DNAzyme-Mediated Silencing of Ornithine Decarboxylase†

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ABSTRACT: The value of reducing the activity of ornithine decarboxylase (ODC), a key enzyme in the biosynthesis of polyamines, is well-appreciated. Polyamines are necessary components for cell growth, and manipulation of polyamine homeostasis may be an effective strategy for the treatment of a number of disorders, including neoplastic diseases. An approach to develop an effective DNAzyme, using the 10−23 model, against ODC is described in these studies. DNAzymes able to cleave the target ODC RNA were identified in vitro and further characterized by the effect each had on ODC protein and activity levels using in vitro translated ODC RNA. ODC protein levels and activity correlated well with the RNA cleavage activity of the DNAzyme. One of the DNAzymes, DZ IV, which exhibited good activity, was optimized for use in cell culture studies. The DNAzyme hybridization arms were altered from equal length arms varying in length (8, 9, 10, or 11 nucleotides) or to unequal length arms (7/11 nucleotides), and kinetic analyses were performed to identify the most catalytically efficient configuration. DZ IV with equal arms nine nucleotides in length proved to be the most catalytically efficient. In HEK 293 cells, DZ IV was able to reduce the amount of translated ODC protein, resulting in ∼80% reduction in ODC activity—a statistically significant enhancement over the apparent antisense effect of a catalytically inactive DNAzyme. These results indicate that this DNAzyme may be a useful tool to study the function of ODC and may have potential therapeutic uses.

RNA-cleaving DNA enzymes have been successfully used for a number of applications (*1*). The 10−23 model DNA enzyme (referred to as DNAzyme or DZ[1]) has a relatively small catalytic domain (15 nucleotides) flanked by hybridization arms (generally 6−12 nucleotides each) that target it to bind to a cognate RNA substrate via Watson−Crick base pairing, and it subsequently catalyzes cleavage of a phosphodiester bond of the RNA (*2*, *3*). An approach to develop an effective DNAzyme to silence ornithine decarboxylase (ODC), an enzyme involved in polyamine biosynthesis, is described.

Several methods of silencing mRNA exist (*4*), including antisense oligodeoxynucleotides (AS-ODNs), ribozymes, siRNA, and DNA enzymes. Although each of the silencing strategies has merits, the DNAzyme was chosen for these studies for several reasons. A DNAzyme is comprised of DNA, so it is inherently more stable than the RNA-based ribozyme and siRNA. DNAzymes possess catalytic activity, whereas AS-ODNs and siRNA rely on cellular machinery to achieve silencing (AS-ODNs designed for clinical use are generally designed to work by an RNase H-dependent mechanism (*5*)). Moreover, the 10−23 DNAzyme has a favorable catalytic efficiency compared to the hammerhead and hairpin ribozymes. The lower K_m of DNAzymes (*2*) is advantageous because ODC mRNA is not abundant.

The biosynthesis of putrescine, spermidine, and spermine, collectively referred to as polyamines, is well-studied. All known living organisms contain polyamines, with the exception of two orders of *Archaea* (*6*), and in fact, polyamines are essential for cell growth in humans and other mammals. Ornithine decarboxylase (ODC) is the enzyme that decarboxylates ornithine to putrescine, a rate-limiting step in the biosynthesis of polyamines. Deletion of the ODC gene in mice results in lethality at early embryonic stages (*7*), and the importance of ODC in cellular physiology has been further demonstrated in numerous cell culture (*8*, *9*) and animal models (reviewed in refs *10*, *11*). These studies demonstrate that ODC plays a role in cell transformation and that ODC overexpression renders mouse models more susceptible to tumor development, which can be reversed by reducing ODC activity by either pharmacological intervention or overexpression of antizyme, a protein that targets ODC for degradation (*12*). Therefore, there is keen interest in the development of ODC inhibitors.

Great promise was held for α-difluoromethylornithine (DFMO, eflornithine), a specific, irreversible inhibitor of ODC, as a chemotherapeutic and chemopreventative agent in humans (*13*). Although clinical trials with DFMO continue more than 25 years after its development and some therapeutic usages have been established, the anticipated impact of DFMO to this end has yet to be realized. However, this is most likely a reflection of the pharmacokinetic properties of DFMO and the need for more appropriate adjuvant agents,

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[1] Abbreviations: ODC, ornithine decarboxylase; DZ, DNAzyme, DNA enzyme; AS-ODNs, antisense oligodeoxynucleotides; DFMO, eflornithine, α-difluoromethylornithine; UTR, untranslated region; DTT, dithiothreitol.

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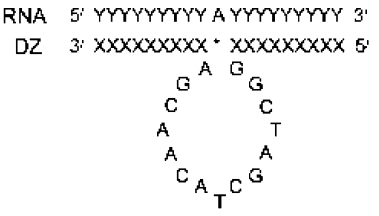
rather than the validity of ODC as a target. DFMO has proven to be a clinically relevant drug, but for uses not initially foreseen. DFMO is FDA approved for the treatment of West African trypanosomiasis (sleeping sickness) transmitted by *Trypanosoma brucei gambiense*, but due to poor oral bioavailability, it must be administered intravenously every 6 h for 2 weeks—a less than practical proposition in rural hospitals (*14*). DFMO is the only FDA-approved drug (labeled as Vaniqa, 13.9% eflornithine; Bristol-Myers Squibb) for the treatment of unwanted facial hair. It is likely that there are other unrealized uses for ODC inhibitors. The poor pharmacokinetic profile of DFMO, the limitations of it as a chemotherapeutic/preventative in humans, and the scope of "alternative uses" for an ODC inhibitor make the development of improved ODC inhibitors attractive.

ODC is a good candidate for gene silencing, because the mammalian protein has a very short half-life (10−60 min (*15*, *16*)) and ODC is highly regulated at the translational level (for a review, see ref *17*). Due to the rapid turnover of ODC protein, cells are reliant on translation of new protein to maintain or elevate ODC activity. Therefore, targeting ODC mRNA, rather than protein, may be a better strategy. ODC mRNA has many characteristics of translationally repressed mRNAs that are generally associated with mRNAs that encode factors related to cell proliferation, such as growth factors and proto-oncogenes (reviewed in ref *18*): a long 5′ untranslated region (UTR) that contains a GC-rich region that has been shown to repress translation, a small internal open reading frame (ORF) in the 5′ UTR (*17*), and an internal ribosome entry site (IRES) for cap-independent translation (*19*). Little to no change in ODC mRNA content is often detected upon substantial increases or decreases in ODC protein content and activity (*20*−*24*). Indeed, NIH-3T3 cells that overexpress eIF-4E, the limiting component of the eIF4F complex needed for cap-dependent translation, exhibit elevated ODC levels up to 30 times higher than wild-type NIH-3T3 cells with no significant change in ODC mRNA (*22*). Polyamines themselves are also known to regulate the translation of ODC through a putative polyamine response element in the 5′ UTR, with low levels of polyamines stimulating ODC translation and higher levels reducing it (reviewed in ref *17*).

