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Viral replicons as valuable tools for drug discovery

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RNA viruses can cause severe diseases such as dengue, Lassa, chikungunya and Ebola. Many of these viruses can only be propagated under high containment levels, necessitating the development of low containment surrogate systems such as subgenomic replicons and minigenome systems. Replicons are self-amplifying recombinant RNA molecules expressing proteins sufficient for their own replication but which do not produce infectious virions. Replicons can persist in cells and are passed on during cell division, enabling quick, efficient and high-throughput testing of drug candidates that act on viral transcription, translation and replication. This review will explore the history and potential for drug discovery of hepatitis C virus, dengue virus, respiratory syncytial virus, Ebola virus and norovirus replicon and minigenome systems.

Introduction

Vaccines and therapeutics are the main weapons deployed to combat infectious diseases. Vaccines have been successful in dramatically lowering infection rates for diseases like measles, mumps, rubella, diphtheria, pertussis and many more, and have led to the complete eradication of smallpox and near-eradication of polio [1–4]. However, safe and effective vaccines for some of the most prevalent and crippling diseases known to mankind remain elusive, despite decades of research [5–7]. Against a backdrop of a rise in vaccine hesitancy across the world, the development of effective drug treatments for patients suffering from infectious diseases is imperative [8]. However, drug lead discovery and optimisation for infectious agents can be technically challenging for a variety of reasons: (i) no cell culture or adequate animal model exists [9–11]; (ii) little is known about the biological activity of the agent's targets; (iii) the agent requires category 3 or 4 containment facilities not available to most academic or industrial researchers and institutions. The 2018 WHO Blueprint List of Priority Diseases is exclusively populated by RNA viruses that fulfil one, two or all three of the criteria mentioned above [12]. Therefore, the development of low containment systems for high-throughput hit discovery has become a necessity.

Small-compound drugs act on proteins involved in the viral life cycle: entry into host cells, virus uncoating, replication and translation of the viral genome or suppression of the innate host immune response. The development of viral pseudotypes and virus-like particles (VLPs) has made research and drug discovery into virus entry and uncoating possible at a reduced containment level [13–15]. By contrast, analysis of subgenomic replicons enables uncoupling of viral replication, transcription and translation from virus assembly, host cell egress, entry and uncoating. Replicons are broadly defined as autonomously replicating DNA or RNA molecules, whereas viral subgenomic replicons are usually produced by deletion of one or more genes coding for structural proteins or insertion of or replacement by a reporter gene and/or selectable marker [16–19]. Replicons have been made possible by the advent of reverse genetics: a process of storage and manipulation of entire viral genomes hosted on plasmids, and the recreation of viral RNA genomes by *in vitro* or *in vivo* transcription of these plasmids. In cases where replicons cannot be established, plasmid-driven co-expression of replication factors necessary to drive replication of a reporter RNA create minimal replication systems termed minigenomes. These reporter RNAs usually contain the genomic 5′ and 3′ untranslated regions (UTRs) which are essential for replication, transcription and translation. Whereas most replicons can be maintained in cell lines by antibiotic selection

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and passed on during cell division, minigenomes based on plasmid transfection have a limited lifespan and need to be recreated before experimentation. Although not exhaustive, Table 1 lists established replicons and minigenome systems for many medically relevant viruses. Since their conception, replicons and minigenomes have been used in low- and high-throughput screens of compound libraries, to elucidate the biological mechanisms of drug action and to screen for drug escape-mutants arising in the replicon-harbouring cell pool owing to generally poor fidelity of viral RNA-dependent RNA polymerases (RdRp).

This review will briefly cover the modern success story of the establishment and use of subgenomic replicons in the development of drugs against hepatitis C virus (HCV) and then illustrate the potential of dengue virus, togavirus, respiratory syncytial virus and norovirus replicons, and the Ebola virus minigenome, in the discovery of small-molecule inhibitors. In recent years replicons have been derived from viruses such as hepatitis E virus, enterovirus 71, severe acute respiratory syndrome virus, among others, but their description is beyond the scope of this review [20–22].

