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Review

Opportunities and challenges for microRNA-targeting therapeutics for epilepsy

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Epilepsy is a common and serious neurological disorder characterised by recurrent spontaneous seizures. Frontline pharmacotherapy includes small-molecule antiseizure drugs that typically target ion channels and neurotransmitter systems, but these fail in 30% of patients and do not prevent either the development or progression of epilepsy. An emerging therapeutic target is microRNA (miRNA), small noncoding RNAs that negatively regulate sets of proteins. Their multitargeting action offers unique advantages for certain forms of epilepsy with complex underlying pathophysiology, such as temporal lobe epilepsy (TLE). miRNA can be inhibited by designed antisense oligonucleotides (ASOs; e.g., antimiRs). Here, we outline the prospects for miRNA-based therapies. We review design considerations for nucleic acid-based approaches and the challenges and next steps in developing therapeutic miRNA-targeting molecules for epilepsy.

Highlights

MicroRNAs are small noncoding RNAs that suppress the translation of mRNAs and have emerged as therapeutic targets in epilepsy and other diseases.

Inhibition of certain miRNAs with antisense oligonucleotides (ASOs) is therapeutic in preclinical temporal lobe epilepsy (TLE) models.

Chemical modifications can be made to improve the therapeutic properties of ASOs.

The blood–brain barrier is an obstacle in delivering ASOs as therapies in neurological disease, and ASOs currently have to be administered by direct central injection.

miRNA-targeting ASOs are in clinical trials for other diseases, raising the prospect that they could also be safely applied in epilepsy.

Current versus future drugs for epilepsy: the need for change

Epilepsy (see Glossary) is a common, often life-long brain disease characterised by recurrent, spontaneous seizures that are the result of hypersynchronous discharges of neurons [1]. There are more than 20 different small-molecule drugs in clinical use that reduce or prevent seizures in people with epilepsy. This is a remarkable achievement, certainly relative to the lack of treatments for many neurological disorders, and is owed to a solid mechanistic understanding of how seizures arise through imbalances between excitation and inhibition and the availability of good disease models that identify molecules with antiexcitability properties [2]. However, antiseizure drugs likely do not substantially alter the underlying pathophysiology of epilepsy, and one-third of patients are refractory to current treatments. Addressing this treatment gap is a major priority [1,3], and, increasingly, researchers are looking at substantially different targets.

Acquired forms of epilepsy, such as TLE, have a limited genetic basis and probably arise from an earlier brain insult that leads to select cell loss, gliosis, neuroinflammation, and vascular and microscopic as well as macroscopic reorganisation of brain networks [4,5]. Because of this, single targets, such as ion channels, may be unsuitable or insufficient to overcome drug resistance, achieve disease modification, or prevent epileptogenesis [6] (Box 1). Novel approaches that can modify multiple targets may be necessary. Additionally, suitable target(s) may not reside on the outside of neurons but instead be intracellular. Both challenges could be solved by using RNA-based medicines such as **ASOs**, which offer virtually unlimited potential to target any gene, and small noncoding RNAs called **microRNAs** (miRNAs), which are 'multi-pathway' regulatory molecules (for a recent review on the general topic of RNA medicines, the reader is referred elsewhere [7]).

The first miRNA medicine reached human trials in 2013 [8], and several others are in clinical testing. The versatility of ASO-based medicines was elegantly demonstrated by the recent design and clinical trial, in a single year, of an ASO (Milasen) for an individual with a rare genetically defined neurodegenerative disease [9]. More recently, ASOs have been used to treat a rare genetic epilepsy

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Box 1. Definition of disease modification and prevention of epileptogenesis

Current antiseizure drugs are symptomatic agents and do not modify the underlying pathophysiology. Disease-modifying drugs are ideally sought, which change the disease. Disease modification is defined as curing epilepsy, or at least modifying its severity or responsiveness to antiseizure drugs, by addressing the underlying disease pathophysiology. Prevention of epileptogenesis or antiepileptogenic drugs refers to agents that can prevent the development of epilepsy following a precipitating insult. Such drugs could be given to individuals at risk of epilepsy, for example, after traumatic brain injury, stroke, or infection. However, since only a percentage of such patients would normally develop epilepsy, biomarkers of epileptogenesis are needed so that only the at-risk patients are treated.

