Carboranyl Oligonucleotides. 3. Biochemical Properties of Oligonucleotides Containing 5-(o -Carboranyl-1-yl)-2‘-deoxyuridine † , ‡

ARTICLE *in* BIOCHEMISTRY · JUNE 1996

Impact Factor: 3.02 · DOI: 10.1021/bi953078e · Source: PubMed

5 AUTHORS, INCLUDING:

Zbigniew J Lesnikowski
Polish Academy of Sciences
128 PUBLICATIONS **1,363** CITATIONS
SEE PROFILE

Raymond F Schinazi
Emory University
711 PUBLICATIONS **18,613** CITATIONS
SEE PROFILE

Carboranyl Oligonucleotides. 3. Biochemical Properties of Oligonucleotides Containing 5-(*o*-Carboranyl-1-yl)-2′-deoxyuridine[†,‡]

Zbigniew J. Lesnikowski, Géraldine Fulcrand, Robert M. Lloyd, Jr., Amy Juodawlkis, and Raymond F. Schinazi*

Veterans Affairs Medical Center and Georgia Research Center for AIDS and HIV Infections, Medical Research 151, Decatur, Georgia 30033-4004, and Laboratory of Biochemical Pharmacology, Department of Pediatrics, Emory University School of Medicine, Atlanta, Georgia 30322

Received December 29, 1995; Revised Manuscript Received March 8, 1996[⊗]

ABSTRACT: Boronated oligonucleotides are potential candidates for boron neutron capture therapy, antisense technology, and as tools in molecular biology. The biological properties of dodecathymidylic acids containing one or more 5-(*o*-carboran-1-yl)-2′-deoxyuridine residues at different locations within the oligonucleotide chain were studied. 5-(*o*-Carboran-1-yl)-2′-deoxyuridine containing oligonucleotides manifested marked increased lipophilicity and resistance to 3′- or 5′-phosphodiesterases compared to the corresponding unmodified oligomer. They were substrates for T4 polynucleotide kinase and primers for *Escherichia coli* polymerase I and human immunodeficiency virus type 1 reverse transcriptase but not for human DNA polymerase α and *β*. They also formed heteroduplexes that were substrates for *E. coli* RNase H, an essential property for antisense technology. These studies indicate that the carboranyl-containing oligonucleotides have desirable properties that need to be exploited further in the design of novel biopharmaceuticals.

The carboranyl cluster is a new modifying entity for oligonucleotides potentially useful as boron carriers for boron neutron capture therapy (BNCT),[1] as antisense agents for antisense oligonucleotide therapy (AOT) and as probes for tumor diagnosis and virology. Recently, we described the synthesis of thymidine-(3′,5′)-thymidine (*o*-carboran-1-yl)-methylphosphonate, the first oligonucleotide analogue bearing 3′,5′-*O*,*O*-[(*o*-carboran-1-yl)]methylphosphonate internucleotide linkage instead of natural 3′,5′-*O*,*O*-phosphodiester residue (Lesnikowski & Schinazi, 1993), and dodecathymidylate containing 5-(*o*-carboran-1-yl)-2′-deoxyuridine (CDU) instead of thymidine, at different locations within the oligonucleotide chain (Fulcrand-El Kattan et al., 1994). The physicochemical properties of these CDU-oligonucleotides were studied. The thermostability of duplexes formed by these new oligonucleotides with natural complementary strand $d(A)_{12}$ was affected by the location of the carboranyl nucleotide within the chain. The melting temperature (T_m) of the duplexes was highest when the CDU was located at the 5′-end than at the 3′-end or in the middle of the oligonucleotide. The T_m studies were supported by molecular modeling studies. In addition, the presence of one or more carborane clusters in these new compounds increased their lipophilicity. This desirable property and the high boron content make them useful leads for designing oligonucleotides for BNCT, AOT, and as probes for the diagnosis of malignancies, viruses, and other pathogens (Schinazi et al., 1994). Herein, we report some of the pertinent biological properties of this new class of oligonucleotide analogues (Figure 1), focusing on their stability to enzyme hydrolysis, phosphorylating potential, formation of the heteroduplex substrates for RNase H, and their use as primers by various polymerases.

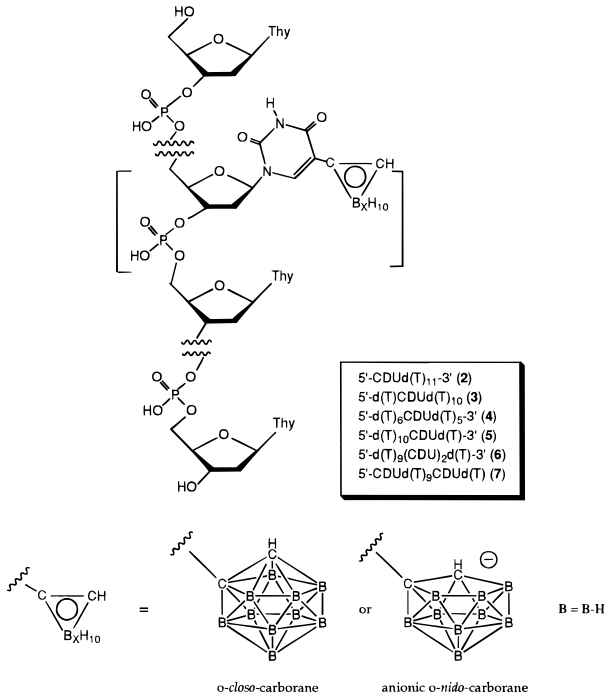

FIGURE 1: Dodeca(thymidine phosphates) containing 5-(*o*-carboranyl-1-yl)-2′-deoxyuridine.

