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Direct In Vitro Selection of a 2′-*O*-Methyl Aptamer to VEGF

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Summary

Aptamers (protein binding oligonucleotides) have potential as a new class of targeted therapeutics. For applications requiring chronic systemic administration, aptamers must achieve high-affinity target binding while simultaneously retaining high in vivo stability, tolerability, and ease of chemical synthesis. To this end, we describe a method for generating aptamers composed entirely of 2′-*O*-methyl nucleotides (mRmY). We present conditions under which 2′-*O*-methyl transcripts can be generated directly and use these conditions to select a fully 2′-*O*-methyl aptamer from a library of 3×10^{15} unique 2′-*O*-methyl transcripts. This aptamer, ARC245, is 23 nucleotides in length, binds to vascular endothelial growth factor (VEGF) with a Kd of 2 nM, and inhibits VEGF activity in cellular assays. Notably, ARC245 is so stable that degradation cannot be detected after 96 hr in plasma at 37°C or after autoclaving at 125°C. We believe ARC245 has considerable potential as an antiangiogenesis therapeutic.

Introduction

Aptamers are oligonucleotides that interact highly specifically with molecular targets such as proteins, and in this respect are similar to antibodies. Aptamers are generated using a process called either in vitro selection [1] or SELEX [2]. Over 100 aptamers have been generated using this process to targets as diverse as small molecules, peptides, proteins, and whole cells [3]. Aptamers can be made that specifically and selectively interact with, and often inhibit the function of, protein targets. There is considerable interest in the use of aptamers as therapeutics in a manner similar to that of therapeutic antibodies.

In order for an aptamer to be suitable for use as a therapeutic it must be inexpensive to synthesize, safe, and stable in vivo. Wild-type RNA and DNA aptamers are not stable in vivo because of their susceptibility to degradation by nucleases. Resistance to nuclease degradation can be greatly increased by the incorporation of modifying groups at the nucleotide 2′ position, and 2′-fluoro- and 2′-amino-substituted libraries have been successfully used to select partially stabilized aptamers [4]. However, these particular modifications increase the cost of synthesis of the resultant aptamer, and may introduce safety concerns, as these modified nucleotides could potentially be recycled into host DNA.

Aptamers that contain 2′-*O*-methyl (2′-OMe) nucleotides overcome many of these drawbacks. Oligonucleotides containing 2′-OMe pyrimidines are nuclease resistant [5] and inexpensive to synthesize. For example, fully 2′-*O*-alkylated nucleic acids have been shown to be completely stable in the rat gastrointestinal tract over an 8 hr period, in contrast to partially 2′-*O*-alkylated nucleic acids [6]. Additionally, 2′-OMe nucleotides are ubiquitous in biological systems (there are about 100 of these residues in each ribosome [7]). Probably as a consequence of their presence in biological systems, natural polymerases do not accept 2′-OMe nucleotide triphosphates (NTPs) as substrates [8], and so there are no significant safety concerns regarding the recycling of 2′-OMe nucleotides into host DNA.

There are several examples of 2′-OMe-containing aptamers in the literature (see, for example, [9]). These aptamers were generated by the in vitro selection of libraries of modified transcripts in which the C and U residues are 2′-fluoro substituted and the A and G residues are 2′-OH (rRfY). Once functional sequences are identified, each A and G residue is then tested for its tolerance of 2′-OMe substitution, and the aptamer is ultimately synthesized with all A and G residues that tolerate 2′-OMe substitution as 2′-OMe residues. Most of the A and G residues of aptamers generated in this fashion tolerate substitution with 2′-OMe residues, although on average approximately 10%–20% do not. Consequently, aptamers generated using this method tend to contain from two to four 2′-OH residues, and have their stability and cost of synthesis compromised as a result.

One example of a 2′-OMe-containing aptamer is Macugen (Pegaptanib sodium) [10] which binds to vascular endothelial growth factor (VEGF). This is an aptamer that is 27 nucleotides long and binds to VEGF in a calcium-dependent manner with a K_d of 49 pM. Macugen inhibits the binding of VEGF to its receptors Flt-1 and KDR. Macugen has successfully completed phase III clinical trials for wet age-related macular degeneration and was recently approved for use as a human therapeutic by the U.S. Food and Drug Administration (FDA). Macugen was originally selected from a 2′-fluoro pyrimidine, 2′-OH purine (rRfY) library, and subsequent stabilization efforts successfully replaced 12 of the 14 2′-OH purines with 2′-OMe purines. Macugen is a good example of an aptamer that has its stability and cost of synthesis compromised as a result of the fact that it contains two 2′-OH nucleotides. It would be beneficial if conditions could be found under which 2′-OMe transcripts could be generated enzymatically, and then reverse transcribed; in this manner, fully 2′-OMe aptamers (mRmY) could be selected directly.

Recently, two studies have been published that report the discovery of polymerase variants that can incorporate 2′-OMe NTPs [11, 12]. Fa et al. report the directed evolution of a DNA polymerase that can incor-

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porate up to five dNTPs after a single 2′-OMe NTP, at which point polymerization ceases. Chelliserrykattil and Ellington report mutants of T7 RNA polymerase that can introduce 2′-OMe A, C, and U into transcripts, but not 2′-OMe G. Unfortunately, neither of these enzymes can be used to generate fully 2′-OMe (mRmY) transcripts that are long enough to contain aptamers.

Results and Discussion

Screening Polymerases for Production of 2′-OMe-Containing Transcripts

In order to generate libraries of 2′-OMe transcripts, it was first necessary to find conditions under which a template-dependent polymerase would accept 2′-OMe NTPs as substrates. The T7 RNA polymerase mutant Y639F [13] was screened using various combinations of concentrations of divalent magnesium and manganese to establish conditions under which transcripts could be produced from solutions containing one 2′-OMe NTP and three 2′-OH NTPs. Transcription requires the presence of magnesium, and it is well known that the addition of manganese to the reaction mixes of other polymerases can increase their error rates [14]. Different combinations of concentrations of magnesium chloride and manganese chloride were investigated for their effect upon transcription yields. The greatest yields of transcripts were obtained with 5 mM magnesium chloride and 1.5 mM manganese chloride, although departures from these concentrations of up to 2-fold will still give significant amounts of modified transcripts. It should be noted that because NTPs complex divalent metal ions, the concentration of free metal ions in solution will be affected by changes in the concentration of NTPs. The experiments and optimal concentrations of metal ions described here apply to transcriptions in which each of the four NTPs is at a concentration of 0.5 mM.