[10], indicating another potential therapeutic avenue (see Concluding remarks and future perspectives). Ambitious programmes, such as LifeTime to map the molecular basis of various common diseases at the single-cell level [11], will undoubtedly generate additional RNA targets for medicine that may synergise with advances in chemical modifications and delivery carriers to increase the scope for deployment in the future. In this review, we highlight the key advances, and also outstanding challenges, in the use of miRNA-targeting therapies in brain diseases with a specific focus on epilepsy.

miRNA biogenesis and mechanism of action

Mature miRNAs are generated via a multistep process. miRNAs are initially transcribed as relatively large (can be more than 1 kb) hairpin structures known as pri-miRNA. This undergoes cleavage in the nucleus by the enzyme Drosha to produce a 60–70-nucleotide (nt) stem loop pre-miRNA [12,13], which is subsequently transported from the nucleus to the cytoplasm [14,15]. The enzyme Dicer recognises pre-miRNA and cleaves the stem loop [12], leaving an imperfect ~21–23-nt miRNA duplex with a ~2-nt 3′ overhang at each end [16]. The less thermodynamically stable end of the pre-miRNA duplex is then uploaded to a binding pocket within an Argonaute (Ago) protein [17] to form the RNA-induced silencing complex (RISC) [18]. The miRNA-loaded RISC then traffics along mRNAs searching for complementary binding sites and, upon finding mRNA targets containing a ~7–8-nt seed match [typically within the 3′ untranslated region (UTR)], triggers either target degradation or translational repression [19] (Figure 1). miRNAs, therefore, play a key role in regulating the cellular transcriptomic landscape. Individual miRNAs can have dozens of targets, so they are seen as network molecules that limit cellular protein 'noise' within pathways [20]. (Readers are referred elsewhere for more detailed reviews of miRNA biogenesis, targeting, and regulatory functions [16,21–25].) Because of this short seed requirement, individual miRNAs have dozens and potentially hundreds of potential targets. An individual miRNA can act upon several different pathways or a single mRNA target may contain sites for multiple miRNAs, which can increase the strength of negative regulation. Various mechanisms restrict the potential target pool, including cell-type-specific expression of miRNAs and mRNA and RNA compartmentalisation. Notably, several neuron-enriched miRNAs are concentrated at synapses, which brings them into close contact with mRNAs involved in shaping dendritic spines and synaptic plasticity [26,27]. Crosslinking techniques have enabled researchers to identify which miRNAs are bound to which mRNA targets [28] to better understand how they regulate key functions in the brain [29]. This approach, and functional studies manipulating miRNAs, have demonstrated key roles for miRNAs, including in gliosis [30], neuroinflammation [31], ion channel function [32–34], synaptic connectivity [35,36], myelination [37], neuronal differentiation [38], and neuronal morphology [39].

'Drugging' miRNAs

There is accumulating evidence that the miRNA landscape is dysregulated in epilepsy [29,40–43] and other brain diseases [44–48], raising prospects for treating disease by over- or underexpressing key miRNAs. Key experimental tools for miRNA targeting are summarised in Figure 1.

Glossary

AntimiR: an ASO designed to specifically target an miRNA.
Antisense oligonucleotide (ASO): a synthetic single strand of DNA or RNA designed with complementarity to target RNA sequences. ASOs can also be chemically modified to improve their performance.
Epilepsy: a serious, disabling, and life-shortening brain disease characterized by recurring, unprovoked seizures. Epilepsy can be caused by many factors, including genetic mutation, infection, or brain injury. Epilepsy is often accompanied by comorbidities, including memory deficits, sleep disorders, and poor mental health.
MicroRNA (miRNA): naturally occurring 21–23-nt noncoding RNA molecules that suppress the translation of target mRNAs through sequence-specific binding.

