Synthetic agonists of Toll-like receptors 7, 8 and 9

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Abstract

TLRs (Toll-like receptors) are a family of innate immune receptors that induce protective immune responses against infections. Single-stranded viral RNA and bacterial DNA containing unmethylated CpG motifs are the ligands for TLR7 and TLR8 and 9 respectively. We have carried out extensive structure–activity relationship studies of DNA- and RNA-based compounds to elucidate the impact of nucleotide motifs and structures on these TLR-mediated immune responses. These studies have led us to design novel DNA- and RNA-based compounds, which act as potent agonists of TLR9 and TLR7 and 8 respectively. These novel synthetic agonists produce different immune response profiles depending on the structures and nucleotide motifs present in them. The ability to modulate TLR-mediated immune responses with these novel DNA- and RNA-based agonists in a desired fashion may allow targeting a broad range of diseases, including cancers, asthma, allergies and infections, alone or in combination with other therapeutic agents, and their use as adjuvants with vaccines. IMO-2055, our first lead candidate, is a TLR9 agonist that is currently in clinical evaluation in oncology patients. A second candidate, IMO-2125, is also a TLR9 agonist that has been shown to induce high and sustained levels of IFN (interferon) in non-human primates and is being evaluated in HepC-infected human subjects.

Introduction

The host defence system primarily relies on the ability of the innate immune system, which is the first line of defence, to recognize danger signals associated with pathogens and activate immediate, rapid and appropriate immune response to fight the infection [1]. Besides limiting the early spread of infection, the innate immune system also induces a delayed pathogen- or antigen-specific adaptive immunity and immunological memory responses. The innate immune system consists of highly conserved receptors called PRRs (pattern-recognition receptors), which sense danger signals based on the cellular constituents present in the pathogens. TLRs (Toll-like receptors) are one class of PRR of which ten have now been identified in humans [2]. TLRs recognize PAMPs (pathogen-associated molecular patterns) such as lipoproteins/lipopeptides, flagellin, lipopolysaccharides, and nucleic acids of bacteria and viruses. Unmethylated CpG motifs are one type of PAMP that are present in bacterial and viral DNAs.

TLRs

TLRs are transmembrane receptors comprising an extracellular leucine-rich repeat and a cytoplasmic TIR [Toll/IL-1 (interleukin-1) receptor] domain, connected through a transmembrane domain [3]. The TIR domain has structural homology with the IL-1 receptor. In general, upon encountering a PAMP, TLRs recruit an appropriate adaptor protein to the TIR domain [4]. This leads to the engagement of IRAK (IL-1-receptor-associated kinase), TLR-specific additional adaptor proteins, and TRAF6 (tumour-necrosis-factor-receptor-associated factor 6) in the signalling pathway, resulting in the activation of transcription factors AP-1 (activator protein 1), ELK-1 [ETS (E twenty-six)-like kinase 1], NF-κB (nuclear factor κB), and others, depending on the signalling pathway activated [5].

Of the ten TLRs identified in humans, TLR3, 7, 8 and 9, referred to as intracellular TLRs, are expressed on the membranes of the endosomes and recognize nucleosides, nucleotides and oligo- and poly-nucleotides derived from intracellular viral and bacterial pathogens [6]. TLR3 is the receptor for viral and synthetic dsRNAs (double-stranded RNAs) [7]. TLR7 and 8 are the receptors for viral ssRNAs (single-stranded RNAs) and nucleosides [8–10]. Bacterial DNAs containing unmethylated CpG motifs comprise the ligand for TLR9 [11]. The other six TLRs are referred to as cell-surface TLRs. They are expressed on the cell or plasma membrane and recognize cell-wall constituents such as lipoproteins/lipopeptides, flagellin and lipopolysaccharides of extracellular microbial agents [6].

Intracellular TLRs

All of the nucleic acid-recognizing TLRs are expressed on endosomal membranes, and their expression is cell-specific. In humans, TLR3 is expressed in mDCs (myeloid dendritic cells) and NK cells (natural killer cells). TLR7 and 9 are expressed in human B-cells and pDCs (plasmacytoid dendritic cells). TLR8 is expressed in mDCs, monocytes and

Key words: agonist, immune stimulation, interferon α (IFNα), intracellular Toll-like receptor, pathogen-associated molecular pattern, unmethylated CpG motif.

