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(71) Applicant: **THE UNITED STATES OF AMERICA, AS REPRESENTED BY THE SECRETARY, DEPARTMENT OF HEALTH & HUMAN SERVICES** [US/US];
National Institutes of Health, Office of Technology Transfer, 6011 Executive Boulevard, Suite 325, MSC 7660, Bethesda, MD 20852-7660 (US).

(72) Inventor: **KLINMAN, Dennis M.**; Lab Experimental Immunology (HNC7U), Building 567 - FCRDC, Room 205, Fort Detrick, Frederick, MD 21702 (US).

(74) Agent: **SIEGEL, Susan, Alpert**; Klarquist Sparkman, LLP, One World Trade Center, Suite 1600, 121 SW Salmon Street, Portland, OR 97204 (US).

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(54) Title: METHODS FOR THE TREATMENT OR PREVENTION OF ISCHEMIC TISSUE DAMAGE

(57) Abstract: Methods are disclosed for inhibiting or reducing ischemic tissue damage in a subject. These methods include selecting a subject having ischemic tissue damage or at risk of developing ischemic tissue damage and administering to the subject a therapeutically effective amount of an imidazoquinoline compound. In some embodiments, the method also includes administering to the subject a therapeutically effective amount of an immunostimulatory composition comprising an immunostimulatory oligodeoxynucleotide and a pharmaceutically acceptable carrier prior to administering the imidazoquinoline compound to the subject. The immunostimulatory composition includes either a D-type CpG oligodeoxynucleotide or a K-type CpG oligodeoxynucleotide.



WO 2016/183371 A1

- 1 -

METHODS FOR THE TREATMENT OR PREVENTION OF ISCHEMIC TISSUE DAMAGE

CROSS REFERENCE TO RELATED APPLICATION

5 This claims the benefit of U.S. Provisional Application No. 62/160,836, filed May 13, 2015, which is incorporated by reference herein.

FIELD OF THE DISCLOSURE

 This relates to the field of ischemia, specifically to the use of an imidazoquinoline
10 compound and an immunostimulatory CpG oligodeoxynucleotide (ODN) to treat ischemic disorders such as, but not limited to, a stroke and/or a transient ischemic attack.

BACKGROUND

 Stroke is the second most common cause of death and the third most common cause of
15 disability-adjusted life-years (DALYs) worldwide. The global burden of stroke as measured by the number of people affected every year, stroke survivors, related deaths, and DALYs lost is increasing (Feigin et al., 2014, *Lancet Infect Dis* 14, 869-880). The immune system plays a critical role in the development and pathobiology of stroke. Inflammation and immunity have been linked to multiple risk factors in stroke including hypertension (Trott and Harrison, 2014, *Adv Physiol*
20 *Educ* 38, 20-24), atherosclerosis (Hansson and Hermansson, 2011, *Nat Immunol* 12, 204-212.), diabetes (Atkinson and Eisenbarth, 2001, *Lancet* 358, 221-229; Seijkens et al., 2014, *Diabetes* 63, 3982-3991), atrial fibrillation (Guo et al., 2012, *J Am Coll Cardiol* 60, 2263-2270), and tobacco smoke-induced vascular impairment (Mazzone et al., 2010, *Int J Environ Res Public Health* 7, 4111-4126). Stress and danger signals generated by strokes promote inflammatory and immune
25 responses that contribute to brain damage and neurological deficits (Iadecola and Anrather, 2011, *Nat Med* 17, 796-808; Fang et al., 2013, *J Neuroinflammation* 10, 27).

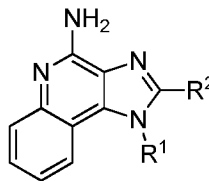
 Although therapies directed at early restoration of reperfusion have shown clear efficacy in Phase III clinical trials (Saver, 2011, *J Thromb Haemost* 9 Suppl 1, 333-343), decades of research focused on mechanisms of cytoprotection that permit brain cells to maintain homeostasis under
30 ischemic stress have uniformly failed to translate in clinical trials (O'Collins et al., 2006, *Ann Neurol* 59, 467-477). Despite the difficulty in effectively translating forms of cytoprotective therapy into the clinical arena, the basic and translational efforts of the international stroke basic

- 2 -

and translational science community have, over the years, massively advanced the understanding of stroke pathobiology. A need remains for therapeutic agents that can be used to treat stroke and other ischemic tissue damage.

SUMMARY OF THE DISCLOSURE

Methods are disclosed for inhibiting or reducing ischemic tissue damage in a subject. These methods include selecting a subject having ischemic tissue damage or at risk of developing ischemic tissue damage and administering to the subject a therapeutically effective amount of an imidazoquinoline compound having a formula:



wherein, R¹ is selected from -C₁₂₋₂₄alkyl, -OC₁₁₋₂₄alkyl, -NHC₁₁₋₂₄alkyl, and -X-Y-Z-R⁶, wherein

X is selected from the group consisting of a bond, -O-, and -NH-;

Y is selected from the group consisting of C₁₋₁₀alkyl and C₁₋₅alkyloxyC₁₋₅alkyl

Z is selected from the group consisting of:

-NHC(O) -,

-NHS(O)₂ -,

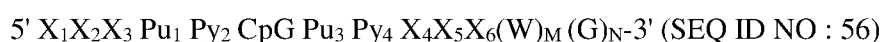
-NHC(O)NH-; and

R⁶ is -C₁₁₋₂₃alkyl;

R² is selected from the group consisting of hydrogen, alkyl, alkylaminoalkyl, alkoxyalkyl, and hydroxyalkyl, thereby inhibiting or reducing the ischemic tissue damage in the subject. In some embodiments, X is optional and/or R¹ is -COOH.

In some embodiments, the method also includes administering to the subject a therapeutically effective amount of an immunostimulatory composition comprising an immunostimulatory oligodeoxynucleotide and a pharmaceutically acceptable carrier prior to administering the imidazoquinoline compound to the subject. The immunostimulatory composition includes either:

a) a D-type CpG oligodeoxynucleotide that is least 18 nucleotides and no more than 30 nucleotides in length and comprises a sequence represented by the formula:



- 3 -

wherein the central CpG motif is unmethylated, Pu is a purine nucleotide, Py is a pyrimidine nucleotide, X and W are any nucleotide, M is any integer from 0 to 10, and N is 6, wherein $X_1X_2X_3$ and $X_4X_5X_6$ are self-complementary; or

b) a K-type CpG oligodeoxynucleotide that has a nucleic acid sequence set forth as:

5' $N_1N_2N_3D$ -CpG- $WN_4N_5N_6$ 3' (SEQ ID NO: 22)

wherein the central CpG motif is unmethylated, D is T, G or A, W is A or T, and N_1 , N_2 , N_3 , N_4 , N_5 , and N_6 are any nucleotide, wherein the CpG oligodeoxynucleotide is 10 to 30 nucleotides in length.

The foregoing and other features and advantages of the invention will become more apparent from the following detailed description of several embodiments which proceeds with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE FIGURES

Figs. 1A-1F. A151 reduced proinflammatory cytokine production and cell death in BMDM subjected to lipopolysaccharide (LPS) and oxygen glucose deprivation (OGD). Bone marrow derived macrophages (BMDM) were treated with OGD, with or without 1 ng/ml LPS, A151 or C151 for 18 hours. Interleukin (IL)- 1β (Fig. 1A), IL- 1α (Fig. 1B), IL-6 (Fig. 1C), cytokine induce neutrophil chemoattractant (CINC)-1 (Fig. 1D), tumor necrosis factor (TNF) α (Fig. 1E), and lactose dehydrogenase (LDH) (Fig. 1F) in cell culture supernatant were measured by Elisa. Data are presented as mean $\pm$ SEM from three replicates representative of three experiments (*, $P < 0.05$ compared with LPS treatment; **, $P < 0.05$ compared with LPS or C151 treatment).

Figs. 2A-2D. A151 reduced IL- 1β and caspase 1 maturation, and the expression of *NOD*-like receptor family, pyrin domain containing 3 (NLRP3) and inducible nitrous oxide synthetase (iNOS) in BMDM subjected to LPS and OGD. (Fig. 2A) A151 reduced mature IL- 1β in supernatant. (Fig. 2B) A151 reduced mature caspase 1 in supernatant. (Fig. 2C) A151 reduced NLRP3 in cell lysate. (Fig. 2D) A151 reduced iNOS in cell lysate. Data are presented as mean $\pm$ SEM from three replicates representative of three experiments (**, $P < 0.05$ compared with LPS or C151 treatment).

Figs. 3A-3E. A151 reduced brain ischemic injury in SHR-SP rats 48 hours after pMCAO. The rats in saline groups were combined for analysis as they were not statistically different. (Fig.

- 4 -

3A) Representative coronal brain sections stained with cresyl violet. (Fig. 3B) A151 reduced infarct volumes in male rats. (Fig. 3C) A151 reduced infarct volumes in female rats. (Fig. 3D) A151 improved performance in forepaw test in female rats. (Fig. 3E) A151 reduced brain NLRP3 mRNA 48 hours after pMCAO, the error bars represent the 95th upper and lower confidence intervals of gene expression. (n = 7-17 per group; *, $P < 0.05$ compared with saline control; **, $P < 0.05$ compared with saline control or C151).

Figs. 4A-4B. A151 (SEQ ID NO: 1) reduced depolarization of mitochondrial membrane potential (MMP) in BMDM subjected to LPS and OGD. (A) FACS analysis of cells stained with JC-1. (B) The percentage of cells with depolarized MMP was reduced by A151 treatment. Data are presented as mean $\pm$ SEM from three replicates representative of three independent experiments (**, $p < 0.05$ compared with control or C151 treatment).

SEQUENCE LISTING

The nucleic and amino acid sequences are shown using standard letter abbreviations for nucleotide bases, and three letter code for amino acids, as defined in 37 C.F.R. 1.822. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included by any reference to the displayed strand when appropriate. The Sequence Listing is submitted as an ASCII text file [Sequence_Listing, May 12, 2016, 19,819 bytes], which is incorporated by reference herein. In the accompanying sequence listing:

SEQ ID NOs: 1-20 are nucleic acid sequences of suppressive ODN.

SEQ ID NOs: 21 is the nucleic acid sequences of a control ODN.

SEQ ID NO: 22 is a consensus nucleic acid sequence for K-type CpG ODNs.

SEQ ID NOs: 23-53 are nucleic acid sequences of K-type CpG ODNs.

SEQ ID NOs: 54 and 55 are the nucleic acid sequences of control ODNs.

SEQ ID NO: 56 is a consensus nucleic acid sequence for D-type CpG ODN.

SEQ ID NOs: 57-82 are nucleic acid sequences of D-type CpG ODN.

SEQ ID NOs: 83-87 are nucleic acid sequences of C-type ODN.

SEQ ID NO: 88 is the nucleic acid sequence of a control ODN.

DETAILED DESCRIPTION OF SEVERAL EMBODIMENTS

Methods are disclosed herein for inhibiting or reducing ischemic tissue damage in a subject.

- 5 -

The methods include administering to a subject a therapeutically effective amount of a TLR-9 agonist, such as a CpG ODN, and a therapeutically effective amount of a TLR-7 and/or TLR-8 agonist, such as an imidazoquinoline compound, such as a lipophilic imidazoquinoline compound. These compounds act synergistically to inhibit ischemic tissue damage in the subject.

5 In some embodiments, the methods include selecting a subject having ischemic tissue damage or at risk of developing ischemic tissue damage and administering to the subject an amount of an immunosuppressive composition comprising a therapeutically effective amount of an imidzoquinoline compound and a pharmaceutically acceptable carrier. In some embodiments, the methods can also include administering to the subject a therapeutically effective amount of an
10 immunostimulatory composition comprising an immunostimulatory ODN and a pharmaceutically acceptable carrier prior to administering the imidzoquinoline compound to the subject. In some examples, the methods include administering one or more K-type or D-type CpG oligodeoxynucleotides (ODNs). In further examples, the CpD ODN is a C-type ODN.

The methods can also include administering to the subject an immunosuppressive
15 composition comprising a therapeutically effective amount of a suppressive ODN and a pharmaceutically acceptable carrier.

Terms

Alkyl: A saturated or unsaturated monovalent hydrocarbon radical having a number of
20 carbon atoms ranging from one to 30 (e.g., C₁₋₃₀ alkyl), which is derived from removing one hydrogen atom from one carbon atom of a parent compound (e.g., alkane, alkene, alkyne). An alkyl group may be branched, straight-chain, or cyclic.

Alkenyl: An unsaturated monovalent hydrocarbon radical having a number of carbon atoms ranging from two to 30 (e.g., C₂₋₃₀ alkenyl), which has at least one carbon-carbon double bond and
25 is derived from removing one hydrogen atom from one carbon atom of a parent alkene. An alkenyl group may be branched, straight-chain, cyclic, cis, or trans.

Alkynyl: A unsaturated monovalent hydrocarbon radical having a number of carbon atoms ranging from two to 30 (e.g., C₂₋₃₀ alkynyl), which has at least one carbon-carbon triple bond and is derived from removing one hydrogen atom from one carbon atom of a parent alkyne. An alkynyl
30 group may be branched, straight-chain, or cyclic.

Administration: Administration of an active compound or composition can be by any route known to one of skill in the art. Administration can be local or systemic. Examples of local

- 6 -

administration include, but are not limited to, topical administration, subcutaneous administration, intramuscular administration, intrathecal administration, intrapericardial administration, intra-ocular administration, topical ophthalmic administration, or administration to the nasal mucosa or lungs by inhalational administration. In addition, local administration includes routes of

administration typically used for systemic administration, for example by directing intravascular administration to the arterial supply for a particular organ. Thus, in particular embodiments, local administration includes intra-arterial administration and intravenous administration when such administration is targeted to the vasculature supplying a particular organ. Local administration also includes intra-theal injection, intra-cranial injection or delivery to the cerebral spinal fluid.

Systemic administration includes any route of administration designed to distribute an active compound or composition widely throughout the body via the circulatory system. Thus, systemic administration includes, but is not limited to intra-arterial and intravenous administration. Systemic administration also includes, but is not limited to, oral administration, subcutaneous administration, intramuscular administration, or parenteral administration, when such administration is directed at absorption and distribution throughout the body by the circulatory system.

Anti-Inflammatory Agent: Any of various medications that decrease the signs and symptoms (for example, pain, swelling, or shortness of breath) of inflammation. Corticosteroids are exemplary potent anti-inflammatory medications. Nonsteroidal anti-inflammatory agents are also effective exemplary anti-inflammatory agents and do not have the side effects that can be associated with steroid medications.

Animal: Living multi-cellular vertebrate organisms, a category that includes, for example, mammals and birds. The term mammal includes both human and non-human mammals. Similarly, the term “subject” includes both human and veterinary subjects.

Atherosclerosis: The progressive narrowing and hardening of a blood vessel over time.

Atherosclerosis is a common form of arteriosclerosis in which deposits of yellowish plaques (atheromas) containing cholesterol, lipoid material and lipophages are formed within the intima and inner media of large and medium-sized arteries. Treatment of atherosclerosis includes reversing or slowing the progression of atherosclerosis, for example as measured by the presence of atherosclerotic lesions and/or functional signs of the disease, such as improvement in cardiovascular function as measured by signs (such as peripheral capillary refill), symptoms (such as chest pain and intermittent claudication), or laboratory evidence (such as that obtained by EKG, angiography, or other imaging techniques).

- 7 -

“C” class oligodeoxynucleotides (ODNs): ODNs that resemble K ODNs and are composed of only phosphorothioate nucleotides. Typically, C class ODNs have a TCGTCG motif at the 5' end and have a CpG motif imbedded in a palindromic sequence. Backbone modifications like 2'-O-methyl modifications especially in the 5' part of the ODN influence IFN- α -producing capacity of these ODN. C class ODNs have combined properties of D- and K-type CpG ODNs. This class of ODNs stimulates B cells to secrete IL-6 and stimulates plasmacytoid dendritic cells to produce interferon- α . C class ODNs also induce IP-10 production and strong NK activation.

Cerebral ischemia or stroke: A condition that occurs when an artery to the brain is partially or completely blocked such that the oxygen demand of the tissue exceeds the oxygen supplied. Deprived of oxygen and other nutrients following an ischemic stroke, the brain suffers damage and cell death as a result of the stroke.

Ischemic stroke can be caused by several different kinds of diseases. The most common problem is narrowing of the arteries in the neck or head. This is most often caused by atherosclerosis, or gradual cholesterol deposition. If the arteries become too narrow, blood cells may collect in them and form blood clots (thrombi). These blood clots can block the artery where they are formed (thrombosis), or can dislodge and become trapped in arteries closer to the brain (embolism). Also cerebral stroke can occur when atherosclerotic plaque separates away partially from the vessel wall and occludes the flow of blood through the blood vessel.

Another cause of stroke is blood clots in the heart, which can occur as a result of irregular heartbeat (for example, atrial fibrillation), heart attack, prior mural clot formation within a heart chamber and abnormalities of the heart valves such as occurs from rheumatic fever. While these are the most common causes of ischemic stroke, there are many other possible causes. Examples include use of street drugs, traumatic injury to the blood vessels of the neck, or disorders of blood clotting.

Ischemic stroke is by far the most common kind of stroke, accounting for about 80% of all strokes. Stroke can affect people of all ages, including children. Many people with ischemic strokes are older (60 or more years old, or 65 years or older), and the risk of stroke increases with older ages. At each age, stroke is more common in men than women, and it is more common among African-Americans than white Americans. Many people with stroke have other problems or conditions which put them at higher risk for stroke, such as high blood pressure (hypertension), heart disease, smoking, or diabetes. Subjects with cerebral ischemia can benefit from angiogenic therapy and therapies which prevents or reduces atherosclerotic plaque in the major arteries of the

- 8 -

body.

Chemokine: A type of cytokine (a soluble molecule that a cell produces to control reactions between other cells) that specifically alters the behavior of leukocytes (white blood cells). Examples include, but are not limited to, interleukin 8 (IL-8), platelet factor 4, melanoma growth stimulatory protein, etc.

Circular Dichroism (CD) value: The formation of G-tetrads yields a complex with different physical properties than the individual oligonucleotides. Spectroscopically, this is manifested by an increase in circular dichroism (CD), and an increase in peak absorbance to the 260-280 nm wavelength owing to the formation of secondary structures. In one embodiment, a method for identifying oligonucleotides that form G-tetrads is to assess the **CD values**. An increase in peak ellipticity values to greater than 2.0 is typical of a G-tetrad forming oligonucleotide. The higher the ellipticity value, the greater the tetrad-forming capacity of the oligonucleotide.

CpG or CpG motif: A nucleic acid having a cytosine followed by a guanine linked by a phosphate bond in which the pyrimidine ring of the cytosine is unmethylated. The term "methylated CpG" refers to the methylation of the cytosine on the pyrimidine ring, usually occurring at the 5-position of the pyrimidine ring. A CpG motif is a pattern of bases that include an unmethylated central CpG surrounded by at least one base flanking (on the 3' and the 5' side of) the central CpG. Without being bound by theory, the bases flanking the CpG confer a significant part of the activity to the CpG oligodeoxynucleotide. A CpG oligodeoxynucleotide is an oligodeoxynucleotide that is at least about ten nucleotides in length and includes an unmethylated CpG. CpG oligodeoxynucleotides include both D and K-type oligodeoxynucleotides (see below). CpG oligodeoxynucleotides are single-stranded. The entire CpG oligodeoxynucleotide can be unmethylated or portions may be unmethylated. In one embodiment, at least the C of the 5' CG 3' is unmethylated.

Coronary Artery Disease: In coronary artery disease, the coronary arteries become narrowed (stenosed) or blocked (occluded) by a gradual build-up of fat (cholesterol) within or on the artery wall, which reduces blood flow to the heart muscle. This build-up is called atherosclerotic plaque or simply plaque.

If plaque narrows the lumen or channel of the artery, it may make it difficult for adequate quantities of blood to flow to the heart muscle. If the build-up reduces flow only mildly, there may be no noticeable symptoms at rest, but symptoms such as chest pressure may occur with increased activity or stress. Other symptoms include heartburn, nausea, vomiting, shortness of breath and

- 9 -

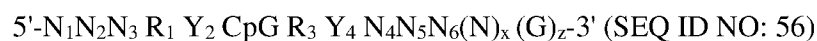
heavy sweating.

When flow is significantly reduced and the heart muscle does not receive enough blood flow to meet its needs (cardiac ischemia), severe symptoms such as chest pain (angina pectoris), heart attack (myocardial infarction), or rhythm disturbances (arrhythmias) may occur. A heart attack usually is the result of a completely blocked artery, which may damage the heart muscle.

There are three conventional ways to treat atherosclerotic disease: medication, surgery, and minimally invasive interventional procedures such as stent implantation, percutaneous transluminal coronary angioplasty (PTCA), intravascular radiotherapy, atherectomy and excimer laser. The purpose of these treatments is to eliminate or reduce atherosclerotic narrowing of the coronary blood vessels and hence eliminate or reduce symptoms, and in the case of coronary artery disease, decrease the risk of heart attack.

Cytokine: The term “cytokine” is used as a generic name for a diverse group of soluble proteins and peptides that act as humoral regulators at nano- to picomolar concentrations and which, either under normal or pathological conditions, modulate the functional activities of individual cells and tissues. These proteins also mediate interactions between cells directly and regulate processes taking place in the extracellular environment. Examples of cytokines include, but are not limited to, tumor necrosis factor α (TNF α), interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-12

D-type Oligodeoxynucleotide (D ODN): A D-type ODN is at least about 16 nucleotides in length, such as 16 to 30 nucleotides in length, and includes a sequence represented by the following formula:



wherein the central CpG motif is unmethylated, R is a purine nucleotide, Y is a pyrimidine nucleotide, N is any nucleotide, X is any integer from 0 to 10, and Z is any integer from 4 to 10.

Additional detailed description of D ODN sequences and their activities can be found in Verthelyi et al., *J. Immunol.* 166:2372-2377, 2001, which is herein incorporated by reference. Generally D ODNs can stimulate a cellular immune response.

Elderly: An aged subject, who has passed middle age. In one embodiment, an elderly mammalian subject is a subject that has survived more than two-thirds of the normal lifespan for that mammalian species. In a further embodiment, for humans, an aged or elderly subject is more than 65 years of age, such as a subject of more than 70, more than 75, more than 80 years of age. In

- 10 -

yet another embodiment, for mice, an elderly mouse is from about 14 to about 18 months of age.

