Immunomodulatory spherical nucleic acids

Aleksandar F. Radovic-Moreno1, Natalia Chernyak1, Christopher C. Mader2, Subbarao Nallagatla3, Richard S. Kang3, Liangliang Hao2, David A. Walker2, Tiffany L. Halo3, Timothy J. Merkel3, Clayton H. Rische2, Sagar Anantatmula3, Merideth Burkhart4, Chad A. Mirkin1,2, and Sergei M. Gryaznov1,2

Immunomodulatory nucleic acids have extraordinary promise for treating disease, yet clinical progress has been limited by a lack of tools to safely increase activity in patients. Immunomodulatory nucleic acids act by agonizing or antagonizing endosomal toll-like receptors (TLR3, TLR7/8, and TLR9), proteins involved in innate immune signaling. Immunomodulatory spherical nucleic acids (SNAs) that stimulate (immunostimulatory, IS-SNA) or regulate (immunoregulatory, IR-SNA) immunity by engaging TLRs have been designed, synthesized, and characterized. Compared with free oligonucleotides, IS-SNAs exhibit up to 80-fold increases in potency, 700-fold higher antibody titers, 400-fold higher cellular responses to a model antigen, and improved treatment of mice with lymphomas. IR-SNAs exhibit up to eightfold increases in potency and 30% greater reduction in fibrosis score in mice with nonalcoholic steatohepatitis (NASH). Given the clinical potential of SNAs due to their potency, defined chemical nature, and good tolerability, SNAs are attractive new modalities for developing immunotherapies.

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Significance

We show that by organizing immunomodulatory nucleic acids into spherical nucleic acid (SNA) form, significant increases in activity are observed. Treatment of mice with cancer using immunostimulatory SNAs and nonalcoholic steatohepatitis (NASH) using immunoregulatory SNAs leads to improved disease outcomes vs. their unstructured counterparts. These improvements derive from several key SNA properties, including rapid cellular uptake, endosomal delivery, and multivalent binding. Overall, this work underscores the importance of the spatial orientation and presentation of oligonucleotides in the design of novel immunomodulators.

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1A.F.-R.M. and N.C. contributed equally to this work.

2To whom correspondence may be addressed. Email: chadnano@northwestern.edu or sgryaznov@aurasense.com.

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Similarly to C values) was observed and ± Fig. S1 March 31, 2015 PNAS E and μ | SD (– all-PO oligonucleotide (5 | following 48-h incubation with RAW and | E receptor-mediated endocytosis and accumulate primarily in endosomes.

We next sought to evaluate whether 3D organization of IS and IR-SNAs (Fig. S2). These data suggest that IS-SNAs enter immune cells via receptor-mediated endocytosis and accumulate primarily in endosomes.

TLR agonists or antagonists, respectively. In addition to this modularity, we demonstrate that the properties of SNAs lead to significant activity improvements compared with free oligonucleotide counterparts for both IS-SNAs and IR-SNAs in vitro and in vivo. Furthermore, through systematic modification of multiple components that comprise the SNA structure, including oligonucleotide sequence, sugar-phosphate backbone chemistry, orientation, and core template, we are able to elucidate the key structural factors that provide for enhanced immunomodulatory function.

Results and Discussion

Cellular Internalization Pathway for IS- and IR-SNAs. Similarly to previous observations involving other cell lines (25, 26), immunomodulatory SNAs (Fig. 1A) are taken up by RAW 264.7 macrophages, a model antigen-presenting cell (APC) line, via endocytosis (Fig. 1B). Using confocal fluorescence microscopy, we observed rapid internalization of fluorophore-labeled SNAs bearing immunostimulatory motifs in RAW 264.7 macrophages (Fig. 1C and D and Fig. S1). We also found that the SNA constructs associate with cells via class A scavenger receptors (SRA), as IS-SNA internalization was dramatically decreased in the presence of a SRA inhibitor, fucoidan (Fig. 1D). Furthermore, immunostaining studies show that the SNAs are found primarily in the endosomes at early time points (Fig. 1F). These data suggest that IS-SNAs enter immune cells via receptor-mediated endocytosis and accumulate primarily in endosomes.