Abbreviations used: EGFR, epidermal growth factor receptor; HEK-293 cell, human embryonic kidney cell; IFN, interferon; IP-10, IFNγ-inducible protein 10; IL-1, interleukin-1; mDC, myeloid dendritic cell; MIP, macrophage inflammatory protein; NF-κB, nuclear factor κB; NK cell, natural killer cell; OVA, ovalbumin; PAMP, pathogen-associated molecular pattern; PBMC, peripheral blood mononuclear cell; pDC, plasmacytoid dendritic cell; PRR, pattern-recognition receptor; SIMRA, stabilized immune modulatory RNA; ssRNA, single-stranded RNA; TIR, Toll/IL-1 receptor; TLR, Toll-like receptor.

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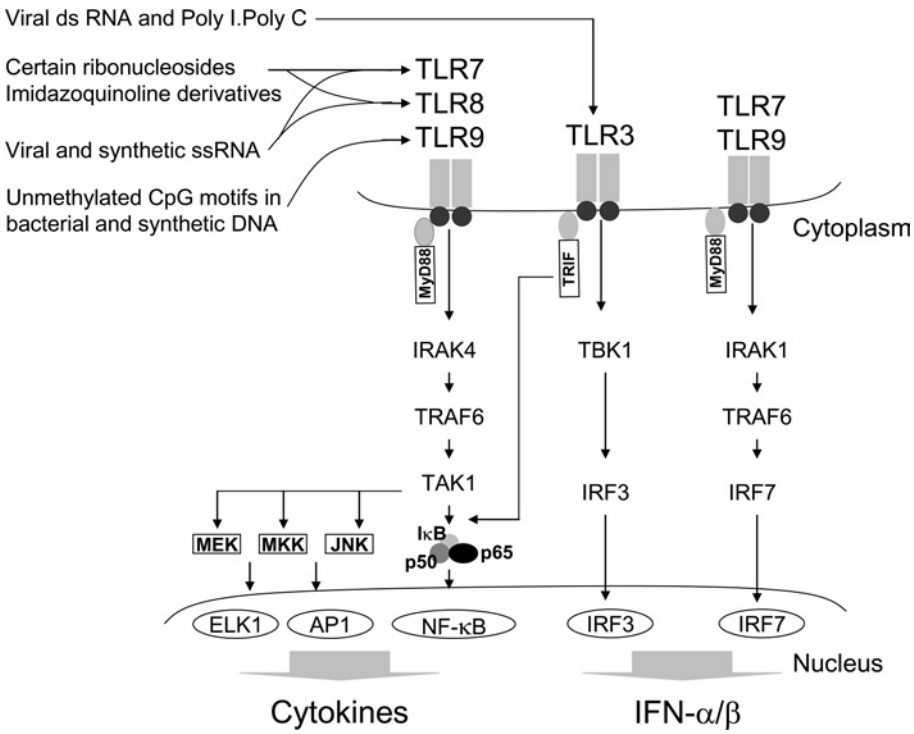
Figure 1 | **Brief intracellular signalling pathways activated following stimulation of TLR3, 7, 8 and 9 with appropriate agonists**
Agonists known to stimulate each TLR are indicated.



neutrophils. In general, the TLR-mediated immune responses include cytokine/chemokine production and activation of surface molecules. However, the cytokine profile produced by activation of each TLR is dependent on the PAMP it recognizes (type of micro-organism encountered), the cell type it is expressed in, and the signal transduction cascades the specific TLR activates (Figure 1). In general, nucleic acid-based agonists of TLRs induce Th1-type immune responses.

