

Therapeutic Applications of Synthetic CpG Oligodeoxynucleotides as TLR9 Agonists for Immune Modulation

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Contents

Abstract	387
1. Classes of CpG Oligodeoxynucleotide	388
2. Mode of Action	390
3. Route of Administration, Pharmacokinetics, and Biosafety	390
4. Infectious Diseases	391
5. Treatment of Cancer	393
6. Vaccination	394
7. Treatment of Asthma and Allergy	395
8. Conclusion	396

Abstract

Vertebrate toll-like receptors (TLRs) sense invading pathogens by recognizing bacterial and viral structures and, as a result, activate innate and adaptive immune responses. Ten human functional TLRs have been reported so far; three of these (TLR7, 8, and 9) are expressed in intracellular compartments and respond to single-stranded nucleic acids as natural ligands. The pathogen structure selectively recognized by TLR9 in bacterial or viral DNA was identified to be CpG dinucleotides in specific sequence contexts (CpG motifs). Short phosphorothioate-stabilized oligodeoxynucleotides (ODNs) containing such motifs are used as synthetic TLR9 agonists, and different classes of ODN TLR9 agonists have been identified with distinct immune modulatory profiles. The TLR9-mediated activation of the vertebrate immune system suggests using such TLR9 agonists as effective vaccine adjuvants for infectious disease, and for the treatment of cancer and asthma/allergy. Immune activation by CpG ODNs has been demonstrated to be beneficial in animal models as a vaccine adjuvant and for the treatment of a variety of viral, bacterial, and parasitic diseases. Antitumor activity of CpG ODNs has also been established in numerous mouse models. In clinical vaccine trials in healthy human volunteers or in immunocompromised HIV-infected patients, CpG ODNs strongly enhanced vaccination efficiency. Most encouraging results in the treatment of cancers have come from human phase I and II clinical trials using CpG ODNs as a tumor vaccine adjuvant, monotherapy, or in combination with chemotherapy. Therefore, CpG ODNs represent targeted immune modulatory drugs with a broad range of potential applications.

Almost 25 years ago, Tokunaga et al.^[1] described the beneficial effects of the DNA fraction of *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) for antitumor applications, leading to the discovery of immunostimulatory DNA.^[2,3] Years later, Krieg and colleagues^[4] reported that unmethylated CpG motifs within the

bacterial DNA are responsible for this effect. Since its discovery, the therapeutic potential of CpG DNA and its synthetic counterpart, CpG oligodeoxynucleotides (ODNs), has been reported by numerous researchers, both in animal studies and in several clin-

ical trials, to show that CpG ODNs have beneficial effects in vaccination and for the treatment of a variety of diseases.

The immunostimulatory effect of synthetic CpG ODNs is based in part on the difference between the genomic DNA of vertebrates and pathogens: in vertebrates, CpG dinucleotide motifs are methylated and their frequency is suppressed, whereas in viral and bacterial DNA, CpG motifs are unmethylated and occur with a much higher frequency.^[4] Therefore, distinction between self (vertebrate) and non-self (pathogenic) DNA due to structural differences is possible, although another factor contributing to the ability of eukaryotes to discriminate between self and foreign DNA is the intracellular localization of the nucleic acid receptor. Recognition of pathogenic DNA occurs through specific pathogen recognition receptors (PRRs). PRRs sense pathogenic invasion through the detection of so-called pathogen-associated molecules, and activate signaling cascades leading to the stimulation of innate immune responses.^[5]

A member of the toll-like receptor (TLR) family, TLR9, is responsible for the recognition of bacterial DNA and unmethylated CpG motifs. The human TLR family consists of ten members (for a review see Akira and Takeda^[6]). TLRs can be classified by their cellular localization into two groups: TLR1, 2, 4, 5, 6, and 10 are located on the cell surface where they sense extracellular pathogen-associated molecules, whereas TLR3, 7, 8, and 9 are located in the endosomal compartment where they are capable of sensing intracellular pathogen-associated molecules, such as viral RNA (in the case of TLR3, 7, and 8), and bacterial or viral DNA (TLR9).^[7-9] TLR3 has been reported to be located on the cell

surface as well as in the endosomes.^[10-12] All TLRs consist of an extracellular leucine-rich repeat region responsible for pathogen-associated molecular pattern (PAMP) recognition, a transmembrane domain, and a TLR/interleukin (IL)-1 β receptor-like (TIR) signaling domain. Binding of the ligand to the receptor initiates signaling events that are characteristic of the individual TLRs (for reviews see Honda et al.^[13] and Akira^[5]). In addition, the different expression patterns of the TLRs allow for a perfectly adjusted immune response to each individual pathogen.

Human TLR9 expression is mainly confined to B cells and plasmacytoid dendritic cells (pDCs).^[14] Recently, it has also been suggested that TLR9 is expressed in other cell types such as T cells^[15-18] and astrocytes.^[19,20] In addition, mucosal epithelial cells of the lower female reproductive tract express TLR9, which indicates a protective role of TLR9 during viral or bacterial infection.^[21]

1. Classes of CpG Oligodeoxynucleotide

Since the description of CpG ODNs, three different classes have been established.^[22-24] The first CpG ODN described belongs to the so-called B class (e.g. PF-3512676 [ODN 7909], table I). These ODNs contain an optimized TLR9 recognition motif that comprises a CpG dinucleotide motif surrounded by additional bases.^[25] The optimal motif for human TLR9 stimulation (GTCGTT) differs from the described murine TLR9 recognition motif (GACGTT).^[4,26-28] In B-class ODNs, two such hexameric motifs are usually embedded within thymidine-rich stretches ideally containing additional CpG dinucleotide motifs at the 5' end.

