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Aggregation and Secondary Loop Structure of Oligonucleotides do not Determine Their Ability to Inhibit TLR9

Robert F. Ashman^a, J. Adam Goeken^a, and Petar S. Lenert^a

^aDepartment of Internal Medicine, Carver College of Medicine, The University of Iowa, Iowa City, IA 52242, USA

Abstract

Toll-like receptor 9 (TLR9) is an endosomal DNA sensor that warns us of the presence of infectious danger and triggers a rapid pro-inflammatory response in dendritic cells, macrophages and B cells. The consequences of uncontrolled TLR9 activation can be detrimental for the host, contributing to the pathogenesis of bacterial septic shock or autoimmune diseases, such as Systemic Lupus Erythematosus. Therefore, we need to develop TLR9 antagonists. We and others have created inhibitory oligonucleotides (INH-ODN) that are capable of sequence-dependent inhibition of TLR9-induced activation in both human and mouse cells. But it is not clear whether marked differences in INH-ODN activity related to base sequence derived from polymerization of INH-ODNs, or their ability to complex with stimulatory CpG-oligonucleotides (ST-ODN). Furthermore, the 5' end of INH-ODNs may assume a particular loop configuration that may be needed for binding to a critical site on TLR9. Here we show that: 1) G-tetrads required for ODN stacking were compatible with INH-ODN activity, but were not necessary; 2) There was no relationship between activity and self-association at endosomal pH; 3) There was no evidence for direct binding between ST-ODNs and INH-ODNs; 4) When a 3 G sequence was disrupted, despite a preserved stem-loop formation, INH-ODN activity was abolished. These results support the conclusion that certain features of the primary linear sequence are critical for TLR9 inhibition, but changes in secondary structure or in ODN aggregation are irrelevant.

Keywords

Toll like receptor 9; B lymphocytes; Inhibitory oligonucleotides; CpG-DNA

1. Introduction

An important early warning of the presence of bacteria and viruses is provided by the recognition of their DNA and RNA by Toll-like receptors (TLRs) 3, 7, 8 and 9 [1]. These TLRs differ from the other TLRs in that they are active in endosomes, rather than on the cell surface [2,3]. Thus the response of TLRs to nucleic acids in the environment depends on delivery of nucleic acids to endosomes either by passive uptake [4], via B cell receptor for antigen [5,6], or (as immune complexes) by the Fc receptors of dendritic cells [7,8]. A main reason why self DNA and RNA fail to trigger TLRs in B cells is their normal inability to

Corresponding author: P. Lenert, M.D., Ph.D., Department of Internal Medicine, Carver College of Medicine, 200 Hawkins Drive, C428-2GH, Iowa City, IA 52242, USA, petar-lenert@uiowa.edu, Phone: 1-319-353-7698, Fax: 1-319-353-6290.

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access endosomes. When this barrier is overcome, as through the co-engagement of B cell receptor for antigen, they become stimulatory [6,9,10]. Once stimulatory DNA is in the endosome, TLR9 is recruited there from storage in the endoplasmic reticulum [11] an event facilitated by the cytoplasmic protein UNC93b1 [12,13].

The structural requirements for triggering TLR9 have been elucidated, largely by the study of synthetic single-stranded DNA [14]. Base sequence and demethylation of certain cytosines proved critical as an optimal stimulatory motif was described as “purine, purine, unmethylated cytosine, guanine, pyrimidine, pyrimidine (The “CpG motif”) [15]. Six base sequences containing this motif appear in mammalian DNA about 1/25 as frequently as in bacterial DNA, whereas other six-base sequences had roughly equal frequency [15]. Other sequence features were described that further increased the rarity of the CpG motif [16] but never as much as 2 logs, raising the fear that high concentrations of mammalian DNA might still stimulate TLR9 (as in crush injuries). It would be comforting to have a protective inhibitor in the system to prevent activation by self DNA.

