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European Journal of Medicinal Chemistry 193 (2020) 112238

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Review article

Agonist and antagonist ligands of toll-like receptors 7 and 8: Ingenious tools for therapeutic purposes

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ARTICLE INFO

Article history:
Received 13 February 2020
Received in revised form
11 March 2020
Accepted 12 March 2020
Available online 17 March 2020

Keywords:
TLRs 7/8
Immunological modulators
Heterocycle
Oligonucleotide
Peptide

ABSTRACT

The discovery of the TLRs family and more precisely its functions opened a variety of gates to modulate immunological host responses. TLRs 7/8 are located in the endosomal compartment and activate a specific signaling pathway in a MyD88-dependant manner. According to their involvement into various autoimmune, inflammatory and malignant diseases, researchers have designed diverse TLRs 7/8 ligands able to boost or block the inherent signal transduction. These modulators are often small synthetic compounds and most act as agonists and to a much lesser extent as antagonists. Some of them have reached preclinical and clinical trials, and only one has been approved by the FDA and EMA, imiquimod. The key to the success of these modulators probably lies in their combination with other therapies as recently demonstrated. We gather in this review more than 360 scientific publications, reviews and patents, relating the extensive work carried out by researchers on the design of TLRs 7/8 modulators, which are classified firstly by their biological activities (agonist or antagonist) and then by their chemical structures, which total syntheses are not discussed here. This review also reports about 90 clinical cases, thereby showing the biological interest of these modulators in multiple pathologies.

Contents

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https://doi.org/10.1016/j.ejmech.2020.112238
0223-5234/

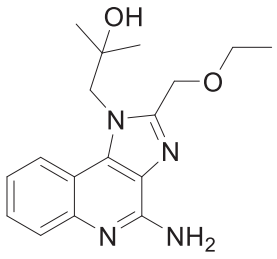
Abbreviations

A	Adenosine
AIDS	Acquired Immuno-Deficiency Syndrome
ART	Antiretroviral therapy
BCC	Basal Cell Carcinoma
BCG	Bacillus Calmette Guerin
DAMP	Damage-associated molecular pattern
DNA	Deoxyribonucleic acid
dG	2′-deoxyguanosine
ds-RNA	Double-stranded RNA
EC_{50}	Half maximal effective concentration
EMA	European Medicine Agency
FDA	Food and Drug Administration
G	Guanosine
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HIV	Human Immunodeficiency Virus
HSV-I, HSV-II	Herpes Simplex Virus
IC	Immune complex
IC_{50}	Half maximal inhibitory concentration
IFN	Interferon
IL	Interleukin
IRAK	IL-1 receptor-associated kinase
IRF	Interferon regulatory factor
IRO	Immunoregulatory oligonucleotide
LRR	Leucine-rich repeat motif
MDC	Myeloid dendritic cell
MEC	Minimum effective concentration
MPL	Monophosphoryl lipid A
miRNA	Micro RNA
NK	Natural Killer
NSCLC	Non-small cell lung cancer
OA	Osteoarthritis
ODN	Oligodeoxynucleotide,
ORN	Oligoribonucleotide
PAMP	Pathogen-associated molecular pattern
pDC	Plasmacytoid dendritic cells
PBMC	Peripheral blood mononuclear cell
PPR	Pattern recognition receptor
RA	Rheumatoid arthritis
Renca	Renal cancer
RNA	Ribonucleic acid
SAR	Structure-activity relationship
s. c.	sub cutaneous
SLE	Systemic lupus erythematosus
siRNA	Small-interfering RNA
ssRNA	Single-stranded RNA
T	Thymidine
TIR	Toll/IL-1 receptor
Th1	T helper cell type 1
Th2	T helper cell type 2
TLR	Toll-like Receptor
TNF	Tumor Necrosis Factor
TRAF	TNF receptor associated factors
TRIF	TIR-domain-containing adapter-inducing interferon-β
TRAIL	TNF-related apoptosis-inducing ligand
U	Uridine
8-OHdG	8-hydroxydeoxyguanosine
8-OHG	8-hydroxyguanosine

1. Introduction

The innate immunity is composed of several families of pattern recognition receptors (PPRs). The latter receptors serve to identify pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs). The PPRs act as the primary defense against pathogenic entities and control the activation and progression of the adaptive immunity by activating the production not only of pro-inflammatory cytokines, chemokines and interferons, but also B and T cells. Among the PPRs, the Toll-like Receptors (TLRs) are of special interest. Their discovery more than 30 years ago has improved knowledge in the regulation of innate immunity, inflammation and cytokines induction.

TLRs are divided into two groups depending on their subcellular localization, which largely correlate with the type of molecular patterns they are able to recognize. Among endosomal TLRs, TLR7



resiquimod (**1**)

Fig. 1. A synthetic tricyclic derivatives belonging to the imidazo[4,5-*c*]quinoline.

and TLR8 have a high degree of sequence homology and function similarity. Both receptors recognize viral single-stranded RNAs, as well as synthetic tricyclic derivatives belonging to the imidazo[4,5-*c*]quinoline series such as resiquimod (**1**), a TLRs 7/8 agonist (Fig. 1). Many novel TLRs 7/8 agonists from different chemical series have been developed these last years. The immunostimulating activity of these agonists allows them to intervene in the therapy of several diseases and to be valuable vaccine adjuvant candidates. In contrast, chronic immune activation by a sustained TLR activation is a hallmark of several infectious, autoimmune and malignant diseases. Therefore, the development of TLRs 7/8 antagonists may also play an important role in the therapy of these diseases. The evidence of the therapeutic potential of TLRs 7/8 ligands has prompted many pharmaceutical companies and research institutes to design, synthetize and optimize TLRs modulators.

Our research team has attached great importance to the immunomodulatory potential of heterocycles known for their antitumoral and antiparasitic activities [1–8]. The structural analogies between our synthetized imidazo[1,5-*a*]quinoxalines, pyrazolo[1,5-*a*]quinoxalines, imidazo[1,2-*a*]pyrazines and imiquimod, set the future lines of our research both in chemistry and biology, allowing us to focus on the TLRs 7/8 immunomodulatory effects of bi- and tri-cyclic derivatives [9–11]. In this review, we gather more than 340 publications until January 2020 relating to TLR7 and TLR8 modulators. By the extent of these data, we show in this review that the modulation of TLR activity is not easy despite many SAR studies around various recognized chemical cores. Not only minimal structures but also macromolecules have been considered and prepared by researchers. In the first chapter, we briefly present data concerning the expression, the structure and the signaling pathways of TLR7 and TLR8. This information will help to better understand the importance of targeting these receptors and the interest of preparing compounds able to modulate the inherent biological responses. We then focus in a second and a third chapter on the implication of TLR7 and TLR8 agonists and antagonists, respectively, in therapeutic treatments, and describe the natural and synthetic ligands focusing mainly on small organic compounds. In a final chapter, we have exposed all the clinical cases using agonist and antagonist ligands clearly referring to receptors.

2. Presentation of the TLR7 and TLR8 receptors

2.1. Localization and expression

TLRs are expressed on immune cells such as macrophages, dendritic cells, B and T cells, neutrophils, natural killer cells (NK), monocytes, eosinophils and non-immune cells such as fibroblasts, keratinocytes, the epithelial cells of the intestinal, respiratory and urogenital tracts [12,13]. TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10, identified in mice and humans, are expressed on the cellular plasma membrane while TLR3, TLR7, TLR8 and TLR9 are expressed on the endosome membrane in mice and humans [14,15]. TLR11, TLR12 and TLR13 are endosomal receptors specific for mice [16–19].

TLR7 is predominantly expressed in plasmacytoid dendritic cells (pDC) and B cells [13,20,21]. Low levels of TLR7 have also been observed in non-immune cells such as hepatocytes, epithelial cells and keratinocytes [22–25]. Contrary to TLR7, TLR8 is more strongly expressed in myeloid dendritic cells, monocytes and to a lesser extent in pDC [13,26]. Under certain circumstances, TLR7 expression is inducible in cells expressing a basal, undetectable level of TLR7, including immune cells such as macrophages, myeloid dendritic cells and non-immune cells such as hepatocytes and keratinocytes [27–29]. For example, TLR7 mRNA expression is induced by the stimulation of IFNγ in macrophage level. Similarly, viral infections such as hepatitis C, the human immunodeficiency virus (HIV) and the influenza A virus lead to upregulation of TLR7 expression in hepatocytes, circulating immune cells and primary macrophages respectively [27,30]. Under these circumstances, it seems that the cytokines induced by the viral infection are responsible for triggering the induction of TLR7 expression. In fact, infection of primary macrophages by the influenza virus leads to upregulation of TLR7 mRNA in a type I IFN dependent manner [30]. In addition, the increased expression of TLRs 7 and 8 is observed in cancer and autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus (SLE) [28,31,32].

2.2. Composition and signaling pathways

Each TLR is composed of an extracellular domain with leucine-rich repeat motifs (LRRs) that are responsible for the recognition of pathogen elements PAMPs and/or DAMPs, a transmembrane domain, and a cytoplasmic domain comprising a Toll/IL-1 receptor (TIR) that is homolog to the IL1 receptor and is consequently responsible for signal transduction. TLRs interact with their PAMPs or DAMPs as a homo or heterodimer along with a co-receptor or an accessory molecule [33,34]. Both TLRs 7 and 8 receptor types have a high degree of sequence homology and function similarity [35–38].

Basically, TLR signaling is divided into two pathways: the MyD88-dependent pathway and the TRIF-dependent pathway [33]. Activation of TRIF/TRAF3 pathway leads to the recruitment of IKKε/TBK1, the phosphorylation of IRF3 and the expression of IFNβ. As this way is not involved in the signaling pathway of TLR7/8, we will not give more details. In fact, the signaling pathways of TLR7 and TLR8 are dependent on MyD88 protein (Fig. 2).

MyD88 is composed of a TIR domain and a death domain. Following the activation of TLRs 7/8, MyD88 interacts, through its death domain, with the death domains of members of the IRAK (IL-1 receptor-associated kinase) protein kinase family, including IRAK1, IRAK2, IRAK4 and IRAK-M [39,40]. IRAK4 phosphorylates and activates IRAK1. After phosphorylation of IRAK4 and IRAK1, these two kinases dissociate from MyD88 and interact with TRAF6 with E3 ubiquitin ligase activity. TRAF6, together with the ubiquitin E3: UBC13 and UEV1A conjugation enzyme, favor the lysine-related polyubiquitination of TRAF6 itself and the TAK1 protein kinase complex [41]. The latter is a member of the MAPKKK family and forms a complex with the regulatory subunits TAB1, TAB2 and TAB3 that will interact with the TRAF6-generated polyubiquitin chains to allow activation of TAK1 [41]. TAK1 then activates two signaling pathways: NF-κβ and MAPK pathways. The IKK complex is composed of two catalytic subunits: IKKα and IKKβ and a regulatory subunit NEMO (or IKKγ). TAK1 binds to the IKK complex *via* ubiquitin chains, allowing it to phosphorylate and activate IKKβ. The IKK complex phosphorylates the inhibitory protein of NF-κβ: Iκβα, which will undergo degradation in the cytoplasm, thus

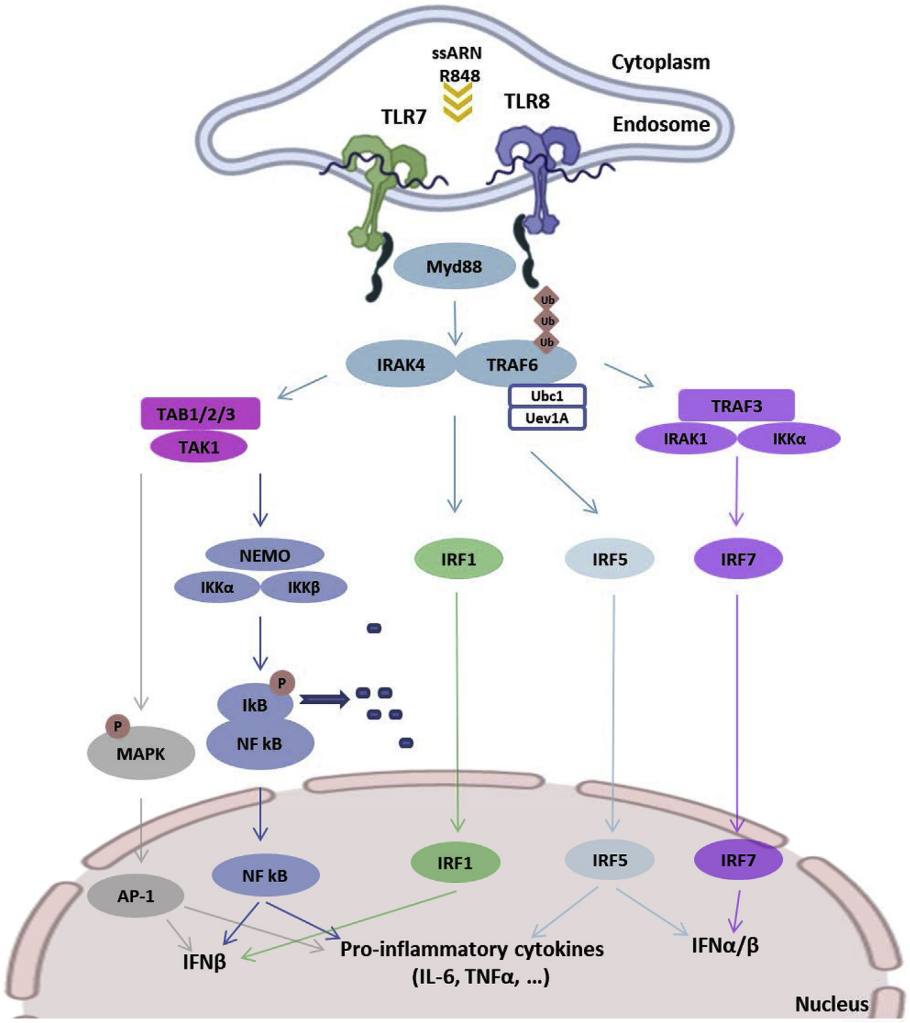


Fig. 2. Signal transduction of TLRs 7/8 receptors leading to the production pro-inflammatory cytokines.

allowing NF-κβ to translocate to the nucleus to induce the expression of pro-inflammatory genes. In addition, TAK1 also activates members of the MAPKs family such as ERK1/2, p38 and JNK, which mediate the activation of the AP-1 transcription factor, responsible for the expression of pro-inflammatory cytokines and IFNβ (Fig. 2) [42,43].

Moreover, Ito et al. showed in 2002 that TLR7 is expressed in myeloid and plasmacytoid dendritic cells. They studied the production of IL12 and IFNα by dendritic cells during TLR7 agonist stimulation. They found that the cytokine induction pattern differs between myeloid dendritic cells (mDCs) and pDCs. pDCs produce IFNα while mDCs produce IL12 [44]. Given the large amounts of IFNα produced by pDCs expressing TLRs 7 and 9, much work has been published in the literature to elucidate the signaling pathway leading to activation and secretion of IFNα especially by dendritic cells. TLR7, TLR8 and TLR9 induce antiviral responses by the production of IFNα as well as pro-inflammatory cytokines. These three receptors use the MyD88 adapter protein to initiate the signaling pathways. The IRF7 transcription factor (Interferon regulatory factor 7) is responsible for the expression and production of IFNα. MyD88 interacts directly with IRF7 at the endosome [45]. IRF7 also interacts with TRAF6, another adapter molecule that operates downstream of MyD88, and after receptor activation (TLRs 7, 8 or 9), IRF7 is activated in a MyD88 and TRAF6 dependent manner. Splenic pDCs from IRF7-deficient mice show a significant decrease in IFNα induction following viral infection or exposure to synthetic TLR7 or 9 ligands [46]. On the other hand, this induction is normal in IRF1, IRF3 or IRF5-deficient pDCs. This shows that induction of IFNα in pDCs requires IRF7 [46]. In addition, MyD88 mutation studies have shown that this protein interacts with IRF7 *via* its death domain. This death domain also interacts with the serine/threonine kinase family (IRAK), which will transduce the signal between MyD88 and TRAF6, indicating that IRAKs are involved in the signaling of IRF7 [47]. pDCs from IRAK1 or IRAK4-deficient mice are unable to produce IFNα upon activation of TLRs 7, 8 or 9 [46]. In addition, one study has shown that IKKα is also essential for activation of IRF7 [48], indicating that activation of IRF7 requires a cascade of IRAK4-IRAK1-IKKα protein kinases. Studies have also shown that TRAF3 plays an important role in this IRF7-dependent signaling [46].

In addition to IRF7, IRF5 also interacts with MyD88 and TRAF6. Unlike IRF7, which binds to the MyD88 death domain, IRF5 interacts with the middle region (known as the intermediate domain) and part of the MyD88 TIR domain [49]. Activation of the MyD88-dependent signaling pathway by TLR7 or TLR9 ligands leads to translocation of IRF5 to the nucleus where it will activate the expression of pro-inflammatory cytokines [50]. In 2005, Schoenemeyer et al. have shown that stimulation of TLR7 and TLR8 by

resiquimod induces the activation of IRF5 as well as IRF7, and they also found that IRF5 is a central mediator in TLRs 7/8 signaling pathway. IRF5 contributes to the induction of IFN type I in human cells, and in addition, is important not only for IFNα induction but also for IFNβ induction [50]. In 2009, a study showed that mDCs, and not pDCs or macrophages, are capable of inducing a large amount of IFNβ after bacterial degradation in phagolysosomes and such response requires the intervention of TLR7, MyD88 and IRF1 [51].

Consequently, those signaling pathways lead to the activation of transcription factors NF-κβ and AP1, which regulate the expression of inflammatory cytokines, and IFN inducible genes. Briefly, despite the phylogenetic and structural similarities between TLR7 and TLR8, these TLRs differ functionally in their cytokine profiles induced by human PBMCs as well as in PBMC-isolated cell populations. TLR7 is functionally associated with the production of IFNα. TLR8 is involved in the production of pro-inflammatory cytokines such as TNFα and IL12.

3. Agonist ligands

TLR signaling pathways activate innate immunity and help in modulating adaptive immunity. Hence, they are essentially useful and developed in immunotherapy. In clinical trials, two third of TLR agonist ligands are first exploited as adjuvants versus one third as potent drugs for various therapies. Herein, the therapeutic purposes of agonist ligands of TLRs 7/8 are discussed, and natural and synthetic ligands are then presented.

3.1. Therapeutic purposes

3.1.1. Antiviral and antibacterial actions

In response to viral infections, the host activates its innate immune system whose response is characterized by IFNs, inflammatory cytokines and chemokines production. This response to viral infection is mediated through several TLR signaling pathways including TLR3, TLRs 7/8/9, TLR4 and TLR2. The contribution of each TLR is different depending on the virus, cell type and infection model examined. TLR7 and 8 are able to detect GU-rich and AU-rich ssRNA sequences of RNA viruses [36,52]. They are known to serve as endosomal PRRs for a number of ssRNA viruses as influenza, HIV-1, vesicular stomatitis virus, Sendai virus, flaviviruses, and coronaviruses [36,53–60]. Moreover, TLR7 signaling is well described in human and mice for type I IFN production by pDCs in response to ssRNA viral stimuli [52], and TLR8 signaling mainly results in NF-κB activation and subsequent inflammatory cytokine expression [61]. Shortly after TLR7 and TLR8 description, it was shown that both receptors respond to potent antiviral imidazoquinoline compounds. These low molecular weight synthetic compounds have been shown to mediate the TLR7 and TLR8 inflammatory responses [62]. TLRs 7/8 play key roles in antiviral and antibacterial responses.