G-tetrad: G-tetrads are G-rich DNA segments that can accommodate complex secondary and/or tertiary structures (see Fig. 1). A G-tetrad involves the planar association of four Gs in a cyclic Hoogsteen hydrogen bonding arrangement (this involves non-Watson Crick base-pairing).

5 In general, either a run of four or more contiguous Gs or a hexameric region in which >50% of the bases are Gs, is needed for an ODN to form a G-tetrad. The longer the run of contiguous Gs, and the higher the G content of the ODN, the higher the likelihood of G-tetrad formation, as reflected by higher CD or ellipticity values.

Oligonucleotides that form G-tetrads can also form higher-level aggregates that are more easily recognized and taken up by immune cells, for example, through scavenger receptors or by nucleolin.

Guanosine-rich sequence: A hexameric region of a nucleotide sequence in which >50% of the bases are Gs.

Hypoxia: Deficiency in the amount of oxygen reaching body tissues. Hypoxia may occur concurrently with ischemia (lack of blood flow) due to loss of circulation to a specific tissue, organ or complete circulatory collapse; or without ischemia, as when secondary to a problem in ventilation alone.

Immune response: A response of a cell of the immune system, such as a B cell or T cell to a stimulus. In one embodiment, the response is an inflammatory response.

20 **Immunostimulatory CpG motifs:** Immunostimulatory sequences that trigger macrophages, monocytes and lymphocytes to produce a variety of pro-inflammatory cytokines and chemokines. CpG motifs are found in bacterial DNA. The innate immune response elicited by CpG DNA reduces host susceptibility to infectious pathogens, and can also trigger detrimental inflammatory reactions. Immunostimulatory CpG motifs are found in "D" and "K" type ODNs
25 (see, for example PCT Publication No. WO 01/51500, published on July 19, 2001).

Infarct: An area of tissue death due to a local lack of oxygen.

Infiltration: The diffusion or accumulation of a substance, such as a neutrophil, in a tissue or cell.

Inflammation: A localized protective response elicited by injury to tissue that serves to sequester the inflammatory agent. Inflammation is characterized by the appearance in or migration
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- 11 -

into any tissue space, unit or region of any class of leukocyte in numbers that exceed the number of such cells found within such region of tissue under normal (healthy) circumstances. Inflammation is orchestrated by a complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells, or irritants. It is a protective attempt by the organism to remove the
5 injurious stimuli as well as initiate the healing process for the tissue. An **inflammatory response** is an accumulation of white blood cells, either systemically or locally at the site of inflammation. The inflammatory response may be measured by many methods well known in the art, such as the number of white blood cells, the number of polymorphonuclear neutrophils (PMN), a measure of the degree of PMN activation, such as luminal enhanced-chemiluminescence, or a measure of the
10 amount of cytokines present. Inflammation can be classified as either acute or chronic. Acute inflammation is the initial response of the body to harmful stimuli and is achieved by the increased movement of plasma and leukocytes from the blood into the injured tissues. A cascade of biochemical events propagates and matures the inflammatory response, involving the local vascular system, the immune system, and various cells within the injured tissue. Prolonged inflammation,
15 known as **chronic inflammation**, leads to a progressive shift in the type of cells which are present at the site of inflammation and is characterized by simultaneous destruction and healing of the tissue from the inflammatory process.

Inflammosomes: Multi-protein complexes activated as part of the innate immune response to stress or infection that trigger the maturation of caspase-1 followed by the production of IL-1 β
20 and IL-18.

Ischemia: A vascular phenomenon in which a decrease in the blood supply to a bodily organ, tissue, or part is caused, for instance, by constriction or obstruction of one or more blood vessels. Ischemia sometimes results from vasoconstriction or thrombosis or embolism. Ischemia can lead to direct ischemic injury, tissue damage due to cell death caused by reduced oxygen
25 supply. Ischemia can occur acutely, as during surgery, or from trauma to tissue incurred in accidents, injuries and war settings, for instance. It can also occur sub-acutely, as found in atherosclerotic peripheral vascular disease, where progressive narrowing of blood vessels leads to inadequate blood flow to tissues and organs.

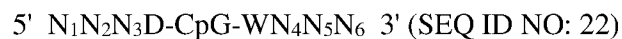
Ischemia/reperfusion injury: In addition to the immediate injury that occurs during
30 deprivation of blood flow, ischemic/reperfusion injury involves tissue injury that occurs after blood flow is restored. Without being bound by theory, it is believed that much of this injury is caused by chemical products and free radicals released into the ischemic tissues.

- 12 -

When a tissue is subjected to ischemia, a sequence of chemical events is initiated that may ultimately lead to cellular dysfunction and necrosis. If ischemia is ended by the restoration of blood flow, a second series of injurious events ensue, producing additional injury. Thus, whenever there is a transient decrease or interruption of blood flow in a subject, the resultant injury involves two components - the direct injury occurring during the ischemic interval and the indirect or reperfusion injury that follows. When there is a long duration of ischemia, the direct ischemic damage, resulting from hypoxia, is predominant. For relatively short duration ischemia, the indirect or reperfusion mediated damage becomes increasingly important. In some instances, the injury produced by reperfusion can be more severe than the injury induced by ischemia *per se*. This pattern of relative contribution of injury from direct and indirect mechanisms has been shown to occur in all organs.

Isolated: An “isolated” biological component (such as a nucleic acid, peptide or protein) has been substantially separated, produced apart from, or purified away from other biological components in the cell of the organism in which the component naturally occurs, *i.e.*, other chromosomal and extrachromosomal DNA and RNA, and proteins. Nucleic acids, peptides and proteins which have been “isolated” thus include nucleic acids and proteins purified by standard purification methods. The term also embraces nucleic acids, peptides and proteins prepared by recombinant expression in a host cell as well as chemically synthesized nucleic acids.

K-type CpG Oligodeoxynucleotide (K ODN): An ODN including an unmethylated CpG motif that has a sequence represented by the formula:



wherein the central CpG motif is unmethylated, D is T, G or A, W is A or T, and N₁, N₂, N₃, N₄, N₅, and N₆ are any nucleotides. In one embodiment, D is a T. Additional detailed description of K ODN sequences and their activities can be found in the description below. Generally K ODNs can stimulate a humoral response. For example, K ODNs stimulate the production of immunoglobulins, such as IgM and IgG. K ODNs can also stimulate proliferation of peripheral blood mononuclear cells and increase expression of IL-6 and/or IL-12, amongst other activities. In several embodiments, K ODNs are about 10 to about 30 nucleotides in length.

Macrophage: A monocyte that has left the circulation and settled and matured in a tissue. Macrophages are found in large quantities in the spleen, lymph nodes, alveoli, and tonsils. About 50% of all macrophages are found in the liver as Kupffer cells. They are also present in the brain as microglia, in the skin as Langerhans cells, in bone as osteoclasts, as well as in seous cavities and

breast and placental tissue.

Along with neutrophils, macrophages are the major phagocytic cells of the immune system. They have the ability to recognize and ingest foreign antigens through receptors on the surface of their cell membranes; these antigens are then destroyed by lysosomes. Their placement in the peripheral lymphoid tissues enables macrophages to serve as the major scavengers of the blood, clearing it of abnormal or old cells and cellular debris as well as pathogenic organisms. Macrophages also serve a vital role by processing antigens and presenting them to T cells, activating the specific immune response. They also release many chemical mediators that are involved in the body's defenses, including interleukin-1.

Nucleic acid: A deoxyribonucleotide or ribonucleotide polymer in either single or double stranded form, and unless otherwise limited, encompasses known analogues of natural nucleotides that hybridize to nucleic acids in a manner similar to naturally occurring nucleotides.

Oligonucleotide or "oligo": Multiple nucleotides (*i.e.*, molecules comprising a sugar (*e.g.*, ribose or deoxyribose) linked to a phosphate group and to an exchangeable organic base, which is either a substituted pyrimidine (Py) (*e.g.*, cytosine (C), thymine (T) or uracil (U)) or a substituted purine (Pu) (*e.g.*, adenine (A) or guanine (G)). The term "oligonucleotide" as used herein refers to both oligoribonucleotides (ORNs) and oligodeoxyribonucleotides (ODNs). The term "oligonucleotide" also includes oligonucleosides (*i.e.*, an oligonucleotide minus the phosphate) and any other organic base polymer. Oligonucleotides can be obtained from existing nucleic acid sources (*e.g.*, genomic or cDNA), but are preferably synthetic (*i.e.*, produced by oligonucleotide synthesis).

A "stabilized oligonucleotide" is an oligonucleotide that is relatively resistant to *in vivo* degradation (for example via an *exo*- or *endo*-nuclease). In one embodiment, a stabilized oligonucleotide has a modified phosphate backbone. One specific, non-limiting example of a stabilized oligonucleotide has a phosphorothioate modified phosphate backbone (wherein at least one of the phosphate oxygens is replaced by sulfur). Other stabilized oligonucleotides include: nonionic DNA analogs, such as alkyl- and aryl- phosphonates (in which the charged phosphonate oxygen is replaced by an alkyl or aryl group), phosphodiester and alkylphosphotriesters, in which the charged oxygen moiety is alkylated. Oligonucleotides which contain a diol, such as tetraethyleneglycol or hexaethyleneglycol, at either or both termini have also been shown to be substantially resistant to nuclease degradation.

- 14 -

An “immunostimulatory oligonucleotide,” “immunostimulatory CpG containing ODN,” “CpG ODN,” refers to an ODN, which contains a cytosine, guanine dinucleotide sequence. In one embodiment, CpG ODN stimulates (e.g. has a mitogenic effect or induces cytokine production) vertebrate immune cells. CpG ODN can also stimulate angiogenesis. The cytosine, guanine is
5 unmethylated. This includes K and D ODN.

An “oligonucleotide delivery complex” is an oligonucleotide associated with (e.g., ionically or covalently bound to; or encapsulated within) a targeting means (e.g., a molecule that results in a higher affinity binding to a target cell (e.g., B-cell or natural killer (NK) cell) surface and/or increased cellular uptake by target cells). Examples of oligonucleotide delivery complexes include
10 oligonucleotides associated with: a sterol (e.g., cholesterol), a lipid (e.g., cationic lipid, virosome or liposome), or a target cell specific binding agent (e.g., a ligand recognized by a target cell specific receptor). Preferred complexes must be sufficiently stable *in vivo* to prevent significant uncoupling prior to internalization by the target cell. However, the complex should be cleavable or otherwise accessible under appropriate conditions within the cell so that the oligonucleotide is functional.

15 (Gursel, *J. Immunol.* 167: 3324, 2001).

Parenteral: Administered outside of the intestine, e.g., not via the alimentary tract.

Generally, parenteral formulations are those that will be administered through any possible mode except ingestion. This term especially refers to injections, whether administered intravenously, intrathecally, intramuscularly, intraperitoneally, intraarticularly, or subcutaneously, and various
20 surface applications including intranasal, intradermal, and topical application, for instance.

Peripheral Vascular Disease (PVD): A condition in which the arteries and/or veins that carry blood to and from the arms, legs, soft tissues and vital organs of the body, including the heart and brain, become narrowed or occluded. This interferes with the normal flow of blood, sometimes causing pain but often causing no readily detectable symptoms. With progression of PVD,
25 significant loss of blood flow to tissue and organs can lead to tissue death, necrosis and organ death.

The most common cause of PVD is atherosclerosis, a gradual process in which cholesterol and scar tissue build up, forming plaques that occlude the blood vessels. In some cases, PVD may be caused by blood clots that lodge in the arteries and restrict blood flow.

- 15 -

PVD affects about one in 20 people over the age of 50, or 8 million people in the United States. More than half the people with PVD experience leg pain, numbness or other symptoms, but many people dismiss these signs as a normal part of aging and do not seek medical help.

5 The most common symptom of PVD is painful cramping in the leg or hip, particularly when walking. This symptom, also known as claudication, occurs when there is not enough blood flowing to the leg muscles during exercise, such that ischemia occurs. The pain typically goes away when the muscles are rested.

10 Other symptoms may include numbness, tingling or weakness in the leg. In severe cases, people with PVD may experience a burning or aching pain in an extremity such as the foot or toes while resting, or may develop a sore on the leg or foot that does not heal. People with PVD also may experience a cooling or color change in the skin of the legs or feet, or loss of hair on the legs. In extreme cases, untreated PVD can lead to gangrene, a serious condition that may require amputation of a leg, foot or toes. People with PVD are also at higher risk for heart disease and stroke.

15 Typically most symptomatic PVD is ascribed to peripheral artery disease (PAD) denoting the above described pathology predominantly in arteries. The term PVD includes this symptomology and pathology in all classes of blood vessels.

Pharmaceutical agent or drug: A chemical compound or composition capable of inducing a desired therapeutic or prophylactic effect when properly administered to a subject.

20 Pharmaceutical agents include, but are not limited to, anti-infective agents, anti-inflammatory agents, bronchodilators, enzymes, expectorants, leukotriene antagonists, leukotriene formation inhibitors, and mast cell stabilizers.

Pharmaceutically acceptable carriers: The pharmaceutically acceptable carriers useful in this disclosure are conventional. *Remington's Pharmaceutical Sciences*, by E. W. Martin, Mack
25 Publishing Co., Easton, PA, 15th Edition (1975), describes compositions and formulations suitable for pharmaceutical delivery of the suppressive ODNs herein disclosed.

In general, the nature of the carrier will depend on the particular mode of administration being employed. For instance, parenteral formulations usually comprise injectable fluids that include pharmaceutically and physiologically acceptable fluids such as water, physiological saline,
30 balanced salt solutions, aqueous dextrose, glycerol or the like as a vehicle. For solid compositions (e.g., powder, pill, tablet, or capsule forms), conventional non-toxic solid carriers can include, for example, pharmaceutical grades of mannitol, lactose, starch, or magnesium stearate. In addition to

- 16 -

biologically-neutral carriers, pharmaceutical compositions to be administered can contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate.

Preventing or treating a disease: Inhibiting a disease refers to inhibiting the full

5 development of a disease, for example in a person who is at risk for a disease such as stroke. An example of a person at risk for stroke is someone with a family or personal history of stroke, with atherosclerosis, hypertension, or with other vascular disease. Inhibiting a disease process includes preventing the development of the disease. "Treatment" refers to a therapeutic intervention that ameliorates a sign or symptom of a disease or pathological condition, such as after it has begun to
10 develop.

Self-complementary nucleic acid sequence: A nucleic acid sequence that can form Watson-Crick base pairs. The four bases characteristic of deoxyribonucleic acid unit of DNA are the purines (adenine and guanine) and the pyrimidines (cytosine and thymine). Adenine pairs with thymine via two hydrogen bonds, while guanine pairs with cytosine via three hydrogen bonds. If a
15 nucleic acid sequence includes two or more bases in sequence that can form hydrogen bonds with two or more other bases in the same nucleic acid sequence, then the nucleic acid includes a self-complementary sequence. In several embodiments, a self-complementary nucleic acid sequence includes 3, 4, 5, 6 or more bases that could form hydrogen bonds with 3, 4, 5, 6 or more bases, respectively, of the same nucleic acid sequence.

Specific binding: Binding which occurs between such paired species as enzyme/substrate, receptor/agonist, receptor/ligand, antibody/antigen, and lectin/carbohydrate which may be mediated by covalent or non-covalent interactions or a combination of covalent and non-covalent interactions. When the interaction of the two species produces a non-covalently bound complex, the binding that occurs is typically electrostatic, hydrogen-bonding, or the result of lipophilic
25 interactions. Accordingly, "specific binding" occurs between a paired species where there is interaction between the two that produces a bound complex having the characteristics of an antibody/antigen or enzyme/substrate interaction. In particular, the specific binding is characterized by the binding of one member of a pair to a particular species and to no other species within the family of compounds to which the corresponding member of the binding member belongs. Thus,
30 for example, an antibody preferably binds to a single epitope and to no other epitope within the family of proteins. A TLR-9 agonist binds to TLR-9 and not to other TLRs, such as TLR-7 or TLR-8. Similarly, a TLR-7 agonist binds to TLR-7 and not to other TLRs, such as TLR-9.

- 17 -

Substituted: A fundamental compound, such as an aryl or aliphatic compound, or a radical thereof, having coupled thereto, typically in place of a hydrogen atom, a second substituent. For example, substituted aryl compounds or substituents may have an aliphatic group coupled to the closed ring of the aryl base, such as with toluene. Again solely by way of example and without
5 limitation, a long-chain hydrocarbon may have a substituent bonded thereto, such as an aryl group, a cyclic group, a heteroaryl group or a heterocyclic group.

Suppressive ODN: DNA molecules of at least eight nucleotides in length, such as about 8 to about 40 nucleotides in length or about ten to about 30 nucleotides in length, wherein the ODN has at least four guanosines, and has a CD value of greater than about 2.9 and suppresses an
10 immune response in a subject. Generally, a suppressive ODN has at least four guanonsines. In additional embodiment, a suppressive ODN includes repeats of the nucleic acid sequence TTAGGG. Exemplary suppressive ODN are described below. In one embodiment, a suppressive ODN inhibits the generation of reactive oxygen intermediates, such as by macrophages.

Therapeutic agent: Used in a generic sense, it includes treating agents, prophylactic
15 agents, and replacement agents.

Therapeutically effective amount: A quantity of a specified compound such as an imidazoquinoline compound, ODN sufficient to achieve a desired effect in a subject being treated. For instance, this can be the amount of a suppressive ODN necessary to suppress CpG-induced immune cell activation in a subject, or a dose sufficient to prevent advancement, or to cause
20 regression of a disease, or which is capable of relieving symptoms caused by a disease.

A therapeutically effective amount of a therapeutic agent can be administered systemically or locally. In addition, an effective amount of a therapeutic agent can be administered in a single dose, or in several doses, for example daily, during a course of treatment. However, the effective amount of the agent will be dependent on the preparation applied, the subject being treated, the
25 severity and type of the affliction, and the manner of administration of the compound. For example, a therapeutically effective amount of an ODN can vary from about 0.01 mg/kg body weight to about 1 g/kg body weight in some specific, non-limiting examples, or from about 0.01 mg/kg to about 60 mg/kg of body weight, based on efficacy.

The methods disclosed herein have equal applications in medical and veterinary settings.
30 Therefore, the general term “subject” is understood to include all animals, including, but not limited to, humans or veterinary subjects, such as other primates, dogs, cats, horses, and cows.

- 18 -

Vasculopathy: A disease of the blood vessels. An “age-related vasculopathy” is a disease of the blood vessels that is associated with advanced age. One specific, non-limiting vasculopathy is atherosclerosis. Other vasculopathies include, but are not limited to, diabetic associated vasculopathy, hypertension associated vasculopathy, Burger’s disease associated vasculopathy and scleroderma associated vasculopathy. It is understood that “endothelial dysfunction” typically refers to an insufficiency in the production or response to nitric oxide.

Unless otherwise explained, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. The singular terms “a,” “an,” and “the” include plural referents unless context clearly indicates otherwise. Similarly, the word “or” is intended to include “and” unless the context clearly indicates otherwise. It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of this disclosure, suitable methods and materials are described below. The term “comprises” means “includes.” All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including explanations of terms, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Description of Several Embodiments

Methods are disclosed herein for inhibiting or reducing ischemic tissue damage in a subject. The method includes selecting a subject having ischemic tissue damage or at risk of developing ischemic tissue damage and administering to the subject an immunosuppressive composition including a therapeutically effective amount of an imidazoquinoline compound and a pharmaceutically acceptable carrier, thereby inhibiting the ischemic tissue damage in the subject. Exemplary imidazoquinoline compounds are disclosed below. The method can inhibit development of ischemic damage, or can reduce ischemic tissue damage from an event. In some embodiments, the disclosed methods are of use to reduce the size of the tissue damage and/or reducing the symptoms caused by the tissue damage. The subject can be male or female.

In some embodiments, the subject is at risk for ischemic tissue damage, and the imidazoquinoline compound is administered from about six hours prior to the ischemic tissue

- 19 -

damage to about 24 hours after the ischemic tissue damage. In other embodiments, the subject is at risk for ischemic tissue damage, and the imidazoquinoline compound is administered at any time from about three hours prior to the ischemic tissue damage to about 24 hours after the ischemic tissue damage. In further embodiments, the subject is at risk for ischemic tissue damage, and the imidazoquinoline compound is administered at any time from about three hours prior to the ischemic tissue damage to about 12 hours after the ischemic tissue damage. In yet other examples, the subject is at risk for ischemic tissue damage, and the imidazoquinoline compound is administered at any time from about three hours prior to the ischemic tissue damage to the time of the ischemic tissue damage.

Thus, in some examples, the imidazoquinoline compound can be administered at about 6, about 5, about 4, about 3, about 2 hours prior to the ischemic tissue damage. In other examples, the imidazoquinoline compound can be administered about 60 minutes, about 45 minutes, about 30 minutes, about 15 minutes, about 10 minutes, or about 5 minutes prior to ischemic tissue damage. In further examples, the imidazoquinoline compound can be administered about 5 minutes, about 10 minutes, about 15 minutes, about 30 minutes, about 45 minutes, or about 60 minutes after ischemic tissue damage. In other examples, the imidazoquinoline compound is administered about 1, about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, about 20, about 21, about 22, about 23 or about 24 hours after ischemic tissue damage.

In some embodiments, the method can include administering to the subject a therapeutically effective amount of an immunostimulatory composition including an immunostimulatory ODN and a pharmaceutically acceptable carrier prior to administering the suppressive ODN to the subject. The immunostimulatory composition includes either a) a D-type CpG ODN, b) a K-type CpG ODN or c) a C-type CpG ODN. Exemplary ODNs are disclosed below, as are combinations of ODNs that are of use in the methods disclosed herein. Usually, such an immunostimulatory composition is administered about three days before an ischemic event or activity that leads to ischemia. In additional embodiments, the immunostimulatory composition is administered two to five days prior to an immunosuppressive composition, such as two to three days prior to an immunosuppressive composition.