Indeed it was shown that poly G-containing deoxyribonucleotides (ODN) could block DNA-induced activation, including that driven by stimulatory (ST-) ODN bearing the CpG motif [17,18]. This effect was otherwise ODN sequence non-specific, required high micromolar concentrations of inhibitory (INH-) ODN, and also blocked signaling pathways other than TLR9 [19]. INH-ODNs with greater sequence and TLR9 specificity were then described by Krieg et al [20,21], culminating in our extensive structure-function studies of single-stranded ODN to define the differences between INH-ODN and ST-ODN [16,22-25]: 1) ST-ODNs require the unmethylated CpG, whereas INH-ODNs do not. 2) For inhibition or stimulation a CC is required at the 5' end. An extra 5' T is required for stimulation, but not for inhibition. 3) A sequence of 3G's is required for INH-ODN, with 3 to 5 covalently-linked bases intervening between the 3G's and the 5' CC [16]. At two of these intervening positions, Cs greatly reduce activity [23]. 4) Base choice at the other positions is much less important [23]. 5) The shortest ODN with substantial inhibitory activity is thus a 10-mer: CC x (not C) (not C) x GGGG where x is any base [23]. 6) Duplication of the 5' end can enhance activity 3-6 fold [25]. 7) Orientation is critical, for when the order of bases was reversed, INH-ODN activity was 99.9% lost [24].

What do these strict sequence requirements tell us about the mechanism of action of INH-ODN? There is evidence that avidity of TLR9 binding is not strictly dependent on base sequence and is a poor predictor of biologic activity [26]. So, could the sequence requirements be necessary for INH-ODN to polymerize, complex with ST-ODN, or assume a particular secondary structure needed to bind to a critical site?

2. Materials and Methods

2.1. Oligonucleotides

Sigma Genesis (St Louis, MO) and Integrated DNA Technologies (Coralville, IA) synthesized and purified our phosphorothioate (pS) and phosphodiester (pO) ODN. The prototype ST-ODN sequence for human cells is 5'-TCGTCGTTTTGTCGTTTTGTCGTT-3', numbered 2006 with a phosphorothioate (pS-) backbone and 2059 with a native phosphodiester (pO-) backbone [27,28]. The standard concentrations used were 100nM for 2006 and 10 μ M for 2059, chosen for their ability to provide equal suboptimal stimulation.

The prototype inhibitory sequence is 2114: 5'-TCCTGGAGGGGAAGT-3'. The pS- form of this sequence is a strong inhibitor of ST-ODN in both mouse and human cells used in previous studies [23,24]. Titrations were routinely performed with 8 concentrations 0.5 log

apart from 0.3 to 1000 nM. The pO- form of this same sequence, 4228, was also used at 8 concentrations 0.5 log apart, but the range used was higher: 185 to 15000 nM. Sequences of the other INH-ODN used appear in Figs 1 and 3.

2.2. Definitions

“Potency” is the term for the concentration of an INH-ODN which inhibits the optimal stimulation by 50%. “% activity” refers to potency of the prototype INH-ODN (2114 or 4228) / potency of the test INH-ODN. Thus the % activity of 2114 is set at 100%. “Critical areas” refer to 3 pairs of bases in the INH-ODN sequence where single base changes caused the most profound decrease in activity in mouse B cells [16,23] underlined in the following sequence of 2114:

CC is required at area 1. Anything except C will work at area 2. One additional adjacent G is required at area 3, though it may be 5' or 3' to the G pair underlined. ST-ODN share with INH-ODN the requirement for areas 1 and 2, but differ at area 3 by requiring CGTT in place of xGGG (x is any base, A in 2114). Whereas base changes in critical areas may reduce activity by 2 logs or more, changes at other locations generally produce <2 fold activity changes [23]. In the nM range sequence-specific inhibition is restricted to TLR9 [22], but backbone dependent sequence-independent inhibition of TLR7 is seen [29]. At concentrations of pS-INH-ODN over 25 μ M, backbone mediated inhibition may be observed. Δ G (Gibbs free energy of bonding) and T_m (temperature of dissociation) were derived from m-fold software (<http://mfold.rna.albany.edu/?q=mfold/download-mfold>).

2.3. Cells

The Namalwa human B cell line from ATCC (Manassa, VA) was maintained in RPMI 1640 with phenol red (GIBCO-BRL, Life Technologies, Gaithersburg, MD), 7.5% FBS (Atlanta Biologicals, Atlanta, GA), 10 mM Hepes, 2mM l-glutamine, 1mM sodium pyruvate, and 1 $\times$ penicillin-streptomycin at 37° and 5%CO₂. Mouse B cells were isolated from the spleens of 8-12 week old B₆D₂F₁ mice obtained from Jackson Laboratories, Bar Harbor, ME. Following RBC lysis of whole splenocytes, B cells were negatively selected with anti-CD43-coated magnetic beads (Midi-Macs from Miltenyi Biotec, Auburn, CA). The yield of 25 $\times$ 10⁶ total B cells per spleen consisted of (>97% CD19⁺ cells, of which 1% was apoptotic, 1% was in G₁ and 98% was in G₀ by acridine orange flow cytometry. Purified B cells were cultured at 1.5 $\times$ 10⁶ ml in RPMI 1640 (GIBCO-BRL, Life Technologies, Gaithersburg, MD), 5% FCS.