3.1.2. Adjuvant strategies

Vaccination imprints the immune system with the experience of pathogen exposure resulting in the production of humoral immune response by Ag-specific memory B cells. The efficacy of the resulting immunization strongly varies with the ingredients of the vaccine. As the vaccine employed in the early stages of vaccinology were live attenuated or killed organisms, adjuvants were not systematically required. Currently employed adjuvants in human vaccines are aluminum salts, oil in water emulsions (*e.g.* MF59) and TLR9 adjuvants which have limitations and require multiple administrations to induce protection and drive Th2- over Th1-polarized immunity. Moreover, vaccinology moves toward the use of inactivated antigens which lack the immunological information needed to enhance an adaptive immunity response. Hence, the development of novel adjuvants and delivery systems to replace the ones actually used has become crucial to improve vaccine-induced protective immunity [63]. TLR7 and TLR8 together mediate recognition of purine-rich ssRNA to elicit an immune response to pathogens recognized in the endosome. TLRs 7 and 8 are implicated in the recognition of naturally derived uridine−rich ssRNAs of influenza and HIV [36]. In addition TLR7 and TLR8 recognize bacterial RNA [51,64]. Furthermore, TLR7 and 8 are expressed in human plasmacytoid DCs (pDCs), in T and B cells, monocytes and macrophages [65,66]. Naive human B cells express low levels of TLR7 and, whereas activated and memory human B cells also express a broader range of TLRs including TLR7. B cell intrinsic TLR7 signaling may play a role in B cell responses during chronic infections which could be used to activate memory B cells and boost humoral immune responses during immunization [67]. Synthetic small molecules agonists, which activate TLRs 7 and 8 and could be used as adjuvants, have been identified and presented herein. However, the challenge in the development of these molecules remains their formulation.

3.1.3. Cancer targeting

Specific and potent immunostimulatory molecules targeting TLRs 7/8 lead to stimulate innate and adaptive immunity, product cytokines and activate cytotoxic cells. TLRs agonists could be promising elements for novel therapies. Whereas many studies suggest that the activation of TLRs may have potential applications in oncotherapy, others have proved that TLRs signaling is also implicated in tumorigenesis. Even if fighting cancers by TLRs targeting is always under investigation, human clinical trials often failed. Many patients are immunosuppressed due to the traditional anticancer therapies. By this way, it is difficult to induce a positive immune response able to help organism to combat tumors. Understanding the role of TLRs in all cancer cells types remains the main obstacle for clinical therapeutic application. Combination of modulators and traditional approaches (chemotherapy or radiotherapy) could be the key for a successful anticancer therapy [68–70].

3.1.4. Allergy and asthma treatments

The primary step of type I allergic diseases (such as allergic rhinitis, asthma, drug-induced anaphylaxis) is usually a strong pro-inflammatory Th2 and IgE-mediated responses against antigens. It can be counterbalanced by the induction of TLR signaling inducing a cytokine response and activating antigen presenting cells, leading to a Th1 response. Agonists that activate TLRs, particularly TLR7, TLR8 and TLR9 would be expected to induce a strong Th1 response and have been a focus for novel therapeutics to treat asthma and allergies [71,72].

Among allergic diseases, asthma is a complex and chronic inflammatory disease of the airways characterized by airway hyperresponsiveness, eosinophilic infiltration, reversible airflow obstruction, airway remodeling, mucus hypersecretion, and goblet cell hyperplasia. Asthma affects more than 350 million people worldwide. Allergy and atopy are the main causes of asthma. Genetic and environmental triggers modulating the activation and regulation of the immune system are also implicated in the pathophysiology of asthma. Bronchial inflammation, smooth muscle spasm, and mucus production in allergic asthma are triggered by IL-4, IL-5, and IL-13, which are released by Th2 cells. TLR7 ligands play an important role in reduction of airway inflammation, promoting Th1 responses in immune cells, reversing airway hyperresponsiveness, and preventing airway remodeling (including smooth muscle proliferation and goblet cell hyperplasia). Acute and chronic asthma attacks can be managed by precisely identifying the

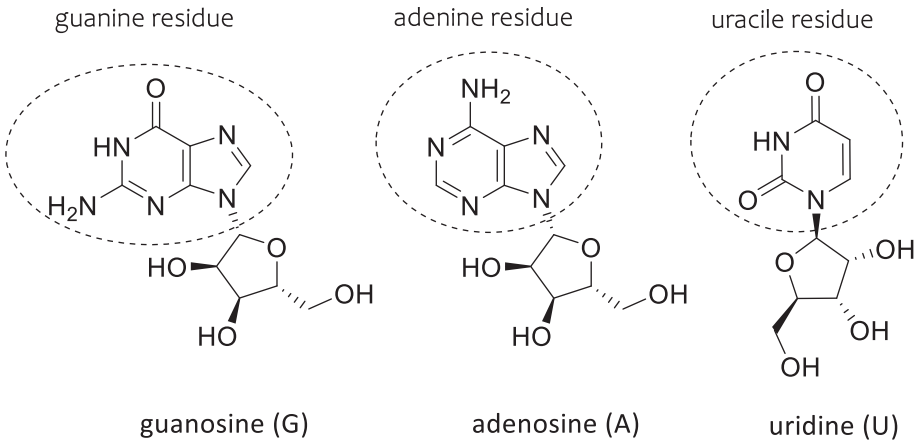


Fig. 3. Nucleobases and nucleosides.

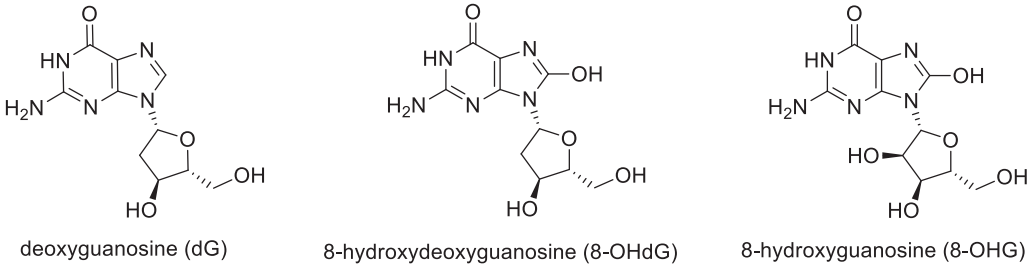


Fig. 4. Guanosine derivatives.

regulators of these pathogenic pathways and targeting their molecular mediators [73,74].

3.2. Natural ligands

Given the structural similarity of the firstly described agonists of TLRs 7/8 with nucleic acids, Heil et al. studied the effect of single-stranded ribonucleic acids (ssRNAs) on the activation of antigen-presenting cells *via* TLR7 and TLR8. They showed that guanosine (G) and uridine (U)-rich oligonucleotides induce the production of TNFα by human PBMCs and that ssRNAs are the natural ligands of TLR7 and TLR8 (Fig. 3) [36]. The same year, Diebold et al. have shown that the ssRNA of influenza virus is a TLR7 agonist, and that TLR7 has a preference for a particular RNA motif that is uridine [62]. In addition, studies have shown that adenosine- and uridine-rich oligoribonucleotides (ORNs) are capable of activating TLR8, without any effect on TLR7, while guanosine-rich ORNs activate TLR7 and TLR8-dependent signaling [52]. In quest of TLR7 and TLR8 agonists, a range of chemical modifications was done to natural ORN ligands in order to increase their selectivity for TLR7 and/or TLR8 and stability against nucleases [75–78].

In 2015, Tanji et al. showed for the first time, that TLR8 recognizes the ssRNA degradation products at two different sites: the first site recognizes uridine while the second recognizes short ORNs such as UG and UUG. Uridine alone is not able to activate TLR8 in the absence of the ORNs at the second site. The ORN binding to the second site increases the affinity of uridine at the first site and therefore leads to activation of TLR8 [79].

Based on the above results, Shibata et al. showed that TLR7 responds to guanosine analogues: guanosine, 2′-deoxyguanosine (dG), 8-hydroxydeoxyguanosine (8-OHdG), in the presence of oligoribonucleotides (Fig. 4) [38]. Guanosine alone or ssRNA alone (polyU) are not capable of activating TLR7, however a combination of both leads to receptor activation. Similarly, ssRNA enhances the affinity of guanosine, dG, 8-OHG, and 8-OHdG to TLR7. Considering that these nucleotides are metabolites present in the physiological or pathological state, they can be considered as endogenous ligands of TLR7. These results were confirmed by Zhang et al. by showing in 2016 that TLR7 also recognizes the degraded form of ssRNA: guanosine binds to the first site while the oligonucleotide (polyU) binds to the second site, and the combination of these two products activates the dimerization of TLR7 in a synergistic way [80].

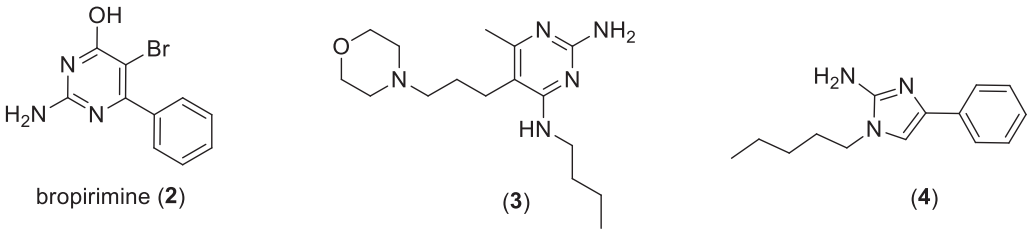


Fig. 5. Pyrimidine derivatives.

Furthermore, many articles were published showing the role of small-interfering RNA (siRNAs) in stimulating the immune system. After siRNAs recognition by TLR7 and TLR8, cytokines are released [81,82].

In 2012, Fabbri et al. show that tumor-secreted miRNA-21 and miRNA-29 can function as ligands by binding to murine TLR7 and human TLR8 receptors in immune cells, triggering a TLR-mediated pro-metastatic inflammatory response that ultimately may lead to tumor growth and metastasis [83].

3.3. Synthetic ligands

Many synthetic ligands as potent agonists of TLRs 7/8 have been designed worldwide. They have been classified in this review by increasing number of (hetero)cycles.

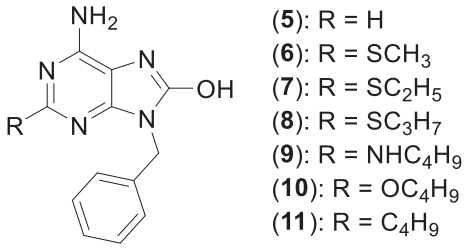
3.3.1. Pyrimidine and purine base derivatives

3.3.1.1. Pyrimidine derivatives and smaller fragment. First described by Skulnick et al. [84], **Bropirimine** (**2**, also known as ABPP for 2-amino-5-bromo-6-phenylpyrimidin-4-ol, Fig. 5) is an orally active immunostimulant acting as IFN inducer in several species, immunomodulator of macrophages, natural killer cells and cytokines [85–87]. Bropirimine is described as antiviral and antineoplastic agent [88]. It was tested as agent against melanoma [89], bladder tumor [90–92], prostate cancer [93] and renal cell carcinoma [94].

Beesu et al. have undergone SAR studies in the 2,4-diamine pyrimidine series, focusing on C5 substituents modulation [95]. Compound **3** (*N*-4-butyl-6-methyl-5-(3-morpholinopropyl)-pyrimidine-2,4-diamine, Fig. 5) exhibits important simultaneous TLR7 and TLR8 agonist activities, with remarkable activation of several subsets of dendritic cells (EC_{50} (TLR7) = 0.046 μM, EC_{50} (TLR8) = 0.280 μM).

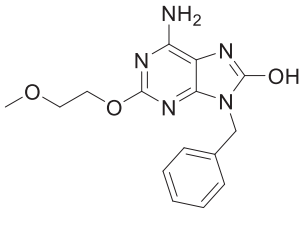
In an attempt to design a TLR8 agonist containing benzimidazole core as vaccine adjuvant, Beesu et al. found that the smallest possible fragment of the benzimidazole core allowing retention of TLR8-agonistic activity corresponds to 1-pentyl-4-phenyl-1*H*-imidazol-2-amine (**4**, Fig. 5) [96]. The crystal structure of this compound bound to the TLR8 ectodomain displayed binding interactions that are common to other TLR8 agonists. This compound showed marked attenuated pro-inflammatory properties in *ex vivo* human blood models.

3.3.1.2. Adenine derivatives. 8-hydroxyadenine derivatives as TLRs agonists have attracted significant interest. In 2002, Hirota et al. synthesized a wide variety of compounds based on the 8-hydroxyadenine scaffold (Fig. 6), to test their potential to secrete IFN-α for the treatment of HCV [97]. SAR studies showed that the presence of a benzyl group at the N9 position as well as a C8 hydroxyl group in compound **5** is essential for IFN induction. The introduction of a R substituent such as alkyl, alkylthio, alkylamino and alkoxy groups to the 8-hydroxyadenine moiety resulted in a remarkable increase in activity. The 2-alkylthio (**6** to **8**), 2-butylamino (**9**) and 2-butoxy (**10**) analogues showed important activities after oral administration in mice. IFN-inducing activity of compound **11** is 10 times higher than that of imiquimod after oral administration in mice. Furthermore, compound **11** has a better oral tolerance compared to imiquimod (Fig. 20) in mice. These results indicate that compound **11** has a better efficacy and safety profile compared to imiquimod.

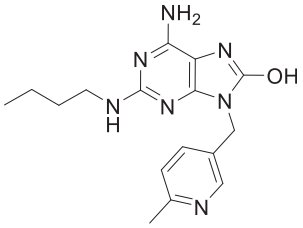


(**5**): R = H
(**6**): R = SCH_3
(**7**): R = SC_2H_5
(**8**): R = SC_3H_7
(**9**): R = NHC_4H_9
(**10**): R = OC_4H_9
(**11**): R = C_4H_9

Fig. 6. General structure of 9-benzyl-8-hydroxyadenine derivatives.



SM-360320 or CL087 (**12**)



SM-276001 (**13**)

Fig. 7. SM derivatives.

Based on these data, Kurimoto et al. synthesized a series of 8-hydroxyadenines having various alkoxy or C2 alkylthio groups and evaluated them for their ability to induce IFN both *in vitro* and *in vivo* [98]. They found that **SM-360320** (**12**, also known as **CL087**, Fig. 7) is a potent inducer of IFN and has good oral bioavailability. SM-360320 is a TLR7 agonist and is able to activate immune responses against the HCV, as shown by Lee et al. [99] It induces IFNα in human peripheral blood mononuclear cells with an EC_{50} value of 0.14 μM [100]. At 10 μM, SM-360320 was shown to reduce HCV levels by 60% in 24 h in the human hepatocyte cell line Huh7, which carries a HCV replicon [99]. It induces IFN in mice with a minimal effective dose of approximately 0.03 mg/kg, making it approximately 100-fold more potent than imiquimod [98]. The mechanism of action is still studied, notably by Russo et al., which showed a higher concentration of this TLR7 agonist in the MIIC of pDC, the acidic compartment protonating and charging positively the compound [101]. SM-360320 is not only a potential treatment for hepatitis C [102] but also for colon cancer [103].

SM-276001 is a selective and potent TLR7 agonist (**13**, Fig. 7), displaying a minimum effective concentration (MEC) of 3 nM, which is comparable to resiquimod (Fig. 1). SM-276001 demonstrated potent at a dose of 0.1 mg/kg by oral administration in mice, a dose dependent manner IFN-inducing activity in monkeys and did not cause emesis in ferrets even at a dose of 30 mg/kg. Isobe et al. also showed that the maximum plasma concentration of SM-276001 was 1019 ng/mL (ca. 3.1 μM), which was approximately 1000-fold higher than the MEC value [104]. Oral administration of SM-276001 leads to the induction of IFNα, TNFα and IL-12p40, the activation of T and B lymphocytes, NK and NKT cells and a reduction in tumor burden in the Balb/c syngeneic Renca and CT26 models. In an ovarian model cancer that spontaneously metastasizes, SM-276001 is able to reduce pulmonary metastasis and a spreading in lymph nodes when dosed either intratracheally or orally [105].

Telormedix, a clinical-stage biopharmaceutical company, proposes diverse prodrugs or formulations of potent TLRs 7/8 agonists. The first generation was represented by **TMX-101**, which is a liquid formulation of imiquimod originally developed as alternative for BCG to produce a local immune response leading to antitumor activity but circumventing the use of live attenuated mycobacteria preventing side effects. Prepared for intravesical instillation of imiquimod and developed by UroGen Pharma (formerly TheraCoat), TMX-101 (Vesimune™) was already tested for pharmacokinetics and pharmacodynamics [106] and was the subject of clinical trials [107–109]. **TMX-202** (**14**, Fig. 8) is a second-generation SM-360320 prodrug, which conjugates the TLR7 ligand to a C12

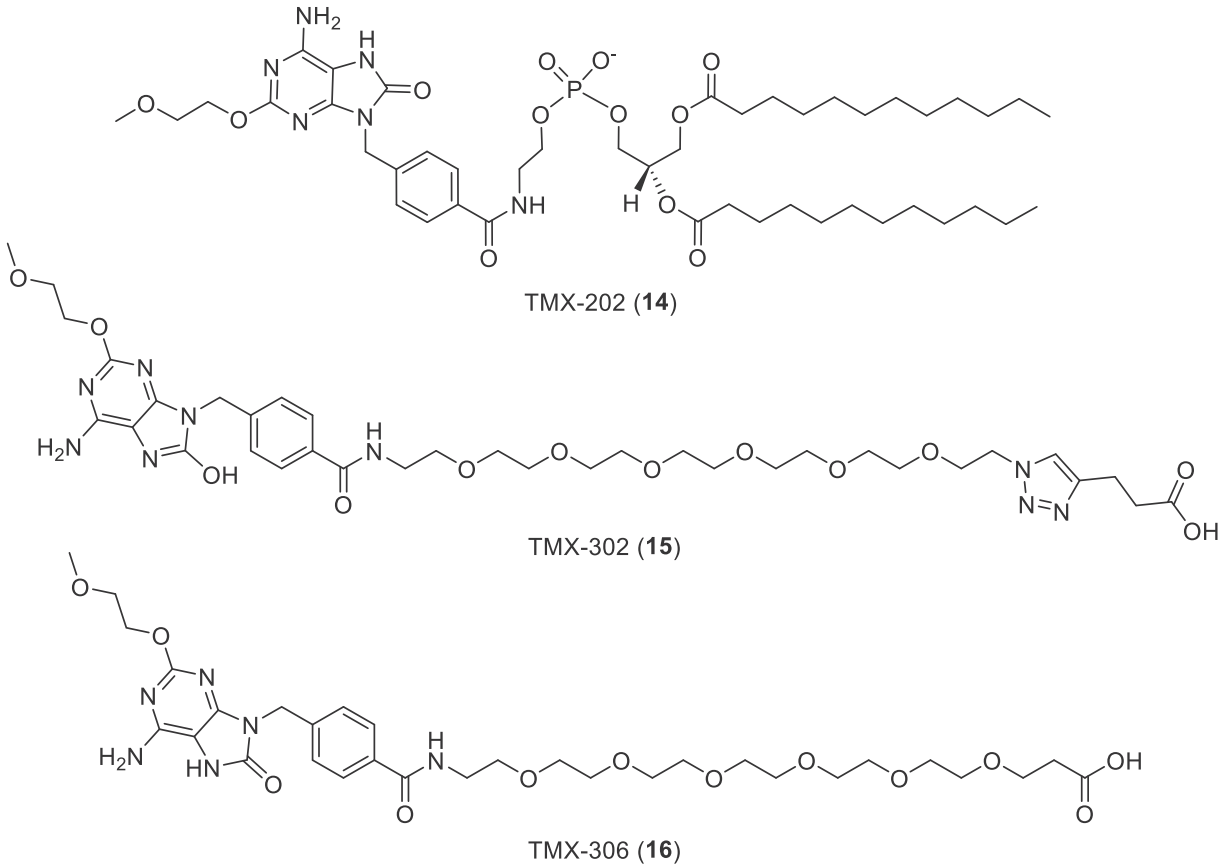


Fig. 8. TMX derivatives.

