EXPERT REVIEWS

RNA-based drugs and vaccines

Expert Rev. Vaccines 14(2), 253–263 (2015)

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RNA-based approaches have provided novel alternatives for modern drug discovery. The application of RNA as therapeutic agents has, until recently, been hampered by issues related to poor delivery and stability, but chemical modifications and new delivery approaches have increased progress. Moreover, the discovery of the importance of RNA in gene regulation and gene silencing has revealed new drug targets, especially related to treatment of cancer and other diseases. Recent engineering of small molecules designed from RNA sequences to target miRNAs opens up new possibilities in drug development. Furthermore, RNA-based vaccines have been engineered applying RNA virus vectors and non-viral delivery for vaccine development.

KEYWORDS: gene silencing • miRNA • RNA interference • RNA-based drugs • RNA-based vaccines • viral RNA replicons

Traditional science-driven drug discovery has been mostly based on screening for small molecule drugs [1]. More recent technology development has allowed expansion into targeting biotherapeutics [2], and during the past few years, we have seen some signs of breakthrough in gene therapy [3]. Despite major development on all fronts of drug discovery and development, bringing novel drugs to the market has not been highly successful. Evidently, there remains a large proportion of the 'chemical space' still to be explored in existing chemical libraries [4]. However, the main reasons for the low success rate can be found in difficulties related to delivery, unwanted side effects and drug inefficiency. Obviously, the increase in safety requirements and demands of significant benefit over existing drugs has further limited the success of bringing new drugs to the market. Therefore, the introduction of novel drug targets might provide the means for the discovery of better medicines.

In this context, targeting RNA-based drugs is definitely an interesting approach. Particularly, RNA interference (RNAi) plays an important role as potential molecules for gene silencing, which could provide new means for not only drug target identification and validation, but also therapeutic interventions [5]. In principle, RNA-based gene silencing relates to cleavage of double-stranded RNA into 21–23 nucleotides siRNA by the cellular endonuclease Dicer followed by binding to and degradation of mRNA [6]. Furthermore, miRNA and short hairpin RNA (shRNA) can contribute to gene silencing [7], for instance, miRNA and chimeric shRNA/miRNA molecules can function at the post-transcriptional level [8]. Especially, the reversibility of gene silencing has made miRNA sequences attractive tools for drug development [9]. Moreover, significant effort has been committed to apply RNA for vaccine development. In this context, *in vitro* transcribed RNA, particularly based on viral replicon sequences have been used for immunization to provide protection against challenges with lethal doses of pathogens and cancer cells [10]. Additionally, mRNA has been employed for *ex vivo* modification of APCs, for instance, dendritic cells (DCs), which have proven to be safe and well tolerated and capable of inducing immune responses against tumor antigens [11]. Direct application of mRNA for APC *in situ* modification has been demonstrated feasible and efficient in preclinical studies. In this review, emphasis is put on the different aspects of applying RNA approaches for both RNA-based drugs and vaccines.

RNA-based target identification & validation

Related to drug discovery, RNAi can provide substantial assistance in target gene identification. For instance, a reliable quantitative reporter-based siRNA validation system has

 10.1586/14760584.2015.959932 ISSN 1476-0584

been established based on short synthetic DNA fragment containing an RNAi-targeted sequence of interest and two expression vectors for targeting reporter expression and triggering siRNA expression [12]. As only short synthetic DNA fragments are required, the system provides a powerful strategy for screening of RNAi-targeted sequences from mammalian genes as well as application of RNAi-based dsRNA reagents for reverse functional genomics and therapeutic use. Much attention needs to be invested in siRNA design as factors such as low G/C content and low internal stability favor silencing efficiency [13]. Application of RNAi for target validation also requires strong engagement of an RNAi library construction. For instance, enzymatic production of RNAi library, in which cDNA is converted by a sequence of enzymatic treatment into an RNAi library consisting of an array of different shRNA constructs has been applied [14]. When the RNAi library was subjected to high-throughput screening, it was possible to identify the shRNA constructs. It was also possible to construct an RNAi library from a cDNA library, which will enable future whole-genome phenotypic gene screening.

RNA delivery

As for any type of drug, delivery is one of the most essential questions also for RNA-based drugs. RNA molecules, in general, are known to be sensitive to degradation, which has to a large extent hampered direct administration. For this reason, chemical modifications have the potential of enhancing nuclease resistance, prevention of immune activation, lowering off-target effects and improvement of pharmacokinetic and pharmacodynamic properties [15]. For example, 2′-*O*-methyl-modified antisense oligonucleotide RNAs called antagomirs demonstrated improved resistance to degradation [16].

