LIPIDATED IMIDAZOQUINOLINE DERIVATIVES

Background

The present invention relates to novel adjuvant compounds, processes for their preparation, compositions containing them, and their use as vaccine adjuvants.

The refinement and simplification of microbial vaccines and the use of synthetic and recombinant subunit antigens to improve vaccine manufacturability and safety has resulted in a decrease in vaccine potency. This has led to studies on the co-administration of adjuvants with antigens to potentiate vaccine activity and the weak immunogenicity of synthetic and recombinant epitopes. Adjuvants are additives that enhance humoral and/or cell mediated immune responses to a vaccine antigen. The design of vaccine adjuvants, however, has historically been difficult because of the complex nature of the molecular mechanisms involved in immune system function. Although the addition of microbial components has long been known to enhance adaptive immune responses, only recently was it shown that toll-like receptors (TLRs) on cells involved in immune surveillance, such as epithelial and dendritic cells, engage many of these microbial products via so-called "pathogen-associated patterns" or PAMPs. Many vaccine adjuvants and stand-alone immunomodulators appear to interact with members of the TLR family.

Of the 10 known TLRs that have been identified in humans, five are associated with the recognition of bacterial components (TLRs 1, 2, 4, 5, 6) and four others (TLRs 3, 7, 8, 9) appear to be restricted to cytoplasmic compartments and are involved in the detection of viral RNA (TLRs 3, 7, 8) and unmethylated DNA (TLR9) (Iwasaki, A., *Nat Immunol* **2004**, *5*, 987) Activation of TLRs regulates intracellular signaling pathways and leads to gene expression via interaction with intracellular adapter molecules such as MyD88, TRIF, TIRAP, and TRAM (Akira, S. *Nat Rev Immunol* **2004**, *4*, 499; Takeda, K. *Semin Immunol* **2004**, *16*, 3). These adapter molecules can differentially regulate the

expression of inflammatory cytokines/chemokines and type I interferons (IFNa/b), which can lead to the preferential enhancement of antigen-specific humoral and cell-mediated immune responses (Zughaier, S. *Infect Immun* **2005**, *73*, 2940). Humoral immunity is the major line of defense against bacterial pathogens, whereas the induction of cytotoxic T lymphocytes (CTLs) appears to be crucial for protective immunity in the case of viral disease and cancer.

Currently, a group of aluminum salts known as alum are the dominant adjuvants used in human vaccines. But alum typically only enhances humoral (Th2) immunity and is generally used intramuscularly due to local toxicity by other routes (e.g., subcutaneous or intradermal inoculation leads to granulomas) (Aguilar, J. *Vaccine* **2007**, *25*, 3752). Other potential side effects of alum include increased IgE production, allergenicity and neurotoxicity. Thus, new safe and effective vaccine adjuvants are needed which are able to stimulate both antibody and Th1-type immune responses and that are compatible with different routes of administration and antigen formulations.

In the case of TLR7 and TLR8 activation, a few different classes of small molecule mimetics of the natural (U- and/or G-rich) viral ssRNA ligands have been identified. These include certain antiviral compounds related to oxidized guanosine metabolites (oxoguanosines), which primarily interact with TLR7 (Heil, F. *Eur J Immunol* **2003**, *33*, 2987; Hemmi, 2002) and derivatives of adenine which engage TLR7 and/or TLR8. The immune stimulating ability of these compounds has been attributed to the TLR/MyD88-dependent signaling pathways and the production of cytokines, including IL-6 and type I (particularly interferon-a) and II interferons. TLR7 or TLR8 activation leads to the upregulation of co-stimulatory molecules (e.g. CD-40, CD-80, CD-86) and class I and II MHC molecules on dendritic cells (DCs). DCs are the principal cells of the immune system involved in uptake and presentation of antigens to T lymphocytes. Plasmacytoid dendritic cells (pDCs), which preferentially express TLR7, are professional interferon-a producing cells; whereas mDCs express TLR8 only. TLR8 activation on mDCs leads to the preferential production of pro-inflammatory cytokines such as IL-12, TNF-a, and IFN-g and cell-mediated immunity (CMI).

One class of adenine derivatives that has received a considerable amount of attention are the 1H-imidazo[4,5-c]quinolines (IQs). The prototypical member of this

class imiquimod (R847, S-26398) was found to be effective against genital papilloma virus infections, actinic keratosis, and basal cell carcinoma when applied topically in cream form. However, imiquimod has relatively low interferon-inducing activity and both oral and topical preparations are not without side-effects. In fact, serious side effects were reported in an HCV clinical trial with imiquimod. The large immunological "footprint" of TLR7 agonists in general has led concerns over toxicity: Clinical trials with another TLR7 agonist ANA-975, an oxoguanosine derivative, were recently suspended due to toxicity issues.

Another member of the IQ class of TLR7/8 ligands and a derivative of a metabolite of imiquimod is resiquimod. Resiquimod (R-848, S-28609) also activates TLR7 in macrophages and DCs in a MyD88-dependent manner either directly or indirectly via an accessory molecule and upregulates co-stimulatory molecules and MHCI/II in DCs. But in contrast to imiquimod, the more potent and toxic resiquimod is also a ligand for TLR8 signaling, which leads to the reversal of CD4+ regulatory (Treg) cell function. Using transfected HEK293 cells, it was recently shown that TLR7 agonists are more effective at generating IFN-a and IFN-regulated cytokines, whereas TLR8 agonists were more effective at inducing proinflammatory cytokines such as TNF-a and IL-12, suggesting that TLR7 activation may be more important for antibody responses (Th2-type responses) while TLR8 activation should drive CMI or Th1-type immune responses. However, as mentioned above, many TLR7/8 agonists often display toxic properties, are unstable, and/or have unsubstantial immunostimulatory effects. Thus, the discovery and development of effective and safe adjuvants that activate TLR7 and/or TLR8 is essential for improving the efficacy and safety of existing and new vaccines via helping to control the magnitude, direction, and duration of the immune response against antigens.

