Nucleic Acid Therapeutics for Hematologic Malignancies—Theoretical Considerations

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ABSTRACT: Our work is motivated by the belief that RNA targeted gene silencing agents can be developed into effective drugs for treating hematologic malignancies. In many experimental systems, antisense nucleic acids of various composition, including antisense oligodeoxynucleotides (AS ODNs) and short interfering RNA (siRNA), have been shown to perturb gene expression in a sequence specific manner. Nevertheless, our clinical experience, and those of others, have led us to conclude that the antisense nucleic acids (ASNAs) we, and others, employ need to be optimized with regard to intracellular delivery, targeting, chemical composition, and efficiency of mRNA destruction. We have hypothesized that addressing these critical issues will lead to the development of practical and effective nucleic acid drugs. An overview of our recent work which seeks to addresses these core issues is contained within this review.

KEYWORDS: leukemia; lymphoma; antisense; oligonucleotides

INTRODUCTION

While the advent of antibodies and small molecules has made an extraordinary difference in the lives of patients with chronic myelogenous leukemia (CML), and many lymphomas, patients with other hematologic malignancies have yet to enjoy the benefits of these types of targeted therapies, and the issue

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TABLE 1. Important issues to address in the development of improved RNA targeting drugs

1. Target biology—Physiologic role, expression level, half-life
2. Delivery
3. mRNA target—physical structure
4. Chemical composition of the molecule

of resistance to drugs like imatinib and rituximab is becoming increasingly important.[1–3] The ability to eliminate proteins that have heretofore escaped specific targeting, or have demonstated the ability to evolve resistant forms is the strength of an RNA-targeted, protein-eliminating, therapeutic strategy. In addition, an ever-expanding knowledge of the molecular pathogenesis of hematologic malignancies continues to suggest new therapeutic targets.[4] Finally, gene-silencing therapies are in principle highly specific, so that if the target gene is thoughtfully chosen, damage to nontargeted tissues should be minimized and a high therapeutic index should result.[5–7]

Numerous "gene-silencing" strategies have evolved over the years, and these have been primarily directed either to the genes themselves,[8–10] or to their messenger RNAs. Some exceptionally clever techniques for direct gene targeting have been developed[10,11] but they have not proven simple or reliable enough, at least thus far, for therapeutic applications. In contrast, the perceived ease with which mRNA can be targeted has resulted in most therapeutic efforts being directed to this approach.[12,13] A number of modalities are available for mRNA targeting and of these, the "antisense" strategies have been the most widely applied.[13–15] All are based on delivery of a reverse complementary, i.e., "antisense," nucleic acid strand into a cell expressing the gene of interest. By processes still unknown, the antisense nucleic acid (ASNA) strand and the mRNA target come into proximity and then hybridize if the strands are physically accessible to each other. Stable mRNA–ASNA duplexes can interfere with the splicing of heteronuclear RNA into mature messenger RNA,[16,17] block translation of mature mRNA,[18,19] or can lead to the destruction of the mRNA by the binding of endogenous nucleases, such as RNase H,[20,21] or by intrinsic enzymatic activity engineered into the sequence as is the case with ribozymes[22,23] and DNAzymes.[24,25] More recently, posttranscription gene silencing or RNA interference (RNAi)[26,27] has emerged as an exciting potential alternative to these more classical approaches.[14,15,28] However, it is quite clear that many of the therapeutic considerations that apply to the use of traditional antisense molecules will also apply to RNAi as evoked by short interfering RNA (siRNA), microRNA (miRNA), and short hairpin RNA expressed in viral vectors (shRNA). These issues are listed in TABLE 1 and include (1) choice of gene target; (2) development of rational, reliable targeting strategies; (3) stability of nucleic acid molecules in body fluids, and cells; and (4) ability to deliver improved molecules into cells.[29–34] The so-called "off target," or unintended, gene silencing is also being increasingly recognized with siRNA.[35–39]

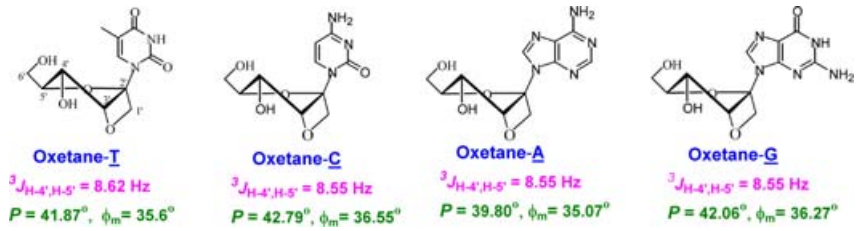

FIGURE 1. Structure of oxetane modified bases.