Another commonly used approach has been to employ nanoparticles for RNA delivery [17]. For instance, polymers provide protection for siRNAs from serum nuclease degradation and can aid in cell targeting [18]. Application of polyethylenimine polymers allows the formation of complexes with DNA and siRNA, which creates a proton sponge effect and results in accumulation in the liver, lung, spleen and kidney [18,19]. Polyethylenimine polymers can be modified by polyethylene glycol, which provides higher transfection efficiency, better solubility and stability as well as longer circulation time *in vivo* [20]. In a nanoplatform approach, tumor-targeted and pH-responsive nanoparticles were engineered by the combination of an artificial RNA receptor and Zn(II)-DPA hyaluronic acid for the delivery of siRNA, miRNA and oligonucleotides [21]. The nanoparticles were further coated with calcium phosphate and showed efficient delivery of siRNA and miRNA for gene silencing or miRNA replacement into different human cancer cells *in vitro* and into tumor-bearing mice after intravenous (iv.) administration. Another core-shell micelleplex delivery system based on block copolymer bearing polyethylene glycol, matrix metalloproteinase 2-degradable peptide PLG*LAG, cationic cell penetrating peptide polyarginine r_9 and poly(ε-caprolactone) has been used for siRNA delivery [22]. The micelles carrying siRNA demonstrated prolonged circulation time in blood, increased accumulation at tumor sites and cell penetration for siRNA uptake. Furthermore, the inhibition of breast tumor growth was increased.

Alternatively, liposomes have been designed for RNA delivery, which has provided additional protection against nucleases and facilitated penetration of cell membranes [23]. Also, fusion of siRNA to a short peptide of the rabies virus glycoprotein (GP) allowed delivery across the blood–brain barrier after intracranial injections [24]. Successful delivery of siRNA to neurons was obtained in mouse brain and resulted in inhibition of protein expression and protection against viral encephalitis [25]. Likewise, the liposome-siRNA rabies virus glycoprotein peptide complex was able to knock down the cellular prion protein expression and substantially decreased the protease-resistant isoform in neurons infected with transmissible spongiform encephalopathy [26]. Furthermore, complexes of a mixture of fusogenic and cationic lipids with nucleic acids named stable nucleic acid-lipid particles (SNALPs) have demonstrated efficient siRNA delivery [27]. Coating SNALPs with polyethylene glycol has provided a neutral or hydrophilic surface, stabilizes the particles and protects the cationic bilayer from rapid systemic clearance. SNALPs have been used iv. in mice carrying replicating HBV [27]. The outcome was improved efficacy, increased half-life and reduced HBV DNA concentration in the serum. SNALP-based delivery has also been used in clinical trials for the treatment of hypercholesterolemia [28]. Another application of SNALPs has been immunization with siRNAs targeting the Ebola virus [29]. Two of three vaccinated macaques showed protection against lethal challenges with Ebola virus.

Gold nanoparticles have shown excellent biocompatibility and modifiable surface chemistry, which has allowed their application in radiotherapy, photothermal cancer therapy and drug delivery and siRNA silencing [30,31]. In this context, cationic lipid-coated gold nanoparticles have been employed in HBV treatment with siRNA targeting the open reading frame of the HBV X protein, which has been evaluated in HepG cells [32]. It resulted in a significant decrease in HBV surface antigen and efficient inhibition of HBV replication.

An attractive alternative to nanoparticle-based delivery of RNA is application of viral vectors. Especially, lentivirus vectors have demonstrated high efficiency of shRNA delivery, and it has also been established in bone marrow cells and blood [33]. For instance, lentivirus vectors have been used for delivery of shRNA engineered to silence the Cu/Zn superoxide dismutase (SDS1) mutant $SOD1^{G93A}$ in mice [34]. Intraspinal administration of lentiviral SOD1-shRNA provided long-term and significant decrease in mutant SOD1 protein and delay in the onset and progression of amyotrophic lateral sclerosis. Similarly, lentivirus vectors were applied for the expression of prion protein-specific shRNAs for efficient knock down of the cellular prion protein and prolonged survival of scrapie-infected mice [35]. However, there is a limitation to clinical applications as the silencing of the cellular prion protein is irreversible. Another application of

lentivirus-based shRNA delivery has been the knock down of chemokine receptor CCR5 in a humanized bone marrow-liver-thymus model [36]. As individuals with deletions in the *CCR5* gene are resistant to HIV infections, this might provide a possibility for the development of an anti-HIV therapy. In this context, the lentivirus-based bone marrow/liver/thymus-HIV (rHIV7-shl-TAR-CCR5RZ) has entered a Phase Ib clinical trial for an anti-HIV drug [37].