HCV replicons

It is estimated that 185 million people are infected with HCV globally [23]. Infected individuals have a 75–85% likelihood of developing chronic infection, which when left untreated can lead to liver cirrhosis and hepatocarcinoma [24]. Previous to 2011 [when the first direct-acting antivirals (DAAs) were released into the market], pegylated interferon (IFN)-α combined with ribavirin was used as a standard therapy, with aviraemia 24 weeks after completion of antiviral therapy of 40–80%, depending on viral genotype [25].

HCV is a hepacivirus within the *Flaviviridae* family and has a single-stranded, positive-sense RNA genome of ~9.6 knt (kilo nucleotides). The HCV genome encodes a polyprotein that is proteolytically cleaved by the viral NS3/4A and cellular proteases into the three structural proteins: Core, Envelope protein 1 and Envelope protein 2, and the seven nonstructural proteins: p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B. Most HCV strains will not establish productive infection in cell culture and it was not until 2005 that JFH-1 was shown to be the first strain that could be

TABLE 1

Replicon and minigenome systems established for medically relevant viruses

	Virus family	Replicon	Minigenome	Refs
Positive-sense single-stranded RNA viruses	*Flaviviridae*	Dengue virus (DENV)		[45]
		West Nile virus (WNV)		[41]
		Kunjin virus (KUNV)		[17]
		Tick-borne encephalitis virus (TBEV)		[105]
		Yellow fever virus (YFV)		[42]
		Japanese encephalitis virus (JEV)		[106]
		Hepatitis C virus (HCV)		[18]
		Bovine viral diarrhoea virus (BVDB)		[107]
	Togaviridae	Sindbis virus (SINV)		[58]
		Chikungunya virus (CHIKV)		[61]
		Venezuelan equine encephalitis virus (VEEV)		[19]
		Western equine encephalitis virus (WEEV)		[67]
		Semliki forest virus (SFV)		[108]
	Coronaviridae	Severe acute respiratory syndrome (SARS) virus		[22]
		Middle eastern respiratory syndrome (MERS) virus		[109]
	Caliciviridae	Human norovirus (NOV)		[92]
	Hepeviridae	Hepatitis E virus		[20]
	Picornaviridae	Polio virus		[16]
		Foot-and-mouth disease virus (FMDV)		[119]
		Enterovirus 71 (EV71)		[21]
	Astroviridae	Human astrovirus		[120]
Mononegavirales	*Rhabdoviridae*	Vesicular stomatitis virus (VSV)	Vesicular stomatitis virus (VSV)	[115,116]
	Paramyxoviridae		Nipah virus	[113]
			Human metapneumovirus (HMPV)	[114]
		Respiratory syncytial virus (RSV)	Respiratory syncytial virus (RSV)	[76,78]
	Filoviridae		Ebola virus (EBOV)	[99]
			Marburg virus (MARV)	[117]
Bunyavirales	*Phenuiviridae*		Rift Valley fever virus (RVFV)	[110]
			Severe fever with thrombocytopenia virus (STFSV)	[111]
	Hantaviridae		Hantaan virus	[118]
	Arenaviridae		Lassa virus (LASV)	[112]

