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Expanding RNA Silencing Approaches by U1 Adaptors

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Since the realization of the therapeutic potential of RNAi as an endogenous catalytic machinery to inactivate specific RNA targets according to Watson–Crick base-pairing rules, other antisense-based strategies have been marginalized. Yet, in a recent publication, Gunderson and co-workers[1] described an approach to recruit the abundant U1 snRNP, a splicing subparticle, to the last exon of a pre-mRNA. This mediates interaction of the U1-70K protein subunit with poly(A) polymerase, resulting in blockage of polyadenylation, followed by pre-mRNA degradation. The endonucleolytic cleavage step prior to polyadenylation is not affected by U1 snRNP recruitment. Thus, cryptic downstream poly(A) sites are removed from the pre-mRNA (Figure 1).

The novelty of the recent work[1] lies in the recruitment of endogenous U1 snRNP to the last pre-mRNA exon through a bifunctional oligonucleotide, roughly 25 nt in length, that base pairs with the target mRNA's terminal exon as well as with the 5′-end of U1 RNA (Figure 1). Such oligonucleotides have been termed U1 adaptors and their inhibitory effect as U1 interference (U1i). Preceding related studies employed a plasmid-borne U1 snRNA variant in which the natural U1 targeting domain was replaced with a 10 nt sequence complementary to the target's terminal exon.[2,3] So far, the *U1A* and *MARK1* genes are the only known genes with natural U1 snRNP binding sites in their terminal exons. The U1A protein negatively autoregulates its expression by polyadenylation inhibition. The mechanism involves U1 snRNP binding to a U1 site in the 3′-UTR of the U1A pre-mRNA; however, the U1 snRNP remains inactive because roughly half of the sequence complementary to U1 RNA is trapped in a stem structure. Only the binding of a second U1A protein (in addition to the one being part of the U1 snRNP) to a downstream conserved *cis*-element (PIE = polyadenylation inhibitory element) mediates activation of the U1 snRNP at the upstream U1 site, resulting in U1-70K-mediated inhibition of poly(A) polymerase.[4]

Two aspects of the U1 adaptor approach merit consideration: 1) U1snRNA, which functions early in splicing, hybridizes to 5′ splice sites through an 8 nt stretch at its 5′ terminus.[5] Thus, the 5′-terminal region of U1 RNA is naturally devoted to forming a duplex structure, thus minimizing the potential that interaction of the adaptor oligonucleotide with U1 RNA may cause any interference with U1 snRNP function. 2) The U1 snRNP, at concentrations of about 0.5 μM in a HeLa cell (with even higher concentrations in the nucleus), is in about tenfold stoichiometric excess over the spliceosome.[5] Thus, sequestering a small fraction of all U1 snRNP complexes by interaction with low-nM amounts of U1 adaptors is not expected to deplete the pool of U1 snRNP complexes to such an extent that the splicing machinery's capacity is compromised. To confirm this assumption, Gunderson and co-workers[1] analyzed the relative splicing patterns of four endogenous genes, known to undergo alternative splicing; in HeLa cells transfected with a U1 adaptor that effectively silenced its target pre-mRNA (*PCSK9*), the ratio of alternatively spliced products remained unaffected by U1 adaptor targeting in all four cases.

The U1 adaptor system was tested in HeLa cells with a dual-luciferase reporter setup. Initially, the 3′-UTR, poly(A) signal region and about 150 nt 3′ of the