SNA 3D Structure Leads to Enhanced Immunomodulatory Activity in Vitro. We next sought to evaluate whether 3D organization of IS oligonucleotides into SNAs substantially changes their baseline activity in vitro. To do this, we synthesized a family of compounds based on the TLR9 agonist oligonucleotide, CpG 1826, with either phosphodiester (PO) or phosphorothioate (PS) backbones, in free or SNA forms, using the conventional 13-nm gold core template (Au-SNA) (21). Following an overnight incubation of IS-SNAs with RAW-Blue macrophages, an 80-fold increase in potency (as measured by EC50 values) was observed for PO CpG SNA compared with the free oligonucleotide counterpart (Fig. 2A). For PS CpG 1826, the differences in activity of IS-SNAs compared with the corresponding free oligonucleotides were highly time-dependent and in all cases higher than the PO counterparts, as expected (29). At relatively short incubation times (30 min), a 94-fold increase in potency (EC50 value) was observed, which decreased to a 1.8-fold higher potency after 4 h of the treatment, ultimately becoming comparable in potency after overnight incubation (Fig. 2A and B and Fig. S2). This marked kinetic difference, which likely is the result of differences in uptake rates, may be beneficial with regard to in vivo (vide infra) applications of these SNA constructs. We also synthesized the corresponding CpG 1826-based compounds with
a liposomal core (L-SNA) instead of a gold core and found that the IS activity was nearly identical and independent of core, for both PO and PS backbones (Fig. 2C). We also found that free 3′-tocopherol oligonucleotides showed similar activity to the SNAs (Fig. S2G); this led us to hypothesize that the 3′-tocopherol oligonucleotides were forming small micellar structures under these conditions. We confirmed formation of the micellar structures in PBS by dynamic light scattering (see SI Materials and Methods for details).

We corroborated our findings of enhanced NF-κB activation in the APC reporter system by IS-SNAs by demonstrating that (i) IS-SNAs in RAW 264.7 murine macrophages generally induce higher levels of several proinflammatory cytokines regulated by NF-κB (TNF-α, IL-12, and IL-6) compared with free oligonucleotides at the same dose (Fig. 2D and Fig. S2B, C, and F); (ii) NF-κB activation by IS-SNAs was dependent on the presence of functional TLR9 (Fig. S2D and E); (iii) TLR9 activation by IS-SNAs could be replicated in a similar human B-cell reporter system (Ramos-Blue cells; Fig. S2D and G); (iv) inversion of the oligonucleotide orientation relative to SNA core by placement of the functionally important 5′-terminus (30) internally and immediately adjacent to the SNA core greatly reduced the compound’s activity (Fig. S2H); (v) use of a different TLR9 agonist oligonucleotides, all-PO CpG 7909 (more optimized for interacting with human TLR9), against human PBMCs, resulted in markedly higher cytokine activation in SNA form (∼1,400-fold higher production of TNF-α compared with linear either all-PO or all-PS oligonucleotides (Fig. S2F)). This improvement in activity in human cells (for CpG 7909 constructs) vs. murine cells (for CpG 1826 counterparts) may be due to the previously described differences in TLR receptor affinities and the preferences of oligonucleotide phosphodiester vs. phosphorothioate backbones for human and murine TLR ligands, respectively (31); and (vi) the studied IS-SNAs did not induce significant production of IFN-α in hPBMCs in vitro under the conditions studied (Fig. S2J-L). Taken together, these results strongly suggest that the 3D structured oligonucleotide pharmacophore shell, and not the core, are the main determinant of IS activity.