An interesting variation on the theme of viral vectors is based on bacteriophage delivery systems. For instance, *Bacillus subtilis* phage phi29 has the capacity of efficient packaging siRNA [38]. Engineering of 70 nm phi29-based nanoparticles with transferrin protein incorporated allowed systemic delivery of siRNA with enhanced accumulation in tumor cells [39]. Furthermore, the integration of a lymphocyte function-associated antigen-1 integrin in nanoparticles provided selective uptake of siRNA by T cells and macrophages, the principal target cells for HIV. Delivery of anti-CCR5 siRNA silenced leukocyte-specific genes and prevented HIV infection in bone marrow/liver/thymus mice [40].

In the field of RNA vaccines, both non-viral and viral delivery has been applied. In this context, RNA has been delivered in naked form, encapsulated in liposomes or as virus-like particles [11,41,42]. For instance, ultrasound-based delivery and small amphiphilic compounds inducing nucleic acid uptake have been engineered [43]. In several studies, electroporation [44] and gene gun [45] technologies have been applied to facilitate RNA delivery. Related to viral delivery, self-amplifying RNA replicons harbored by single-strand RNA viruses of both positive and negative polarity have been used. Typically, replicons of West Nile virus [46] and Kunjin virus [47] belonging to the flavivirus family have been applied. In this context, Kunjin virus replicons expressing GM-CSF demonstrated cure in <50% of mice with established CT26 colon carcinoma and B16-OVA melanoma after intratumoral injections [48]. CT26 tumor regression correlated with the induction of anticancer CD8 T cells. Furthermore, treatment of subcutaneous CT26 tumors showed regression in CT26 lung metastasis. Additionally, Kunjin virus replicons have been applied for vaccine development against simian immunodeficiency virus [49].

Similarly, positive-strand RNA alphaviruses such as Semliki Forest virus (SFV) [50], Sindbis virus (SIN) [51] and Venezuelan equine encephalitis virus (VEE) [52] have been frequently used as delivery vehicles. Furthermore, naked replicon RNA has been administered intramuscularly (im.) [53] as well as encapsulated in liposomes [54]. There are a number of applications on cancer therapy (Table 1) and vaccine development (Table 2) applying alphavirus vectors.

Table 1. Examples of RNA-based drugs.

Disease	Target	Delivery	Phase	Ref.
AMD/DME	VEGF	Direct ocular injection of siRNA	III	[64]
Solid tumors	PLK1	Lipid NPs	I	[65]
Ebola	Ebola virus	Lipid NPs	I	[66]
Eye neuropathy	Caspase 2	Intravitreal siRNA administration	I	[67]
HBV	HBV replication	SNALPs	Preclinical	[27]
	HBV X	siRNA-gold NPs	*In vitro*	[32]
Hypercholesterolemia	ApoB	Lipid NP-siRNA	I	[28]
Kidney carcinoma	IL-12	Encapsulated SFV	I	[61]
Melanoma	IL-12	Encapsulated SFV	I	[58]

AMD: Age-related macular disease; Apo: Apolipoprotein; DME: Diabetic macular edema; NPs: Nanoparticles; SFV: Semliki Forest virus; SNALPs: Stable nucleic acid-lipid particles.

RNA-based drugs

There are currently a number of RNA-based drugs under development in both the preclinical phase and in clinical trials (Table 1). A large number of RNA-based drugs relate to RNAi in some composition. For instance, the growth arrest specific 5 (GAS5) gene encodes long non-coding RNAs and its expression is downregulated in breast cancer [55]. When breast cancer cell lines were transfected with siRNA to GAS5 or with plasmid DNA coding for GAS5 long non-coding RNAs, the apoptosis of triple-negative and estrogen receptor-positive cells was promoted and the extent of cell death was directly proportional to cellular GAS5 levels.

Several attempts have been made to use viral vectors for cancer therapy. Flavivirus vectors based on Kunjin virus have provided excellent means of delivery of DNA, RNA and virus-like particles [56] and studies on tumor regression have been carried out in rodent models [48]. Similarly, alphavirus replicons have been subjected to a number of applications to tackle tumor regression in various cancer models [57]. These studies include intratumoral injections with recombinant particles [58] and im. particle and RNA administration [53]. Generally, significant tumor regression has been obtained even for rapidly growing K-BALB and CT26 tumors [59]. Oncolytic SFV vectors have been applied in attempts to improve delivery and therapeutic efficacy [60]. A single injection (intratumoral, intraperitoneal and iv.) of 10^6 virus particles resulted in significant tumor regression in severe combined immunodeficiency mice implanted with human melanoma xenografts. Another approach has been to engineer liposome-encapsulated SFV particles for tumor targeted systemic delivery [61]. Intraperitoneal administration in severe combined immunodeficiency mice of encapsulated SFV particles carrying the *LacZ* gene resulted in enhanced β-galactosidase expression in implanted LNCaP tumors [62]. Furthermore, encapsulated SFV particles expressing the p40 and p35 subunits of IL-12 were subjected to a Phase I

Table 2. Examples of RNA-based vaccines.

