Pergamon

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry 11 (2003) 2503–2509

BIOORGANIC & MEDICINAL CHEMISTRY

Specific Alkylation of Human Telomere Repeats by Hairpin Pyrrole-Imidazole Polyamide

Ryoko Takahashi, Toshikazu Bando and Hiroshi Sugiyama*

Division of Biofunctional Molecules, Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, 2-3-10 Surugadai, Kanda, Chiyoda, Tokyo 101-0062, Japan

Received 20 January 2003; revised 14 March 2003; accepted 18 March 2003

Abstract—A novel hairpin polyamide-cyclopropapyrroloindole (CPI) conjugate PyImImIm-γ-PyPyPyLDu86 (conjugate **11**), which targets human telomere repeats $d(TTAGGG)_n/d(CCCTAA)_n$, was synthesized. High resolution denaturing polyacrylamide gel electrophoresis using 44 bp DNA fragments and HPLC product analysis of a synthetic nonanucleotide demonstrated that conjugate **11** alkylates the target adenine in the telomere repeats, 5′-CCCTAA-3′. Examination of the antitumor activity of conjugate **11** using a panel of 39 cancer cell lines demonstrated that the average concentration of conjugate **11** required for 50% growth inhibition was 5.75 μM, which is superior to pepleomycin and bleomycin and comparable to cisplatin.

Introduction

The most telomeric ends of chromosomes, which consist of tandem repeats of double-stranded base pair sequence, d(TTAGGG)/d(CCCTAA), for many kilobases,[1] are essential for chromosomal stability. As normal somatic cells divide, telomeres shorten due to the inability of DNA polymerase to replicate the ends of linear molecules. The ribonucleoprotein, telomerase, provides the necessary enzymatic activity to restore and maintain telomere length.[2] Telomerase activity has been found in almost all human tumors, but not in adjacent normal cells.[3] The correlation between telomerase activity and human tumors has led to the hypothesis that tumor growth requires reactivation of telomerase and that telomerase inhibitors represent a class of chemotherapeutic agents.[4] Therefore, relatively extended telomere repeats, d(TTAGGG)/d(CCCTAA), of cancer cells could be a good target for a new class of antitumor agent.

Minor groove-binding *N*-methyl pyrrole (Py) and *N*-methyl imidazole (Im) polyamide uniquely recognize each of the four Watson–Crick base pairs.[5] Antiparallel pairing of imidazole opposite pyrrole (Im/Py) recognizes a G–C base pair, whereas a Py/Py pair recognizes A–T or T–A base pairs.[6]

Recently, we have demonstrated that hybrid molecules derived from duocarmycin A and Py–Im hairpin polyamides alkylate a predetermined seven-base pair sequence using a 450 bp DNA fragment.[7] In addition, we have previously shown that Py–Im hairpin polyamide (cyclopropapyrroloindole) CPI conjugates containing a vinyl linker efficiently alkylate a five-base pair sequence, 5′-AGTCA-3′, in double-stranded DNA.[8] Using the ring-pairing rule of Py–Im polyamides, we designed and synthesized a conjugate PyImImIm-γ-PyPyPyLDu86 (conjugate **11**), which targets adenine of 5′-CCCTAA-3′ in the double-stranded human telomere repeats and extends sequence recognition to a six-base pair sequence (Fig. 1). In this report, the synthesis, DNA alkylation and antitumor activity of conjugate **11** are described.

Results and Discussion

Synthesis of conjugate 11

The synthesis of conjugate **11** is shown in Scheme 1. The reduction of compound **1** by Pd/C and H_2, followed by coupling with compound **2** using pentafluorophenyl diphenylphosphinate (FDPP) produced compound **3**.

*Corresponding author. Tel.: +81-3-5280-8032; fax: +81-3-5280-8127; e-mail: sugiyama.chem@tmd.ac.jp

0968-0896/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0968-0896(03)00176-7

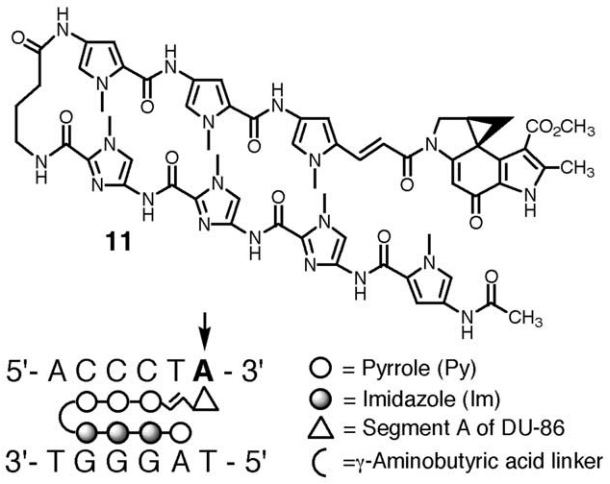
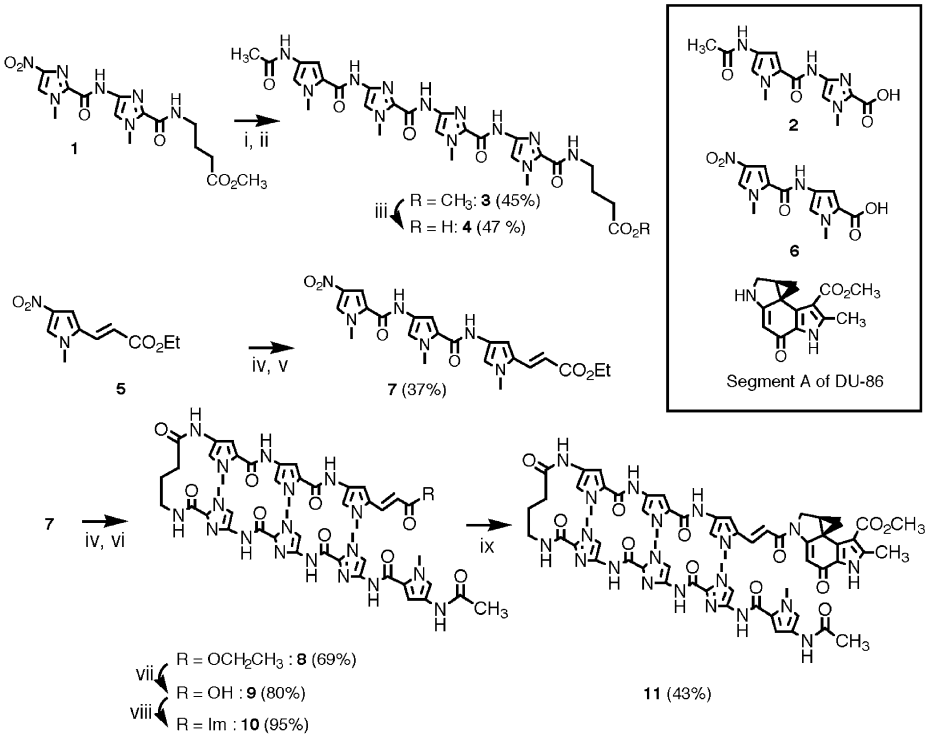