† Supported by the Department of Energy grant DE-FG02-96ER62156, NIH Grants RO1-CA 53892 and R44-CA 65434, the Department of Veterans Affairs, and the Georgia Research Center for AIDS and HIV Infections.

‡ Part of this work was presented in outline at the XIII[th] Conference on Phosphorus Chemistry, Jerusalem, Israel, July 16−21, 1995.

* Corresponding author. Telephone: 404-728-7711. Fax: 404-728-7726. E-mail: schinazi.raymond@atlanta.med.va.gov.

⊗ Abstract published in *Advance ACS Abstracts,* April 15, 1996.

[1] Abbreviations: AOT, antisense oligonucleotide therapy; BNCT, boron neutron capture therapy; BSA, bovine serum albumin; BSPDE, bovine spleen phosphodiesterase II; CDU, 5-(*o*-carboran-1-yl)-2′-deoxyuridine; DTT, DL-dithiothreitol; HIV-1, human immunodeficiency virus type 1; PAGE, polyacrylamide gel electrophoresis; SVPDE, snake venom phosphodiesterase I; RT, reverse transcriptase; T_m, melting temperature; TCA, trichloroacetic acid; TEAA, triethylammonium acetate buffer.

S0006-2960(95)03078-9 CCC: $12.00

MATERIALS AND METHODS

Materials

A C_{18} reverse-phase column (Partisphere C_{18}, 5 μm, 4.6 $\times$ 235 mm) was obtained from Whatman Co. (Hillsboro, OR). Phosphodiesterase II (EC 3.1.16.1) from bovine spleen was purchased from Sigma Co. (St. Louis, MO). Polyacrylamide was purchased from International Biotechnologies Inc. (New Haven, CT). Bispolyacrylamide and urea were bought from Fischer Scientific (Fair Lawn, NJ). Polyadenylic acid r(A) (400−600 bases), $d(A)_{60}$, poly d(A) (ca. 300 bases) homopolymer template, and *Escherichia coli* poly r(A) polymerase were obtained from Pharmacia (Piscataway, NJ). Calf thymus DNA (activated, type XV) was from Sigma Co. (St. Louis, MO). T4 polynucleotide kinase was purchased from New England BioLabs (Beverly, MA). Human DNA polymerases α and β were purchased from Molecular Biology Resources (Milwaukee, WI); *E. coli* DNA polymerase I was obtained from Boehringer Mannheim (Indianapolis, IN), and recombinant p66/51 reverse transcriptase was generously provided by Biotechnology General (Rehovot, Israel). *E. coli* RNase H and placental ribonuclease inhibitor were purchased from Gibco BRL (Gaithersburg, MD). [α-^{32}P]ATP (10−25 Ci/mmol) was purchased from ICN (Irvine, CA). [5-*methyl*-^{3}H]TTP (60 mCi/mmol) was purchased from Moravek Biochemicals (Brea, CA). X-Omat AR film for autoradiography was obtained from Eastman Kodak (Rochester, NY). Polyacrylamide gel electrophoresis was performed using a BRL Model S2 apparatus (Gaithersburg, MD). The Quick Spin Column G-50 Sephadex used for purification of radiolabeled poly r(A) was purchased from Boehringer Mannheim (Indianapolis, IN).

Methods

Oligonucleotide Synthesis. Dodecathymidylic acid (**1**) was prepared according to standard procedure using an oligonucleotide synthesizer (Applied Biosystems USER Bulletin, 1987). Dodecathymidylates **2**−**7** containing CDU (see Figure 1) were synthesized according to the procedure developed in this laboratory (Fulcrand-El Kattan et al., 1994).

HPLC Analysis of Oligonucleotides Containing CDU and Their Enzymatic Digests. HPLC analysis was performed on Hewlett-Packard 1050 system, equipped with a Whatman Partisphere C_{18} 5 μm, 4.6 $\times$ 235 mm column maintained at room temperature. Typically, a gradient of CH_3CN from 0% to 50% or from 5% to 30% in 0.05 M triethylammonium acetate buffer (TEAA), pH 7.0, was used as eluant at a flow rate of 1.0 mL/min. The HPLC characteristics of dodecathymidylates **2**−**7** containing CDU were previously reported (Fulcrand-El Kattan et al., 1994).

Resistance to Calf Spleen Phosphodiesterase II (BSPDE). To 100 mM NaOAc buffer, pH 4.8 (180 μL), containing $ZnCl_2$ (1 mM), was added a mixture of $0.2A_{260}$ ODU of oligonucleotide **2**−**7**, and $0.2A_{260}$ ODU of 2′-deoxycytidine (dC) used as an internal standard (10 μL). The resultant mixture was divided into two fractions (95 μL each). To one fraction, 1.5×10^{-3} unit (5 μL) of calf spleen phosphodiesterase II (EC 3.1.16.1) was added. As a control, the blank reaction contained water instead of enzyme (5 μL). A control reaction with unmodified $d(T)_{12}$ (**1**) was also assayed simultaneously. Reactions were maintained at 37 °C for 10 min and then kept at 80 °C for 3 min and immediately frozen at −70 °C prior to analysis by HPLC. The degree of degradation of the modified oligonucleotides **1**−**7** was calculated according to the following formula: *D* (degree of degradation after 10 min) = (area of T_P signal/area of dC internal standard)$_{assay}$/(area of oligonucleotide signal/area of dC internal standard)$_{blank}$.

