Investigating Toll-Like Receptor Agonists for Potential To Treat Hepatitis C Virus Infection

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Toll-like receptors (TLRs) are key mediators of innate immunity, and their activation by microbial components leads to the production of cytokines and interferons. Recombinant alpha interferon has been used to treat several viral diseases and is the current standard of care for hepatitis C virus (HCV) infection. Recently, agonists of TLR7 and TLR9 have been shown to have clinical efficacy in HCV patients, and this is correlated with their ability to induce endogenous type I interferon production. We have carried out a comprehensive study of agonists of TLRs 1 to 9 to determine if any additional TLRs can induce antiviral molecules from human peripheral blood mononuclear cells (PBMCs). The agonists were incubated with PBMCs, and the supernatant was then removed and added to HCV replicon cells to assess antiviral activity. Agonists of TLRs 3, 4, 7, 8, and 9 were found to be potent inducers of antiviral activity in PBMC supernatants, and the activity correlated with the induction of alpha interferon and the interferon-induced antiviral biomarker 2',5'-oligoadenylate synthase. Antiviral activity of TLR7 and TLR8 agonists was blocked by an antibody that binds to the type I interferon receptor, confirming that the antiviral activity results from type I interferon induction. TLR4 and TLR8 agonists were found to strongly induce the proinflammatory cytokines interleukin 1β and tumor necrosis factor alpha at concentrations similar to those inducing antiviral activity. This raises concerns about adverse side effects if these were to be used as antiviral agents. We therefore conclude that TLRs 3, 7, and 9 represent the most attractive targets for the development of new HCV therapies.

The World Health Organization reports that ~170 million people worldwide are infected with hepatitis C virus (HCV) (29). The disease affects primarily the liver, with 10 to 20% of chronic infections leading to cirrhosis and 1 to 5% leading to liver cancer. The recommended standard therapy for HCV is a once-weekly injection of pegylated interferon alpha 2a (IFN-α2a) plus daily oral ribavirin. The sustained viral response is approximately 50% (23), and the therapy is associated with significant side effects including flu-like symptoms, depression, and injection site reactions (9). In addition, some patients develop neutralizing antibodies to recombinant IFN, which may contribute to treatment failures (2, 13). There is therefore a need to discover new drugs with improved efficacy and reduced side effects.

Toll-like receptors (TLRs) are a family of receptors that play a key role in innate immunity. On binding microbial ligands, they induce a signaling cascade resulting in the induction of type I IFNs and other cytokines, which drive an inflammatory response and activate the adaptive immune system (for a review, see reference 27). Whereas current IFN therapy involves the administration of a single IFN-α subtype (2a or 2b), TLR activation induces a range of different IFN subtypes. For example, the TLR7 agonist imiquimod has been shown to induce IFN-α1, -α2, -α5, -α6, and -α8 (24). This could offer improved antiviral efficacy, since different IFN subtypes have been shown to have different antiviral potencies against HCV, and some subtypes have synergistic activities in combination with others (18, 30). It is also possible that different subtypes could have differences in side effects.

Recently, TLR7 and TLR9 agonists have been shown to have clinical efficacy against HCV (16, 22). Both agonists induced type I IFN and antiviral biomarkers such as 2',5'-oligo-adenylate synthase (OAS). Given that the activation of other TLRs can induce type I IFN production, we have examined ligands of TLRs 1 to 9 to see which ones are capable of inducing antiviral activity against HCV in human peripheral blood mononuclear cells (PBMCs). We have also profiled the induction of the proinflammatory cytokines interleukin 1β (IL-1β), IL-6, and tumor necrosis factor alpha (TNF-α) by the ligands to assess potential undesirable side effects.

MATERIALS AND METHODS

HCV replicon cell culture. Human hepatoma cells (Huh-7) with the persistent replicon PFKI389 Luc Ubi Neo NS3-3'-ET (ReBLikon GmbH) were maintained in phenol red-free Dulbecco’s modified Eagle’s medium (catalog no. 21063; Invitrogen) containing 10% fetal calf serum (FCS) (Sigma), 1% nonessential amino acids (Invitrogen), 1 IU/ml penicillin (Sigma), 0.1 mg/ml streptomycin (Sigma), and 50 μg/ml G418 (Gibco) at 37°C in 5% CO2 humidified incubator. Five hours prior to the assay, the required number of PBMCs were recovered into RPMI 1640 with 10% (vol/vol) FCS and 1% (wt/vol)L-glutamine (Sigma), penicillin (1 IU/ml), and streptomycin (0.1 mg/ml) and incubated at 37°C in a 5% CO2 humidified incubator.