Figure 1. Chemical structure of conjugate **11** and a schematic representation of the recognition of the d(TAGGGT)/d(ACCCTA) sequence by conjugate **11**. Arrow indicates the site of alkylation.

Compound **7** was prepared by the reduction of compound **5**[8] with Pd/C and $NaBH_4$, followed by coupling with compound **6** using FDPP. The reduction of **5** with Pd/C and H_2 leads to an undesired reduction of vinyl linker. Analogous reduction of **7**, followed by coupling with compound **4** using FDPP, produced **8**. After subsequent deprotection using DBU, the carboxylic acid **9** was activated with CDI to give **10**. The alkylating moiety, segment A of DU-86 (CPI), was prepared using a previously reported procedure.[9] Finally, the synthesis of conjugate **11** was accomplished by coupling **10** and segment A of DU-86 by sodium hydride in DMF, obtaining a moderate yield. The structure of conjugate **11** was fully characterized by proton nuclear magnetic resonance (^{1}H NMR) spectroscopy and electrospray ionization time-of-flight mass spectrometry (ESI-TOF).

Alkylation of the 44-bp DNA fragment

Sequence-selective alkylation by conjugate **11** was investigated using an automated DNA sequencer, as previously described.[10] After annealing with the complementary strand, alkylation of 5′-Texas Red-labeled-44 bp DNA fragments, which contained four repeats of d(TTAGGG), was carried out at 37 °C for 24 h, and quenched by addition of calf thymus DNA. The sample was heated at 94 °C under neutral conditions for 20 min. The sites of alkylation were visualized by thermal cleavage of the DNA strand at the alkylated sites. Under these heating conditions, all purine N3 alkylated sites in the DNA produced cleavage bands almost quantitatively on the gel. As shown in Figure 2, alkylation of DNA by conjugate **11** mainly occurred at four sites of sequence 5′-CCCTAA-3′ in double-stranded DNA. Single stranded 5′-Texas Red-labeled-44 bp DNA fragment was not alkylated at all by conjugate **11** indicating that conjugate **11** would be good sequence-specific alkylating agent for the double-stranded telomere repeats. The relatively moderate efficiency of alkylation compared with the analogous hairpin polyamide with a vinyl linker might partly be explained by the poor recognition ability of consecutive guanines by polyamide.[10] Although there are several agents that specifically bind to quadruplex telomere repeats, to the best of our knowledge, the hairpin polyamide–CPI conjugate **11** is the first example of a sequence-specific alkylating agent for human telomere repeats in double-stranded DNA.



Scheme 1. Reagents and conditions: (i) Pd/C, H_2, MeOH–AcOEt; (ii) **2**, FDPP, *i*Pr_2NEt, DMF; (iii) NaOH, H_2O; (iv) Pd/C, $NaBH_4$, MeOH–AcOEt; (v) **6**, FDPP, *i*Pr_2NEt, DMF; (vi) **4**, FDPP, *i*Pr_2 NET, DMF; (vii) DBU, H_2O; (viii) 1,1′-carbonyldiimidazole, DMF; (ix) segment A of DU-86, NaH, DMF.

