SURVEY AND SUMMARY

Managing the sequence-specificity of antisense oligonucleotides in drug discovery

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ABSTRACT

All drugs perturb the expression of many genes in the cells that are exposed to them. These gene expression changes can be divided into effects resulting from engaging the intended target and effects resulting from engaging unintended targets. For antisense oligonucleotides, developments in bioinformatics algorithms, and the quality of sequence databases, allow oligonucleotide sequences to be analyzed computationally, in terms of the predictability of their interactions with intended and unintended RNA targets. Applying these tools enables selection of sequence-specific oligonucleotides where no- or only few unintended RNA targets are expected. To evaluate oligonucleotide sequence-specificity experimentally, we recommend a transcriptomics protocol where two or more oligonucleotides targeting the same RNA molecule, but with entirely different sequences, are evaluated together. This helps to clarify which changes in cellular RNA levels result from downstream processes of engaging the intended target, and which are likely to be related to engaging unintended targets. As required for all classes of drugs, the toxic potential of oligonucleotides must be evaluated in cell- and animal models before clinical testing. Since potential adverse effects related to unintended targeting are sequence-dependent and therefore species-specific, *in vitro* toxicology assays in human cells are especially relevant in oligonucleotide drug discovery.

INTRODUCTION

Hypothesis-driven drug discovery is based on the premise that disease states can be modulated in a desirable manner by perturbing the function of carefully chosen molecular targets. It is therefore a pragmatic goal of drug discovery to ensure that the chemical compounds developed interact specifically with their intended biomolecular targets and do not perturb the functions of any other molecules. In essence, the fewer unintended targets a compound has, the less likely it is to have adverse events related to unintended targeting. Indeed, recent experiments in mice suggest that the number of unintended RNA targets that are effectively reduced in the liver after systemic administration of antisense oligonucleotides (AONs), can be correlated with the hepatotoxic potential of the oligonucleotides, as measured using biochemical markers in the blood (1–3). In addition, highly specific compounds developed during such discovery efforts can help to identify the effects of modulating the intended target more clearly.

All drugs modulate cellular processes that affect the transcriptome

Highly specific drugs acting exclusively on their intended target, as well as those that also have a number of unintended targets, will, as a consequence of their downstream effects, eventually have an impact on the expression of multiple genes that are themselves not directly targeted. This can be measured using global transcriptome analysis in a straightforward manner. As an example, we retrieved gene expression profiles from the connectivity map (4), a public data resource containing information on the transcriptome changes in cells induced by 1309 small molecule compounds (SMCs) and other bioactive molecules. These profiles of drug-induced transcript-level changes have been used to uncover new effects of known drugs, which have subsequently

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been experimentally verified ([5]). A simple, high level analysis of the 315 Food and Drug Administration (FDA)-approved SMC drugs in this dataset (Supplementary Table S1) reveals that most of these drugs, when given at pharmacologically relevant doses, change the expression level of between 25 and 130 genes by >50% (first and third quartiles, respectively), with the median being ∼60 genes (see Figure 1A). Notably, anti-parasitic and oncology drugs tend to have a larger impact than other classes of drugs, with a median of ∼140 genes, and are therefore presented separately in Figure 1A.

We wanted to compare the transcriptome profiles for small molecules to those for AONs. AONs are short, single-stranded, DNA molecules that have been chemically modified to confer drug-like properties. They modulate the function of their RNA targets through various post-binding mechanisms such as protein blocking or RNase H-mediated cleavage. We therefore retrieved transcriptome data from public repositories for 25 AONs (Supplementary Table S2). The AONs were assayed under conditions similar to those for the SMCs featured in the connectivity map. We found that the AONs elicit transcriptome changes in the same range as non-antiparasitic and non-oncology FDA-approved drugs (median of ∼50 genes), as shown in Figure 1A. This suggests that the gross impact on the transcriptome is similar for AONs and approved SMCs, even though oligonucleotides can potentially affect RNA directly, whereas small molecules presumably have more indirect effects on transcript levels. That is, all drugs modulate cellular processes that eventually affect the transcriptome.

It also follows from this analysis that the potential for toxicological effects, derived from intracellular binding events that perturb the transcriptome, is not different for oligonucleotides and approved small molecules. The same thorough preclinical toxicological studies must therefore be performed for oligonucleotides as for other drug classes ([6]).

Oligonucleotide specificity can be evaluated using sequence analysis and transcriptomics

AON interactions with unintended targets are typically divided into hybridization-dependent interactions with RNA, and hybridization-independent interactions with proteins ([7]) (Figure 1B). The hybridization-independent interactions are often related to AONs with a phosphorothioate backbone. Indeed, interactions with intracellular proteins ([8]), plasma proteins ([9]), cell surface proteins ([10–12]) growth factor proteins ([13]), or with components of the immune system ([14]), are examples of hybridization-independent interactions that have been observed for some oligonucleotides with phosphorothioate backbones. In fact, a class of oligonucleotides termed aptamers with protein-specific binding properties can be produced through an *in vitro* selection process. As has been reviewed elsewhere ([15,16]), such aptamers can modulate the activity of the proteins they specifically bind to and provide therapeutic benefit. As with some small molecule pharmacophores, hybridization-independent interactions between some AONs and specific proteins can also lead to unwanted toxicities (Figure 1B) ([7,9,17]). Here, we will focus on those AONs termed gapmers that can cleave RNA by recruiting RNase H, and

discuss methods for evaluating hybridization-dependent effects on unintended RNA targets. The sequence-specificity of siRNAs has been reviewed elsewhere ([18,19]). As will be reviewed here, the sequence-specificity of gapmers differs markedly from that of siRNAs. Contrary to most other drug modalities, AONs have advantages in terms of both the computational predictability and experimental measurability of unintended targets. The sequences of both AONs and RNA are composed of a small set of different nucleotides, and can be analysed using methods from the most mature of all bioinformatic subdisciplines, sequence analysis ([20]). Moreover, the predictable binding properties of any nucleic acid through Watson–Crick base pairing have facilitated the development of quantitative measurement methods such as qRT-PCR ([21]), rapid amplification of cDNA ends (RACE) ([22,23]), serial analysis of gene expression ([24]), microarrays ([25]), and RNA sequencing ([26]), which allows cost-effective measurements of the global transcriptome, including all unintended RNA targets. Although small molecules and oligonucleotides appear to impact the transcriptome to the same degree (Figure 1A), computational and experimental methods therefore exist for oligonucleotides that allow discrimination between effects on intended and unintended RNA targets, as well as their respective downstream secondary effects (Figure 1B), enabling a more precise assessment of specificity.

Defining specificity

For gapmers we divide effects from unintended RNA targets into those which are subject to the same mechanism as the intended effect (here RNase H-induced target degradation) and those where hybridization to the unintended target elicits effects through other mechanisms (such as splice modulation, blocking of binding sites for microRNAs or RNA binding proteins, and more). We expect degradation of unintended RNA targets to be the dominating mechanism influencing the specificity of gapmers (see the subsection headed "Specificity of the same gapmer for different targets"), and we focus on this mechanism here. In this case, specificity can be defined as the rate of cleavage of the intended RNA target, V_{int}, relative to the rate of cleavage of all other, unintended, RNA targets ([27,28]):

$$\frac{V_{int}}{\sum_{i \in unint} V_i} \qquad \text{(definition based on rates)}$$

When cleavage rates cannot be easily measured or inferred, specificity can alternatively be defined as the ratio between the total number of different RNA targets reduced below some pre-determined level after treatment, $N_{reduced}$, intended as well as unintended, relative to all RNA molecules measured, $N_{measured}$:

$$\frac{N_{reduced}}{N_{measured}} \qquad \text{(definition based on counts)}$$

This definition of specificity was originally proposed for kinase inhibitors interacting with panels of protein kinases in competition binding assays ([29]). Both definitions of sequence specificity are dependent on the concentration of gapmer used. When using either of these definitions for calculating specificity, we therefore suggest the evaluation of
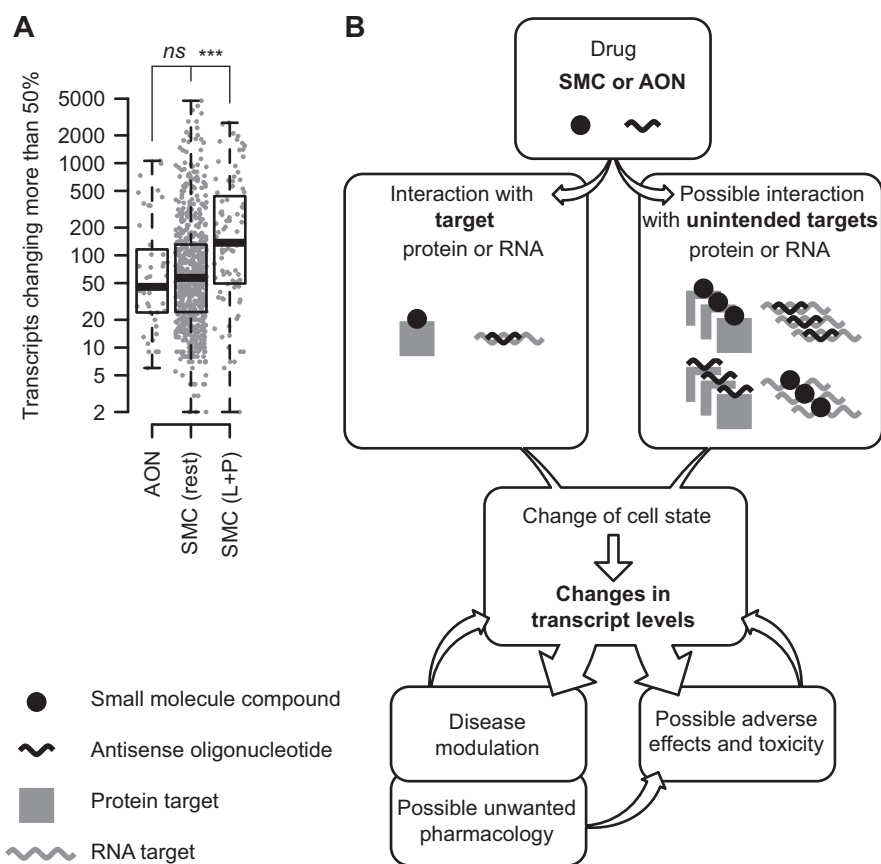
Figure 1. All drugs affect the transcriptome. (**A**) Global transcriptome effects *in vitro* upon treatment with drugs. Microarray data for 25 antisense oligonucleotides (AONs) were retrieved from public repositories (Supplementary Table S2), and for 315 FDA-approved small molecule compounds (SMCs) from the connectivity map repository (4) (Supplementary Table S1). Analysis of the SMCs revealed that those with first level Anatomical Therapeutic Chemical codes L (antineoplastic and immunomodulating agents) and P (antiparasitic products) affected significantly more genes than the others ($P < 0.001$, Wilcoxon test). They are therefore shown in a separate column. Both AONs and SMCs were typically evaluated at several different doses and in multiple cell lines (Supplementary Tables S1 and S2). For this analysis, all microarray data were preprocessed in the same manner, using robust multiarray averaging (129). Data from each study were preprocessed individually, except for the connectivity map data, where each SMC with its designated vehicle controls was preprocessed individually. Microarray data derived from seven different Affymetrix platforms. To allow comparisons across microarray types, a set of 11 367 genes assayed on all platforms was identified and the number of genes that changed expression by >50% in this common set of genes was calculated for each AON or SMC. (**B**) Schematic connecting drugs with their primary intended and unintended targets and the net downstream secondary effects. Here, we only consider RNA molecules as intended targets of AONs, and proteins as intended targets of small molecule compounds. In principle, and as unintended targets, AON binding to proteins and SMC binding to RNAs are also possible.

several different concentrations to choose the concentration that best allows discrimination between different gapmers.