TLR activation assay. A human TLR ligand kit was obtained from Invivogen. Purified PBMCs from single donors or from a mixture of four donors were frozen in liquid nitrogen in 90% FCS (Sigma)–10% dimethyl sulfoxide (DMSO). Five hours prior to the assay, the required number of PBMCs were recovered into RPMI 1640 with 10% (vol/vol) FCS and 1% (wt/vol)L-glutamine (Sigma), penicillin (1 IU/ml), and streptomycin (0.1 mg/ml) and incubated at 37°C in a 5% CO2 humidified incubator.

Antiviral assay. Individual ligands were diluted according to the manufacturer’s instructions and added to 96-well tissue culture-grade clear plates (Perkin-Elmer) in a volume of 20 μl. Resiquimod, SM360320, and 3M002 were synthesized at Pfizer, dissolved in DMSO, and diluted to give a final DMSO concentration in the assay of 0.1%.

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Control experiments showed that this concentration of DMSO had no effect on the assay (data not shown). The preincubated PBMCs were spun down and resuspended in fresh RPMI medium. Approximately 1 × 10^6 PBMC were added to each well in a volume of 180 μl. The plates were incubated for 24 h at 37°C in a 5% CO₂ humidified incubator. The supernatant was removed and used for HCV replicon and cytokine assays. The remaining cell pellet was used to isolate RNA for the OAS assay.

**HCV replicon assay.** HCV replicon cells in Dulbecco’s modified Eagle’s medium containing 10% FCS without a G418 selection marker were plated in white-well tissue culture-grade 96-well plates (Perkin-Elmer) at 1 × 10^4 cells/well in a volume of 90 μl. Following TLR stimulation, 10 μl of PBMC supernatant was added to the HCV replicon cells, and the cells were incubated for 48 h. Replicon levels were quantified using a Promega Bright Glo luciferase detection kit according to the manufacturer’s instructions. Data are expressed as relative light units.

**Cytokine assays.** Following TLR stimulation, 100 μl of PBMC supernatant was added to a four-spot multiplex cytokine detection plate (Meso Scale Discoveries), and amounts of IFN-α2a, IL-1β, IL-6, and TNF-α were quantified according to the manufacturer’s instructions. Limits of detection were 0.1 pg/ml for IL-1β and TNF-α, 1 pg/ml for IL-6, and 2 pg/ml for IFN-α2a.

**OAS assay.** RNA was extracted from PBMC cell pellets using a Mag Max Blood 96 kit (Ambion) according to the manufacturer’s instructions. cDNA was prepared using an Applied Biosystems cDNA Archive kit. Quantitative PCR was carried out using 5 μl of cDNA with OAS primers (human Hs00159719_m1; Applied Biosystems Assays on Demand) multiplexed with beta actin primers (4326315E; Applied Biosystems Assays on Demand). Reactions were performed in a TaqMan 7900 real-time PCR machine (Applied Biosystems) with denaturation at 95°C for 10 min, followed by 40 cycles with annealing at 60°C for 45 s, primer extension at 72°C for 1 min, and denaturation at 94°C for 20 s. Relative quantities of OAS were calculated using the ddct method (http://docs.appliedbiosystems.com/pebiodocs/04371095.pdf).

**Data analysis.** Microsoft Excel with a Lab Stats add-in was used to plot data and generate 50% effective concentration (EC50) values. For statistical comparisons, a t test was used with a cutoff at the 5% significance level.

**RESULTS**

**Assay development.** To assess the antiviral potential of TLR ligands, we treated human PBMCs with ligands and then used the supernatants and PBMC cell pellets to measure antiviral activity, cytokines, and the IFN-induced antiviral biomarker OAS. Before commencing TLR ligand screening, we carried out several controls to validate the methodology.

