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Review

Understanding the alphaviruses: Recent research on important emerging pathogens and progress towards their control

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ABSTRACT

The alphaviruses were amongst the first arboviruses to be isolated, characterized and assigned a taxonomic status. They are globally very widespread, infecting a large variety of terrestrial animals, insects and even fish, and circulate both in the sylvatic and urban/peri-urban environment, causing considerable human morbidity and mortality. Nevertheless, despite their obvious importance as pathogens, there are currently no effective antiviral drugs with which to treat humans or animals infected by any of these viruses. The EU-supported project—VIZIER (Comparative Structural Genomics of Viral Enzymes Involved in Replication, FP6 Project: 2004-511960) was instigated with an ultimate view of contributing to the development of antiviral therapies for RNA viruses, including the alphaviruses [Coutard, B., Gorbalenya, A.E., Snijder, E.J., Leontovich, A.M., Poupon, A., De Lamballerie, X., Charrel, R., Gould, E.A., Gunther, S., Norder, H., Klempa, B., Bourhy, H., Rohayemj, J., L'hermite, E., Nordlund, P., Stuart, D.I., Owens, R.J., Grimes, J.M., Tuckerm, P.A., Bolognesi, M., Mattevi, A., Coll, M., Jones, T.A., Åqvist, J., Unger, T., Hilgenfeld, R., Bricogne, G., Neyts, J., La Colla, P., Puerstinger, G., Gonzalez, J.P., Leroy, E., Cambillau, C., Romette, J.L., Canard, B., 2008. The VIZIER project: preparedness against pathogenic RNA viruses. Antiviral Res. 78, 37–46]. This review highlights some of the major features of alphaviruses that have been investigated during recent years. After describing their classification, epidemiology and evolutionary history and the expanding geographic distribution of Chikungunya virus, we review progress in understanding the structure and function of alphavirus replicative enzymes achieved under the VIZIER programme and the development of new disease control strategies.

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1. Introduction

1.1. Classification

The VIIIth edition of the International Committee for the Taxonomy of Viruses (ICTV) currently lists 29 species in the genus *Alphavirus* that together with the genus *Rubivirus* forms the family *Togaviridae* (Weaver et al., 2005). The alphaviruses are arthropod-borne (arboviruses), whereas the rubiviruses are transmitted via the respiratory tract. All arthropod-borne alphaviruses are antigenically related but most can be distinguished in cross-reactivity tests (Chanas et al., 1976; Clarke and Casals, 1958; Karabatsos, 1975; Porterfield, 1961) with which they have been divided into 8 antigenic complexes: Eastern, Western, and Venezuelan equine encephalitis, Trocara (complex assigned based only on genetic divergence), Middelburg, Ndumu, Semliki Forest and Barmah Forest. In addition, there are two non-arthropod-borne species, Salmon pancreatic disease virus and Southern elephant seal virus. Based on comparative sequence analysis, the arthropod-borne alphaviruses share a minimum of about 40% amino acid identity in the more divergent structural proteins and 60% in the non-structural proteins.

1.2. Structure, genome strategy and replication

Alphavirus virions are approximately 70 nm in diameter. They are spherical with a lipid bilayer containing heterodimeric protein spikes composed of two envelope glycoproteins E1 and E2. Many alphaviruses also contain a third envelope protein E3. The heterodimers are organized in a T = 4 icosahedral lattice consisting of 80 trimers of E1–E2 complex. The enclosed nucleocapsid core consists of 240 copies of capsid protein and a single copy of the genomic RNA, although Aura virus is reported also to enclose the 26S subgenomic RNA (Rumenapf et al., 1995). The one-to-one relationship between glycoprotein heterodimers and nucleocapsid proteins is important in virus assembly. E1 is the fusion protein for virus entry into the acidic cytoplasmic endosomes. The structure of the E1 glycoprotein of Semliki Forest virus has been determined by crystallography (Lescar et al., 2001), revealing a fold closely related to the flavivirus envelope protein. The E2 glycoprotein extends outwards from the envelope and forms the petals of the spike that cover the underlying E1 protein fusion peptide at neutral pH (Mukhopadhyay et al., 2006).

The four non-structural proteins are defined as nsP1, nsP2, nsP3 and nsP4. The genomic RNA is positive-stranded and serves as the mRNA for translation of the polyprotein precursor that is autocatalytically processed to the four non-structural viral proteins by the virus-encoded protease in nsP2 (Fig. 1). The non-structural proteins form the transcription/replication complex that mediates the synthesis of diverse viral RNAs of both polarities. The nsP1 protein was implicated in capping of viral RNAs (Ahola and Kääriäinen, 1995; Scheidel et al., 1989) and in initiation of negative-strand RNA synthesis (Sawicki and Sawicki, 1994). It is bound to the cytoplasmic membrane via a central amphipathic alpha helix located in the middle of the protein (Lampio et al., 2000). The nsP2 gene encodes a putative helicase domain at the 5′end and a protease domain at the 3′end, which presents a unique fold distantly related to that of known cysteine proteases (Russo et al., 2006). This protease domain is linked to the downstream domain of the O′-methyltransferase fold, that may be enzymatically active only in non-arthropod-borne alphaviruses (Feder et al., 2003). In its free form, the nsP2 protein induces cytotoxicity and is responsible for transcriptional shut-off, which is dependent on the integrity of the carboxy-terminal peptide located downstream of its helicase and protease domains. The nsP3 protein is required for RNA replication. It carries three domains: the N-terminal sequence reveals a *macro* domain, the crystal structures of which have recently been determined for Venezuelan equine encephalitis virus (VEEV) and Chikungunya virus (CHIKV) (Malet et al., 2009). It is followed by a serine/threonine-rich sequence that may be phosphorylated. The C-terminal region is poorly conserved in both size and sequence (Strauss and Strauss, 1994). The nsP4 protein carries the viral RNA polymerase motif (Kamer and Argos, 1984). During RNA replication, a negative-stranded copy is produced and used as a template for the synthesis of genome-sized positive-strand RNA and subgenomic 26S mRNA corresponding to the 3′ third of the viral genome and encoding the viral structural proteins (Garmashova et al., 2006; Hahn et al., 1988; Lemm et al., 1994; Mukhopadhyay et al., 2006; Weaver, 2005).

The non-structural proteins function in the cytoplasm of infected cells in association with membrane surfaces, and attachment appears to be mediated by nsP1 palmitoylation (Ahola et al., 2000). For reasons not yet explained, the nsP2 protein migrates to the infected cell nuclei and this has also recently been shown to be the case for the nsP3 protein (Gorchakov et al., 2008). The capsid protein is cotranslationally cleaved from the structural polyprotein by its own protease activity and assembles with the viral genomic

