Small Molecule and Biologic Modulators of the Immune Response to Hepatitis C Virus

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Abstract: Hepatitis C virus represents a major global health problem, with approximately 3% of the world population infected. Immune-response modifiers represent the standard of care, given the lack of approved antiviral agents having direct activity against the viral proteins. Although in recent years, improvements in therapy have been attained by combined treatment with pegylated interferon and ribavirin, the discovery and development of next-generation small molecule and biologic agents is ongoing. Several of these newer therapeutics are focused on modulating Toll-like receptors, interferon-alpha signaling, and the pro-inflammatory cytokine balance. A comprehensive account of the lead compounds in development, the bioassays used for optimization of these immune response modifiers and their clinical status is presented.

Keywords: Imiquimod, isatoribine, cytosine-guanine oligonucleotides (CpG), immunomodulatory oligonucleotides (IMOs), toll-like receptors (TLR), inteferon (IFN).

INTRODUCTION

Hepatitis C virus (HCV) is the primary cause of non-A, non-B hepatitis and accounts for greater than 200 million hepatitis cases worldwide. Although few infections are symptomatic, chronic infection persisting for decades is established in up to 80% of infected individuals [1, 2]. Significant progress in understanding HCV replication and the host-response to infection has been made in the past decade. Despite achieving significant reductions in HCV genotype 2 or genotype 3 RNA using the standard of care, pegylated interferon-alpha and ribavirin combination therapy, major unmet medical need still exists. Limitations associated with this treatment are poor efficacy in patients with genotype 1 virus (approximately 50% sustained viral response [SVR] at 72 weeks post-treatment initiation) and adverse events including flu-like symptoms and neuropsychological complications that often require dosage adjustment or discontinuation of therapy [1-3]. Thus, the identification of improved agents having superior clinical efficacy against HCV genotype 1 with enhanced tolerability are needed.

The viral genome encodes several nonstructural proteins with enzymatic properties amenable to inhibition, including viral protease (NS3/NS4a), helicase (NS3), and an RNA-dependent RNA polymerase (NS5B; RdRp) [4]. Although substantial progress has been made in identifying small molecule inhibitors of HCV enzymes, this review focuses on 2 newly introduced classes of immune-modulating agents targeting toll-like receptors (TLRs). In addition, a comparison of these new classes of therapeutics with interferon-alpha and imiquimod, clinically-utilized immune response modifiers for HCV and human papilloma virus (HPV) respectively, is presented.

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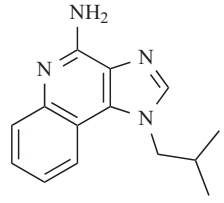
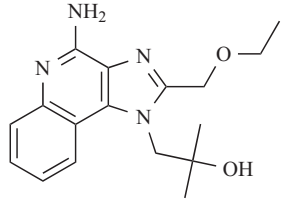
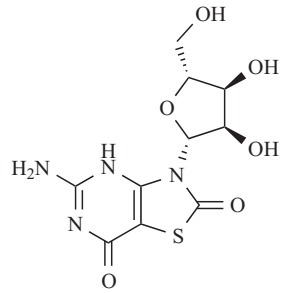
Imidazoquinolines

Imiquimod (1-[2-methylpropyl]-1H-imidazo[4,5c] quinoline-4-amine) and resiquimod (4-amino-α,α-dimethyl-2-ethoxylmethyl-1H-imidazo[4,5-c]-quinoline-1-ethanol; oral formulation of imiquimod) represent two compounds that have antiviral and anti-tumor properties in pre-clinical models, but lack direct antiviral or anti-proliferative properties (Table **1**) [5]. Their biologic activity is presumed

Table 1. Immune Response Modifiers

Compound	Company	Structure
Imiquimod	3M	
Resiquimod	3M	
ANA-245	Anadys	

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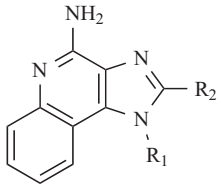
to be mediated through stimulation of innate and adaptive immune responses to produce soluble immune mediators including interferon alpha (IFN-α), tumor necrosis factor alpha (TNF-α), interleukin 12 (IL-12) and interferon gamma (IFN-γ) [6]. Antiviral effects are presumed to be primarily mediated by IFN-α, which in turn induces a range of host-protective proteins, such as PKR, RNase L, 2'-5'-oligoadenylate synthetase (2'-5'-OAS), RIG-1, and MxA [7].