CHEMICAL MODIFICATIONS

Several promising new chemical modifications have been described recently.[40–44] Two relatively novel chemistries are of particular interest to us. One is the ribose sugar constraining oxetane modification[45,46](FIG. 1), the other is the 2′-deoxy-2′-fluoro-D-arabinonucleic acid (2′F-ANA) modification[47–49] (FIG. 2). The oxetane modification [oxetane, 1-(1′,3′-*O*-anhydro-ß-D-psicofuranosyl nucleosides)] imparts a number of highly desirable characteristics to oligodeoxynucleotides (ODN). These include enhanced nuclease stability and T_ms similar to those predicted for ODN/RNA hybrids. Since modification of all bases is not required to impart these characteristics, the ability of modified ODN to recruit RNase H is not impaired. We have examined the efficiency with which oxetane-modified antisense ODN inhibited *c-myb* gene expression in living cells and compared the results to standard phosphorothioate (PS)-modified ODN. We found that Myb mRNA and protein levels were equally diminished by oxetane and PS ODN, but the latter were delivered to cells with ~6× greater efficiency suggesting that oxetane-modified ODN were more potent on a molar basis.

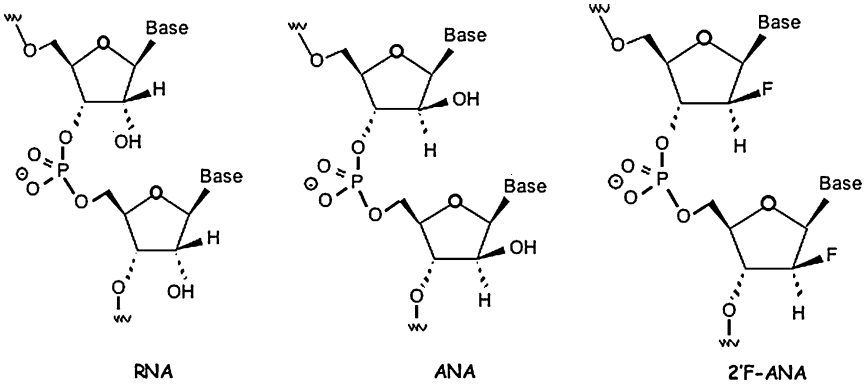

FIGURE 2. Derivation of 2′F-ANA.

The attraction of 2′F-ANA and 2′F-ANA-DNA chimeras derives from their nuclease resistance and their ability to simultaneously increase the strength of oligonucleotide:mRNA hybrids and elicit efficient RNase H-mediated degradation of target mRNA. In detailed studies we found that antisense oligonucleotides (AS ONs) containing a 2′F-ANA sugar modification not only performed as well as PS ODN, but had the added advantage of maintaining high intracellular concentrations for prolonged periods of time, which appears to promote longer-term gene silencing. To demonstrate this, we targeted the *c-myb* protooncogene's mRNA in human leukemia cells with fully PS 2′F-ANA-DNA chimeras (PS-2′FANA-DNA) and compared their gene-silencing efficiency with AS ON containing unmodified nucleosides (PS-DNA). When delivered by nucleofection, chemically modified ON of both types effected a greater than 90% knockdown of *c-myb* mRNA and protein expression, but the PS-2′F-ANA-DNA were able to accomplish this at 20% of the dose of PS-DNA, and in contrast to the PS-AS DNA, their silencing effect was still present 4 days after a single administration. This led us to conclude that PS-2′F-ANA-DNA chimeras are efficient gene-silencing molecules, and suggest that they could have significant therapeutic potential.

GENE TARGET SELECTION

With regard to gene target selection, our group has focused on short-lived mRNA molecules that encode proteins whose functions are critical to the targeted cell and that are equally short lived. We have hypothesized that such mRNA and protein targets are most efficiently eliminated from cells using the present methodologies, and importantly, are likely to have a considerable biologic impact on the cell that is being targeted. Examples of such targets include proteins encoded by the *c-myb* transcription factor gene, the transcriptional repressor *BCL-6*, the src family kinase lyn, and the *c-kit* receptor.[50–54] Of these, we have focused most of our efforts on *c-myb*, and an antisense DNA molecule targeting this transcription factor has been employed in the clinic for *ex vivo* bone marrow purging,[55] for systemic infusion into refractory leukemia patients, and will shortly be entering the clinic again in Phase I studies involving patients with a variety of hematologic malignancies.