Unlike TLR2 and TLR4, which recognize PAMPs on cell surfaces, TLR7/8 PAMPs are sensed in the endosomal/lysosomal compartments and require endosomal maturation. Cellular uptake is prerequisite for cellular activation in the case of natural and zenobiotic TLR7/8 ligands such as imiquimod and resiquimod. Thus, strategies that

would increase the penetration of the TLR7/8 ligand into DCs and other immune cells could enhance TLR activation and vaccine efficacy as well as ameliorate toxic effects.

Lipid conjugates of nucleoside drugs are known in the art to enhance oral bioavailability in general as well as permit incorporation of the resulting "nucleolipid" into lipid membranes of liposomes. Incorporating unstable and/or toxic drugs in liposomes establishes a slow-release carrier system or molecular depot, which protects the drug from degradation and decreases toxic side effects. The potency of such "lipid prodrugs" has been reported to be comparable to that of the non-derivatized drugs (US 5,827,831 – NeXstar). Depot preparations of imidazoquinolines and fatty acylated IQs have been reported in the art for the purposes of maintaining the IQ for an extended period within a localized tissue region to decrease metabolism and toxicity (WO 2005/001022 – 3M). However, conjugating an imidazoquinoline to a phospho- or phosphonolipid in a specific manner in order to facilitate uptake into immune cells, when administered alone or in depot formulation with an antigen, and enhance endosomal TLR7/8 activation and antigen presentation is not known in the art. Enhanced immune responses with compounds of the subject invention are possibly due to direct interaction of compounds of formula (I) with endosomal TLR7 and/or TLR8 and/or interaction of an active metabolite after enzymatic action.

Brief Description of the Invention

The compounds of the invention have been shown to be inducers of interferon-a, IL-12 and other immunostimulatory cytokines and may possess an improved activity-toxicity profile in comparison to known cytokine inducers when used as adjuvants for vaccine antigens in the therapeutic or prophylactic treatment of infectious diseases and cancer. These compounds are also novel per se.

Description of the Drawings

Figure 1 shows a schematic representation of study design.

Figure 2 shows the p27-specific CD8 response.

Figure 3 shows p27-specific cytotoxic activity detected in vivo.

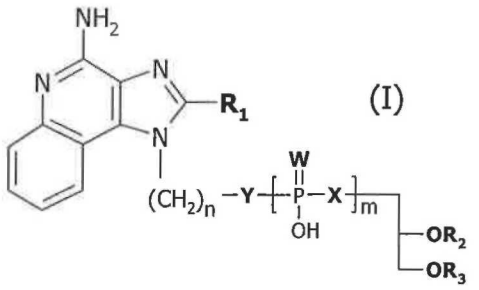
Figure 4 illustrates the antigen- specific CD4 T cell response.

Fig 5 shows serum cytokine response in groups immunized with the liposome -based formulations without (-) or with various amounts of TLR7/8 ligands together with QS21 and MPL.

Fig 6 shows serum cytokine response in groups immunized with the emulsion-based formulations without (-) or with various amounts of TLR7/8 ligands together with QS21 and MPL.

Summary

The compounds of the subject disclosure are adjuvant molecules that comprise a imidazoquinoline molecule which may be covalently linked to a phospho- or phosphonolipid group. The compounds of the subject disclosure are described generally by Formula I:



wherein

$\mathbf{R_1}$ = H, $C_{1\text{-}6}$ alkyl, $C_{1\text{-}3}$ alkoxy$C_{1\text{-}3}$ alkyl, $C_{3\text{-}6}$cycloalkyl$C_{1\text{-}3}$alkoxy, $C_{3\text{-}6}$cycloalkyl$C_{1\text{-}3}$alkyl. . .

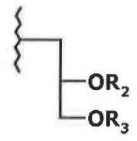
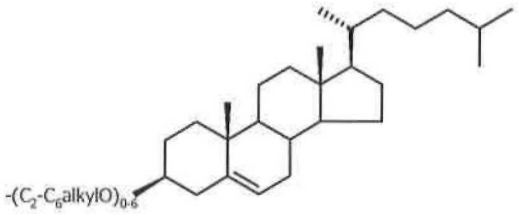
n = 1-6

Y = O, NH

X = O, CH_2, CF_2

W = O or S

m = 1-2,

A =  or 

wherein

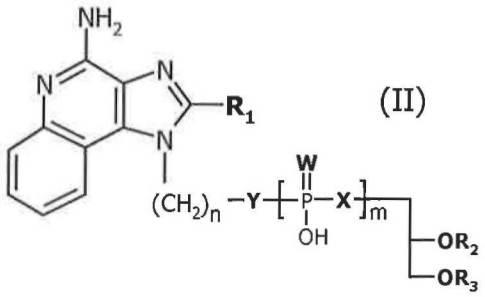
$\mathbf{R_2}$ = H or straight/branched/unsaturated C_4-C_{24} alkyl or acyl

$\mathbf{R_3}$ = straight/branched/unsaturated C_4-C_{24} alkyl or acyl

$\mathbf{R_4}$, $\mathbf{R_5}$ = independently H, C_1-C_6alkyl, C_1-C_6 alkoxy, halogen, or trifluoromethyl; or taken together alternatively form a 6-membered aryl, heteroaryl containing one nitrogen atom, cycloalkyl, or heterocycloalkyl ring containing one nitrogen atom; unsubstituted or substituted by one or more of C_1-C_6alkyl, C_1-C_6 alkoxy, halogen, or trifluoromethyl,

or pharmaceutically acceptable salts thereof.

