

Small molecules in an RNAi world

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In this *Opinion*, we compare and contrast small molecules and RNAi. We discuss the advantages and disadvantages of both technologies focusing on timing, specificity, dose and therapeutic use.

Introduction

Small molecules have been used successfully to probe biological mechanisms for many years. An expansion of this powerful approach, coupled with advances in combinatorial chemistry, has led to a new method to study function in mammalian cells, which is often orthogonal to traditional approaches. In the last decade, researchers have begun systematic efforts to develop cell-permeable small molecule tools and to use them to study a variety of biological processes.¹ Much excitement has surrounded this approach, which is called 'chemical genetics'.² The ultimate goal of chemical genetics is to provide a small molecule tool for every protein. More recently, another powerful method, RNA interference (RNAi), has become widely used

to study function both at the protein and genome level. In this piece, we discuss the advantages and disadvantages of both technologies.

The major advantages of small molecules are that they allow high temporal and some spatial control and their effects can be observed in real time by live imaging. Despite their great promise, only a limited number of bioactive small molecules with known mechanisms of action exist at this point. How can one obtain a small molecule tool? Most *de novo* tools are found by high-throughput screening of libraries of synthetic compounds, or lower throughput testing and fractionation of natural product extracts. In either case, small molecules that produce the desired effect in a cell-based screen or inhibit protein function in a pure-protein screen are identified. Screening hits from synthetic libraries often work in the micromolar range, where the risk of off-target effects is an important concern.³ Extensive optimization is used to make more potent, and presumably more specific, derivatives in industry, but such

resources are typically not available in academic settings. Screening hits from collections of natural products, which have been optimized by evolution, are sometimes much more potent and specific. Nevertheless, synthetic small molecules discovered by academic screening have been used to learn more about dynamic biological processes. For example, monastrol, an inhibitor of the mitotic kinesin Eg5, was used to probe the movement of microtubules in the mitotic spindle,⁴ while blebbistatin, a non-muscle myosin II inhibitor,⁵ has been used to address a number of aspects of cell division and motility.

If small molecules are such useful tools, why are so few available? The most obvious answer is that high-throughput screening has only recently become possible in academic screening centers and many screens have not yet been carried out. Small molecules developed by the pharmaceutical industry target specific disease proteins and many interesting molecules may not be disclosed for commercial reasons. Also certain classes of proteins like enzymes

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or receptors, which have active site binding pockets easily occupied by a small molecule, are more amenable to small molecule inhibition. With a few notable exceptions, it has been difficult to develop small molecules that target other classes of proteins. For example, few small molecule inhibitors target transcription factors, whose activity depends on interactions between surfaces that are larger than an average small molecule. Most transcription factors, however, have a high turnover rate and are readily depleted by RNAi.

Since its discovery about 10 years ago, RNAi has revolutionized biology because it allows depletion of any protein as long as its sequence is known.⁶ The mechanism of RNAi is not yet completely understood.^{7,8} It involves the interaction of short interfering RNAs (siRNA) with the RISC (RNAi-induced silencing complex),⁹ resulting in destruction of mRNA corresponding in sequence to the siRNA and subsequent failure to produce the targeted protein (see Fig. 1). As this protein is turned over during normal cell cycle progression, its amount is reduced and eventually depleted completely. RNAi was first used to great effect in *C. elegans*, where it can be used to observe protein function at cellular and whole-organism levels, which is especially useful in developmental studies. Long pieces of double-stranded RNA, which are processed into siRNAs by cellular machineries, can be used in *C. elegans* and *Drosophila* cultured cells. In mammalian cells, long pieces of dsRNAs trigger the interferon response, and siRNAs need to be delivered directly to the cell, usually by

