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Drug Discovery Today: Therapeutic Strategies
Vol. 3, No. 4 2006

Nervous system disorders

RNA interference as a therapeutic strategy for treating CNS disorders

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RNA interference (RNAi) controls gene silencing in most living organisms. The potential clinical applications of RNAi represent a strategy with unsurpassed selectivity, with the ability to target multiple disease-related genes, independent of their perceived drugability. The design of highly selective and efficacious small interfering (siRNAs) and short hairpin RNAs (shRNAs) has become routine, owing to significant progress in modeling and chemistry. RNAi significantly downregulates gene expression and function both *in vitro* and *in vivo*, including in the brain. This essay highlights recent findings and how the pharmaceutical industry intends to apply RNAi for the treatment neuropsychiatric and other diseases.

Introduction

RNAi (RNA interference) is a cellular surveillance mechanism that not only represses viral infections, transposable elements and repetitive genes (e.g. transgenes) but also regulates gene expression as well as normal cell development. We will refer to RNAi, although post-transcriptional gene silencing (PTGS) is another very close way of describing it. It becomes almost embarrassing to explain what RNAi is: over the past 4 years, RNAi has been the subject of numerous reviews, meetings and even more publications. RNAi was the 'scientific breakthrough of the year' in Science Magazine in 2002; the Fortune magazine labeled RNAi 'Biotech's Billion Dollar Breakthrough' in 2003. In 2004, a whole issue of Nature Magazine was dedicated to RNAi, and the RNAi concept can even be found in the New York Times list of bestsellers. Last, but not least, RNAi was granted the 2006 Nobel Prize for Medicine or Physiology, only 8 years after the first publication. This is reminiscent of the hype experienced when the oligonucleotide antisense technology led to numerous publications, reviews, meetings and the creation of Biotechs, all of which were 'leading in their field'. Similar to what one can read today on the websites of several companies specialized in RNAi. Nevertheless, there are numerous attempts to validate targets preclinically using RNAi, and first attempts to treat disorders that have been resistant to the small molecular entity approach are close to be or are already in the clinic. This essay will primarily deal with RNAi in the context of CNS disorders, although it is clear that the route to success will be a long one in most cases.

The chemistry of RNAi

Effective gene silencing by RNA interference depends on the nature and/or structure of the siRNA or shRNA and the mRNA sequence of the target gene [1]. siRNA design has become very efficient, although the methods used can vary widely [2–8]. Cell penetration and stability remain the key factors for which again various approaches can be taken, but we have seen very good penetration and stability using both naked siRNA as well as modified ones both *in vitro* and *in vivo* [9,10]. Investigating experimental genome-wide siRNA libraries and high throughput siRNA screens have greatly increased our

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1740-6773/$ DOI: 10.1016/j.ddstr.2006.10.008

ability to design and validate potent siRNAs, especially in combination with the use of predictive algorithms guided by artificial neural networks [11–14]. Currently, it is common to generate siRNAs for a single target, which produce close to 100% downregulation in mRNA at least *in vitro* with a 70–80% success rate. *In vivo*, the levels of downregulation can vary markedly depending on RNAi, target, tissue, dose, duration and route of administration, but so far, pronounced and long lasting knock down is rather the exception than the rule.

Side effects of RNA interference

As stated in the introduction (see Box 1), an early concern about RNAi relates to the nonspecific 'off-target' gene activation and induction of the innate immune system, that is the interferon response, although, this seems to be of little relevance when applying siRNAs, for example 19–21 nt long. Another issue relates to the endogenous small regulatory RNAs, known as microRNAs (miRNAs) of which a multitude may exist and may be involved in regulating embryonic development, cell fate determination and other unknown functions [15]. Inhibition of some miRNAs, for example miR15, miR16 and miR17, has been linked to cancer. More generally, the use of high doses of siRNA/shRNA may saturate the RISC complex and lead to profoundly toxic or even lethal effects as shown recently [16] with shRNA directed against hepatitis B virus; however in the same model, siRNAs had no toxic effects.