To compare the two definitions, let us say that two different gapmers each reduce an unintended target to the same extent, but reduce the intended target to different extents. According to the definition based on cleavage rates, the gapmer that reduces the intended target the most will be the one judged as the most specific. However, from the definition based on counting the number of targets reduced, if the threshold level of reduction has not been chosen carefully, the two gapmers could be judged as being equally specific. Conversely, say that for two different gapmers, one reduces a single unintended target to the same extent as the intended target, whereas the other reduces two unintended targets, but only half as much as the intended target. The specificity definition based on cleavage rates would rank the two gapmers as equally specific, whereas the counting-based definition would rank the gapmer with only one unintended target as the most specific.

Pragmatically, the definition based on counts is the one most easily applied to transcriptomics studies, the main way of studying gapmer specificity, and will therefore be the definition of choice in most cases. As defined above, however, it does not differentiate between intended and unintended targets. Many of the RNAs measured as reduced, and therefore contributing to $N_{reduced}$, may not be unintended targets, but merely secondary effects of engaging the intended target. It will therefore be more precise to only count the subset of reduced RNAs that are indeed unintended targets. Methods for identifying this subset in transcriptomics studies are discussed further in Section 3.2. Furthermore, as discussed above, the introduction of a threshold level of reduction is somewhat arbitrary. It may, in many cases, be helpful to consider the magnitude of the difference between the effect on the intended target and the unintended targets. For example, by measuring the knockdown of the intended target and unintended targets at multiple gapmer concentrations, so that it can be estimated at which concentrations half-

maximal effects are achieved on both intended and unintended targets, EC50$_{int}$ and EC50$_{unint}$, respectively, a specificity definition based on such potency estimations could be

$$\frac{EC50_{int}}{\sum_{i \in unint} EC50_i} \qquad \text{(definition based on potency)}$$

In the subsections headed "RNA target dynamics" and "Target degradation by RNase H", the concepts of gapmer potency and EC50 estimations are discussed further.

Clearly, the definition of specificity matters when deciding how to optimize for it in discovery projects. As a final remark, for experimental biologists working with oligonucleotide probe hybridization to membrane-bound RNA or DNA (30), stringency is another often-used term to denote the extent to which hybridization can occur between nucleic acids with mismatched sequences. When washing at high-stringency conditions, typically achieved by reducing salt concentrations or increasing temperature, one can ensure that only nucleic acids that are perfectly complementary to each other will hybridize. Conversely, under low-stringency conditions, nucleic acids can hybridize despite some base mismatches. In contrast to the many experimental variables that can be changed to approach high-stringency conditions in such experiments, when dealing with oligonucleotides in animals or humans, it is only variables in the oligonucleotide itself that may be altered to ensure that mostly nucleic acids that are perfectly complementary to each other will hybridize. This is the reason we do not use the term stringency in this review, but focus on the inherent variables that influence such sequence-specificity of oligonucleotides. Also, in contrast to stringency, when considering the sequence-specificity of gapmers, the properties of the duplex between gapmer and RNA as a substrate for the RNase H enzyme also matters, as discussed in the next section.

DETERMINANTS FOR RNASE H-ACTIVITY ON UNINTENDED TARGET RNA

The factors that determine gapmer activity, i.e. binding and cleavage, at the level of the unintended target RNA are exactly the same as those that determine activity at the intended target RNA molecules. Hence, understanding how to design gapmers with high activity contributes to understanding how to design for high specificity and vice versa. Within the intracellular compartments where the RNA targets reside, the major factors that are known to be relevant for gapmer binding and cleavage are summarized in Figure 2, and will be described in more detail in the following subsections.

Pharmacokinetic properties, such as absorption, distribution, metabolism, and excretion, which govern how much gapmer ends up in different tissues, and in the intracellular compartments where the RNA targets reside, also affect specificity. For example, a gapmer distributed to several tissues and cell types may manifest a different sequence-specificity in each cell type, since expression of potential unintended targets and their dynamical behavior can be tissue- and cell-type specific (6). In addition, the effect of degrading unintended targets may be highly dependent on the duration of exposure, which may also differ between tissues and
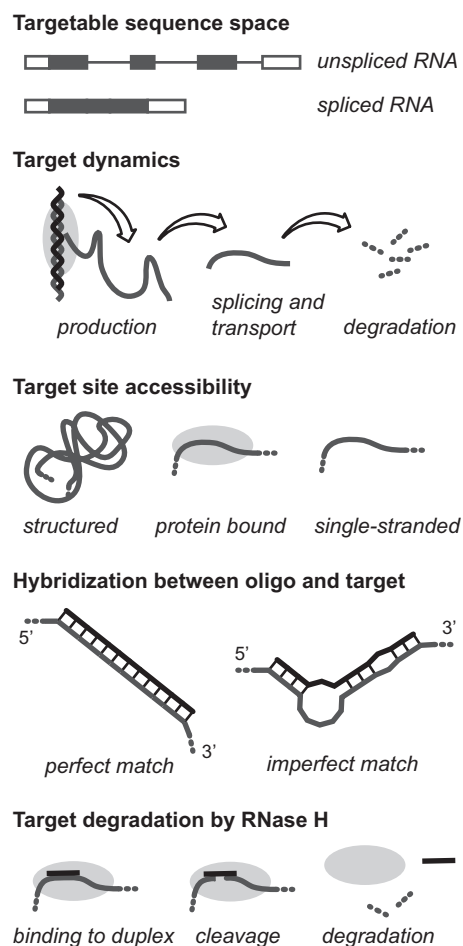

Targetable sequence space

unspliced RNA

spliced RNA

Target dynamics

production   *splicing and transport*   *degradation*

Target site accessibility

structured   *protein bound*   *single-stranded*

Hybridization between oligo and target

5'   3'
5'
perfect match   3'   *imperfect match*

Target degradation by RNase H

binding to duplex   *cleavage*   *degradation*

Figure 2. Summary of major factors affecting gapmer activity on intended and unintended RNA targets: Targetable sequence space, target dynamics, target site accessibility, hybridization between gapmer and target, and target degradation by RNase H. Refer to subsections in the section headed "Determinants for RNase H-activity on unintended target RNA" for details.

cell types. Pharmacokinetic considerations may therefore help to determine in which tissues and cell types specificity needs to be evaluated. However, it is beyond the scope of this review to discuss pharmacokinetics. Instead, we focus on the properties that determine specificity once the gapmer has reached the subcellular compartments, where RNA targets, intended and unintended, reside.

The targetable sequence space

The activity of gapmers is primarily mediated by the RNase H1 enzyme (31). Mammalian RNase H1 is present in the nucleus and mitochondria (32). In mouse liver, using subcellular fractionation and RNase H gel renaturation assays, RNase H1 was found to be relatively more abundant in nuclei than in mitochondria (33). Therefore, the most efficient RNase H-mediated cleavage takes place in the nucleus. Indeed, it has been demonstrated that both nuclear-retained noncoding RNA (34) and coding RNA at both intronic and exonic target sites (35–37) can be efficiently targeted by gapmers and cleaved by RNase H.

Direct measurements of RNase H localized in the cytoplasm are scarce (38). However, RNase H-mediated cleavage also takes place in the cytoplasm (39). Exemplifying this, potent gapmers have been designed, which effectively silence the hepatitis C viral RNA genome (40), residing in the cytoplasm (41–43). Importantly, iso-sequential oligonucleotides with a locked nucleic acid (LNA)-modification pattern not allowing RNase H-recruitment, termed mixmers, did not reduce viral RNA, indicating that the reduction was indeed mediated by RNase H (40).

Taken together, these results demonstrate that the transcriptome residing in both nucleus and cytoplasm can be targeted by gapmers, and therefore must include both unspliced and spliced RNA (Figure 2).

RNA target dynamics

Cellular RNA steady-state levels are determined by the interplay of RNA production, processing and degradation, and the turnover rates of these mechanisms can vary widely between different RNA molecules (44). Studies in mammalian cells have used metabolic labeling of RNA with derivatives of uridine, such as 4-thiouridine (45) or bromouridine (46), to allow capture and separation of recently transcribed RNA from the overall RNA population, followed by sequencing. Such transcriptome-wide evaluations of production and degradation dynamics have revealed significant variations between genes, as well as coordinated and complex regulatory control in response to, for example, lipopolysaccharide- (45) and tumor necrosis factor-induced inflammation (46). Recruitment of RNase H to the oligonucleotide-RNA duplex, and subsequent cleavage of the RNA, introduces an additional mechanism by which RNA can be degraded. This is because the two fragments of the cleaved RNA are not protected by a 5′-cap or poly-A tail at the cleaved ends, and they are therefore rapidly degraded by exoribonucleases present in both nucleus and cytoplasm (44,47). For any RNA molecule, upon introduction of such an RNase H-mediated mechanism of degradation, the relative contribution of antisense-mediated degradation to the overall RNA degradation naturally depends on the magnitude of the endogenous degradation rate for that RNA. That is, for targeted RNA, where the endogenous degradation rate is high, the relative contribution of the antisense-mediated degradation will be comparatively smaller than for targeted RNA that is degraded endogenously at a much slower rate. This exemplifies the importance of target dynamics in gapmer activity. It is usually easier to discover active gapmers against targets with a low turnover rate.

Recently, we modelled the reactions between gapmer, RNA target and RNase H as a four-step process (48). First, the gapmer hybridizes to the RNA target. Second, the RNase H enzyme binds to the gapmer/RNA duplex. Third, the enzyme cleaves the target to yield a complex of gapmer, cleaved target and enzyme. And fourth, the complex dissociates, releasing gapmer and enzyme for a new cycle, and exposing the cleaved RNA fragments to rapid degradation by exonucleases. This process was written as a set of ordinary differential equations and solved numerically (48). In the subsection headed "Relating overall free energy of binding with potency" below, we present a simplified version of this model to explore the relationship between binding affinity and potency. The model predicts that the endogenous rates of RNA production and removal (Figure 2) influence both the potency and efficacy of gapmers (48). Here, potency is defined as the inverse of the concentration at which a half-maximal knockdown effect is achieved, and efficacy is defined as the maximal knockdown that can be achieved. For example, keeping the target production rate constant, an increase in the rate of endogenous target removal results in the gapmer being less efficacious and not as potent. However, lowering the production rate while keeping the endogenous removal rate constant, results in the gapmer being not as potent but with unchanged efficacy (48). Any endogenous mechanism that removes RNA, so that it is not available as a target for the gapmer, is covered by the model (48). That is, the rate of disappearance of target RNA matters, but the reason for this disappearance does not. Indeed, the dominating mechanism will be different for RNase H-mediated degradation taking place in the nucleus and in the cytoplasm, respectively. For gapmers where the RNase H-mediated degradation primarily takes place in the cytoplasm, the endogenous mechanism for RNA disappearance will be RNA degradation in P-bodies via ribonucleases. However, for gapmers where the RNase H-mediated degradation primarily takes place in the nucleus, the endogenous mechanism for RNA disappearance will be the processing and transport of RNA out of the nucleus.

