Review
Future Virology

Novel therapeutic targets for arenavirus hemorrhagic fevers

Cybele C García[1], Claudia S Sepúlveda[1] & Elsa B Damonte†[1]
[1]Laboratorio de Virología, Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Pabellón 2, Piso 4, 1428 Buenos Aires, Argentina
†Author for correspondence: ■ Tel.: +54 114 576 3334 ■ Fax: +54 114 576 3342 ■ edamonte@qb.fcen.uba.ar

Several members of the family *Arenaviridae* can cause severe hemorrhagic fevers in humans, representing a serious public health problem in endemic areas of Africa and South America. Lassa virus is the most dangerous arenavirus, causing over 300,000 infections per year with several thousand deaths. Furthermore, pathogenic arenaviruses are considered category A potential agents for bioterrorism. Based on the danger of arenaviruses for human health, the increased emergence of new viral species in recent years and the lack of effective tools for their control or prevention, the search for novel antiviral compounds effective against these pathogenic agents is a continuous demanding effort. This article focuses on novel strategies to identify inhibitors for arenavirus therapy, analyzing viral and host proteins essential for virus infection as potential targets for antiviral development.

Viral hemorrhagic fevers (HFs) are zoonoses able to cause dramatic and devastating local outbreaks in man. The etiological agents are four very different types of RNA viruses classified in the families *Arenaviridae, Bunyaviridae, Filoviridae* and *Flaviviridae*. These viruses can often produce a subclinical infection or a mild febrile syndrome, but the more severe forms of hemorrhagic disease are associated with extremely high morbidity and mortality. Despite this threat for human health and although different types of compounds were evaluated for HF inhibition [1–4], no specific and safe chemotherapy for any viral HF is currently available for clinical use.

HF are mainly tropical diseases, endemic in developing countries, that received little research attention until recently. The requirement of biosafety level 4 facilities for handling of highly pathogenic HF agents has aggravated this problem. Thus, the deficiency of information about these pathogens has been a real challenge for development of control and prevention strategies. Recently, several HF viruses were included in the Pathogen List of the Centers for Disease Control as potential agents of bioterrorism, reinforcing the importance of and need for adequate tools to combat these lethal microorganisms [5,6].

Arenaviridae is the largest family of viruses causing HF. The arenaviruses Lassa (LASV), Junin (JUNV), Machupo (MACV), Guanarito (GTOV) and Sabia (SABV) are the known agents of HF in West Africa, Argentina, Bolivia, Venezuela and Brazil, respectively, whereas Chapare [7] and Lujo virus [8] were recently isolated from severe cases in Bolivia and South Africa, respectively, and proposed as new tentative species of the family. The most important pathogen among arenaviruses is LASV, which infects over 300,000 individuals in endemic areas per year with several thousands deaths [9].

In this article, we shall briefly summarize the health impact, epidemiology and present treatment of arenavirus HF and give an overview of potential virus and cellular novel targets with promising perspectives for specific chemotherapy.

The impact of arenaviruses as emerging agents

Arenaviruses are included in a unique genus, *Arenavirus*, currently composed of 22 recognized species that are classified into two distinct groups: the New World (NW) or Tacaribe complex, including 17 virus species indigenous to America, and the Old World (OW), or Lassa–Lymphocytic choriomeningitis complex, comprising four viruses from Africa and the worldwide distributed lymphocytic choriomeningitis virus (LCMV) [10] (**Figure 1**). This classification was initially based on the geographical region of isolation and serological cross-reactivity among virus species [11]. With the only exception of Tacaribe virus (TCRV), which infects bats [12], each arenavirus is associated with a rodent species wherein persistent infection without overt disease is established. Consequently, the regional distribution of the viruses is restricted to the areas that are populated by their reservoir. LCMV, the protype species of the family, is the only one with a wide geographic range because its natural host is the house mouse *Mus musculus/Mus domesticus*, introduced in the five continents.

Keywords
antiviral agent ■ arenavirus ■ junin virus ■ lassa virus ■ therapeutic target ■ viral hemorrhagic fevers