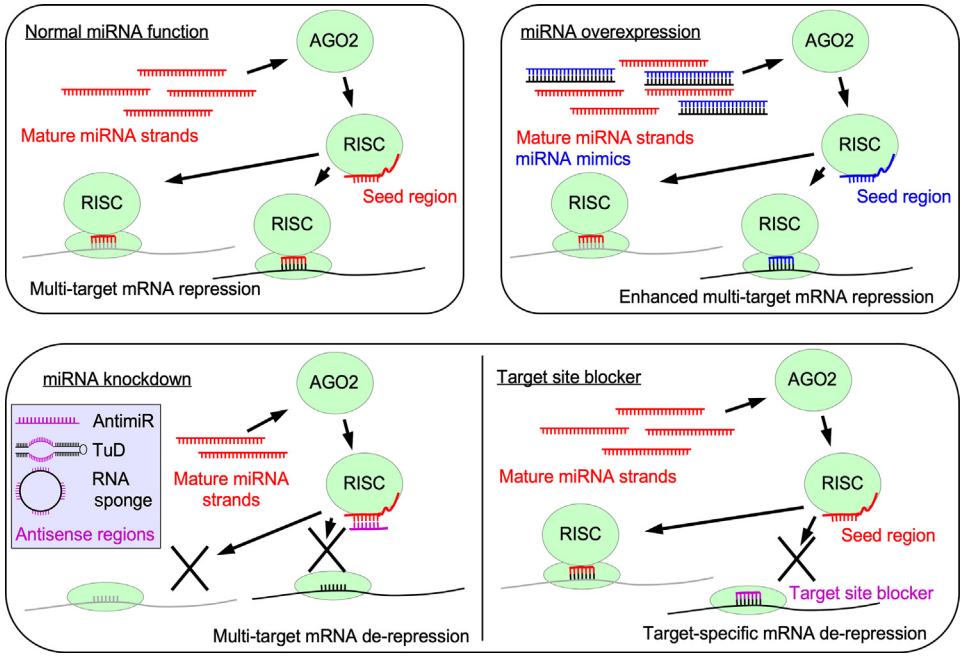

Figure 1. Key experimental tools for manipulating miRNA. Normal miRNA function is mediated when mature miRNA strands are uploaded to the RNA-induced silencing complex (RISC) in conjunction with Argonaute (AGO) proteins. The seed region of the miRNA guides the RISC to target mRNA with sufficient complementarity, mediating translational repression (upper left panel). Synthetic double-stranded miRNA mimics can be used to increase the available pool of a specific miRNA, therefore enhancing repression of all of its mRNA targets (upper right panel). miRNA function can be blocked by antisense molecules, such as antimiRs, tough decoy (TuD) molecules, and RNA sponges, which bind to miRNAs and prevent them from repressing translation of all of their mRNA targets (left, lower panel). Specific miRNA:mRNA interactions can be blocked using target site blockers, which derepress the translation of one target mRNA whilst leaving the miRNA functional and able to continue to repress its other mRNA targets.

Experimental overexpression of miRNAs has typically been achieved using double-stranded synthetic miRNA 'mimic' molecules. However, these small molecules can saturate the miRNA pathway [49], can cause aberrant expression of the miRNA in cells where it is not usually present [50], and are associated with toxicity and mortality when delivered to the brain *in vivo*. As such, this approach has received less attention in the development of miRNA-based therapeutics. A potential solution may be the use of gene therapy vectors to deliver miRNAs in a more controlled and spatially restricted manner to specifically targeted cell types in the brain (as in [51] and see Delivery of miRNA-targeting therapeutics).

The leading approach to reduce miRNA expression is using ASO **antimiRs** (see Table 1 for a summary of preclinical antimiR therapies in epilepsy to date). Oligonucleotide therapies offer lasting, but not permanent, gene targeting, require infrequent dosing due to their target binding and slow elimination, and are increasingly reaching the clinic [7,52]. In its most basic form, an antimiR is simply a synthetic ASO with perfect complementarity to a region (8-mer to 23-mer) of its miRNA target [53]. This basic antimiR unit can be stabilised by the addition of a terminal hairpin at either side of the targeting sequence [54]. RNA sponges are larger constructs containing multiple miRNA binding sites [55]. Tough decoy (TuD) molecules contain two miRNA binding sites, each containing a central bulge, which creates a targeting molecule that no longer has perfect complementarity and therefore favours translational repression over target degradation [56]. A similar modification can be made to RNA sponge constructs [57]. Other strategies for inhibition of endogenous miRNA

Table 1. Overview of antimiR treatment parameters used in studies showing a therapeutic effect on *in vivo epilepsy models*[a,b]

MicroRNA targeted	Species	Linkage structure	Ribose modification	Other specifications	Dose	Delivery method	Timing of treatment and seizure model	Primary therapeutic outcome	Refs
10a, 21a, 142a	Mouse	PO/PS mix	LNA		0.5 nmol in 2 μL	icv	Pretreatment: IAKA, PILO, PPS Post-treatment: IAKA	65% reduction in spontaneous recurrent seizure frequency	[42]
134	Mouse	PS	LNA	3′-chol tag	0.12 nmol in 1 μL (icv) / 0.24 nmol in 5 μL (intranasal)	icv/intranasal	Pre- and post-treatment: IAKA	87% reduction in spontaneous recurrent seizure frequency	[88]
134	Rat	PS	LNA		0.36 nmol in 6 μL	icv	Pretreatment: PPS	91% reduction in spontaneous recurrent seizure frequency	[99]
134	Mouse	PS	LNA		0.12 nmol in 2 μL	icv	Pretreatment: PTZ	69% in acute seizure power	[99]
134	Rat (*ex vivo* slice model)	PS	LNA		0.12 nmol in 1 μL	icv	Pretreatment: High K^+	57% increase in time to acute seizure onset	[99,100]
134	Mouse	PS	LNA	3′-chol tag	0.12 nmol in 2 μL	icv	Pretreatment: PILO	58% reduction in acute seizure power	[98]
134	Rat	PO/PS mix	2′-MOE		0.12 nmol in 2 μL	icv	Pretreatment: ICVKA	44% reduction in spontaneous recurrent seizure frequency	[103]
135	Mouse	PS	LNA	3′-chol tag	1 nmol in 2 μL	icv	Post-treatment: IAKA	~60% reduction in spontaneous recurrent seizure frequency	[101]
199a	Rat	PS	Not specified	3′-chol tag	1 nmol in 5 μL	icv	Pretreatment: PILO	~50% reduction in acute seizure amplitude	[109]
203	Mouse	Not specified	2′-OMe		5 nmol in 24 μL	intranasal	Post-treatment: PILO	72% reduction in spontaneous recurrent seizure frequency	[87]
324	Mouse	PO/PS mix	LNA		0.5 nmol in 2 μL	icv	Post-treatment: PILO	Average 50% reduction in spontaneous recurrent seizure frequency	[34]

