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The pharmacokinetics of Toll-like receptor agonists and the impact on the immune system

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Abstract

Toll-like receptor (TLR) ligation activates both the innate and adaptive immune systems, and plays an important role in antiviral and anti-tumor immunity. Therefore, a significant amount of effort has been devoted to exploit the therapeutic potential of TLR agonists. Depending on the therapeutic purpose, either as adjuvants to vaccine, chemotherapy or standalone therapy, TLR agonists have been administered via different routes. Both preclinical and clinical studies have suggested that the route of administration has significant effects on pharmacokinetics, and that understanding these effects is critical to the success of TLR agonist drug development. This article will summarize the pharmacokinetics of TLR agonists with different administration routes, with an emphasis on clinical studies of TLR ligands in oncologic applications.

Keywords

CpG; imiquimod; immune response; immunotherapy; pharmacokinetics; poly I:C; resiquimod; TLR agonist; Toll-like receptor

Toll-like receptors (TLRs) are a family of evolutionarily conserved pathogen recognition receptors that play a critical role in activating both innate and adaptive immunity. So far, ten and 12 functional TLRs have been identified in humans and mice, respectively [1]. TLR-1, -2, -4, -5 and -6 are located on the cell surfaces, while TLR-3, -7/8 and -9 are located in the endosomal compartments with their ligand-binding domains facing the lumen of the vesicle [2]. The ligands of TLRs have been identified as microbial components from fungi, bacteria or viruses. For example, the natural ligands for TLR-2 are peptidoglycans from Gram-positive bacteria where the heterodimer TLR-1/2 recognizes triacylated lipopeptides and TLR-2/6 recognizes diacylated lipopeptides; TLR-3 recognizes dsRNA; TLR-4 recognizes lipopolysaccharide in the outer membrane of Gram-negative bacteria; TLR-5 recognizes flagellin of motile bacteria [3]; the natural ligands for TLR-7/8 are guanosine- and uridine-rich ssRNA [4-6]; TLR-9 recognizes unmethylated juxtaposed cytosine and guanine nucleotides (CpG) of bacterial DNA [7,8]. Representative ligands for each TLR are listed in Table 1. It should be noted that there are many more known ligands for each TLR and the

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list keeps growing, especially owing to the fact that small synthetic molecules that can activate TLRs are actively being developed and widely pursued for therapeutic purposes. For example, imiquimod and resiquimod, which can activate TLR-7 and TLR-7/8, respectively, have been extensively evaluated in preclinical and clinical studies for their antiviral and anti-cancer effects [9-11]. TLRs are expressed on different immune subsets, especially monocytes, dendritic cells (DCs) and macrophages. The activation of TLR by natural or synthetic ligands results in cytokine secretion (e.g., IFN- α , TNF- α and IL-12) and increased phagocytosis by macrophages and cytolytic activity by natural killer (NK) cells. TLR activation also results in enhanced antigen presentation via upregulating the expression of major histocompatibility complex and costimulatory molecules (CD80 and CD86) on DCs, thus activating an adaptive immune response, including antigen-specific CD8⁺ cytotoxic T lymphocytes. The proinflammatory properties of TLR ligands have made them useful as vaccine adjuvants and antiviral and anti-tumor therapeutics. TLR agonists have shown great promise in preclinical studies, and some of them are entering human trials. With the emergence of clinical trials using TLR agonists, it is becoming evident that understanding the pharmacokinetic properties of a specific TLR agonist, including bioavailability, half-life, clearance and toxicity, are critically important in improving the therapeutic effect. This article aims to summarize the pharmacokinetic information of TLR agonists, highlighting challenges with the current delivery methods and discussing potential solutions. Depending on the therapeutic purposes, TLR ligands have been given via different routes, either systemically, via oral or intravenous administration, or locally as topical cream or subcutaneous injection, as summarized in Table 2. The toxicity, bioavailability and other pharmacokinetic parameters vary greatly depending on the route of administration. Oral dosing can be the most convenient for the patient, but the drug is often subject to first-pass metabolism, limited solubility and bioavailability problems [12,13]. Intravenous injections are ideal for systemic treatment, but can be problematic for patients if multiple doses are necessary [14]. Topical creams are useful for skin diseases, and have been found to be effective for some cancers and viral diseases [15], but may not have the desired systemic immune response. Subcutaneous administration of TLR agonists is frequently used in the vaccine adjuvant setting to enhance the immunogenicity of both viral and tumor vaccines [8,16-25]. Direct intratumoral injection of TLR agonists has also been used with the hope of generating a proinflammatory, anti-tumoral immune microenvironment [25-29]. In the following sections, we will review the pharmacokinetics and immunological responses from systemic administration (intravenous and oral) compared with local administration (topical, subcutaneous/intratumoral).

Systemic administration of TLR agonists

Intravenous TLR agonists

The intravenous administration appears most appropriate when the targeted tissue is either difficult to reach from the skin surface, has highly permeable vasculature or the treated disease is systemic. To date, relatively few TLR agonists have been administered intravenously (Table 2). Most information on the pharmacokinetics, toxicity, and efficacy of intravenously administered TLR agonists are from TLR-9 and TLR-7 agonists, although TLR-3 agonists have also been tested. In general, the immunological effects have been disappointing when compared with other administration routes. The short half-lives of the drugs and suboptimal delivery to the target site could explain the lack of effect, and novel ways of improving drug efficacy, via altering the chemical structure or delivery vehicles, are being developed.

TLR-9—Interstitial fluid and serum contain abundant nucleases that rapidly degrade oligonucleotides preventing the CpG oligodeoxynucleotides (ODNs) from interacting with

the cognate intra cellular TLR-9 receptor. Modification of the oligonucleotide back-bone from the natural phosphodiester bond to a phosphorothioate bond exchanges a nonbridging oxygen molecule for a sulfur moiety that is not recognized by the nucleases and renders it more resistant to degradation (Figure 1). This alteration of the back-bone increases the stability of the resultant oligonucleotides from 30 min for phosphodiester bonds to over 24 h for phosphorothioate bonds when incubated in mouse serum [30]. The elimination half-life within the tissues reveals an increase to between 25 and 50 h for the CpG ODNs with phosphorothioate backbones, with the majority of the drug taken up by the liver and kidney in *in vivo* rodent studies [31].

