The impact of RNAi on drug discovery

RNAi (RNA interference) is an important target validation tool that has risen to prominence since 2001. For a number of reasons, it has supplanted other pre-existing target validation tools and is now the method of choice for target validation in cell culture systems. It also has the potential to be an important technology for target validation *in vivo*, but there are several key challenges that must be addressed before RNAi has the same impact *in vivo* as it has *in vitro*. Most major pharmaceutical companies now have internal RNAi capabilities being applied to drug discovery. RNAi is increasing the efficiency and throughput of target validation and is starting to play a role in target identification. Furthermore, RNAi has the potential to play a role in hit selection, lead optimisation and development of animal models, and in so doing, impact drug discovery and development further downstream. Finally, RNAi-based therapeutics may be on the horizon. Initial successes in using RNAi for *in vivo* target validation suggested that administration of RNAi reagents to human patients may be useful to treat various diseases. This review summarises where RNAi is impacting drug discovery today and highlights how RNAi may impact drug discovery and development over the next few years.

By Dr Christopher P. Miller

RNAi refers to the ability of short double-stranded RNA molecules to silence target genes by inducing sequence-specific degradation of mRNAs. RNAi occurs by an intracellular mechanism that exists in every multicellular organism thus far examined. The molecular mechanisms involved in RNAi have been described elsewhere and the reader is referred to excellent reviews by Dykxhoorn et al, 2003[1] and Hannon, 2002[2] for more detailed information. In brief, double-stranded RNA molecules are taken up by cells and processed by intracellular protein complexes known as Dicer and RISC (RNA-Induced Silencing Complex). Dicer is responsible for chopping up larger double-stranded RNA molecules into shorter molecules (20-22 nucleotides in length) that are then loaded into RISC. Once in RISC, the double-stranded RNA molecules are unwound with one of the two strands remaining associated with RISC and the other being released and degraded. The strand that remains with RISC acts to guide RISC to target mRNAs by sequence-specific base pairing. RISC has an endonucleolytic activity that cuts the target mRNAs, rendering them unable to be translated into proteins and susceptible to further degradation. The cleavage and degradation of the mRNA result in silencing of the gene.

In theory, the goal is to achieve silencing of a target gene without affecting other genes. In practice, other genes can also be inhibited. These so-called 'off-target effects' can occur due to sequence similarity between the intended target mRNA and collaterally-affected

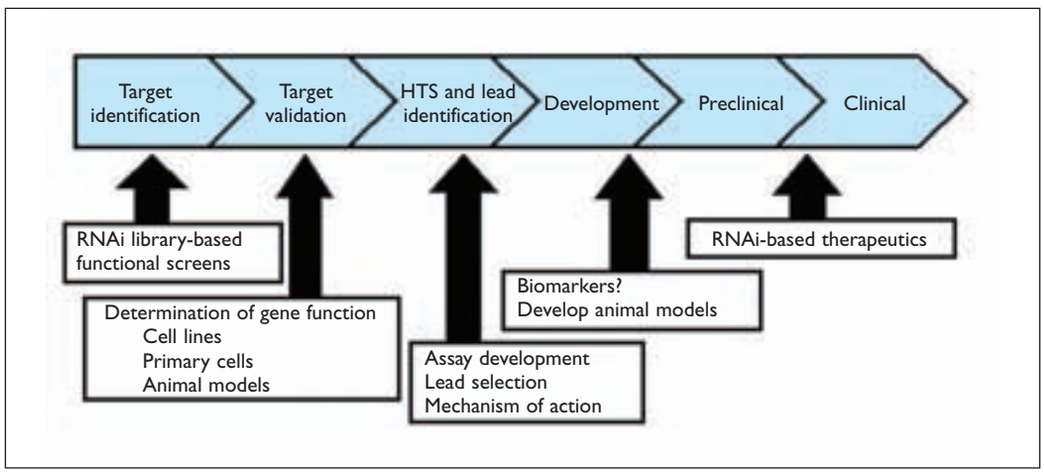


Figure 1
RNAi can impact multiple stages of drug discovery, but impact to date has mainly been in target identification and validation

mRNAs. This can result in cleavage and degradation, or translational suppression, of off-target genes. Cleavage and degradation is due to activation of the RNAi pathway, whereas translational suppression occurs due to activation of the related microRNA pathway (He and Hannon, 2002[3]). Also, some double-stranded RNA molecules can activate the interferon alpha pathway, leading to non-specific cellular stress responses (Sledz et al, 2003[4]). These non-specific inhibitory effects can be minimised by using the lowest possible concentrations of RNAi reagent, testing the effects of multiple independent RNAi reagents per target gene and performing well-designed, carefully controlled experiments. It's important to note that some gene expression changes perceived as being 'off-target' may in fact be true downstream events associated with inhibition of the intended target gene.