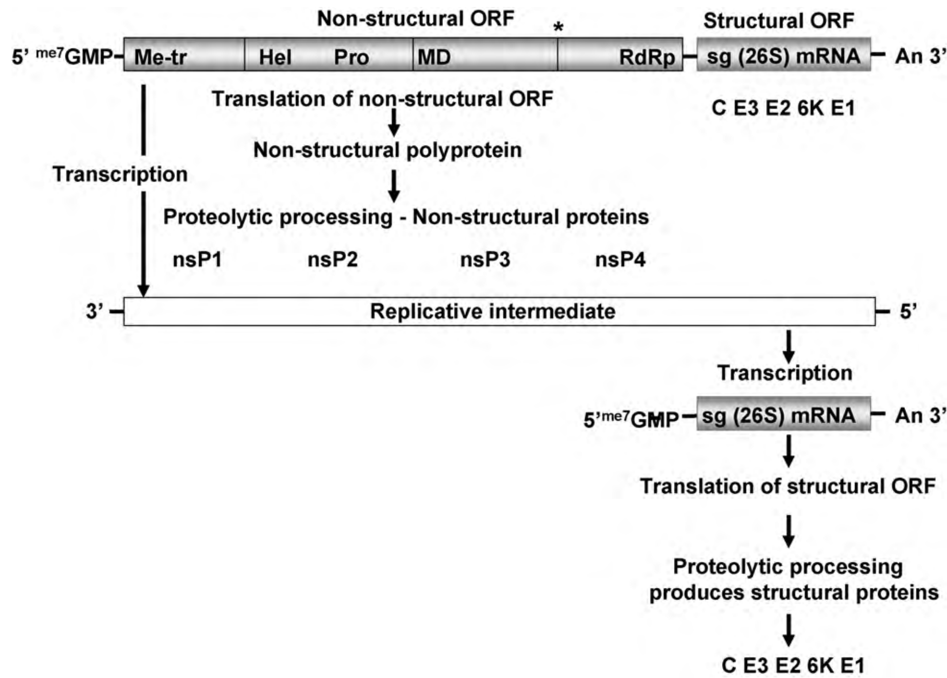

Fig. 1. Alphavirus genome coding strategy (adapted and updated from Strauss and Strauss, 1994). Open-reading frame (ORF) represented as open box, and untranslated regions as solid black lines; sg (subgenomic), asterisk between nsP3 and nsP4 identifies the position of the stop codon that is present in some alphaviruses and is read through to produce the precursor nsP1,2,3,4 polyprotein, Me-tr (methyltransferase), Hel (helicase), Pro (protease), MD (macro domain—exhibits adenosine di-phosphoribose 1″-phosphate phosphatase activity), RdRp (RNA-dependent RNA polymerase), C (capsid), E (envelope).

RNA to form the viral nucleocapsids in the cytosol. Glycoproteins are translocated, via the Golgi apparatus, to the plasma membrane and assembled nucleocapsids bud through these membranes, thus acquiring a lipid envelope containing the integral membrane glycoproteins, E1 and E2.

In this review, we analyse (a) progress in genomics, towards understanding alphavirus evolution, taxonomy, and vector–host relationships in the context of their epidemiology and pathogenesis, and (b) the structures of active domains in alphavirus replicative enzymes with the ultimate objective of identifying suitable targets for molecules that can inhibit their function and thereby serve as antivirals.

2. The alphaviruses as human pathogens

Ten of the arthropod-borne alphaviruses are considered to be of significant importance in terms of public health. Indeed with the recent emergence of chikungunya fever (see later) as a major human disease in Asia and potentially globally, the alphavirus profile has been significantly raised. Alphaviruses that circulate in the Old World most commonly cause febrile illness and painful arthralgias or polyarthralgias, particularly in the small joints. A characteristic macular-papular rash often appears 3–5 days after illness onset. In severe cases the joints are swollen and tender, and rheumatic signs and symptoms may persist for weeks or months following the acute illness. In general, infections with Old World alphaviruses such as CHIKV, or O'nyong nyong virus (ONNV) in Africa/Asia, Sindbis virus (SINV) and closely related viruses (Ockelbo, Whataroa) which are widespread throughout the Old World, or Ross River (RRV), and Barmah Forest virus (BFV), which are confined to Australia, are rarely fatal and only infrequently result in encephalitic disease (Lewthwaite et al., 2009).

In contrast to these Old World diseases, the New World alphaviruses VEEV, Eastern equine encephalitis virus (EEEV), and Western equine encephalitis virus (WEEV) present a different epidemiological and clinical picture. VEEV is one species in the VEE antigenic complex, which is divided into six distinct antigenic subtypes (Walton and Grayson, 1988; Young, 1972; Young and Johnson, 1969). Subtypes IAB and IC are associated with major epidemics and equine epizootics during which equine mortality due to encephalitis can reach 83%. In 1995, a major outbreak in Venezuela and Colombia, was associated with the VEEV subtype IC. This epidemic resulted in roughly 100,000 human cases, with more than 300 fatal encephalitis cases (Diaz et al., 1997). Other epidemics indicate that VEEV still represents a serious public health problem (Weaver et al., 1996). In humans, whilst the overall mortality rate is low (<1%), neurological disease, including disorientation, ataxia, mental depression, and convulsions, can be detected in up to 14% of infected individuals, especially children (Johnson and Martin, 1974). Neurological sequelae in humans are also common (Leon, 1975). However, most human infections present as a non-specific febrile illness or aseptic meningitis. In rare cases, the fever and headache may progress through nausea and vomiting to somnolence or delirium and coma with seizures, impaired sensorium, and paralysis being commonly observed. The severity of neurologic involvement and sequelae is greater with decreasing age.

Horses are more susceptible than humans to neurological disease caused by these VEEV subtypes IAB and IC, but are considered to be dead-end hosts for EEEV and WEEV. Moreover, veterinary vaccines are available to reduce the risk of clinical disease. EEEV and WEEV, are widespread throughout the eastern and western regions of North America, including Canada, and both are also found in South America and Cuba. In North America, they are transmitted to horses by infected ornithophilic (bird-biting) mosquitoes that thrive in wetland habitats. Highlands J virus (HJV), a close relative of WEEV, is not known to be pathogenic for humans but appears to be an important pathogen of several domestic bird species. VEEV also causes encephalitic disease in horses and, occasionally, humans bitten by mosquitoes normally associated with the horses (Weaver, 2005; Weaver and Barrett, 2004; Weaver et al., 1997). The natural life cycle of VEEV involves small mammals, particularly rodents in forest environments more frequently found in South America.

Other closely related alphaviruses are recognised in the Americas but in most cases they are not known to cause disease in humans or animals.

3. Molecular epidemiology and biogeography

3.1. Evolutionary origins of the alphaviruses

In 1988, nucleotide sequence comparisons of several alphaviruses revealed that WEEV was a recombinant virus that had apparently arisen through mixed infection of cells by ancestral EEEV-related and SINV-related viruses (Hahn et al., 1988). Subsequent phylogenetic analyses confirmed this interpretation (Levinson et al., 1990) and it was estimated that recombination had occurred prior to the divergence of EEEV to produce the extant South American lineages (Weaver et al., 1993). These observations were important milestones in our understanding of alphavirus diversity and raised an interesting issue that remains to be satisfactorily resolved, viz., the geographic origin of the arthropod-borne viruses in the genus *Alphavirus*.

Phylogenetic trees based on alphavirus sequences that include the envelope (E1, E2) genes, are incongruent with those based solely on non-structural gene sequence. All trees based on E1 gene sequence show that SINV, an Old World virus, diverges with the WEEV-complex viruses, i.e. New World viruses (Fig. 2a), whereas the other New World equine encephalitis viruses, i.e. EEEV and VEEV diverge independently of the WEEV/SINV complex and also independently of the indigenous Old World viruses that include Semliki Forest virus (SFV), Ross River virus (RRV) and CHIKV. However, trees based on non-structural, and/or capsid genes, show that both SINV and the antigenically closely related New World virus, Aura virus (AURAV) diverge from the New World viruses and are included in a clade of Old World alphaviruses that contain Bebaru virus (BEBV), Getah virus (GETV), SFV, RRV and other related viruses (Fig. 2b). In other words, in trees based on non-structural genes, SINV, and AURAV diverge independently from the WEEV-complex viruses which are now seen to have diverged with EEEV and VEEV.