Corresponding *in vitro* potency analyses of CpG ODNs found expected improvements in the ability of the phosphorothioate stabilized species to promote longer survival of human DC pre-cursors [32], stimulation of murine NK cell-mediated lysis [33], proliferation of murine B cells [34], and murine splenocyte proliferation and production of IL-12 with upregulation of the early activation marker CD69 [35]. This increased potency is partly due to improved stability, but *in vitro* kinetic studies also demonstrated a faster uptake into murine macrophages, providing higher concentrations of the drug to the intracellular TLR-9 receptor of the endosomes [36].

The clinical use of intravenous CpG ODNs is limited to one Phase I trial of patients with non-Hodgkin lymphoma who had progressed through at least one round of therapy [37]. Patients were given two infusions of increasing doses of a phosphorothioate-modified CpG ODN, PF-3512676. Safety analysis demonstrated serious adverse events including anemia, thrombocytopenia and neutropenia that were attributed to disease; however, in light of more recent data, may also have been at least partly due to the CpG ODN [38]. No clinical effects were seen with this infusion and immunological effects were limited to mild increases in NK cell number, anecdotal improvement in NK cell activity and transient increases in circulating IL-6. The lack of effects seen in this Phase I trial is unlikely to reflect underdosing as the rate of serious side effects is troubling and markedly reminiscent of the trial of subcutaneously injected CpG ODN in patients with non-small-cell lung cancer (NSCLC) that was stopped early owing to a combination of serious adverse events and poor efficacy by the data safety monitoring committee [38].

More to the point, the lack of clinical and immunological effects of the intravenous formulation may be secondary to a failure of the pharmacokinetics in terms of targeting the drug to the proposed site of action. As these drugs are meant to positively influence the immune system response at the site of the tumor, systemic distribution may only serve to increase global side effects while decreasing active drug to the effector cell's cognate receptor. Incorporating nuclease-resistant phosphorothioate bonds in the drug's backbone improves the drug's half-life; however, with indiscriminant delivery, this may only serve to increase the duration of the adverse effects. The widespread distribution of drugs via this route of administration may be improved through the use of strategies to focus delivery to the intended targets, thus improving efficacy and decreasing side effects. Drug-delivery systems employing lipid encapsulation and peptides with particular homing characteristics have been developed and take advantage of their unique targeting ability to concentrate active drugs at the proposed sites of action (Figure 1) [39].

Lipid encapsulation: Encapsulation of free drugs within lipid nanoparticles creates a depot effect of a drug, which improves its half-life but also has important effects on tissue distribution [39]. Creation of multilamellar liposomes surrounding a nucleic acid core of CpG ODNs, known as a lipoplex, improves bioavailability when measured immediately after intravenous injection into mice where the encapsulated form was entirely present in the blood compared with only 40% for the free drug, a testament to the speed of the nucleases

present in the serum [40]. Clearance is also documented to be two-to-threefold faster for the free drug versus the encapsulated form. Importantly, infusion of a lipid-encapsulated drug deposits 71% of the CpG ODNs within the liver and 1.6% in the spleen 4 h after injection compared with 20 and 0.4% for infusion of free drug, respectively, and these levels remain fairly constant over the next 24 h in mice [40]. This is a markedly different pattern of uptake compared with a subcutaneously injected drug, where the majority is taken up by the draining lymph nodes and represents a difference that may be exploited in the treatment of diseases of particular organ systems.

In vivo, lipid-encapsulated drugs demonstrate a propensity to deposit in areas of active inflammation given the increased permeability of the blood vessels at these sites [41] and may allow a favorable accumulation of drug at the site of infection or cancer given their associated 'leaky' blood vessels. As TLR agonists are important in influencing the inflammatory state at the intended site of action, this aspect of the lipid complex carrier makes its use highly beneficial. Lipid encapsulation also takes advantage of the tendency of the DCs and macrophages of the reticular endothelial system to phagocytose lipoplex complexes thus directly delivering the drug to the cells expressing the cognate TLR-9 receptor [42]. The dual action of increasing drug delivery to sites of inflammation and concentrating drugs in the endosomes of the cells with the cognate receptor makes lipid encapsulation an extremely attractive strategy for future experimentation.

In an *in vivo* mouse model, immune cells demonstrate increased uptake of the lipoplex CpG ODNs over free drug with elevated levels seen in murine macrophages/DCs positive for CD11c⁺, Mac-3⁺ or CD11b⁺, or B lymphocytes positive for CD45/B220⁺, with peak levels reached at 7.5 h and slight decreases over 24 h [40]. This is in stark contrast to subcutaneously injected lipoplex CpG ODNs, where there is a comparative four- to 13-fold decrease in uptake per cell compared with intravenous injection. The effect of the intravenous infusion on immune activation and inflammation is rapid, with elevation of IL-6 and IFN- β within 2 h, and peak numbers of neutrophils and macrophages accumulating within the lung within 30 min, a nonspecific indicator of inflammation [43]. Intravenous administration into recipient mice also induces higher NK cytolytic function and circulating levels of the Th1 cytokines IFN- γ , IL-12, IL-6, monocyte chemoattractant protein-1 (MCP-1) and TNF- α when using lipoplex CpG ODNs versus free drug [44].

These results reveal the effect lipid encapsulation has on immune parameters through prolonging its duration of action, depositing drug at vasculature associated with disease, and taking advantage of the phagocytes ability to concentrate drugs within endosomes expressing the required intracellular receptor. These attributes appear suited to deliver drugs to the site of disease and thus incorporation may serve to augment the drug's efficacy while decreasing side effects associated with indiscriminant delivery. Special consideration should be made to the use of this strategy in treating infectious diseases of the liver, as the intravenous formulation of the lipoplex formulated drug predominantly accumulates there and shows some benefit in mouse models of hepatitis B virus infections [45].

