# Antisense Gene Inhibition by C-5-Substituted Deoxyuridine-Containing Oligodeoxynucleotides†

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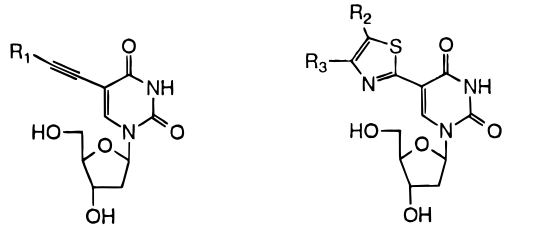
ABSTRACT: Antisense oligodeoxynucleotides (ODNs) are capable of inhibiting gene expression via a RNase H mechanism in which the complementary RNA is degraded by RNase H. C-5 propyne dU phosphorothioate ODNs bind selectively and with high affinity to RNA within cells leading to potent antisense inhibition of RNA translation. The effect that increasing steric bulk of C-5-substituted deoxyuridine analogs has on affinity for RNA and ability to inhibit gene expression is discussed. The relative binding affinity was measured by thermal denaturation (Tm) analysis, and antisense activity was determined by inhibition of SV40 T-antigen (TAg) expression in CV1 cells. The results show that antisense activity is not directly correlated to Tm measurements. *In vitro* analysis (RNase H cleavage, on-rates, and off-rates) and pre-formed ODN/RNA experiments indicate that RNase H activity and intracellular dissociation appear to be major determinants of the antisense potency of the various substituted ODNs. The results of our analysis point to the unique ability of C-5 propyne dU ODNs to selectively bind to RNA within cells and activate cleavage of RNA by RNase H leading to potent inhibition of gene expression.

Antisense oligodeoxynucleotides (ODNs)[1] have generated wide interest because of their potential to inhibit expression of specific genes (Milligan et al., 1993; Wagner, 1994). New techniques for monitoring gene inhibition have resulted in precise demonstrations of antisense effects by putting in place suitable controls to detect gene specific and sequence specific inhibition as well as monitor nonspecific effects (Wagner, 1994; Wagner et al., 1993; Moulds et al., 1995). C-5 propynyl-2′-deoxyuridine (pdU)-substituted phosphorothioate ODNs have been shown to be potent and specific antisense inhibitors of protein expression in cells (Wagner et al., 1993; Fenster et al., 1994; Moulds et al., 1995). The inhibition is due to an RNase H-mediated mechanism (Wagner et al., 1993, 1996; Moulds et al., 1995) and the activity of propyne ODNs correlated with melting temperature and length of ODN (Wagner et al., 1993).

The enhancement in the binding affinity to complementary RNA provided by the C-5 propyne is likely due to increased base stacking interactions between adjacent base pairs (Froehler et al., 1992), and certain methyl-substituted thiazole ODNs increase these hydrophobic interactions yielding ODNs that bind RNA with affinities greater than propyne ODNs (Gutierrez & Froehler, 1996). A microinjection assay (Wagner, 1994; Wagner et al., 1993; Moulds et al., 1995), which removes the question of differential ODN permeability, was used to assess the effect that these C-5-substituted dU-containing ODNs (see Figure 1) have on the ability to inhibit gene expression in cells. The importance of various parameters (equilibrium binding constant, intracellular dissociation and RNase H cleavage of RNA) on the potency of antisense gene inhibition was determined by *in vitro* analysis of the ODNs.




**1** $R_1 = CH_3$
**2** $R_1 = CH_2CH_3$
**3** $R_2 = R_3 = H$
**4** $R_2 = R_3 = CH_3$

FIGURE 1: C-5 2′-deoxyuridine analogs.

## MATERIALS AND METHODS

*General Procedures.* All reactions were performed under an argon atmosphere. Anhydrous tetrahydrofuran and anhydrous dioxane in Sure/Seal bottles and 4,5-dimethylthiazole were used as purchased from Aldrich Chemical Co. Butyne (Wiley Organics) was used without additional purification. 5-Iodo-2′-deoxyuridine was obtained from Chem Impex International. 5-Propynyl-2′-deoxyuridine, **1** (Hobbs, 1989), and 5-(thiazol-2-yl)-2′-deoxyuridine, **3** (Gutierrez et al., 1994), were prepared according to published procedures. The synthesis of additional butyne-dU and 4,5-dimethylthiazole-dU analogs are given below. $^1$H NMR spectra were recorded using a General Electric QE 300 spectrophotometer in deuterated solvent.

