A Prospective Study in the Rational Design of Efficient Antisense Oligonucleotides for Exon Skipping in the *DMD* Gene

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Abstract

Antisense oligonucleotide (AON)-mediated exon skipping to restore dystrophin expression in Duchenne muscular dystrophy (DMD) therapy shown promise in a number of human clinical trials. Current AON design methods are semi-empirical, involving either trial-and-error and/or preliminary experimentations. Therefore, a rational approach to design efficient AONs to address the wide spectrum of patients' mutations is desirable. Retrospective studies have extracted many AON design variables, but they were not tested prospectively to design AONs for skipping *DMD* exons. Not only did the variables differ among the various studies, no numerical cutoff for each variable was inferred, which makes their use in AON design difficult. The challenge is to thus select a minimal set of key independent variables that can consistently design efficient AONs. In this prospective study, a novel set of design variables with respective cutoff values was used to design 23 novel AONs, each to skip one of nine *DMD* exons. Nineteen AONs were found to be efficacious in inducing specific exon skipping (83% of total), of which 14 were considered efficient (61% of total), i.e., they induced exon skipping in >25% of total transcripts. Notably, the satisfactory success rates were achieved by using only three design variables; namely, co-transcriptional binding accessibility of target site, presence of exonic splicing enhancers, and target length. Retrospective analyses revealed that the most efficient AON in every exon targeted has the lowest average cumulative position (ACP) score. Taking the prospective and retrospective studies together, we propose that design guidelines recommend using the ACP score to select the most efficient AON for each exon.

Introduction

SPLICING MODULATION TO INDUCE specific exon skipping to restore the reading frame and/or remove nonsense mutations of the *DMD* gene is now becoming a viable therapeutic strategy for Duchenne muscular dystrophy (DMD) (reviewed in Aartsma-Rus, 2010). In this approach, antisense oligonucleotides (AONs) are designed to bind to appropriate sequences within the target exon pre-mRNA during transcription to induce its skipping. Efficient AONs are critical to pass clinical trials and also to lower effective dosage, which would reduce side effect and costs. Unlike splice site AON target sequences, identifying exon-internal AON target sites that lead to efficient exon skipping is not straightforward due to the vast number of

possible sites. For instance, the average exon in the *DMD* gene is 140 bases long and contains 1276 possible exon-internal AON target sequences of 20 to 30 nucleotides, which are impractical to validate experimentally. Because effective target sites constitute a small fraction of the entire pool of possible sequences and AON efficiency can be sensitive to even a single nucleotide change (Aartsma-Rus *et al.*, 2005; Harding *et al.*, 2007; Popplewell *et al.*, 2009, 2011), a trial-and-error approach would expend significant resources. Therefore, a rational approach for designing efficient AONs targeting exon-internal sequences is desirable.

The prerequisite for rational AON design is a prior knowledge of the design variables and their effects on AON efficiency. Owing to the lack of such knowledge, prior

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approaches used in designing AONs for *DMD* exon skipping are semi-empirical (Aartsma-Rus *et al.*, 2005, 2009b; Popplewell *et al.*, 2009; Wilton *et al.*, 2007), entailing a mix of trial-and-error and/or preliminary experimentation (Supplementary Table S1; Supplementary Data are available online at www.liebertonline.com/hum). Nevertheless, these pioneering efforts have generated experimental data for retrospective studies in which tens of design variables that correlate with observed AON efficiencies in exon skipping were extracted (Aartsma-Rus *et al.*, 2005, 2009b; Popplewell *et al.*, 2009; Wee *et al.*, 2008; Wilton *et al.*, 2007). The extracted variables, however, differed among the various studies, and it is unclear which variable is dominant among non-independent variables that impinge on each other. Furthermore, no numerical cutoff for each extracted variable was inferred, which makes their application in AON design difficult. Most crucially, the effect of each variable on AON efficiencies was not tested experimentally. Therefore, as also remarked by Aartsma-Rus *et al.* (2009b), a prospective study is essential to validate a set of design variables wherein new AONs are designed and their respective exon skipping efficiencies are measured. Since every variable influences AON efficiencies unequally, the challenge is to select a minimal set of key independent variables that can consistently design efficient AONs.

In this prospective study, we proposed and experimentally validated a novel set of three design variables, namely *co-transcriptional* binding accessibility of target site, presence of exonic splicing enhancers (ESEs), and AON target length, to design efficient AONs targeting exon-internal sequences. Respective cutoff values were specified for the design variables that were subsequently used as criteria to select AON target sites. This combination of design variables and the associated cutoff criteria have not previously been applied in AON design, and the rationale for their selection is explained as follows.