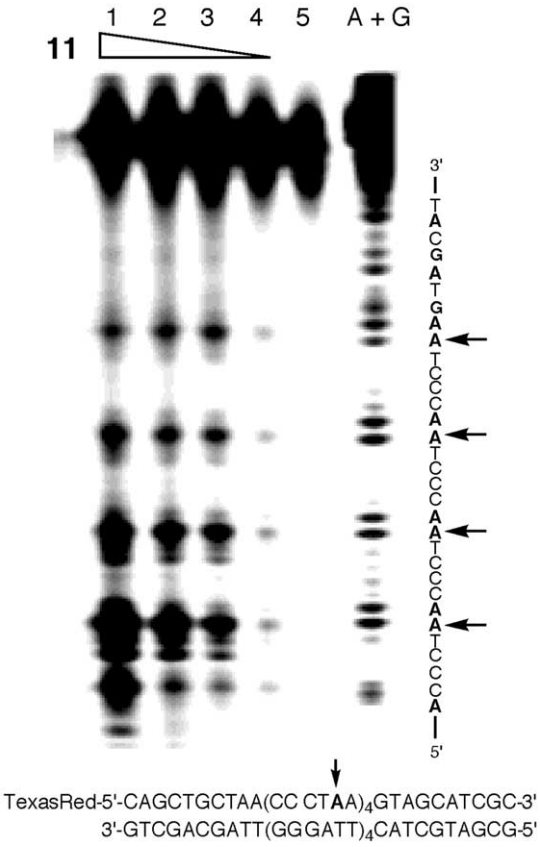


Figure 2. Thermally induced strand cleavage of 5′-Texas Red-labeled-44 bp DNA fragment by conjugate **11**. Lanes 1–4, 100, 10, 1, 0.1 μM of conjugate **11**; lane 5, DNA control. Arrows indicate the sites of alkylation by conjugate **11**.

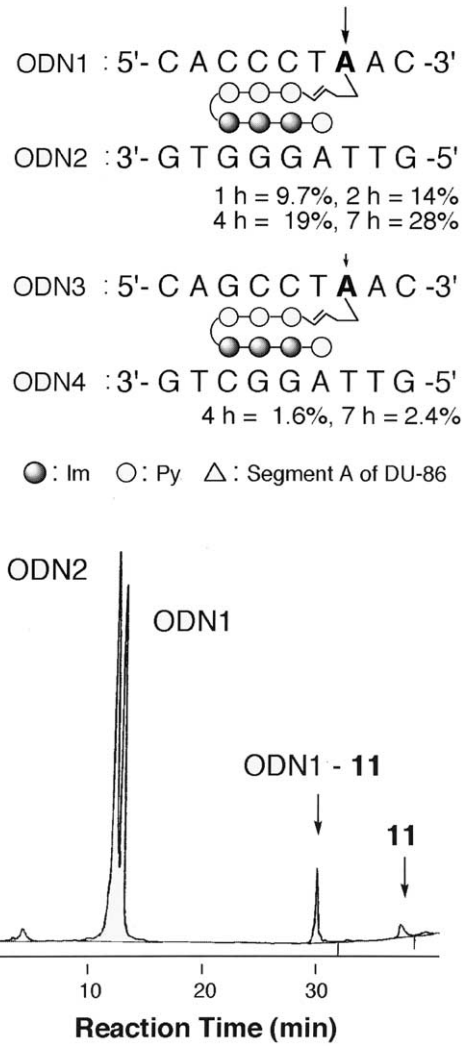


Figure 3. Schematic representation and HPLC analysis of ODN1–ODN2 with conjugate **11** after 7–h incubation.

Alkylation of a deoxynonanucleotide

To clarify the site of DNA alkylation by conjugate **11**, we investigated the alkylation of a duplex nonanucleotide, 5′-CACCCTAAC-3′ (ODN1)/5′-GTTAGGGTG-3′ (ODN2), which contains one telomere sequence d(TTAGGG) in the middle of the duplex (Fig. 3). Alkylation was carried out at 37 °C, and HPLC analysis revealed that a new peak appeared at 30 min with consumption of ODN1 and **11**, indicating conjugate **11** alkylated the adenine base of ODN1. The site of alkylation was determined by HPLC product analysis using a previously reported procedure.[7,8] The ODN1-conjugate **11** alkylation complex in Figure 3 was collected and heated at 90 °C for 10 min, and the cleavage of the abasic site with hot alkali (0.1 N NaOH, 90 °C, 10 min) produced cleaved oligonucleotide fragments. Elimination of the phosphates with alkaline phosphatase (AP) produced d(AC) and d(CACCCT) fragments. Quantitative analysis indicated that 28% of the ODN1–**11** complex was produced after 7–h incubation. These results clearly demonstrated that compound **11** alkylates target A_7 with high sequence specificity. In clear contrast, mismatched duplex 5′-CAGCCTAAC-3′ (ODN3)/5′-GTTAGGCTG-3′ (ODN4) was not efficiently alkylated by **11**: only 2.4% of ODN3–**11** complex produced after 7–h incubation.

Examination of antitumor activity using a panel of 39 cancer cell lines

Using 39 human cancer cell lines, we investigated the cell growth inhibition profile of conjugate **11**. Figure 4 shows the mean graph of conjugate **11** based on the IC_{50} growth inhibition parameter. Conjugate **11** shows differential growth inhibition. The results indicate that conjugate **11** is more effective against melanoma (LOX-IMVI), lung cancer (DMS114) and colon cancer (HCT-116, HCC2998). In fact, it was difficult to explain why telomere sequence-specific alkylation showed better antitumor activity against LOX-IMVI and DMS114. The mean log IC_{50} of conjugate **11** was −5.24 (5.75 μM), which is in the middle of the range of anticancer drugs used in the present study (Fig. 5).[11] Conjugate **11** is more effective than cisplatin and bleomycin in several human cancer cell lines. The relationship between telomere repeats, sequence-specific alkylation and specific antitumor activity, using both biological and chemical approaches, is currently under investigation.