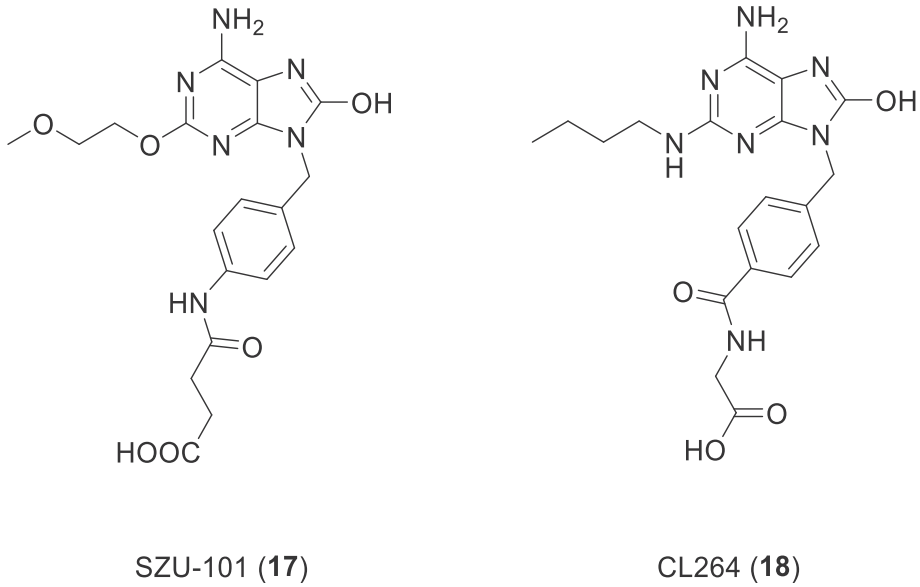


Fig. 9. 8-hydroxyadenine derivatives.

phospholipid *via* a benzoic acid functional group [110]. Discovered through a collaboration with the University of California San Diego, TMX-202 has already successfully completed many *in vivo* studies. TMX-202 is safe and efficacious without causing excessive adverse effects and could be an alternative to imiquimod for the treatment of proliferative skin disorders [111]. It is in preclinical development for the topical treatment of skin cancers, bladder cancers, and other indications. **TMX-302** and **TMX-306** (**15** and **16** respectively, Fig. 8) are PEGylated SM-360320 purine-like compounds characterized by TLR7 partial agonist activity. The clinical application of TMX-302 in lung disorders should be examined with caution because of its direct pro-inflammatory effects. TMX-306 seems to be

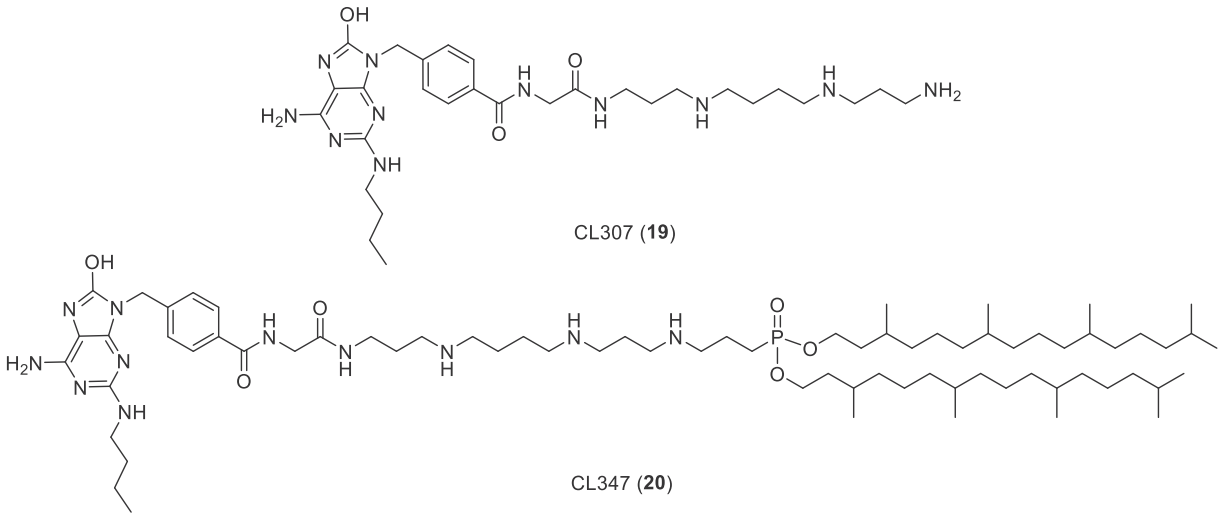


Fig. 10. 8-hydroxyadenine conjugated derivatives.

comparatively more effective and safer, deserving further investigations in drug development particularly for silicosis. This third generation is focused on developing candidates for treating autoimmune diseases [112].

Zhu et al. synthesized a novel 8-hydroxyadenine TLR7 agonist, **SZU-101** (**17**, Fig. 9). Intratumoral administration of **SZU-101** increased the efficacy of doxorubicin, and improved tumor clearance in a T-cell lymphoma mouse model. The new combination of **SZU-101** and doxorubicin administered intratumorally induced high cytokine production and improved cytotoxic T cell response, leading to the eradication of local and distal tumors in tumor-bearing mice [113]. Gao et al. demonstrated the enhancing tumor

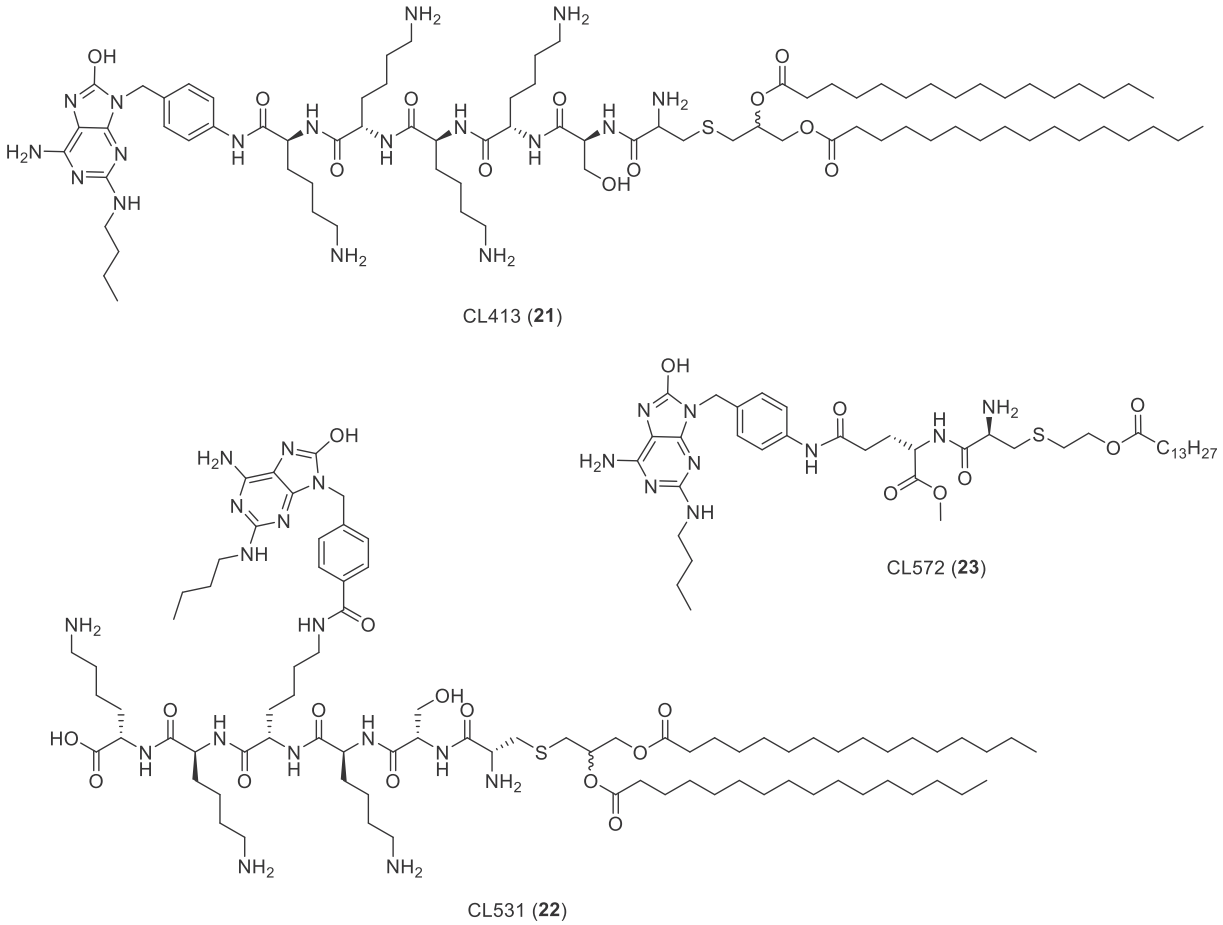


Fig. 11. TLRs 2/7 agonists based on the 8-hydroxyadenine moiety.

clearance in 4T1 human breast cancer mouse model when combined SZU-101 and the tyrosine kinase inhibitor Lapatinib. Moreover, Diao et al. demonstrated that intratumoral SZU-101 injections can reduce both injected and distant tumors in the 4T1 murine breast tumor model by generation of systemic antitumor immunity [114]. In addition, SZU-101 is a promising candidate as adjuvant for inactivated H1N1 influenza vaccines. It induces in mice a strong Th1 response represented by high IgG2a levels [115].

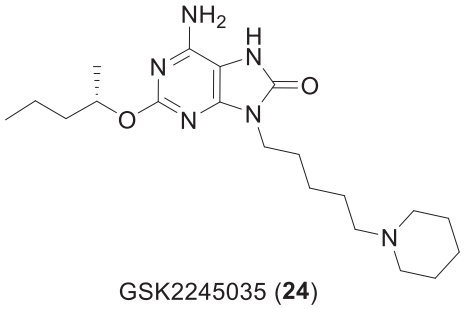
CL264, a 9-benzyl-8-hydroxyadenine derivative, containing a glycine moiety on the benzyl group and similar to SZU-101, induces NF-κB activation and IFNα secretion in TLR7 expressing cells (**18**, Fig. 9). **CL264** is a TLR7 specific agonist, which does not activate TLR8 even at high concentrations [97].

Another base analogue commercialized by Invivogen™ is **CL307** (**19**, Fig. 10) which is prepared by linking a spermine to CL264. CL307 is a very potent TLR7 agonist inducing NF-κB activation even at low concentrations (10 ng/mL).

AdiFectin™ (**20**, also known as **CL347**, Fig. 10), a derivative obtained by conjugation of CL307 with a bis(phytanyl)phosphonate group, is a weaker TLR7 agonist than CL307, but in contrast to CL307, is able to efficiently complex nucleic acids resulting in a strong IFN response. The compound is able to form positively charged liposomes, which can encapsulate DNA (or RNA) thanks to the lipid moiety. Repeated *in vivo* studies showed that pDNA/AdiFectin™ complexes display robust anti-tumor activity. Tumor growth was markedly reduced resulting in a 50% survival rate. Notably, mice that achieved long-term clearance of tumor following AdiFectin™ treatment were protected from subsequent tumor rechallenge suggesting the generation of a tumor-specific memory immune response.

Adilipoline™ (**21**, also known as **CL413**, Fig. 11), a derivative obtained by linearly linking a hydroxyadenine derivative with the terminal acid function of Pam2CSK4 (a TLR2 agonist), is a good ligand for TLRs 2/7. *In vivo* tumor studies have demonstrated that CL413 is a potent antitumor agent. Intratumoral injection of CL413 in established B16 tumors resulted in tumor regression. However, in contrast to CL347, no protection after tumor rechallenge was observed. **CL531** (**22**) corresponds to the conjugation of a hydroxyadenine derivative to the lateral chain of the second lysine of Pam2CSK4, resulting in a dual TLRs 2/7 agonist (Fig. 11). **CL572** (**23**) contains a monoacyl-ethyl-cystein group grafted to a hydroxyadenine *via* a glutamic acid derivative and is also described by Invivogen™ as a dual TLRs 2/7 agonist (Fig. 11).

Among the 8-oxoadenine family, Biggadike et al. prepared derivatives bearing saturated oxygen or nitrogen heterocycles as N-9 substituting group [116]. To treat allergic asthma, **GSK2245035** (**24**) is selected as intranasal candidate (Fig. 12). It resulted *in vitro* in suppression of Th2 cytokine response to allergens and *in vivo* to local upregulation of TLR7-mediated cytokine IP-10. Clinical evaluations on patients confirm the pharmacological interest of this compound.



GSK2245035 (**24**)

Fig. 12. 8-oxoadenine derivative.

DSR-6434 (**25**, Fig. 13) is a specific TLR7 agonist (EC_{50} = 7.2 nM) which activates several immune effectors following intravenous administration. Nakamura et al. demonstrate a strong antitumor effect of DSR-6434 in a melanoma lung metastasis tumor mice model [117]. Systemic treatment with DSR-6434 enhances efficacy of radiation therapy in a model of colorectal carcinoma. Combination therapy with DSR-6434 and ionizing radiation generates antigen-specific memory T cells, leads to a reduction in the incidence of lung metastasis and improves survival in the KHT fibrosarcoma model [118]. By confirming the effectiveness of DSR-6434 on a mouse renal cell carcinoma model, Koga-Yamakawa et al. also showed that the dosing schedules of systemically administered TLR7 agonists significantly affect TLR tolerance and antitumor activity, offering a potential solution to overcome TLR tolerance [119].

DSR-29133 (**26**, Fig. 13) is a TLR7-selective agonist that leads to the induction of IFNα/γ, IP-10, TNFα, IL-1Ra and IL-12p70, and after intravenously injections to a reduction in tumor burden in syngeneic models of renal cancer (Renca), metastatic osteosarcoma (LM8) and colorectal cancer (CT26). Moreover, the efficacy of DSR-29133 was significantly improved when administered in combination with low-dose fractionated radiotherapy. Long-term surviving mice originally treated with DSR-29133 and radiotherapy were protected by a tumor-specific memory immune response which could prevent tumor growth [120].

3.3.1.3. Guanine derivatives. In 2003, Lee et al. proved that guanosine analogues such as **loxoribine** (**27,** also known as **RWJ 21757**) and **isatoribine** (**28**, also known as Thiaoxoguanosine, **TOG**, 7T80G, **ANA-225**, ANA-245, ICN-10146, ICN25, Immunosine, **Immusine**, KN 25, N10146, N12143, N14316, NARI-10146) activate immune cells exclusively *via* TLR7 by a pathway that requires endosomal maturation (Fig. 14) [121]. Previous work showed *in vitro* NF-κβ activity as immunomodulator agents but it could not be linked to *in vivo* observations [122].

Loxoribine (**27**, Fig. 14) displays interesting anti-tumor activities. Significant inhibition of B16 melanoma lung tumor metastasis was observed in mice receiving a single injection of 2 mg loxoribine at day 3 of tumor growth. The greatest inhibition (96%) corresponds to four injections of loxoribine on alternate days starting the day before tumor injection. The combination of loxoribine and IL-2 significantly greater inhibition of metastasis. As guanosine analogues display adjuvant activity in B cell systems, loxoribine was evaluated as adjuvant in a tumor protection model. Mice immunized with both irradiated tumor cells and loxoribine developed a significantly lower number of lung tumors when challenged by live B16 tumor cells, whereas mice injected with either vaccine or loxoribine alone were not protected [123]. Loxoribine was also tested in the treatment of chronic lymphocytic leukemia [124–126]. By this way, loxoribine may be useful in tumor therapy as an immunomodulator or as an adjuvant.

Isatoribine (**28**, Fig. 14), which synthesis was described by Nagahara et al. [127], enhances IFNα levels through its interaction with TLR7 and stimulate of the patient's own immune system. With a potent toxicity profile in multiple species, this compound displays antiviral activity in a variety of murine systems (semliki forest, san angelo, banzi, rat-corona, EMC viruses) [128–131]. Moderate to absent antiviral effects were observed against HSV-2, *VSV* infections or influenza B pneumonia. Isatoribine was also evaluated on chronic hepatitis C infection [132]. On February 2007, this drug was no longer listed on Anadys Pharmaceuticals, Inc. development pipeline.

3.3.2. (Hetero)Bicyclic compounds

3.3.2.1. [5 + 6] fused systems. Yoo et al. synthesized new imidazo

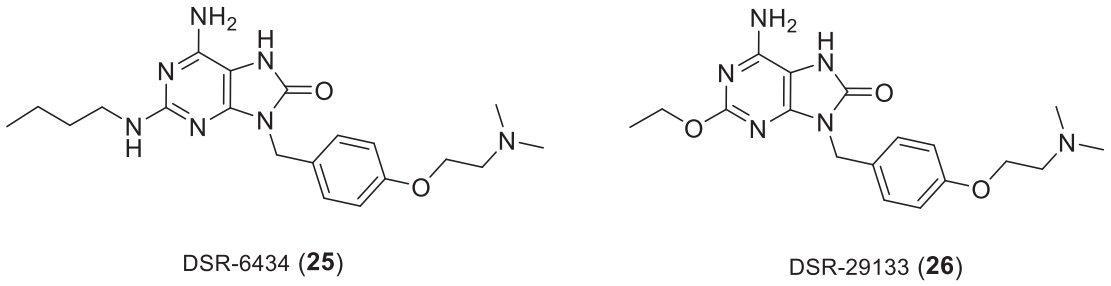


Fig. 13. DSR derivatives.

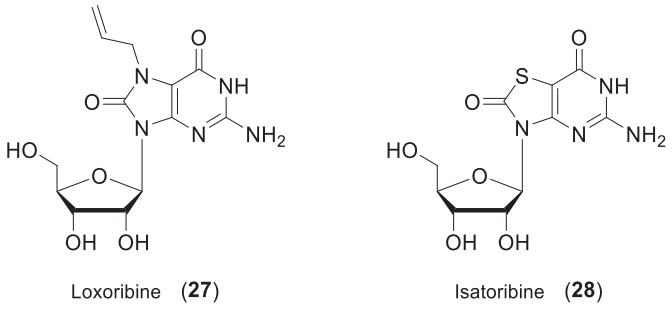


Fig. 14. Guanosine derivatives.

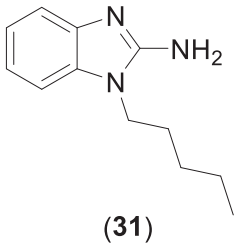


Fig. 16. Benzimidazol-2-amine derivative.

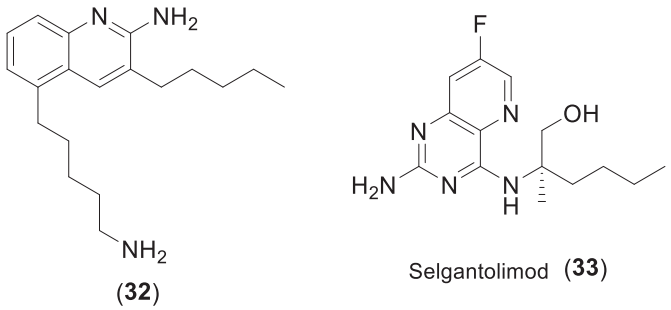


Fig. 17. Pyridine derivatives.

[4,5-*c*]pyridine compounds by incorporating substituents which were previously determined to be optimal agonists (N1-benzyl and C2-butyl, Fig. 15) in the imidazoquinoline series. These compounds were studied for their potency to induce the IFNα production [133]. Compound **29** showed TLR7 agonistic activity (EC50 = 1.57 μM) with negligible activity on TLR8. Direct aryl-aryl bonds at the R1 position abolish all activities, but the TLR7 agonist effect is restored with analogues having a benzyl or phenethyl group. For example, the specific TLR7 agonist compound **30** (EC50 = 0.26 μM) displayed high IFNα induction in human PBMCs. Since some of these compounds are potent inducers of type I IFN, with an attenuated pro-inflammatory profiles, they could be potent adjuvants without local or systemic inflammation reactions [134].