In some embodiments, the immunostimulatory composition is administered to the subject about two to about five days prior to the ischemic tissue damage. In some examples, the immunostimulatory composition is administered to the subject about three to about five days prior

- 20 -

to the ischemic tissue damage. In other examples, the immunostimulatory composition is administered to the subject about three days prior to the ischemic tissue damage. Other administration regimens are disclosed for example, in PCT Publication No. 2007/030580, which is incorporated by reference herein. The immunosuppressive composition, and optionally an immunostimulatory composition, can be administered systemically, such as orally or parenterally. The immunosuppressive composition, and optionally an immunostimulatory composition, can be administered locally, such as intra-theccally, intra-cranially, or by injection into the cerebral spinal fluid.

In another example, the immunostimulatory compositions is administered prior to an event or activity associated with (e.g., that increases the risk of) ischemia. For example, at least one dose of the imidazoquinoline compound can be administered about three to about five days prior to the event or activity, in order to better realize the preconditioning effect of administration. Usually, the composition is administered about three days before the event or activity. In the case of an isolated event, that is, an event that is not predicted to be a recurring event, such as a surgical operation, the composition is given prior to the commencement of the event, such as about five, about four, or about three days prior to the event or activity. Optionally, multiple doses of the composition are administered prior to the commencement of the event (e.g., surgery), such as at about five, about four and about three days prior to the event. For example, two, or three, or more doses can be administered on separate occasions preceding the event. Several doses can be administered on the same day, such as two or more doses administered about five, four or three days prior to an event.

In yet other embodiments, the method also includes selecting a subject having ischemic tissue damage or at risk of developing ischemic tissue damage and administering to the subject another immunosuppressive composition including a therapeutically effective amount of a suppressive ODN and a pharmaceutically acceptable carrier, thereby inhibiting the ischemic tissue damage in the subject. Exemplary suppressive ODNs are disclosed below. The method can inhibit development of ischemic damage, or can reduce ischemic tissue damage from an event. In some embodiments, the disclosed methods are of use to reduce the size of the tissue damage and/or reducing the symptoms caused by the tissue damage. In specific, non-limiting examples, immunosuppressive composition can be administered at the same time as the imidazoquinoline compound.

- 21 -

In some embodiments, the subject is at risk for ischemic tissue damage, and the immunosuppressive composition is administered from about six hours prior to the ischemic tissue damage to about 24 hours after the ischemic tissue damage. In other embodiments, the subject is at risk for ischemic tissue damage, and the immunosuppressive composition is administered at any time from about three hours prior to the ischemic tissue damage to about 24 hours after the ischemic tissue damage. In further embodiments, the subject is at risk for ischemic tissue damage, and the immunosuppressive composition is administered at any time from about three hours prior to the ischemic tissue damage to about 12 hours after the ischemic tissue damage. In yet other examples, the subject is at risk for ischemic tissue damage, and the immunosuppressive composition is administered at any time from about three hours prior to the ischemic tissue damage to the time of the ischemic tissue damage.

In further embodiments, the immunosuppressive composition is administered about three days prior to about three hours after ischemic tissue damage. In another embodiment, the immunosuppressive composition is administered about one day prior to about three hours after ischemic tissue damage. In one specific non-limiting example, the immunosuppressive composition is administered at the time of the ischemic tissue damage or any time at or after about 3 hours after ischemic tissue damage.

Thus, in some examples, the immunosuppressive composition can be administered at about 6, about 5, about 4, about 3, about 2 hours prior to the ischemic tissue damage. In other examples, the immunosuppressive composition can be administered about 60 minutes, about 45 minutes, about 30 minutes, about 15 minutes, about 10 minutes, or about 5 minutes prior to ischemic tissue damage. In further examples, the immunosuppressive composition can be administered about 5 minutes, about 10 minutes, about 15 minutes, about 30 minutes, about 45 minutes, or about 60 minutes after ischemic tissue damage. In other examples, the immunosuppressive composition is administered about 1, about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, about 20, about 21, about 22, about 23 or about 24 hours after ischemic tissue damage.

The timing of the use of the immunosuppressive composition is disclosed above. An immunostimulatory composition can be administered with any of these specific timings.

Exemplary timings for the use of the immunostimulatory composition are disclosed herein, see below.

- 22 -

In any of these embodiments, the immunosuppressive composition can be administered at the same time as the imidazoquinoline compound. However, the immunosuppressive composition also can be administered at a different time than the imidazoquinoline compound. Multiple administrations of the immunosuppressive composition and the imidazoquinoline compound are contemplated.

Without being bound by theory, the immunosuppressive composition can reduce the production of reduced the levels of IL-1 β , IL-1 α , IL-6, CINC-1, and/or TNF α . The suppressive composition can reduce the production of caspase-1, and NLRP3. In specific non-limiting examples, the immunosuppressive composition can attenuate NLRP3 inflammasome activity.

Ischemia-reperfusion injury is a well-known condition that generally includes damage to a tissue caused when the blood supply returns to the tissue (reperfusion) after a period of ischemia (restriction in blood supply). The absence of oxygen and nutrients from the blood creates a condition in which the restoration of circulation results in inflammation and oxidative damage, rather than restoration of normal function. Ischemia-reperfusion injury can cause increases in the production of or oxidation of various potentially harmful compounds produced by cells and tissues, as well as inflammation, which can lead to oxidative damage to and/or death of cells and tissues. For example, renal ischemia-reperfusion injury can result in histological damage to the kidneys, including kidney tubular damage and changes characteristic of acute tubular necrosis. The resultant renal dysfunction permits the accumulation of nitrogenous wastes ordinarily excreted by the kidney, such as serum urea nitrogen (SUN). Ischemia-reperfusion may also cause injury to other organs, such as the lung, and is associated with a wide variety of diseases and conditions involving inflammation.

In additional embodiments, the subject has or is at risk of an ischemic reperfusion injury, a cardiac, kidney, liver, or brain reperfusion injury. In further embodiments, the subject has atherosclerosis. An ischemia-reperfusion injury that can be prevented or treated includes any injury due to one or more ischemic events and reperfusion that occurs in any organ or tissue and in the context of a healthy individual or in any disease or condition. The subject can be elderly. Ischemia-reperfusion injuries include, but are not limited to, intestinal ischemia-reperfusion injury, renal ischemia-reperfusion injury, cardiac ischemia-reperfusion injury, ischemia-reperfusion injury of other internal organs such as the lung or liver, peripheral, or central nervous system ischemia-reperfusion injury, ischemia-reperfusion injury of the limbs or digits, trauma-induced hypovolemia, or ischemia-reperfusion injury of any transplanted organ or tissue. Ischemia-reperfusion injury can

- 23 -

also occur in conjunction with a variety of other conditions including, but not limited to, stroke, traumatic brain injury (see above), spinal cord injury, and trauma-induced hypovolemic shock. Other conditions and diseases in which ischemia-reperfusion injury occurs will be known to those of skill in the art.

5 Risk can be indicated by a variety of medical as well as non-medical indicators, as would be recognized by one of ordinary skill in the art. For example, various cardiovascular signs and symptoms, such as atrial fibrillation, angina pectoris, hypertension, transient ischemic attacks and prior stroke, are all indicators of risk that can be used to select a subject for administration of preconditioning agent according to the methods disclosed herein. Similarly, surgical procedures,
10 especially those specifically involving the cardiovascular system, such as endarterectomy, pulmonary bypass and coronary artery bypass surgeries, are indicators of risk that can be used to select a subject.

The methods can be used to treat ischemic tissue damage in any organ. In some embodiments, the methods are of use to inhibit or reduce ischemic tissue damage in the central
15 nervous system. Hypoxia in the central nervous system (CNS) can be associated with ischemic events (such as cerebrovascular ischemia, transient ischemic attacks or stroke, myocardial ischemia due to narrowing or blockage of the vessels of the heart, iatrogenic ischemia, due to surgical procedures, and the like).

The methods can involve selecting a subject (1) who has or (2) is at risk for, an ischemic
20 event. For example characteristic symptoms of stroke are sudden weakness, numbness, or paralysis (usually unilateral and in the arm, leg, or face), also a sudden and severe headache, full or partial loss of vision, dizziness and loss of balance, loss of memory, loss of consciousness, and difficulty speaking or understanding language may be observed. Diagnosis of a stroke can be readily made by one of ordinary skill in the art. The diagnosis can be confirmed by cerebral angiography and by a
25 computed axial tomography (CT) scan of the brain. Biological signs of ischemic events may be observed using magnetic resonance imaging (MRI) or other techniques. In one embodiment, the subject has, or is at risk for having, an acute myocardial infarction. In other embodiments, the subject has, or is at risk for having, a disease or condition associated with focal or global ischemia, such as myocardial infarction, stroke, pulmonary embolism, mesenteric ischemia, Budd-Chiari
30 syndrome, peripheral vascular disease, solid organ transplantation, cardiac arrest, cardiopulmonary bypass and trauma.

- 24 -

In the context of the methods described herein, risk is indicated by a variety of medical as well as non-medical indicators, as would be recognized by one of ordinary skill in the art. For example, various cardiovascular signs and symptoms, such as atrial fibrillation, angina pectoris, hypertension, transient ischemic attacks and prior stroke, are all indicators of risk that can be used to select a subject for use of the methods disclosed herein. Similarly, invasive procedures, such as surgical or intravascular procedure, especially any surgery involving the cardiovascular system, such as endarterectomy, pulmonary bypass and coronary artery bypass surgeries, stent or valve replacement, or abdominal surgeries are indicators of risk that can be used to select a subject for administration.

In specific non-limiting examples, the subject has or is at risk of having a stroke or a transient ischemic attack. Causes of ischemic stroke are thrombosis, vasoconstriction and embolism. The disclosed methods can be used to treat, prevent, inhibit or reduce tissue damage from a stroke or a transient ischemic attack.

In other embodiments, the subject has or is at risk of having ischemia resulting from atherosclerosis or a vasculopathy. In further embodiments, the subject is at risk of ischemia from a surgical procedure, such as, but not limited to, a vascular surgical procedure or any procedure that carries a risk of cerebrovascular embolism. The surgical procedure can be an endarterectomy, a pulmonary bypass, stent placement, or a coronary artery bypass. The subject can have peripheral vascular disease.

Administration can be systemic or local. In some embodiments, the immunosuppressive composition, and/or the immunotimulatory composition is administered orally. In other embodiments, parenteral administration is utilized. For subjects with cerebral ischemia, administration is, for example, by intra-arterial or intrathecal injection, or by direct injection of ischemic brain areas. Intra-arterial injection can be directed to ischemic regions, for example, by injection into the basilar artery to administer the agent to the occipital cortex. In some embodiments, administration is by systemic intravenous or intra-arterial injection following osmotic disruption of the blood brain barrier (see, for example, U.S. Patent No. 5,124,146). In some embodiments, administration is, for example, by injection into the basilar, carotid, or cerebral arteries. Administration can be by intra-theal or intra-cranial injection, by by administration to the cerebral spinal fluid.

- 25 -

For subjects with peripheral artery disease (and other systemic and arterial diseases), administration is, for example, by intra-arterial (particularly intracoronary), or intrapericardial injection. In some embodiments, the therapeutic agent(s) are administered systemically, such as by intravenous injection. Additionally, in some embodiments the therapeutic agents may be

5 incorporated into or on an implantable device, such as vascular stents placed directly in diseased blood vessels in the coronary or cerebral circulation, and undergo slow release providing regional sustained release of the therapeutic agents. Efficacy of treatment is demonstrated, for example, by a regression of symptoms, for example chest pressure or pain.

The imidazoquinoline compound, immuostimulatory composition, and optionally the

10 immunosuppressive composition, can also be administered directly as part of a surgical procedure, or at the bedside by a treating physician. Drug quality ODNs can be diluted for instance in sterile saline and given by injection using sterile 1 cc syringes and small bore needles (25 gauge and less) to ischemic soft tissue units. Alternatively, a surgical wound can be irrigated for instance with a saline or other therapeutically effective solution containing a known concentration (dosage).

15 Precise control and localization of therapeutic effects can thus be obtained.

Any suitable route of administration can be utilized for the imidazoquinoline compound, immunostimulatory composition, and the immunosuppressive composition. Suitable pharmaceutical compositions, and additional information on routes of administration, are disclosed below.

20 The subject can be administered other agents. For example, the subject can be administered a thrombolytic agent (an anti-clotting agent) or a surgical procedure such as balloon angioplasty or stenting. Thrombolytic agents include, but are not limited to streptokinase, urokinase, anistreplase, alteplase and reteplase. The use of more than one type of reperfusion therapy is also contemplated.

25 *Imidazoquinoline Compounds*

Imidazoquinolines are of use in the methods disclosed herein. Imidazoquinolines are synthetic immunomodulatory drugs that act by binding toll-like receptors 7 and 8 (TLR7/TLR8) on dendritic cells, structurally mimicking these receptors' natural ligand, viral single-stranded RNA. Imidazoquinolines are heterocyclic compounds comprising a fused quinoline-imidazole skeleton.

30 Derivatives, salts (including hydrates, solvates, and N-oxides), and prodrugs thereof also are contemplated by the present disclosure. Particular imidazoquinoline compounds are known in the

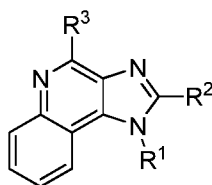
- 26 -

art, see for example, U.S. Patent No. 6,518,265; and U.S. Patent No. 4,689,338.

In some embodiments, the imidazoquinoline compound is not imiquimod and/or is not resiquimod. In additional embodiments, the imidazoquinoline compound is a lipophilic imidazoquinoline compound.

- 5 Additional compounds can be used, such as alkoxy-substituted imidazoquinolones (see, for example, Published U.S. Patent Application No. 2015/0023990, which is incorporated herein by reference) and hydrazine 1H-imidazoquinolin-4-amines (see, for example, Published U.S. Patent Application No. 2014/0286988, which is incorporated herein by reference), and imidazoquinoline compounds substituted at the 1-position with a cyclic substituent (see, for example, Published U.S. Patent Application No. 2014/0011777 and Published U.S. Patent Application No. 2014/0194628, which are both incorporated herein by reference).

Certain embodiments of the compound may have a formula as illustrated below:



(Formula I)

- 15 With reference to Formula I, R^1 may be selected from hydrogen, C_{12-24} alkyl, C_{11-24} heteroalkyl, substituted C_{1-10} heteroalkyl, carboxyl, and hydroxyl; R^2 may be selected from hydrogen, C_{1-10} alkyl, C_{1-10} heteroalkyl, and substituted C_{1-10} alkyl; and R^3 may be selected from hydrogen, amino, aminoacyl, hydroxyl, and C_{1-10} alkoxy.

- In particular disclosed embodiments, R^1 may be C_{11-24} heteroalkyl, such as C_{11-24} alkoxy (e.g., $-OC_{11-24}$ alkyl), C_{11-24} thioalkyl (e.g., $-SC_{11-24}$ alkyl), and C_{11-24} aminoalkyl (e.g., $-NR^5C_{11-24}$ alkyl, wherein R^5 is selected from hydrogen, alkyl, cycloalkyl, aryl, and the like). In other disclosed embodiments, R^1 may be C_{1-10} heteroalkyl substituted with one or more substituents selected from amino (e.g., $N(R^5)_2$, wherein each R^5 independently is selected from hydrogen, alkyl, cycloalkyl, aryl, and the like), aminoacyl (e.g., $-NC(O)C_{1-30}$ alkyl, $-NC(O)C_{1-23}$ alkyl; such as $-NC(O)C_{11-23}$ alkyl; $-NC(O)C_{15-23}$ alkyl; $-NC(O)C_{15-19}$ alkyl, and $-NC(O)C_{17}$ alkyl), aminocarbonylamino (e.g., $-NC(O)NR^5C_{1-30}$ alkyl, $-NC(O)NR^5C_{1-23}$ alkyl, such as $-NC(O)NR^5C_{11-23}$ alkyl, $-NC(O)NR^5C_{15-23}$ alkyl, $-NC(O)NR^5C_{15-19}$ alkyl, and $-NC(O)NR^5C_{17}$ alkyl), aminocarbonyloxy (e.g., $-NC(O)OC_{1-30}$ alkyl, $-NC(O)OC_{1-23}$ alkyl, such as $-NC(O)OC_{11-23}$ alkyl, $-NC(O)OC_{15-23}$ alkyl, $-NC(O)OC_{15-19}$

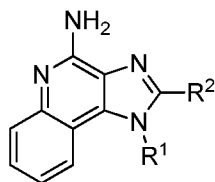
- 27 -

alkyl, and $-\text{NC}(\text{O})\text{OC}_{17}$ alkyl), ether (e.g., $-\text{OC}_{1-30}$ alkyl, $-\text{OC}_{1-23}$ alkyl, $-\text{OC}_{11-23}$ alkyl, $-\text{OC}_{15-23}$ alkyl, $-\text{OC}_{15-19}$ alkyl, and $-\text{OC}_{17}$ alkyl), ester (e.g., $-\text{OC}(\text{O})\text{C}_{1-30}$ alkyl, $-\text{OC}(\text{O})\text{C}_{1-23}$ alkyl, such as $-\text{OC}(\text{O})\text{C}_{11-23}$ alkyl, $-\text{OC}(\text{O})\text{C}_{15-23}$ alkyl, $-\text{OC}(\text{O})\text{C}_{15-19}$ alkyl, and $-\text{OC}(\text{O})\text{C}_{17}$ alkyl), aldehyde (e.g., $-\text{OC}(\text{O})\text{H}$), carboxyl (e.g., $-\text{OC}(\text{O})\text{H}$), thioether (e.g., $-\text{SC}_{1-30}$ alkyl, $-\text{SC}_{1-23}$ alkyl, such as $-\text{SC}_{11-23}$ alkyl, $-\text{SC}_{15-23}$ alkyl, $-\text{SC}_{15-19}$ alkyl, and $-\text{SC}_{17}$ alkyl), and thioester (e.g., $-\text{SC}(\text{O})\text{C}_{1-30}$ alkyl, $-\text{SC}(\text{O})\text{C}_{1-23}$ alkyl, $-\text{SC}(\text{O})\text{C}_{11-23}$ alkyl, $-\text{SC}(\text{O})\text{C}_{15-23}$ alkyl, $-\text{SC}(\text{O})\text{C}_{15-19}$ alkyl, and $-\text{SC}(\text{O})\text{C}_{17}$ alkyl).

In particular disclosed embodiments, R^1 is $-\text{X}-\text{Y}-\text{Z}-\text{R}^6$ wherein X is selected from the group consisting of a bond, $-\text{O}-$, and $-\text{NH}-$; Y is selected from the group consisting of C_{1-10} alkyl and C_{1-5} alkyl OC_{1-5} alkyl; Z is selected from the group consisting of $-\text{NHC}(\text{O})-$, $-\text{NHS}(\text{O})_2-$, $-\text{NHC}(\text{O})\text{NH}-$; and R^6 is $-\text{C}_{11-23}$ alkyl. In particular disclosed embodiments, Y is C_{2-10} alkyl or Y is C_{2-4} alkyl OC_{2-4} alkyl, with certain embodiments being C_{2-5} alkyl. X is selected from a group consisting of a bond and $-\text{O}-$ in certain embodiments, with particular disclosed embodiments having X as $-\text{O}-$. In certain embodiments, Z is $-\text{NHC}(\text{O})-$. Particular disclosed compounds may have an R^6 substituent that is C_{15-23} alkyl, more typically R^6 is C_{15-19} alkyl. In some embodiments, X is optional.

In particular disclosed embodiments, R^1 is selected from the group consisting of $-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NHC}(\text{O})\text{C}_{11-23}$ alkyl, $-\text{OCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NHC}(\text{O})\text{C}_{11-23}$ alkyl, $-\text{NHCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NHC}(\text{O})\text{C}_{11-23}$ alkyl, $-\text{CH}_2\text{CH}_2\text{OCH}_2\text{CH}_2\text{NHC}(\text{O})\text{C}_{11-23}$ alkyl. More typically, R^1 is selected from the group consisting of $-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NHC}(\text{O})\text{C}_{15-23}$ alkyl, $-\text{OCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NHC}(\text{O})\text{C}_{15-23}$ alkyl, $-\text{NHCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NHC}(\text{O})\text{C}_{15-23}$ alkyl, $-\text{CH}_2\text{CH}_2\text{OCH}_2\text{CH}_2\text{NHC}(\text{O})\text{C}_{15-23}$ alkyl.

In another disclosed embodiment, the imidazoquinoline compound has the formula:



wherein, R^1 is selected from $-\text{C}_{12-24}$ alkyl, $-\text{OC}_{11-24}$ alkyl, $-\text{NHC}_{11-24}$ alkyl, and $-\text{X}-\text{Y}-\text{Z}-\text{R}^6$, wherein X is selected from the group consisting of a bond, $-\text{O}-$, and $-\text{NH}-$; Y is selected from the group consisting of C_{1-10} alkyl and C_{1-5} alkyl OC_{1-5} alkyl; Z is selected from the group consisting of:

- 28 -

-NHC(O) -,

-NHS(O)₂ -,

-NHC(O)NH-; and

R⁶ is -C₁₁₋₂₃alkyl;

- 5 R² is selected from the group consisting of hydrogen, alkyl, alkylaminoalkyl, alkoxyalkyl, and hydroxyalkyl.

In some embodiments, X is optional and/or R¹ is -COOH

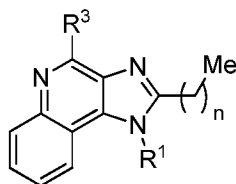
- In particular disclosed embodiments, R² may be C₁₋₁₀ alkyl, such as methyl, ethyl, propyl, butyl, pentyl, hexyl, heptyl, octyl, and the like. In other disclosed embodiments, R² may be C₁₋₁₀ alkyl substituted with one or more substituents selected from halogen (e.g., chloro, iodo, bromo, fluoro), trihaloalkyl (e.g., trifluoromethyl), aryl (e.g., C₆₋₁₄ aryl), heteroaryl (e.g., C₆₋₁₄ aryl wherein one or more carbon atoms is replaced with a heteroatom selected from oxygen, sulfur, and nitrogen), amino (e.g., -N(R⁵)₂, wherein each R⁵ independently is selected from hydrogen, alkyl, cycloalkyl, aryl, and the like), aminoacyl (e.g., -NC(O)C₁₋₁₀ alkyl), aminocarbonylamino (e.g., -NC(O)NR⁵C₁₋₁₀ alkyl), aminocarbonyloxy (e.g., -NC(O)OC₁₋₁₀ alkyl), ether (e.g., -OC₁₋₁₀ alkyl), ester (e.g., -OC(O)C₁₋₁₀ alkyl), hydroxyl (-OH), aldehyde (e.g., -OC(O)H), carboxyl (e.g., -OC(O)H), thioether (e.g., -SC₁₋₁₀ alkyl), and thioester (e.g., -SC(O)C₁₋₁₀ alkyl).