Our experience with targeting the obligate hematopoietic transcription factor *c-myb*, whose mRNA and protein have very short half-lives of ~30–60 min each,[56] and which is required for G1/S transition, as well activation of other critical cellular genes,[53,55,57–61] would seem to be a good example.

To complement this strategy, we have also examined the utility of targeting an upstream signaling protein and a transcription factor simultaneously. In a specific instance, using cell lines and primary CML cells, we found that targeting *c-myb* and proto-Vav signaling protein can give additive cell inhibitory effects.[50]

mRNA MAPPING

How to select sequence targets within the mRNA of the candidate genes described above has been highly problematic. The physical structure of mRNAs is known to play an important role in target accessibility for both classical AS ONs (reviewed in Ref. 62), and more recently for siRNA molecules as well.[31] We have developed a novel approach to solving this problem, which depends on the use of fluorescent self-quenching reporter molecules (SQRM) to probe mRNA and signal the presence of hybridization accessible regions[51,63,64] (FIG. 3). We have used this method to target classical,[63] as well as chemically modified oligonucleotides.[42,51]

SQRM are DNA stem loops with fluorophore on the 5′ end (EDANS) and a quenching molecule (DABCYL) on the 3′ end.[51,63] To make the search more rational, and to ensure that strong hybrids were formed, we wrote a

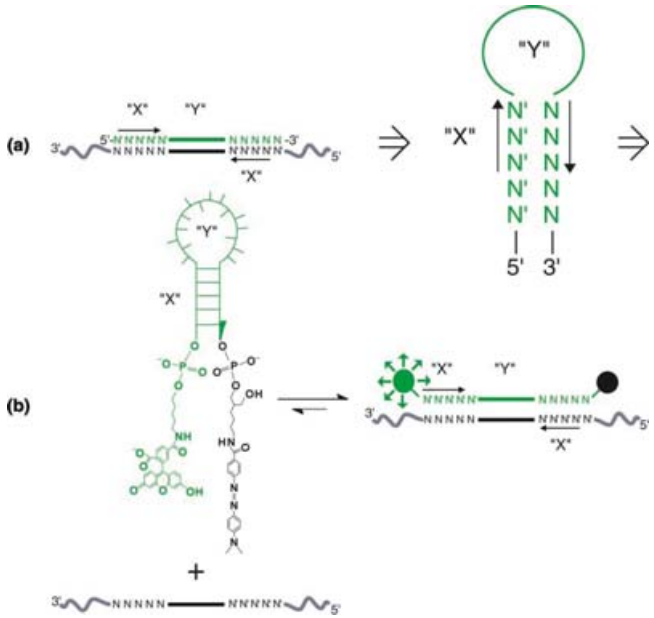

FIGURE 3. SQRM design and reaction. (**A**) Concept: to exploit the traditional stem–loop structure of the SQRMs, a computer algorithm ('AccessSearch') searches an entire sequence of mRNA for complementary sequences of a desired length (stems) that are separated by a proscribed distance (loop). (**B**) Chemistry: the complementary sequences are synthesized as SQRM possessing 50-fluorescein and 30 DABCYL groups. In the absence of target, quenching of fluorescence occurs. Once hybridization of the loop sequence to a complementary target takes place, the moieties are separated and fluorescence can be detected. From Gifford *et al.,* 2005. Nucleic Acids Res. 33: No. 3 e28. (Shown in color in online version).

simple computer algorithm that allowed us to search for inverted repeats within mRNA sequences downloaded from the NCBI site. Repeats of between 4 and 6 nucleotides were specified, along with intervening sequence of ~18–20 bases. Numerous sites compatible with these criteria could be found in any message we examined. SQRM corresponding to several such sites were synthesized, and then tested for hybridization as reported.[51] Of the probes studied, only two (+321, +964) demonstrated significant hybridization above background. Based on these results we would predict that a 26nt oligonucleotide targeted to *c-myb* beginning at +321 would effectively target the mRNA and silence gene expression. This prediction was evaluated in tissue culture and found to be correct (FIG. 4 A, B).