Further increases in modified transcript yield were observed by priming transcription with either 0.5 mM GMP or guanosine. This effect presumably results from the specificity of the polymerase for the initiating nucleotide. Additionally, double-stranded transcription templates generated by PCR were found to be greatly superior to single-stranded synthetic DNA with a double-stranded promoter. Yet further improvements were made to the yield of these partially modified transcripts by including polyethylene glycol (PEG) in the transcription mixture.

Improving the Yield of 2′-OMe-Containing Transcripts

Transcription can be divided into two phases: initiation and elongation [15, 16]. During initiation, GTP (or another guanosine derivative) is substituted at its 3′-OH to yield a dinucleotide and this is then extended by a further eight or so nucleotides. During elongation, transcription proceeds beyond the first 10–12 residues of the transcript. There is structural evidence to support a qualitative distinction between the initiation and elongation phases of transcription [17]. Further consideration of the conformational distinction between the initiation and elongation stages of transcription leads us to consider the utility of a leader sequence. It is well known that transcript yields are greatly influenced by the composition and sequence of the first three to six nucleotides of the transcript, and that purines, especially guanosine, are preferred in these positions [18]. We reasoned that the incorporation of 2′-OMe nucleotides into transcripts might be further facilitated by the use of an all-purine leader sequence that extends beyond the point at which the elongation conformation is achieved at approximately position +10. Large increases in the yields of modified transcript were, in fact, observed when the following leader sequence was incorporated into the sequence of the DNA template: GGGAGAGGAGAGAA. Using this leader sequence, it is possible to produce up to 1 μM transcripts in solutions containing only 2′-OMe ATP, UTP, and CTP with 2′-OH GTP (rGmH). Unfortunately, the yields of 2′-OMe G-containing transcripts under these conditions are still low.

We also hypothesized that these successive stages of transcription were likely to be increasingly insensitive to the incorporation of 2′-substituted NTPs. For example, it is known that T7 RNA polymerase is more prone to incorporate dNTPs once the polymerase is in the elongation phase [19]. Consequently, we hoped that small amounts of 2′-OH GTP added to the transcription mixture would be sufficient to enable the polymerase to initiate, but that once transcription was in the elongation phase the reduced discrimination between 2′-OMe and 2′-OH GTP, and the excess of 2′-OMe GTP over 2′-OH GTP in the transcription mixture, would allow the incorporation of principally 2′-OMe GTP. A titration of 2′-OH GTP into a transcription mixture containing only all four 2′-OMe NTPs (r/mGmH) showed up to a 10-fold increase in yield of modified transcript as compared to the same transcription without any added 2′-OMe GTP (data not shown). These results suggest that this combination of reaction components results in the incorporation of significant proportions of 2′-OMe G into transcripts. Ultimately the yields of r/mGmH transcripts were further increased by a factor of five by the use of the T7 RNA polymerase double-mutant Y639F/H784A [20].

Ultimately, the effect of each of these changes to the transcription conditions was assessed by comparing the yields of r/mRmY transcripts in the presence and absence of each change. The double-stranded nature of the entire transcription template, as opposed to just the promoter region, and the presence of manganese were of critical importance. Their absence decreased the yield by factors of 200 and 100, respectively. The shortening of the purine-rich leader sequence from fourteen to five purines reduced the yield by a factor of eight and the exclusion of the 30 μM 2′-OH GTP "spike" reduced the yield by a factor of ten. The exclusion of PEG and GMP are responsible for relatively minor reductions in r/mRmY transcript yield under the conditions studied. The yield of r/mRmY transcripts under these optimized conditions is approximately 250 nM or 5 μg/ml, which is a reduction of approximately 100-fold from an RNA transcription using 2′-OH NTPs and wild-type T7 RNA polymerase.

The T7 RNA polymerase double-mutant Y639F/H784A [20] was used for the transcriptions performed during SELEX, whereas the T7 RNA polymerase single-

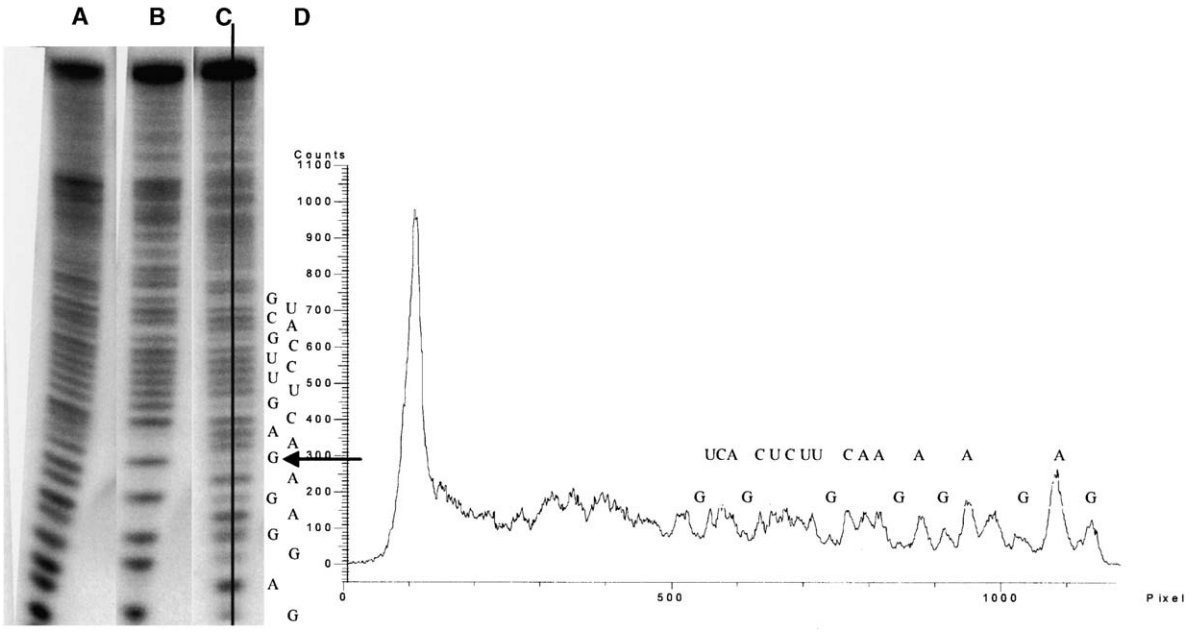

Figure 1. Alkaline Hydrolysis Data Demonstrating the Incorporation of 2′-OMe Nucleotides into Transcripts

(A) An alkaline hydrolysis ladder of a 5′-^{32}P-labeled transcript generated by transcription from a mixture containing 2′-OH ATP, CTP, GTP, and UTP (RNA), each at 0.5 mM, and the single-mutant T7 RNA polymerase Y639F [13]. This ladder shows bands corresponding to all positions in the transcript.