Disease	Target	Delivery	Response	Ref.
Viral				
Ebola	GP	Kunjin VLPs	Ebola protection	[49]
	GP	VEE VLPs	Ebola protection	[86]
	NP	VEE VLPs	Ebola protection	[87]
SIV	Gag-pol	Kunjin VLPs	SIV protection	[47]
Influenza	NP	SFV VLPs	Immune response	[80]
	HA	VEE VLPs	Influenza protection	[81]
HCV	nsPs	SFV VLPs	$CD8^+$ T-cell response	[91]
HIV	env	SFV VLPs	Humoral response	[83]
	gp41	SFV VLPs	CTL response	[84]
	MA/CA	VEE VLPs	CTL response	[85]
SARS-CoV	SARS-CoV GP	VEE VLPs	SARS-CoV protection	[88]
RSV	siRNA	VEE-LNPs	RSV protection	[91]
RVFV	Gn	VEE VLPs	RVFV protection	[89]
Vaccinia	A33R, B5R	VEE VLPs	Vaccinia protection	[90]
Non-viral				
Plasmodium falciparum	Pf332 antigen	SFV VLPs	Immunological memory	[92]
Malaria	CS	SIN VLPs	Malaria protection	[96]
Prion	PRNP	SFV VLPs	Monoclonal antibodies	[97]
Cancer				
Brain cancer	Endostatin	SFV VLPs	Tumor inhibition	[108]
	gp100, IL-18	SIN DNA	Tumor protection	[109]
Breast cancer	neu	SIN DNA	Tumor protection	[101]
	neu	VEE VLPs + DCs	Tumor regression	[102]
	VEGFR-2	SFV VLPs	Tumor inhibition	[103]
Cancer	LacZ	SFV RNA	Tumor protection	[53]
Cervical cancer	HPV E7	VEE VLPs	Tumor protection	[100]
Colon carcinoma	VEGFR-2	SFV VLPs	Tumor inhibition	[103]
Melanoma	TRP-2	VEE VLPs	Therapeutic effect	[98]
Prostate cancer	PSMA	VEE VLPs	Tumor response	[105]
	STEAP	VEE DNA	Antitumor response	[106]
	PSCA	VEE VLPs	Tumor protection	[107]
Tumor antigen	P815	SFV VLPs	Tumor protection	[99]

Env: Envelope; GP: Glycoprotein; HA: Hemagglutinin; NP: Nucleoprotein; nsPs: Non-structural proteins; PMSA: Prostate-specific membrane antigen; PSCA: Prostate stem cell antigen; RSV: Respiratory syncytial virus; RVFV: Rift Valley fever virus; SARS-CoV: Serial acute respiratory syndrome corona virus; SFV: Semliki Forest virus; SIN: Sindbis virus; STEAP: Six-transmembrane epithelial antigen of the prostate; TRP: Tyrosine-related protein; VEE: Venezuelan equine encephalitis; VEGFR: VEGF receptor; VLPs: Virus-like particles.

trial in kidney carcinoma and melanoma patients [61]. The iv. administration was well tolerated and generated a transient 5- to 10-fold increase in IL-12 plasma levels.

Related to RNAi-based drugs, a number of clinical trials are in progress (Table 1). For instance, siRNAs directed toward VEGF provided positive results in VEGF inhibition in human cell lines and animal models for age-related macular degeneration [63]. These results encouraged to conduct a clinical trial by direct ocular injection of siRNAs in age-related macular degeneration patients [64]. Moreover, lipid nanoparticles (LNPs) have been subjected to RNAi delivery to target solid tumors by silencing polo-like kinase I in clinical trials [65]. Similarly, LNPs have been applied to target Ebola virus [66]. In another approach, synthetic siRNA was subjected to intravitreal administration in a Phase I trial related to eye neuropathy [67].

