

# RNA Interference: A New Targeted Tumour Therapy?

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**Abstract:** RNA interference (RNAi) is a mechanism in which double-stranded (ds) RNA acts as a guide to sequence-specifically suppress gene expression. To achieve targeted 'knock-downs' of gene function in mammalian cells, *in vitro* synthesised small interfering RNAs (siRNAs) can be introduced transiently into cells or, for more stable suppression by RNAi, various vector strategies can be employed to achieve prolonged *in vivo* synthesis of targeting RNA sequences.

RNAi, beyond being a powerful experimental tool, has been widely promoted as a future gene-targeted therapeutic strategy of exquisite specificity. In the context of cancer, in which mutation, over-expression and *de-novo* acquisition of tumour-promoting genes are of central importance to the pathology, multiple molecular targets have been proposed for RNAi-based therapies. In this review, we will summarise what is known about the biology of RNAi in mammalian cells, outline the various expression and delivery strategies that have been developed, and discuss the features of possible therapeutic gene targets. We will also highlight the present technical limitations of RNAi that will need to be addressed if it is to be developed therapeutically, including the important issues of effective delivery and the potential for development of tumour resistance.

**Keywords:** Cancer, RNAi, RNA interference, therapy, tumor.

The term RNA interference (RNAi) describes several mechanisms of sequence-specific gene silencing initiated by double stranded RNA (dsRNA) and resulting in cleavage or translational repression of single stranded RNAs (ssRNAs) e.g. mRNA with a homologous sequence. dsRNA can also direct transcriptional silencing via DNA methylation at homologous sequences [for recent reviews see 1; 2; 3]. RNAi was first described in plants [4; 5] where it was known as post-transcriptional gene silencing [6] and similar mechanisms have now been identified in many eukaryotic organisms including fungi [7], flies [8], nematode worms [9] and mammals [10]. The functional molecules of RNA interference are short (~21 nucleotide) RNA duplexes which are generated from several types of naturally occurring precursor, and it is these that are generally exploited experimentally to reduce expression of specific genes in mammalian cells.

## MECHANISMS OF RNAI

The initial findings that RNAi could be initiated by dsRNA led researchers to propose that it was an evolutionarily conserved anti-viral strategy, as most viruses produce long dsRNA at some stage of their life cycle. It may also protect cells from the mutagenic effects of randomly integrating transposable elements whose aberrant mRNAs are copied by endogenous RNA-dependent RNA polymerases to form long dsRNA [11]. More recently it has been shown that RNAi can play a role in developmental gene regulation through endogenous non-coding RNAs called microRNAs (miRNAs). These transcripts have internal complementarity and can therefore form intra-molecular base pairs resulting in

a dsRNA hairpin [reviewed in 12]. The hairpin is excised from the primary transcript in the nucleus by a dsRNA-specific RNase-III-type endonuclease known as Drosha [13] to form a miRNA precursor which is exported to the cytoplasm. Whatever their initial source, dsRNAs are all eventually processed in the cytoplasm by another RNase-III-type enzyme called Dicer [14].

Dicer cleaves dsRNA to ~21 nucleotide RNA duplexes which are phosphorylated at their 5' ends and have 2-nucleotide (nt) overhangs at their 3' ends [15]. These duplexes are known as small interfering RNAs (siRNAs) if they have been generated from long dsRNA, or miRNAs if they have been processed by Drosha from microRNA precursors. In humans only one Dicer gene has been identified to date [14] although other organisms e.g. *Drosophila*, express multiple Dicer proteins which have preferences for dsRNA from particular sources [16]. In mammals Dicer-interacting proteins may confer such selectivity. Cleavage of dsRNA in *Drosophila* is ATP-dependent [17] although this does not appear to be the case in humans [18; 19].