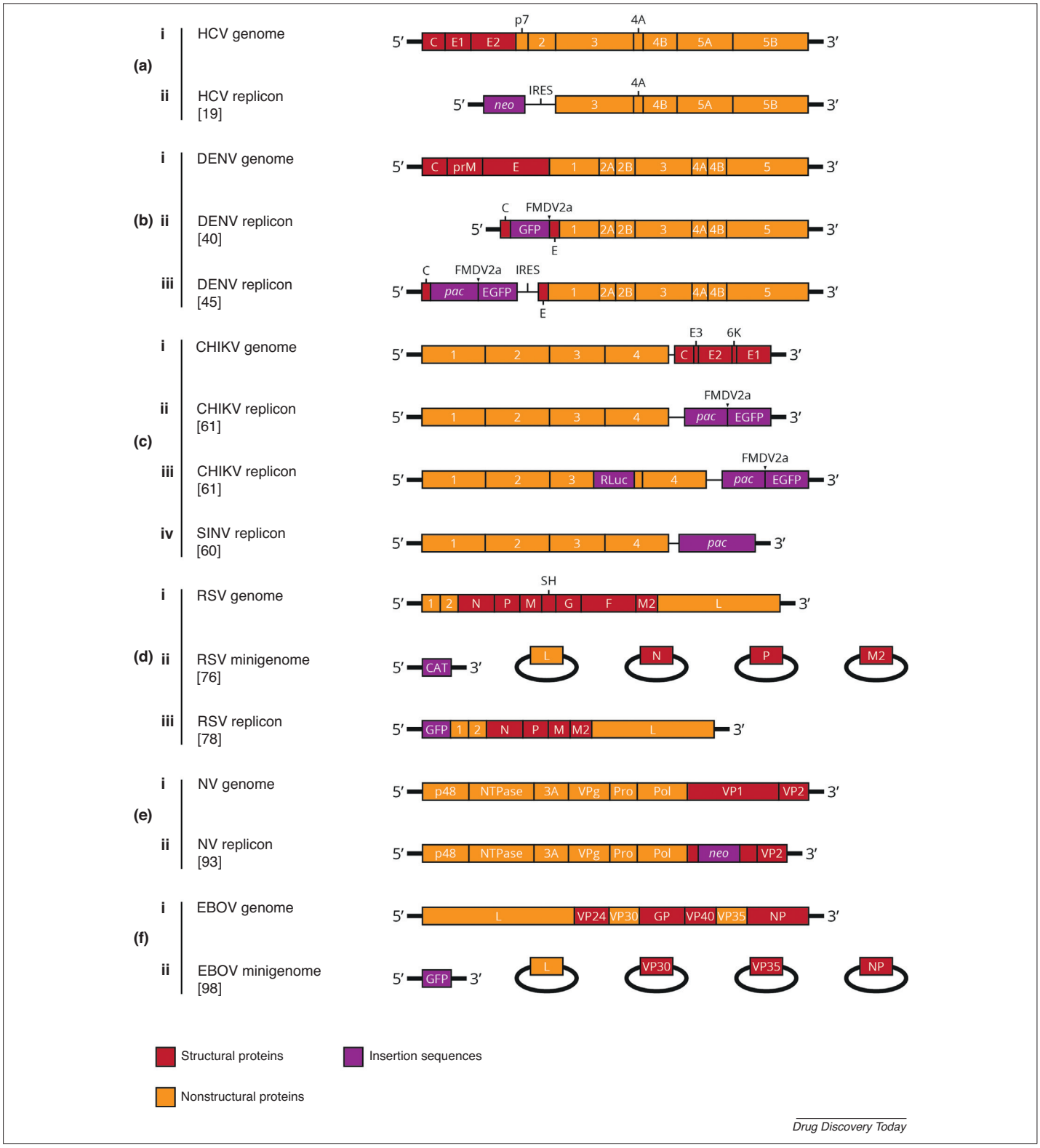

FIGURE 1

Graphic representation of viral genomes and their derived replicons and minigenomes. Each region coding for a protein is shown as a box in red if the region codes for a structural protein, in yellow if it codes for a nonstructural (NS) protein or in purple if it codes for a nonviral protein. Internal ribosome entry sites (IRES) and the foot-and-mouth disease virus 2A autoprotease sequences (FMDV2A) are marked by lines or arrows, respectively. For minigenomes (d,f) plasmid-driven expression of viral genes is shown by circularized lines. **(a)** Hepatitis C virus (HCV) genome (ai) and its derived replicon (aii). The HCV replicon was established by replacement of C, E1, E2 and NS2 by the neomycin phosphotransferase (*neo*). Translation of NS3–NS5B was driven by an IRES sequence. **(b)** Dengue virus (DENV) genome (bi) and its derived replicons (bii and iii). Replicons for dengue virus have been produced by replacement of the C-terminal part of C, full length prM and the N-terminal part of E with either green fluorescent protein (GFP), which is cleaved by from NS1 by the FMDV2A sequence (bii), or with a polyprotein cleaved by the FMDV2A sequence producing puromycin-N-acetyl transferase (*pac*) and enhanced GFP (EGFP). Translation of NS1–NS5 is driven by an IRES sequence (biii). **(c)**

propagated in a cell culture system without adaptive mutations [26,27]. To overcome this limitation, in 1999 Lohmann *et al.* defined NS3, NS4A, NS4B, NS5A and NS5B as the minimal set of HCV proteins necessary to facilitate autonomous replication by replacement of the coding region for Core, Envelope protein 1 and 2, p7 and NS2 with a neomycin resistance cassette. Expression of NS3–NS5B was driven by the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES), establishing a bicistronic HCV replicon [18] (Fig. 1a). This development started DAA discovery which eventually led to the licensing in 2011 of first-generation DAAs boceprevir and telaprevir, both NS3/4A protease inhibitors. Replicons were used to assess efficacy and bioavailability of drug candidates, as well as analysis of escape mutants during discovery and optimisation of candidates, reviewed in Refs. [28,29]. However, boceprevir and telaprevir act on HCV genotype 1 only, making development of subgenomic replicons of other genotypes necessary to facilitate discovery of drugs acting on some or all genotypes. To date, subgenomic replicons have been developed for HCV strains 1a, 1b, 2, 3, 4, 5a and 6a to ensure testing of pan-genotype effectiveness of drug candidates [30–35]. These replicon systems have aided in the development of second-generation DAAs such as ledipasvir, acting on the viral NS5A regulatory protein, and sofosbuvir, a nucleotide phosphoramidate prodrug inhibiting the NS5B polymerase [36,37]. Without the development of HCV replicons DAA development would have been severely delayed if not impossible and, for the first time in history, patients can now be cured of HCV by DAA combination therapy.