Box 1. The basics of RNA interference

RNAi is evolutionarily-conserved in plants, planaria, Hydras, *Trypanosomes*, *Drosophila*, and mammals [33–36]. A post-transcriptional gene-silencing (PTGS) event is triggered by cytoplasmic long double-stranded (ds)RNAs, which are cleaved by the RNase III enzyme, Dicer, to generate short interfering (si)RNAs. siRNAs are ~21–25 nucleotides (nt)-long dsRNAs containing 5′ phosphorylated ends and 3′ 2-nt overhangs on each strand. siRNAs get incorporated into a multiprotein RNA-inducing silencing complex (RISC) that unwinds the siRNA duplex. The siRNA strand retained in RISC then guides the entire complex to a target mRNA. Depending on the complimentarity of the target mRNA sequence with that of the siRNA guiding strand, RISC initiates either an endonucleolytic cleavage or a translational arrest of the target mRNA. Furthermore, RISC may facilitate the formation of heterochromatin such that RNAi induces gene silencing even at the transcriptional level. RNAi regulates the endogenous gene expression via similar mechanisms, however, using a single-stranded RNA with imperfectly self-complementary sequences that fold back to form a hairpin RNA. Hairpin RNAs provide a source of dsRNAs that are cleaved by Dicer to generate single-stranded micro (mi)RNAs. miRNAs, like siRNA guiding strands, then steer a protein complex to target endogenous mRNAs to initiate RNAi-mediated silencing of endogenous genes [37,38,26]. RNAi offers a rapid and efficient means of somatic gene knock down which is more potent than the conventional antisense oligodeoxynucleotide or ribozyme approaches, and importantly, mimics pharmacological target validation by only partially inhibiting target gene function. Thus, RNAi has been increasingly used, since its discovery in 1998, to generate dsRNA-mediated loss-of-function phenotype for deciphering the function(s) of novel genes. dsRNAs, longer than 30 nt, effectively downregulate gene expression in invertebrates, including *Aplysia*, planaria and *Drosophila*. However, in mammalian cells, these dsRNAs induce a toxic interferon response that leads to cell death, following (1) global mRNA translational arrest, which results from the protein kinase R-mediated phosphorylation of the translational initiation factor, eIF2α, or (2) non-specific mRNA degradation by 2′-5′oligoadenylate synthase-RNase L. In marked contrast to long dsRNAs, siRNAs do not induce the interferon response, although they produce sequence-specific gene knockdown in mammalian cells. An effective knockdown of exogenous as well as endogenous genes has been demonstrated in several mammalian organs (liver, lung, spleen, kidney, pancreas, skeletal muscles and even brain) using intravascular injections, nasal instillations or local injections/perfusions. In addition, RNAi has been used in a multitude of studies to explore the pathophysiology and potential therapeutic applications in animal models of cancer, viral infections, autoimmune, inflammatory, neurologic and psychiatric diseases. Obviously, in the latter case, the route of application is a key hurdle and one has to rely on i.c.v. or repeated local injections.

RNA interference, formulation, biovailability, pharmacokinetics, metabolism

One of the major challenges to overcome to create effective siRNAs is that of compound delivery and stability, both *in vitro* and *in vivo*. Foreign dsRNA which cells recognize as viral pathogens are rapidly eliminated requiring rather high dosing schedules of siRNA or shRNA. Protein or liposome conjugate systems and chemically modified or protected siRNAs show better stability and longer elimination half lives than naked siRNAs [17,18]. By further shielding the 2′F, 2′O-Me or 2′H stabilized siRNA in specialized liposomes, these compounds show higher potency and longer duration of action and can be used at lower doses, potentially avoiding oligonucleotide-related side effects [18]. Coupling naked siRNA to carrier molecules of various types will help to target the active siRNA to sites where they are needed and further reduce the amount of siRNA intake necessary for therapeutic effect. Another improved targeted delivery principle for siRNA is the modified cyclodextrin polymer-based nucleic acid approach. This nanoparticle technology polymer system contains transferrin taken up by cancer cells that overexpress the transferrin receptor [19]. Currently, approaches applied to antibodies, for example local injection and/or perfusion strategies to discrete compartments, for example within the eye and spine, appear to be best suited for siRNA drug delivery. Future delivery modalities may be adapted to patient and/or diseases, such as implantation of minipumps connected to deep brain delivery devices. On the contrary, we have noticed that naked or slightly modified siRNAs can be quite stable, for example up to 2 weeks in cerebrospinal fluid or in minipumps implanted into mice for 2 weeks.