Resistance to Snake Venom Phosphodiesterase I (SVPDE). The assay was described previously (Fulcrand-El Kattan et al., 1994). The fraction of undigested oligonucleotide was calculated using formula: *R* (area of oligonucleotide remaining intact after 10 min) = (area of intact oligonucleotide/area of dC internal standard)$_{assay}$/(area of intact oligonucleotide/area of dC internal standard)$_{blank}$.

*Phosphorylation of CDU-Containing Oligonucleotides **1**−**7** by T4 Polynucleotide Kinase.* Modified oligonucleotides **2**−**7** and unmodified $d(T)_{12}$ (**1**) (20 pmol) were incubated at 37 °C in the presence T4 polynucleotide kinase (0.5 μL, 5 unit) and 10 μCi of [γ-^{32}P]ATP (5000 Ci/mmol) in a Tris-HCl (70 mM) buffer (pH 7.6) containing $MgCl_2$ (10 mM) and dithiothreitol (5 mM). The final volume of the reaction mixture was 10 μL. After 30 min, the reaction mixtures containing the enzyme were heat inactivated for 2 min at 92 °C. A 10× tracking dye (0.5% bromophenol blue, 0.5% xylene cyanol FF, 30% glycerol in water) (5 μL) was added to the reaction mixture, and 5 μL aliquots were analyzed by polyacrylamide gel electrophoresis (PAGE). RT-MT6 (18-mer) was used as the unmodified control. Additional controls with enzyme, buffer, and [γ-^{32}P]ATP only (with no oligonucleotide added) were also included in the gel.

PAGE Analysis. ^{32}P-Labeled or unlabeled samples of modified oligonucleotides **2**−**7**, and dodeca(thymidine phosphate) (**1**), prepared as described above, were separated by electrophoresis using a 20% polyacrylamide denaturing gel containing urea (7 M) for 45 min at 50 mA. The samples were visualized using standard autoradiography on X-Omat AR film or, in the case of unlabeled oligonucleotides, by means of UV shadowing.

Primers for Polymerases. The enzymes tested were human DNA polymerases α and β, *E. coli* DNA polymerase I, and recombinant p66/51 HIV-1 reverse transcriptase. Extension assays were performed using a $d(A)_{60}$ (for polymerase β), poly d(A) (ca. 300 bases, for polymerase α and polymerase I), poly r(A) (ca. 450 bases, for HIV-1 RT) as homopolymer template, CDU modified oligonucleotides **2**−**7**, and unmodified $d(T)_{12}$ oligonucleotide (**1**). Briefly, oligonucleotides were annealed to the DNA template using a Perkin-Elmer 480 thermocycler with the following cycle parameters: 30 min at 95 °C, 10 min at 80 °C, 10 min at 70 °C, 5 min at 50 °C, 5 min at 30 °C, 10 min at 15 °C, 5 min at 12 °C, 10 min at 14 °C, 30 min at room temperature, and then stored at 4 °C before use. Extension assays were performed in 50 or 100 μL reaction mixtures in a 96-well plate containing appropriate buffer, respective oligonucleotide, template, [^{3}H]dTTP (1 μM, 60 Ci/mmol), and respective enzyme. Other assay conditions for each enzyme used were as follows: for human DNA polymerase α, Tris-HCl (60 mM) pH 8.0, $Mg(OAc)_2$ (5 mM), bovine serum albumin (BSA, 0.3 mg/mL), DTT (1 mM), spermine (0.1 mM), respective oligonucleotide (10 pmol), poly d(A) (0.25 pmol), and 1 unit of enzyme; for human DNA polymerase β, Tris-HCl (50 mM) pH 8.7, $MgCl_2$ (10 mM), BSA (0.4 mg/mL), DTT (1 mM), KCl (100 mM), glycerol (15% v/v), the

Table 1: CDU-Modified Oligonucleotides **2**−**7** and Unmodified Dodecathymidylic Acid **1** Resistance against Nucleases, Substrate Activity for T4 Kinase, Induction of RNase H Activity, and as Primers for Polymerases

		resistance against exonucleases[b]				polymerase[f]			
oligomer[a]	base composition	BSPDE[c]	SVPDE[d]	T4 kinase phosphorylation	induction of RNase H activity[e]	α DNA (human)	β DNA (human)	I DNA (*E. coli*)	HIV-1 RT (recombinant)
1	$d(T)_{12}$	−	−	+	+	−	−	+	+
2	5′-CDUd$(T)_{11}$-3′	++[g]	nd	+	+	−	−	+	++
3	5′-d(T)CDUd$(T)_{10}$-3′	++[g]	nd	+	+	−	−	++	++
4	5′-d$(T)_6$CDUd$(T)_5$-3′	+	nd	+	++	−	−	++	+
5	5′-d$(T)_{10}$CDUd(T)-3′	−	+	+	+	−	−	+	+
6	5′-d$(T)_9(CDU)_2$d(T)-3′	−	++	+	+	−	−	+	−
7	5′CDUd$(T)_9$CDUd(T)-3′	++[g]	+	+	+	−	−	+	+