To ensure consistent data that were comparable across all ligands, we aimed to use the same batch of PBMCs for the entire study. Given the number of samples required for the study, the most convenient way to achieve this was to pool PBMCs from several donors. In doing this, there is a possibility of inducing activation of the PBMCs, resulting in the production of IFNs and cytokines that could alter the antiviral activity of the supernatants. In order to investigate this, supernatants from untreated pooled PBMCs were incubated with HCV replicon cells for various times, and the level of HCV was quan-
tified by measuring luciferase activity (Fig. 1A). Over time, there was a slight reduction in the level of HCV, although this did not reach statistical significance. Similarly, there was no statistically significant increase in the IFN-induced antiviral biomarker OAS over time (Fig. 1B). There was no significant difference in HCV levels when untreated supernatants of PBMCs from a single donor were compared to those from pooled donors ($P = 0.41$) (Fig. 1C). When the antiviral activity of the TLR7 ligand resiquimod in a single donor was compared with that of pooled PMBCs, the antiviral potency was similar across the four individual donors, with 50% effective concentrations ($EC_{50}$s) ranging from 5 to 53 nM (Fig. 2). When the PBMCs from the four donors were mixed, there was no change in either the antiviral potency ($EC_{50}$ of 24 nM versus a mean $EC_{50}$ of 26.5 nM for single donors), nor was there a change in the maximum or minimum signals in the assay. 

Taken together, these data suggest that untreated PBMCs do not secrete significant levels of antiviral molecules, that the PBMC supernatant alone does not have any significant antiviral or cytotoxic activity, and that mixing PBMCs from different donors does not result in an activation that leads to the induction of antiviral molecules over the time course of our experiments. All further studies were therefore carried out using PBMCs pooled from four donors.

We next explored the optimal time of incubation. PBMCs were incubated with the TLR7 ligand resiquimod for 1, 6, or 24 h, and the supernatant was then removed and used to quantify cytokine levels and antiviral activity. The remaining cell pellet was used to quantify OAS. TNF-$\alpha$ levels were significantly higher at 6 h than at 24 h ($P = 0.01$) (Fig. 3C) but only by a factor of 2.5-fold. There were no statistically significant differences in antiviral activity and levels of OAS, IFN-$\alpha$, and IL-6 between 6 h and 24 h (Fig. 3), although the mean values were higher at 24 h in all cases. For convenience, 24 h was chosen as the incubation time for further experiments.

**Antiviral activity of TLR ligands.** PMBCs were treated with ligands of TLRs 1 to 9 (Table 1). Supernatants were removed and added to HCV replicon cells to assess the degree of anti-

![Antiviral activity of resiquimod in PBMCs from a single donor versus PBMCs pooled from four donors. Resiquimod was incubated with PBMCs from four separate donors and a mixture of the four donors for 24 h, and supernatants were then transferred to HCV replicon cells to determine antiviral activity. Duplicate data points at each concentration are plotted. RLU, relative light units.](http://aac.asm.org/)
viral activity (Fig. 4). All TLR ligands tested induced some degree of antiviral activity in PBMC supernatants. Supernatants from PBMCs treated with agonists of TLRs 3, 4, 7, 8, and 9 demonstrated potent and complete inhibition of HCV replication, whereas agonists of TLRs 1/2, 2, 5, and 6/2 induced only weak and partial inhibitory activity. None of the ligands had any effect on HCV replication at the concentrations tested when added directly to the HCV replicon cells (Fig. 4).

**Induction of IFN and OAS.** The amount of IFN-α in PBMC supernatants treated with the different TLR ligands was quantified (Fig. 5A and Table 2) using an antibody specific to the IFN-α2a subtype. The highest amounts of IFN-α were induced by SM360320, 3M002, and resiquimod. On the whole, the most antiviral TLR ligands induced 0.4 ng/ml IFN, whereas Pam3CSK, HKLM, flagellin, and FSL induced 0.2 ng/ml IFN. Surprisingly, single-stranded RNA40 (ssRNA40) induced only very low levels of IFN-α (0.2 ng/ml) despite being a potent inducer of antiviral activity.