In one embodiment, the compounds of the subject invention are more specifically described by Formula II:



(II)

wherein

$\mathbf{R_1}$ = H, $C_{1\text{-}6}$alkyl, $C_{1\text{-}6}$alkylamino, $C_{1\text{-}6}$alkoxy, $C_{3\text{-}6}$cycloalkyl$C_{1\text{-}6}$alkyl, $C_{3\text{-}6}$cycloalkyl$C_{1\text{-}6}$alkylamino, $C_{3\text{-}6}$cycloalkyl$C_{1\text{-}6}$alkoxy, $C_{1\text{-}6}$alkoxy$C_{1\text{-}6}$alkyl, $C_{1\text{-}6}$alkoxy$C_{1\text{-}6}$alkylamino, $C_{1\text{-}6}$alkoxy$C_{1\text{-}6}$alkoxy; branched or unbranched and optionally terminally substituted with a hydroxyl, amino, thio, hydrazino, hydrazido, azido, acetylenyl, carboxyl, or maleimido group,

n = 1-6

Y = O, NH

X = O, CH_2, CF_2

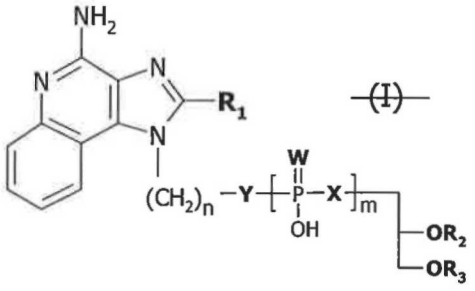
W = O or S

m = 1-2,

$\mathbf{R_2}$ = H or straight/branched/unsaturated C_4-C_{24} alkyl or acyl

$\mathbf{R_3}$ = straight/branched/unsaturated C_4-C_{24} alkyl or acyl *(e.g. phosphatidyl, lysophosphatidyl ether or ester when W=O, X=O, m=1)*

In accordance with one aspect, there is provided a compound having the following formula:



wherein

$\mathbf{R_1}$ = H, $C_{1\text{-}6}$ alkyl, $C_{1\text{-}3}$ alkoxy$C_{1\text{-}3}$ alkyl, $C_{3\text{-}6}$cycloalkyl$C_{1\text{-}3}$alkoxy, $C_{3\text{-}6}$cycloalkyl$C_{1\text{-}3}$alkyl,

n = 1-6,

X = O, CH_2, CF_2,

W = O or S,

m = 1-2,

$\mathbf{R_2}$ = H or straight/branched/unsaturated C_4-C_{24} alkyl or acyl,

$\mathbf{R_3}$ = straight/branched/unsaturated C_4-C_{24} alkyl or acyl

Y=O, NH.

In accordance with another aspect, there is provided a method for improving the adjuvant activity of an imidazoquinoline comprising

conjugating the imidazoquinoline to a phospho- or phosphonolipid to obtain a compound as disclosed herein.

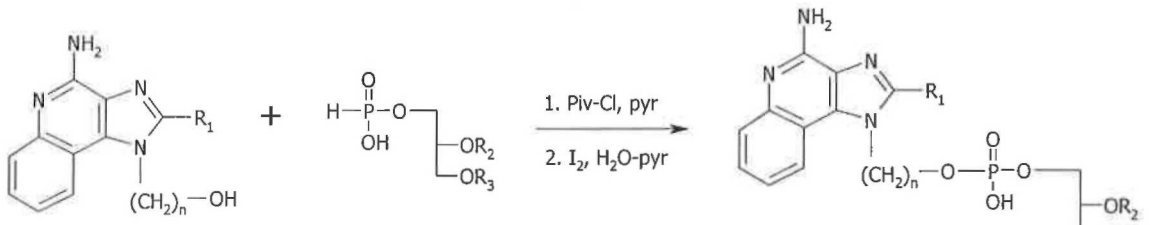
TABLE 1

Example	Ref. No.	*CRX#*	R_1	n	m
1	—	—	—	—	—
2	—	*664*	—	—	—
3	**L1**	*650*	H	2	1
4	**L2**	*652*	n-Bu	2	1
5	**L3**	*651*	CH_2OEt	2	1
6	**L4**	*665*	CH_2OEt	4	1
7	—	—	—	—	—
8	**L5**	*658*	CH_2OEt	2	2

For all Examples shown: Y = W = X = O; $R_2 = R_3$ = hexadecanoyl

EXAMPLE 1

General procedure for the preparation of 4-Amino-1-[2-(1,2-dipalmitoyl-*sn*-glycero-3-phospho)alkyl]-1H-imidazo[4,5-c]quinolines (Compound (I), Y=W=X= O, m=1)



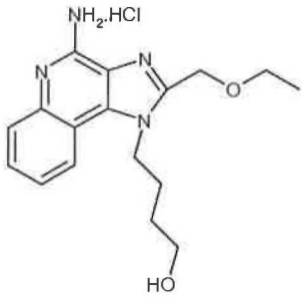
II **III** **IV**

Imidazoquinoline monophosphate diglycerides **IV** were prepared by coupling 4-amino-1-hydroxyalkyl-imidazoquinolines **II** (Gerster et al. *J Med Chem* **2005**, *48*, 3481; Izumi et al. *Bioorg Med Chem* **2003**, *11*, 2541) with 1-H phosphonate **III** according to methods known in the art (Crossman, et al. *J Chem Soc, Perkin Trans 1*, **1997**, 2769; Westerduin,

et. al. *Tet Lett*, **1986**, *15*, 6271; Nikolaev, et al., *Carbohydr Res*, **1990**, *204*, 65) as follows: Imidazoquinoline **III** (1 eq) and H-phosphonate **IV** (2 eq) were suspended in n-heptane and, after evaporation of the solvent, dried overnight under high vacuum. The resulting residue was dissolved in pyridine (0.01 M compound **III**), treated with pivaloyl chloride (12.4 eq), and then stirred at room temperature for 6 h. A solution of iodine (4 eq) in 19:1 pyridine-water (0.04 M) was added and the resulting mixture stirred at room temperature for 1 h and then partitioned between $CHCl_3$ and 1 M aq $Na_2S_2O_5$. The layers were separated and the aqueous layer was extracted twice with $CHCl_3$. The combined organic extracts were washed with 1 M triethylammonium borate buffer (pH 8), dried (Na_2SO_4), and concentrated. The residue obtained was purified by flash chromatography on silica gel (gradient elution, 0→25% MeOH-$CHCl_3$) and then by reverse phase chromatography (Bakerbond C8 in CH_3CN containing 1% TEA, eluting with 0→60% MeOH-CH_3CN containing 1% Et_3N) to provide compound **V** as a colorless solid.

