006, Curr. Opin. Struct. Biol., 16, 27.
96. De Clercq, E. 2004, J. Clin. Virol., 30, 115.
97. De Clercq, E. 2013, Biochem. Pharmacol., 85, 727.
98. Clegg, J. C. 1975, Nature, 254, 454.
99. Schlesinger, S. and Schlesinger, M. J. 1972, J. Virol., 10, 925.
100. Melancon, P. and Garoff, H. 1987, J. Virol., 61, 1301.
101. Garmashova, N., Atasheva, S., Kang, W., Weaver, S. C., Frolova, E. and Frolov, I. 2007, J. Virol., 81, 13552.
102. Garmashova, N., Gorchakov, R., Volkova, E., Paessler, S., Frolova, E. and Frolov, I. 2007, J. Virol., 81, 2472.
103. Atasheva, S., Fish, A., Fornerod, M. and Frolova, E. I. 2010, J. Virol., 84, 4158.
104. Atasheva, S., Garmashova, N., Frolov, I. and Frolova, E. 2008, J. Virol., 82, 4028.
105. Peltier, D. C., Lazear, H. M., Farmer, J. R., Diamond, M. S. and Miller, D. J. 2013, J. Virol., 87, 1821.
106. Simmons, J. D., White, L. J., Morrison, T. E., Montgomery, S. A., Whitmore, A. C., Johnston, R. E. and Heise, M. T. 2009, J. Virol., 83, 10571.
107. Sanz, M. A., Rejas, M. T. and Carrasco, L. 2003, Virology, 305, 463.
108. Rice, C. M. and Strauss, J. H. 1981, Proc. Natl. Acad. Sci. USA, 78, 2062.
109. Tucker, P. C. and Griffin, D. E. 1991, J. Virol., 65, 1551.
110. Metcalf, T. U., Baxter, V. K., Nilaratanakul, V. and Griffin, D. E. 2013, J. Virol., 87, 2420.
111. Selvarajah, S., Sexton, N. R., Kahle, K. M., Fong, R. H., Mattia, K. A., Gardner, J., Lu, K., Liss, N. M., Salvador, B., Tucker, D. F., Barnes, T., Mabila, M., Zhou, X., Rossini, G., Rucker, J. B., Sanders, D. A., Suhrbier, A., Sambri, V., Michault, A., Muench, M. O., Doranz, B. J. and Simmons, G. 2013, PLoS Negl. Trop. Dis., 7, e2423.
112. Goh, L. Y., Hobson-Peters, J., Prow, N. A., Gardner, J., Bielefeldt-Ohmann, H., Pyke, A. T., Suhrbier, A. and Hall, R. A. 2013, Clin. Immunol., 149, 487.
113. Warter, L., Lee, C. Y., Thiagarajan, R., Grandadam, M., Lebecque, S., Lin, R. T., Bertin-Maghit, S., Ng, L. F., Abastado, J. P., Despres, P., Wang, C. I. and Nardin, A. 2011, J. Immunol., 186, 3258.
114. Dorr, P., Westby, M., Dobbs, S., Griffin, P., Irvine, B., Macartney, M., Mori, J., Rickett, G., Smith-Burchnell, C., Napier, C., Webster, R., Armour, D., Price, D., Stammen, B., Wood, A. and Perros, M. 2005, Antimicrob. Agents Chemother., 49, 4721.
115. Reeves, J. D., Gallo, S. A., Ahmad, N., Miamidian, J. L., Harvey, P. E., Sharron, M., Pohlmann, S., Sfakianos, J. N., Derdeyn, C. A., Blumenthal, R., Hunter, E. and Doms, R. W. 2002, Proc. Natl. Acad. Sci. USA, 99, 16249.

116. Harrison, S. C. 2008, Nat. Struct. Mol. Biol., 15, 690.
117. De Clercq, E., Bernaerts, R., Shealy, Y. F. and Montgomery, J. A. 1990, Biochem. Pharmacol., 39, 319.
118. Julander, J. G., Bowen, R. A., Rao, J. R., Day, C., Shafer, K., Smee, D. F., Morrey, J. D. and Chu, C. K. 2008, Antiviral. Res., 80, 309.
119. Malinoski, F. and Stollar, V. 1981, Virology, 110, 281.
120. Jordan, I., Briese, T., Fischer, N., Lau, J. Y. and Lipkin, W. I. 2000, J. Infect. Dis., 182, 1214.
121. Sidwell, R. W., Huffman, J. H., Barnett, B. B. and Pifat, D. Y. 1988, Antimicrob. Agents Chemother., 32, 331.
122. McJunkin, J. E., Khan, R., de los Reyes, E. C., Parsons, D. L., Minnich, L. L., Ashley, R. G. and Tsai, T. F. 1997, Pediatrics, 99, 261.