- In particular embodiments, R² is selected from the group consisting of hydrogen, alkyl, alkylaminoalkyl, alkoxyalkyl, and hydroxyalkyl. For example, R² may be selected from hydrogen, methyl, ethyl, propyl, butyl, ethoxymethyl, methoxymethyl, 2-methoxyethyl, hydroxymethyl, and 2-hydroxyethyl. In certain embodiments, R² is selected from the group consisting of ethyl, propyl, butyl, methoxyethyl, and ethoxymethyl. In exemplary embodiments, R² is selected from the group consisting of butyl and ethoxymethyl.

- In particular disclosed embodiments, R³ may be amine (-NH₂), amino (e.g., -N(R⁵)₂, wherein each R⁵ independently is selected from hydrogen, alkyl, cycloalkyl, aryl, heterocyclyl, heteroaryl, and the like), or aminoacyl (e.g., -NC(O)R⁶ wherein R⁶ is selected from C₁₋₁₀ alkyl, C₁₋₁₀ haloalkyl, hydrogen, C₆₋₁₄ aryl, and the like).

- 29 -

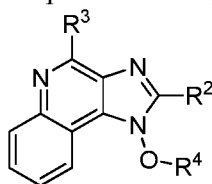
In other embodiments, the imidazoquinoline compound has a formula:



(Formula II)

wherein n ranges from zero to 10; and R^1 and R^3 may be selected from any of the particular groups recited above for Formula I.

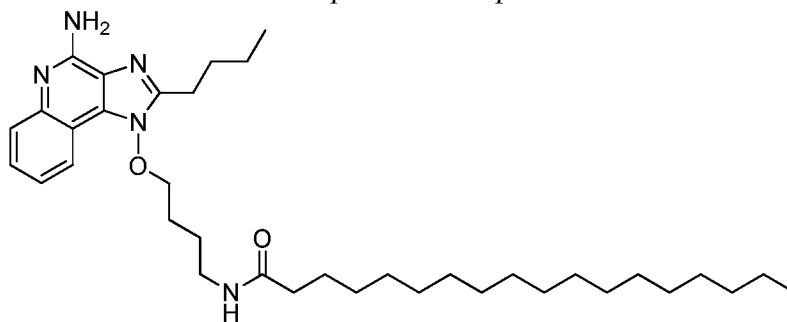
In further embodiments, the imidazoquinoline compound has a formula:



(Formula III)

wherein R^2 and R^3 may be selected from any of the particular groups recited above for Formula I, and R^4 may be selected from C_{1-10} alkyl substituted with one or more of the substituents provided for substituted heteroalkyl, such as those provided for R^1 in Formula I, above.

In further embodiments the imidazoquinoline compound is:

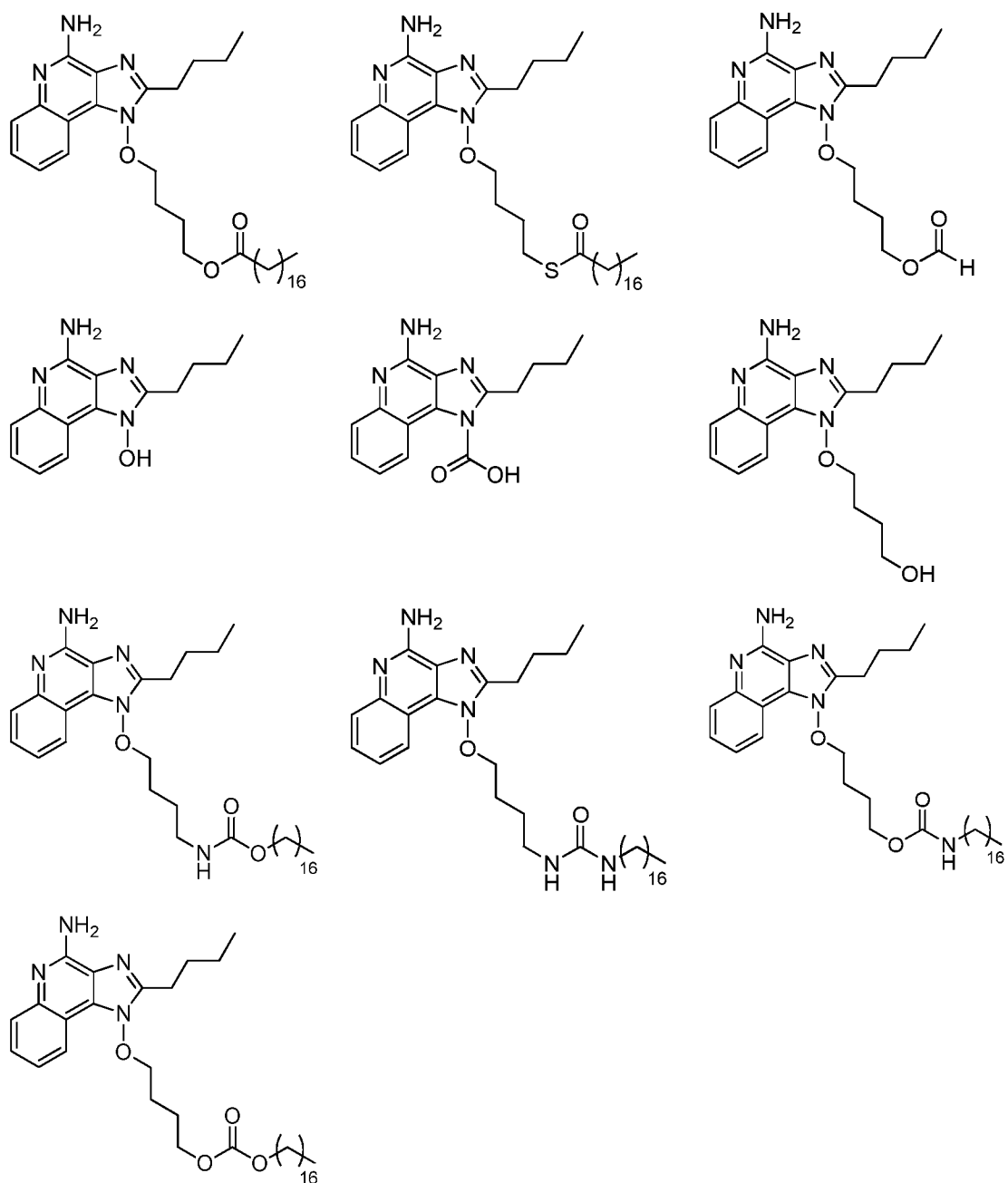


(Formula IV)

(i.e., N-(4- {[4-amino-2-butyl-1H-imidazo[4,5-c]quinolin-1-yl]oxy}butyl) octadecanamide, also known as 3M-052).

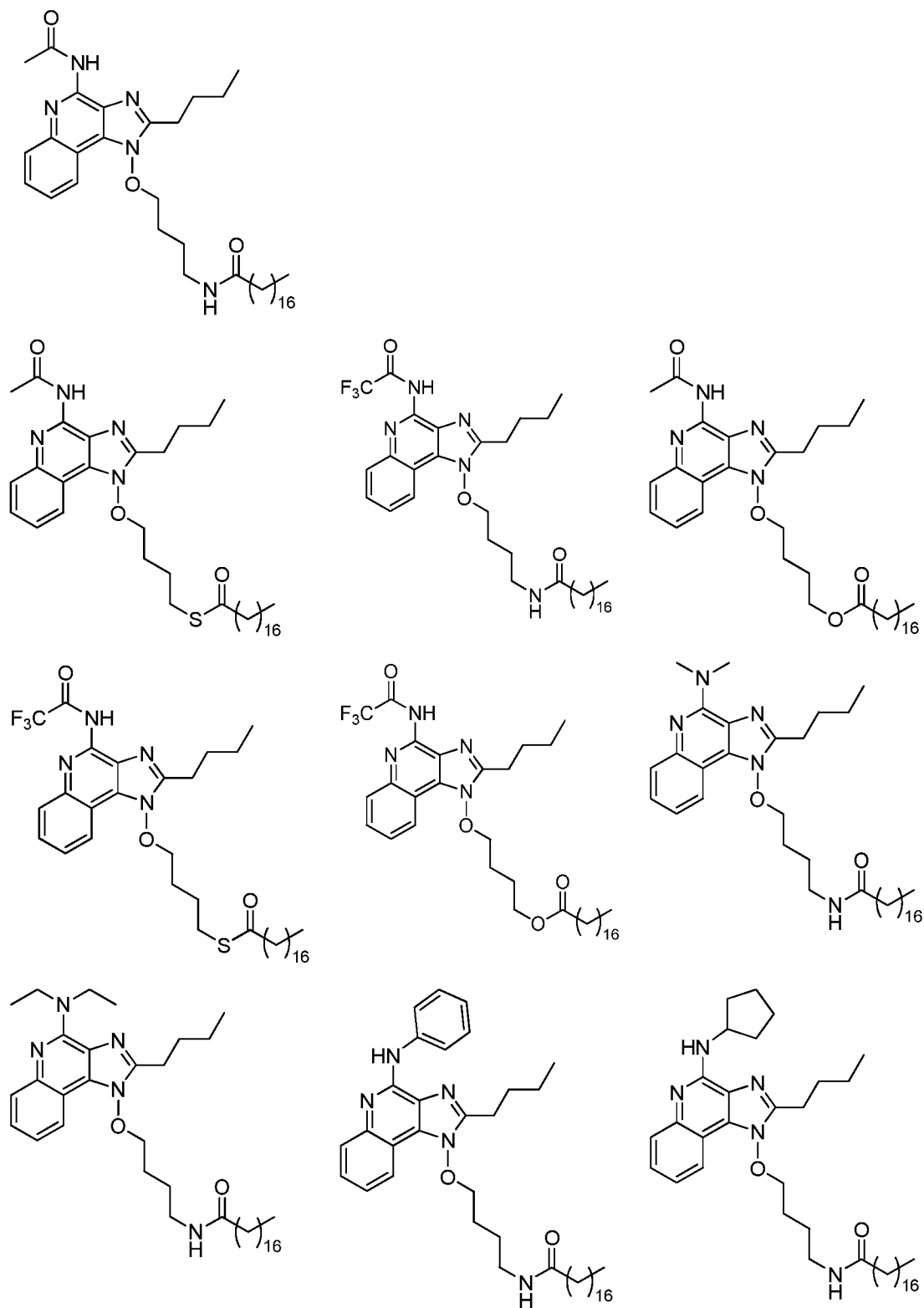
Other exemplary imidazoquinoline compounds include the following:

- 30 -



In other disclosed embodiments, the imidazoquinoline compounds can be selected from the following compounds:

- 31 -



This imidazoquinoline compound can be used in any of its pharmaceutically acceptable forms including solid, semi-solid, solvate (e.g., hydrate), wholly or partially dissolved (such as in a

- 32 -

pharmaceutical composition), a prodrug, or dispersed in a pharmaceutically acceptable carrier. Any pharmaceutically acceptable salt of the imidazoquinoline compound can also be used, see PCT Publication No. WO 2012/024284, which is incorporated herein by reference. Additional compounds are disclosed for example, in U.S. Patent No. 7,799,800, which is incorporated herein
5 by reference.

In particular disclosed embodiments, the pharmaceutically acceptable salt may be selected from any suitable salt known in the art, such as (but not limited to) salts of organic and inorganic counter ions and salts of organic or inorganic acids. In particular disclosed embodiments, the pharmaceutically acceptable salt may be a hydrochloride, hydrobromide, hydroiodide, nitrate,
10 sulfate, bisulfate, phosphate, acetate, lactate, salicylate, citrate, tartrate, bitartrate, ascorbate, succinate, ammonium, potassium, sodium, calcium, magnesium, maleate, fumarate, gluconate, glucuronate, saccharate, formate, benzoate, glutamate, mesylate, tosylate, or besylate salt. One or more of the functional groups provided in any one of Formulas I-III may be manipulated to provide the desired pharmaceutically acceptable salt. Methods of forming pharmaceutically acceptable salts
15 are readily recognized by a person of ordinary skill in the art.

Also disclosed herein are prodrugs of the disclosed compounds. Exemplary prodrug moieties that may be used to functionalize one or more of the functional groups provided in any one of Formulas I-III include, but are not limited to, carbonates, esters, amides, carbamates, oximes, imines, phosphates, and ethers. Methods of forming prodrugs are readily recognized by a person of
20 ordinary skill in the art.

Suppressive Oligodeoxynucleotides

In some embodiments, the disclosed methods include administration of an imidazoquinoline compound and a suppressive ODN. These suppressive ODN utilize a class of DNA motifs that
25 selectively inhibits or suppresses immune activation and are of use of in any of the methods disclosed herein. Optimal activity is observed using multimers of these motifs, which are rich in G bases. The suppressive ODNs of the disclosure are highly specific (*i.e.*, are neither toxic nor non-specifically immunosuppressive), and are useful for inhibiting an immune response. One or more suppressive ODNs can be administered to the subject at the same time as the imidizaquinoline
30 compound

In some embodiments, the ODNs of use in the methods disclosed herein are capable of forming G-quadruplexes (G-tetrads). G-tetrads are G-rich DNA segments that can accommodate

- 33 -

complex secondary and/or tertiary structures (see Fig. 1 of U.S. Patent Publication No. US-2004-0132682-A1, herein incorporated by reference). A G-tetrad involves the planar association of four Gs in a cyclic Hoogsteen hydrogen bonding arrangement (this involves non-Watson Crick base-pairing). In general, either a run of two or more contiguous Gs or a hexameric region in which
5 >50% of the bases are Gs, is needed for an ODN to form a G-tetrad. The longer the run of continuous Gs, and the higher the G content of the ODN, the higher the likelihood of G-tetrad formation, as reflected by higher ellipticity values. Oligonucleotides that form G-tetrads can also form higher-level aggregates that are more easily recognized and taken up by immune cells, for example, through scavenger receptors or by nucleolin.

10 The circular dichromatism (CD) value is an increase in peak absorbance to the 260-280 nm wavelength, generally owing to the formation of secondary structures. Thus, a convenient method for identifying suppressive oligonucleotides is to study their CD values. An increase in peak ellipticity values to greater than 2.0 is typical of a suppressive oligonucleotide, such as a ODN with at least four guanosines.

15 In some embodiments, suppressive ODNs can have CD values of about 2.2, 2.4, 2.6, 2.8, 3.0, 3.2, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, or higher. The higher the ellipticity value, the greater the tetrad-forming capacity of the oligonucleotide, so an ODN with a CD value of 8.5 is typically more suppressive than an ODN with a CD value of 2.9. Generally, a suppressive ODN includes at least four guanosines. In some embodiments, the ODN forms a G-tetrad.

20 In some embodiments, the ODN is from about 8 to about 120 nucleotides in length, such as 8 to 100 nucleotides in length. In particular examples, the ODN is from about 8 to about 40 nucleotides in length, or from about 10 to about 30 nucleotides in length such as 18, 20, 22, 24, 26, or 28 nucleotides in length. Optionally, the suppressive ODN has multiple guanosine-rich sequences, for example, in certain embodiments the ODN has from about two to about 20
25 guanosine-rich sequences, or, more particularly, from about two to about four guanosine-rich sequences. In some embodiments, the suppressive ODN is 18, 24 or 30 nucleotides in length. These short ODNs are non-naturally occurring.

In one embodiment, the suppressive ODNs have a sequence comprising at least one of the human telomere-derived TTAGGG suppressive motifs. In some examples, the ODN has at least
30 one TTAGGG motif, and in certain examples, the ODN has multiple TTAGGG motifs. For example, in particular embodiments, the ODN has from about two to about 20 TTAGGG motifs. In

- 34 -

this context, “about” refers to a difference of an integer amount. Thus, in some examples, the suppressive ODNs have from two to five TTAGGG motifs, such as three or four TTAGGG motifs.

In some embodiments, the ODN includes or consists of three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen or fifteen TTAGGG motifs. Single TTAGGG motifs are

5 suppressive only when incorporated into larger ODNs with greater than 10 bases. In several examples, the suppressive ODN is from about 18 to about 30 nucleotides in length and includes three or four TTAGGG motifs.

The methods utilize an ODN with a G-tetrad-forming sequence that imposes the two-dimensional structure necessary for G-tetrad formation. Examples of suppressive ODN include, but

10 are not limited to, those shown in Table 1. However, any ODN capable of forming G-tetrads may be used. In particular examples, the ODN has a sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 15 18, SEQ ID NO: 19, and SEQ ID NO: 20 (see Table 1). Combinations of one or more of these ODNs are also of use.

Control ODNs can be ODNs that include 7-deazaguanine. Due to the presence of 7-deazaguanine, GAGCAAGCTG*G*ACCTTCCAT (SEQ ID NO: 21, wherein G* is 7-deazaguanine) is an inactive form of ODN 1502 (SEQ ID NO: 20).

- 35 -

TABLE 1

List of suppressive ODNs		
A151	SEQ ID NO: 1	(TTAGGG) ₄ (4 repeats of the TTAGGG base sequence)
A152	SEQ ID NO: 2	(TTAGGG) ₃
A153	SEQ ID NO: 3	(TTAGGG) ₂
A156	SEQ ID NO: 4	(TGGGCGGT) ₃
A157	SEQ ID NO: 5	(TGGGCGGT) ₂
A1	SEQ ID NO: 6	TCAACCTTCATTAGGG
A161	SEQ ID NO: 7	TTAGGGTTAGGGTCAACCTTCA
A162	SEQ ID NO: 8	TCAACCTTCATTAGGGTTAGGG
A163	SEQ ID NO: 9	GGGTAGGGTTATCAACCTTCA
A164	SEQ ID NO: 10	TCAACCTTCAGGGTTAGGGTTA
A15	SEQ ID NO: 11	GGGTGGGTGGGTATTACCATTA
A16	SEQ ID NO: 12	ATTACCATTAGGGTGGGTGGGT
A17	SEQ ID NO: 13	TGGGCGGTTCAAGCTTGA
A18	SEQ ID NO: 14	TCAAGCTTCATGGGCGGT
A19	SEQ ID NO: 15	GGGTGGGTGGGTAGACGTTACC
A20	SEQ ID NO: 16	GGGGGGTCAAGCTTCA
A21	SEQ ID NO: 17	TCAAGCTTCAGGGGGG
A22	SEQ ID NO: 18	GGGGGGTCAACGTTCA
H154	SEQ ID NO: 19	CCTCAAGCTTGAGGGG
1502	SEQ ID NO: 20	GAGCAAGCTGGACCTTCCAT

Combinations of these ODNs are also of use in any of the methods disclosed herein.

- 5 In particular embodiments, the ODN is modified to prevent degradation. In one embodiment, suppressive ODNs can include modified nucleotides to confer resistance to degradation. Without being bound by theory, modified nucleotides can be included to increase the stability of a suppressive ODN. Thus, because phosphorothioate-modified nucleotides confer resistance to exonuclease digestion, the suppressive ODNs are “stabilized” by incorporating
- 10 phosphorothioate-modified nucleotides. The pharmacokinetics of phosphorothioate ODN show that they have a systemic half-life of forty-eight hours in rodents and are useful for *in vivo*

- 36 -

applications (Agrawal *et al. Proc. Natl. Acad. Sci. USA* 88:7595-7599, 1991). Phosphorothioates may be synthesized using automated techniques employing either phosphoramidate or H phosphonate chemistries. In some embodiments, the ODN has a phosphate backbone modification, and in particular examples, the phosphate backbone modification is a phosphorothioate backbone modification.

In one embodiment, the guanosine-rich sequence and its immediate flanking regions include phosphodiester rather than phosphorothioate nucleotides. In one specific non-limiting example, the sequence TTAGGG includes phosphodiester bases. In some examples, all of the bases in an ODN are phosphodiester bases. In other examples, the ODN is a phosphorothioate/phosphodiester chimera.

As disclosed herein, any suitable modification can be used to render the ODN resistant to degradation *in vivo* (such as resistant to degradation by an *exo*- or *endo*-nuclease). In one specific, non-limiting example, a modification that renders the ODN less susceptible to degradation is the inclusion of nontraditional bases such as inosine and queosine, as well as acetyl-, thio- and similarly modified forms of adenine, cytidine, guanine, thymine, and uridine. Other modified nucleotides include nonionic DNA analogs, such as alkyl or aryl phosphonates (*i.e.*, the charged phosphonate oxygen is replaced with an alkyl or aryl group, as set forth in U.S. Patent No. 4,469,863), phosphodiesters and alkylphosphotriesters (*i.e.*, the charged oxygen moiety is alkylated, as set forth in U.S. Patent No. 5,023,243 and European Patent No. 0 092 574). ODNs containing a diol, such as tetraethyleneglycol or hexaethyleneglycol, at either or both termini, have also been shown to be more resistant to degradation.

The suppressive ODNs can be synthesized by standard methods well known in the art. Most commonly, synthesis is performed on an oligonucleotide synthesizer using the standard cyanoethyl phosphoramidite chemistry. These include, but are not limited to, phosphodiester, phosphorothioate, peptide nucleic acids, synthetic peptide analogues, and any combination thereof. Those skilled in the art will recognize that any other standard technique may be used to synthesize the suppressive ODN.

In one embodiment, a suppressive ODN is included in a delivery complex. The delivery complex can include the suppressive ODN and a targeting agent. Any suitable targeting agent can be used. For example, in some embodiments, a suppressive ODN is associated with (*e.g.*, ionically or covalently bound to, or encapsulated within) a targeting means (*e.g.*, a molecule that results in

- 37 -

higher affinity binding to a target cell, such as a neuronal cell). A variety of coupling or cross-linking agents can be used to form the delivery complex, such as protein A, carbodiimide, and N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP). Examples of ODN delivery complexes include a suppressive ODN associated with a sterol (*e.g.*, cholesterol), a lipid (*e.g.*, a cationic lipid, anionic lipid, virosome or liposome), and a target cell specific binding agent (*e.g.*, a ligand recognized by target cell specific receptor). Without being bound by theory, the complex is sufficiently stable *in vivo* to prevent significant uncoupling prior to delivery to the target cell. In one embodiment, the delivery complex is cleavable such that the ODN is released in a functional form at the target cells.