[a]Studies that showed a therapeutic effect but did not specify antimiR details were not included. Studies where antimiRs were given during or immediately after status epilepticus are considered pretreatment.
[b]Abbreviations: chol, cholesterol; High K^+, 9 mM potassium (slice model); IAKA, intra-amygdala kainic acid; icv, intracerebroventricular; ICVKA, intracerebroventricular kainic acid; LNA, locked nucleic acids; MOE, *O*-methoxyethyl; OMe, *O*-methyl; PILO, pilocarpine; PO, phosphodiester; PPS, perforant pathway stimulation; PS, phosphorothioate; PTZ, pentylenetetrazole.

activity include miRNA-directed short hairpin RNAs (shRNAs) [58] and target site blockers [59], short sequences that compete with the miRNA for binding to one of its target mRNAs, offering more precise targeting of a single miRNA–mRNA interaction. Small molecules, such as antimiRs, can be directly injected into the brain, whereas larger antisense constructs, such as TuD molecules and RNA sponges, require viral vectors for effective cellular delivery [60].

AntimiR chemistry: modifications to enhance stability and potency

ASOs have unconventional pharmacokinetic (PK) and pharmacodynamic (PD) properties that have important implications in relation to their efficacy and dosing as drugs [61]. These properties, and therefore the therapeutic potential of antimiR molecules, can be greatly impacted by chemical modifications to their molecular structure. DNA and RNA nucleotides are naturally linked by phosphodiester (PO) bonds. However, ASOs composed of only PO bonds with unmodified sugar structures are easily degraded by endo- and exonucleases, severely limiting their efficacy. Indeed, exposed ASOs can be degraded by a 3′ to 5′ exonuclease in serum in less than 30 minutes, and degradation occurs even faster in the presence of intracellular exo- and endonucleases [62]. This is not therapeutically viable; therefore, chemical modifications are made to ASOs to confer greater stability. Modifications are typically made to the PO bond of the ASO or to the 2′-carbon of the sugar ring [63].

PO linkage modifications

A key modification that provides protection from nucleases is the phosphorothioate (PS) backbone, created by replacing one of the non-bridging oxygen atoms in the PO backbone of the DNA/RNA molecule with a sulfur atom (Figure 2). This modification gives ASOs greater stability, reportedly allowing for a 9-hour half-life in human serum and a 19-hour half-life in rat cerebral spinal fluid (CSF) [64], and improves cellular uptake [63]. The PS modification also creates an