In quest of new vaccine adjuvants, Beesu et al. synthesized a wide variety of heterocyclic compounds that showed good selectivity for TLR8, such as compound **31** (Fig. 16) belonging to the benzimidazol-2-amine series (EC50 (TLR8) = 1.13 μM) [135–137].

3.3.2.2. [6 + 6] fused systems

3.3.2.2.1. Unsaturated derivatives. Among all the prepared heterocyclic derivatives, Beesu et al. found that the quinoline derivative **30** (Fig. 17) displays a remarquable TLR8 agonist activity with an EC50 value of 9 nM [135–137].

Selgantolimod (**31**, also known as GS-9688, Fig. 17), a TLR8 agonist, induces cellular immune mediators, such as IL-12 and IL-8, as well as antiviral cytokines TNF-α and IFN-γ *in vitro* in human PBMCs. Selgantolimod activates NK and mucosal associated invariant T cells, stimulates cluster of differentiation (CD)-8+ T-cell proliferation, while lowering programmed cell death protein 1 expression by HBV-specific CD8+-T cells *in vitro*. Selgantolimod-induced cytokines reduce HBV DNA, RNA, and antigen levels in HBV-infected primary human hepatocytes. The agonist is actually under investigation to treat HIV infection and hepatitis B virus [138].

3.3.2.2.2. Saturated derivatives. Pteridinone-based compounds were identified as potent and selective TLR7 agonists, leading to the discovery of **GS-9620** (**34**, also known as vesatolimod, Fig. 18), a drug with multiple potent applications [139,140]. This orally

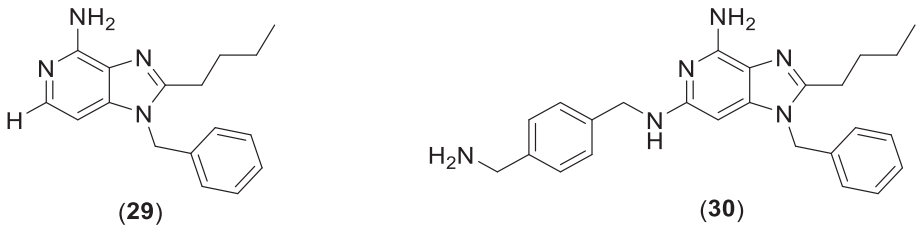


Fig. 15. Core-structure of the imidazo[4,5-*c*]pyridine compounds.

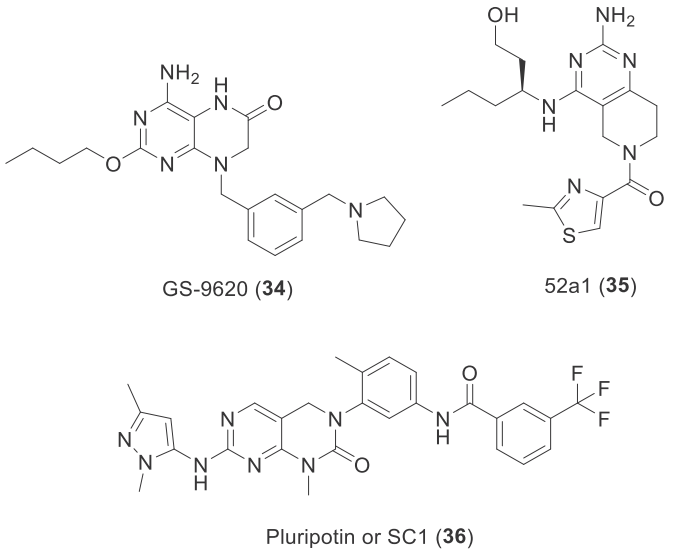


Fig. 18. Annulated pyrimidine derivatives.

bioavailable small molecule has 30-fold selectivity over TLR8 (EC_{50} (TLR7) = 291 nM, EC_{50} (TLR8) = 9 μM). GS-9620 selectively induces IFN-α, cytokines and chemokines. TLR7 activation by GS-9620 is likely associated with compound-induced conformational changes. Activation of NF-κβ and Akt pathways in primary plasmacytoid dendritic cells occurs as immediate downstream cellular responses to GS-9620 stimulation. GS-9620 possesses high absorption properties in the gastrointestinal tract but has moderate clearance during first-pass hepatic metabolism. These properties minimize systemic exposure of GS-9620 following oral administration while targeting exposure to, and stimulation of, innate immune cells within the gastrointestinal tract and/or liver [141–143].

Furthermore, GS-9620 could activate TLR7 signaling in immune cells to induce clearance of virus infected cells. The minimum effective concentrations for IFN-α induction were similar in pDCs and in PBMCs from HCV-positive donors. It has been shown to stimulate a TLR7 immune response in different animal models of chronic hepatitis B infection (mice, woodchuck, nonhuman primates, …) [141,144]. GS-9620 was administered to HBV infected three chimpanzees for 8 weeks with an interval of 1 week [145]. Consequently, serum concentrations of HBV surface antigen and HBV antigen, and the number of HBV antigen–positive hepatocytes, were decreased as hepatocyte apoptosis increased. In a phase 1 clinical trial to evaluate the safety and tolerability of GS-9620, treatment of GS-9620 results in dose dependent increases in select cytokines, chemokines, and ISGs beginning at 2 mg and is safe in a single dose up to 12 mg. Increases in percentages of immunocytes, like T cells, B cells and NK cells, expressing CD69 were also noted in subjects receiving GS-9620 treatment [146–148]. A dose-dependent pharmacodynamic induction of interferon-stimulated genes (ISGs) was demonstrated without a significant systemic induction of IFNα expression or related symptoms. No significant hepatitis B surface antigen decline was observed [149,150].

Moreover, GS-9620 is able *in vitro* to inhibit HIV-1 infection in human peripheral blood mononuclear cells [151]. It was also demonstrated that GS-9620 induced HIV RNA expression *ex vivo* in PBMC isolated from patients on suppressive antiretroviral therapy [152–154]. Although GS-9620 was inactive against HIV in purified $CD4^+$ T cells and macrophages, HIV replication was potently inhibited by conditioned medium derived from GS-9620-treated pDC cultures when added to $CD4^+$ T cells prior to infection. GS-9620-treated PBMCs primarily showed increased production of IFN-α, and cotreatment with IFN-α-blocking antibodies reversed the HIV-1-inhibitory effect of GS-9620. Supplementary studies demonstrated that GS-9620 inhibited a post-entry event in HIV replication at a step coincident with or prior to reverse transcription. GS-9620 induced phagocytic cell maturation and improved effector-mediated killing of HIV-infected CD4 T cells by the HIV envelope-specific broadly neutralizing antibody PGT121. All data show that the compound enhanced antibody-mediated clearance of HIV-infected cells by improving immune effector functions targeting specifically those cells. GS-9620 is under clinical evaluation for reducing latent reservoirs in virally suppressed HIV-infected patients. The viral reservoir in latently infected cells is responsible for viral rebound in the vast majority of HIV-1-infected individuals who stop taking antiretroviral therapy. The key challenge to cure for HIV-1 infection is represented by the more extensively studied therapeutic strategies known as “Shock and kill”. These strategies are aimed toward the reactivation of the latent reservoir using a latency-reversal agent (LRA) with the subsequent killing of the reactivated cell either by the cytotoxic arm of the immune system, including NK and CD8 T cells, or by viral cytopathic mechanisms [155,156]. Borducchi et al. show that administration of the V3 glycan-dependent bNAb PGT121 together with GS-9620 during ART delayed viral rebound following discontinuation of ART in simian–human immunodeficiency virus (SHIV)-SF162P3-infected rhesus monkeys in which ART was initiated during early acute infection [157]. More contrasted results were obtained by Del Prete et al. [158] **GS-986**, another structurally-unpublished TLR7 agonist, was also evaluated to reduce latent reservoir in SIV-infected rhesus monkeys [159].

Among a panel of tetrahydropyridopyrimidines, MacGowan et al. identified **52a1** (**35**) a potent dual TLRs 7/8 agonist (least effective concentrations: 1.5 μM hTLR7, 3.4 μM hTLR8, Fig. 18), as demonstrated by the induction of interferon-γ induced protein (IP-10) [160]. The pharmacokinetic profile of 52a1 in mice confirmed the low systemic exposure. In addition, 52a1 showed low plasma protein binding (30% bound in mice plasma), no inhibition of major CYP450 isozymes (CYP450 > 10 μM: 3A4, 2C8, 2C9, 2D6, 1A2, 2C19) and lacked off-target activity across a multi-receptors panel (>10 μM against histamine, dopamine, and serotonin subtypes).

SC1 (**36**, also known as **Pluripotin**, Fig. 18), a TLR7 agonist, is an inhibitor of extracellular signal-regulated kinase 1 (ERK1, MAPK3) and RasGAP with Kd values of 98 nM and 212 nM respectively. It maintains embryonic stem cell self-renewal in the absence of feeder cells and exogenous factors. Long-term SC1-expanded murine embryonic stem cells can be differentiated into cells of the three primary germ layers *in vitro* and also can generate chimeric mice and contribute to the germ line *in vivo* [161]. Intravenously administered SC1 mediates systemic release of type I IFN, but not of pro-inflammatory cytokines such as TNFα and IL6, and results in activation of circulating immune cells. Intratumoral $CD8^+$ T cells and CD11b + are significantly increased, pDCs are strongly activated and macrophages are M1 phenotype polarized, whereas myeloid-derived suppressor cells are decreased. SC1 prevents spontaneous lung metastasis, retards tumor growth, results in tumor rejection, prolongs survival and, most importantly, protects from tumor rechallenge, as demonstrated by Vascotto et al. in melanoma and colorectal cancer tumor models. SC1 treatment of mice inhibits the growth of established syngeneic tumors and results in significantly prolonged survival. Tumor-free mice treated with SC1 are protected from subsequent tumor rechallenge [162,163]. SC1 is also efficient in preventing lung metastases in a pulmonary metastatic Renca model [164]. Mice bearing the NK cell-sensitive lymphoma RMA-S are cured by repeated *s. c.* SC1

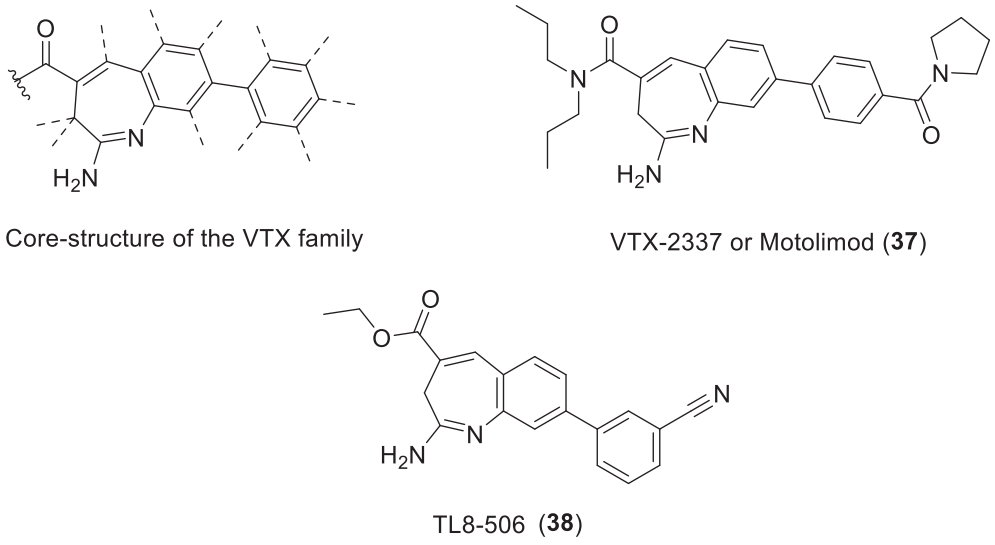


Fig. 19. VTX series and analogues.

administrations as efficiently as with resiquimod. No relevant toxicities were observed. Mechanistically, SC1 reverses NK cell anergy and restores NK cell-mediated tumor cell killing in an IFN-dependent manner [165]. **SC-2** (unpublished structure) is a dual-specific TLRs 7/8 agonist which displays a more pronounced pro-inflammatory cytokines release than SC-1. Additional experiments showed a significant up-regulation of activation marker CD69 on NK cells and increased cytolytic activity of peripheral blood cells [164].

3.3.2.3. [6 + 7] fused systems. **VTX-2337** (**37**, also known as **Motolimod**, Fig. 19) is a selective and potent benzazepine small molecule agonist of TLR 8 with EC_{50} of 100 nM and displays 50-fold selectivity over TLR7. By coordinated activation of TLR8 and NLRP3, VTX-2337 activates NK cells and augments ADCC [166,167]. When treated with VTX-2337, TLR8 stimulates TNF-α and IL-12 production at lower concentrations in human PBMCs. It also induces TNF-α and IL-12 secretion from monocytes and myeloid DCs through the NF-κB pathway. IFNγ secretion was observed when NK cells were treated with VTX-2337, which can enhance the lytic capability and antibody-dependent cell-mediated cytotoxicity of NK cells. VTX-2337 also improves the efficacy of pegylated liposomal doxorubicin in treatment of ovarian cancer in a mouse model with humanized immune system that has been reconstituted with human $CD34^+$ cells. This compound is evaluated for treatment of a variety of cancers, including head and neck cancer, colorectal, pancreatic, melanoma, breast, renal cell carcinoma, non small—cell lung carcinoma, and other solid neoplasms. Evaluated as a stand-alone drug for treatment of lymphoma, VTX-2337 was essentially used in combination with other drugs [166,168]. Thanks to its potential immunostimulating and anticancer activities, VTX-2337 is currently under clinical development as an immunotherapy for multiple oncology indications such as ovarian cancer and squamous cell carcinoma of the head and neck [169]. **VTX-1463** is an intranasal formulation of VTX-2337 under phase I clinical trial for allergic rhinitis [170,171].

Among the VTX family (Fig. 19) [172], **VTX-294** (also known as **VTX-763**) was defined as an ultra-potent TLR8 agonist that activates newborn and adult leukocytes and is a candidate vaccine adjuvant in both early life and adulthood. VTX-294 was 100x more active on TLR8-than TLR7-transfected HEK cells (EC50 (TLR8) = 50 nM *vs*. EC50 (TLR7) = 5700 nM) defining by this way its selectivity for TLR8. VTX-294-induced TNF and IL-1β production were comparable in newborn cord and adult peripheral blood and was more potent than MPLA, R848 or CL075. Combination of VTX-294 and MPLA induced greater blood TNF and IL-1β responses than combination of R-848 and MPLA. VTX-294 also potently induced expression of cytokines and co-stimulatory molecules HLA-DR and CD86 in human newborn MoDCs [173]. **VTX-763**, another TLR8 agonist, is in preclinical study to treat autoimmune inflammation. **VTX-463,** a dual TLRs 7/8 agonist, is in preclinical study to treat allergies.

TL8-506, a benzoazepine analogue to motolimod (**38**, Fig. 19), is also a TLR8 ligand more potent than resiquimod and 3M-002. TL8-506 is 500x more active on HEL-Blue hTLR8 than TLR7-transfected HEK293 (HEK-Blue™ hTLR7) cells (EC50 = 30 nM *vs* 15 μM). TL8-506 is 50x and 25x more potent in inducing NF-κB activation in TLR8-transfeceted HEK293 cells than resiquimod and 3M − 002 (EC50 = 30 nM *vs* 1500 nM and 800 nM respectively) [174,175].

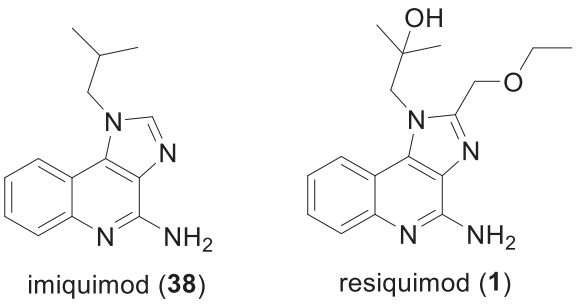


Fig. 20. Imidazo[4,5-*c*]quinoline derivatives.

3.3.3. (Hetero)Tricyclic compounds

3.3.3.1. Imidazo[4,5-c]quinoline series. Among the imidazo[4,5-*c*] quinolines series, imiquimod and its derivative resiquimod (Fig. 20) were the first synthetic molecules described in literature as TLR8 and/or TLR7 agonists [37,176]. Various derivatives have been then designed.

Imiquimod (**39**, also known as **R-837**, Fig. 20) is a tricyclic nitrogen molecule belonging to the imidazo[4,5-*c*]quinoline series, developed 3M Pharmaceuticals laboratories (Saint Paul, MN, USA). It is commercialized as Aldara® or Zyclara®, respectively 5% and

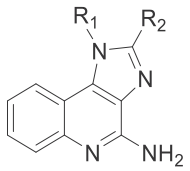
3.75% imiquimod-containing creams. It was first described for its antiviral activity against certain viruses such as herpes simplex II (HSV-II) [177], sendai virus [178] and papilloma virus [179]. This molecule does not directly inhibit viral replication, it is an immunomodulator whose viral activity is related to the inducing power of IFNα and other cytokines. It was approved in 1997 by the Food and Drug Administration (FDA) for the treatment of external genital and perianal warts (*condylomata acuminata*). In 2004, its use was extended to the treatment of actinic keratosis and superficial basal cell carcinoma [180,181].

In 2002, Hemmi et al. have shown that imiquimod and its derivative resiquimod (Fig. 20) exert their antiviral and antitumor properties *via* TLR7 (EC50 = 10.7 μM) [176]. They showed that TLR7-deficient mice were not able to produce pro-inflammatory cytokines following stimulation with imiquimod and resiquimod. Once bound to its TLR7 receptor, imiquimod triggers a Myd88-dependent signaling pathway that drives activation of NF-κB. Once activated, NF-κB migrates into the nucleus and induces the transcription of pro-inflammatory cytokines: TNFα, IL-1, IL-6, IL-8 and IL-12 as well as IFNα. The consequence of this effect is a marked stimulation of the immune response against infected and tumor cells [182]. The effect of imiquimod was also demonstrated by skin recruitment and activation of pDCs [183]. In addition, topically applied imiquimod induces the maturation of epidermal Langerhans cells *in vivo* and stimulates the migration of these antigen-presenting cells to the lymph nodes where they induce a specific T-cell response [184]. In addition, imiquimod can also enhance adaptive immunity by indirectly stimulating the production of IFNγ, a cytokine involved in a Th1 response [185,186]. At high concentrations, imiquimod induces apoptosis of tumor cells *via* the activation of Bcl-2 proteins and the caspase family [187]. Besides FDA approved indications, topical imiquimod has been used in the treatment of several infectious skin disorders (non-anagenital cutaneous warts [188], *Molluscum Contagiosum* [189], cutaneous leishmaniasis [190], etc.), neoplastic (fungoid mycosis [191], vascular tumors [192], cutaneous metastases [193], etc.) and other disorders such as ring granuloma [194], primary follicular mucinosis [195]. Moreover, topical imiquimod might be considered as adjuvant for vaccination [191,196]. Imiquimod was also tested as potent agent against prostate [197], bladder [107,198,199], breast [200–203] cancers and squamous carcinoma [204,205].

Resiquimod (**1**, also known as **R-848**, Fig. 20) is an imidazo[4,5-*c*]quinoline compound stimulating the immune system by activating TLR7 and TLR8 (EC50 (TLR7) = 1.5 ± 0.3 μM, EC50 (TLR8) = 4.5 ± 3.2 μM). It was developed by 3M pharmaceuticals laboratories in the early 1980s in attempts to identify nucleoside-like structures inhibiting infection with HSV-2 [206].