2.4. Assays for B cell Activation

At a concentration of 2 $\times$ 10⁵ cells/ml Namalwa cells were plated in 24-well tissue culture plates with ST-ODN and a panel of INH-ODN at the concentrations listed above, and then incubated for 48h. Cells were harvested and washed 2 times with phosphate-buffered saline (PBS) 2% fetal bovine serum (FCS) by centrifugation at 300 $\times$ g for 10 min. The resulting pellets were resuspended, incubated for 15 min in 60 μ l PBS, 2% FCS and 20 μ l affinity purified human Fc γ R-binding inhibitor (eBioscience, San Diego, CA), and then stained with PE conjugated anti-human CD86. Flow cytometric analysis to determine the expression of the co-stimulatory molecule CD86 was performed on a Becton Dickinson FACScan. Namalwa CD86 expression was selected as the assay providing the most reproducible data in pilot experiments, which included the measurement of IL-6 and IgM production. Mouse B

cell culture supernatants were tested for IL-6 using antibodies obtained from eBioscience (San Diego, CA). Clones MP5-20F3 anti-IL-6 and MP5-32C11 anti IL-6 biotin were used. Assay sensitivity was 16 pg/ml.

2.5. Native Polyacrylamide Gel Electrophoresis (PAGE)

To study aggregation of INH-ODN and ST-ODN with themselves and each other, ODN were analyzed by native polyacrylamide gel electrophoresis. Lyophilized oligonucleotides were dissolved in sterile distilled water to a concentration of 1 µg/µl. Two µl of each were loaded in 1xTris-HCl pH 6.8, 10% (v/v) glycerol on a native gel (20% polyacrylamide 1xTBE) at pH 8.0, 7.5, 7.0, 6.5, or 6.0, and were electrophoresed at 150 V, 80 mA for 120 minutes. The gel was stained overnight using Stains-All (Sigma-Aldrich) 0.005% (w/v) 25% isopropanol, 20 mM Tris-HCl pH 8.0, and 75% (v/v) H₂O.

To reduce secondary structure, ODN were exposed to high heat (100°C) for 10 minutes then rapidly cooled (-80°C for 30 minutes), and immediately loaded on to a gel and electrophoresed as previously described. The possibility of delayed INH-ODN and ST-ODN interaction was determined by co-incubating 10 µl (50 µg/ml concentration) of each in PBS pH 6.0 at 37°C for 48 hours.

3. Results

3.1. Homotypic and Heterotypic Aggregation

Since TLR9 exists largely as a dimer in unstimulated cells [26], it is possible that ODN self aggregation could permit interaction with TLR9 not possible for the monomer form. Furthermore, sequence requirements for INH-ODN activity derived first in mouse B cells showed the importance of GGGG and CC areas properly spaced and oriented [22-24], suggesting the possibility that activity required head-to-tail dimerization. By native PAGE at a typical endosomal pH of 6.0, we saw ODN varied in their degrees of aggregation (Fig 1A). The fastest band (on the right) should be the monomer with the various multimers migrating more slowly. For example, 2114 would be expected to form multimers by planar stacking of the poly G region [30,31]. 4347/4378 is 4 bases longer than 2114/4228 and therefore migrates more slowly; it is also 3 to 4 times more powerful than 2114/4228 (Table 1). 2114R and 4325 are biologically inactive ODN (<1% of the activity of 2114 or 4347) containing the same base sequence as 2114 and 4347 (respectively) in reverse order. 2114R has a pS backbone and 4325 a pO-backbone; as expected the pO form migrates faster than the pS. No relationship of aggregation to activity was seen in Fig 1A, nor in other experiments performed at pH 6.5 (Fig 1B) or higher pHs (not shown), although pH changes did affect the degree of INH ODN self-aggregation. When ST-ODN and INH-ODN were mixed using pS ODN at pH 6.0 (Fig 1C) or pS ODN at pH 7.0 (Fig 1D), or pO ODN at pH 6.0 (Fig 1E) no evidence of interaction between stimulator or inhibitor was seen. Further studies at other pHs show that this conclusion was not confined to a narrow pH range. The activity of ODN 4033 and 4329 were around 5 to 6 times higher than the prototype INH ODNs 2114 and 4228 [32]. There was still no evidence of ST-ODN aggregation with INH-ODN after incubating them together for 48h (Fig 1F).