Next, we sought to evaluate whether it is advantageous to organize IS-oligonucleotides into IS-SNAs vs. encapsulating them in liposomes of comparable size. To do this, we prepared an IS L-SNA (external CpG 7909-PO L-SNA) and a corresponding liposome with the IS-oligonucleotides encapsulated (internal CpG 7909-PO liposome) and compared them in terms of their ability to activate signaling in the NF-κB reporter cells. Our results show that the outward-facing L-SNA structure led to an approximate threefold improvement in potency following overnight incubation (Fig. 2E). To test whether this improvement was simply the result of greater SNA-mediated uptake, we took the internal CpG 7909-PO liposome structures and postfunctionalyzed them with immunologically inactive d-A40 all-PO oligonucleotide and tested their activity. The internal CpG/external d-A40 L-SNAs showed no difference compared with the oligonucleotide-encapsulated liposome, suggesting that external presentation of oligonucleotides in SNA format confers other advantages in addition to uptake. These advantages may include presentation of the oligonucleotide pharmacophore in a more available conformation for interactions with the target receptor.

Next, we tested the IR-SNAs (both Au-SNA and L-SNA) as potential negative regulators of TLR activity in vitro. IR-SNAs were designed based on the TLR9 antagonist oligonucleotide, 4084F (32). To evaluate TLR antagonist activity, cells were first activated by incubation with PS CpG 1826 (at 0.5 μM) for 2 h. The cells were then treated with various antagonists: 4084F-derivatized Au-SNA, free 4084F all-PS oligonucleotide, or mismatched oligonucleotide controls. The results demonstrate that 4084F-derivatized Au-SNAs show an approximate eightfold increase in potency compared with the free 4084F all-PS counterpart (Fig. 2F). Similar trend in the activity was observed with L-SNA antagonist constructs designed with 4084F sequences (Fig. S3A).

These data further suggest that the outer oligonucleotide shell, and not the SNA core, is the defining factor for the increased IR properties. Last, we confirmed that the administration of IR-SNAs

![Fig. 3. IS-SNAs induce systemic responses. IS-SNAs (Au-SNA) were administered into the footpad and the draining (popliteal) LN was collected aseptically at (A) t = 1 h and (B) t = 24 h and placed into RPMI-1640 growth medium for overnight incubation. The LNs were silver stained to reveal the presence of the Au nanoparticle core and counterstained with eosin. (C) The supernatants from LNs collected at t = 4 h and incubated overnight were probed for cytokines, including IL-12 at multiple doses. (D) Full cytokine panel showing specificity for TLR9 oligonucleotide motifs. (E) Cytokine panel collected from serum at t = 4 h post footpad administration. (F) Elevated and prolonged induction of IL-12 following systemic administration (n = 6 mice per group pooled to n = 3 to increase sample volume, points or bars show mean ± SD).](image-url)
(Au-SNA) inhibited production of a key proinflammatory protein downstream of NF-κB, TNF-α (Fig. S38).

IS-SNA 3D Structure Leads to More Potent and Durable Activation of Innate Immune Cells in Vivo. Next, we sought to evaluate the potential of PS CpG 1826 Au-SNA (IS-SNA) constructs to activate innate immune cells in vivo. We first confirmed that Au-based IS-SNAs accumulate in draining lymph nodes in mice (LNs) at short time points following intradermal injection (t = 1, 4, and 24 h postinjection). The histological sections show evidence of significant accumulation of Au-based IS-SNAs in the cortical and medullary LN regions as early as 1 h postinjection (Fig. 3A). Progressive enlargement of lymph nodes with time was also observed, which is consistent with IS-SNA–induced local cellular activation (Fig. 3B and Fig. S4).

To test which formulation of nucleic acid induces more robust draining LN responses, we compared the levels of cytokines produced by the aseptically collected LNs at varying oligonucleotide doses. The results demonstrate that IS-SNA formulation of PS CpG 1826 produces a ∼10-fold increase in the amounts of several secreted proinflammatory cytokines compared with free IS-oligonucleotide, particularly for IL-12 and IFN-γ (Fig. 3B and Fig. S5). Importantly, the increased IS potency did not appear to be an intrinsic feature of the SNA 3D structure itself, because a control SNA (containing TLN9 inactive CpG replacing CpG motif) was essentially inactive (Fig. 3D); given the robust local LN cytokine response, we sought to evaluate whether this led to elevations in a representative set of cytokines in the serum. Despite the profound IS effect in the draining LN, we saw no notable increases in serum levels for a panel of 12 different cytokines (Fig. 3E).