*5-(Butyn-1-yl)-2′-deoxyuridine, **2**.* To a solution of 5-iodo-2′-deoxyuridine (0.708 g, 2.0 mmol) and triethylamine (0.40 g, 4.0 mmol) in *N*,*N*-dimethylformamide (10 mL) was added copper(I) iodide (0.076 g, 0.40 mmol) and tetrakis(triph-

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[1] Abbreviations: ODN, oligodeoxynucleotide; RNA, ribonucleic acid; Tm, thermal denaturation; TAg, SV40 T-antigen; DMT, 4,4′-dimethoxytrityl; PAGE, polyacrylamide gel electrophoresis; $\beta$-gal, $\beta$-galactosidase; CAT, chloramphenicol acetyltransferase; IdU, 5-iodo-2′-deoxyuridine; dU, 2′-deoxyuridine; pdU, 5-propynyl-2′-deoxyuridine.

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Table 1: Biophysical Data and Biological Activity of C-5-Substituted dU ODNs (ODN Sequence, 5′-CUUCAUUUUUUCUUC-3′)[a]

| U | ODN | Tm[b] (°C) | $k_a$[c] ($s^{-1}$) (rel) | $k_d$[d] ($s^{-1}$) (rel) | $K_d$[e] ($k_a/k_d$) (rel) | RNase H[f] (%) (rel) | pre-form[g] (%) | $IC_{50}$[h] ($\mu$M) |
|---|---|---|---|---|---|---|---|---|
| **1** | **5** | 66.5 | $5.7 \times 10^5$ (1.0) | $1.13 \times 10^{-3}$ (1.0) | $5.0 \times 10^8$ (1.0) | $32 \pm 7$ (1.0) | 100 | 0.25 |
| **2** | **6** | 66.0 | $7.7 \times 10^5$ (1.3) | $1.08 \times 10^{-3}$ (1.0) | $7.1 \times 10^8$ (1.4) | $27 \pm 5$ (0.84) | 100 | 1.0 |
| **3** | **7** | 67.5 | $5.1 \times 10^5$ (0.9) | $4.44 \times 10^{-4}$ (0.4) | $1.1 \times 10^9$ (2.2) | $14 \pm 3$ (0.44) | 0 | 5.0 |
| **4** | **8** | 69.0 | $1.3 \times 10^6$ (2.3) | $4.83 \times 10^{-4}$ (0.4) | $2.7 \times 10^9$ (5.4) | $5 \pm 3$ (0.16) | 0 | 5.0 |

[a] All ODNs contain phosphorothioate linkages; C = 5-methyl-2′-deoxycytidine, A = 2′-deoxyadenosine. [b] Tm values were assessed in 140 mM KCl/5 mM $Na_2HPO_4$/1 mM $MgCl_2$, pH = 7.2, at 260 nm, and the final concentration of ODN was ~2 $\mu$M (RNA concentration = ~2 $\mu$M). [c] Psuedo-first-order association rate constants for antisense ODNs to RNA (see Materials and Methods). Relative values (rel) are based on the values of the propyne ODN, **5**. [d] The dissociation rate constants of antisense ODNs at 37 °C. [e] The equilibrium binding constants for the ODNs to RNA was determined from the quotient of $k_a$ and $k_d$. [f] The percent of cleavage of RNA by RNase H in HeLa cell nuclear extract after 10 min at 37 °C. [g] The percent inhibition of SV40 TAg translation in CV-1 cells by antisense ODNs pre-bound to 5′-capped, unspliced TAg RNA. [h] The concentration of antisense ODN necessary for 50% inhibition of SV40 TAg after nuclear microinjection.

enylphosphine)palladium(0) (0.231 g, 0.20 mmol). To this solution was quickly added butyne (0.270 g, 5.0 mmol), and the mixture was stirred at room temperature for 16 h. Dowex 1 × 8−100 ($HCO_3^-$) (2.0 g), $CH_3OH$ (10 mL), and $CH_2Cl_2$ (10 mL) were added, and the mixture was stirred 1 h. The resin was filtered, and the filtrate evaporated. Silica gel chromatography yielded 0.402 g (1.5 mmol, 75%) of **2**. $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 11.50 (br s, 1H, N*H*), 8.11 (s, 1H, *H*6), 6.11 (t, $J$ = 6 Hz, 1H, *H*1′), 5.24 (d, $J$ = 4 Hz, 1H, 3′O*H*), 5.09 (t, $J$ = 5 Hz, 1H, 5′O*H*), 4.23 (m, 1H, *H*3′), 3.79 (m, 1H, *H*4′), 3.58 (m, 2H, *H*5′), 2.36 (q, $J$ = 7 Hz, 2H, C$H_2$CH$_3$), 2.11 (m, 2H, *H*2′), 1.11 (t, $J$ = 7 Hz, 3H, CH$_2$C$H_3$).