The role of RNAi in target validation

RNAi has the potential to impact multiple stages of drug discovery (**Figure 1**), but to date the greatest impact has been on target validation in cell culture systems. Target validation is a broad term that encompasses several aspects of understanding the roles of specific genes in disease and making the case that modulation of specific gene products will result in amelioration of disease phenotypes. Often, target validation is performed in cell culture systems prior to performing studies in animals but this isn't always the case. Target validation refers to demonstrating a functional link to a disease rather than a correlative link. Target validation in cell culture systems has several requirements. First, it is critical that the cell-based systems to be used faithfully model relevant aspects of the disease under study. Typically, there is an inverse relationship between the ease of working with a particular cell system and its relevance to disease. Many robust and easily transfectable cell lines are different enough from primary cells that they have limited utility other than in initial first pass experiments. Conversely, many important primary cells do not have robust growth and survival characteristics and are not easily transfectable. Second, readouts must be established that reflect the activity of the target and are connected to the disease phenotype. Readouts can range from transcriptional reporters, cytokine production, protein phosphorylation, proliferation, apoptosis, or more recently, high content cell imaging. The issue is not so much of whether readouts exist and can be set up and optimised, but rather that they are truly meaningful and are relevant in the context of what is known about the disease under study. The third requirement is that methods must exist for modulating the activity or level of the candidate target genes. This is where RNAi has made its biggest impact. By using RNAi, it is now possible to get at least a first pass approximation of how a given gene may be functioning in various cell culture systems. By knocking down a target mRNA using RNAi, one can begin to understand whether the gene product encoded by that mRNA is involved in a pathway or process and begin to determine whether the gene or gene product may be suitable as a target for small molecule intervention. Before RNAi, target validation in cell culture systems relied on a handful of tools and approaches that were fraught with problems and never achieved widespread use. These tools included engineered transcription factors, ribozymes, antisense DNA oligonucleotides, peptide nucleic acids (PNAs), locked nucleic acids (LNAs), inhibitory peptides, neutralising antibodies, and wild-type, dominant negative or constitutively active proteins. There are numerous examples where these tools have been useful but they have not been as broadly adopted as RNAi. RNAi has become widely used

because it has a relatively high success rate, is easy to use, is reasonably priced, has a rapid time to results, is amenable to high throughput applications and is adaptable to multiple systems. RNAi also has some degree of selectivity (see above) and acts through an endogenous mechanism of action that is becoming well understood. None of the other tools listed above can claim these advantages.