RNA interference *in vivo*

There is a wealth of *in vitro* RNAi publications. *In vivo* data are, by comparison, more sparse; however over the last 3–4 years, much convincing evidence has been published showing that RNAi application can indeed result in downregulation of mRNA levels and produce selective and potent functional

effects in a range of very different spheres and in different animal species. Thus, local administration of siRNAs by intranasal, intrathecal and intravitreal routes show protection in models of lung ischemia [20], neuropathic pain [21] and neovascularization in the eye [22]. Intravenous administration of siRNA against apolipoprotein B (apoB) reduced apoB mRNA and protein levels, and total cholesterol in mice [23]. Stable siRNA given by the i.v. route resulted in long lasting knockdown of hepatitis B virus (HBV) replication in mice [18]. There are numerous reports on successful viral delivery of shRNA to the brain; however, we will not discuss this approach because of space constraints [24,25].

More surprisingly, exogenously applied siRNA has been successfully used to silence gene function in mouse brain [9,10,26], in spite of the perceived limitations of a CNS approach (see Box 2). Thus, direct perfusion of EGFP-siRNA (400 μg/day) for 1 or 2 weeks into the third ventricle of EGFP transgenic mice, knocked down EGFP mRNA and protein levels [9]. The effects were more pronounced after 2 weeks of treatment compared to one week, they were region selective, although relatively extensively distributed throughout the brain. There were clearly lesser effects in structures distant from the injection site (e.g. the olfactory bulb, the pons and the cerebellum were only minimally affected). Interestingly, there was a good relationship between EGFP mRNA and protein knock down, both after 1 or 2 weeks. These data provided a good case to pursue the approach. Similarly, siRNAs against the mouse dopamine (DAT) and the serotonin transporter (SERT) specifically downregulated the respective mRNAs and proteins [9,10]. Further, the siRNAs produced behavioral effects, namely hyperlocomotor activity and antidepressant-related behavior respectively, that paralleled the pharmacological blockade of DAT and SERT function, as produced by potent DAT inhibitor (GBR 12099) and a selective inhibitor of serotonin uptake (SSRI, paroxetine) [9,10,26]. Interestingly, although downregulation of mRNA and protein were rather limited, 40–60%, siRNA treatment produced full blown behavioral effects comparable to those observed following maximally effective pharmacologic intervention (DAT inhibitor, SSRI) or full KO as observed in transgenic mice [27]. Along the same lines, Salahpour *et al.* [28], used siRNA against DAT (35 μg/14 days) or tyrosine hydroxylase (TH) (15 μg/3 days), injected into the ventral tegmental/substantia nigra areas of the brain of adult wild type or DAT-knockout mice, respectively. siRNA resulted in a 35–40% reduction of DAT and TH protein levels in the striatum, respectively. DAT knockdown had little effect on novelty-induced locomotion, but the locomotor response of DAT siRNA treated animals to amphetamine was blunted, similar to what is observed in the DAT heterozygote animals. TH siRNA experiments were carried out in DAT-knockout animals that show increased dependence on newly synthesized dopamine. The knockdown of TH in these animals resulted in reduced basal locomotion. Wang *et al.* [29] used siRNA directed against the huntingtin gene to repress the transgenic mutant huntingtin expression in a Huntington's disease mouse model, R6/2. A single intraventricular injection of siRNA (0.2 μg) with lipofectamine at postnatal day 2 knocked down transgenic huntingtin expression (50%) and induced a decrease in the numbers and sizes of intranuclear inclusions in striatal neurons at week 8. siRNA treatment significantly prolonged mice longevity (by 2 weeks), improved motor function and slowed down the loss of body weight. Our data became even more intriguing when the mGlu7 receptor was targeted by siRNA: very limited mRNA knock down (20–25%) was observed with two different siRNAs, yet the behavioral effects were very marked and more pronounced that those observed in the mGlu7 receptor knock out mouse. These results clearly suggest that a partial target mRNA or protein downregulation can produce very marked functional effects, which parallel those obtained by the best available pharmacologic treatments. If more generally applicable, this point becomes very interesting. Indeed, although siRNAs or shRNAs appear to be very efficacious *in vitro* (with target knock down efficacy close to 100%), the situation *in vivo* looks so far, much less promising as several investigators report *in vivo* RNAi-induced knock down of various targets limited to about 30–40%. Thus, keeping in mind the limitations of CNS targets and drug delivery, the number of possible targets in primarily neurological disorders is rather extensive (see Table 1), especially targets with a strong genetic component, which have been resistant to classical pharmaceutical approaches.