[a] A mixture of *closo/nido*-oligomers was used. [b] Resistance to nucleolytic digestion was calculated as described in Materials and Methods. (−) Lack of resistance against enzymatic digestion ($D > 0.9$), (+) increased resistance ($0.3 < D < 0.9$), (++) good resistance ($D < 0.3$). [c] Calf spleen phosphodiesterase (SPDE), phosphodiesterase II (EC 3.1.16.1). [d] Snake venom phosphodiesterase (SVPDE) from *Crotalus durissus terrificus*, phosphodiesterase I (EC 3.1.4.1), from Fulcrand-El Kattan et al. (1994). [e] Individual *nido*-1, *nido*-2, and *closo*-forms of the oligonucleotide were used. No effect on the carboranyl group form was detected except for the 4 *nido*-1 (++). Descriptors *nido*-1 and *nido*-2 correspond to diastereoisomeric oligonucleotides containing carboranyl cage in *nido*-form, and characterized by lower and higher retention time, respectively, as established by HPLC (Fulcrand-El Kattan et al., 1994). [f] (+) or (++) describes oligonucleotide efficiency as a primer for the specified polymerases; (−) indicates that the oligonucleotide was not a primer for the polymerase under conditions described in Materials and Methods. [g] No T_P detected by HPLC.

respective oligonucleotide (20 pmol), $d(A)_{60}$ (0.5 pmol), and 2 units of enzyme; for *E. coli* polymerase I, Tris-HCl (100 mM) pH 8.0, $MgCl_2$ (6 mM), DTT (5 mM), respective oligonucleotide (20 pmol), poly d(A) (0.5 pmol), and 0.1 unit of enzyme; and for HIV-1 reverse transcriptase, Tris-HCl (100 mM) pH 8.0, $MgCl_2$ (2 mM), KCl (50 mM), the respective oligonucleotide (20 pmol), poly r(A) (0.5 pmol), and 1 unit of enzyme. The reactions were incubated for 1 h at 37 °C and then precipitated with TCA (5%) for 1 h at 4 °C. Finally, reactions were transferred to filter paper using a Skatron micro-cell harvester (Skatron, Lier, Norway) or Packard manual harvester, and radioactive substrate incorporations or extensions were determined with a Beckman LS 3801 scintillation counter (Beckman Instruments, Fullerton, CA) or Packard 9600 direct Beta counter.

Induction of RNase H Activity. Poly r(A) template (400−600 bases, 270 pmol) was incubated for 30 min at 37 °C with poly r(A)polymerase (40 units/mL) and [α-^{32}P]ATP (40 μCi) in buffer containing $MgCl_2$ (10 mM), $MnCl_2$ (2.5 mM), Tris-HCl (50 mM), pH 7.9, NaCl (250 mM), and BSA (0.5 mg/mL). The reaction mixture (50 μL) was then maintained for 10 min at 65 °C in order to inactivate the enzyme. The radiolabeled poly r(A) template was purified by Quick Spin Column G-50 Sephadex and the eluate lyophilized. CDU modified oligonucleotides **2**−**7**, unmodified $d(T)_{12}$ oligonucleotide (**1**) (200 pmol of each), and poly r(A) template (5 pmol) were added to the annealing mixtures (44 μL) containing Tris-HCl (40 mM), pH 7.5, $MgCl_2$ (4 mM), DTT (1mM), BSA (30 μg/mL), and glycerol (4% v/v). The samples were heated to 85 °C, cooled slowly to room temperature, and then maintained for 30 min at 4 °C. To these solutions, 20 units/mL of placental ribonuclease inhibitor (1 μL) and 10 units/mL RNase H (5 μL) were added, and then the reactions were maintained at 4 °C overnight. Blank reactions without RNase H and controls without deoxyoligonucleotide were included. After 20 h, aliquots (10 μL) of each reaction were removed and mixed with loading solution (6 μL) containing glycerin, bromophenol blue (BPB), and xylene cyanole FF (XCFF). The samples were then loaded on a 0.4 mm 8% denaturing polyacrylamide gel containing 7 M urea, and electrophoresis was performed at 1550 V (75 W, 50 mA) for 90 min. The gel was dried and autoradiographed on Kodak X-Omat film.

RESULTS

Resistance to Bovine Spleen Phosphodiesterase II (BSPDE). A pronounced effect of CDU modification on the oligonucleotide stability in the presence of phosphodiesterase II, 3′-exonuclease from calf spleen (BSPDE) was observed (Table 1). No thymidine 3′-monophosphate release was detected by HPLC after 10 min treatment of the oligonucleotides **2** or **7** with the enzyme. Under the same conditions, the unmodified $d(T)_{12}$ was almost all digested (only 2.5% remained intact). In general, the resistance of the CDU-oligonucleotide toward BSPDE, as measured by the amount of thymidylate released, depended on the CDU location within the oligonucleotide chain. Resistance to the enzyme increased in the order **1** < **5** ∼ **6** < **2** ∼ **3** ∼ **7**.