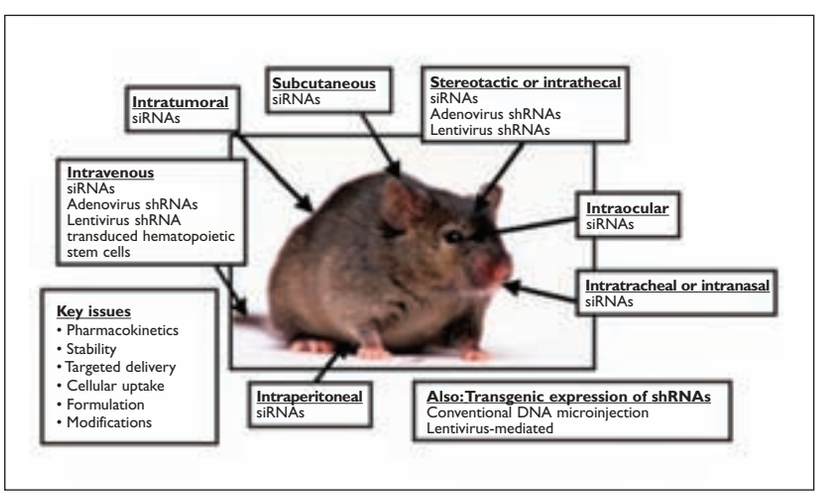
There are at least two different ways that RNAi is used in cell culture systems. The most commonly used approaches are chemically-synthesised short double-stranded RNA molecules and expressed short hairpin RNA molecules. Chemically-synthesised RNA duplexes are commonly referred to as siRNAs, or short interfering RNAs. They can be synthesised or ordered from several commercial vendors. Expressed short hairpin RNAs are commonly referred to as shRNAs. They are generated inside cells after transfection with plasmid or viral expression vectors. The initial choice of which RNAi approach to use is dependent on how transfectable the cells are and how long after transfection the cells will be cultured before harvest. For short-term experiments (48-72 hours) in easily transfectable cells, it makes sense to attempt to use siRNAs first. They are readily obtained and modestly priced. The only real challenge can be to work out transfection conditions for a cell type for which it hasn't previously been done. Finding the right combination of transfection reagent (type and concentration), RNAi concentration, time point and assay readout can be time-consuming, but is a relatively straightforward exercise. shRNA expression systems are useful when it is necessary to do functional studies in many primary cells or when sustained or inducible silencing is required. shRNAs can be expressed from plasmids or viruses, depending on target cell type. Adenoviruses and lentiviruses are proving to be workhorse shRNA expression systems because they can transduce many important cell types.

An important current area of technology development for RNAi is in moving from *in vitro* to *in vivo* applications. There have been a limited number of successes in using RNAi *in vivo*, and these have included several different routes of local and systemic administration (**Figure 2**) (Van den Haute, 2003[5], Xia et al, 2002[6]). The key challenges related to improving the pharmacokinetics, stability and cellular uptake of RNAi reagents, whether they be chemically-synthesised siRNAs or vector-expressed shRNAs. Improvements are being made in chemical modifications, delivery formulations and vector design, such that *in vivo* RNAi will likely soon be routinely possible. Moving RNAi to animal systems is important for several reasons. Target validation in animal models is mostly done using transgenic and knockout mice. There are a number of model organisms that are also used (flies, worms, fish, etc…) and these have generated meaningful information. Also, tools such as neutralising antibodies have been used in a range of organisms, but for the most part, the workhorse for *in vivo* target validation has been over-expression and gene ablation in genetically modified mice. The problem is that the timelines required to generate transgenic and knockout mice are quite long (12-18 months from idea to initial experimental cohort under the best of circumstances). Use of RNAi *in vivo* has the potential to generate target validation information under more rapid timelines. The same challenges that face RNAi-based target validation *in vivo* need to be addressed for RNAi-based therapeutics to be successful. During the course of the next 12-18 months, initial results will become available for proof of concept in clinical trials using RNAi as a therapeutic agent.

The role of RNAi in target identification

Target identification refers to the initial results suggesting that a gene may be involved in a disease. Target identification data are often correlative in nature, rather than causative. For example, targets are sometimes identified by molecular profiling studies that indicate a particular gene or protein is upregulated in disease samples relative to normal samples. These types of results raise the hypotheses that the dysregulated genes or proteins may be involved in, or associated with, the disease, but they do not demonstrate that the mRNA or protein dysregulation is causing the disease.

Figure 2
In vivo applications of RNAi for target validation and potential



References

1 Dykxhoorn, DM, Novina, CD, Sharp, PA. Nature Reviews: Molecular Cell Biology. 4:457-467, 2003.
2 Hannon, GJ. RNA interference. Nature. 11;418(6894):244-51, 2002.
3 He, L, Hannon, GJ. MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet. 5(7):522-31, 2004.
4 Sledz, CA, Holko, M, de Veer, MJ, Silverman, RH, Williams, BR. Activation of the interferon system by short-interfering RNAs. Nat Cell Biol. 5(9):834-9, 2003.
5 Van den Haute, C, Eggermont, K, Nuttin, B, Debyser, Z, Baekelandt, V. Lentiviral vector-mediated delivery of short hairpin RNA results in persistent knockdown of gene expression in mouse brain. Hum Gene Ther. 10;14(18):1799-807, 2003.
6 Xia, H, Mao, Q, Paulson, HL, Davidson, BL. siRNA-mediated gene silencing *in vitro* and *in vivo*. Nat Biotechnol. 20(10):1006-10, 2002.