Although Imiquimod and its derivative resiquimod belong to the same chemical family, resiquimod can activate both TLR7 and TLR8, whereas imiquimod is only TLR7 agonist [37,176]. These differences in TLR binding presumably explain the important cytokine induction subsequent TLRs activation. The cytokine profile observed following exposure of human PBMCs to resiquimod is similar to that of imiquimod, nevertheless the level of expression of pro-inflammatory cytokines (TNFα, IL-1β and IL-6) as well as IFNα is approximately 100 times higher when using the 1 μg.mL-1 of resiquimod compared to 1.2 μg.mL-1 of imiquimod [207,208]. In response to resiquimod, dendritic cells secrete IL-6, IL-12, TNFα and IFNα [209], monocytes secrete IL-1, IL-6, IL- 8, IL-12, TNFα and IFNα [210].

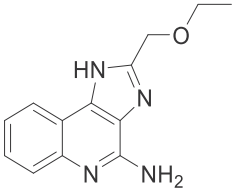
Even if the development of topical resiquimod for the treatment of genital herpes in humans has been discontinued due to inconsistent results in clinical trials, many studies have shown the interest of resiquimod in the treatment of cutaneous tumors. In 2001, resiquimod was considered as potential anti-HSV-2 treatment but due to a lack of efficacy in clinical trials, its development in this indication was stopped [211,212]. Nevertheless, this compound proved to be effective in the treatment of actinic keratosis. At skin level, the target cells of resiquimod are mainly monocytes and dendritic cells. PDCs express TLR7 and TLR9, while MDCs express TLRs 2, 3, 4, 5 and 8, therefore resiquimod by stimulating both TLR 7 and TLR 8 can induce a strong cytokine response including type-I IFN and pro-inflammatory cytokines, which may explain the anti-tumor effect of resiquimod in skin cancer [213]. In basal cell carcinomas (BCC) tumor cells, imiquimod showed direct and independent of death receptor binding pro-apoptotic activity. This activity was also observed with resiquimod, however, to a much smaller extent compared with imiquimod [214]. Resiquimod can indirectly provoke an apoptosis induction in tumor cells by inducing the expression of TRAIL (TNF-related apoptosis-inducing ligand) on dendritic cells and thus increasing their cytotoxic potency [215,216]. In 2015, Rook et al. have shown that topical resiquimod can also improve effector T cell functions in cutaneous T-cell lymphoma and consequently induce regression of the disease [217]. Furthermore, resiquimod has proven to be an effective adjuvant against several diseases as cancer given its ability to induce local activation of immune cells, stimulate the production of pro-inflammatory cytokines and improve the presentation of antigen by dendritic cells leading to the activation of cellular immune responses like CD8 + cytotoxic T lymphocytes [212,218,219]. Resiquimod is also tested as potent agent in glioma [220], acute myeloid leukemia [221,222] and breast cancer [223] studies.



Compounds	R_1	R_2
40	$-CH_2CH(CH_3)_2$	-butyl
41	$-CH_2CH(CH_3)_2$	-pentyl

Fig. 21. Imidazo[4,5-*c*]quinolone derivatives developed by 3M pharmaceuticals.

3.3.3.1.1. Other imidazo[4,5-c]quinoline derivatives. In 2005, Gerster et al. of 3M pharmaceuticals laboratory published an article showing the different synthetic strategies of imidazo[4,5-*c*]quinoline compounds as well as structure-activity relationship studies that influence IFN induction [224]. The presence of an alkyl or hydroxyalkyl chain at R_1 position and no substitution at R_2 show good IFN induction in human PBMCs (Fig. 21). However, the activity is not improved compared to the unsubstituted compound at R_1. When longer alkyl chains (heptyl or more) or bulkier alkyl substituents (*i.e.*, *tert*-butyl) are considered at R_1, the activity is abolished. The activity is also lost when a phenyl group is directly linked to the R_1 position. The substitution by an alkyl group at the R_2



CL097 (**42**)

Fig. 22. Imidazoquinoline developed by Invivogen™.

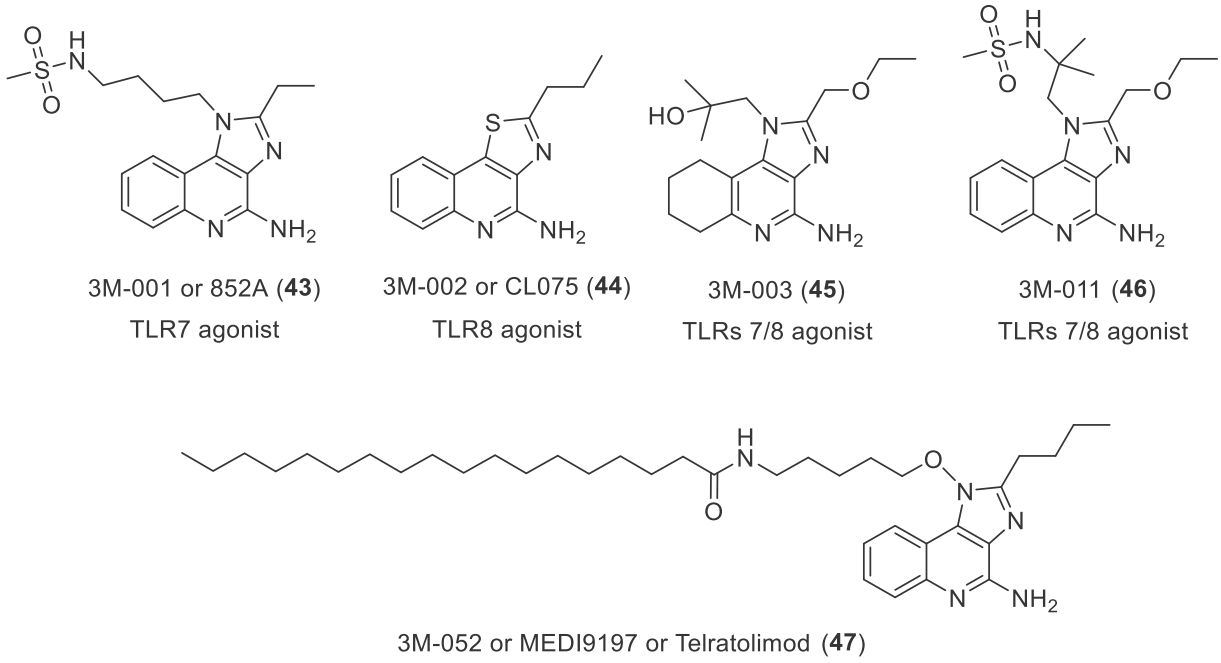


Fig. 23. 3M compounds.

position improves the IFN induction for small chains, butyl bringing the better activity (MEC = 0.01 μg/mL or 0.05 μg/mL for butyl (**40**) or pentyl (**41**) respectively). Phenoxymethyl and benzyl groups at R_2 also increase activity. The amine in C4 position is essential for the activity. All other modifications on this position rule out the IFN production. Overall, a simple substitution on the benzene ring appears to be detrimental since most of the synthesized compounds are not efficient in inducing IFN. However, C7-substituted compounds preserve the activity of the unsubstituted scaffold.

Among TLRs 7/8 ligands commercialized by Invivogen™, **CL097** (**42**) is a water-soluble imidazoquinoline derivative. Similarly to resiquimod, CL097 is TLR7 and TLR8 agonist (Fig. 22). It induces activation of NF-κβ at 0.1 μM in TLR7 transfected HEK293 cells and at 4 μM in TLR8-transfected HEK293 cells [225]. CL097 also induces pro-inflammatory cytokines in macrophages [54].

In 2005, Gorden et al. have studied the selectivity towards TLR7 and TLR8 of compounds belonging to imidazo[4,5-c]quinoline (**3M-001** (**43**) and **3M-003** (**45**)) and thiazolo[4,5-*c*]quinoline (**3M-002** (**44**)) series (Fig. 23) [62]. Compounds **3M-001** and **3M-002** are selective agonists for TLR7 and TLR8 respectively.

Compound **3M-001** (also known as **852A**, 3M-852A, PF-04878691, PF-4878691, S-32865, Fig. 23) was used in preclinical studies for ovarian, cervix and breast cancers [226]. It has undergone clinical trials for melanoma (phase II) [227] and lymphocytic leukemia (phase I/II) [124].

Compound **3M-002** (also known as **CL075,** Fig. 23) has low water solubility and is a highly potent immunostimulant that can be systemically toxic. A polymersome nanocarrier encapsulating CL075 (CL075-PS) mimicked the immunomodulatory effects of live BCG vaccine and improved innate and adaptive neonatal immune responses [228]. The *in vitro* immunostimulatory activities of CL075-PS on human newborn and adult monocyte-derived dendritic cells (MoDCs) were benchmarked against conventional adjuvants and human vaccines, including the live attenuated BCG vaccine, which elicits moderate TH1 immunity in neonates and is safe and effective at birth. Compared with BCG, CL075-PS induced greater production of IL-12p70, a cytokine that enhances TH1-polarized immune responses and promotes cytotoxic T-cell proliferation and survival. Considering all their results, Dowing et al. suggest a strong potential for CL075-PS to serve as a dual antigen/adjuvant vaccine delivery system for human neonatal vaccines.

Compound **3M-003** is an agonist of both TLR7 and TLR8 (Fig. 23).

In the same structural family, **3M-011** (**46**, Fig. 23) is described as a potent TLRs 7/8 agonist. It stimulates type I IFN and other cytokines such as TNF-α, IL-12, and IFN-γ from rat peripheral blood mononuclear cells, induces IL-12 and COX-2 expression in mDC from HIV+ and HIV- individuals, and inhibits H3N2 influenza viral replication in the nasal cavity. It potentiates NK cytotoxicity, and shows antitumor effects in scid/B6 mice and scid/NOD mice. That is why, 3M − 011 is evaluated not only in pancreatic, gastro-intestinal and colon cancers [229,230], but also in bladder and renal cancers [231–233].

3M-052 (**47**, also known as **MEDI9197** or **Telratolimod**, Fig. 23) is an injectable TLRs 7/8 agonist corresponding to a lipid modified imidazoquinoline able to form a tissue depot with gradual, sustained release, allowing local TLR triggering activity without systemic cytokine release to finally modify the tumoral microenvironment. 3M − 052 displays potential immunostimulating and antitumor activities [234,235] Compound 3M − 052 was used as potent agent in melanoma studies [236,237]. Even if 3M − 052 is retained at the sight of injection limiting systemic exposure upon intratumoral administration, it also generated systemic antitumor immunity. The agonist is able to suppress not only both injected and distant uninjected wild-type poorly immunogenic B16–F10 melanomas in mice, but also in the more immunogenic version B16.OVA melanoma. Treated tumors showed increased level of CCL2 chemokines and infiltration of M1 phenotype-shifted macrophages, which could kill tumor cells directly through production of nitric oxide and CCL2. 3M − 052 therapy potentiated checkpoint blockade therapy (with anti-CTLA-4 or anti-PD-L1 antibodies), that was also confirmed by Mullins et al. [238] Zhao et al. showed that combining 3M − 052 with CpG ODN increases cytotoxic T cell activity and Th1 cytokine production while downregulating the activity of immunosuppressing myeloid derived suppressor cells. This combination allows tumor

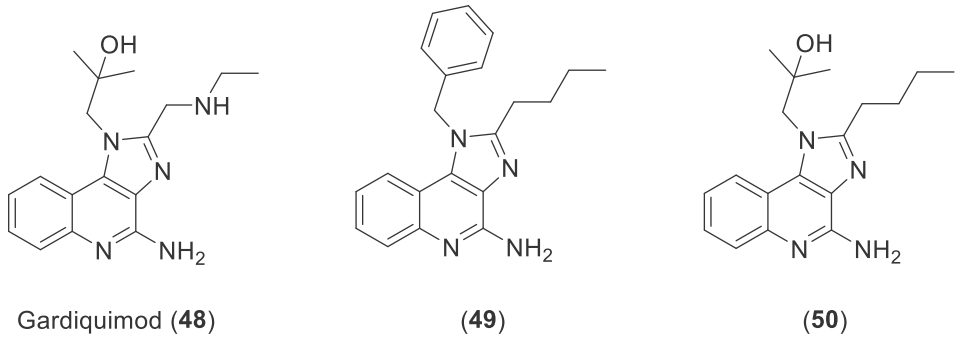


Fig. 24. Gardiquimod and derivatives.

suppression in CT26 colon cancer bearing mice and establishment of long-term immunity [239]. In 2017, Hoeven et al. described **3M-052** formulation development studies [240]. The hydrophobicity of 3M − 052 allows its incorporation in lipid-based formulations (anionic, cationic, PEGylated or neutral liposomes or oil-in-water emulsion), which were able to protect both mice and ferrets from infections caused by lethal H5N1 homologous virus. Furthermore, they demonstrated the ability of **3M-052** adjuvant formulations to broaden responses to H5N1 hemagglutinin-based antigens.

Gardiquimod (**46**, Fig. 24) is an agonist of TLR7 but not of human TLR8, although it activates both porcine TLR7 and TLR8 [241]. It enhances the immunosuppressive activity of T regulatory cells at 0.5−2 μg/mL [242]. Gardiquimod is effective as a mucosal adjuvant for Norwalk virus-like particles and inhibits infection of macrophages and T cells by HIV-1 [243,244]. It inhibits proliferation and migration while inducing apoptosis in pancreatic cancer cells *in vitro* [245]. Guardiquimod was also evaluated on melanoma [246].

It should be noted that gardiquimod is a more active TLR7 agonist than imiquimod. Based on these data, Shukla et al. hypothesized that the C2 ethylaminomethylene substituent of the gardiquimod brings sufficient polarity to prevent its efficient transcutaneous penetration and stimulation of plasmacytoid dendritic cells. Hydrophobic analogues of gardiquimod were later synthesized and their TLR7 and TLR8 agonist activities were studied using HEK293 cells expressing either TLR7 or TLR8 [247]. Compounds with modifications of the secondary amine at the C2 position are not convenient to keep the agonist activity. Replacing the imidazole ring with a triazole ring leads to a total loss of activity. The 4-amino group is absolutely necessary to obtain maximum activity. In a series of synthesized lipophilic compounds carrying a benzyl group in the N1 position and a C2 alkyl chain show a link between the length of the alkyl chain and the TLR7 agonist activity. The optimal activity was observed for compound **47** carrying a C2 butyl chain (Fig. 24) with a minimum effective concentration at which IFN induction was observed of 0.5 μg/mL as suggested by Gerster et al. [224] The rearrangement of the substituents in N1 and C2 of gardiquimod and of compound **47** leads to the identification of the highly active compound **48**, agonist TLR7 with an EC_{50} of 8.6 nM (Fig. 24). These compounds are selective for TLR7 without any agonistic activity for TLR8.

In 2012, Shi et al. synthesized an imidazo[4,5-*c*]quinoline series by replacing hydrogen of the C7 carbon with a methoxycarbonyl group (Fig. 25) [248]. They showed that the elimination of the C2 ethoxymethyl chain of resiquimod results in a significant decrease in the TLR7 agonist activity and a complete loss of the TLR8 agonist activity. The activity is restored and even improved, by the addition of a butyl chain in C2 position (EC50 TLR7 = 0.1 ± 0.02 μM, EC50 TLR8 = 0.1 ± 0.003 μM). The unsubstituted in C2 position and carrying a methoxycarbonyl group in C7 position compound lacked TLRs 7/8 agonist activity, however, it was able to induce a high production of pro-inflammatory cytokines (TNFα, IL12, IL1β). This suggests the existence of an alternative mechanism of action by which imidazoquinolines can stimulate the production of cytokines. The addition of the alkyl (butyl) chain in C2 position restored TLR7 and TLR8 agonist activity. All this indicates that C7 substitution may be important for cytokine production, whereas TLRs 7/8 agonist activity requires C2 substitution.

Based on these data, Schiaffo et al. synthesized imidazo[4,5-*c*] quinolines by introducing substituents in R2 position (Fig. 26). The results show that the TLRs 7/8 agonist activity is correlated with the length of the alkyl chain in C2 position, with maximum TLR 7 activity for derivatives with butyl and TLR8 activity with pentyl. A similar SAR study also identified that the production of IL1β, IL12 and IFNγ depends both on the length of the C2 alkyl chain and on the N1 substitution. The compounds also stimulate the production of IFNα and IL-10, but with less pronounced structural correlations [249].

Larson et al. proposed imidazo[4,5-*c*]quinoline derivatives substituted in N1 position as TLR7 and TLR8 agonists (Fig. 27). While the selectivity of the compounds for TLR8 can be obtained by the introduction of an ethyl, propyl or butylamino group on N1 position [250], analysis of the SAR indicates that TLR7 activity is less sensitive to N1 modification. However, a high TLR7 affinity can be obtained by extending the aminoalkyl chain length to pentyl and p-

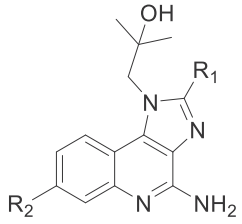


Fig. 25. Core-structure of the imidazo[4,5-*c*]quinoline series [248].

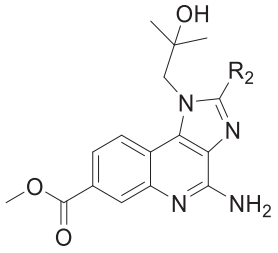


Fig. 26. Core-structure of the imidazo[4,5-*c*]quinoline series [249].

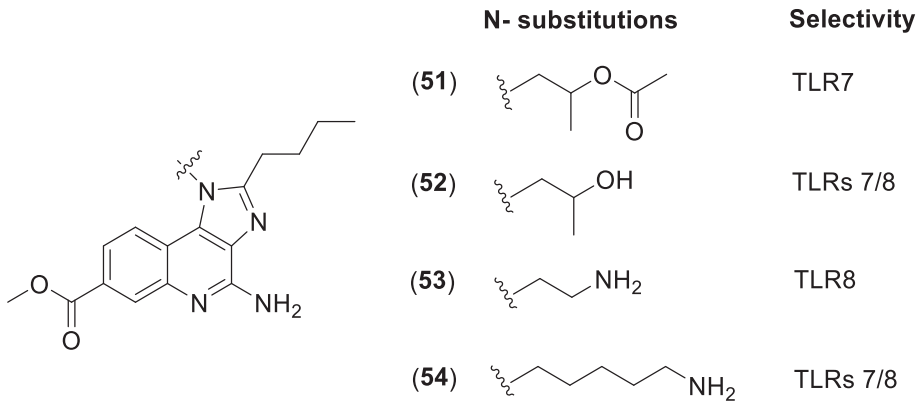


Fig. 27. Imidazo[4,5-*c*]quinoline derivatives substituted in N1 position.

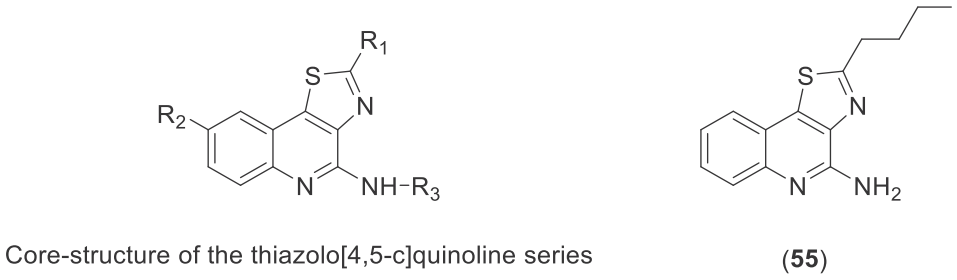


Fig. 28. CL075 analogues.

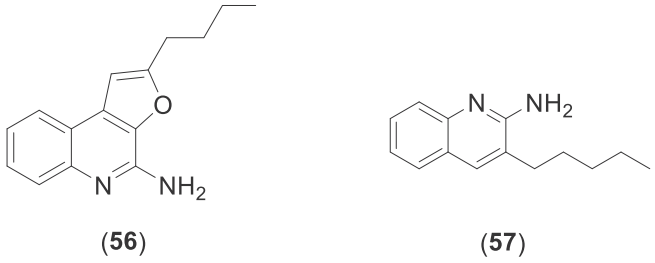


Fig. 29. Quinoline derivatives.