Dengue virus replicons

Dengue serotype 1–4 viruses (DENV1–4) are arthropod-borne flaviviruses spread by *Aedes* mosquitos. It is estimated that 390 million people are infected with dengue virus every year. Despite a high proportion of infections being asymptomatic, 96 million cases of dengue disease are diagnosed annually. Roughly 5% of symptomatic patients progress to dengue haemorrhagic fever and/or dengue shock syndrome, which require hospitalisation and can be fatal. To date, there is no approved specific drug treatment available against dengue virus [38].

Dengue virus has a single-stranded, positive-sense RNA genome with a size of roughly 11 knt that is translated into a ~3400 amino acid polyprotein. The polyprotein is then processed by the viral NS2b/3 and cellular proteases into the three structural proteins Capsid, pre-Membrane and Envelope, and the seven nonstructural (NS) proteins NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5, with NS5 harbouring N-terminal methyltransferase and C-terminal RdRp activity. In addition to the 5′ and 3′ UTRs, the first 65 nucleotides of the Capsid coding region contain a hairpin element and the 5′ cyclisation motif, which are essential for replication [39]. A DENV2 replicon was established by Pang *et al.* in 2001 by deletion of pre-Membrane and Envelope protein coding regions, showing that DENV is amenable to analysis by replicons [40]. Around the same time, replicons were established for closely related viruses like Kunjin, West Nile and yellow fever virus [17,41,42]. In 2011, selectable DENV replicons harbouring reporter genes were reported by multiple groups. These were constructed by replacement of Envelope or by excision of the coding region from the C-terminal portion of Capsid to the end of Envelope and replacement with a variety of reporter genes, including fluorescent proteins, luciferase and a combination of reporter genes and antibiotic resistance cassettes. In replicons with two foreign genes (e.g., reporter and resistance cassette) translation of nonstructural proteins was driven by an IRES sequence, and translation of the reporter was driven by the viral 5′ UTR. The reporter is separated from the antibiotic resistance by the foot-and-mouth disease virus (FMDV) 2A autoprotease, enabling post-translational cleavage of the reporter from the resistance marker [43–46] (Fig. 1b).