These experiments describe the development and evaluation of the 10−23 model DNAzyme directed against the sequence for ODC mRNA. All DNAzymes designed were active in cleaving synthetic ODC RNA in vitro. The length of the hybridization arms for one DNAzyme, DNAzyme IV, which exhibited good activity, was optimized, and this optimized DNAzyme effectively silenced ODC in a cell culture model. These findings suggest that this DNAzyme will be an effective modulator of ODC for research and, potentially, therapeutic uses.



DNAzyme	5' Arm	Catalytic Domain	3' Arm
I	AGCTGCTCA	GGCTAGCTACAACGA	GGTTCTCGA
II	CATCAAGGA	GGCTAGCTACAACGA	GTGGCAGTC
III	GGTCCAGAA	GGCTAGCTACAACGA	GTCCTTAGC
IV	TCCGCAACA	GGCTAGCTACAACGA	AGAACGCAT
IV-mut	TCCGCAACA	GGCTA*C*CTACAACGA	AGAACGCAT
8/8	CCGCAACA	GGCTAGCTACAACGA	AGAACGCA
10/10	GTCCGCAACA	GGCTAGCTACAACGA	AGAACGCATC
11/11	GGTCCGCAACA	GGCTAGCTACAACGA	AGAACGCATCC
7/11	CGCAACA	GGCTAGCTACAACGA	AGAACGCATCC
V	TGCTCACTA	GGCTAGCTACAACGA	GGCTCTGCT
VI	GCCTTTGGA	GGCTAGCTACAACGA	GTGCTCTGG
UR	CTCCACGGCA	GGCTAGCTACAACGA	CAGCTGCAG-T
SCR-T	ACAACGCCT	GGCTAGCTACAACGA	TACGCAAGA-T
NS-T	NNNNNNNNN	NNNNNNNNNNNNNNN	NNNNNNNNN-T

FIGURE 1: Sequences and schematic representation of DNAzymes and controls. The 10−23 DNAzyme (DZ) is comprised of a 15-nucleotide catalytic domain flanked by target-specific hybridization arms. Cleavage of the RNA occurs 3′ to the unpaired purine (A, adenine). The catalytic domain of the DNAzyme is inserted in place of the unpaired A (denoted by asterisk). The sequences of all the DNAzymes used in this study are listed in the table. Six DNAzymes (I, II, III, IV, V, and VI) were evaluated in vitro, and DNAzyme IV and derivatives of it (listed within the same box) were used in subsequent studies. The sequence for DNAzyme IV-mut, a negative control, is the same as that of DNAzyme IV, except for a G→C nucleotide change (underlined, italicized C) in the catalytic domain which abolishes cleavage activity. DNAzymes IV-8/8, -10/10, -11/11, and -7/11 are variations of DNAzyme IV in which the length of the 9/9-nucleotide hybridization arms has been altered. A DNAzyme, DNAzyme UR, which effectively cleaves a RNA sequence unrelated to ODC, but whose hybridization arms do not target it to ODC, served as another negative control. DNAzymes and controls used for cell culture studies (DNAzyme IV-T, IV-mut-T, and IV-SCR-T and the oligo NS-T) contained an inverted 3′ T (denoted in sequence by "-T") to enhance stability. The sequences for DNAzyme IV-T and IV-mut-T (not shown) are the same as the sequences for DNAzyme IV and IV-mut, except that each contains an inverted 3′ T. The hybridization arms of DNAzyme IV-SCR-T are comprised of the same nucleotide composition as DNAzyme IV, except that the sequence is scrambled, so the arms do not target the DNAzyme to base pair with the sequence for ODC mRNA. NS-T is a degenerate 33mer (plus the inverted 3′ T) oligo that does not contain a catalytic domain or ODC-specific hybridization arms. (Y represents the targeted sequence of ODC RNA specific to each DNAzyme; X represents the nucleotide sequence for the DNAzymes, which is defined in the table; N = A, C, G, or T).

MATERIALS AND METHODS

Preparation of Oligonucleotides. Seven 33-mer oligonucleotides (DZ I, II, III, IV, V, VI, IV-mut; see Figure 1 for sequences) comprised of a 15 nucleotide domain flanked by arms of equal, nine-nucleotide length were purchased from Invitrogen Life Technologies (Carlsbad, CA) for use in in vitro cleavage studies. A DNAzyme designed against a target unrelated to ODC (DZ-UR) that exhibited extensive in vitro cleavage activity against the intended target was purchased from Qiagen Inc. (Valencia, CA; see Figure 1). Four additional derivatives of DZ IV (DZ IV-8/8, -10/10, -11/11, -7/11; see Figure 1) with varying hybridization arm lengths were synthesized (Invitrogen Life Technologies) to examine the effect of altering the length of the arms of DZ IV. For cell culture studies, the following oligonucleotides were

purchased from Qiagen Inc. (Valencia, CA) with an inverted 3′ T (3′-3′ phosphodiester linkage, denoted by "-T") to protect the DNA from degradation (*25*): DZ IV-T, DZ IV-mut-T, DZ IV-SCR-T, and NS-T (see Figure 1).

Preparation of in Vitro Transcript. To ensure that only full-length RNA substrate was used in the cleavage reaction, the sequence coding for a poly(A)$_{24}$ tail was added 3′ to the stop codon of the cDNA encoding for a truncated mouse ODC (425 out of 461 amino acids) lacking the 5′ and 3′ UTR. Poly(A) purification of the in vitro transcribed RNA removed premature transcripts, thereby reducing background in DNAzyme cleavage experiments. To introduce the poly-(A)$_{24}$ tail, PCR was performed on pGEM-ODC (425) (*26*) with the sense primer 5′-CATCTGCTTGATATTGGTG-GTGGC-3′ and antisense primer 5′-GAACTTGGATCC-(T)$_{24}$CTAGGCGCCATGGCTC-3′ (*Bam*HI site underlined). The PCR product was precipitated, electrophoresed, extracted, and gel-purified. pGEM-ODC (425) and the PCR product were digested with *Pfl*MI and *Bam*HI. The digests were precipitated, and the PCR product was ligated into pGEM-ODC (425) to make pGEM-ODC (425)-poly(A)$_{24}$. The construct was verified by sequence analysis. The plasmid, pGEM-ODC (425)-poly(A)$_{24}$, linearized by *Bam*HI digestion, was transcribed from the T7 promoter using the AmpliScribe T7 High Yield Transcription kit (Epicentre, Madison, WI) following the manufacturer's instructions. In vitro transcription was carried in the presence or absence of [α-^{32}P]-UTP (3 μCi/pmol, Perkin-Elmer, Boston, MA). The full-length transcript was purified from premature transcripts by poly(A) purification using Ambion's (Austin, TX) Poly-(A) Purist reagents and procedure. This synthetic ODC RNA contained 1381 nucleotides—1274 nucleotides coding for ODC with an additional 104 nucleotides resulting from cloning the gene into the vector and the addition of the poly-(A)$_{24}$ tail. The ^{32}P-labeled synthetic RNA substrate was used to evaluate DNAzyme-mediated cleavage of the RNA and for kinetic analysis, while the unlabeled synthetic RNA was used as the substrate for experiments in which the DNAzyme-incubated RNA was subsequently translated in vitro (ODC translation and ODC activity experiments).