Another important observation is that trees based on either E1 or non-structural genes show that the New World alphaviruses, Mayaro virus (MAYV) and Una virus (UNV), always cluster with the Old World alphaviruses, RRV, SFV, GETV in the SFV antigenic complex, confirming that transoceanic alphavirus introductions must have occurred, as previously proposed (Powers et al., 2001; Weaver et al., 1997). In the case of MAYV the supposition of transoceanic introductions is also supported by the evidence that this New World virus causes arthritic disease in humans, as is characteristic of the Old World alphaviruses, rather than encephalitic disease as typified by New World viruses such as EEEV and VEEV (Poidinger et al., 1997; Russell, 1998). Weaver et al. (1997) proposed that these alphavirus dispersal patterns could be most readily explained if the origin of these viruses was the New World. However, in order to support this argument, it was necessary to propose that several transoceanic crossings (i.e. at least three) must have taken place, in both directions, i.e. westwards and eastwards (Powers et al., 2001). Moreover, it was estimated that recombination, as seen in WEEV and closely related descendant viruses, is likely to have occurred between 1300 and 1900 years ago (Weaver et al., 1997).

Assuming this to be a reasonable estimate for the time of the recombination event, and assuming a New World origin for the alphaviruses, it would have been necessary for an ancestral SINV-related virus to be present in the New World, either by evolutionary origination or by introduction from the Old World. This virus would then have to encounter an ancestral EEEV-related virus in the same ecological habitat in the New World and then produce mixed infections in rodents, or other animal species such as birds. Moreover, before or after this recombination event, it must also be assumed that one or more of these ancestral alphaviruses (not the recombinant virus) was then dispersed to the Old World, for the alphaviruses to establish in the new environments and to continue their global dispersal. Whilst this scenario may be theoretically possible, it is hard to imagine that these transoceanic movements would occur very frequently more than 1000 years ago, since little if any commercial movement of ships took place during the first millennium AD.

If one argues that birds could have moved the viruses across the oceans more than 1000 years ago, then one is inevitably obliged to ask why is this not a common occurrence today? On the other hand, during the past 500–600 years, transoceanic crossings by ships for commercial and/or immigration and slave-trading purposes have been the major factor for the introduction into the Americas of many different Old World mosquito species, including *Aedes aegypti*, the *Culex pipiens* complex, *Aedes albopictus* (Calder and Laird, 1994) and African arboviruses including Yellow fever virus, Dengue virus, St. Louis encephalitis virus, West Nile virus and Powassan virus (reviewed in Bloom, 1993; de Lamballerie et al., 2008b; Gould et al., 2003; Lounibos, 2002; Strode, 1951; Tabachnick, 1991; Tatem et al., 2006). Whilst in the modern era, introductions do occur in the reverse direction (New World to Old World), they are usually importations of recognised arboviruses by viraemic individuals returning to their country of origin, following a business or leisure trip in the Americas. In temperate regions of the Old World, these individual introductions from the New World rarely, if ever, result in the establishment of endemic/epidemic arboviruses. There is a detailed record of YFV being introduced into South Wales (United Kingdom) from Cuba; the virus caused a small localised outbreak of yellow fever, but rapidly disappeared from circulation (Smith and Gibson, 1986) presumably, because the habitat lacked a suitable vector for YFV to become established.

The alternative possibility, i.e. that the alphaviruses originated in the Old World, is also recognised (Lavergne et al., 2006; Powers et al., 2001; Weaver et al., 1997). In Africa, a wide range of alphaviruses such as SFV, SINV, CHIKV, ONNV and others circulate in geographically overlapping environments, where they have relatively similar ecological requirements. Thus, the possibility of ancestral alphaviruses in Africa causing mixed infections of vertebrates and recombining to produce an ancestral lineage of a virus such as WEEV, is at least plausible. Secondly, during the centuries of intensive commercial trading between Africa and the Americas, some or many of these alphaviruses could have been carried to the Americas, in the way that many flaviviruses and mosquitoes were transported to the New World (Bloom, 1993; Bryant et al., 2007; Gould et al., 2003, 2001; Lounibos, 2002; Tabachnick, 1991). Under this scenario, transoceanic alphavirus dispersal would not need to occur in both directions. Moreover, the extremely low probability of a mixed infection occurring between an enzootic New World virus and an Old World virus, introduced to the Americas more than 1000 years ago, no longer needs to be explained. In addition, the geographically wide distribution of the alphaviruses in natural cycles in the Old World is entirely consistent with Old World anthropological distribution and commerce, and bird migratory patterns. Clearly, more alphaviruses need to be isolated and studied in detail to fill the gaps in our knowledge and to resolve the question of how these viruses have evolved and dispersed during the past few millennia. However, the ideas related above, appear to favour an Old World origin for the alphaviruses that are currently circulating.

3.2. Alphavirus emergence in the New World

In the New World, prior to late 2004 or early 2005, alphaviruses were known to cause spasmodic human outbreaks in different geographic regions of the world. For example, in equatorial South