*5-(4,5-Dimethylthiazol-2-yl)-5′-O-dimethoxytrityl-2′-deoxyuridine, **4***. To a solution of 4,5-dimethylthiazole (0.567 g, 5.0 mmol) in tetrahydrofuran (5 mL), at −38 °C, was added *tert*-butyllithium in pentane (1.7 M, 3.5 mL, 6.0 mmol) over 20 min. The solution was stirred for 30 min at −38 °C and then cooled to −78 °C. A solution of tributyltin chloride (1.61 g, 4.95 mmol) in THF (2.5 mL) was added dropwise over 15 min, and the mixture warmed to room temperature over 16 h. The solvent was evaporated, and the liquid was dissolved into ether (50 mL), washed with $H_2O$ (20 mL), dried ($Na_2SO_4$), and evaporated. To this red liquid was added 5-iodo-3′,5′-di-*O*-toluyl-2′-deoxyuridine (Robins & Barr, 1982) (1.16 g, 1.96 mmol), dioxane (30 mL), and bis-(triphenylphosphine)palladium(II) chloride (0.14 g, 0.20 mmol). The mixture was heated at 90 °C for 48 h, and the solvent was evaporated. To the residue was added $CH_3OH$ (40 mL) and $K_2CO_3$ (0.66 g, 4.8 mmol), and the mixture stirred at room temperature for 4 h. Acetic acid (0.290 g, 4.8 mmol) was added, and the solvent was evaporated. Silica gel chromatography (10% $CH_3OH$/1% $NH_4OH$/$CH_2Cl_2$) yielded 1.06 g of solid which was evaporated from pyridine (5 mL) and dissolved into pyridine (15 mL). To the solution was added 4,4′-dimethoxytrityl chloride (0.800 g, 2.95 mmol), and the mixture was stirred 24 h, and quenched with $CH_3OH$ (2 mL). The solvent was evaporated to 1−2 mL, dissolved into $CH_2Cl_2$ (80 mL), washed with saturated $NaHCO_3$ (20 mL), dried ($Na_2SO_4$), and evaporated. Silica gel chromatography (2−5% $CH_3OH$/$CH_2Cl_2$) yielded 0.710 g (1.11 mmol, 56%) of **4**. $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 8.86 (br s, 1H, N*H*), 8.64 (s, 1H, *H*6), 7.46−7.15 (m, 9H, DMT-*H*), 6.74 (dd, $J$ = 2, 9 Hz, 4H, DMT-*H*), 6.28 (t, $J$ = 6 Hz, 1H, *H*1′), 4.39 (m, 1H, *H*3′), 4.02 (dd, $J$ = 4, 8 Hz, 1H, *H*4′), 3.74 (s, 6H, (OC$H_3$)$_2$), 3.51 (dd, $J$ = 4, 11 Hz, 1H, *H*5′), 3.42 (dd, $J$ = 4, 11 Hz, 1H, *H*5″), 2.46 (ddd, $J$ = 4, 6, 13 Hz, 1H, *H*2′), 2.32 (m, 1H, *H*2″), 2.30 (s, 3H, C$H_3$), 1.91 (s, 3H, C$H_3$).

*ODN Synthesis, Purification, and Tm Analysis.* Automated synthesis of ODNs was performed on a Milligen-Biosearch 8750 using H-phosphonate methodology (Froehler, 1993). ODNs were removed from the solid support with concentrated $NH_4OH$ at 55 °C for 2 h and evaporated. All ODNs were purified by PAGE, the bands were excised and extracted with $H_2O$, and the volume reduced by n-butanol extraction. The ODNs were desalted (Sephadex NAP-25), converted to the $Na^+$ counterion (AG 50W-X8($Na^+$)), and evaporated. ODNs **5**−**8** were prepared as phosphorothioate diesters (Table 1). Tm analysis was carried out as described (Froehler et al., 1992) in 140 mM KCl, 5 mM $Na_2HPO_4$, and 1 mM $MgCl_2$ (pH 7.2) at 260 nm. The final concentration of all ODNs was ~2 $\mu$M (RNA concentration = ~2 $\mu$M).

*Antisense Assays via Microinjection.* Nuclear microinjection of African green monkey kidney cells [CV1 (ATCC)] with plasmids expressing mutant SV40 TAg and *Escherichia coli* $\beta$-galactosidase with or without ODNs (Wagner et al., 1993) followed by immunofluorescence staining and fluorescence microscopy was performed to detect specific antisense gene inhibition as described (Moulds et al., 1995). The ODNs **5**−**8** were targeted to a sequence ~150 nucleotides downstream of the TAg initiation codon. $IC_{50}$ values were estimated by scoring for TAg expression vs untargeted $\beta$-gal expressing cells relative to control injections containing no ODN (Wagner et al., 1993). All experiments were repeated in triplicate, and $IC_{50}$ values are ±10%.

