The pharmacology of endosomal TLR agonists in viral disease

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Abstract
The discovery of endosomal TLRs (Toll-like receptors) and their natural ligands has accelerated efforts to exploit them for therapeutic benefit. Importantly, this was preceded by clinical exploration of agents now known to be endosomal TLR agonists. Clinical effects in viral disease have been reported with agonists of TLR3, TLR7, TLR7/8 and TLR9, and the TLR7 agonist imiquimod is marketed for topical use against warts, a papillomavirus disease. The observed pre-clinical and clinical profiles of agonists of each of these TLRs suggest induction of a multifaceted innate immune response, with biomarker signatures indicative of type 1 interferon induction. However, these agents differ in both their pharmaceutical characteristics and the cellular distribution of their target TLRs, suggesting that drugs directed to these targets will display differences in their overall pharmacological profiles.

Introduction
Pharmacological enhancement of immune responses for the treatment of viral disease is attractive for several reasons. Beyond the obvious observation that the immune response is generally successful in clearing natural infections, the breadth of the immune response offers the potential to respond to infections by any virus, with low risk of development of resistance to the drug. Also, immune-based therapy offers the potential to address the treatment of infections where direct replication inhibitors are either absent or unsatisfactory. Some viruses, such as HPV (human papillomavirus), offer only poor prospects for development of effective agents directed at virally encoded targets due to their replication strategies; others, such as HCV (hepatitis C virus), are so genetically plastic that the inhibitory effect of a direct replication inhibitor is lost rapidly, unless effective strategies are taken to suppress the evolution of resistant variants.

Antiviral immunotherapy exists. The advent of molecular biology has enabled products based on IFN (interferon) α, an immune cytokine, and these form the backbone of the current standard of care for chronic HCV infection. Also, the biological importance of IFN in antiviral host defence has spawned many efforts to identify agents that stimulate endogenous IFN production. Several types of agents have been discovered and advanced to clinical investigation. Of these drugs, imiquimod was successfully developed and approved for treatment of HPV in its clinical manifestation as genital and perianal warts.

Imiquimod and other investigational ‘IFN inducers’ were subsequently shown to be agonists of certain TLRs (Toll-like receptors) thought to be localized to endosomes within cells. TLRs are one class of pattern-recognition receptors that mediate the initial identification of immunological danger. The localization of TLRs to membranes is consistent with their role in sampling the extracellular environment both directly and following endocytosis. Various specific TLRs are the focus of efforts to develop either agonists or antagonists for a range of clinical uses. The scope of potential therapies that target TLRs has been reviewed elsewhere [1,2].

The conceptual division of TLRs into endosomal and non-endosomal groups is helpful, because members of each group share multiple characteristics in addition to their location within the cell. From a pharmacological perspective, endosomal TLRs offer the prospect of antiviral effects via the production of abundant type 1 IFNs. In this brief overview, we focus on efforts to create drugs that stimulate the immune response via endosomal TLRs for intervention in ongoing viral infections, in an effort to highlight knowledge that suggests areas for future investigation. Given this pharmaceutical perspective, the characteristics of the agonist are at least as important as the characteristics of the target.

Broadly stated, all endosomal TLRs recognize nucleic acids as their natural agonists. Importantly, low-molecular-mass agonists of endosomal receptors have been reported, albeit for TLR7 and TLR8 only, offering the prospect of greater flexibility with regard to dosage form and medicinal use.

TLR3
The ligand for TLR3 was shown to be dsRNA (double-stranded RNA) [3], several decades after a report that intravenous administration of dsRNA potently induced IFN in rabbits and protected mice from viral infections [4]. The IFN-inducing activity of heterogeneous dsRNA was confirmed and extended by the in vitro use of a synthetic complex of polyriboinosinic and polyribocytidylic acids [poly(I:C)] to inhibit viral replication in association...
with evidence of IFN induction [5]. The activity was dependent on the double-stranded character of the polymer; single-stranded RNA was at least 1000-fold less active.