Table I. Sequence characteristics and properties of several classes of immune modulating oligodeoxynucleotide (ODN)

Therapeutic ODN	Sequence and backbone	Immunologic properties
CpG ODN		
B-class (e.g. PF-3512676 [ODN 7909], 1018-ISS, Hyb-2055 [IMO-2055])	T-rich sequences with embedded CpG motifs All phosphorothioate backbone	Strong B-cell activation Strong pDC maturation Moderate induction of IFN α by pDCs
A-class (e.g. ODN 2216, 2336)	Palindromic sequence surrounded by G-rich (5' and 3') stretches Mixed backbone (central phosphodiester and stretches of phosphorothioate) Form complexes, higher ordered structures	Strong induction of IFN α by pDCs Poor B-cell activation Moderate IL-10 and IL-6 production Moderate pDC maturation
C-class (e.g. ODN 2395, C272)	All phosphorothioate 5' part B-Class like, 3' part palindromic sequence Form duplexes	Good B-cell activation Good pDC maturation Good induction of IFN α by pDCs Good IL-10 and IL-6 levels
Non-CpG ODN		
S-class (e.g. ODN 2088)	All phosphorothioate 5' CCT and 3' G-rich region	Suppresses TLR7-, TLR8-, and TLR9-mediated activation

IFN = interferon; IL = interleukin; pDC = plasmacytoid dendritic cell; TLR = toll-like receptor.

The most prominent B-class ODN is PF-3512676 (formerly termed CPG 7909), which is currently in use in human clinical trials (see sections 5, 6, and 7). B-class ODNs usually contain a phosphorothioate (PS) backbone rendering them more nuclease resistant than the naturally occurring phosphodiester (PO) backbone. In addition to their improved stability, PS-based ODNs show enhanced B-cell stimulation and other activities compared with ODNs with PO backbones. B-class ODNs (also termed K-type ODNs)^[29] induce moderate production of interferon (IFN)- α by pDCs and induce their maturation, as measured by markers such as CD83 and the chemokine receptor CCR7.^[30] B-class ODNs strongly activate B-cell proliferation and upregulate co-stimulatory molecules on B cells and antigen-presenting cells (APCs), as well as the production of IL-6 and IL-10.^[24,25]

A-class ODNs (or D-type ODNs)^[29] differ strikingly in their properties from B-class ODNs. They induce very strong production of IFN α by pDCs, whereas their capacity for B-cell activation is very limited. A-class ODNs consist of a mixed PO/PS backbone: G-rich sequences at the 5' and 3' end with a PS backbone, and a central part comprising a palindromic sequence with PO backbone containing at least one CpG dinucleotide motif.^[22,29,31] The contribution of the poly-G sequences to the immunostimulatory profile of A-class ODNs is not completely clear. Several recent reports suggest that the runs of G nucleotides are responsible for the A-class immune responses.^[32-35] The poly-G runs at the end of A-class ODNs have also been hypothesized to confer either a structural benefit, or to improve the efficiency of uptake. A-class ODNs form higher-ordered structures due to the presence of the G-rich stretches known to form G tetrads. A number of scavenger receptors recognize structures containing G tetrads, and only recently it has been demonstrated that a membrane-bound scavenger receptor, chemokine (C-X-C motif) ligand (CXCL)16, indeed influences the uptake and intracellular localization of A-class ODNs.^[36] Therefore, the G tetrads appear to be responsible for an enhanced uptake of A-class ODNs and for an intracellular distribution mostly confined to early endosomes^[33] resulting in different cellular immune stimulatory outcomes. However, other reports describe the potential of poly-G motifs themselves in the absence of CpG motifs to either directly signal the outgrowth of macrophage progenitors or co-stimulate antigen-specific CD8⁺ T cells contributing to the immune stimulatory effects mediated by A-class CpG ODNs.^[37,38]

C-class ODNs conveniently combine the properties of A- and B-class ODNs;^[22,39,40] they induce significantly higher amounts of IFN α than B-class ODNs together with a B-cell activation comparable to B-class ODNs. Their ODN sequences usually consist of a 3' palindromic sequence combined with a B-class-like 5' se-

quence. Because of their palindromic nature these ODNs can form specific secondary structures (duplexes) that are most likely responsible for the improved IFN α induction similar to A-class ODNs. C-class ODNs are found in early (as A-class) and late endosomes, whereas the B-class, which induces only moderate IFN α activation, can be found mainly in late endosomes, suggesting differences in signaling properties between the different intracellular locations.^[33] Examples of C-class ODNs are ODN 2395^[22] and CPG 10101.

Although not stimulatory ODNs, it is noteworthy to mention another class, suppressive or S-class ODNs, because of their potential therapeutic interest. S-class ODNs, depending on their exact sequences, inhibit TLR7-, 8-, and/or TLR9-mediated effects. For suppression of TLR9-dependent effects, the ODNs must contain a 5'-CC and a G-rich sequence with slight differences between motifs found to inhibit murine or human TLR activation.^[41,42] The formation of G tetrads does not seem to be a prerequisite for the inhibitory function of S-class ODNs.^[43] Another class of suppressive sequences is represented by repetitive elements present at high frequency in mammalian telomeres (5'-TTAGGG-3' repeats).^[44] Although still incompletely understood, initial studies support the notion that these ODNs act through direct binding to TLRs.^[45] Suppressive ODNs are able to inhibit TLR7 and TLR8, although some reports^[46,47] describe that activation of TLR7 can also be inhibited by the PS ODN sequence in a nonspecific manner. Suppressive ODNs have been shown to interfere with numerous forms of immune stimulation *in vivo* in animal disease models, including collagen-induced arthritis, systemic lupus erythematosus (SLE) and lipopolysaccharide-induced toxic shock.^[48] Moreover, recent reports suggest that these ODNs also act on multiple points during signal transduction.^[49-52]