*RNase H Analysis of ODN/RNA Complexes.* A 5 pM solution of 5′-end labeled [$^{32}$P]TAg 20-mer RNA (6 $\mu$L) was mixed with 10 nM antisense ODNs **5**−**8** (1 $\mu$L) in RNase H buffer [50 mM Tris·HCl (pH 8.0), 10 mM $MgCl_2$, 20 mM KCl, 3 mM dithiothreitol] containing 20 units of RNasin and 1 $\mu$g/$\mu$L tRNA. HeLa cell nuclear extract (100 ng) was added (final volume, 10 $\mu$L), and after 10 min aliquots were removed and added to loading solution [90% formamide, 0.025% xylene cyanol, 50 mM EDTA (pH 8.0)], heated to 95 °C, and analyzed by denaturing (8.3 M urea) PAGE (Moulds et al., 1995).

*Kinetics of Hybridization.* A gel shift assay was used to analyze rates of hybridization of ODNs **5**−**8** to a 20-mer sense RNA that contained the 15-mer TAg target sequence (Moulds et al., 1995). ODNs **5**−**8** (final concentrations of 2.5, 5, 10, and 20 nM) were mixed with 5′-end labeled [$^{32}$P] target RNA (final concentration 0.5 nM) in buffer [40 mM MOPS (pH 7.2), 140 mM KCl] at 37 °C (final volume 80 $\mu$L). Aliquots (8 $\mu$L) were removed and quenched by addition of excess unlabeled sense RNA (~2 $\mu$M) after 15 s and 1, 2, 4, 8, 16, and 32 min. 20% native PAGE of the

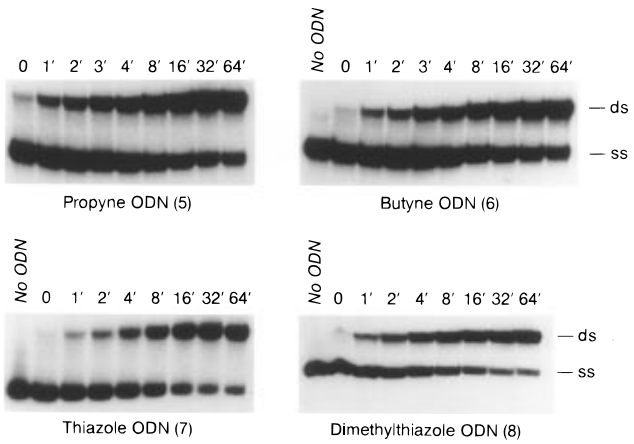


FIGURE 2: Gel shift analysis of the hybridization rates of ODNs **5**−**8** to 5′-end labeled sense [$^{32}$P]RNA. ODN (2.5 nM) was added to the RNA (0.5 nM), and the extent of association was observed over time. At each time point the reactions were quenched by addition of ~2 $\mu$M unlabeled sense RNA followed by PAGE. The top bands (ds) refer to the ODN/RNA duplex formed, and the lower bands (ss) are the unhybridized RNA. The zero band actually represents approximately 15 s.

samples (Figure 2) and quantitation (Ambis 4000) of labeled ODN/RNA duplex and labeled single-strand RNA yielded the results in Figure 3A. The first-order rate expression is

$$-kt = \ln[\alpha_o - x_t/\alpha_o]$$

where $\alpha_o$ is the initial concentration of ODN and $x_t$ is the extent of hybridization of the ODN to RNA at time, $t$.

Since particle decay counts are measured and not concentrations, this expression may take the form

$$-kt = \ln[(1 - X_t)/(1 - X_o)]$$

where $X_t$ is the fraction of duplex formed at time, $t$ and $X_o$ is the fraction of duplex at time zero.

From the fractions of the double-strand and single-strand RNA, $k_{obs}$ for each concentration was determined from the psuedo-first-order rate plots as in Figure 3B (Moore, 1972). The association rate constants, $k_a$, was determined from a plot of $k_{obs}$ vs ODN concentrations, Figure 3C.

Off-rate analysis was determined by a similar experiment. The 20-mer 5′-end labeled [$^{32}$P]RNA (0.5 nM) [80 000 cpm/experiment] was prebound to 10 nM antisense ODN **5**−**8** for 24 h at 37 °C under the same buffer conditions described above. To aliquots of the prebound complexes was added unlabeled 20-mer sense DNA (final concentration 10 $\mu$M) at time points 0, 2 min, and 0.25, 0.5, 1, 2, 4, 8, and 24 h, cooled to 0 °C, and analyzed by 20% native PAGE. Mole fraction of double-strand and single-strand RNA was determined as described above.