Targeted delivery via peptides: The localization of intravenously administered lipoplex CpG ODNs can be further improved by the covalent addition of peptides with affinity for receptors of certain tissue types. Evaluation of phage libraries in a transgenic mouse model of islet cell tumors revealed one such peptide with sequence similarity to the β -subunit of PDGF that preferentially bound both the angiogenic islet cells and tumors [46] and was previously shown to deliver anti-CD40 antibodies and IL-2 to the tumor vasculature of these mouse models [47]. In mice, covalent binding of this peptide to the lipid molecules targeted the encompassed CpG ODN lipoplex to the tumor vasculature and was taken up by endothelial cells and CD68⁺ resident tumor macrophages by a far greater extent than the

lipoplex CpG ODNs without the peptide or free CpG ODNs after intravenous administration. In a mouse model of *de novo* arising pancreatic tumors, use of the peptide-tagged lipoplex CpG ODNs induced 30% survival versus no effect when untagged lipoplex CpG ODNs or naked CpG ODNs were infused [48].

It remains to be seen whether the improvements in drug delivery, by improvements in organ distribution, duration of action and disease specificity imbued through lipid encapsulation or addition of peptides with specificity for particular organs, will improve clinical outcomes. The preclinical data demonstrate improvements in drug delivery that theoretically should concentrate drugs to the desired sites of action and decrease the widespread distribution to unaffected tissues. These advantageous attributes are important enough to demand their evaluation in future clinical trials to hopefully allow these drugs to realize their to-date unmet potential in affecting clinical outcomes.

TLR-7

ssRNA & its derivative: The natural ligand for TLR-7 is ssRNA that needs to be endocytosed to interact with the intracellular receptor. Unfortunately, RNA is also very susceptible to the abundant nucleases present in the serum and is quickly degraded when exposed to human serum [49] or serum-free media [50], regardless of phosphorothioate backbones. To improve stability, covalent linkage of two ssRNA species by their 3' ends or substitution of the 5' guanosine nucleotides with nuclease-resistant 7-deazaguanosines greatly increased stability and increased circulating levels of IP-10, MCP-1 and IL-12 when given intravenously to mice [49].

Use of lipoplex-complexed RNA or the polycationic peptide, protamine, to protect the RNA has also been used, with improvements seen in both duration and efficacy. Infusion of lipid encapsulated TLR-7 agonist ssRNA in mice stimulated peripheral and intratumoral NK cells and delayed the growth of NK-sensitive tumors by an IL-12-dependent mechanism [51]. The role that lipid encapsulation plays in efficacy was proven in separate studies where induced IFN- α production was completely abrogated when a free drug was infused without the lipoplex complex [52]. Use of protamine to condense RNA that serves to protect nucleic acids from degradation [53] is also an effective agent to improve efficacy *in vivo*, as experiments demonstrating the induction of cytotoxic T-lymphocyte responses by protamine-complexed RNA species was lost when using free drug; however, the role of the RNA species as a TLR-7 agonist was not evaluated [54]. Both strategies for improving RNA stability, although seemingly successful, are being replaced by small-molecule mimetics of ssRNA, for example, 852A, with improved stability for intravenous administration [14].

Synthetic TLR-7 agonist: 852A (N-[4-(4-amino-2-ethyl-1*H*-imidazo[4,5*c*]quinolin-1-yl)butyl] methanesulfonamide, 3M-001) is a small-molecule imidazoquinoline that is related to imiquimod but is a more potent and more selective activator of TLR-7 [12]. 852A is approximately 40-times more water soluble than imiquimod at physiologic pH, allowing easier formulation as an injectable systemic agent [12]. 852A also has slower elimination and less extensive metabolism than imiquimod in animal pharmacokinetic studies or when incubated in the presence of human liver enzymes [12]. Comparison of the pharmacokinetics of the drug when given intravenously, subcutaneously or orally demonstrated that mean serum concentrations were equivalent for the subcutaneous or intravenous route but greater than the oral route [14]. The subcutaneous route demonstrates a slightly prolonged half-life of the drug over the intravenous route; however, urinary excretion, safety and production of systemic IFN- α levels were similar between routes, concluding either intravenous or subcutaneous administration as a viable option, although a total of only 18 patients were enrolled in these studies.

852A has been employed in a Phase I clinical trial in patients diagnosed with advanced cancers, including renal cell carcinoma, melanoma and NSCLC [12]. During the trial, most patients suffered at least one grade 3 drug-related adverse event, most frequently reported as fever and fatigue. Use of doses at or above 1.2 mg/m² were associated with dose limiting toxicity; however, the short half-life of 10 h made most of these events self-limited. The higher doses also induced higher levels of IFN- α , IL-1 receptor antagonist (IL-1RA) and IP-10, with no change in circulating levels of IL-12. Using Response evaluation criteria in solid tumors criteria, 24% of the patients had disease stabilization after 2 weeks of therapy, but most went on to develop progressive disease thereafter.

In a Phase II trial with 852A in patients with melanoma, intravenous application of the synthetic TLR-7 agonist was evaluated through intravenous administration of the drug in a dose-escalation manner three-times a week [55]. A total of 14% of study participants had severe adverse events during the trial and 24% withdrew secondary to these effects, where 19% of patients had disease stabilization for at least 100 days. Lack of a control group makes these comparisons difficult and subjective; however, the side effects were significant. Evaluation of the immune parameters revealed drug-related increases in circulating type I interferons and IP-10 within 6 h of infusion and upregulation of the maturation marker CD86 on monocytes.