ODNs not containing any CpG motif are also able to stimulate immune responses.^[53-56] However, stimulation of immune cells *in vitro* and *in vivo* with CpG-free ODNs results mostly in a T helper-2 (Th2)-biased but TLR9-dependent immune response. The capability of these ODNs to stimulate immune responses seems to be associated with the presence of thymidine-rich regions. Recent reports also suggest a difference in the activation pattern of naturally occurring PO ODNs and PS ODNs. Recognition of ODNs with a PO backbone and consecutive immune activation seems to be less restricted to the presence of CpG motifs than for PS ODNs. However, PO ODNs appear to require enforced liposomal uptake to result in CpG-independent immune responses.^[57] TLR9-independent activation by double-stranded B-form DNA has also been reported, and recent data suggest a role for the DNA-dependent activator of IFN-regulatory factors (DAI) in the response to B-form DNA.^[55,58,59]

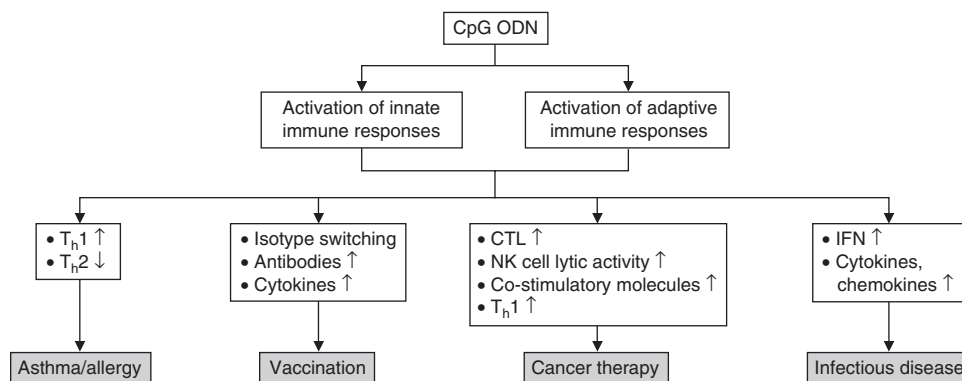


Fig. 1. CpG oligodeoxynucleotides (ODNs) targeting toll-like receptor (TLR)9 are immune modulatory drugs activating innate and adaptive immune responses and addressing a multiplicity of disease indications alone, as vaccine adjuvants or in combination with other therapies. CTL = cytotoxic T lymphocyte; IFN = interferon; NK = natural killer; Th1 = T helper-1; Th2 = T helper-2; ↑ indicates increase; ↓ indicates decrease.

2. Mode of Action

Activation of TLR9 appears to occur through the direct binding of CpG ODN to the receptor.^[45] For the activation of TLR9, endosomal uptake of the CpG ODN is imperative^[33,60,61] and can be blocked by endosomal maturation inhibitors such as bafilomycin.^[9] The uptake and endosomal maturation appears to be CpG-independent but is influenced by backbone modifications and the presence of G-rich regions.^[62–65] For example, PS modification of CpG ODNs enhances their uptake approximately 100-fold, and poly-G motifs have also been reported to result in increased uptake of ODNs, probably through binding to the scavenger receptor CXCL16.^[36]

So far, the TLR9 signaling mechanism seems to be divided into two different cascades, one leading to activation of the IFN α pathway and the other leading to the activation of the nuclear factor (NF)- κ B pathway, which initiates the transcription of several genes, including maturation markers and cytokines. How the two pathways are activated differently on stimulation with ODNs is poorly understood, although some studies have shed some light on the mechanism.^[5,6,66] The first downstream protein necessary for both pathways is the adaptor protein myeloid differentiation factor (MyD)88. IFN α signaling is dependent on IL-1 receptor-associated kinase (IRAK)-1 and tumor necrosis factor (TNF) receptor-associated factor (TRAF)-6, and occurs through phosphorylation of IFN regulatory factor (IRF)-7,^[67–69] which activates type I IFN gene transcription. In contrast, the signaling pathway for the activation of NF- κ B is independent of IRAK-1 and IRF-7 but requires TRAF-6 and IRAK-4. A very recent report proposed that a physical association between the polytopic endoplasmic reticulum (ER)-resident membrane protein UNC93B and TLRs in the ER is essential for proper TLR signaling.^[70]

CpG ODNs primarily activate the innate immune system by the direct and indirect induction of IFNs, chemokines, and ILs result-

ing in a Th1-biased immune response (figure 1). Cells of the innate immune system, such as pDCs and natural killer (NK) cells, are efficiently activated by CpG ODNs. In addition, CpG ODNs stimulate adaptive immune responses by activating B-cell proliferation, antibody production, and isotype switching or activation of antibody-dependent cell-mediated cytotoxicity (ADCC).

The overall immune responses mediated by these activation cascades should be beneficial for the treatment of a number of diseases where intact and strong innate and adaptive immune responses are a requirement for improvement or even cure of the disease (figure 1). For example, the decreased capacity of many types of tumor cells to induce an appropriate immunogenic signal often leads to an insufficient activation of innate immunity and, therefore, to inadequate adaptive immune responses resulting in a failure to eliminate or control the tumor. Therefore, CpG ODNs that induce the activation of critical components of the innate and adaptive immune system may stimulate an orchestrated immune response that is sufficient to eradicate tumors or control tumor growth, either alone or in combination with other antitumor therapies.