INH-ODN with pS or pO backbones were boiled for 10 minutes and rapidly cooled to reduce ODN aggregation and secondary structure. When these INH-ODN were tested as inhibitors of CD86 induction in Namalwa cells, their activity was actually a little greater than the un-boiled, partly multimeric preparation (Table 1).

3.2. Secondary Structure

An alternative to dimerization compatible with monomer activity is the possibility that the CC (area 1) and GGGG (area 3) interact to form a hairpin structure, in which case the loop might be the key structure for binding, and resistance to dissociation of the hairpin might relate to activity. Because our usual prototype, 2114, has 4Gs and therefore can engage in G-tetrad stacking (see aggregation in Fig 1A-D), to eliminate G-tetrad stacking we designed a similar ODN 4024 with T substituted for one of the 4 Gs leaving 3 Gs (diagrammed in Fig 3A). Fig 2 shows that in Namalwa cells, INH-ODNs 4024 and 2114 showed identical activity.

Fig 3 A-B presents 5 variants of 4024 which share the same base composition, loop sequence and lengths. INH-ODNs 4024 A, B, C, and D have stronger bonding (ΔG) between the critical areas than 4024 because they have 3 C-G bonds instead of 2 (Fig 3B). As expected, the ΔG s and melting temperature of 4024A, B, C and D were very similar. Polyacrylamide gels showed all the variants had the same mobility pattern as 4024 showing a single band (Fig 3C). However, as inhibitors of ST-ODN 2084-driven IL-6 secretion in mouse B cells (Fig 3D), or of ST-ODN 2006 driven CD86 expression in human Namalwa cells (Fig 3E), INH-ODNs 4024 and 4024A showed equally strong activity, 4024B was 1 to 1.5 logs weaker, and 4024C and D were even weaker (3 logs weaker according to the human cell data). Thus the primary sequence rules previously described [23, 25] determined activity in both mouse and human B cells, not G-stacking, the loop sequence, or the strength of hairpin formation.

4. Discussion

4.1. Association between ODNs

Acidification of endosomes is thought to be essential for TLR9 activity. One mechanism by which anti-malarial drugs (chloroquine) block TLR9 activation [33] is their ability to prevent endosomal acidification/maturation [2]. Many INH-ODNs exist as both monomers and multimers in the pH range 6.0 to 7.0 probably as a result of G-tetrad-stacking [31]. Even with a small number of comparisons, the lack of relationship of INH-ODN aggregation to activity or of pH to activity was convincing (Fig 1A-D). That 4024 (a monomer in Fig 3C which has only 3 consecutive Gs) and 2114 (which has the G-tetrad and was self-aggregated in Fig 1A-D) have identical INH-ODN activity (Fig 2) further reinforces this conclusion.

There is evidence that self-aggregating pO ST-ODN tend to be more active than linear monomeric forms, perhaps because they can crosslink dimer TLR9 ectodomains [34]. In contrast, for INH-ODN, physical separation of monomers from multimers demonstrates that monomers are active [35], in agreement with the lack of correlation between inhibitory activity and multimerization shown in Figs 1,2 and 3. An extensive comparison of different INH-ODN sequences showed that ST-ODN and INH-ODN must share areas 1 and 2 in the 5' half, but have contrasting requirements in the central CG and adjacent area 3. Since inhibition is competitive, these results suggested a model where areas 1 and 2 compete for binding to a critical site, whereas area 3 determines the type of signal [16,23], recalling the 2-site model suggested for ST-ODN [34]. Evidence that the linear monomeric form of INH-ODN is the active form suggest that covering one site on TLR9 may prevent the formation of signaling configuration of the ST-ODN/TLR9 complex. Alternatively, if monomer INH-ODNs can link molecules with ODN- binding properties, they would have to do so using different areas of the same linear sequence (sample models in ref. [16,23]. This could be the role of the activity-enhancing 5' extension seen in 4347/4378 (Table 1,ref [25,32]).

Another way that INH-ODN could exert sequence-specific inhibition is for the strongest inhibitors to bind more avidly to ST-ODN, blocking their ability to bind or bend TLR9 [26].

However, at least in the absence of TLR9, we observed no association between INH-ODN and ST-ODN at molar ratios that would have shown major inhibition in a biologic assay (Fig 2) and at endosomal pH (Fig 1C and E), even when co-incubated for 48h (Fig. 1F). A range of pH from 6.0-8.0 was studied without altering that conclusion (data not shown).