Phosphorylation by T4 Polynucleotide Kinase. The phosphorylation experiments with T4 polynucleotide kinase showed that CDU-oligonucleotides **1**−**7** were efficiently phosphorylated at their 5′-ends by the enzyme (Table 1). The efficacy of the 5′-end phosphorylation was comparable to the phosphorylation of unmodified, standard oligonucleotide $d(T)_{12}$ (**1**).

Priming the DNA Polymerization. CDU-modified oligonucleotides **2**−**7** and unmodified dodecathymidylic acid **1** were tested for their ability to serve as primers for DNA polymerases. Four different enzymes were studied, namely, human polymerase α and β, *E. coli* bacterial polymerase I, and human immunodeficiency virus type 1 (HIV-1) viral reverse transcriptase (RT). All oligonucleotides **1**−**7** functioned as primers for *E. coli* bacterial polymerase I and HIV-1 RT but not for human polymerase α and β (Table 1). The DNA-polymerizing activity of *E. coli* polymerase I and HIV-1 RT was affected to some extent by the location of the CDU modification within the primer oligonucleotide chain. In general, oligonucleotides **2** and **3** modified at the 5′-end were elongated more efficiently, as measured by [5-^{3}H]thymidine incorporation, than oligonucleotides **4**−**7** modified in the middle or at the 3′-end. For oligonucleotides **2** and **3**, the efficacy of the elongation was 5−10 times and about 2 times lower than elongation of commercial standard calf thymus activated DNA using *E. coli* polymerase I or poly $r(A)_n \cdot d(T)_{12-18}$ using HIV-1 RT, respectively.

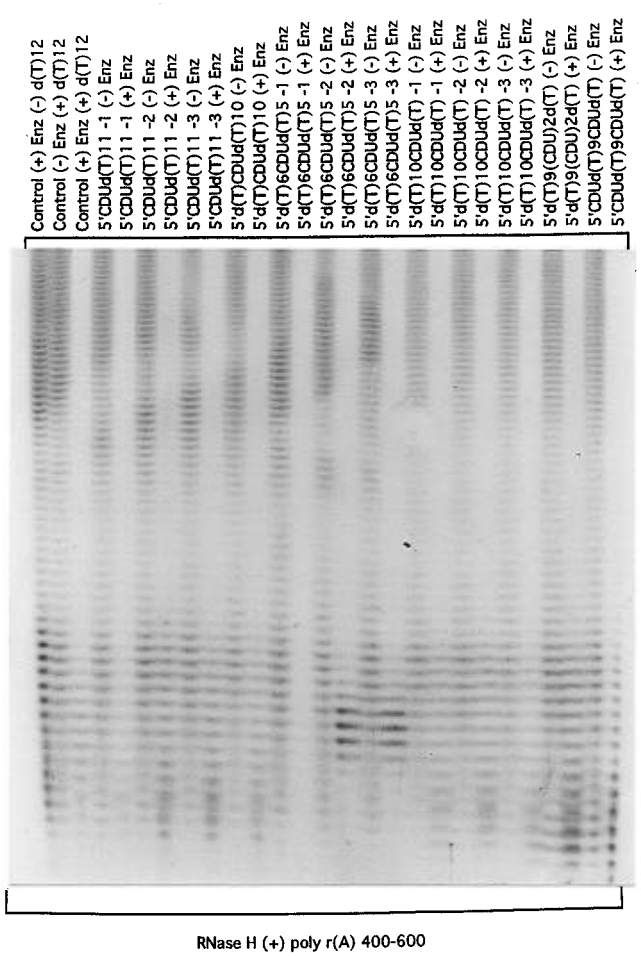

FIGURE 2: Effect of RNase H on CDU-containing oligonucleotides **2**−**7** heteroduplexes; PAGE analyses. CDU modified oligonucleotides, unmodified $d(T)_{12}$ oligonucleotide (200 pmol of each), and [^{32}P] radiolabeled poly (A) template (400−600 bases, 5 pmol) were annealed. To these, 20 units/mL placental ribonuclease inhibitor and 10 units/mL RNase H were added, and then the reactions were maintained at 4 °C overnight. After 20 h, aliquots of each reaction were loaded on a 0.4 mm 8% denaturing polyacrylamide gel containing 7 M urea, and electrophoresis was performed at 1550 V (75 W, 50 mA) for 90 min. The gel was dried and autoradiographed on Kodak X-Omat film.

Induction of RNase H Activity. All CDU-modified oligonucleotides **2**−**7** formed RNA-DNA complexes which were substrates for *E. coli* RNase H with a poly r(A) template of 400−600 bases in length (Table 1). These heteroduplexes were digested by RNase H in a fashion comparable to the digestion of the unmodified duplex formed by dodecathymidylic acid **1** (Figure 2). The relationship between the *closo*- and *nido*-form of CDU carboranyl residue (Fulcrand-El Kattan et al., 1994) and susceptibility of their heteroduplex toward enzymatic digestion by RNase H were also studied. No clear effect of CDU location on efficacy of digestion was detected except that the heteroduplex formed by the dodecamer containing CDU residue at position 6 of the 12-mer (in the middle of oligonucleotide chain, **4**) and the carboranyl group in *nido*-1 form was digested more efficiently (Figure 2).