3. Route of Administration, Pharmacokinetics, and Biosafety

Various routes of administration have been tested and shown to be effective in animal models and humans.^[24,71–73] Depending on the therapeutic application, different routes of administration are employed. For vaccination studies, usually parenteral delivery (e.g. subcutaneous and intramuscular) has been used; direct delivery to the mucosal surface has also been shown to be effective.^[71] For infectious disease studies, it has been suggested that the route of delivery should be identical to the route of infection for optimal efficacy.^[73–76] In cancer studies, CpG ODN has been administered effectively by intratumoral, peritumoral, and subcutaneous injection.

tion.^[77-88] In the majority of cancer disease models, intravenous CpG ODN injections have turned out to be less effective than other routes of administration^[88] with some exceptions.^[89,90] The route of administration, therefore, appears to be an important factor in the success of treatment. Subcutaneous delivery of B-class CpG ODNs has been shown to induce a systemic Th1-like immune response in healthy volunteers, whereas intravenous application did not yield a strong effect.^[91] Recently, induction of the rate-limiting tryptophan-catabolizing enzyme indoleamine 2,3-dioxygenase (IDO) in mice by intravenous injection of CpG has been reported.^[92] Upregulation of IDO was not found following subcutaneous delivery of CpG ODNs. The induction of IDO is thought to be associated with the suppression of T-cell expansion.^[92] However, induction of IDO appeared to be dose dependent, as another report using lower doses of CpG ODNs did not observe IDO induction with intravenous administration of CpG ODN.^[93]

Characterization of the pharmacologic properties of CpG ODNs benefits greatly from the already well characterized properties of antisense ODNs, as both drug compounds are based on PS backbones.^[94,95] As with antisense ODNs, the major catabolic pathway of CpG ODNs involves cleavage by 3' exonucleases. Subcutaneous injection of CpG ODN results in a high concentration in the draining lymph node and the ODN is then distributed to the tissues, especially liver, kidney, and spleen.^[95]

The safety of PS ODNs has also been studied extensively in animal models. Some of the observed effects were sequence-independent, backbone-related effects and have been studied over a long period of time in the antisense field.^[96,97] Chronic administration of PS ODNs in rodents results in dose-dependent infiltration of mononuclear cells in the organs where the ODNs are cleared from circulation, i.e. liver, kidney, spleen, and bone marrow. However, such effects have so far not been reported for antisense ODNs in monkeys or humans.^[98] CpG-specific adverse effects in rodents have usually been reported to be related to high serum levels of proinflammatory cytokines (e.g. TNF α , which can result in a lethal cytokine storm).^[99] The high systemic levels of pro-inflammatory cytokines seen in mice have also been thought to be associated with a broad range of other pathogenic effects, such as the destruction of lymphoid follicles or extramedullary hematopoiesis following repeat administration of the highest CpG ODN dosages (60 μ g/day which is approximately 2.5 mg/kg).^[100] However, in humans the serum levels of pro-inflammatory cytokines such as TNF α remain mainly unchanged following CpG ODN administration,^[91] lower dosages than those applied by Heikenwalder et al.^[100] are used at much lower frequency in human clinical trials (0.0025–0.81 mg/kg),^[24] the route of administration of CpG ODNs is usually via subcutaneous injection, and extramedullary hematopoiesis was not observed in primates, even

after twice-weekly administration at 5 mg/kg for 6 months.^[91] In summary, injection of CpG ODNs in humans has so far not been reported to result in the same effects as observed in mice, suggesting that toxicities described in rodents are unlikely to occur in humans. This is most probably also due to the different TLR9 expression pattern in human and rodent immune cells (for reviews see Krieg,^[24] Vollmer,^[66] and Uhlmann and Vollmer^[101]).

Because of the recently investigated role of TLR9 in autoimmune diseases such as SLE,^[102] one concern appears to be the possibility of immunostimulatory ODNs inducing autoimmune reactions by stimulation of TLR9-dependent IFN α . Administration of a CpG B-class ODN alone to healthy volunteers was not found to increase autoantibody levels, and no evidence of autoimmune disease was observed.^[91] However, CpG ODNs can exacerbate ongoing inflammation or early autoimmune disease in certain mouse models, such as lupus,^[103] arthritis,^[104] multiple sclerosis,^[105] and colitis,^[106] although there are also animal models suggesting a beneficial impact of CpG ODNs (e.g. on colitis when the CpG ODN is given prior to the onset of inflammation).^[107]

It now appears from a variety of studies that not CpG DNA alone but its presence in self-immune complexes has pathogenic potential in lupus-prone mice, through synergistic engagement of the B-cell receptor and TLR9.^[102,108,109] Therefore, if making predictions based on murine models, precaution should be taken in the administration of CpG ODNs to patients with a diagnosed systemic or organ-specific autoimmune disease, because of the risk of flaring the disease. Extensive experience from human clinical trials is also available, and so far no incidence of autoimmunity has been reported over a wide dosage range (0.0025–0.81 mg/kg). CpG ODNs are usually well tolerated in healthy volunteers and patients, and reported adverse events are consistent with the mechanism of action of CpG ODNs, ranging from influenza-like symptoms to transient injection site reactions.^[24,110]

4. Infectious Diseases

Activation of the innate immune system by CpG ODNs has been demonstrated to be beneficial for the treatment of viral, bacterial, and parasitic diseases. Various animal models (for more details see earlier studies reviewed in Klinman,^[110] Krieg,^[111] and Dittmer and Olbrich^[112]) have been used to study the effects of CpG ODNs. Mouse models are the most commonly used, although some reports are available describing the role of CpG ODNs in other nonprimates (e.g. rats,^[77,113,114] chicken,^[115,116] and sheep),^[117,118] or in primates.^[119-121] Some reports also demonstrate an immunoprotective activity of CpG ODNs against infections in fish.^[122,123]

CpG ODNs protect mice from lethal doses of *Listeria monocytogenes*^[124,125] and *Francisella tularensis* live vaccine strain^[125] when administered prior to exposure. The protective effect in animal models was dependent on IL-12 and IFN γ .^[126] The presence of the transcription factor T-bet is also required for the protective mechanism, indicating the absolute requirement for a T_H1-driven immune response.^[127] In the case of *Mycobacterium tuberculosis* infection, intraperitoneal administration of CpG ODNs reduced bacterial growth in the lungs of infected mice through an IFN γ dependent mechanism.^[128] In addition, a CpG B-class ODN administered intraperitoneally 48 hours before bacterial challenge protected mice from a low aerosol dose of *Burkholderia mallei*, a category B biothreat agent causing glanders.^[129]