123. Ravichandran, R. and Manian, M. 2008, J. Infect. Dev. Ctries., 2, 140.
124. Fox, J. L. 2007, Nat. Biotechnol., 25, 1395.
125. Ikeda, M. and Kato, N. 2007, Adv. Drug Deliv. Rev., 59, 1277.
126. NIH/NIAID Updated Strategic Plan for Biodefense Research 2007, available at http://www3.niaid.gov/topics/BiodefenseRelated/Biodefense/PDF/biosp2007.pdf.
127. Smee, D. F., Alaghamandan, H. A., Kini, G. D. and Robins, R. K. 1988, Antiviral Res., 10, 253.
128. Scheidel, L. M. and Stollar, V. 1991, Virology, 181, 490.
129. Puig-Basagoiti, F., Tilgner, M., Forshey, B. M., Philpott, S. M., Espina, N. G., Wentworth, D. E., Goebel, S. J., Masters, P. S., Falgout, B., Ren, P., Ferguson, D. M. and Shi, P. Y. 2006, Antimicrob. Agents Chemother., 50, 1320.
130. Sindac, J. A., Yestrepsky, B. D., Barraza, S. J., Bolduc, K. L., Blakely, P. K., Keep, R. F., Irani, D. N., Miller, D. J. and Larsen, S. D. 2012, J. Med. Chem., 55, 3535.
131. Sindac, J. A., Barraza, S. J., Dobry, C. J., Xiang, J., Blakely, P. K., Irani, D. N., Keep, R. F., Miller, D. J. and Larsen, S. D. 2013, J. Med. Chem., 56, 9222.
132. Thompson, L. A. and Ellman, J. A. 1996, Chem. Rev., 96, 555.
133. Dobson, C. M. 2004, Nature, 432, 824.
134. Schneider, G. and Fechner, U. 2005, Nat. Rev. Drug Discov., 4, 649.
135. Verdine, G. L. 1996, Nature, 384, 11.
136. Walsh, C. T. 2004, Science, 303, 1805.
137. Newman, D. J. and Cragg, G. M. 2007, J. Nat. Prod., 70, 461.
138. Verpoorte, R. 2000, J. Pharm. Pharmacol., 52, 253.
139. Li, J. W. and Vederas, J. C. 2009, Science, 325, 161.
140. Harvey, A. L. 2007, Curr. Opin. Chem. Biol., 11, 480.
141. Laakkonen, P., Hyvonen, M., Peranen, J. and Kaariainen, L. 1994, J. Virol., 68, 7418.
142. Vasiljeva, L., Valmu, L., Kaariainen, L. and Merits, A. 2001, J. Biol. Chem., 276, 30786.
143. Gomez de Cedron, M., Ehsani, N., Mikkola, M. L., Garcia, J. A. and Kaariainen, L. 1999, FEBS Lett., 448, 19.
144. Takehara, M. 1971, Arch. Gesamte Virusforsch, 34, 266.
145. Barton, D. J., Sawicki, S. G. and Sawicki, D. L. 1991, J. Virol., 65, 1496.
146. Li, M. L., Lin, Y. H. and Stollar, V. 2005, Virology, 341, 24.
147. Bourai, M., Lucas-Hourani, M., Gad, H. H., Drosten, C., Jacob, Y., Tafforeau, L., Cassonnet, P., Jones, L. M., Judith, D., Couderc, T., Lecuit, M., Andre, P., Kummerer, B. M., Lotteau, V., Despres, P., Tangy, F. and Vidalain, P. O. 2012, J. Virol., 86, 3121.
148. Lucas-Hourani, M., Lupan, A., Despres, P., Thoret, S., Pamlard, O., Dubois, J., Guillou, C., Tangy, F., Vidalain, P. O. and Munier-Lehmann, H. 2013, J. Biomol. Screen., 18, 172.
149. Herrmann, E. C. Jr., Gabliks, J., Engle, C. and Perlman, P. L. 1960, Proc. Soc. Exp. Biol. Med., 103, 625.
150. Takatsuki, A., Arima, K. and Tamura, G. 1971, J. Antibiot., 24, 215.
151. Schwarz, R. T., Rohrschneider, J. M. and Schmidt, M. F. 1976, J. Virol., 19, 782.
152. Gong, E., Ivens, T., Van den Eynde, C., Hallenberger, S. and Hertogs, K. 2008, J. Virol. Methods, 151, 121.

153. Kaur, P., Thiruchelvan, M., Lee, R. C., Chen, H., Chen, K. C., Ng, M. L. and Chu, J. J. 2013, Antimicrob. Agents Chemother., 57, 155.