The ability of topical application of 852A to eradicate skin surface lesions in chronic lymphocytic leukemia (CLL) [56] led to the recent Phase I/II trial in CLL patients using intravenous administration of the drug in the hope of producing more of a systemic effect [57]. A total of 14 patients who met criteria for systemic therapy and had previously received at least two courses of chemotherapy were given intravenous 852A at one of three doses and followed for safety and clinical effect. Using a highest dose of 0.6 mg/m², the drug was well tolerated with only grade 1 and 2 adverse events, mostly confined to chills, fatigue and 'hot flashes', and one patient achieved a complete response while all other patients either required additional therapeutic intervention or died. Analysis of circulating levels of cytokines demonstrated modest treatment related increases in TNF- α , IL-6 and IL-1 β that were not responsive to dosing. Unfortunately, the predicted increases in B-cell activation markers seen by treatment of CLL cells *in vitro* with a different TLR-7 agonist, S28690 [58], were not demonstrated after 852A infusion in this trial when evaluated at 4 h after injection.

These clinical trials demonstrate that an effect on the immune system can be achieved using a synthetic analog of ssRNA that avoids complications occurring via the instability of RNA. Use of such synthetic analogs in the future is likely to supplant experimentation with the natural compounds due to stability. The finding of elevated adverse effects that limits dosing without seeing clinical effect is reminiscent of the TLR-9 agonists and demonstrates a need to focus delivery of drug only to the intended site of action.

TLR-3—The classic TLR-3 agonist is polyribosinic:polyribocytidylic acid (poly[I:C]), a synthetic dsRNA species that with the addition of mismatched uracil and guanosine residues creates poly(I:C₁₂U) trademarked as Ampligen®. The hypersensitivity, renal failure and coagulopathy seen in human studies using the parent compound poly(I:C) was abrogated through the addition of unpaired bases within the sequence [59,60]. Evaluation of the intracellular receptors for both forms revealed that poly(I:C) bound to both the TLR-3 receptor and the melanoma differentiation-association antigen-5, whereas poly(I:C₁₂U) only bound TLR-3 and this difference may be responsible for the decrease in systemic toxicity [61]. Both drugs induced DC maturation, however, poly(I:C₁₂U) induced a greater elaboration of the Th1 cytokine IL-12 with less IL-10 production and was effective in both healthy and cancer patients cells *in vitro* [62]. In animal experiments, mice were protected

from a lethal virus infection when treated with the poly(I:C₁₂U) form and not the poly(I:C) parent drug [61]. The decreased toxicity, superior cytokine profile and animal data made poly(I:C₁₂U) the optimal formulation for use in clinical trials.

Intravenous poly(I:C₁₂U) was used in early trials to study its ability to treat HIV infection in 36 patients receiving zidovudine in a randomized placebo-controlled double-blinded crossover trial [63]. Patients treated with poly(I:C₁₂U) had a trend towards delayed CD4 T-cell loss that reversed itself upon the patient crossing over to placebo. This study did show a statistically significant decrease in the number of patients who progressed to develop category C HIV infection upon receiving the drug versus placebo. Importantly, the drug was well tolerated with no difference in adverse events reported in placebo versus the experimental arm.

Based on abnormalities in the immune system of patients diagnosed with chronic fatigue syndrome (CFS) thought to cause the neurocognitive changes associated with the disease, clinical trials have been performed to test the efficacy of intravenous poly(I:C₁₂U) on rectifying the immunologic aberrancies [60]. In all trials, the drug was well tolerated as the number and severity of adverse events in the experimental arm was similar to patients who received the placebo. Compared with placebo, patients in the experimental arm had a 12.9% improvement in Karnofsky Performance Score ($p = 0.052$), increased exercise tolerance in the subset of patients with greater baseline exercise capacity and decreased medication usage, all metrics of disease activity. However, the US FDA denied the New Drug Application for poly(I:C₁₂U) for the treatment of CFS owing to a lack of clinical efficacy.

The parent drug, poly(I:C), was initially used in a clinical trial of patients with cancer in 1976 where the lack of clinical efficacy and increased toxicity negated further experimentation [59]. The modified drug poly(I:C₁₂U) has generated new excitement as an adjuvant for cancer vaccines through its *in vitro* ability to induce positive effects on DC maturation and induction of Th1 immune responses [62]. In mice, infusion of poly(I:C₁₂U) in a lipid complex reduced the amount of lung metastasis via induction of IFN- β [64]. These reports, in addition to the drug's experience in HIV and CFS, have made it an attractive adjuvant for further study in cancer applications [65]; however, whether it also will require manipulation to focus drug delivery to the intended site of action to avoid side effects and maximize efficacy remains to be determined.

In summary, intravenous delivery of TLR agonists faces the problem of rapid metabolism by serum enzymes resulting in short drug half-lives. The nonspecific distribution may also result in activation of immune cells in normal tissue begetting side effects while delivering subtherapeutic concentrations of a drug to the targeted tissue (i.e., the tumor). Optimization strategies for intravenous delivery include variation of chemical structure and lipid encapsulation to increase stability, and targeted delivery via homing peptides.

Oral TLR agonists

Oral administration of a drug is one of the easiest dosing regimens for patients to follow for the systemic treatment of disease. Most of the information about orally administered TLR agonists in humans is generated from TLR-7 agonist studies. Imiquimod (1-isobutyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine) is a small synthetic molecule that can activate TLR-7 on plasmacytoid DCs [15,66] and stimulates IFN- α production [15,67-69]. Although imiquimod was most commonly used as a topical agent for the treatment of skin diseases [15,70-74], it has been shown that orally administered imiquimod is also effective in inducing IFN- α , the major mediator of imiquimod-induced inflammatory responses [67,75]. A study in healthy volunteers showed that imiquimod has a half-life of 2–3 h orally and a total body clearance of approximately 970 ml/h*kg [14,75]. Food did not have a major

effect on the rate, extent of absorption or bioavailability of oral imiquimod [75], however, there can be large variability in metabolism, bioavailability and toxicity among individuals [13,14,75]. For instance, the total area under the curve ($AUC_{0-\infty}$) for oral dosing of imiquimod while fasting was 673 ± 615 ng*h/ml while the $AUC_{0-\infty}$ for subcutaneous dosing of imiquimod was 405 ± 64 ng*h/ml, demonstrating higher intersubject variability in oral administration due to first-pass metabolism [75]. A mouse pharmacokinetic and pharmacodynamic model can be useful to estimate oral bioavailability, half-life and clearance as well as predict the agonist interaction with receptors [76]. By using the TLR-7 agonist 9-benzyl-8-hydroxy-2-(2-methoxyethoxy) adenine (BHMA), Benson *et al.* discovered that the pharmacokinetics of BHMA followed a two-compartment model with first-order uptake from the GI tract and linear elimination [76]. Immune activation measured by IFN- α production showed peak levels at 1–2 h, and they were able to calculate a degradation constant for IFN- α of 0.96 h^{-1} and a half-life of 0.7 h, and complete clearance from the plasma was estimated at 3–4 h [76]. Knowing these parameters for human studies could help understand the doses and dosing schedules necessary to sustain the required immune responses for treatment success while minimizing toxicity.