EXAMPLE 2

Preparation of 4-Amino-1-(4-hydroxybutyl)-2-ethoxymethyl-1H-imidazo[4,5-c]quinoline hydrochloride (Compound (**III**), R_1=$CH_2OCH_2CH_3$, n=4)



(1) A suspension of 4-hydroxy-3-nitroquinoline (Gerster et al. *J Med Chem* **2005**, *48*, 3481) in DMF (0.7 M) was treated dropwise with $POCl_3$ (1.2 eq) and stirred at 50 °C for 30 min. The reaction mixture was poured into ice-water and extracted twice with CH_2Cl_2. The combined organic layers were washed with water, dried (Na_2SO_4) and concentrated. The crude product obtained was added to a solution of 4-amino-butanol

(1.3 eq) and triethylamine (1.9 eq) in EtOH and heated to reflux for 15 min. After concentration, flash chromatography on silica gel (gradient elution, 2→4% MeOH-$CHCl_3$) afforded 4-(4-hydroxybutyl)amino-3-nitroquinoline as a yellow solid in 97 % yield.

(2) A solution of the compound prepared in (1) above in EtOAc (0.1 M) was hydrogenated in the presence of 5 % Pt/C (5 % w/w) and $MgSO_4$ (1.5 eq) at 50 psig for 6 h. The reaction mixture was filtered through celite and concentrated. The orange oil obtained was heated with ethoxyacetic acid (11 eq) at 150 °C for 1h. The reaction mixture was cooled to 0 °C, basified to pH 10 with conc NH_4OH, and extracted twice with CH_2Cl_2. The combined organic layers were dried (Na_2SO_4) and concentrated. Flash chromatography on silica gel (1:60 MeOH-$CHCl_3$) gave the ethoxyacetate derivative which was treated with 2.6 M NaOH (5.0 eq) in EtOH (0.20 M) at room temperature for 1h. Ethanol was removed under reduced pressure and the aqueous layer was extracted several times with AcOEt and CH_2Cl_2. The combined organic layers were dried (Na_2SO_4), and concentrated. Flash chromatography on silica gel (gradient elution, 1:50→1:15 MeOH-$CHCl_3$) afforded 1-(4-hydroxybutyl)-1H-imidazo[4,5-c]quinoline as a solid in 74 % yield. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.29 (s, 1H), 8.25 (dd, 2H), 7.67 (m, 2 H), 4.89 (s, 2H), 4.71 (t, 2H), 3.79 (m, 2H), 3.62 (dd, 2H), 2.12 (m, 2H), 1.82 (m, 2H), 1.25 (t, 3H).

(3) A solution of the compound prepared in (2) above and peracetic acid (1.2 eq) in ethanol (0.4 M) was heated at 60 °C for 2.5 h. After concentration, the crude product obtained was purified by chromatography on silica gel (gradient elution, 1:30→1:6 MeOH-$CHCl_3$) to afford 1-(4-hydroxybutyl)-1H-imidazo[4,5-c]quinoline 5-N-oxide as a yellow solid in 94 % yield

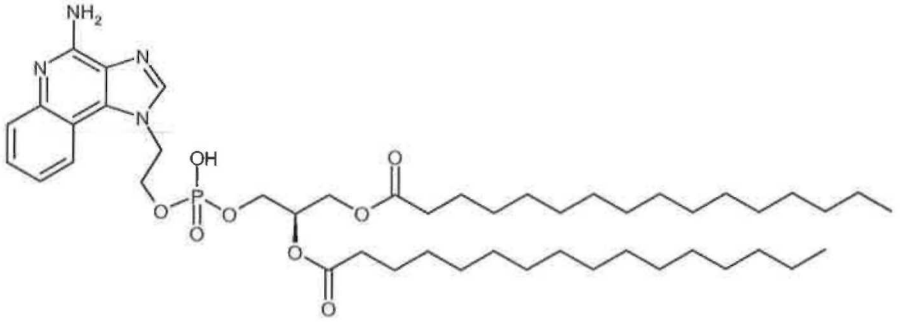
(4) A suspension of the compound prepared in (3) above in CH_2Cl_2 (0.43 M) was treated with NH_4OH (30 % aq solution, 2.7 mL) followed by *p*-toluenesulfonyl chloride (1.0 eq) dropwise. The resulting mixture was stirred at room temperature for 1.5 h and then concentrated. Flash chromatography on silica gel (gradient elution, 1:30→1:9 MeOH-$CHCl_3$) afforded 4-amino-1-(4-hydroxybutyl)-1H-imidazo[4,5-c]quinoline as an orange solid in quantitative yield.

(5) A solution of the compound prepared in (4) above in dioxane (0.12M) at 50 °C

was treated dropwise with 4N HCl in dioxane (1.5 eq) and then allowed to cool to room temperature. The solid precipitate was collected, washed with dioxane, and dried to give 4-amino-1-(4-hydroxybutyl)-1H-imidazo[4,5-c]quinoline hydrochloride salt in 89 % yield: ^{1}H NMR ($CDCl_3$-CD_3OD, 400 MHz) δ 8.13 (d, 1H), 7.97 (d, 1H), 7.65 (t, 1 H),7.55 (t, 1H), 4.89 (bs, 2H), 4.68 (m, 2H), 3.75 (m, 2H), 3.68 (dd, 2H), 2.10 (m, 2H), 1.80 (m, 2H), 1.29 (t, 3H). ^{13}C NMR ($CDCl_3$-CD_3OD, 100 MHz) δ 151.9, 148.1, 135.8, 133.7, 130.2, 128.7, 125.8, 125.4, 122.5, 121.2, 118.8, 112.1, 66.8, 64.0, 60.8, 46.8, 28.6, 26.6, 14.5. HRMS calcd for $[M+H]^+$ 315.1821, found 315.1839.

