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Potent CpG oligonucleotides containing phosphodiester linkages: in vitro and in vivo immunostimulatory properties

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Abstract

Bacterial and synthetic DNAs, containing CpG dinucleotides in specific sequence contexts, activate the vertebrate immune system. Unlike phosphorothioate (PS) CpG DNAs, phosphodiester (PO) CpG DNAs require either palindromic sequences and/or poly(dG) sequences at the 3′-end for activity. Here, we report 'PO-immunomers' having two PO-CpG DNA molecules joined through their 3′-ends. These PO-imunomers permitted us, for the first time, to assess immunostimulatory properties of PO-CpG DNAs in vitro and in vivo without the need for palindromic and/or poly(dG) sequences. In medium containing 10% fetal bovine serum, PO-immunomers were more resistant than PO-CpG DNAs to nucleases. Compared to PS-CpG DNA in BALB/c and C3H/HeJ mice spleen cell culture assays, PO-immunomers showed increased IL-12 secretion and minimal amounts of IL-6 secretion. PO-immunomers activated NF-κB and induced cytokine secretion in J774 cell cultures. In addition, PO-immunomers showed antitumor activity in nude mice bearing human breast (MCF-7) and prostate (DU145) cancer xenografts.

Keywords: Anticancer; CpG DNA; IL-12; IL-6; Immunostimulatory; 3′–3′-linked; Oligonucleotides; Phosphodiester; Phosphorothioate

Bacterial DNA and synthetic oligodeoxynucleotides containing CpG dinucleotides (CpG DNA) activate the innate immune system. Tokunaga and colleagues [1,2] were the first to report that mycobacterial DNA and phosphodiester (PO) oligodeoxynucleotides containing CpG dinucleotides in certain palindromic sequences stimulate NK cells, induce secretion of interferons (IFNs), and show antitumor activity. Subsequent studies showed that phosphorothioate (PS) DNAs containing CpG dinucleotides (PS-CpG DNAs) within appropriate flanking sequences induce proliferation of murine B cells and stimulate innate immune cells similar to bacterial DNA [3–5]. Upon CpG DNA uptake, the activation of immune cells (B-cells, macrophages, and dendritic cells) could occur through a recently identified receptor, TLR9 [6], or other receptors [7], resulting in the activation of the stress kinase and NF-κB pathways [8,9] and induction of various cytokines including IFN-γ, IL-12, IL-6, and TNF-α [10–13].

PS-CpG DNAs have been studied extensively for their immunostimulatory activity in various assays and hosts. In general, PS-CpG DNAs, with [5,14] or without [3,4] palindromic sequences, activate B cells, leading to the induction of cytokines and Ig. Innate immune cells, including macrophages and dendritic cells, are also activated. Several PS-CpG DNAs have been studied extensively in animal models, either alone or in combination with antigens, allergens or antibodies, and human clinical trials are ongoing [15–17].

PO-CpG DNAs are inherently prone to rapid degradation by nucleases and, generally, have half-lives of less than two minutes in vivo [18,19]. PO-CpG DNAs containing palindromic and/or poly(dG) sequences can induce immune stimulation in vitro and in vivo [20–27]. These structural features provide some nuclease stability but also result in the formation of secondary structures.

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Due to the ability of PO-CpG DNAs to enhance CTL (cytotoxic T lymphocyte) activity, they elicit efficient antitumor activity in vivo [20–22]. Unlike the phosphorothioate analogs, PO-CpG DNAs induce higher levels of IFN-γ in human PBMC cultures and in vivo. As a result, they act as superior adjuvants by promoting Th1-immune responses when used in combination with vaccines and allergens [25–27].

Recently, we have shown that the 5′-end of CpG DNA should be accessible for immunostimulatory activity [28]. Studies using 3′–3′- and 5′–5′-linked CpG DNAs showed that the former linkage facilitates while the latter significantly interferes with activity [28]. Blocking the 3′-end with chemical modifications [29,30] or linking to another oligonucleotide through 3′–3′-linkage increases the stability against nucleases [31,32]. In the present study, we used 3′–3′-linked PO-CpG DNAs, referred to here as 'PO-immunomers,' to evaluate immunostimulation properties. PO-immunomers described in this paper do not contain self-complementary or poly(dG) sequences that might form secondary structures (Table 1). This enabled us, for the first time, to evaluate the immune responses of PO-CpG DNAs without the need of palindromic or poly(dG) sequences.