An alternative explanation of sequence-specific effects on INH-ODN activity is that active sequences bind more avidly to TLR9. Latz and coworkers have shown that there is no relationship between avidity for TLR9 and ST-ODN activity [11,26] as binding avidity and the ability to effect a TLR9 conformational change are both sequence-insensitive. Our recent data show that this is also true for INH-ODN with pO backbones tested in Namalwa cells [32].

4.2. Secondary Structure

The polyacrylamide gel experiments ruled out important activity-related sequence-specific effects of ODN-ODN interaction, but left open the possibility that certain secondary structures are required for activity. Indeed, the requirement for CC at critical area 1 and at least 3 sequential Gs in area 3 [23] suggested that the forbidden CC at area 2 might displace the CC at area 1, altering the loop into an inactive conformation. In that case, both the base sequence of the loop and the stability of the hairpin would be expected to relate to activity. INH-ODN 4024 has activity indistinguishable from that of 2114 in both mouse and human B cells (Fig 2). It was selected because it lacked the G-tetrad; furthermore it was possible to keep its loop sequence constant while varying the strength and pattern of C-G bonds (Fig. 3A, B). Mouse B cell IL-6 secretion and human Namalwa cell CD86 expression showed a similar pattern of relative inhibition by the 4024 variants (Fig 3D, E). Wide differences in inhibitory activity were shown by ODN with similar ΔG s and identical loop sequences (compare Fig 3D, E), demonstrating the strong dominance of linear sequence differences over secondary structure in determining activity. Importantly, all 4024 variants tested also had 4 nucleotide long 3' overhangs that could have allowed easier access to certain helicases, opening the 5' loop. Evidently, activity is independent of this feature as well.

Brief boiling has been shown to dissociate hairpin and dimer forms of INH-ODN [36]. While the exact changes in secondary structure caused by boiling 2114 remain unknown, they resulted in an increase rather than a decrease in activity (Table 1). This result is consistent with previous evidence that isolated monomer INH-ODN are more active than multimers formed by G-stacking [35].

Together with the evidence that INH-ODN and ST-ODN bind TLR9 (and perhaps an array of other DNA sensors in the cell) by their backbone [26,37], these data imply that once mounted on TLR9, the linear sequence must be recognized by another molecule. Two candidates for this molecule already known include UNC93b1 [12,13], which recruits TLR9 to the endosome (that INH-ODN only act within the endosome has not been proven), and an endopeptidase that cleaves TLR9 into its active form [38,39]. Interference with gene regulatory molecules downstream of TLR9 is unlikely to be the target since INH-ODN block the activation of NF- κ B [40] and AP-1 [41].