**Induction of the antiviral gene OAS** was measured by quantitative PCR in PBMC pellets. Figure 6A shows the profile of IFN-α and OAS from cells treated with the most potent antiviral TLR ligands. All of the antiviral TLR ligands induced significant amounts of IFN-α and OAS. The antiviral EC50 coincided with the concentration at which IFN started to become detectable. At this concentration, there were already significant amounts of detectable OAS. Ligands of TLRs 3, 7, 8, and 9 induced a dose-dependent increase in IFN-α and OAS. The amount of IFN-α and OAS decreased significantly at higher concentrations of the TLR7 ligand SM360320 and the TLR9 ligand ODN, suggesting that a down-regulation mechanism may be induced. OAS induction with lipopolysaccharide (LPS) treatment appeared to be biphasic, with an initial peak at low concentrations and a second increase at
higher concentrations. Ligands of TLRs 1/2, 2, 5, and 6/2 induced small amounts of IFN-α (0.1 ng/ml for Pam3CSK, 0.2 ng/ml for HKLM, 0.1 ng/ml for flagellin, and 0.03 ng/ml for FSL) and no statistically significant induction of OAS (Fig. 6B).

**Induction of proinflammatory cytokines.** TLR signaling pathways are known to result in the activation of the NF-κB transcription factor, which leads to the induction of proinflammatory cytokines. An ideal antiviral therapy would result in a maximal induction of IFN and a minimal induction of proinflammatory cytokines, since the latter could lead to unwanted side effects. We therefore measured the amount of IL-6, IL-1β, and TNF-α induced by ligands of different TLRs (Fig. 5b, c, and d and Table 2). The cytokine induced to the highest levels was IL-6 (Fig. 5b). Despite being weak inducers of IFN-α, HKLM and flagellin induced significant amounts of all three proinflammatory cytokines. TLR4 and TLR8 ligands induced the highest levels of proinflammatory cytokines, whereas TLR3, TLR7, and TLR9 ligands induced the lowest levels of proinflammatory cytokines.

We next compared the dose response for the induction of TNF-α with the antiviral activity of TLRs 3, 4, 7, and 8 ligands (Fig. 7). Ligands of TLR4 and TLR8 caused a significant induction of TNF-α at concentrations coinciding with the antiviral activity, indicating that a strong proinflammatory response is likely to accompany the antiviral effects of these ligands. In contrast, ligands of TLRs 3, 7, and 9 did not show any significant induction of TNF-α, in some cases at concentrations up to 1,000 times the antiviral EC\(_{50}\), suggesting that antiviral and proinflammatory effects may be differentiated for these TLRs.

**Antiviral activity is due to type I IFNs.** The correlation of the antiviral activity of TLR ligands with the induction of IFN-α and OAS led us to explore the possibility that the
antiviral activity results from type I IFN induction. In order to test this, we used an antibody that blocks the type I IFN receptor. To check that the antibody itself had no significant effects on HCV replication, supernatants from untreated PBMCs were added to replicon cells in the presence or absence of antibody. In the absence of any compound treatment, the antibody slightly increased the levels of HCV replicon (Fig. 8). This is consistent with the slight antiviral effect that was previously observed in PBMC supernatants (Fig. 1A) and suggests that the PBMCs are secreting low constitutive levels of type I IFN.

As a positive control in the assay, IFN-α/H9251 was added to untreated PBMC supernatants. The supernatants were transferred to HCV replicon cells in the presence or absence of antibody, and the levels of HCV replication were quantified. As expected, in the absence of antibody, IFN-α/H9251 resulted in an almost complete inhibition of HCV replication (Fig. 8). The addition of antibody completely prevented the inhibition, confirming that in our assay system, the antibody can fully block the type I IFN receptor.

The effect of the antibody on the activity of the TLR7/TLR8 ligands resiquimod, 3M002, imiquimod, and SM360320 was tested in the same assay system. In all cases, the presence of the antibody blocked the antiviral effects of the ligands (Fig. 8), confirming that the antiviral activity is due to the induction of type I IFNs.