Combinations of these ODNs are also of use in the methods disclosed herein. Thus, two, three, four, five, six, seven, eight, nine or more than ten of the suppressive ODNs can be administered to a subject. These ODNs can be administered as parts of a single nucleotide molecule, or on different nucleotide molecules. One exemplary combination of use is D35, D29 and D19 (see Table 1 above).

Pre-Administration of Immunostimulatory CpG ODNs

In some embodiments of the methods disclosed herein an effective amount of a CpG ODN including an unmethylated CpG motif is administered to the subject, prior to the administration of the imidazoquinoline compound and prior to the exposure to the ischemic event. In additional embodiments of the methods disclosed herein an effective amount of a CpG ODN including an unmethylated CpG motif is administered to the subject, prior to the administration of the imidazoquinoline compound and the suppressive ODN, and prior to the exposure to the ischemic event.

The unmethylated CpG motifs present in bacterial DNA stimulate cells that express Toll-like receptor 9 (TLR9). This interaction triggers a short-lived innate immune response characterized by the production of pro-inflammatory cytokines and chemokines (Krieg et al., Nature 374:546-548, 1995; Klinman Nat.Rev.Immunol. 2004, 4 (4):249-258). Microarray identification of the genes triggered by CpG ODN showed that the inflammatory response peaked on day 1, but that >96% of these genes were suppressed by a counter-regulatory process that peaked on day 5 (Klaschik et al, J Leukocyte Biol 85 (5):788-795, 2009; Klaschik et al., Mol.Immunol. 47 (6):1317-

- 38 -

1324, 2010). Any immunostimulatory CpD ODN can be used in the methods disclosed herein, such as K-type ODN or D-type ODN. Combinations of K-type ODN or D-type ODN are also of use.

In specific non-limiting examples, a CpG ODN is administered about 1 day to about 5 days prior to the suppressive ODN, such as about 1 to about 3 days prior to the suppressive ODN, such as about 2 to 3 days prior to the suppressive ODN, for example 48, 60 or 72 hours prior to the suppressive ODN. One of skill in the art can readily determine the optimum timing for administering one or more CpG ODNs.

K-Type CpG ODN

The present methods can include administering a therapeutically effective amount of a K-type CpG oligodeoxynucleotide (ODN). A CpG ODN is an ODN including a CpG motif, wherein the pyrimidine ring of the cytosine is unmethylated. Two types of CpG ODNs have been identified: K-type and D-type ODNs. In several embodiments, the CpG ODN is at most 100 nucleotides or at most 80 nucleotides in length. In other embodiments the CpG ODN is in the range of about 8 to 30 nucleotides in length. In another embodiment, the CpG ODN is at least 10 nucleotides in length, such as about 10 to about 30 nucleotides in length.

K-type CpG ODN of use that are disclosed, for example, in published PCT Application No. WO 98/18810A1 (K-type), which is incorporated by reference herein in its entirety. In some embodiments, only K-type CpG ODNs (or combinations of K-type ODNs) are used in the methods disclosed herein. Thus, in several embodiments, the methods do not include the use of D-type ODNs.

Combinations of K-type CpG ODNs are of use, such as the use of at least two, at least three, at least four, at least five, at least six at least seven, at least eight or at least ten ODNs, each with a different nucleic acid sequence. In several embodiments, two, three, four, five or six K-type CpG ODNs, each with a different nucleic acid sequence, are utilized in the methods.

A single K ODN can be used in the methods disclosed herein, or mixtures of K ODN can also be used in the methods disclosed herein. Specific combinations of ODNs are disclosed, for example, in U.S. Patent Application No. 10/194,035, which is incorporated herein by reference.

In several embodiments, a K-type CpG ODN or a mixture of K-type CpG ODNs are utilized. Briefly, the K-type nucleic acid sequences useful in the methods disclosed herein are represented by the formula:

- 39 -



wherein at least one nucleotide separates consecutive CpGs; D is adenine, guanine, or thymidine; Y is cytosine or thymine, N is any nucleotide and $N_1 + N_2$ is from about 0-26 bases. In one
 5 embodiment, N_1 and N_2 do not contain a CCGG quadmer or more than one CGG trimer; and the nucleic acid sequence is from about 8-30 bases in length, such as about 10 to 30 nucleotides in length. However, nucleic acids of any size (even many kb long) can be used in the methods disclosed herein if CpGs are present. In one embodiment, synthetic oligonucleotides of use do not include a CCGG quadmer or more than one CCG or CGG trimer at or near the 5' or 3' terminals
 10 and/or the consensus mitogenic CpG motif is not a palindrome. A "palindromic sequence" or "palindrome" means an inverted repeat (i.e., a sequence such as ABCDEE'D'C'B'A', in which A and A' are bases capable of forming the usual Watson-Crick base pairs).

In another embodiment, the methods include the use of an ODN which contains a CpG
 15 motif represented by the formula:



wherein at least one nucleotide separates consecutive CpGs; RD is selected from the group
 20 consisting of GpT, GpG, GpA, ApT and ApA; YT is selected from the group consisting of TpT or CpT; N is any nucleotide and $N_1 + N_2$ is from about 0-26 bases, such that the ODN is about 8 to 30 nucleotides in length.

In several embodiments, the methods disclosed herein include the use of an effective amount of at least one K-type CpG ODN, wherein the K-type CpG ODN includes an unmethylated
 25 CpG motif that includes a sequence represented by the formula:



wherein the central CpG motif is unmethylated, D is T, G or A, W is A or T, and $N_1, N_2, N_3, N_4, N_5,$
 and N_6 are any nucleotides. In one embodiment, D is a T. The K ODN(s) can be 10 to 30 nucleotides in length. A K-type CpG ODN can include multiple CpG motifs. In some
 30 embodiments, at least one nucleotide separates consecutive CpGs; N_3D is selected from the group

- 40 -

consisting of GpT, GpG, GpA, ApT and ApA; WN₄ is selected from the group consisting of TpT or CpT; N is any nucleotide and N₁ + N₂ is from about 0-26 bases

In one embodiment, N₁, and N₂ do not contain a CCGG quadmer or more than one CCG or CGG trimer. CpG ODN are also in the range of 8 to 30 bases in length, but may be of any size

5 (even many kb long) if sufficient motifs are present. In several examples, the CpG ODN is 10 to 20 nucleotides in length, such as 12 to 18 nucleotides in length. In one embodiment, synthetic ODNs of this formula do not include a CCGG quadmer or more than one CCG or CGG trimer at or near the 5' and/or 3' terminals and/or the consensus CpG motif is not a palindrome. Other CpG ODNs can be assayed for efficacy using methods described herein. It should be noted that exemplary K-
10 type CpG ODNs are known in the art, and have been fully described, for example in PCT Publication No. WO 98/18810A1, which is incorporated herein by reference.

Exemplary K-type CpG ODN are listed below:

15 **Table 2: K ODN**

CpG 7909	5'-TCGTCGTTTTGTCGTTTTGTCGTT-3'	(SEQ ID NO: 23)
CpG10103	5'-TCGTCGTTTTTCGGTCGTTTT-3'	(SEQ ID NO: 24)
20 K X	ATAATCGACGTTCAAGCAAG	(SEQ ID NO: 25)
K22	CTCGAGCGTTCTC	(SEQ ID NO: 26)
K21	TCTCGAGCGTTCTC	(SEQ ID NO: 27)
K82	ACTCTGGAGCGTTCTC	(SEQ ID NO: 28)
25 K30	TGCAGCGTTCTC	(SEQ ID NO: 29)
k31	TCGAGGCTTCTC	(SEQ ID NO: 30)
K39	GTCGGCGTTGAC	(SEQ ID NO: 31)
K16	TCGACTCTCGAGCGTTCTC	(SEQ ID NO: 32)
K3	ATCGACTCTCGAGCGTTCTC	(SEQ ID NO: 33)
30 k23	TCGAGCGTTCTC	(SEQ ID NO: 34)
K40	GTCGGCGTCGAC	(SEQ ID NO: 35)

- 41 -

	K34	GTCGACGTTGAC	(SEQ ID NO: 36)
	K83	ACTCTCGAGGGTTCTC	(SEQ ID NO: 37)
	K19	ACTCTCGAGCGTTCTC	(SEQ ID NO: 38)
	K73	GTCGTCGATGAC	(SEQ ID NO: 39)
5	K46	GTCGACGCTGAC	(SEQ ID NO: 40)
	K47	GTCGACGTCGAC	(SEQ ID NO: 41)
	K72	GTCATCGATGCA	(SEQ ID NO: 42)
	K37	GTCAGCGTCGAC	(SEQ ID NO: 43)
	k25	TCGAGCGTTCT	(SEQ ID NO: 44)
10	K82	ACTCTGGAGCGTTCTC	(SEQ ID NO: 45)
	K83	ACTCTCGAGGGTTCTC	(SEQ ID NO: 46)
	K84	ACTCTCGAGCGTTCTA	(SEQ ID NO: 47)
	K85	CATCTCGAGCGTTCTC	(SEQ ID NO: 48)
	K89	ACTCTTTCTTCTC	(SEQ ID NO: 49)
15	K109	TCGAGCGTTCT	(SEQ ID NO: 50)
	K123	TCGTTCTTCTC	(SEQ ID NO: 51)
	K1555	GCTAGACGTTAGCGT	(SEQ ID NO: 52)
	K110	TCGAGGCTTCTC	(SEQ ID NO: 53)

20 Exemplary Control ODNs are:

K1612 TAGAGCTTAGCTTGC (SEQ ID NO: 54)

C163 TTGAGTGTTCTC (SEQ ID NO: 55)

As noted above, combinations of K-type CpG ODN can also be used. Exemplary combinations include 1) K3, K19, K110; 2) K19, K23, K123; K3, 3) K110, K123; 4) K3, K23, K123; 5) K3, 25 K19, K123; and 6) K19, K110, K123. Additional exemplary combinations include at least two

- 42 -

different K-type CpG ODNs, wherein one of the K-type CpG ODNs is K1555, and/or wherein one of the K-type CpG ODNs is K3.

D-type CpG ODN

5 In some embodiments, the methods include administering an effective amount a “D type” CpG ODN (see Verthelyi et al, *J. Immunol.* 166:2372, 2001; U.S. Patent No. 7,960,356, both of which are herein incorporated by reference in their entirety). D type ODNs differ both in structure and activity from K type ODNs. The unique activities of D type ODNs are disclosed below. For example, as disclosed herein, D ODNs stimulate the release of cytokines from cells of the immune
10 system. In specific, non-limiting examples D type oligonucleotides stimulate the release or production of IP-10 and IFN- α by monocytes and/or plasmacitoid dendritic cells and the release or production of IFN- γ by NK cells. The stimulation of NK cells by D ODNs can be either direct or indirect.

In some embodiments, only D-type CpG ODNs (or combinations of D-type ODNs) are used
15 in the methods disclosed herein. Thus, in several embodiments, the methods do not include the use of K-type ODNs.

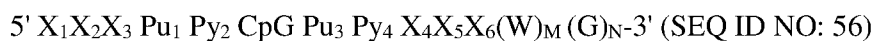
Combinations of D-type CpG ODNs are of use, such as the use of at least two, at least three, at least four, at least five, at least six at least seven, at least eight or at least ten ODNs, each with a different nucleic acid sequence. In several embodiments, two, three, four, five or six D-type CpG
20 ODN are utilized. Exemplary combinations of D ODNs of use are D19, D29 and D35.

With regard to structure, in one embodiment, a CpG motif for a D type oligonucleotides can have the structure:



wherein the central CpG motif is unmethylated, R is A or G (a purine), and Y is C or T (a
25 pyrimidine). D-type oligonucleotides include an unmethylated CpG dinucleotide. Inversion, replacement or methylation of the CpG reduces or abrogates the activity of the D oligonucleotide.

In one embodiment, a D type ODN is at least about 16 nucleotides in length and includes a sequence represented by:



- 43 -

wherein the central CpG motif is unmethylated, Pu is a purine nucleotide, Py is a pyrimidine nucleotide, X and W are any nucleotide, M is any integer from 0 to 10, and N is any integer from 4 to 8.

The region Pu₁ Py₂ CpG Pu₃ Py₄ is termed the CpG motif. The region X₁X₂X₃ is termed the 5' flanking region, and the region X₄X₅X₆ is termed the 3' flanking region. If nucleotides are included 5' of X₁X₂X₃ in the D ODN these nucleotides are termed the 5' far flanking region. Nucleotides 3' of X₄X₅X₆ in the D ODN are termed the 3' far flanking region.

In one specific non-limiting example, Py₂ is a cytosine. In another specific, non-limiting example, Pu₃ is a guanidine. In yet another specific, non-limiting example, Py₂ is a thymidine and Pu₃ is an adenine. In a further specific, non-limiting example, Pu₁ is an adenine and Py₂ is a tyrosine. In another specific, non-limiting example, Pu₃ is an adenine and Py₄ is a tyrosine.

In one specific not limiting example, N is from about 4 to about 8. In another specific, non-limiting example, N is about 6.

D-type CpG ODNs can include modified nucleotides. These modified nucleotides can be included to increase the stability of a D-type oligonucleotide. Without being bound by theory, because phosphorothioate-modified nucleotides confer resistance to exonuclease digestion, ODN are "stabilized" by incorporating phosphorothioate-modified nucleotides. In one embodiment, the CpG dinucleotide motif and its immediate flanking regions include phosphodiester rather than phosphorothioate nucleotides. In one specific non-limiting example, the sequence Pu₁ Py₂ CpG Pu₃ Py₄ includes phosphodiester bases. In another specific, non-limiting example, all of the bases in the sequence Pu₁ Py₂ CpG Pu₃ Py₄ are phosphodiester bases. In yet another specific, non-limiting example, X₁X₂X₃ and X₄X₅X₆(W)_M(G)_N include phosphodiester bases. In yet another specific, non-limiting example, X₁X₂X₃ Pu₁ Py₂ CpG Pu₃ Py₄ X₄X₅X₆(W)_M(G)_N (SEQ ID NO: 1) includes phosphodiester bases. In further non-limiting examples the sequence X₁X₂X₃ includes at most one or at most two phosphothioate bases and/or the sequence X₄X₅X₆ includes at most one or at most two phosphothioate bases. In additional non-limiting examples, X₄X₅X₆(W)_M(G)_N includes at least 1, at least 2, at least 3, at least 4, or at least 5 phosphothioate bases. Thus, a D type ODN can be a phosphorothioate/phosphodiester chimera.

As disclosed herein, any suitable modification can be used in the present invention to render the ODN resistant to degradation in vivo (e.g., via an exo- or endo-nuclease). In one specific, non-

- 44 -

limiting example, a modification that renders the ODN less susceptible to degradation is the inclusion of nontraditional bases such as inosine and quesine, as well as acetyl-, thio- and similarly modified forms of adenine, cytidine, guanine, thymine, and uridine. Other modified nucleotides include nonionic DNA analogs, such as alkyl or aryl phosphonates (i.e., the charged phosphonate oxygen is replaced with an alkyl or aryl group, as set forth in U.S. Patent No. 4,469,863), phosphodiester and alkylphosphotriesters (i.e., the charged oxygen moiety is alkylated, as set forth in U.S. Patent No. 5,023,243 and European Patent No. 0 092 574). Oligonucleotides containing a diol, such as tetraethyleneglycol or hexaethyleneglycol, at either or both termini, have also been shown to be more resistant to degradation. The D type ODNs can also be modified to contain a secondary structure (e.g., stem loop structure). Without being bound by theory, it is believed that incorporation of a stem loop structure renders an ODN more effective.

In a further embodiment, Pu₁ Py₂ and Pu₃ Py₄ are self-complementary. In another embodiment, X₁X₂X₃ and X₄X₅X₆ are self-complementary. In yet another embodiment X₁X₂X₃ Pu₁ Py₂ and Pu₃ Py₄ X₄X₅X₆ are self-complementary.

Specific non-limiting examples of a D type oligonucleotide wherein Pu₁ Py₂ and Pu₃ Py₄ are self-complementary include, but are not limited to, ATCGAT, ACCGGT, ATCGAC, ACCGAT, GTCGAC, or GCCGGC. Without being bound by theory, the self-complementary base sequences can help to form a stem-loop structure with the CpG dinucleotide at the apex to facilitate immunostimulatory functions. Thus, in one specific, non-limiting example, D type oligonucleotides wherein Pu₁ Py₂ and Pu₃ Py₄ are self-complementary induce higher levels of IFN- γ production from a cell of the immune system (see below). The self-complementary need not be limited to Pu₁ Py₂ and Pu₃ Py₄. Thus, in another embodiment, additional bases on each side of the three bases on each side of the CpG-containing hexamer form a self-complementary sequence (see above).

Exemplary D type ODNs are well known in the art. Specific non-limiting examples include, but are not limited to:

GGTGCGTCGATGCAGGGGGG (D28, SEQ ID NO: 57)

GGTATATCGATATAGGGGGG (D142, SEQ ID NO: 58)

5'XXTGCATCGATGCAGGGGGG 3' (SEQ ID NO: 59)

5'XXTGCACCGGTGCAGGGGGG3' (SEQ ID NO: 60),

- 45 -

5'XXTGCGTCGACGCAGGGGGG3' (SEQ ID NO: 61),

5'XXTGCGTCGATGCAGGGGGG3' (SEQ ID NO: 62),

5'XXTGCGCCGCGCAGGGGGG3' (SEQ ID NO: 63),

5'XXTGCGCCGATGCAGGGGGG3' (SEQ ID NO: 64),

5 5'XXTGCATCGACGCAGGGGGG3' (SEQ ID NO: 65),

5'XXTGCGTCGGTGCAGGGGGG3' (SEQ ID NO: 66),

wherein X any base, or is no base at all. In one specific, non-limiting example, X is a G.

Further examples include (underlined bases are phosphodiester):

10 **Table 3: D-type CpG ODN**

D104	GGT <u>GCATCGATGC</u> AGGGGGG	(SEQ ID NO: 67)
D19	GGT <u>GCATCGATGC</u> AGGGGGG	(SEQ ID NO: 67)
D29	GGT <u>GCA</u> CCGGTGCAGGGGGG	(SEQ ID NO: 68)
D35	GGT <u>GCATCGATGC</u> AGGGGGG	(SEQ ID NO: 69)
15 D28	GGT <u>GCGT</u> CGATGCAGGGGGG	(SEQ ID NO: 57)
D106	GGT <u>GTGT</u> CGATGCAGGGGGG	(SEQ ID NO: 70)
D116	TG <u>CATCGATGC</u> AGGGGGG	(SEQ ID NO: 71)
D113	GGT <u>GCATCGATAC</u> AGGGGGG	(SEQ ID NO: 72)
D34	GGT <u>GCATCGATGC</u> AGGGGGG	(SEQ ID NO: 73)
20 D102	GGT <u>GCATCGTTGC</u> AGGGGGG	(SEQ ID NO: 74)
D32	GGT <u>GCGT</u> CGA <u>CGC</u> AGGGGGG	(SEQ ID NO: 75)
D117	GGT <u>CGATCGATGC</u> ACGGGGG	(SEQ ID NO: 76)
D37	GGTGC <u>ATCGATGC</u> AGGGGGG	(SEQ ID NO: 77)
D25	<u>GGTGCATCGATGC</u> AGGGGGG	(SEQ ID NO: 77)
25 D30	GGT <u>GCATCGACGC</u> AGGGGGG	(SEQ ID NO: 78)

- 46 -

d120	GGTGCATCGATAGGCGGGGG	(SEQ ID NO: 79)
D27	GGTGCACCGATGCAGGGGGG	(SEQ ID NO: 80)
d119	CCTGCATCGATGCAGGGGGG	(SEQ ID NO: 81)
D142	GGTATATCGATATAGGGGGG	(SEQ ID NO: 58)
5 d143	GGTGGATCGATCCAGGGGGG	(SEQ ID NO: 82)

Combinations of these ODNs are also of use. One exemplary combination is D19, D29 and D35. However, other combinations of ODN can be used, such as any two or three ODN from the Table above. In a specific non-limiting example, an immunostimulatory composition comprises a) a D19 (and/or D35) ODN comprising the nucleic acid sequence set forth as SEQ ID NO: 67; b) a D29
 10 ODN comprising the nucleic acid sequence set forth as SEQ ID NO: 68.

C-type CpG ODN

C-type ODNs also can be utilized in the methods disclosed herein. Typically, C class ODNs have a TCGTCG motif at the 5' end and have a CpG motif imbedded in a palindromic sequence. M362 is an exemplary C-type CpG ODN that contains a 5'-end 'TCGTCG-motif' and a 'GTCGTT-
 15 motif'. C-type ODNs resemble K-type as they are composed entirely of phosphorothioate nucleotides, but resemble D-type in containing palindromic CpG motifs. This class of ODNs stimulates B cells to secrete IL-6 and pDCs to produce IFN- α (see Hartmann et al., Eur. J. Immunol. 33: 1633-41, 2003, incorporated herein by reference). A palindromic sequence of at least 8
 20 nucleotides increases activity, for example a palindrome of at least 12, such as 14, 16, 18 or 20 nucleotides, increases activity. In some embodiments, the CpG-C ODNs include one to two TCG trinucleotides at or close to the 5' end of the ODN and a palindromic region of at least 10–12 bases, which contains at least two additional CG dinucleotides preferably spaced zero to three bases apart. The CG dinucleotides in the palindrome are preferably spaced 1, 2, or 3 nucleotides apart, although sequences with four nucleotide spacings retained low levels of IFN- α -inducing activity (see
 25 Marshall et al., J. Leukocyte Biol. 73: 781-792, 2003, incorporated herein by reference). C-type ODNs are present in both early and late endosomes, and thus express properties in common with both K- and D-type CpG ODNs. C-type CpG ODNs include ODN2216 (ggGGGACGATCGTCgggggg, SEQ ID NO: 83, wherein the bases shown in capital letters are phosphodiester, and those in lower case are phosphorothioate) ODN M362
 30 (tcgtcgtcgttcgaacgacgttgat, SEQ ID NO: 84) , ODN 1668 (tccatgacgttcctgatgct, SEQ ID NO: 85),

- 47 -

and ODN2395 (tcgtcgttttcggcgcgcgcgcg, SEQ ID NO: 86), which are available from Invivogen and C274 (tcgtcgaacgttcgagatgaT, SEQ ID NO: 87, wherein all the bases are phosphorothioate except the last base), which is available from Novusbio. C-type ODNs also can be modified to be resistant to degradation, as disclosed herein.