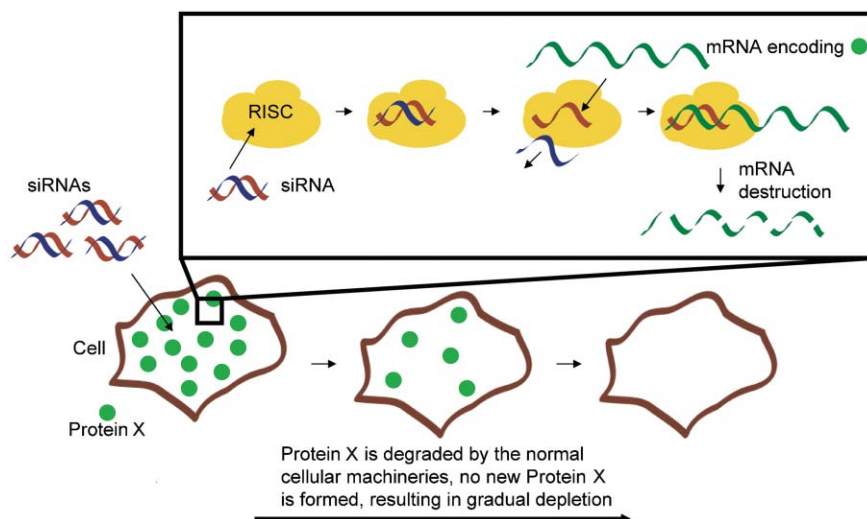


Fig. 1 How does RNAi work? This figure cartoons a simplified mechanism. After siRNAs enter a cell, they associate with the RISC complex. mRNAs with sequences corresponding to the siRNAs enter the RISC complex and are destroyed. No more mRNA is present, therefore new protein X cannot be produced. Since protein X is degraded normally during the cell cycle and no new protein can be made, the amount of protein X in the cell decreases, eventually resulting in the complete absence of protein X. Parts of this figure are adapted from www.cenix-bioscience.com.

transfection. Because of these technical challenges, a number of genome-wide screens have been reported in *C. elegans* and *Drosophila*, but are only just becoming possible in mammalian cells.¹⁰

Genome-wide capability and the ability to target any protein are the major advantages of RNAi over small molecules. A smaller scale analogy to a genome-wide RNAi library would be a collection of small molecules with known biological activity. When comparing the effects of small molecules and RNAi, it is important to note that, while the end results are the same in many cases, there are major differences in mechanism. An RNAi experiment results in protein

depletion, whereas a small molecule inhibits protein function while the protein is still present. Many proteins have domains with a variety of additional functions. If the enzyme activity is inhibited without affecting other functions, for example a scaffolding function in a complex, the phenotype observed might be very different from a phenotype caused by complete removal (reviewed in 3). In contrast, small molecule and RNAi phenotypes are expected to be the same if the small molecule inhibits a protein that does not have other significant functions. In addition to inhibiting a protein, a small molecule can also activate proteins or result in a scenario that would be comparable to a dominant negative mutation. The differences between small molecules and RNAi for specific topics are discussed below and summarized in Table 1.

Timing

Small molecules act quickly, and their effect can often be reversed rapidly by simply washing. RNAi results in a gradual depletion of the target protein (e.g. Protein A, see Fig. 1) that is only slowly reversible after several cell cycles. Most small molecules affect cells within minutes, making them ideal tools for live imaging. One can compare a small



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Table 1 Comparative summary of small molecules and RNAi

	Small molecules	RNAi
Generality	Not available for every target Significant screening effort needed to obtain initial small molecule	Available for every target siRNAs readily available
Mechanism	Inhibition, activation or partial inhibition of enzymes or other proteins	Depletion or partial depletion of any protein
Timing	Fast, often reversible inhibition	Gradual protein depletion
Effect on multidomain proteins or complexes	Protein inhibited, can still be present, other domains may be functional	Protein might be depleted in less stable structures first, complexes might dissociate
Specificity	Non-specific effects possible—use structurally distinct small molecules	Off-target effects possible—use multiple oligos
Dose	Dose–response is informative	Difficult to determine dose during gradual depletion
Therapeutic use	Very commonly used as therapeutic drugs	High potential, not yet used

molecule to a switch that turns off a function and allows us to observe the immediate effects. Depending on the stability of the target protein and the average length of the cell cycle, RNAi depletion normally takes from 24 h to several days. When RNAi is used as a screening tool, this can lead to false negatives because one might miss a protein that has not been sufficiently depleted, but rarely to false positives because one usually observes a phenotype only if enough protein has been knocked-down. If RNAi is used to study function, incomplete depletion can be a big issue because it is very difficult to assess if the function observed is due to complete ablation of the protein or a small but crucial amount of remaining protein (also see the 'Dose' section). Gradual depletion can give unexpected results because a cell might adapt to slow change, for example some other protein might compensate or other proteins are up- or down-regulated leading to more complex phenotypes.

Specificity

Both small molecules and RNAi can have non-specific effects. With small molecules, these are usually related to structure, *i.e.* a small molecule might slot into a similar binding pocket in multiple proteins. Non-specific effects due to chemical functionality, such as electrophilic groups that can alkylate biomolecules, is another potential concern. The origin and nature of off-target effects in RNAi is a rapidly expanding research

area, and both sequence-dependent and -independent effects have been described.^{11,12} Non-specific effects can be optimized for both small molecules and RNAi, but this is typically easier for RNAi because many different 20–30 base pair sequences are present in a given gene, and they can all be synthesized using the same chemistry. Dosage considerations (see below) are also critical for maximizing the specificity of both classes of inhibitor, and in general it is important to work with the minimum dose that gives the required degree of inhibition.