For siRNAs, similar observations have been made based on modeling, which have been supported to some extent by experimental evidence (49,50). To the best of our knowledge, only a single published study has investigated the effect of RNA target production rate on gapmer activity (51). In this study, RNA levels and transcription rates were systematically varied in cells, both for an exogenous gene expressed after transfection and an endogenous gene induced using a cytokine. Surprisingly, these variations did not appear to affect the potency of the gapmers. The gapmers used in the study were only phosphorothioate-modified, however, and therefore of low affinity and stability. A plausible explanation for these results, which was also suggested by the authors, may be that the number of cell-associated gapmers needed to achieve an effect was in vast excess of the RNA copy numbers present during the experiments. In this case, it can indeed be shown that primarily affinity, and not target RNA levels, determine potency (see the subsection headed "Target degradation by RNase H"). Repeating such a study with high-affinity gapmers dosed at pharmacologically relevant levels would help to confirm this.

In general, further experiments are needed in this field to clarify to what extent, and in which compartments, transcriptome dynamics influence the amenability of particular RNA targets to gapmer treatment.

Target site accessibility

RNA folds into complex secondary and tertiary structures, where some segments hybridize to neighboring or more distal segments to form hairpins and other RNA–RNA interactions (52,53). Most RNA (including mRNA) in the cell is also bound by a large variety of RNA-binding proteins (54).

Gapmers have to compete with these structures and proteins to access the target site (Figure 2). The more structured and inaccessible a given target site is, the lower the activity of a gapmer against that site, while more accessible targets can facilitate higher levels of gapmer activity. The role of RNA secondary structure in gapmer activity has been demonstrated directly by Vickers *et al.* (55), by cloning a specific target site into a luciferase reporter gene along with different adjoining sequences that formed secondary structures to varying degrees. When analyzing the same gapmer against each of these constructs, they were able to show that the activity of the gapmer was reduced when the target site was part of a double strand in a secondary structure in the RNA.

There are well-established experimental methods based on chemical probing that allow the structure of specific transcripts to be determined (53,56). Methods that probe the secondary structure of RNA on a transcriptome-wide scale are also being developed (57–60). Furthermore, local secondary structure can be predicted from sequences using dynamic programming algorithms that take experimentally measured values of stacking and destabilizing energies into account, such as mfold (61). Based on such algorithms, accessibility predictors that compute probabilities of short stretches of RNA being unpaired have been developed, such as sfold (62) or RNAplfold (63). Indeed, on two sets of 573 and 360 siRNAs, RNAplfold showed reasonable success in correlating siRNA activity with predicted local accessibility (63). Recently, RNAplfold was also used to successfully correlate large differences in target RNA reduction by gapmers to differences in predicted local accessibility (64). In the same study, the impact on target inaccessibility due to protein binding was compared with the impact due to predicted target RNA structure. Here, gapmer activities on target RNA in cells, where proteins were bound to the RNA, and in a cell-free system, where proteins were not bound, were seen to be relatively similar, leading to the conclusion that RNA structure has a significantly greater effect on gapmer activities than protein binding (64). To more comprehensively evaluate the effects of protein binding to RNA, large transcriptome-wide occupancy maps are starting to appear. These maps are based on UV crosslinking and immunoprecipitation of mRNA–protein complexes in combination with sequencing, to identify RNA binding sites (65,66). These global maps are based on the spliced transcriptome, however, which limits their usefulness for rational specificity optimization, since gapmers also target the unspliced transcriptome (subsection headed "The targetable sequence space").

In principle, the competing interactions from higher order structures in RNA and protein binding should be assessed for every potential target site to evaluate the likelihood of oligonucleotide binding at that site. Alternatively, methods to directly evaluate target site accessibility to gapmers have also been investigated. By using random libraries of 10^6–10^{12} different gapmers of defined length against a single RNA target, the target sites most accessible to RNase H-mediated cleavage by oligonucleotides from the library can then be determined by measurement of cleavage fragments (67). This method and other similar methods have been reviewed elsewhere (68).

As a final note, just as the RNA molecule can form secondary structures with itself, so can the oligonucleotide. Such oligonucleotide structures can be divided into duplexes formed between two oligonucleotides, and folding of a single oligonucleotide into a stable hairpin structure. Oligonucleotides forming self-structures first need to break those structures before hybridizing with RNA. Gapmers predicted to form duplexes have been associated with decreased activity (69). However, although oligonucleotide structures are detrimental to the binding between oligonucleotides and the RNA target site, they may improve other drug-properties, such as transport or uptake. For splice-modulating oligonucleotides transfected into human myotube cultures, those predicted to form duplexes have been associated with increased activity (70), although this observation may be an artifact of the delivery procedure (71).

Hybridization between oligonucleotides and (unintended) target sites

The most important determinant of the effect of an oligonucleotide on a target site is the annealing reaction between them, resulting in their hybridization.

Hybridization between two complementary nucleic acid strands is governed by hydrogen bonding between base pairs on opposite strands and base stacking (72,73). The stability of the duplex is mainly driven by stacking effects in the double helix (74), but water exclusion and counterion structuring also contribute (75). The hydrogen bonds between opposing bases are primarily responsible for base pairing selectivity (76). Indeed, the Watson–Crick base pairs between adenine (A) and uridine or thymine (U or T), and between guanine (G) and cytosine (C), where hydrogen bonds are optimally aligned sterically (73), generally result in the strongest binding, although many other hydrogen bonded base pairs are possible (77). A base pair that is not a Watson–Crick pair, between A and U/T, or between G and C, is here called a mismatched base pair. The binding affinity of fully complementary and partially mismatched nucleic acid duplexes can be measured in melting experiments (78,79). Thermodynamic parameters based on nearest-neighbor approximations (80) can then be calculated from UV absorbance versus temperature curves. The major assumption in such nearest-neighbor models is that the contribution to overall binding affinity for a base pair is dependent only on the identity of adjacent base pairs, since the major interactions involved, hydrogen bonding and stacking, are both short-range interactions. In this manner, the contribution to the standard free energy of binding for any dinucleotide base pair, such as the 16 possible fully complementary dinucleotide base pairs (such as AA/TT and AT/TA), as well as the 64 possible dinucleotide base pairs where one mismatch is allowed (such as GA/TT and GT/TA), can be reliably determined (81). Since the standard free energy of binding at 37°C, $\Delta G°$, is logarithmically proportional to the dissociation constant for oligonucleotide duplexed to an RNA target (79), $\Delta G°$ is considered a physiologically relevant representation of binding affinity (78).

As an example, we can calculate the effect on affinity as a function of changing a G/C base pair to either a G/G mis-
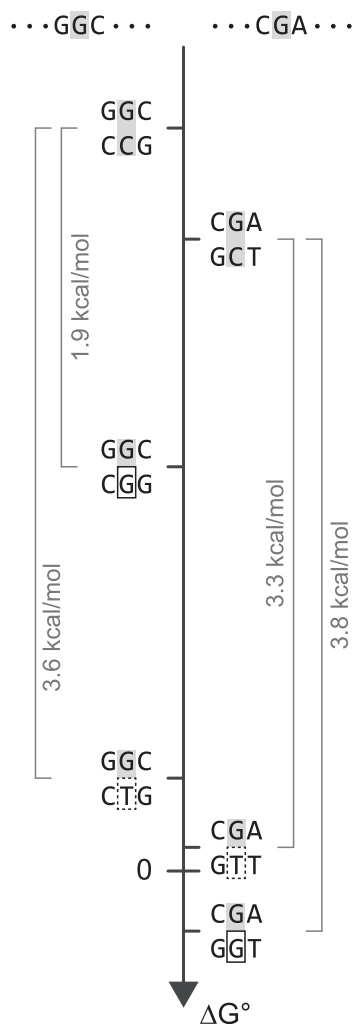
Figure 3. The effect of mismatches on affinity depends on the identity of the mismatched base pairs and the sequence context. The vertical axis indicates standard free energy, $\Delta G°$. On each side of the axis are indicated $\Delta G°$ values for three examples of trinucleotide bases, which are paired either fully matched or with one central mismatch. On the left-hand side, the trinucleotide is GGC, and on the right-hand side it is CGA. The two mismatch-examples are G/G and G/T on both the left- and right-hand sides. Thermodynamic parameters for DNA–DNA binding were used to calculate $\Delta G°$ values (81).

match or a G/T mismatch, and show that it depends on the surrounding base pairs. For this calculation, we use thermodynamic parameters for DNA–DNA binding (81). First, we consider the case with a G immediately upstream and a C immediately downstream, so that the sequence reads (from 5′ to 3′) GGC. The fully complementary binding region is then CCG (from 3′ to 5′), and the free energy of binding $\Delta G°$ for this region is calculated to be −4.1 kcal/mol, as shown in Figure 3 at the left-hand side of the vertical axis. If a G instead of a C is introduced in the central position of the binding motif, giving CGG, $\Delta G°$ increases by 1.9 kcal/mol (lower affinity). If instead, the central mismatch is a T, giving CTG, a much larger reduction in affinity is seen, with $\Delta G°$ increasing by 3.6 kcal/mol (Figure 3, left). That is, the reduction in binding strength introduced with a single mis-

match depends on the type of mismatch, where a G/G is less detrimental than a G/T.

If a different sequence context, CGA, is considered, the fully complementary binding region is then GCT (from 3′ to 5′), and $\Delta G°$ for this region is calculated to be −3.5 kcal/mol, as shown in Figure 3 at the right-hand side of the axis. If, as in the first example, a mismatched G is introduced at the central position, the increase in free energy is twice as large as for the first sequence context (GGC), increasing by 3.8 kcal/mol. Interestingly, whereas for the first sequence context, a G/T mismatch was more detrimental than a G/G mismatch, with the CGA sequence context, the G/T mismatch only increases $\Delta G°$ by 3.3 kcal/mol, which is less than the effect of the G/G mismatch (Figure 3, left-hand side).

Thermodynamic parameters are different for DNA–RNA binding compared with DNA–DNA binding (82,83) and DNA–RNA binding is also affected by chemical modifications such as LNA (84,85). This will change the numerical values for the thermodynamic parameters in the nearest-neighbor model, but not the conclusion, which is that oligonucleotides will bind with highest affinity to their fully complementary intended RNA target regions, but they can also bind, albeit with lower affinity, to unintended regions of RNA where one or more bases are mismatched. The strength and extent of mismatched binding will depend on the identity of the involved base pairs as well as the neighboring nucleobases (Figure 3).