Dengue replicon systems have been successfully used for hit discovery by Lu *et al.*, who identified phthalazinone derivatives as potent inhibitors of DENV2 replication [47]. Frabasile *et al.*, using a similar screen, showed that naringenin, a citrus flavonone, greatly impaired genome replication using human Huh7.5 cells harbouring DENV1 and DENV3 replicons [48]. Hernandez-Morales *et al.* tested a HCV compound library in a high-throughput DENV2 replicon screening assay, identifying JNJ-1A as an effective lead [49]. This publication showed how the subgenomic replicon system can be used to detect drug-induced resistance-associated mutations. In the case of JNJ-1A, resistance-associated mutations were found to cluster in the NS4b coding region. Interestingly, NS4b has no known enzymatic activity but is essential for replication, and is known to interact with viral and cellular proteins to form the dengue replication complex and subvert the cellular innate immune response [50]. Quinic acid derivatives were shown to reduce the amount of NS3 in treated DENV1 and DENV3 replicon-containing cells by FACS analysis [51]. Interestingly, genome replication and translation were excluded as the mode of action for some drug candidates, such as hirsutine, using the dengue subgenomic replicon system [52]. Dengue virus drug discovery is a hotbed of activity as recently reviewed [53]. However, none of the aforementioned drug candidates has been taken to the clinic.

Togavirus replicons

Chikungunya virus (CHIKV), western, eastern and Venezuelan equine encephalitis virus (WEEV, EEEV, VEEV) and Sindbis virus (SINV) are the most prominent members of the *Togaviridae* family. CHIKV is transmitted by *Aedes* mosquitos and can cause symptoms

Togaviridae genome (ci) and derived replicons for Chikungunya virus (CHIKV) (cii and ciii) and Sindbis virus (SINV) (civ). Replicons have been established by replacement of the structural polyprotein (C-E3-E2-6K-E1) with the polyprotein cleaved by FMDV2A producing *pac* and EGFP (cii) or an additional insertion of *Renilla* luciferase (RLuc) within nonstructural protein (nsp) 3 (ciii). The SINV replicon was established by replacement of the structural polyprotein with *pac*. **(d)** Respiratory syncytial virus genome (di) and its derived minigenome system (dii) or replicon (diii). The minigenome system was established by tagging the coding sequence of chloramphenicol acetyl-transferase (*cat*) with the 5′ leader and the 3′ trailer sequences of the viral genome and plasmid-driven co-expression of the viral polymerase (L), nucleoprotein (N), phosphoprotein (P) and matrix protein 2 (M2). The replicon (diii) was established by deletion of the coding regions of the small hydrophobic protein (SH) and the two glycoproteins G and F, and insertion of GFP at the 5′ end of NS1. **(e)** Human norovirus (NV) genome (ei) and its derived replicon (eii). The replicon was established by replacement of the majority of the viral protein (VP)1 coding region with *neo*. **(f)** Ebola virus (EBOV) genome shown in (+) sense (fi) and its derived minigenome system (fii). The minigenome was established by tagging GFP with the (+) sense 5′ leader and 3′ trailer sequences of the viral genome and plasmid-driven co-expression of the viral polymerase (L), VP30, VP35 and the nucleoprotein (NP).

such as fever, rash and joint and muscle pain [54]. VEEV is confined to Central and South America and can infect humans causing flu-like symptoms in healthy individuals, and severe illness and death in the immunosuppressed. This virus has been weaponised by the USA and the Soviet Union [55]. SINV has a wide geographic distribution and can cause fever, rash, polyarthritis and myalgia in humans [56].