The first two design variables are associated with the two respective key biological processes during AON-mediated exon skipping—(1) specific binding of an AON to an accessible target site, and (2) the binding thereafter prevents the spliceosome from identifying the target as an exon, and it will be removed along with the introns during splicing. As exon recognition and splicing is *co-transcriptional* (Maniatis and Reed, 2002; Tennyson *et al.*, 1995), AONs need to compete with splicing factors for binding to the target exon during transcription. Throughout this process, however, the target exon secondary structure is dynamic as newly transcribed nucleotides create new combinations of complementary Watson-Crick base-pairings within the pre-mRNA. As a result, the target exon accessibility is dynamic and is accounted for by the first design variable, *co-transcriptional* binding accessibility of target site. To prompt target exon removal, AONs must bind to target sites possessing ESE motifs, as considered in the second design variable. These ESE motifs are exon-internal sequences to which splicing factors bind for exon recognition. ESE-dependent exon recognition is particularly important in the *DMD* gene due to the presence of long introns (Gravely *et al.*, 1999; Lam and Hertel, 2002). The third design variable, AON target length, is required for practical considerations because many short AON target sites (<10 bases) with high *co-transcriptional* binding accessibilities and

ESE motifs are generated. Besides the issue of nonspecific binding to other sites, they may not anneal stably to the target due to low annealing temperatures. Generally, AON target length between 20 to 30 nt correlates with efficient exon skipping using AONs with either 2'-O-methyl phosphorothioate (2OMePS) or phosphorodiamidate morpholino (PMO) chemistries (Aartsma-Rus *et al.*, 2010; Arechavala-Gomez *et al.*, 2007; Harding *et al.*, 2007; Heemskerk *et al.*, 2009; Popplewell *et al.*, 2009, 2010). For 2OMePS AONs, Aartsma-Rus *et al.* (2009b) concluded that the optimal length is about 20 nt, while Harding *et al.* (2007) reported an optimal range of 25 to 30 nt. Whereas Popplewell *et al.* (2009, 2010) reported an optimal length of 30 nt for PMO AONs.

A total of 23 novel 2OMePS AONs, each targeting exon-internal sequences of one of nine exons, were designed based on the proposed set of design variables. Of these, 19 AONs (83% of total) were validated to be efficacious in inducing specific skipping of their targeted exons. Fourteen of the efficacious AONs (61% of total) were deemed efficient, inducing specific skipping of 25% or more transcripts; this standard classification criteria of AON efficiencies is used by prior studies (Aartsma-Rus *et al.*, 2009b; Popplewell *et al.*, 2009; Wilton *et al.*, 2007). At least one efficient AON was obtained per targeted exon. The satisfactory success rates validated the selected set of design variables as major factors influencing AON efficiencies, and hence supported the plausibility of rational AON design. Retrospective analyses revealed that the most efficient AON in every exon targeted had the lowest average cumulative position (ACP) score. Taking the prospective and retrospective studies together, the proposed design guidelines will expedite the development of efficient AONs for therapy.

Materials and Methods

Computational prediction of the dynamical pre-mRNA secondary structure

The methodology for quantifying and analyzing the dynamics of the pre-mRNA structures in the progression of transcription did not depend on the choice of the prediction tool as long as *co-transcriptional* structures were obtainable. The mfold algorithm was eventually chosen because of its relative efficiency in computing long RNAs. It also has the advantage of being used in most published experimental results on AONs that target the *DMD* gene, and therefore results of this work can be compared with others on a common basis. Each window of analysis sequence was fed into mfold version 3.1 (Mitrant *et al.*, 2009; Zuker, 2003), which was executed on a Dell PowerEdge SC1420 server running Red Hat Enterprise Linux 4.0 OS (Fig. 1a). Since it was highly probable that the nascent pre-mRNA may not have the chance to assume optimal structures, we accepted suboptimal structures whose energies lie within 5% of the optimum.

Using the predicted secondary structures at each step of transcription, a particular nucleotide of the exon is termed as an *engaged* nucleotide when all the structures indicate that it is paired to another complementary base. Figure 1b depicts the occurrences of all *engaged* nucleotides (shown as black spots) of exon 2. The L3 score of an AON target site is then computed from the *engaged* nucleotides plot as follows: the sum of total number of *engaged* nucleotides (black spots)

FIG. 1. Co-transcriptional binding accessibility of target exon. **(a)** To approximate the transcription elongation process, a "window of analysis" is shifted one nucleotide at a time along the pre-mRNA sequence from the 5' to the 3' end. Each window, which includes the full target exon sequence and part of its flanking introns encompassing the donor and acceptor splice sites and branch point, corresponds to a step of transcriptional analysis. The possible secondary structures of the entire sequence in a window were then predicted. **(b)** Occurrences of *engaged* nucleotides at each step of transcriptional analysis for the case of exon 2. The horizontal axis denotes sequential steps of transcriptional analysis while the vertical axis denotes numbered nucleotides within the target exon. At each step of transcriptional analysis, an *engaged* nucleotide is depicted as a black spot.

divided by the product of the target length and total number of transcriptional steps of analysis.