Biological properties of imidazoquinolines include activation of the local immune response, which is readily observed during treatment of human papilloma virus (HPV) infections. Specifically, treatment results in increased antigen presentation by Langerhans cells and the subsequent proliferation and maturation of antigen-specific B lymphocytes. Altogether, this led to speculation that these agents might function through pattern-recognition, or TLR stimulation [8-10]. These suggestions were later confirmed when it was shown that the imidazoquinoline compounds imiquimod and resiquimod signal through TLR7 and TLR7 and TLR8 respectively [11, 12]. Subsequently, single-stranded RNA was identified as a native ligand for TLR7 and TLR8 [13]. Stimulation of these TLRs activates the transcription factor NF-κB through a MyD88 dependent signaling pathway resulting in the activation of innate immune cells and the secretion of pro-inflammatory cytokines [11, 13, and reviewed in 14]. Because of its immunostimulatory potential, topical formulations of imiquimod were seen as an advancement in the treatment of HPV infections adding to the repertoire of options which include surgical excision, cryotherapy and IFN-α treatment, although lesion clearance and recurrence rates remain less than satisfactory.

Although a great deal of work was done to understand the structure-activity relationship with respect to interferon production, the utility of imiquimod will most likely be limited to topical applications because of toxicity issues and poor oral bioavailability. To measure IFN-α production, human peripheral blood mononuclear cells (hPBMCs) were cultured overnight with test compound and supernatants were analyzed for their ability to protect A549 human lung carcinoma cells from cytolysis by encephalomyelitis virus (EMCV). Compound activity was measured as the minimally effective concentration required to induce protective levels of IFN-α in this bioassay. Structure Activity Relationship (SAR) of the 1H-imidazo[4,5-c]quinoline core with variable substituents at the R_1 and R_2 positions (Table **2**) was found to be critical for IFN-α induction [15]. Compounds with alkyl substituents at R1 induced IFN at similar concentrations (MEC = 0.5 ug/ml; Table **2**), although longer straight-chain analogues, attachment of a phenyl group at N-1 or a bulky tert-butyl group were inactive (Table **3**). Improvements were found upon addition of a phenyl group at the terminus of an alkyl chain (MEC = 0.1 – 0.5 ug/ml; Table **2**). SAR on R_2 was further explored keeping R_1 constant as $CH_2CH(CH_3)_2$. Here, straight chain alkyl substituents (C_{1-5}) enhanced IFN induction activity up to 50-fold (MEC = 0.01 – 0.05 ug/ml), with losses in bioactivity observed with alkyl chains longer than C-5 (Table **3**). Several modifications such as trifluoromethly or aryl substituents at C-2 abrogated IFN induction (Table **3**), whereas phenoxymethyl and benzyl derivatives conferred up to a 10-fold increase in activity. Modification of both R_1 and R_2 resulted in the preferred profile (Table **2**) [15].

Table 2. Imiquimod Structure Activity Relationship



R_1	R_2	MEC (ug/ml)
$CH_2CH(CH_3)_2$	-	0.5
CH_2Ph	-	0.1
$CH_2CH_2CH_2CH_2Ph$	-	0.1
$CH_2CH(CH_3)_2$	CH_2OPh	0.1
$CH_2CH(CH_3)_2$	CH_2Ph	0.05
$CH_2C(CH_3)_2OH$	$CH_2CH(CH_3)_2$	0.01

Resiquimod advanced toward clinical studies for chronic hepatitis C, and at a 0.02 mg/kg biweekly dose induction of 2'-5'-OAS, IL-12, IL-6, TNF-α and IFN-α were evident [16]. A mean 1.5 log reduction in HCV RNA was obtained after 24 weeks of therapy, however significant IFN-like side effects were apparent including flu-like symptoms and malaise. Although the clinical utility of imiquimod and resiquimod validates TLR modulation as an approach for treating infections, limitations regarding bioavailability, potential selectivity, dose-selection and toxicity highlight the medicinal chemistry challenges for orally bioavailable immune response modifiers.

Table 3. Imiquimod Structure Activity Relationship

R_1	R_2	MEC (ug/ml)
$CH_2(CH_2)_6CH_3$	-	NI
$CH_2(CH_2)_{14}CH_3$	-	NI
$C(CH_3)_3$	-	NI
Ph	-	NI
$CH_2CH(CH_3)_2$	$CH_2(CH_2)_4CH_3$	0.5
$CH_2CH(CH_3)_2$	$CH_2(CH_2)_5CH_3$	1.0
$CH_2CH(CH_3)_2$	CF_3	NI
$CH_2CH(CH_3)_2$	Ph	NI

NI = no induction of IFN at the highest concentration tested (5.0 ug/ml).