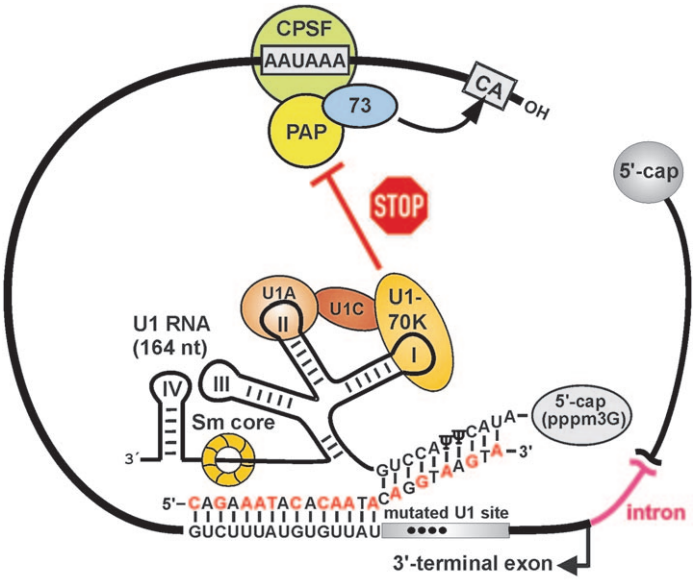

Figure 1. Mechanistic basis of the U1 adaptor approach that results in inhibition of poly(A) polymerase (PAP) activity by the U1 snRNP subunit U1-70K. A U1 snRNP is recruited to the 3′-terminal exon of a target pre-mRNA via a bridging oligonucleotide that simultaneously base pairs with the 5′-end of U1 RNA and the target sequence. After CPSF-dependent cleavage by the CPSF subunit 73, the U1-70K protein blocks polyadenylation by PAP. USE: upstream element, CPSF: cleavage and polyadenylation specificity factor, CA: cleavage site. The U1 adaptor design is exemplified for the UA6 variant (LNA residues in red) directed against the *MARK1*mt 3′-UTR (see text).

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poly(A) site of the *MARK1* gene were transferred into the *Renilla* luciferase reporter gene. A construct with four consecutive mutations in this U1 RNA binding site (*MARK1* mt; Figure 1) served as control. The *Renilla* reporter harbouring the wild-type U1 target sequence showed a 17-fold lower expression than the one with the mutated sequence. After this test system had been established, a chemically synthesized 25 nt U1 adaptor (UA6) was designed with ten nucleotides at its 3′-end complementary to the 5′-end of U1 RNA and 15 nucleotides complementary to the *MARK1* sequence immediately 3′ to the mutated U1 binding site (Figure 1; Table 1). This adaptor was a "mixmer" of ten DNA and 15 locked nucleic acid (LNA) residues, the latter increasing duplex stability with the two target RNAs. The UA6 adaptor decreased *Renilla* luciferase expression with an IC_{50} of 6.6 nM, whereas a control adaptor with four scattered mutations in the U1 RNA binding domain failed to inhibit reporter expression. In a next step, the authors transferred the 15 nt exon binding site of adaptor UA6 into another context, the 3′-UTR and poly(A) signal region of SV40. A very similar inhibition efficiency was observed (IC_{50} = 7.4 nM) and no effect was seen with the control adaptor. Inserting a second UA6 binding site into the SV40 3′-UTR even increased knockdown efficiency (IC_{50} = 2.2 nM vs. 7.4 nM; Table 2), thus demonstrating additive suppression if more than one binding site exists on the same target. Relative to the original design (ten LNA and five DNA residues in the target domain), UA6 adaptor efficacy was increased when the target domain was all-LNA; even an all-LNA target domain reduced to 12 residues was more efficient than the ten LNA/five DNA format.

Prompted by the possibility that LNA residues in some tailored U1 adaptors might impair suppression efficiency owing to self-dimerization or intramolecular hairpin formation of adaptor oligonucleotides, the authors tested U1 adaptor variants with the original exon binding domain design (ten LNA, five DNA residues), but with a U1 RNA binding sequence varying in length from seven to 19 nucleotides and composed of 2′-OMe-RNA residues only (all-2′-OMe variants of the entire UA6 adaptor were inactive). Knockdown efficiency peaked with a 13 nt 2′-OMe U1 domain (variant UA17-13; IC_{50} = 1.5 nM), being three times more potent than the original UA6 adaptor with its 10 nt LNA/DNA sequence. A 13 nt 2′-OMe U1 domain was also shown to be optimal for a different target sequence. Replacing five of the 13 2′-OMe residues with LNA improved the IC_{50} of adaptor UA17-13 from 1.5 to 0.5 nM (Table 2). U1 adaptors with all-phosphorothioate backbones were effective as well. The mismatch discrimination of U1 adaptors was as for high-affinity antisense oligonucleotides, with three mismatches in the 15 nt target domain essentially abrogating target suppression at 2.5 nM adaptor concentration in HeLa cell transfection experiments. With LNA modifications, and depending on sequence and target context, a reduction of target domain length to 10–14 nt might allow an improvement in mismatch discrimination without compromising efficiency.[6]