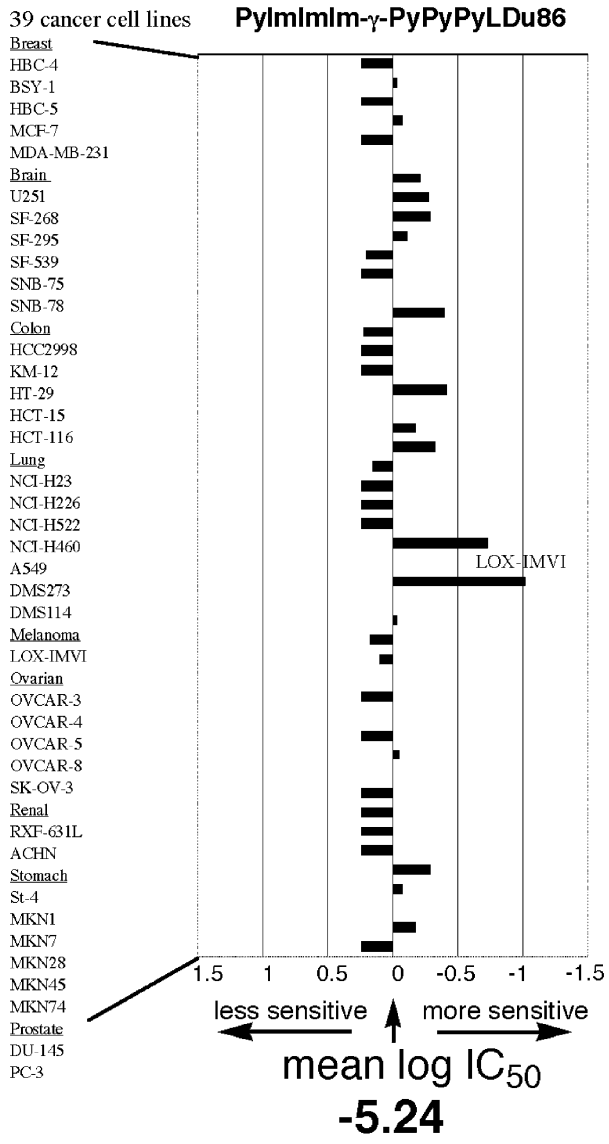


Figure 4. Growth inhibition in a panel of 39 human cancer cell lines. The log IC_{50} for each cell line is indicated. Columns extending to the *right*, sensitivity to conjugate **11**; columns extending to the *left*, resistance to conjugate **11**. One scale represents one logarithm difference. MG-MID, the mean of log IC_{50} values for 39 cell lines.

Conclusion

We synthesized a new hairpin polyamide-CPI, conjugate **11**, which targets human telomere repeats. We evaluated the DNA alkylating activity of conjugate **11** in detail by high resolution denaturing gel electrophoresis using 44 bp DNA fragments and by HPLC analysis with nonanucleotides. These results indicate that conjugate **11** specifically alkylates the adenine in telomere repeats. In comparison with clinically active drugs, conjugate **11** was more effective than cisplatin and bleomycin in several human cancer cell lines. The results clearly demonstrate that Py/Im polyamide CPI conjugate alkylates telomere repeats and exhibits antitumor activity. The effective DNA alkylating agent developed in the present investigation provides a promising approach for developing new types of antitumor agents.

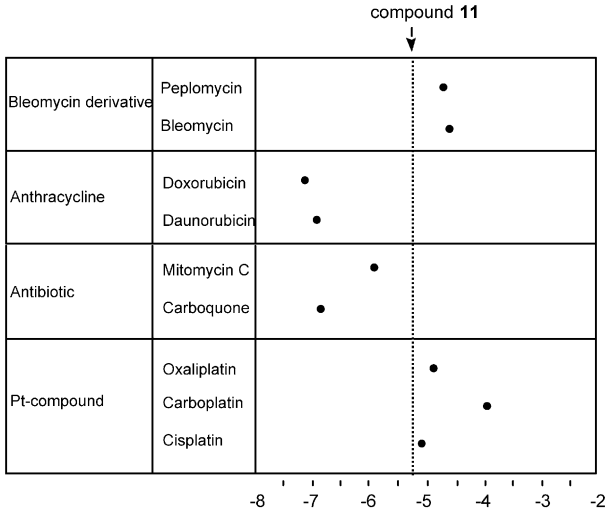


Figure 5. Comparison of the mean log IC_{50} of conjugate **11** with the mean log IC_{50} of clinically used anticancer agents, determined from a panel of 39 cell lines. Plots indicate mean log IC_{50} of each drug.

Further improvement of telomere specificity and alkylating activity is currently underway.

Experimental

General methods

Reagents and solvents were purchased from standard suppliers and used without further purification. Abbreviations of some reagents: DBU, 1,8-diazabicyclo [4.3.0] undec-7-ene; DIEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; FDPP, pentafluorophenyl diphenylphosphinate. Reactions were monitored by thin-layer chromatography (TLC) using 0.25-mm silica gel 60 plates impregnated with 254-nm fluorescent indicator (from Merck). Plates were visualized by UV illumination. NMR spectra were recorded with a JEOL JNM-A 500 nuclear magnetic resonance spectrometer, and tetramethylsilane was used as the internal standard. Proton NMR spectra were recorded in parts per million (ppm) downfield relative to tetramethylsilane. The following abbreviations apply to spin multiplicity: s (singlet), d (doublet), t (triplet), q (quartet), qu (quintet), m (multiplet), br (broad). Electrospray ionization time-of-flight mass spectra (ESI-TOF) were produced on a BioTOF II (Bruker Daltonics) mass spectrometer. Polyacrylamide gel electrophoresis was performed on a Hitachi 5500-S DNA Sequencer. A loading dye (dimethylformamide with fuschin red) from Amersham Co. Ltd; 5′-Texas Red-modified DNA oligomer (18-mer) from Kurabo Co. Ltd; and 50% Long Ranger™ gel solution from FMC Bioproducts. Calf intestine alkaline phosphatase (AP, 1000 units/mL) were purchased from Roche Diagnostics. The deoxynonanucleotides, 5′-CACCCTAAC-3′, 5′-GTTAGGGTG-3′ and the 5′-Texas Red-modified 44-bp DNA fragments were purchased from Jbios Co Ltd. The following precursors were prepared by the reported procedures.[7,8]