DISCUSSION

Resistance to Nucleases. The enzyme SVPDE successively hydrolyzes 5′-mononucleotides from deoxyribooligonucleotides with free 3′-OH groups, and digestion of the DNA proceeds in the 3′→5′ direction (Laskowski et al., 1971). In contrast, BSPDE requires free 5′-OH termini, and digestion proceeds in the opposite 5′→3′ direction (Bernardi & Bernardi, 1971). Both SVPDE (Nossal & Singer, 1968; Brody, 1986) and BSPDE (Thomas & Oliviera, 1978) are nonprocessive enzymes.

The nucleolytic activity of both enzymes toward oligonucleotides is substantially decreased by nucleic base modification for single-stranded as well as double-stranded substrates (Stezowski et al., 1987; Mao et al., 1993). The location of the modified base (3′- or 5′-terminal versus 3′- or 5′-penultimate) within the oligonucleotide chain affects the rate of nucleolytic hydrolysis (Biltonen & Lumry, 1965; Stezowski et al., 1987; Bernardi & Bernardi, 1971). Both enzymes are affected by modification and the stereochemistry of the internucleotide linkage (Uhlmann & Peyman, 1990; Frey, 1989). It is noteworthy that BSPDE activity is affected not only by chemical oligonucleotide modifications but also by intercalating agents forming complexes with double-stranded substrates (Lesnikowski, 1988).

A substantially pronounced effect on the oligonucleotide **2**−**7** stability was observed for the 3′-exonuclease from calf spleen (BSPDE), which truncates the oligonucleotides from the 5′-end. The presence of one CDU residue at the 5′-end protected the oligonucleotide against digestion. No thymidine 3′-monophosphate release was detected by HPLC after treatment of the oligonucleotides **2** or **7** with the enzyme. It is likely that the nucleolytic hydrolysis of the oligionucleotides **3**−**6** proceeded fast until the first CDU residue or nucleoside directly preceding the CDU was reached (Biltonen & Lumry, 1965; Stezowski et al., 1987). Inhibition of the enzymatic activity is probably due to a diminished ability of the enzyme to bind to the modified portion of the oligonucleotide and/or to inhibit hydrolysis at the binding site. These results are consistent with our previous findings which indicated notable effect of CDU modification on oligonucleotide resistance against digestion by SVPDE (Fulcrand-El Kattan et al., 1994).

The presence of two CDU residues at the 3′-end of the oligonucleotide substantially increased the oligonucleotide stability toward 5′-exonucleolytic activity of SVPDE (Table 1). After 10 min, unmodified oligonucleotide **1** was ≥ 97.5% digested while oligonucleotides containing one or two CDU residues at the 3′-end (position 2, or 2 and 3, oligonucleotides **5** and **6**) were 60% and 20% digested, respectively. This substantially higher stability of **6** compared to **5** may be due to the slow hydrolysis of two internucleotide linkages beyond the modified base 5′-CDU_P-CDU-3′ and 5′-T_PCDU-3′ or one very slow cleavage of the phosphodiester linkage beyond modified dimer 5′-T_PCDU_P-CDU-3′ or trimer 5′-$T_PT_PCDU_P$CDU-3′. The second possibility is supported by the finding that action of the BSPDE on oligonucleotides containing thymidine dimers produced by UV irradiation generates trinucleotides 5′-$_PN_PT_P$T-3′ which are resistant to further enzymatic digestion (Setlow et al., 1964). It should be noted that, due to the synthetic procedure used for the modified oligonucleotide, the CDU residue was located at the penultimate position of the oligomer, and thymidine was the 3′-terminal nucleoside. Presumably 3′-thymidylate was removed in the first stage of enzymatic digestion, and then the resultant undecamer terminating with CDU was slowly digested by the enzyme. This reasoning was supported by additional enzyme stability

studies of oligonucleotide with two adjacent CDU residues at the 3′-end which demonstrated notably higher resistance than the oligonucleotide with one modification, suggesting further disturbance of oligonucleotide-enzyme interaction.

Phosphorylation of CDU-Containing Oligonucleotides by T4 Polynucleotide Kinase. A variety of modified nucleic acids can be phosphorylated in the polynucleotide kinase reaction provided they have a nucleotide bearing a free 5′-hydroxyl group with a phosphoryl group at the 3′-position (Richardson, 1981; Sande van de & Bilsker, 1973).

As anticipated, phosphorylation of CDU-containing oligonucleotides with T4 polynucleotide kinase was shown for all oligonucleotides **2**−**7**. The efficacy of phosphorylation was comparable to unmodified oligonucleotide **1**, even for the oligonucleotide **2** bearing CDU modification at the 5′-end. The data demonstrate that CDU-oligonucleotides can be labeled at the 5′-end, which is of practical importance.

Priming the DNA Polymerization. CDU-modified oligonucleotides **2**−**7** and unmodified dodecathymidylic acid **1** were tested for their priming activity in the DNA polymerization process catalyzed by four different DNA polymerases: human polymerases α and *β*, *E. coli* polymerase I, and HIV-1 RT. The polymerases studied varied in polypeptide size and differ in term of template requirement (DNA, or RNA for RT), processivity, and the optimum condition for the polymerization process (Burgers, 1989; Mizrachi et al., 1989). RNA priming is the general mechanism for starting DNA chains *in vivo* (Loeb et al., 1986), but in *vitro* DNA primers are accepted as well (Gross & Krauss, 1984). It was found that all oligonucleotides **1**−**7** were primers for the *E. coli* polymerase I and HIV-RT but not for human polymerase α and *β*, indicating the greater discriminating activity of these important human enzymes.