EXAMPLE 3 (L1)

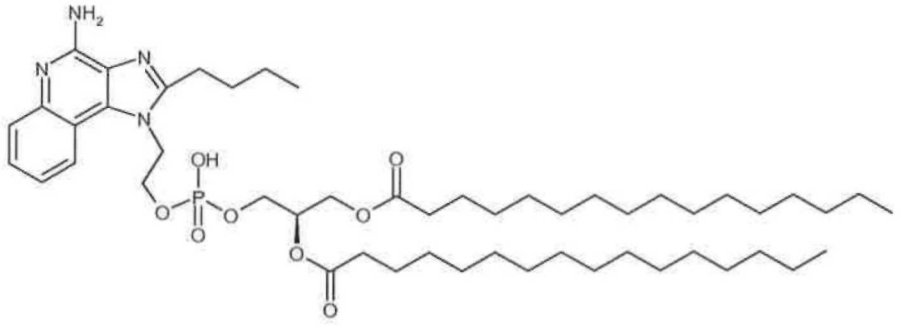
Preparation of 4-Amino-1-[2-(1,2-dipalmitoyl-*sn*-glycero-3-phospho)ethyl]-1H-imidazo[4,5-c]quinoline (Compound (I), R_1= H, Y=W=X= O, n=2, m=1, R_2=R_3= n-$C_{15}H_{31}CO$)



Compound **L1** was prepared in 80% yield following the general procedure described in Example 1 above: ^{1}H NMR ($CDCl_3$-CD_3OD, 400 MHz): δ 8.22 (s, 1H), 8.16 (d, 1H), 7.41 (t, 1H); 7.21 (t, 1H), 6.92 (d, 1H), 5.26 (m, 1H), 4.82 (bs, 2H), 4.67(bs, 2H), 4.42 (dd, 1H), 4.20 (dd, 1H), 4.05 (t, 2H), 3.14 (q, 1H), 2.31(m, 4H), 1.59 (m, 4H), 1.25 (m, 48H), 0.88(m, 6H); ^{13}C NMR ($CDCl_3$-CD_3OD, 100 MHz): δ 173.6, 173.2, 148.1, 145.8, 134.5, 133.9, 129.3, 125.5, 124.5, 118.4, 112.3, 100.3, 77.2, 70.1, 70.0, 63.5, 62.3, 45.9, 34.1, 33.9, 31.7, 29.5, 29.5, 29.3, 29.2, 29.1, 29.1, 28.9, 28.9, 24.7, 24.7, 22.5, 13.9, 8.3. HRMS calcd for $[M+H]^+$ 859.5714, found 859.5688.

EXAMPLE 4 (L2)

Preparation of 4-Amino-1-[2-(1,2-dipalmitoyl-*sn*-glycero-3-phospho)ethyl]-2-butyl-1H-imidazo[4,5-c]quinoline (Compound (I), R_1= n-C_4H_9, Y=W=X= O, n=2, m=1, R_2=R_3= n-$C_{15}H_{31}CO$)

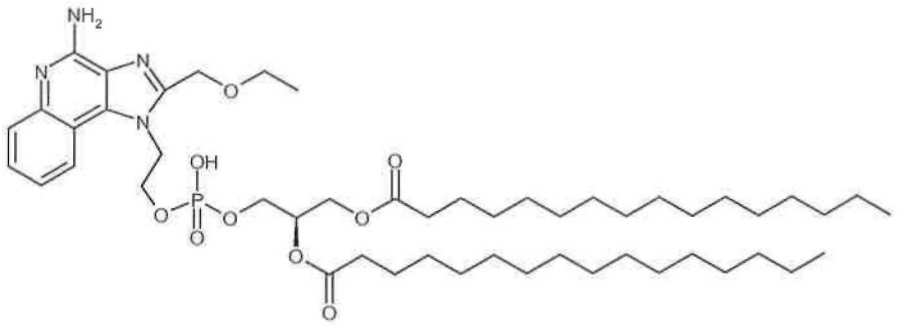


Compound **L2** was prepared in 78% yield following the general procedure described in Example 1 above: ^{1}H NMR ($CDCl_3$-CD_3OD, 400 MHz): δ 8.23 (bs, 1H), 7.39 (t, 1H), 7.22 (bs, 1H), 6.93 (bs, 1H), 5.25 (m, 1H), 4.7 (bs, 2H), 4.6 (bs, 2H), 4.42 (dd, 1H), 4.19 (dd, 1H), 4.04 (t, 2H), 3.06 (bs, 2H) 2.32 (m, 4H), 1.96 (p, 2H) 1.59 (m, 6H) 1.26 (m, 48H), 1.07 (t, 3H), 0.88 (m, 6H); ^{13}C NMR ($CDCl_3$-CD_3OD, 100 MHz): δ 173.6, 173.2, 157.2, 147.4, 135.2, 133.6, 128.8, 124.2, 123.6, 120.9, 118.2, 112.2, 77.2, 70.0, 69.9, 63.2, 62.2, 46.3, 33.9, 33.7, 31.6, 29.3, 29.3, 29.3, 29.1, 29.0, 28.95, 28.9, 28.8, 28.7, 28.6, 27.0, 24.5, 24.5, 22.3, 22.1, 13.6, 13.4. HRMS: calcd for $[M+H]^+$ 915.6340, found 915.6309.

EXAMPLE 5 (L3)

Preparation of 4-Amino-1-[2-(1,2-dipalmitoyl-*sn*-glycero-3-phospho)ethyl]-2-

ethoxymethyl-1H-imidazo[4,5-c]quinoline (Compound (I), R_1= $CH_2OCH_2CH_3$, Y=W=X= O, n=2, m=1, R_2=R_3= n-$C_{15}H_{31}CO$)

