EXPERT OPINION

Immunological and hematological toxicities challenging clinical translation of nucleic acid-based therapeutics

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Introduction: Nucleic acid-based therapeutics (NATs) are proven agents in correcting disorders caused by gene mutations, as treatments against cancer, microbes and viruses, and as vaccine adjuvants. Although many traditional small molecule NATs have been approved for clinical use, commercialization of macromolecular NATs has been considerably slower, and only a few have successfully reached the market. Preclinical and clinical evaluation of macromolecular NATs has revealed many assorted challenges in immunotoxicity, hematotoxicity, pharmacokinetics (PKs), toxicology and formulation. Extensive review has been given to the PK and toxicological concerns of NATs including approaches designed to overcome these issues. Immunological and hematological issues are a commonly reported side effect of NAT treatment; however, literature exploring the mechanistic background of these effects is sparse.

Areas covered: This review focuses on the immunomodulatory properties of various types of therapeutic nucleic acid concepts. The most commonly observed immunological and hematological toxicities are described for various NAT classes, with citations of how to circumvent these toxicities.

Expert opinion: Although some success with overcoming immunological and hematological toxicities of NATs has been achieved in recent years, immunostimulation remains the main dose-limiting factor challenging clinical translation of these promising therapies. Novel delivery vehicles should be considered to overcome this challenge.

Keywords: drug safety, hematotoxicity, immunotoxicity, nucleic acid-based therapeutics

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1. Introduction

Nucleic acid-based therapeutics (NATs) consist of two diverse categories, small molecule agents and macromolecular NATs. The former includes nucleotide and nucleoside analogues, which interfere with DNA replication, RNA formation and stability of DNA or RNA or modulate function of enzymes, receptors or structural proteins necessary for DNA replication, reparation and RNA synthesis. There have been many small molecule agent NATs approved for clinical use. Macromolecular NATs are much more diverse than small molecule NATs. They include antisense oligonucleotides (ASOs), triplex-forming oligodeoxyribonucleotides (TFOs), immunostimulatory oligonucleotides (IMO), catalytic oligonucleotides, inhibitory DNA (DNAi), inhibitory RNA (RNAi) and aptamers, which defer by the

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Article highlights.

- Nucleic acid-based therapeutics (NATs) are broadly considered for the intervention of conditions associated with gene mutations, for cancer therapy, treatment of infection diseases as well as applications as vaccine adjuvants.
- Preclinical and clinical studies revealed many challenges including hematological and immunological dose-limiting toxicities restricting delivery of therapeutic benefits of these concepts to patients.
- Induction of cytokine and interferon responses, activation of complement system and effects on coagulation cascade are responsible for undesirable immunostimulation by macromolecular NATs, while immunosuppression is a common side effect of small molecule NATs.
- Sequence optimization, chemical modifications of individual nucleotides and backbone helped reducing immuno- and hematotoxicities but did not eliminate them completely.
- Inhibiting or eliminating Toll-like receptors (TLRs) from test systems reduced inflammatory cytokines and interferon induction by NATs but were insufficient to prevent these events due to the presence of other, non-TLR proteins and receptors sensing nucleic acids.
- Strategy focusing on delivery vehicles masking NATs from the immune surveillance has to be considered to overcome translational challenges arising from immunostimulatory properties of NATs.

This box summarizes key points contained in the article.

mechanism of action (**Table 1**) [1]. Each of these classes also includes multiple subtypes of oligonucleotides (**Table 2**).

Macromolecular NATs affect flow of genetic information at the DNA, mRNA and protein levels, as well as stimulate the immune system causing either suppression of disease-specific genes or stimulation of gene expression in response to an antigen. In spite of decades of research involving macromolecular NATs, including many clinical trial evaluations [2], only three have successfully reached the market: i) fomivirsen (Vitravene), an ASO approved by the US FDA and EMA for the treatment of HIV-related cytomegalovirus infection [3]; ii) mipomersen (KYNAMRO), an ASO approved by the US FDA for the treatment of homozygous familial hypercholesterolemia [2] and iii) pegaptanib (Macugen), a DNA aptamer approved by both the US FDA and EMA for therapy of age-related macular degeneration [4]. All of these approved formulations are given to patients through routes that avoid systemic exposure of oligonucleotides to circulating blood. Vitravene and Macugen are given through intravitreous injection and KYNAMRO is given subcutaneously (**Table 3**). In 2002, Vitravene was voluntarily withdrawn from the market in the EU by the manufacturer and has since been discontinued in the USA as well, driven by a decline in demand for the drug (**Table 3**).

Preclinical and clinical studies of macromolecular NATs have revealed numerous challenges, including pharmacokinetics (PKs), toxicology and instability in blood [3,5-11]. Many of these hurdles have been successfully addressed through chemical modifications of the backbone, changes in sequences or by altering the dose regimen, and have been reviewed elsewhere [8,9,12]. Additional strategies for overcoming some of these obstacles have also been recently discussed, such as *in silico* screening for potential off-target toxicities [13]. Both desirable and undesirable adverse changes in immunological and hematological parameters have been commonly reported for various types of NATs, although concerns regarding the chemistry and pharmacology of these compounds have often overpowered the immunotoxicity and hematotoxicity concerns. As such, little attention has been given to this subject in the literature.

Herein, we focus on immunomodulatory properties of various types of therapeutic nucleic acid concepts, providing a review of immunological and hematological properties of various types of NATs as well as approaches attempted to circumvent these toxicities.

2. Nucleosides and nucleotides

Therapeutic nucleosides and nucleotides (**Figure 1A**) are generated by chemical modification to the basic structure of purines or pyrimidines allowing them to distribute to and interfere with DNA and RNA synthesis. Most compounds in this category are immunosuppressive due to their ability to inhibit DNA replication of proliferating cells. Leukocyte proliferation is an essential part of the normal immune response, while uncontrolled proliferation results in hematological cancer. These agents are toxic to normal leukocytes, but offer a beneficial treatment option against leukemias and lymphomas. Toxicity to normal cells manifests as leukopenia and depending on the compound may affect some cells lineages more than others, for example, be more toxic to T cells than B cells [1].

Chemical structures of the trade formulations mentioned below are summarized in **Table 4**. Beneficial anti-proliferative effects of these compounds have been reported for hematological cancers. For example, Lanvis is approved for treatment of acute myelocytic leukemia, chronic myelocytic leukemia and acute lymphocytic leukemia. Cladribine is approved to treat leukemic reticuloendotheliosis, hairy cell leukemia, macroglobulinemia and several other types of hematological cancers (**Table 3**). Fludarabine is used for chronic lymphocytic leukemia and non-Hodgkin's lymphomas. Pentostatin is used to treat hairy cell leukemia. Clofarabine (also known as Clofazic, Clolar and Evoltra), an analogue of cladribine and fludarabine, is used for leukemia therapy. Pyrimidine analogues cytarabine and 5-fluorouracil (also known as Adrucil, Carac, Efudex) are used for various forms of non-hematological cancers.

Undesirable immunological and hematological effects reported from clinical studies of these anti-cancer compounds include myelosuppression, neutropenia, thrombocytopenia, anemia, thrombosis, lymphoadenopathy and autoimmune hemolytic anemia (**Table 3**). Other common side effects

Table 1. Mechanism of action of nucleic acid-based therapies.

Type of nucleic acid therapeutic agent	Main mechanism of action
Nucleotides and nucleosides	Distributing to and interfering with DNA and RNA synthesis
Antisense ODN	Complementary binding to target gene mRNA and inhibiting splicing, arresting translation, modulating polyIC site selection or disrupting regulatory RNA structure
Immunostimulatory ODN	Internalization and interaction with receptors and proteins initiating signaling cascade(s) leading to expression of inflammatory cytokines and interferons
DNAi	Binding to 5′-promoter region of the target gene and inhibiting transcription
TFO	Hybridization with double-stranded DNA containing target gene and forming triplex preventing transcription and formation of mRNA
RNAi	Upon delivery into cytoplasm of target cells these agents are enzymatically processed by DICER to form activated RISC; the latter interacts with complementary mRNA of target gene and stimulates its degradation
Bacterial and pDNA	Delivery of coding region of target gene as well as interaction with the immune receptors to stimulate production of inflammatory cytokines and interferons
Catalytic ODN	Hybridization to and cleaving target mRNA
Aptamers	High-affinity binding to a variety of ligands (receptors, peptides, hormones, coagulation factors and other proteins) and modulating their function

Known mechanisms by which small or macromolecular nucleic acid-based therapeutics act to achieve therapeutic effects are summarized. Chemical structures of some of these therapeutics is provided in **Figure 1**.
DICER: Endoribonuclease; DNAi: Inhibitory DNA; ODN: Oligonucleotide; pDNA: Plasmid DNA; RISC: RNA-induced silencing complex; RNAi: Inhibitory RNA; TFO: Triplex-forming oligodeoxyribonucleotide.

include suppression of immune responses to antigens, decrease in the number of T-helper cells, allergic reactions as well as cytokine release syndrome which may progress to life-threatening systemic inflammatory response syndrome (**Table 3**). Anaphylaxis and delayed-type hypersensitivity reactions have also been reported for certain therapeutic nucleotides and nucleosides. Septicemia and opportunistic infections are also very common due to decreased function of normal immune cells affected by these compounds.

In addition to cancer therapy, many nucleotides and nucleosides are successfully used as anti-viral compounds to treat HIV, human hepatitis B virus, human papilloma virus, cytomegalovirus, polyoma, adenovirus and poxvirus infections. Examples of approved drugs include acyclovir, ganciclovir, cidofovir, AZT, lamivudine, ribavirin, adefovir (Hepsera), entecavir (Baraclude, Entaliv), tenofovir (Viread, Truvada). Immunological and hematological toxicities observed with these anti-viral compounds during clinical studies and post-marketing investigations include many of the same side effects as observed with nucleoside and nucleotide hematological cancer treatments. For example, neutropenia, thrombocytopenia, anemia and allergic reaction have all been reported for this class of therapies as well. Anaphylaxis, leukopenia, thrombotic thrombocytopenic purpura, fever and flu-like reactions, immune reconstitution syndrome and autoimmunity are additional toxicities and side effects associated with anti-viral nucleosides and nucleotides (**Table 3**) [14].