Next, we sought to determine whether IS-SNA structures could be used to increase the activity of systemically administered (i.v. administration) oligonucleotides. To evaluate the effect of IS-SNA compounds, we administered PO or PS CpG 1826 in either free or in Au-SNA form, and then measured the serum cytokines over a 6-h period. The results show that SNAs, particularly PS-containing SNAs, induce up to 10-fold higher levels of serum proinflammatory cytokines, particularly for IL-12 (Fig. 3F and Fig. S6). In addition, the duration of the stimulatory effect appeared to be extended by several hours compared with free oligonucleotides.

**IS-SNAs Elicit Enhanced Humoral and Cellular Immune Responses to Model Antigens in Vivo.** We next sought to examine whether the observed increases in the key cytokines could adjuvant improved humoral and/or cellular immune responses to a model antigen. We selected the model antigen ovalbumin (OVA) due to its widespread use in vaccine efficacy and antibody production studies. To maximize the immune response, we have attached the antigen to the surface of the IS-SNA ([Ag]IS-SNA); to achieve this, OVA or SIINFEKL peptides were conjugated to the oligonucleotide sequence complementary to the adjuvant oligonucleotide strands on the nanoparticle. The obtained peptide–oligonucleotide conjugates were hybridized to the surface of an SNA to achieve loading of ∼30 OVA or SIINFEKL per particle (see SI Materials and Methods for details). We tested by immunizing mice into the footpad with [OVA]IS-SNA or matched unformulated oligonucleotide mixed with OVA, using alum as a benchmark for potent antibody responses and evaluated both IgG1 and IgG2a titers at day 30 postimmunization (Fig. 4A). Remarkably, formulations containing IS-SNA–based adjuvants with OVA antigen led to ∼700-fold higher median IgG2a serum titers than unformulated CpG oligonucleotide at the same dose (Fig. 4B), with comparable levels of IgG1 compared with alum and unformulated CpG oligonucleotide (Fig. S7). The latter observation demonstrates that IS-SNA–based adjuvants induce significantly higher Th1-like memory response to antigen peptide, in contrast to free CpG oligonucleotide or alum under the conditions tested.

Next, we sought to evaluate the adjuvant properties of [Ag]IS-SNAs. We compared the overall cellular response by immunizing mice with PS CpG 1826 containing [OVA]IS-SNAs, and free OVA antigen in the presence of unformulated linear 1826 PS CpG oligonucleotide, or alum as adjuvants. Phosphate buffer saline was used as an adjuvant control in this experiment. At the end of the immunization, mice were killed and the collected splenocytes were restimulated with the major histocompatibility complex class I–restricted peptide determinant OVA257–264 (SIINFEKL) in an IFN-γ ELISPOT assay ex vivo (Fig. 4C). The results demonstrate that IS-SNA–based adjuvants induce significant desired memory response to SIINFEKL peptide, in contrast to free CpG oligonucleotide or alum under the conditions tested (Fig. 4D).