NO_2ImIm-γ-CO_2CH_3 (1). ^{1}H NMR (DMSO-d_6) δ 12.20 (brs, 1H), 10.19 (s, 1H), 8.62 (s, 1H), 8.28 (brt, J = 6.0 Hz, 1H), 7.51 (s, 1H), 4.04 (s, 3H), 3.95 (s, 3H), 3.57 (s, 3H), 3.24 (dt, J = 6.0, 7.0 Hz, 2H), 2.33 (t, J = 7.5 Hz, 2H), 1.75 (qu, J = 7.0 Hz, 2H).

AcPyImCO_2H (2). ^{1}H NMR (DMSO-d_6) δ 10.59 (s, 1H), 9.81 (s, 1H), 7.61 (s, 1H), 7.26 (d, J = 2.0 Hz, 1H), 6.93 (d, J = 2.0 Hz, 1H), 3.92 (s, 3H), 3.83 (s, 3H), 1.96 (s, 3H).

NO_2PyPyCO_2H (6). ^{1}H NMR (DMSO-d_6) δ 12.22 (brs, 1H), 10.22 (s, 1H), 8.18 (d, J = 2.0 Hz, 1H), 7.55 (d, J = 2.0 Hz, 1H), 7.41 (d, J = 1.5 Hz, 1H), 6.83 (d, J = 1.5 Hz, 1H), 3.95 (s, 3H), 3.83 (s, 3H).

AcPyImImIm-γ-CO_2CH_3 (3). To a solution of compound **1** (200 mg, 0.508 mmol) in MeOH–AcOEt (1:1, 10 mL) was added 10% Pd/C (70 mg), and the reaction mixture was stirred for 2 h at room temperature under an H_2 atmosphere. The catalyst was removed by filtration through Celite. The filtrate was concentrated in vacuo to produce crude amine (174 mg), which was used in the next step without further purification. To the solution of crude amine (174 mg, 0.48 mmol) in 5 mL DMF was added compound **2** (146 mg, 0.48 mmol) and FDPP (276 mg, 0.82 mmol), followed by ^{i}Pr$_2$NEt (0.25 mL, 1.44 mmol). The solution was stirred for 24 h and concentrated to a residue, which was subjected to column chromatography (silica gel, 0–10% MeOH in $CHCl_3$, gradient elution) to produce compound **3** (162 mg) at a yield of 48%. ^{1}H NMR (DMSO-d_6) δ 10.43 (s, 1H), 9.83 (s, 1H), 9.74 (s, 1H), 9.66 (s, 1H), 8.31 (brt, 1H), 7.65 (s, 1H), 7.63 (s, 1H), 7.52 (s, 1H), 7.26 (d, J = 1.5 Hz, 1H), 6.94 (d, J = 1.5 Hz, 1H), 4.02 (s, 3H), 4.00 (s, 3H), 3.96 (s, 3H), 3.84 (s, 3H), 3.59 (s, 3H), 3.57 (dt, J = 6.0, 7.0 Hz, 2H), 2.35 (t, J = 7.7 Hz, 2H), 1.98 (s, 3H), 1.77 (qu, J = 7.5 Hz, 2H). ESI-TOF m/e calcd for $C_{28}H_{34}N_{12}O_7$ (M + H) 651.2740, found 651.2673.

AcPyImImIm-γ-CO_2H (4). To a suspension of compound **3** (284 mg, 0.44 mmol) in 20 mL H_2O was added NaOH (400 mg, 10 mmol). The solution was stirred for 24 h at room temperature and the aqueous solution was acidified to pH 2 at 0 °C. The precipitate was collected by filtration, washed with water, and dried to produce compound **4** (131 mg) at a yield of 47%. ^{1}H NMR (DMSO-d_6) δ 10.43 (s, 1H), 9.85 (s, 1H), 9.79 (s, 1H), 9.71 (s, 1H), 8.30 (brt, 1H), 7.65 (s, 1H), 7.63 (s, 1H), 7.52 (s, 1H), 7.27 (d, J = 1.5 Hz, 1H), 6.94 (d, J = 1.5 Hz, 1H), 4.02 (s, 3H), 4.00 (s, 3H), 3.96 (s, 3H), 3.84 (s, 3H), 3.25 (dt, J = 6.0, 7.0 Hz, 2H), 2.26 (t, J = 7.7 Hz, 2H), 1.98 (s, 3H), 1.75 (qu, J = 7.0 Hz, 2H). ESI-TOF m/e calcd for $C_{27}H_{32}N_{12}O_7$ (M + H) 637.2568, found 637.2571.