Target degradation by RNase H

RNase H1 is a non-sequence-specific endonuclease that recognizes RNA–DNA heteroduplexes and specifically cleaves the RNA strand (86). The molecular structure of RNase H1 complexed with an RNA/DNA substrate has been identified using crystallography (87). Since most of the modifications that increase affinity, such as LNA, 2′-*O*-methoxyethyl and 2′-*O*-methyl (2′OMe), do not recruit RNase H (7), modified oligonucleotides are typically designed with high-affinity nucleotides in the flanks and a central gap of DNA, hence the name gapmers. As discussed in the subsection headed "The targetable sequence space", it is the RNase H1 enzyme variant that primarily contributes to oligonucleotide-mediated degradation. Here, we focus on the effects of mismatched base pairs on human RNase H1 cleavage rates, which is of particular interest when evaluating specificity. In a recent study, RNase H1 was incubated with a gapmer duplexed with a mismatched RNA (88). The subsequent RNA cleavage fragments were measured using phosphor imaging. About half of the gapmers tested with single mismatches at various positions had decreased cleavage rates compared with the fully complementary gapmer. The other half, however, had increased rates of cleavage. Clearly, therefore, RNase H1 is able to cleave mismatched duplexes. The variability in the position of the cleavage sites and the rate of cleavage, as reported in the study (88), also demonstrate that the RNase H enzyme has some degree of sequence-motif-dependence, probably relating to the overall structure of the gapmer when duplexed with RNA. The correlation between the structure of the RNA-DNA duplex and its properties as a substrate for RNase H has been re-
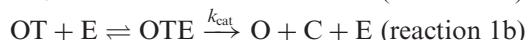
viewed by Zamaratski *et al.* (89). In a recent study, we explored the sequence-specificity of gapmers after transfection into HeLa cells, using massively parallel reporter analysis of short RNA motifs (28). The HeLa cells were first transfected using a library of plasmids, expressing identical reporter genes except for a degenerate 7mer subsequence in the 3′ UTR. The gapmers were designed with LNA-flanks perfectly complementary to the 3 nt-flanking regions up- and downstream of the degenerate 7mer sequence in the reporter genes. By sequencing cDNA derived from the plasmid library, the effects of targeting the degenerate region with each gapmer could be determined. In effect, for each gapmer, quantitative information was obtained for over 15 000 partially mismatched target sequences in parallel. This study confirmed, as expected, that the free energy of binding between gapmer and mismatched target RNA affects the extent of the observed knockdown (28). The study also showed that the positions of mismatches or bulges in the duplex relative to the RNase H cleavage site influence the extent of the knockdown as well (28). More *in vitro* and *in vivo* work is needed to further evaluate the properties of mismatched duplexes as substrate for RNase H when considering the sequence-specificity of gapmers.

Relating overall free energy of binding with potency

Since the free energy of binding is a thermodynamic state function, ΔG° values are additive for sequential reactions, and the overall free energy of binding between gapmer and target RNA can therefore be written as a sum of all the reactions involved (Figure 2)

$$\begin{aligned} \Delta G^\circ(\text{overall}) = {} & \Delta G^\circ(\text{RNA structure}) + \\ & \Delta G^\circ(\text{protein occlusion}) + \\ & \Delta G^\circ(\text{oligo structure}) + \\ & \Delta G^\circ(\text{hybridization}) \end{aligned}$$

In this section, we will demonstrate how the overall free energy of binding, or overall binding affinity, can be related to the potency of the gapmer. The enzyme reaction scheme for gapmer binding to target RNA, and subsequent cleavage by RNase H, can be written

$$O + T \underset{}{\overset{K_d}{\rightleftharpoons}} OT \qquad \text{(reaction 1a)}$$

$$OT + E \rightleftharpoons OTE \xrightarrow{k_{cat}} O + C + E \text{ (reaction 1b)}$$

where gapmer oligonucleotide, O, binds to RNA target, T, giving the duplex, OT, which is a substrate for the RNase H enzyme, E, that can cleave the RNA, indicated as C in reaction (1b) (90). This reaction scheme could be expanded, for example to take into account that RNA is produced at a constant rate and degraded following first-order kinetics (48), or that gapmer is continuously excreted through urine. Here, the simple reaction scheme described by reactions (1a) and (1b) suffice for exploring the possible relations between binding affinity and potency.

The dissociation constant, $K_d = [O][T] / [OT]$, in reaction (1a) is related to the binding affinity, ΔG°, through the basic thermodynamic relationship $\Delta G^\circ = RT\ln(K_d)$, where R is the gas constant and T the absolute temperature (79). To relate this to the potency of the gapmer, we first apply the law of mass action to the reactions (1a) and (1b), and

write the changes in concentrations over time as a set of five coupled differential equations (one for each of O, T, OT, E and OTE). The numeric solutions to these equations (91), for physiologically relevant reaction parameters (48), are shown in Figure 4.

The relative concentrations of free and uncleaved target RNA, [T], free gapmer, [O] and the duplex between gapmer and RNA, [OT], as they develop over time, are shown in Figure 4A. After administration of the gapmer, the duplex between gapmer and target rapidly forms, and the RNA levels start to decline, as it is being cleaved by RNase H, and the simple model does not include the production of new RNA. For clarity, the concentrations of RNase H enzyme alone, and when it is complexed with OT, are not shown. Also, the increasing concentration of cleaved (and degraded) RNA, [C], is not shown.

At a fixed time of evaluation, denoted by the grey vertical line in Figure 4A, the concentration of RNA is recorded and plotted as a function of the total concentration of gapmer administered, as shown in Figure 4B. This traces out the familiar concentration–response curve (CRC) relationship that is often approximated by the Hill–Langmuir equation (92). From the CRC, the concentration of gapmer at which the half maximal effect (EC50) is achieved, can be identified. The potency of the gapmer can be defined as 1/EC50 (93).

In Figure 4C, EC50 is plotted as a function of the dissociation constant, K_d (solid line). As discussed, Figure 4C also traces out the relationship between binding affinity and potency. As can be seen from Figure 4C, for weak binding affinities, there is a linear relationship between binding affinity and potency. However, as the binding affinity is increased, typically only achievable by high-affinity modifications such as LNA, the potency reaches a low plateau (solid line in Figure 4C). Mechanistically, this can correspond to a situation where the gapmer binds so strongly to the RNA, that practically all of the RNA is in duplex. Increases in the binding affinity beyond this point will then not result in increased potency. As argued in Pedersen *et al.* (48), a low plateau can also be reached when the factor limiting potency is the rate at which RNase H cleaves the target, rather than the affinity between gapmer and target RNA.

Also argued in Pedersen *et al.* (48), using a more detailed reaction scheme than the one presented here, is the concept that a parabolic relationship may appear, where there exists an optimal binding affinity (sketched in Figure 4C as a dashed line). This can happen when the affinity between gapmer and RNA target is so high, that the gapmer also has appreciable binding to the RNA fragments after cleavage, thereby limiting their catalytic effectiveness. This situation is particularly relevant if the target RNA has a high turnover rate, since the gapmer is then simply sequestered by RNA that is quickly replaced by newly produced RNA. Such a parabolic relationship between affinity and potency was demonstrated experimentally for four different RNA targets *in vitro* (48). In summary, there may only be linearity between gapmer binding affinity and potency up to a certain point, after which increased affinity has no effect, or may even reduce the potency.

As a final remark, in the discussion above, a gapmer was considered to only have a single binding site in the tar-
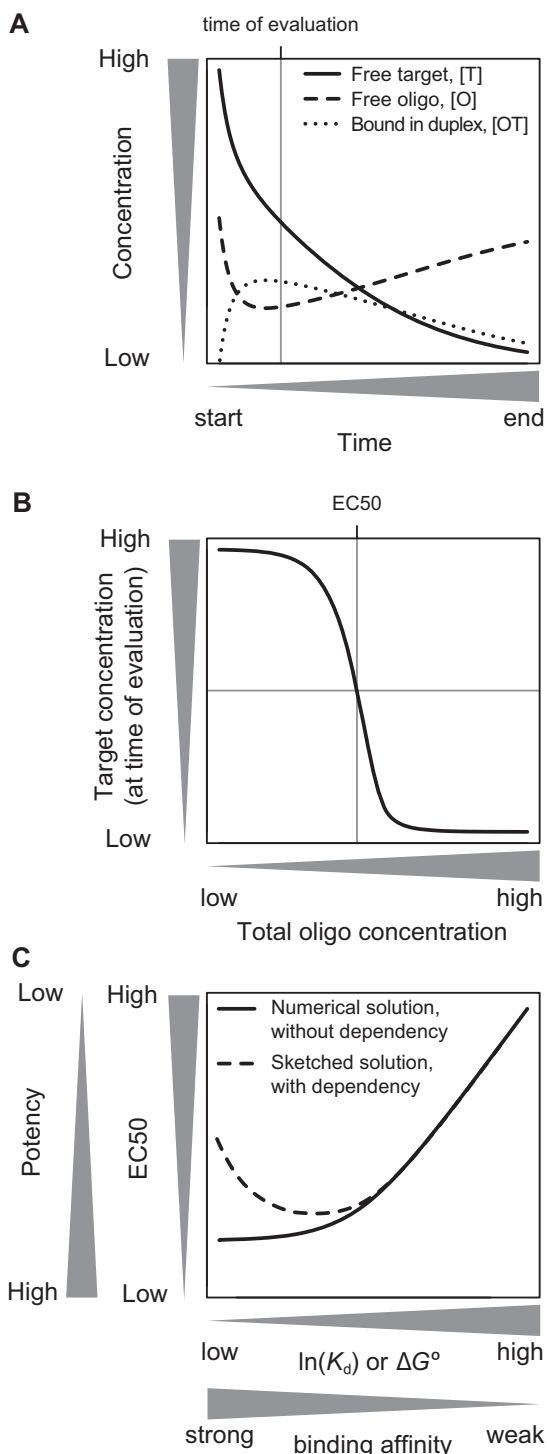
Figure 4. Model solutions of reactions (1a) and (1b) leading up to the relationship between binding affinity and potency. (**A**) Time-resolved numerical solution of the relative concentrations of free target RNA, [T], free gapmer oligonucleotide, [O], and the duplex between gapmer and RNA, [OT]. At a fixed time point, denoted by the vertical grey line, the concentration of RNA target is recorded, and in (**B**) plotted as a function of the total concentration of gapmer. From this curve, the gapmer concentration at which a half-maximal effect is achieved can be identified, and in (**C**) plotted as a function of the dissociation constant, K_d, between free gapmer and target RNA, and duplex (solid line). The relationship between binding affinity and potency presented in Pedersen *et al.* (48), is sketched as a dashed line.

get RNA. Gapmers with multiple mismatched binding regions in the same RNA, each of these weakly binding regions being slightly active, may still have an overall potent effect on that RNA. This is supported by the observation that gapmers with multiple fully matched binding regions in the same RNA demonstrate significantly increased potency compared with single-region targeting controls (94). Therefore, when assessing the potency of an oligonucleotide, ideally all possible target regions, and not just the region of strongest binding, must be taken into account.