The adverse events from oral imiquimod include vomiting, fatigue, lymphopenia and fever [67,77], with hepatic or renal impairment seen at doses of 100 and 200 mg [77]. Some of these issues can be remedied with modifying synthetic drugs or co-administration with additional drugs or adjuvants designed to increase the therapeutic index [13,78]. Two derivatives, ANA975 and 852A, were developed for treating cancer and viral infections as oral therapies [14,79]. ANA975 is a prodrug that is converted to its active metabolite by first-pass metabolism [13] and as such is designed to increase the tolerability and efficacy of the oral drug [13]. However, in a Phase I trial of ANA975 for hepatitis C viral infection, the study was discontinued owing to ‘inadequate therapeutic index’ and further preclinical evaluation of dosing schedules for toxicity would need to be determined [201].

The imidazoquinoline 852A is several fold more potent and more selective for TLR-7 versus TLR-8 than imiquimod, and the increased specificity for TLR-7 and decreased metabolism could potentially improve problems with toxicity [14]. As was seen for imiquimod [75], 852A had only moderate bioavailability of $26.5 \pm 7.84\%$ and also had highly variable absorption and lower availability in oral dosing compared with intravenous and subcutaneous injection [14]. Overall, the toxicity was similarly low for the three routes of administration, but cytokine induction was lowest and not significant via oral administration [14], thus the recommendation was for intravenous or subcutaneous treatment.

Resiquimod (R-848, S-28463 or 1-[4-amino-2-(ethoxymethyl) imidazo[4,5-c]quinolin-1-yl]-2-methylpropan-2-ol) has also been tested orally. Resiquimod has a similar structure and stimulates immune reactions via TLR-7 in a similar way to imiquimod, but resiquimod also stimulates TLR-8 [68,80]. A clinical trial of oral resiquimod for treatment of hepatitis C virus infection found that 0.01 mg/kg was tolerated but had minimal antiviral effect [81]. While the 0.02 mg/kg dose had better induction of cytokines (IFN- γ , IFN- α , IL-1RA, IL-6, TNF- α and IL-12p40) and nearly half the subjects at that dose had a 1-log reduction in hepatitis C virus RNA levels, there were increases in adverse reactions and the decrease in viral RNA was not sustained [81]. Resiquimod was originally developed for potential as an antiviral treatment for genital herpes, but was reportedly discontinued owing to a lack of significant therapeutic value [13,82].

In summary, orally given TLR agonists, mainly the TLR-7 family of agonists, have been tested in the clinic but have met limited success. Low bioavailability may account for the lack of therapeutic effects and novel drug formulations remain to be developed.

Local administration

Topical administration of TLR agonists

Some of the toxicity and adverse effects of systemic TLR agonist treatment can be avoided through local administration. One of the most successful of these is the topical application of imiquimod cream that has been used for multiple diseases, from viral infections to cancer [15,79,83,84]. Topical imiquimod is absorbed through the skin with minimal systemic distribution, as estimated by urinary excretion of imiquimod and its metabolites that were found to be 0.6% or less [85]. The half-life of topical imiquimod at the treatment site is between 21 and 27 h, with little variation between application to the face, scalp or hands [85]. Side effects were minimal and localized to the site of application [85]. Topical imiquimod is currently approved for external genital warts, actinic keratosis and superficial basal cell carcinoma, and is being tested for melanoma [15,70-74,86-88]. The low toxicity and efficient immune stimulation has led to the exploration of topical imiquimod in novel adjuvant applications [89].

Imiquimod has been used as an adjuvant to multiple cancer vaccines [90-92]. In conjunction with a NY-ESO-1 vaccine for metastatic melanoma, imiquimod increased CD4⁺ T-cell secretion of IFN- γ , activated NK cells, DCs and T cells, and had no significant adverse events [90]. However, the NY-ESO-1 vaccine and topical imiquimod did not increase CD8⁺ T-cell responses as was seen in the more potent immune activation from CpG plus montanide (Montanide ISA-51, an oil-in-water emulsified adjuvant) [90,91]. Topical imiquimod monotherapy and topical imiquimod as an adjuvant to a human papilloma virus therapeutic vaccine have both been tested in a clinical trial for vulvar intraepithelial neoplasia, a disease where surgical treatment has a high rate of recurrence [92,93]. Topical imiquimod monotherapy increased the number of DCs, CD8⁺ T cells and NK cells in the epidermis, and 81% of patients had reduced lesion size at 20 weeks, with 35% of patients treated with imiquimod who achieved a complete response remained disease free at the 12-month follow-up [93]. The recent study by Daayana *et al.* reported a correlation between immune parameters and clinical response after topical imiquimod and human papillomavirus vaccination [92]. They reported that two populations were found in the trial: 'lesion responders', who had increased infiltration of CD4⁺ and CD8⁺ T cells, and the 'non-responders' who had an increase in regulatory T cells, indicating that the phenotype of the infiltrating immune cells can impact treatment outcome. Overall, the treatment shows promise, with 63% of patients demonstrating a lesion response at week 52 after 8 weeks of topical imiquimod treatment and vaccination [92].