In another example, we have been developing antisense ODN and siRNA to knock down *BCL-6* expression in Diffuse Large B cell Lymphomas (DLCL). *BCL-6* is a zinc finger protein, which acts as a sequence-specific transcriptional repressor. Although *BCL-6* mRNA is ubiquitous, its expression is highest in germinal center B cells where it is thought to repress the expression of genes involved in B cell activation, cell cycle progression, and terminal differentiation.

In non-Hodgkin lymphomas, *BCL-6* is the most frequently deregulated gene and abnormal expression is found in ~30–40% of DLCL, and ~14% of follicular lymphomas (FL). Accordingly, we sought to develop gene-silencing antisense molecules targeted to *BCL-6* mRNA. Our strategy was based on the use of SQRM to rationally probe for hybridization accessible regions within a specific mRNA species. We found an accessible sequence within the *BCL-6* mRNA (SQRM-1310). An AS ON corresponding to SQRM-1310,

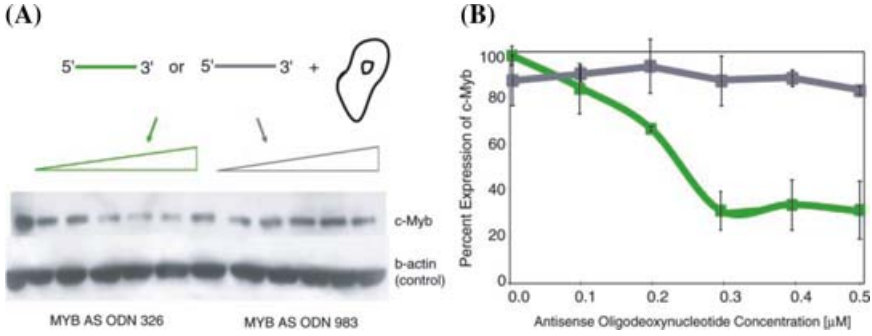

FIGURE 4. *c-myb* AS ODNs *in vivo*. An AS ODN corresponding to the SQRM321 was synthesized and transfected into hamster fibroblast Tkts 13 cells engineered to express human *c-myb*. (**A**) The Western blot shows a decrease in protein expression following treatment with the AS ODN 326–345 as compared with AS ODN 983–1000 (negative control). (**B**) Graphical representation of the western blot data: AS ODN 326 345 (dark gray); AS ODN 983–1000 (light gray). From Gifford *et al.*, 2005. Nucleic Acids Res. 2005, **33:** No. 3 e28. (Shown in color in online version).

were transfected into *BCL-6* (+) Louckes Cells using an Amaxa nucleoporator (Gaithersburg, MD). As a control, five other molecules were also transfected into Louckes Cells. Effects of these on *BCL-6* mRNA is shown in FIGURE 5. Cell viability was determined for 4 consecutive days. We found that cells transfected with Sequence +1310 exhibited an ~50% drop in viability within 24 h, while three other sequences were largely ineffective. Coincident with the viability drop, we found a seven-fold decrease in *BCL-6* mRNA in cells transfected with 1310, and little change in cells transfected with control ON. Corroborating Western Blot data on *BCL-6* expression were also obtained (FIG. 5).

DELIVERY

It is straightforward that without the ability to deliver material into cells, even the most cleverly designed molecule cannot be effective. As a general rule, oligonucleotides are taken up primarily through a combination of adsorptive and fluid-phase endocytosis.[65] After internalization, confocal and electron microscopy studies have indicated that the bulk of the oligonucleotides enter the endosome/lysosome compartment where most of the material either becomes trapped or degraded. Biologic inactivity is the predictable result of these events. Nevertheless, oligonucleotides can escape from the vesicles intact, enter the cytoplasm, and then diffuse into the nucleus where they presumably acquire their mRNA target. Colocalization of the effector strand and target mRNA in nucleoli,[23] or cytoplasmic P-bodies[66] appears important for AS ON and siRNA, respectively. In our hands and those of others,[67] lipid-transfecting agents have proven toxic to hematopoietic cells. Accordingly, we have begun to develop alternate means for delivering AS ON and siRNAs to hematopoietic cells including the use of electroporation for *ex vivo* delivery[52] (FIG. 6), as well as virosomes[68] and chitosan polymers[69,70] for systemic delivery.