RNASE H-ACTIVITY ON UNINTENDED TARGET RNA *IN VIVO*

In the case where unintended RNA targets are present that are fully complementary to the gapmer, the extent to which the gapmer will be active will be determined by the factors reviewed in the section headed "Determinants for RNase H-activity on unintended target RNA". These factors are the same as those that govern the effect on the intended RNA target of interest, where some gapmers will be highly active and others will not, even though they are all fully matching (36,95). In this section, we focus on the experimental evidence that RNA to which the gapmer has a mismatched binding region, can be unintentionally targeted *in vivo* and degraded as a consequence of this binding. Degradation of mismatched RNA in vitro by gapmers has been amply demonstrated, as reviewed elsewhere (96,97).

When investigating the effects of mismatched binding to RNA, a direct approach is to introduce mismatches in a gapmer, and compare it with a gapmer that fully matches the RNA target. Examples of such studies are presented in the subsection headed "Specificity of different gapmers for the same target". The results from these studies, however, are limited to demonstrating only that unintended cleavage of mismatched RNA is possible, and not the extent to which it actually happens transcriptome-wide in various tissues when dosing at therapeutic levels. Studies analyzing such global effects of gapmers with respect to specificity are reviewed in the subsection headed "Specificity of the same gapmer for different targets".

Specificity of different gapmers for the same target

Using Xenopus oocytes as a model system for investigating the specificity of gapmers *in vivo*, it was demonstrated already in 1992 that unmodified gapmers can cause degradation of transcripts at mismatched target sites (98). More recently, this observation has been extended to LNA- and 2′-OMe-modified gapmers with phosphorothioate backbones (99). In the study by Lennox *et al.* (99), when microinjecting 40 nM LNA-modified gapmer in Xenopus oocytes, a 90% knockdown of the target mRNA *survivin* was observed after 4 h. Gapmers with one to three mismatches retained the ability to reduce transcript amounts by as much as 40–50%. When modifying the gapmers with 2′OMe instead of LNA, at the same dose level and duration, fully complementary gapmers reduced transcript levels by 70%, one mismatch by 40%, whereas two or three mismatches did not reduce transcript levels appreciably below control levels in uninjected oocytes. These results were correlated with the affin-

ity of the gapmers towards the target region on the transcript, as measured by melting temperatures. The melting temperature (T_m) is defined as the temperature at which half of the oligonucleotides are duplexed with target RNA. Although not strictly proportional to the binding affinity at physiological conditions, $\Delta G°$ (78), the T_m is a frequently used experimental measure of duplex stability. The LNA-modified gapmers in the study (99) had melting temperatures ranging from 78°C to 59°C, depending on the number of mismatches for the target site in *survivin*. The melting temperatures of iso-sequential but 2′-OMe-modified gapmers ranged from 60°C to 33°C. The generally higher affinities obtained with the LNA-modified gapmers, both fully matched and mismatched variants, compared with the 2′-OMe-modified gapmers, were suggested as an explanation for the more pronounced effects on target knockdown. This relationship between affinity and potency has also been observed *in vitro*, as reviewed elsewhere (97), and can be predicted theoretically, as discussed in the subsection headed "Relating overall free energy of binding with potency".

The effects of single basepair mismatches have also been investigated in mice using LNA-modified gapmers with a phosphorothioate backbone (100). Intravenous injection of a 12 nucleotide (nt) long gapmer, dosed at 2.5 mg/kg, with a fully complementary target site in apolipoprotein B (*Apob*), demonstrated >95% knockdown in whole kidney tissue. A single mismatch version of this gapmer resulted in only a 30% reduction. To explore possible length-dependent effects, 14 nt and 16 nt long gapmers targeting the identical, but extended, region in *Apob* were compared with the 12 nt gapmer. As explained by the authors, since the longer versions were found to be less potent *in vitro*, in order to have a similar effect in mice across all lengths, they were dosed at higher levels, 5 and 25 mg/kg (14 nt and 16 nt, respectively), compared with the 12 nt gapmer dosed at 2.5 mg/kg. Interestingly, the difference between target knockdown for fully matched and one-mismatch versions became successively smaller for the 14 nt gapmer and the 16 nt gapmer. For the 14 nt gapmer, the fully matched version reduced *Apob* by 90%, and the mismatched by 40%, and for the 16 nt gapmer, the fully matched reduced *Apob* by 85%, and the mismatched by 50%. The reason for this apparent improved mismatch-specificity for shorter gapmers, it was argued by the authors, is because differences in binding affinity between fully matched and mismatched gapmers are larger for shorter gapmers than for longer (100). However, to demonstrate that this is the case, free energies of binding, $\Delta G°$, must be measured, and not just melting temperatures, T_m. The reason for this is that whereas $\Delta G°$ is logarithmically proportional to binding affinity (78), the relationship between T_m and binding affinity is more complex and not linear. According to You *et al.* (101), there will always be a larger gap in T_m between fully matched and mismatched gapmers for shorter lengths, compared with longer lengths. This, however, does not necessarily reflect a larger difference in binding affinity between a fully matched and mismatched gapmer for shorter lengths, compared with longer. If such a length-dependence on affinity changes could be shown by direct measurement of free energies of binding, it would be a deviation from nearest neighbor assumptions (subsection headed "Hybridization between oligonucleotides and (un-

intended) target sites"), which would have important implications for how to calculate binding affinity for oligonucleotides. An alternative explanation of these results, could be that the 12 nt gapmer has a binding affinity where there is optimal potency for the fully matched version (refer to the subsection headed "Relating overall free energy of binding with potency"). For the 14 nt gapmer and 16 nt gapmer, the binding affinities for the fully matched versions are higher than for the 12 nt gapmer, so they are on the left side of the affinity/potency parabola in Figure 4C, and the potency thus becomes lower as the affinity increases (Figure 4C). In contrast, the reduced binding affinities for the three mismatched versions could place them on the right side of the parabola in Figure 4C, where increases in binding affinities result in higher potency.

At some point, enough mismatches are introduced that the binding affinity is reduced below the level needed to elicit activity. As an example, a 20 nt long phosphorothioate gapmer with seven mismatches was unable to cleave v-raf-1 murine leukemia viral oncogene homolog 1 (*RAF1*) mRNA compared with a fully matching version that reduced mRNA by >90% (102). The seven-mismatch gapmer also did not affect tumor growth in nude mice, whereas the fully matching version significantly affected growth (102). There are other examples where multiple mismatches in a 20 nt gapmer completely abolished the activity (103). To the best of our knowledge, the most mismatches reported for a gapmer that still retained some ability to reduce target RNA, is a 20 nt long gapmer targeted against tumor necrosis factor (*TNF*) (104). In this study, in macrophages isolated from the adipose tissue of db/db mice, at 5 mg/kg, the fully matched gapmer reduced transcript levels by 65%, whereas a six-mismatch version was able to reduce transcript levels by 30%, compared with a completely sequence-scrambled gapmer.

These examples demonstrate that under certain conditions gapmers can bind to and degrade transcripts with mismatched target regions *in vivo*. This fact has been used to design 20 nt gapmers that target two apoptosis regulators, B-cell CLL/lymphoma 2 (*BCL2*) with full complementarity, and BCL2-like 1 (*BCL2L1*) with three mismatches (105). When dosing at 200 nM *in vitro* by transfection, after 7 h *BCL2* mRNA was reduced by 75% by both LNA- and 2′-MOE-modified iso-sequential versions, whereas the *BCL2L1* mRNA was reduced by 70% by the LNA-modified version, and by 40% by the 2′-MOE-modified version. Dosing with the 2′-MOE-modified version at 20 mg/kg in nude mice caused marked growth inhibition of human colon cancer cell xenografts compared with a scrambled control gapmer (106).

Specificity of the same gapmer for different targets

Work on gapmers targeting single nucleotide polymorphisms for allele-selective inhibition of mutant RNA demonstrates how varied the effect of a single mismatch in the target region can be (107). For a set of gapmers designed to fully match a mutant version of *Huntingtin* RNA, reduction of mutant protein levels by 80% relative to control levels in mouse brain were reported. However, when the effects on wild-type *Huntingtin* RNA were measured, towards

which the gapmers had a single mismatch in the RNase H-recruiting gap-region, anything between no measureable effect and reduction in protein levels by up to 80% relative to control were observed. Based on the design of the gapmers, these differences in the sensitivity to the single mismatch were inferred to depend on factors such as the binding affinity of the gapmer, the length and position of the gap-region, the position of the mismatched nucleotide within the gap-region, and the type of chemical modifications used (107).

Since gapmers catalyze cleavage of target transcripts, the ability to study transcriptome-changes on a global scale using microarrays or RNA sequencing, allows direct measurement of global intended and unintended target reductions and downstream effects. This was first demonstrated *in vivo* by Cho-Chung *et al.* (108) in a mouse model of human prostate cancer. Treatment with human- and mouse-specific gapmers targeting the cAMP-dependent protein kinase regulatory subunit RIα (*PRKAR1A*) was found to alter the expression of hundreds of genes >2-fold, as measured using microarrays. This included genes involved in proliferation and differentiation, as would be expected, but also genes that appeared to have no relationship to *PRKAR1* and cAMP-dependent signaling (109).

In a more direct microarray-based analysis of the sequence-specific effects of gapmers targeting the ABC transporter *ABCB1 in vitro*, Fisher *et al.* (110), identified 37 genes consistently affected >2-fold, eight of which were repressed by treatment with both conjugated and unconjugated gapmer, and not by a mismatched control gapmer. Among the transcripts encoded by these eight genes, besides the intended target *ABCB1* (also known as *MDR1*), sequence analysis identified potential unintended target sites in three of them. These potential unintended target sites had four or five mismatches, but predicted melting temperatures were sufficiently high to allow binding (110). Although the sequence analysis therefore suggests that some of these genes are unintended targets, the experimental design did not allow clear discrimination between effects from unintended targeting and secondary effects due to reduction of the target gene.