Definition of ACP

The ACP score is the average of the sum of each target nucleotide position from exon 5'.

$$ACP = \frac{\frac{P_1}{L_{\text{Exon}}} + \frac{P_2}{L_{\text{Exon}}} + \dots + \frac{P_{L_{\text{AON}}}}{L_{\text{Exon}}}}{L_{\text{AON}}} = \frac{2P_1 + L_{\text{AON}} - 1}{2L_{\text{Exon}}}$$

where P_i is relative position of the i th target nucleotide from exon 5'

L_{AON} is AON target length

L_{Exon} is target exon length

Cell cultures

Primary myoblast cells from DMD patients (kindly provided by Prof. Francesco Muntoni, UK) and primary human fibroblast cells from normal individual (Coriell, USA) were used. Fibroblast cells were used to test the AONs for their effect in restoring dystrophin mRNA reading frame when suitable patient myoblast cells are not available. To obtain a measurable amount from the low dystrophin mRNA in fibroblast cells, a higher amount of template was used in the nested PCR amplification of harvested dystrophin transcript.

Transfection into human fibroblast cells

AONs were synthesized by Sigma (Singapore) and modified with 2OMePS. Human fibroblast cells were cultured in

six-well plates containing a growth medium of Dulbecco's modified Eagle's medium supplemented with 2 mmol/L L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% fetal calf serum. All media, supplements, and Lipofectamine2000 were purchased from Invitrogen (Singapore). When the cells reached 80% confluence, transfection was performed with either 100 or 200 nM AONs and Lipofectamine2000 at a ratio of concentrations following manufacturers' recommendations and protocol. To ensure reproducibility, transfection was done in duplicate, triplicate, or quadruplicate. After 5 hr of incubation, the transfection medium containing AON-Lipofectamine2000 was replaced with growth medium to maintain the cells. Twenty-four hours after transfection, the cells were harvested and subjected to mRNA analysis to assess the efficacy of the AONs in inducing exon skipping. Total RNA was isolated using Trizol (Invitrogen, Singapore). Single step RT-PCR was performed on 400 ng total RNA using a single step RT-PCR analysis kit, Access RT-PCR system (Promega, Singapore), according to the manufacturer's instructions but with modified PCR amplification of 30 cycles. Four microliters from the 50 μ l PCR products were then used for nested PCR of 32 cycles in a 20- μ l total volume reaction. Sequences of *DMD* exon-specific primers used for single step RT-PCR and nested PCR are available upon request. The PCR products were then verified by agarose gel electrophoresis and DNA sequencing to confirm skipping of the specific target exon. Exon skipping efficiency was estimated by densitometry analysis of the gel images comparing the density of amplicons from *DMD* mRNA with the targeted exon skipping to the native *DMD* mRNA.

Transfection into primary cultures of DMD patients' myogenic cells

Two primary cell cultures derived from skeletal muscle of DMD patients carrying deletion of exons 45–50 and deletion of exons 48–52 were used to test the efficacy and efficiency of the AONs targeting exons 51 and 53, respectively. AONs were synthesized with identical chemistry as described above. The cells were seeded in six-well cell culture plates coated with collagen (Becton Dickinson, USA), and grown in supplemented muscle cell growth medium. Once confluent, the cultures were switched to supplemented muscle cell differentiation medium. Both media were purchased from Promocell (Germany). Transfection of AON was then performed when myoblasts fused to form visible myotubes (elongated cells containing multiple nuclei and myofibrils). Transfection was performed using protocols identical to those used in fibroblast cells except for the following. After 5-hr incubation, the transfection medium containing AON-Lipofectamine2000 was replaced with differentiation medium to maintain the cells. The cells were harvested 24 hr after transfection. Total RNA was isolated using Trizol (Invitrogen, Singapore) followed by first-strand cDNA synthesis using 1 μ g of the total RNA, oligo random hexamer primers and the SMARTTM cDNA Synthesis Kit (Clontech, USA) in a total reaction volume of 20 μ l. First PCR was performed using 4 μ l of cDNA in a total volume of 20 μ l with 30 cycles. One microliter from the 20 μ l of PCR products was then used for nested PCR of 32 cycles in a total 20- μ l volume reaction. Exon skipping efficiency was also estimated by densitometry.