Agonists of TLR7 and/or TLR8

Viral and synthetic ssRNAs act as agonists of TLR7 and TLR8 [9,10]. Certain nucleoside analogues, such as loxoribine, 7-thia-8-oxo-guanosine and 7-deazaguanosine, also activate TLR7 at concentrations greater than 100 μM [8]. Imidazoquinoline-based compounds also act as agonists of TLR7 and TLR8 [8] and interact with adenosine receptor signalling pathways to induce immune responses [12]. Recent studies have shown that synthetic RNAs that act as gene expression-control agents [siRNAs (small interfering RNAs)] also induce TLR3- [13], TLR7- and TLR8- [14–16] mediated immune responses. The recognition of RNA by TLR7 and TLR8 is dependent on the nucleotide composition.

RNA is rapidly degraded by nucleases *in vivo*, which limits its use as an immune modulator. Nuclease degradation of RNA primarily occurs from the 3′-end by exonucleases, with degradation at internal sites by non-specific and dinucleotide-specific endonucleases, and to a lesser degree from the 5′-end. All the studies reported to date have used formulation of ssRNA-based TLR7/8 agonists with lipids to increase their nuclease stability and enhance delivery [17].

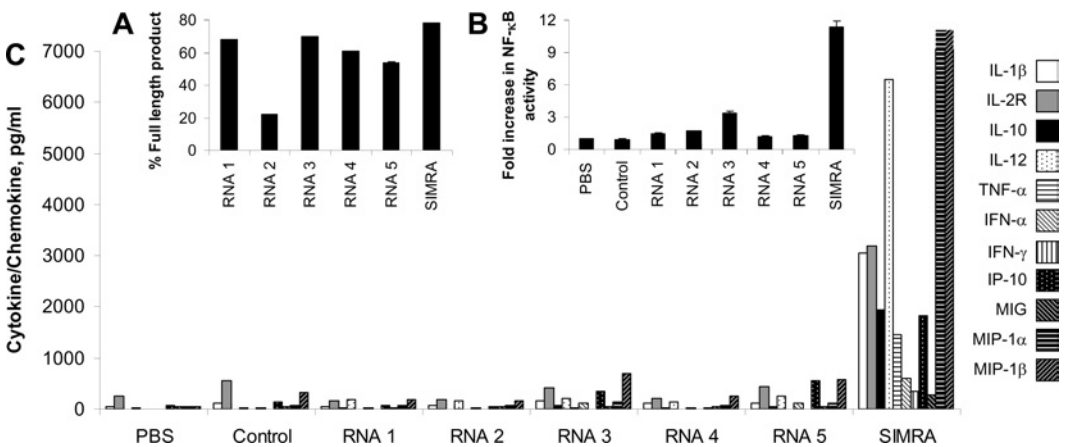
We have recently reported the use of RNAs linked through their 3′-ends to prevent 3′-exonuclease degradation, referred to as SIMRA (stabilized immune modulatory RNA) compounds [18]. SIMRA compounds are more stable in human serum than are ssRNA. We have also observed that the stability of ssRNA depends on their nucleotide composition: specific dinucleotide motifs such as UA and CA are susceptible to rapid degradation by endonucleases [18]. Avoiding these dinucleotide motifs enhances stability of ssRNA against endonucleases.

We have synthesized several ssRNAs with a phosphorothioate backbone and examined their stability against nucleases in human serum. These ssRNAs showed varying levels of stability, ranging from 1 to 70% full-length product remaining after 10 min of incubation in human serum without using cationic lipids (Figure 2A) [18]. In the absence of cationic lipids, even the most stable ssRNAs showed no or weak activation of human TLR8-transfected HEK-293 cells (human embryonic kidney cells; Figure 2B) and in human PBMC (peripheral blood mononuclear cell) cultures (Figure 2C) compared with a SIMRA compound. These results suggest that nucleotide composition and nuclease stability of ssRNA are both important for the activation of TLR8.