Similar to *Listeria* and *Francisella* infection, CpG ODNs can protect mice against parasitic infections with *Leishmania major* and malaria in an IL-12 and IFN γ -dependent manner.^[75,119,130] A protective effect could also be demonstrated in a primate model of *Leishmania*: lesions resulting from the treatment of macaques with *Leishmania amazonensis* were significantly reduced when the animals were treated with an A-class ODN 3 days before and 3 days after the infection challenge. This reduction of *Leishmania* lesions was also seen in simian immunodeficiency virus (SIV)-infected macaques, making CpG ODNs an interesting tool for potential therapeutic intervention in immunosuppressed patients.^[131] With a few exceptions,^[128,130] animals have usually been treated with CpG ODNs prior to pathogenic exposure in these disease models. Protection usually lasts from several days to up to 2 weeks, depending on the challenge model.^[73,132] An exception is *Leishmania* infection, where CpG ODN treatment was still beneficial when administered 20 days following infection.^[130]

In addition to these bacterial diseases, protective effects of CpG ODNs to viral challenges in murine models have been demonstrated for orthopoxviruses,^[133] foot and mouth disease virus,^[134] and Friend leukemia virus.^[135] In the case of the Friend virus model, treatment schedule seemed to be critical, and CpG ODNs appeared to be beneficial only after infection. The mechanism of protection involved CD8⁺ T cells and IFN γ production.^[136] In contrast, CpG ODN pretreatment rendered the mice more susceptible to Friend virus infection.^[137] B-class ODNs protected mice from lethal doses of influenza virus even in a senescence-accelerated mouse strain by restoring senescence-associated impaired T_H1 immune response, suggesting a potential therapeutic application especially in elderly people.^[138] Interestingly, the immunoprotective mechanism of baculovirus administered to mice prior to influenza challenge was demonstrated to be TLR9 dependent,^[139,140] and involved activation of TLR9-mediated immune responses by baculovirus DNA. A more recent study demonstrated the effec-

tiveness of CpG ODNs in protection from a neurotrophic viral disease (Tacaribe arenavirus) on the treatment of newborn mice with CpG ODNs (simultaneously or up to 3 days post challenge).^[141] Some members of the arenavirus family cause South African hemorrhagic fever and have been listed as potential biological warfare agents. The protection required inducible nitric oxide synthase (iNOS) expression and NO production and seemed to be mediated by an increase in specific antibodies.

Sexually transmitted infections are of special interest as they represent a major public health concern. Studies have shown the protective function of CpG ODNs against genital herpes in murine models.^[76,142-144] The best protection was achieved with the intravaginal injection of CpG ODN prior to herpes simplex virus (HSV)-2 challenge and required IFN γ , IL-12, and IL-18, and the presence of T cells.^[145] However, protection independent of IFN γ has also been reported.^[176] The protective mechanism was TLR9-dependent and based on the presence of cells from both hematopoietic and stromal compartments.^[146] pDCs were recruited rapidly to the vaginal mucosa following intravaginal CpG ODN delivery. In another study, pDC-depleted mice treated with CpG ODN were as susceptible to HSV-2 infection as nontreated wild-type mice, and protection was independent of IFN γ and IL-12.^[146] However, this discrepancy might have been due to differences in timing and administration in the individual challenge models.

In vitro studies have also demonstrated the inhibition of viral replication by CpG ODNs in human cells, such as in the hepatitis B virus (HBV) replication model. The inhibitory effect appeared not to be direct but secondary to CpG ODN treatment, as only supernatants of CpG ODN-treated peripheral blood mononuclear cells (PBMCs) inhibited HBV replication, whereas CpG ODNs alone did not result in inhibition.^[147] Interestingly, the inhibition of viral replication can also be observed with supernatants of CpG ODN-treated PBMCs derived from patients with chronic hepatitis B.^[148] In addition, a therapeutic effect of CpG ODNs was observed in hepatitis B animal models and appears to be mediated, at least in part, by the induction of type I IFN responses.^[149] Therefore, therapeutic CpG ODN treatment may be effective in chronic viral diseases such as hepatitis B or C.

CpG ODNs appear also to be an option for the treatment of HIV. Indeed, several reports demonstrate antiviral activity of CpG ODNs, especially of the A-class that have a high IFN α -producing capacity.^[150-152] However, some groups have also reported an induction of the HIV transcriptional regulatory elements in long terminal repeats when spleen cells of HIV-a transgenic mice were treated with CpG ODNs or bacterial DNA *in vitro*.^[153] Another group has reported the induction of antiviral cytokines after intravaginal administration of CpG ODN in Rhesus macaques, al-

Table II. Therapeutic use of CpG oligodeoxynucleotides (ODNs) in a number of selected clinical trials

CpG ODN (developing company)	Phase	Clinical trial details	References
Cancer			
PF-3512676 [CPG 7909] (Pfizer Coley)	I	Non-Hodgkin lymphoma; monotherapy	156
PF-3512676	II	Non-small-cell lung cancer; combination therapy	24,157
PF-3512676	III	Non-small-cell lung cancer; combination therapy	24
PF-3512676	Ib	Renal cell carcinoma; monotherapy	24,158,159
PF-3512676	Ib	Cutaneous T-cell lymphoma; monotherapy	24,160
PF-3512676	II	Melanoma; monotherapy and combination therapy	24,160,161
ODN 1018-ISS (Dynavax)	I	Non-Hodgkin lymphoma; combination therapy with rituximab	162
CpG-28	I	Recurrent glioblastoma; monotherapy	163
Hyb-2055 [IMO-2055] (Idera)	II	Renal cell carcinoma	164
Infectious disease			
CPG 10101 (Coley)	I	Hepatitis C	24
Asthma/allergy			
Ave0675 (Sanofi-Aventis/Coley)	I	Asthma/allergic rhinitis	24
Vaccines			
PF-3512676 (Coley with partner)	I	Cancer vaccination (renal cell carcinoma, melanoma)	24,158,165
PF-3512676	I	Hepatitis B, influenza, anthrax, and other	166-168
ODN 1018-ISS	III	Hepatitis B, asthma/allergy (ODN conjugated to protein)	169,170

though vaginal transmission of the SIV with this route of administration could not be prevented.^[121]