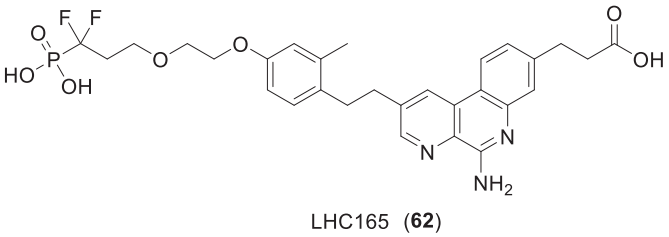


Fig. 31. A TLR7 agonist belonging to Novartis pipeline.

methyl benzyl. Compared to selective TLR7 agonists and mixed TLRs 7/8 agonists, the TLR8 agonist reported better cytokine induction with higher levels of IL1β, IL12 and IFNγ.

3.3.3.2. Other chemical series. Apart from the development of imidazo[4,5-*c*]quinolines and 8-hydroxyadenines, the interest for small agonist molecules of TLR7 and TLR8 has been represented in the last five years by numerous papers focusing on the development of new molecules belonging to different chemical series.

In the perspective of developing new vaccine adjuvants, Kokatla et al. synthesized thiazolo[4,5-*c*]quinolines (Fig. 28), analogous to **CL075** (Fig. 23) [251]. Synthesized alkylthiazoloquinolines with methyl, ethyl, propyl (**CL075**) and butyl groups in the R1 position have comparable TLR8 agonist activities. The activity moderately decreases with the pentyl-bearing compound at R1 position and is completely lost for the compounds with a longer alkyl chain.

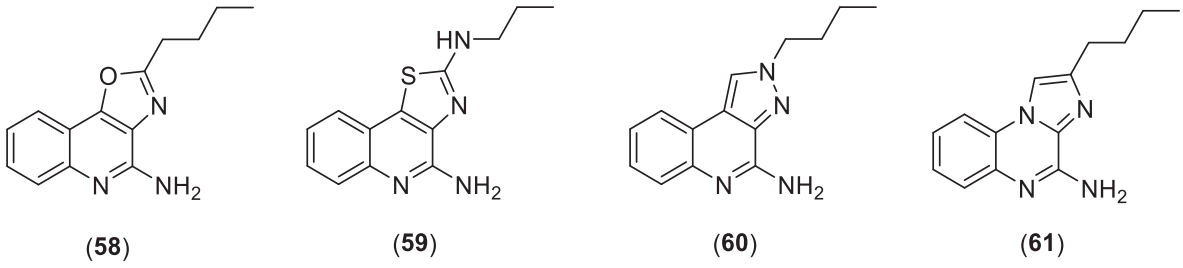


Fig. 30. Quinoline and quinoxaline derivatives.

Virtually all changes in the R2 position led to loss of agonist activity. The alkylation of the amine at position R3 is not tolerated. The compound **55** with a butyl in R1 was also endowed with TLR7 agonist activity. Immunization in rabbits with an antigen adjuvated with compound **55** showed significant improvements in antigen-specific antibody titers. Given the pledge of TLR8 agonists in the production of pro-inflammatory cytokines including TNFα and IL12 and activation of a Th1 response in neonatal antigen presenting cells, researchers focused on the synthesis of selective TLR8 agonists for use as vaccine adjuvants, in particular to improve the immune responses in newborns.

Furo[2,3-*c*]quinolines, as well as alkylated aminoquinolines, were synthesized by Kokatla et al. to evaluate their TLR8 agonist activities (Fig. 29) [252,253]. 2-butylfuro[2,3-*c*]quinoline (**54**) and 3-pentyl-quinolin-2-amine (**55**) showed TLR8 selectivity with EC50 of 1.6 and 0.2 μM respectively. Interactions of compound **54** with the ectodomain of human TLR8 was evaluated by docking studies.

Yoo et al. analyzed the electronic effects of heterocyclic modifications while maintaining the butyl at C2 position since most of the active compounds carry this substitution [134]. They synthesized 13 different tricyclic structures including thiazolo/oxazolo [4,5-*c*]quinolin-2-amines, thiazolo/oxazolo[5,4-*c*]quinolines, imidazo[1,2-*c*]quinazolines [1,2,4],triazolo[1,5-*c*]quinazolines, imidazo [1,2-*a*] quinoxalines [1,2,4],triazolo[1,5-*a*]quinoxalines [1,2,4],-triazolo[4,3-*a*]quinoxalines, and pyrazolo[3,4-*c*]quinolines. Among all of these compounds, oxazolo[4,5-*c*]quinolin-2-amine (**58**), thiazolo [4,5-*c*]quinoline (**59**) and pyrazolo[3,4-*c*]quinoline (**60**) showed TLR7 agonist activities with EC_{50} of 0.55, 0.73 and 0.19 μM respectively, as well as TLR8 agonist activities with respective EC_{50} of 0.18, 3.94 and 0.056 μM (Fig. 30). Imidazo[1,2-*a*]quinoxaline (**61**) showed a selectivity towards TLR8 with an EC_{50} of 7.99 μM. This activity was confirmed by a study of the secretion of cytokines by human PBMCs. Compounds **58**, **59** and **60** showed an ability to produce IFNα, whereas compound **61**, a TLR8 agonist, was not able to produce it. In addition, these compounds have been able to produce pro-inflammatory cytokines (TNFα, IL6, IL1β, …), type II interferon (IFNγ) as well as chemokines. Docking studies were performed with the most active compound **60** and the ectodomain of human TLR8.

LHC165 (**62**) is a TLR7 agonist belonging to the Novartis global pipeline and defined as an antineoplastic agent (Fig. 31). In mice, LHC-165 induced immune response, reduced tumor growth, and showed signs of an abscopal effect [254]. It is currently under evaluations in patients with advanced malignancies, either as a single agent or in combination with checkpoint inhibitor [255].

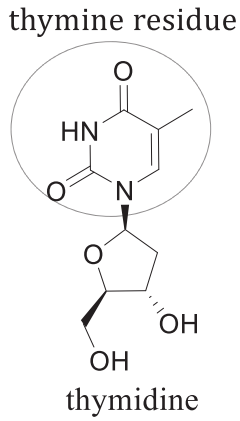


Fig. 32. Thymidine nucleoside.

3.3.4. Macromolecules

3.3.4.1. Thymidine (dT) derivatives. **Poly(dT)** is a thymidine 17-mer homopolymer phosphorothioate ODN commercialized by Invivogen™ and described as a modulator of human TLRs 7/8 (Fig. 32). Poly(dT) alone has no significant effect on TLRs 7/8. Nevertheless, in combination with imidazoquinolines such as resiquimod or CL075, poly(dT) increases TLR8 mediated signaling and abolishes TLR7 mediated signaling [256,257]. Moreover, co-incubation of poly(dT) and an imidazoquinoline induces NF-κβ activation in HEK293 cells transfected with murine TLR8-and primary TLR8-expressing mousse cells [258].

3.3.4.2. RNA-based agonists. **MCT465,** developed by MultiCell Technologies Inc., is a high molecular weight synthetic double-stranded RNA (dsRNA) designed to activate the innate immune system through TLRs 3/7/8 signaling. with immune enhancing properties. In preclinical studies, MCT-465 successfully reduced pulmonary influenza virus levels 1,000-fold in animal models. It also demonstrated effectiveness in reducing virus levels of HIV and HCV in animal models. MCT465 is currently ongoing preclinical trials for liver cancer (hepatocellular carcinoma), breast cancer (triple-negative breast cancer TNBC), and others [259]. According to the company, MCT-465 could be indicated as cancer adjuvant therapy alone or combined with currently available or novel therapies. MCT-465 can also be combined with MCT-475, a patent pending antigen-presenting immunoglobulin therapeutic, for the treatment of various cancers such as breast and metastatic colorectal carcinoma.

CV8102 is a ssRNA-based TLRs 7/8 and retinoic acid-inducible gene I (RIG-I) agonist containing (non-coding and uncapped) several polyU-repeats complexed with a polymeric carrier formed by arginine-rich disulfide-crosslinked cationic peptides. It was developed by CureVac company as adjuvant to enhance immunogenicity of poorly immunogenic antigens. Upon intratumoral injection, CV8102 stimulates a Th1 response and activates a systemic cytotoxic T-lymphocyte-mediated immune response against the tumor cells when simultaneously exposed to tumor-associated antigens. CV8102 is actually under clinical investigations for oncological indications [260–262].

3.3.5. Structurally-unpublished potent agonists

The researches and developments of agonist ligands are clearly booming. Certain referenced products display interesting activities without detailed chemical structure. Indications on their belonging to chemical families are sometimes given (Table 1).

4. Antagonist ligands

Beside their role as defensive tools of the immune system, over-activation or dysregulation of the TLR signaling are causing a panel of diseases. TLRs 7 and 8 are today well known for their implication in the development of autoimmune diseases, cancers and in the progression of the acquired immunodeficiency syndrome (AIDS). Countering uncontrolled TLR-mediated signaling has been a major subject of research in the last decades by designing TLR agonist ligands and to a much lesser extent TLR antagonist ligands. TLR antagonists are regulators that inhibit or reduce activation of TLR-mediated cytokine cascades and check over-reactive uncontrolled adaptive immune responses. TLR antagonists are generally modified agonists that bind TLRs but fail to induce the signal transduction [69,283]. Recently highlighting the existence of antagonist pockets on TLRs 7/8 will also allow specific structure-based design using molecular modeling tools to produce new potent structures [9,284]. They represent novel therapies to treat or prevent diseases cited herein [285].

Table 1
Potent agonists.

Ligands	Chemical family	Agonist	Biological evaluations	References
S28690	Imidazoquinoline derivative	TLR7	Immunogeneicity effect on chronic lymphocytic leukemia	[263–266]
SB 9922	Nucleic acid hybrid	TLR7	Potent vaccine adjuvant used in combination with BCG vaccine against *Mycobacterium tuberculosis*	[267,268]
RO6870868 and **RO6864018** (ANA773 or RG7795)	Tosylate prodrug of isatoribine	TLR7	Treatment for hepatitis C and cancers	[269–271]
RO7020531		TLRs 7/8	Cure chronic HBV infection	[272]
AZD8848 (DSP-3025)	–	TLR7	Long-term remission in allergic disorders such as bronchial asthma and allergic rhinitis	[273]
DSP-0509		TLR7	Potent immunostimulatory and antineoplastic activities	[274,275]
BNT411	–	TLR7	Combination with chemotherapy and checkpoint inhibitors in preclinical development	[276]
JNJ-4964 (AL-034 or TQ-A3334)	–	TLR7	Clinical development for the treatment of chronic hepatitis B (CHB)	[277–280]
NKTR-262		TLRs 7/8	Induction of antigen-specific immunity and tumor regression effects	[281,282]

4.1. Therapeutic purposes

4.1.1. Implications in autoimmune diseases

Scientific literature show that in autoimmune diseases, such as lupus and psoriasis, TLRs 7 and 8 recognize endogenous immune complexes (IC) containing nucleic acids, and induce pro-inflammatory cytokines contributing to the progression of the disease [273,286].

TLRs 7 and 8 activation by ICs leads to IL12, IL6, TNFα, IL1β and IFNα expression and is associated with the presence of anti-DNA and anti-RNA autoantibodies in patients with systemic lupus erythematosus (SLE) [287,288]. These autoantibodies cause organ damage by binding directly to host tissues or forming IC that deposit in vascular tissue, thereby inducing destructive inflammation [289]. Several studies using TLR-deficient mice have investigated the role of TLRs 7, 8 and 9 in the development of SLE in mice prone to lupus. The deletion of TLR7 in mice prone to lupus led to failure in antibody production, decreased lymphocyte activation, decreased serum IgG levels and attenuation of the disease [290].

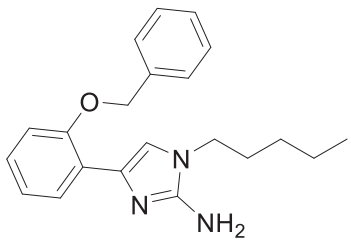
The role of TLR8 in autoimmune diseases has not been extensively studied, perhaps because of a lack of response to TLR8 ligands in murine models. There is a difference in the response immune profile after TLR 7 or TLR8 stimulation [37]. TLR7 responses are characterized by a high production of type I IFNs such as IFNα, which appears to play a major role in autoimmune diseases, while TLR8 stimulates very low levels of IFNα but induces a strong production of other pro-inflammatory cytokines [62,256,291]. However, TNFα produced during TLR8 activation may exert pro-inflammatory effects in autoimmune diseases [292,293]. TLR8 being stimulated by ribonucleoproteins [294] and TLR8 mRNA over-regulated in patients with Sjögren's syndrome [295] indicates a possible role of TLR8 in autoimmune processes. In psoriasis, a chronic autoimmune inflammatory disease of the skin, extracellular RNA forms complexes with the cationic antimicrobial peptide LL37. These complexes are highly protected against degradation of RNAs and have access to the endosomal compartments of pDCs and mDCs. The RNA-LL37 complexes induce the activation of TLR7 in PDCs and trigger IFNα secretion. RNA-LL37 complexes also trigger direct activation of mDCs to secrete TNFα and IL6 and differentiate into mature dendritic cells. This maturation of MDCs is triggered by endosomal TLR8 and is enhanced by the concomitant activation of pDCs to produce IFNα. These data identify RNA-LL37 complexes as endogenous triggers of TLR7 and TLR8 in human dendritic cells thus leading to the development of psoriasis [296].

4.1.2. Implications in cancers

In contrast to the therapeutic benefits of TLRs 7/8 agonists on immune cells, several studies have shown that TLRs 7/8 stimulation promote tumor progression. In 2010, Cherfils-Vicini et al. have shown that TLR7 and TLR8 are highly expressed on primary tumor cells of patients with non-small cell lung cancer (NSCLC). Additionally to the chemoresistance in patients with NSCLC, TLR7 agonist induce an important protumurogenic effect *in vitro* [297]. These effects were also verified in immunodeficient (NOD/SCID) and immunocompetent (C57BL/6) murine models, using subcutaneously grafted lung carcinoma cells [298]. In both models, the repeated administration of TLR7 agonists, such as CL264, loxoribine or imiquimod resulted in increased tumor volume. This protumorigenic effect could be mediated by direct stimulation of tumor cells expressing TLR7 or by increased recruitment and differentiation of immunosuppressive cells into the tumor microenvironment. Furthermore, research teams find similar results in various tumor models. Stimulation of TLR7 in a pancreatic cancer model expressing TLR7 induces tumor growth acceleration and reduction in antitumor molecules expression [299,300]. Similarly, TLR7 stimulation in hepatocellular carcinoma model expressing TLR7 boost malignant cells proliferation [301].

4.1.3. TLR7 implication in AIDS

Chronic immune activation is a leading cause of progressive immunodeficiency of human immunodeficiency virus type 1 (HIV-1) infection. HIV ssRNA is a potent immune activator that triggers TLR7-mediated signaling. The HIV virus itself directly contributes to the activation of the immune system and its dysfunction by stimulating TLR7 [302]. Recent studies have shown that activation of TLR7 in human $CD4^+$ T cells triggers an anergic state that may contribute to $CD4^+$ T cell hyporeactivity after infection with HIV-1



(**63**)

Fig. 33. Benzimidazole moiety containing agonist.

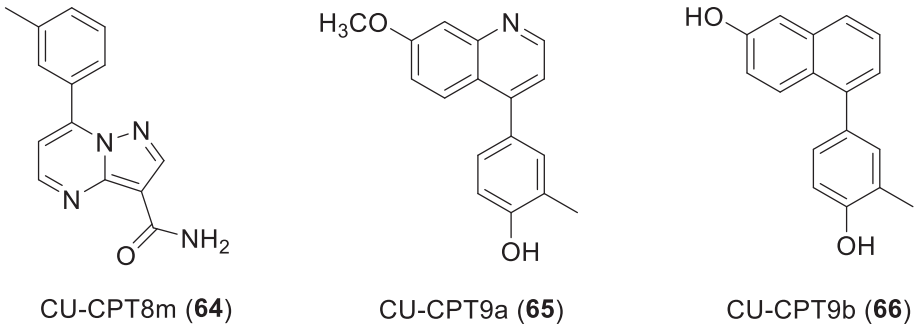


Fig. 34. CU-CPT derivatives.

and may also increase the spread of this virus. This unexpected role of TLR7, whose activation facilitates infection of $CD4^+$ T cells by HIV-1, was blocked using a TLR7 inhibitor. The presence of this inhibitor has led to an attenuation of the infection. The authors concluded that HIV-1 use the anergic state induced by TLR7 activation to support its own spread [303].

The inhibition of TLRs 7 and 8 activation by synthetic compounds is therefore a promising research area for potential treatment of autoimmune diseases, cancers and AIDS. TLRs 7/8 antagonists bind to the receptor and inhibit its activation, thereby suppressing the inherent immune response. Till 2020, antagonist molecules are compounds developed generally as structural analogues to TLRs 7/8 agonists. As in the case of TLR7 and TLR8 agonists, currently known antagonists can be divided into two groups (oligonucleotides and bi- and tricyclic heterocyclic compounds) that we discus below.

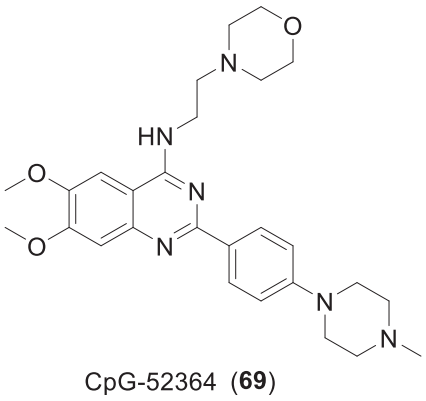


Fig. 36. Quinazoline derivative.

4.2. Synthetic ligands

4.2.1. Imidazole derivatives

In their search for TLR8 agonists containing a benzimidazole moiety, Beesu et al. have prepared an inactive agonist (**63**, Fig. 33) that competitively inhibited TLR8, while inhibited TLRs 2, 3, 4, 5, 7, and 9 by an unknown mechanism [96]. The micromolar affinity of this pan-TLR antagonist toward multiple TLRs in various cell lines suggests that it could be further a promising anti-inflammatory agent. A kinase-screening of this compound showed relative specificity for calmodulin kinases.

4.2.2. Bicycles analogues

Recently, Zhang et al. identified, for the first time, human TLR8 small antagonist molecules with a novel inhibition mechanism [304]. SAR studies on the pyrazolo[1,5-*a*]pyrimidine series led to identification of the compound: **CU-CPT8m** (**64**), which proved to be the most active with an IC50 of 67 nM and a strong ability to inhibit the production of pro-inflammatory cytokines (Fig. 34). Docking studies reveal that CU-CPT8m is sandwiched between the two monomers of TLR8 and is localized in a hydrophobic pocket at the protein-protein interface of the two monomers TLR8. This new binding site is close, but distinct from the identified agonist site, whose occupation prevents the activation of TLR8. This implies a new inhibitory mechanism by CU-CPT8m.