(B) An alkaline hydrolysis ladder of a 5′-^{32}P-labeled transcript generated by transcription from a mixture containing 2′-OH CTP, GTP, UTP, and 2′-OMe ATP (rBmA), each at 0.5 mM, and the single-mutant T7 RNA polymerase Y639F. The absence of hydrolysis at positions corresponding to A residues indicates that the transcript contains 2′-OMe A at these positions, and that 2′-OMe nucleotides, within oligonucleotides, are highly resistant to alkaline hydrolysis.

(C) An alkaline hydrolysis ladder of a 5′-^{32}P-labeled transcript generated by transcription from a mixture containing 2′-OH ATP, CTP, and UTP and 2′-OMe GTP, each at 0.5 mM, and 2′-OH GTP, at 30 μM (r/mGmH). Positions corresponding to G residues show greatly reduced intensities after position +10 (arrow); this observation indicates the incorporation of largely 2′-OMe G residues at G positions once the polymerase has attained its elongation conformation. The increased intensity of the hydrolysis ladder bands at positions corresponding to G residues at positions before +10 suggests that the polymerase has a higher discrimination against 2′-OMe GTP before it attains the elongation conformation.

(D) The integrated intensity of the hydrolysis ladder shown in (C). This more clearly shows the difference in intensity between the bands corresponding to G residues in the initiation and elongation phases of polymerization. Hydrolyses were conducted for 5 min at 95°C in 50 mM Na_2CO_3 at pH 9.

mutant Y639F [13] was used for the transcription optimization work. After the transcription optimization and SELEX phases of this project had been completed it was discovered that both the Y639F single-mutant and Y639F/H784A double-mutant T7 RNA polymerases used inadvertently additionally contained the K378R mutation.

Demonstration of 2′-OMe Incorporation

We were able to demonstrate that each of 2′-OMe A, C, and U was being incorporated into modified transcripts simply by comparing the yield of radiolabeled transcript ([α-^{32}P]GTP was included in the transcription mixture), as observed by phosphorimaging of a denaturing gel, in the presence and absence of the nucleotide being considered (data not shown). Because the transcription mixtures used to incorporate 2′-OMe G additionally contained 2′-OH GTP, this approach could not be used to prove the incorporation of 2′-OMe G. In this instance, a modified transcript of a fixed sequence was generated from a mixture of 2′-OH ATP, CTP, and UTP and 2′-OMe GTP, each at 0.5 mM, and 2′-OH GTP, at 30 μM (r/mGmH). This transcript was 5′ end labeled with ^{32}P and subsequently subjected to partial alkaline hydrolysis before analysis by denaturing PAGE. A ladder of bands was generated in which the intensity of each band is proportional to the proportion of 2′-OH nucleotide at the corresponding position in the transcript (Figure 1C). Integration of the intensity of each band clearly shows that up to position +10 in the transcript, substantial proportions of 2′-OH G are present at each G position in the transcript (Figure 1D). After this point, presumably once the polymerase has transitioned to the elongation conformation, each G in the transcript is principally 2′-OMe, and as a consequence, the extent of hydrolysis at each of these positions is greatly reduced (Figure 1D). An alkaline hydrolysis ladder is shown for a transcript generated from a mixture of 2′-OH CTP, GTP, and UTP and 2′-OMe ATP (rBmA) (Figure 1B). This image shows the absence of bands corresponding to A residues in the modified transcript, thus demonstrating the incorporation of 2′-OMe ATP into the transcript and the hydrolysis-resistant nature of 2′-OMe nucleotides. Additionally, an alkaline hydrolysis ladder is shown for a transcript generated from a mix-

Table 1. The Fidelity with which Sequence Information Is Transmitted to the Next Generation of Transcripts

	Deletions	Insertions	Substitutions	Perfect N30	N
2′-OH RNA	0.7%	0.0%	0.3%	74%	1936 bases
2′-OMe RNA	0.6%	0.6%	1.2%	49%	1584 bases

Sequence data for a single fixed sequence were used to calculate deletion, insertion, and substitution rates after transcription from DNA into 2′-OMe RNA (r/mRmY) using the T7 RNA polymerase single-mutant Y639F [13], reverse transcription into cDNA, and subsequent PCR amplification. Perfect N30 refers to the probability that a sequence of 30 contiguous positions outside of the designed primer binding regions is perfectly transmitted to the next generation. Thirty nucleotides was the length of the degenerate region of the library used in this study. N is the number of nucleotides sequenced. Data for transcription into 2′-OH RNA are shown for comparison. These data are likely to overestimate error rates because no allowance is made for errors occurring during the sequencing process itself.

ture of 2′-OH ATP, CTP, GTP, and UTP (RNA; Figure 1A). This image shows the presence of bands corresponding to all positions in the sequence.

Reverse Transcription of 2′-OMe-Containing Transcripts

Modified transcripts were reverse transcribed using Thermoscript Reverse Transcriptase (Invitrogen, Grand Island, NY) at 65°C. Primer extension experiments showed that the yield of full-length cDNA reverse transcribed from a synthetic fully 2′-OMe RNA (mRmY) sequence is about 10% of that obtained by the reverse transcription of the corresponding synthetic DNA (Supplemental Figure S1). This yield is consistent with the observation that PCR amplification of the resultant cDNA lagged about four cycles behind the amplification of an equal amount of synthetic DNA (data not shown). No efforts were made to further increase this yield, and no other reverse transcriptases were assessed for their ability to reverse transcribe fully 2′-OMe RNA (mRmY). Fully 2′-OMe RNA (mRmY) does not act as a template for PCR using Taq polymerase and a standard PCR protocol. Also, 2′-OMe NTPs do not act as substrates for DNA synthesis during PCR, nor do they inhibit PCR (data not shown). Transcription with 2′-OMe ATP, CTP, GTP, and UTP at 0.5 mM and 2′-OH GTP at 30 μM (r/mGmH) as in Figure 1 gives concentrations of transcript of approximately 250 nM in the transcription mixture after an overnight incubation at 37°C.