We then sought to examine whether the observed increases in activity in vivo by the SNAs depend on the type of SNA core. For this, we compared the ability of PS CpG 1826 [Ag]Au-SNA and [Ag]L-SNAs for their ability to boost humoral or cellular immune responses to OVA antigen. The results show that both [Ag]Au-SNAs and [Ag]L-SNAs induce production of comparable levels of IgG2a antibody titers, as well as comparable secretion of IFN-γ following restimulation with SIINFEKL peptide ex vivo (Fig. 4E and F). In addition, we found that immunizations with OVA peptide in the presence of 3′-tocopherol–modified CpG oligonucleotide or with [Ag]IS-SNAs resulted in comparable humoral and cellular immune responses. However, the general ability of 3′-tocopherol–modified CpG DNA, PS 1826 [Ag]Au-SNA, and PS 1826 [Ag]L-SNA to boost the anti-OVA responses was higher than that of the free PS CpG 1826 oligonucleotide adjuvant (Fig. 4E).
We next sought to determine whether [Ag]IS-SNAs could demonstrate improved efficacy in cancer immunotherapy disease model. For this purpose, we used the well-established E.G7-OVA lymphoma syngeneic flank model as a model for a cancer expressing a well-defined antigen (OVA). To produce OVA-expressing tumors, we injected 2 x 10^6 E.G7-OVA lymphoma cells into the flank of C57BL/6 mice. Following the tumor cell injection (on day 0), we treated mice with either [Ag]IS-SNAs or appropriate controls on days 3, 5, and 7 (Fig. S4). The results show that SNA-boosted anti-OVA responses were able to induce profound and durable tumor growth re-
mission (Fig. 5B) that translated into a doubling of mouse sur-
vival rates (Fig. 5C). Use of the full OVA protein or the major antigenic determinant SIINFEKL peptide in vaccination appeared to show no difference in either tumor growth rates or survival under these conditions (Fig. S8). Interestingly, OVA-free formulations also exhibited a significant reduction in tumor growth rates and improvements in mouse survival. The latter observation could be attributed to the induction of innate immune responses. Moreover, significant differences were observed in tumor growth rates in vaccinations containing L-SNAs and 3'-tocopherol-modified oligonucleotides. Thus, results show a significant reduction in tumor growth rates by the 3D struc-
tured L-SNAs compared with free 3'-tocopherol-modified CpG oligonucleotides, which also correlates with positive differences in mouse survival. Importantly, for all adjuvant formulations, and in all animal groups, no signs of gross toxicity were observed (Fig. S8C).

IR-SNAs Demonstrate Enhanced Antifibrotic Activity in a Mouse Model of NASH. To further elucidate therapeutic potential of IR-SNAs, we sought to determine whether SNA structure exhibited advantages over sequence- and chemistry-matched free unformulated TLR antagonist oligonucleotides in vivo. Non-
alcoholic steatohepatitis (NASH) is a fatty liver disease with prevalence as high as 3–5% in the United States. It is charac-
terized by pathologic changes in livers of patients, including steatosis and inflammation, which ultimately progress in a subset of patients to nodular fibrosis, full-blown cirrhosis, and hepatocellular carcinoma (HCC). Currently, there are no FDA-approved treat-
ments for this disease.

The STAM mouse model has been developed as a small ani-
mal model that recapitulates important features of the human disease (33). Nearly 100% of STAM mice follow disease pro-
gression from steatosis to NASH to fibrosis to HCC, making this a model that is well suited to evaluate the ability of compounds to prevent disease progression. In this model, mice that are 6 wk old demonstrate evidence of NASH, and from 6 to 9 wk old progress to histological evidence of fibrosis.

To assess whether IR-SNAs could affect disease progression, we began treatment at week 6 with IR-SNAs (L-SNA-4084F-Ext, L-SNA-4084F), sequence-matched unformulated free oligonucleotides (4084F-Ext, 4084F), or controls (L-SNA-CTL, PBS) and continued treatment following an every-other-day schedule for the 3-wk period to week 9 (Fig. 5D). After 9 wk, the mice in all groups were killed, and their livers were collected, processed for histology (H&E and Sirius Red), and scored with regards to fibrosis (Fig. 5E and Fig. S9) and NASH (Fig. 5F and Fig. S9). The results demonstrate that IR-SNA administration leads to a 40–51% reduction in fibrosis relative to control; this is in contrast to unformulated free oligonucleotides, which showed more modest anti-inflammatory effects (21–25% reduction). Neither IR-SNAs nor unformulated oligonucleotides showed any significant effect on nonalcoholic fatty liver disease score in this study. These results suggest that IR-SNAs can potentially be developed into compounds that modify rates of NASH progression to fibrosis, an advance that could reduce mortality from this disease.

Conclusion

The results presented in this manuscript are significant for the following reasons. The data convincingly show that SNAs can be used as constructs for sequence-specific, potent, and therapeu-
tically relevant immunostimulation and immunoregulation. Importantly, such constructs outperform linear nonlipidated
phosphodiester deoxyribonucleic acids due to a rapid cellular uptake, predominant accumulation in endosomes, and increased resistance to nucleases. Moreover, the data suggest that many of the attractive SNA properties are defined primarily by the 3D arrangement and orientation of the oligonucleotide shell for both IS and IR applications. Taken together, the presented data underscore the importance of spatial orientation of the immunomodulatory pharmacophores, and highlight the modular nature of the SNA therapeutic constructs.