**DISCUSSION**

It has been previously shown that ligands of TLRs 7, 8, and 9 induce antiviral activity against HCV (16, 20, 22). It has also been shown that TLR ligands could have utility in other viral diseases. For example, resiquimod, a dual TLR7/8 ligand, protects against influenza virus in a rat model (12); TLR 3, 4, 5, 7, and 9 ligands have been shown to inhibit hepatitis B virus replication in mice (17); and LPS treatment inhibits human immunodeficiency virus replication in human macrophages (6). We have carried out a comprehensive study to evaluate the antiviral potential of TLR ligands for treating HCV infection. Our data show that agonists of TLRs 3, 4, 7, 8, and 9 are the most potent inducers of anti-HCV molecules from PBMCs.

**TABLE 2. Concentrations of TLR ligands that induce maximal cytokine levels**

<table>
<thead>
<tr>
<th>Ligand</th>
<th>IFN-α2 (μM)</th>
<th>IL-6 (μM)</th>
<th>IL-1β (μM)</th>
<th>TNF-α (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pam3CSK</td>
<td>0.3</td>
<td>1</td>
<td>1</td>
<td>0.1</td>
</tr>
<tr>
<td>HKLM</td>
<td>1E8 cells/ml</td>
<td>1E8 cells/ml</td>
<td>1E8 cells/ml</td>
<td>1E8 cells/ml</td>
</tr>
<tr>
<td>Poly(LC)</td>
<td>25 μg/ml</td>
<td>25 μg/ml</td>
<td>25 μg/ml</td>
<td>25 μg/ml</td>
</tr>
<tr>
<td>LPS</td>
<td>0.1 μg/ml</td>
<td>0.1 μg/ml</td>
<td>0.1 μg/ml</td>
<td>0.3 ng/ml</td>
</tr>
<tr>
<td>Flagellin</td>
<td>1 μg/ml</td>
<td>1 μg/ml</td>
<td>1 μg/ml</td>
<td>1 μg/ml</td>
</tr>
<tr>
<td>FSL-1</td>
<td>1 μg/ml</td>
<td>0.1 μg/ml</td>
<td>1 μg/ml</td>
<td>1 μg/ml</td>
</tr>
<tr>
<td>Imiquimod</td>
<td>1 μg/ml</td>
<td>1 μg/ml</td>
<td>1 μg/ml</td>
<td>1 μg/ml</td>
</tr>
<tr>
<td>SM360320</td>
<td>0.1 μM</td>
<td>1 μM</td>
<td>1 μM</td>
<td>1 μM</td>
</tr>
<tr>
<td>ssRNA40</td>
<td>1 μg/ml</td>
<td>1 μg/ml</td>
<td>1 μg/ml</td>
<td>1 μg/ml</td>
</tr>
<tr>
<td>3M002</td>
<td>10 μM</td>
<td>3.3 μM</td>
<td>3.3 μM</td>
<td>10 μM</td>
</tr>
<tr>
<td>Resiquimod</td>
<td>0.1 μM</td>
<td>1 μM</td>
<td>1 μM</td>
<td>1 μM</td>
</tr>
<tr>
<td>ODN2006</td>
<td>0.2 μM</td>
<td>0.2 μM</td>
<td>5 μM</td>
<td>5 μM</td>
</tr>
</tbody>
</table>
FIG. 6. Induction of IFN-α and OAS by TLR ligands. TLR ligands were incubated with PBMCs at various concentrations for 24 h. The supernatant was removed and used to quantify IFN-α, and the remaining cell pellet was used to quantify OAS by quantitative PCR. Induction (n-fold) was calculated relative to untreated samples. Squares, OAS; triangles, IFN-α. (A) The eight ligands that induced complete and potent antiviral activity. The dashed line indicates the antiviral EC50. (B) The four ligands that induced only weak, partial antiviral activity. All data points are means of duplicate samples ± standard deviations. The TLR ligand is indicated at the top of each graph, with the TLR that it activates following in parentheses.
The antiviral activity of these ligands correlates with the induction of IFN-α and the IFN-induced antiviral OAS gene, and in addition, an antibody that blocks the type I IFN receptor was able to block the antiviral activity of TLR7 and TLR8 ligands. Taken together, our data suggest that the antiviral activity of TLR ligands is due to the induction of type I IFNs. This is consistent with the fact that TLRs 3, 4, 7, 8, and 9 signal via IRF3, IRF5, and IRF7 transcription factors, resulting in the direct induction of type I IFNs (15, 25). In contrast, TLRs 1/2, 4/2, 5, and 6/2 signal via AP1 and NF-κB transcription factors, resulting in the induction of other cytokines and chemokines (1). All of the TLR ligands also induced IL-1β, which has been shown to have antiviral activity against HCV (31). However, we did not find a correlation between IL-1β levels and the antiviral activity of TLR ligands in this study. In fact, the ligands that induced the least antiviral activity (Pam3CSK4, HKLM, flagellin, and FSL) all induced high levels of IL-1β.