DNAzyme-Mediated Cleavage Reaction. Poly(A)$_{24}$ purified, ^{32}P-labeled ODC RNA substrate was heat denatured at 65 °C for 5 min and then immediately cooled in an ice water bath. Then 16 μL of the substrate mix (containing ^{32}P-labeled RNA substrate) was added to 4 μL of the enzyme mix [respective DNAzyme or nuclease-free H_2O (no DZ reaction)] to initiate the reaction. The reactions were incubated at 37 °C for 60 min, at which time 20 μL of stop solution (71% formamide, 22 mM EDTA, and loading dye) was added to terminate each reaction, and the reactions were placed on ice. The incubated reactions contained 1 pmol of poly(A)$_{24}$ purified, ^{32}P-labeled ODC RNA and 10 pmol of respective DNAzyme (or nuclease-free H_2O) in DNAzyme buffer (final concentration: 5 mM Tris, pH 7.5, 30 mM NaCl, 10 mM $MgCl_2$). A 12 μL aliquot of each reaction was electrophoresed on a 5% denaturing polyacrylamide gel containing 7 M urea. Gels were dried and the radiolabeled RNA was detected using a phosphorimager and quantified with ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Protein Translation and Activity Determination of Translated ODC RNA Preincubated with DNAzyme. ODC RNA treated with DNAzyme was further analyzed by translating the RNA into protein. The percent translated full-length protein and the activity of the translated protein were measured. The DNAzyme-mediated cleavage reactions were carried out as described above, except unlabeled ODC RNA was substituted for the ^{32}P-labeled RNA. Increased reaction volumes were used; however, the concentrations and ratios of all reactions components remained constant. Following the DNAzyme-mediated cleavage reaction, the RNA was precipitated, heated at 65 °C for 3 min, cooled in an ice water bath, and translated into protein using the Flexi Rabbit Reticulocyte Lysate System (Promega, Madison, WI) according to the manufacturer's instructions. One 25 μL reaction contained the precipitated DNAzyme-treated RNA substrate, 70 mM KCl, 2 mM DTT, 0.2 mM amino acids minus methionine, 20 units RNasin ribonuclease inhibitor, 16 μL Flexi Rabbit Reticulocyte Lysate, and either 1.5 μL of L-[^{35}S]-methionine (15 μCi, 12.5 μmol, Perkin-Elmer, for use in the protein translation experiments) or 40 μM of unlabeled methionine (for use in ODC activity experiments). The reactions were incubated at 30 °C for 90 min. An aliquot of the reactions incubated with the L-[^{35}S]-methionine was electrophoresed on a 12.5% polyacrylamide gel to determine the relative amount of ODC protein translated from the DNAzyme-treated ODC RNA template. The gels were dried and the protein bands representing ODC were quantified with a phosphorimager and ImageQuant software (Molecular Dynamics). ODC activity was determined on an aliquot of the translation reaction in which unlabeled methionine was used. ODC activity was determined by measuring the release of $^{14}CO_2$ from L-[1-^{14}C]ornithine as previously described (*27*). The data are reported as the average of two or more experiments.

Kinetic Assays. The amount of substrate cleaved by DNAzyme IV was determined by comparison to a standard curve created using a fragment of RNA (referred to as DZ standard). DZ standard was constructed by PCR using pGEM-ODC (425) as the template. PCR was performed on the template linearized with *Bam*HI and/or *Sal*I using a sense primer (5′-CACGACGTTGTAAAACGACGGC-3′) upstream of the promoter and start codon and a downstream antisense primer (5′-(T)$_{18}$TAGAACGCATCCTTATCGTCAG-3′). When transcribed, the resulting RNA was the same size and sequence as ODC RNA cleaved by DNAzyme IV, except for a poly(A)$_{18}$ nucleotide tail at the 3′ end used to purify full-length DZ standard. The PCR product was gel-purified and precipitated. Linearized pGEM-ODC (425)-poly(A)$_{24}$ and the PCR product coding for DZ standard were transcribed in the presence of [α-^{32}P]UTP and poly(A) purified as described above. Both were transcribed side-by-side under the same conditions using the same lot of [α-^{32}P]UTP so that equimolar amounts of the 5′ cleavage product from ODC RNA reacted with DZ IV and DZ standard would give equal signal intensity. The kinetic assays were performed under multiple-turnover conditions. The reaction was initiated by adding equal volumes (10 μL) of enzyme mix and substrate mix. The enzyme mix contained 0.5 pmol of DZ IV-8/8, -9/9, -10/10, -11/11, or -7/11 or no DNAzyme (nuclease-free H_2O). The substrate mix consisted of one of seven varied concentrations of labeled ODC RNA. In the final reaction mix, ODC RNA concentrations ranged from 0 to 5 μM and the final buffer concentration was the same as for the in vitro

cleavage reactions. After incubation for 20 min at 37 °C, the reaction was terminated with the addition of 180 μL of DNAzyme stop solution and placed on ice. For any given substrate concentration, <10% of the substrate was converted to product. Cleavage products were resolved from substrate by gel electrophoresis. Each gel contained the entire range of points for the kinetic analysis of one DNAzyme along with a standard curve. The standard curve consisted of six concentrations of DZ standard ranging from 0 to 2 pmol, which bracketed the concentration of product formed. Bands representing product and standard were quantified as described before. Background directly above and below each band of interest was subtracted from that band to negate the difference in background due to the varying substrate concentrations. The amount of product formed was converted to picomoles using the standard curve, and rectangular hyperbola plots were generated in SigmaPlot 2000 (ver. 6.00, SPSS Inc., Chicago, IL) to fit the Michaelis−Menten equation