The relatively low yields of both the transcription and reverse transcription of 2′-OMe RNA lead us to consider that these steps might introduce composition biases into libraries of nucleic acids that they act upon. A secondary concern was that the rate of misincorporation of nucleotides for either process might be too high for the SELEX process to be able to operate successfully. We examined these concerns by synthesizing DNA of a fixed sequence, and then subjecting this to PCR amplification to add a T7 promoter and render it double stranded. This transcription template was then transcribed, DNase treated, reverse transcribed, PCR amplified, cloned, and sequenced. Transcriptions were performed both under conditions yielding 2′-OMe (r/mGmH) and 2′-OH RNA, and the statistics of substitutions, deletions, and insertions were compared as shown in Table 1. The deletion rate for 2′-OMe RNA (r/mGmH) amplification is not higher than for 2′-OH RNA, but the insertion and substitution rates are higher. These decreases in fidelity will result in an approximately 1.5-fold reduction in enrichment rates for an N30 library during the selection process. This relatively minor reduction in enrichment rate is unlikely to significantly affect the trajectory of an in vitro selection.

Direct Selection of Fully 2′-OMe-Modified Aptamers against VEGF

A library of 3×10^{14} unique transcription templates, each containing a random region of 30 contiguous nucleotides, was generated by the PCR amplification of synthetic DNA. Cloning and sequencing of this library demonstrated that the composition of the random region in this library contains approximately 25% of each nucleotide (data not shown). This DNA library was purified away from unincorporated dNTPs by gel filtration and ethanol precipitation. Modified transcripts were then generated from a mixture containing 0.5 mM of each of the four 2′-OMe NTPs and 30 μM 2′-OH GTP (r/mGmH). After gel purification and DNase treatment, these modified transcripts were dissolved in PBS and incubated for 1 hr in an empty well on a hydrophobic multiwell plate in an effort to subtract plastic binding sequences. The supernatant solution of modified transcripts was then transferred to a well that had previously been incubated for 1 hr with $VEGF_{165}$ (Peprotech, Rocky Hill, NJ) at room temperature in PBS. After a subsequent 1 hr incubation, this well was washed with PBS and bound sequences were reverse transcribed in situ using Thermoscript Reverse Transcriptase (Invitrogen) at 65°C for 1 hr. The resultant cDNA was then PCR amplified, separated from dNTPs by gel filtration, and used to generate modified transcripts for input into the next round of selection. An independent initial library of 3×10^{15} synthetic 2′-OMe oligonucleotides with sequences that correspond to the library of 2′-OMe transcripts was similarly subjected to a single round of selection and amplification, and the two selected libraries were then combined. After ten rounds of selection and amplification, the ability of the resultant library to bind to VEGF was assessed by a nitrocellulose filter binding assay [21] to be 35% at 64 nM VEGF. At this point, the library was cloned and sequenced and individual clones were assayed for their ability to bind VEGF. Using a combination of sequence and clone binding data, sequence motifs were identified. These were used to design smaller synthetic constructs of mRmY composition, which were also assayed for binding to VEGF. Ultimately, minimized mRmY aptamers to VEGF were identified, ARC245 and ARC259, both of which are 23 nucleotides long and bind VEGF with K_d values of 2 and 1 nM, respectively,

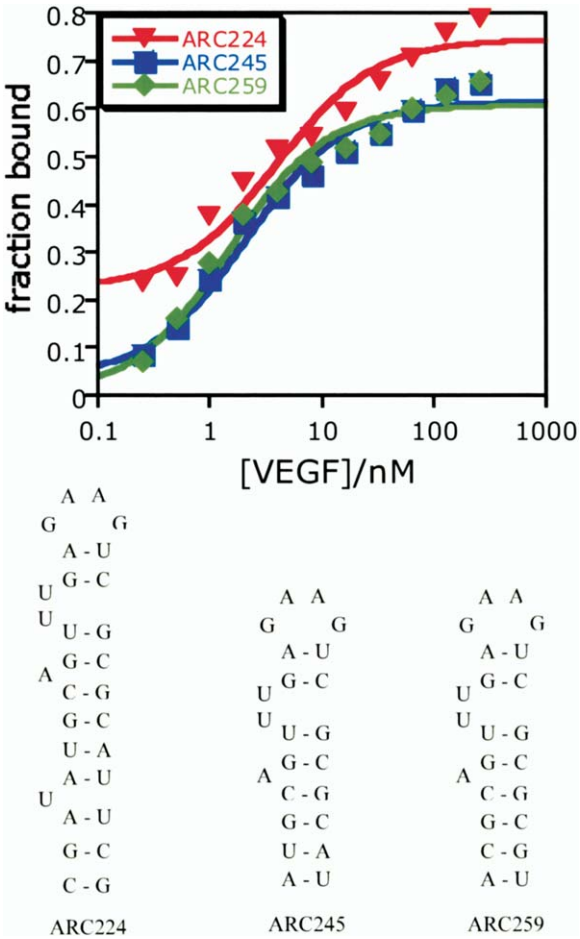

Figure 2. VEGF Binding Characteristics and Sequences and Putative Secondary Structures of Aptamers Featured in the Text

All residues are 2′-OMe (mRmY). All constructs were generated by solid-phase chemical synthesis. These data were generated by nitrocellulose filter binding assay [21] in PBS. These data give K_d values as follows: ARC224, 3.9 nM; ARC245, 2.1 nM; ARC259, 1.4 nM.

as measured by nitrocellulose filter binding assay. ARC259 differs from ARC245 only in the substitution of an A-U base pair in the terminal stem for a G-C base pair. ARC224 is an incompletely minimized version of ARC245 which is 30 nucleotides long and has similar VEGF binding characteristics. ARC225 has the same sequence as ARC224, but has seven 2′-OMe G nucleotides replaced by 2′-OH G nucleotides and does not bind VEGF. Putative secondary structures of these synthetic 2′-OMe aptamers, and their VEGF binding curves, are shown in Figure 2.