Immunocytochemistry

To show the restoration of dystrophin expression, immunohistochemistry staining was performed on the patients' muscle cells after treatment with AONs targeting exons 51 and 53. To analyze the effect of AON therapy, the expression of dystrophin was detected by immunocytochemistry staining of both AON-treated and untreated muscle cells. Primary antibody anti C-terminal dystrophin was used to assess the restoration of the dystrophin expression. Since DMD patients carry exon deletions that lead to an mRNA reading frame shift with a premature stop codon/truncation of the protein, the antibody against C-terminal (3') of dystrophin should not be able to detect any dystrophin in their muscle cells. The success of the AON in restoring dystrophin expression should be marked by the detection of the dystrophin in the patients' muscle cells. The efficiency of the AON was reflected by the number of dystrophin-positive cells compared to dystrophin-negative myogenic cells.

On the third day after transfection, the muscle cells on the cover slips were fixed with methanol for 5 min followed by acetone treatment for 1 min. A double-staining immunocytochemistry method was used to detect dystrophin and desmin. Mouse monoclonal anti-dystrophin antibody (Novocastra Laboratories Ltd., UK) and rabbit polyclonal anti-desmin antibody (Sigma, Singapore) were used as the respective primary antibodies. Following the primary antibody, Alexa Fluor 594 goat anti-mouse IgG (Invitrogen, Singapore) and Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, Singapore) were applied to stain dystrophin and desmin, respectively. 6'-Diamino-2-phenylindole was used to visualize the nucleus.

Results

Rational AON design

Twenty-three novel AONs targeting exon-internal sequences of exons 2, 43, 45, 46, 47, 50, 51, 53, and 57 of the *DMD* gene were designed based on the three design variables—*co-transcriptional* binding accessibility of target site ($L3 < 0.23$), presence of ESEs ($R_{ESE} > 0.44$) and AON target length (20–35 nt). The target exons were chosen based on the therapeutic potential of their skipping on known *DMD* mutations (Aartsma-Rus *et al.*, 2009a). Table 1 lists the AON target sequences and the numerical values of each of the respective design variables.

The *co-transcriptional* binding accessibility of each target exon was determined as previously described (Wee *et al.*, 2008) and is outlined in Fig. 1a (see *Materials and Methods*). As an illustrative example, the *co-transcriptional* binding accessibility of exon 2 is depicted in Fig. 1b. A black spot on the figure indicates that the nucleotide of a target exon (as indicated by the vertical axis) is paired with a complementary nucleotide in all predictions of secondary structure at the transcriptional step (as indicated by the horizontal axis). Such a nucleotide is termed an *engaged nucleotide* at that transcriptional step. Thus, the black spots indicate the occurrences of potentially inaccessible nucleotides during transcription. The $L3$ score (Wee *et al.*, 2008), which denotes the average number of *engaged* nucleotides over all the transcriptional steps manifested by the nucleotides of an AON target site, was used to identify target sites with high *co-transcriptional* binding accessibility; a low score indicates