The efficacy of the primer elongation was affected by the location of the CDU-modification within the primer chain. Oligonucleotides modified at the 5′-end (**2** and **3**) were elongated more efficiently than oligonucleotides **4**−**7** modified in the middle or at the 3′-end of the oligomer. This finding is in agreement with the 3′→5′ direction of primer elongation, the polymerase requirement for proper base-pairing of the primer 3′-end to the template, and the recently described unfavorable effect of CDU-modification on base pairing and homo- and heteroduplex stability, when located at the oligonucleotide 3′-end (Fulcrand-El Kattan et al., 1994). The observed lower efficacy of extension of the primer containing CDU-modification at 3′-end catalyzed by *E. coli* polymerase I and HIV-RT is of interest in comparison with an effect of N^2-propanyl-2′-deoxyguanosine modification on human polymerase α activity. It was found that the presence of N^2-propanyl-2′-deoxyguanosine in the primer region at the 4 position or closer, relative to the 3-primer terminus, prevents primer elongation (Weiss & Fisher, 1992). A similar effect was described in extension assays in the presence of *E. coli* polymerase I, for N^2-(p-*N*-butylphenyl)-2′-deoxyguanosine located at the 3′-end of the primer (Misra et al., 1992).

DNA-dependent DNA polymerase activity of HIV-1 RT was also examined using poly d(A) template and oligonucleotide **1**−**7** primers. In contrast to RNA-dependent DNA polymerase activity (RT activity), no elongation of **2**−**7** was detected. However, **1** was effectively elongated with polymerase T7 used as a control, under the same conditions (data not shown).

Polymerase α is a major enzyme responsible for replicative DNA synthesis in higher eucaryotes, whereas polymerase *β* is believed to play role in repair of DNA (Siedlecki et al., 1980). Elongation of CDU-modified oligonucleotide **2**−**7** primers as well as unmodified $d(T)_{12}$ (**1**), using poly d(A) template, was not observed with either human polymerase α or *β*.

A catalytic hallmark of the polymerases α is their preferential interaction with single-stranded DNA. Unfortunately, the disparity of results obtained with enzymes from different cell types does not allow a simple unifying concept as to its involvement in different types of template-primer DNA. Nevertheless, the finding that oligonucleotide **1**−**7** failed to elongate, in the presence of poly d(A) template under the condition used, suggests that the resultant poly d(A)·oligonucleotide **1**−**7** complex is not an optimal substrate for the enzyme. This is supported by the observation that commercial activated calf thymus DNA used as a control was elongated efficiently under the same conditions.

For the human polymerase *β*, the observed phenomenon can be tentatively explained by the enzyme's absolute requirement for the phosphate presence at the template 5′-end in the short gap (less than six nucleotides) filling process (Murakami et al., 1985; Abbots et al., 1988). None of the oligonucleotides **1**−**7** was phosphorylated at the 5′-end, and all were used in 4-fold excess (per base) relative to the template, which favors short gap formation.

The observed selectivity of human DNA polymerases α and *β* compared to *E. coli* bacterial polymerase I and HIV-1 RT toward the CDU-modified oligonucleotide primers may have practical implications, for example, by limiting potential nonspecific activities *in vivo* (Kregenow et al., 1995).

Induction of RNase H Activity. RNase H recognizes RNA−DNA hybrids as a substrate and cleaves only the RNA strand in an endonucleolytic manner. The substrate requirements for RNase H are not clearly recognized. Most of the information regarding the enzymatic character of the enzyme comes from studies on rather simple, homopolymeric substrates. At least four base pair heteroduplex stretches are necessary for recognition by the enzyme as a substrate. RNase H displays low sequence specificity, and it is presumed that it recognizes some general structural features of the heteroduplex, such as sugar−phosphate backbone (Hogreffe et al., 1990). Of note is that heteroduplexes formed by modified oligonucleotides containing an uncharged internucleotide linkage such as methylphosphonate, phosphotriester, and dephosphono linkage (Uhlmann & Peyman, 1990) or containing modified sugar such as 2′-*O*-methylribose (Inoue et al., 1987) or α-anomeric nucleoside (Bloch et al., 1988) are not substrates for RNase H. Therefore, it may be of interest that all CDU-modified oligonucleotides **2**−**7** RNA−DNA heteroduplexes with a 400−600 bases poly rA template were found to be substrate for RNase H. This observation is in agreement with the present knowledge on RNase H substrate specificity since CDU-oligonucleotides, although containing a modified base, retain an unchanged, charged backbone. Additionally, the efficacy of poly rA template digestion seems independent from T_m of the duplex formed with CDU-oligonucleotide despite the finding that the T_m of duplexes with CDU-oligonucleotides varied from 15 to 29 °C (Fulcrand-El Kattan et al., 1994).

The data presented in this paper represent the initial studies and controls for more in depth work on an approach that might have clinical relevance. Based on the favorable physicochemical and biological properties of certain oligomers described here, the design of carboranyl oligonucleotides bearing one or more carboranyl moieties that could be targeted against overexpressed genes in cancer and in virally infected cells is being investigated.