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References

1. Takeda K, Kaisho T, Akira S. Toll-like receptors. *Annu Rev Immunol*. 2003; 21:335–76. [PubMed: 12524386]

2. Hacker H, Mischak H, Miethke T, Liptay S, Schmid R, Sparwasser T, et al. CpG-DNA-specific activation of antigen-presenting cells requires stress kinase activity and is preceded by non-specific endocytosis and endosomal maturation. *EMBO J*. 1998; 17:6230–40. [PubMed: 9799232]
3. Ahmad-Nejad P, Hacker H, Rutz M, Bauer S, Vabulas RM, Wagner H. Bacterial CpG-DNA and lipopolysaccharides activate Toll-like receptors at distinct cellular compartments. *Eur J Immunol*. 2002; 32:1958–68. [PubMed: 12115616]
4. Stacey KJ, Sweet MJ, Hume DA. Macrophages ingest and are activated by bacterial DNA. *J Immunol*. 1996; 157:2116–22. [PubMed: 8757335]
5. Chaturvedi A, Dorward D, Pierce SK. The B cell receptor governs the subcellular location of Toll-like receptor 9 leading to hyperresponses to DNA-containing antigens. *Immunity*. 2008; 28:799–809. [PubMed: 18513998]
6. Leadbetter EA, Rifkin IR, Hohlbaum AM, Beaudette BC, Schlomchik MJ, Marshak-Rothstein A. Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. *Nature*. 2002; 416:603–7. [PubMed: 11948342]
7. Boulé MW, Broughton C, Mackay F, Akira S, Marshak-Rothstein A, Rifkin IR. Toll-like receptor 9-dependent and -independent dendritic cell activation by chromatin-immunoglobulin G complexes. *J Exp Med*. 2004; 199:1631–40. [PubMed: 15197227]
8. Means TK, Latz E, Hayashi F, Murali MR, Golenbock DT, Luster AD. Human lupus autoantibody-DNA complexes activate DCs through cooperation of CD32 and TLR9. *J Clin Invest*. 2005; 115:407–17. [PubMed: 15668740]
9. Brummel R, Roberts TL, Stacey KJ, Lenert P. Higher-order CpG-DNA stimulation reveals distinct activation requirements for marginal zone and follicular B cells in lupus mice. *Eur J Immunol*. 2006; 36:1951–62. [PubMed: 16791898]
10. Barton GM, Kagan JC, Medzhitov R. Intracellular localization of Toll-like receptor 9 prevents recognition of self DNA but facilitates access to viral DNA. *Nat Immunol*. 2006; 7:13–5. [PubMed: 16357851]
11. Latz E, Schoenemeyer A, Visintin A, Fitzgerald KA, Monks BG, Knetter CF, et al. TLR signals after translocating from the ER to CpG DNA in the lysosome. *Nat Immunol*. 2004; 5:190–8. [PubMed: 14716310]
12. Brinkmann MM, Spooner E, Hoebe K, Beutler B, Ploegh HL, Kim YM. The interaction between the ER membrane protein UNC93B and TLR3, 7, and 9 is crucial for TLR signaling. *J Cell Biol*. 2007; 177:265–75. [PubMed: 17452530]
13. Tabeta K, Hoebe K, Janssen EM, Du X, Georgel P, Crozat K, et al. The Unc93b1 mutation 3d disrupts exogenous antigen presentation and signaling via Toll-like receptors 3, 7 and 9. *Nat Immunol*. 2006; 7:156–64. [PubMed: 16415873]
14. Krieg AM, Yi AK, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R, et al. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature*. 1995; 374:546–9. [PubMed: 7700380]
15. Krieg AM. CpG motifs in bacterial DNA and their immune effects. *Annu Rev Immunol*. 2002; 20:709–60. [PubMed: 11861616]
16. Lenert P, Goeken JA, Ashman RF. Extended sequence preferences for oligodeoxyribonucleotide activity. *Immunol*. 2006; 117:474–81.
17. Halpern MD, Pisetsky DS. In vitro inhibition of murine IFN gamma production by phosphorothioate deoxyguanosine oligomers. *Immunopharmacology*. 1995; 29:47–52. [PubMed: 7768671]
18. Pisetsky DS, Reich CF. Inhibition of murine macrophage IL-12 production by natural and synthetic DNA. *Clin Immunol*. 2000; 96:198–204. [PubMed: 10964537]
19. Zhu FG, Reich CF, Pisetsky DS. Inhibition of murine dendritic cell activation by synthetic phosphorothioate oligodeoxynucleotides. *J Leukoc Biol*. 2002; 72:1154–63. [PubMed: 12488497]
20. Krieg AM, Wu T, Weeratna R, Efler SM, Love-Homan L, Yang L, et al. Sequence motifs in adenoviral DNA block immune activation by stimulatory CpG motifs. *Proc Natl Acad Sci U S A*. 1998; 95:12631–6. [PubMed: 9770537]
21. Chen Y, Lenert P, Weeratna R, McCluskie M, Wu T, Davis HL, et al. Identification of methylated CpG motifs as inhibitors of the immune stimulatory CpG motifs. *Gene Ther*. 2001; 8:1024–32. [PubMed: 11438837]