$$k_{obs} = (k_{cat}[S])/(K_m + [S])$$

Cell Culture Studies. Human embryonic kidney (HEK 293) cells were cultured in complete growth medium (Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum and 100 μg/mL penicillin and streptomycin) at 37 °C with 5% CO_2. One day prior to transfection, 1.2×10^6 cells were plated in 60 mm plates. The following day, the cells were washed twice with PBS and each plate was incubated with a transfection mix containing no DNAzyme or 600 pmol (6.2 μg) of DZ IV-T, DZ IV-mut-T, or DZ IV-SCR-T, 0.1 μg of pCMV-Zeo-ODCtr [mammalian expression vector coding for truncated mouse ODC (425 of 461 amino acids) lacking the 5′ and 3′ UTR; a generous gift from Dr. L. M. Shantz, Pennsylvania State College of Medicine, Hershey, PA], 12 μL of Lipofectamine Reagent (Invitrogen, Carlsbad, CA), and 8 μL of Plus Reagent (Invitrogen) in Opti-MEM I Reduced Serum Medium (Invitrogen) for 1.5 h. After the incubation period, the transfection mix was aspirated, and the cells were washed once with PBS and incubated in complete growth medium for 24 h, after which the cells were harvested in ODC buffer (25 mM Tris-HCl, pH 7.5, 2.5 mM DTT, 0.1 mM EDTA). Harvested cells were lysed by freeze−thawing three times in liquid nitrogen and centrifuged at 4 °C at 12 000*g* to isolate ODC in the cytosolic fraction. An aliquot of the supernatant was assayed for protein concentration and ODC activity as described before. ODC specific activity was calculated in pmol $^{14}CO_2$/30 min/μg protein. The activity of the different treatment groups was expressed as a percentage of ODC activity compared to that of NS-T, a nonsense oligo containing the same number of nucleotides. Each data point was derived from two independent experiments performed in triplicate. Error bars represent standard deviation and *p*-values were calculated using Student's *t*-test.

Western blotting was also performed on the cell lysate. An equal protein amount (75 μg) of cell lysate was resolved by SDS−PAGE for each treatment and transferred to a PVDF membrane (Pall Corp., Pensacola, FL). The membrane was probed with an affinity-purified ODC polyclonal antibody (0.5 μg/mL) (*8*) and detected using a chemiluminescent detection system (Cell Signaling Technology, Beverly, MA). To control for protein loading, the membrane was stripped in stripping buffer (100 mM 2-mercaptoethanol, 2% (w/v) SDS, 62.4 mM Tris-HCl, pH 6.7) for 30 min at 60 °C, reprobed with a monoclonal antibody to GAPDH (Biodesign, Saco, ME), and detected using a chemiluminescent detection system (Cell Signaling Technology). Bands representing ODC and GAPDH were quantified by densitometry using a GeneGenius BioImaging System (Syngene, Federick, MD). The signal obtained for ODC was normalized to the signal of GAPDH, and the normalized ODC protein for each treatment group was expressed as a percentage of ODC protein for NS-T treatment.

RESULTS

In Vitro Characterization of DNAzymes. Six 10−23 DNAzymes targeted to cleave mouse ODC mRNA were designed and evaluated in vitro (Figure 1). The initial evaluation was performed with 33-mer oligonucleotide DNAzymes comprised of a central 15 nucleotide catalytic domain (5′-GGCTAGGTACAACGA-3′) and flanked by two nine-nucleotide arms (Figure 1). Nine-nucleotide-length arms were chosen on the basis of previously determined kinetics (*3*). The variable flanking arms confer the specificity of the DNAzymes to target the sequence for ODC mRNA through Watson−Crick base pairing. Two additional DNAzymes, DZ IV-mut and DZ-UR, served as negative controls. The hybridization arms of DZ IV-mut are complementary to ODC RNA, but DZ IV-mut contains a G→C mutation at position 6 of the catalytic domain, which has been previously demonstrated to abolish the cleavage activity of the 10−23 DNAzyme (*3*). DZ-UR is an unrelated DNAzyme that effectively cleaves RNA unrelated to ODC (data not shown). The substrate recognition arms of DZ-UR are not complementary to ODC, but it contains an intact catalytic domain. The 18 nucleotide substrate recognition arms span a 19 nucleotide stretch of the target RNA, with the asterisk representing a purine of the target RNA that is unpaired in the DNAzyme (Figure 1). The cleavage reaction occurs at the phosphodiester bond between this unpaired purine and the adjacent 3′ pyrimidine; A−U and G−U are reportedly the preferred sites (*28*). Therefore, the six ODC DNAzymes were designed to target A−U sites toward the 5′ end of a long RNA (1381 nucleotides), which encodes for the first 425 of 461 amino acids of mouse ODC. Truncations near the C-terminus of ODC stabilize the protein by decreasing the rate of degradation, while not affecting activity of the enzyme (*26*, *29*). Although DNAzymes exhibit better activity against short RNA substrates (*30*), the long RNA was chosen over a short fragment of ODC RNA for in vitro experiments because it more closely represents the endogenous ODC mRNA.

Initial in vitro characterization determined that all six DNAzymes were capable of cleaving in vitro transcribed ODC RNA. Figure 2A represents the cleavage sites in ODC RNA targeted by the DNAzymes and the expected cleavage products upon successful cleavage. All six DNAzymes cleaved the target RNA and the size of the cleavage products agreed with the targeted site of DNAzyme cleavage of the ODC RNA (Figure 2B). No cleavage of ODC RNA was detectable for the negative control reactions: no DZ, DZ IV-mut, and DZ-UR. This suggests that (1) the reaction