REFERENCES

Abbots, J., SenGupta, D. N., Zmudka, B., Widen, S. G., Notario, V., & Wilson, S. H. (1988) *Biochemistry 27*, 901−909.

Applied Biosystems USER Bulletin No. 43 (1987) Applied Biosystems, Foster City, CA.

Bernardi, A., & Bernardi, G. (1971) in *The Enzymes* (Boyer P. D., Ed.) Vol. IV, 3rd ed., pp 329−336, Academic Press, New York.

Biltonen, R. L. & Lumry, R. (1965) *J. Am. Chem. Soc*. *87*, 4209−4210.

Bloch, E., Lavignon, J. R., Bertrand, J. R., Pognan, F., Morvan, F., Malvy, C., Rayner, B., Imbach, J.-L., & Paoletti, C. (1988) *Gene 72*, 349−360.

Brody, R. S., Doherty, K. G., & Zimmerman, P. D. (1986) *J. Biol. Chem*. *261*, 7136−7143.

Burgers, P. M. J. (1989) *Prog. Nucleic Acid Res. Mol. Biol*. *37*, 235−280.

Frey, P. (1989) *Adv. Enzymol. Relat. Areas Mol. Biol*. *62*, 119−201.

Fulcrand-El Kattan, G., Lesnikowski, Z. J., Yao, S., Tanious, F., Wilson, W. D. & Schinazi, R. F. (1994) *J. Am. Chem. Soc*. *116*, 7494−7501.

Gross, F. & Krauss, G. (1984) *Eur. J. Biochem*. *141*, 109−114.

Hogreffe, H. H., Hogreffe, R. I., Walder, R. Y. & Walder, J. A. (1990) *J. Biol. Chem*. *265*, 5561−5566.

Hostomsky, Z., Hostomska, Z. & Matthews, D. A. (1993) in *Nucleases* (Linn, S. M., Lloyd, R. S., & Roberts, R. J., Eds.) 2nd ed., pp 341−376, Cold Spring Harbor Laboratory Press, New York.

Inoue, H., Hayase, Y., Iwai, S., & Ohtsuka, E. (1987) *FEBS Lett*. *215*, 327−330.

Kregenow, D., Ratajczak, M. Z., & Gewirtz, A. M. (1995) in *Delivery Strategies for Antisense Oligonucleotide Therapeutics* (Akhtar, S., Ed.) pp 1−15, CRC Press, Boca Raton, FL.

Laskowski, M., Sr. (1971) in *The Enzymes* (Boyer P. D., Ed.) Vol. IV, 3rd ed., pp 313−328, Academic Press, New York.

Lesnikowski, Z. J. (1988) *Biochem. Biophys. Res. Commun. 152*, 477−483.

Lesnikowski, Z. J., & Schinazi, R. F. (1993) *J. Org. Chem. 58*, 6531−6534.

Lesnikowski, Z. J., & Schinazi, R. F. (1995) *Polish J. Chem. 69*, 827−840.

Mao, B., Li, B., Amin, S., Cosman, M., & Geacintov, N. E. (1993) *Biochemistry 32*, 11785−11793.

Miller, P. S., Cushman, C. D., & Levis, J. T. (1991) in *Oligonucleotides and Analogues: A Practical Approach* (Eckstein, F., Ed.) pp 137−154, IRL Press, New York.

Misra, H. K., Khan, N. N., Agrawal, S., & Wright, G. E. (1992) *Nucleic Acids Res. 20*, 4547−4551.

Mizrachi, V., Lazarus, G. M., Miles, L. M., Meyers, C. A., & Debouck, C. (1989) *Arch. Biochem. Biophys. 273*, 347−358.

Murakami, Y., Yasuda, H., Miyazawa, H., Hanaoka, F., & Yamada, M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1761−1765.

Nossal, N. G., & Singer, M. F. (1968) *J. Biol. Chem. 243*, 913−922.

Richardson, C. C. (1981) in *The Enzymes* (Boyer, P. D., Ed.) Vol. XIV, 3rd ed., pp 299−314, Academic Press, New York.

Sande van de, J. H., & Bilsker M. (1973) *Biochemistry 12*, 5056−5062.

Schinazi, R. F., Lesnikowski, Z. J., Fulcrand-El Kattan, G., & Wilson, W. D., (1994) *American Chemical Society Symposium Series*, Vol. 580, Carbohydrate Modifications in Antisense Research (Sanghvi, Y. S., & Cook, P. D., Eds.) Chapter 11, pp 169−182, American Chemical Society, Washington, D.C.

Setlow, R. B., Carrier, W. L., & Bollum, F. J. (1964) *Biochim. Biophys. Acta 91*, 446−461.

Siedlecki, J. A., Szysko, J., Pietrzykowska, I., & Zmudzka, B. (1980) *Nucleic Acids Res. 8*, 361−375.

Stezowski, J. J., Joos-Guba, G., Schonwalder, K.-H., Straub, A., & Glusker, J. P. (1987) *J. Biomol. Struct. Dyn. 5*, 615−637.

Thomas, K. R., & Oliviera, B. M. (1978) *J. Biol. Chem. 253*, 424−429.

Uhlmann, E., & Peyman, A. (1990) *A. Chem. Rev. 90*, 543−584.

Weiss, S. J., & Fisher, P. A. (1992) *J. Biol. Chem. 267*, 18520−18526.

BI953078E