Another aspect of applying RNAs in medicine has been the use of miRNAs as biomarkers. In this context, miRNAs have been linked to a large number of diseases for which they may serve as biomarkers [68]. For instance, miRNA binding site polymorphism in the 3′ end untranslated regions of KRAS rs6174370, SET8 rs16917496 and MDM4 rs4245739 are highly promising cancer biomarkers [69]. Similarly, miRNAs have been identified as potential diagnostic biomarkers in renal carcinoma [70] and multiple myeloma [71]. The application of miRNAs as biomarkers has also been suggested for other disease indications such as chronic obstructive pulmonary disease, as specific expression of seven miRNAs was observed in chronic obstructive pulmonary disease patients [72]. Moreover, miR-196a, miR-30a-5P and miR-490 have been associated with disease activity among patients with membranous nephropathy and diabetic nephropathy and may serve as biomarkers [73]. Likewise, miR-223 and miR-143 provide important systemic biomarkers for psoriasis activity [74]. Interestingly, a panel of five miRNAs has demonstrated the potential as biomarkers for diagnosis and assessment of male infertility [75]. In addition to direct applications of miRNAs as biomarkers,

they can also be used for target and drug efficacy assessments, with a special emphasis on prediction of clinical responses in patients, promoting personalized medicines [76]. Particularly, in oncology, this approach has allowed prescreening for disease-specific therapy without subjecting patients to drugs showing no clinical benefit [77].

Recently, an interesting application for RNA-based drugs has been the development of the design of lead small molecules for RNA based uniquely on sequence information [78]. The approach termed INFO-RNA has been applied to all human miRNA hairpin precursors, which identified bioactive small molecules that inhibit biogenesis by binding nuclease-processing sites with a hit rate of 44%. The most avid interaction was discovered between a benzimidazole compound and precursor miRNA-96. Selective inhibition of miRNA-96 biogenesis upregulated the protein target FOXO1 and induced apoptosis in cancer cells. Furthermore, when the FOXO1 mRNA expression was silenced by siRNA, apoptosis was inhibited.

RNA-based vaccines

Viral targets

The majority of RNA vaccines are based on viral vectors, both negative and positive strand viruses such as flaviviruses [49] and alphaviruses [57], respectively (Table 2). As described, for RNA delivery, RNA replicons can be administered as RNA molecules [53] and recombinant particles [50]. In a number of cases, the targets have been viral envelope proteins to provide protection against challenges with lethal doses of pathogenic viruses. Furthermore, cancer vaccines have been developed by immunization with various types of cancer antigens (Table 2).

The flavivirus Kunjin replicons have been subjected to SIVmac239gag vaccine development in mice [49]. Four vaccines encoding the wild-type gag-pol, an RNA-optimized gag, a codon-optimized gag and a modified gag-pol were evaluated in the study. The outcome was significant differences in induction and effector memory and central memory responses, protection and insert stability. Clearly, the SIV gag-pol vaccine provided the best results. In another study, virus-like particles containing the Kunjin virus replicon and the Ebola virus wild-type GP, a membrane anchor-truncated GP (GP/Ctr) and a mutated GP (D637L) with enhanced shedding capacity were evaluated for Ebola virus protection in guinea pigs [47]. The wild-type GP and D637L mutant GP induced dose-dependent protection against challenges with lethal doses of Ebola virus and a complete clearance of virus was observed in surviving animals.

A number of studies have been conducted on applying alphavirus vectors for vaccine development as recently reviewed [79]. For example, SFV particles expressing influenza virus nucleoprotein generated strong immune responses in mice [80]. Moreover, VEE particles expressing influenza virus hemagglutinin demonstrated protection against challenges with H5N1 virus in chicken [81]. Likewise, when VEE particles expressing the cluster swine influenza virus hemagglutinin H3N2 gene was administered to swine, it resulted in protection against influenza virus challenges [82]. Furthermore, HIV vaccines have been developed using SFV particles expressing the HIV envelope [83] and gp41 [84] and VEE particles carrying HIV MA/CA [85] showing humoral and cytotoxic T-lymphocyte responses in mice. Additionally, protection against challenges with lethal doses of Ebola virus in mice and guinea pigs was observed after administration of VEE particles expressing Ebola nucleoprotein [86] and GP [87], respectively. Likewise, VEE particles expressing the severe acute respiratory syndrome coronavirus GP provided protection against challenges with lethal doses of severe acute respiratory syndrome coronavirus in vaccinated mice [88]. In another approach, alphavirus particles with the Rift Valley fever virus GP Gn was fused to the C3d complement protein elicited neutralizing antibodies and provided protection against Rift Valley fever virus challenges in mice [89]. VEE particles have also been subjected to expression of the *A33R*, *B5R*, *A27L* and *L1R* genes for the development of smallpox vaccines [90]. Protective immunity was obtained in vaccinated mice. Vaccination of macaques generated strong antibody responses, which neutralized and inhibited the spread of vaccinia virus. Furthermore, HCV has been targeted for vaccine development applying alphavirus vectors. In this context, all or part of the HCV non-structural proteins were expressed from SFV vectors resulting in a strong long-lasting NS3-specific $CD8^+$ T-cell response [91]. Immunization of mice demonstrated significant delay of HCV-expressing EL4 tumor growth.