The combination of TLR agonist therapy with cytokines or other treatment modalities may be more effective in developing new disease treatments. For instance, using topical imiquimod to treat malignant melanoma in conjunction with IL-2 intratumoral injection produced a complete response in 41% of the patients treated, with no return of responding lesions once therapy was stopped [94]. The combined treatment promoted Th1-type immune responses characterized by increased CD4⁺ and cytotoxic T cells [15]. Further investigation of the immune response to imiquimod and IL-2 treatment found an increase in CD4⁺CD25⁺ effector T-helper cells and IFN- γ production with no increase in regulatory T cells [95]. In addition to cytokine therapy, topical imiquimod has been used in combination with other local treatments, including cryosurgery in mice where it augmented anti-tumor immunity and a case report with radiotherapy in Merkel cell carcinoma [96,97]. The results, although promising, remain to be validated in clinical trials.

While imiquimod is the most widely used and studied topical TLR agonist, resiquimod has also been explored as treatment for various diseases including herpes virus infections and as a cancer adjuvant [98,99]. It has been shown that 0.01% resiquimod gel is effective in

decreasing herpes simplex virus shedding in a randomized clinical trial [100]. Topical resiquimod has also been shown to improve the priming of cytotoxic T-cell responses to subcutaneously injected antigen and augment anti-tumor immunity in mice [80]. The dose is important, as multiple doses at 0.01 or 0.05% were tolerated but multiple doses of 0.25% was not [101]. The study also found serum levels of resiquimod of 5–6 ng/ml are necessary for systemic cytokine production [101], which could explain their observation that only one patient had measurable serum concentrations of cytokines after topical dosing since local administration produced little systemic absorption accounting for <1% of topical dose of resiquimod [101]. Currently, an active Phase II clinical trial of gp100 vaccine plus topical resiquimod is set to determine whether antigen-specific T cells can be enhanced by vaccination in melanoma patients (Table 3) [202].

In summary, topical TLR agonists, especially imiquimod, have been shown to be effective in treating different viral diseases and superficial cancers with acceptable adverse events. The combination of imiquimod with cytokine therapy appears to augment efficacy and deserves continued clinical evaluation. Topical TLR agonists are also promising as vaccine adjuvants and further explorations of topical applications are listed in Table 3.

Subcutaneous administration of TLR agonists

Since subcutaneous injection of TLR agonists is more convenient than intravenous injection, many TLR agonists have been administered subcutaneously in different disease settings, as listed in Tables 2 & 3. Certain TLR agonists have been shown to induce greater systemic cytokine production when given subcutaneously rather than intravenously for unclear reasons, but this highlights the appropriateness of matching the route of administration with the intended clinical effect [102].

Subcutaneous administration of TLR agonists can induce systemic production of Th1 cytokines with low or no tolerance to repeated injection, and can produce long-lasting immune protection [20,103,104]. A study comparing subcutaneously and intravenously administered 852A showed that serum concentrations after 30 min following subcutaneous injection are nearly identical to those after intravenous administration [11]. A study in normal volunteers showed that subcutaneous dosing of CpG 7909 increases Th1 cytokines, including IL-12p40 and IFN- α , while intravenous does not stimulate cytokine production at any time point despite increasing the intravenous dose to four-times the highest subcutaneous dose [102]. Subcutaneous administration can also avoid the bioavailability problems [14]. Reports of toxicity are often minimal and localized injection-site reactions [16-18,22,24,105,106]. Use of subcutaneously administered TLR agonists in combinatorial vaccines and as adjuvants are showing success [8,107,108] and continue to be a focus of new research (Table 3).

TLR-7—Subcutaneous dosing of the TLR-7 agonist 852A was recommended over oral administration owing to favorable pharmacokinetic parameters in patients [14]. Dosing 852A subcutaneously had approximately 80% bioavailability, which is greatly improved over oral dosing, which had approximately 27% bioavailability [14]. The $C_{\max}$ and AUC were dose dependent, and the half-life was approximately 8 h for each of the three doses (0.5, 1.0 and 2.0 mg) [14].

One preclinical study tested the gene-gun injection of a DNA vaccine combined with subcutaneous administration of imiquimod or resiquimod at the vaccination site in a murine model [78]. Both imiquimod and resiquimod were effective in enhancing CD4⁺ and CD8⁺ T-cell responses to the antigen encoded by the DNA vaccine and activating cytokine production, however, resiquimod produced maximal cytokine induction at 1 mg/kg and imiquimod peaked at 10 mg/kg [78]. For imiquimod therapy, evaluation of splenocytes

revealed a greater increase in T-helper cells expressing IFN- γ than IL-4, signifying a polarization of the immune response towards a favorable Th1 milieu [78].

TLR-9—Subcutaneously administered CpG 7909, a type B CpG ODN with the sequence 5'-TCGTCGTTTTGTCGTTTTGTCGTT-3', induced production of Th1 cytokines (IFN- α , IL-12p40, IL-6 and IP-10) and was maintained at relatively high levels at the injection site and lymph nodes for up to 2 weeks, while intravenous infusion showed no immune response even with a 128-fold higher dose [102]. Cytokine IP-10 could be detected with the lowest dose of 0.0025 mg/kg CpG 7909 and adverse reactions were tolerable up to 0.08 mg/kg [102]. Follow-up subcutaneous injection of CpG 7909 yielded similar immune responses, which demonstrated that TLR-9 could be repeatedly administered without inducing tolerance [102].

When combined with taxane therapy for NSCLC, CpG 7909 induced a trend towards improved median survival compared with the chemotherapy alone in a Phase II trial (6.8 months vs 12.3 months; $p = 0.19$) [18]. The subsequent Phase III trial, of similar design, was prematurely stopped due to a lack of clinical efficacy and unacceptable toxicity [38]. In a separate Phase I trial of treatment-naïve Japanese patients with advanced NSCLC, the combination of CPG 7909 with chemotherapy produced objective responses in 33% and stable disease in 58%; however, almost all patients experienced significant hematologic toxicity similar to previous trials of subcutaneous CpG 7909 [105]. The time to maximum plasma concentration for CpG 7909 was found to be 2–3 h, but the peak concentration and the half-life was dose dependent [105]. The pharmacodynamics measurements of IFN- α , IL-12p40, IL-6, IP-10, C-reactive protein and MCP-1 tracked the response to CpG 7909 and found that while cytokine levels rose approximately 3 h after administration, peak amounts for each varied from 24 to 96 h [105]. Clearance and return to the pretreatment cytokine profile took approximately 168 h [105].