Materials and methods

CpG DNA synthesis and purification. The CpG DNAs used in the study were synthesized on a PerSeptive Biosystems 8909 Expedite DNA synthesizer on 1–2 μmol scale as described earlier [31,32] using iodine or Beaucage's reagent [33] as an oxidant to obtain phosphodiester or phosphorothioate backbone modification, respectively. Final products were purified by HPLC and dialyzed against USP quality sterile water for irrigation (Braun). All CpG DNAs were characterized by CGE and MALDI-TOF mass spectrometry (PerSeptive Biosystems Voyager DEStar MALDI-TOF mass spectrometer with 337 nm N_2 laser) for purity and molecular mass (Table 1), respectively. The purity of full-length CpG DNAs ranged from 89% to 95% with the remainder found to lack one or two nucleotides by CGE and/or denaturing PAGE. All CpG DNAs contained <0.075 EU/mL of endotoxin by the Limulus assay (Bio-Whittaker).

Nuclease digestion assay. Purified CpG DNA samples (1 AU, 20 μL) were incubated in 500 μL medium (1× DPBS) containing 10% FBS (Atlas Biologicals, Fort Collins, CO) at 37 °C for 4, 24, and 48 h. Following dilution with 1.0 mL of 0.1 M ammonium acetate, they were desalted on C_{18} SEP-PAK cartridges (Waters) and analyzed by CGE on a Beckman P/ace system 5010 instrument operating at 14 kV.

Cell culture conditions and reagents. BALB/c mouse (4–8 weeks) or C3H/HeJ mouse (4–8 weeks) spleen cells were cultured in RPMI complete medium as described earlier [34,35]. Murine macrophage-like cells, J774 (American Type Culture Collection, Rockville, MD), were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) FCS and antibiotics (100 IU/mL of penicillin G/streptomycin). All other culture reagents, were purchased from Mediatech (Gaithersburg, MD).

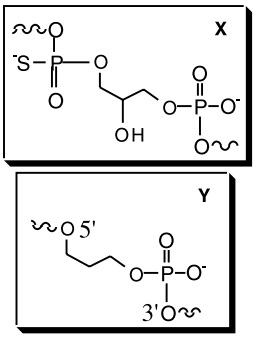
ELISAs for IL-12 and IL-6. BALB/c or C3H/HeJ mouse spleen or J774 cells were plated in 24-well dishes using 5×10^6 or 1×10^6 cells/mL, respectively. The CpG DNA dissolved in TE buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA) was added to a final concentration of 0.03, 0.1, 0.3, 1.0, 3.0, or 10.0 μg/mL to the cell cultures. The cells were then incubated at 37 °C for 24 h and the supernatants were collected for ELISA assays. The experiments were performed two or three times for each CpG DNA in triplicate for each concentration. The secretion of IL-12 and IL-6 was measured by sandwich ELISA as described previously [36]. The required reagents, including cytokine antibodies and standards were purchased from PharMingen.

Preparation of J774 cell nuclear extracts and EMSA. For NF-κB activation studies, J774 cells were plated at a density of 5×10^6 cells/well in six-well plates, treated for 1 h with CpG DNAs at a concentration of 10 μg/mL and nuclear extracts were prepared as described earlier [37]. Protein concentrations were determined [38] and the samples were either used immediately or stored frozen at −70 °C. Reaction mixtures (25 μL) containing 10 μg nuclear protein, 5 mM Tris (pH 7.5), 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 4% glycerol (v/v), and 0.08 mg/mL salmon-sperm DNA were preincubated on ice for 15 min. Then, 1×10^6 cpm of 5′-^{32}P-labeled oligonucleotide probe (5′-AGTTGAGGGGACTTTCCCAGGC-3′) was added and the binding reaction was allowed to proceed for 20 min at room temperature. DNA–protein complexes were then resolved in a 6% native polyacrylamide gel in TBE buffer (22.5 mM Tris, 22.5 mM boric acid, and 0.5 mM EDTA, pH 8.3) at 140 V for 2–3 h. Gels were dried and exposed to Kodak Biomax MR film at −70 °C. Films were scanned and the images were processed using Adobe imaging software.