TABLE 1. SUMMARY OF 23 NOVEL ANTISENSE OLIGONUCLEOTIDES DESIGNED AND VALIDATED IN THIS STUDY

AON	Target sequence (5' to 3')	Position	Exon	Exon length	L3	R _{ESE}	AON length	AON efficiency	Third ^a	ACP ^b
AON2-1	ugu uuu cuu uug aac auc uuc ucu uu	4–29	2	62	0.069	0.96	26	++ (64.6%)	1	0.266
AON2-2	uac cca uuu ugu gaa ugu uuu cuu uug a	17–44			0.068	0.89	28	++ (64.5%)	1	0.492
AON43-1	gag cuu ugu ugu aga cua ucu uuu aua uu	1–29	43	173	0.049	0.86	29	++ (25.4%)	1	0.087
AON43-2	cuu ccu gua gcu uca ccc uuu cca c	88–112			0.130	0.92	25	+ (6.0%)	2	0.578
AON45-1	uua uuu cuu ccc cag uug cau uca aug uuc u	40–70	45	176	0.049	0.94	31	++ (28.8%)	1	0.313
AON45-2	cca auu uuu ccu gua gaa ua	99–118			0.036	0.80	20	+ (13.0%)	2	0.616
AON45-3	cuu uuu ucu guc uga cag cug uu	152–174			0.026	1	23	– (0%)	3	0.926
AON46-1	uga caa gau auu cuu uug uuc uuc uag c	1–28	46	148	0.063	0.75	28	++ (33.0%)	1	0.098
AON46-2	ucu uuu cca ggu uca agu ggg aua cu	89–114			0.010	0.81	26	++ (25.2%)	2	0.686
AON47-1	acg ggu ccu cca guu uca uuu aa ugu uug	39–68	47	150	0.015	0.87	30	++ (32.6%)	1	0.357
AON47-2	uua ucu ugc ucu ucu ggg cuu aug gga	81–107			0.017	0.89	27	+ (14.0%)	2	0.627
AON47-3	uua ucc acu gga gau uug ucu	128–148			0.001	1	21	– (0%)	3	0.920
AON50-1	acu cag agc uca gau cuu cua ac	6–28	50	109	0.096	1	23	++ (37.6%)	1	0.156
AON51-1	ggu ugu guc acc aga gua aca guc uga gua	5–34	51	233	0.024	1	30	++ (100%, 54.9% c)	1	0.084
AON51-2	uuu cua guu ugg aga ugg cag uuu ccu uag u	42–72			0.032	0.61	31	++ (100%, 32.3% c)	1	0.245
AON51-3	ccu cug uga uuu uau aac uug	162–182			0.003	0.62	21	+ (30.8%, 1.7% c)	2	0.738
AON53-1	gug uuc uug uac uuc auc cca cug a	17–41	53	212	0.030	0.96	25	++ (34.9%)	1	0.137
AON53-2	cgg uuc uga agg ugu ucu ugu acu uca	26–52			0.054	0.96	27	++ (59.0%) ^c	1	0.184
AON53-3	cug uug ccu ccg guu cug aag gug uuc uug u	32–62			0.079	0.81	31	++ (55.5%) ^c	1	0.222
AON53-4	ugg uuu cug uga uuu ucu uuu gga uu	185–210			0.008	1	26	– (0%)	3	0.932
AON57-1	guc aga acu ggc uuc caa aug g	4–25	57	157	0.009	0.73	22	++ (25.4%)	1	0.092
AON57-2	uaa uuc auc auc uuu cag cug ua	72–94			0.027	0.91	23	+ (5.0%)	2	0.529
AON57-3	ugu aca ucg uuc ugc uuc uga	133–153			0.014	1	21	– (0%)	3	0.911

^aIn the “Third” column, 1, 2, and 3 indicate that the first nucleotide of the AON target site is located at the first, second, and last third of the exon 5', respectively.

^bACP (last column) refers to average cumulative position that is defined in *Materials and Methods*.

^cAON efficiency obtained using patient myoblast cells.

an AON target site is highly accessible for AON binding during transcription.

Next, target exon sequences were predicted for ESE motifs using ESE-finder (Cartegni *et al.*, 2003) and Rescue-ESE (Fairbrother *et al.*, 2002). The ESE prediction tools were chosen because they were widely used in prior AON studies (Aartsma-Rus *et al.*, 2005, 2009b; Harding *et al.*, 2007; Popplewell *et al.*, 2009; Wilton *et al.*, 2007), which facilitates the comparisons of our results with previously reported AON datasets. R_{ESE} score, which denotes the fraction of nucleotides within a target sequence with ESE motifs, was computed from merging the predicted motifs from the two

sources with equal weight. The predicted ESE motifs were then merged with the *co-transcriptional* binding accessibility plot; see Fig. 2 for the case of exon 45 (see Supplementary Fig. S1 for the other target exons).

Lastly, AON target sequences with lengths between 20 and 35 nucleotides long were subsequently identified first by having the lowest possible L3 score (<0.23) and then by the highest possible R_{ESE} score (>0.44). The range of AON target length, upper limit of L3 score, and lower limit of R_{ESE} score used were inferred from prior studies wherein efficient AON target sequences lie between this range (Aartsma-Rus *et al.*, 2005, 2009b; Popplewell *et al.*, 2009; Wilton *et al.*, 2007).

FIG. 2. Rational identification of antisense oligonucleotide (AON) target sites for the case of exon 45. AON target sequences were determined by first identifying the sequences with the lowest possible L3 score, followed by selecting those among them with the highest possible R_{ESE} score. A low L3 score means that there are minimal *engaged* nucleotides (or black spots on the plot). Segments of the target exon sequence that are predicted to contain exonic splicing enhancer (ESE) motifs are indicated on the vertical axis—bar charts (ESE-finder) and short sequences (Rescue-ESE). The three AON target sites selected are indicated in the plot. See Supplementary Fig. S1 for the remaining target exons. R_{ESE} score denotes the fraction of nucleotides within a target sequence with ESE motifs.