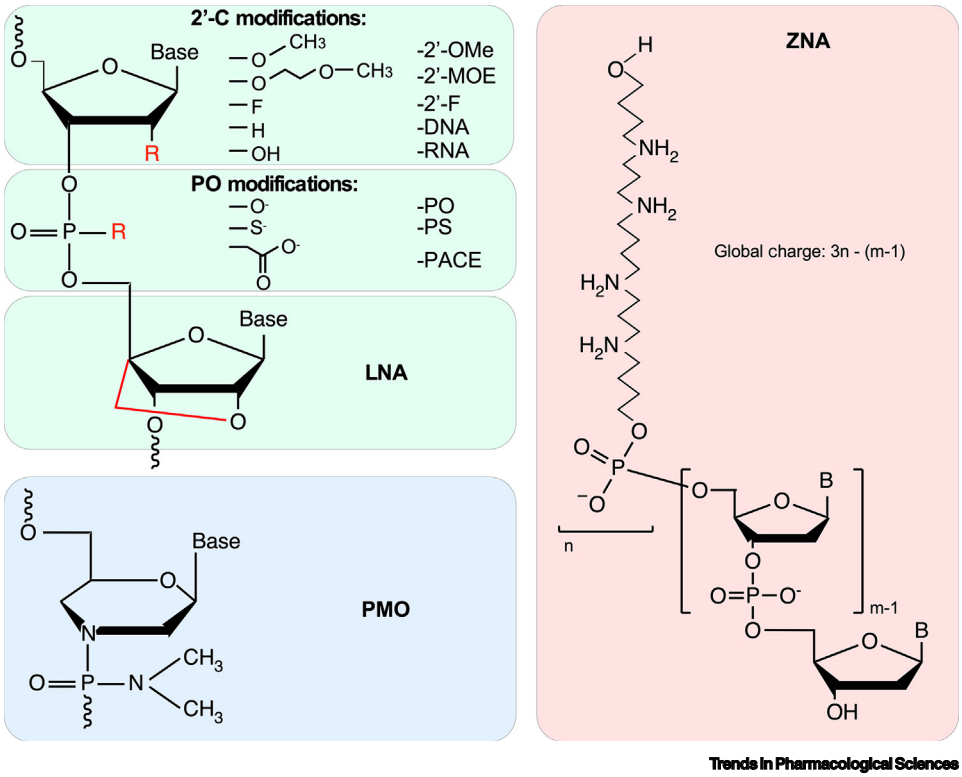

Figure 2. Chemical modifications can be made to enhance antisense oligonucleotide (ASO) properties. A number of chemical modifications can change and enhance ASO properties. In a standard DNA/RNA-based ASO (green boxes), the functional group attached to the 2′-carbon can be substituted to create 2′-*O-methyl* (2′-OMe), 2′-*O-methoxyethyl* (2′-MOE), or 2′-deoxy-2′-fluoro-nucleosides (2′-F) modifications. The phosphodiester (PO) linkage between ribose units can also be altered from PO to phosphorothioate (PS) or phosphonoacetate (PACE). Finally, a bridge between the 2′-oxygen and 4′-carbon of a ribose unit creates a locked nucleic acids (LNA). Additional ASO modifications can include the inclusion of phosphorodiamidate Morpholino oligomer (PMO) rings (blue box) and the addition of an oligocationic tail to create a zip nucleic acid (ZNA) (red box).

association with carrier proteins in the blood, thus ensuring the ASO remains in the bloodstream for longer, improving absorption and half-life [65]. Another benefit of the PS modification is that it can activate ribonuclease H (RNase H), which is required for degradation of target mRNA [66]. PS backbone modifications are, however, associated with potential toxicity due to off-target protein binding and also lead to a slight reduction in binding affinity to target RNA [63]. A similar ASO backbone modification is phosphonoacetate (PACE) in which the non-bridging oxygen of the PO link is instead replaced by an acetate group, creating ASOs that are reported to be completely resistant to nucleases [63]. Phosphorodiamidate Morpholino oligomers (PMOs) are created by replacing the PO linkage groups with phosphorodiamidate and feature a six-membered morpholine ring in place of the ribose moiety. PMOs are more resistant to nucleases and also show slight improvements in binding affinity, but show poor uptake in mammalian cells [63]. Although chemical modifications to the backbone of oligonucleotides greatly improve their therapeutic properties, multiple chiral centres arise as a result, leading to mixtures of many stereoisomers following standard synthesis methods. Only a handful of these stereoisomers are effective in their interactions with RNA or DNA. Efforts to reduce this problem and thus increase potency include synthesis methods that yield stereopure oligonucleotides [67].

Ribose modifications

The stability of ASOs can also be enhanced by modifying the functional group at the 2′-carbon of the ribose ring (summarised in Figure 2). 2′-*O*-methyl (2′-OMe) modifications use a methylated hydroxyl group at the 2′-C position. They are nontoxic and slightly increase both nuclease resistance and RNA-binding stability [68,69], but remain vulnerable to exonucleases in serum [63]. 2′-*O*-methoxyethyl (2′-MOE) modifications contain a methoxyethyl modification at the 2′ position, again slightly increasing nuclease degradation resistance [68,69] and the hybridization affinity of the ASO to target RNA [68,70,71]. The 2′-O modifications also reduce sequence-independent toxicity caused by the PS backbone [72]. Oligonucleotides with full 2′ modifications have an inability to activate RNase H [73], which impairs degradation of target mRNA. 2′-Deoxy-2′-fluoro-nucleosides (2′-F) have a fluorine at the 2′ position in the ribose. This mediates slightly greater improvements in binding affinity than 2′-OMe and 2′-MOE modifications, but, with unmodified PO backbone structures, is very susceptible to exonucleases [63].

Locked nucleic acids (LNAs) are a ribose sugar moiety modification in which the 2′-O is connected to the 4′-carbon via a methylene bridge. LNAs mediate superior improvements in binding stability when compared with the other ribose modifications [63,74,75] and also provide high resistance to nucleases [76]. LNAs do not induce RNase H-mediated downregulation [77]. The disadvantage of LNAs in comparison to other 2′ modifications is that, when systemically administered, these oligonucleotides have higher toxicity [78]; however, it is unclear if this is the case in the central nervous system (CNS). Also, target specificity could be hindered as a result of the high affinity of the LNAs [79]. LNA-based antimiRs are the most commonly used ASO to target miRNAs in models of epilepsy (see Table 1 for summary). A further possible ASO modification is the addition of oligospermine nucleobases to form an oligonucleotide–oligospermine conjugate, termed zip nucleic acids (ZNAs) [80]. ZNAs lack a negative charge, mediating greater binding affinities. The global charge of a ZNA molecule can be modulated by selecting the number of cationic units (Figure 2).