In vivo nude mice models and treatment plan. The human breast cancer MCF-7 and prostate cancer DU145 models were established

Table 1
CpG DNA sequences, modifications used and analytical data

CpG DNA	Sequence[a]	Backbone[b]	Molecular weight	
			Calculated	Found[c]
1	5'-CTATCTGA$\overrightarrow{CG}$TTCTCTGT-3'	PS	5702	5704
2	5'-CTATCTGA$\overrightarrow{CG}$TTCTCTGT-3'	PO	5432	5428
3	5'-CTGA$\overrightarrow{CG}$TTCTCTGT-**X**-TGTCTCTT$\overleftarrow{GC}$AGTC-5'	PO	8656	8649
4	5'-**YY**CTGA$\overrightarrow{CG}$TTCTCTGT-**X**-TGTCTCTT$\overleftarrow{GC}$AGTC**YY**-5'	PO	9208	9214



[a]Arrows indicate 5'-3' directionality of CpG dinucleotide in each DNA molecule and structures of X and Y are shown in boxes.
[b]PS and PO stand for phosphorothioate and phosphodiester backbones, respectively.
[c]As determined by MALDI-TOF mass spectrometry.

using the protocols described previously [39,40]. Female (for MCF-7) or male (for DU-145) athymic nude mice (nu/nu, 4–6 weeks old) were used. The animal use and care protocols were approved by the Institutional Committee on Animal Use and Care of the University of Alabama at Birmingham. All mice were obtained from Frederick Cancer Research and Development Center (Frederick, MD, USA). To establish MCF-7 xenografts, each of the female nude mice was implanted a 60-day subcutaneous slow release estrogen pellet (E-121, 1.7 mg of 17β-estradiol/pellet; Innovative research of America, Sarasota, FL), prior to cell inoculation. Cultured cells were harvested from monolayer cultures, washed twice with serum-free media (MEM for MCF-7 and EMEM for DU-145), and re-suspended in the same medium. The suspensions (5×10^6 cells) were then injected into the left inguinal area of the mice. The mice were monitored by general clinical observation as well as by body weight and tumor growth. Tumor growth was recorded with the use of calipers, by measuring the long and short diameters of the tumor. Tumor mass (in gram) was calculated using the formula $1/2a \times b^2$, where "a" and "b" are the long and short diameters (in centimeter), respectively [39–41]. When the tumor mass reached 50–150 mg, the animals were randomly divided into various treatment groups and treated by subcutaneous injection with PO-immunomer **4** at a dose of 0.5 mg/kg or saline (control) every other day.

Results

Design of PO-immunomers

PS-CpG DNA **1** (Table 1) was found to induce an immune response in mice [36,42,43] with PO-CpG DNA **2** serving as a control. PO-immunomers **3** and **4** each contain two identical, truncated copies of the parent CpG DNA **2** joined through their 3′-ends via a glyceryl linker, **X** (Table 1). While **3** and **4** each contain the same oligonucleotide segments of 14 bases, the 5′-ends of **4** were modified by the addition of two C3-linkers, **Y** (Table 1). All oligonucleotides **1–4** contain a 'GACGTT' hexameric motif known to activate the mouse immune system [4].