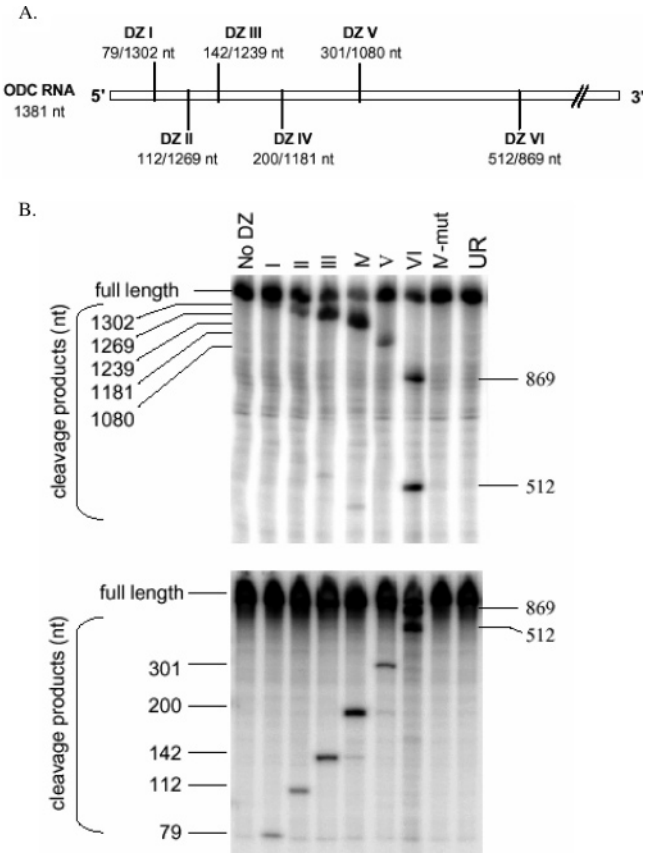


FIGURE 2: In vitro cleavage of ODC RNA by DNAzymes. A schematic representation of ODC RNA indicating the targeted cleavage sites for each DNAzyme. Labels indicate the size of the expected RNA products (in nucleotides) after successful cleavage by the different DNAzymes (A). Ten picomoles of DNAzyme I, II, III, IV, V, and VI or controls was incubated with 1 pmol of ^{32}P-labeled ODC RNA for 60 min at 37 °C (B). The smaller cleavage product was resolved on a second gel (B, lower panel). Each DNAzyme cleaved the target at the intended site. No cleavage was detected in the absence of DNAzyme (no DZ) or with negative controls IV-mut (DNAzyme IV with mutation in catalytic domain) and UR (unrelated DNAzyme).

components, minus a DNA oligo, are not sufficient for RNA degradation (Figure 2B, no DZ); (2) an intact catalytic domain is necessary for cleavage to occur, the mutation in the catalytic domain of DZ IV-mut abolishes cleavage activity (Figure 2B, IV-mut), and (3) arms that target the DNAzyme to Watson−Crick base pair with a specific region of the substrate are necessary for the cleavage reaction, as no cleavage was observed with DZ-UR (Figure 2B).

Translation of ODC RNA cleaved by a DNAzyme is expected to result in lower protein yield and less ODC activity compared to translation of untreated ODC RNA. To test this, ODC RNA treated with DNAzymes or controls was translated in vitro either in the presence or absence of L-[^{35}S]-methionine. An aliquot of the L-[^{35}S]-methionine reaction was electrophoresed and the band representing the labeled protein translated from the uncleaved ODC RNA was quantified (Figure 3, black bars). The results are presented as a percentage of expressed protein compared to the translation of ODC RNA that was not treated with a DNAzyme (no DZ), which was taken as 100% translation. All six of the DNAzymes (DZ I, II, III, IV, V, VI) reduced the percent protein translated by at least 40%. DNAzyme II, III, and IV showed the greatest reduction (84%, 91%,

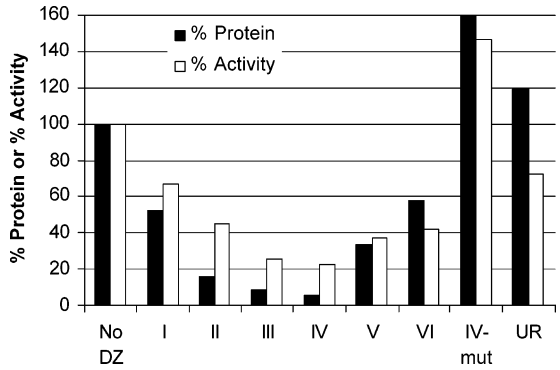


FIGURE 3: In vitro translation of DNAzyme-treated ODC RNA. Following DNAzyme treatment, ODC RNA was translated in vitro to determine the relative amount of ODC protein translated (black bars) and ODC activity (white bars). No DZ (no DNAzyme) was taken to be 100% for both measurements. The trend reflects the in vitro cleavage data. Data represents the average of at least two experiments.

and 94%, respectively). The two negative controls, DZ IV-mut and DZ-UR, did not decrease the amount of protein translated compared to no DZ and actually increased it slightly (particularly DZ IV-mut). It is possible that these controls stabilize the RNA template and facilitate translation and/or reduce degradation.

The sites of cleavage of the DNAzymes are such that, even if the cleaved 5′ fragment of the RNA was translated, the truncated polypeptides would not have ODC activity. A decrease in translated protein would be expected to result in decreased ODC activity, and to determine this, L-[^{35}S]-methionine was replaced with unlabeled methionine in the translation mix and ODC activity was determined for an aliquot of the translation reaction. The ODC activity profile for the DNAzymes and controls (Figure 3, white bars) closely follows the percentage of translated protein results. DNAzyme III (26%) and IV (22%) show the lowest ODC activity compared to no DNAzyme control (no DZ). These results establish that DNAzyme-mediated cleavage of ODC RNA, for all six DNAzymes, functionally results in reduced ODC protein, thereby silencing ODC activity. The degree of protein and activity reduction correlates well, and reduction in both these measures is dependent upon the ability of each DNAzyme to cleave ODC RNA.

Kinetic Analysis. Previously, the activity of DNAzymes has been demonstrated to be dependent upon hybridization arm length (*3*, *25*). The optimal length of the hybridization arms is dependent on the RNA substrate, specifically the composition and structure. We sought to determine the optimal length arms for DNAzyme IV—a promising candidate based upon the previous experiments. A different approach was taken to determine enzyme kinetics than previous studies (*31*, *32*), mainly to avoid potential issues with the dynamic range of the densitometer when quantitating bands at higher substrate concentrations. This approach utilized a standard curve to quantitate the cleavage product. A linear DNA template coding for the first 79 nucleotides of ODC RNA plus a poly(A)$_{18}$ tail at the 3′ end was constructed. Transcription of this template produced RNA, referred to as DZ standard, the same size and sequence as that produced by the cleavage of ODC RNA by DNAzyme IV, plus the addition of a poly(A)$_{18}$ tail at the 3′ end. Kinetic experiments were carried out under steady-state, multiple-