Delivery of miRNA-targeting therapeutics

ASOs do not readily cross the blood–brain barrier (BBB) [61], with <1% of intravenously injected oligonucleotides in rats reaching the brain [81]. Thus, miRNA-targeting therapeutics must generally be given via a route that circumvents the BBB or use modifications that facilitate uptake via a systemic route, such as encapsulation and attachment of certain brain-penetrating

molecules. One mechanism that may allow oligonucleotides to cross the BBB is receptor-mediated endocytosis following conjugation to a transport vector. For example, it has been shown that a radioactively labelled ASO conjugated to a monoclonal antibody against the transferrin receptor was able to reach the brain through the endogenous transferrin transport pathway in a preclinical model. This resulted in a ~tenfold increase in brain uptake of the ASO, owing to an enhanced ability to permeate the BBB and brain cell membranes [82]. Another solution is the cell-penetrating peptide (CPP)-based delivery mechanism. These cationic peptide chains may be up to 30 amino acids in length and may enhance cellular ASO uptake by endocytosis. It has been shown that when tagged with arginine-rich CPPs, systemically delivered ASOs can reach the brain in mice [83]. However, concerns remain about possible toxicity of CPP ASOs, which may cause mild tubular degeneration in the kidneys [84], lethargy, and weight loss [85]. Alternatively, ASOs can be packaged within a poly(lactic-co-glycolic acid) (PLGA)-based nanoparticle formulation, which enhances cellular uptake [86]. Of note, few of these approaches have been specifically developed for miRNA-targeting ASOs.

Currently, only direct delivery techniques that bypass the BBB are in use for ASO delivery to the brain. This includes injecting the small molecule ASO into the CSF, either by the intracerebroventricular (ICV) or intrathecal route (IT) into the spinal cord. These direct delivery methods result in rapid and high ASO concentrations in the brain and spinal cord, minimizing toxicity to other organs and allowing for the use of lower doses. However, they are also invasive and inconvenient for patients, of which a portion may be young children. Tolerance of such invasive approaches must be balanced against the benefit the treatment brings and against other options, such as surgical resection of seizure foci. Another drawback of direct small-molecule injection is lack of cell-type specificity, as they are taken up by all cells. For example, increasing the neuron-specific miR-124 using a small-molecule approach resulted in aberrant gliosis [50]. A possible solution is the use of viral vectors (e.g., [51]), where specific promoters can be used to restrict ASO expression to targeted cell types, likely reducing adverse effects as certain miRNA dysregulations are restricted to specific cell types. Viral vectors can also be used to deliver larger antisense molecules such as TuD molecules and RNA sponges [60] (Figure 1), which are too large for direct delivery approaches. A less invasive solution may be intranasal delivery. Molecules can take advantage of the olfactory and trigeminal nerve pathway and the rostral migratory stream to enter the CNS. Intranasal administration of antimiRs is less invasive than direct central injection and has been reported to alter seizures in animal studies [87,88]. However, it remains to be seen how effective intranasal delivery routes will be for the administration of an miRNA-based treatment to a seizure focus in epilepsy. Finally, recent work from our lab has shown that antimiRs can cross the BBB when it is more permeable following seizures, which can permit timed systemic delivery of ASOs for therapeutic CNS delivery [89].

Pharmacological properties of ASO-based therapies

Distribution and uptake of ASOs is increasingly well understood following systemic administration (the reader is referred elsewhere for a detailed review [90]). Briefly, ASOs are absorbed quickly following subcutaneous administration, with peak plasma concentrations within 3–4 hours that then decline rapidly due to a distribution phase, which sees absorbed molecules transferred into tissues in a few hours. Following this, a much slower terminal elimination phase occurs in which an ASO may have a half-life of up to several weeks. Protein binding in the plasma assists tissue bioavailability, as it limits glomerular filtration of antimiRs and urinary excretion. Backbone modifications such as PS further influence plasma protein binding and plasma stability, maximising bioavailability.

We know less about the PK of ASOs, such as antimiRs, after direct delivery to the CNS. Recent ASO imaging studies show the progressive uptake after IT injection over time, including accumulation in

neurons [91]. The highest ASO concentration is achieved in tissues adjacent to the CSF [92], and ASO concentration was found to be lowest in regions most distal to CSF sources along a gradient [93]. The CNS regions that typically experience the highest ASO concentration in large primate brains are the hippocampus, spinal cord, cerebellum, cortex, and areas of the caudate proximal to the ventricle, while ASO concentrations are lower in deeper areas of the brain [92,93].