Once an siRNA/miRNA duplex has been formed a single strand may be incorporated into a multiprotein complex known as RNA-induced silencing complex [RISC; 20]. This process requires ATP, probably to unwind the two RNA strands [17; 21] and the proteins responsible for this may be RNA helicases. Armitage is thought to perform this role in *Drosophila* [22], however a human homologue has yet to be identified. In addition to helicases all RISCs contain an Argonaute (Ago) protein family member. Eight of these have so far been identified in humans although only three (Ago 1-3) have been characterised [23; 24; 25]. Argonaute is thought to help transfer small RNAs from Dicer to the RISC complex through its PAZ (PIWI-Argonaute-Zwille) domain [23]. Ago proteins also contain a PIWI domain which resembles RNase H and whose function may be RNA target cleavage [26]. In some organisms different Ago proteins

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appear to process differently sourced small RNAs [27], however in humans only Ago-2 containing complexes have been shown to have endonuclease activity [28; 25]. Other components of RISC complexes in humans remain to be identified and functionally characterised.

RISC complexes use the single stranded siRNA they contain as a template for degrading complementary or near-complementary RNAs, with target cleavage occurring at a single site 10 nucleotides from the 5' end of the guide siRNA [15]. Cleavage does not require ATP, but does require magnesium ions and hydrolyses the target-RNA phosphodiester linkage to produce 5' phosphate and 3' hydroxyl ends [17; 29; 30]. Endogenous miRNAs tend to be near complementary rather than identical to their natural target sequences, and in animals interact with the 3' untranslated regions of mRNAs to induce translational repression rather than RNA cleavage [reviewed in 31]; the RNA/protein complexes mediating translational repression have been termed miRNP as distinct from the RNA-cleaving RISC complexes. The mechanism of translational repression is not as well understood as RNA cleavage however, since the target and miRNA are found associated with polyribosomes in *Caenorhabditis elegans*, it appears to be elongation or termination that is blocked rather than initiation of protein synthesis [32]. miRNAs can act as siRNAs, and vice-versa, and whether a particular function is carried out does not appear to be solely due to the degree of complementarity between the template and the target [33; 34].

A further mechanism of gene silencing induced by dsRNA, but occurring at the transcriptional level, was originally identified in plants [35] and has recently been shown in mammalian cells [36; 37]. In this process the silencing trigger induces methylation of genomic DNA with a homologous sequence, and if this target sequence is in a promoter then the gene can become transcriptionally silenced [38].

In *C.elegans* silencing can be amplified in a process known as transitive RNAi, whereby siRNAs are produced that target regions of an mRNA away from the original site of homology with the 'inducing' dsRNA [reviewed in 39]. In this organism, silencing can also spread systemically from cell to cell and tissue to tissue [9] and be passed to progeny through the germ line [9; 40]. Since none of these processes have been identified in mammals so far, we will not consider them further.

## EXPERIMENTAL APPROACHES AND SEQUENCE REQUIREMENTS FOR EFFICIENT RNAi IN MAMMALIAN CELLS

Plants and lower eukaryotes, e.g. *C. elegans*, can be 'treated' (by various protocols) with long (>30nt) stretches of dsRNA to initiate specific gene silencing. However in mammals long dsRNA can result in non-specific off-target effects by inducing interferon synthesis and activating the dsRNA-dependent protein kinase PKR and 2', 5'-oligoadenylate synthase (OAS). PKR phosphorylates translation elongation initiation factor 2, blocking protein synthesis, and OAS activates RNase L to degrade RNA non-specifically [41]. The breakthrough for the use of RNAi in

mammalian cells came when it was discovered that this interferon response could be circumvented by the use of 21-nt siRNA duplexes which incorporate directly into RISC [42; 10]. Gene knockdown by this method is relatively simple if the targeting sequence is effective, but it is transient and the effects may be limited if the target protein is highly expressed or has a long half life. In order to achieve sustained RNAi-mediated suppression in mammalian cells, a variety of vector systems have been devised that allow genomic integration and therefore maintenance and heritability, and the important shared feature of each of these is that they direct the accurate *in vivo* transcription of short RNA species that can enter the RNAi pathways. A commonly-employed strategy is the transcription of an RNA comprising self-complementary sequences joined by a linker sequence, which intramolecularly base-pairs to produce a short hairpin (sh)RNA, and is then processed by Dicer to produce an siRNA [1].