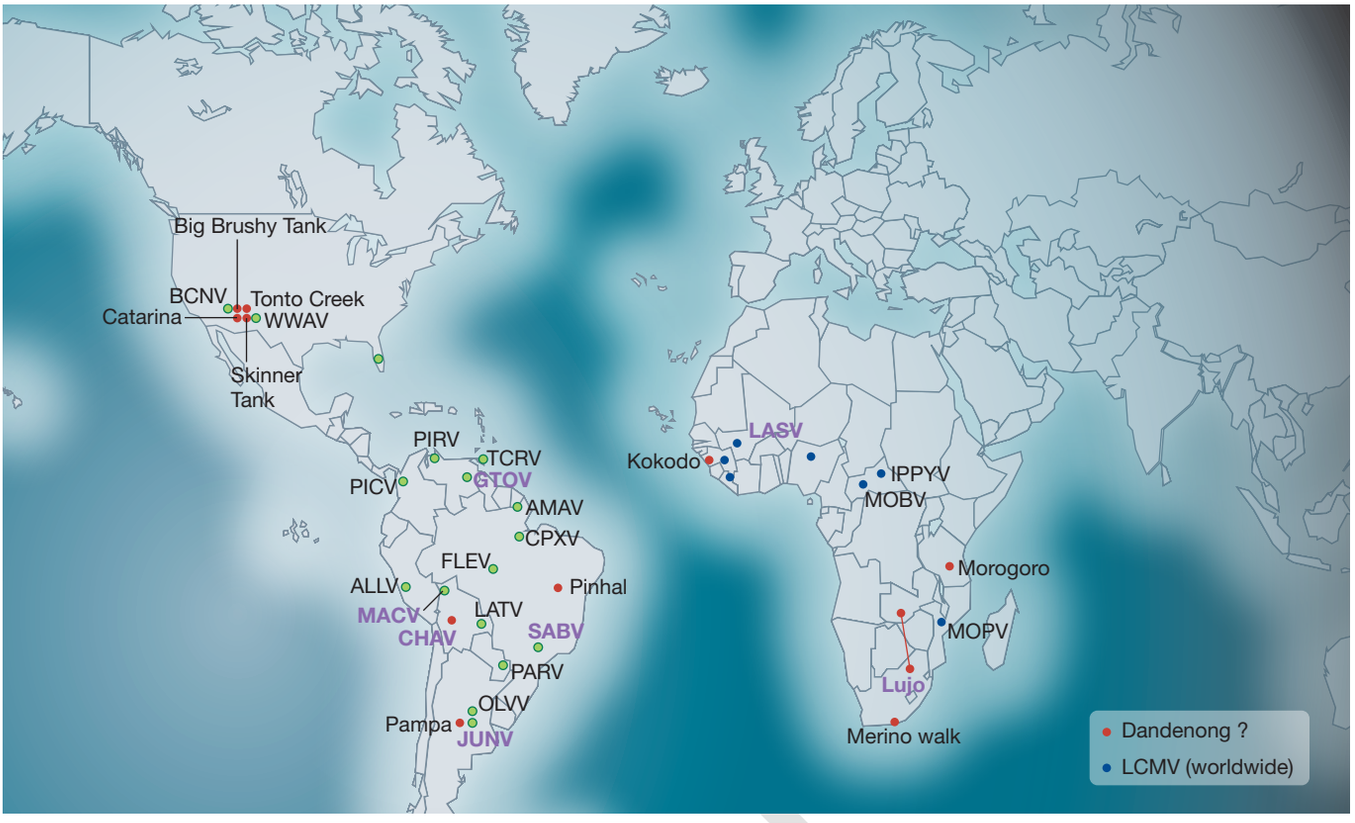

Figure 1. Geographical distribution of arenaviruses. The points indicate the geographical location of each virus, except for LCMV (with worldwide distribution) and Dandenong virus, with still unknown (?) reservoir and geographical origin.
Old World arenaviruses; New World arenaviruses; tentative new species; the names of HF viruses are highlightened
ALLV: Allpahuayo virus; AMAV: Amapari virus; BCNV: Bear Canyon virus; CHAV:.....; CPXV: Cupixi virus; FLEV: Flexal virus; GTOV: Guanarito virus; IPPYV: Ippy; JUNV: Junin virus; LASV: Lassa virus; LATV: Latino virus; MACV: Machupo virus; MOBV: Mobala virus; MOPV: Mopeia virus; OLVV: Oliveros virus; PARV: Parana virus; PICV: Pichindé virus; PIRV: Pirital virus; SABV: Sabiá virus; TAMV: Tamiami virus; TCRV: Tacaribe virus; WWAV: Whitewater Arroyo virus;

More recent phylogenetic analysis of genome sequence data supported the OW–NW division and it also allowed the classification of both complexes in lineages. The OW complex consists in a unique lineage formed by LCMV, and the African viruses LASV, Mopeia (MOPV), Mobala and Ippy. In the NW complex, 14 South American viruses were grouped in three lineages, designated A, B and C [13,14]. The pathogenic arenaviruses JUNV, MACV, GTOV and SABV, which cause severe HF in South America, are included in lineage B together with TCRV, Amapari and Cupixi. Lineage A contains Allpahuayo, Flexal, Parana, Pichinde (PICV) and Pirital viruses and clade C is formed by Latino and Oliveros viruses. Finally, the North American arenaviruses Bear Canyon, Tamiami and Whitewater Arroyo are distinguished from South American arenaviruses in a separate clade due to the nature of the sRNA genome of these three viruses derived by intrasegmental recombination [15,16].

Due to their ability to establish chronic viremic infections in specific rodent hosts, arenaviruses are typical agents of endemic emerging diseases. In fact, an increased emergence of new arenaviruses has frequently occurred in recent years either because humans became accidentally infected, causing an alarming disease, or as result of a systematic survey for the presence of virus, virus genome or antibodies in native rodents. As a result of this last approach, four new virus species were isolated from *Neotoma* rodents in Southwestern USA, with the proposed names of Catarina [17], Skinner Tank [18], Tonto Creek and Big Brushy Tank [19] viruses, phylogenetically related to the group of North American arenaviruses (Figure 1). In Guinea, the proposed Kodoko virus was genetically detected during an arenavirus screening of rodents, but virus isolation must still be confirmed [20]. A serosurvey of small mammals from Tanzania identified arenavirus circulation and by molecular screening through RT-PCR of L gene the Morogoro virus was identified, then found closely related

but distinct to MOPV [21,22]. Merino Walk virus was isolated from a *Myotomys* rodent collected in Eastern Cape, South Africa, and was genetically characterized as an arenavirus related distantly to the African members of the OW complex [23]. By RT-PCR amplification, the partial genome of a novel arenavirus isolate named Pampa virus was also obtained from a rodent trapped in Central Argentina [24].

New virus isolates were also obtained from severe human disease. Chapare virus was isolated in Bolivia from the unique fatal case of a small focus of HF and was classified as a new member of NW clade B like all other South American HF arenaviruses [7]. In 2008, from five cases of HF with a high fatality rate (4/5) recognized in South Africa after air transfer of a critically ill index case from Zambia, a new member of the family provisionally named Lujo virus was identified, representing the first HF-associated OW arenavirus from Africa discovered in the past three decades [8]. Finally, Dandenong virus, a new LCMV-related arenavirus that caused fatal disease in three recipients of organs from a single donor in Australia, was detected through unbiased high-throughput sequencing and then confirmed by isolation from tissue specimens [25,26].

In conclusion, eleven new tentative species, two of them (Lujo and Chapare viruses) agents of severe HF and one (Dandenong virus) lethal for immunocompromised patients, have been proposed to be included in the family. Although their precise taxonomic status must still be addressed, it is to be expected that the number of *Arenaviridae* members will grow as far as new human–reservoir contact that may occur or improved epidemiological surveys and tools for the discovery of pathogens will be developed. On the basis of the appearance of new species, it was estimated that a new arenavirus may emerge and be recognized on average every three years, a prediction that appears to have been surpassed in the last few years.

Apart from the high risk for humans to acquire a severe arenavirus infection in endemic regions, increasing air international travel has contributed to transport of LASV from its niche in West Africa to other geographic areas, posing a hazard to the local population. So far, 27 imported cases of Lassa fever were reported in Europe, USA and Israel [27–30].

Clinical significance of arenaviruses

From the recognized human HF arenaviruses, SABV, MACV and GTOV produced very sporadic cases in their endemic regions, in contrast to JUNV and LASV, which generate periodic annual outbreaks of disease and represent the main health threat in the families.

Unlike many other viral HF Lassa fever is an ever-present and likely increasing threat to large communities in Africa, representing one of the most neglected and harmful tropical diseases. Although its real incidence is probably underestimated due to inadequate surveillance, available data reveal that over 300,000 cases occur annually and in some areas 20–30% LASV infection rates have been detected in the adult population [31]. The highest incidence is reported in Sierra Leone, Guinea, Liberia and Nigeria, due to the presence of the reservoir, the multimammate rat *Mastomys natalensis* [32]. The main route of human transmission is contact with excreta from the infected rodent. But, uniquely among arenaviruses, person-to-person spread of LASV can often occur by close contact in the same household or nosocomial exposure to contaminated body fluids [9,33].