HCV, with approximately 170 million infected patients worldwide, has become a promising indication for treatment with immunostimulatory CpG ODNs. CPG 10101 is a C-class ODN, inducing high levels of IFN α together with activating B cells and pDCs. Indeed, recent data from a phase Ib hepatitis C clinical trial indicated that CPG 10101 shows antiviral activity as monotherapy. Standard-of-care treatment for chronic HCV is currently the combination of pegylated IFN α with ribavirin. In a phase Ib trial involving patients who were relapsed responders to pegylated IFN and ribavirin, the addition of CPG 10101 improved the antiviral activity of these agents^[154,155] (for an overview on clinical trials with CpG ODNs also see table II).

5. Treatment of Cancer

The antitumor activity of CpG ODNs has been established in numerous mouse models (see review by Krieg^[88]) and continues to be evaluated in various clinical trials treating different tumors.^[24,72] The mechanism of action is thought to be associated with the stimulation of NK-cell activity and cytotoxic T lymphocytes together with the induction of cytokines and chemokines. In addition, CpG ODNs may also act as maturation activators of professional APCs to induce tumor antigen presentation to tumor-

specific effector T cells, shifting them from tolerance to activation.^[24,171]

Depending on the tumor, monotherapy or therapy with CpG ODNs in combination with chemotherapy or radiotherapy appear to be promising treatment options. The combination of chemotherapy with CpG ODNs may show synergy and is thought to increase the effectiveness of treatment by boosting the immune system and promoting the development of an antitumor T-cell response able to control the tumor and improve survival, while the reduction of tumor burden is achieved by the conventional therapy. Tumor vaccination approaches also utilize the adjuvant properties of CpG DNA (see section 6).

In mice, B-class ODNs strongly enhance the response of tumors to either a variety of chemotherapeutic agents, such as docetaxel,^[171] SBP-002 (coramsine)^[172] cyclophosphamide,^[173] and topotecan,^[82,173,174] or to radiotherapy.^[171,175-177] However, in certain murine cancer models the presence of T cells and B cells is not necessary for the beneficial effect of CpG ODNs.^[178-180] Indeed, it has recently been shown that activation of murine macrophages by CD40 ligation and subsequent stimulation with CpG ODNs provides antitumor activity in the absence of T cells or NK cells.^[181-184] In other tumor models, the necessity for T cells and/or NK cells has been similarly demonstrated.^[77,81] Radiotherapy combined with CpG ODNs has been shown to be a successful

combination for the treatment of malignant glioma in rats.^[77] However, this therapeutic approach did not work in nude mice, most likely due to the absence of T cells. The radiotherapy combination is currently being evaluated in a phase I trial for patients with recurrent glioblastoma using a B-class ODN, CpG-28.^[163] Expression of TLR9 within the glioma cells might also contribute to the beneficial effect observed in the animal models. TLR9 is overexpressed not only in murine but also in human glioma cell lines. CpG ODNs were demonstrated to induce TLR9 downregulation in TLR9-expressing cells,^[14,39] and also in glioma cells *in vitro* as well as *in vivo*. The subsequent apoptosis of glioma cells most probably results in prolonged survival of mice with experimental brain tumors.

A recent report^[178] compared the activity of CpG ODNs with the anti-Bcl-2 antisense ODN oblimersen (G3139) tested in phase II and III clinical trials for various tumors in combination with chemotherapy. In this model, B-class ODNs and oblimersen were effective in the treatment of human tumor xenografts in nude mice. Nevertheless, B-class ODNs required a much smaller dose than the antisense oblimersen. Methylation of cytidine within the CpG motif of oblimersen completely abolished its antitumor activity, indicating that the activity in human tumor grafts was CpG-dependent and not based on the anti-Bcl-2 antisense effect.

In a phase I clinical trial for the treatment of non-Hodgkin lymphoma (NHL)^[156] the safety and tolerability of the CpG B-class ODN PF-3512676 (CPG 7909) was determined as well as its effects on ADCC, NK-cell activity, and cytokine production. NK-cell concentration and activity was increased in most of the patients. Patient response was formally assessed on day 42, at which time no patient met the National Cancer Institute consensus criteria for response. However, two patients showed evidence of disease regression without further therapy and met the International Workshop Criteria for Complete Response several months after the study endpoint.^[156] Treatment of relapsed NHL patients has also been assessed in a phase I clinical trial using a combination of ODN 1018-ISS and rituximab, an anti-CD20 chimeric monoclonal antibody.^[162] 1018-ISS was administered subcutaneously and induced a systemic effect in patients, which appeared to be largely mediated by the production of type-I IFNs. One concern in the treatment of lymphoma with CpG ODNs was their potential to induce B-cell proliferation, but this was not observed in the combination therapy study^[162] or monotherapy trial.^[156] Moreover, *in vitro* B-cell chronic lymphoma (B-CCL)-cell proliferation was induced by treatment with CpG ODNs but was followed by an increased rate of apoptosis leading to a lower overall survival of B-CCL cells.^[185,186]

Recently, the results of a phase II trial with PF-3512676 in patients with unresectable stage IIIB/c or stage IV metastatic

melanoma were published.^[187] Two patients experienced a confirmed partial response, one ongoing for more than 140 weeks, and three patients experienced stable disease. An increase in the number of activated pDCs, increased serum levels of 2',5'-oligoadenylate synthetase, a surrogate marker for IFN α , and significant stimulation of NK-cell cytotoxicity were observed. PF-3512676 has also been tested in a phase Ib trial in advanced renal cell carcinoma^[159] and cutaneous T-cell lymphoma (CTCL), with encouraging results demonstrating significant antitumor activity in CTCL^[160].