NO_2PyPyPyL$CO_2CH_2CH_3$ (7). To a solution of compound **5** (150 mg, 0.67 mmol) in MeOH–AcOEt (3:1, 4 mL) was added 10% Pd/C (50 mg). After $NaBH_4$ (50 mg, 1.34 mmol) in H_2O (0.1 mL) was added dropwise at 0 °C, the reaction mixture was stirred for 20 min at room temperature under an N_2 atmosphere. The catalyst was removed by filtration through silica gel. The filtrate was concentrated in vacuo to produce crude amine (103 mg), which was used in the next step without further purification. To a solution of crude amine (103 mg, 0.53 mmol) in 4 mL DMF was added compound **6** (232 mg, 0.80 mmol) and FDPP (305 mg, 0.80 mmol), followed by ^{i}Pr$_2$NEt (0.28 mL, 1.59 mmol). The solution was stirred for 20 h and concentrated to a residue, which was subjected to column chromatography (silica gel, 0–2% MeOH in $CHCl_3$, gradient elution) to produce compound **7** (117 mg) with a yield of 37%. ^{1}H NMR (DMSO-d_6) δ 10.27 (s, 1H), 9.97 (s, 1H), 8.18 (d, J = 16.0 Hz, 1H), 7.58 (d, J = 16.0 Hz, 1H), 7.51 (d, J = 15.0 Hz, 1H), 7.41 (d, J = 16.0 Hz, 1H), 7.26 (d, J = 16.0 Hz, 1H), 7.06 (d, J = 16.0 Hz, 1H), 6.75 (d, J = 16.0 Hz, 1H), 6.07 (d, J = 15.0 Hz, 1H), 4.16 (q, J = 7.0 Hz, 2H), 3.97 (s, 3H), 3.86 (s, 3H), 3.69 (s, 3H), 1.25 (t, J = 7.0 Hz, 3H). ESI-TOF m/e calcd for $C_{22}H_{24}N_6O_6$ (M + H) 469.1767, found 469.1757.

AcPyImImIm-γ-PyPyPyL$CO_2CH_2CH_3$ (8). To a solution of compound **7** (129 mg, 0.28 mmol) in MeOH–AcOEt (3:1, 4 mL) was added 10% Pd/C (25 mg). After $NaBH_4$ (60 mg, 1.12 mmol) in H_2O (0.2 mL) was added dropwise at 0 °C, the reaction mixture was stirred for 2 h at room temperature under an N_2 atmosphere. The catalyst was removed by filtration through silica gel. The filtrate was concentrated in vacuo to produce crude amine (87.4 mg), which was used in the next step without further purification. To a solution of crude amine (87.4 mg, 0.2 mmol) in 0.5 mL DMF was added compound **4** (72 mg, 0.11 mmol) and FDPP (127 mg, 0.33 mmol), followed by ^{i}Pr$_2$NEt (117 μL, 0.66 mmol). The solution was stirred for 18 h and concentrated to a residue, which was subjected to column chromatography (silica gel, 0–5% MeOH in $CHCl_3$, gradient elution) to produce **8** (80 mg) at a yield of 69%. ^{1}H NMR (DMSO-d_6) δ 10.40 (s, 1H), 9.89 (s, 1H), 9.86 (s, 1H), 9.84 (s, 1H), 9.81 (s, 2H), 9.74 (s, 1H), 8.31 (brt, 1H), 7.64 (s, 1H), 7.60 (s, 1H), 7.51 (s, 1H), 7.49 (d, J = 15.5 Hz, 1H), 7.38 (s, 1H), 7.23 (s, 1H), 7.20 (s, 1H), 7.14 (s, 2H), 7.03 (s, 1H), 6.92 (s, 1H), 6.85 (s, 1H), 6.73 (s, 1H), 6.05 (d, J = 15.5 Hz, 1H), 4.14 (q, J = 7.5 Hz, 2H), 4.00 (s, 3H), 3.99 (s, 3H), 3.95 (s, 3H), 3.82 (s, 6H), 3.81 (s, 3H), 3.67 (s, 3H), 3.27 (m, 2H), 2.27 (t, J = 7.5 Hz, 1H), 1.95 (s, 3H), 1.81 (m, 2H), 1.23 (t, J = 7.0 Hz, 3H). ESI-TOF m/e calcd for $C_{49}H_{56}N_{18}O_{10}$ (M + H) 1057.4352, found 1057.4472.

AcPyImImIm-γ-PyPyPyLCO_2H (9). To a suspension of compound **8** (96.3 mg, 0.091 mmol) in 0.4 mL H_2O was added DBU (0.4 mL, 2.68 mmol). The solution was stirred for 3 h and concentrated to a residue, which was subjected to trituration with Et_2O and AcOEt. After column chromatography (silica gel, 0–20% MeOH in $CHCl_3$, gradient elution), the crude amine was acidified with 1% AcOH. The precipitate was collected by filtration, washed with water, and dried to produce **9** (74.5 mg) at a yield of 80%. ^{1}H NMR (DMSO-d_6) δ 10.40 (s, 1H), 9.86 (s, 1H), 9.85 (s, 1H), 9.83 (s, 2H), 9.82 (s, 2H), 8.30 (brt, 1H), 7.63 (s, 1H), 7.61 (s, 1H), 7.51 (s, 1H), 7.37 (d, J = 15.0 Hz, 1H), 7.33 (s, 1H), 7.23 (s, 1H), 7.20 (s, 1H), 7.14 (s, 1H), 7.03 (s, 1H), 6.93 (s, 1H), 6.85 (s, 1H), 6.63 (s, 1H), 5.98 (d, J = 15.0 Hz, 1H), 4.00 (s, 3H), 3.99 (s, 3H), 3.95 (s, 3H), 3.82 (s, 6H), 3.81 (s, 6H), 3.21

(m, 2H), 2.38 (m, 2H), 1.98 (s, 3H), 1.90 (m, 2H). ESI-TOF *m/e* calcd for $C_{47}H_{52}N_{18}O_{10}$ (M + H) 1029.4074, found 1029.4114.