shRNAs are normally delivered by the viral approach; however, Zhang *et al.* [30] used a very original strategy: they developed an expression plasmid encoding an shRNA directed at the human EGFR mRNA. The shRNA was encapsulated in pegylated immunoliposomes and targeted at brain cancer (Human U87 glioma) with 2 receptor-specific monoclonal antibodies (MAb), the murine 83-14 MAb to the human insulin receptor and the rat 8D3 MAb to the mouse transferrin

Box 2. Limitations of targeting and delivering RNAi to the CNS

Issues

- Blood brain barrier
- Diversity of cell types and complex neuronal networking
- Choice of disease model, duration of treatment, choice of target(s)

Possible delivery modalities

- Intracranial injections
- Transfection (oncoretrovirus, lentivirus, adenovirus, adeno associated virus, others)
- Electroporation
- Osmotic minipump infusions, i.c.v. or intrathecal

Table 1. Examples of neurological disorders and their respective molecular target(s) for RNAi

Neurological disorder	Mutated protein(s)
Amyotrophic lateral sclerosis	Cu/Zn superoxide dismutase
Spinocerebellar ataxia type 1	Ataxin-1
Huntington's disease	Huntingtin
Spinobulbar muscular atrophy (Kennedy's disease)	Androgen receptor
Familial Alzheimer's disease	Amyloid precursor protein, presenilin 1 or 2
Parkinsonian frontotemporal dementia	Tau
DYT1 dystonia	TorsinA
Spinal muscular atrophy	Survival motor neuron protein
Machado-Joseph disease	MJD1
Prion-based disease	Prion
Chronic pain	Various ligand or ion-gated channels

receptor. Weekly i.v. RNAi gene therapy caused reduced tumor expression of immunoreactive EGFR and an 88% increase in survival time of mice with advanced intracranial brain cancer. This nonviral gene transfer technology, which delivers liposome-encapsulated plasmid DNA across cellular barriers with receptor-specific targeting ligands is a first. Clearly, this shRNA approach is highly promising.

The large majority of the initial work establishing RNAi has been carried out *in vitro* and in rodents. In non-human primates, RNAi is making progress too: intranasal siRNA against the SARS coronavirus (SCV) was shown to treat SARS in a rhesus macaque model [31]. In cynomolgus monkeys, single i.v. specific siRNAs against apolipoprotein B (ApoB) and encapsulated in stable nucleic acid lipid particles (SNALP) produced dose-dependent silencing of ApoB mRNA expression in the liver 48 h after administration, with maximal silencing of >90% [32]. Significant reductions in ApoB protein, serum cholesterol and low-density lipoprotein levels were observed 24 h after treatment and lasted for 11 days at the highest siRNA dose, demonstrating an immediate, potent and lasting biological effect of siRNA treatment. Thus, clinically relevant RNAi-mediated gene silencing and concomitant functional effects in both rodents and non-human primates, support RNAi as a viable therapeutic principle.

RNA interference in the clinic

Both Biotech and pharmaceutical companies, often in partnership deals, are planning or already performing clinical trials based on siRNA as the therapeutic principle (see Table 2). The number of potential diseases to be targeted using RNA interference, be it by siRNA or shRNA, using a viral approach or just regular dsRNA is almost unlimited, especially for diseases which have escaped 'classical' pharmaceutical treatment, such as complex viral, autoimmune, neurologic, metabolic, oncologic and/or genetic diseases.