Approximately 80% of human LASV infections are asymptomatic. In clinical cases Lassa fever is difficult to diagnose clinically because symptoms and signs are indistinguishable from those of febrile illnesses such as malaria or yellow fever. The incubation period is 7–21 days, followed by fever, general malaise, sore throat and muscle aches, progressing to gastrointestinal manifestations, conjunctivitis, severe chest and abdominal pain [9,31]. In severe cases leading to death, pulmonary edema, respiratory distress, shock, encephalopathy and hemorrhages are seen [9,31]. Patients with a fatal outcome have very high viral load at the initial stages of disease and are unable to limit virus spread due to a marked immunosuppresion and lack of an adaptive immune response. The overall case–fatality rate is approximately 1%, but is estimated to be 20–30% in hospitalized patients, and sensorineural deafness is the major chronic sequela in recovered patients [34].

JUNV is the agent of Argentine HF in the humid pampas, the fertile farmland of Central Argentina. Human exposure occurs through skin lesions or inhalation of aerosols contaminated with secretions from the main reservoir, the rodent *Calomys musculinus*, in coincidence with the time of crop harvest (April–July), typically as a seasonal and occupational disease. In general, the incidence and severity of JUNV human infection is significantly lower in comparison to LASV, with 100–1000 notified cases per year and a case–fatality rate about 15% in the absence of treatment [35]. After 1–2 weeks

of incubation, the initial symptoms are nonspecific, with fever, muscular pain, asthenia, lymph node enlargement, cutaneous petechiae and retroocular pain. Over 80% of the patients improve during the second week of disease mounting a detectable humoral immune response with virus clearance, whereas the remainders are prone to worsen presenting severe hemorrhagic or neurological manifestations, shock and superimposed bacterial infections [35].

Besides the HF-producing viruses, the prototype arenavirus LCMV can also infect humans, generally resulting in an asymptomatic course or a mild, transient illness. However, LCMV has also been implicated as the etiologic agent of aseptic meningitis in humans. In particular, human LCMV infection is of considerable concern in pediatrics in cases of congenital infection when human-to-human vertical transmission can result in death or neurological sequelae and mental retardation [36,37]. Human-to-human horizontal infection has only been documented in a few unusual circumstances in which LCMV was acquired through transplantation of infected tissues with fatal outcomes, posing a new potential risk to immunocompromised patients [38].

Disease control: prevention & treatment

As seen, the occurrence of arenaviral HF is largely confined to developing countries with a limited medical infrastructure. This consideration, combined with the sizeable disease burden in Lassa fever, makes vaccination the method of choice for prevention. Development of an arenavirus vaccine has been attempted during the last 40 years employing diverse approaches.

An effective live attenuated JUNV vaccine, Candid 1, was developed through a cooperative international effort. The vaccination of at-risk population with Candid 1, initiated in Argentina in 1991, showed a protective efficacy greater or equal to 84% and has led to a consistent reduction in Argentine HF in recent years [39,40]. For LASV, the situation appears to be more complex, perhaps due to the different contribution of antibodies and T cell response to the control of infection by NW and OW arenaviruses. The most recent projects in progress include replication-competent vaccines based on attenuated recombinant vesicular stomatitis virus vector expressing the LASV glycoprotein [41], the attenuated MOPV/LASV reassortant [42], and a recombinant yellow fever 17D vaccine expressing LASV glycoproteins [43]. These three recombinant vaccines have elicited a protective response when assayed in animal models. To date, no human LASV vaccine trials have taken place.

The current therapy for arenavirus infection includes treatment with ribavirin, a guanosine analog with a broad spectrum of antiviral activity against RNA viruses, and the passive administration of high-titer specific antibodies through convalescent serum. For LASV, the recommended treatment is the intravenous administration of ribavirin within the first 6 days of illness [44]. However, the drug is not effective for the treatment of advanced LASV infections and is also less effective if given orally. It is also advised as a prophylactic agent in cases of possible exposure to LASV, but its usefulness has not been systematically studied [45–47]. Furthermore, it must also be remarked that undesirable secondary reactions such as thrombocytosis and anemia have been recorded for ribavirin treatment [44,48,49]. Although multiple mechanisms of action have been proposed for the antiviral action of ribavirin [4,50], the primary target is thought to be the cellular enzyme inosine monophosphate dehydrogenase (IMPDH), which converts IMP to xanthosine monophosphate [51]. The blockade of IMPDH decreases the level of the intracellular GTP pool, with a consequent reduction of viral RNA synthesis and virus yield inhibition, an effect reversed by exogenous addition of guanosine. To overcome the disadvantages recorded for human treatment with ribavirin, other related analogues and other types of IMPDH inhibitors were screened for antiarenavirus activity with interesting results [52–55], but further research is required to assess the potential for *in vivo* utilization.

The clinical evaluation of ribavirin in Argentine HF patients demonstrated that the drug had an antiviral effect but did not show efficacy in reducing mortality [49]. The transfusion of immune convalescent plasma with defined doses of JUNV-neutralizing antibodies is the present therapeutic intervention against this HF, effective to attenuate disease and reduce mortality to less than 1% [35]. However, many considerations argue for the need for alternative treatments. First, plasma therapy is not so efficient when initiated after 8 days of illness; second, a late neurological syndrome is observed in 10% of plasma-treated patients; third, troubles in maintaining adequate stocks of plasma; fourth, the risk of transfusion-borne diseases. By contrast, immune plasma did not improve recovery from Lassa fever [44].

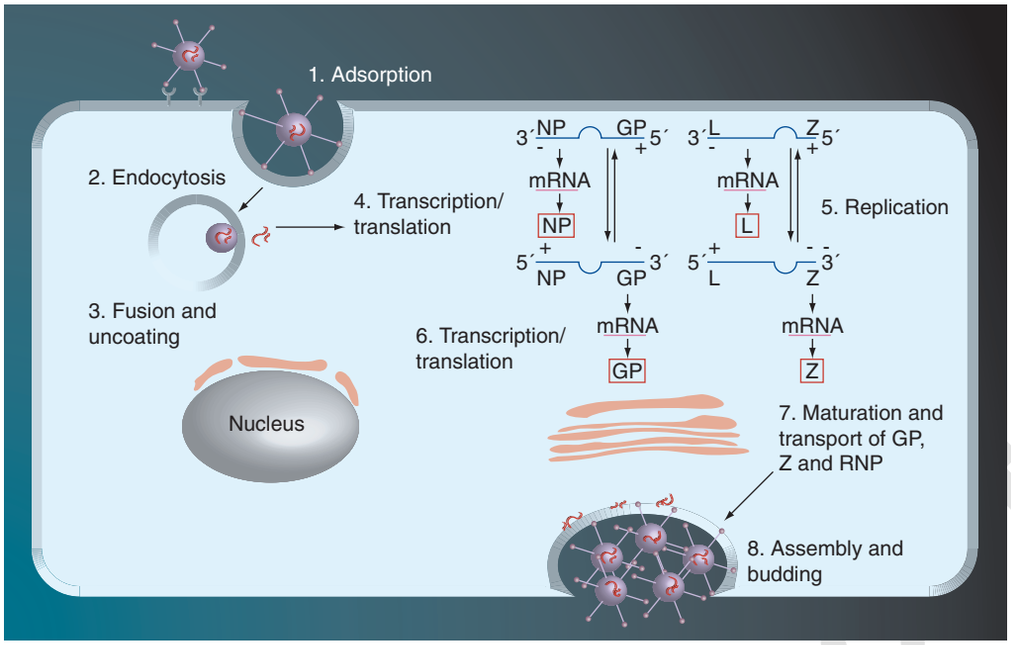

Figure 2. Arenavirus life cycle.