Nuclease stability of PO-immunomers

The stability of PO-immunomers against nucleases was assessed by incubating CpG DNAs **1–4** in cell culture medium containing 10% fetal bovine serum (FBS) (non-heat-inactivated) at 37 °C for 4, 24, and 48 h. Intact CpG DNA remaining in the reaction mixtures was then determined by CGE. Figs. 1A–D show the nuclease digestion profiles of CpG DNAs **1–4** incubated in 10% FBS for 24 h. The amount of full-length CpG DNA remaining at each time point is shown in Fig. 1E. As expected, the parent PS-CpG DNA **1** is the most resistant to serum nucleases. About 55% of 18-mer **1** remained undegraded after 48 h incubation. In contrast, only about 5% of full-length PO-immunomer **2** remained after 4 h under the same experimental conditions, confirming that DNA containing phosphodiester linkages undergoes rapid degradation [18,19]. As expected, both PO-immunomers **3** and **4** were more resistant than **2** to serum nucleases. After 4 h, about 62% and 73% of **3** and **4**, respectively, were intact compared with about 5% of **2** (Fig. 1E). Even after 48 h, about 23% and 37% of **3** and **4**, respectively, remained undegraded. As well as showing that 3′–3′-linked PO-immunomers are more stable against serum nucleases, these studies indicate that chemical modifications at the 5′-end can further increase nuclease stability. This is consistent with our earlier report that oligonucleotides are degraded from the 5′-end also, though to a lesser extent than that from the 3′-end [44].

Immunostimulatory activity of PO-immunomers in mouse spleen cell cultures

The immunostimulatory activity of CpG DNAs was studied in BALB/c and C3H/HeJ mice spleen cell cultures by measuring levels of cytokines IL-12 and IL-6 secreted. All CpG DNAs induced a concentration-dependent cytokine secretion in BALB/c mouse spleen cell cultures (Fig. 2). At 3 μg/mL, PS-CpG DNA **1** induced 2656 ± 256 and 12234 ± 1180 pg/mL IL-12 and IL-6, respectively, as reported earlier [36,42,43]. The parent PO-CpG DNA **2** did not raise cytokine levels above background, except at a concentration of 10 μg/mL.

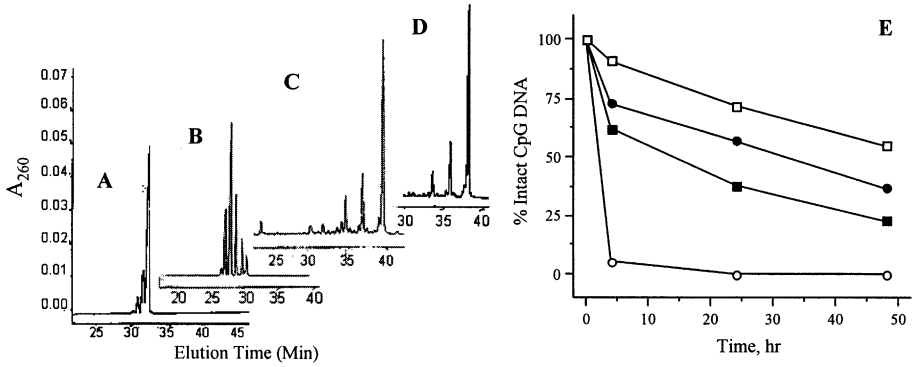


Fig. 1. (A)–(D) Nuclease digestion profiles of CpG DNAs **1–4**, respectively, and (E) stability of CpG DNAs **1** (open squares), **2** (open circles), **3** (filled squares), and **4** (filled circles) in 10% fetal bovine serum as determined by CGE analysis.

This observation is consistent with the nuclease stability assay results. In contrast, PO-immunomers **3** and **4** induced both IL-12 and IL-6 secretion in BALB/c mouse spleen cell cultures.

The results presented in Fig. 2 show a clear distinction in cytokine induction profiles of PS- and PO-CpG DNAs. PO-immunomers **3** and **4** induced higher levels of IL-12 than did PS-CpG DNA **1** in BALB/c mouse spleen cell cultures (Fig. 2A). In contrast, at concentrations up to 3 μg/mL, they produced negligible amounts of IL-6 (Fig. 2B). Even at the highest concentration (10 μg/mL), PO-immunomer **3** induced significantly less IL-6 than did PS-CpG DNA **1**. The presence of C3 linkers at the 5′-terminus of PO-immunomer **4** resulted in slightly higher levels of IL-6 secretion compared with **3**. However, importantly, the levels of IL-6 produced by PO-immunomer **4** are much lower than those induced by PS-CpG DNA **1**. The inset of Fig. 2A shows the ratio of IL-12 to IL-6 secreted at 3 μg/mL concentration. In addition to increasing IL-12 secretion, PO-immunomers **3** and **4** induced higher levels of IFN-γ than did PS-CpG DNA **1** in BALB/c mouse spleen cell cultures (data not shown).