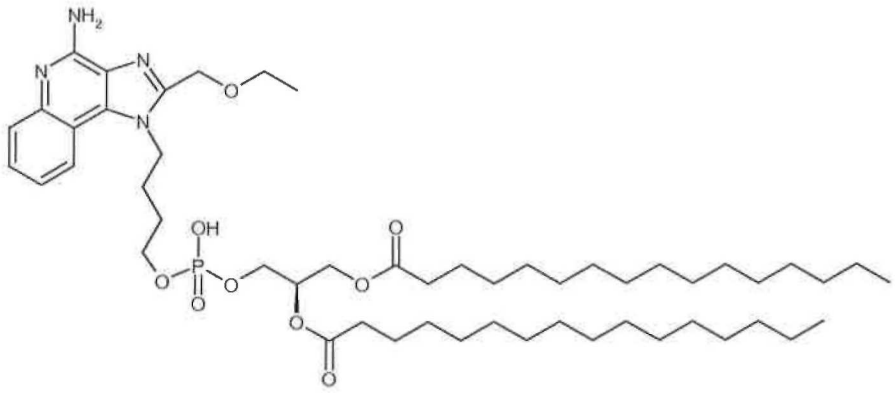


Compound **L3** was prepared in 86% yield following the general procedure described in Example 1 above: ^{1}H NMR ($CDCl_3$-CD_3OD, 400 MHz) δ 8.05 (bs, 1H), 7.29 (t, 1H), 7.09 (bs, 1H), 6.78 (bs, 1H), 5.11 (m, 1H), 4.80 (bs, 4H), 4.60 (bs, 2H), 4.28 (dd, 1H), 4.07 (dd, 1H), 3.90 (t, 2H), 3.54 (q, 2H), 2.18 (m, 4H), 1.59 (m, 4H), 1.16 (m, 51H), 0.76(m, 6H); ^{13}C NMR ($CDCl_3$-CD_3OD, 100 MHz): δ 173.4, 173.0, 153.3, 148.2, 135.7, 134.7, 129.1, 124.4, 124.2, 121.1, 119.1, 112.8, 77.2, 70.2, 70.2, 66.6, 65.4, 64.2, 63.5, 62.5, 57.7, 47.1, 45.7, 34.3, 34.1, 31.9, 29.7, 29.7, 29.6, 29.5, 29.3, 29.3, 29.1, 29.1, 24.9, 22.7, 15.0, 14.1, 8.6. HRMS calcd for $[M+H]^+$ 917.6132, found 917.6162.

EXAMPLE 6 (L4)

Preparation of 4-Amino-1-[2-(1,2-dipalmitoyl-*sn*-glycero-3-phospho)butyl]-2-

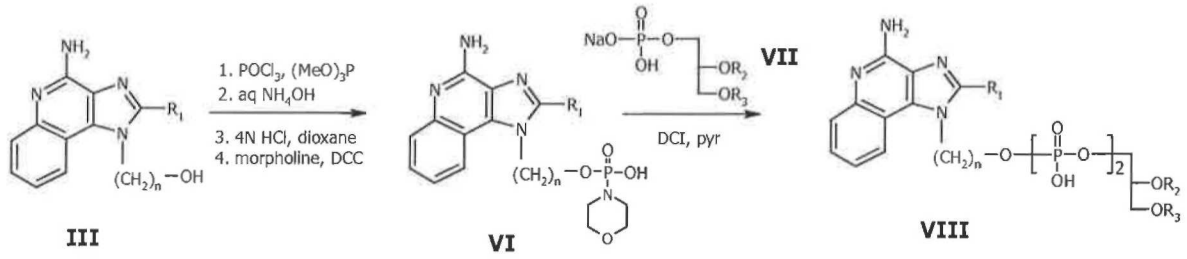
ethoxymethyl-1H-imidazo[4,5-c]quinoline (Compound (I), R_1= H, Y=W=X= O, n=4, m=1, R_2=R_3= n-$C_{15}H_{31}CO$)



Compound **L4** was prepared in 26% yield following the general procedure described in Example 1 above: ^{1}H NMR ($CDCl_3$, 400 MHz): δ 11.2 (bs, 1H), 7.78 (d, 1H), 7.30 (t, 1H), 7.20 (d, 1H), 6.78 (t, 1H), 6.39 (bs, 1H), 5.28 (m, 1H), 4.79 (s, 2H), 4.43-4.50 (m, 3 H), 4.11-4.27 (m, 5H), 3.67 (dd, 2H), 2.41 (bs, 2H), 2.30 (dd, 4H), 1.96 (bs, 1H), 1.60 (m, 4H), 1.25 (m, 54H), 0.88 (1, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 173.4, 173.0, 150.9, 148.9, 134.7, 134.2, 128.0, 124.3 (2), 120.5, 118.4, 111.8, 70.3, 70.2, 66.8, 64.7, 64.4, 64.3, 63.4 (2), 62.4, 46.6, 34.2, 34.1, 31.9, 29.6 (3), 29.4, 29.3 (2), 29.2, 29.1, 28.2, 27.4, 24.8 (2), 22.6, 15.1, 14.1. HRMS calcd for $[M-H]^-$ 943.6289, found 943.6251.

EXAMPLE 7

General procedure for the preparation of 4-Amino-1-[2-(1,2-dipalmitoyl-*sn*-glycero-3-diphospho)alkyl]-1H-imidazo[4,5-c]quinolines (Compound (I), Y=W=X= O, m=2)