Subsequently, many groups have investigated various poly(I:C) preparations in a range of non-clinical studies, including potential uses outside of antiviral utility. Observations of benefit and toxicity varied. Formulation or structural modification enhanced the stability of the poly(I:C) structure against plasma nucleases [6,7]. Route of administration, dose and schedule were factors for consideration [8,9], and activity indicative of potential benefit in hepatitis B-infected chimpanzees was reported [10]. Importantly, reports of organ-specific IFN induction and antiviral protection suggest that the tissue distribution of either poly(I:C) or cells responsive to it may also play a role [11].

Clinical exploration of single intravenous doses of partially mismatched poly(I:C) [poly(I:C12U), also known as Ampligen™] confirmed the induction in blood of the IFN-responsive intracellular enzyme 2′,5′-oligoadenylate synthetase and of circulating neopterin, which is a useful biological marker of immune activation. Circulating IFN was not detected, although adverse events similar to those seen with IFN administration were observed [12]. Notably, there are practical limitations to the use of this drug, because it must be administered by intravenous infusion in a relatively large volume (∼400 mL). This may have restricted the clinical exploration of this agent, particularly as multiple orally administered antiviral agents became available.

Early reports suggested immunological activity of repeated twice weekly intravenous doses of poly(I:C12U) in patients infected with HIV, based on observations indicating retention of immune reactivity to recall antigens [13]. A blinded controlled study of this drug reported slowing of the decline of CD4+ cells in infected patients who entered the study with baseline CD4 counts ≥300/mm3, but a benefit in terms of reduction in viral load was not described [14]. Two clinical studies that explore the use of poly(I:C12U) in HIV disease currently continue to recruit patients (http://www.clinicaltrials.gov/ct/show/NCT0035893 and http://www.clinicaltrials.gov/ct/show/NCT00035581).

Poly(I:C12U) also is the subject of clinical investigation for the treatment of chronic fatigue syndrome (http://www.clinicaltrials.gov/ct/show/NCT00215813). The aetiology of this syndrome is unclear and may not involve viral infection; these studies are noted here for completeness.

In summary, the TLR3 agonist poly(I:C) has demonstrated antiviral effects in animals, and these effects are related to IFN induction. In humans, immunological effects are observed that are consistent with TLR3 agonism, and these are suggestive of potential clinical benefit in selected situations. However, 40 years after the discovery of the biological activity of dsRNA, its clinical use remains under investigation.

**TLR9**

Two lines of investigation have led to the identification of specific DNA sequences as immune stimulants. Pursuit of bioactive fractions from bacterial products [15] resulted in identification of specific palindromic DNA sequences that induce IFN [16]. Independently, study of the immunostimulatory activity of bacterial DNA has led to the recognition that unmethylated CpG motifs stimulate B-cell responses and thus has rationalized a range of off-target effects of antisense DNA [17]. These motifs can interact directly with dendritic cells to stimulate cytokine release [18,19]. Subsequent to the early recognition of its immune effects, the biological activity of bacterial CpG DNA was shown to derive from interactions with TLR9 [20].

Substantial experience exists regarding the biological impact of the pharmacokinetics and distribution of oligonucleotides generally, and these have been discussed elsewhere [21–23]. Although there is some variability with chemical structure, particularly with regard to stability of oligonucleotides in plasma, careful characterization of specific compounds indicates non-uniform distribution across organs and suggests a reasonable ability to extrapolate animal pharmacokinetics to humans [24,25]. Because of the short plasma half-life of oligonucleotides, the observed pharmacodynamics may be significantly affected by drug-distribution parameters. Most work with designed TLR9 agonists has focused on subcutaneous injection, although other routes have been explored [26,27]. In contrast with the cross-species parallels in pharmacokinetic parameters, the chemical structural requirements for immunological activity differ between mouse and humans [28]. Thus work in murine systems, although helpful, requires caution when extrapolating results to humans. Also, TLR9 agonists may differ with regard to their immunological profile, even within a species [29].

Clinical exploration of TLR9 agonists in viral disease has focused on chronic HCV infection. In a dose-escalation study in patients with poor prior responses to IFNα, once- or twice-weekly subcutaneous injection of CPG10101 over a period of 28 days resulted in a dose-related increase in the proportion of patients with an antiviral response. In general, antiviral effects correlate with immune parameters [30]. Adverse events observed resemble those seen with IFNα-based products, including injection site reactions, flu-like symptoms, headache and nausea.