5 *Pharmaceutical Compositions and Administration*

For use in the methods disclosed herein, ODNs (either immunosuppressive or immunostimulatory) can be synthesized *de novo* using any of a number of procedures well known in the art. For example, the b-cyanoethylphosphoramidite method (Beaucage et al., *Tet. Let.* 22:1859, 1981) or the nucleoside H-phosphonate method (Garegg et al., *Tet. Let.* 27:4051, 1986; Froehler et al., *Nucl. Acid Res.* 14:5399, 1986; Garegg et al., *Tet. Let.* 27:4055, 1986; Gaffney et al., *Tet. Let.* 29:2619, 1988) can be utilized. These chemistries can be performed by a variety of automated oligonucleotide synthesizers available in the market.

Alternatively, ODNs can be produced on a large scale in plasmids, (see Sambrook, et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York, 1989) which after being administered to a subject are degraded into oligonucleotides. ODNs can be prepared from existing nucleic acid sequences (e.g., genomic or cDNA) using known techniques, such as those employing restriction enzymes, exonucleases or endonucleases (see PCT Application No. PCT/US98/03678).

As noted above, for use *in vivo*, nucleic acids can be utilized that are relatively resistant to degradation (such as by endo-and exo-nucleases). Secondary structures, such as stem loops, can stabilize nucleic acids against degradation. Alternatively, nucleic acid stabilization can be accomplished via phosphate backbone modifications of immunosuppressive and immunostimulatory ODNs. In one embodiment, a stabilized nucleic acid has at least a partial phosphorothioate modified backbone. Phosphorothioates may be synthesized using automated techniques employing either phosphoramidate or H-phosphonate chemistries. Aryl- and alkyl-phosphonates can be made (e.g., as described in U.S. Patent No. 4,469,863) and alkylphosphotriesters (in which the charged oxygen moiety is alkylated, as described in U.S. Patent No. 5,023,243 and European Patent No. 092,574), and can be prepared by automated solid phase synthesis using commercially available reagents.

In one embodiment, the phosphate backbone modification occurs at the 5' end of the ODN. One specific, non-limiting example of a phosphate backbone modification is at the first two

- 48 -

nucleotides of the 5' end of the nucleic acid. In another embodiment, the phosphate backbone modification occurs at the 3' end of the nucleic acid. One specific, non-limiting example of a phosphate backbone modification is at the last five nucleotides of the 3' end of the nucleic acid.

Methods for making other DNA backbone modifications and substitutions have been described (Uhlmann et al., *Chem. Rev.* 90:544, 1990; Goodchild, *Bioconjugate Chem.* 1:1, 1990). 2'-O-methyl nucleic acids with CpG motifs also cause angiogenesis, as do ethoxy-modified CpG nucleic acids. In fact, no backbone modifications have been found that completely abolish the CpG effect, although it is greatly reduced by replacing the C with a 5-methyl C.

For administration *in vivo*, nucleic acids (including immunostimulatory ODNs or immunosuppressive ODNs) can be associated with a molecule that results in higher affinity binding to target cell (such as an epithelial cell) surfaces and/or increased cellular uptake by target cells to form a "nucleic acid delivery complex." Nucleic acids can be ionically or covalently associated with appropriate molecules using techniques which are well known in the art (see below). Nucleic acids can alternatively be encapsulated in liposomes or virosomes using well-known techniques.

An ODN, including either a suppressive or an immunostimulatory ODN, can be associated with (for example, ionically or covalently bound to, or encapsulated within) a targeting moiety. Targeting moieties include any molecule that results in higher affinity binding to a target cell. For example, for an immunostimulatory CpG ODN (D-type or K-type), a targeting molecule can target the ODN to cells that express TLR9, including B cells and plasmacytoid dendritic cells.

A variety of coupling or cross-linking agents can be used to form the delivery complex, such as protein A, carbodiimide, and N-succinimidyl (2-pyridyldithio) propionate (SPDP). Examples of delivery complexes include ODNs associated with a sterol (such as cholesterol), a lipid (such as a cationic lipid, virosome or liposome), and a target cell specific binding agent (such as a ligand recognized by target cell specific receptor). In one embodiment, the complexes are sufficiently stable *in vivo* to prevent significant uncoupling prior to internalization by the target cell. However, these complexes can be cleavable under appropriate circumstances such that the ODN can be released in a functional form (see, for example, PCT Application No. WO 00/61151).

Generally, the methods include selecting a subject and administering to the subject a therapeutically effective amount of (1) an imidazoquinoline compound and (2) a CpG ODN, such as K-type CpG ODN, D-type CpG ODN, C-type CpG ODN. Optionally, the method includes administering a suppressive ODN.

The administration can be local. In some embodiments, the imidazoquinoline compound and the CpG ODN are administered intrathecally or intra-cranially. In any of the embodiments disclosed herein, the imidazoquinoline compound can be a lipophilic imidazoquinoline compound. In some embodiments, the imidazoquinoline compound is formulated with a suppressive ODN.

5 The inclusion of the suppressive ODN is optional.

Pharmaceutical compositions can include (1) an imidazoquinoline compound or (2) one or more CpG ODN, such as K-type CpG ODN, D-type CpG ODN, C-type CpG ODN as active ingredients. Pharmaceutical compositions can also include a suppressive ODN, or a combination of an imidazoquinoline compound and a suppressive ODN as active agents. These compositions can also include an additional agent(s). Pharmaceutical compositions are thus provided for both local use and for systemic use, formulated for use in human or veterinary medicine.

An effective amount of at least one ODN is included in the pharmaceutical compositions. Suitable concentrations include, but are not limited to, about 1 to about 100 $\mu\text{g/gm}$ ODN, such as about 5 to about 50 $\mu\text{g/gm}$, such as about 50 $\mu\text{g/gm}$ ODN. Additional suitable concentrations include 1 to 100 mg/kg, such as about 5 to about 50 mg/kg, such as about 10 mg/kg. The ODN can be immunostimulatory or immunosuppressive.

In additional embodiments, a therapeutically effective amount of ODN (either a CpG ODN or a suppressive ODN) can vary from about 0.01 μg per kilogram (kg) body weight to about 1 g per kg body weight, such as about 1 μg to about 5 mg per kg body weight, or about 5 μg to about 1 mg per kg body weight. The exact dose is readily determined by one of skill in the art based on the potency of the specific compound (such as the ODN utilized), the age, weight, sex and physiological condition of the subject.

In other embodiments, about 0.1 to about 10 mg/kg of the imidazoquinoline compound is included in a composition, such as about 0.1 to about 1 mg/kg. In specific non-limiting examples, about 0.5 to about 5 mg/kg, about 1 to about 5 mg/kg, about 0.5 to 2 mg/kg, or about 1 ng/kg, about 2 mg/kg, about 3 mg/kg, about 4 mg/kg, or 5 mg/kg of the imidazoquinoline compound is included in the composition and administered to the subject.

The imidazoquinoline compound and any of the ODNs can be formulated in a variety of ways for use in the methods disclosed herein. Pharmaceutical compositions are thus provided for both local (such as intra-theal or intra-cranial) use and for systemic (such as parenteral) use. Therefore, the disclosure includes within its scope pharmaceutical compositions comprising at least one CpG ODN, or at least one immunosuppressive ODN, formulated for use in human or veterinary

- 50 -

medicine. While the ODNs will typically be used to treat human subjects they may also be used to treat similar or identical diseases in other vertebrates, such as other primates, dogs, cats, horses, and cows.

Pharmaceutical compositions include at least one ODN as an active ingredient and can optionally include additional agents. The pharmaceutical composition can be formulated with an appropriate solid or liquid carrier, depending upon the particular mode of administration chosen. As an example, additional immunosuppressive agents can be formulated with suppressive ODNs and/or an imidazoquinoline compound.

A suitable administration format may best be determined by a medical practitioner for each subject individually. Various pharmaceutically acceptable carriers and their formulation are described in standard formulation treatises, *e.g.*, *Remington's Pharmaceutical Sciences* by E. W. Martin. See also Wang, Y. J. and Hanson, M. A., *Journal of Parenteral Science and Technology*, Technical Report No. 10, Supp. 42: 2S, 1988. The pharmaceutical compositions that comprise an ODN, in some embodiments, will be formulated in unit dosage form, suitable for individual administration of precise dosages. The amount of active compound(s) administered will be dependent on the subject being treated, the severity of the affliction, and the manner of administration, and is best left to the judgment of the prescribing clinician. Within these bounds, the formulation to be administered will contain a quantity of the active component(s) in amounts effective to achieve the desired effect in the subject being treated.

When locally administered into cells in an affected area or a tissue of interest, such as the central nervous system, the (1) the imidazoquinoline compound, such as a lipophilic imidazoquinoline compound (for example, 3M-052), (2) the one or more CpG ODN, such as K-type CpG ODN, D-type CpG ODN, C-type CpG ODN, (3) the suppressive ODN, or (4) a combination of the imidazoquinoline compound and the suppressive ODN can be administered in a composition that contains a synthetic or natural hydrophilic polymer as the carrier. Examples of such polymers include hydroxypropyl cellulose and polyethylene glycol. The active ingredients can be mixed with a hydrophilic polymer in an appropriate solvent. The solvent is then removed by methods such as air-drying, and the remainder is then shaped into a desired form (for example, a sheet) and applied to the target site. Formulations containing such hydrophilic polymers keep well as they have a low water-content. At the time of use, they absorb water, becoming gels that also store well. In the case of sheets, the firmness can be adjusted by mixing a polyhydric alcohol with a hydrophilic polymer similar to those above, such as cellulose, starch and its derivatives, or synthetic

- 51 -

polymeric compounds. Hydrophilic sheets thus formed can be used.

Compositions also can be administered by any route, including parenteral administration. Exemplary routes include intravenous, intraperitoneal, intramuscular, intraperitoneal, intrasternal, or intraarticular injection or infusion, or by intra-tracheal, intra-cranial, sublingual, oral, topical, intranasal, transmucosal administration, endotracheal instillation, or by pulmonary inhalation. The compositions can be administered into the cerebral spinal fluid.

The dosage form of the pharmaceutical composition will be determined by the mode of administration chosen. For instance, in addition to injectable fluids, inhalational and oral formulations can be employed. The pharmaceutically acceptable carriers and excipients useful in this invention are conventional. For instance, parenteral formulations usually comprise injectable fluids that are pharmaceutically and physiologically acceptable fluid vehicles such as water, physiological saline, other balanced salt solutions, aqueous dextrose, glycerol or the like. Excipients that can be included are, for instance, proteins, such as human serum albumin or plasma preparations. If desired, the pharmaceutical composition to be administered may also contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate. Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in the art.

For example, for parenteral administration, ODNs can be formulated generally by mixing them at the desired degree of purity, in a unit dosage injectable form (solution, suspension, or emulsion), with a pharmaceutically acceptable carrier, *i.e.*, one that is non-toxic to recipients at the dosages and concentrations employed and is compatible with other ingredients of the formulation. A pharmaceutically acceptable carrier is a non-toxic solid, semisolid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type.

When ODNs are provided as parenteral compositions, such as for injection or infusion, they are generally suspended in an aqueous carrier, for example, in an isotonic buffer solution at a pH of about 3.0 to about 8.0, preferably at a pH of about 3.5 to about 7.4, 3.5 to 6.0, or 3.5 to about 5.0. Useful buffers include sodium citrate-citric acid and sodium phosphate-phosphoric acid, and sodium acetate-acetic acid buffers. A form of repository or "depot" slow release preparation may be used so that therapeutically effective amounts of the preparation are delivered into the bloodstream over many hours or days following transdermal injection or delivery.

For oral administration, the pharmaceutical compositions can take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients

- 52 -

such as binding agents (for example, pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (for example, lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (for example, magnesium stearate, talc or silica); disintegrants (for example, potato starch or sodium starch glycolate); or wetting agents (for example, sodium lauryl sulphate). The tablets can be coated by methods well known in the art. Liquid preparations for oral administration can take the form of, for example, solutions, syrups or suspensions, or they can be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations can be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (*e.g.*, sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (*e.g.*, lecithin or acacia); non-aqueous vehicles (*e.g.*, almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (*e.g.*, methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations can also contain buffer salts, flavoring, coloring, and sweetening agents as appropriate. For solid compositions, conventional non-toxic solid carriers can include pharmaceutical grades of mannitol, lactose, starch, or magnesium stearate.

Actual methods of preparing such dosage forms are known, or will be apparent, to those of ordinary skill in the art.

ODNs can be adsorbed onto calcium based nanoparticles. Without being bound by theory, these nanoparticles protect the ODN from degradation by acids in the stomach. Thus, in one embodiment, the ODNs are adsorbed onto nanoparticles, and are delivered orally to the subject.

The use of nanoparticles for delivery of ODNs, such as calcium based nanoparticles and silica based nanoparticles is known in the art, see Hanagata, In. J. Nanomedicine 7: 2181-2195, 2012, and Sokolova et al., Biomaterials (Impact Factor: 8.31). 04/2010; 31(21):5627-33, DOI: 10.1016/j.biomaterials.2010.03.067, incorporated herein by reference.

Optionally, the carrier for any composition used in the methods of the present disclosure is a parenteral carrier, and in some embodiments it is a solution that is isotonic with the blood of the recipient. Examples of such carrier vehicles include water, saline, Ringer's solution, and dextrose solution. Non-aqueous vehicles such as fixed oils and ethyl oleate are also useful herein, as well as liposomes.

The pharmaceutical compositions may be in the form of particles comprising a biodegradable polymer and/or a polysaccharide jellifying and/or bioadhesive polymer, an amphiphilic polymer, an agent modifying the interface properties of the particles and a pharmacologically active substance. These compositions exhibit certain biocompatibility features

- 53 -

which allow a controlled release of the active substance. See U.S. Patent No. 5,700,486.

Generally, the formulations are prepared by contacting the ODNs each uniformly and intimately with liquid carriers or finely divided solid carriers or both. Then, if necessary, the product is shaped into the desired formulation. Optionally, the carrier is a parenteral carrier, and in some embodiments it is a solution that is isotonic with the blood of the recipient. Examples of such carrier vehicles include water, saline, Ringer's solution, and dextrose solution. Non-aqueous vehicles such as fixed oils and ethyl oleate are also useful herein, as well as liposomes.

A suitable administration format may best be determined by a medical practitioner for each subject individually. Various pharmaceutically acceptable carriers and their formulation are described in standard formulation treatises, *e.g.*, *Remington's Pharmaceutical Sciences* by E. W. Martin. See also Wang, Y. J. and Hanson, M. A., *Journal of Parenteral Science and Technology*, Technical Report No. 10, Supp. 42: 2S, 1988. The pharmaceutical compositions that comprise a ODN, in some embodiments, will be formulated in unit dosage form, suitable for individual administration of precise dosages. The amount of active compound(s) administered will be dependent on the subject being treated, the severity of the affliction, and the manner of administration, and is best left to the judgment of the prescribing clinician. Within these bounds, the formulation to be administered will contain a quantity of the active component(s) in amounts effective to achieve the desired effect in the subject being treated.

EXAMPLES

Inflammasomes are multi-protein complexes activated as part of the innate immune response to stress or infection that trigger the maturation of caspase-1 followed by the production of IL-1 β and IL-18 (Schroder and Tschopp, 2010, *Cell* 140, 821-832; Latz et al., 2013, *Nat Rev Immunol* 13, 397-411). Caspase-1 and IL-1 β promote inflammation and cell death and IL-1 β has been implicated in a number of diseases including ischemic injury (Bergsbaken et al., 2009, *Nat Rev Microbiol* 7, 99-109; Dinarello, 2011, *Eur J Immunol* 41, 1203-1217; Denes et al., 2012, *Cell Death Dis* 3, e338). In experimental stroke, IL-1 β expression increases following brain ischemia and multiple studies show that blocking IL-1 β can be neuroprotective (Lambertsen et al., 2012, *J Cereb Blood Flow Metab* 32, 1677-1698). In humans, IL-1 β levels increase in both the cerebrospinal fluid and the blood after ischemic stroke (Tarkowski et al., 1995, *Stroke* 26, 1393-1398; Mazzotta et al., 2004, *Eur J Neurol* 11, 377-381; Sotgiu et al., 2006, *Eur J Neurol* 13, 505-513). In murine models, levels of inflammasome proteins increase after ischemic brain injury and

- 54 -

inhibition of inflammasome activity reduces the extent of injury (Benchoua et al., 2004, *J Cereb Blood Flow Metab* 24, 1272-1279; Abulafia et al., 2009, *J Cereb Blood Flow Metab* 29, 534-544; Deroide et al., 2013, *J Clin Invest* 123, 1176-1181; Fann et al., 2013, *Cell Death Dis* 4, e790; Zhang et al., 2014, *Mediators Inflamm* 2014, 370530).

5 Telomeres cap the ends of linear chromosomes, protecting them from fusion, degradation, or recombination (Shampay et al., 1984, *Nature* 310, 154-15). Mammalian telomeres are composed of repetitive TTAGGG motifs (Meyne et al., 1989, *Proc Natl Acad Sci U S A* 86, 7049-7053). These motifs are released from dying host cells and serve to down-regulate inflammatory responses that can cause tissue destruction (as in autoimmune disease) (Gursel et al., 2003, *J Immunol* 171, 10 1393-1400; Yamada et al., 2004, *Crit Care Med* 32, 2045-2049). The synthetic ODN A151 is composed of four TTAGGG motifs on a phosphorothioate backbone. A151 blocks inflammation, including the production of IL-6, IL-12, IFN γ , MIP-2, and TNF α (Shirota et al., 2004, *J Immunol* 173, 5002-5007; Yamada et al., 2004, *Crit Care Med* 32, 2045-2049; Shirota et al., 2005, *J Immunol* 174, 4579-4583). It has been used as an anti-inflammatory agent, specifically in animal 15 models of arthritis (Zeuner et al., 2003, *Arthritis Rheum* 48, 1701-1707), endotoxic shock (Shirota et al., 2005, *J Immunol* 174, 4579-4583), concanavalin A induced hepatitis (Li et al., 2013, *Immunol Lett* 151, 54-60), ocular inflammation (Fujimoto et al., 2009, *Clin Exp Immunol* 156, 528-534), lupus nephritis (Dong et al., 2005, *Arthritis Rheum* 52, 651-658), atherosclerosis (Cheng et al., 2008, *J Mol Cell Cardiol* 45, 168-175), and silica-induced pulmonary inflammation (Sato et al., 20 2008, *J Immunol* 180, 7648-7654). The pharmacokinetics, pharmacodynamics and safety of phosphorothioate ODNs have been established in multiple clinical trials (Jahrsdorfer and Weiner, 2008, *Update Cancer Ther* 3, 27-32; Koo and Wood, 2013, *Hum Gene Ther* 24, 479-488; Bedikian et al., 2014, *Melanoma Res* 24, 237-243).

 The effect of A151 on IL-1 β production and brain ischemia was examined, as disclosed 25 below. Macrophages were selected for study because macrophages/microglia are major sources of IL-1 β in the ischemic brain (Lamberts et al., 2012, *supra*). In addition to perivascular macrophages, after brain ischemia, monocytes rapidly infiltrate the brain and become macrophages (Iadecola and Anrather, *Nat Med* 17, 796-808, 2011; Chiba and Umegaki, 2013, *Mediators Inflamm* 2013). The effect of A151 on the development of ischemic lesions in stroke-prone 30 spontaneously hypertensive rats was investigated by means of permanent middle cerebral artery occlusion, which is a common animal model of ischemic stroke. It was found that A151 reduces

- 55 -

the maturation of caspase-1 and IL-1 β and the production of the inflammasome sensor protein, NLRP3. A151 also repressed levels of iNOS. Of particular importance, A151 reduced ischemic brain injury and NLRP3 mRNA in SHR-SP rats. This type of anti-inflammatory agent represents a novel approach to the prevention and treatment of ischemic stroke.

5

Example 1

Materials and Methods

Reagents: Phosphorothioate oligodeoxynucleotide (ODN) A151 (5'-

TTAGGGTTAGGGTTAGGGTTAGGG-3', SEQ ID NO: X) and control ODN C151 (5'-

10 TTCAAATTCAAATTCAAATTCAAA-3', SEQ ID NO: Y) were synthesized by the FDA CBER Core Facility (Silver Spring, MD). These ODNs were free of detectable protein and endotoxin contaminations. The ODNs were reconstituted in saline for intraperitoneal injection in rats. For cell culture, the ODNs were reconstituted in PBS. Lipopolysaccharide from *E. coli* OIII:B4 was purchased from Invivogen (San Diego, CA; #LPS-EB). Rat macrophage colony stimulating factor (M-CSF) was from GenScript (Piscataway, NJ). All cytokine Elisa kits were purchased from R&D Systems (Minneapolis, MN). LDH kits were from Abcam (Cambridge, MA).

Animals: Male and female offspring (5-7 months of age) of spontaneously hypertensive rat stroke-prone (SHR-SP) breeders were used. The rats were randomly divided into treatment groups and each group contains 7-18 rats. Each group received one intraperitoneal injection of
20 oligodeoxynucleotides or saline 3 days before, 1 day before, or 3 hours post permanent middle cerebral artery occlusion (pMCAO). Oligodeoxynucleotide doses of 1 mg or 3 mg per rat were tested.