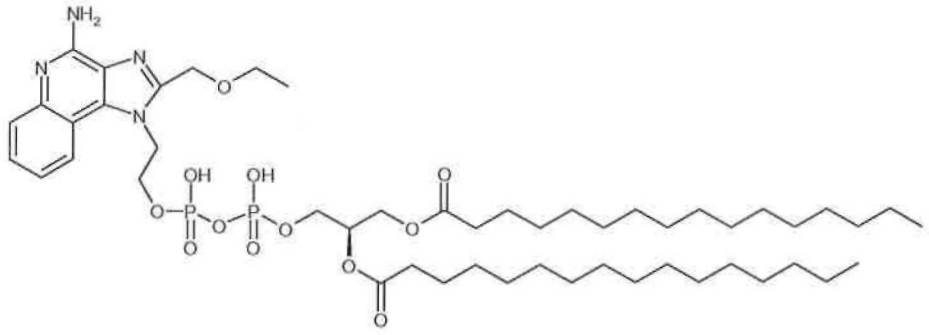


III **VI** **VIII**

Imidazoquinoline diphosphate diglycerides **VIII** were prepared by coupling the imidazoquinoline monophosphomorpholidate **VI**, prepared in crude form from imidazoquinoline **III**, with 1,2-diacyl-sn-glycerol-3-phosphate sodium salt **VII** according to methods known in the art (*Biochim. Biophys. Acta* **1980**, *619*, 604, *J. Biol. Chem.*, **1990**, *265*(11), (6112-6117) *J. Org. Chem.* **1997**, *62*, 2144-2147) as follows: $POCl_3$ (2.0 eq) and imidazoquinoline **III** (1.0 eq) were added to trimethyl phosphate (0.38 M) at 0 °C. After stirring 15 h at 0 °C, the reaction mixture was partitioned between H_2O and Et_2O and the layers separated. The organic layer was extracted three times with H_2O and the pH of combined aqueous layers was adjusted to pH 9 with aq NH_4OH. The aqueous solution was concentrated and dried under high vacuum and the residue obtained purified by chromatography on silica gel with $CHCl_3$-MeOH-H_2O-Et_3N (gradient elution, 90:10:0.5:0.5→60:40:5:1). The product obtained was dissolved in dioxane (0.12M) at 50 °C and treated with 4N HCl (1.5 eq). The HCl salt that precipitated was collected, rinsed with dioxane, and dried under high vacuum. Morpholine (5.0 eq) was added to a suspension of the salt in 1:1 *t*-BuOH-H_2O (0.5M) and the reaction mixture was heated to 90 °C and treated with a solution of 1,3-dicyclohexylcarbodiimide (DCC, 5.0 eq) in *t*-BuOH (0.33 M). After 1 h at 90 °C, the cooled reaction mixture was partitioned between H_2O and Et_2O and the layers separated. The organic layer was extracted twice with H_2O and the combined aqueous layers concentrated and dried under high vacuum. A suspension of the crude phosphomorpholidate **VI** obtained (1.5 eq) and **VII** (1.0 eq) in a small volume of pyridine was concentrated under vacuum, and then co-evaporated twice with toluene, and dried under high vacuum; this procedure was repeated twice more. 4,5-Dicyanoimidazole (DCI, 3.0 eq) was then added to a suspension of the dried solids in pyridine (0.10 M) and the reaction mixture was stirred at room temperature for 10 days. The resulting mixture was concentrated and the residue obtained partitioned between H_2O-CH_2Cl_2 and the layers separated. The aqueous layer was extracted twice with CH_2Cl_2 and the combined organic layers were dried (Na_2SO_4), and concentrated. Chromatography on silica gel with $CHCl_3$-MeOH-H_2O (gradient elution, 90:10:0.5 → 70:30:2) afforded compound **VIII** as a colorless solid.

EXAMPLE 8 (L5)

Preparation of 4-Amino-1-[2-(1,2-dipalmitoyl-*sn*-glycero-3-diphospho)ethyl]-2-ethoxymethyl-1*H*-imidazo[4,5-c]quinoline (Compound (I), R_1= $CH_2OCH_2CH_3$, Y=W=X= O, n=2, m=2, R_2=R_3= n-$C_{15}H_{31}CO$)



Compound **L5** was prepared in 22% yield following the general procedure described in Example 6 above: ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.17 (bs, 1H), 7.10-7.40 (2-3 m, 2-3H), 5.25 (bs, 1H), 4.60-5.00 (bm, 3H), 4.38 (m, 1H), 4.05-4.22 (m, 3H), 3.60-3.82 (m, 4H), 3.41 (bs, 1H), 3.10 (dd, 2H of Et_3N), 2.28 (m, 4H), 1.84 (dd, 1H), 1.56 (m, 5H), 1.25 (m, 54H), 0.88 (t, 7H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 173.5, 173.1, 152.4, 147.7, 135.8, 134.2, 128.8, 124.5, 123.6, 122.0, 118.8, 112.2, 77.2, 70.0, 68.1, 66.5, 63.8, 62.4, 54.6, 46.5, 45.5, 38.5, 33.9, 33.0, 29.5, 29.4, 29.1, 28.9, 28.7, 25.0, 24.6, 23.5, 22.7, 22.5, 14.6, 13.8, 13.7, 13.2, 10.7, 8.1. HRMS calcd for $[M+H]^+$ 997.5796, found 997.5776.

EXAMPLE 9

In vivo testing of lipidated TLR7/8

TLR7/8 ligands may promote various aspects of the immune response in mice, noticeably the CD8 response. The difference in response between a TLR7/8 ligand (the "core" compound), and its corresponding lipidated derivative are investigated using techniques such as those described below.

Formulation of the compounds for a comparison study requires taking into account the different molecular weights of the core and lipidated molecules, (e.g. 45 and 4.5μg of the lipidated compound L3 corresponds to approximately to 15 and 1.5μg of the corresponding core compound, "L3 core"), allowing for the side by side comparison of the corresponding groups in the study. Higher doses of L3 (200μg) and of L3 core (150μg) are also tested. In one such study formulations summarized and described below (table 1) are used to vaccinate 6 -8 week old C57BL/6 (H2Kb), female mice (10/group). The mice receive two injections 14 days apart and are bled during weeks 1, 3 and 4 (for precise bleed days see Figure 1). The mice are vaccinated intramuscularly. A heterologous prime/boost using recombinant adenovirus coding for the SIV-p27 protein and adjuvanted p27 are used as control groups, the adenovirus is injected at a dose of 5 x 10^8 VP. The study design is represented in Figure 1.

description	p27	QS21	MPL	SB62c[b]	L3	L3 Core
QS/MPL (liposome based formulation)	5	5	5	-	-	-
QS/MPL+ 200μg L3	5	5	5	-	200	-
QS/MPL+ 45μg L3	5	5	5	-	45	-
QS/MPL+ 4.5μg L3	5	5	5	-	4.5	-
QS/MPL+ 150μg L3 core	5	5	5	-	-	150
QS/MPL + 15μg L3 core	5	5	5	-	-	15
QS/MPL + 1.5μg L3 core	5	5	5	-	-	1.5
QS/MPL/SB62C (emulsion-based formulation)	5	5	-	5 μl		
QS/MPL/SB62C+ 200μg L3	5	5	5	5 μl	200	-
QS/MPL/SB62C+ 45μg L3	5	5	5	5 μl	45	-
QS/MPL/SB62C+ 4.5μg L3	5	5	5	5 μl	4.5	-
QS/MPL/SB62C+ 150μg L3 core	5	5	5	5 μl	-	150
QS/MPL/SB62C + 15μg L3 core	5	5	5	5 μl	-	15
QS/MPL/SB62C + 1.5μg L3 core	5	5	5	5 μl	-	1.5
p27	5	-	-	-	-	-
naive	-	-	-	-	-	-

Table 1: Summary of the formulations
All compounds are in μg unless otherwise stated.
[b] SB62c contains the oil in water SB62 and cholesterol

In the study design molecules are formulated in either a liposome-based or an oil-in-water–based adjuvant composition containing QS21 and MPL immunostimulants. To assess the added value of TLR7/8L, the innate and adaptive immune responses induced by the formulations containing the TLR7/8L, QS21 and MPL are compared to the one induced by the corresponding QS21 and MPL containing formulations.