*Translation of Preformed ODN/RNA Complexes.* ODNs **5**−**8** were hybridized at room temperature to 5′-capped, unspliced TAg RNA for 2 h, and these complexes were microinjected with chloramphenicol acetyltransferase (CAT) RNA into the nucleus CV1 cells. The cells were incubated for 4 h at 37°C, fixed, immunolabeled, and scored visually by fluorescence microscopy for TAg and CAT expression (Moulds et al., 1995). These experiments were repeated in duplicate.

## RESULTS

*Nucleoside Synthesis.* Both the C-5 alkyne- and C-5 thiazole-dU analogs (Figure 1) were prepared by palladium-

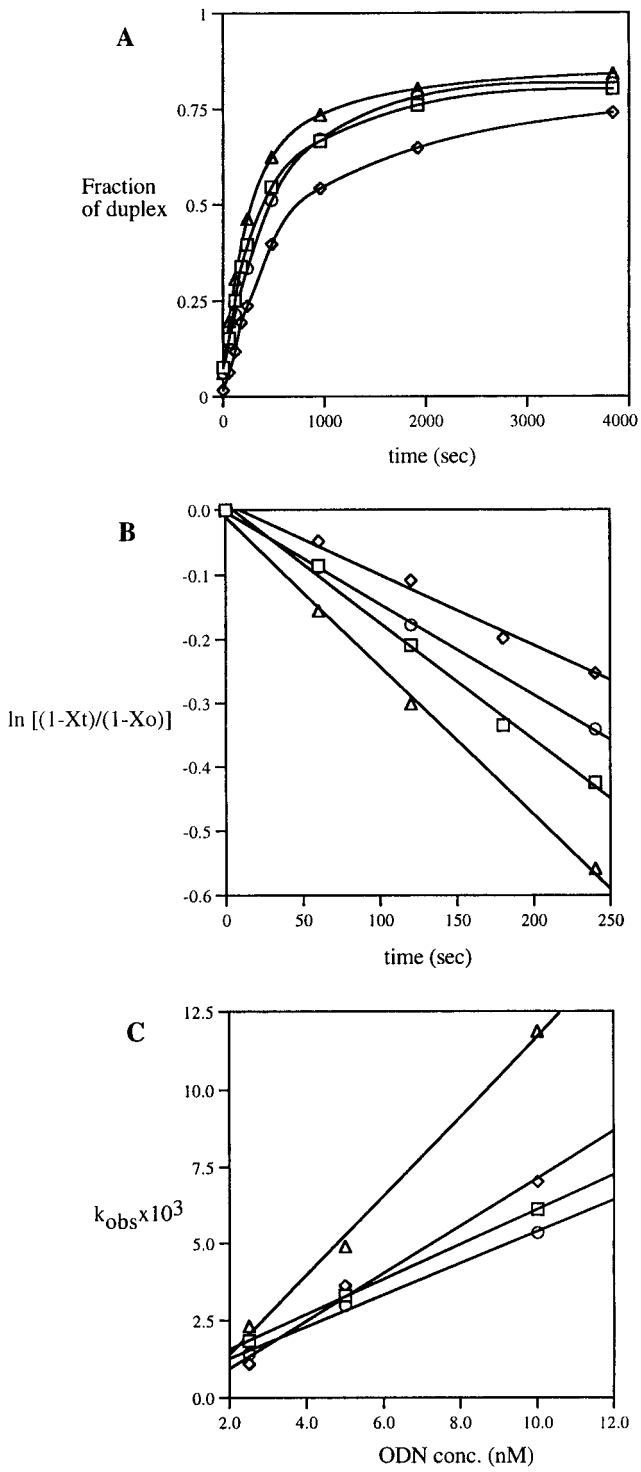


FIGURE 3: (A) Hybridization over time of ODNs **5**−**8** (2.5 nM) to 5′-end labeled sense [$^{32}$P]RNA, resulting from quantitation of gel shifts by particle detection (Ambis 4000). Fraction of duplex is the ratio of particle counts of double-stranded ODN/RNA (ds) to the total of ds and ss. The symbols in the plot represent the following: **(□)** propyne ODN **5**; (◇) butyne ODN **6**; (○) thiazole ODN **7**; (△) dimethylthiazole ODN **8**. (B) The determination of $k_{obs}$ for the association of ODNs **5**−**8** at 2.5 nM. $X_t$ is the mole fraction of (ds) at time, $t$, and $X_o$ is the mole fraction of (ds) at time, zero. (C) Determination of the association rate constants, $k_a$, for ODNs **5**−**8** from plot of $k_{obs}$ vs ODN concentration.

catalyzed coupling reactions. The alkynes **1** and **2** were synthesized in high yield by direct coupling with 5-iodo-2′-deoxyuridine (IdU) in the presense of palladium(0) catalyst (Hobbs, 1989). Alternatively, the thiazole-dU analogs **3** and **4** required palladium-catalyzed coupling of 2-tributylstan-

nylthiazoles with 5′,3′-di-*O*-*p*-toluyl-IdU (Gutierrez et al., 1994, 1996).