The virion & cell infection

Knowledge of the virion structure and the viral lifecycle is essential to elucidate potential targets of antiviral therapy.

The virions are pleomorphic particles with an average diameter of 90–110 nm, composed of two helical nucleocapsids enclosed in a lipid envelope. A variable number of cell ribosomes, not required for virus multiplication, are also packaged in the particle and this peculiar granular structure originated the family name (*arenosus*, Latin for sandy). The genome consists of two ssRNA molecules known as L (large, average 7.1 kb) and S (small, average 3.4 kb), both with an ambisense coding strategy consisting of two genes with opposite polarity separated by a loop-like noncoding sequence. The S RNA encodes the nucleocapsid protein (NP) at its 3′half from an mRNA in the genome-complementary sense and the glycoprotein precursor (GPC) at the 5′half from an mRNA in the genome sense. GPC undergoes post-translational cleavage to generate the components of the GP (glycoprotein) complex: a small signal peptide that is unusually retained in this mature complex [56], the external glycoprotein GP1 and the transmembrane GP2. Similarly, the L RNA encodes the RNA-dependent RNA polymerase (L) in the genome-complementary sense, and a small matrix protein with a RING finger motif (Z) in opposite sense.

An overview of arenavirus multiplication cycle is outlined in Figure 2. GP1 is the virion attachment protein that mediates interaction with host cell receptors for initial adsorption. Within *Arenaviridae*, α-dystroglycan (α-DG) is the cellular receptor for OW viruses LCMV, LASV, MOPV and Mobala virus as well as for the NW clade C Latino virus and Oliveros virus [57–59]. Another molecule, transferrin receptor 1 (TfR1), was recently identified as the receptor for the NW clade B HF viruses JUNV, MACV, GTOV and SABV [60,61]. By contrast, the non-pathogenic clade B viruses AMPV and TCRV can enter cells in a TfR1-independent manner, although the receptor has not been identified [62]. The North American arenavirus Whitewater Arroyo infects cells also independently of both TfR1 and α-DG [63], revealing a complex pattern of receptor use within NW complex with a possible relationship between receptor usage and disease potential [64].

Upon cell binding, arenaviruses are internalized by endocytosis into low pH vesicles where fusion between the viral envelope and endosome membrane is triggered after conformational alteration in GP exposing a fusogenic peptide present in GP2 [65–67]. As occurred with receptor use, OW and NW arenaviruses also differed in the endocytic pathway for penetration: JUNV is taken by a classical clathrin-mediated endocytosis dependent on an intact actin network, whereas LCMV and LASV enter cells predominantly via an unusual route independent of clathrin, caveolin, dynamin or actin [68–72].

After fusion, the ribonfucleoproteins are

Table 1. Arenavirus-targeted inhibitors.

Target	Agent	Virus	Refs.
Enveloped glycoproteins	ST-193, ST-194	TCRV, JUNV, MACV, GTOV, LASV	[80,81,82]
	Iminodiacetic acid- and pyrrolidine-based peptidomimetics	LASV, JUNV, GTOV, MACV	[83,84]
	Heterocyclic compounds	LASV, JUNV, GTOV, MACV	[83,84]
	Amphipathic DNA polymers	LCMV	[79]
	Aryl methyldiene rhodamine derivative	JUNV	[86]
	mAb	JUNV	[87]
Z protein	Azoic compounds, hydrazide derivatives	JUNV, TCRV, LCMV	[105,106,107]
	Thiuram and aromatic disulfides	JUNV, TCRV, LCMV	[106,108,109,110]
RNA	Favipiravir (T-705)	JUNV, PICV, TCRV	[115,120]
	Imidazole nucleoside/ nucleotide analogues	LASV	[113]
	Acridones	JUNV, LCMV, TCRV	[114]
	Fluorouracil	LCMV	[53,121]
Other targets	Dehydroepiandrosterone, epiandrosterone	JUNV	[124]
	Azoles	JUNV, TCRV	[125]
	Brassinosteroids	JUNV	[122,123]
	siRNA	LASV, JUNV	[127,128]

GTOV: Guanarito virus*; JUNV:* Junin virus; *LASV:* Lassa virus*; LCMV: Lymphocytic choriomeningitis Virus; MACV:* Machupo virus*; PICV:* Pichindé virus*; TCRV:* Tacaribe virus.

released into the cytoplasm and the associated RNA polymerase L starts the macromolecule biosynthesis. Primary transcription of the mRNAs for NP and L from S and L segments, respectively, is the first event (Figure 2). The noncoding intergenic region serves as a transcription terminator signal [73,74], but after NP synthesis has occurred replication would proceed by synthesis of full-length antigenomic RNAs. These antigenomes serve both as replication intermediates in the synthesis of full-length genome RNAs and as templates for the transcription of S and L subgenomic mRNAs for GPC and Z, respectively. Although both genome fragments contain positive sense sequences at their 5' regions, they are not directly translated and thus arenaviruses behave at this point like true negative-strand viruses with transcription as the first biosynthetic event.

Finally, the formation and release of the progeny virions involve the intracellular transport and assembly of the ribonfucleoproteins with the GP complex inserted in the cell surface. This morphogenetic process requires the correct processing of GPC to generate infective virions [56,75–77] and the participation of Z as a matrix protein that interacts with viral and specific cellular proteins to promote virus budding from the plasma membrane [78–80].

Novel therapeutic targets

As illustrated above, the incidence, human health threat, increased emergence and lack of effective control of arenavirus disease highlight the need of novel effective antiviral agents. This search is centered on viral or cellular target-based approaches.

Viral targets

Envelope glycoproteins

GP1 and GP2 are involved in arenavirus entry, which is the first step of infection and a crucial determinant for cellular tropism, host range and virus pathogenesis. Entry has become a very attractive antiviral target for different human viruses because it represents a barrier to suppress the beginning of infection. It is clear that there is a close competition between virus spread and the patient's antiviral immune response. While the rapid viral dissemination critically depends on efficient attachment of the virus to host cell and subsequent entry, drugs targeting these steps will give the host's immune system an advantage by providing a wider window of opportunity for the generation of an efficient antiviral immune response [81]. In recent years, several projects have been developed for the finding of new anti-arenavirus agents, which potentially target the viral GPs (Table 1).