An interesting approach for vaccine development has been the use of VEE replicons for the delivery of siRNA using LNPs [92]. The LNP encapsulated RNA demonstrated a substantially increased immunogenicity in vaccinated mice in comparison to unformulated replicon RNA. The immunization elicited broad and potent immune responses and provided protection against respiratory syncytial virus challenges in mice. The potency was comparable to delivery by viral particles, however, excluding the safety risks related to the use viral vectors.

Non-viral targets

In addition to viral targets a number of non-viral infectious targets have been subjected to vaccine development (Table 2). SFV vectors expressing the *Plasmodium falciparum* Pf332 antigen generated immunological memory in vaccinated mice [93]. Furthermore, immunizations with SIN and SFV particles expressing the *Bacillus anthracis* protective antigen [94] and *Brucella abortus* translation initiation factor 3 [95], respectively, provided protection against challenges with the corresponding pathogen. Moreover, subcutaneous administration of SIN particles expressing the class I major histocompatibility complex-restricted-9-mer epitope of the malaria *Plasmodium yoelii circumsporozoite* protein induced large epitope-specific $CD8^+$ T-cell responses and provided a high degree of protection against malaria [96]. In another immunization study, SFV particles expressing the prion protein PRNP elicited monoclonal antibody production available for basic research and diagnostics [97].

Table 3. Examples of alphavirus and flavivirus RNA-based vaccines.

Virus	Gene	Delivery	Immunization	Response	Ref.
CHIK	IRES	CHIK infection	Vero, insect	Mosquito resistance	[119]
	nsP3, E1 siRNA	CHIK infection	Vero cells	Reduced CHIK titer	[120]
	miRNAs	CHIK infection	Mouse	Reduced CHIK replication	[121]
	TSI-GSD-218	CHIK infection	Phase II	Neutralizing antibodies	[123]
	Glycoprotein	CHIK infection	Macaques	Neutralizing antibodies	[127]
	C, E1VLPs	CHIK infection	Primates	CHIK protection	[128]
Dengue	env gp DIII	VLPs	Mouse	Neutralizing antibodies	[115]
EEE	EEE/WEE	EEE infection	Mouse	EEE protection	[118]
WNV	env gp DIII	VLPs	Mouse	Neutralizing antibodies	[115]
VEE	RdRp miRNA	VEE infection	BHK cells	Inhibition of VEE replication	[122]

BHK: Baby hamster kidney; CHIK: Chikungunya virus; EEE: Eastern equine encephalitis virus; IRES: Internal ribosomal entry site; RdRp: RNA-dependent RNA polymerase; VEE: Venezuelan equine encephalitis virus; VLPs: Virus-like particles; WEE: Western equine encephalitis virus; WNV: West Nile virus.

Tumor targets

In addition to viral targets, vaccine development has focused on protection against cancer. Naked SFV RNA replicons expressing the *LacZ* gene were im. administered in mice [53]. Interestingly, a single injection of 1 μg of SFV-LacZ RNA was able to provide complete protection against challenges with tumor cells, and even in animals with existing tumors, the survival was extended by 10–20 days. Alphavirus vectors have also been applied for the expression of the tyrosine-related protein 2, which showed inhibition of the growth of B16 transplantable melanoma and potent therapeutic effect on melanoma in vaccinated mice [98]. Likewise, immunization with alphavirus particles expressing the *P1A* gene [99] and the HPV *E7* gene [100] from SFV and VEE vectors, respectively, provided tumor protection in mice. Furthermore, successful tumor vaccination has been established after administration of SIN vectors carrying the neu gene in a murine breast tumor model [101]. An interesting approach has been to combine DC-based immunotherapy with the administration of VEE particles expressing the neu gene [102]. This approach induced both cellular and humoral immunity against mice bearing human breast tumors and also demonstrated a significant inhibition of tumor growth. In another study, immunization with SFV particles expressing the VEGF receptor 2 showed substantial inhibition of both tumor growth and pulmonary metastatic spread in mice with pre-existing tumors [103]. Similarly, immunization with SFV-VEGF receptor 2 resulted in inhibition of CT26 colon carcinoma and metastatic 4T1 mammary carcinoma growth.