AcPyImImIm-γ-PyPyPyLCOIm (10). To a solution of compound **9** (36.3 mg, 35.3 μmol) in DMF (0.4 mL) was added 1,1′-carbonyldiimidazole (40 mg, 247 μmol). The mixture was stirred for 14 h at room temperature. Evaporation of the solvent gave a yellow residue, which was triturated with ethyl ether (5 mL×3) to produce **10** (36 mg, 95%). ESI-TOF *m/e* calcd for $C_{50}H_{54}N_{20}O_9$ (M + H) 1079.4307, found 1079.4383.

AcPyImImIm-γ-PyPyPyLDu86 (11). To a solution of sodium hydride (12 mg, 266 μmol, 60% oil suspension) in DMF (0.2 mL) was added segment A of DU-86 (3.7 mg, 55 μmol) in DMF (0.1 mL). Compound **10** (36 mg, 33 μmol) in DMF (0.4 mL) was added at 0 °C, and the reaction mixture was then stirred for 1 h at 0 °C. The reaction mixture was quenched by the addition of 50 mM sodium phosphate buffer (4 mL, pH 6.86) at 0 °C. Evaporation of the solvent gave a yellow residue, which was subjected to column chromatography (silica gel, 0–5% MeOH in $CHCl_3$, gradient elution) to produce compound **11** (18 mg) with a yield of 43%. After further purification by HPLC using a Chemcobond 5-ODS-H column (0.1% AcOH/CH_3CN 0–50% linear gradient, 35.1 min/40 min, 254 nm), **11** was used in the DNA alkylation reaction. ^{1}H NMR (DMSO-d_6) δ 12.34 (brs, 1H), 10.38 (s, 1H), 9.91 (s, 1H), 9.86 (s, 1H), 9.80 (s, 1H), 9.69 (s, 1H), 9.63 (s, 1H), 8.28 (brt, 1H), 7.94 (s, 1H), 7.64 (s, 1H), 7.61 (s, 1H), 7.57 (d, J = 15.0 Hz, 1H), 7.51 (s, 1H), 7.37 (s, 1H), 7.24 (s, 1H), 7.21 (s, 1H), 7.14 (s, 1H), 7.05 (s, 1H), 6.93 (s, 2H), 6.88 (s, 1H), 6.86 (s, 1H), 6.55 (d, J = 15.0 Hz, 1H), 4.03 (m, 2H), 4.00 (s, 3H), 3.99 (s, 3H), 3.95 (s, 3H), 3.83 (s, 6H), 3.72 (s, 3H), 3.70 (s, 3H), 3.3 (m, 2H, overrap with DMSO-d_6), 2.88 (s, 3H), 2.74 (m, 1H, overlap with DMSO-d_6), 2.72 (s, 3H), 2.28 (t, J = 7.5 Hz, 2H), 1.95 (s, 3H), 1.82 (m, 2H), 1.55 (m, 2H) ESI-TOF *m/e* calcd for $C_{61}H_{64}N_{20}O_{12}$ (M + H) 1269.4958, found 1269.5013.

High-resolution gel electrophoresis

The 5′-Texas Red-labeled DNA fragments (20 nM) were alkylated by various concentration of **11** in 10 μL of 2 mM sodium phosphate buffer (pH 7.0) containing 10% DMF at 23 °C. The reaction was quenched by the addition of calf thymus DNA (1 mM, 1 μL) and heating for 5 min at 90 °C. The DNA was recovered by vacuum centrifugation. The pellet was dissolved in 8 μL loading dye (formamide with fuschin red), heated at 94 °C for 20 min, and then immediately cooled to 0 °C. A 2-μL aliquot was subjected to electrophoresis on a 15% denaturing polyacrylamide gel using a Hitachi 5500-S DNA Sequencer. The lanes of sequencing (G + A) were prepared according to published protocol.[15]

Alkylation of oligonucleotides by conjugates 11, as monitored by HPLC

A reaction mixture (20 μL) containing conjugates **11** (200 μM) and the duplex oligonucleotide (200 μM duplex concentration) in 500 μM sodium cacodylate buffer (pH 7.0) was incubated at 37 °C for the indicated periods. The progress of the reaction was monitored by HPLC using a Chemcobond 5-ODS-H column (4.6×150 mm). Elution was performed with 50 mM ammonium formate and a 5–12% (0–20 min), 12–50% (20–40 min) acetonitrile linear gradient at a flow rate of 1.0 mL/min. Products were detected at 254 nm.

Characterization of ODN-conjugate 11 alkylation complex. The alkylation product ODN1-conjugate **11** shown in Figure 3 was collected by HPLC (elution with 50 mM ammonium formate and a 5–12% (0–20 min), 12–50% (20–14 min) acetonitrile linear gradient at a flow rate of 1.0 mL/min). The collected fractions were evaporated, then heated at 90 °C for 10 min and heated in the presence of 0.1 N NaOH at 90 °C for 10 min. After neutralization, the solution was analyzed by HPLC [elution with 50 mM ammonium formate and a 5–10% acetonitrile linear gradient (0–20 min) at a flow rate of 1.0 mL/min]. The composition of the cleaved oligonucleotides was confirmed by enzymatic digestion. Oligonucleotides were digested with AP (5 units/mL) in 5 mM Na cacodylate buffer (pH 7.0) at 37 °C for 30 min, and analyzed by HPLC [elution with 50 mM ammonium formate and a 5–10% acetonitrile linear gradient (0–20 min) at a flow rate of 1.0 mL/min].