A quick look at the portfolio of for example Isis and Alnylam, illustrates the breadth of the RNAi targets and diseases which are currently in the works: vascular endothelial growth factor (VEGF) in age-related macular degeneration (AMD), diabetic retinopathy and diabetic macular edema; the

Table 2. Industrial efforts towards clinical applications of RNAi

Company	Partners	Indications
Acuity Pharmaceuticals	Alcon	VEGF in AMD, diabetic retinopathy.
Alnylam	Merck, Mayo Clinic, Medtronics, Novartis	AMD, diabetic retinopathy and diabetic macular edema; cystic fibrosis; respiratory syncytial virus (RSV); spinal cord injury; pandemic flu; other programs at Huntington's, Parkinson's diseases and neuropathic pain.
Atugen	Sanofi Aventis, Quark Biotech	AMD, prostate, lung, pancreas, hepatocellular carcinoma.
Benitec	Center for Biomedicine and Genetics	HIV and AIDS-related lymphoma with siRNA, using lenti virus; Hepatitis-C.
CombiMatrix		Diagnostics, early drug development
CytRx	MGH, Imperial college, London	Obesity, type 2 diabetes, CMV, ALS
Genta		Technology platform, cancer targets
Intradigm		VEGF in colon cancer and ocular neovascularization, SARS coronavirus.
International Therapeutics	City of Hope National Medical Center and Beckman Research Institute	HIV
Isis	Lilly, GSK, Novartis, Oncogenex, OSI, Rosetta, Pfizer, ALS Association	Type 2 diabetes, homozygous familial hypercholesterolemia, amyotrophic lateral sclerosis, ulcerative colitis, irritable bowel disease, Crohn's disease, asthma, multiple sclerosis, prostate cancer and other solid tumors, psoriasis, age related macular degeneration and diabetic retinopathy.
Sirna Therapeutics	Allergan, Targeted Genetics, GSK	VEGF in AMD, Huntington's disease, Respiratory diseases (COPD)
ToleroTech		Transplant rejection and autoimmune diseases

transmembrane conductance regulator (CFTR) in cystic fibrosis; respiratory syncytial virus (RSV) in upper airways infections (phase II); the Nogo protein in spinal cord injury; influenza virus for pandemic flu; the protein tyrosine phosphatase PTP-1B in Type 2 diabetes (positive phase II); the miRNA miR-122 to lower cholesterol and triglycerides in diabetes; the apolipoprotein (apoB-100) in homozygous familial hypercholesterolemia (orphan drug status, phase II); Cu/Zn superoxyde dismutase1 (SOD1) in amyotrophic lateral sclerosis; the cellular adhesion molecule ICAM-1 in ulcerative colitis, irritable bowel disease and Crohn's disease (Phase III); the interleukin receptor IL4-alpha in asthma/rhinitis; the C-Reactive protein (CRP) in coronary heart disease; the glucagon receptor in diabetes; VLA-4 (very late antigen-4) in multiple sclerosis (phase II?); clusterin in prostate cancer and other solid tumors, for example non-small cell lung cancer and breast cancer (phase II); the insulin-like growth factor 1 receptor (IGF-1R) in psoriasis (phase I); survivin and eukaryotic initiation factor 4 E (EiF-4E) in various cancers (both in phase I); the heat shock protein 27 (Hsp27) in various cancers; the cRAF kinase in age-related macular degeneration and diabetic retinopathy.

Thus, clinical trials are already under way, some of which target the same diseases as for instance AMD and certain viruses, for example hepatitis B and C, HIV, RSV, papilloma virus. It is clear that CNS disorders are not first line in the treatment priorities, but neurologic/neurodegenerative disorders are considered very seriously. This is largely because to the fact that the genomics of neurodegenerative disorders are much more advanced that those of psychiatric disorders. Results of phase I, phase II studies are being made available at scientific meetings and/or at press conferences or releases. Very recently (August 2006), siRNA therapeutics announced first positive results in a clinical trial (26 patients) against the wet form of AMD. Further down the road, targets like Parkinson's, Alzheimer's, Huntington's or prion diseases, where delivery will represent the real challenge will have their place (see Box 2). This is why companies like Alnylam enter partnerships with Medtronics, to apply siRNAs directly into the brain with deep brain delivery devices. The latter diseases are ideally suited, because the RNAi treatment should only affect the mutated protein, whereas the normal forms should remain unaffected. This is only possible because RNAi is so exquisitely selective, that is one can target a protein in its variant forms whereas the parent protein remains in principle untouched. It is even suggested that RNAi may work specifically on certain SNPs (single nucleotide polymorphism) from preliminary reports in meetings. Obviously, this remains to be confirmed *in vivo*, just as other claims need confirmation. Finally, it remains to be seen whether some of these treatments do not interfere with the natural RISC pathway given the high doses that are needed to produce a full blown knock down of gene expression.