Peak ASO concentrations in CSF occur within 30–60 minutes of dosing and then quickly decline over the next 24–48 hours [94]. This is due to rapid distribution of drug to the CNS tissues, and also a portion of the drug is moved to the systemic circulation during CSF turnover. Similar to administration of ASOs to plasma, CSF concentrations show multiphasic kinetics with a long terminal half-life at low concentrations that show an equilibrium between postdistribution CSF concentration and ASO concentration in CNS tissues [93]. There appears to be an uptake of ASOs broadly in multiple cell types in the CNS. One week after administration of either 1, 3, or 7 mg of PS/2′-MOE-modified ASO to adult cynomolgus monkeys by IT injection, the highest concentration was observed to be in large and small cell bodies in the grey matter, consistent with a pattern of neuronal and glial cell uptake [93]. It had been demonstrated that negligible nuclease activity occurred in the CSF, and also that the CSF was an immune-privileged zone, allowing ASOs to be highly stable. These characteristics could explain the long half-lives of ASOs in the CNS. Notably, the ASO nusinersen, used to treat spinal muscular atrophy, was still detectable in motor neurons 1 year after a week-long ICV infusion. The tissue half-life of ICV-infused 2′-MOE-modified ASOs was 71–126 days in the brain and 145–191 days in the spinal cord [93]. The relatively slow rate of ASO degradation and removal from the CNS makes it possible to use decreased doses for IT administration in CNS disorders, reducing the possibility of adverse events occurring in patients. The extended CNS half-life also allows for an infrequent dose schedule, providing an advantage from the perspective of safety, tolerability, and manufacturing.

Clinical trials of miRNA-targeting therapeutics

Miravirsen was the first miRNA-targeting ASO tested in a clinical trial [8]. There are now a small number of other clinical trials underway that are testing miRNA therapeutics, including for CNS indications [7]. While there have been no clinical trials of an miRNA-based ASO for epilepsy, the era of miRNA therapies for neurological disorders has begun.

SOD1-related familial amyotrophic lateral sclerosis (ALS)

A recent exploratory study in ALS used a miRNA-based approach, delivered via an IT infusion of adeno-associated virus (AAV-miR-SOD1), in an attempt to suppress overexpression of *SOD1* arising from gene mutations in two patients [95]. In patient 1, SOD1 levels in spinal cord tissue were lower than corresponding levels in untreated patients (comparable data were not obtained for patient 2). SOD1 levels in CSF were transiently slightly lower in patient 1, with no change observed in patient 2. Patient 1 developed meningoradiculitis, likely as an immune response to the AAV capsids; however, patient 2 was pretreated with immunosuppressive drugs and did not experience the same complication. Whilst AAV-miR-SOD1 did not alter the clinical course of ALS in the two patients, this study did indicate broad safety of the approach. Therefore, IT miRNA administration can potentially be a beneficial treatment for SOD1-related familial ALS.

Other miRNA-targeting ASOs in clinical trials

Miravirsen, a 15-nt antimiR targeting miR-122 for the treatment of hepatitis C, was the first antimiR trialled in humans [96]. More recently, several clinical trials of antimiRs have been undertaken for non-CNS diseases (detailed in [7]). A clinical trial is currently active on an antimiR targeting miR-21 for Alport syndrome, a kidney disease, and for cutaneous T cell lymphoma, in which miR-155 is the target. Limited data are available on the results of these trials, while the

clinical trial of AZD-4076, which targets miR-103/107, has been halted. Results from these trials should generate a broader understanding of the therapeutic potential and any potential safety issues of antimiRs in humans.

Preclinical targeting of miRNAs with antimiRs in epilepsy

There have been more than 300 studies on miRNA and epilepsy, and over 100 different miRNAs have found to be altered in experimental models and human samples (EpimiRBase [97]). Of these, there are 10–20 miRNAs that appear consistently dysregulated and for which there is functional evidence for effects on seizures (see Table 1 for summary). We have the most preclinical evidence from studies targeting miR-134-5p (miR-134). It was first shown by our laboratory in 2012 [88] that ICV injection of an LNA antimiR targeting miR-134 (Ant-134) powerfully reduced seizures in two mouse models of epilepsy in which the excitotoxin kainic acid was used to trigger seizures. When given after an epilepsy-inducing insult, Ant-134 mediated seizure reductions that lasted at least 2 months following a single direct injection to the brain [88,89]. Antiseizure effects of targeting this miRNA have since been replicated in a mouse model that used the cholinergic mimetic pilocarpine [98] and subsequently in other models across both mice and rats [99]. The therapeutic mechanism of action of Ant-134 is not known but may involve the derepression of *LimK1* [88,89], a regulator of dendritic spines and known target of miR-134 [27]. No adverse effects of Ant-134 on brain function have been noted to date [100].