When VEE particles carrying the HPV *E7* gene were administered subcutaneously 2 weeks prior to cancer cell inoculation, tumor formation was prevented in mice [100]. The immunization also provided long-term protection, which lasted for 3 months post-vaccination. However, the therapeutic efficacy was only 67%, which could be significantly enhanced by co-expression of HPV *E6* and *E7* [104]. In a prostate tumor model, strong cellular and humoral immunity was observed after subcutaneous administration of VEE particles expressing the human prostate-specific membrane antigen (PSMA) [105]. Additionally, the prostate tissue-specific six transmembrane epithelial antigen of the prostate was expressed from VEE vectors, which induced a specific immune response and significantly prolonged overall survival of mice bearing TRAMPC-2 tumors [106]. Antitumor protection was achieved in 90% of vaccinated TRAMP mice when prophylactically immunized with a prostate stem cell antigen DNA plasmid followed by VEE-prostate stem cell antigen particle administration [107]. Finally, brain tumors have been targeted in several studies. For instance, a significant reduction of intratumoral vascularization was observed after intratumoral delivery of SFV particles expressing endostation [108]. Additionally, SIN vectors expressing the human melanoma-associated antigen gp100 and IL-18 induced specific antitumor cytotoxic T-lymphocyte responses and provided tumor protection [109]. Immunization prevented B16-hgp100 tumor formation and showed significant survival prolongation in mice with established B16-hgp100 tumors.

Related to RNA-based cancer immunotherapy, it has been demonstrated that the combination of non-packaged tumor antigen mRNA with protamine-packaged mRNA provides potent immune responses [110]. In particular, for the Toll-like receptor 7, a two-component vaccine containing free and protamine-complexed mRNA induced adaptive immune response of antigen-specific $CD4^+$ T-helper cells and cytotoxic $CD8^+$ cells [111]. Furthermore, intranodal immunization with antigen-encoding mRNA showed efficient mRNA uptake by DCs, modulation of DCs and superior therapeutic immune responses [112]. Also, the delivery of tumor-associated antigen mRNA with TriMix, a mixture of mRNA encoding CD40 ligand, constitutive active Toll-like receptor 4 and CD70, resulted in the *in situ* modification and maturation of DCs [113]. Recently, the TRiMix formula has been evaluated for the treatment of melanoma [114].

Vaccines against flaviviruses and alphaviruses

As members of both the flavivirus and alphavirus family have been classified as pathogens, they have been subjected to vaccine development (Table 3). In this context, the CD16 ectodomain of the CD16-RIgE chimera was replaced by the envelope GP domain III of dengue virus or West Nile virus Kunjin and were expressed on the surface of human and insect cells [115]. Displayed on VLPs domain III were capable of inducing specific neutralizing antibodies against dengue virus and West Nile virus in mice. Although the response was modest, this approach could be developed as a vaccine against different flaviviruses.

Alphaviruses have also been subjected to vaccine development. For example, when BALB/c mice were vaccinated with an attenuated VEE strain protection was obtained against airborne virus [116]. In another study, a live attenuated V3526 VEE vaccine demonstrated improved protection against VEE challenges [117]. Likewise, vaccination of C57BL/6 mice with a chimeric Eastern equine encephalitis and Western equine encephalitis virus showed complete protection against lethal challenges with a virulent Eastern equine encephalitis strain [118]. The association of Chikungunya (CHIK) alphavirus to recent epidemics has accelerated vaccine development programs. For instance, mosquito transmission of CHIK virus was inhibited by making the replication dependent on internal ribosome entry sites [119]. In the context of RNAi, siRNAs were engineered against two conserved regions in the *nsP3* and *E1* genes of CHIK, which showed a significant reduction of CHIK virus titers in Vero cells after 24 h (99%) and 48 h (65%) [120]. Furthermore, miRNA-specific sequences targeting replicon particle production were introduced into alphavirus helper RNA [121]. Cellular miRNAs downregulated helper RNA replication, but addition of miRNA-specific inhibitors allowed efficient CHIK particle production. Cellular miRNA further prevented the replicon RNA replication in mice after inoculation of replicon RNA carrying engineered miRNA sequences. This approach demonstrated the feasibility of applying miRNAs for therapeutic interventions. Additionally, VEE RNA-dependent RNA polymerase was targeted by artificial miRNAs for inhibition of VEE replication [122]. Three out of five miRNAs showed significant inhibition of VEE replication in BHK cells.