After these encouraging results, the authors carried out a structural optimization which led to two new TLR8 inhibitors belonging to the quinolines series structurally similar to CU-CPT8m: **CU-CPT9a** (**65**, IC50 = 0.5 nM) and **CU-CPT9b** (**66**, IC50 = 0.7 nM) (Fig. 34). By blocking the newly identified site, these TLR8 inhibitors appear to antagonize the binding of TLR8 agonists such as R848 and uridine, and/or block a conformational change necessary for the receptor activation. In addition, authors explored the effects of **CU-CPT8m** and **CU-CPT9a** in human samples from various patients with inflammatory and autoimmune diseases. The study showed that treatment with **CU-CPT9a** has potent anti-inflammatory effects in the samples of patients with osteoarthritis (OA), rheumatoid arthritis (RA), and adult Still's disease, thus

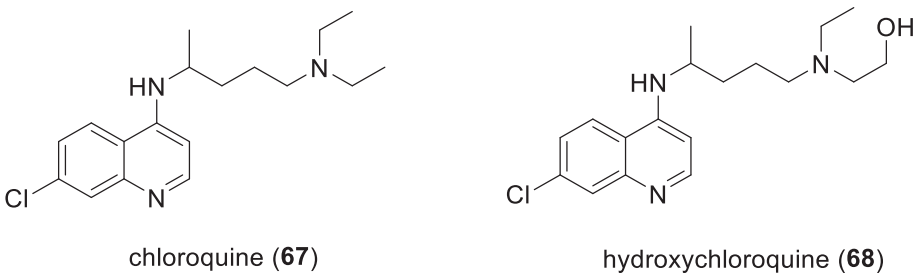
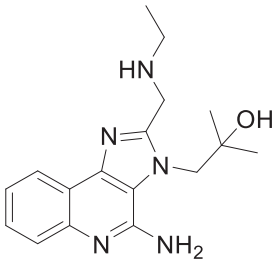
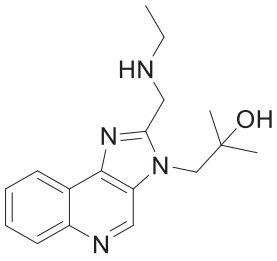


Fig. 35. Worldwide-known antimalarial drugs.

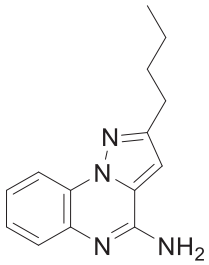


(70)

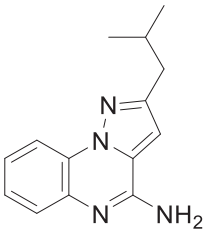


(71)

Fig. 37. Quinoline compounds.

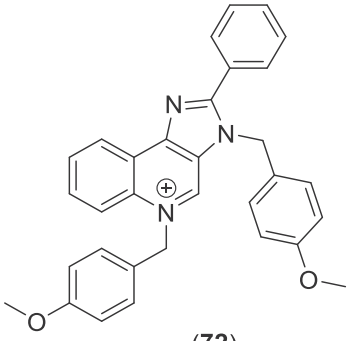


(76)

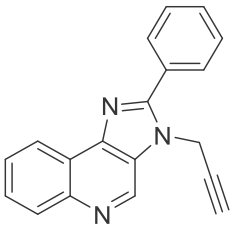


(77)

Fig. 40. Pyrazolo[1,5-*a*]quinoxaline derivatives.



(72)



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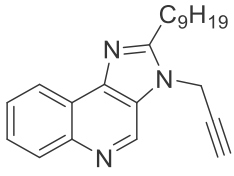


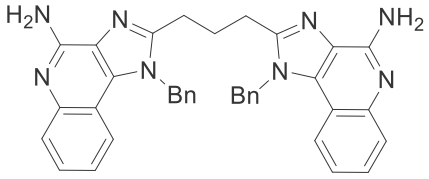
(74)

Fig. 38. Quinoline and quinolinium derivatives.

confirming previously published work showing that TLR8 may play a role in inflammatory disorders and autoimmune diseases.

Known as antimalarial drugs **chloroquine** (**67**, CQ) and **hydroxychloroquine** (**68**, HCQ) (Fig. 35), have been used to treat autoimmune diseases (arthritis and SLE) after World War II [305,306]. These weak bases tend to accumulate in the acidic intracellular compartments of endosomes and lysosomes and modulate their pH leading to suppression of autoantigen presentation, blockade of endosomal TLR signaling, and decrease in cytokine production [307,308]. Other mechanisms include inhibition of MAPK signaling and phospholipase A2, antiproliferation, photoprotection as well as reduction of matrix metalloproteinase-9 activities [309–312]. All these mechanisms of action highlight the anti-inflammatory and immuno-suppressive activity of this antimalarial drugs by acting on endosomal TLR signaling (TLRs 7/8/9). Moreover, CQ was under investigation for diseases associated with uncontrolled acute or chronic inflammation (severe sepsis [313]), to prevent and treat viral infections (HIV, influenza, and dengue) [314,315], cardiovascular diseases [316] or cancer by notably modulating autophagy [317]. Long term HCQ administration ameliorate hypertension and aortic endothelial dysfunction in a SLE mouse model [318].

The quinazoline derivative **CpG-52364** (**69**) from Coley Pharmaceutical Group Inc. (Fig. 36), a TLRs 7/8/9 antagonist (TLRs 7/8 ratio = 0.8), inhibits disease progression of SLE, rheumatoid arthritis and other autoimmune diseases in animal models [319–321]. It also increases potency compared to HCQ alone. In combined therapy, it added efficacy by preventing the formation of antiDNA antibodies in SLE-prone mice. CpG-52364 is evaluated in a phase I clinical trial for SLE therapy (NCT00547014).



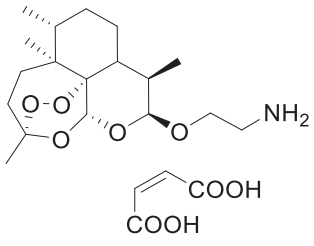
(75)

Fig. 39. Dimer compound.

4.2.3. Imidazoquinoxaline derivatives

In 2009, Shukla et al. synthesized **gardiquimod** (**46**, TLR7 agonist) and its 3H regioisomer **70** in order to explore the SAR of substituents on the imidazoline ring (Fig. 37). Compound **70** revealed to be completely inactive as a TLR7 agonist, having surprisingly weak antagonistic effect. A *des*-amino precursor of the 3H-regioisomer **71** was more potent as TLR7 antagonist, with an IC50 of 7.5 μM [322].

Among the synthesized compounds [322], the **72** quinolinium bearing *p*-methoxybenzyl substituents at the N3 and N5 positions was identified as active antagonist of TLR7 and TLR8 with IC_{50} of



(78)

Fig. 41. Artemisinin analogue.

2.79 μM and 4.55 μM, respectively (Fig. 38). Compounds **73** and **74** carrying the propargyl group on N3, have also shown a good TLRs 7/8 antagonist activity (10 μM range) and they may prove to be useful supports for additional alkylation in the N5 position with electron-rich substituents as for compound **72**.

Dimers linked to C2, C4, C8 and N1-aryl positions were also evaluated [323]. C4, C8 and N1-aryl linked dimers were TLR7 agonists. Only N1-aryl dimer with a spacer of 12 carbon atoms was a double agonist of TLR7 and TLR8. C2 dimer with a propylene spacer (**75**) was a maximal antagonist of both TLR7 and TLR8 with IC50 of 3.1 and 3.2 μM respectively (Fig. 39). This compound also showed inhibition of pro-inflammatory cytokine production in human PBMCs.

Recently, Deleuze-Masquéfa et al. synthesized and characterized a variety of compounds belonging to three heterocyclic chemical series: imidazo[1,2-*a*]pyrazine, imidazo[1,5-*a*]quinoxaline, and pyrazolo[1,5-*a*]quinoxaline [9]. These compounds have been tested for their TLR7 or TLR8 agonistic and antagonistic activities. Several of them are shown to be selective TLR7 antagonists without any TLR7 or TLR8 agonistic activity. The selectivity was confirmed by a comparative ligand-docking study suggesting a specific binding mode in a novel TLR7 antagonist site. Two compounds of the pyrazolo[1,5-*a*]quinoxaline series (**76** and **77**, IC_{50} = 8.2 and 10.0 μM respectively, Fig. 40) are potent selective TLR7 antagonists and may be considered as promising starting points for further rational development of low molecular TLRs 7/8 ligands.

4.2.4. Other chemical series

SM934 (**78**), a TLRs 7/9 antagonist and a water-soluble analog of artemisinin, is used as antimalarial drug (Fig. 41). SM934 possesses better bioavailability and better immunosuppressive activity than traditional arteminisin derivatives, and displays antiproliferative and anti-inflammatory properties [324,325]. *In vitro*, SM934 inhibited IFNγ and IL-17 production from polyclonal $CD4^+$ T cells activated by T cell receptor engagement and the differentiation of naive $CD4^+$ T cells into Th1 and Th17 cells, but not Treg cells. *Ex vivo*, SM934 treatment elevated the percentage of Treg cells, impeded the comprehensive activation of STAT-1, STAT-3, and STAT-5 proteins in splenocytes, suppressed the TLR-triggered activation and proliferation of B cells, as well as antibody secretion. SM934 interfered with the B-cell intrinsic pathway by downregulating TLRs 7/9 mRNA expression, MyD88 protein expression and NF-κβ phosphorylation. Studies have shown the therapeutic effects of the orally administered SM934 on lupus-prone MRL/lpr mice by inhibiting Th1 and Th17 cell responses suppressing the B cell activation and plasma cell production, ameliorating proteinurea and renal lesion severity. SM934 exhibited protective effects in two mouse models of SLE, MRL/lpr and NZB/W F1 mice by suppressing pathogenic T-cell development [326–328]. On a murine model of Multiple Sclerosis (EAE), SM934 is able to stop the progression and to reverse the clinical and histopathological signs, *via* modulating the balance of T cell subsets, especially by promoting Treg cell generation [329]. On the collagen-induced arthritis in DBA/1 mice, SM934 ameliorated the severity of arthritis by inhibiting development of Tfh and Th17 cells as well as autoantibodies production. SM934 could prohibit the IL-21-mediated signaling pathway by preventing the activation of STAT3 [330].

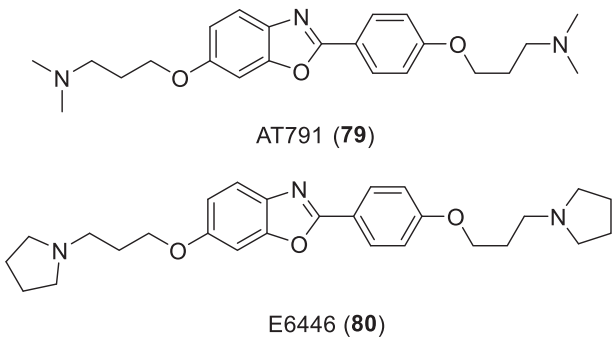


Fig. 42. Antagonist compounds interacting with nucleic acids.

4.2.5. Indirect targets

Targeting different domains of a TLR could block its activation. This inhibition could also be done indirectly by targeting effectors in relationship with these TLRs. This part will not be explicit due to its remoteness with our main researches.

4.2.5.1. Nucleic acids. Lamphier et al. found two TLR7 antagonist small compounds, **AT791** (**79**, Fig. 42) and **E6446** (**80**, Fig. 42), able to interact with nucleic acid (DNA or RNA) agonists in acidic intracellular compartments. These orally bioavailable compounds can inhibit short-term induction of inflammatory cytokines by DNA. In a mouse MRL/lpr spontaneous model of lupus, E6446 slowed the development of circulating antinuclear antibodies and modestly suppressed anti-dsDNA with no observable impact on proteinuria or mortality. E6446 is effective in preventing hyper-inflammation and lethality caused by the parasite *Plasmodium berghei* in a mouse model of cerebral malaria [331]. AT791 is evaluated on SLE or autoimmune disease [332].

4.2.5.2. D helix. Piao and *al.* found that **2R9 peptide** (PQRFCKLRKIMNT sequence) derived from helix D of the TIR domain of TLR2 specifically targets TIRAP and blocks TLRs 2/4/7/9, but not TLR3 and TNF-α signaling. As confirmed by *in vitro* studies, cell imaging and co-immunoprecipitation, 2R9 binds TIRAP with nanomolar affinity as a result of a fast association rate coupled with slow dissociation of the complex. 2R9 binds albumin with micromolar affinity. 2R9 diminished systemic cytokine responses elicited *in vivo* by synthetic TLR2 and TLR7 agonists. It inhibited the activation of macrophages infected with influenza strain A/PR/8/34 (PR8) and significantly improved the survival of PR8-infected mice. Thus, 2R9 represents a TLR-targeting agent that blocks protein interactions downstream of activated TLRs [333].

4.3. Macromolecules

4.3.1. Modified nucleic acids

In 2006, Judge et al. have shown that the incorporation of 2′-O-methyl (2′OMe)-uridine or 2′OMe-guanosine residues in siRNA or in ssRNA eliminates their immunostimulatory activity both *in vitro* and *in vivo* [334]. It has also been reported that chemical modifications of nucleotides in mammalian RNA, including 2′OMe modifications, prevents them from activating dendritic cells [335]. In 2007, Robbins et al. tested the ability of 2′OMe RNA to antagonize TLR7 immune stimulation [336]. They reported that 2′OMe RNA directly inhibits cytokines induction in murine and human systems by immunostimulatory RNA and by a TLR7 agonist Loxoribine. The inhibitory activity of 2′OMe RNA has also been shown *in vivo*. These results indicate that 2′OMe RNA acts as a TLR7 antagonist.

Anti-miRNA target TLRs 7/8 binding off viral and bacterial infections [337].

4.3.2. Oligonucleotides

Oligodeoxynucleotides (ODNs) containing unmethylated CpG dinucleotides act as TLR9 agonists and induce Th1 type immune responses [338]. On the other hand, the substitution of cytosine in

the CpG motif by 2′-O-methyl-cytosine, 2′-O-methyl-5-methyl-cytosine or 5-methyl-deoxycytosine or the substitution of guanosine by 2′-O-methyl-guanosine leads to loss of immunostimulatory activity; these oligonucleotides therefore appear to be TLR7 and TLR9 antagonists [339].

Dynavax Technologies has designed immunoregulatory oligonucleotides (IROs) having 2′-O-methyl or methoxyethyl sugar modifications as inhibitors of TLR7 and/or TLR9 [340]. Further, Dynavax Technologies has developed IROs with unique inhibitory sequences for TLR7, 8, and 9 that suppress autoimmune and inflammatory diseases [341]. The novel approach can be applied to treat a variety of autoimmune diseases, such as SLE or cutaneous lupus erythematosus (CLE), systemic sclerosis, polymyositis, dermatomyositis, RA, and Sjorgren's syndrome.

Based on this work, several recent studies have focused on the synthesis of oligonucleotides using the chemical modifications mentioned above [342–344]. Idera Pharmaceuticals has designed unique CpG oligonucleotide sequences containing an immune stimulatory motif with a 7-deaza-dG or arabino-G modification and an immune regulatory motif with 2′-O-methylribonucleotides that act as antagonists of TLRs 7, 8, and 9 [345]. IROs have shown promising results in preclinical studies and effectively prevented hyperactivation of these TLRs in response to agonists. They are foreseen as future therapies for TLRs 7, 8, and 9-mediated inflammatory disorders, including various autoimmune and inflammatory diseases and cancers initiated by pathogens.

Chemical modifications and/or internucleotide linkages introduced into the IROs were found to increase inhibition or suppression of TLRs 7, 8, and 9. Their leader, **IMO-8400** (also known as Bazlitoran, sequence: CTATCTNNNNTTCTCTNN), is an oligonucleotide antagonist of TLR7, TLR8 and TLR9, currently in clinical development (phase IIa) for the treatment of plaque psoriasis (NCT01899729) and dermatomyositis (NCT02612857) [346]. Treatment with this antagonist regulates IL-23-induced gene expression as demonstrated by Suarez-Farinas et al. [347] This compound was very effective in preventing inflammation and disease development in both lupus and psoriasis mouse models [348,349].

Similar to IMO-8400, **IMO-9200**, a TLRs 7/8/9 antagonist, is evaluated on auto-immune diseases. From a phase I clinical trial, it showed a safe and well-tolerated profile in healthy subjects. IMO-9200 was evaluated as potent treatment against inflammatory bowel disease [350].

IMO-3100, dual antagonist of TLR7 and TLR9, is being tested for the treatment of inflammatory and autoimmune disease, such as SLE, rheumatoid arthritis, multiple sclerosis, psoriasis, lupus and colitis. Evaluation of the effect of IMO-3100 on gene expression in human peripheral blood monocytes showed that the drug could significantly reduce the expression of inflammatory genes, such as IL-17A, β-defensin, CXCL1, and keratin 16, inhibited TLR ligand-induced expression of TNF-α and IFNα [286,347,351].

IMO-4200, a dual agonist of TLR7 and TLR8, is a synthetic RNA-based dual agonist of TLR7 and TLR8, identified as a lead drug candidate for the treatment of hematological malignancies. IMO-4200 stimulates immune responses mediated through TLR7 and TLR8, which are expressed in human dendritic cells, B-cells, monocytes, and macrophages. In preclinical mouse models of cancer, IMO-4200 has shown anticancer activity involving both innate and adaptive immune responses. IMO-4200, when administered in combination with approved cancer therapy drugs, rituximab (an anti-CD20 antibody) or bortezomib (a proteasome inhibitor), showed significantly increased antitumor activities compared to the single-agent effects in several preclinical lymphoma models and increased survival compared to treatment with either agent alone. Notably with rituximab, IMO-4200 is able to increase activation of natural killer cells, indicating an improved immune response and to enhance clearance of circulating tumor cells compared to treatment with agent alone [352,353].

Based on the inhibitory oligodeoxynucleotide 2088 (INH-ODN 2088), **INH-ODN-24888** was designed (T*C*C*T*G*G*C*mE*G*G*G*A*A*G*T sequence, * = phosphorothioate-binding) as a guanine-modified inhibitory oligonucleotide TLRs 3/7/9 antagonist. This compound demonstrated a promising therapeutic effect in SLE through the suppression of both TLR7 and TLR9, potentiated the inhibitory activity and efficiently improved its capacity to reduce TLR7/9-mediated immune signaling in human immune cells [354,355]. Among their derivatives, Römmler et al. designed diverse antagonists as described below (Table 2).

Several oligonucleotides antagonists of TLRs 7 and 9 have been developed such as immunoregulatory sequences (IRS) that are short DNA sequences. The most promising compound is **IRS 954** (5′-TGCTCCTGGAGGGGTTGT-3′ sequence, also known as **DV-1079**), which was developed for the treatment of SLE [356]. This compound was found to block IFN-α production by human plasmacytoid predendritic cells. IRS-954 treatment showed a reduction of serum levels of nucleic-acid specific autoantibodies and improvement of disease symptoms in lupus-prone mice (decrease of proteinurea, glomerulonephritis and end-organ damage) resulting in an increasing survival [357]. IRS-954 could also reverse TLR7/9-mediated glucocorticoid resistance of SLE, and thus could be potentially used as corticosteroid-sparing drug [358]. Administration of the drug to HIV-stimulated peripheral blood monocyte cells also led to a decrease in IFNα production, suggesting a potential therapeutic opportunity for treating HIV infection [359].

IRS 661 (5′-TGCTTGCAAGCTTGCAAGCA-3′ sequence) is a specific TLR7 antagonist evaluated by Pawar et al. on a lupus mouse model [356,360]. Studies on autoimmune diabetes were also conducted [361,362].

DV-1179, another TLRs 7/9 dual antagonist, entered a phase Ib/IIa study for its safety in both healthy volunteers and patients with active SLE. Although DV-1179 administration was well tolerated, it failed to achieve the endpoints of reducing the IFNα-regulated genes. Such an unfortunate outcome ended the clinical development of this compound ever since [363].

5. Clinical trials

Herein, we gather all the clinical trials where TLRs 7/8 are mentioned and in which studies relate to the biological activities of the named compound (last update on January 2020, Table 3) [69,364–368]. Among the three FDA or EMA-approved TLR agonists, only one of them targets a TLR which is discussed in this review: the TLR7 agonist imiquimod. Regarding others, BCG is agonist of TLRs 2/3/4 and possibly 9, and monophosphoryl lipid A (MPL) is agonist of TLR 4. Indeed, despite efforts from researchers,

Table 2
INH-ODN sequences.