Additionally, non-nucleoside modifications incorporated at the 5′-end of SIMRA compounds increase their stability

Figure 2 | *In vitro* nuclease stability and immune stimulatory activity of synthetic agonists of TLR7 and TLR8

(**A**) Nuclease stability of phosphorothioate-modified ssRNA and a SIMRA compound in human serum for 10 min. Samples were analysed by anion-exchange HPLC and the results are presented as percentage of intact full-length compound compared with before digestion (see [18] for experimental details). (**B**) Activation of HEK-293 cells expressing human TLR8 by RNAs and a SIMRA compound at 150 μg/ml concentration without cationic lipid formulation. Results shown are representative of three or more independent experiments. (**C**) Cytokine and chemokine induction by RNAs and a SIMRA compound in 24 h human PBMC cultures at 150 μg/ml concentration without cationic lipids. Cytokine and chemokine levels in culture supernatants were determined by the luminex multiplex assay [18]. MIP (macrophage inflammatory protein)-1α and MIP-1β values are out of the scale and the values are 86358 and 161402 pg/ml respectively. Sequence compositions of RNAs used in the study are: RNA 1, 5′-UGCUGCUUCUGUGCUGCUUCUG-3′; RNA 2, 5′-UGCUGCUUCUGGUCUUCGUCGU-3′; RNA 3, 5′-UUGGUUGUUUGGUUUGUUGGUU-3′; RNA 4, 5′-UGCUGCCUUUGGUUUCCGUCGU-3′; RNA 5, 5′-UGCUGCUUGUGGUG-UUCGUCGU-3′; SIMRA compound, 5′-YUGCUGCUUGUG-X-GUGUUCGUCGUY-5′; control, 5′-AAAAAAAAAAA-X-AAAAAAAAAAA-5′, where X and Y stand for 1,2,3-propanetriol and 1,3-propanediol linkers respectively.



against nucleases and further enhance their ability to activate TLR8 [18]. In addition, when the guanosines in TLR8-activating SIMRA compounds are replaced with 7-deazaguanosine, the compounds activate TLR7 in addition to TLR8, providing for the first time RNA-based compounds that activate both TLR7 and TLR8 [18]. Thus we have identified SIMRA compounds that activate TLR8 only and both TLR7 and TLR8 depending on the chemical modifications incorporated.

The SIMRA compounds that activate TLR7, but not TLR8, induce cytokine production in mouse splenocyte cultures and *in vivo* in mice [18], as mice lack a functional TLR8. Both TLR8- and TLR7- and TLR8-activating SIMRA compounds induce dose-dependent Th1-type cytokine production in human PBMC cultures [18], but only the latter induce IFN (interferon) α production in human PBMC and pDC cultures [18]. pDCs, which express TLR7 and 9, but not TLR8, are the primary producers of IFNα upon stimulation with appropriate TLR agonists [19].

Administration of a single dose of SIMRA compounds to Rhesus monkeys induces lymphocyte migration, activation of CD69 on T-lymphocytes, monocytes and NK cells, and elevation of cytokine and chemokine levels in the plasma [18]. A SIMRA compound that activates both TLR7 and 8 produces higher levels of IFNα than does a TLR8-only-activating SIMRA compound [18].

Agonists of TLR9

Bacterial and synthetic DNA containing unmethylated CpG motifs act as agonists of TLR9 and induce Th1-type immune response profiles. The immune-stimulatory effects of TLR9 agonists are multifactorial and depend on the nucleotide sequence, the nature of the backbone and the presence of specific structural motifs. Based on the cytokine profiles induced, three distinct types of TLR9 agonists, class A, B and C, have been described in the literature [20]. Each class of TLR9 agonist is composed of a different nucleotide sequence that allows formation of structures (or no structures) that generate different immune profiles.

We have systematically studied the structure–activity relationships of oligonucleotides that act as agonists of TLR9 [21]. The presence of a CpG motif in oligonucleotides is required for TLR9 stimulation. Oligonucleotides with phosphodiester and phosphorothioate backbone stimulate TLR9-mediated immune responses. Phosphorothioate backbone oligonucleotides are commonly used because they are less susceptible to degradation by ubiquitous nucleases than are phosphodiester oligonucleotides. Introduction of a sulfur atom on the internucleotide phosphodiester bond results in the formation of Rp and Sp diastereoisomers; the Rp diastereomer of phosphorothioate linkage stimulates a stronger TLR9-mediated immune response than does the Sp diastereomer [22]. The

negative charges on phosphates between and adjacent to cytosine (C) and guanine (G) are also required for TLR9-mediated activity. Neutralization of charges by incorporation of methylphosphonate linkages at these positions results in the loss of immune-stimulatory activity [23–25]. Moreover, TLR9 activation is also dependent on the sequences flanking the CpG dinucleotide, the nature of the nucleotide backbone and the secondary structures [25–27].