*Tm Analysis of ODNs Containing C-5-Substituted dUs.* Stability of the C-5-substituted dU phosphorothioate ODN/RNA complexes were determined by Tm analysis (Table 1), and all ODNs had an increased stability relative to the thymidine control ODN (Tm = 56.0 °C). The propyne-substituted ODN (**5**) and butyne-substituted ODN (**6**) led to stable ODN/RNA duplexes (ΔTm = +10.5 °C and +10.0 °C, respectively), and thiazole-dU-substituted ODN (**7**) and the dimethylthiazole-substituted ODN (**8**) formed the most stable duplexes with RNA (ΔTm = +11.5 and +13.0 °C, respectively).

*Nuclear Microinjection of ODNs Containing C-5-Substituted dU Analogs.* ODNs containing C-5-substituted dU analogs **5**−**8**, targeted to a site downstream of the TAg initiation codon, were microinjected into the nuclei of CV-1 cells. Expression of $\beta$-galactosidase was used as an internal control in each experiment to detect nonspecific effects of the ODNs (Wagner et al., 1993). All ODNs contained phosphorothioate internucleotide linkages to increase nuclease stability while retaining the ability to activate RNase H cleavage of the complementary RNA strand (Bennett et al., 1992; Monia et al., 1993; Wagner et al., 1993). Gene specific inhibition of TAg expression was observed with all ODNs (Table 1), propyne having the greatest potency ($IC_{50}$ = 0.25 $\mu$M). The more bulky butyne-substituted ODN (**6**) led to a 4-fold decrease in antisense activity ($IC_{50}$ = 1.0 $\mu$M) even though there was a minimal effect on Tm. The thiazole-substituted ODN (**7**) exhibited a 20-fold decrease in potency relative to the propyne-substituted ODN (**5**) even though it exhibited higher affinity (Tm = 67.5 °C). The 4,5-dimethylthiazole-substituted ODN (**8**) had the highest affinity of all ODNs tested (Tm = 69.0 °C) but was not very potent ($IC_{50}$ = 5.0 $\mu$M). The lack of correlation between ODN/RNA stability and ability to inhibit gene expression with these dU analogs prompted us to examine a number of *in vitro* parameters.

*Association and Dissociation Kinetics of C-5-Substituted dU ODNs.* In order to determine if the decreased activity of ODNs **7** and **8** was due to slow rate of association to RNA, the *in vitro* hybridization of ODNs containing propyne (**5**), butyne (**6**), thiazole (**7**), and dimethylthiazole (**8**) to the target RNA was observed over time under "psuedo-first-order" conditions with respect to ODN (Figures 2 and 3) (Moulds et al., 1995). The data in Table 1 indicate that all ODNs had comparable hybridization rates to RNA; the butyne-substituted ODN (**6**, $k_a = 7.7 \times 10^5$ $s^{-1}$) being slightly faster than the propyne substituted ODN (**5**, $k_a = 5.7 \times 10^5$ $s^{-1}$), and the dimethylthiazole-substituted ODN (**8**) exhibiting the fastest "on-rate" ($k_a = 1.3 \times 10^6$ $s^{-1}$). The rate of dissociation of the thiazole-substituted ODNs (**7** and **8**) were 2.5 times slower relative to the propyne- and butyne-substituted ODNs (**5** and **6**). The resulting equilibrium dissociation contants, $K_d$, indicate that the thiazole substituted ODNs (**7** and **8**) form the most thermodynamically stable duplexes with RNA (Table 1), but this increased stability does not result in increased potency. Therefore, the lower potency of these ODNs relative to propyne substituted ODN (**5**) is probably unrelated to the *in vitro* thermodynamic stability of the ODN/RNA duplex.

*RNase H Cleavage of ODN/RNA Complexes.* ODNs **5**−**8** were tested for their ability to activate RNase H cleavage of the complementary RNA (Figure 4). The ODNs were complexed to 5′-end labeled [$^{32}$P]RNA and treated with HeLa cell nuclear extract for 10 min at 37 °C. Reaction conditions were adjusted so that ∼30% cleavage of the RNA was observed for all ODNs representing single-hit kinetics (Moulds et al. 1995). The propyne-substituted ODN (**5**) facilitated RNase H cleavage of 32% of the RNA at several sites (Figure 4), and the butyne-substituted ODN (**6**) led to a slight decrease (27%) in the amount of RNA cleaved (Table 1). In contrast, the thiazole-containing ODNs (**7** and **8**) activated RNA cleavage predominantly at different sites relative to both the propyne- and butyne-substituted ODNs (**5** and **6**, Figure 4), and the rates of *in vitro* RNase H cleavage of the RNA were slower. The thiazole-substituted ODN (**7**) led to cleavage of 14% of the RNA (44% relative to ODN **5**), and the dimethylthiazole-substituted ODN (**8**) led to cleavage of 5% of the complementary RNA (16% relative to ODN **5**). Therefore, the rates of RNA cleavage by mammalian RNase H likely account, at least in-part, for the low potency of ODNs **7** and **8** relative to the propyne-substituted ODN (**5**), and the decreased RNase H activity of the butyne substituted ODN (**6**, 84% relative to the ODN **5**) is likely a major determinant of the decreased activity of this ODN.