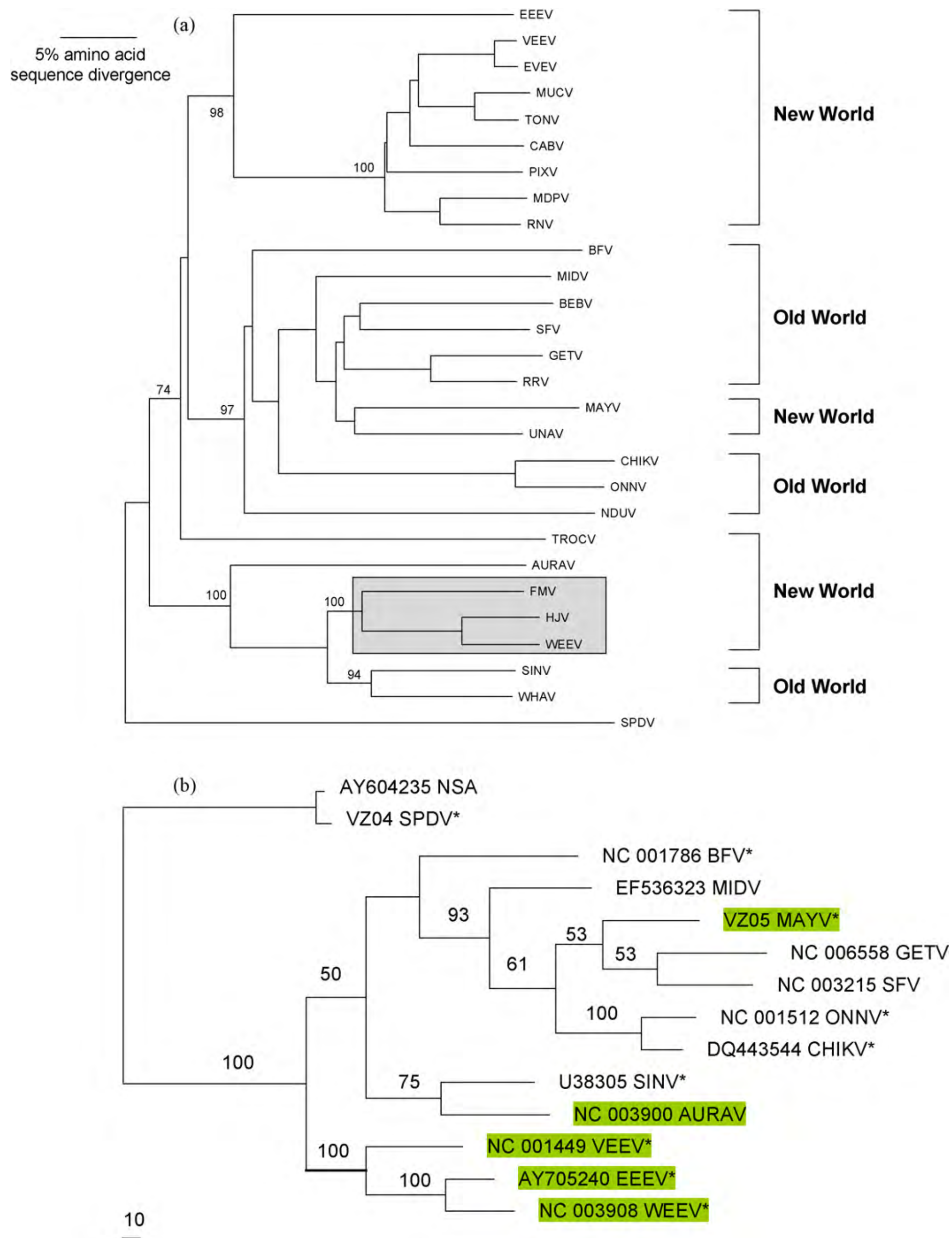

Fig. 2. Phylogenetic analyses of selected alphaviruses. (a) Midpoint rooted tree generated using partial E1 envelope glycoprotein amino acid sequences and the neighbour joining program implemented in PAUP 4.0 (Swofford, 1998). Numbers indicate bootstrap values generated using 1000 resamplings. Scale indicates 5% amino acid sequence divergence. Gray box shows recombinant alphaviruses that were derived from ancestors of EEEV and SINV. Shaded background indicates recombinant viruses. (b) Most parsimonious alphavirus nsP4 tree, rooted with NSA/SPDV outgroup Bootstrap percentage numbers on nodes with 50% or more support from 1000 resamplings, are indicated. Asterisk (*) indicates studies in VIZIER; shaded background indicates New World viruses.

and Central America, VEEV exists in two epidemiological forms: (i) enzootic viruses that are transmitted continuously in sylvatic habitats between mosquitoes and rodent reservoir hosts and (ii) epidemic/epizootic viruses (Weaver and Barrett, 2004; Weaver et al., 2004), that emerge periodically to cause outbreaks involving large numbers of humans and horses (Weaver et al., 1996). A Mexican equine epizootic, involving a proven epizootic vector *Ochlerotatus* (formerly *Aedes*) *taeniorhynchus*, serves as an excellent example. When compared with closely related enzootic strains, VEEV isolates from infected horses exhibited significantly greater infectivity for the vector that was associated with horses, suggesting that adaptation to the appropriate vector contributed to disease emergence. It was subsequently demonstrated that a Ser-Asn substitution in the E2 gene was the sole determinant of the increased vector infectivity (Brault et al., 2004). Similar patterns of enzootic and epizootic behaviour have also been observed with EEEV and WEEV although these viruses are not known to show increased patterns of virulence during these outbreaks.

3.3. Recent emergence of Chikungunya virus

In the Old World, spasmodic febrile/arthritic outbreaks are associated with infections due to SINV, CHIKV, ONNV, RRV, or BFV in Africa, and/or Europe, Asia, South East Asia/Australasia. Prior

Table 1
Number of non-structural alphavirus protein targets sequenced expressed and under further analysis in the VIZIER project.

	nsP1	nsP2	nsP3	nsP4	Crystallized proteins	Crystal structures
Aura virus				1		
Barmah Forest virus	2	16	4	4		
Chikungunya virus (2 strains)	0	10	9	11	2	3
Eastern equine encephalitis virus	2	17	4	2	1	1
Mayaro virus	0	14	4	3	1	
O'nyong nyong virus	2	16	5	15		
Salmon pancreatic disease virus	0	3	0	1		
Semliki Forest virus	1	4	3	1	1	
Sindbis virus	2	18	4	2		
Venezuelan equine encephalitis virus	2	15	4	4	1	2
Western equine encephalitis virus	2	14	4	2	1	
Total (227)	13	127	41	46	7	6

to 2005, these alphaviruses were considered to be of relatively minor importance by most government health departments, largely because they were associated with localised outbreaks involving relatively few individuals and in general they were considered to be insignificant agents of encephalitic disease or fatal infections. Moreover, clinical misdiagnosis of many of these alphavirus infections in the dengue-endemic locations where they circulate is probably another reason that they have been underappreciated as causes of febrile illness. However, this attitude changed, from 2005, onwards when CHIKV unexpectedly arose as a major epidemic human pathogenic arbovirosis throughout, central and southern Africa, the Islands of the southern Indian Ocean, India, Indonesia and Malaysia. As the result of these spreading epidemics, CHIKV is now frequently being introduced into other regions of Asia, Europe, Australia and the Americas by travellers returning from CHIKV epidemic areas of Asia. Indeed in Northern Italy the introduced virus became established in *A. albopictus* and caused localised outbreaks of chikungunya fever (Rezza et al., 2007). Although case fatality rates resulting from CHIKV infection are considered to be low, the very large numbers of clinical cases in Asia (now unofficially estimated to be millions) have almost certainly resulted in significantly more mortality than is currently recognised, particularly in countries where health services and recording procedures are poorly developed. Moreover, it has now become apparent that thousands of excess deaths, with many involving neurological disease, have accompanied recent CHIKV outbreaks (Josseran et al., 2006; Mavalankar et al., 2008; Robin et al., 2008). Additionally, recent studies on children in India, reported that at least 14% of CHIKV infections may result in neurological complications (Lewthwaite et al., 2009).

Until 2005, CHIKV was usually described as a virus that circulated in a natural cycle amongst forest-associated simians and sylvatic mosquito species (*Aedes furcifer, Aedes luteocephalus, Aedes taylori*), in the central and west African jungles, spasmodically causing outbreaks of varying size and intensity amongst the local populations living near the jungles. Human outbreaks were associated with domestic mosquito species such as *A. aegypti* and it has always been assumed that the virus is transmitted down the chain of sylvatic/domestic mosquito species via humans and simians that come into contact with the virus at the edges of the jungles and in the nearby local villages/towns (Gould and Higgs, 2009). CHIKV was also known to cause spasmodic outbreaks in many parts of Asia, although a forest reservoir cycle has never been identified in Asia (Powers and Logue, 2007). However, as indicated above, this spasmodic pattern of outbreaks changed dramatically from 2005 onwards, when a major CHIKV fever epidemic was first identified on La Réunion Island in the southern Indian Ocean (Charrel et al., 2007; de Lamballerie et al., 2008b; Schuffenecker et al., 2006). Epidemics were also reported on many neighbouring islands and the virus dispersed rapidly to India, and south-east Asia (de Lamballerie et al., 2008b). This virus is continuing to cause major epidemics throughout Asia involving millions of humans.

It is now considered possible that CHIKV could spread even more widely, reaching and becoming firmly established in Europe, and tropical America. This potential global emergence of CHIKV appears to be at least partly attributable to its adaptation to the Asian tiger mosquito *A. albopictus* (Calder and Laird, 1994; de Lamballerie et al., 2008b; Schuffenecker et al., 2006; Tsetsarkin et al., 2007), which has dispersed widely in the past two decades. These conclusions were derived from a combination of genomic, phylogenetic, and vector transmission studies, from which it was conclusively demonstrated that the adaptation of CHIKV to high vector competence in *A. albopictus* resulted directly from the substitution of the amino acid alanine by valine at position 226 (A226V) in the envelope gene (E1) of the virus (Schuffenecker et al., 2006; Tsetsarkin et al., 2007). Even more surprisingly, based on phylogenetic evidence and local knowledge of CHIKV epidemiology, it is clear that this selective adaptation for the tiger mosquito has occurred on several independent occasions (de Lamballerie et al., 2008b).