22. Stunz LL, Lenert P, Peckham D, Yi AK, Haxhinasto S, Chang M, et al. Inhibitory oligonucleotides specifically block effects of stimulatory CpG oligonucleotides in B cells. *Eur J Immunol.* 2002; 32:1212–22. [PubMed: 11981808]
23. Ashman RF, Goeken JA, Drahos J, Lenert P. Sequence requirements for oligodeoxyribonucleotide inhibitory activity. *Int Immunol.* 2005; 17:411–20. [PubMed: 15746247]
24. Lenert P, Rasmussen W, Ashman RF, Ballas ZK. Structural characterization of the inhibitory DNA motif for the type A(D)-CpG-induced cytokine secretion and NK-cell lytic activity in mouse spleen cells. *DNA Cell Biol.* 2003; 22:621–31. [PubMed: 14611683]
25. Ashman RF, Lenert P. Structural requirements and applications of inhibitory oligodeoxyribonucleotides. *Immunol Res.* 2007; 39:4–14. [PubMed: 17917051]
26. Latz E, Verma A, Visintin A, Gong M, Sirois CM, Klein DC, et al. Ligand-induced conformational changes allosterically activate Toll-like receptor 9. *Nat Immunol.* 2007; 8:772–79. [PubMed: 17572678]
27. Hartmann G, Krieg AM. Mechanism and function of a newly identified CpG DNA motif in human primary B cells. *J Immunol.* 2000; 164:944–53. [PubMed: 10623843]
28. Hartmann G, Weeratna RD, Ballas ZK, Payette P, Blackwell S, Suparto I, et al. Delineation of a CpG phosphorothioate oligodeoxynucleotide for activating primate immune responses in vitro and in vivo. *J Immunol.* 2000; 164:1617–24. [PubMed: 10640783]
29. Lenert P, Yasuda K, Busconi L, Nelson P, Fleenor C, Ratnabalasuriar RS, et al. DNA-like Class R inhibitory oligonucleotides (INH-ODNs) preferentially block autoantigen-induced B-cell and dendritic cell activation in vitro and autoantibody production in lupus-prone MRL-Fas^{lpr/lpr} mice in vivo. *Arthritis Res Ther.* 2009; 11:R79. [PubMed: 19476613]
30. Laughlan G, Murchie AIH, Norman DG, Moore MH, Moody PCE, Lilley DMJ, et al. The high resolution crystal structure of a parallel-stranded guanine tetraplex. *Science.* 1994; 265:520–5. [PubMed: 8036494]
31. Benemetskaya L, Berton M, Kolbanovsky A, Benimetsky S, Stein CA. Formation of a G-tetrad and higher order structures correlates with biological activity of the RelA (NF- κ B p65) “antisense” oligodeoxynucleotide. *Nucl Acids Res.* 1997; 25:2648–56. [PubMed: 9185577]
32. Ashman RF, Goeken JA, Latz E, Lenert P. Optimal Oligonucleotide Sequences for TLR9 Inhibitory Activity in Human Cells: Lack of correlation with TLR9-binding. *Int Immunol.* 2011 in press.
33. Yi AK, Peckham DW, Ashman RF, Krieg AM. CpG DNA rescues B cells from apoptosis by activating NF κ B and preventing mitochondrial membrane potential disruption via a chloroquine-sensitive pathway. *Int Immunol.* 1999; 11:2015–24. [PubMed: 10590267]
34. Wu CC, Lee J, Raz E, Corr M, Carson DA. Necessity of oligonucleotide aggregation for toll-like receptor 9 activation. *J Biol Chem.* 2004; 279:33071–8. [PubMed: 15184382]
35. Duramad O, Fearon KL, Chang B, Chan JH, Gregorio J, Coffman RL, et al. Inhibitors of TLR-9 act on multiple cell subsets in mouse and man in vitro and prevent death in vivo from systemic inflammation. *J Immunol.* 2005; 174:5193–200. [PubMed: 15843514]
36. Goeken JA, Layer T, Fleenor S, Laccheo M, Lenert P. B-cell receptor for antigen modulates B-cell responses to complex TLR9 agonists and antagonists: implications for systemic lupus erythematosus. *Lupus.* 2010; 19:1290–301. [PubMed: 20605877]
37. Haas T, Metzger J, Schmitz F, Heit A, Müller T, Latz E, et al. The DNA sugar backbone 2' deoxyribose determines toll-like receptor 9 activation. *Immunity.* 2008; 28:315–23. [PubMed: 18342006]
38. Ewald SE, Lee BL, Lau L, Wickliffe KE, Shi GP, Chapman HA, et al. The ectodomain of Toll-like receptor 9 is cleaved to generate a functional receptor. *Nature.* 2008; 456:658–62. [PubMed: 18820679]
39. Sepulveda FE, Maschalidi S, Colisson R, Heslop L, Ghirelli C, Sakka E, et al. Critical role for asparagine endopeptidase in endocytic Toll-like receptor signaling in dendritic cells. *Immunity.* 2009; 31:737–48. [PubMed: 19879164]
40. Lenert P, Stunz L, Yi AK, Krieg AM, Ashman RF. CpG stimulation of primary mouse B cells is blocked by inhibitory oligodeoxyribonucleotides at a site proximal to NF- κ B activation. *Antisense Nucleic Acid Drug Dev.* 2001; 11:247–56. [PubMed: 11572601]