A second clinical study explored the utility of adding a low dose of CPG10101 to ribavirin and PEGylated IFNα, the two elements of current standard of care, in patients who had responded previously to prior IFNα-based treatment, but had relapsed after cessation of drug therapy. Interim results show an increase in the proportion of patients with a reduction in circulating virus while being treated with the combination therapy compared with the control [31]. In spite of these promising early results, the combination treatment offered no apparent advantage in suppressing virological relapse: 6 months after cessation of treatment, only two of seven patients who completed 48 weeks of treatment were free of detectable virus. CPG10101 was not evaluated in the treatment-naïve patient population as a replacement for PEGylated IFNα. Clinical development of CPG10101 in HCV has been stopped.
Clinical studies of other TLR9 agonists in viral disease are under consideration; however, none is underway at the time of writing.

**TLR7**

Although the natural ligand for TLR7 is single-stranded RNA [32,33], there are several distinct chemical classes of low-molecular-mass molecules that effectively and selectively agonize this receptor [34,35]. Similarly to the history of TLR3 and TLR9 agonists, these low-molecular-mass TLR7 agonists were first discovered as IFN inducers [36–38], and clinical investigation predated knowledge of their molecular mechanism.

Low-molecular-mass TLR7 agonists exhibit similar cytokine signatures in vitro (Figure 1A), suggesting that observed biological differences result from factors other than interaction with TLR7 itself. Although potency differences exist between imiquimod and isatoribine in terms of the molar concentration required to elicit an immune response in vitro, this is readily managed with applied dose. In contrast with the nearly identical cytokine profiles for these TLR7 agonists, comparison of a TLR7 agonist with a TLR8 agonist [39] highlights the much higher levels of pro-inflammatory cytokines that result from TLR8 agonism (Figure 1B). This marked difference may be a result of the differential distribution of TLR7 and TLR8 on certain cell populations, such as myeloid and plasmacytoid dendritic cells [40].

Also apparent in Figure 1(A) is the variable magnitude of in vitro induction across individual donors. This is reminiscent of variability observed in clinical studies of isatoribine [41]. The sources of this variability are not fully understood, but must involve factors independent of the compounds because identical variability is seen for both TLR7 agonists. The concentrations used in this in vitro experiment were chosen to achieve maximal IFNα induction in this system, and so the variability at least for this cytokine is not simply a result of donor-specific variation in dose–response relationships. However, polymorphism does exist in TLR7, and this may affect the maximum cytokine response magnitude in vitro [42], perhaps contributing to variability. Interestingly, similarly large donor-specific variation in IFNα induction has also been observed for a TLR9 agonist, although this was not attributable to the subset of polymorphisms examined [43].

Clinical studies of topical imiquimod resulted in initial regulatory approval for use in the treatment of genital and perianal warts, a condition caused by HPV. The activity of imiquimod has been reviewed previously [44–46]. Interestingly, although the agent is reported to have activity against topical HSV (herpes simplex virus) lesions, it did not increase the interval between recurrences. Nevertheless, the growing breadth of approved indications for this agent highlights the potential of immunotherapy as an approach that can address multiple clinical needs.

Orally administered imiquimod on either daily or less frequent schedules has been studied in patients suffering from...
Table 1 | Stage of clinical development of TLR agonists for antiviral indications

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Agonist</th>
<th>Approx. molecular mass (Da)</th>
<th>Route administered</th>
<th>Viral disease</th>
<th>Last reported development status</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR3</td>
<td>Poly(I:C 12U)</td>
<td>&gt; 8000</td>
<td>Intravenous</td>
<td>HIV</td>
<td>Phase 2</td>
</tr>
<tr>
<td>TLR7</td>
<td>Imiquimod</td>
<td>240</td>
<td>Topical</td>
<td>HPV</td>
<td>Marketed</td>
</tr>
<tr>
<td>TLR7/8</td>
<td>Resiquimod</td>
<td>314</td>
<td>Oral</td>
<td>HIV</td>
<td>Phase 1b</td>
</tr>
<tr>
<td>TLR7</td>
<td>Isatoribine</td>
<td>340</td>
<td>Subcutaneous</td>
<td>HCV</td>
<td>Phase 1b†</td>
</tr>
<tr>
<td>TLR9</td>
<td>CPG10101</td>
<td>∼14000</td>
<td>Oral delivery of isatoribine</td>
<td>HCV</td>
<td>Phase 2a‡</td>
</tr>
</tbody>
</table>