After high-throughput screening (HTS) in which 400,000 molecules were studied

by antiviral action in a TCRV-induced cytopathic effect assay, the small molecule inhibitor ST-294 arose as a lead compound. In addition, this compound was a potent specific inhibitor of other NW arenaviruses, including the Category A HF viruses JUNV, MACV and GTOV. Interestingly, ST-294 also demonstrated good oral bioavailability and protective efficacy in a TCRV newborn mouse model [82]. Given the safety challenge represented by the requirement for biosafety level 4 containment for highly pathogenic arenaviruses, the development of pseudoparticles or vectors that express only envelope GPs represent a powerful tool to screen for new viral entry inhibitors. In this context, lentiviral pseudotypes expressing arenavirus GPs were used against ST-193, a benzimidazole derivative identified from a HTS and subsequent lead optimization, which then also showed a potent antiviral activity against LASV *in vitro* and in a small animal model [83]. This compound inhibited the entry of pseudotypes associated with HF arenaviruses and TCRV but, remarkably, showed no antiviral activity against a pseudotype expressing the LCMV GP. Sensitivity to ST-193 is dictated by a segment of approximately 30 amino acids within the GP2 subunit, a region including the C-terminus of the ectodomain and the predicted transmembrane domain required for fusion [83]. Mechanistic studies determined that both ST-294 and ST-193 interfered with GP-mediated membrane fusion by targeting the interaction of GP2 with small signal peptide and stabilizing the prefusion GP complex against acidic pH [84].

Retroviral pseudotypes bearing the GPs of LASV, JUNV, GTOV and MACV were used in another HTS of a synthetic combinatorial small molecule library including iminodiacetic acid- and pyrrolidine-based peptidomimetics as well as heterocyclic compounds [85]. This screening resulted in the identification of the molecules designated 8C1, 16G8 and 17C8, which also exerted potent inhibitory action against live infectious OW and NW HF arenaviruses in human and primate cells. The characterization of the mechanism of action revealed that the compound 16G8 efficiently blocked pH-dependent membrane fusion triggered by GP2 without significantly affecting cell surface expression of the known receptors. The examination of the individual enantiomers of the inhibitor 16G8 reported that (-)-enantiomer was 15-fold more active than the corresponding (+)-enantiomer [86].

Amongst other approaches for entry blockade, the phosphorothioate oligonucleotides emerged as potent antiviral substances that could effectively obstruct receptor binding and fusion of HIV, and it was determined that the antiviral action of these drugs was dependent on their amphipathic polymer structure, which allows it to interact with HIV-1 gp41 [87]. In this context, amphipathic DNA polymers were evaluated against a number of LCMV isolates *in vitro*, proving to be potent inhibitors in a form dependent on the size and hydrophobicity [81]. Amphipathic DNA polymers disrupted the interaction between LCMV GP and its cellular receptor α-DG, blocking entry and cell–cell propagation of the virus and acting as prophylactic agents to prevent infection.

Recently, the search for new broad-spectrum antiviral therapies, aryl methyldiene rhodamine derivative LJ001 emerged, which inhibited entry and infectious cellular spread of a wide variety of lipid-enveloped viruses including influenza A, HIV, hepatitis C virus and a large number of category A–C priority pathogens, such as Ebola and JUNV HF viruses. This compound did not affect nonenveloped viruses, suggesting that the compound targets an invariant component among all enveloped viruses [88]. It was seen that LJ001 was inserted specifically in the viral lipid membrane and virions were irreversibly inactivated. The envelope GPs appeared to be maintained intact, but the lipid membrane alteration prevented the viral entry at a step after binding but before virus–cell fusion. The compound specifically inhibited virus–cell fusion, but not cell–cell fusion by exploiting the lack of biogenic reparative capacity of viral membranes, which leaves them susceptible to specific disruption.

The fusion of viral and endosomal membranes proceeds through a structural reorganization in GP in which the ectodomain of GP2 engages the host cell membrane and subsequently refolds to form a highly stable six-helix bundle structure that brings the two membranes into apposition for fusion. A GP2-directed monoclonal antibody, F100G5, recognized a pH-induced intermediate of JUNV GP and prevented GP2-mediated membrane fusion. This antibody binds at or near the internal fusion peptide of GP2 and may act by interfering with its penetration into the host cell membrane [89]. Although F100G5 is not an immediate candidate for therapeutic use, the structures identified by the antibody at or near the GP2 fusion peptide may serve as targets for development of small molecule entry inhibitors.

Z protein

Another arenaviral target studied is the Z protein (Table 1), a small 11-kDa protein of approximately 90–100 amino acids, with the ability to bind zinc through a conserved RING finger motif. Recent studies performed with reverse genetics systems have shown that Z exerted a dose-dependent inhibitory effect on both viral transcription and RNA replication [90–93]. Through this inhibitory activity, Z might contribute to the known restricted replicative ability and noncytopathic properties of many arenaviruses. Since Z is a structural component of the virion closely associated to the nucleocapsid protein NP, it has been proposed as the arenavirus counterpart of the matrix protein, found in most enveloped RNA negative strand viruses. Evidence for this structural function was provided by studies showing that LCMV and LASV Z proteins are strongly membrane-associated and are sufficient, in the absence of all other viral proteins, to release enveloped virus-like particles [78,79,94–96]. Thus, it was assumed that Z is responsible for driving arenavirus budding through the recruitment of NP, complexed in the ribonucleoprotein, to the patches in the cellular membranes enriched in envelope GPs where virus assembly takes place [97,98].

Another outstanding property of Z is its ability to interact with several cellular proteins, such as the oncoprotein promyelocytic leukemia protein, the ribosomal protein P0, the eukaryotic translation initiation factor eIF4E and the proline-rich homeodomain protein [99–102]. These interactions of Z may provide mechanisms to elucidate a viral strategy for the establishment of chronic infections, a typical property of arenaviruses. It was found that the integrity of certain sequence motifs and the RING finger domain is necessary for Z-mediated regulatory and structural functions [78,94,103,104] , making this protein a very promising target for arenavirus chemotherapy.

In the search for Z-targeted agents, antiretroviral zinc-finger compounds with diverse chemical structures, including azoic compounds, hydrazide derivatives, disulfide-based reagents and others were screened *in vitro* against various arenaviruses. These compounds had previously been shown to target the retroviral zinc-finger motifs of HIV nucleocapsid protein NCp7, causing zinc ejection from the protein and inhibition of virus multiplication [105,106]. One of the most active arenavirus inhibitors, the aromatic disulfide NSC20625, was a very potent virucidal agent that destroyed virion infectivity, generating particles that entered the host cell but were unable to complete the viral biosynthetic processes [107–109]. Posterior studies with this compound showed that LCMV and JUNV inactivated particles retained the biological functions of the virion GP in virus binding and uptake, but were blocked in virus uncoating remaining associated within the endosome vesicles [110]. Electrophoretic profile of the Z protein in these inactivated particles was altered under nonreducing conditions and the compound was able to induce metal-ion ejection from purified recombinant LCMV Z protein, with the consequent loss of its native structure and stability and oligomerization to high-molecular-mass aggregates [111]. The screening against JUNV, TCRV and LCMV of a extended series of thiuram and aromatic disulfides allowed the identification of even more effective virucidal and antiviral agents in the range of submicromolar to low micromolar concentrations [112], prompting the promising perspectives of these agents for prophylactic and therapeutic intervention against HF arenaviruses.