Conclusions

RNA interference has the potential of targeting the entire genome, that is all proteins will soon be drugable: siRNA is limited to cytoplasmic targets, whereas shRNA will have to reach the nucleus. The chemical design of siRNAs has become almost flawless, as at least *in vitro*, very high knockdown specificity and potency can be regularly achieved. The main issues still remain a shared feature with all potential drugs: bioavailability, delivery, resistance to metabolism, tissue and/or brain penetration, elimination and toxicology. Several recent papers show that siRNAs can downregulate gene expression and protein function *in vivo* with very specific functional outcomes, including in blood, various organs, and both the peripheral and central nervous systems. Clinical trials are being run or are at least planned, by both big pharma and biotech. With the so far limited success of immunotoxins, gene therapies, antisense agents and ribozymes, great hopes for RNAi therapeutics have emerged supported by numerous pre clinical findings reported over the last 3–4 years. It is hoped that a better knowledge of disease mechanisms, which is certainly more advanced than say 15 years when the antisense era started, combined with significant expertise in siRNA- design and delivery, will meet success, especially because numerous aspects of biosciences, genetics, chemistry, genomics, bioinformatics, modeling and formulation have significantly progressed. However, as is common in clinical trials and due to the ever-increasing demands of the public and regulatory authorities, one has to be pragmatic and expect a few successes and many failures. Whereas viral approaches look very promising in animals, it is to be expected that such a route will only be taken in the clinical situation with extremely great care, so as not to repeat some of the tragedies that have occurred in the gene therapy field. Thus, one will have to see to a stepwise approach, using siRNAs probably targeting intractable diseases and viruses or other infectious agents, or in very local settings (such as the eye) before more common diseases will be targeted. The right balance will have to be found between gene silencing and the possible side effects, such as interferon responses and saturation of the RISC complex and thus off target effects. Possibly, one will soon be able to modulate the expression of the endogenous RNAi system, namely microRNAs. The area is indeed very promising, but like with all other drug like candidates (chemicals, proteins, immunizations, radiochemicals), the therapeutic window will dictate the feasibility of the approach. Eventually, success will come from carefully designed studies, where the target is well validated and accessible to treatment, that is where the pharmacokinetic and metabolic aspects are well controlled and no major toxicology alerts. As with other new technological advances, there will be successes and failures, especially because the early trials will be conducted by Biotech. As is common, many of these trials will be stopped because of unexpected side

effects, lack of efficacy, safety issues, poor bioavailabilty, metabolism, lack of brain penetration or more pragmatically because of cash is running out. It should be remembered that antisense therapy or gene therapy in spite of great promises have encountered difficulties, which were beyond the capacities of most of the Biotechs who launched such candidate treatments and as of today, there are still only very few antisense treatments that have been approved. There is no doubt that RNA interference will represent a major therapeutic advance. This approach comes in very timely, as the human genome is being functionalized, the links between targets and diseases are becoming better known, and importantly, because regulatory authorities, society and industry are prepared to move toward personalized medicine, with smaller target populations, but better genetics or biomarkers, better links to disease and ultimately greater chances of success to cure entire patient populations, which had no or very little therapeutic hopes. This is particularly true for neurodegenerative disorders, but once the genetic causes for psychiatric disorders are better established, RNAi may represent a primary strategy for target validation and possibly therapy, at least in drug treatment resistant populations. It is clear that in animal neuropsychiatric disorder models, RNAi produces marked behavioral effects at least as pronounced as those of established pharmacologic treatments or complete gene knock out [9,10,26,27].

Acknowledgments

We are grateful to Dr Graeme Bilbe (Head of Neurosciences, NIBR) for the careful reading of an early version of this script.

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