More than a dozen other miRNAs have been targeted in at least one *in vivo* model (see [26] for detailed review). Recently, antimiR knockdown of miRNA-135a was shown to reduce seizures in the mouse intra-amygdala kainic acid model [101]. Inhibition of miR-324-5p rescued seizure frequency in the mouse systemic kainic acid [32] and pilocarpine [34] models, an effect mediated at least in part by blocking miRNA targeting of Kv4.2, which carries the A-type potassium current. It cannot be ruled out that other targets of miR-324-5p play roles in this effect. Agonism of miR-137, through direct injection of a mimic construct into the hippocampus, prolonged seizure onset in both pilocarpine and pentylenetetrazol-induced seizures in mice, possibly by increasing presynaptic release of inhibitory neurotransmitters [102]. Recently, our laboratory led a systems approach based on the sequencing of Ago2-bound, functionally engaged miRNAs that revealed three additional miRNAs with effects on seizures, miR-10a-5p, miR-21a-5p, and miR-142a-5p. AntimiRs against these miRNAs appear to suppress seizures in TLE models via a convergent effect on the TGFβ signalling pathway [42]. Therapeutic studies in epilepsy have typically used DNA/LNA mixmer ASOs with mixed PO and PS [42] or full PS linkages [34,88,98,99,101], although other studies have used 2′-MOE [103] or 2′-OMe [87] modifications (Table 1), and direct comparisons of backbone modification performance on the same miRNA have not been undertaken. Additionally, some studies have used ASOs with a 3′-cholesterol tag to enhance cellular uptake [88,98,99], although it has also been reported that this modification can reduce ASO solubility [34].

Concluding remarks and future perspectives

The present review summarises the key arguments for why new targets are needed for drug-resistant epilepsy and the potential suitability of miRNAs. AntimiR targeting of miRNAs has potent and lasting antiseizure, and even disease-modifying, effects in rodent models of drug-resistant epilepsy. Treating patients with antimiRs will require, however, invasive procedures or advances in formulation as well as other safety-related risks that arise from the difficulty in predicting off-target effects of miRNA inhibition.

With several antimiRs having passed proof-of-concept stage and some tested in multiple models, what are the next steps (see also ‘Outstanding questions’)? A priority is to obtain evidence of efficacy in a human model. While the mature sequences of miRNAs, such as miR-134, are fully

Outstanding questions

Which of the various miRNAs is the best target for TLE?

What is the safety profile of antimiRs and how can this be improved?

Do antimiRs reduce epileptic activity or hyperexcitability in human models?

Can miRNA-based therapies be used for genetic epilepsies?

conserved between rodents and humans, the 3′ UTRs of mRNAs to which they bind display variation between species and within organs, including the brain [104]. If key targets of an miRNA are not the same in humans, this could render a rodent-developed miRNA therapy ineffective. Suitable test models could include human-derived neurons (e.g., induced pluripotent stem cell-derived neurons) or resected human brain tissue [105]. Large-animal testing is another potential next step. Notably, drug-resistant epilepsy occurs in various breeds of dogs, and a trial of an ASO in such a model would be a major translational opportunity [106]. We know relatively little about the efficacy of antimiRs in the immature brain. As the transcriptional landscape changes during brain development [107], so too can an miRNA's target pool and functional role. Interference in miRNA function could produce different effects with age. For example, as neural stem cells divide and differentiate, there are changes to miRNA expression that are critical for producing the correct gene expression that drives the changes in neurons during maturation and migration. There may also be changes in the cell types that express miRNAs during development. This will clearly have implications for safety. Introduction of an miRNA manipulation would need to be performed with full knowledge of potential interactions. Our laboratory has made recent progress in this regard, observing that while the antiseizure effects of Ant-134 are retained in juvenile mice, these animals were less tolerant of higher doses [108]. Beyond this, clinical development will likely require partnering with biotechnology or pharmaceutical companies. Unfortunately, many pharma companies dropped their epilepsy research programmes, and most of the miRNA therapy companies are not targeting CNS diseases [7]. Successful development of an ASO may encourage more companies to explore targets such as miRNA. Extensive work remains to determine (i) the safety profile of antimiR design and backbone chemistry in the brain, (ii) whether factors such as age and sex affect efficacy and (iii) optimisation of delivery route and duration of action. Finally, would an miRNA-targeting approach also work for other forms of epilepsy, including genetic epilepsy? It should be possible to upregulate any protein in a patient haploinsufficient due to a mutation by targeting the miRNA(s) sequestering the remaining functional transcript. Indeed, there are ASOs under development for certain rare epilepsies [10]. This approach could be flexible enough to be used for any genetic epilepsy. The miRNAs known to bind a specific transcript could be identified, and then individual or combination antimiRs could be delivered to derepress the remaining functional copy, thus compensating for haploinsufficiency. In summary, miRNAs represent a broad class of molecules that can be targeted using ASOs and could potentially become treatments of the future for epilepsy and possibly other complex and chronic brain diseases.

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Declaration of interests

D.C.H. declares patent US 9,803,200 B2 'Inhibition of microRNA-134 for the treatment of seizure-related disorders and neurologic injuries'. The other authors have no interests to declare.

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