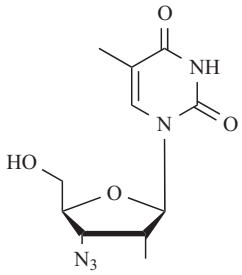
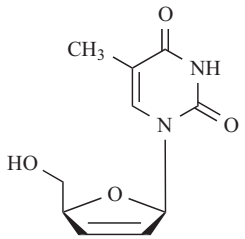
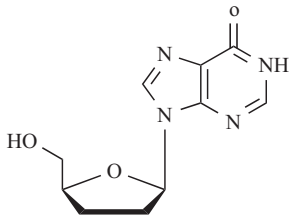
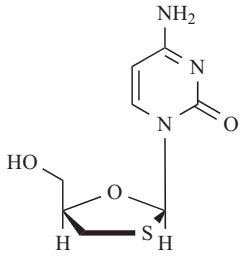
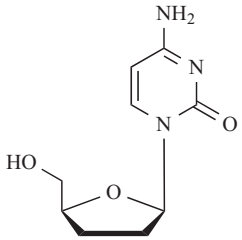
Novel Immune Response Modifiers

Recently, several groups have initiated efforts to identify orally-bioactive immune response modifiers to replace interferon injection as the standard of care for HCV, with a focus on modulating the TLR-dependent signals regulating innate and adaptive immune responses.

TLR7 Agonists

Anadys Pharmaceuticals has recently completed a multi-component Phase 1b clinical trial of isatoribine (ANA245), derived from a new class of drugs to regulate innate

Table 4. HIV Reverse Transcriptase Antivirals

Drug	Structure	Drug	Structure
AZT (Zidovudine)		d4T (Stauvudine)	
ddI (Didanosine)		3TC (Lamivudine)	
ddC (Zalcitabine)			

immunity by interacting with TLR7 (Table **1**) [17]. This agent was recently reported at the American Association for the Study of Liver Diseases Annual meeting to be safe, well tolerated and biologically active confirming that a TLR7 agonist can reduce serum levels of HCV. The dose-escalating, open-label study evaluated the impact of daily intravenous administration for seven days in 32 patients, with doses ranging from 200 to 800 mg. Using 2'-5'- OAS as a surrogate marker of IFN-α antiviral activity, ANA245 demonstrated bioactivity in the highest dose cohort, with a decrease in viral load of 0.76 log units.

Therapeutic uses of D- and L-purine nucleoside analogs such as AZT, ddI, ddC, d4T and 3TC, have been exploited most notably as HIV reverse transcriptase inhibitors (Table **3**). A subset of nucleoside analogs have also been reported to possess immune-modulating properties, a property exploited by Anadys using 3-β-D-ribofuranosylthiazolo[4,5-d]pyrimidine nucleosides as a starting point. SAR was evaluated for oral delivery characteristics and induction of immune responses in mice. This group showed that the 5'-valine ester of val-isatoribine elicits a substantially improved interferon response over oral administration of isatoribine, with measurable IFN concentrations at 1 and 2 hr post-dosing (~50-300 pg/ml plasma concentration). Furthermore, an increase in the proportion of mice exhibiting an IFN response (from 4% to 30%) and in the magnitude of the response (2-fold) was observed with val-isatoribine in comparison with isatoribine. Additional improvements on gastrointestinal (GI) tolerability were claimed with the use of the prodrug. This particular aspect was investigated because of the extensive immune-cell rich tissue present in the GI tract. Specific details on antiviral activity and *in vitro* assays used to develop SAR of these prodrug derivatives was not provided. Subsequent to progression of ANA245, Anadys has initiated Phase I studies with two oral prodrug derivatives of ANA245, namely ANA971 and ANA975 [18].

TLR9 Agonists

Similar to the discovery of imiquimod as an immune response modifier having bioactivity as an anti-tumor and anti-infective agent, cytosine-guanosine oligodeoxynucleotides were found by William Coley to have immunomodulatory activities. Bacterial DNA itself was initially identified as the active component responsible for the anti-tumor activity of streptococci injection into a tumor mass. This bacterial DNA stimulated natural killer cell activity, activated B-cells and enhanced expression of co-stimulatory and MHC molecules by antigen-presenting cells leading to the induction of IFN-α and a Th1-type cytokine response [19-21]. TLR9 was subsequently identified as the receptor mediating this response [22]. The unmethylated CpG dinucleotide within the bacterial DNA in a particular sequence context was found to be preferred ($R_1R_2CGY_1Y_2$, where R_1 represents a purine with preference for G, R_2 a purine or thymidine, and Y_1/Y_2 are pyrimidines). Studies utilizing these ODNs have demonstrated that TLR9 signals through a MyD88-dependent signaling pathway to activate

NF-κB leading to the activation of DCs and the secretion of IFN-α and pro-inflammatory cytokines [22, 23].