Cancer patients immunized with CpG 7909, NY-ESO-1 and montanide have responded well, with an overall increase in CD8⁺ T-cell responses [20,22,91], and one patient showing a significant percentage of antigen-specific CD8⁺ T cells circulating 3 years after vaccination [20]. Another CpG analogue, 1018 ISS (sequence 5'-TGACTGTGAACGTTTCGAGATGA-3'), was combined with rituximab for treating lymphoma [24,109]. Weekly doses up to 0.5 mg/kg 1018 ISS were tolerated and type 1 interferons were dose-dependently increased [24]. Similarly, subcutaneous administration of 1018 ISS increased tumor infiltration of CD8⁺ T cells and 45% of patients showed increased T cells and DCs, which was associated with increased survival and positive clinical outcome [109].

These clinical trials show mixed results concerning the subcutaneous application of TLR agonists; however, as delivery systems and the biology become better elucidated, improvements in efficacy should be realized.

Other administration routes for TLR drugs

Subcutaneous injection is the most frequently used route for local administration, but injection of TLR agonists intravesically and intratumorally have also been explored. *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) has been used as a preventative vaccine for TB and therapy for bladder cancer, but recently has been found to stimulate TLR-2 [110]. Intravesical infusion of BCG onto the bladder wall generates a localized induction of TNF-related apoptosis-inducing ligand [110], which is associated with better survival and delayed progression [111]. Combination therapy of intravesical BCG and IFN- α 2b is well tolerated and effective at preventing recurrence of bladder cancer at a dose of 80

mg BCG plus 10 million units of IFN- α 2b for 8 weeks post-transurethral resection of bladder cancer [112,113].

Intratumoral injection can be important in the local treatment of cancers. A Phase I/II trial of intratumoral injection of PF-3512676 with low-dose radiation induced a systemic antilymphoma immune response, where in some cases a protective T-cell response occurred up to 1 year postvaccination [114]. The study also found patients whose Treg numbers increased ('Treg inducers') had shorter progression-free survival than patients with lower Treg numbers after PF-3512676 treatment [114]. A Phase I trial of intracranial administration of CpG was conducted in patients with recurrent glioblastoma and treatment was well tolerated at doses up to 20 mg per injection [115]. The main side effects were limited to transient worsening of neurological condition and fever [115].

Preclinical studies also showed that injection of the TLR-4 agonist, lipopolysaccharides and the TLR-2/6 agonist, zymosan A, could induce anti-tumor immune responses in a rat glioma model and that multiple doses increased the percentage of complete tumor regression [29]. In addition, poly(I:C) was able to increase the activation of CD8⁺ T cells in the tumor, leading to 40% complete tumor regression in murine mesothelioma [26]. The pharmacokinetics of these locally administered TLR agonists remain to be investigated.

In summary, locally administered TLR agonists via topical, subcutaneous or intratumoral injections have shown some promising results in both preclinical and clinical studies. These treatments not only modulated the local microenvironment, but also elicited systemic immune responses in some cases. There is great individual variability in clinical response, and preliminary data suggest a partial correlation between the induction of immune response and clinical outcomes [92,114].

Although we categorized the administration routes of TLR agonist into systemic versus local, it should be noted that there is no clear boundary between the two as the goal of topically or subcutaneously administered TLR agonists is to not only modulate the local immune environment, but also enhance systemic immunity and generate long-term immune responses. It should also be recognized that individual TLR agonists are different from each other in terms of their receptor location (cell surface vs endosomal receptor), specific immune cells targets (e.g., TLR-7 ligands activate plasmacytoid DCs and TLR-8 ligand activates myeloid DCs [116]), and signaling pathways (MyD88-dependent or -independent). Therefore, the pharmacokinetic property of one TLR agonist cannot be generalized to another TLR agonist and each of them has to be investigated individually.

Expert commentary

Toll-like receptor agonists maintain great promise in the therapeutic intervention of both infectious and neoplastic diseases as such diseases only become clinically relevant when they overcome the host immunity often through immune escape mechanisms that suppress innate and adaptive immunity. These drugs have convincing evidence in the literature that they can induce the appropriate microenvironment to create effective immune responses capable of eradicating underlying disease. We have summarized the current clinical applications of these agonists and can conclude that although the potential for TLR agonists to influence disease remains strong, it has yet to be demonstrated in clinical scenarios. The underachievement of TLR agonist therapy in the clinic is due, in part, to the impressive strength of the induced immune reactions that is applied in a nonspecific manner in the current bevy of clinical trials. The systemic effects seen by the widespread distribution of these drugs convey the toxicity and thus limits the dosing without allowing them to focus their effect on diseased tissue where the change in microenvironment is truly needed to affect outcomes. Strategies to target the sites of disease or application of the drug at

cutaneous diseases improves the outcomes by focusing the alteration of the microenvironment solely to the intended site of action, thus decreasing the side effects induced from the indiscriminate activation of immune cells at nondiseased tissues. The therapeutic efficacy is also hindered by the short half-life and poor bioavailability of some TLR agonists. In addition to delivery problems, preclinical and clinical data have suggested that the induction of regulatory pathways, such as IL-10 and Treg induction, may limit the proinflammatory effect of TLR agonists, and thus blocking the regulatory pathways may augment the therapeutic effect. In summary, TLR agonist-based therapy could be improved by better delivery vehicles, targeted delivery and combinations with other therapeutic modalities.