* The aetiology of CFS (chronic fatigue syndrome) is unclear, but may be related to human herpesvirus infections.
† Clinical development stopped by sponsor.
‡ Clinical development stopped by sponsor.

from cancer [47,48] or HIV infection [49]. Circulating IFN levels increased over time on a daily schedule [47], and other markers of immune activation were evident. Imiquimod induced circulating IFN in asymptomatic HIV-infected persons, with variable effects on virus load. The side-effect profiles observed in these studies overlap those customarily seen with IFNα, including flu-like symptoms, nausea and lymphopenia, suggesting that these are mechanism-related. However, non-TLR7 causes for these adverse events cannot be excluded. No other clinical studies of imiquimod for systemic viral infection have been reported.

Intravenously administered isatoribine was evaluated in a dose-escalation proof-of-concept study in patients chronically infected with HCV [41]. Although the treatment duration was short, plasma virus concentration declined over the course of treatment, achieving statistical significance at a dose of 800 mg once daily. Notably, this agent was well tolerated and elicited few of the flu-like symptoms classically associated with IFN inducers. Pre-clinical studies showed that avoidance of exposure of the gastrointestinal epithelium to isatoribine improved tolerance [50], and this was achieved by intravenous infusion in the clinical proof-of-concept study. However, intravenous administration is not a preferred dosage form for longer-term dosing, leading to the development of the orally bioavailable pro-drug, ANA975. ANA975 is not itself an agonist of TLR7, but efficiently delivers isatoribine to the systemic circulation, with a pharmacokinetic profile that resembles intravenous infusion [51].

After appropriate pre-clinical toxicology studies, clinical investigation of ANA975 began. Work in volunteers indicated a generally good tolerability profile for ANA975, similar to intravenous isatoribine [51], and a dose-escalation study of 28 days of daily oral ANA975 in HCV-infected patients was initiated. However, this study was halted after enrolling only the lowest planned dose group to allow time for assessment of findings from 13-week pre-clinical toxicology studies of daily administration of this compound, which indicated intense immune responses (http://www.secinfo.com/d17WEy.v4Ac.d.htm). The IND (investigational new drug application) for ANA975 was placed on clinical hold, and ANA975 was subsequently discontinued in development (http://www.secinfo.com/d17WEy.u4bd.d.htm).

**TLR8**

Selective TLR8 agonists have not been investigated in the clinic. However, an interesting clinical study evaluated two dose levels of orally administered resiquimod, an agonist of both TLR7 and TLR8, in HCV-infected patients. This drug produced a dose-dependent effect on plasma HCV concentration [52], but tolerability of this agent was poor. Possible reasons for this poor tolerability include the strongly pro-inflammatory cytokine profile that results from TLR8 agonism (Figure 1B), the magnitude of the applied dose, and the oral route of administration of this agonist. Resiquimod also was investigated as a topical agent in HSV infection, with findings similar to those reported for imiquimod [53].

**Conclusions**

Pharmacological administration of agonists of endosomal TLRs is clearly associated with the induction of immunological responses, and these can be shown to translate into antiviral effects. Multiple agents, routes and viruses have been investigated clinically, and the status of these is summarized in Table 1. Agonists vary with regard to the ease with which they can be administered and also their distribution within the body, and this must be considered in association with the physiological role and distribution of each TLR within the organism. Also, the rates at which the various elements of the immune cascade react to signalling events may have an impact on the best timing for repeated pharmacological stimulation.

Notwithstanding the history summarized in the present paper, much work remains to be done to explore dose...
interval, dosing duration and dose routes at varied applied doses to maximize benefit in viral infections, while limiting potential toxicities that may accompany excessively robust and/or prolonged induction of innate immunity. The pharmaceutical properties of the agonists clearly place limits on this exploration; the TLR3 and TLR9 agonists identified to date require parenteral administration. Topical application of the TLR7 agonist imiquimod provides clear clinical benefit, but full clinical success in systemic disease requires additional investigation. The availability of active and selective agents that can be delivered by convenient dosage forms now enables further exploration of pharmacological control of the innate immune response for the treatment of disease.

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References

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