Flanking sequences play a significant role in TLR9 stimulation

Chemical modifications introduced at the 2′-position of the sugar ring of a C or G nucleotide in the CpG motif result in the loss of immune-stimulatory activity of TLR9 agonists [23]. In addition, studies of TLR9 agonists containing chemical modifications such as methylphosphonate linkages [24,25], 2′-alkyl or 3′-deoxy or -alkyl ribonucleosides [28–30], non-nucleotide linkers [31] or abasic nucleotides [32,33] in the flanking sequences indicate that substitutions incorporated at the fourth to sixth nucleotide positions 5′ to the CpG dinucleotide significantly enhance immune-stimulatory activity. In general, modifications incorporated in the 3′-flanking sequence distal to the CpG dinucleotide have effects dependent on the nature of the modification [24–33].

TLR9 requires a free 5′-end of agonist for stimulation

Two CpG oligonucleotides linked through their 5′-ends do not activate immune cells despite the availability of two CpG motifs [34,35]. When the same oligonucleotides are linked through their 3′-ends, they produce higher and distinct cytokine profiles than the parent CpG oligonucleotide with a single 5′-end. These are the first studies demonstrating the requirement of an accessible or free 5′-end for TLR9 activation and that the receptor reads the sequence from the 5′-end. The transcription factor NF-κB is rapidly activated by TLR9 agonists that contain two 5′-ends, but these compounds have the same activity as conventional TLR9 agonists on the MAPK (mitogen-activated protein kinase) pathway in J774 cells [36].

These studies suggest that agonists containing two 5′-ends facilitate dimerization of the receptor, leading to rapid activation of immune responses. Moreover, TLR9 activation can be modulated through appropriate presentation of the free 5′-ends and synthetic immune-stimulatory motifs, leading to changes in the downstream cytokine induction profiles. Consistent with these results, recent studies have shown that TLR9 exists in dimer form and binds to single-stranded oligonucleotides [37]. However, only oligonucleotides containing the CpG motif cause conformational changes in the receptor, leading to the activation of immune signalling pathways [37].

The attachment of oligonucleotides through their 3′-ends not only provides two 5′-ends for optimal activation of TLR9, but also increases the stability against 3′-exonucleases. Oligonucleotides with a phosphodiester backbone and as short as 5 and 6 nt linked through their 3′-ends act as potent TLR9 agonists and produce immune responses [38,39]. Moreover, oral administration of the novel structure containing TLR9 agonists induces potent mucosal immune responses, acts as an adjuvant with antigens, and prevents and reverses peanut allergy in mouse models because of their greater stability in the gastrointestinal tract [40,41].

Functional groups of cytosine and guanine required for TLR9 stimulation

As described above, certain chemical modifications introduced within the CpG dinucleotide that alter structure and conformation lead to the loss of immune-stimulatory activity of agonists. One such modification is a replacement of the methyl group at the 5-position of cytosine in the CpG motif of TLR9 agonists [23]. Vertebrates use this feature to distinguish self-DNA from that of bacterial DNA, which contains more unmethylated CpG motifs.