Members of the *Togaviridae* family are single-stranded positive-sense RNA viruses with genomes of ~10–12 knt in size, featuring a 5′ cap and a 3′ poly-A tail. Togavirus genomes code for two polyproteins, the 5′ nonstructural and the 3′ structural polyprotein. The nonstructural polyprotein is produced by direct translation of the genome. The structural polyprotein is produced by translation of a 26S subgenomic RNA produced by the viral RdRp from a promoter located between the two coding regions. The structural polyprotein is processed into four structural proteins: Capsid, E2/3, E1 and 6K. The nonstructural polyprotein is proteolytically processed into four nonstructural proteins: nsP1, nsP2, nsP3 and nsP4, which all interact to form the replication complex (reviewed in Ref. [57]).

The first togavirus replicon was established in 1989 for SINV by replacing the region coding for the structural polyprotein with the chloramphenicol transferase (*cat*) gene [58]. However, replicons of the SINV and CHIKV old world togaviruses, using wildtype viral sequences, caused cytopathic effect in host cells, and could not be propagated in cell culture for a prolonged period of time. This was found to be caused by nuclear translocation of nsP2, resulting in cytotoxicity [59]. In the case of CHIKV and Semliki Forest virus replicons, mutations such as P718G in the nuclear localisation sequence of nsP2 are essential to achieve a noncytopathic replicon phenotype [60–62]. Interestingly, replicons of wildtype sequence VEEV, a new-world togavirus, did not show a cytopathic effect [63]. As with DENV replicons, several reporter genes and/or antibiotic selection markers have been used to replace *cat*, such as fluorescent proteins or luciferase, either alone or in conjunction with an antibiotic resistance gene separated by the FMDV 2A autoprotease (Fig. 1b).

These replicons have been used to screen for drug candidates acting on the transcription and replication machinery of *Togaviridae* family members. Lead compounds identified in the past years include abamectin, ivermectin and berberine – a plant-derived isoquinoline alkaloid [64]. Abamectin and ivermectin are widely used antihelminthics but no follow-up studies were conducted on their antiviral effect against CHIKV. However, a Phase II/III trial is ongoing to assess safety and efficacy of ivermectin use in DENV infections [65]. In a follow-up study, berberine was shown to inhibit the MAP kinase pathway activated by CHIKV infection, showing that it was effective in alleviating symptoms of CHIKV infection in a mouse model [66]. However, no clinical studies followed. The WEEV replicon has been used in a high-throughput study using a library of 2206 extracts of marine organisms from diverse geographic regions. Thirty-seven primary hits were identified and, from these primary hits, an antimycin A derivative from the marine actinomycete *Streptomyces kaviengensis* was isolated as the most promising. Antimycin A is a prominent broad-spectrum antiviral that inhibits the cellular mitochondrial electron transport chain and *de novo* pyrimidine synthesis [67]. Besides drug discovery, togavirus replicons have been modified and successfully used as platforms for the expression of heterologous recombinant proteins and as vaccine platforms [68–71].

Respiratory syncytial virus (RSV) replicon and minigenome

RSV is a common cause of acute lower respiratory infections. In 2015 an estimated 33 million cases were reported, with ~60000 deaths of hospitalised children under 5 years of age [72]. Modern vaccine efforts are well under way by several major pharma companies after the disastrous failure of a first-generation formalin-inactivated whole virus vaccine in the 1960s [73]. To date, only ribavirin, a small-molecule broadband antiviral, and palivizumab, a monoclonal antibody targeting the viral fusion protein, have been approved for treatment and prevention of RSV [74]. RSV is a member of the *Paramyxoviridae* family. It is an enveloped virus with a nonsegmented, single-stranded, negative-sense RNA genome of ~15.2 knt. The genome codes for 11 proteins: small hydrophobic protein (SH), attachment protein (G), fusion protein (F), matrix protein (M), Nucleoprotein (N), phosphoprotein (P), Large protein (L) harbouring RdRp activity, transcriptional regulator M2.1, transcription/replication regulatory protein M2.2, and the two nonstructural proteins: NS1 and NS2.