Five-year view

We speculate that the delivery of TLR agonists could be much improved in the next 5 years by the use of nanoparticles that are now being actively pursued as the optimal delivery vehicle for various kinds of drugs. Better ways of homing the TLR agonists to disease sites or the specific immune subsets that need to be activated may be developed in the near future. Continued effort will be devoted to develop combinational therapies that combine TLR agonists with chemotherapy or other immunotherapy, such as cytokine or monoclonal antibody-based therapy. In addition to these, we speculate that a significant amount of effort will be spent towards a better understanding of the individual variability in TLR response. Although the individual variability in response to TLR agonists could partially be explained by drug metabolism, it may also reflect the underlying polymorphism in TLR genes, which may result in varied sensitivity to the drug. This topic is not included in the current article, but we believe it is an important factor to consider for the development of personalized medicine using TLR agonists.

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Figure 1. Strategies used in CpG formulations to improve the drug's pharmacokinetics
TLR: Toll-like receptor.

Table 1

Ligands for Toll-like receptors.

Receptor	Location	Ligand
TLR-1/2	Cell surface	Peptidoglycan, triacylated lipopeptide
TLR-3	Endosomal	dsRNA
TLR-4	Cell surface	Lipopolysaccharide
TLR-5	Cell surface	Flagellin
TLR-2/6	Cell surface	Peptidoglycan, diacylated lipopeptide
TLR-7/8	Endosomal	Uridine/guanidine-rich ssRNA
TLR-9	Endosomal	Unmethylated CpG dinucleotide

TLR: Toll-like receptor.

Table 2

Published preclinical and clinical studies on Toll-like receptor agonists administered via different routes for various therapeutic purposes.

TLR ligands	Therapeutic purpose	Ref.
<i>Intravenous administration</i>		
CpG 7909 (TLR-9)	Non-Hodgkin lymphoma	[37]
852A (TLR-7)	Melanoma and other cancer	[12,55]
852A (TLR-7)	Chronic lymphocytic leukemia	[57]
Ampligen (TLR-3)	Chronic fatigue syndrome	[60]
Ampligen (TLR-3)	HIV	[63]
<i>Oral administration</i>		
Resiquimod (TLR-7/8)	Hepatitis C	[81]
ANA975 (TLR-7)	Hepatitis C	[13]
<i>Topical administration</i>		
Imiquimod (TLR-7)	Basal cell carcinoma	[73,86]
Imiquimod (TLR-7)	Squamous cell carcinoma	[74]
Imiquimod (TLR-7)	Melanoma	[88,95]
Imiquimod (TLR-7)	Vulvar intraepithelial neoplasia	[93]
Imiquimod (TLR-7)	Cutaneous lymphoma	[117]
Imiquimod (TLR-7)	HPV vaccine	[92]
Resiquimod (TLR-7/8)	Herpes simplex virus 2	[100]
<i>Subcutaneous/intramuscular administration</i>		
MPL (TLR-4)	HPV vaccine	[104]
MPL (TLR-4)	Non-small-cell lung cancer vaccine	[17]
CpG 7909 (TLR-9) (PF-3512676)	Non-small-cell lung cancer	[18,38]
CpG 7909 (TLR-9)	Metastatic melanoma	[118]
CpG 7909 (TLR-9)	Melan-A peptide vaccine for melanoma	[19,119]
CpG 7909 (TLR-9)	NY-ESO-1 protein vaccine	[91]
CpG 1018 ISS (TLR-9)	Lymphoma	[24,109]
Imiquimod (TLR-7)	NY-ESO-1 protein vaccine for melanoma	[90]
<i>Intratumoral administration</i>		
BCG (TLR-2)	Bladder cancer	[110]
LPS (TLR-4) and Zymosan A (TLR-2)	Glioma	[29]
Poly I:C (TLR-3)	Mesothelioma	[26]
CpG (TLR-9)	Lymphoma	[114]
CpG (TLR-9)	Glioblastoma	[115]

BCG: Bacillus Calmette–Guérin; CpG: Cytosine and guanine nucleotides; HPV: Human papillomavirus; LPS: Lipopolysaccharide; MPL: Monophosphoryl lipid A; NY-ESO-1: New York esophageal squamous cell carcinoma 1 cancer antigen; Poly I:C: Polyinosinic:polycytidylic acid; TLR: Toll-like receptor.

Table 3

Pending clinical trials of Toll-like receptor agonists.

Clinical trial ID [†]	TLR target	Route of administration	Disease target	Status of trial	Ref.
NCT00810758	TLR-7	Oral	Hepatitis C virus	Completed [‡]	[203]
NCT00941811	TLR-7	Topical	Human papillomavirus	Recruiting	[204]
NCT00899574	TLR-7	Topical	Breast cancer and metastases	Recruiting	[205]
NCT00453050	TLR-7	Topical	Melanoma	Ongoing, NR	[206]
NCT00821652	TLR-7/8	Topical	Melanoma	Ongoing, NR	[207]
NCT00960752	TLR-7/8	sc./intradermal injection + topical	Melanoma	Recruiting	[202]
NCT00669292	TLR-9	sc. injection	Esophageal cancer	Recruiting	[208]
NCT00990938	TLR-9	sc. injection	Hepatitis C	Recruiting	[209]
NCT01079741	TLR-3	sc. injection	Melanoma	Recruiting	[210]
NCT01040832	TLR-9	sc. injection	Squamous cell carcinoma of the head and neck	Recruiting	[211]
NCT00728936	TLR-9	sc. injection	Hepatitis C	Recruiting	[212]
NCT00780988	TLR-9	sc. injection	Metastatic colorectal cancer	Not yet recruiting	[213]
NCT00860821	TLR-4	Nasal challenge	Chronic obstructive pulmonary disease	Completed [‡]	[214]
NCT00226993	TLR-9	Intratumoral	Recurrent mycosis fungoides	Terminated	[215]
NCT00185965	TLR-9	Intratumoral + sc. injection	Lymphoma	Completed [‡]	[216]
NCT00823862	TLR-9	Intramuscular injection	Hepatitis C	Completed [‡]	[217]

[†]Table indicates current listings of clinical trials for TLR agonists as of 10 October 2010.

[‡]Completed studies included in this table do not have publications as of 10 October 2010.

NR: Not recruiting; sc.: Subcutaneously; TLR: Toll-like receptor.

Data taken from [203-217].