Other targets

Beside the strategies focused on GP and Z proteins, in recent years different classes of agents were evaluated for their inhibitory effects on arenavirus replication *in vitro*, displaying modest and rather nonspecific effects without, at present, a complete identification of the antiviral target (Table 1). Synthetic brassinosteroids adversely affected viral RNA replication by preventing the synthesis of full-length antigenomic RNA JUNV, and also showed antiviral action on later events in the replicative cycle [113,114]. Dehydroepiandrosterone, epiandrosterone and 16 synthetic derivatives were screened *in vitro* against JUNV, and a partial inhibitory action on the surface expression of GP1 in dehydroepiandrosterone- and epiandrosterone-treated infected culture was observed, an effect probably related to the capacity of steroid hormones to alter membrane fluidity and the insertion of viral GPs into cell membrane [115]. Azoles obtained from carbohydrates were also able to inhibit JUNV replication [116].

The pyrazine derivative T-705 (6-fluoro-3-hydroxy-2-pyrazinecarboxamide), first found active *in vitro* and *in vivo* against influenza virus [117–119], was also tested against a panel of arenaviruses (JUNV, PICV and TCRV) showing high level of inhibition by cytopathic effect and virus yield reduction cell-based assays. Studies on the mechanism of action of T-705, also known as

favipiravir, have shown that this compound is converted to the ribofuranosyl-triphosphate derivative by host enzymes, and this metabolite selectively inhibits the influenza RNA-dependent RNA polymerase without toxicity to mammalian cells. Interestingly, this compound did not inhibit host DNA and RNA synthesis and IMPDH activity [120]. The mode of action of T-705 against arenaviruses has not been determined but it is a promising drug since its oral administration was efficacious in treating PICV infection in hamsters [121,122].

A novel approach intended the use of specific siRNA to investigate if arenaviruses are amenable to RNA interference [123]. The therapeutic use of this class of agents was first evaluated with siRNA targeting the conserved RNA termini upstream of NP and L gene. They were found to reduce reporter gene expression from LASV replicon and LASV mRNA expression construct and to inhibit replication of different LASV virus strains, LCMV and MOPV *in vitro* [124]. Four Z-specific siRNAs (Z1- to Z4-siRNAs) were tested showing variable efficacy against JUNV [125]. The most effective inhibitor was Z2-siRNA targeted at the region encompassed by NT 179–197 of Z-JUNV gene. Further studies are required to ascertain the potential of RNA interference in therapy.

Targeting host cell functions

As an alternative to the traditional virus-based approach, some of the novel targets for antiviral drugs are cellular, not viral, proteins. There are a number of virus-specific processes within the infected cell that involve cellular proteins and have proven to be attractive targets for chemotherapeutic intervention against several unrelated viruses. In addition, this antiviral strategy should be active even against viral mutants that are already resistant to conventional viral-targeted antiviral drugs. On the negative side, targeting cellular proteins can certainly result in cytotoxic or other undesirable side effects. To minimize this problem, inhibition needs to be targeted with pinpoint accuracy.

It is worth mentioning that the search for new antiviral molecules is extremely valuable; however, all existing licensed drugs should be considered as a ready-made pharmacy of antimicrobial agents with defined safety data profiles and available clinical use histories, requiring only assessment for new or 'off target' second use.

The evidence that inhibiting host cell functions is effective works now extends across many diverse virus types. In the last few years, numerous reports of host cell targeting strategies against arenaviruses have been reported, and some of them will be discussed in this section.

N-myristolyltransferase

Like many other enveloped viruses, arenaviruses are able to use host cell strategies for processing and transport of GPs from their site of synthesis in ER to their proper location in the plasma membrane [126,127]. Modifications such as glycosylation, cleavage, oligomerization and fatty acylation take place in this process. The two predominant types of fatty acylation known to occur on viral proteins are palmitoylation and myristoylation [128,129]. Myristic acid is a 14-carbon saturated fatty acid that is cotranslationally transferred from myristoyl-CoA to the penultimate glycine residue found in the N-terminal sequence of the protein, a reaction catalyzed by the soluble cellular enzyme N-myristolyltransferase (NMT) [130]. As myristoyl proteins are frequently essential to virus function, this protein modification has become an attractive target for antiviral strategy [131].

Few years ago, it has been reported the antiviral effect of two myristoylation analogs on arenavirus replication [132]. The compounds studied were the DL-2-hydroxymyristic acid, an inhibitor of NMT, which binds the enzyme and blocks protein myristoylation, and the 13-oxamyristic acid, a competitive inhibitor of NMT which incorporates into the protein instead of myristic acid. Both types of analogs achieved dose-dependent inhibition of viral multiplication at concentrations not affecting cell viability. Surprisingly, the cytoplasmic and surface expression of JUNV GPs was not affected in the presence of the compounds, suggesting that JUNV GP myristoylation would not be essential for the intracellular transport of the envelope proteins, but it may have an important role in their interaction with the plasma membrane during virus budding. Also Z protein needs to be myristoylated for efficient virus budding [133]. At present, there are various studies describing the importance of this protein modification and the NMT as a key host-based antiviral target that deserves some more consideration.

Proprotein convertase site 1 protease/ subtilisin kexin isozyme-1

Numerous reports have described the importance of the S1P/SK1–1 for the post-translational maturation cleavage of arenavirus GPC [134–137]. Moreover, due to the essential roles of S1P/SK1–1 in diverse cellular reactions, this

enzyme has attracted great attention from the pharmaceutical industry. A successful approach to inhibit proprotein convertases involves genetically engineered antitrypsins, which are derived from α_1-antitrypsin (α_1-AT). This approach has been used for the generation of highly selective α_1-AT variants by introducing various S1P/SK1–1 recognition motifs into the reactive center loop of α_1-AT [138]. The adaptation of α_1-AT towards S1P/SK1–1 by the generation of recombinant α_1-AT variants mimicking the S1P/SK1–1 recognition peptide motifs RRLL and RRIL efficiently blocked proteolytic maturation of the LASV precursor GPC. Also, α_1-AT variants RRVL and RRYL were found to be inhibitory, although to a lesser extent.