RSV can be handled at Biosafety Level 2; however, the majority of virus progeny in cell culture are filamentous and extremely fragile [75]. Therefore, in the mid-1990s a minigenome system was developed based on plasmid-driven expression of the L, N, P and M2 proteins that form a ribonucleoprotein complex within transfected cells, together with an RNA molecule consisting of the leader and trailer sequences of the RSV genome and a region coding for *cat* (Fig. 1c) [76]. This system was mainly used to elucidate replication and transcription of the RSV genome, although some drug leads were found, including an inhibitor of co-transcriptional RNA guanylylation [77]. In 2011 a true RSV replicon was established by replacing the SH, G and F coding regions with the selectable marker blasticidin S deaminase (bla). The replicon was found to be stable and non-cytopathic in several cell lines. It could be packaged into VLPs by co-expression of structural proteins SH, G and F in *trans*, and transferred to different cell lines by infection with resulting *trans*-packaged VLPs (Fig. 1d) [78].

This replicon system was then used in high-throughput screening for specific anti-RSV drugs acting on the replication machinery of this virus. The small-molecule compound AZ-27 was found to inhibit transcription and replication initiation [79]. This finding was later confirmed using the minigenome system [80]. In 2014, Laganas *et al.* further screened the Astra Zeneca compound library in a high-throughput approach using the RSV replicon and identified three new lead compounds – nucleoside analogues and non-nucleoside inhibitors of the RSV RdRp [81]. In 2015 > 100 nucleoside analogue polymerase inhibitors were screened using the HeLa395-RV replicon cell line. Compound ALS-8176 was found to be a first-in-class small-molecule inhibitor of RSV replication, acting by chain termination during replication [82]. The compound was abandoned by Johnson & Johnson in March 2019 after Phase IIb trials [83].

Norovirus replicon

Norovirus was originally identified in 1968 from an outbreak of 'winter vomiting disease' in Norwalk, Ohio. The disease manifests in self-limiting fulminant vomiting, diarrhoea and low-grade fever lasting from 24 to 48 h [84]. It is estimated that, per year, norovirus prompts ~900000 hospital visits in the developed world and an estimated 200 000 deaths of children under 5 years of age in the developing world [85].

Despite very high virus titers in stools during infection and successful culture of murine noroviruses in RAW264.7 and primary murine cells, human noroviruses have eluded efficient propagation in cell culture so far. Human noroviruses have been found to produce low virus titers in B cells and 3D models of differentiating Caco-2 cells; however, these data are controversial [9,86,87]. To circumvent this lack of virus propagation in cell culture, norovirus replicons have been established.

Human noroviruses are non-enveloped, positive-sense, single-stranded RNA viruses belonging to the family of *Caliciviridae*. Their genome size varies between 7.2 and 7.5 knt and they contain three open reading frames. ORF1 codes for a polyprotein that is post-translationally processed by the NS6 protease into the six non-structural proteins NS6 (protease), NS7 (RdRp), NS5/VPg (capping of viral RNA), NS3 (RNA helicase) and NS1/2 and NS4, which have been implicated in the formation of the replication complex. ORF2 and ORF3 are translated from subgenomic RNAs into the major and minor capsid proteins VP1 and VP2, respectively (reviewed in Ref. [88]). The short 5′ and 3′ UTRs flanking the ORFs contain secondary structures that stretch into the coding regions and are essential for replication, transcription and pathogenesis [89,90].