Experimental validation of AON efficiency in inducing exon skipping

Twenty-one AONs were tested for their efficiency in exon skipping in primary cultures of human fibroblast cells, while five AONs each targeting exon 51 or 53 were tested in primary cultures of patient muscle cells (see *Materials and Methods*). Their efficacy was determined by RT-PCR analysis, followed by sequencing analysis, to show the target exon skipping induced in *DMD* transcripts. Their efficiency was measured by densitometry of the gel images that semiquantified the amplicons with exon skipping and the amplicons of native transcripts. AON efficiency is presented as a percentage of the amplicons with exon skipping relative to total amplicons and are classified as ++ (>25%), + (<25%), and – (0%); these standard classification criteria of AON efficiencies were used in prior studies (Aartsma-Rus *et al.*, 2005, 2009b; Popplewell *et al.*, 2009; Wilton *et al.*, 2007). An efficient or “+++” AON is defined as one that can consistently induce transcription of more than 25% of total dystrophin mRNAs based upon the observation that 22% of human and 20% of mice native dystrophin transcripts are sufficient to either avoid or ameliorate *DMD* symptoms (Faulkner *et al.*, 1997; Neri *et al.*, 2007; Sharp *et al.*, 2011). The agarose gel electrophoresis of nested PCR products from the cDNA derived from *DMD* patients’ muscle cells, which were treated with AONs targeting exons 51 and 53, showed a band with a molecular weight between the native-transcript amplicons and the exon-skipping-carrying-transcript amplicons (Figs. 3c and 3d). Bidirectional sequencing confirmed that this band is a hetero-duplex formed between the native-transcript amplicons with the exon-skipping-carrying-transcript amplicons. In such hetero-duplex, the contributions from native transcripts and transcripts with exon skipping to the band were considered

to be 50%/50%. Table 1 lists the average efficiency of each AON; the standard deviation of the average efficiency is given in Supplementary Table S2.

Of the 23 validated AONs, 19 AONs were efficacious in inducing skipping of their specific target exon, among which 14 AONs induced specific skipping of 25% or more transcripts (efficiency score ++) and were considered efficient AONs. Notably, each target exon could be skipped by at least one efficient AON. Skipping of exons 47 and 57, which have been deemed unskippable by single target AONs (Aartsma-Rus and Van Ommen, 2007), was induced by at least one of our efficient AONs (Figs. 3a and 3b). Figures 3c and 3d show the RT-PCR products gel images demonstrating specific exon skipping induced by AONs targeting exons 51 and 53, respectively, in *DMD* patients’ muscle cells. Immunocytochemical staining showing the restoration of dystrophin expression in *DMD* patients’ muscle cells induced by AONs targeting exons 51 and 53, respectively, are shown in Fig. 4.

It is possible that the efficiency of AON57-1 reported here is underestimated. This is because the skipping of exon 57 generates an out-of-frame transcript that could be subjected to nonsense-mediated decay (NMD), possibly reducing the amount of skipped transcripts for amplification. Possible future work to offset the NMD effect is to transfect AON57-1 into *DMD* patient myoblasts. Although the reading frame of the intact transcript cannot be restored, the NMD effect of the skipped transcript can be offset by the NMD of the intact transcript as long as both transcripts undergo NMD at similar rates. However, because the locations and number of premature termination codons, which could influence the initiation and/or rate of NMD (McGlinchey and Smith, 2008), differ in the intact and skipped transcripts, it is possible that they may not undergo NMD at similar rates.

FIG. 3. Efficiency of novel AONs targeting exons 47, 51, 53, and 57. AON at 100 nM concentration was used to treat cells to induce specific exon skipping of *DMD* transcripts. AON efficiency was assessed by comparing amplicons of *DMD* transcripts harboring exon skipping (lower arrow) with amplicons of native *DMD* transcript (upper arrow). AONs were able to induce exon skipping efficiently in human primary fibroblast cells for exons (a) 47, (b) 57, and in DMD patient skeletal muscle cells for exons (c) 51 and (d) 53. *Hetero-duplex formation between amplicons carrying exon skipping with native transcripts that appear as an additional band with molecular weight of slightly lower than those of native transcript amplicons.