It is not clear whether the observed differences between the fully 2′-OMe (mRmY) VEGF aptamers discovered in the present study and the 2′-ribo/methyl purine, 2′-fluoro pyrimidine (r/mRfY) VEGF aptamers, including Macugen, discovered by Ruckman et al. [10] are significant. For example, these mRmY aptamers are more stable, are unusually small, and bind VEGF in a divalent metal ion-independent manner, while Macugen binds with a lower K_d value (approximately 50 pM versus approximately 1 nM) and does so in a calcium-dependent fashion. It is possible that mRmY composition aptamers generally bind less well to VEGF than rRfY and r/mRfY composition aptamers.

Aptamer Tolerance to 2′-OH G Substitution

During the selection, modified transcripts were generated by transcription in the presence of 0.5 mM of each 2′-OMe NTP and additionally 30 μM 2′-OH GTP (r/mGmH). As a consequence, each time selected sequences are amplified and transcribed for entry into the next round of selection, a small number of 2′-OH G residues are incorporated into the modified transcripts in a stochastic fashion. Any selected sequence could therefore, in principle, be dependent upon the presence of one or more 2′-OH G nucleotides for VEGF binding. In order to test the effect of the presence of 2′-OH G residues in the selected sequence, substitution variants of ARC224 were synthesized in which single 2′-OMe G nucleotides were substituted by 2′-OH G nucleotides. Each of these constructs was then assayed for its ability to bind VEGF by nitrocellulose filter binding. In every instance, the ability of the 2′-OH-substituted variant to bind VEGF was inferior to that of fully 2′-OMe ARC224 (mRmY) as shown in Figure 3. In addition, a construct in which all seven of these nucleotides were simultaneously substituted for 2′-OH G nucleotides (rGmH; ARC225) showed no binding to VEGF at all.

Aptamer Specificity

To explore the specificity of these VEGF aptamers, the affinity of ARC259 for various related proteins was measured by nitrocellulose filter binding assay. Murine $VEGF_{164}$, which is highly homologous to human $VEGF_{165}$, bound ARC259 with the same affinity as human $VEGF_{165}$, whereas human $VEGF_{112}$ and the human growth factors bFGF, PDGFbb, and PlGF-1 showed no binding to ARC259 at all. $VEGF_{112}$ lacks the heparin binding domain to which ARC259 presumably binds.

Inhibition of VEGF Activity by Fully 2′-OMe Composition Aptamers

In order for an aptamer to have potential therapeutic utility, it needs to bind to a therapeutic target in a manner that inhibits the function of that target. To assess whether the synthetic aptamers ARC224 and ARC245 inhibit VEGF activity, two different assays were performed. Initially, the ability of ARC224 to inhibit the binding of VEGF to VEGF receptors upon the surface of human umbilical vascular endothelial cells (HUVEC) was assessed. ^{125}I-labeled VEGF was incubated with ARC224 and the resultant mixture was then added to HUVEC; after incubation, the cells were washed and ^{125}I was then counted in a scintillation counter. These data are presented in Figure 4, which shows that ARC224 inhibits the binding of VEGF to the HUVEC VEGF receptors with an IC_{50} of approximately 2 nM. In an independent approach to the demonstration of VEGF inhibition, 293 cells were transfected with a VEGFRII (KDR)-encoding plasmid and a KDR-luciferase reporting system. Incubating these cells with VEGF in the presence of ARC224 and ARC245, and assaying for luciferase using a luminometer, clearly indicates the ability of these aptamers to bind to VEGF in a manner that

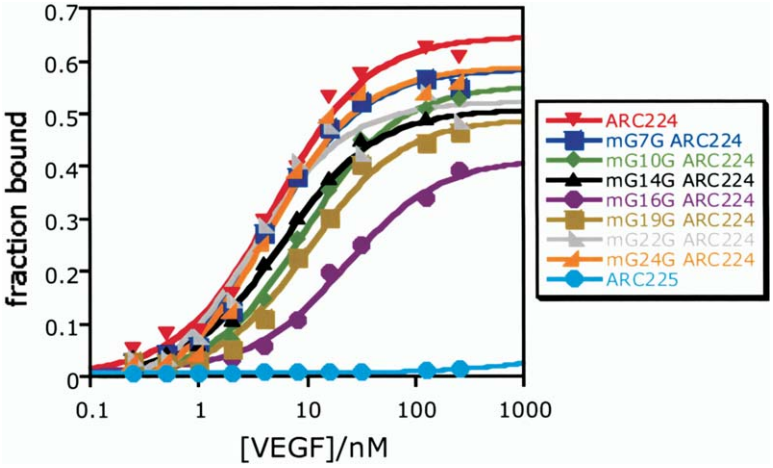

Figure 3. VEGF Binding Characteristics of Various 2′-OH G Variants of ARC224

In each instance, the nomenclature mGXG indicates the substitution of 2′-OH G for 2′-OMe G at position X numbered from the 5′ terminus. ARC225 has the same sequence as ARC224 and 2′-OMe to 2′-OH substitutions at positions 7, 10, 14, 16, 19, 22, and 24 numbered from the 5′ terminus. All constructs were generated by solid-phase chemical synthesis. These data were generated by nitrocellulose filter binding [21] in PBS. The fully 2′-OMe aptamer, ARC224, has superior VEGF binding characteristics (lower K_d) when compared to any of the 2′-OH G substitution variants studied here.

inhibits its ability to bind KDR at aptamer concentrations as low as 10 nM. These data are presented in Figure 5.