In 2004, the plasmid-based infectious clone NV FL101 (based on the 1968 Norwalk strain) was established, which contained the cDNA of the full virus genome under the control of the T7 promoter [91]. Based on NV FL101, Chang *et al.* established a replicon by replacing the majority of the VP1 coding region with the neomycin phosphotransferase (*npt*) gene (Fig. 1e). Transfection of BHK cells expressing T7 polymerase with the replicon-coding plasmid resulted in cell lines that maintained the replicon under G-418 selection. The replicon could then be transferred to Huh-7 cells by RNA extraction and transfection with the resulting RNA, and maintained for 100 passages under G-418 selection [92].

The Groutas lab at Witchita State University rationally designs protease inhibitors of norovirus and other viruses, and tests compounds using replicon systems (reviewed in Ref. [93]). Four nucleoside analogues were tested on the norovirus replicon, with 2′-C-methylcytidine showing good antiviral activity [94]. Rupintrivir, a protease inhibitor of rhinovirus 3C protease, was shown to clear cells of the norovirus replicon [95]. However, owing to the fact that norovirus infections are short-lived and self-limiting, the interest of pharma companies to develop small-molecule inhibitors has been limited, and none of the mentioned candidates has been taken forward into clinical trials.

Ebola virus minigenome

Although not a true replicon, the Ebola virus minigenome has been an integral part of Ebola virus basic research and inhibitor discovery. The 2013–2016 EBOV outbreak in Sierra Leone, Liberia and Guinea, and the ongoing epidemic in the Democratic Republic of Congo, led to a sharp increase in funding and efforts to develop vaccines and therapeutics against Ebola and related filoviruses. EBOV causes viral haemorrhagic fever with mortality rates of up to 80%, and the virus has to be handled in BSL 4 conditions [96].

EBOV is a negative-sense, single-stranded RNA virus. The EBOV genome is ~19 knt in size and codes for eight major viral proteins: Nucleoprotein (NP), glycoprotein (GP) and soluble glycoprotein (sGP), VP35, VP40, VP24, VP30 and Large protein (L) [97]. The L protein, harbouring RdRp activity, together with NP, VP30 and VP35, constitutes the ribonucleoprotein complex, with the former three proteins being sufficient for replication, and VP30 essential for transcription [98]. The minigenome system consists of an RNA molecule carrying the native 5′ leader and 3′ trailer sequences of the genome, with an expression cassette inserted in the antisense direction coding for either *cat*, luciferase or green fluorescent protein (GFP) (Fig. 1f) [15,98]. Classically, cells are co-transfected with plasmids coding for L, VP30, VP35 and NP. These cells are then transfected with the *in vitro* transcribed minigenome RNA, leading to replication and transcription of the minigenome within transfected cells, and ultimately to expression of the reporter gene. Over the years improvements have been made to this system, such as cell lines stably expressing L, NP, VP30 and VP35 to minimise variability introduced during co-transfection of plasmids [99], a selectable marker that is co-expressed with the reporter for creation of a stable cell line continuously replicating the minigenome and T7-polymerase-driven expression of the minigenome within cells transfected with a plasmid coding for the minigenome and T7 polymerase [100].

These minigenome systems have been used on either small-scale or in high-throughput systems for identification of lead compounds such as angelicin derivates and benzoquinolones [101], MCCB4-8 [102], the two anticancer drugs 6-azauridine and 2′-deoxy-2′-fluorocytidine [103], and VER-155008, a heat-shock protein (hsp)70 inhibitor [104]. However, to date, none of these compounds has been taken into the clinical phase for use as antivirals.

Concluding remarks

The advent of reverse genetics and the establishment of stable replicon-harbouring cell lines and minigenomes have furthered our understanding of the molecular biology of viruses and facilitated the advancement of antiviral drug discovery in the absence of viable cell culture systems and for viruses that require high-containment facilities. Replicons have been and still are invaluable tools for drug discovery.

Conflicts of interest

No conflicts of interest are declared.

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