Cell culture: Bone marrow derived macrophages (BMDM) were derived from femur and tibia of SHR-SP rats as previously described with minor modifications (Davis, 2013). Bone
25 marrow cells were cultured in DMEM supplemented with 10% FBS and 10 ng/ml rat M-CSF. Two million cells in 11 ml culture medium were seeded in one 100 mm tissue culture dish. Three days later, 5.5 ml medium with 30 ng/ml rat M-CSF was added to each dish. On day 6, the cells were washed twice with PBS and scraped in the presence of cold HBSS. After centrifugation and resuspension, the cell density was adjusted to 2×10^5 cells per ml and 2 ml cells were seeded in each
30 well of six-well plates. Iba staining showed that >99% cells are macrophages. On day 7, the cells were treated with 1 ng/ml LPS and oxygen glucose deprivation with or without oligodeoxynucleotides for 18 hours. Supernatants were collected and used for Elisa and LDH

- 56 -

assays.

Oxygen glucose deprivation (OGD): 4×10^5 BMDM cells were seeded in each well of six-well plates. After overnight incubation, the cells were washed twice in PBS and 1 ml culture medium without glucose (Life Technologies, Grand Island, NY) was added to each well. The plates were placed in modular incubator chambers (Billups-Rothenberg, Del Mar, CA) with anaerobic colorimetric indicator strips that detected a 0.2% oxygen threshold (Becton Dickinson). The chamber was flushed with a gas mixture of 95% N₂ and 5% CO₂ for 20 min at room temperature at 6 L/min. After flushing, the chambers were sealed and maintained at 37°C for 18 hours.

Western blot: Total cell lysate was prepared by using IP lysis buffer (Thermo Fisher Scientific, Rockford, IL) to each well of six-well plates. After fifteen minutes incubation on ice and five seconds sonication, the lysate was centrifuged at 10,000 g for 15 min at 4 °C. The supernatant was collected and protein concentration was determined by BCA assay (Thermo Fisher Scientific, Rockford, IL). All samples were heated for 5 min at 95 °C. Fifteen micrograms of total cell lysate was used for each SDS-PAGE analysis. The following primary antibodies were used for WB analyses: anti-IL-1 β (R&D Systems, Minneapolis, MN; #AF-501-NA), anti-caspase-1 (Abcam, Cambridge, MA; #ab108362), anti-caspase 8 (Cell Signaling, Danver, MA; #4790), anti-NLRP3 (AdipoGen, San Diego, CA; #AG-20B-0014), anti-ASC (AdipoGen, San Diego, CA; #AG-25B-0006), anti-AIM2 (Santa Cruz Biotechnology, Santa Cruz, CA; #SC137967), anti-NLRP1 (Cell Signaling, Beverly, MA; #4990), anti-NLRC4 (Santa Cruz Biotechnology, Santa Cruz, CA; #SC49395), anti-iNOS (Abcam, Cambridge, MA; #ab3523). Signals were detected using a chemiluminescent substrate – Immobilon Western (Millipore, Billerica, MA) followed by digital imaging with Fluor Chem camera (Alpha Innotech, San Leandro, CA) or C-Digit (LI-COR, Lincoln, NE).

The supernatant was concentrated by using methanol/chloroform precipitation as described (Westwell-Roper et al., 2013). Briefly, 500 μ l supernatant was mixed with 500 μ l methanol and 125 μ l chloroform. After centrifuging at 16,000 g for 5 min and removal of the top layer, 500 μ l methanol was added to the sample. Following centrifugation for 5 min at 16,000 g, the pellet was dried at 50°C for 5 min. The pellet was resuspended in 50 μ l SDS loading buffer and heated at 95°C for 15 min.

Permanent middle cerebral artery occlusion (pMCAO): Rats were anesthetized with 5% isoflurane for induction and 2-2.5% isoflurane for maintenance in 30% O₂/70% N₂O via face mask.

- 57 -

The rats were placed in the lateral position, and a curved vertical 2-cm skin incision was made in the midpoint between the left orbit and the external auditory canal. One mm hole was drilled into the skull 1 mm posterior and 6 mm lateral from the midline of skull. The filament probe was attached to the cerebral blood flow (CBF) monitor, followed by one small burr hole (1.5-2 mm) made with a high-speed microdrill through the outer surface of the skull at the junction between the medial wall and the roof of the inferotemporal fossa. The dura was opened with a 30-gauge needle to expose the middle cerebral artery (MCA), and the MCA was occluded between the inferior cerebral vein and the lateral olfactory tract by bipolar electrocoagulator. The coagulated MCA segment was then transected with microscissors to ensure that the occlusion was permanent. During pMCAO, the rectal temperatures were measured and maintained at $37\pm 0.5^{\circ}\text{C}$ with a heating plate (TCAT-2DF controller, Physitemp Instruments, Clifton, NJ). The rats were allowed to recover for 48 hours. The surgeon was blinded to the treatment the rats received.

Neurological assessment: Forty-eight hours post pMCAO, the rats were evaluated by methods described by Hunter (Hunter et al., 2000). The forepaw test was modified to assess the function in detail. The animal was held by the base of the tail and allowed to grasp a horizontal bar. If both of the forepaws are able to grasp the bar immediately at the same time and the grip-strength is equal, score 0; if both of the forepaws grasp the bar immediately at the same time, but with decreased right forepaw grip-strength while the animal is gripping the bar as its tail is pulled up, score 0.5; if both of the forepaws are able to grasp the bar, but with delayed motion and decreased strength of the right forepaw, score 1.0; if the right forepaw is not able to grasp the bar, score 1.5; if the rat has no motion to grasp the bar, score 2. The person evaluating the neurological deficit score was blinded to the treatment of the rats.

Infarct volume determination: Rats were euthanized forty-eight hours after pMCAO. The brains were removed, frozen on dry ice, and stored at -80°C . Frozen brains were sectioned coronally at 20- μm thickness. One section was collected at every 800- μm , the leftover sections were used for RNA isolation and real-time RT PCR. The sections were fixed in paraformaldehyde vapor and stained with cresyl violet. Image J (NIH, Bethesda, MD) was used to quantitate infarct area. Infarct volume was calculated by summing cross-sectional areas and multiplying these areas by the distance between sections, followed by correction for brain swelling (edema) as described (Chen et al., 2003). The person calculating infarct volume was blinded to the treatment of the rats.

Blood test and tissue homogenization: Rat blood was collected 48 hours after pMCAO at euthanization. Blood chemistry and complete blood count were performed by Department of

- 58 -

Laboratory Medicine, NIH (Bethesda, MD). Brain, lung, heart, spleen, kidney, and liver were rapidly freezed on dry ice. Frozen tissues were crushed into powder and homogenized in IP lysis buffer (Thermo Fisher Scientific, Rockford, IL). After centrifugation at 10,000g for 15 min, supernatant was collected and used for Elisa.

5 *RNA isolation and real-time RT-PCR:* The frozen rat brain sections were homogenized in Qiazol lysis reagent (Qiagen, Valencia, CA). All of the reagents and primers used for RNA isolation and real time RT-PCR were purchased from Qiagen. Total RNA was isolated by using Qiagen miRNeasy mini kit according to the manufacturer's instructions. RNA was quantitated by NanoDrop spectrophotometer (Thermo Fisher Scientific Inc). 1 µg RNA was used for cDNA
10 synthesis by using QuantiTect Reverse Transcription Kit. Real-time PCR was performed by using QuantiTect SYBR Green PCR Kit and the following PCR primers: caspase 1 (#QT00191814), IL-1β (#QT00181657), NLRP3 (#QT01568448), iNOS (#QT00178325), sdha (#QT00195958). LightCycler 480 II (Roche, Indianapolis, IN) was used for reverse transcription and PCR. Melt-curve analysis was performed to affirm the single-band production. The gene expression was
15 normalized to housekeeping gene sdha and delta delta Ct method was used to compare treatment groups.

Statistical Analysis: All data are expressed as mean and standard error of the mean (SEM). Cytokine, LDH release, and real-time PCR results were compared by student's t test. Comparisons of infarct volume, neurological deficit score, blood tests of two different treatment groups in rats
20 were analyzed by Mann-Whitney U test. Differences were considered significant when $P < 0.05$.

JC-1 assay: The MITOPROBE™ JC-1 assay kit was obtained from Life Technologies (Grand Island, NY). BMDM cells were cultured in 100 mm dishes and subjected to 1 hour OGD in the presence of 1 ng/ml LPS + 50 ug/ml A151 or C151. After OGD, the cells were incubated with 2uM JC-1 in colorless DMEM medium at 37°C for 30 min. The cells were washed twice with cold
25 PBS supplemented with 1.8 mM CaCl₂, 0.8mMMgCl₂, 10mM glucose, and 1 mg/ml BSA. The cells were scraped, centrifuged and resuspended in 500 ul colorless DMEM with 1uM DAPI for and prepped for FACS analysis.

Example 2**A151 reduces the release of inflammatory factors and death of BMDM induced by LPS and oxygen glucose deprivation (OGD)**

Brain ischemia is characterized by oxygen and glucose deprivation and inflammation in the brain. To investigate the immunomodulatory potential of A151 under ischemic conditions, BMDM were treated with A151 or C151, LPS, and OGD. A151 dramatically reduced the levels of IL-1 β , IL-1 α , IL-6, CINC-1, and TNF α in culture supernatants (Fig. 1). This contrasted to the effect of control ODN C151, which reduced IL-6 and CINC-1, but no other cytokine. Neither ODN altered levels of CINC-3, IFN γ , IL-10, and TGF β .

As ischemia induces cell death and one form of proinflammatory cell death, pyroptosis, is associated with release of proinflammatory cytokines from macrophages (Bergsbaken et al., 2009) and also occurs in various forms of organ ischemia (Takahashi, 2011; Sagulenko et al., 2013), the effect of A151 on the survival of oxygen and glucose deprived BMDM was explored. Compared with LPS treatment, A151 at 50 ug/ml reduced LDH release from $94.3 \pm 4.2\%$ to $62.6 \pm 7.3\%$ ($P < 0.05$). C151 did not affect LDH release. The effect of A151 on proinflammatory cytokines was not due to cytotoxicity (see also Gursel et al., 2003; Shirota et al., 2005).

Example 3**A151 reduces the maturation of IL-1 β and caspase 1 and the expression of NLRP3 and iNOS in response to OGD and LPS stimulation**

The effect of A151 on IL-1 β expression and maturation was explored further by Western blot. A151 treatment of oxygen and glucose deprived BMDM reduced mature IL-1 β in cell culture supernatant (Fig. 2a). Since the inflammasome multiprotein complex is a key regulator of IL-1 β production, the regulatory potential of A151 on the expression of additional inflammasome components was studied. A151 reduced mature caspase 1 (Fig. 2b) and NLRP3 (Fig. 2c), but did not affect ASC, AIM2, NLRP1 or NLRC4. IL-1 β can induce iNOS expression (Kim et al., 2006) and iNOS expression can influence stroke-induced cellular damage (Iadecola et al., 1996). We therefore analyzed iNOS levels and found that A151 reduced iNOS expression (Fig. 2d).

- 60 -

Example 4

A151 reduces the depolarization of mitochondrial membrane potential in BMDM

Mitochondrial dysfunction has been linked to NLRP3 inflammasome activation and it was observed that A151 reduces NLRP3 protein expression (Elliott et al., 2015, Immunol Rev 265: 35–52). It was determined whether A151 could ameliorate BMDM mitochondrial dysfunction. To accomplish this, the JC-1 assay was used to study the mitochondrial membrane potential (MMP). It was found that A151 reduced the depolarization of MMP (Fig. 4). Compared with C151 treatment, A151 reduced the percentage of cells with depolarized MMP from $15.8 \pm 2.8\%$ to $7.4 \pm 0.9\%$ ($p < 0.05$).

Example 5

A151 reduces permanent middle cerebral artery occlusion (pMCAO) induced brain ischemic injury in SHR-SP rats

The ability of A151 to prevent/treat ischemic injury was evaluated in SHR-SP rats using the pMCAO model. A single dose of 3 mg A151 was administered by i.p. injection 3 days prior to (-3d), 1 day prior to (-1d), or 3 hours post (+3h) pMCAO. 1 mg A151 administered 1 day prior to pMCAO was also tested. Each of these treatment regimens significantly reduced infarct volume ($P < 0.05$, Fig. 3). In male rats (Fig. 3B), at 48 h after MCAO, the infarct volumes (corrected for edema) of saline treated ($145.7 \pm 6.6 \text{ mm}^3$) and C151 treated ($141.3 \pm 7.6 \text{ mm}^3$) animals were similar; the infarct volumes in 3 mg A151 -3d treated animals ($119.5 \pm 5.8 \text{ mm}^3$) averaged 15.4% smaller than in C151 treated rats; infarct volume was decreased by 26.9% and 23.9%, respectively, in 3 mg A151 -1d group ($103.2 \pm 9.3 \text{ mm}^3$) and +3h group ($107.5 \pm 11.7 \text{ mm}^3$); 1 mg A151 -1d reduced infarct volume ($101.5 \pm 14.0 \text{ mm}^3$) by 28.1%. In female rats (Fig. 3C), compared with saline group ($116.8 \pm 7.1 \text{ mm}^3$), infarct volume was decreased in 3 mg A151 -1d group ($94.3 \pm 3.9 \text{ mm}^3$) and 3 mg A151 +3h group ($89.2 \pm 4.7 \text{ mm}^3$); 3 mg C151 +3h did not affect infarct volume ($118.8 \pm 10.3 \text{ mm}^3$).

Neurological deficits were also evaluated 48 hours after surgery. 3 mg of A151 improved the forepaw grasp and grip performance in female rats if administered 3 hours post surgery (Fig. 3D). A151 (3 mg) -1d showed a trend to improve the forepaw performance. In male rats, A151 did not improve the forepaw grasp and grip performance. Rats' circling, swing, and side push responses were also tested; and A151 did not affect these functions in either gender.

- 61 -

Example 6**A151 reduced NLRP3 mRNA in the brain of post ischemia SHR-SP rats**

Total RNA was purified from the brain of SHR-SP rats 48 hours after pMCAO. All data were normalized against sdha mRNA level and the expression of NLRP3 mRNA in 3 mg A151 –1d rats were compared to rats treated with saline and 3 mg C151 –1d. Compared with saline and 3 mg C151 -1d treatments, the expression of NLRP3 was reduced by about 6.9-fold and 2-fold in 3 mg A151 –1d treated rats, respectively ($P < 0.05$, Fig 3E).

The immune system influences susceptibility to ischemic stroke and the size of the resulting lesion. Inflammation has been shown to trigger strokes and to magnify subsequent brain damage (Emsley and Tyrrell, 2002, *J Cereb Blood Flow Metab* 22, 1399-1419; Fugate et al., 2014, *Lancet Infect Dis* 14, 869-880). Conversely, brain ischemia has been shown to trigger local inflammation that exacerbates brain damage and promotes brain recurrence (Iadecola and Anrather, 2011, *Nat Med* 17, 796-808; Courties et al., 2014, *JAMA Neurol* 71, 233-236). Thus, systemic inflammation can influence patient prognosis and survival (Emsley and Hopkins, 2008, *Lancet Neurol* 7, 341-353; McColl et al., 2009, *Neuroscience* 158, 1049-1061). The work disclosed herein evidences that immunosuppressive oligodeoxynucleotides such as A151, a synthetic oligodeoxynucleotide containing telemeric TTAGGG motifs, suppresses the production of inflammatory factors by BMDM subjected to OGD (including CINC-1, IL-1 α , mature IL-1 β , IL-6, TNF α , and mature caspase 1) and reduces ischemic brain injury in SHR-SP rats. The regulation of inflammasome sensors and adaptors was explored, and it was determined that A151 reduces the expression of NLRP3 protein in BMDM and NLRP3 mRNA in the ischemic brain of SHR-SP rats.

Inflammasomes act as sensors of both host-derived danger signals and infectious agents. Thus they play an important role in mediating inflammation in diseases including cancer, ischemia and reperfusion injuries, and metabolic and autoimmune disorders (Davis et al., 2011, *Cell Death Dis* 3, e338; Henao-Mejia et al., 2012, *Nat Immunol* 13, 321-324). Stroke involves increased extracellular ATP abundance, reactive oxygen species production, and necrotic cell death (Iadecola and Anrather, 2011, *supra*) and all of these factors are implicated in the activation of the NLRP3 inflammasome (Iyer et al., 2009, *Proc Natl Acad Sci U S A* 106, 20388-20393; Walsh et al., 2014, *Nat Rev Neurosci* 15, 84-97). Besides inducing bioactive IL-1 β production and subsequent inflammation, caspase 1 and NLRP3 also mediate cell death (Broz et al., 2010; Satoh et al., 2013, *Cell Death Dis* 4, e644). Immunoglobulin treatment, intermittent fasting, and intraperitoneal

- 62 -

injection of the fungal isolate, chrysophanol, attenuate NLRP3 inflammasome activity and reduce ischemic brain damage (Fann et al., 2013, *Cell Death Dis* 4, e790; Fann et al., 2014, *Exp Neurol* 257, 114-119; Zhang et al., 2014). The results disclosed herein evidence that ischemic stroke-induced brain damage can be ameliorated by modulating NLRP3 inflammasome activity.

5 The studies show that A151 reduced iNOS expression in BMDM subjected to LPS and OGD. In murine macrophages or microglia, iNOS is induced by LPS primarily through NF- κ B activation (Lowenstein and Padalko, 2004, *J Cell Sci* 117, 2865-2867; Wen et al., 2011, *J Neuroinflammation* 8, 38). Since NF- κ B also plays a key role in NLRP3 expression (Bauernfeind et al., 2009, *J Immunol* 183, 787-791; Qiao et al., 2012, *FEBS Lett* 586, 1022-1026) and NLRP3
10 expression was reduced by A151 treatment, it suggests that A151 can modify intracellular signaling pathways involving NF- κ B when cells are subjected to oxygen and glucose deprivation.

Without being bound by theory, it is possible that A151 may protect against brain ischemia by additional mechanisms. Multiple sensor molecules, including NLRP1, NLRP3, NLRP6, NLRP7, NLRP12, NLRC4, AIM2, and IFI16 can trigger inflammasome formation (Latz et al.,
15 2013, *Nat Rev Immunol* 13, 397-411). It has not been excluded that A151 regulates the expression of inflammasome sensor molecules in addition to NLRP3 or influences the multimolecular assembly of the inflammasomes. A151 binds to AIM2 and competes for binding of immune-stimulatory DNA (Kaminski et al., 2013, *J Immunol* 191, 3876-3883). Since A151 promotes the generation of regulatory T cells (Bode et al., 2014, *Int Immunopharmacol* 23, 516-522) and
20 regulatory T cells have been shown to protect brain from ischemia in mice (Liesz et al., 2009, *Nat Med* 15, 192-199), regulatory T cells may play a role in A151 brain protection from ischemia.

Phosphorothioate ODNs administered by intraperitoneal (i.p.) or intravenous (i.v.) injection distribute systemically and reach most tissues including the bone marrow (Saijo et al., 1994, *Oncol Res* 6, 243-249; Zhang et al., 1995, *supra*; Peng et al., 2001, *Antisense Nucleic Acid Drug Dev* 11,
25 15-27). Monocytes, macrophages, lymphocytes, dendritic cells, and endothelial cells internalize and respond to phosphorothioate ODNs following parenteral administration (Bijsterbosch et al., 1997; Klinman, 2004, *Nat Rev Immunol* 4, 249-258; Wilson et al., 2005, *Mol Ther* 12, 510-518). Thus, A151 could influence cell types other than microglia/macrophages and regulatory T cells.

Other studies examined the effect of administering immunostimulatory CpG ODNs to
30 animals with MCAO. CpG ODNs trigger TLR9 receptors and activate the innate immune system (Shirota and Klinman, 2014, *Expert Rev Vaccines* 13, 299-312) unlike A151 which suppresses

- 63 -

immunity and blocks TLR9 mediated signaling (Gursel et al., 2003). Systemic delivery of CpG ODNs prior to temporary MCAO reduced infarct volumes in mice and rhesus macaques (Stevens et al., 2008; Bahjat et al., 2011). In the murine model, TNF α levels increased 1 hour post CpG ODN injection and TNF α induction was required to reduce ischemic injury (Stevens et al., 2008). TNF α induction was not observed in SHR-SP rats after administering A151 nor was TNF α production reported by other groups following A151 treatment. In addition, unlike CpG ODN1826, A151 did not reduce OGD induced cell death in mixed cortical cultures. In combination with the findings that A151 reduced caspase 1 and IL-1 β maturation and NLRP3 expression, it was concluded that A151 ameliorates brain ischemic injury through mechanisms that differ from CpG ODNs.

Synthetic ODN A151 mimics the inhibitory activity of telemeric TTAGGG motifs and slow or prevent the development of diseases characterized by excessive immune activation (Klinman et al., 2009, *Ann N Y Acad Sci* 1175, 80-88). Available evidence suggests that A151 promotes the maturation of Th17 effector cells and improves host resistance to fungal pathogens (Bode et al., 2013, *Int Immunopharmacol* 23, 516-522). The reduced infarct volume in the brains of SHR-SP rats with middle cerebral artery occlusion demonstrated that a single administration of A151 is safe in an animal model of brain ischemia. No adverse effect was observed on weight, serum chemistry, and blood composition. Indeed, administering A151 for up to 32 weeks did not adversely impact health in mice (Dong et al., 2005, *Arthritis Rheum* 52, 651-658).

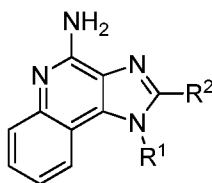
The development of stroke and post-ischemic inflammation involve multiple cell types and inflammatory pathways. Blocking upstream components of inflammatory signaling or targeting inflammatory pathways can be effective in animal stroke models (Iadecola and Anrather, 2011, *Nat Med* 17, 796-808). The study disclosed herein documents that oligodeoxynucleotides containing telemeric TTAGGG motifs reduce IL-1 β and caspase 1 maturation, NLRP3 expression, and ischemic brain injury.

In view of the many possible embodiments to which the principles of our invention may be applied, it should be recognized that illustrated embodiments are only examples of the invention and should not be considered a limitation on the scope of the invention. Rather, the scope of the invention is defined by the following claims. We therefore claim as our invention all that comes within the scope and spirit of these claims.