41. Lenert P, Yi AK, Krieg AM, Stunz LL, Ashman RF. Inhibitory oligonucleotides block the induction of AP-1 transcription factor by stimulatory CpG oligonucleotides in B cells. *Antisense Nucleic Acid Drug Dev.* 2003; 13:143–50. [PubMed: 12954114]

Abbreviations

TLR	Toll-like receptor
INH-ODN	inhibitory oligonucleotide
ST-ODN	stimulatory CpG oligonucleotide
pO	phosphodiester
pS	phosphorothioate
nM	nanomolar

A

2114	TCCTGGAGGGGAAGT	pS
2114R	TGAAGGGGAGGTCCT	pS
4228	TCCTGGAGGGGAAGT	pO
4325	TGAAGGGGAGGTCCT	pO
4347	CCTATCCTGGAGGGGAAGT	pS
4378	CCTATCCTGGAGGGGAAGT	pO

B

2114	TCCTGGAGGGGAAGT	pS
2114R	TGAAGGGGAGGTCCT	pS
4228	TCCTGGAGGGGAAGT	pO
4325	TGAAGGGGAGGTCCT	pO
4347	CCTATCCTGGAGGGGAAGT	pS
4378	CCTATCCTGGAGGGGAAGT	pO

C

2006	TCGTCGTTTTGTCGTTTTGTCGTT
2114	TCCTGGAGGGGAAGT
4033	CCTGGAGGGGAAGT
4347	CCTATCCTGGAGGGGAAGT
2006+2114
2006+4033
2006+4347

D

2006	TCGTCGTTTTGTCGTTTTGTCGTT
2114	TCCTGGAGGGGAAGT
4033	CCTGGAGGGGAAGT
4347	CCTATCCTGGAGGGGAAGT
2006+2114
2006+4033
2006+4347

E **F** **Fig. 1. Search for Molecular Associations between ODN**

To investigate the self-association of INH-ODN or their inactive variants (panel A, pH 6.0; panel B, pH 6.5) and their ability to bind ST-ODN (Panel C, pS backbone, pH 6.0; Panel D, pS backbone, pH 7.0; Panel E, pO backbone, pH 6.0), ODN preparations were assayed by native 20% PAGE. Origin is at the left. In Panel F, pH 6.0, ST-ODN 2059 and INH-ODN 4228, both with pO backbones, were mixed and co-incubated or incubated separately for 48h at 37° before assay on PAGE. For comparison, fresh 2059 and 4228 are also shown.

Fig. 2. Similar Inhibitory Potency of INH-ODN 2114 and 4024 for ST-ODN stimulated Namalwa Cells

Namalwa cells were stimulated with 100 nM ST-ODN 2006 with a range of concentrations of either INH-ODN 2114 or 4024. Inhibition of CD86 expression was measured by flow cytometry.

Fig. 3. Predicted Secondary Structure of a Panel of INH-ODN: Relationship to Biologic Activity
 Structure of INH-ODN 4024 (Panel A), and four variants of the 4024 sequence (Panel B) presenting with the same loop sequence and 3' tail as 4024, but with more stable hairpin structure (lower ΔG and higher T_m) than 4024 by virtue of an extra hydrogen bonded base pair. ΔG and T_m were derived from mFold software. Migration of ODN 4024 variants on 20% PAGE is shown in Panel C. Panels D and E show their biologic activity in mouse B cells stimulated with 100 nM ST-ODN 2084 and assayed at 18h for IL-6 secretion by ELISA (panel D), or in human Namalwa cells assayed for CD86 expression driven by 100 nM ST-ODN 2006 (panel E).

Table 1
Effects of Boiling on INH-ODN Activity

	Normal		Disaggregated	
	Potency (μ M)	% activity	Potency (μ M)	% activity
pS backbone				
2114	0.051	100	0.029	175
4347	0.018	290	0.011	470
pO backbone				
4228	4.600	100	2.400	193
4378	1.300	360	0.560	830

¹The preparations shown in Fig. 1B were tested as inhibitors of CD86 induction in Namalwa cells as described with and without being boiled for 10 minutes at 100°C and rapidly cooled. INH-ODN were tested in cells stimulated with 0.1 μ M 2059 (same sequence as 2006). The sequence of pS ODN 4347 and pO ODN 4378 are given Fig 1A. Potency is the concentration of INH-ODN required for 50% inhibition, whereas % activity is potency of prototype (2114 or 4228) $\div$ potency of test INH-ODN.