INH-ODN	Sequences	TLRs inhibitory activity
2088	T*C*C*T*G*G*C*G*G*G*A*A*G*T	3, 7, 9
21595	T*C*C*T*G*G*C*E*G*G*G*A*A*G*T	7, 9
20844	T*C*C*T*G*G*C*G*E*G*G*A*A*G*T	7, 9
24888	T*C*C*T*G*G*C*mE*G*G*G*A*A*G*T	3, 7, 9
24991	C*C*T*G*G*C*mE*G*G*G	3, 7, 9
105870	T*A*A*T*G*G*C*E*G*G*G*A*A*G*T	7
105871	T*A*A*T*G*G*C*mE*G*G*G*A*A*G*T	3, 7

* = phosphorothioate-binding, E = 7-deaza-2′-deoxyguanosine, mE = 7-deaza-2′-O-methyl-guanosine.

Table 3
Clinical trials involving TLRs 7/8 agonists and antagonists.

Ligands (synonyms or explanations given once)	In combination with	TLRs	Investigators	Pathologies Applications	Type[a]	Phase	Clinical Trial Identification	Status[b]
Agonists								
852A PF-04878691 S-32865 3M852A		7	3M Pharmaceuticals	Inoperable melanoma (Unresectable metastatic cutaneous melanoma)	D	II	EudraCT2004-003910-41	C
852A		7	Masonic Cancer Center University of Minnesota, Pfizer	Breast, ovarian, endometrial, and cervical cancer	D	II	NCT00319748	C
852A		7	Pfizer	Hepatitis C Virus (Multiple-dose escalation study, safety, tolerability, pharmacokinetics and pharmacodynamics evaluation in healthy volunteers)	D	I	NCT00810758	C
852A		7	Pfizer	Inoperable metastatic Melanoma	D	II	NCT00189332	C
Imiquimod R837	radiotherapy	7	University of Oklahoma, NCI	Melanoma and metastatic cancer	D	I	NCT00453050	O
Imiquimod	cyclophosphamide and radiotherapy	7	NYU Langone Health, New York University School of Medicine, NCI	Metastatic and recurrent breast cancer	A	I and II	NCT01421017	C
Imiquimod		7	New York University School of Medicine, NYU Langone Health	Breast cancer Breast neoplasms	D	II	NCT00899574	C
Imiquimod		7	The University of Hong Kong	Influenza viral infection	A	III	NCT02103023	C
Imiquimod		7	Medical University of Vienna	Human Papillomavirus	D	II	NCT00941811	C
Imiquimod		7	Graceway Pharmaceuticals, LLC	Actinic keratosis	D	III	NCT00894647	C
Imiquimod		7	MEDA Pharma GmbH & Co. KG	Actinic keratosis	D	IV	NCT00777127	C
Imiquimod		7	Mochida Pharm. Co Ltd, 3 M Pharm., Valeant Pharm. International Inc, iNova Pharm. Pty Ltd, Intendis GmbH, Meda AB	Keratosis, mycosis fungoides, verruca vulgaris, condyloma, basal cell carcinoma, and molluscum contagiosum infection	A	Approved	NCT01453179	
Imiquimod		7	3M Pharmaceuticals	Papilloma viruses		Approved		
Imiquimod		7	University of Michigan	Photoaged skin and normal skin	A	Unknown	NCT02889159	O
Imiquimod		7	3M Pharmaceuticals	Anogenital Human Papillomavirus Infection, Anal Condyloma	D	III	NCT03289260	O
Imiquimod	intradermal HBVv	7	The University of Hong Kong	Renal failure	A	II and III	NCT02621112	C
Imiquimod		7	The University of Hong Kong	Intra-dermal with Topical Imiquimod Pretreatment *vs*. intra-muscular Hepatitis B Vaccination in Inflammatory bowel disease Patients	A	II and III	NCT04083157	O
Imiquimod	influenza vaccine	7	The University of Hong Kong	Influenza Viral Infections	A	III	NCT02103023	C
Imiquimod	influenza vaccine	7	The University of Hong Kong	Chronic illness	A	Unknown	NCT01508884	C
Imiquimod	5-FU	7	3M Pharm. National Cancer Institute	high-grade cervical intraepithelial neoplasia	A	I	NCT03196180	O
Imiquimod	PD-1 blockade	7	University of Virginia, Theraclion	Advanced Solid Tumors (Melanoma, Breast, Merkel Cell, Squamous Cell, Non-Small Cell Lung, Cervical, Urothelial, Ovarian, Hepatocellular, Small-cell Lung, Gastric and Esophageal Cancers)	A	I	NCT04116320	O
Imiquimod	doxepin	7	Aalborg University	Itch (to verify if imiquimod mechanism of action follows the histaminergic or non-histaminergic pathway by using the anti-histamine drug doxepin)	A	Unknown	NCT03943407	O
Imiquimod	Abraxane	7		Breast Cancer	D	II	NCT00821964	C

Table 3 (*continued*)

Ligands (synonyms or explanations given once)	In combination with	TLRs	Investigators	Pathologies Applications	Type[a]	Phase	Clinical Trial Identification	Status[b]
			University of Washington					
Imiquimod		7	Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins	Cervical Cancer	D	I	NCT00788164	O
Imiquimod		7	Massachusetts General Hospital	Neurofibromatosis	D	I	NCT00865644	C
Imiquimod		7	St. Justine's Hospital	Hemangioma	D	II	NCT00601016	C
Imiquimod	tazarotene	7	University of Utah	Lentigo Maligna	D	Not applicable	NCT00707174	C
Imiquimod	Peptide-based vaccine	7	University of Virginia	Melanoma	D	I	NCT01264731	C
Imiquimod	Peptide-based vaccine	7	Ludwig Institute for Cancer Research	Melanoma	A	I	NCT00142454	C
Imiquimod	Peptide-based vaccine	7	National Cancer Institute	Melanoma	A	I	NCT00118313	C
Imiquimod	Melan-A VLP vaccine, Montanide	7	Cytos Biotechnology AG	Melanoma	A	II	NCT00651703	C
Imiquimod	Dendritic cell/ tumor fusion vaccine, GM-CSF	7	Beth Israel Deaconess Medical Center	Ovarian Cancer Primary Peritoneal Cancer Fallopian Tube Cancer	D	II	NCT00799110	O
Imiquimod		7	Memorial Sloan Kettering Cancer Center	Vulvar Cancer	D	Non Applicable	NCT00504023	C
Imiquimod		7	Medical University of Vienna	Cervical Intraepithelial Neoplasia	D	II, III	NCT00941252	C
Imiquimod		7	Medical University of Graz	Lentigo Maligna	D	II, III	NCT01088737	O
Imiquimod		7	Jerry Marsden, University Hospital Birmingham NHS Foundation Trust	Lentigo Maligna	D	IV	NCT01161888	C
Imiquimod		7	Medical University of Graz	Vulvar intraepithelial Neoplasia	D	III	NCT01861535	O
Imiquimod		7	University Medical Center Nijmegen	Paget Disease	D	III	NCT02385188	C
Imiquimod	Radiotherapy	7	Melanoma and Skin Cancer Trials Limited	Lentigo Maligna	D	III	NCT02394132	O
TMX-101 (liquid formulation of imiquimod) **Vesimune** **TMX-101**		7	Telormedix SA	non-invasive bladder cancer, including patients with CIS bladder cancer. Non-Muscle-Invasive Bladder Cancer	A D	II I	NCT01731652 EudraCT2009-014757-33	C C
Resiquimod **R848**		7/8	3M Pharmaceuticals	Hepatitis C Infection Herpes	D	II/III	—	S
Resiquimod		7/8	University of British Columbia	Influenza vaccination in seniors	A	I	NCT01737580	C
Resiquimod		7/8	Vincent S. Fan, VA Puget Sound Health Care System, University of Washington, Novartis Pharmaceuticals	Chronic Obstructive Pulmonary Disease, Chronic Bronchitis	A	Unknown (observational non-interventional trial)	NCT02637219	C
Resiquimod	NY-ESO-1 protein	7/8	CRI New York City, Icahn School of Medicine at Mount Sinai	Tumors expressing NY-ESO-1 protein	A	I	NCT00821652	C
Resiquimod		7/8		Melanoma	A	II	NCT00960752	O

(*continued on next page*)

Table 3 (*continued*)

Ligands (synonyms or explanations given once)	In combination with	TLRs	Investigators	Pathologies Applications	Type[a]	Phase	Clinical Trial Identification	Status[b]
			M.D. Anderson Cancer Center, Houston, Texas					
Resiquimod	poly-ICLC	7/8	Jonsson Comprehensive Cancer Center	Glioma, anaplastic astrocytoma, anaplastic astro-oligodendroglioma	A	II	NCT01204684	O
Resiquimod		7/8	University of Virginia	Melanoma and its metastatic mucosal variants	A	I and II	NCT02126579	C
AZD8848 **DSP-3025** **AZD-3025**		7	Sumitomo Dainippon Pharma Co., Ltd.	Healthy volonteers	D	I	NCT01124396	C
AZD8848		7	AstraZeneca	Allergy Asthma	D	II	NCT00999466	C
GSK2245035		7	GSK, PATH	Asthma Rhinitis	D	II	NCT01607372	C
GSK2245035		7	GSK	Mild asthma and allergic rhinitis (to examine the safety and effects on the immune system)	D	II	NCT01788813	C
GSK2245035		7	GSK	Allergy rhinitis, asthma, and respiratory tract allergy	D	I and II	NCT02833974	C
GSK2245035		7	GSK	Allergy rhinitis	D	II	EudraCT2015-005645-31	C
GSK2445035		7	GSK	Allergic Rhinitis (to collect tolerability, pharmacokinetic and pharmacodynamic information)	D	I	NCT01480271	C
GS-9620 **vesatolimod**		7	Gilead Sciences	Chronic hepatitis B in virally suppressed patients	A	II	NCT02166047 GS-US-283-1059	C
GS-9620	Tenofovir Disoproxil Fumarate	7	Gilead Sciences	Chronic hepatitis B	D	II	NCT02579382	C
GS-9620		7	AELIX Therapeutics	HIV vaccine	A	II	EudraCT2018-002125-30	S
Single or multiple GS-9620		7	Gilead Sciences	Hepatitis B	A	I	NCT01590654	C
GS-9620	ARV Regimen	7	Gilead Sciences	Latent reservoir HIV (suppressive ART)	D	I	NCT02858401	C
GS-9620	ART	7	Gilead Sciences	Latent reservoir HIV (HIV infected controllers)	D	I	NCT03060447	O
RO7020531		7	Hoffmann-La Roche	Healthy volunteers	D	I	NCT03530917	C
RO7020531		7	Hoffmann-La Roche	Chronic Hepatitis B	D	I	NCT02956850	O
RO6864018 (aka-ANA773, ANA773, RG7795)		7	Hoffmann-La Roche	Healthy volunteers	D	I	NCT02015715	C
ANA773		7	Anadys Pharmaceuticals	Cancer	A	I	EudraCT2011-000728-14	O
ANA773		7	Anadys Pharmaceuticals	Hepatitis C	A	I	EudraCT2011-000728-14	O
ANA773		7	Hoffmann-La Roche	Chronic Hepatitis B	D	II	NCT02391805	C
RO6871765 or RO7011785		7	Hoffmann-La Roche	Chronic Hepatitis B	D	Unknown	NCT02498275	T
DSP-0509	Pembrolizumab	7	Boston Biomedical, Inc Syneos Health	Advanced Solid Tumors	D	I and II	NCT03416335	O
NJH395		7	Novartis Pharmaceuticals	non-breast HER2+ Malignancies (NJH395 = Immune stimulator antibody conjugate (ISAC), consisting of a monoclonal antibody which targets HER2 conjugated to an immune-stimulatory agent)	A	I	NCT03696771	O
BNT411	with or without atezolizumab, carboplatin and etoposidz	7	BioNTech Small Molecules GmbH	Solid Tumor Extensive-stage Small Cell Lung Cancer (combination with combination with cytotoxic therapies and immune checkpoint inhibitors)	D	I and II	NCT04101357	O
TQ-A3334 JNJ-4964 AL-034	entecavir	7	Chia Tai Tianqing Pharmaceutical Group Co., Ltd.	Chronic Hepatitis B	D	II	NCT04180150	O
LHC165	PDR-1	7	Novartis Pharmaceuticals	Advanced Solid Tumors	D	I	NCT03301896	O
Hydroxychloroquine		7/9	Seoul National University Hospital	Dry Eye/dry mouth of Primary Sjogren's Syndrome, Autoimmune Diseases	D	III	NCT01601028	C
MEDI9197		7/8	MedImmune LLC		D	I	NCT02556463	

Table 3 (*continued*)

Ligands (synonyms or explanations given once)	In combination with	TLRs	Investigators	Pathologies Applications	Type[a]	Phase	Clinical Trial Identification	Status[b]
	durvalumab and/or palliative radiation			Solid tumor CTLC				T (compagny strategy)
NKTR-262	bempegaldesleukin with or without nivolumab	7/8	Nektar Therapeutics	Locally Advanced or Metastatic Solid Tumor Malignancies (Melanoma, Merkel Cell, Triple Negative Breast, Head and Neck Squamous Cell, Renal Cell and Colorectal Cancers)	D	I and II	NCT03435640	O
CV8102	IMA970A Cyclophosphamide	7/8	CureVac	Early and intermediate stages hepatocellular carcinoma	A	I and II	NCT03203005	O
CV8102	anti-PD-1	7/8	CureVac	Solid tumors (Melanoma, Squamous Cell Carcinoma of the Skin, Squamous Cell of Head and Neck Carcinoma, Adenoid Cystic)	D	I	NCT03291002	O
VTX-1463		8	VeniRx Pharmaceuticals Inc	Allergy	D	I		O
VTX-2337 Motolimod	cetuximab	8	University of Washington, NCI	Different stages of locally advanced, recurrent, or metastatic squamous cell cancer of the head and neck	A	I	NCT01334177	C
VTX-2337	Cetuximab, Nivolumab	8	Celgene	Squamous Cell Carcinoma of the head and neck	D	I	NCT02124850	T
VTX-2337	cyclophosphamide	8	Mayo Clinic, NCI	Metastatic, persistent, recurrent or progressive solid tumors (various types and stages of colorectal, pancreatic, breast, melanoma, non-small cell lung carcinoma, pancreatic, renal cell carcinoma and solid neoplasm)	A	I	NCT02650635	T
VTX-2337	paclitaxel or doxorubicin	8	Gynecologic Oncology Group, NCI	Recurrent or persistent ovarian epithelial, fallopian tube, or peritoneal cavity cancer	A	I	NCT01294293	C
VTX-2337	radiotherapy	8	VentiRx Pharmaceuticals Inc, Stanford University, Celgene	Low grade B-Cell lymphoma	A	I and II	NCT01289210	T (The study was stopped due to slow rate of recruitment)
VTX-2337	cisplatin	8	VentiRx Pharmaceuticals Inc	Carcinoma, squamous cell of head and neck	A	II	NCT01836029	C
VTX-2337	doxorubicin	8	Gynecologic Oncology Group, Celgene	Recurrent or persistent, epithelial ovarian, fallopian tube or primary peritoneal cancer	A	II	NCT01666444	C
VTX-2337	anti-PD-L1 antibody durvalumab (MEDI4736)	8	Ludwig Institute for Cancer Research, MedImmune LLC, VentiRx Pharmaceuticals Inc, CRI New York City	Ovarian cancer	A	I and II	NCT02431559	O
VTX-2337	anti-PD-L1 antibody nivolumab	8	Celgene	Squamous cell carcinoma of the head and neck	A	I	NCT03906526	O
GS-9688	TAF	8	Gilead Sciences	Chronic Hepatitis B	D	II	NCT03615066	O
Antagonists								
IMO-8400 Bazlitoran		7/8/9	Idera Pharmaceuticals, Inc	Dermatomyositis		II	NCT02612857	O
IMO-8400		7/8/9	Idera Pharmaceuticals, Inc	Diffuse large B-cell lymphoma	D	I/II	NCT02252146	C
IMO-8400		7/8/9	Idera Pharmaceuticals, Inc	Waldenstrom's macroglobulinemia	A	I/II	NCT02092909	T (lack of efficacy)
IMO-8400		7/8/9	Idera Pharmaceuticals, Inc	Plaque psoriasis	D	II	NCT01899729	C
IMO-3100		7/8	Idera Pharmaceuticals, Inc	SLE Rheumatoid Arthritis Multiple sclerosis		Preclinical	–	–
IRS-954 DV-1079		7/9	Dynavax Technologies	SLE		Preclinical	–	–
IRS-954		7/9	Dynavax Technologies	HIV		Preclinical	–	–
CPG-52364		7/8/9	Pfizer	SLE	D	I	NCT00547014	O

[a] D for drug, A for adjuvant.
[b] C for completed, T for terminated, O for ongoing, S for suspended.

few pharmacological modulators reach final stages of clinical studies either as drugs or as adjuvants, due to obstacles that are difficult to overcome. For example, toxicity related to immunomodulating compounds could be modulated by delivering potent therapies locally (intratumorally or intradermally) thus directly targeting pathological sites and/or cell populations [369]. Moreover, animal models do not reflect exactly the human immunological pathways, which did not allow appreciating the potentialities of certain compounds. With less genetic variability in animal models, a margin of error is possible to predict safety but active doses in humans. Specific molecular alterations or mutations due to the evolution of pathologies could also prevent potent modulators to mediate the expected therapeutic effect, in addition of host-related characteristics, such as gender, age or gene polymorphic variants. Despite all the barriers mentioned, the number of clinical trials relating to the biological activities of TLRs 7/8 ligands perfectly illustrates the interest of researchers in modulating them for therapeutic purposes.

6. Conclusion

Toll-like receptors 7/8 demonstrate a key role in the immunology by protecting the host body against invading pathogens or endogenously released hazardous molecules. By improving or blocking their activities, TLRs are capable of modifying the inherent signaling pathways and thus mediating host responses, rendering them ingenious tools to be targeted for therapeutic purposes. A wide spectrum of diseases is associated with TLRs, which directly or indirectly aggravate the pathological conditions. Many pharmaceutical companies and research institutions are thereby developing specific TLR modulators. Some of them have been preclinically and clinically evaluated.

TLRs ligands are primarily used as adjuvants to boost the immunity system and to trigger humoral and/or cell-mediated responses. Agonists are also developed as drugs to fight viral infections and to prevent and/or treat cancers. Antagonists are developed for downregulating TLRs overactivity as tools for the treatment of inflammatory and autoimmune diseases. Synthetic TLRs 7/8 ligands are not only small molecules belonging to diverse chemical series, but also macromolecules, such as oligonucleotides, peptides and proteins. Rational drug design of small molecule agonists and antagonists may be helped by the recent knowledge of TLRs three-dimensional structures. The potential of non-traditional approaches such as the use of miRNAs should not be overlooked.

Trials involving TLRs ligands as adjuvants are numerous than those considering them as drugs. Moreover, the imbalance in the number of agonists and antagonists under clinical investigations could be explained by the recent understanding of the implication in pathologies of signaling dysregulation. After an explosion in the number of clinical studies launched, we have witnessed a decrease in recent years. Even if some ligands have performed well in advanced clinical trials, clinical success is generally limited. Researchers still have to overcome some obstacles. Indeed, if animal models allow to appreciate biological activities of the TLRs 7/8 modulators, they do not reflect exactly what happen in human trials due to genetic variability among others. Specific molecular alterations or mutations may also prevent potent modulators to mediate the expected therapeutic effect. Researchers aim to reduce systemic toxicity of immunomodulating compounds by modulating their formulation (liposome, hydrogel, nanoparticle instigations) or way of administration (intratumorally or intradermally). Many studies revealed the importance of combined therapy including TLRs modulators to obtain convenient results. Immunotherapy has been revealing as a good approach to complete traditional clinical management.

Even if there are similarities in the TLRs 7/8 mechanism of actions, specificities remain to be elucidated to specifically induce a response from a single receptor. Moreover, animal models do not reflect the potent human response to a drug because of the differences in TLRs expression and activity between humans and mice. Continuing researches around TLR biology would allow finding novel therapies to address unmet medical needs, such as cancers, diabetes, rheumatoid arthritis, systemic lupus erythematosus, Alzheimer's disease, and chronic neuropathic pain.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Authors would thank Laurianne Adin for helping to find additional detailed data during the preparation of this manuscript.

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