The induction of antigen-specific CD8 and CD4 responses is assessed by measuring intracellular cytokines 7 days after the second injection. Peripheral blood lymphocytes (PBLs) are stimulated in the presence of a pool of peptides encompassing the whole p27 antigen (15-mers peptides, overlap by 11). The secretion of cytokines is blocked by Brefeldin A and the presence of 3 cytokines (IFNγ, TNFα and IL2) is evaluated by flow cytometry after intracellular staining with appropriate antibodies.

Studies similar to that described above were performed using CRX-642 and its lipidated counterpart L3. Fig 2 and 3 show the p27-specific T-cells frequency observed 7 days after the second immunization. In a dose response manner, the p27- specific CD8 frequency was clearly increased when liposomes containing L3 were co-administered with MPL and QS-21 formulations as compared to the control formulation without TLR7/8L (Figure 2). Noticeably both the liposome-based formulation and the oil-in-water based formulations allowed for an increased in the CD8 response in presence of the lipidated TLR7/8L as compared to the corresponding control formulations without TLR7/8L. Furthermore the ability of generating cytokine-producing CD8 T cells was dependent on the lipidated nature of the TLR7/8 ligand, as the core molecule L3 core in contrast to L3 did not increase the response.

In complement to the evaluation of the induced CD8 response, antigen-specific cytotoxic activity may be assessed in vivo. Briefly, targets pulsed with p27 peptides spanning the whole protein and control unpulsed targets are injected into immunized mice and 24hrs after injection, the p27-specific cytotoxicity is assessed by the disappearance of the pulsed target.

Complementary cytotoxic activity studies were performed with the evaluation of induced CD8 response of L3 core and L3 explained above. A higher cytotoxic activity was detected in mice immunized with lipidated TLR7/8 ligand- based formulations than with in mice receiving a core TLR7/8 ligand-based formulations (Figure 3). This activity was higher than the one induced by control formulations based on QS21 and MPL only,

especially when high doses of lipidated TLR7/8L were used.

As shown for the CD8 response, the p27- specific CD4 frequency increased when liposomes containing L3 were co-administered with the liposome-based MPL and QS-21-containing formulation as compared to the control formulation, the response being dependent on the dose TLR7/8L injected (Fig. 4). As for the CD8 response, the core molecule L3 core was not able to induce a CD4 response over the one induced by the control formulations.

When administered in an emulsion-based formulation, the lipidated TLR7/8L was also able to increase the CD4 T-cell response over the level reached by the control formulation.

When lipidated, the added value of the TLR7/8 ligands within different formulations is shown by the increase up to 5-fold in cytokine-producing T cell frequency (both CD8 and CD4 T cells). Interestingly the cytokine profile of T-cell response was characterized by the high frequency of double positive T-cells ($IFN\gamma^{+}$ $TNF\alpha^{+}$).

Additional investigation illustrates the ability of lipidated TLR7/8 compounds to induce innate chemokines and pro-inflammatory cytokines among which type I IFN is known to be required for the programming of naïve CD8- T-cell (survival, differentiate and memory development). These cytokines are measured in the sera of mice 3 and 24hrs after the first injection (figures 5 and 6).

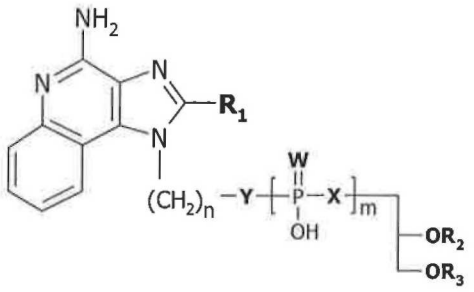
Results of cytokine profiles for L3 core and L3 show similar profile of cytokines are observed between the liposome-based and emulsion-based formulations. TLR7/8 ligands are known to induce IFNα due to their ability to stimulate plasmacytoid dendritic cells and IFNα was indeed detected in the serum of mice immunized with L3 in a dose-dependent manner and at higher level than for its core counterpart, L3 core. Low IL-12p70 production, close to background level, was also detected. Level of INFγ increased at low dose of L3 while other inflammatory cytokines such as TNFα or IL-6 were

enhanced when both L3 core and L3 were added to QS21 and MPL. The chemokines MCP-1 and MIG were both increased up to 10 fold with both compounds. Altogether, these data show that the tested lipidated molecule is as effective if not more potent than the corresponding core molecules to induce cytokine production in vivo.

5

CLAIMS

1. A compound having the following formula:



wherein

$\mathbf{R_1}$ = H, $C_{1\text{-}6}$ alkyl, $C_{1\text{-}3}$ alkoxy$C_{1\text{-}3}$ alkyl, $C_{3\text{-}6}$cycloalkyl$C_{1\text{-}3}$alkoxy, $C_{3\text{-}6}$cycloalkyl$C_{1\text{-}3}$alkyl,

n = 1-6,

X = O, CH_2, CF_2,

W = O or S,

m = 1-2,

$\mathbf{R_2}$ = H or straight/branched/unsaturated C_4-C_{24} alkyl or acyl,

$\mathbf{R_3}$ = straight/branched/unsaturated C_4-C_{24} alkyl or acyl
Y=O, NH.

2. A method for improving the adjuvant activity of an imidazoquinoline comprising conjugating the imidazoquinoline to a phospho- or phosphonolipid to obtain a compound as claimed in claim 1.

LIPIDATED IMIDAZOQUINOLINE DERIVATIVES

Abstract

The compounds of the subject invention are adjuvant molecules that comprise an imidazoquinoline molecule covalently linked to a phospho- or phosphonolipid group. The compounds of the invention have been shown to be inducers of interferon-a, IL-12 and other immunostimulatory cytokines and possess an improved activity profile in comparison to known cytokine inducers when used as adjuvants for vaccine antigens.

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