Finally, three different microarray-analyses have recently been reported, which all compare gene expression changes in the liver of mice, after administration of LNA-modified phosphorothioate gapmers with different hepatotoxic potentials (1–3). In a study by Kakiuchi-Kiyota *et al.* (1), three different gapmers were evaluated. The gapmer with the lowest hepatotoxic potential produced no significant changes in various biochemical markers after dosing. This gapmer had no fully matched- or one-mismatch target sites, and only a single unintended target with two mismatches. The gene harboring that two-mismatch target site did not exhibit reduced expression at any of the time points evaluated in the study. A gapmer with high hepatotoxic potential also had no fully matching target sites, but three genes with one-mismatch target sites, and six genes with two-mismatch target sites. Out of these, only one of the genes with a one-mismatch target site, and one of the genes with a two-mismatch target site, were significantly reduced. The last gapmer, with the highest hepatotoxic potential of the three gapmers tested in the study, was also the one that had the most unintended target sites: three genes with

fully matched target sites, one of which was found to be significantly reduced; ten genes with one-mismatch target site, two of which were significantly reduced; and 22 genes with two-mismatch target sites, three of which were significantly reduced. As discussed in the section headed "Determinants for RNase H-activity on unintended target RNA", the mismatch-tolerance of a gapmer is related to its binding affinity. However, the authors did not measure binding affinities to the intended and unintended targets that were identified. This limits the extent to which general conclusions on mismatch-tolerance can be drawn from these results. The results reported by Kakiuchi-Kiyota *et al.* (1) do however suggest that the more unintended target sites a gapmer exhibits, the higher the risk of increased hepatotoxic potential. Such a relationship between hepatotoxicity and number of unintended targets is also supported by the work of Burel *et al.* (2). Here, across 13 different gapmers, a clear correlation between the number of genes with lowered expression in the liver of mice, as measured 1 day after dosing, and hepatotoxic potential, as measured using alanine transaminase (ALT) levels in serum 4 days after dosing, was observed. Moreover, knockdown of RNase H1 prior to treatment with the gapmers attenuated the observed hepatotoxicity. That the observed hepatotoxicity is mediated through an RNase H1-dependent mechanism is also supported by the work of Kasuya *et al.* (3). Here, two LNA-modified gapmers known to have a high hepatotoxic potential were re-designed by introducing two additional LNA modifications in the central gap-region, thereby reducing the possibilities for RNase H to bind. Whereas the original gapmers both resulted in increased ALT levels 4, 7 and 10 days after dosing at 10 mg/kg, the re-designed oligonucleotides, which were not expected to recruit RNase H effectively, did not result in increased levels of ALT after dosing. Similarly to what has been reported by Burel *et al.* (2), siRNA-mediated knockdown of RNase H1 prior to treatment using three other LNA-modified gapmers, significantly attenuated the observed hepatotoxicity (3). Early changes in liver RNA levels were evaluated for one of the five hepatotoxic gapmers using a microarray analysis. After 24 h, when dosed at 20 mg/kg, the intended target mRNA Acyl-CoA synthetase long-chain family member 1 (*ACSL1*) was reduced almost two-fold, and an additional 16 genes were significantly reduced >2-fold. Interestingly, the most likely target regions identified using a sequence analysis of the 16 genes all had as many as three to five mismatches to the gapmer. When evaluating the 185 mRNAs where sequence analysis identified potential target regions with two or fewer mismatches, nine were found to be significantly reduced, out of which seven could be confirmed using qRT-PCR (3). These results are all consistent with a model where the more unintended targets, the higher the risk of some of these targets being involved in critical cellular functions leading to hepatotoxicity.

The microarray studies on gapmer specificity mentioned above (1–3,108,110) do not conclusively identify the unintended RNA targets. For future transcriptomics studies on specificity, we suggest modifying the study design to include at least two gapmers with different sequences, but with largely the same effect on the intended target. This is based on the well-established rule-of-thumb for relating
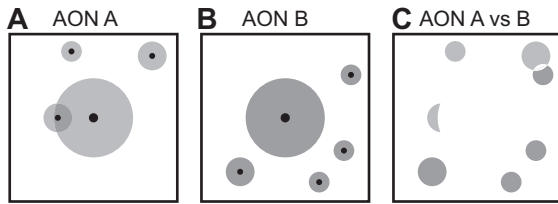
Figure 5. Experimental design to identify effects of unintended RNA targets using transcriptomics. Each box represents the entire transcriptome. Black dots and filled circles indicate targets and secondary effects. (**A**) Upon treatment with AON A, the central dot and largest circle indicate intended target and secondary effects. In addition, three unintended targets with corresponding secondary effects are shown. (**B**) When treating with AON B, the intended target and secondary effects are similar to AON A (compare A and B), but the unintended targets are different. (**C**) When comparing transcriptome changes upon treatment with AON A versus AON B, pharmacologically-induced changes derived from silencing the intended target are similar, whereas unintended effects are unique to each oligonucleotide.

gamper target engagement to the phenotypic changes observed (95). Essentially, if two or more gapmers of different sequences, but both complementary to the same RNA target, result in a similar phenotype, whereas a control gapmer does not, it strongly suggests that the phenotype is mediated by target degradation (95). Such a study design will also allow identification of unintended targets of gapmers from transcriptome-wide profiling (Figure 5). Since hybridization-dependent unintended targets are, by definition, sequence dependent, two different gapmers targeting the same transcript at different positions will allow separation of the effects of intended and unintended targeting: The effects of targeting the intended transcript will be the same for the two gapmers, but effects from unintended targets will be different, because the two gapmers have different sequences. The only theoretical limitation to this setup, as indicated in Figure 5, is when unintended effects overlap with intended effects, as seen in Figure 5A, or when parts of the unintended effects from the two gapmers overlap, as seen for the effects in the upper right corner in Figure 5C.

Two recent transcriptome-wide profiling studies have applied such a design (34,111), and while the focus in these studies was on pharmacological effects derived from silencing the intended target, re-analysis of the data should, in principle, allow identification of unintended targets, if such exist, for these gapmers.

To correlate whether the observed reductions in transcript levels can be explained by oligonucleotide-directed cleavage, one can apply character- or energy-based searching (discussed in the section headed "History and developments in computational specificity assessments") of the RNA sequences to identify the most likely binding regions. Subsequently, these short RNA sequences can be synthesized to test whether they can activate RNase H when incubated together with the gapmer (112). To conclusively demonstrate that the observed reductions in transcript levels are due to oligonucleotide-directed cleavage, for each RNA, the specific cleavage products can be characterized using 5′-RACE (23). From such fragments, it can then be confirmed if the cleavage occurred at the predicted binding site of the oligonucleotide.

For gapmers, we divide effects from unintended RNA targets into those which are subject to the same mechanism as the intended effect (here RNase H-induced target degradation) and those where hybridization to the unintended target elicits effects through other mechanisms (such as splice modulation, blocking of binding sites for microRNAs or RNA binding proteins, and more). We expect degradation of unintended RNA targets to be the dominating mechanism influencing the specificity of gapmers (see the subsection headed "Specificity of the same gapmer for different targets"), and we focus on this mechanism here.

As mentioned in the subsection headed "Defining specificity", in this review we focus on the RNase H-induced degradation of unintended target RNA, since we consider other effects stemming from hybridization to unintended target RNA, for example steric blocking of interactions with ligands, to be negligible in comparison. To illustrate this, consider oligonucleotides that bind fully to part of the microRNA mature sequence, but do not recruit RNase H and instead sequester the microRNA. Because of the high binding affinity of LNA, it is possible to design oligonucleotides consisting of as few as seven or eight LNAs, termed tinyLNAs, which are fully complementary to the seed region of a microRNA, and which effectively sequester the microRNA target with melting temperatures in the range 55–80°C (113). As a result of their short length, they will naturally also be fully complementary to regions in many other RNAs besides the intended microRNAs. For a tinyLNA targeting the seed region of miR-122, we have identified fully matched regions in ∼12 000 mouse transcripts (∼20% of the unspliced transcriptome in mice). By comparison, for a longer, 15 nt oligonucleotide also targeting miR-122, and with similar binding affinity, we identified fully matched regions in only two transcripts. However, upon treatment with either the tinyLNA or the longer version in mice, measurements of transcriptome- as well as proteome-changes in liver, relative to a control oligonucleotide, revealed highly similar impacts (113). This similarity suggests that secondary effects derived from the inhibition of miR-122 dominate the measured transcript- and protein changes. Indeed, the only sequence-specific effect that could be inferred was derepression of transcripts with predicted miR-122 binding sites in their 3′-untranslated region (113). No effect on the many transcripts with fully matched binding sites for the tinyLNA could be detected at the RNA or proteome levels (113). This suggests that the probability that a binding event between a transcript and the tinyLNA would sterically block an important interaction with other ligands is small. For example, splice-modulating oligonucleotides need to bind at precisely the right position to have an effect (114). Finally, protein translation from the ribosome is not affected by tinyLNA binding in the coding regions of transcripts (113).

HISTORY AND DEVELOPMENTS IN COMPUTATIONAL SPECIFICITY ASSESSMENTS

In this section, we will discuss computational strategies that have been used to evaluate the specificity of gapmers. Normally, specificity evaluations are performed on a large number of theoretical oligonucleotide sequences tiled along an

RNA target of interest. Those oligonucleotide sequences likely to be most specific, as identified by the computational analysis, can then be selected for actual synthesis and experimental testing.

As already discussed in the section headed "Determinants for RNase H-activity on unintended target RNA", multiple factors are known to influence the activity of gapmers on a target RNA. Some of these, such as protein occupancy, are not easily predicted computationally. Even for those that are, it is not straightforward to integrate them in a model that gives accurate predictions across multiple RNA targets (36). Therefore, the hybridization between putative unintended target sites and the oligonucleotide is the single factor that currently dominates how computational specificity assessments are performed in practice. The computational identification of unintended targets follows developments in bioinformatics algorithms for character-based sequence searching and more recently searching using thermodynamic models for predicting free energies of binding and hybridization affinities (Table 1). The Table catalogs the approximate points in time where new algorithms in antisense oligonucleotide discovery came into routine use, and represents our best estimates based on personal experience.

Character-based search strategies

Sequence searching is one of the earliest, and arguably most mature, disciplines in bioinformatics. Character-based search algorithms (Table 1), such as BLAST (115) and FASTA (116) for finding homology between sequences have been employed since the early days of antisense drug discovery. These algorithms were constructed primarily to find homology (understood evolutionarily as common ancestry) between longer sequences, and employ heuristics that trade sensitivity for speed. Although character-based methods do not differentiate between types of base pairs, so A/T and G/C are weighted equally and stacking is not taken into account (see the subsection headed "Hybridization between oligonucleotides and (unintended) target sites"), there is nevertheless an overall correlation between the matching of characters and affinity. Even though the heuristic method is faster than basic pairwise alignment, in 2007, Freier and Watt considered it too slow to guide the design of the early large screening libraries (36) and instead postponed computational specificity analysis to later stages of drug discovery, when a lower number of drug candidates are left.

Later character-based algorithms took advantage of the increased working memory of computers and construct efficient indexes (117,118) of the database sequence (e.g., all the possible unintended target transcripts), which allows for exhaustive searching at speeds orders of magnitude faster than BLAST. Using these new character-based data structures and search algorithms it is feasible to perform computational specificity analysis on all possible oligonucleotide sequences before synthesizing any of them.

Regardless of the algorithm used, the analyst has to apply rules and cutoffs to determine search hits that are relevant as potential unintended targets. This relationship may be phrased in two ways, which can lead to qualitatively very different results:

1. More matches between characters (A with T, G with C) indicates higher affinity leading to assumed higher activity
2. More mismatches between oligo and RNA targets indicates lower affinity leading to assumed lower activity

Ostensibly, 1 and 2 appear equivalent. However, the search strategies that they often lead to yield qualitatively quite different results.