Some research studies have described cell-mediated toxicities which may help explain the immunological and hematological toxicities reported in the clinic. For example, acyclovir is selectively cytotoxic to T cells evidenced by a reduction in the number of $CD3^+$, $CD8^+$ and $CD25^+$ cells *in vitro*, and a reduction in Thy 1.2+ and $CD8^+$ cells *in vivo*, without affecting the number of Ig+ cells [15]. Acyclovir also affected the $CD4^+/CD8^+$ ratio both *in vitro* and *in vivo* [15]. Ribavirin is potent in suppressing proliferation of normal human peripheral blood leukocytes [16]. Cidofovir has been shown to affect vaccine efficacy [17]. Lamivudine has been shown to affect both T- and B-cell proliferation without affecting metabolic pathways in these cells. Proliferation of ConA or anti-CD3-stimulated T cells and pokeweed mitogen (PWM)-stimulated B cells was suppressed *in vitro* by lamivudine. In addition, no effect on production of IL-2, TNF-α, IFN-γ, IL-6 and GM-CSF by α-CD3 primed T cells was detected. Secretion of IgM by PWM-stimulated B cells was affected only by high concentrations of lamivudine [18]. Alteration of the T-reg profile and number of T cells has also been reported for both adefovir and entecavir [19].

3. Antisense oligonucleotides

ASOs bind to target gene mRNAs and inhibit splicing, arrest translation, modulate polyA site selection or disrupt regulatory RNA structure, all of which result in silencing of target gene expression. Therapeutic oligonucleotides from this category are commonly developed for cancer and anti-viral indications, although other indications have included cardiovascular and metabolic disorders [2].

There are several different types of ASOs, including phosphodiester oligodeoxyribonucleotides (ODNs) with a native DNA backbone and phosphorothioate ODNs with a sulfur substitution on the phosphate moiety, termed first-generation phosphorothioate ODNs. Second-generation

Table 2. Common immunological and hematological reactivity of macromolecular nucleic acid-based therapies.

Category	Subcategory	Known immunological and hematological reactivity	Innate immune receptors involved in recognition
Antisense oligonucleotides	Phosphodiester DNA oligos	Complement activation	n.s.
	First-generation phosphorothioate DNA oligos	Anemia APTT and PT prolongation Complement activation Cytokine induction Fever Leukopenia Monocytosis Thrombocytopenia	TLR9
	Second-generation phosphorothioate DNA oligos	Cytokine induction	TLR9, MDA-5, IPS-1
	Morpholino oligos	Complement activation	n.s.
	DNA/RNA oligos	Cytokine secretion	TLR9
	PNA	Immunologically inert	n.a.
	LNA	Cytokine secretion	TLR9
Immunostimulatory oligonucleotides	Class A CpG	Activation of pDC B-cell proliferation IFN-α, IFN-γ, TNF-α, IL-12 secretion Indirect NK cell activation T-cell activation and proliferation	TLR9
	Class B CpG	Low IFN-α, IL-6, IL-10, IL-12 secretion B- and T-cell activation and proliferation Direct NK cell activation	TLR9
	Class C CpG	IFN-α secretion T-cell activation and proliferation Direct B-cell proliferation Indirect NK cell activation pDC activation	TLR9
	Class P CpG	Strong IFN-α secretion Primary pDC activation	TLR9
	Synthetic CpR	B-cell proliferation and activation Cytokine and chemokine secretion by PBMC IL-12 and IFN-γ secretion	TLR9
	Synthetic RpG	High levels of IL-12 and IFN Low levels of IL-6	TLR9
	Non-CpG	B cell proliferation Cytotoxicity of NK cells IL-6 secretion by PBMC	TLR9
	RNA oligos	Cytokine and chemokine inductionDC activation Interferon type I and type II induction	TLR3, TLR7, TLR8
	dsDNA/RNA oligos	Cytokines and type I interferon induction DC activation	TLR9

Various types of macromolecular nucleic acid-based therapeutics, and where available, their subtypes are summarized. Only receptors that have been experimentally proven to activate an immune response to therapeutic NATs are included into this table. This table was prepared using information extracted from the following references: [36,38-40,63,64,73,86-88,90,91,110].

APTT: Activated partial thromboplastin time; DC: Dendritic cell; DNAi: Inhibitory DNA; IPS-1: IFN-β promoter stimulator-1; LNA: Locked nucleic acid; MDA-5: Melanoma-differentiation associated-5; miRNA: MicroRNA; n.a.: Not applicable; NK: Natural killer; n.s.: Not studied; pDC: Plasmacytoid dendritic cell; PNA: Peptide nucleic acid; RNAi: Inhibitory RNA; shRNA: Short hairpin RNA; siRNA: Small interfering RNA; TFO: Triplex-forming oligodeoxyribonucleotide; TLR: Toll-like receptor.

Table 2. Common immunological and hematological reactivity of macromolecular nucleic acid-based therapies (continued).

Category	Subcategory	Known immunological and hematological reactivity	Innate immune receptors involved in recognition
DNAi	n.a.	Thrombocytopenia Lymphopenia Fever	n.s.
TFO	n.a.	n.s.	n.s.
RNAi	siRNA	Cytokines and type I interferon induction	TLR3, TLR7, TLR8
	shRNA	Cytokines and type I interferon induction	TLR3
	miRNA	Cytokines and type I interferon	TLR7, TLR8
DNA	Bacterial	Cytokine and type II interferon induction	TLR9
	Plasmid	Cytokine and type II interferon induction	TLR9
Catalytic oligos	DNAzyme	None detected in clinical trials when DNAzyme was injected intratumorally	n.a.
	RNAzyme	Fever Injection site reaction	n.s.
Aptamers	DNA aptamers RNA aptamers	Anemia Changes in lymphocyte, leukocyte and platelet counts Hypoalbuminemia Rash	n.s.

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phosphorothioate ODNs include chemical modifications at the 2′ site (e.g., 2′-*O*-methyl, 2′-*O*-methoxyethoxy, bromo- or iodo-modifications). Third-generation phosphorothioate ODNs include single-stranded DNA/RNA (ssDNA/ssRNA) oligonucleotides with 2′-modifications and methylphosphonate flanking moieties, as well as structures with much greater variation from the classical nucleotide structure, including locked nucleic acids (LNAs), peptide nucleic acids (PNA) and morpholino oligonucleotides. LNAs contain 2′-*O*,4′-C-intranucleotide methylene bridges. PNA have the entire phosphate deoxyribose backbone substituted with *N*-(2-aminoethyl)-glycine [5,20]. Morpholino oligonucleotides replace the deoxyribose with a morpholine ring connected through a phosphorodiamidate linkage (**Figure 1B**). In this section, we will focus on therapeutic ASOs intended for silencing disease-specific genes and for which immunostimulation is undesirable.

3.1 Phosphosdiester and first-generation phosphorothioate ASOs

Most current knowledge about NAT toxicological profiles, including immunological and hematological toxicities, originates from studies investigating phosphodiester and phosphorothioate oligonucleotides. Despite the pronounced variations in chemical structures, several physicochemical properties of ASO are similar. Within a class of given oligonucleotides they have similar length, charge-to-mass ratio and solubility. This is believed to be the reason for comparable PKs and tissue uptake. Several key factors reported for influencing immunological and hematological toxicity of ASOs include: i) composition and linkage of the backbone; ii) sequence; iii) dose and dose regimen; iv) plasma concentration and clearance rate and v) oligo length [6,7,9,21-25]. It is generally accepted, and has been demonstrated in numerous studies, that toxicity of oligonucleotides can be sequence-specific, sequence-independent, hybridization-dependent and hybridization-independent [8].

In human clinical trials, commonly reported side effects following systemic administration of phosphorothioate ASOs include fever and fever-like reactions, activated partial thromboplastin time (APTT) prolongation, thrombocytopenia and leukopenia (**Table 2**) [3,26-34]. Common immunological and hematological abnormalities in rodent studies

Table 3. URL reference summary.

Drug	Type of report	URL
KYNAMRO	Administration route	http://ir.isispharm.com/phoenix.zhtml?c=222170&p=irol-newsArticle&ID=1779162&highlight
Vitravene	Administration route	http://www.rxlist.com/vitravene-drug/indications-dosage.htm
Macugen	Administration route	http://www.drugs.com/pro/macugen.html
Vitravene	Market withdrawal	http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/medicines/000244/human_med_001148.jsp&mid=WC0b01ac058001d125http://www.isispharm.com/Pipeline/Therapeutic-Areas/Other.htm
Cladribine	Indication	http://www.nlm.nih.gov/medlineplus/druginfo/meds/a693015.html http://www.mayoclinic.org/drugs-supplements/cladribine-intravenous-route/description/drg-20062966
Cytarabine	Immuno- and hematotoxicity	http://www.drugs.com/pro/cytarabine.html
Pentostatin	Immuno- and hematotoxicity	http://www.drugs.com/sfx/pentostatin-side-effects.html
Clofarabine	Immuno and hematotoxicity	http://dailymed.nlm.nih.gov/dailymed/lookup.cfm?setid=c35903cd-e9eb-4841--93d1-fc14249d1e9f#section-1
Adefovir	Immuno and hematotoxicity	http://www.drugs.com/sfx/adefovir-side-effects.html
Entecavir	Immuno and hematotoxicity	http://www.drugs.com/sfx/entecavir-side-effects.html
Lamivudine	Immuno and hematotoxicity	http://www.drugs.com/sfx/lamivudine-side-effects.html
Tenofovir	Immuno and hematotoxicity	http://www.drugs.com/sfx/tenofovir-side-effects.html
Ribavirin	Immuno and hematotoxicity	http://www.mayoclinic.org/drugs-supplements/ribavirin-oral-route/side-effects/drg-20071471
IMO-2125	Clinical trials	http://clinicaltrials.gov/ct2/show/NCT00990938?term=IMO-2125&rank=1 http://clinicaltrials.gov/ct2/show/NCT00728936?term=IMO-2125&rank=2
IMO-2055	Clinical trials	http://clinicaltrials.gov/ct2/show/NCT00729053?term=IMO-2055&rank=1 http://clinicaltrials.gov/ct2/show/NCT00719199?term=IMO-2055&rank=2 http://clinicaltrials.gov/ct2/show/NCT00633529?term=IMO-2055&rank=3
IMO-8400	Clinical trials	http://clinicaltrials.gov/ct2/show/NCT02092909?term=IMO-8400&rank=1 http://clinicaltrials.gov/ct2/show/NCT01899729?term=IMO-8400&rank=2
DNAi	Preclinical development pipeline	http://www.pronai.com/pipeline/index.htm
Herzyme	Clinical trial	http://www.sec.gov/Archives/edgar/data/892112/000091205702020355/a2080095zex-99_1.htm
Ribozymes	Administration route	http://clinicaltrials.gov/ct2/results?term=ribozyme&Search=Search
DNAzymes	Administration route	http://clinicaltrials.gov/ct2/results?term=DNAzyme&Search=Search