The effective strand in an siRNA/miRNA can be denoted the antisense strand as this is complementary to the target RNA sequence and can therefore base pair with it to cause interference. The use of synthetic siRNAs for gene silencing in mammals is becoming widespread and, based on siRNAs which silence effectively, a variety of characteristics of efficiently targeting sequences have been identified [reviewed in 43; 44]. The siRNA duplexes with the most efficient silencing effect appear to have reduced thermodynamic stability (i.e. A/U pairing or base mismatches rather than G/C) at the 5' end (first five nucleotides) of their antisense strand compared to the 3' end (last five nucleotides). It is hypothesised that this results in the duplex becoming accessible to an RNA helicase first at this end so the 5' end of the antisense strand is free to enter RISC before the 5' end of the sense strand [45; 46]. In order to enter RISC the antisense strand should also be 5' phosphorylated. Highly functional duplexes have a G/C content of between 36-52%, presumably to balance efficient separation of the two siRNA strands with target affinity. Both 3' overhangs appear to be important for activity and it may be best if these are deoxythymidines as they may protect strands from ribonucleases [10]. Sequence designs should avoid internal repeats or palindromes to reduce the formation of hairpins within the siRNA which may result in reduced binding to the target RNA. There are also several positions where specific nucleotides appear to affect knockdown. The most important of these is at position 10 of the sense strand where a uridine is favoured. Activated RISC cleaves target RNA between bases 10 and 11 and RISC (like many endonucleases) preferentially cleaves 3' of a uridine. In addition an adenosine is preferred at position 3 of the sense strand, a guanosine at position 13 and an adenosine rather than a guanosine or cytidine at position 19. Many companies supplying siRNAs have designed computer algorithms which take these characteristics into account when designing targeting sequences [for a list of companies selling RNAi-related products see 47]. For short hairpin RNA design the rules appear to be much the same as for siRNAs with the obvious addition of the terminal loop which can range in size from 3-23 nucleotides. In 75% of naturally occurring miRNA precursors a uridine is present at position 1 which corresponds to position 19 of the siRNA sense strand where an adenosine is preferred. This may

reflect the reduced duplex stability required at this end for preferential incorporation of the targeting strand into RISC.

## RNAi AS A FUTURE TUMOUR THERAPEUTIC APPROACH?

There is no doubt that the advent of RNAi as a tool for gene function analysis in mammals has already had a major impact on therapeutic target identification and validation. Individual molecules implicated by various criteria as potentially having roles in tumour development (e.g. aberrant expression) have been specifically targeted to dissect their functions [for example our own studies on a protein kinase over-expressed in breast tumours; 48], and high-throughput knock-down screens coupled with robust functional assays raise the possibility of increasingly unbiased searches for novel regulatory pathways. For example, in a gene family-based RNAi screen targeting 50 deubiquitinating enzymes, the product of the *CYLD* cylindromatosis tumour suppressor gene was found to be a regulator of the NF- $\kappa$ B transcription factor [49]. Notably, on the basis of these findings, the authors proposed that aspirin derivatives may be of therapeutic value in treating cylindromatosis. In a much larger screen employing a library of retroviral vectors expressing shRNAs capable of targeting 7,914 different human genes, several novel regulatory interactions with the p53 pathway were discovered, with implications for a better understanding of tumour development in a large number and variety of cancers [50]. While few would doubt the value of RNAi as an experimental tool, its relative potency and specificity in suppressing gene function has led a number of groups to investigate the possibility of harnessing it as a therapeutic approach.

### A Variety of Potential Targets

A comprehensive survey of those molecules that have been suggested as therapeutic RNAi targets in cancer is beyond the scope of this mini-review, and we will provide an overview of the field. From the point of view of specificity alone, the most tractable targets will be expressed sequences that are unique to tumour cells, whether they be exogenously derived or the products of mutated genes, and we will therefore focus primarily on these.