The different cytokine profiles induced by PO- and PS-CpG DNAs in BALB/c mouse spleen cell cultures prompted us to study the pattern of cytokine induction of CpG DNAs in C3H/HeJ mouse spleen cell cultures (an LPS hyporesponsive strain). All three CpG DNAs tested in this assay induced concentration-dependent cytokine secretion (Figs. 3A and B). Since PO-CpG DNA **2** failed to induce cytokine secretion in BALB/c mouse spleen cell cultures, it was not further tested in C3H/HeJ spleen cell cultures. Both PO-immunomers **3** and **4** induced higher IL-12 production than did PS-CpG DNA **1** (Fig. 3A). However, at concentrations up to 3 μg/mL, neither induced IL-6 production. At the

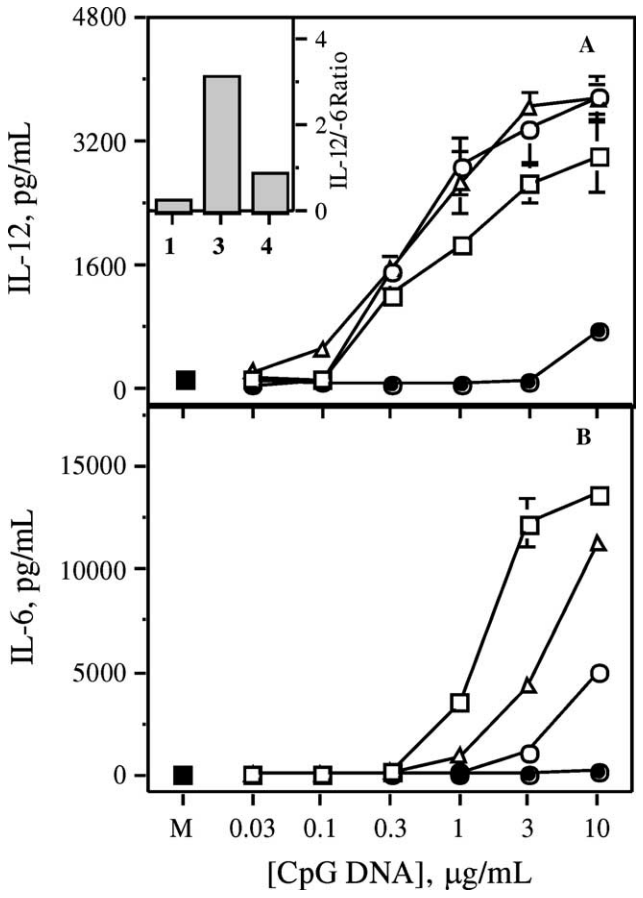


Fig. 2. Immunostimulatory activity of PO- and PS-CpG DNAs in BALB/c mouse spleen cell culture assays. (A) IL-12 and (B) IL-6 secretion after 24 h incubation with CpG DNAs or medium (M) alone. In both panels, filled squares, open squares, filled circles, open circles, and triangles represent medium and CpG DNAs **1**, **2**, **3**, and **4**, respectively. Each value is an average of four replicate samples. Inset shows the ratio of IL-12 to IL-6 induced by PS-(**1**) and PO-CpG DNAs (**3** and **4**) at 3.0 μg/mL concentration.