Aptamer Stability and Pharmacokinetics

Aptamers that are entirely comprised of 2′-OMe nucleotides (mRmY) are highly resistant to chemical, physical, thermal, and enzymatic insults. We were unable to observe any degradation or decrease in the ability of synthetic ARC224 to bind to VEGF after exposure to a 25 min autoclave cycle with a peak temperature of 125°C (Supplemental Figure S2). The robust nature of 2′-OMe aptamers opens up a host of possible applications for which monoclonal antibodies are not suitable. One such example is the coating of stents and other medical devices while retaining the ability to sterilize the coated device using an autoclave. The nuclease-resistant nature of these aptamers additionally confers pharmacokinetic benefits. The clearance half-life of ARC247 (synthetic ARC245 5′ substituted with 40 kDa poly[ethylene glycol]) and administered to CD-1 mice intravenously was 23 hr (Figure 6). This compares favorably with aptamers containing nuclease-susceptible residues such as 2′-OH and 2′-deoxy that have in vivo half-lives in the range of 2–10 hr when bearing PEG groups, and less than 1 hr when not functionalized with PEG. An in vivo clearance half-life of 23 hr introduces the possibility of dosing frequencies of once every several days.

Partially 2′-OMe Aptamers

The nuclease resistance of RNA is greatly increased by 2′ substitution at pyrimidine residues [5], although fully 2′-substituted RNA is even more stable. Notably, transcriptions with 2′-OMe CTP, 2′-OMe UTP, 2′-OH ATP, and 2′-OH GTP (rRmY) or 2′-OMe CTP, 2′-OMe UTP, 2′-OMe ATP, and 2′-OH GTP (rGmH) give greater yields of transcript than transcriptions from mixtures containing all four 2′-OMe NTPs (r/mGmH) (1 μM compared to 250 nM for transcriptions with all four 2′-OMe NTPs [r/mGmH]). We additionally used both of these transcription conditions to select partially 2′-OMe aptamers to VEGF. Some of these partially 2′-OMe-modified aptamers have K_d values in the high pM range (data not shown). However, inclusion of any 2′-OH residues in an aptamer is likely to increase cost of synthesis, and negatively impact physical, chemical, and enzymatic stabilities and pharmacokinetic characteristics.

Significance

VEGF induces angiogenesis [22], the growth of new blood vessels, and as such is implicated in several disease states. Early interest focused upon VEGF as a cancer target because tumor growth is consequent upon angiogenesis and the use of the resultant vasculature for the delivery of nutrients to the tumor.

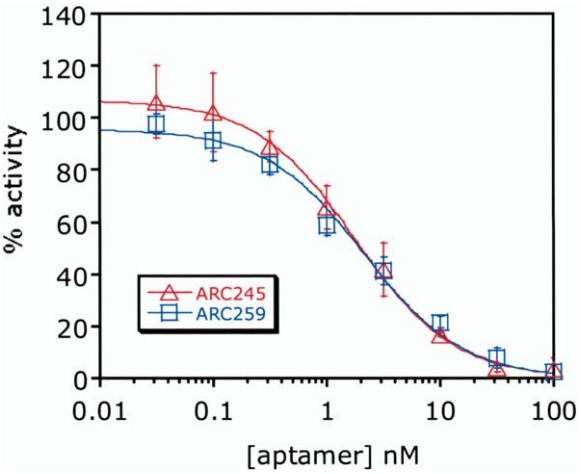

Figure 4. ARC245 and ARC259 Bind to VEGF in a Manner that Inhibits Its Ability to Bind to VEGF Receptors on HUVEC

^{125}I-labeled VEGF was incubated with the synthetic aptamer and this mixture was then incubated with human umbilical vascular epithelial cells (HUVEC). The supernatant was removed, the cells were washed, and bound VEGF was counted in a scintillation counter. These data indicate that the IC_{50} values of ARC245 and ARC259 are approximately 2 nM.

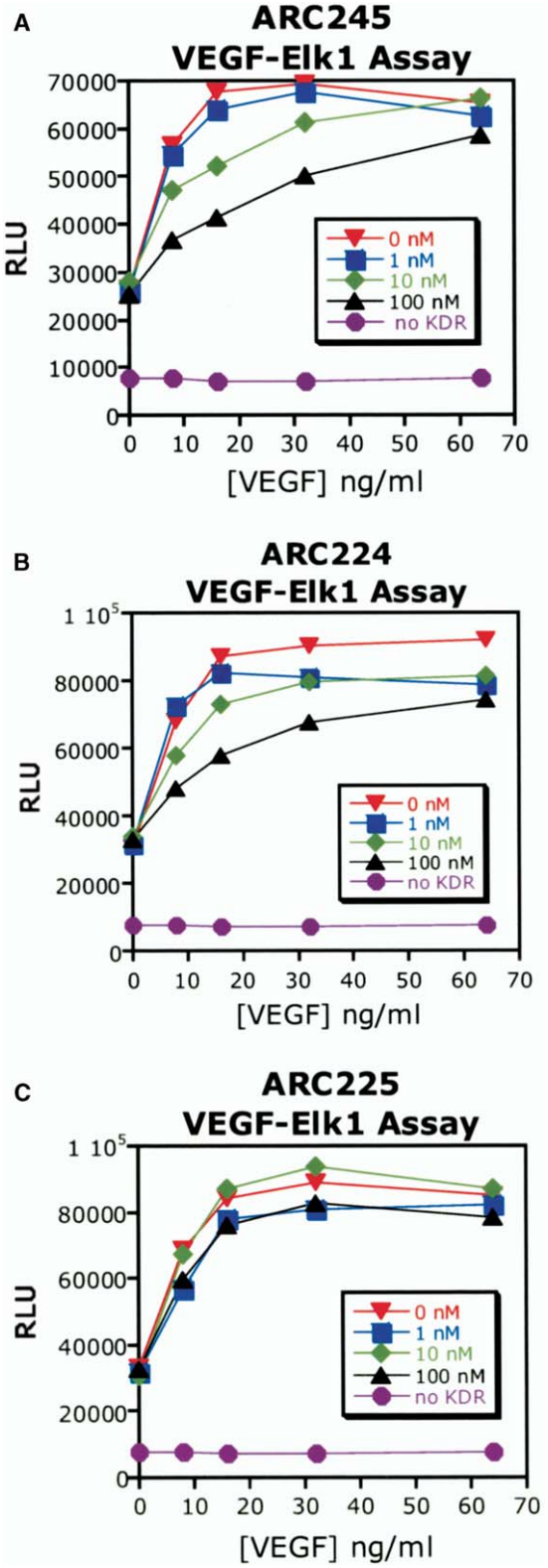

Figure 5. ARC224 and ARC245 Bind to VEGF in a Manner that Inhibits Its Biological Function

293 cells were transiently transfected with a VEGFRII (KDR)-expression plasmid and a KDR-luciferase reporter system. These data indicate that significant inhibition of KDR occurs as a result of ARC224 (A) and ARC245 (B) synthetic aptamer concentrations as low as 10 nM. ARC225 (C) has the same sequence as ARC224, but has seven 2′-OMe G nucleotides replaced by 2′-OH G nucleotides and does not bind VEGF.