The purpose of this table is to summarize URL to clinical trials and companies' websites referring to (pre)clinical development of corresponding nucleic acid-based therapeutics and commonly reported immunological/hematological side effects.
DNAi: Inhibitory DNA; IMO: Immunostimulatory oligonucleotide.

include the same conditions observed clinically plus complement activation, prothrombin time prolongation and mononuclear cell infiltrates in multiple organs (predominantly lung, kidney and liver). Of interest, variability in the immunological response to ASOs has been previously reported for different species used in preclinical studies [11]. For example, rodents strongly respond to ASOs by lymphoid hyperplasia, splenomegaly, hypergammaglobulinemia and at high doses by thrombocytopenia, anemia and monocytosis [35-39]. Monkeys show similar immunostimulatory effects, but to a much weaker extent [40]. Prolongation of APTT and complement activation is observed similarly in non-human primates, rodents and humans and correlates with plasma levels of ASOs. It was suggested that hematological toxicity of phosphorothioate ASOs is greater than that of phosphodiester ASOs due to the higher lipophilicity and greater protein binding of phosphorothioates [12,40]. Binding of phosphorothioate ASOs to plasma proteins is believed to be ionic and reversible, yet sufficient to cause alterations in protein conformation, enzymatic properties and function [12]. **Table 5** provides a summary of the various immunological and hematological toxicities observed in rodent, non-human primates and humans [32].

Clinical evaluation of several ASOs have shown that dosing schedule can also affect some immunostimulatory properties, including complement activation, coagulation time, fever and myelosuppression [3]. Holmlund *et al.* found in two different dose schedules (2 h intravenous infusions 3 times weekly for 3 weeks, and 24 h intravenous infusion repeated weekly for 3 weeks), the most prominent side effects were

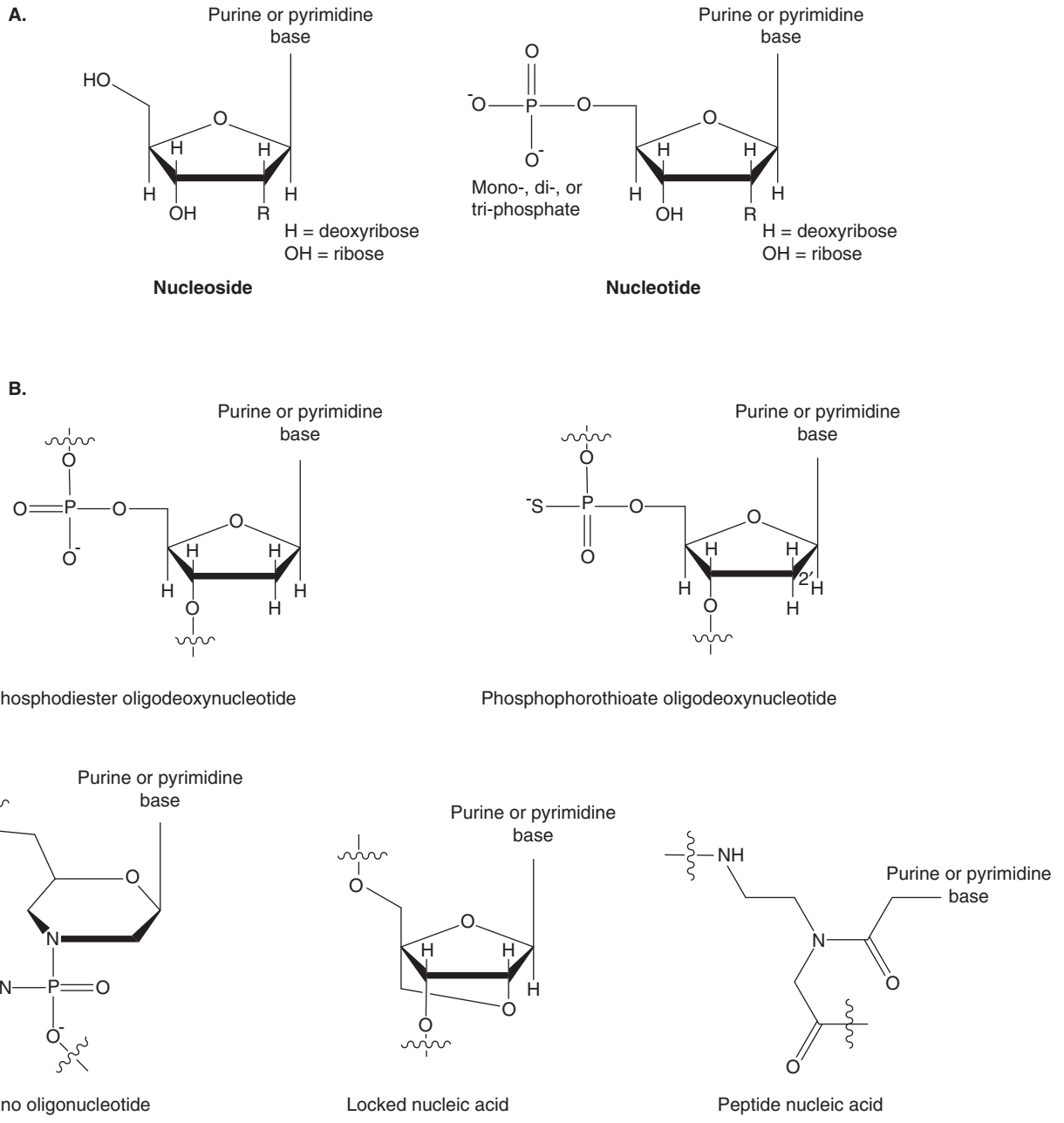
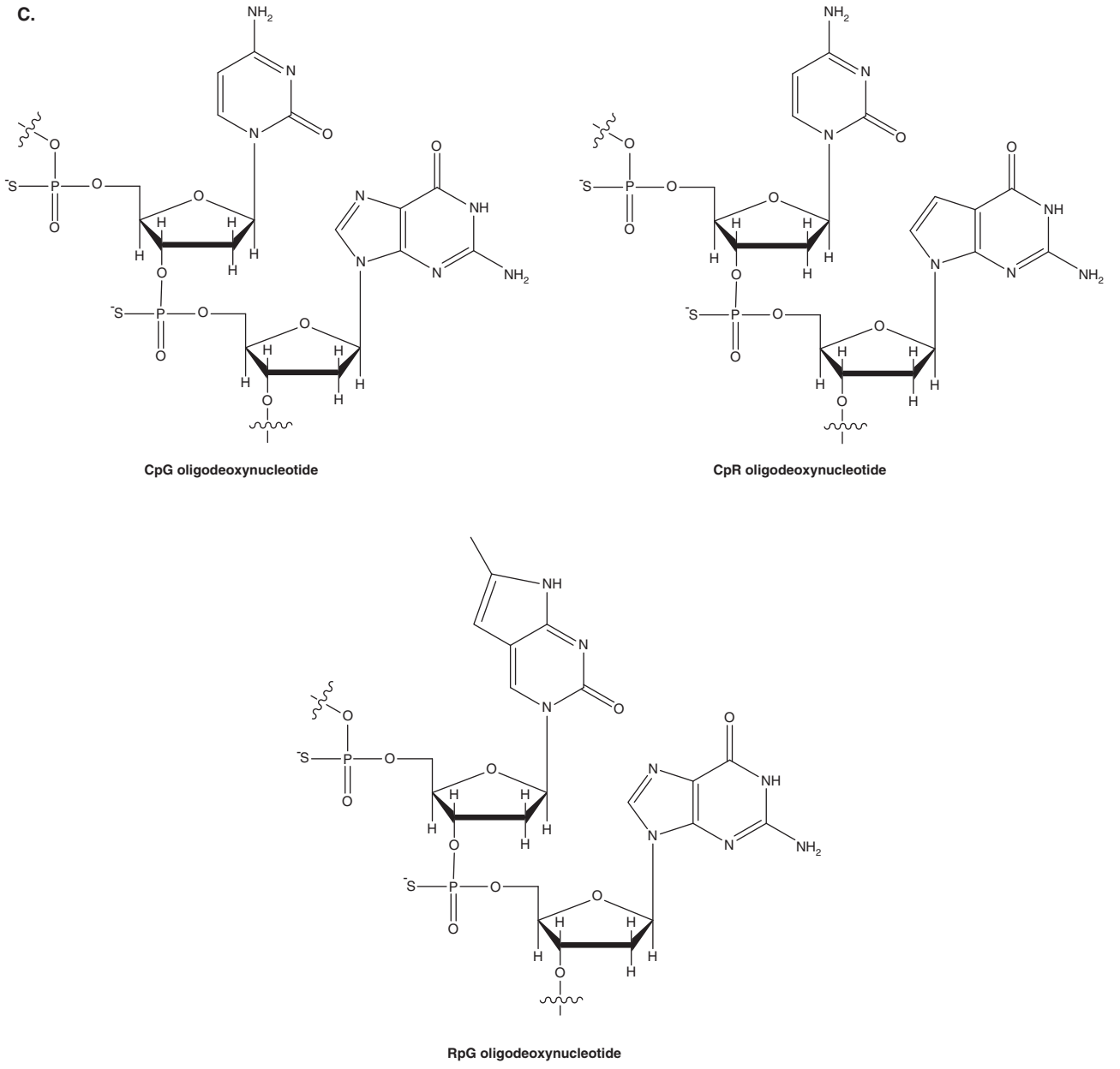