The global invasion by *A. albopictus*, mainly facilitated by tyre shipments, is regarded as the "third wave" of human aided dispersal of mosquito vectors of human disease, following the previous cosmotropical spread of *A. aegypti* and *C. pipiens* (Benedict et al., 2007; Calder and Laird, 1994). *A. albopictus* is now established in virtually all tropical, sub-tropical and southern temperate regions (Lounibos, 2002). Moreover, local species of *A. albopictus* in Florida, USA, have now been shown to be susceptible to infection by CHIKV and competent to transmit this virus (Reiskind et al., 2008). Thus, it is almost certainly only a matter of time before CHIKV invades the Americas, being introduced by infected humans returning from endemic areas of Africa or Asia, possibly causing greater problems in the tropical regions due to the behavioural advantages of *A. aegypti*. Clearly, there is a pressing need for effective antivirals and vaccines with which to develop CHIKV disease control strategies.

4. Studies on the active domains of alphavirus replicative enzymes

Although crystallization of alphavirus proteins provided early data concerned with the mechanism of virus entry into susceptible cells (Lescar et al., 2001) progress has been much slower in the case of non-structural proteins. The major difficulty to overcome has been the problem of expressing suitable recombinant target proteins. This is reflected by the fact that the literature reports the structures of only two non-structural protein domains, viz., the amphipathic helix of nsP1, a 20 amino acid peptide (Lampio et al., 2000) and the protease-methyltransferase subdomain of nsP2 (Russo et al., 2006). Therefore, alphavirus nsP enzyme domains were identified as potentially important targets for structural studies in VIZIER, and Table 1 summarises the current numbers and

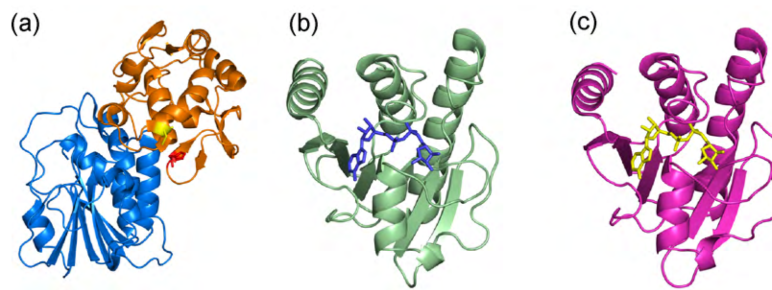

Fig. 3. Known structures of alphavirus nsP2 (protease) and nsP3 (*macro* domain). (a) Cartoon representation of the VEEV protease (C-terminal part of nsP2) (Russo et al., 2006). The domain coloured in orange corresponds to the catalytic domain where the catalytic cysteine and histidine are highlighted respectively in yellow and red. (b) Cartoon representation of the CHIKV *macro* domain (in green) complex with ADP-ribose (in blue). (c) Cartoon representation of the VEEV *macro* domain (in pink) complex with ADP-ribose (in yellow) (Malet et al., 2009).

status (in terms of protein solubility and crystal formation) of expressed alphavirus nsP targets. The decision to produce and study at the structural level, non-structural protein domains active in viral replication has proved to be highly beneficial since genomics progressed at a rapid rate, and most of the predicted (identified) structural folds appear to be original (Fig. 3).

Four non-structural proteins (nsP1, 2, 3 and 4), corresponding to the four domains resulting from maturation (post-translational processing by protease enzymatic function of nsP2) of the polyprotein (Fig. 1), are associated with the virus replication complex. They are synthesized as a short polyprotein nsP123, the translation termination of which is terminated at a UGA stop codon, found in most alphavirus genomes, and located near the junction between nsP3 and nsP4 (Fig. 1). Alternatively, the ribosome can "read-through" this codon, using a rare suppressor tRNA to produce the complete nsP1234 polyprotein (Li and Rice, 1989) which is subsequently processed to the mature products in regulated sequential order (Vasiljeva et al., 2003). The current state of our knowledge concerning the structural and enzymatic properties of these four processed non-structural alphavirus proteins is summarised in Table 2.

4.1. Alphavirus nsP1 protein

The indication that the nsP1 gene carries RNA methyltransferase activity was derived using a SINV nsP1 mutant engineered to allow replication in insect cells depleted of methionine (Mi et al., 1989). This finding was extended in studies in which *in vitro* methyltransferase activity of the SINV nsP1 (Mi and Stollar, 1991) and the SFV nsP1 (Laakkonen et al., 1994) was demonstrated. The active site residues of MT were identified by comparative sequence analysis which demonstrated that this enzyme is conserved in many virus families forming an alpha-like supergroup (Rozanov et al., 1992). The substrate of the reaction is GTP, which becomes methylated at its N7 position (^{me7}GTP). The product ^{me7}GTP is then covalently bound to nsP1 to generate a covalent ^{me7}GMP-nsP1 adduct and inorganic pyrophosphate (Ahola and Kääriäinen, 1995). However, guanylyltransfer of the ^{me7}GMP onto RNA remains to be demonstrated. nsP1 activity is dependent on membrane association (Ahola et al., 1999). This interaction is mediated by an amphipathic helix located in the middle of the nsP1 sequence (Spuul et al., 2007). This helix contains hydrophobic amino acids that can interact with acyl groups of the membrane and a cluster of positively charged residues that contact the phospholipid polar heads (Lampio et al., 2000). It remains unclear if palmitoylation of several cysteines contributes to membrane binding (Ahola et al., 2000), but this post-translational modification plays a role in the cellular localization of the replication complex (Laakkonen et al., 1996).

4.2. Alphavirus nsP2 protein

The N-terminal region of the nsP2 gene was predicted to encode a helicase domain (Gorbalenya et al., 1988; Hodgman, 1988) that was later classified as the helicase Superfamily 1 (Gorbalenya and Koonin, 1993; Singleton et al., 2007). Using *in vitro* tests, the nsP2 gene of SFV exhibits nucleoside triphosphatase activity utilizing GTP and ATP (Rikkonen et al., 1994) and stimulated by RNA. This activity is stimulated in the presence of RNA. nsP2 could thus act at least at two stages in the infected cell. Firstly, as with the NS3 protein of flaviviruses, the nsP2 nucleotide triphosphatase may produce 5′ diphosphate mRNA, the expected final acceptor of the nsP1-mediated guanylyltransferase reaction catalysed by nsP1 (Vasiljeva et al., 2000). Secondly, the nsP2 ATPase activity is believed to be necessary to fuel RNA helicase activity. The dsRNA unwinding is magnesium-dependent in SFV (Gomez de Cedrón et al., 1999). The C-terminal region of nsP2 is a cysteine protease which is responsible for viral polyprotein processing (Strauss et al., 1992; Vasiljeva et al., 2001).