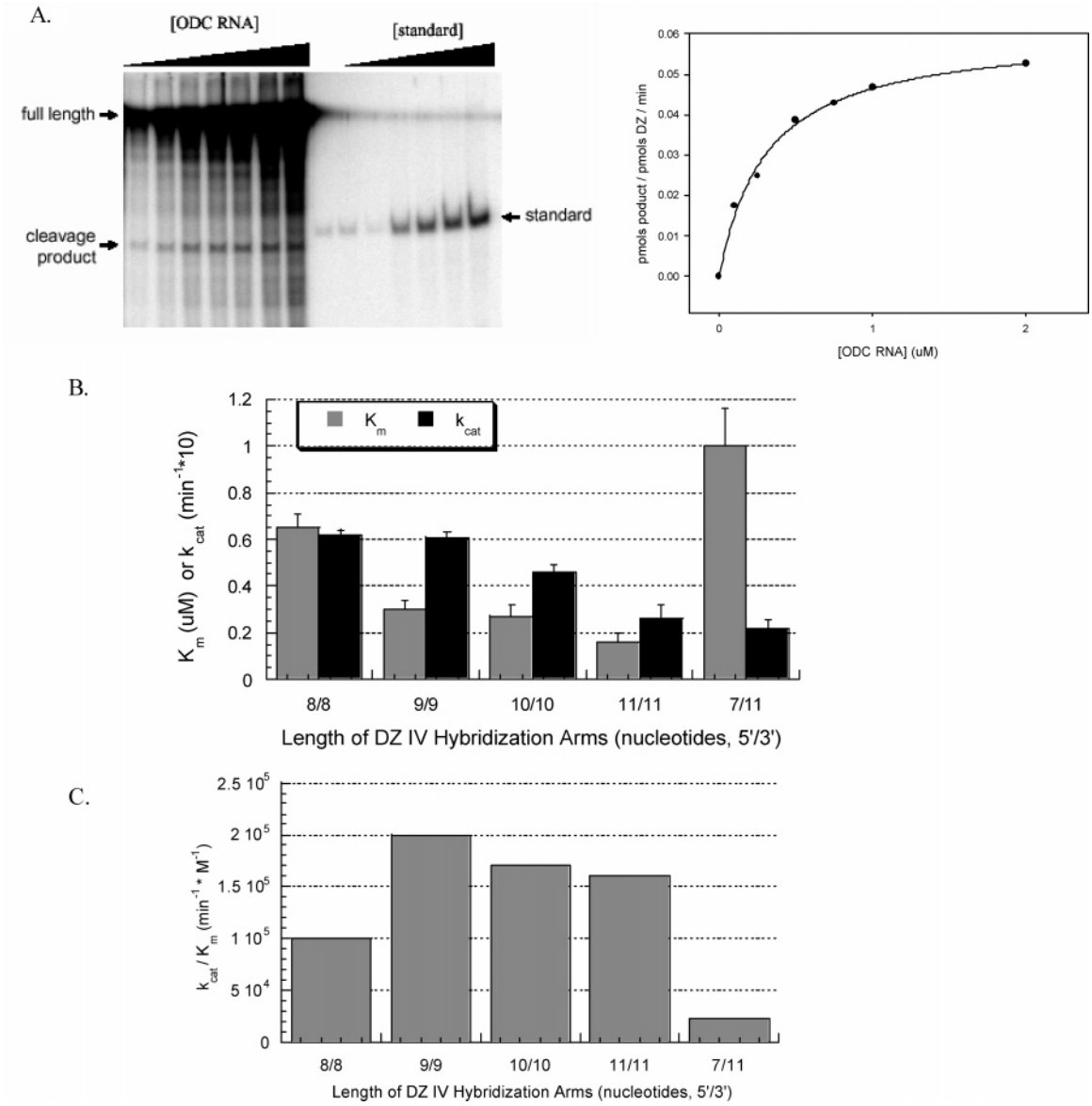


FIGURE 4: Effect of altering hybridization arm length on DNAzyme IV kinetics. K_m and k_{cat} values were determined under multiple turnover conditions in the presence of 10 mM $MgCl_2$. The 5′ cleavage product was separated on a gel from the uncleaved ^{32}P-labeled ODC RNA and the signal of the cleavage product was converted into picomoles by comparison to a standard curve (representative gel in A, left panel). The standard used to create the curve was the same sequence and size as the 5′ cleavage product, but had the addition of a poly(A)$_{18}$ tail used to purify it, which caused the standard to run slightly higher. K_m and k_{cat} were determined from a rectangular hyperbola plot fitted to the Michaelis−Menten equation (A, right panel). The resulting K_m and k_{cat} values for each arm configuration are plotted in B. Error bars represent standard deviation derived from the curve fit for a representative experiment. Catalytic efficiency (k_{cat}/K_m) was calculated for the DNAzymes (C).

turnover conditions for DNAzyme IV with varying length hybridization arms: 8/8, 9/9, 10/10, 11/11, and 7/11 (where the first number represents the 5′ hybridization arm and the second number the 3′ arm). Figure 4A (left panel) is a representative gel of a kinetic experiment for DNAzyme IV-9/9. Aliquots of reactions with increasing concentrations of ODC RNA and DZ standard were resolved by electrophoresis. The bands representing the 79-nucleotide product and DZ standard were quantitated by densitometry. The DZ standard runs higher than the cleavage product because of the additional 18 nucleotides of the poly(A)$_{18}$ tail of the DZ standard. A standard curve, generated from the DZ standard data, was used to convert the product band into picomoles, and these data were plotted using the Michaelis−Menten equation to determine the apparent K_m and k_{cat} (Figure 4A, right panel).

Lengthening the hybridization arms can affect the kinetics of the DNAzyme, in theory, by lowering the K_m (assuming this does not alter the DNAzyme's accessibility to the RNA). However, this may also stabilize the interaction of the DNAzyme and RNA substrate, hence slowing the rate of product release. DNAzyme IV with arm lengths that result in the optimal balance between the K_m and k_{cat} will be the most catalytically efficient. It was found that the K_m for DNAzyme IV decreases by ∼50% when the arm lengths are increased from 8/8 (0.65 μM) to 9/9 (0.30 μM) nucelotides, with a further decrease to 0.16 μM at 11/11 nucleotides (Figure 4B). No change in the k_{cat} is detected between DNAzyme IV-8/8 (0.062 min^{-1}) and -9/9 (0.061 min^{-1}), but it does decrease stepwise when the arm length is increased to 10/10 (0.046 min^{-1}) and 11/11 (0.026 min^{-1}) nucleotides. The offset 7/11-length arms displayed the highest K_m and

lowest k_{cat}. By virtue of combining the highest k_{cat} and moderate K_m, DZ IV with 9/9-nucleotide-length arms attains the greatest catalytic efficiency (measured by k_{cat}/K_m; Figure 4C). Despite having a k_{cat} equal that of DNAzyme IV-9/9, the catalytic efficiency of DNAzyme IV-8/8 is less favorable due to the high K_m. The differences in the K_m and k_{cat} for 10/10 and 11/11 offset each other, resulting in similar catalytic efficiencies.

Cell Culture Studies. Ultimately, the goal is to develop useful DNAzymes to silence ODC in cell culture and animal models. This end was pursued by transiently cotransfecting the catalytically optimized DZ IV-9/9, along with a plasmid coding for ODC, into HEK 293 cells. HEK 293 cells were chosen because this cell line is conducive to transfection. Although these cells naturally express endogenous ODC, the activity and protein levels are too low (using the detection methods described) to accurately evaluate the DNAzymes. It is possible to induce ODC activity by chemical or other means (*33*), but the observed effect of the DNAzyme on ODC activity would be limited by the transfection efficiency. In the cotransfection method, cells with the potential to express detectable ODC activity will also be transfected with DNAzyme.