Figure 1. Structure of various types of NATs. A. Nucleosides and nucleotides. Representation of a nucleoside and a nucleotide. Both structures consist of a purine or pyrimidine base connected to a five-membered ring sugar moiety. Nucleosides contain a hydroxyl (-OH) group at the 5′ carbon, whereas nucleotides contain a phosphate group (-PO_4^-). **B.** Antisense oligonucleotides. Phosphodiester and phosphorothioate ODN are shown. Phospohosdiester ODNs comprised traditional nucleotides. Phosphorothioate ODNs replace one of the oxygen molecules on the phosphate group with a sulfur. Phosphorothioate ODNs have evolved into several generations over the years. Generation two contains modifications at the 2′ carbon, such as 2′-*O*-methyl or 2′-*O*-methoxyethoxy. Generation three comprises more complex modifications and includes structures such as morpholino ODN, locked nucleic acids and peptide nucleic acids. **C.** Immunostimulatory oligonucleotides. The most common immunostimulatory ODN are CpG ODNs that contain multiple cytosine (C) and guanine (G) nucleotides linked to one another. CpR and RpG are synthetic analogues of the traditional CpG ODN. **D.** Triplex-forming DNA. Triplex-forming DNA consists of a triple-stranded DNA stretch. A single DNA strand hybridizes with a traditional Watson–Crick paired double-stranded DNA helix to form the triplex DNA. **E.** RNAi. Different types of siRNA are depicted. siRNA is a short double-stranded RNA, typically 19 – 24 units in length. shRNA is similar in length, but has a small hairpin loop connecting the 5′ end of one strand to the 3′ end of the second strand. miRNA is also similar in length, but is single-stranded RNA (**continued**).

miRNA: MicroRNA; NATs: Nucleic acid-based therapeutics; ODN: Oligonucleotide; RNAi: Inhibitory RNA; shRNA: Short hairpin RNA; siRNA: Small interfering RNA.



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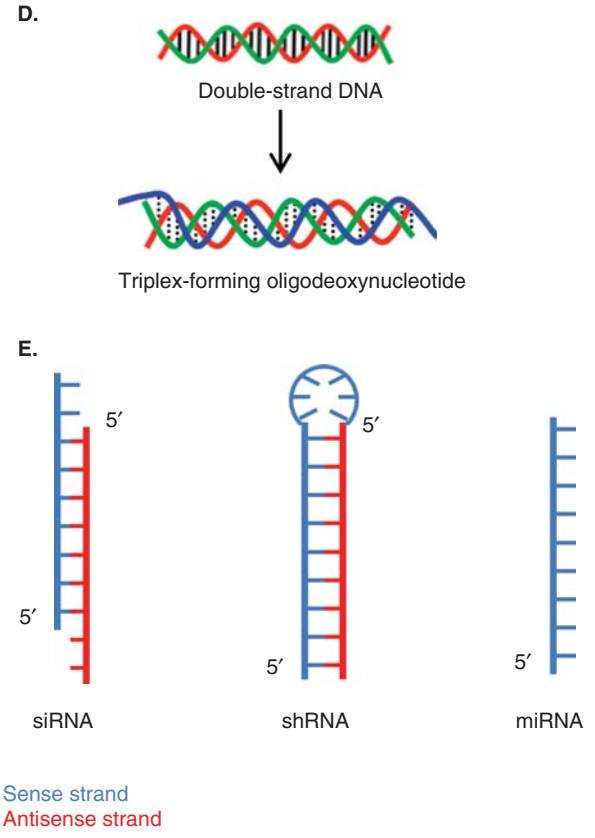

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prolongation of plasma coagulation, low-grade fever and thrombocytopenia. There was transient elevation of complement split products, but was not accompanied by clinical signs of complement activation. However, in a third dose schedule (3-week continuous infusion followed by 7 days rest), the only side effect noted was thrombocytopenia [3]. Hematological toxicities often correlate with plasma levels of ASOs. All three studies described here were designed to maintain low plasma levels of ASOs, but there were still noted differences in immunotoxicities. The mechanism responsible for these changes is unclear.

Complement activation-related toxicity is a very common, undesirable side effect of the first-generation phosphorothioate ASOs [6-8,22,41-43]. ASOs with a phosphodiester backbone generally do not activate complement, although weak activation has been reported at high ASO concentrations [44]. Interestingly, while both phosphodiester and phosphorothioate ASOs can activate complement through interaction with the C4 component of complement in the classical and lectin pathways, C1q-specific (i.e., classical pathway specific) interaction has been reported for phosphodiester ASOs while activation of the alternative pathway through

Table 4. Summary of small molecule nucleic acid therapeutics approved for clinical use.

Trade name	Chemical name
Lanvis	Thioguanine
Cladribine	2-Chloro-2′-deoxyadenosine-5′-monophosphate
Fludarabine	9-β-D-Arabinofuranosyladenine
Pentostatin	2′-Deoxycoformycin
Clofarabine	
Clofazic	(2*R*,3*R*,4*S*,5*R*)-5-(6-amino-2-chloro-9H-purin-9-yl)-4-fluoro-2-(hydroxymethyl)oxolan-3-ol
Clolar	
Evoltra	
Cytarabine	1β-Arabinofuranosylcytosine (cytarabine)
5-Fluorouracil (also known as Adrucil, Carac, Efudex)	5-fluoro-1H,3H-pyrimidine-2,4-dione
Acyclovir	9-(2-Hydroxyethoxymethyl)guanine
Gancyclovir	9-[(1,3-Dihydroxy-2-propoxy)methyl]guanine
Cidofovir	[1-(4-amino-2-oxo-pyrimidin-1-yl)-3-hydroxy-propan-2-yl]oxymethyl phosphonic acid
AZT	3′-Azido-3′-deoxy-thymidine
Lamivudine	2′3′-Dideoxy-3′thiacytidine
Ribavirin	1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboxamide
Adefovir	((2-(6-Amino-9H-purin-9-yl)ethoxy)-methyl) phosphonic acid
Hepsera	
Entecavir	2-Amino-9-[(1*S*,3*R*,4*S*)-4-hydroxy-3-(hydroxy-methyl)-2-methylidenecyclopentyl]-6,9-dihydro-3*H*-purin-6-one
Baraclude	
Entaliv	
Tenofovir	[(2*R*)-1-(6-aminopurin-9-yl)propan-2-yl]oxymethylphosphonic acid
Viread	
Truvada	

The purpose of this table is to align chemical name of each compound to the trade name this compound is distributed by and known as commercially.

selective inhibition of Factor H has been reported for phosphorothioate ASOs [41,44]. Complement activation-mediated toxicities of phosphorothioate ASOs can be mitigated by changing the infusion rate, dose regimen and/or lowering the dose such that plasma concentration does not exceed ~ 40 – 50 μg/ml [41].

Phosphorothioate ASOs also commonly affect coagulation. They can induce transient prolongation of APTT, and to a lesser extent prothrombin times, which is associated with increased bruising in non-human primates [45,46]. Similar anti-coagulant effects are also observed when phosphorothioate ASOs are added to plasma *in vitro* [46]. Both complement and anti-coagulant activity of phosphorothioate ASOs can be reversed by the heparin-neutralizing drug protamine sulfate which binds to phosphorothioate ASOs in a concentration-dependent manner with saturation binding at a 0.9 molar ratio. Initially, it was attributed to the polyanionic nature of ASOs and heparin, and was suggested by Shaw *et al.* to be non-specific electrostatic neutralization [44]. However, another study demonstrated that anti-coagulant activity of ASO ISIS 2302 (a phosphorothioate ODN inhibiting intercellular adhesion molecule 1) was inhibited by protamine sulfate, but the mechanism of inhibition was different than that of heparin. They found no direct association between the phosphorothioate ASO and coagulation factors Xa, XIa and thrombin [46]. Later, Sheehan and Lan showed that ISIS 2302 selectively inhibited intrinsic tenase complex (Factor IXa_B, Factor VIIIa, phospholipid and calcium) [47]. The inhibition was independent of sequence but required a phosphorothioate backbone. The authors suggested that inhibition of the tenase complex is a general property for the entire class of phosphorothioate ASOs [47].

Leukopenia, neutropenia and thrombocytopenia have been commonly noted in clinical trials investigating safety and efficacy of phosphorothioate ASO therapies [3,29]. It has also been shown that cells have different preferences to ASO uptake. Normal bone marrow, healthy peripheral blood mononuclear and B cells demonstrate greater uptake than healthy T cells. Transformed myeloid leukemia cells have a greater uptake than their normal counterparts [48]. Preclinical studies conducted in rodents have also shown thrombocytopenia, anemia and splenomegaly in response to phosphorothioate ASOs [49,50]. Lymphoid and reticuloendothelial hyperplasia were proposed as a possible mechanism for the splenomegaly [49]. Kupffer cell hyperplasia and hypertrophy resulting from increased phagocytosis of ASO–protein complexes has also been described [51,52]. Interestingly, preclinical studies in rodents revealed extramedullary hematopoiesis in the spleen which was suggested to compensate for erythrocyte loss [3,49-55]. The most noteworthy histopathological findings with phosphorothioate ASOs in rodents are mononuclear cell infiltrates in multiple organs, including the liver, lungs and kidney [49-55]. The infiltrates consisted of macrophages and lymphocytes. The degree of splenomegaly and lymphocyte activation is dependent upon sequence and chemical modification of the ASO backbone, as well as on the presence of immunomodulatory agents such as cyclodextrin [9,56-58].

Henry *et al.* suggested complement activation by ASOs as a potential mechanism responsible for transient neutropenia observed during preclinical and clinical studies with various phosphorothioate constructs [41]. The proposed mechanism is related to binding of the C5a component of complement to relevant receptors on neutrophils, followed by an increase in expression of adhesion molecules on the cell surface eventually leading to loss of cell deformability. This change subsequently leads to the sequestration of neutrophils in capillary beds, decreasing the number of circulating cells. Once C5a is cleared and neutrophils regain their deformability, the number of these cells in circulation is restored [41].

Fever and fever-like reactions are commonly reported side effects of ASO therapies in human clinical trials [3,26,28,29,32-34,59]. Interestingly, although cytokines (TNF-α, IL-1 and IL-6) are a known cause of fever, and certain ASOs possess pro-inflammatory properties including cytokine induction, fever and fever-like reactions to some ASOs were

Table 5. Hematological and immunological toxicities of ASOs with phosphorothioate and phosphodiester backbones.