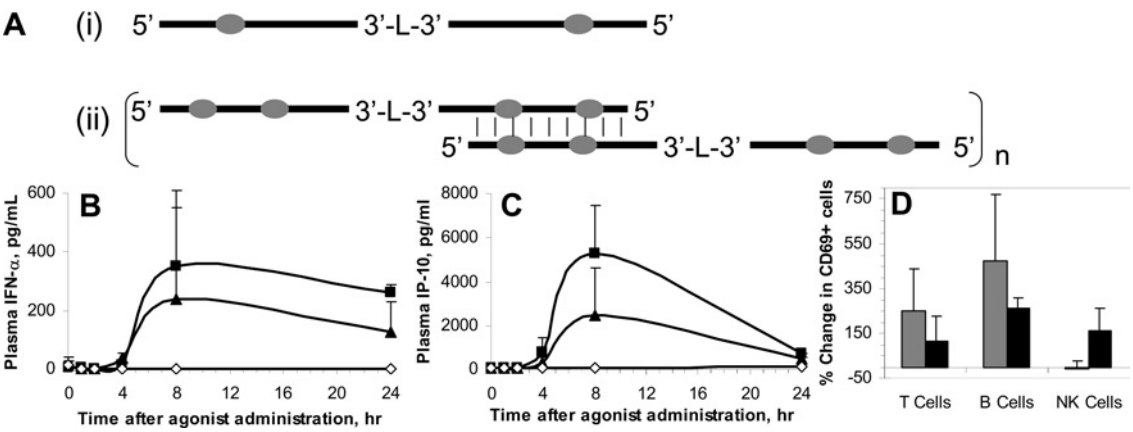
We have studied the effects of various pyrimidine analogues (Y), such as 5-OH-dC, dU, dP, 1-(2′-deoxy-β-D-ribofuranosyl)-2-oxo-7-deaza-8-methyl-purine, N^3-Me-dC and N^4-Et-dC, in place of cytosine [42–44]. To understand the role of different functional groups of guanine in the recognition of TLR9, several purine nucleobases (R) such as 7-deaza-dG, N^1-Me-dG, 2-amino-D-purine, nebularine, 2-amino-dA, 7-deaza-D-xanthine, K-base and dI were examined in place of guanine in the CpG [42–46]. These studies led to the development of alternative synthetic nucleotide motifs (YpG, CpR) for immune modulation and have demonstrated acceptance by TLR9 of certain heterocyclic base variants.

Novel synthetic agonists of TLR9

The combinations of novel structures and synthetic immune-stimulatory motifs described above provided us with tools to generate combinatorial libraries of novel synthetic agonists of TLR9. Systematic studies of several TLR9 agonists that have two 5′-ends and contain synthetic CpR dinucleotides in different nucleotide compositions in mouse, human and monkey systems suggest that nucleotide sequence and secondary structures play a role in modulating the immune response. Based on these studies, we have broadly identified two different groups of synthetic agonists of TLR9 (Figure 3A). Both types of TLR9 agonist contain two short segments of phosphorothioate oligonucleotides with a synthetic immune-stimulatory motif (CpR, YpG or R′pG) attached through their 3′-ends (Figure 3A). The first type of compound (**i**) does not form a secondary structure, whereas the second type (**ii**) forms intermolecular secondary structures as a result of the presence of a palindromic sequence (Figure 3A). Both types of TLR9 agonist that contain a CpR dinucleotide motif activate HEK-293 cells expressing TLR9, but not TLR3, 7 or 8, suggesting that the CpR motif is recognized by TLR9. Although both types of agonist induce surface marker expression and proliferation of human B-cells, only compounds of the second type produce IFNα in human pDC and PBMC cultures. Consistent with *in vitro*

Figure 3 | *In vivo* immune stimulatory activity of synthetic agonists of TLR9 in non-human primates

(**A**) A pictorial depiction of the two types of compound described in the literature. Solid line represents nucleotide sequence and oval bulge represents synthetic immune-stimulatory motif, CpR, YpG or R'pC. L represents a non-nucleotidic linker. In structure (**ii**), palindromic sequence can be incorporated into either one or both the branches, resulting in dimeric or multimeric intermolecular secondary structures. Immune response profiles of representative compounds from the two types of compound depicted in (**A**) in non-human primates. Plasma (**B**) IFNα, (**C**) IP-10 and (**D**) changes in CD69$^+$ cell levels in peripheral blood in Rhesus monkeys following subcutaneous administration of 1 mg/kg of TLR9 agonists. Filled triangles and squares represent data for the first (**i**) and second (**ii**) types of compound shown in (**A**) respectively. Open diamonds represent a control TLR9 agonist without an immune-stimulatory motif but with the same structure and length as agonists. In (**D**) the values are at 24 h post dosing compared with 0 h (pre-dosing). Data used in (**B** and **D**) are taken from [46].



results, both types of compound induce cytokine production *in vivo* in mice and non-human primates. In contrast with *in vitro* results, both types of agonist induce IFNα *in vivo* in monkeys; however, the agonists that form a secondary structure induce higher and sustained levels of IFNα (Figure 3B), IP-10 (IFNγ-inducible protein 10) (Figure 3C) and activation of NK cells (Figure 3D). Both *in vitro* and *in vivo*, TLR9 agonists that have two 5′-ends and contain synthetic immune-stimulatory motifs induce significantly lower levels of IL-6, but similar or higher levels of IL-12, than do conventional TLR9 agonists [42–46].