Clinical trials for alphavirus-based vaccines

A limited number of clinical trials have been conducted for alphavirus-based vaccines. In this context, a Phase II, randomized, double-blind, placebo-controlled safety and immunogenicity study was conducted for a live CHIK vaccine [123]. A single subcutaneous injection of vaccine was administered to 59 volunteers while 14 individuals received placebo. The only adverse event was transient arthralgia in five CHIK vaccinated individuals. CHIK neutralizing antibodies were elicited in 57 (98%) of the volunteers by day 38 and 85% remained seropositive 1 year after the vaccination. In contrast, none of the placebo recipients was seropositive.

A two-component vaccine expressing CMV gB or pp65/E1 fusion protein was tested in a Phase I trial [124]. The vaccine was administered im. or subcutaneously in CMV seronegative volunteers. Neutralizing antibodies and multifunctional T cells were induced against CMV antigens. The vaccination caused only mild-to-moderate local reactions and no clinical changes of importance. In another clinical trial, VEE particles expressing the carcinoembryonic antigen were subjected to repeated administration to patients with metastatic cancer [125]. The induction of carcinoembryonic antigen-specific antibodies contributed to antibody-dependent cellular toxicity against tumor cells from human colorectal cancer metastases. Encouragingly, patients with carcinoembryonic antigen-specific antibodies showed an extended overall survival. VEE replicons carrying the PSMA have also been subjected to a Phase I dose escalation trial in patients with metastatic cancers [126]. Two doses of 0.9×10^7 and 3.6×10^7 IU were used at weeks 1, 4, 7, 10 and 18. No PSMA-specific cellular response was observed at the lower dose, although a weak PSMA-specific signal was detected by ELISA. Disappointingly, no PSMA-specific response could be demonstrated for the higher dose, which suggested that the dosing was suboptimal. However, despite the absence of robust immune responses and clinical benefit, no toxicities were associated with the vaccination.

Expert commentary

The urgent need of finding new drug targets and mechanisms of drug action has accelerated the interest in RNA-based drugs. An essential part of the development has relied on the discovery of the importance of RNAi in gene silencing and gene regulation. A number of applications of siRNA and shRNA have showed great promise in specifically targeting genes related to disease. The reversibility of the process is a further attraction. Needless to say, the discovery of the importance of miRNAs in gene regulation and their association with cancer and other diseases have made them important targets for drug development. The approach of designing lead small molecules for RNA by targeting human miRNA hairpin precursors is also of great interest.

In relation to RNA-based vaccines, the possibilities to use flavivirus and alphavirus RNA replicons have a great potential. It has been demonstrated that naked RNA replicons, RNA replicons associated with LNPs as well as recombinant virus-like particles can elicit strong immune responses in immunized animals. Furthermore, protection against challenges with lethal doses of infectious agents and cancer cells has been established in rodents and primates. It is also encouraging to see that several clinical trials are in progress for vaccine development.

Five-year view

Futuristic predictions, especially in the area of science are extremely difficult. For instance, it was not until the beginning of the millennium when miRNAs were recognized as a distinctive class of regulators. Today, miRNAs represent the most promising targets for RNA-based drugs. In 5 years, we might see novel applications and new targets for both drug and vaccine development. However, for the time being the two most

important issues to address remain delivery and efficacy. Related to delivery, the stability of RNA and targeting to the appropriate cell/tissue of choice are the most important issues. Chemical modifications have significantly enhanced the stability of RNA molecules. Furthermore, nanoparticles and their modifications including pegylation has improved the circulation of systemically delivered vehicles. The field of viral vectors has seen tremendous development during the past decade, especially related to targeting, which has improved both efficacy and safety. Furthermore, the number of RNA-based drugs and vaccines subjected to clinical trials is a promising sign. Taking the above comments into account, RNA-based drugs and vaccines may play an important role as the medicines of the future.

Financial & competing interests disclosure

The author has no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Key issues

- Proof of concept both *in vitro* and *in vivo* of siRNA, short hairpin RNA and miRNA with potential therapeutic efficacy.
- Development of nanoparticle and lipid-based technologies for optimized targeted delivery of RNA molecules.
- Design of lead small molecules targeting RNA, specifically miRNA hairpin precursors.
- Application of naked RNA replicons, RNA replicon–lipid nanoparticles complexes and recombinant viral-like particles based on flaviruses and alphaviruses for immunization studies in animal models.
- Immunization with flavivirus and alphavirus RNA replicons provide protection against challenges with lethal doses of viruses and cancer cells in rodents and primates.
- Clinical trials for RNA-based drugs and vaccines in progress.

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