Nor do they suggest that inhibition of the target will be useful to treat the disease. Genetic studies in model organisms are an exception to this in that genes can be identified by the functional consequences of activation or inhibition in living systems, but translating the results to mammalian systems and human diseases is not always straightforward. RNAi is starting to provide a new paradigm for target identification based on consequences of functional inhibition in mammalian cell systems. Arrayed collections of siRNA reagents are available from the major RNAi vendors (Qiagen, Ambion, Dharmacon) and shRNA libraries are being generated by several groups (Galapagos Genomics (Belgium), Broad Institute (Boston, MA), Cold Spring Harbor Laboratories (Long Island, NY)). These collections cover the major druggable gene families and in some cases are aiming for complete genome coverage. The arrayed RNAi libraries are introduced into microtiter plate cell-based assays then cellular function determined by assaying various readouts. The key point here is that high throughput RNAi allows genes to be identified based on the consequences of functional inhibition. This is a much more powerful and direct way of identifying targets than first looking for which genes are differentially expressed, then testing those for function.

Additional roles for RNAi

There are several additional opportunities for RNAi to impact drug discovery beyond target identification and validation. For example, RNAi can be a useful tool for assay development, hit selection and specificity testing. In assay development, RNAi can be used as a positive control to 'calibrate' the assay readout based on the effect of known levels of mRNA or protein knockdown. Effects of test compounds can then be benchmarked against the effects of known inhibitory effects of RNAi reagents. The selectivity of certain RNAi reagents can also be used to advantage in assay development. By using RNAi reagents with known specificity for individual mRNA isoforms or closely related family members, one can estimate what the effects would be for compounds with similar specificity. This can also be a useful way to establish whether compound specificity is required. In hit selection, RNAi can be used as a benchmark for small molecules identified in HTS. Those compounds with activities most similar to known, high quality RNAi reagents may be assigned a higher priority than compounds behaving differently than the RNAi reagents. It has been suggested that RNAi may also play a role in biomarker discovery and validation. The simplest example would be to look for mRNAs or proteins that are differentially expressed after RNAi treatment and to use them as markers for testing activities of compounds in cell systems and animal models. However, the RNAi-derived biomarkers would have to be validated against the small molecule compounds, and so in practice it probably makes more sense to wait until one has good lead compounds to identify biomarkers.

The impact of RNA on drug discovery

The true measure of any technology or approach in drug discovery is whether the information derived from it has a tangible impact on go/no-go decisions along the pipeline. RNAi has improved the efficiency and throughput of target validation and is starting to improve target identification. There are now numerous examples where RNAi-derived data played a role in initiating or advancing drug discovery projects by providing key functional data. Often, these data include demonstration of functional effects in cell lines, or more importantly in relevant primary human cells. Prior to RNAi, these data were lacking, or were much harder to generate. It should be noted that, although RNA is playing an important role in identifying and validating targets, there are many other criteria that influence whether a target will advance beyond the initial stages of drug discovery. Factors such as target class and druggability, therapeutic rationale, market size and strategic fit, risk analysis and IP position all come into play, and in fact will over-ride even the most solid target validation data. For all of the criteria that go into decision-making, it is important to have the best possible information on which to base the decisions. RNAi provides a tool for making good decisions regarding target validation. As RNAi continues to evolve and as it starts to be applied to other steps in drug discovery (eg, assay development and hit selection), it will likely help make good decisions at other stages as well. DDW

Dr Christopher Miller *is Associate Director of Applied Genomics for Wyeth Research, based in Cambridge MA. The Wyeth Applied Genomics group performs target identification and validation experiments in support of Wyeth's five Discovery Research Areas (Oncology, Neuroscience, Inflammation, Cardiovascular and Metabolic Diseases and Women's Health and Bone). Major current efforts in the group include RNAi and dominant negative strategies, viral shRNA expression systems, high content cell-based assays, and phenotype screening using RNAi libraries.*