Since glycoprotein processing by the endoprotease S1P/SK1–1 is not only critical for infectivity of LASV and other HF arenaviruses [139], but also for members of the *Bunyaviridae* family [140], further optimization based on these findings could lead to a potent and specific S1P/SK1–1 inhibitor for treatment of viral HF [141,142].

Fatty acids

Since the replicative cycle of enveloped viruses is closely dependent on the characteristics of the host cell membrane, at the viral entry and at the assembly and budding stages, it is expected that alterations in the fluidity and/or the permeability of the plasma membrane may affect infection with these viruses. On this basis, several agents disturbing the lipid composition of the cell membrane have been proposed as potential antiviral compounds [143].

In particular, fatty acids have a prominent role in the lipid bilayer of the cell membrane as components of phospholipids, glycolipids and triacylglycerols. Using saturated fatty acids of variable chain length, the functional involvement of cellular membrane properties on arenavirus infection have been analysed [144]. Results have shown that lauric acid reduced virus yields of several strains of JUNV in a dose-dependent manner without affecting cell viability. In addition, while viral protein synthesis was not affected by the compound, the expression of GPs in the plasma membrane was diminished. From mechanistic studies, it was concluded that lauric acid inhibited a late maturation stage in the replicative cycle of JUNV. A direct correlation between the inhibition of JUNV production and the stimulation of triacylglycerol cell content was also demonstrated, and both lauric acid induced effects were dependent on the continued presence of the fatty acid. Thus, the decreased insertion of viral GPs into the plasma membrane, apparently due to the increased incorporation of triacylglycerols, seems to cause an inhibition of virus maturation and release, and could be considered as an strategy for the treatment of viral HF.

Phospholipids

Phosphatidylserine (PS), the most abundant anionic phospholipid of the plasma membrane, is segregated to the inner leaflet of the plasma membrane of resting mammalian cells [145,146]. This internal position changes the exposure of PS, and possibly other anionic phospholipids, to cell surface in virus-infected cells.

Recently, the detection of target exposed anionic phospsolipids on PICV-infected cells using a human–mouse chimeric version of a monoclonal IgG3 antibody, which binds with high affinity to complexes of the PS-binding plasma protein B2-glycoprotein I and anionic phospholipids has been described [147]. In collaboration with Peregrine Pharmaceuticals Inc, this antibody has been recently developed and patented under the name of 'bavituximab', and it has been demonstrated that bavituximab-coated magnetic beads specifically removed infectious PICV, confirming that infectious virions carry external PS [148]. Furthermore, the therapeutic effectiveness of bavituximab was demonstrated against advanced PICV infections in guinea pigs. In addition, the treatment combining bavituximab and ribavirin had an additive activity, as expected for drugs with nonoverlapping mechanism of action. Two mechanisms appear to explain the protective effect: First, bavituximab causes opsonization and clearance of infectious virus from bloodstream, leaving less virus to infect other tissues; second, bavituximab induces antibody-dependent cellular cytotoxicity of virus infected cells. Since PS exposure is an early event during virus infection, antibody-dependent cellular cytotoxicity may limit virus spread. Also, bavituximab treatment may mask PS on virus-infected cells and/or viruses, leading to the development of effective antiviral immune responses. It seems that targeting PS on cells infected with different viruses and on virions themselves represent a promising antiviral strategy.

Cholesterol

It has been shown that entry of PICV, LASV and LCMV particles is susceptible to cholesterol depletion of the target host cell membrane using methyl-β-cyclodextrin treatment [149]. Moreover, analyses of the distribution of viral proteins in

cholesterol-rich, detergent-resistant membrane areas showed that LASV buds from membrane areas other than those responsible for impaired infectivity due to cholesterol depletion of lipid rafts [150]. Thus, derivation of the viral envelope from cholesterol-rich membrane areas is not a prerequisite for the impact of cholesterol on virus infectivity. More studies are needed to elucidate the potential utility of this cellular component as a therapeutic target against viral diseases.

Tetherin

Cellular factors that inhibit viral replication through interactions with viral components at various steps have also been studied. Recently, tetherin (also known as BST2, CD317, or HM1.24) was identified as a cellular factor that inhibits the release of HIV-1 from infected cells [151]. Tetherin is a membrane-associated protein with an N-terminal transmembrane domain, a central extracellular domain with two potential N-linked glycosylation sites, and a C-terminal glycosylphosphatidylinositol anchor [152,153], which appears to prevent virus release by retaining fully formed progeny virions on the surface of infected cells [154]. Tetherin is constitutively present on the surfaces of HeLa and CEM cells, while its expression is induced by IFN-α in other different cells and is stimulated by IFN in various tissues, suggesting that it may function as part of IFN-induced innate immunity against enveloped viruses *in vivo*. It was recently shown that the production of virus-like particles induced by the matrix Z protein of LASV was markedly inhibited by the expression of tetherin and that N-linked glycosylation of tetherin was dispensable for this antiviral activity [155]. This report also suggests that Z or one or more cellular components are targets of tetherin but that viral surface GPs are not.

Thus, tetherin may represent a potential antiviral strategy against a variety of enveloped viruses by inhibiting their release from host cells through a common mechanism that has still to be fully elucidated. Furthermore, analyses of the expression pattern of tetherin *in vivo* may aid in understanding the susceptibility of tissues or cells to virus replication.

Endosomal sorting complexes required for transport

The tumor suppressor gene 101 (TSG101) is a component of the class E vacuolar protein sorting cellular machinery involved in the routine recycling/ degradation of cellular proteins. Class E proteins are cytoplasmic multidomain proteins that transiently attach to the endosomal membrane, where the inward invagination of cargo-laden vesicles takes place. When a cell is infected with a virus, the TSG101 is hijacked to orchestrate the release of viral particles from the infected cell. TSG101 has been reported to interact with the late domain motif of Ebola virus VP40 and HIV-Gag proteins. This interaction recruits TSG101 to sites of particle assembly, where it is required for efficient virion formation [156,157].

The first arenavirus TSG101-related report has shown that the silencing of TSG101 by siRNA caused a strong inhibition of VLP production [94]. Both LCMV and LASV Z proteins are colocalized with TSG101 in the proximity of the plasma membrane. Compelling evidence indicates that budding of viruses with a PTAP-containing late domain requires interaction between TSG101 and the tetrapeptide PTAP [158]. However, PTAP is present in LASV Z, but not in LCMV Z, suggesting that recruitment of TSG101 by LCMV Z may not be due to a direct interaction, but, rather, is mediated by a third protein capable of binding to both TSG101 and Z [94]. In this regard, the ubiquitin ligase Nedd4 protein may be a candidate since it has been involved in the budding of several viruses through its interaction with PPXY motifs, and Nedd4 also interacts with TSG101 [159]. Finally, it was also shown that TCRV Z does not utilize TSG101 but does depend on another endosomal sorting complex required for transport component (Vps4A/B) activity for budding [79], suggesting that, as with LCMV and LASV, TCRV budding requires the participation of the endosomal sorting complex required for transport machinery but involves different specific components that remain to be determined. These interactions appeared to be a key stage of successful arenavirus budding, and could be used to impair viral spread.