After 24 h treatment, ODC activity and ODC protein were determined for the treated cells. Cells were treated with one of four oligos, all 34-mers containing a 3′ inverted T (denoted by "-T" in the oligo name)—a modification that has been shown to protect the DNAzyme from degradation (*25*). Transfection of 0.1 μg of pCMV-Zeo-ODCtr alone only nominally increases ODC activity (under the conditions described in Materials and Methods; data not shown). Additional DNA is needed to enhance transfection efficiency; therefore, a degenerate oligo, NS-T, acted as the control to which the other treatments were compared. DZ IV-T is the active DNAzyme IV, while DZ IV-mut-T and DZ IV-SCR-T served as negative controls. DZ IV-mut-T is identical in sequence to DZ IV-T, except for a G→C mutation in the catalytic domain that renders it catalytically inactive (see Figure 1). DZ IV-SCR-T has an intact catalytic domain but scrambled hybridization arms that do not target it to ODC (see Figure 1). After DZ IV-T treatment, ODC activity was reduced by 79% compared to NS-T—a significant decrease in activity (Figure 5A). No change in ODC activity was detected in DZ IV-SCR-T treated cells, thereby showing that complementary target arms are necessary for target inhibition by DNAzymes and an intact catalytic domain alone is not sufficient. DZ IV-mut-T treatment reduced ODC activity by 68%, due to an antisense effect of the hybridization arms. Although this is a substantial decrease in ODC activity, the intact catalytic domain of DZ IV-T leads to further, and highly statistically significant, reduction ($p < 0.0001$) compared to the catalytically inactive mutant, DZ IV-mut-T.

Treatment with an effective DNAzyme reduces the amount of ODC mRNA available for translation, so the ODC protein profile should reflect the activity measurements. Indeed, Western blots of the cytosolic fraction for ODC protein levels revealed a trend similar to the activity results. DZ IV-T decreased the amount of translated ODC protein (normalized to GAPDH expression) by 75% compared with NS-T control, again representing a more complete reduction in ODC expression than DZ IV-mut-T treatment (58% reduction; Figure 5B). DZ IV-SCR-T had little effect on translated ODC protein (8% reduction; Figure 5B). Together, the activity data and Western blot results provide evidence that the DNAzyme IV-T effectively silences ODC and that catalytic activity enables it to be more effective in cell culture than an inactive DNAzyme (DZ IV-mut-T) that is complementary to the same region of ODC.

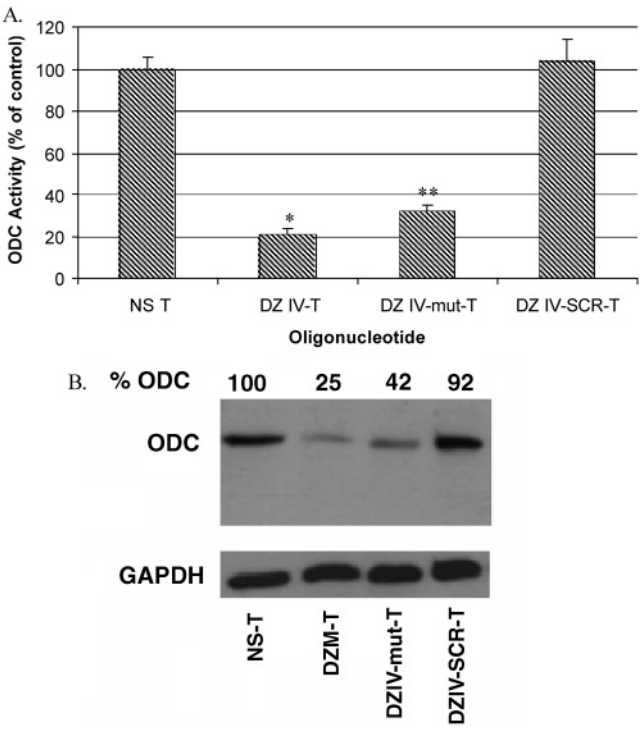


FIGURE 5: Effect of DNAzyme IV-T in cell culture. HEK 293 cells were transiently cotransfected with 6 μg of DNAzyme or control and 0.1 μg of a plasmid coding for ODC (pCMV-Zeo-ODCtr) for 24 h. Cells were harvested and ODC activity (A) or protein (B) was assayed. ODC activity: The activity of each treatment group is expressed as a percentage of NS-T (a degenerate oligo) activity. Error bars represent standard deviation of two independent experiments performed in triplicate ($n = 6$). The decrease in ODC activity for DZ IV-T was statistically significant compared with both NS-T and DZ IV-mut-T (A). ODC protein: Equivalent protein concentrations were analyzed by Western blotting for ODC and GAPDH. A representative blot is shown (B). Bands were quantitated by densitometry, and percent ODC protein relative to NS-T was determined for each treatment group. ODC was normalized to GAPDH. NS-T, degenerate oligo; DZ IV-T, catalytically active DZ IV; DZ IV-mut-T, catalytically inactive mutant; DZ IV-SCR-T, intact catalytic domain with scrambled hybridization arms. All oligos are 33-mers plus an inverted 3′ T (represented by "-T"). [(A) *DZ IV-T vs NS-T and DZ IV-T vs DZ IV-mut-T, $p < 0.0001$; **DZ IV-mut-T vs NS T, $p < 0.0001$; two-tailed Student's *t*-test].

DISCUSSION

ODC is a validated drug target, and the ability to modulate its activity has proven (i.e. African sleeping sickness and unwanted facial hair) and potential applications (e.g. chemopreventative/therapeutic uses). ODC is tightly regulated at the translational level and the protein has a very short half-life, thereby making it an attractive target for gene silencing via mRNA cleavage. The 10−23 model DNAzyme was chosen as the method to silence ODC based on the stability, catalytic activity, and the diverse applications of this method.

Six DNAzymes were designed and all exhibited the ability to cleave an in vitro transcribed ODC transcript at the

targeted site (Figure 2), but the extent to which each cleaved ODC RNA differed. This is illustrated in Figure 3, in which the DZ-reacted ODC RNA was translated in vitro. The extent of cleavage is reflected by the amount of protein translated and the resulting ODC activity. Even though successful DNAzyme cleavage sites are much more readily identified in short RNA substrates versus long RNA, a long RNA substrate (1381 nucleotides) was chosen for these studies to better recapitulate the mRNA that the DNAzyme would encounter in the cell. Previous studies have reported as few as 10% of DNAzymes designed to putative target sites were very effective at cleaving long (>700 nucleotides) RNA substrates (*34*, *35*).