Strategy 1 leads to the following search and selection strategy:

```
for each considered oligonucleotide se-
 quence
count the number of sites in the sequence
 database with at least x complementary
 characters to the oligonucleotide se-
 quence
choose oligonucleotide sequences with low
 counts as more specific
```

Strategy 2 leads to the following search and selection strategy:

```
for each considered oligonucleotide se-
 quence
count the number of sites in the sequence
 database allowing y mismatching charac-
 ters
choose oligonucleotide sequences with low
 counts as more specific
```

If the oligonucleotide sequences under consideration are of different lengths, strategy 1 will conclude that shorter oligonucleotides are more specific, whereas strategy 2 will lead to the opposite conclusion, as shown in Figure 6.

In reality, both short and long AONs can be specific. In order to reconcile the apparently contradictory strategies for evaluating specificity using character-based searching, as presented above, we need to consider the binding affinity between oligonucleotide and target RNA more closely, as discussed in the next two sections.

Energy-based search strategy

Energy-based searching addresses some of the shortcomings of the purely character based methods (119). These methods are based on the significant accomplishments of *de novo* RNA secondary structure-based methods (120). Contrary to character-based methods, they are based on the biophysics of hybridization (see the subsection headed "Hybridization between oligonucleotides and (unintended) target sites") and incorporate both stacking, and the fact that some base pairs (G/C) contribute more to the free energy of binding than others (A/T). This allows a more physically accurate method of finding possible hybridization sites (unintended targets). One can search for sites where the oligonucleotide is likely to form a thermodynamically stable duplex with the target site (low free energy of hybridization), rather than sites with the highest number of matching characters. Originally, these methods were introduced primarily to predict the optimal target site(s) on the intended RNA target (121,122). Only later were energy models adapted to

Table 1. Sequence search algorithms and methods used for specificity assessments of oligonucleotides

Time of introduction	Method	Advantages	Disadvantages
1990	BLAST individual drug candidates on a webpage	Can be performed by anyone. No programming skills or specialist software packages needed	Very low throughput, interpretation subjective, uses a heuristic algorithm, i.e. it is not exhaustive
2005	Scripted BLAST or FASTA on local databases	Can be performed on many RNA targets in a standardized fashion	BLAST and FASTA are designed and optimized to detect evolutionary relationships, not hybridization
2007	Suffix-array matching and Burrows–Wheeler Transforms	Fast and exhaustive. Can be performed for all possible gapmers against a target	Character matching is unphysical, large memory requirement
2010	Search with energy based affinity model	Thermodynamic scoring based on binding affinity between gapmer and RNA targets	Slow, parameters not publicly available for most chemical modifications
Future (nothing published yet)	Activity model trained on transcriptomics data and historic screening results	Driven by actual activity measurements on unintended RNA targets	Requires expertise and high-quality, needs comprehensive data to build

The times of introduction represent estimates based on our own experience, and lag between 1 and 10 years behind the time of publication in scientific journals. The methods listed are referenced in the main text when mentioned.
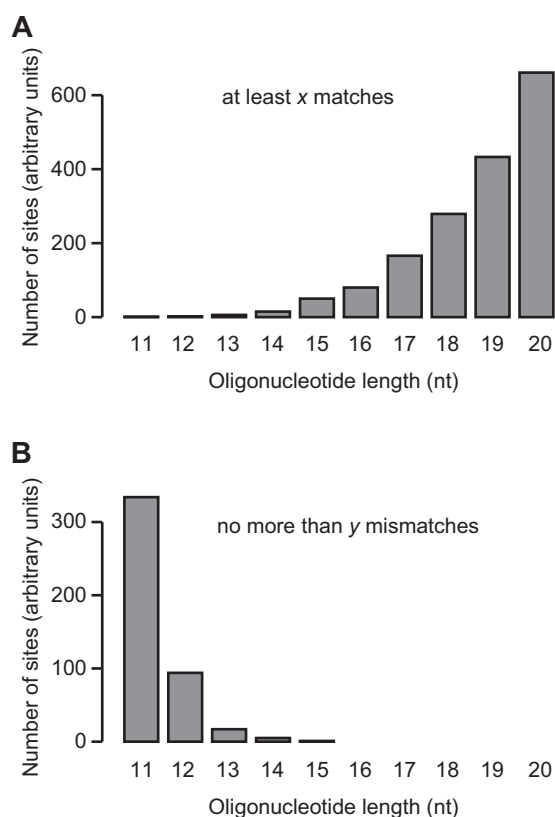

Figure 6. Different search paradigms yield very different results when studying the effects of oligonucleotide length on specificity. (**A**) When searching for matches with at least a certain number, *x*, of matching characters, specificity appears to decrease with length, whereas (**B**) when searching for hits with no more than a certain number, *y*, of mismatching characters, specificity appears to increase with the length of the oligonucleotide. Simulated data based on 100 oligonucleotides of each length. The *y*-axis in both A and B depicts arbitrary units, reflecting that any choice of *x* and *y* will result in the same overall shape of the distribution, although actual counts will differ.

allow scanning of large sequence databases for putative unintended targets (123,124).
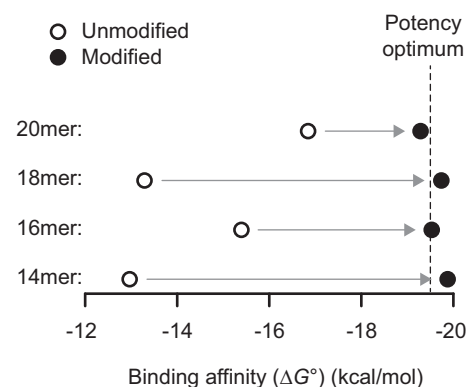

Figure 7. Examples of modifications to gapmers with LNA to increase binding affinity for optimal potency (here chosen as $\Delta G° = -19.5$ kcal/mol, which is approximately the average of the optimal affinities identified Pedersen *et al.* (48) and matches our own experience as well). For the 14 nt gapmer, from atcgccgtactatg to ATCGccgtactATG (lowercase: DNA, uppercase: LNA); for the 16 nt gapmer, from tcagaagaccgctact to TCagaagaccgctACT; for the 18 nt gapmer, from ggcaagactgaatatgaa to GGcaagactgaataTGAA; and for the 20 nt gapmer, from taagcaaattagcgcgtatg to TaagcaaattagcgcgtaTG. Approximated thermodynamic parameters for LNA/DNA-RNA binding were used to calculate $\Delta G°$ values (48).

However, energy-based specificity evaluation has to be applied with caution, since potency scales with affinity only within a limited range (refer to the subsection headed "Relating overall free energy of binding with potency"). Beyond a given threshold, any further increase in affinity does not improve potency—it might even make it worse, as suggested by Pedersen *et al.* (48). From this observation, it follows that there is an optimal affinity that designers of gapmers should aim for. Affinity can be modulated by changing the length of the gapmer or by adding and removing affinity-enhancing chemical modifications such as LNA. To reach optimal affinity, short gapmers generally need more affinity enhancing modifications than longer versions (see Figure 7).

In our experience, the optimum affinity is quite broad, meaning that there is a range of optimal affinities where other factors contribute more to potency than affinity does

(48). When evaluating the specificity of a gapmer using an energy model, the pertinent question therefore is: how many sites exist in the targetable sequence space (subsection headed "Target site accessibility") that have an affinity in the optimal range (or close to it)?

One possible way to address this question is to consider that the potency of gapmers does not increase with affinity ad infinitum (subsection headed "Target degradation by RNase H"). For any gapmer with a number of mismatched unintended targets, the affinity towards these unintended target sites will generally be lower than the affinity for the intended target (subsection headed "Hybridization between oligonucleotides and (unintended) target sites"). By increasing the overall affinity of the gapmer, using chemical modifications such as LNA, both affinities to intended and unintended targets are increased. When the affinity/potency relationship plateaus (Figure 4C), the gapmer can become unspecific at the point where increased affinity does not increase potency for the intended target (where it has plateaued), but only on the unintended targets (where it is still in the linear range). Therefore, one has to be careful not to increase the affinity beyond what is needed for maximal potency, when working with high-affinity modifications.

Figure 8 illustrates that the number of unintended target sites decreases with the length of the gapmer, given a hypothetical set of gapmers of different lengths, all adjusted using chemical modifications to have the same optimal affinity towards the intended target site (Figure 8, black bars). As a corollary, it follows that if affinity to the intended target is increased beyond the optimum for maximal potency, the specificity will decrease, because more mismatched target sites will be in the optimal affinity range (Figure 8, gray bars).

As it is rooted in thermodynamics and experimentally determined binding parameters, we believe that energy-based specificity evaluation has the potential to be much more informative than character-based methods. Currently, however, energy-based searching has two major practical disadvantages: slow speed and lack of easily available parameters for modified nucleotides. The more complex energy-based scoring is not compatible with the current indexing technology (suffix-arrays and -trees) and transforms that have vastly improved the speed of character-based searches. Furthermore, nucleotide modifications often have a large impact on the hybridization energy, but the nearest-neighbor parameters for modified nucleotides are not readily available for most modifications except for LNA (84,85).

Nevertheless, it is our opinion that an energy-based search with RNA parameters for a modified oligonucleotide is still more relevant than the simpler character-based methods. A pragmatic solution for the slow speed of energy-based searches is to perform a two-step process: first, a low-stringency by fast character-based searching, followed by post-scoring of hits with a nearest-neighbor energy model.

Computational evaluation of specificity in a discovery process

As a concluding example, we will now examine the transcriptome-wide specificity-profile for gapmers between 12 nt and 20 nt in length targeted to the human pre-
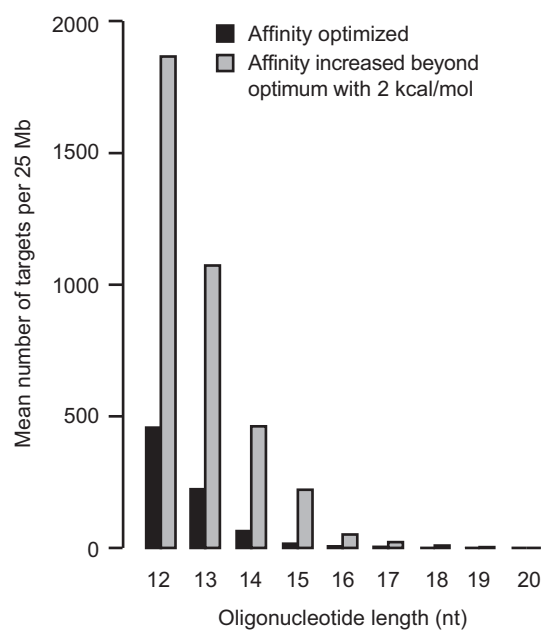

Figure 8. The relationship between the length of oligonucleotides with equal affinity to an intended target and specificity according to computational predictions based on an energy model. Simulated data of 100 oligonucleotides of each length used to search a target space of 250 kb using RNAhybrid (123). A (partially mismatched) target site was included in the count if it had a free energy of binding to the oligonucleotide no more than 3 kcal/mol higher (weaker) than for the oligonucleotide to the fully matched intended target site (black bars). About half of all possible single mismatches will be within this range. The grey bars capture the effect of oligonucleotides binding 2 kcal/mol more strongly than what is needed for optimal potency. In this case, all partially mismatched sites with free energy of binding to the oligonucleotide of no more than 5 kcal/mol higher (weaker) than that of the oligonucleotide to the fully matched intended target site were included in the count. The numeric simulation was performed using thermodynamic parameters for RNA–RNA interaction (123), but the qualitative conclusions will be the same with parameters for DNA and modified nucleotides.

mRNA for proprotein convertase subtilisin/kexin type 9 (*PCSK9*). An LNA-modified gapmer targeting *PCSK9* has been shown to induce a sustained reduction of low-density lipoprotein cholesterol in nonhuman primates (125). For this example, we will evaluate specificity towards the unspliced human transcriptome, where each gene is represented by the longest pre-mRNA variant as annotated by the Ensembl gene builds release 70 (126). This unspliced human transcriptome covers around 56 000 different genes. For simplicity, we will only consider gapmers designed with three LNAs in each flank and a central gap of DNA. Therefore, in this example, only a single gapmer can be designed against each target site in *PCSK9*.