Retrospective analysis

Retrospective analysis of the AON efficiencies obtained was performed to uncover additional design variables that may explain the basis for our inefficient and/or ineffective AONs. As expected, since all the AONs designed have low L3 scores, high R_{ESE} scores, and target length within the range, there is no statistically significant difference in L3 score, R_{ESE} , and AON length between efficient (++) and both inefficient (+) and ineffective (-) AONs (Supplementary Fig. S2). However, correlation between AON efficiency and the starting position of the AON target site was discerned. AONs whose first target nucleotide lies in the first third of the exon (from 5' to 3') are all efficient whereas for inefficient (+) and nonefficient (-) AONs, the first target nucleotide lies in the middle and last third of the exon, respectively (Table 1). The ACP score (see *Materials and Methods* for its mathematical definition) is the average of the sum of each target nucleotide position from exon 5' (Table 1). Statistical analysis revealed that efficient AONs have the lowest ACP scores ($p = 2E-5$, see Fig. 5); i.e., they target sites nearest to the exon 5' end. This finding is not due to lower L3 score or presence of more R_{ESE} motifs near the 5' of the target exon (Supplementary Fig. S3).

Other variables were used to test for statistical significance between efficient and nonefficient AONs. With the exception of AON-target Gibbs energy (Fig. 5), no statistical significance was detected (Supplementary Fig. S4).

To determine possible variables that can be used to discern the most efficient AON per target exon, we sorted all AONs that target the same exon by their measured average efficiencies to examine whether their rankings correlate with any of the variables tested above. The AON with the lowest

ACP score consistently correlated with the efficiency rankings in all exons targeted. That is, AON efficiency increases when ACP score decreases among AONs targeting the same exon (Supplementary Fig. S5). This suggests that the most efficient AON is one with the lowest ACP score.

Discussion

In this prospective study, a new combination of three design variables—*co-transcriptional* binding accessibility of target site, presence of ESE motifs, and AON length, were used to design 23 novel 20MePS AONs each targeting one of nine exons of *DMD* pre-mRNA. Upon experimental validation, 14 AONs were found to be efficient among 19 efficacious AONs, with at least one efficient AON obtained per target exon. This translates to success rates of 83% and 61% for designing efficacious and efficient AONs, respectively. This study demonstrates the plausibility of attaining satisfactory success rates in designing AONs through an effective AON design guideline. Notably, this is achieved with only three design variables, which underscores the importance of selecting independent design variables appropriately and defining a specific numerical boundary for each variable. Retrospective analyses of the validated AON efficiencies revealed that the most efficient AON in every exon targeted had the lowest ACP score. This suggests that the most efficient AON could be selected from the pool of AONs by satisfying the design guideline of having the lowest ACP score. In contrast, reported design guidelines, which were inferred from retrospective analyses of published AONs (Aartsma-Rus *et al.*, 2009b; Popplewell *et al.*, 2009), have not been validated in a prospective study to design new AONs for exon skipping in the *DMD* gene. Furthermore, because

AON 51

AON 53

Lipofectamin

FIG. 4. Immunocytochemistry of dystrophin protein in DMD patient skeletal muscle cells. AON induced restoration of dystrophin expression in DMD patient skeletal muscle cells. Immunocytochemistry were performed to stain desmin that mark (a) myogenic cells, (b) dystrophin, (c) nucleus, and (d) double staining of desmin and dystrophin. Restoration of dystrophin expression were observed in DMD patients' skeletal muscle cells treated with AONs targeting exons 51 (top) and 53 (middle). DMD patients' muscle cells treated with lipofectamine did not show any dystrophin expression (bottom).

these guidelines do not define numerical cutoffs of each design variable to limit the number of predicted AON target sites, some form of trial-and-error is inevitable in selecting AON candidates for validation.

Statistical analysis of the validated AONs efficiencies reveals that efficient AONs have the lowest ACP scores ($p=2E-5$) and the lowest AON-target Gibbs energy ($p=0.008$) (see Fig. 5). The ACP score quantifies the position of each target nucleotide from the target exon 5', and our results show that AONs whose target sites had their first starting (5') nucleotide in the first third of the exon (5' to 3') were efficient, whereas those that lay in the last third were all nonefficient. In fact, several labs have reported the corre-

lation of AON efficiency with position of target site from target exon 5' end in their retrospective studies (Aartsma-Rus *et al.*, 2005, 2009b; Popplewell *et al.*, 2009; Wilton *et al.*, 2007). The success rate for designing efficient AONs can be improved with ACP score as the fourth design variable; i.e., among candidate AON target sites with similar L3 score, AON length, and R_{ESE} , the one nearest to the exon 5' should be selected. It is an independent variable since it is not correlated with the first three design variables (Supplementary Fig. S3). However, the effect of AON-target Gibbs energy is less clear because it is associated with both AON-target binding energy and GC content. In fact, the three variables have been reported to correlate with efficiency of mRNA

FIG. 5. Boxplots of average cumulative position (ACP) score and AON-target Gibbs energy. ACP and AON-target Gibbs energy of 14 efficient (++) and 9 inefficient and non-efficacious (+ and -) AONs were compared. Kolmogorov-Smirnov (K-S) tests were performed for each score and the *p*-values are given on the respective boxplots.