Infection with viruses has been associated with several human malignancies, and the functions of virally-encoded genes have been implicated in the development and maintenance of those diseases. For example, the great majority of cervical carcinomas contain genome sequences derived from specific types of human papillomavirus (HPV), and the viral E6 and E7 genes are presumed to co-operate in mediating altered cell proliferation and survival. Targeting of these genes with either synthetic siRNAs or hairpin-encoding vectors transfected into cultured HPV-positive carcinoma cell lines resulted in selective suppression of their expression. The resulting phenotypes were attenuation of cell proliferation, increased apoptosis, and reduced tumour size after transplantation into immunodeficient mice, and these phenotypic outcomes were not manifested in HPV-negative cells [51; 52; 53]. The development of several other types of

cancer are also associated with viral infection, for example Epstein-Barr Virus (EBV) in nasopharyngeal carcinoma, Kaposi's sarcoma-associated herpesvirus (KSHV) in primary effusion lymphoma, and human T-cell leukaemia virus type 1 (HTLV-1) in adult T-cell leukaemia, and in models of each of these diseases RNAi targeting of virally-encoded genes resulted in changes in invasive behaviour, tumour cell apoptosis or reduced tumorigenicity after transplantation into immunodeficient rats [54; 55; 56]. Viral genes are, therefore, potentially ideal targets against which to develop RNAi-based therapies, and the applicability of targeting viral sequences therapeutically is clearly not restricted to cancer.

The other class of genes that represent tumour cell-specific targets are those cellular genes that are mutationally activated. Prominent among these are the *RAS* genes, mutations of which are commonly found in a variety of cancers, notably carcinomas of the pancreas and colon. These mutations, frequently causing mis-sense changes at specific amino acid residues, result in the synthesis of *RAS* proteins that constitutively activate signalling pathways regulating diverse processes, including cell proliferation and survival. Infection of a pancreatic carcinoma cell line, carrying a mutant endogenous allele of the *K-RAS* gene, with a retrovirus directing the expression of an siRNA spanning and fully complementary with the mutant site, resulted in suppressed expression of the protein, almost completely abrogated anchorage-independent growth, and failure to produce tumours after transplantation into immunodeficient mice. Importantly, expression of the wild-type *K-RAS* gene was unaffected by the retrovirus in another cell line that carries mutant *H-RAS*, and these control cells retained their anchorage-independent proliferation after retroviral infection [57]. Comparable results have also been reported targeting mutant *H-RAS* [58]. These findings are notable in that they report discrimination between transcripts differing by single base differences, a feature that is likely to be necessary in developing mutant *RAS*-directed RNAi therapies that do not impinge on the anticipated important functions of unaltered *RAS* genes in normal cells. This potential for RNAi to manifest exquisite sequence specificity would at first sight appear to be one of its strengths, however absolute specificity is not always the rule and, we will argue, may in fact need to be avoided to target tumours effectively. The potential downside of allele-specific targeting (in common with fusion gene targeting, see below) is that the options for alternative siRNA design are severely limited should problems of efficacy or off-target effects arise.

Perhaps some of the most promising altered endogenous genes for RNAi targeting are those that encode fusion transcripts, due to the juxtaposition of portions of distinct genes as the result of chromosomal translocations. The breakpoints in the fusion transcripts are in one respect ideal targets for RNAi targeting, in that siRNA sequences can be designed that are only complementary to the respective normal cellular transcripts over ~50% of their length. The *Bcr-Abl* fusion gene is characteristic of chronic myelogenous leukaemia (CML) and some cases of acute lymphoblastic leukaemia (ALL), and encodes a protein tyrosine kinase with aberrant activity and intracellular localisation when compared with the unaltered cellular *Abl* enzyme. *Bcr-Abl* breakpoint sequences have been targeted using chemically synthesised siRNAs and an shRNA-encoding lentiviral vector [59; 60;