The unspliced transcript for *PCSK9* is 25k nt in length (Ensembl ID ENST00000302118). For gapmers of length l, the total number of unique but overlapping target sites that can be targeted by gapmers is therefore $25000 - l + 1$. That is, gapmers can be designed for just below 25k target sites of each length. Since we consider gapmers of nine different lengths (between 12 nt and 20 nt), in all we need to evaluate the specificity of close to $9 \times 25\,000$ gapmers $= 225\,000$ gapmers.

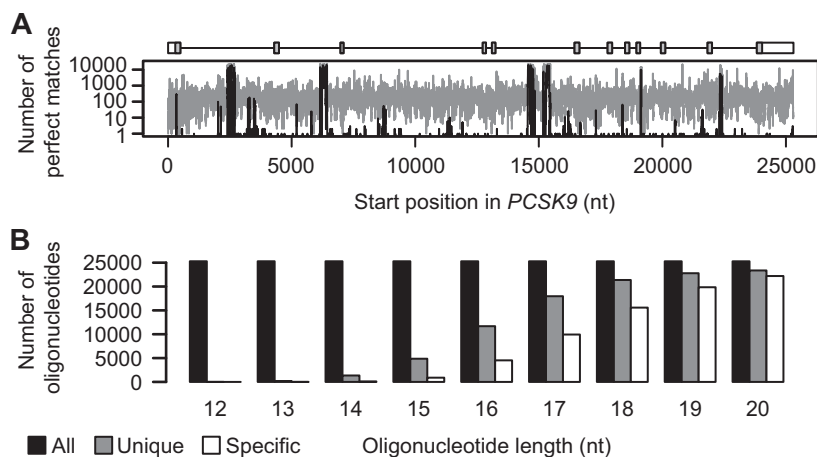Figure 9A shows the number of fully matched, unin-

Figure 9. Specificity profile for gapmers targeting the pre-mRNA for *PCSK9*. (**A**) For each 12 nt (gray) and 20 nt (black) gapmer that can be designed, the number of fully matching unintended target sites in the transcriptome are shown as a function of the starting position for that oligonucleotide in *PCSK9*. At the top, introns (line) and exons (box) are shown for *PCSK9*. For exons, untranslated regions are in white, and coding regions in grey. (**B**) All possible gapmer sequences of each length complementary to the target are shown (black), along with the subset that are unique to *PCSK9*, i.e. have no fully matched target regions anywhere else in the transcriptome (gray bars), and the number of unique gapmers with no unintended targets in the human transcriptome with a binding energy within 3 kcal/mol of the fully matching intended target site.

tended RNA targets found in the human transcriptome, for each possible gapmer of 12 nt (grey line) and 20 nt (black line) in length, as a function of the position along the *PCSK9* transcript. For clarity, only the 12 nt and 20 nt lengths are shown in the Figure. For 12 nt gapmers, the median number of unintended fully matching targets is 160. That is, in general a 12 nt gapmer can be expected to bind with perfect complementarity to hundreds of transcripts besides the intended target transcript. In contrast, most 20 nt gapmers are unique and only target the *PCSK9* transcript (see Figure 9A, black line). Notably, there are four regions of around 0.5 kb within which both 12 nt and 20 nt gapmers have >2000 unintended targets. These regions have a highly repetitive nucleotide composition.

In Figure 9B, we summarize the observations discussed so far. First, the black bars show the total number of gapmers of each length that can be designed, which is just below 25k. Second, the grey bars show the number of gapmers of each length that are unique, that is, have no fully matched unintended targets. As seen, for 12 nt gapmers almost none are unique whereas for 20 nt gapmers most are. For gapmers of length 16, around half of them can be expected to be unique.

Similarly to the analysis presented in the subsection above, we next calculate the minimal free energy of binding for each of the gapmers that can be designed to the intended target sites in *PCSK9*, against all pre-mRNA molecules in the human transcriptome. We judge those gapmers that have no unintended, fully matched as well as mismatched, targets with binding energy within 3 kcal/mol of the intended target site in *PCSK9*, as specific. In Figure 9B, we summarize these observations in the white bars. As seen, only a few 15 nt gapmers are specific whereas most 20 nt gapmers are.

CONCLUSION AND RECOMMENDATIONS

We have shown that RNase H-recruiting oligonucleotides (gapmers), taken as a class, are not different from approved small molecules in terms of the magnitude of their impact on the transcriptome (discussed in the Introduction section). However, the simple sequence-based nature of gapmers promises predictability of specificity and thus also the opportunity to maximize specificity when designing gapmers. Still, although the basic principles for hybridization with unintended targets through base pairing interactions are well understood (section headed "Determinants for RNase H-activity on unintended target RNA"), we are not yet at the point where computational predictions alone can ensure specificity. This may in part be because the reaction between RNase H and the gapmer/RNA target duplex needs to be better understood. We have discussed the tolerance for mismatches between gapmer and unintended target sites (section headed "RNase H-activity on unintended target RNA in vivo") and presented algorithms that allow computational prediction of these tolerances (section headed "History and developments in computational specificity assessments"). A pragmatic way to apply these algorithms in drug discovery is to de-select sequences that are obviously un-specific before they are ever synthesized, keeping in mind that such computational screens certainly do not guarantee specificity.

We have extensively reviewed the thermodynamics of gapmer binding to unintended RNA target sites and how this relates to potency. Only for weakly binding gapmers can we expect a linear relationship between affinity and potency. When high-affinity inducing chemistry is introduced, the relationship can become non-linear, plateauing or even reversing. This is important, because if the affinity/potency relationship plateaus, specificity will decrease if affinity to the intended target site is too high. Furthermore, the thermodynamics of gapmer-binding to target predicts that long gapmers will be more specific than short ones of equal affinity.

Should we then always design gapmers of 20 nt in length or even longer? In our opinion, the answer is no. Other properties that gapmers must have to be drug-like may be more easily, or only, realized with shorter lengths. A simple example could be, that the preferred target region in the intended target transcript is only accessible to gapmers that are 17 nt in length or shorter, due to stable secondary structures in the target. Another example could be toxicity mechanisms where shorter lengths are better tolerated than longer, such as the heparin-like effect observed for some phosphorothiate oligonucleotides, where the negatively charged phosphorothioate linkages has been implicated as a potential modulating factor on blood clotting times (17). In the discovery process, we usually start out with gapmers of length 14 to 20 nt and between 2 and 4 LNAs in each flank, which have been computationally identified as having an acceptable sequence-specificity. Through several rounds of screening in cellular assays for activity, specificity, and tolerance, followed by re-designing preferred gapmers, we end up with a small set of optimized gapmers with measured properties that fulfil all our criteria for drug-likeness. Notably, in these small sets of drug-like gapmers, all lengths from 14 to 20 nt can still be present. Because of all the different properties that drug-like gapmers must fulfil, and because of the complex relation between these properties and the chemical structure of the gapmers, in our experience there are no simple heuristics for length or modification pattern that can be generally recommended.

For computational specificity evaluation, energy-based searching promises better performance than simpler character matching. Nevertheless, there are many other factors that determine if an unintended RNA target is downregulated by a gapmer. A carefully designed experimental global transcriptome measurement (refer to Figure 5) directly measures the effect on possible unintended targets, and remains an important validation of current computational predictions. However, regardless of whether experimental and/or computational methods are used to assess specificity, the result will be a list of genes whose expression is predicted to change when cells are exposed to the gapmer. The list of genes will also be species-specific, since the transcriptomes of rodents and even primates are different from the transcriptome of man (especially in regions of the transcriptome not under strong evolutionary pressure, such as introns). In most cases, it is not simple to infer the risk of actual clinical toxicity from such a list of affected genes (127). Conversely, if a gapmer is not well tolerated, it is not straightforward to establish if one or more unintended targets are the cause. Indeed, these uncertainties emphasize the need to develop gapmers with few or no unintended targets. As discussed in the subsection headed "Specificity of the same gapmer for different targets", one mechanism of hepatotoxicity leading to increased levels of plasma ALT following treatment with several different high-affinity modified gapmers, was demonstrated to be correlated to the overall number of genes with significantly reduced transcript levels (2). These results are consistent with a model where the more unspecific a gapmer is, the higher the chance of hepatotoxicity, again emphasizing the need to control the sequence-specificity of gapmers at the design stage. Finally, the industry consensus recommendation (6) reminds us that

preclinical toxicological assays in animal models supersede the specificity assessments discussed here in terms of de-risking drug candidates. There are, to the best of our knowledge, no reports connecting toxic events observed in human clinical trials to measured hybridization-induced effects derived from unintended targets. Nevertheless, since such effects are inherently species-specific, we believe it is prudent to put extra emphasis on developing in vitro toxicology assays based on human cells in antisense oligonucleotide drug discovery (128,130). It may be that for some gapmers, their critical unintended targets are only present in the human transcriptome, and their toxic liability therefore only revealed when evaluated against a human transcriptome. Pragmatically, for gapmers having a few unintended RNA targets that are reduced to some extent at therapeutically relevant doses, relevant human in vitro toxicology assays can help to evaluate the actual toxic liability associated with these unintended targets.

We recommend applying global transcriptome profiling to understand the sequence-specificity of antisense oligonucleotide drug candidates. Our reasons are: (i) experiments are generally more reliable than computational predictions. Knowing which unintended targets are affected can, on a case-by-case basis, help decide between candidates, and (ii) as more transcriptome-wide experiments are performed, the accumulating data can be utilized to develop algorithms that are better at predicting the experimental results (refer to Table 1). Importantly, the examination of the effect of one gapmer on many putative targets (understanding specificity) and the examination of the effects of many different gapmers on a single target (understanding potency and knockdown to find potent drug candidates) are governed by the same principles and are mutually beneficial. Hence, learning how to construct and select the most specific gapmers will also teach us how to design the most potent ones. Doing so exploits the biggest advantage antisense oligonucleotide drugs have over other classes of drugs - their sequence-based nature.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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