Identifier	Backbone/modification	Indication & target	Immune-mediated reactions in:			Ref.
			Rodents	Non-human primates	Humans	
ISIS 5132	PTO	Cancer: C-raf	Extramedullary hematopoiesis in spleen and liver Decreased platelets, RBC, HCT Increased bone marrow cellularity Increased WBC Kupffer cell hyperplasia Mononuclear cell infiltrates in multiple organs	APTT prolongation Complement activation	APTT prolongation Complement activation Fever Thrombocytopenia	[3,33,52]
ISIS 3521 ISIS 2503	PTO	Cancer: PKCa Cancer: H-ras	n.s.	n.s.	APTT prolongation Complement activation Fever Thrombocytopenia	[3]
OGX-11	2′-Methoxyethyl PTO	Cancer: clusterin	n.s.	n.s.	Fever Leukopenia Thrombocytopenia	[29]
ODN 1668 ODN 1720	PTO	Research: CG versus GC sequence	Extramedullary hematopoiesis Splenomegaly (CG more potent than GC)	n.s.	n.s.	[54]
25 different CpG ODNs	PTO	Research: CG versus non-CG sequence	B-cell proliferation Increased IgM (CG active, non-GC inactive)	n.s.	n.s.	[82]
ISIS 3082	PTO	Colitis: ICAM-1	Lymphoid hyperplasia Monocytosis Mononuclear inflammatory infiltrates in multiple organs and tissues Splenomegaly Thrombocytopenia	n.s.	n.s.	[22,50]
ISIS 9044	PDE	ISIS 3082 analogue	Splenomegaly	n.s.	n.s.	[22]
ISIS 9045	2′-propoxy-PTO	ISIS 3082 analogue	Basophilic granules in Kupffer cells Decreased platelets Kupffer cell hypertrophy Splenomegaly	n.s.	n.s.	[22]
ISIS 9046	Chimeric ODN (2′-propoxy wings and PTO deoxy center)	ISIS 3082 analogue	Splenomegaly Decreased RBC, HCT	n.s.	n.s.	[22]
ISIS 9047	5′ C18 amine PTO	ISIS 3082 analogue	Basophilic granules in Kupffer cells Decreased platelets, RBC, HCT Kupffer cell hypertrophy Splenomegaly	n.s.	n.s.	[22]

The toxicities are dose dependent and are shown for rodents (mice or rats), non-human primates and humans.

APTT: Activated partial thromboplastin time; HCT: Hematocrit; HPV: Human papilloma virus; ODN: Oligonucleotide; ICAM-1: Intercellular adhesion molecule 1; n.s.: Not studied or not reported; PDE: Phosphodiester; PTO: Phosphorothioate oligodeoxyribonucleotide; RBC: Red blood cells; WBC: White blood cells.

Table 5. Hematological and immunological toxicities of ASOs with phosphorothioate and phosphodiester backbones (continued).

Identifier	Backbone/modification	Indication & target	Immune-mediated reactions in:			Ref.
			Rodents	Non-human primates	Humans	
ISIS 8005	5′ cholesterol PTO	ISIS 3082 analogue	Basophilic granules in Kupffer cells Decreased platelets, RBC, HCT Kupffer cell hypertrophy Splenomegaly	n.s.	n.s.	[22]
ISIS 1082	PTO	Anti-viral: HSV	Anemia Bone marrow hypocellularity Decreased platelets, RBC Mononuclear inflammatory infiltrates at injection site Necrotizing dermatitis Widespread perivascular and interstitial infiltrates of macrophages and lymphocytes in multiple organs Splenomegaly	n.s.	n.s.	[49]
ISIS 2105	PTO	Anti-viral: HPV	Anemia Bone marrow hypocellularity Decreased platelets, RBC Mononuclear inflammatory infiltrates at injection site Necrotizing dermatitis Widespread perivascular and interstitial infiltrates of macrophages and lymphocytes in multiple organs Splenomegaly	n.s.	n.s.	[49]
ISIS 2302	PTO	Colitis: ICAM-1	Lymphoid hyperplasia Monocytosis Mononuclear inflammatory infiltrates in multiple organs and tissues Splenomegaly Thrombocytopenia	APTT and PT prolongation Decreased platelets	APTT prolongation	[50,52,55]
ISIS 3521 ISIS 5132 ISIS 2922 ISIS 1082 ISIS 11061 ISIS 12959	PTO	Cancer: PKCα Cancer: c-raf Anti-viral: CMV Anti-viral: HSV Murine ICAM-1 Monkey ICAM-1	B-lymphocyte proliferation Hypergammaglobulinemia Lymphoid hyperplasia Mixed mononuclear inflammatory infiltrate in organs and tissues Splenomegaly	n.s.	n.s.	[9]
ISIS 481464	cEt-PTO	Cancer: STAT3	Basophilic granules in kidney and liver Elevated IL-10 and MCP-1 Enlarged lymph nodes Increased spleen weight Kupffer cell hyperplasia, minimal Lymphohistiocytic infiltration in liver	APTT prolongation, minimal and transient Basophilic granules in Kupffer cells and lymph nodes Complement activation Enlarged spleen Injection site inflammation		[53]

The toxicities are dose dependent and are shown for rodents (mice or rats), non-human primates and humans.

APTT: Activated partial thromboplastin time; HCT: Hematocrit; HPV: Human papilloma virus; ODN: Oligonucleotide; ICAM-1: Intercellular adhesion molecule 1; n.s.: Not studied or not reported; PDE: Phosphodiester; PTO: Phosphorothioate oligodeoxyribonucleotide; RBC: Red blood cells; WBC: White blood cells.

Table 5. Hematological and immunological toxicities of ASOs with phosphorothioate and phosphodiester backbones (continued).

Identifier	Backbone/modification	Indication & target	Immune-mediated reactions in:			Ref.
			Rodents	Non-human primates	Humans	
ISIS 3521	PTO	Cancer: PKCα			APTT prolongation Complement activation Elevation of serum cytokines Fever	[26,32]
G3139	PTO	Cancer: bcl-2	n.s.	n.s.	Fever Leukopenia Local skin inflammation Lymphocytic infiltrate Thrombocytopenia	[34]

The toxicities are dose dependent and are shown for rodents (mice or rats), non-human primates and humans.
APTT: Activated partial thromboplastin time; HCT: Hematocrit; HPV: Human papilloma virus; ODN: Oligonucleotide; ICAM-1: Intercellular adhesion molecule 1; n.s.: Not studied or not reported; PDE: Phosphodiester; PTO: Phosphorothioate oligodeoxyribonucleotide; RBC: Red blood cells; WBC: White blood cells.

attributed to their polyanionic nature [29]. Specifically, in a clinical trial of OGX-11 (2′-MOE ASO against clusterin), fever was not a sustained reaction. Fever ensued after the first and second infusions (days 1 and 3), but was continually less prominent or absent in subsequent infusions [29]. In contrast, another clinical study in patients treated with ISIS 5132, ISIS 3521 and ISIS 2503 reported fever and chills occurring 2 – 6 h after injection, and was attributed to a cytokine response [3]. Similarly, ISIS 3521/LY900003 resulted in fever which indeed correlated with elevation of serum levels of TNF-α, IL-6 and IL-1β 8 h post-infusion [26].

It is now widely accepted that a pro-inflammatory response to ASOs is largely determined by their sequence [8]. For example, CpG-containing ASOs are more immunostimulatory than their non-CpG counterparts [8]. CpG ODNs contain multiple cytosine (C) and guanine (G) nucleotides linkages (**Figure 1C**). They are present in both eukaryotic and prokaryotic DNAs but have one major difference – the former is methylated while the latter is unmethylated. The mammalian immune system is armed with a battery of receptors with versatile abilities to recognize specific molecular patterns. Unmethylated CpG motifs represent one such molecular pattern and are effectively sensed by Toll-Like receptor (TLR)9 [40]. Since ASOs developed for therapeutic silencing of disease-specific genes are not methylated, they are recognized by TLR9 resulting in a pro-inflammatory response including production of inflammatory cytokines (TNF-α, IL-1β, etc.), type I (IFN-α and -β) and, indirectly, type II interferons (IFN-γ) [40]. This property is used to benefit development of CpG ODNs containing vaccine adjuvants and will be discussed in Section 4. Excessive activation of TLR9 by CpG oligonucleotides, however, may also lead to loss of peripheral tolerance and result in autoimmunity [40]. Designing ASOs to specifically exclude CpG motifs is one approach to avoid immunostimulation, but is not always possible depending on the sequence of the target gene.

3.2 Second- and third-generation phosphorothioate ASOs

Several approaches have been shown to reduce immunostimulation of ASOs, including modification of the backbone, optimization of the sequence and other approaches as discussed above. Second- and third-generation phosphorothioate ASOs utilize a variety of backbone modifications (**Figure 1B**) to aid in reducing immunostimulation. Second-generation phosphorothioate ASOs introduce chemical modifications specifically at the 2′ site of the sugar moiety. Third-generation phosphorothioate ASOs include much more diverse modifications, including chemical modifications to the native backbone, forming internal bridges within each backbone sugar (LNAs) and completely disrupting the sugar backbone (PNAs).

Complement activation and anti-coagulant properties can be reduced or eliminated by monomerization, chemical

modification of the backbone or other synthetic modifications [46,60,61]. For example, backbone modification of phosphorothioate ODNs through addition of a 2′-MOE moiety [8] or methylphosphonate internucleotide linkage [44] has been shown to reduce complement activation. Synthesis of a mixed backbone consisting of 2′,5′-ribo- and 3′-5′-deoxyribonucleotide segments [23,60], or synthesis of chimeric oligonucleotides containing phosphorothioate ODN flanked on both ends with ethyl (cEt)-modified nucleosides [53] has also been successfully employed. Other approaches shown to mediate complement activation-related toxicities have included changing the sequence to create a self-stabilizing loop at the 3′ end of the ASOs [44], changing both the backbone and sequence creating hybrid oligonucleotides consisting of both phosphorothioate deoxyribonucleosides and 2′-*O*-methylribonucleosides [44], and co-infusion with the complement activation blocker-2 [42].