Next, the catalytic efficiency of the DNAzyme was optimized by altering the length of the hybridization arms. Limitations of the method most widely used to determine DNAzyme kinetics lead us to develop a new method. Most of the reports describing DNAzyme kinetics use a method in which the concentration of product formed is determined by quantitating the fraction of cleaved and uncleaved RNA resolved by gel electrophoresis and by comparing this percentage to the known amount of substrate RNA used in the reaction. This method is not ideal for determining kinetics under multiple turnover (saturating substrate concentrations) conditions in which the reaction is not allowed to surpass 10% completion at the lowest substrate concentration. The difference in the intensity of the bands representing the product formed at the lowest substrate concentration and the uncleaved substrate at the highest substrate concentration makes it difficult to measure both bands accurately. The difference in band intensities may exceed the linear dynamic range of the instrument/software used to quantitate the bands. This problem is circumvented in the method described here because only the bands representing the product formed are quantitated. The intensity of these bands is converted into an amount of product formed by comparison to a standard curve of known concentration (Figure 4A).

It has been demonstrated that the length of the hybridization arms affects the catalytic efficiency of DNAzymes (*3*, *25*, *36*). The optimal length is likely to be target-specific, depending on the GC content and structure of the RNA. DZ IV was chosen for optimization because it showed favorable results in the in vitro experiments. Also, the same region of ODC targeted by DZ IV was identified by a ribozyme SELEX screen (*37*) to be an optimal site for ribozyme targeting, which suggests that this is an accessible site of the RNA molecule (data not shown). It was expected that the k_{cat} and K_m would both decrease as the length of the hybridization arms increased from 8 to 11 nucleotides. In general, this was the case, however no change in the k_{cat} was observed between arm lengths of eight and nine nucleotides. In the kinetic analysis performed by Santoro and Joyce (*3*), similar results were observed with a 27-nucleotide synthetic RNA substrate and DNAzyme unrelated to ODC. They observed that the k_{cat} remained relatively constant for hybridization arms seven to nine nucleotides in length and then decreased stepwise when lengthened beyond nine nucleotides because the rate-limiting step shifts from the rate of cleavage (for arm lengths ≤ 9 nucleotides) to the rate of product release (for arm lengths ≥ 10 nucleotides). Nine nucleotides was the most catalytically efficient length for the hybridization arms for DZ IV. The DNAzyme with the offset arms (7/11) exhibited the least favorable kinetics—the highest K_m and lowest k_{cat}. A previous study that investigated the effect of asymmetric length arms of a DNAzyme on the k_{obs} found no distinct pattern to help predict the effect of arm asymmetry (*38*).

The K_m (0.3 μM) and k_{cat} (0.06 min^{-1}) for DZ IV-9/9 are similar to those previously reported for DNAzymes designed for different targets (*25*, *36*, *39*, *40*), despite differing conditions that can have a profound negative impact on the activity of DNAzymes. Catalytic nucleic acids targeted against long RNA substrates versus short RNA substrates reportedly exhibit activities several orders of magnitude lower (*30*, *41*−*43*). The 1381 nucleotide RNA target used for this study was much longer than the short RNA substrates employed in other DNAzyme kinetic studies (≤27 nucleotides (*25*, *40*), 92, and 121 nucleotides (*36*)). Elevated concentrations of Mg^{2+} have been shown to increase the catalytic rate of the 10−23 DNAzyme (*2*). The 10 mM $MgCl_2$ concentration used in this study was significantly less than the 25 mM concentration used in other studies in which kinetics was determined (*36*, *39*). Despite these differences, the kinetics for DZ IV-9/9 was comparable to that of DNAzymes targeting short RNA targets and some of that used higher concentrations of $MgCl_2$.

Since ODC translation is tightly controlled, it could be argued that it would be more difficult to silence the transcript in cell culture due to complex folding, overall structure, and proteins that may be associated with it. However, catalytically optimized ODC DZ IV (9/9-nucleotide-length arms) with the 3′−3′ inverted T effectively reduced ODC activity (Figure 5A), which correlated well with the reduction in ODC protein (Figure 5B), in HEK 293 cells. A similar reduction of ODC has been shown to revert the transformed phenotype of Ras-transformed NIH/3T3 cells back to normal (*8*). Therefore, DZ IV sufficiently silences ODC to use it as a tool to study the functional effects of ODC. The catalytically active DNAzyme was also able to silence ODC more completely than the antisense effect of DZ IV-mut-T. These data provide the first report that ODC can be silenced by a DNAzyme and provide support that a catalytically active DNAzyme has the potential to be more effective than oligos that work through an antisense mechanism.

The development and identification of a DNAzyme that is able to suppress endogenous ODC mRNA, as well as in vitro transcribed ODC RNA, open many exciting possibilities, especially in the light of recent developments in the DNAzyme field. An approach to express a 10−23 DNAzyme in vivo from a vector has been described and demonstrated to be effective against the intended target in cell culture (*44*−*46*). Expression of DZ IV-T in cells would allow for more flexibility to study the functional effects of silencing ODC than transfecting DNAzyme oligos. This would enable the selection of cells that constitutively express DZ IV-T, thereby permitting the study of the DNAzyme in cells with low transfection efficiency. A tetracycline-regulated DNAzyme expression system has also been developed, which further increases the power of this system (*46*). For example, the transformed phenotype of cells transfected with activated HRas(61L) or RhoA(63L) mutants or cells that overexpress eIF4E can be reverted by reducing ODC activity (*8*, *47*). By controlling expression of DZ IV-T, the direct effect of

silencing ODC on the transformed phenotype of these cells could be examined.

In another recent development, topical application of a DNAzyme targeted to mRNA for the hairless gene recapitulated the hairless phenotype in mice, thereby providing proof of principle that a DNAzyme can be effective when applied topically to the skin (*48*). Since DFMO (Vaniqa) is the only FDA-approved drug for the treatment of unwanted facial hair, future studies will investigate the effect of DZ IV topically applied to mouse skin to determine the effect on ODC activity and hair growth as an alternative to DFMO.

In conclusion, we have identified DNAzymes that cleave a long transcript of ODC and subsequently reduce the amount of translated ODC protein resulting in a concomitant decrease in activity both in vitro and in cell culture. This is also one of relatively few reports that demonstrates that an active DNAzyme can more completely suppress the activity of the target mRNA in a cell culture model compared to a catalytically inactive counterpart, as measured by a direct effect on the target. The development of a DNAzyme that is effective against ODC opens many opportunities to better study ODC and to explore its role in various biological processes.

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