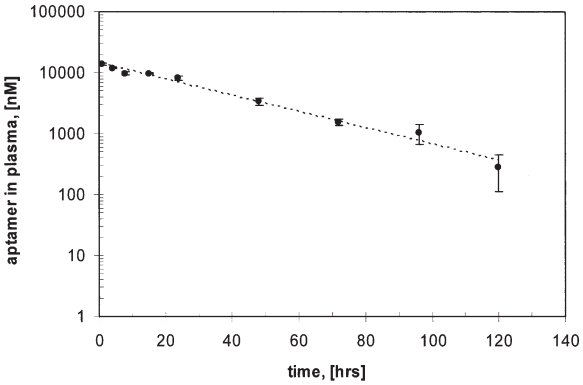

Figure 6. ARC247 (Synthetic ARC245 5′ Substituted with 40 kDa Polyethylene Glycol) Persists In Vivo in the Blood with a Half-Life of 23 hr in the Mouse

ARC247 was formulated to 10 mg/ml (oligonucleotide mass) in 0.9% saline and dosed intravenously to ten groups of three CD-1 mice by single bolus injection into the tail vein to 10 mg/kg (oligonucleotide mass). Blood samples were collected at the indicated time points from groups of three mice and processed into plasma. Aptamer concentrations in these plasma samples were assessed by fluorescence of their Oligreen (Molecular Probes) complexes at 520 nm (excitation at 480 nm). Data were processed using WinNonLin v4.0 (Pharsight). ARC247 has a clearance half-life of 23 hr, a mean residence time of 33 hr, a terminal clearance rate of 0.04 ml/min/kg, and a volume of distribution at steady state (Vss) of 87 ml/kg. The Vss value suggests a moderate degree of binding or sequestration of the aptamer to proteins and/or tissue matrix in the extravascular space.

More recently, the utility of VEGF as a therapeutic target has additionally focused upon other angiogenesis disorders such as retinopathy [23, 24] and macular degeneration, for which an anti-VEGF aptamer (Macugen) was recently approved by the FDA. Avastin, a humanized anti-VEGF antibody, is also newly FDA-approved. When administered with a chemotherapeutic agent, Avastin showed a 50% increase in survival rate, over the duration of the study, of untreated metastatic colorectal cancer patients [25, 26]. Angiogenesis also takes place in other indications such as psoriasis and rheumatoid arthritis [27]. Inhibitors of VEGF, therefore, have the potential to act as potent therapeutics for a range of indications. Aptamers that function as VEGF inhibitors offer many advantages as therapeutics, including the fact that they are generated by chemical synthesis and spontaneously refold after denaturation. A primary attribute of fully 2′-OMe (mRmY) aptamers is their extreme biological stability. Additionally, 2′-OMe nucleotides do not act as substrates for DNA polymerases [8], and the possibility of the recycling of 2′-OMe nucleotides into host DNA is not the concern it may be for 2′-fluoro nucleotides. We anticipate that, in addition to VEGF, the selection methods presented here offer the possibility to gener-

ate highly stable aptamers against a variety of therapeutic targets.

Experimental Procedures

Methods

All reagents were acquired from Sigma (St. Louis, MO) except where stated otherwise. $VEGF_{165}$ was acquired from Peprotech (Rocky Hill, NJ).

Oligonucleotide Synthesis

DNA syntheses were undertaken according to standard protocols using an Expedite 8909 DNA synthesizer (Applied Biosystems, Foster City, CA). The DNA library used in this study had the following sequence: 5′-CATCGATGCTAGTCGTAACGATCCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNCGAGAACGTTCTCTCTCCCTATAGTGAGTCGTATTA-3′, in which N has an equal probability of being each of the four nucleotides. Transcription yield optimization and fidelity experiments were performed using transcription templates prepared by the PCR amplification of the following synthetic DNA sequence: 5′-TAATACGACTCACTATAGGGAGAGGAGAGAACGTTCTCGACTGACGTACCACCATCGGATGGTGGAAGATCGAGCTCGAGCTAGCTAGCTCTACATCGACTGATCG-3′. 2′-OMe RNA syntheses, including those containing 2′-OH nucleotides, were undertaken according to standard protocols using a 3900 DNA synthesizer (Applied Biosystems). All oligonucleotides were purified by denaturing PAGE except PCR and RT primers.

2′-OMe Library Generation

The synthetic DNA library (1.5 nmol, 9×10^{14} unique sequences) was amplified by PCR under standard conditions with the following primers: 3′-primer CATCGATGCTAGTCGTAACGATCC and 5′-primer TAATACGACTCACTATAGGGAGAGGAGAGAAACGTTCTCG. The resultant library of double-stranded transcription templates was precipitated and separated from unincorporated nucleotides by gel filtration. At no point was the library denatured, either by thermal means or by exposure to low-salt conditions. Transcription was performed under the following conditions: 200 nM double-stranded DNA template, 200 mM HEPES, 40 mM DTT, 10% PEG 8000, 0.01% Triton X-100, 2 mM spermidine, 2′-*O*-methyl ATP, CTP, GTP, and UTP, 0.5 μM each (Trilink, San Diego, CA), 30 μM 2′-OH GTP, 0.5 mM GMP, 5.5 mM $MgCl_2$, 1.5 mM $MnCl_2$, 10 U/ml inorganic pyrophosphatase, 200 nM T7 RNA polymerase double-mutant Y639F/H784A [20] (pH 7.5), incubated at 37°C overnight. The resultant transcripts were purified by denaturing 10% PAGE, eluted from the gel, incubated with RQ1 DNase (Promega, Madison, WI), phenol extracted, chloroform extracted, precipitated, and taken up in water. Transcripts for transcription fidelity and yield optimization experiments were prepared similarly using the following primers: 3′-primer CGATCAGTCGATGTAGAGCTAGC and 5′-primer TAATACGACTCACTATAGGGAGAGGAGAGAACGTTCTCGACTGACGTAC and the T7 RNA polymerase single-mutant Y639F [13]. After the transcription optimization and SELEX phases of this project had been completed, it was discovered that both the Y639F single-mutant and Y639F/H784A double-mutant T7 RNA polymerases used additionally contained the K378R mutation. For the initiation of selection, synthetic 2′-OMe oligonucleotides with sequences that correspond to the library of 2′-OMe transcripts were additionally generated by the direct chemical synthesis of 2′-OMe RNA; these were purified by 10% denaturing PAGE.