Other approaches to negate immunostimulatory properties, specifically CpG-containing ASOs, include the use of cytosine derivatives (e.g., 5-methylcytosine) or 2′-MOE backbone modification [21,62]. 2′-MOE-modified oligonucleotides display lower levels of undesirable pro-inflammatory activity than 5′-methylcytosine-modified ASOs [21]. Short (12-mer) ASOs have also been shown to be less immunostimulatory than their longer (15-mer) counterparts [25].

A subset of 2′-MOE-modified ASOs was found to be inflammatory despite the lack of CpG motifs in its sequence. More interestingly, it induced pro-inflammatory cytokines and type I interferons (IFN-α/β) similar to that induced by viral RNA. Senn *et al.* reported that 2′-MOE-modified non-CpG ASO (ISIS 116847) triggered pro-inflammatory responses consisting of high levels of IL-18, RANTES, JE/MCP-1 and MIP1-α *in vitro* and *in vivo* [63]. Interestingly, at low doses (4 mg/kg) the response to 2′-MOE-modified non-CpG ASO was TLR9 and MyD88-dependent. However, at high doses (50 mg/kg) it was observed equally in both wild-type and knockout animals deficient in TLR9 or MyD88 genes. Based on these findings, the authors suggested that 2′-MOE non-CpG ASO induced cytokines through either TLR3 which functions through the My-D88-independent adaptor TRIF (Toll/IL-1R-containing adaptor protein inducing IFN-β), or another unidentified receptor. Another elegant study from this group showed a different 2′-MOE-modified non-CpG ASO (ISIS 147420) induced high levels of type I interferons (IFN-β) independently from TLR3 [64]. Similar to ISIS 116847, no change in inflammatory signaling was observed in knockout animals deficient in MyD88 and TRIF. Interestingly, this study demonstrated that two cytosolic receptors, melanoma-differentiation associated-5 (MDA-5) and IFN-β promoter stimulator-1 (IPS-1) were involved in the inflammatory reaction to ISIS 147420, but retinoic acid inducible gene-1 (RIG-1) was not [64]. Both IPS-1 and RIG-1 are pattern recognition receptors that are capable of recognizing foreign nucleic acids.

Oligonucleotides with phosphorothioate backbones containing internucleotide linkages can be immunostimulatory. This property is dependent on the distance at which the internucleotide linkage is introduced with respect to the CpG motif. For example, introduction of a 2′-5′ nucleotide linkage within the CpG motif or adjacent to the CpG does not induce immunostimulation. However, the same modification introduced distal to the CpG at the 5′ end enhances immunostimulation. A modification distal to the 3′ end results in only a minor change in the immunostimulatory potential [65,66].

In contrast, PNA modification of immunostimulatory CpG-containing ASOs was shown to reduce their ability to induce cytokines (e.g., TNF-α) in macrophages both *in vitro* and *in vivo* [67]. Another study showed PNA ASOs were immunologically inert. They did not activate splenocytes or lymph node resident cells, or induce antigenic responses unless conjugated to keyhole limpet hemocyanin [68,69].

CpG-containing oligonucleotides with phosphodiester backbones can also become potent immunostimulants. Sequences containing palindromic CpG or poly(pG) moieties or two CpG-containing oligos linked together at the 3′ end have proven immunostimulatory in that they resulted in increased IL-12 and IFN-γ secretion, likely due to their greater stability against nucleases [70].

In summary, even though modifications of backbone, optimization of sequence and other approaches discussed above help reducing immunostimulation by ASOs, when undertaken alone they may not eliminate it completely, so systematic approach including various measures is important when imunostimulation-related toxicity is undesirable.

4. Immunostimulatory oligonucleotides

This category of nucleotides contains sequence and backbone modifications specifically designed to activate the innate immune system [71], although the latter contains many receptors for sensing nucleic acids [72]. The best-studied examples include IMOs activating the immune system through TLR9 (CpG-containing oligonucleotides), TLR3 (double-stranded RNA [dsRNA]) and TLR7 and TLR8 (nucleotide analogues and ssRNA) [73]. Oligonucleotides described in this section are undergoing development as vaccine adjuvants [74]. Their primary routes of administration are intradermal and subcutaneous. Several IMOs, including IMO-2125, IMO-2055 and IMO-8400, are in various stages of clinical trials, and in each case the route of administration is subcutaneous (**Table 3**). The majority of published studies are focused on specific effects of these constructs on antigen-presenting cells (APCs) and lymphocytes. Even though some of these constructs have a phosphorothioate backbone, the effects on complement and the coagulation system may not be the same as reported for antisense phosphorothioate oligonucleotides. There are likely differences in biodistribution as a result of the different administration routes which will affect immune responses.

4.1 CpG oligonucleotides

Several classes of CpG-containing oligonucleotides have been described. Class A (also referred to as class D) CpG oligonucleotides consist of mixed backbone (phosphodiester in the center flanked by phosphorothioate) with unmethylated CpG-rich moieties and G-stretches at 3′ and 5′ ends. Class B (also referred to as class K) consists of oligonucleotides with a phosphorothioate backbone and several 5′-Pu-Py-C-G-Py-Pu-3′ motifs. Class C CpG oligonucleotides consist of oligonucleotides with a phosphorothioate backbone without G-stretches, but high in CpG motifs [1,74,75]. Finally, class P oligonucleotides combine the 3′ CG-rich palindrome of class C and a non-CG-rich 5′ palindrome. These nucleotides form hairpins at the 3′ end and concatamers at the 5′ end [75].

Although CpG oligonucleotides of all classes activate the innate immune system through TLR9, the cells activated by these constructs, and the cytokine/interferon induction potency, are different between the classes. Class A oligos induce maturation of APCs, B cells and indirectly activate natural killer (NK) cells through induction of IFN-α [76,77]. Class B oligos are weak stimulators of IFN-α but directly activate both NK and B cells [78]. They also induce maturation of B cells and monocytes [77,79]. Class C strongly stimulates IFN-α production by plasmacytoid dendritic cells (pDCs), stimulate activation and maturation of APCs, indirectly activate NK cells and have direct activation effects on B cells [80]. Oligonucleotides from this class have also been shown to stimulate activation and proliferation of γδT cells [81]. Class P are the strongest inducers of IFN-α due to preferential induction of the interferon regulatory factor 7-signaling pathway [75].

The response to CpG oligos may vary between species [76,79,82]. For example, while a 6-mer motif (5′-GTCGTT-3′) of class B oligos is most optimal for induction of an immune response in humans, 5′-GACGTT-3′ is most potent in the mouse [76,82]. The immunostimulatory activity of class A CpG oligonucleotides is determined by backbone, length and chemical modification of the base and sugar. Replacement of linkages to phosphorothioate bonds across the entire oligonucleotide results in loss of immunostimulation for this class [77,83]. Factors determining the immunostimulatory potential of class B CpG oligos include length, number and position of CpG motifs, spacing between them, nucleotide context around CpG motifs and chemical nature of the backbone [75]. Class B CpG oligonucleotides with a phosphorothioate backbone are more potent than their phosphodiester counterparts due to their enhanced stability in biological matrices [75]. Both phosphodiester and phosphorothioate oligonucleotides are anionic, which is thought to contribute to their immunostimulatory nature, because 'neutralization' of CpG oligonucleotides by addition of methylphosphonate linkages decreases their immunostimulatory potency [48]. Other modifications reducing and suppressing the immunostimulatory potential of CpG oligonucleotides with a phosphorothioate backbone include 2′-*O*-methyl, 2′-*O*-methoxyethyl sugar modifications [21,35], and substitution with RNA derivatives or LNAs, respectively [36]. Immunostimulation by class C and class P oligonucleotides is regulated by their length, content and chemical modifications [75].

Other structural requirements for the uptake and immune recognition of CpG oligonucleotides have been reviewed elsewhere [84]. Due to their immunostimulatory properties, CpG oligonucleotides are generally being pursued as vaccine adjuvants. It has also been suggested that class B CpG oligonucleotides induce extramedullary hematopoiesis, which could be beneficial for recovery of myelosuppression caused by lethal doses of gamma irradiation [54].

4.2 Non-CpG oligonucleotides

Non-CpG-containing oligonucleotides can also induce strong immunostimulation. Vollmer *et al.* reported that thymidine content is critical in achieving an immunostimulatory property for non-CpG oligonucleotides with a phosphorothioate backbone [36,85]. Thymidine-rich phosphorothioate oligos had dramatic stimulatory effects on B cells, induced production of IL-6 by peripheral blood mononuclear cell (PBMC) and enhanced NK-cell cytotoxicity. Interestingly, oligonucleotides with the same backbone, but containing a polythymidine sequence, were more immunostimulatory than their counterparts with similar thymidine content but in which polythymidine stretches were interrupted with guanosine. The immunostimulatory properties of polyT guanosine-containing phosphorothioate oligos were further decreased when guanosine residues were replaced by adenosines [36,85]. An increase in oligonucleotide length in the absence of CpG results in higher immunostimulation by phosphorothioate oligonucleotides. Polythymidine oligonucleotides in which the phosphorothioate backbone is changed to phosphodiester are not immunostimulatory [85]. Therefore, length, backbone, sequence and nucleotide content are critical for immunostimulation by non-CpG oligonucleotides. Non-CpG-containing oligonucleotides were shown to activate the immune system through TLR9 [36].

4.3 Synthetic CpR and RpG oligonucleotides

CpR is cytosine-phosphate-2′-deoxy-7-deazaguanosine. Presence of this motif in phosphorothioate oligonucleotides allows for induction of a different cytokine profile than observed with their CpG-containing counterparts, although both types of oligonucleotides activate immune cells through TLR9 [86-88]. CpR-containing oligos induce higher levels of IL-12 and lower levels of IL-6 [86-88]. Induction of IFN-α and activation of B cells and pDCs by CpR oligos depends on the oligonucleotide length, context and sequence [89]. Interestingly, differences in induction of IFN-α were reported between *in vitro* studies using human blood and *in vivo* studies in monkeys; IFN-α induction was stronger *in vivo* than *in vitro* [89]. Yet it remains unclear whether the difference in TLR9 between the monkey and human contributed to the

distinct IFN-α response. This study also raises an important question regarding the degree of predictability of *in vitro* assays, and suggests that weak interferon induction observed *in vitro* should not be ignored.