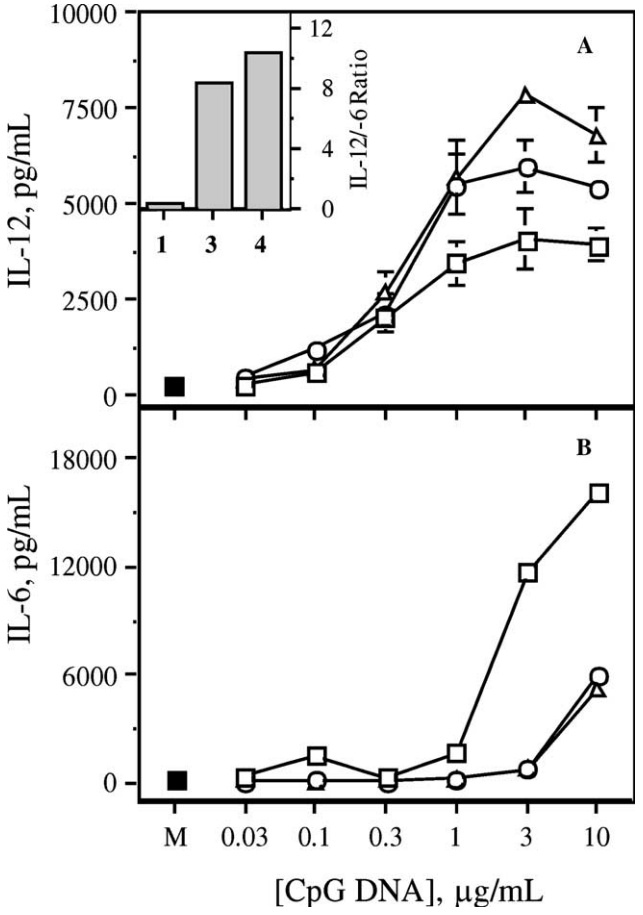


Fig. 3. Immunostimulatory activity of PO- and PS-CpG DNAs in C3H/HeJ mouse spleen cell culture assays. Induction of (A) IL-12 and (B) IL-6 secretion after 24 h incubation with CpG DNAs or medium (M) alone. In both panels, filled squares, open squares, open circles, and triangles represent medium and CpG DNAs **1**, **3**, and **4**, respectively. Each value is an average of four replicate samples. Inset shows the ratio of IL-12 to IL-6 induced by PS-(**1**) and PO-CpG DNAs (**3** and **4**) at 3.0 μg/mL concentration.

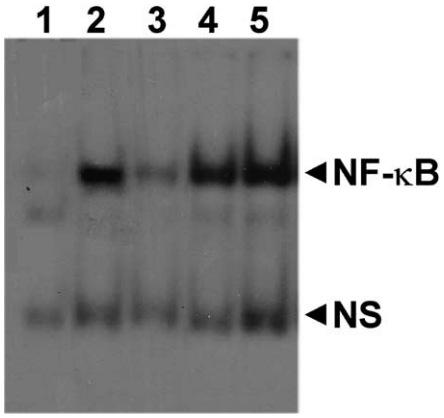


Fig. 4. Effect of PS- and PO-CpG DNAs on NF-κB activatation in J774 cells. J774 cells were stimulated with CpG DNAs **1–4** (lanes 2–5, respectively) at 10 μg/mL concentration for 24 h, nuclear extracts were prepared and analyzed on a native polyacrylamide gel as described in Materials and methods. Lane 1 corresponds to medium control and NS stands for non-specific band. NF-κB band is indicated with an arrow.

highest concentration tested (10 μg/mL), both induced significantly less IL-6 than did PS-CpG DNA **1** (Fig. 3B). The ratio of IL-12 to IL-6 secreted is calculated to distinguish cytokine secretion profiles of PS- and PO-CpG DNAs (Fig. 3A, inset). In addition, the C3H/HeJ spleen cell culture results suggest that the responses observed with CpG DNAs are not due to LPS contamination and that they do not signal through the TLR4 receptor.