Drug and cell culture

Conjugate **11** was dissolved in dimethyl sulfoxide (DMSO) and added to cells with less than 1% DMSO in the final drug dilution with a culture medium. Five breast cancer (HBC-4, BSY-1, HBC-5, MCF-7, MDA-MB-231), six brain cancer (U251, SF-268, SF-295, SF-539, SNB-75, SNB-78), five colon cancer (HCC2998, KM-12, HT-29, HCT-15, HCT-116), seven lung cancer (NCI-H23, NCI-H226, NCI-H522, NCI-H460, A5449, DMS273, DMS114), one melanoma (LOX-IMVI), five ovarian cancer (OVCAR-3, OVCAR-4, OVCAR-5, OVCAR-8, SK-OV-3), two renal cancer (RXF-631L, ACHN), six stomach cancer (St-4, MKN1, MKN7, MKN28, MKN45, MKN74), two prostate cancer (DU-145, PC-3) cancer cell lines were all cultured in RPMI1640 (Sigma) containing 10% heat-inactivated fetal bovine serum (Life Technologies), penicillin (100 U/mL) and streptomycin (100 μg/mL). The cells were maintained at 37 °C in humidified atmosphere of 95% air and 5% CO_2.

Measurements of cell growth inhibition

The details of measuring cell growth inhibition are described elsewhere.[12] Briefly, the cell were plated at proper density in 96-well plates in RPMI 1640 with 5% fetal bovine serum and allowed to attach overnight. The cell were exposed to drugs for 48 h. Then, the cell growth was determined according to the sulforhodamine B assey, described by Skehan et al.[13] Data calculations were made according to method descrived previously.[12a] By using the computer to process % growth values, the 50% growth inhibition parameter (IC_{50}) was determined. The mean graph, which shows

the differential growth inhibition of the drug in the cell line panel, was drawn based on a calculation using a set of IC_{50}.[14]

Acknowledgements

We thank the Screening Committee of New Anticancer Agents supported by Grant-in-Aid for Scientific Research on Priority Area 'Cancer' from The Ministry of Education, Culture, Sports, Science and Technology, Japan.

References and Notes

1. Martens, U. M. *Nat. Genet.* **1998**, *18*, 76.
2. Blackburn, E. H. *Annu. Rev. Biochem.* **1992**, *61*, 113.
3. (a) Kim, N. W.; Piatyszek, M. A.; Prowse, K. R.; Harley, C. B.; West, M. D.; Ho, P. L. C.; Coviello, G. M.; Wright, W. E.; Weinrich, S. L.; Shay, J. W. *Science* **1994**, *266*, 2011. (b) Counter, C. M.; Hirte, H. W.; Bachetti, S.; Harley, C. B. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 2900.
4. (a) Herbert, B.-S.; Pitts, A. E.; Baker, S. I.; Hamilton, S. E.; Wright, W. E.; Shay, J. W.; Corey, D. R. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 14276. (b) Hurley, L. H. *Nat. Rev. Cancer* **2002**, *2*, 188.
5. Pelton, J. G.; Wemmer, D. E. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 5723.
6. White, S.; Baird, E. E.; Dervan, P. B. *Chem. Biol.* **1997**, *4*, 569.
7. Tao, Z.-F.; Fujiwara, T.; Saito, I.; Sugiyama, H. *J. Am. Chem. Soc.* **1999**, *121*, 4961.
8. Bando, T.; Narita, A.; Saito, I.; Sugiyama, H. *Chem. Eur. J.* **2002**, *8*, 4781.
9. Nagamura, S.; Asai, A.; Kanda, Y.; Kobayashi, E.; Gomi, K.; Saito, H. *Chem. Pharm. Bull.* **1996**, *44*, 1723.
10. Swalley, S. E.; Baird, E. E.; Dervan, P. B. *J. Am. Chem. Soc.* **1997**, *119*, 6953.
11. Yamori, T.; Matsunaga, A.; Sato, S.; Yamazaki, K.; Komi, A.; Ishizu, K.; Mita, I.; Edatsugi, H.; matsuba, Y.; Takezawa, K.; Nakanishi, O.; Kohno, H.; Nakajima, Y.; Komatsu, H.; Andoh, T.; Tsuruo, T. *Cancer Res.* **1999**, *59*, 4042.
12. (a) Monks, A.; Scudiero, D.; Skehan, P.; Shoemaker, R.; Paull, K.; Vistica, D.; Hose, C.; Langley, J.; Cronise, P.; Vaigro-Wolff, A.; Gray-Goodrichi, M.; Campbell, H.; Mayo, J.; Boyd, M. *J. Natl. Cancer Inst.* **1991**, *83*, 757. (b) Yamori, T.; Sato, S.; Chikazawa, H.; Kadota, T. *Jpn. J. Cancer Res.* **1997**, *88*, 1205.
13. Skehan, P.; Storeng, R.; Scudiero, D.; Monks, A.; McMahon, J.; Vistica, D.; Warren, J. T.; Bokesch, H.; Kenney, S.; Boyd, M. R. *J. Natl. Cancer Inst.* **1990**, *82*, 1107.
14. (a) Boyd, M. R. Status of the National Cancer Institute Preclinical Antitumor Drug Discovery Screen: Implications For Clinical Trial. In: V. T. Devita, Jr., S. Hellman, S. A. Rosenberg (eds.), Cancer: Principles and Practice of Oncology Update, Vol. 3, p 1–12. Philadelphia: Lippincott, **1989** (b) Paull, K. D.; Shoemaker, R. H.; Hodes, L.; Monks, A.; Scudiero, D. A.; Rubinstein, L.; Plowman, J.; Boyd, M. R. *J. Natl. Cancer Inst.* **1989**, *81*, 1088.
15. Maxam, A. M.; Gilbert, W. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 560.