Based on the immune-stimulatory profiles induced, we have selected two lead candidates representing each class of TLR9 agonist structures (Figure 3A). The first candidate (referred to as IMO-2055) is our clinical candidate for the treatment of cancers. The safety and immunopharmacological activity of IMO-2055 have been studied in Phase I trials in healthy and cancer subjects [17], and the compound is currently being tested in Phase II trials either alone or in combination with targeted therapies for renal cell carcinoma and non-small cell lung carcinoma respectively. The second candidate (referred to as IMO-2125) is a representative of the second type of compound (Figure 3A). IMO-2125 induces high and sustained levels of IFNα and activates NK cells in non-human primates. As it has shown induction of high and sustained levels of IFNα *in vitro* in human cell-based assays and *in vivo* in non-human primates, it has been selected as a candidate for the treatment of hepatitis C, where recombinant IFNα is the standard of care.

Therapeutic potential of TLR9 agonists

A number of preclinical studies have shown that TLR9 agonists provide protection against virus, bacteria, parasites and fungi through the induction of Th1 cytokines (e.g. IL-12, IFNα and IFNγ) [47]. TLR9 agonists have also been shown to boost acquired Th1-type immune responses when used as adjuvants in combination with protein, peptide and DNA vaccines in preclinical and clinical studies [17,48]. Our recent studies have shown the potential application of TLR9 agonists with two free 5′-ends and synthetic immunostimulatory motifs as adjuvants, with HBsAg (hepatitis B virus surface antigen) [49] and gp120-depleted whole killed HIV in mouse models [50].

TLR9 agonists also prevent and reverse allergen-induced Th2 immune responses that are commonly observed in asthma and allergic conditions in a number of preclinical models [51], and clinical studies are under way [52]. In studies in OVA (ovalbumin)-sensitized mouse models of asthma, agonists of TLR9 containing novel structures and synthetic immune-stimulatory motifs prevent the development of allergic airway inflammation and airway hyper-responsiveness when co-administered during OVA-sensitization [53], and reverse established allergic response when administered to OVA-sensitized and -challenged mice [54]. Novel TLR9 agonists can also prevent and treat OVA-induced Eustachian tube dysfunction in rat models, suggesting that they might be useful in treating allergy-associated OME (otitis media with

effusion), a chronic inflammatory condition of the middle ear [55–57].

The Th1-type innate immune responses induced by TLR9 agonists can lead to tumour killing either directly or indirectly via NK cell activation. A number of preclinical studies have shown the use of TLR9 agonists as anticancer agents either alone or in combination with chemotherapeutic agents and a number of clinical trials are in progress [17,58,59]. Our recent studies of TLR9 agonists in combination with EGFR (epidermal growth factor receptor) and VEGF (vascular endothelial growth factor) inhibitors show enhancement of antitumour effects of these inhibitors through interference with EGFR-related signalling pathways and angiogenesis in addition to usual TLR9-depended pathways [60,61].

Conclusions

The novel structures and synthetic immune-stimulatory motifs developed through our rational structure–activity relationship studies have provided numerous novel agonists of TLR7, TLR8 and TLR9 and have allowed us to expand the breadth of ligands for these receptors. The novel agonists designed through variations of structures and synthetic stimulatory motifs can be used to modulate the immune system through TLR-dependent pathways, and can be used to develop novel drugs that are quite distinct from natural ligands. The ability to modulate immune responses in a desired and optimal fashion may allow targeting a broad range of diseases, including cancers, asthma, allergies and infections, alone or in combination with other agents, as well as adjuvants with vaccines.

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