Activation of NF-κB and induction of cytokine secretion in J774 cell cultures by PO-immunomers

CpG DNA recognition by TLR9 has been shown to activate a number of signaling pathways, including the NF-κB pathway, which plays a critical role in the up-regulation of cytokine gene expression [9]. To study the effect of PO-immunomers on activation of NF-κB, J774 cells were stimulated with CpG DNAs **1–4** for 1 h and the nuclear extracts were examined by EMSA for NF-κB activation. The results are presented in Fig. 4. The presence of complexes that correspond to NF-κB in lane 2 suggests that PS-CpG DNA **1** activated the transcription factor NF-κB. The absence of a band in lane 3 suggests that linear PO-CpG DNA **2** failed to activate NF-κB, consistent with the cytokine secretion results obtained in BALB/c mouse spleen cell cultures with this CpG DNA. The failure of **2** to activate NF-κB further suggests that it is degraded in less than 1 h in J774 cells. In contrast, both PO-immunomers **3** and **4** showed strong activation of the transcription factor NF-κB, as indicated by the presence of corresponding bands in lanes 4 and 5, respectively. Consistent with NF-κB activation, PO-immunomers **3** and **4** and PS-CpG DNA **1** induced IL-12 and IL-6 secretion in J774 cell cultures (data not shown).

In vivo antitumor activity of PO-immunomer

PS-CpG DNAs have been shown to induce potent antitumor activity in vivo. Since PO-CpG DNAs exhibited greater nuclease stability and induced higher levels of IL-12 and IFN-γ secretion in in vitro assays, we were interested to see if these desirable properties of PO-immunomers improve the antitumor activity in vivo. We administered PO-immunomer **4** subcutaneously at a dose of 0.5 mg/kg every other day to nude mice bearing MCF-7 breast cancer xenografts that express wild-type p53 or DU-145 prostate cancer xenografts that express mutated p53. PO-immunomer **4** gave 57% growth inhibition of MCF-7 tumors on day 15 compared with the saline control (Fig. 5A). It also produced 52% growth inhibition of DU-145 tumors on day 34 (Fig. 5B). These preliminary antitumor studies suggest that PO-immunomers of the proposed design exhibit potent antitumor activity in vivo as a result of their increased nuclease

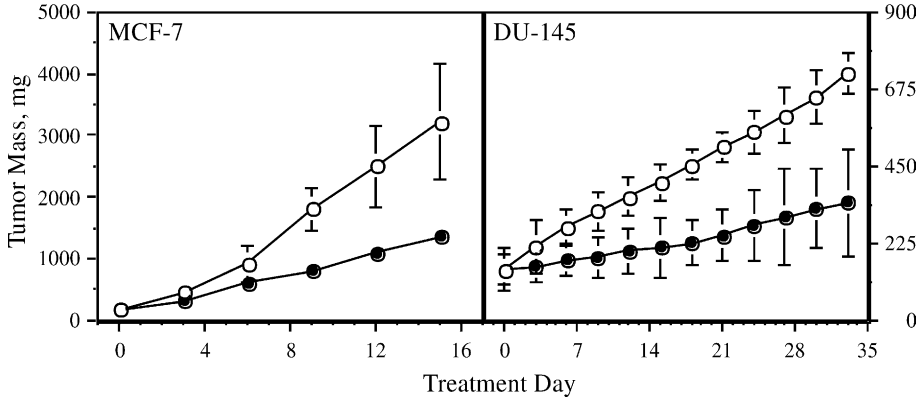


Fig. 5. Antitumor activity of PO-immunomer **4** in mice bearing human breast (MCF-7) and prostate (DU145) cancer xenografts. In both panels, open and filled circles represent saline control and PO-immunomer **4** treated groups, respectively.

stability and perhaps through induction of IL-12 and IFN-γ secretion.

Discussion

PO-CpG DNAs are rapidly degraded by nucleases limiting their utility for in vivo therapeutic applications. PO-CpG DNAs containing palindromic structures, which provide some nuclease stability, have been used in the past [20,21]. The addition of poly(dG) sequences at the 3′-end of PO-CpG DNAs to provide additional nuclease stability have also been studied [22–27]. However, the inclusion of poly(dG) sequences in oligonucleotides could pose a number of problems, including their synthesis and purification [45], unpredictable secondary structure formation, depending on the experimental conditions, and non-sequence-specific protein binding, including immune stimulation [45–47].