Structural analysis of the VEEV nsP2 C-terminal region revealed two distinct domains (Russo et al., 2006). The first, adopting an original a/b-fold very distantly related to that of papain-like proteases, whose two principal catalytic residues, a cystine and a histidine, are

Table 2
Summary of current enzymatic and structural data of the alphavirus proteins nsP1, nsP2, nsP3 and nsP4.

	nsP1	nsP2		nsP3		nsP4
Domain	MTase/GTase	NTPase/helicase	Protease	Macro domain/ADPribosePPase	Cter domain	RdRp/TATase
Structural data	Yes (partly) (Spuul et al., 2007)	No	Yes (Russo et al., 2006)	Yes (Malet et al., 2009)	No	No
Enzymatic data	Yes/(partly) (Ahola and Kääriäinen, 1995)	Yes/yes (Rikkonen et al., 1994)	Yes (Vasiljeva et al., 2001)	Yes (partly) (Malet et al., 2009)	No	Yes (Lemm et al., 1994; Rubak et al., 2009; Tomar et al., 2006)
Inhibition data	Yes (Lampio et al., 1999)	No	Yes (Pastorino et al., 2008)	No	No	No

uniquely located in the N-terminal subdomain. The second domain has a classical methyltransferase fold although it does not exhibit methyltransferase activity. Structural and biochemical data on the proteases of VEEV and CHIKV nsP2 support the hypothesis that this second domain contributes to substrate presentation to the protease active site (Pastorino et al., 2008; Russo et al., 2006). This structural arrangement may also regulate protease activity through possible RNA binding.

4.3. Alphavirus nsP3 protein

The N-terminal region of nsP3 was initially described as a conserved domain with an unknown function (X domain) in large replicative proteins of alphaviruses, coronaviruses and rubiviruses (Gorbalenya et al., 1991). This 160 amino acid domain belongs to a large protein family including more than 1000 domains from all kingdoms of life, dubbed the *macro* domain family after a unique domain in the human macroH2A1 histone (Perhson and Fuji, 1998). This histone association was found only in a small fraction of domains in the family. Several members of this family were shown to possess a diphosphate-ribose 1′′-phosphate activity and can bind ADP-ribose (Kumaran et al., 2005; Shull et al., 2005). For some others, as exemplified by the Infectious Bronchitis coronavirus X domain, ADP-ribose binding was not revealed *in vitro* (Piotrowski et al., 2009). The *macro* domains of VEEV and CHIKV are very similar. Their structures were found to resemble the homologous *Escherichia coli* domain more closely than the coronavirus *macro* domains (Malet et al., 2009).

Both VEEV and CHIKV *macro* domains have a specific affinity for ADP-ribose, via a conserved aspartic acid and they also display diphosphate-ribose 1′′-phosphate activity. A positively charged amino acid patch also enables the binding of oligonucleotides such as poly-ADP-ribose (PAR) or RNA, but the common determinant of substrate binding is a conserved adenosine 5′-monophosphate binding site. It has now been shown that mutation of amino acids 10 and 24 from asparagine to alanine in the ADP-ribose binding region of the SINV *macro* domain impairs replication and viral RNA synthesis particularly in mouse neurones without any alteration in poly(ADP-ribose) binding (Park and Griffin, 2009). Moreover, mutation at position 10 had the greatest effect and caused nsP3 instability in neurones, decreased SINV-induced death of mature, but not immature neurones, and attenuated virulence in 2 weeks old, but not 5-day-old mice. Thus, the nsP3 macro domain appears to be significantly involved in both SINV replication and age-dependent susceptibility to encephalomyelitis. As a part of the structural genomics section in VIZIER, we have also determined the crystal structure of the EEEV macro domain. The level of amino acid conservation of the entire nsP3 when compared with VEEV and EEEV is 43% identity. However, the structural model for EEEV is still under refinement, and was therefore not included in our most recent publication (Malet et al., 2009). Sequence analysis of the C-terminal region of the nsP3 protein does not suggest any putative enzymatic activity. This region encodes a cluster of up to 16 serines and threonines that can be phosphorylated (LaStarza et al., 1994; Li et al., 1990) although it is not catalysed by nsP3 (Vihinen et al., 2001). Its decreased phosphorylation in SINV produces less minus strand RNA compared to wild type (Dé et al., 2003). Moreover, variants of SFV that are poorly phosphorylated or have deletions in this hypervariable domain exhibit a low viral pathogenicity profile in mice and show reduced viral RNA synthesis in cell culture (Galbraith et al., 2005; Vihinen et al., 2001). The absence of the nsP3 C-terminus also alters SFV neurovirulence (Tuittila et al., 2000). The C-terminal region of nsP3 has specific sequence features of natively unfolded proteins, suggesting that this domain is involved in transcription regulation as proposed previously (Wang et al., 1994).

4.4. Alphavirus nsP4 protein

The alphavirus nsP4 contains a canonical GDD motif and is considered to be the RNA-dependent RNA polymerase (RdRp) (Kamer and Argos, 1984). It is believed that this protein synthesizes both negative- and positive-strand RNAs. In SINV, the switch between the production of antisense RNA to genomic and subgenomic RNA is mediated by processing of the nsP123 region of the polyprotein (Lemm et al., 1998, 1994; Shirako and Strauss, 1994). *In vitro*, this activity has been reported to be sequence-specific (Lemm et al., 1998). A peptide sequence containing two key arginines is involved in promoter sequence recognition to initiate the synthesis of subgenomic RNA (Li et al., 2004). The N-terminal region of nsP4 is poorly conserved and natively unfolded. It could be involved in the recruitment of RNA or protein partners. Alone, nsP4 does not replicate RNA. An N-terminally truncated version of SINV nsP4 carries out terminal adenylyltransferase (TATase) activity that might be involved in maintenance of the 3′ poly A tail (Tomar et al., 2006). Very recently, RdRp activity was demonstrated *in vitro* using a purified full-length SINV nsP4 protein (Rubak et al., 2009).

5. Progress towards therapy and prevention

Chikungunya fever commonly presents as a painful febrile illness that is quite often accompanied by relapsing and incapacitating polyarthralgia that may persist for several months. During the past 4–5 years, CHIKV has dispersed widely throughout Africa and Asia causing morbidity amongst millions of infected patients. However, this may be only the tip of the iceberg because the two most important vectors of this virus, *A. aegypti* and *A. albopictus*, are continually expanding their geographic distribution and density amongst domestic and peridomestic human populations. Thus it is the strongly held belief that CHIKV will spread even more widely, and quite possibly into the Americas, during the next few years. There are currently no recognised antiviral therapies or human vaccines with which to control infections due to CHIKV.

5.1. Search for antiviral drugs

Failure to develop approved antiviral therapeutic agents or vaccines has been particularly exposed during the recent outbreaks of CHIKV in the Indian Ocean. Thus, patient treatment has been based on non-salicylate analgesics and non-steroidal anti-inflammatory drugs (Pialoux et al., 2007). However, the elaboration of mouse (Couderc et al., 2008; Ziegler et al., 2008) and non-human primate models (Roques et al., 2007) together with antivirals currently undergoing clinical trials, and new approaches involving natural products of plants (Li et al., 2007) provide the stimulus for improving development of antiviral candidates.

5.1.1. Chloroquine

Chloroquine was first reported to inhibit SINV and SFV infectivity *in vitro* more than 35 years ago (Cassell et al., 1984; Coombs et al., 1981; Helenius et al., 1982; Inglot, 1969; Shimizu et al., 1972) but studies in mice suggested that the drug might enhance viral replication and aggravate the disease (Maheshwari et al., 1991). Recent research on the efficacy of Chloroquine has focused on the dosage used to treat acute CHIKV infections (de Lamballerie et al., 2008a; Savarino et al., 2007). Chloroquine phosphate has also been used to treat chronic chikungunya arthritis (Brighton, 1984) by utilising the anti-inflammatory properties of the molecule, rather than possible antiviral effects. Based on experiments in cell culture, results comparable with those obtained using CHIKV, were also observed with the SARS coronavirus. Chloroquine was therefore proposed as a potential antiviral molecule for the treatment of humans infected

with the SARS coronavirus (Keyaerts et al., 2004; Leyssen et al., 2006).