61; 62]. In a CML-derived cell line, or factor-dependent haematopoietic cell lines modified to factor-independence by experimental Bcr-Abl expression, targeting resulted in selective suppression of Bcr-Abl expression (sparing the endogenous Bcr and Abl products), a loss of viable cell number/increased apoptosis, and in one study an increase in sensitivity to -irradiation-induced cell death. Imatinib mesylate (Gleevec, Glivec, STI571) [reviewed in 63] is a small molecule that inhibits the tyrosine kinase catalytic domains of Bcr-Abl, c-Abl and several other kinases. Treatment with imatinib has resulted in complete cytogenetic (but rarely molecular) remission in a majority of chronic phase CML patients, and in lower proportions of patients with advanced disease. However, a significant proportion of patients suffer a relapse, often as a result of mis-sense mutations in the Bcr-Abl kinase domain [reviewed in 64]. There is, therefore, a need to develop further therapeutic approaches for drug-resistant CML. In cell line models artificially expressing Bcr-Abl, or two resistance-associated mutants, RNAi targeting the oncogenic gene product resulted in loss of cell viability, and an increased sensitivity to imatinib was observed after targeting the 'original' oncogene and one of the resistance-associated mutants [61], lending preliminary support to the hypothesis that RNAi might be employed in the future to overcome imatinib resistance. Promising though these findings are, one important point that has to be highlighted is that the only study that reported attempts to determine whether RNAi targeting of Bcr-Abl influences primary CML cell behaviour failed to detect any response [60]. Indeed, data on the outcome of RNAi targeting in primary human tumour cells (as opposed to cell lines) are notably lacking, and this must remain an important goal for future research.

The over-expression of numerous structurally un-altered gene products is associated with tumour development, and many of these are thought to have important effects on cell behaviour that could provide opportunities for therapeutic intervention. One example of this is the human epidermal growth factor receptor-2 (HER2) that is over-expressed in approximately one in three breast and ovarian carcinomas, and against which a therapeutically active monoclonal antibody has been developed. Targeting the HER2 mRNA with synthetic or retrovirally-encoded siRNAs resulted in compromised proliferation, survival, and tumourigenicity in immunocompromised mice, of carcinoma cell lines over-expressing the molecule; results that were consistent with expectations based on the findings of previous studies employing alternative experimental approaches [65; 66]. In an exciting recent development dsRNA-dependent gene silencing through DNA methylation, previously characterised in plants, has been shown to be inducible by siRNAs in mammalian cells [36; 37]. In the latter study, plasmids encoding shRNAs targeting regions rich in CpG dinucleotides (CpG islands) within the promoter of the *HER2* gene were transfected into a breast carcinoma cell line. This resulted in sequence-specific promoter methylation, suppression of *HER2* mRNA levels, and attenuation of cell proliferation. Although studies into this phenomenon are at a very early stage, it is possible that the induction of promoter silencing by RNAi may provide a route to attaining sustained changes in cell phenotypes after the introduction of only transiently maintained siRNAs, since

epigenetic gene silencing through DNA methylation can be heritable.

The paragraphs above have described studies on the RNAi targeting of genes that contribute to the tumourigenic phenotype per se, however it is worth highlighting the possibility of targeting a somewhat different class of gene for clinical benefit in cancer. Failure of chemotherapy is often as the result of a cross-agent tumour cell resistance phenotype called multidrug resistance (MDR), due to the over-expression of the P-glycoprotein product of the *MDR1* gene. In recent studies, *MDR1*-directed shRNA vectors and siRNAs introduced into drug-resistant tumour cell lines caused a suppression of P-glycoprotein expression, increased intracellular drug accumulation, and reversal of the MDR phenotype [e.g. 67; 68; 69], suggesting that RNAi could be employed in combination with 'classical' chemotherapies to overcome refractory disease.