Cellular signalling pathways

Many viruses have evolved mechanisms to gain control of key cellular signalling pathways that affect broad aspects of cellular macromolecular synthesis, metabolism, growth and survival. The phosphatidylinositol 3-kinase/protein kinase B (Akt) is one of such pathways promoting cell survival by phosphorylation and inhibition of a number of pro-apoptotic proteins. Viruses must regulate this pathway, either by activating or inactivating activity, in order to achieve an efficient replication process [160,161]. Recently, this pathway has been assoicated with arenavirus replication [162]. The authors observed that infection

of cells with UV-irradiated JUNV redeemed the same pattern of Akt stimulation obtained for infectious virus, indicating that an early stage would be enough to trigger activation. In addition, the treatment of cells with chlorpromazine abrogated phosphorylation of Akt upon JUNV infection, suggesting that virus internalization is responsible for activation. On the other hand, inhibition of Akt phosphorylation by Ly294002 impaired viral protein synthesis and expression, leading to a reduced infectious virus yield. It is clear that once virus has bound to the cell receptor, protein phosphorylation likely plays an important role and downstream cell signalling events may be required to prime the cell to facilitate viral replication. Thus, the impairment could be linked to a reduced amount of cell-bound virus to cells, probably due to a blockage on the recycling of TfR1 cell receptor.

Another group has also investigated the global kinase/phosphorylation response to PICV infection [163]. By comparing the activity of the macrophage kinome following PICV infection of guinea pigs, they have shown the predicted phosphorylation state of a number of proteins from cell surface receptors to downstream transcription factors. Other studies also specifically described PICV-induced phosphorylation of the activating transcription factor-2 protein and cyclic adenosine monophosphate response element-binding protein, and that these phosphorylation pathways were inhibited following genistein treatment [164]. Altogether, these results lead us to speculate that activation and stabilization of different cellular proteins by phosphorylation might be critical to arenavirus multiplication.

Innate defense against HF arenaviruses

The modulation of the host immune response to a pathogen may well prove to be an efficacious form of treatment against a number of infections, and current strategies are focussed on a broad-spectrum 'boosting' of this response [165]. However, immunopathological virus infections may be exacerbated by this approach. It is also known that viruses modulate cell signalling pathways to induce a cellular state that can facilitate productive infection or to evade the immune response.

In this context, numerous reports suggest that the severity of arenavirus pathogenesis may be due to dysregulation of innate immune signalling and the cytokine response [166]; however, it is not yet fully understood how arenaviruses induce this dysregulation or how this leads to disease. Assessment of transcriptome profiles in monkey models of arenavirus infection has shown up- and down-regulation in the transcription of multiple genes before the viremic stage and during the first few days of disease [167]. In other studies using a guinea pig model, the gene profile changes of two variants of PICV, the attenuated variant P2 and the virulent variant P18, was analyzed [168]. As the host was able to effectively clear P2, but not P18 infection, the authors suggested that P18 suppresses the signalling events that lead to a protective immune response. By using microarray systems, three signalling networks (p53, c-Myc, and Akt) were identified as the central nodes of the different response and differences were also shown in IκB kinase, IκB epsilon, phospholipase C (involved in NF-κB activation), and protein kinase C-iota (involved in a number of pathways, including NF-κB signalling) [169]. As arenaviruses are not likely to activate cell signalling pathways to enhance viral replication, it is probable that P18 virus variant may be inducing an active suppression of host cell signalling. Furthermore, these results are consistent with those seen with other arenaviruses: the nonpathogenic MOPV activates macrophages following infection, but LASV does not [170].

These gene profile studies with microarrays are relevant to identify key proteins involved in signalling networks that are differentially regulated in attenuated and lethal arenavirus disease, and may provide useful targets for future development of antiviral agents, perhaps also effective against other HF virus infections.

Future perspective

The information here reviewed has shown the existence of a range of viral and cellular targets for new antiviral drugs against arenaviruses. Several projects focused on the viral GP and Z proteins are presently under study with the potential to block infection at early or late stages of the virus life cycle. Furthermore, the innovative technologies for drug targeting by HTS, recently developed for arenaviruses, may lead in the near future to an accelerated identification of novel hits with greater sensitivity and application. Although the search for host target inhibitors appears more challenging than virus target inhibitors, the understanding of arenavirus–cell interactions has also advanced greatly in recent years with the identification of key host factors in regulatory networks that are important for pathogen survival. Further complete understanding

of these interactions may be achieved by analysis of transcriptome profiles of infected cells now in progress, which will allow delineation novel of antiviral cell targets. Finally, it must also be considered that arenavirus HF is an acute disease with a short period of viremia and, consequently, a rapid and precise diagnosis will be essential to the success of any treatment. Since early clinical diagnosis is difficult, improvement in accessible diagnostic tests should proceed in parallel with new therapies.

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Executive summary

Introduction

- *Arenaviridae* is the largest family of viruses causing lethal hemorrhagic fever in humans. The most harmful member, Lassa virus, is agent of Lassa fever, a highly neglected and severe tropical disease with over 300,000 annual cases in West Africa.
- It was estimated that a new arenavirus species may emerge and be recognized on the average every 3 years, a prediction that appeared to be surpassed in the last few years in Africa and America.
- There is no specific antiviral therapy for prophylaxis or treatment. Thus, the incidence, human health threat, increased emergence and lack of specific control of arenavirus disease are factors that highlight the need for effective antiviral agents.

Viral targets

- Selective inhibition of *in vitro* infection with live viruses or pseudotypes expressing arenavirus glycoprotein was achieved with diverse entry inhibitors targeted to the envelope glycoproteins. The most effective compounds prevented glycoprotein 2-mediated fusion between virus envelope and endosome membrane.
- Z, a matrix protein with ability to bind zinc through a conserved RING finger domain, was also the target of different zinc-finger-reactive compounds with virucidal and antiviral activities against Junin virus, lymphocytic choriomeningitis virus, Pichindé virus and Tacaribe virus. Aromatic disufides were the most effective inactivating agents inducing blockade of virus uncoating and oligomerization of Z.

Host cellular targets

- Various cellular proteins essentially required for virus replication have also become potential targets for arenavirus inhibition. Membrane- and lipid-associated proteins and enzymes, components of cell signalling pathways such as kinases and proteases, cytokines are among the host cell factors involved in arenavirus infection and potentially useful for chemotherapy.

Future perspective

- The innovative technologies for drug targeting by high-throughput screening, recently developed for arenaviruses, as well as analysis of transcriptome profiles of infected cells, may lead in the near future to an accelerated identification of novel viral hits with greater sensitivity and key host factors in regulatory networks important for virus propagation.

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