CONCLUSIONS

The use of reverse complementary nucleic acid drugs to inhibit gene expression originated from studies that were initiated almost a quarter of a century ago.[71,72] Even though the mechanism by which these drugs modulate gene expression is not always clear,[73–75] the clinical development of nucleic acid drugs has proceeded to the point at which several of these drugs have entered Phase I/II, and in a few cases, Phase III trials. Results to date for most of these trials have been disappointing from the point of view of clinical efficacy. Nonetheless, the attraction for drugs of this class remains very strong and has been revitalized the development of RNAi.[76] This very exciting approach to gene silencing is, at the end of the day, also and "antisense-based

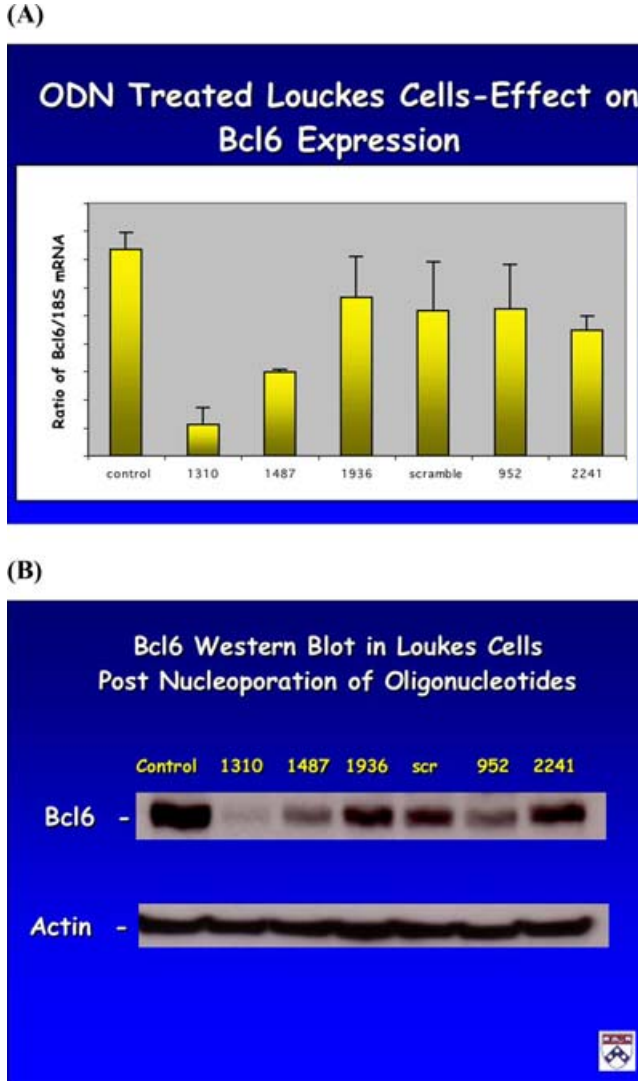

FIGURE 5. BCL-6 mRNA (**A**) and protein levels (**B**) in Loukes Cells treated with ODN targeted to different sequences within the *BCL-6* mRNA.

methodology" whose robustness in a clinical setting also needs to be determined. Therefore, despite the fact that only one ASNA drug has received FDA approval to date,[77] there is reason to remain optimistic that the problems that slow down progress in this field will be overcome, and that many very useful drugs for the treatment of a variety of human and animal diseases will result.

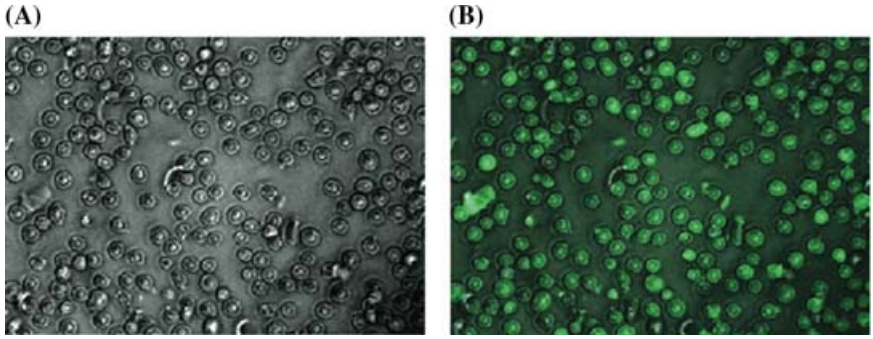

FIGURE 6. Delivery of fluorescein labeled unmodified ODNs into K562 cells with the nucleoporation technique: phase (**A**) and fluorescent (**B**) low power (200x) photomicrographs. From Opalinska *et al.*, 2004. Nucleic Acids Res. **32:** 5791-5799. (Shown in color in online version).

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