154. Spurgers, K. B., Hurt, C. R., Cohen, J. W., Eccelston, L. T., Lind, C. M., Lingappa, V. R. and Glass, P. J. 2013, J. Virol. Methods, 193, 226.
155. Pohjala, L., Barai, V., Azhayev, A., Lapinjoki, S. and Ahola, T. 2008, Antiviral Res., 78, 215.
156. Raveh, A., Delekta, P. C., Dobry, C. J., Peng, W., Schultz, P. J., Blakely, P. K., Tai, A. W., Matainaho, T., Irani, D. N., Sherman, D. H. and Miller, D. J. 2013, PLoS One 8, e82318.
157. Lietava, J. 1992, J. Ethnopharmacol., 35, 263.
158. Verpoorte, R. 1998, Drug Development Today, 3, 232.
159. Bradley, D. 2005, Nat. Rev. Drug Discov., 4, 945.
160. Ghosh, S., Chisti, Y. and Banerjee, U. C. 2012, Biotechnol. Adv., 30, 1425.
161. Jassim, S. A. and Naji, M. A. 2003, J. Appl. Microbiol., 95, 412.
162. Li, Y., Wang, L., Li, S., Chen, X., Shen, Y., Zhang, Z., He, H., Xu, W., Shu, Y., Liang, G., Fang, R. and Hao, X. 2007, Proc. Natl. Acad. Sci. U.S.A., 104, 8083.
163. Allard, P. M., Leyssen, P., Martin, M. T., Bourjot, M., Dumontet, V., Eydoux, C., Guillemot, J. C., Canard, B., Poullain, C., Gueritte, F. and Litaudon, M. 2012, Phytochemistry, 84, 160.
164. Allard, P. M., Martin, M. T., Dau, M. E., Leyssen, P., Gueritte, F. and Litaudon, M. 2012, Org. Lett., 14, 342.
165. Fresno, M., Jimenez, A. and Vazquez, D. 1977, Eur. J. Biochem., 72, 323.
166. Pelaez, F. 2006, Biochem. Pharmacol., 71, 981.
167. Magarvey, N. A., Keller, J. M., Bernan, V., Dworkin, M. and Sherman, D. H. 2004, Appl. Environ. Microbiol., 70, 7520.
168. Mincer, T. J., Jensen, P. R., Kauffman, C. A. and Fenical, W. 2002, Appl. Environ. Microbiol., 68, 5005.
169. Jensen, P. R., Dwight, R. and Fenical, W. 1991, Appl. Environ. Microbiol., 57, 1102.
170. Jensen, P. R. and Fenical, W. 1994, Annu. Rev. Microbiol., 48, 559.
171. Jiang, Z. D., Jensen, P. R. and Fenical, W. 1999, Bioorg. Med. Chem. Lett., 9, 2003.
172. Feling, R. H., Buchanan, G. O., Mincer, T. J., Kauffman, C. A., Jensen, P. R. and Fenical, W. 2003, Angew. Chem. Int. Ed. Engl., 42, 355.
173. Kurtboke, D. I. 2005, Antonie Van Leeuwenhoek, 87, 19.
174. Scheidel, L. M., Durbin, R. K. and Stollar, V. 1987, Virology, 158, 1.
175. Khan, M., Dhanwani, R., Patro, I. K., Rao, P. V. and Parida, M. M. 2011, Antiviral Res., 89, 1.
176. Eckardt, K. 1983, J. Nat. Prod., 46, 544.
177. Leavitt, R., Schlesinger, S. and Kornfeld, S. 1977, J. Virol., 21, 375.
178. Ulug, E. T. and Bose, H. R. Jr. 1985, Virology, 143, 546.
179. Steele, K. E., Seth, P., Catlin-Lebaron, K. M., Schoneboom, B. A., Husain, M. M., Grieder, F. and Maheshwari, R. K. 2006, Vet. Pathol., 43, 904.
180. Maheshwari, R. K., Husain, M. M., Attallah, A. M. and Friedman, R. M. 1983, Infect. Immun., 41, 61.
181. Mizrahi, A., O'Malley, J. A., Carter, W. A., Takatsuki, A., Tamura, G. and Sulkowski, E. 1978, J. Biol. Chem., 253, 7612.
182. Dushee, B. R., Leben, C., Keitt, C.W., and Strong, F. M 1949, J. Am. Chem. Soc., 71, 2436.
183. Trumpower, B. L. 1990, J. Biol. Chem., 265, 11409.