SELEX

Selection for VEGF binding aptamers was undertaken using VEGF immobilized by hydrophobic absorption to the surface of 96-well plates (C96 MaxiSorb 430341, Nalge Nunc, Rochester, NY). VEGF (200 nM in 100 μl PBS) was incubated in wells for 1 hr at 23°C; these wells were then washed with PBS six times. 2′-OMe transcripts (1 μM in 100 μl PBS per well) were incubated in empty wells for 1 hr at 23°C; the supernatants were then transferred to the VEGF-containing wells where they were incubated for 1 hr at 23°C. The supernatants were then removed and discarded, wells were washed with PBS six times, adherent sequences were reverse transcribed in situ using Thermoscript Reverse Transcriptase (Invitrogen) at 65°C, resultant cDNA was amplified by PCR, and the resultant DNA was used to program a transcription for the subsequent selection step. Round 1 was undertaken separately for synthetically and enzymatically derived transcripts with 0.6 nmol of unique enzymatically generated transcripts and 5 nmol of unique synthetic transcripts. The corresponding PCR products were transcribed separately and combined.

Partial Alkaline Hydrolysis

Partial alkaline hydrolyses were conducted with trace 5′-^{32}P-labeled 2′-OMe transcripts (prepared with T7 RNA polymerase single-mutant Y639F [13]) at 95°C in 50 mM Na_2CO_3 at pH 9 for 5 min in the presence of 1 mg/ml tRNA. Samples were analyzed by 15% PAGE and phosphorimaging.

VEGF Binding Assays

The binding of 2′-OMe RNA to purified recombinant VEGF was assessed by nitrocellulose filter binding assay [21] in which trace 5′-^{32}P-labeled synthetic 2′-OMe RNA was incubated with a dilution series of VEGF in 40 μl PBS with 0.1 mg/ml tRNA and 1 mg/ml BSA in a silanized plate. This mixture was then passed through a sandwich of a nitrocellulose membrane (Schleicher and Schuell, Keene, NH) over a nylon membrane (Amersham Biosciences, Little Chalfont, UK) over Whatman paper (Whatman, Maidstone, UK) on a vacuum manifold, followed by a single wash of 100 μl of the binding buffer. The membranes were phosphorimaged and Kd values were calculated by fitting the resulting data to $y = b + c(x/K_d + x)$, where y is the proportion binding, x is the protein concentration, and b and c are dimensionless constants.

Cell Surface VEGF Inhibition Assays

The ability of these 2′-OMe aptamers to inhibit the binding of VEGF to VEGF receptors upon the surface of HUVEC (ATCC, Manassas, VA) was assessed by premixing the synthetic 2′-OMe aptamer with ^{125}I-labeled VEGF (Perkin Elmer, Wellesley, MA), incubating the resultant mixture with HUVEC in MEME (ATCC), washing away the supernatant, resuspending the cells with SDS/NaOH, and counting with a scintillation counter.

VEGF Inhibition Assays

The ability of these 2′-OMe aptamers to inhibit the binding of VEGF to VEGFRII (KDR) was also assessed using the PathDetect Trans-Reporting system (Stratagene, La Jolla, CA) in 293 cells transfected with a KDR-expression plasmid. 293 cells (ATCC) were plated (10,000 cells/well) in a 96-well plate and incubated at 37°C in DMEM and 10% FBS (GIBCO, Carlsbad, CA) overnight with 5% CO_2. Three plasmids were then transiently transfected into these cells using FUGENE 6 (Roche, Indianapolis, IN), a KDR-expression plasmid, pCMV6-XL4-KDR (clone number PR1371_H11; OriGene Technologies, Rockville, MD; 25 ng/well), an Elk1-dependent luciferase reporter plasmid, pFR-Luc (Roche; 25 ng/well), an Elk-1 fusion protein-expression plasmid, pFA2-Elk1 (Roche; 10 ng/well), and a second overnight incubation in Opti-MEM (GIBCO). On the third day, the indicated amount of recombinant VEGF premixed with the indicated amount of synthetic 2′-OMe aptamer and this mixture was added to the cells and incubated for 4–6 hr at 37°C with 5% CO_2. The level of luciferase produced was then determined with the Steady Glo Luciferase kit (Promega) and immediately read on a luminometer.

Synthesis of PEG-Aptamer Conjugates

ARC247 was prepared by the 5′ substitution of synthetic ARC245 with a hexylamine linker phosphoramidite (Glen Research, Sterling, VA) and the subsequent functionalization of this amine with 40 kDa PEG (Nektar, San Carlos, CA). Both processes were carried out according to the manufacturers' recommendations.

Murine Pharmacokinetics

ARC247 (5′-40 kDa PEG-terminated synthetic ARC245) was dissolved in 0.9% saline to 10 mg/ml (oligonucleotide mass) and sterile filtered through a 0.2 μm filter. This solution was dosed to ten groups of three CD-1 mice to the tail vein by single bolus injection. Blood samples were collected by terminal bleeds into EDTA-coated

tubes at the indicated time points and processed into plasma by centrifugation at 3000 rpm for 5 min and stored at –80°C until analysis. Mean aptamer concentrations in these plasma samples were assessed by fluorescence of their Oligreen (Molecular Probes, Eugene, OR) complexes after dilution into Tris-EDTA (pH 8) at 520 nm (excitation at 480 nm) by comparison with standard curves. Data were processed using noncompartmental analysis with WinNonLin v4.0 (Pharsight, Mountain View, CA).

Supplemental Data

Supplemental Figures S1–S2 are available at http://www.chembiol.com/cgi/content/full/12/1/25/DC1/.

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