In a phase II trial involving patients with advanced non-small-cell lung cancer (NSCLC), chemotherapy-naïve patients received standard chemotherapy alone or in combination with subcutaneous administration of PF-3512676. The primary endpoint in the trial was response rate, which was improved from 19% in the group receiving chemotherapy alone to 37% in the group receiving PF-3512676 in addition to standard chemotherapy.^[24,157] In addition, overall survival was a median of 6.8 months (chemotherapy) compared with 12.8 months (combination arm).

6. Vaccination

Infectious diseases are still responsible for millions of deaths annually, especially in underdeveloped countries. Current immunization programs greatly reduce the mortality often caused by infectious diseases. Nevertheless, more effective vaccines are needed to reduce the overall costs of vaccinations, particularly in poorer countries, as well as to increase the efficacy of vaccines in cases of hyporesponsiveness. One way to improve vaccination is the use of adjuvants, but the only adjuvant licensed to date for human use is aluminum hydroxide. From all adjuvants currently in preclinical and clinical development, CpG ODNs appear to be one of the most promising.^[71] CpG ODNs induce strong antigen-specific humoral as well as cellular immune responses against a wide variety of different antigens. Remarkably, CpG ODNs create a microenvironment that promotes the induction of Th1-biased, antigen-specific adaptive immune responses. In fact, CpG ODNs trigger the strongest Th1-type responses in mouse models when compared with other known adjuvants or TLR ligands.^[188,189] Synergy between CpG ODNs and several other adjuvants has been observed^[71] and is probably explained, for example, by the enhanced uptake of CpG ODNs and antigen in antigen-presenting cells (e.g. with liposomes).^[57,190] Indeed, enforced uptake of CpG ODNs into endosomal compartments *in vitro* by admixing them with an uptake enhancer results in increased immune stimulation. Recent data suggest that enhanced ligand concentrations in TLR9-containing endosomal compartments or altered intracellular

compartmental distribution allowing for preferred stimulation of IFN signaling may be responsible for the observed effects.^[33,57,190]

CpG B-class ODNs have been evaluated as adjuvants in human immunization. For example, in clinical trials the combination of CpG ODNs (e.g. PF-3512676 or 1018-ISS) with an alum-absorbed HBV vaccine Engerix-B®¹ administered intramuscularly resulted in a significant improvement over the HBV vaccine alone.^[166,167,169,170] Protective antigen-specific antibody levels appeared sooner and were higher at all timepoints in PF-3512676 recipients compared with control subjects. PF-3512676 also enhanced the affinity maturation process, suggesting that CpG ODNs induce circulating antibodies and memory B cells with higher antigen-binding affinity and more potent neutralizing activity.^[191] In another trial, the addition of CpG ODN (PF-3512676) to an anthrax vaccine induced a statistically significant increase in the peak titer of toxin-neutralizing antibody, as well as increasing the proportion of subjects who achieved a strong antigen-specific IgG response.^[24] In addition to these encouraging results obtained in healthy human volunteers, CpG ODNs also overcame vaccination hyporesponsiveness. For example, admixing of CpG ODN with the HBV Engerix-B® vaccine increased seroconversion rates after only two doses to 100% and enhanced antibody titers in orangutans that are hyporesponders to this vaccine.^[192] In human clinical trials, HIV-positive individuals who were previously nonresponsive to the Engerix-B® vaccine developed protective antibody titers when the vaccine was administered together with PF-3512676.^[166] Therefore, CpG ODNs appear not only to be excellent adjuvants for prophylactic and therapeutic vaccination, but also to help vaccine-hyporesponsive populations to benefit from vaccinations.

The adjuvant activity of CpG ODNs has also been exploited extensively for tumor vaccination in therapeutic and prophylactic settings with promising results.^[193,194] CpG ODNs have been reported to be superior in the induction of a CD8+ T-cell response to the major histocompatibility complex class I-restricted TERT peptide and delayed mortality in an induced tumor model.^[195] Study of the CD8+ T-cell response after the antigenic challenge suggested that a functional antitumor response is induced after vaccination with CpG. For effective tumor vaccination, CD8+ T cells seem to play a major role.^[196,197] The effective induction of a CD8+ T-cell response has also been demonstrated in melanoma patients. In a clinical trial, patients were vaccinated with a combination of incomplete Freund's adjuvant (IFA), PF-3512676, and melanoma antigen A analog peptide. All patients exhibited a rapid and strong melan A-peptide-specific T-cell response that was one order of magnitude higher than in the control group where they

only received peptide and IFA.^[198,199] The antigen-specific CD8+ T cells circulating in the blood of the patients showed functional and phenotypic properties similar to those specific for immunodominant viral antigens such as cytomegalovirus or Epstein-Barr virus. However, CD8+ T cells infiltrating the tumor remained functionally different to their circulating counterparts. This observation coincided with the presence of regulatory T cells within the tumor,^[199] highlighting the negative role of regulatory cells in the antitumor response.

