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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 druggability. 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 of 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
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 activa-

tion and induction of the innate immune system, that is the
interferon response, although, this seems to be of little rele-

vance 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,
Tryplosomes, 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
ingcreasingly 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
translation 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/perfu-
sions. 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, autoimmunity, 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, bioavailability, 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 dos-

ing schedules of siRNA or shRNA. Protein or liposome con-
jugate 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 com-
pounds show higher potency and longer duration of action
and can be used at lower doses, potentially avoiding oligo-
nucleotide-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 con-
tains transferrin taken up by cancer cells that overexpress the
transferrin receptor [19]. Currently, approaches applied to
antibodies, for example local injection and/or perfusion stra-
tegies 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 (20–25%) was observed with two different siRNAs against the mGlu7 receptor: very limited mRNA knock down (5–10%) was observed in the cingulate cortex and the hippocampus. The knockdown of TH in these animals and protein were rather limited, 40–60%, siRNA treatment produced full blown behavioral effects comparable to those observe 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.

siRNAs 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 | Possible delivery modalities |
|--------|-----------------------------|
| Blood brain barrier | Intracranial injections |
| Diversity of cell types and complex neuronal networking | Transfection (oncoretrovirus, lentivirus, adenovirus, adenovirus, associated virus, others) |
| Choice of disease model, duration of treatment, choice of target(s) | Electroporation |
| Osmotic minipump infusions, i.v. or intrathecal | |
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, oncolgic 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

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**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    |

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**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                 | MGH, Imperial college, London   | Diagnostics, early drug development.                                       |
| CytRx                       | Genta                           | Technology platform, cancer targets.                                       |
| Intradigm                   | Allergan, Targeted Genetics, GSK | VEGF in colon cancer and ocular neovascularization, SARS coronavirus.      |
| International Therapeutics  | City of Hope National Medical   | VEGF in AMD.                                                                |
|                             | 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 superoxide 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 4E (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 Medtronic, 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 druggable: 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 therapies 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 bioavailability, metabolism, lack of brain penetration or more pragmatically because of cost 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 Biotech 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].

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