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concentrations below those where IFN-α was detected. This might indicate an earlier induction of IFN-β. Consistent with this theory, it has previously been reported that LPS induces much higher levels of IFN-β in human dendritic cells than does resiquimod (10). It should also be noted that we used an antibody specific for the IFN-α2a subtype. TLR7 agonists induce other IFN-α subtypes (24), which may also have contributed to the antiviral activity and OAS induction.

For all of the antiviral ligands, OAS was detected at lower concentrations than IFN-α. This may reflect the different sensitivities of the detection methods used. The assay that we used for IFN-α protein has a detection limit of approximately 10 pg/ml, whereas OAS was quantified by quantitative PCR to measure RNA levels. It is possible that IFN-α concentrations of less than 10 pg/ml could induce significant changes in OAS mRNA levels. These data suggest that OAS is a sensitive biomarker for the induction of IFN-α.

SM360320 and ODN both showed reduced IFN-α and OAS induction at the highest concentrations tested, which may indicate a down-regulation of the response. Several mechanisms for negative regulation of TLR signaling have been identified (21), and it has been previously reported that treatment with high concentrations of the TLR9 ligand CpG1668 results in a reduced cytokine response compared with lower doses (28a). It therefore seems likely that the induction of negative regulators is concentration dependent. Indeed, it has been shown that the degree of tolerance to CpG-ODN repeat treatment is greater when the concentration of the initial treatment is increased (4). This apparent down-regulation of the response at high concentrations could have implications for the selection of optimal doses for the treatment of HCV patients.

Note the differences in profiles between different ligands of the same TLR. For example, SM360320 induced higher levels of IFN-α and proinflammatory cytokines than did imiquimod. This may reflect the different potencies of these molecules against the TLR7 receptor. Similarly, ssRNA40 and 3M002 also showed very different profiles. In molar terms, 3M002 is a weaker agonist of TLR7 than ODN and imiquimod against the TLR7 receptor. Similarly, ssRNA40 and 3M002 showed reduced IFN-α induction at the highest concentrations tested, which may indicate an earlier induction of IFN-α and proinflammatory cytokines than did imiquimod.

Our data show that agonists of TLRs 3, 4, 7, 8, and 9 can induce potent antiviral activity against HCV in vitro. However, TLR4 and TLR8 agonists also induce high levels of the proinflammatory cytokines IL-1β, IL-6, and TNF-α. High levels of these cytokines have been shown to correlate with increased levels of liver damage in HCV patients (7, 8, 19, 28), and elevated levels of IL-6 and TNF-α are associated with a lower response rate to IFN-α therapy (26). When TLRs are considered as targets for potential therapeutics for HCV infection, it is therefore desirable to minimize the induction of these cytokines. In our assay system, TLR3, TLR7, and TLR9 ligands did not induce significant levels of the proinflammatory cytokines tested, although it should be noted that the cytokines measured in this study are by no means an exhaustive list, and we tested only a single time point. A more thorough cytokine profiling for these ligands, including a time course, would be required to fully explore their potential for proinflammatory effects. In addition, consideration should be given to other cell types in the body that express TLRs and the possibility that the spectrum of cytokines induced could vary depending on the cell type that is stimulated.

In summary, based on the data generated in this study, we propose that agonists of TLRs 3, 7, and 9 represent the most attractive TLR targets for pursuing new therapies to treat HCV infection and warrant further investigation. Although this study was based on activity against HCV, there are other viruses that can be inhibited by IFNs (5), and these TLRs may therefore represent attractive targets for a range of viral infections.

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