Another clinical trial using PF-3512676 for vaccination with another melanoma-associated protein, melanoma associated antigen (MAGE) A3, resulted in two patients with stable disease and two with partial responses lasting at least 1 year, out of a total of six patients.^[165] A phase I/II trial with PF-3512676 and granulocyte-macrophage colony-stimulating factor followed by subcutaneous administration of PF-3512676 and IFN α in patients with metastatic renal cell carcinoma has been initiated only recently. Preliminary results appear to suggest a specific antitumor response.^[158]

Tumor vaccination in the presence of CpG ODNs also has very high potential in the treatment of papillomavirus-associated cancer. In a prophylactic setting, a systemic injection of E7 peptide and CpG ODN at day 14 and day 7 prior to injection of tumor cells to mice prevented tumor growth in the treated animals, whereas control mice all developed aggressive tumors. Peptide vaccination in the presence of CpG ODN also induced regression of E7-transformed tumors when given 7, 14, and 21 days after the injection of tumor cells. Neither E7 nor CpG ODN alone was as effective.^[194,200] The introduction of CpG motifs into plasmid-based DNA vaccines has also been used to successfully induce melanoma antigen-specific protective T-cell responses in a melanoma model.^[201] Protection was significantly higher than with vaccination with plasmid-based DNA not containing the CpG motifs.^[202]

7. Treatment of Asthma and Allergy

Allergic diseases affect a high percentage of the population in developed countries. Asthma is the most severe atopic disorder due to inappropriate Th2 responses in the lung resulting in airway hyper-reactivity and remodeling. Interestingly, a study on genetic polymorphisms within *TLR* genes recently suggested a single nucleotide polymorphism (SNP) in the promoter region of the human *TLR9* gene possibly associated with asthma in Europeans.^[203] However, another European study involving 527 healthy human volunteers including more than 150 atopic participants, and another with 32 asthmatic children in Japanese families, could not

1 The use of trade names is for product identification purposes only and does not imply endorsement.

find this or other *TLR9* SNPs likely to be associated with asthma.^[204,205]

Because of the imbalance in the T_H1/T_H2 cytokine ratio with a predominant T_H2 profile found in atopic disorders, the induction of T_H1 responses should influence the development of such diseases by dampening the T_H2 response. Indeed, bacterial infections or the application of bacterial preparations, such as from BCG inducing antibacterial T_H1 responses in the host, can inhibit the development of allergic responses in animal models of asthma.^[206,207] CpG ODNs induce targeted *TLR9*-dependent T_H1 immune responses, and an opposing effect on T_H2 responses in the airways upon CpG ODN administration can be observed.^[208] The treatment with CpG ODNs in animal models of asthma results in the inhibition of the development of airway hyper-responsiveness, as well as the reversal of established disease.^[23] Besides their use as monotherapy, CpG ODNs can dramatically improve the efficacy of allergy vaccines in mouse models when given before or after an allergen challenge, especially when directly linked to the allergen.^[209-211] It is possible that different mechanisms are responsible for the therapeutic and prophylactic effects of CpG ODNs in the treatment of asthma. CpG ODNs induce T_H1 -biased immune effects,^[66,212] and the rebalancing T_H2/T_H1 effect of CpG ODNs may be the result of the inhibition of T_H2 -mediated effects or the inhibition of T_H2 cell activation. Nevertheless, any influence of CpG ODN stimulation on, for example, activities mediated by T_H2 cytokines such as IL-4 appears unlikely.^[213] Therefore, CpG ODNs may have a direct effect on allergic effector cells such as mast cells and basophils and inhibit, for example, the release of T_H2 cytokines. Indeed, human mast cells appear to express *TLR9* and may be responsive to *TLR9* stimulation.^[214] Nevertheless, more recent data indicated an involvement of the pulmonary expressed rate-limiting, tryptophan-catabolizing enzyme IDO in the reduction of local inflammation.^[93] *TLR9* signaling enhances IDO expression, and dendritic cells expressing IDO participate in immunoregulation. Therefore, CpG ODN-mediated induction of IDO activity may contribute to the counter-regulation of T_H2 -mediated inflammatory responses and reduction of lung damage. In addition, a change in the expression of co-stimulatory molecules on antigen-presenting cells involved in allergic airway inflammation by CpG ODNs has been suggested.^[213] A decrease of the overall expression of co-stimulatory or regulatory molecules such as CD86 or programmed cell death 1 (PD-1) and, therefore, a reduction of the stimulation of T_H2 T cells in the airways with CpG ODN treatment, may result in the prevention of T_H2 cytokine release involved in atopic processes in the lung.

Recent studies have demonstrated that repeated inhalation of CpG ODNs can treat or prevent allergic airway responses not only in mouse models but also in primates.^[215] In a small clinical study,

asthmatic subjects received CpG ODN treatment (1018-ISS) weekly for 4 weeks by inhalation. Although the CpG ODN treatment resulted in a T_H1 immune activation pattern in the airways, no obvious clinical benefit was observed.^[216] Nevertheless, these data rather indicate a further requirement for disease-specific optimization of the ODN design, or dose and schedule of ODN treatment. In human vaccine clinical trials with a conjugate of a CpG ODN to a portion of the ragweed allergen, encouraging evidence for a selective and specific redirection of the allergic T_H2 response towards a non-allergic and non-inflammatory T_H1 response was observed.^[217] Although allergy vaccines with CpG ODNs can provide antigen-specific rapid redirection of allergic responses, inhaled CpG ODN monotherapy offers the potential advantage of antigen-independent treatment of a broad spectrum of allergic patients.

8. Conclusion

The stimulation of the immune system by synthetic CpG ODN via *TLR9* leads to a variety of directed effects linking innate to adaptive immune responses. *TLR9* agonists can be used as highly effective targeted immune modulatory drugs with broad potential applications as vaccine adjuvants, and as stand-alone therapy or in combination with other therapies in cancer, infectious diseases, or asthma and allergy. Data from studies in animal models and ongoing phase I–III clinical trials suggest that CpG ODNs have antitumor activities and are effective vaccine adjuvants in a variety of disease indications, and also support the development of CpG ODNs as monotherapy agents for allergic diseases.

Acknowledgments

The authors are employees of Coley Pharmaceutical GmbH and hold company stock options. No external sources of funding were used to assist in the preparation of this review.

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