### Strategies for Therapeutic Delivery

One of the most vexing problems that will face those wishing to exploit RNAi clinically is the problem of efficient delivery *in vivo*. The seriousness of the problem is compounded in the specific case of tumour therapy, since failure to target the great majority of affected cells will almost certainly result in therapeutic failure or rapid relapse. Nonetheless, serious attempts to develop RNAi therapies are underway, and some of the approaches have recently been reviewed [47]. Naked siRNAs, and plasmids encoding shRNAs, have been shown to be active in the tissues of experimental animals after 'hydrodynamic transfection' or electroporation [e.g. 70; 71; 72]. siRNAs complexed with agents such as cationic liposomes [e.g. 73] have been shown to be effective *in vivo* after intravenous injection, the complexes presumably enabling cell entry and possibly extending the half-life of the RNA in the circulation by protecting it from the actions of nucleases. Sensitivity to serum nucleases has also been reduced by developing chemically modified siRNAs which retain RNAi activity [e.g. 74]. Many investigators have produced modified viral vectors that might be suitable for delivering RNAi *in vivo*, including adeno-associated viruses, adenoviruses, retroviruses and lentiviruses [e.g. 75]. Each type of viral vector has its inherent properties with respect to achievable titres, immunogenicity, cell-type tropism, efficiency of infection and sustainability of expression after infection, that might make it more suited to one type of clinical application over another, and ongoing research is constantly improving their design. In those instances where the RNAi-induced phenotype in tumour cells is anything other than efficient induction of cell death, either protracted treatment with synthetic siRNAs or a stable transduction strategy would seem to be required, and for therapeutic efficacy we have already pointed out that the application of RNAi in cancer therapy might require effectively 100% 'hit' of the tumour cell population (unless the strategy enlists a 'bystander' effect or modifies the tumour environment for clinical benefit). In recognition of the fact that such efficient transduction *in vivo* is currently beyond our capabilities, one might consider attempting to develop an '*ex-vivo*' therapy. For example, since some malignancies of the haematopoietic system are already known to be treatable by bone marrow

transplantation, this type of approach could be modified to exploit RNAi. Patient-derived cultures enriched for haematopoietic stem cells could be treated *in vitro* by RNAi targeting and, after treatment of the patient with an ablative regime to remove residual disease, autotransplanted to reconstitute a 'corrected' immune system. Since *in vitro* vector targeting might not reach 100% efficiency, one could include in an RNAi delivery vector the capacity to express a marker allowing cell selection, whereby only transduced cells were ultimately transplanted.

### The Problems of Resistance and Specificity

As anyone working on cancer knows, the spectre of tumour resistance to therapy is ever-present. We envisage at least two general routes to RNAi resistance that will need to be considered, yet very little attention has been paid to these. It has been argued that RNAi may ultimately prove to be a significantly more robust and reliable gene silencing strategy than the various 'antisense' approaches that have been tried, since it enlists 'natural' cell-intrinsic processes. This very feature of RNAi may, however, prove to be one of its disadvantages in the cancer therapy context. Our results, and numerous anecdotal reports, suggest that intact functioning RNAi pathways may not be required for cell viability; thus tumour cells may acquire (or possess at the outset) the ability to escape any RNAi-based therapy. Advancing understanding of the molecular components that underlie RNAi [reviewed in 2] has revealed multiple proteins whose functional inactivation would be predicted to interrupt gene silencing pathways, each of which could potentially be subject to alteration in tumours as a pre-requisite of resistance. Alternatively, there may be inhibitors of RNAi pathways, whose de-regulation could equally be associated with escape from RNAi-targeting. One protein in the latter category might be the probable human orthologue of the *C. elegans* protein ERI-1, an siRNA-degrading exonuclease that is responsible for suppressing RNAi activity in specific tissues of the organism [76]. It is not clear at present how this type of resistance could be circumvented, except possibly in an *ex-vivo* treatment/transplantation strategy (see above), in which the RNAi delivery vector could conceivably be modified such that transduced cells incapable of RNAi were selectively killed. In common with many 'classical' drugs, RNAi may be best employed in combination with other agents to reduce the likelihood of minority populations of resistant cells surviving.