Materials and Methods

Spherical Nucleic Acids, Adjuvants, Antigens, and Reagents. Oligonucleotides were synthesized using automated solid support phosphoramidite synthesis (sequences for activating and regulating immunity are included in SI Materials and Methods). Ovalbumin (Sigma-Aldrich) or SIINFEKL (GenScript) were purchased at their highest purity and used as-is without additional purification. The 13-nm Au-SNAs were prepared as described (21) with important modifications (SI Materials and Methods) and formulated to contain 5.2% (wt/vt) (L-SNA) or 6.3% (wt/vt) (Au-SNA) of oligonucleotide L-SNAs were synthesized as described (27) with modifications (SI Materials and Methods and Fig. S10).

Antibody Titers. All animal studies were conducted according to protocols approved by the local Institutional Animal Care and Use Committees of Explora BioLabs, SBI Biosciences, Stellic Institute & Co., and Ircerca Biosciences, and by the local Ethical Review Process committee of KBS BioTest. Anti-OVA antibody endpoint titers were determined by ELISA. In brief, mice were bled as indicated in the figure captions either on day 28 or day 30 following the first immunization. Either OVA323-339 peptide (Fig. S8 and Fig. S6) or ovalbumin protein (Fig. S5) was used as the capture antigen, and anti-lgG or IgG isotype-specific (IgG1 or IgG2) was used as the secondary antibody linked to HRP. The endpoint titer was defined via linear regression analysis using a curve with six or more points.

Ex Vivo ELISPOT. The OVA-specific cellular response was evaluated by IFN-γ ELISPOT. In brief, at the conclusion of the in-life portion (day 28), mice were killed and spleens were processed to splenocytes by mechanical disruption of the spleen and removal of RBC by hypotonic lysis. Splenocytes were seeded at 4 × 10^6 cells per well of IFN-γ ELISPOT plates together with media alone (negative control), 1 μM OVA323-339, or PMAL-Vinomycin (positive control). Following incubation overnight, the number of IFN-γ-secreting cells was quantified by an automated ELISPOT counter.

In Vivo E.G7-OVA Tumor Growth Study. The 2 × 10^6 E.G7-OVA (American Type Cell Culture Collection) cells were inoculated into the right flank of 4- to 6-wk-old C57BL/6 mice on day 0. On days 3, 5, and 7 post tumor induction, 200 μL of compound were administered. Tumor volumes were measured twice a week by quantifying the length and width of the tumor and applying the formula V = L × W × W/2. If a second tumor occurred in a given mouse, the second tumor was measured via the same method and the two volumes were added together. The general health of animals was monitored daily. Mice were euthanized and autopsied when tumor volume reached or exceeded 2,000 mm^3 or if found to be moribund.

Liver Fibrosis Prevention Study. The activity of IR-SNAs was tested in the STAM mouse model (Stelic Institute & Co., Inc.) of NASH. In brief, pathogen-free, 15-d pregnant C57BL/6 mice were obtained from Japan SLC. NASH was induced by s.c. injection of streptozotocin (Sigma-Aldrich) after birth followed by feeding with a high fat diet (CLEA Japan) ad libitum after 4 wk of age. At 6 wk old, mice were randomized into groups of eight and treated every 2 wk from 6 to 9 wk of age. Compositions or control (PBS) were administered (40 μM oligonucleotide equivalent concentration) in 100 μL by i.p. administration. The impact of treatment administration on NASH and liver fibrosis was determined by collecting livers at 9 wk of age and assessing NASH disease and fibrosis histologically in blinded fashion. In brief, H&E-stained liver sections were scored for steatosis (0–3), lobular inflammation (0–3), and hepatocellular ballooning (0–2), after which a composite non-alcoholic fatty liver disease score was assigned (scale: 0–8). Sections were also stained with Sirius Red for fibrosis and scored as percent positive area according to established protocols.

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