Initially for research and now for therapeutic purposes, short CpG containing oligonucleotide sequences (CpG-ODN) are routinely substituted in place of bacterial DNA to mediate stimulation of TLR9. Different classes or subtypes of CpG-ODN have been described, and the immunostimulatory activity of the CpG-ODN is dependent upon the sequences surrounding the CpG dinucleotide(s) as well as the secondary structure formed by the ODN. Type A ODNs have been shown to activate plasmacytoid dendritic cells to mature, upregulate costimulatory molecules, and to secrete type-I interferons, but they are not potent activators of B cells. These CpG-ODNs contain palindromic sequences allowing for the potential of secondary structure formation, and they may also contain end termini poly(dG) nucleotide sequences for nuclease resistance [24, 25]. Type B CpG-ODNs are potent activators of B cells mediating cytokine and immunoglobulin secretion [26]. These ODNs do not strongly activate plasmacytoid dendritic cells, and therefore are not associated with high levels of IFNα secretion. These latter ODNs lack the secondary-structure forming palindromic sequences, and rather it is the dinucleotide flanking purines and pyrimidines that influence the immunostimulatory activity [26]. Recent data indicate additional structural features that can influence immunopotency [27]. ODNs containing a synthetic CpG analog linked with 3'-3' glycerol linker creating free 5' palindromic ends mediated high levels of IFNα production from plasmacytoid dendritic cells as well as enhanced NF-κB activity relative to CpG-ODN of similar sequence but lacking the linker and free 5' ends. Using this collective knowledge of SAR, both Coley Pharmaceutical Group and Hybridon, Inc. have developed TLR9 agonist ODN therapeutics [28, 29].

In 2002, Coley Pharmaceuticals announced a new "C-Class" oligonucleotides, CpG 10101 (Actilon) that, *in vitro*, was shown to enhance proliferation of B cells and dendritic cells as well as IFN-α secretion from both healthy and HCV infected patients [28]. The mechanism of action is believed to be stimulation of TLR9, which is found on B cells and plasmacytoid dendritic cells. This initial study further demonstrated synergy between Actilon and Intron-A on IFN-α secretion. In 2005, Coley Pharmaceuticals announced the results of their phase 1b clinical study in patients predominantly of genotype 1 and who had previously failed to achieve an SVR after 48 weeks of IFN-α plus ribavirin treatment. In this double blind study, 20mg Actilon given twice weekly resulted in a 1.0 to 1.4 log unit reduction in viral load and increased serum levels of IFN-αpresumably *via* TLR9 stimulation on plasmacytoid dendritic cells. However, if SVR is eventually demonstrated in Actilon-treated patients, this would suggest that the mechanism of action is more complex than simply IFN-α production, as the same patients previously failed to achieve SVR with IFN-α treatment. More detailed mechanism of action studies will be required if we are to fully realize the potential of modulating the immune response to combat chronic HCV infection.

Currently, drug therapy for chronic HCV includes various forms of IFN-α with or without Ribavirin. Yet, significant dose-limiting side effects exist for IFN-α therapy including flu-like symptoms and depression. Therefore, the utility of IFN-α inducing TLR agonist-based therapies could be questioned. Importantly however, TLR agonists are capable of activating and/or enhancing immune responses beyond IFN-α, including stimulation of pro and anti-inflammatory cytokines, DC maturation, activation of NK and cytotoxic T cell activity, and the induction of immunoglobulin secretion [14], all of which are known to be important for effective anti-viral responses. Furthermore, these immunostimulatory activities are present even in the absence of significant levels of IFN-α production, as demonstrated in studies comparing the immunostimulatory potential of TLR agonists with a range of IFN-α producing capabilities [14, 27]. These data challenge the dogma of the necessity of high levels of IFN-α in an HCV therapeutic. In fact, although TLR agonists may trigger high levels of IFN-α *in vitro*, the pharmacokinetic and pharmacodynamic profiles of IFN-α inducers *in vivo* could be very different compared to systemic administration of IFN-α. The current challenge facing the development of TLR therapeutics rests in the identification and analysis of the additional functional properties of TLR agonism (beyond IFN-α induction) and how to screen for and characterize these properties in a robust way with relevant assays.

CONCLUSION

In an attempt to build upon the clinical successes of interferon therapy, drug discovery focus has intensified on immune response modifying agents. As the potential to reduce viral RNA in HCV chronically-infected patients is being realized with next generation IFN-inducers, new challenges will begin to emerge with respect to our understanding of the cross talk between the various TLRs and their collective impact on both innate and adaptive immunity. Further medicinal chemistry efforts will identify novel compounds selectively targeting individual TLRs as well as those which interact with higher order TLR containing complexes consisting of heterodimerized, homodimerized and oligomeric complexes of TLRs with key adaptor or co-receptor proteins which modulate the immune response to infection.

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