To advance the studies with Chloroquine and CHIKV, a double blind placebo-controlled randomized trial (see http://clinicaltrials.gov/ct/show/NCT00391313) (de Lamballerie et al., 2008a; Leyssen et al., 2006) was conducted on Réunion Island (Indian Ocean). No statistical difference was observed between the Chloroquine and placebo groups, either in mean duration of febrile arthralgia or rate of decrease of viraemia (between day 1 and day 3). At day 200 post-infection Chloroquine-treated patients declared more frequently ($p < 0.01$) that they still suffered from arthralgia than patients who had received the placebo. However, the number of patients included in the study was too small to draw definitive conclusions regarding the efficacy of the Chloroquine treatment. Recent experiments in macaques (*Macaca fascicularis*) also failed to identify a detectable antiviral effect of Chloroquine following experimental infections and similar doses of Chloroquine (Le Grand et al., manuscript in preparation). Thus, the use of Chloroquine to treat CHIKV-infected patients does not appear to be justified.

5.1.2. Quinine

The antimalarial drug, Quinine, inhibits replication of CHIKV *in vitro*. Quinine appears to be a more likely candidate for antiviral therapy against CHIKV because the 50% inhibitory concentration (IC_{50}) value is much lower than that of Chloroquine (0.1 μg/ml for Quinine versus 1.1 μg/ml for Chloroquine, using Vero cells and 1×10^2 cell culture infectious doses ($CCID_{50}$)). Also, in contrast with Chloroquine, resistant mutants were obtained by growing CHIKV in increasing concentrations of Quinine. The mutations were detected in the nsP1 protein, suggesting impairment of function of the viral guanylyltransferase enzyme [XdeL; personal observation]. This region of nsP1 has also been indirectly related to methyltransferase activity in SINV isolates (Laakkonen et al., 1994; Mi et al., 1989; Mi and Stollar, 1991; Wang et al., 1996) which has been shown to be an essential component of SINV pathogenicity in mice (Zhu et al., 2009). Thus, the alphavirus nsP1 might prove to be a good target for antiviral therapy.

5.1.3. Ribavirin

Ribavirin shows wide *in vitro* inhibitory activity against RNA viruses with different modes of action depending on the virus (Leyssen et al., 2006). In some cases it inhibits IMP dehydrogenase, depleting cellular GTP pools. In others it is used as a non-canonical substrate for RNA synthesis introducing numerous mutations whose accumulation may lead to virus inactivation due to error catastrophe (Crotty et al., 2001; Severson et al., 2003). In the case of some arenaviruses ribavirin 5′-tri-phosphate interacts with the viral polymerase (Sun et al., 2007). It was also tested as an aerosol for the treatment of paediatric Respiratory Syncytial Virus infections but was not approved and this method has now been discontinued. Ribavirin is used in combination with alpha-interferon for the treatment of hepatitis C virus (HCV) infection but the mechanism of action is multifactorial. The predominant direct mechanism by which ribavirin exerts its antiviral activity *in vitro* against flaviviruses and paramyxoviruses is mediated by inhibition of IMP dehydrogenase (Leyssen et al., 2005), because viral RNA synthesis requires a high GTP concentration, and also because resistance mutations can be isolated that map on the NS5b polymerase (Young et al., 2003). An indirect mechanism via decreasing the GTP pools may also be important (Zhou et al., 2003).

In the case of alphaviruses, resistance to ribavirin was first reported following studies with SINV (Scheidel et al., 1987). Resistant mutants were mapped to the nsP1 protein (Scheidel and Stollar, 1991). It was proposed that the mutant encoded an altered RNA guanylyltransferase enzyme with increased affinity for GTP, enabling it to replicate in cells with reduced levels of GTP. This is consistent with the hypothesis that ribavirin acts mainly as an IMP dehydrogenase inhibitor against alphaviruses.

5.1.4. Interferon and ribavirin

A combination of interferon-alpha and ribavirin shows a synergistic effect on the *in vitro* inhibition of CHIKV (Briolant et al., 2004). However, for clinical application it is expensive and requires parenteral injection, making it unsuitable for large-scale use during epidemics. Human infection with CHIKV appears to induce immunological dysfunction. However, interferon usually boosts the immune response. Therefore caution in its usage is recommended until more extensive non-human primate models have been studied. Pegylated alpha interferon appears to be an effective treatment against infection with VEEV and has profound effects on the host immune response to infection (Lukaszewski and Brooks, 2000). Thus, it might be justified to test pegylated alpha interferon on CHIKV and other pathogenic alphaviruses.

5.1.5. Inhibitors of alphavirus entry and maturation

Molecules that inhibit alphavirus entry into susceptible cells have also been investigated with promising results. A study recently reported the possible inhibition of the replication of VEEV using polyclonal antibodies to laminin-binding protein (Bondarenko et al., 2004). CHIKV infection of cultured human cells was also shown to be inhibited by impairing the maturation of the CHIKV E2 surface glycoprotein using furin inhibitors (Ozden et al., 2008). A similar observation was previously reported using the flavivirus Tick-borne encephalitis virus (Stadler et al., 1997).

5.1.6. Carbodine

Recent studies on the carbocyclic analogue of cytidine (cyclopentylcytosine or carbodine) suggest that it has potential as an antiviral agent against VEEV (Julandera et al., 2008). Carbodine was shown to inhibit cellular cytidine triphosphate (CTP) synthetase, which converts UTP to CTP, resulting in indirect inhibition of virus replication through reduction of CTP pools (de Clercq et al., 1990). In cell culture, carbodine is a broad-spectrum antiviral, with activity against several unrelated viruses (Andrei and De Clercq, 1990; Neyts et al., 1996) although cytotoxicity has been demonstrated. The addition of exogenous cytidine (cyd) or uridine results in reversal of antiviral activity of carbodine in various cell lines (Andrei and De Clercq, 1990). The natural nucleosides are dextrorotatory (d), but both d- and laevorotatory (l)-analogues can inhibit metabolic enzymes (Gumina et al., 2002) and the d-enantiomer may show reduced cytotoxicity. Accordingly, the d-enantiomer was recently used to test the inhibitory qualities of carbodine against VEEV-challenged mice (Julandera et al., 2008). Increased survival rates, increased average time to death and reduced brain virus titres were observed when carbodine treatment was followed by challenge of the mice with the attenuated vaccine strain (TC83) of VEEV. Evidence of inhibitory effects was also detected if the carbodine was administered up to 4 days post-infection.

5.1.7. Small RNA molecules

In common with many other RNA viruses, inhibition of alphavirus replication in cell culture has been demonstrated using interfering RNAs and antisense oligonucleotides. Inhibition of VEEV was observed using a mixture of four short interfering RNAs (O'Brien, 2007), and antisense morpholino oligomers were successfully used for the inhibition of SINV infection, in cell culture and in mice (Paessler et al., 2008) strongly suggesting that similar approaches should be applied to other alphaviruses such as CHIKV. Methods to overcome the difficulties encountered in delivering these antiviral molecules are currently the subject of major research.