Nuclease degradation of oligonucleotides occurs primarily from the 3′-end. In this report, we attached PO-CpG DNAs through their 3′-ends to prevent nuclease degradation and to provide optimal accessible 5′-ends for immunostimulatory activity [28]. The PO-immunomer design proposed in here is the most appropriate for immunostimulatory applications without the need for palindromic structures and poly(dG) sequences. The data presented in this paper clearly demonstrate that PO-immunomers are stable against nuclease degradation and induce potent immunostimulatory activities both in vitro and in vivo.

Upon internalization and endosomal localization through non-sequence-specific mechanisms, CpG DNA is sequence-specifically recognized by receptor(s) [6,48,49]. Following receptor recognition of CpG DNA, signaling pathways are rapidly triggered, leading to the activation of multiple transcription factors, including NF-κB and AP-1 [8,9]. In fact, the activation and nuclear translocation of NF-κB have been reported to occur within minutes of CpG DNA stimulation of the immune cells [8,50]. The present in vitro and in vivo studies suggest that the greater stability of PO-immunomers compared with parent PO-CpG DNA (**2**) is sufficient to induce immunostimulatory activities. Additionally, improved immunostimulatory activity of PO-immunomers could result from the presence of two accessible 5′-ends as reported earlier [28]. Recently, we have shown that the presence of ligands of smaller than a di-or trinucleotide at the 5′-end of a CpG DNA does not block 5′-end accessibility to receptors [51]. Consistent with those reports, the incorporation of C3-linkers at the 5′-ends of PO-immunomer **4** did not interfere with immunostimulatory activity, but provided additional nuclease stability.

CpG DNAs containing PO palindromic sequences and PO or PS poly(dG) sequences have been shown to produce immune responses [20–27] that are distinct from those produced by PS-CpG DNAs. However, it was not clear whether the responses were a result of the presence of palindromic PO-CpG DNA, the poly(dG) sequence, or the secondary structures formed by both. A recent study showed that the substitution of poly(dG) sequences in PO-CpG DNAs with poly(T) sequences diminishes immunostimulatory activity [26], suggesting that the effects observed could be mostly as a result of the presence of G-rich sequences or the secondary structures formed by such sequences. The PO-immunomers described in the present study allowed us to determine the effects of PO-CpG DNA without interference from such structural motifs and without rapid nuclease degradation in vivo. To our knowledge, this is the first study demonstrating in vitro and in vivo immunostimulatory effects of single-stranded PO-CpG DNAs without poly(dG) sequences.

The induction of different cytokine secretion patterns by PO- and PS-CpG DNAs suggests that they either activate different subsets of immune cells or different receptor(s) or pathways. The distinct immunostimulatory properties of these modified novel CpG DNAs may enable the design of immunotherapeutic agents to induce selected cytokines to treat specific diseases. Tumor growth inhibition seen with PO-immunomer **4** in nude mice further suggests that PO-immunomers exhibit potent antitumor activity in vivo consistent with in vitro nuclease stability and selective cytokine secretion properties. In addition, the selective induction of Th1-type cytokines by PO-immunomers could be of an advantage for their use as adjuvants with vaccines [25,27] and as anti-asthmatic/allergic agents.

Previously, we showed that it is possible to substitute natural C and G bases of the CpG dinucleotide in CpG DNA with certain synthetic pyrimidine (Y) or purine (R) analogs and design potent immunostimulatory oligonucleotides containing synthetic YpG or CpR dinucleotides [52,53]. Our preliminary results suggest that PO-immunomers containing these synthetic motifs also show immunostimulatory activity. The combination of these synthetic motifs with other immunomodulatory moieties in the flanking sequences [53] of PO-immunomers, such as those used in **4**, may lead to more potent immunostimulatory agents.

In conclusion, the present study showed that: (i) PO-immunomers with or without certain end chemical modifications have higher nuclease stability, (ii) PO-immunomers induce higher levels of IL-12 and minimal amounts of IL-6 secretion, (iii) self-complementary sequences are not required for immunostimulatory activity of PO-CpG DNAs, and (iv) secondary structure-forming poly(dG) sequences are also not obligatory in PO-CpG DNAs for their nuclease stability, cellular uptake, and subsequent immunostimulatory activity. PO-immunomers containing human-specific CpG motifs are being evaluated currently.

Acknowledgments

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