A U1 adaptor (UA25) directed against another endogenous target, the *RAF1* oncogene, carried an 11 nt all-2′-OMe U1 domain and depleted *RAF1* mRNA and protein levels with an IC_{50} of 8 nM. The best antisense oligonucleotide directed against the same target site in another study[7] had an IC_{50} of only 50 nM. Three more U1 adaptors targeting areas of the terminal *RAF1* exon, predicted to be unstructured, were approximately half as effective as the UA25 adaptor. Two U1 adaptors targeting the *PCSK9* gene had IC_{50} values of 4 to 5 nM, and simultaneous transfection of both adaptors enhanced the knockdown. Moreover, combining siRNAs and U1 adaptors against the same target gene always resulted in enhanced suppression (four different targets tested). This raises the interesting question of how two different siRNAs against the same target perform relative to a single siRNA combined with a U1 adaptor at comparable concentrations. Microarray analysis of HeLa cells transfected with either a U1 adaptor or an siRNA against *PCSK9* (pre-)mRNA suggested that global off-target effects are comparable for siRNA and U1i treatments.

In conclusion, the U1 adaptor approach is a novel promising strategy that expands the arsenal of gene silencing concepts. U1i appears to be almost as potent as RNAi and can be potentiated by simultaneous application of multiple U1 adaptors or by combination with siRNAs. By acting on pre-mRNAs in the nucleus, U1i complements RNAi operating on mature mRNAs in the cytoplasm.

Table 1. Present design of U1 adaptors.

Design	Target domain	U1 domain
length	12/15 nt	10–13 nt
modification	up to 15 LNAs	optimal: 8×2′-OMe, 5×LNA (13 nt)
PS	tolerated	tolerated

target domain U1 domain

5′-NNNNNNNNNNNN(NNN)CAGGUAAGUA(NNN)-3′

U1 adaptor oligo design

LNA: locked nucleic acids; PS: phosphorothioate backbone.

Table 2. Parameters to increase U1 adaptor efficacy.

Parameters	Increase in silencing activity
design (domain lengths, nucleotide modifications)	~14-fold (IC_{50}: ~7 nM→0.5 nM)
tandem adaptors	~threefold (IC_{50}: 7.4 nM→2.2 nM)
U1i + RNAi	~tenfold (see example below)
no silencing	*100% luciferase activity*
U1i-GAPDH (15 nM):	*20% luciferase activity*
siRNA-GAPDH (1 nM):	*7.7% luciferase activity*
U1i + siRNA	*0.8% luciferase activity*

An advantage over RNAi and antisense oligonucleotides recruiting endogenous RNase H is that U1 adaptors are amenable to the full scope of nucleotide modifications, thus facilitating attempts to fine-tune their pharmacokinetic and pharmacodynamic properties. So far, there is no evidence that U1i exacerbates the problem of off-target effects; favourable in this respect is also the fact that U1i is restricted to the last exon of a pre-mRNA, which reduces the potential of non-specific effects at upstream exons. Conceivable drawbacks of U1i may include the fact that only genes that undergo polyadenylation can be targeted and the requirement of accessible target sites for U1 adaptors in the 3′-terminal exon. Yet, LNA modifications in the adaptor likely alleviate the latter limitation, as LNA oligonucleotides are known to be able to invade stable RNA structures.[6,8] Nevertheless, extensive secondary structures masking the U1 binding site (see above;[4]) may better be avoided, and target sequences should be as unique as possible. Conceivably, miRNAs may in some cases compete with U1 adaptors for binding to 3′-UTRs. An important hurdle to be taken is the demonstration of U1i in animal models. Yet, for this purpose the same delivery strategies and formulations developed for RNAi should be readily adaptable to U1i. Future studies are awaited that provide a more comprehensive picture on potential off-target effects and which validate and fine-tune the U1 adaptor design rules. An open question is the spatial relation of the U1 RNA binding and target domains. The present design (Figure 1) lacks any spacer nucleotides between the two; insertion of a short (for example, tetranucleotide) spacer sequence between the two paring segments may avoid mutual interference in duplex formation or may relax topological constraints relevant to interaction of U1-70K and poly(A) polymerase.

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Keywords: LNA · polyadenylation block · RNA silencing · U1 adaptors · U1 snRNP · U1i

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