RpG is a synthetic CpG analogue oligonucleotide in which C is replaced with 1-(2′-deoxy-b-D-ribofuranosyl)-2-oxo-7-deaza-8-methyl purine [90]. Nucleotides with RpG motifs induce high levels of IFN-γ and IL-2, but low levels of IL-6, and stimulate proliferation of leukocytes in various species including human, monkey, pig, horse, goat, sheep, rat, mouse and chicken [90].

4.4 RNA and dsDNA/RNA oligonucleotides

dsRNA and ssRNA induce the immune system through TLR3 and TLR7/8, respectively [91]. Similar to CpG DNA oligonucleotides, the immunostimulatory potential of RNA oligonucleotides depends on sequence and modifications of nucleotides. For example, ssRNA with an unmethylated CpG motif is immunostimulatory, while 5′-C methylation and 2′-*O* methylation abrogated any immunostimulatory effect [37]. Synthetic ssRNA activates human DCs to secrete TNF-α and IL-8. Such activity is impaired by introduction of modified nucleosides N6-methylaladanosine, 5-methylcytidine, 2′-thiouridine or pseudouridine [38]. Double-stranded hybrid DNA:RNA oligonucleotides induce type I interferon, IL-6 and activate DCs via TLR9, not through TLR7, RIG-1 or MDA-5 [39].

5. Inhibitory DNA

This is a novel category of DNAi, which is represented by ssDNA oligonucleotides interfering with expression of a target gene [92]. Several DNAi concepts including PNT-100, PNT-200, PNT-300, PNT-400, PNT-500, PNT-600 and PNT-700 are in various stages of preclinical development for cancer and inflammatory disease indications (**Table 3**). There are no data available yet on the immunological toxicities of this class of therapeutics.

6. Catalytic oligonucleotides

6.1 Ribozymes

Ribozymes are catalytic RNA molecules that hybridize to and cleave target mRNA. Two types of therapeutic RNAzymes (hammerhead and hairpin) have been described [1,93]. Hammerhead ribozymes cleave target RNA containing specific sequences (5′-NUX-3′, where N is any nucleotide and X is C, U or A). They cleave RNA at the 3′ position to the specific sequence generating a 5′ hydroxyl terminus and a 2′3′-cyclic phosphate at the cleavage site [1]. Hairpin ribozymes cleave target RNA containing a 5′-XN*GUC-3′ motif, where X is G, C or U, N is any nucleotide and * is the cleavage site [1].

Therapeutic ribozymes can be produced by either chemical synthesis or through expression of a variety of vectors [1,94]. The former allows introduction of chemical modifications to nucleotides and is used to achieve desirable stability, circulation time and PKs. Ribozyme strategies that reached clinical trials include angiozyme, also known as RPI.4610 (a hammerhead ribozyme targeting VEGF-R1 mRNA) [95-97], herzyme (hammerhead ribozyme targeting HER2/neu mRNA) (**Table 3**) [98], heptazyme (hammerhead ribozyme targeting human hepatitis virus C mRNA) [99] and OZ1 (a ribozyme targeting *tat-vpr* RNA of HIV-1) [100]. None of these concepts is administered systemically. Routes tested in clinical trials include subcutaneous and infusion of hematopoietic cells transduced with ribozyme-encoding vectors *ex vivo* (**Table 3**). Common clinical side effects observed with subcutaneous administered ribozymes include, among others, fever and injection site reactions [95,101]. These reactions may be attributed to immunostimulatory properties of oligonucleotides; however, mechanistic follow-up studies have not been reported. Ribozymes delivered through infusion of autologous *ex vivo* transduced hematopoietic stem cells exhibited thrombocytopenia and neutropenia accompanied by opportunistic infections [100].

6.2 DNAzymes

DNAzymes are catalytic DNAs which can cleave target mRNA. They resemble hammerhead ribozymes and cleave target mRNAs in a sequence-specific manner. Such cleavage results in formation of two products: a 5′-terminal product containing a 2′3′-cyclic phosphate and a 3′-product containing a 5′-hydroxyl [1]. In spite of low cost synthesis and extensive research, only two DNAzyme concepts have reached clinical trials in the USA. They include DZ1 (a DNAzyme targeting EBV-LMP-1 to treat nasopharyngeal carcinoma) and hgd40 (targeting GATA-3 to treat asthma, atopical dermatitis and ulcerative colitis). The clinically evaluated concepts have been administered topically, intratumorally, intrarectally and via inhalation (**Table 3**). None has been administered systemically. Available research data suggest that selection of alternative administration routes is likely driven by the desire to avoid immunostimulation-based side effects, although there are no published studies specifically investigating the immuno/hematotoxicity of systemically administered DNAzymes.

7. Triplex-forming oligodeoxynucleotides

TFOs are single-stranded ODNs that hybridize with dsDNA containing the target gene forming a triplex (**Figure 1D**). This, in turn, interferes with binding of regulatory proteins and transcription factors and prevents formation of mRNA [1]. Extensive work has been done to optimize the synthesis and characterization of TFOs, and has been reviewed in details elsewhere [102]. Some TFOs have been effectively designed to interfere with abnormal coagulation [103]. TFOs have yet to reach the clinical phase and no data are available regarding their immuno- and hematological toxicities.

8. RNAi (small interfering RNA, short hairpin RNA, microRNA)

Inhibitory RNA interference (RNAi) technology includes RNAs with different structures, mechanisms of action and routes of delivery into the cells. This category most commonly includes small interfering RNA (siRNA), short hairpin RNA (shRNA) and microRNA (miRNA) (**Figure 1E**). siRNAs are short RNA duplexes (21 – 23 bps) with two nucleotide 3′ end single-stranded overhangs. siRNAs are typically introduced into the target cells exogeneously. As an alternative, longer dsRNAs can be used, which upon delivery into cytoplasm of target cells are first cleaved by the enzyme DICER, producing siRNAs that are then loaded into the RNA-induced silencing complex (RISC). Within RISC, dsRNA oligonucleotides are processed into single-stranded counterparts, leading to formation of activated RISC. The activated RISC containing target gene-specific RNA (called antisense or guide strand) then interacts with complementary mRNA of the target gene, which culminates with degradation of the target mRNA. shRNA functions similarly to siRNA. Its function depends on DICER and RISC formation, but is delivered into cells encoded in a vector, translation of which results in ssRNA oligonucleotides forming hairpin structures. These hairpin structures are later processed by DICER and loaded into RISC. miRNAs are DICER independent and inhibit translation of mRNA. All three types have been extensively studied *in vitro* [1,104], although siRNA and miRNA are the best studied in terms of their therapeutic applications [105]. siRNAs are further advanced in their clinical development than miRNAs. siRNAs have more concepts in various stages of clinical trials and have been more thoroughly studied in terms of immunological properties and mechanisms of interaction with the immune system.

Induction of the innate immune response to siRNA and shRNA depends on several properties such as length, secondary structure and sequence. Some reports indicate that length is critical, and short siRNA (< 30 bp) are incapable of interferon induction, while their long counterparts are immunostimulatory [106,107]. Another study has suggested that immunostimulation also depends on cell type [108]. Some studies observed immunostimulation by si- and shRNA independent of the oligonucleotide sequence [109], while others have shown it to be sequence specific [110]. For example, Kariko *et al.* reported induction of IL-8, TNF-α, IFN-α and IFN-β by si- and shRNA regardless of their sequence and secondary structure [109], while Judge *et al.* demonstrated that siRNA containing 5′-UGUGU-3′ motifs were the most potent in terms of activation of pDC and monocytes as well as induction of cytokines and type I interferons both *in vitro* and *in vivo* [110]. Substitution of the second U by C, resulting in the sequence 5′-UGCGU-3′, completely eliminated the immunostimulatory activity of siRNA. Kariko *et al.* suggested that recognition of si- and shRNA occurs through TLR3 [109]. Judge *et al.* suggested that other TLRs (TLR7 and TLR8), in addition to TLR3 may also be involved, although the authors could not completely confirm this with HEK293 cells overexpressing these receptors [110]. Interestingly, polyacrylamide gel electrophoresis and high-pressure liquid chromatography performed to purify double-stranded siRNA from single-stranded contaminant oligonucleotides did not have an effect on immunostimulation [110]. The difference in results obtained in these studies may be due to the disparity in experimental models (Kariko *et al.* used monocyte-derived macrophages [109], while Judge *et al.* used pDCs and PBMC [110]) and divergence of the delivery methods (Kariko *et al.* complexed si- and shRNA with transfection reagents [109], while Judge *et al.* encapsulated siRNA into liposomes [110]).

Immunological side effects in response to systemically administered siRNA therapeutics evaluated in preclinical studies were dose-, backbone- and sequence-dependent. They included elevated plasma IL-6 levels, fever, generation of non-neutralizing antibodies to a protein targeting agent [111], elevation of serum levels of IL-6, IL-12 and IFN-α [112,113], elevation of serum levels of IL-6 and IL-12 [114,115], production of neutralizing IgM specific to components of delivery vehicle [116,117], elevation of IL-6, TNF-α and IFN-γ and activation of NF-κB [118]. In addition, dose-dependent but sequence-independent disseminated intravascular coagulation-like reactions were also reported [119]. The majority of studies utilizing siRNA were designed to avoid systemic administration. The routes of administration in these studies were subcutaneous [120], intradermal [121], intraocular [122], intralesional [123], intranasal [124-126], intratracheal [127,128], intrathecal [129-131], intracerebral [132,133], intratumoral [134-137], intravaginal [138,139], intrarectal [140], transdermal [141] and topical [142].

Premedication with immunosuppressive agents such as dexamethasone or pretreatment with inhibitors of Jak, PI3K and p38 pathways involved in the immune response to siRNA eliminated or reduced immunostimulation by therapeutic siRNA [119,143]. Pre-treatment with or co-delivery (but not co-treatment) of siRNA and the cytotoxic oncology drug oxaliplatin was also reported as an effective approach for suppressing immunological toxicity to siRNA [118].