One strategy that can be used to combat non-specific small molecule effects is to develop a second small molecule that has the same biological activity, but a distinct chemical scaffold that contacts different protein residues. Off-target effects in RNAi are still poorly understood, but they mainly involve the RNAi machinery targeting mRNA that has some sequence overlap with the target gene. Non-specific effects are also possible, for example, even short siRNAs may begin to trigger the deleterious interferon response if used at too high a concentration.¹¹ When using siRNA, it is important to confirm results with one or more siRNAs that have non-overlapping sequences, and to use appropriate negative controls. It is good practice to confirm depletion of the target protein by immunofluorescence, but the most rigorous verification that the correct protein has been depleted is a rescue experiment, where the gene is reintroduced into the cell. An add-back

experiment can also be very useful to test the function of a mutated gene, where the wild-type is depleted by RNAi and then the mutant is introduced. Similarly, if overexpression of a target protein requires a higher dose of small molecule to maintain the observed effect, it is a good indication that the small molecule is acting specifically.

Dose

Cells' response to inhibitors is dose-dependent, both in degree and in phenotype. This fact is well known for small molecules, but it also applies to RNAi, though this is often less appreciated. High doses of small molecules can cause cell death, often due to binding of multiple proteins. As the dose is reduced, binding becomes more specific for one or a few related targets. For a single target, dose–response typically generates the hyperbolic curves expected for the reversible, saturatable binding of a small molecule to a protein, although more complex relationships such as cooperative binding are possible. To maximize specificity, it is important to use small molecules at appropriate concentrations, typically only a few fold higher than the EC₅₀ for the response of interest. siRNA experiments do not appear to exhibit the kind of hyperbolic dose–response typically seen for small molecules, rather the response appears to be all-or-none. The reason for this is unclear since the mechanism of RNAi is not fully known, particularly at a quantitative level. We should, however, expect a more familiar

dose–response connecting the remaining amount of protein to the biological response. One of the big challenges in RNAi is to assess how much protein has been depleted at a specific time. The best method currently available is immunofluorescence, which may still miss small amounts of active residual protein. Many proteins have multiple cellular localizations that can change during the cell cycle. This, coupled with imperfect fixation protocols, can make it difficult to determine and quantitate the exact localization of active protein even in untreated cells. In a striking example, Meraldi *et al.* illustrate the importance of quantitating the amount of depleted protein. They show that cells completely lacking the kinetochore protein hNuf2R do not arrest in mitosis, while cells where the protein is depleted incompletely arrest in mitosis for a prolonged period.¹³ Significant cell-to-cell variations have been observed in some RNAi experiments, where some cells could be experiencing a completely different protein level from their neighbors, making methods that average over entire populations, such as a Western blot, less informative.

Another issue related to dose is access to the target protein. If the target protein is buried in a dense structure or is located inside an organelle, it will be more difficult for some small molecules to reach their targets. Similarly, differential stability of protein pools can lead to depletion of one pool, while another remains in a more stable structure. This might create the illusion that most of the protein has been depleted although a majority of functional protein is still present.

Therapeutic use

Both small molecules and RNAi are valuable as tools in cell biological analysis, but they also have great potential as therapeutic agents. The impact of therapeutic drugs in medicine is obvious. Small molecules discovered as tools in an academic setting are rarely potent

enough to be used as drugs. They can provide leads for improvement by medicinal chemists or can prove that a particular protein is a druggable target. siRNA have significant therapeutic potential if they can be engineered to enter a cell safely and effectively. In a very promising start it has been shown that knock-down can be achieved in a mouse model using siRNA conjugated to cholesterol.¹⁴

Combining small molecules and RNAi

We discussed the advantages and disadvantages of small molecules and RNAi above. By combining the two methods some of the disadvantages might be overcome and new uses could be uncovered. One of the major disadvantages of small molecules is that there is a limited number available. Screening strategies that compare small molecule and RNAi phenotypes can be a useful tool in the discovery of new active small molecules.¹⁵ In the future, modifier screens that identify RNAi enhancers or suppressors of small molecule phenotypes or *vice versa* could help to uncover various biological connections and provide tools to study them.

Conclusions

We conclude that RNAi and small molecule inhibition are both very useful technologies for revealing biological mechanisms. The primary advantage of small molecules is temporal control; the primary advantage of RNAi is generality. Both methods suffer from potential lack of specificity, and to minimize this problem it is important to use appropriate doses, and ideally to compare the effect of multiple reagents that include both active inhibitor and inactive controls. Combining the two technologies to probe a given pathway can take advantage of the complementary strengths of the two approaches, and confirm specificity. For that reason, we urge the chemical biology community to embrace

RNAi technology, and to use it to help develop and characterize small molecules with new mechanisms.

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