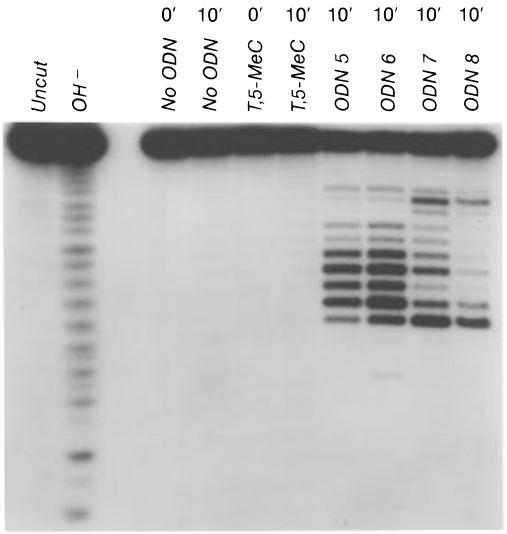


FIGURE 4: RNase H cleavage of ODN/RNA duplexes. 5′-End labeled [$^{32}$P]RNA was prebound to 10 nM ODNs **5**−**8** for 1 h in RNase H buffer. A phosphorothioate ODN containing thymidine (T) and 5-methyl-2′-deoxycytidine (5-MeC) was used as a control. HeLa cell nuclear extract was added, and the extent of RNA cleavage of the ODN/RNA duplexes was determined after 10 min by PAGE. The RNA cleavage ladder was generated with 4 mM NaOH. The "No ODN" lanes show a small amount of hydrolysis of the RNA in all samples containing the extract.

*Gene Inhibition by Preformed ODN/RNA Complexes.* A 2-fold excess of **5**−**8** was preincubated with 5′ capped (unspliced) TAg RNA (Moulds et al., 1995), and the mixture, along with CAT RNA (internal control), was microinjected into the nuclei of CV-1 cells. This "pre-form" experiment allows the assessment of antisense inhibition by an ODN that is pre-hybridized to the target RNA prior to exposure to the intracellular environment. 100% inhibition of TAg expression was observed with the propyne-substituted ODN (**5**) and butyne-substituted ODN (**6**, Table 1). No inhibition was observed with either thiazole-containing ODN (**7** and **8**). Since the thiazole-containing ODNs do facilitate cleavage of RNA by RNase H, although less efficiently than propyne-

substituted ODN (**5**), these results suggest that an additional factor may be contributing to the lower potency of ODNs **7** and **8**. Intracellular dissociation of the ODN/RNA complex is a parameter that may correlate with the decreased antisense activity of these ODNs.

## DISCUSSION

Gene inhibition by propyne-substituted ODNs likely inactivates newly transcribed RNA in the cell nucleus by means of an RNase H degradation pathway (Moulds et al., 1995; Wagner et al., 1993). The antisense gene inhibition data (Table 1) demonstrates that the propyne-substituted ODN (**5**) is the most potent ODN ($IC_{50} = 0.25$ $\mu$M) tested and that the thiazole-substituted ODNs (**7** and **8**) are relatively inactive ($IC_{50} = 5.0$ $\mu$M). The thymidine-substituted ODN showed no antisense activity in this assay (data not shown) likely due to poor binding affinity and susceptibility to intracellular dissociation (Moulds et al., 1995). The most surprising aspect of the antisense inhibition data is the low activity of the thiazole-substituted ODNs (**7** and **8**). Because of their high binding affinity (based upon Tm measurements and *in vitro* binding constants, see Table 1), one would predict potent antisense activities with these ODNs.

$$\mathrm{ODN} + \mathrm{RNA} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ODN{-}RNA} \xrightarrow[\text{RNase H}]{k_2} \mathrm{ODN} + \text{cleaved RNA} \quad (1)$$

Our data suggests that there are two important intracellular parameters that must be considered for potent antisense activity, namely, (i) RNase H cleavage of the RNA strand in the ODN/RNA duplex ($k_2$, eq 1) and (ii) intracellular dissociation of the ODN/RNA duplex ($k_{-1}$, eq 1). The *in vitro* assessment of the ability of substituted ODNs to support RNase H cleavage of complementary RNA was determined with HeLa cell nuclear extracts, and the data show that the thiazole-substituted ODNs (**7** and **8**) are 2−6-fold less active in supporting RNase H cleavage relative to the propyne-substituted ODN (**5**, Table 1). While we cannot directly measure the RNase H activity of these ODNs within CV-1 cells, we assume that it is comparable to the RNase H activity using HeLa cell nuclear extracts. Our *in vitro* results demonstrate that RNase H is very sensitive to slight modifications of the heterocycle and that increased steric bulk at this position may be detrimental to the efficient cleavage of RNA by RNase H.