down-regulation via AON-induced RNaseH degradation (Kinali *et al.*, 2009). However, no statistical significance was detected for the latter two associated variables in our dataset, whereas only AON-target binding energy has been reported to be significant in two datasets (Aartsma-Rus *et al.*, 2009b; Popplewell *et al.*, 2009). Without future work to determine the relative importance and extent of interdependence among the variables, it is unclear whether the inclusion of AON-target Gibbs energy will lead to substantial improvement in the success rates. Furthermore, since the *p*-value of AON-target Gibbs energy is 345 times larger than the *p*-value of ACP score, ACP exerts more influence on AON efficiency.

Nevertheless, the approach used in this study is not without limitations. The effectiveness of the design guidelines depends on the accuracies of the computational tools used for predicting the pre-mRNA secondary structures and the presence of ESE motifs. Secondly, transcription, exon recognition, and exon splicing are complex regulated processes that involve myriad cellular components and not all mechanisms have been elucidated. Thus, the possibility of omitting a critical design variable cannot be ruled out. These limitations do not seem to be involved in this study as all the ineffective AONs can be explained by the fourth design variable.

To evaluate our AON design guidelines against reported AONs targeting exon-internal sequences, the following two assessments were performed: comparison of success rates and classification of AONs.

Comparison of success rates

Reported success rates for designing efficient (++) and efficacious (++) and (+) AONs range from 18% to 38% and from 56% to 79%, respectively (Aartsma-Rus *et al.*, 2005, 2009b; Harding *et al.*, 2007; Popplewell *et al.*, 2009; Wilton *et al.*, 2007) (Supplementary Table S1). If only AONs targeting the same exons as in this study were considered, the reported range of success rates are lower; i.e., from 21% to 27% and from 44% to 73%, respectively (Supplementary Table S1). By comparison, with a success rate of 61%, our design guidelines confer a more substantial improvement in designing efficient AONs. Nevertheless, although the comparison of success rates is relatively less sensitive to the AON efficiencies than a direct comparison of AON efficiencies,

differences in success rates detected between studies could be partially attributed to differences in AON efficiencies, which result from experimentation differences among the studies including, but not limited to transfection method, cell density, incubation time and PCR condition.

Classification of AONs

Our design guidelines were tested for the ability to correctly classify observed AON efficiencies in three reported datasets. Our design guidelines can classify the first dataset (Wilton *et al.*, 2007) reasonably well, whereas only efficient and efficacious AONs in the second dataset (Popplewell *et al.*, 2009) and only ineffective AONs in the third dataset (Aartsma-Rus *et al.*, 2005) were classified well. The respective classification rates of efficient, efficacious, and ineffective AONs for the three dataset are (Supplementary Table S3): {97%, 96%, and 57%}, {80%, 88%, and 21%}, and {22%, 28%, and 68%}. On the other hand, Aartsma-Rus *et al.* (2009b) used their design guidelines encompassing four design variables inferred from retrospective analysis to classify efficacious and ineffective AONs from their dataset and those reported by Wilton *et al.* (2007). The respective classification rates for their and the Wilton *et al.* datasets were {94% and 48%} (Aartsma-Rus *et al.*, 2009b) and {100%, 0%} (Aartsma-Rus *et al.*, 2010). Aartsma-Rus *et al.* (2010) then went on to infer six-variables design guidelines from the Wilton *et al.* (2007) dataset and use it to reclassify the two datasets. The respective classification rates were {19% and 87%} and {92%, 63%}.

In conclusion, we have proposed and validated new design guidelines in this first prospective study to design AONs for efficient exon skipping in the *DMD* gene. The satisfactory success rates obtained through the application of the design guidelines support the use of a rational approach in designing AONs to determine efficient candidates for clinical trials. Since exon skipping has already been shown to be a viable therapy for DMD in clinical trials (Goemans *et al.*, 2011; Kinali *et al.*, 2009; van Deutekom *et al.*, 2007), the determination of a set of validated design variables is timely given that different customized AONs are needed to address specific individual mutations in DMD patients (Aartsma-Rus *et al.*, 2009a). Therefore, by eliminating trial-and-error and/or preliminary experimentations, our approach confers the advantage of reducing cost and resources while speeding up the AON design process. Besides DMD, a rational approach for designing efficient AONs could be applied to other genetic diseases which are amenable to AON therapy (Dominski and Kole, 1993; Gorman *et al.*, 2000; Mercatante *et al.*, 2001; Suwanmanee *et al.*, 2002; Vetrini *et al.*, 2006).

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Author Disclosure Statement

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