We can be almost certain that another type of RNAi resistance would be encountered, that being target gene-specific resistance, and at this point it might be informative to briefly review the experience of CML treatment with the kinase inhibitor imatinib (see above). Patients who suffer relapse of their disease due to imatinib resistance often do so as a result of mis-sense mutations of the *Bcr-Abl* gene, the product of which is the target of imatinib [77], and some mis-sense mutations have been found to exist before the onset of therapy [e.g. 78]. The amino acid substitutions allow the oncogenic protein to retain its biochemical functions whilst decreasing sensitivity to the drug, and the base-change options for this outcome are presumably relatively limited. Nonetheless, *Bcr-Abl* mis-sense mutations present a major clinical problem. In the case of RNAi

targeting employing siRNAs that are sensitive to single base mismatches (such as oncogenic *RAS* gene targeting, see above), it would seem likely that resistance through point-mutation might arise relatively frequently, especially as synonymous base changes would be equally effective escape routes as those that cause coding alterations. Add to this the possibility that the RNAi targeting strategy may need to be directed at a region encoding a portion of the protein on which there are few structural constraints, as may be the case with the breakpoint sequences in some fusion genes, and the prospects for specific RNAi targeting without tumour resistance begin to look bleak. One simple approach applicable in those instances when allele-specific targeting is not required (e.g. viral and over-expressed genes), would be to simultaneously treat with multiple siRNAs directed at distinct regions of the mRNA, thus ensuring that cells would need to have acquired multiple alterations in individual alleles in order to escape RNAi. In those cases of allele-specific targeting (e.g. mis-sense mutations and fusion breakpoints) such an option is not available. For these reasons, we propose that those of us considering developing RNAi for cancer therapy might investigate discarding the, at first sight attractive, idea of maximum specificity, in favour of a deliberate attempt to achieve reduced stringency while retaining targeting efficiency. RNAi has been demonstrated to be effective on target genes with 3-4 base mis-matches between siRNA and mRNA, [34], and while suppression mediated by mismatched siRNAs may frequently be due to efficient translational inhibition it may also direct target cleavage [79]. It remains to be seen whether such observations are of general applicability, or whether particular targeting sequences are inherently less stringent than others in their actions, but we propose that further attention to RNAi specificity may allow the design of 'relaxed' siRNAs that are effective but significantly less prone to escape by target mutation. One obvious counter-argument to this proposal is that reduced specificity for the sequence of the intended target must entail an siRNA being more likely to manifest sequence-dependent off-target effects. Several studies have revealed apparent off-target effects of RNAi in mammalian cells, and experiments documenting transcript profiles resulting from siRNA-induced gene silencing have been interpreted as showing targeting due to sequence identity over only 11 contiguous nucleotides [80]. When one also considers that several studies have suggested that, contrary to the original assumptions, a subset of short (as opposed to long, >~30bp) interfering RNAs seems to non-specifically activate interferon-dependent signalling [e.g. 81; 82], do the accumulating data suggest that RNAi might have to be abandoned as a therapeutic possibility on specificity grounds?

We propose that RNAi-based therapeutic strategies should, indeed, be considered seriously. While some are likely to suffer from un-anticipated off-target effects, as do 'classical' small molecule drugs, this should not necessarily deter us. What RNAi potentially offers is a rational approach to the extremely rapid and cheap design of 'lead agents', which will then need to be tested and refined just as extensively as any drug does currently. What we also have in RNAi is the ability to design active agents irrespective of the specific biochemical function of the target; the important issue being selectivity of the cellular responses to the

therapy. The efforts of the pharmaceutical industry are currently heavily biased to studying members of gene families such as protein kinases for which there are clear precedents of successful targeting with small molecules [e.g. 83], but RNAi holds the promise of significantly broadening the scope for therapeutic development since its target is, in principle, simply nucleic acid sequence. We should not underestimate the advances that are still required, notably in ways to deliver RNAi to a frequently highly disseminated disease, and in strategies to minimise the incidence of tumour resistance, but RNAi may yet have an important place in cancer clinics some time in the future.

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