9. Aptamers

Agents in this category include short ssDNA or ssRNA oligonucleotides, which function by high-affinity binding to a variety of ligands including receptors, neuropeptides, hormones, coagulation factors and other proteins, and modulate the function of the respective targets [12,144,145]. Similar to IMOs, some aptamers are being developed to specifically target the immune system through modulation of receptors and proteins involved in immune response [4,146]. Examples of aptamers which have reached the clinical stage, and which function as active pharmaceutical ingredients (API),

Table 6. Proteins involved in nucleic acids recognition and initializing immune responses.

Receptor/protein	Species	Intracellular localization	Nucleic acid	Induces:
TLR3	Human, Mouse	Endosome Endolysosome	dsRNA	Cytokines Type I interferons
TLR7	Human	Endosome Endolysosome	ssRNA	Cytokines Type I interferons
TLR8	Mouse	Endosome Endolysosome	ssRNA	Cytokines Type I interferons
TLR9	Human, Mouse	Endosome Endolysosome	ssDNA Plasmid DNA Bacterial DNA dsDNA/RNA hybrids	Cytokines Type I interferons Type II interferons
TLR13	Mouse	Endosome Endolysosome	Bacterial RNA Ribozyme	Cytokines Type I interferons
RIG-1	Human, Mouse	Cytosol	Short dsRNA	Type I interferons
LGP-2	Human, Mouse	Cytosol	Long dsRNA ssRNA ssDNA	Regulatory role in MDA-5-initiated signaling; Type I interferons
MDA-5	Human, Mouse	Cytosol	Long dsRNA ssRNA ssDNA	Type I interferons
MAVS (IPS-1, CARDIF, VISA)	Human, Mouse	Mitochondria	ssRNA ssDNA	Cytokines Type I interferons
PKR	Human, Mouse	Cytosol	dsRNA	Type I interferons
OAS	Human, Mouse	Cytosol	dsRNA	Degradation of RNA and type I interferon induction through RIG-1
DNA-dependent RNA polymerase III	Human, Mouse	Cytosol	dsDNA	Conversion of DNA into 5′-triphosphate short dsRNA to initiate type I interferon response through RIG-1
STING	Human, Mouse	Endoplasmic reticulum Golgi	dsDNA	Type I interferons
cGAS	Human, Mouse	Cytosol	dsDNA	Type I interferons
DAI	Human, Mouse	Cytosol	dsDNA	Type I interferons
IFI16/p204	Human, Mouse	Cytosol	dsDNA	Type I interferons
DDX41	Human, Mouse	Cytosol	dsDNA	Type I interferons
DNA-PK	Human, Mouse	Cytosol	dsDNA	Cytokines Type I interferons
MRE11	Human, Mouse	Cytosol	dsDNA	Type I interferons
AIM-2	Human, Mouse	Cytosol	dsDNA	IL-1, IL-18, IL-33, IL-36, IL-37, IL-38

Proteins shown to directly bind nucleic acids and initiate immune responses are summarized here. The table was prepared using information extracted from references: [72,73,84,91].

AIM2: Absent in melanoma 2; CARD: Caspase recruitment domain; CARDIF: CARD adaptor inducing interferon; cGAS: Cyclic GMP-AMP synthase; DAI: DNA-dependent activator of IRF; DDX41: DExD/H-box helicase 41; DNA-PK: DNA-dependent protein kinase; IFI16/p204: Interferon-inducible protein 16/p204; dsRNA: Double-stranded RNA; IPS-1: IFN-β promoter stimulator-1; IRF: Interferon regulatory factor; LGP2: Laboratory of Genetics and Physiology-2; MAVS: Mitochondrial anti-viral signaling; MDA-5: Melanoma-differentiation associated-5; MER 11: Meiotic recombination 11 homolog A; OAS: Oligo adenylate synthetase; PKR: Protein kinase R; RIG-1: Retinoic acid inducible gene-1; ssRNA: Single-stranded RNA; STING: Stimulator of interferon genes; TLR: Toll-like receptor; VISA: Virus-induced signaling adaptor.

are Macugen and AS1411. Macugen (Pegatinib) is a VEGF-inhibiting aptamer which is used for treatment of age-related macular degeneration [147]. The aptamer is administered locally via intravitreal injection and does not show systemic immunological/hematological toxicity in humans, dogs or rabbits [148,149].

AS1411 (AGRO100) is a nucleolin-specific DNA aptamer which is administered intravenously by slow infusion over 4 days [150]. Hematological and immunological toxicities include anemia, rash, hypoalbuminemia and change in lymphocyte, leukocyte and platelet counts [150]. Two other systemically administered aptamers, REG-1 [151] and

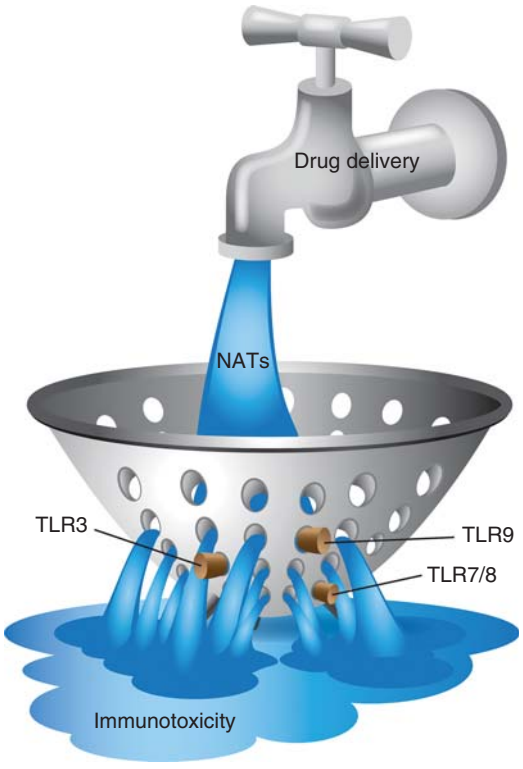

Figure 2. Immunotoxicity of NATs cannot be prevented by blocking or inhibiting TLRs. It is important to recognize that blocking or inhibiting TLR signaling does not eliminate immunostimulation-related toxicities due to the presence of multiple other receptors in various intracellular compartments that sense nucleic acids and trigger robust interferon and cytokine response.
NATs: Nucleic acid-based therapeutics; TLR: Toll-like receptor.

BAX499 [152] promoted plasma coagulation, which was expected due to their intended primary mode of action. Neither of these studies reported immunological/hematological abnormalities related to the aptamers. Aptamers NOX-E36, NOX-A12, NOX-94 and E10030 are in Phase II clinical trials [4], but no data about their immuno- and/or hematocompatibility are available at this time. The vast majority of aptamers in current preclinical and clinical development are not used as the API but rather as 'escort' molecules to specifically deliver siRNAs, toxins, small molecular drugs, enzymes, photodynamic molecules, radionuclides and nanoparticles [12,144,145,153]. Interpretation of immunotoxicity results from these studies is complicated by the complex nature of constructs containing aptamers and study designs.

10. Bacterial and plasmid DNA

Bacterial and plasmid DNA (pDNA) are being studied as adjuvants and antigen-delivery systems for vaccines [154-157]. pDNA is also being investigated as a gene-delivery system for cancer therapy [158-160] and reprogramming of stem cells [161-163]. Immunostimulatory properties of bacterial and pDNA are desirable for vaccine applications while considered adverse for other applications [161]. Bacterial DNA activates B cells, NK cells as well as monocytes and macrophages to produce type I and II interferons and inflammatory cytokines [164]. The induction of IFN-γ in NK cells is indirect and occurs through IL-12 produced by macrophages [164]. The immune activation by bacterial DNA is sequence dependent, where CpG-containing sequences are more potent [164]. The immunostimulation by bacterial and pDNA occurs primarily through TLR9, although several other non-TLR recognition mechanisms have been described recently [72]. Preclinical and clinical trials investigating efficacy and safety of pDNA described generation of antigen-specific $CD4^+$ and $CD8^+$ T cells following intramuscular administration [155-157]. Side-effect profiles observed in these studies were not unexpected and included injection site reactions and elevation in leukocyte count [155-157]. pDNA has also been used to develop tolerogenic vaccines, which aim at turning off the adaptive immunity to an autoantigen. Several approaches have been suggested to succeed with such vaccination: i) addition of immunosuppressive GpG hexamers into the plasmid backbone; ii) including DNA sequences encoding immunosuppressive cytokines such as IL-4 and IL-10 into the pDNA vaccine profile and iii) combination treatment with CD3 and CD20 antibodies to eliminate B cells [154].

11. Expert opinion

Historically, preclinical and clinical translational studies follow basic immunological research approaches to identify, explain and address immunotoxicity concerns of target therapeutics. For NATs, the majority of available studies have focused on TLRs (TLR3, TLR7/8 and TLR9) to explain observed immunostimulation [63,64,165-168]. However in many cases, inhibiting or eliminating TLRs from test systems reduced immunostimulation but did not eliminate it, leaving a gap in the understanding of the mechanism(s) [63,64]. In recent years, several new non-TLR receptors for nucleic acids have also been discovered. In addition to TLRs, many other proteins have been shown to directly bind to RNA or DNA and initiate potent immune responses. These proteins are summarized in **Table 6** and have been reviewed elsewhere [72]. Although there are not enough data linking each of these receptors to NAT immunostimulation, they may explain the unusual immune system activation seen with certain types of NATs and the inability to reduce such immunostimulation with inhibited or knockout TLRs [63,64] (**Figure 2**). Indeed, several studies have already linked MDA-5 and IPS-1 with immune responses to ASO therapeutics [63,64].

Since nucleic acid sensing TLRs are located in endosomes and lysosomes, researchers developing NATs have begun engineering NAT delivery vehicles to include endosome escape units in an effort to improve efficacy and avoid immunostimulation [169]. Note however, that non-TLR sensors of

nucleic acids are located in the cytosol, mitochondria, endoplasmic reticulum, Golgi and even the nucleus [72]. Recent progress in understanding immune system recognition of nucleic acids suggests existence of a thorough surveillance of foreign nucleic acids in essentially all intracellular compartments. Therefore, a strategy focusing on delivery vehicles masking NATs from the immune surveillance system should be considered to overcome translational challenges arising from immunostimulatory properties of NATs. Strategy for selecting nanotechnology carriers to overcome these challenges is reviewed in part II [170].

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