The ODN-directed RNase H-mediated degradation of RNA within the cell can be envisioned as a two-step pathway with an initial equilibrium to form the ODN/RNA duplex ($K_{eq} = k_1/k_{-1}$, eq 1) followed by an irreversible degradation of the RNA ($k_2$, eq 1). For all ODNs tested (**5**−**8**) the *in vitro* equilibrium lies far to the right ($K_d = 5 \times 10^8 - 3 \times 10^9$, Table 1). When the ODN is pre-hybridized to 5′-capped, unspliced TAg RNA and microinjected into cells, the propyne- and butyne-substituted ODNs (**5** and **6**) led to complete inhibition of translation, but the thiazole-substituted ODNs (**7** and **8**) had no effect on translation of the RNA. The "pre-form" ODN/RNA duplex experiment simplifies kinetic analysis by removing the "on-rate" ($k_1$) from the equation above and allows for the relative assessment of $k_2$ and $k_{-1}$ within the cell.[2] The results of this experiment clearly show that in the case of the propyne- and butyne-substituted ODNs (**5** and **6**) $k_2$ is much faster than $k_{-1}$, while with the thiazole-substituted ODNs (**7** and **8**) $k_{-1}$ is much faster than $k_2$. On the basis of the *in vitro* data one would expect the thiazole-substituted ODN (**7**) to have similar activity in this assay as the propyne-substituted ODN (**5**) because, while ODN **7** has a slower rate of RNase H cleavage (∼2 fold) it also shows a slower rate of ODN/RNA duplex dissociation (∼2-fold). If the relative rates of RNase H cleavage in the cell are comparable to the *in vitro* rates, then the results suggest that within the cell the rate of double-helix dissociation ($k_{-1}$) is increased for ODN **7** relative to ODN **5**. Therefore, the fast intracellular dissociation of ODN **7**, relative to ODN **5**, likely accounts for the lower antisense potency ($IC_{50} = 5.0$ $\mu$M) of this ODN and supports the concept of intracellular factors that facilitate dissociation of the ODN/RNA duplex (Moulds et al., 1995).

Previous results with thymidine-containing ODNs, which are known to have much lower affinity for RNA than propyne-substituted ODNs, have suggested the presence of cellular factors that may facilitate the dissociation of an ODN/RNA double-helix within the cell (Moulds et al., 1995). Propyne-substituted ODNs appear to be resistant to intracellular dissociation by these factors (Moulds et al., 1995), but no other high-affinity ODNs were tested at that time. The results above suggest that high binding affinity, measured *in vitro*, is not necessarily a good indicator of intracellular dissociation because of the absence of cellular factors that may be involved with the intracellular dissociation of ODNs. The propyne- and butyne-substituted ODNs (**5** and **6**) were the only high-affinity ODNs tested that are resistant to intracellular dissociation (see pre-form data, Table 1) prior to RNase H cleavage of the target RNA. These results point to the unique ability of propyne substituted ODNs to facilitate cleavage of RNA, by RNase H, prior to intracellular dissociation resulting in potent antisense inhibition of gene expression.

## SUMMARY

The *in vitro* binding affinity, RNase H activity, and antisense activity of C-5-substituted dU phosphorothioate ODNs has been discussed. The propyne-substituted ODN is the most potent antisense inhibitor, and the thiazole-substituted ODNs are the least potent ODNs. This is not a result of binding affinity since the thiazole-substituted ODNs have the highest *in vitro* binding affinity, but is a result of intracellular dissociation and ability to activate RNase H cleavage of the complementary RNA. No correlation between binding affinity and ability to inhibit gene expression was observed. A butyne-substituted ODN was 4-fold less potent than the propyne-substituted ODN, and the reason for this decreased activity is unclear at this time. These results demonstrate the unique ability of propyne ODNs to selectively bind to RNA within cells and activate cleavage of RNA by RNase H, leading to potent inhibition of messenger RNA translation.

[2] In this experiment the concentration of free ODN, generated by duplex dissociation ($k_{-1}$), is very low (<0.1 $\mu$M) and $k_1$ becomes insignificant, therefore $k_2$ and $k_{-1}$ are the only significant kinetics parameters leading to antisense inhibition.

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