5.1.8. Plant compounds

Compounds extracted from plants have also shown very encouraging antiviral effects against certain RNA viruses. The seco-pregnane steroid glaucogenin C and its monosugar-glycoside cynatratoside A extracted from *Strobilanthes cusia*, together with three new pantasugar-glycosides of glaucogenin C from *Cynanchum paniculatum*, can suppress a range of positive-strand RNA viruses including Tobacco mosaic virus (TMV), and the alphaviruses, SINV, EEEV, and Getah virus in cell culture. Moreover, mice were protected from lethal SINV infections with no adverse effects of the compounds on the infected animals (Li et al., 2007).

5.1.9. nsP1 active domains as a target for inhibition

In addition to the research activities described above, recent efforts have focused on the identification of specific inhibitors of viral enzymes involved in replication. Enzyme production processes, activity assays, and structural data are all available, and could therefore provide a basis for inhibitor screening. Since nsP1 is the main enzyme involved in RNA capping, an essential step in promoting viral RNA translation, nsP1 is an excellent target candidate for antiviral therapy. SINV and SFV nsP1s can be produced to high levels in *E. coli* or insect cells, and both methyltransferase activity on GTP and guanylyltransferase activity have been tested *in vitro*. Following this strategy, several GTP analogues have been reported to inhibit the two activities with K_i values below 100 μM (Lampio et al., 1999).

5.1.10. nsP2 active domains as a target for inhibition

It has also been demonstrated that helicases are good antiviral targets in flaviviruses (Goodell et al., 2006), picornaviruses (De Palma et al., 2008) and hepaciviruses (Borowski et al., 2002). The helicase region of SFV nsP2 can be expressed in *E. coli* but the protein is unstable after purification, requiring a high salt concentration (Gomez de Cedrón et al., 1999). Nevertheless, the protein is active and production yields could be compatible with existing helicase HTP assays using non-radioactive readouts (Boguszewska-Chachulska et al., 2004; Tani et al., 2009). Currently, the protease domain of nsP2 is the most promising target for rational inhibitor screening. This protease is responsible for non-structural polyprotein processing, an essential function for virus replication (Balistreri et al., 2007; de Groot et al., 1990; Mayuri et al., 2008). Inhibitors can be selected through a structure-based method, using the VEEV protease structural data as a template (Russo et al., 2006). This strategy could be combined with enzymatic assays already described for several alphavirus proteases (Zhang et al., 2009). Interestingly, leupeptin, a broad-spectrum cysteine protease inhibitor, has no effect on the protease activity of CHIKV nsP2 (Pastorino et al., 2008), suggesting that this protease has structural and functional specificities that could be specifically targeted.

5.1.11. nsP3 active domains as a target for inhibition

Structural data and enzymatic properties of *macro* domains of nsP3 in two alphaviruses have recently been reported (Malet et al., 2009). On the basis of very recently published results (Park and Griffin, 2009), the nsP3 *macro* domain appears to be significantly involved in both SINV replication and age-dependent susceptibility to encephalomyelitis and therefore may become an appropriate target for the development of antivirals.

5.1.12. nsP4 active domains as a target for inhibition

The RdRp is the crucial enzyme in RNA virus replication. Because viral RdRps have no close homologues amongst cellular replicative enzymes, they have been targeted since the earliest times in the search for antivirals. The first demonstration of antiviral activity provided by a nucleoside analogue (reviewed in Leyssen et al., 2008) has contributed significantly to the consideration of replicative enzymes as highly promising targets in the field of antiviral drug discovery and design.

Prior to the commencement of the VIZIER project (Coutard et al., 2008), the alphaviruses had not attracted too much attention in the context of drug discovery. However, as discussed earlier, this situation began to change from 2005 onwards, following the surprisingly large-scale outbreaks of chikungunya fever on many of the inhabited islands of the southern Indian Ocean. Consequently, the recent first demonstration that alphavirus RdRp activity is encoded in the nsP4 gene (Rubak et al., 2009) provides a strong incentive for vigorous studies to identify effective inhibitors of alphavirus RdRps. In alphaviruses, assembly of the multi-subunit replication complex is required for demonstration of RdRp activity. Thus, *in vitro* assays require at least nsP123 and nsP4 in the reaction mixture, and the assays are only effective if recombinant nsP4 is expressed with the N-terminal tyrosine (Rubak et al., 2009; Shirako et al., 2003). Whilst experimental conditions for the generation of authentic nsP4 have been defined, recombinant nsP123 is still expressed exclusively in mammalian cells (Rubak et al., 2009), which could prove to be a limitation in the context of HT inhibitor screening. Clearly many problems remain to be resolved.

5.2. Antiviral vaccines

An alternative approach to virus disease control involves the use of vaccines Whilst some are available to immunise horses against VEEV, WEEV and EEEV, and both live and inactivated vaccines have been used to immunise laboratory workers at risk of exposure to encephalitic alphaviruses, there are no licensed human vaccines against alphaviruses. A live-attenutated vaccine against VEEV was first developed in 1961 (Berge et al., 1961) and has been administered to more than 8000 individuals (Alevizatos et al., 1967; Burke et al., 1977; Pittman et al., 1996). As the result of adverse effects in a significant proportion of these vaccinated individuals, an inactivated vaccine was developed (Pittman et al., 1996) and a more promising highly attenuated vaccine (V3526) based on an infectious cDNA clone of VEEV was also developed (Davis et al., 1995; Hart et al., 2000) but concerns over the possibility of reversion to virulence have not been totally alleviated. Hence, alternative approaches such as the development of chimaeric viruses with the replicative machinery from SINV, and the structural genes from VEEV are still being pursued (Berge et al., 1961; Paessler et al., 2006). The first attempts to develop inactivated vaccines against CHIKV were reported in 1970 (Eckels et al., 1970). However, these early immunogens have not been developed as licensed vaccines.

Later, a live-attenuated vaccine strain was developed by serial, plaque-to-plaque passages of CHIKV (Edelman et al., 2000). This vaccine proved highly immunogenic but some phase II volunteers developed transient arthralgia. At least three different methods are currently under development to produce safe, effective vaccines against CHIKV. One method involves the preparation of purified inactivated virus using methods similar to those that have proved successful for the development of an inactivated vaccine against Tick-borne encephalitis virus (Pavlova et al., 2003). A second method involves the use of cDNA fragments representing the important immunogenic regions of the CHIKV genome (Muthumani et al., 2008). Finally, chimaeric alphaviruses containing the genetic backbone of SINV, TC-83 or a naturally attenuated EEEV strain, and the structural proteins of wild-type CHIKV, have produced promising results in murine efficacy studies (Wang et al., 2008).

6. Conclusion

At the commencement of the VIZIER project, alphaviruses were given a relatively low profile because other, apparently more worrisome RNA viruses, in terms of their potential virulence such as SARS coronavirus, avian influenza virus, Nipah virus, rabies virus and West Nile virus were attracting much more attention globally. However, in 2005, the relative level of importance of alphaviruses as a significant public health problem was raised when CHIKV unexpectedly emerged as a major human pathogen in Africa, the Indian Ocean, India and Malaysia. The continuing epidemic of chikungunya fever, which has now lasted for 4 years and shows little sign of abating, has the potential to reach the Americas, where if past history of other invading RNA viruses is predictive, could lead to high levels of human morbidity. Faced with this prospect and the possibility that as the result of human impact on habitat and global/local distribution of vectors and hosts, other alphaviruses such as ONNV might also emerge to produce much more extensive epidemics than they have done in the past, the search for antivirals to combat these viruses clearly needs to be intensified.

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