- 64 -

We claim:

1. A method of inhibiting or reducing ischemic tissue damage in a subject comprising selecting a subject having ischemic tissue damage or at risk of developing ischemic tissue damage; and administering to the subject a therapeutically effective amount of an imidazoquinoline compound having a formula:



wherein, R^1 is selected from $-C_{12-24}alkyl$, $-OC_{11-24}alkyl$, $-NHC_{11-24}alkyl$, $-COOH$ and $-X-Y-Z-R^6$, wherein

X is selected from the group consisting of a bond, $-O-$, and $-NH-$;

Y is selected from the group consisting of $C_{1-10}alkyl$ and $C_{1-5}alkylOC_{1-5}alkyl$

Z is selected from the group consisting of:

$-NHC(O)-$,

$-NHS(O)_2-$,

$-NHC(O)NH-$; and

R^6 is $-C_{11-23}alkyl$;

R^2 is selected from the group consisting of hydrogen, alkyl, alkylaminoalkyl, alkoxyalkyl, and hydroxyalkyl;

thereby inhibiting or reducing the ischemic tissue damage in the subject.

2. The method of claim 1, wherein R^2 is selected from the group consisting of hydrogen, methyl, ethyl, propyl, butyl, ethoxymethyl, methoxymethyl, 2-methoxyethyl, hydroxymethyl, and 2-hydroxyethyl.

3. The method of claim 1, wherein R^2 is selected from the group consisting of ethyl, propyl, butyl, methoxyethyl, and ethoxymethyl.

- 65 -

4. The method of claim 1, wherein R^2 is selected from the group consisting of butyl and ethoxymethyl.

5. The method of any one of claims 1-4, wherein Y is C_{2-10} alkyl.

6. The method of any one of claims 1-4, wherein Y is C_{2-4} alkylOC $_{2-4}$ alkyl.

7. The method of any one of claims 1-4, wherein Y is C_{2-5} alkyl.

8. The method of any one of claims 1-7, wherein X is selected from a group consisting of a bond and -O-.

9. The method of any one of claims 1-7, wherein X is -O-.

10. The method of any one of claims 1-9, wherein R^6 is C_{15-23} alkyl.

11. The method of any one of claims 1-9, wherein R^6 is $C_{15-C_{19}}$ alkyl.

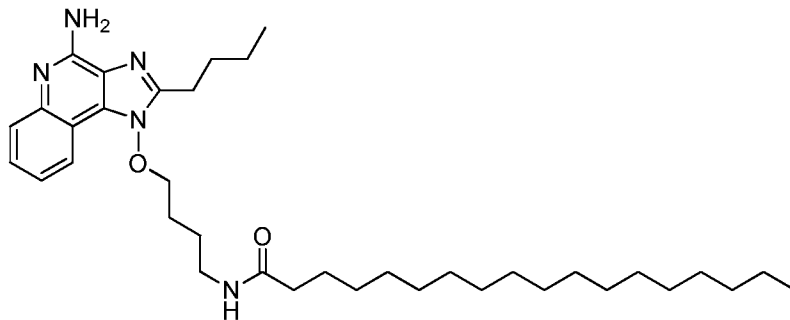
12. The method of any one of claims 1-11, wherein Z is -NHC(O) -.

13. The method of any one of claims 1-12, wherein R^1 is selected from the group consisting of $-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NHC(O)}C_{11-23}\text{alkyl}$, $-\text{OCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NHC(O)}C_{11-23}\text{alkyl}$, $-\text{NHCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NHC(O)}C_{11-23}\text{alkyl}$, $-\text{CH}_2\text{CH}_2\text{OCH}_2\text{CH}_2\text{NHC(O)}C_{11-23}\text{alkyl}$.

14. The method of any one of claims 1-12, wherein R^1 is selected from the group consisting of $-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NHC(O)}C_{15-23}\text{alkyl}$, $-\text{OCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NHC(O)}C_{15-23}\text{alkyl}$, $-\text{NHCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NHC(O)}C_{15-23}\text{alkyl}$, $-\text{CH}_2\text{CH}_2\text{OCH}_2\text{CH}_2\text{NHC(O)}C_{15-23}\text{alkyl}$.

- 66 -

15. The method of any one of claims 1-14, wherein the imidazoquinoline compound is:



16. The method of any one of claims 1-15, wherein the imidazoquinoline compound is
5 administered systemically, intra-cranially or intra-theccally to the subject.

17. The method of any one of claims 1-16, wherein the subject is at risk for ischemic tissue damage, and wherein the imidazoquinoline compound is administered from about six hours prior to the ischemic tissue damage to about 24 hours after the ischemic tissue damage.

18. The method of any one of claims 1-16, wherein the subject is at risk for ischemic tissue damage, and wherein the imidazoquinoline compound is administered at any time from about three hours prior to the ischemic tissue damage to about 24 hours after the ischemic tissue damage.

19. The method of any one of claims 1-16, wherein the subject is at risk for ischemic tissue damage, and wherein the imidazoquinoline compound is administered at any time from about three hours prior to the ischemic tissue damage to about 12 hours after the ischemic tissue damage.

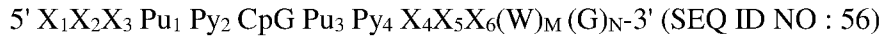
20. The method of any one of claims 1-16, wherein the subject is at risk for ischemic tissue damage, and wherein the imidazoquinoline compound is administered at any time from about three hours prior to the ischemic tissue damage to the time of the ischemic tissue damage.

21. The method of any one of claims 1-20, further comprising administering to the subject a therapeutically effective amount of an immunostimulatory composition comprising an
immunostimulatory oligodeoxynucleotide and a pharmaceutically acceptable carrier prior to
administering the imidazoquinoline compound to the subject, wherein the immunostimulatory

- 67 -

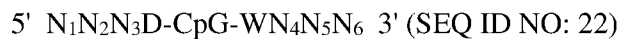
composition comprises either:

a) a D-type CpG oligodeoxynucleotide that is least 18 nucleotides and no more than 30 nucleotides in length and comprises a sequence represented by the formula:



wherein the central CpG motif is unmethylated, Pu is a purine nucleotide, Py is a pyrimidine nucleotide, X and W are any nucleotide, M is any integer from 0 to 10, and N is 6, wherein $X_1 X_2 X_3$ and $X_4 X_5 X_6$ are self complementary; or

b) a K-type CpG oligodeoxynucleotide that comprises a nucleic acid sequence set forth as:



wherein the central CpG motif is unmethylated, D is T, G or A, W is A or T, and N_1, N_2, N_3, N_4, N_5 , and N_6 are any nucleotide, wherein the K-type CpG oligodeoxynucleotide is 10 to 30 nucleotides in length.

22. The method of claim 21, wherein the immunostimulatory composition comprises the D-type oligodeoxynucleotide.

23. The method of claim 22, wherein $Pu_1 Py_2$ and $Pu_3 Py_4$ are self-complementary.

24. The method of any one of claims 20-21, wherein the D-type CpG oligodeoxynucleotide comprises the nucleic acid sequence set forth as any one of SEQ ID NOs:61-85.

25. The method of any one of claims 22-24, wherein the immunostimulatory composition comprises a) a D19 oligodeoxynucleotide comprising the nucleic acid sequence set forth as SEQ ID NO: 67; b) a D29 oligodeoxynucleotide comprising the nucleic acid sequence set forth as SEQ ID NO: 68.

26. The method of claim 25, wherein the immunostimulatory composition comprises the K-type CpG oligodeoxynucleotide.

27. The method of claim 26, wherein the K-type CpG oligodeoxynucleotide comprises the nucleic acid sequence set forth as one of SEQ ID NOs: 23-53.

- 68 -

28. The method of any one of claims 21-27, wherein the immunostimulatory oligodeoxynucleotide comprises at least one phosphate backbone modification.

5 29. The method of claim 28, wherein the immunostimulatory oligodeoxynucleotide comprises at least one phosphodiester base.

30. The method of claims 28 or claim 29, wherein the immunostimulatory oligodeoxynucleotide comprises a plurality of phosphorothioate bases.

10 31. The method of any one of claims 21-30, wherein the immunostimulatory composition is administered to the subject about two to about five days prior to the ischemic tissue damage.

32. The method of any one of claims 21-31, wherein the immunostimulatory composition is administered to the subject about three to about five days prior to the ischemic tissue damage.

15

33. The method of any one of claims 21-32, wherein the immunostimulatory composition is administered to the subject about three days prior to the ischemic tissue damage.

20 34. The method of any one of claims 1-32, wherein the immunosuppressive composition and/or the imidazoquinoline compound are administered parenterally to the subject.

35. The method of any one of claims 1-34, wherein the subject has or is at risk of having ischemia resulting from atherosclerosis or a vasculopathy

25 36. The method of any one of claims 1-34, wherein the subject has or is at risk of having a stroke or a transient ischemic attack.

37. The method of any one of claims 1-36, wherein the subject is at risk of ischemia from a surgical procedure.

30

38. The method of claim 37, wherein the wherein the surgical procedure is a vascular surgical procedure.

- 69 -

39. The method of claim 38, wherein the surgical procedure is an endarterectomy, a pulmonary bypass or a coronary artery bypass.

5 40. The method of any one of claims 1-34, wherein the subject has or is at risk of an ischemic reperfusion injury.

41. The method of claim 40, wherein the ischemic reperfusion injury is a cardiac, kidney, liver, or brain reperfusion injury.

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42. The method of any one of claims 1-41, wherein the subject has atherosclerosis.

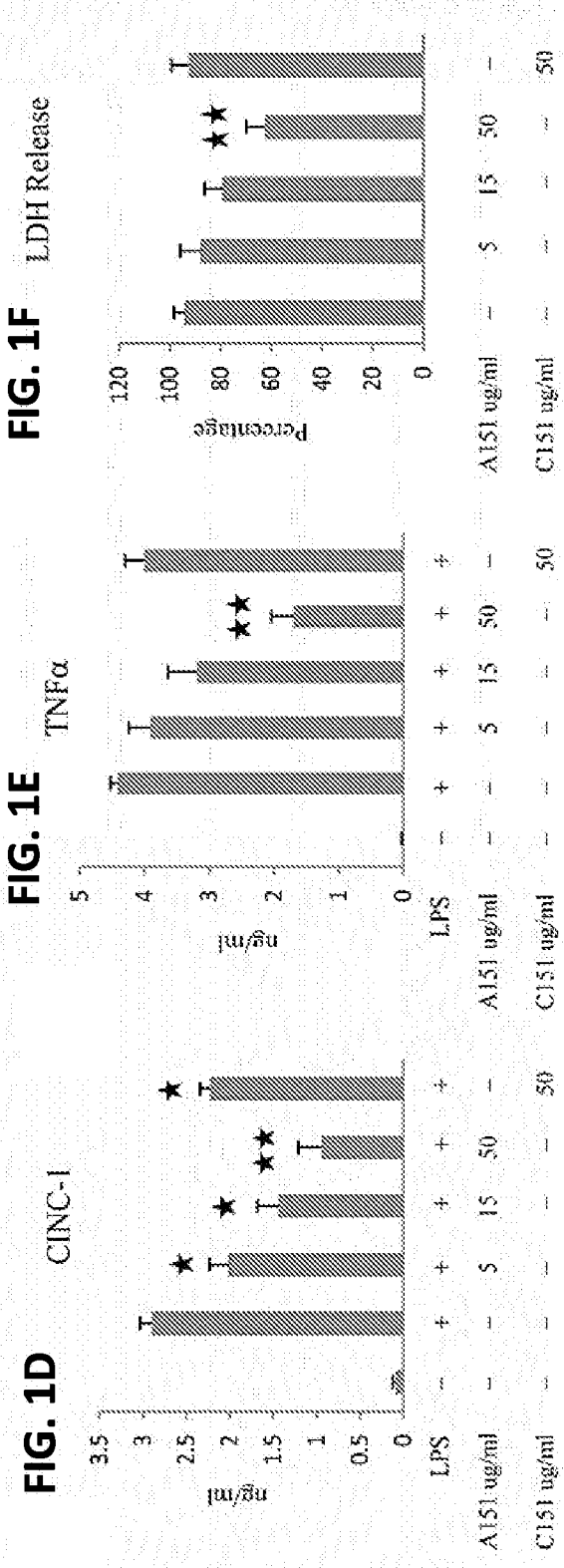
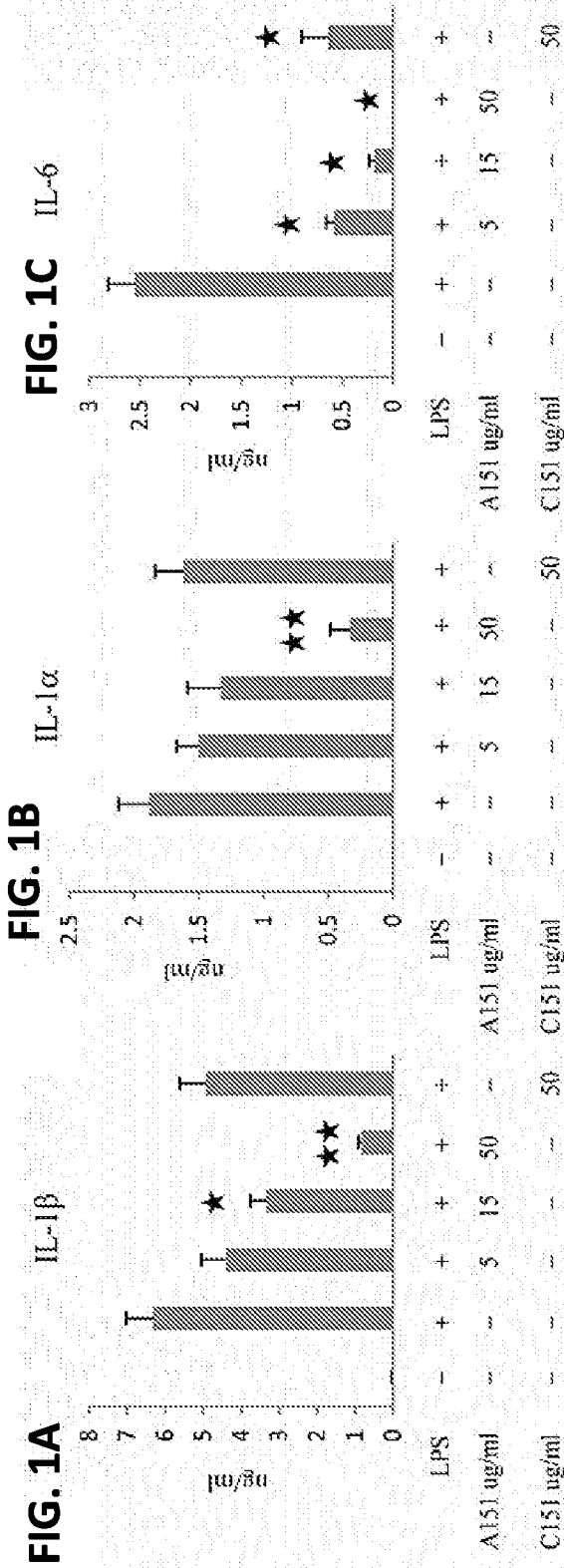


FIG. 2A

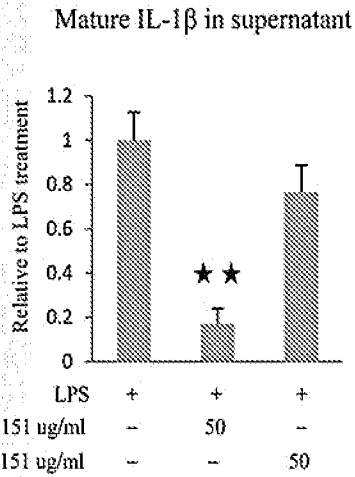
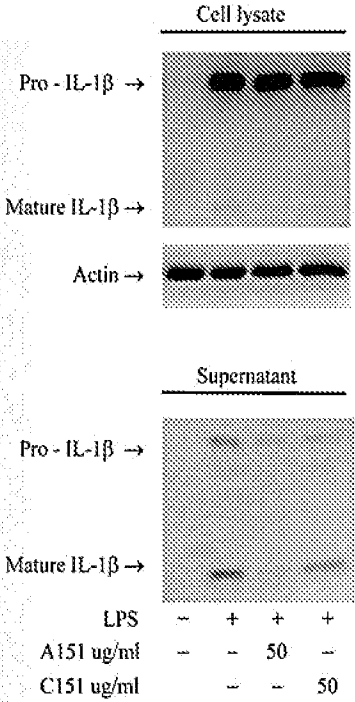


FIG. 2B

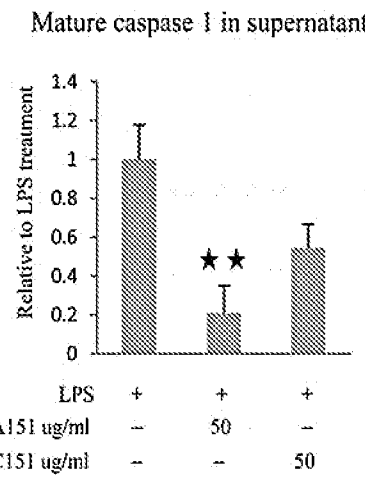
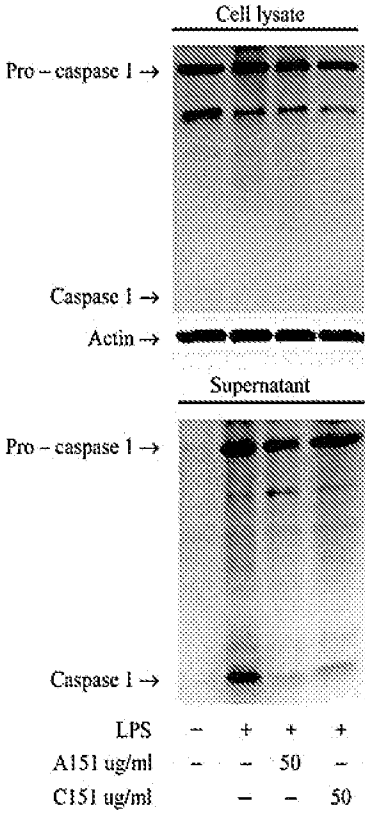


FIG. 2C

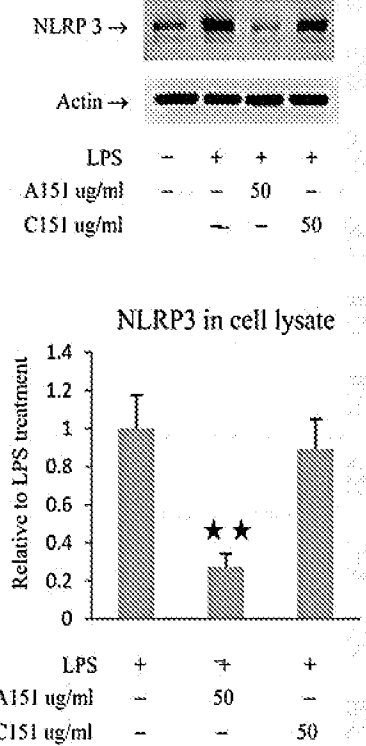


FIG. 2D

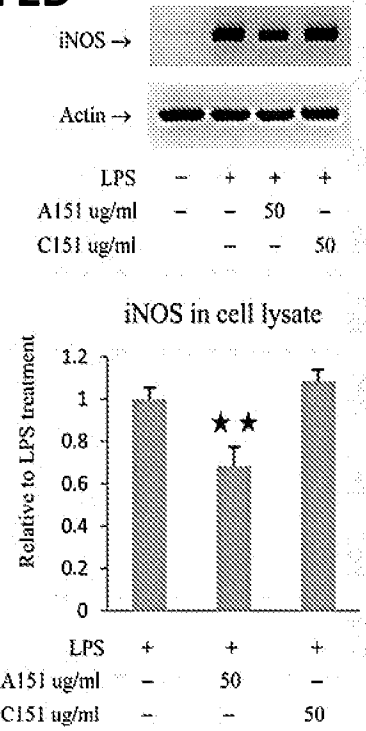


FIG. 3A

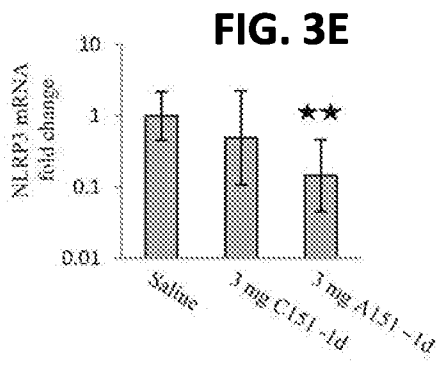
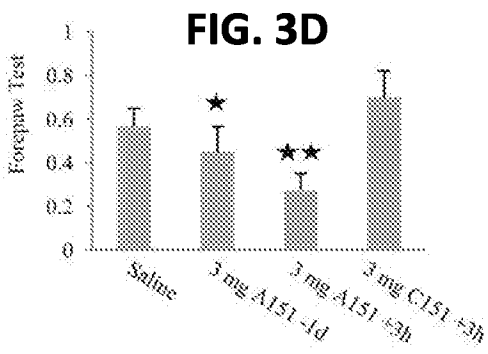
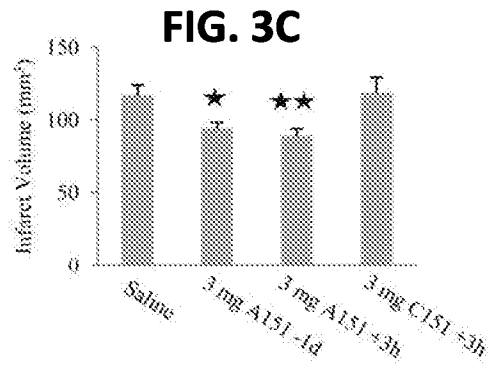
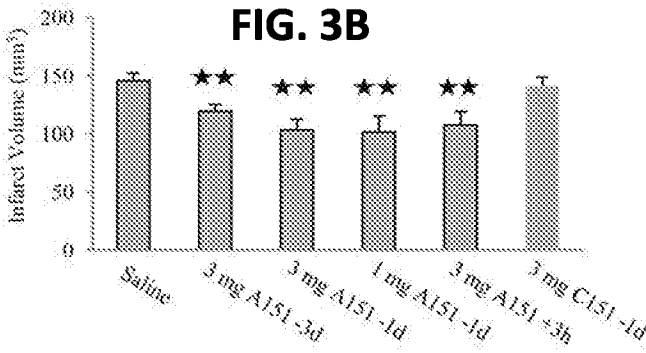
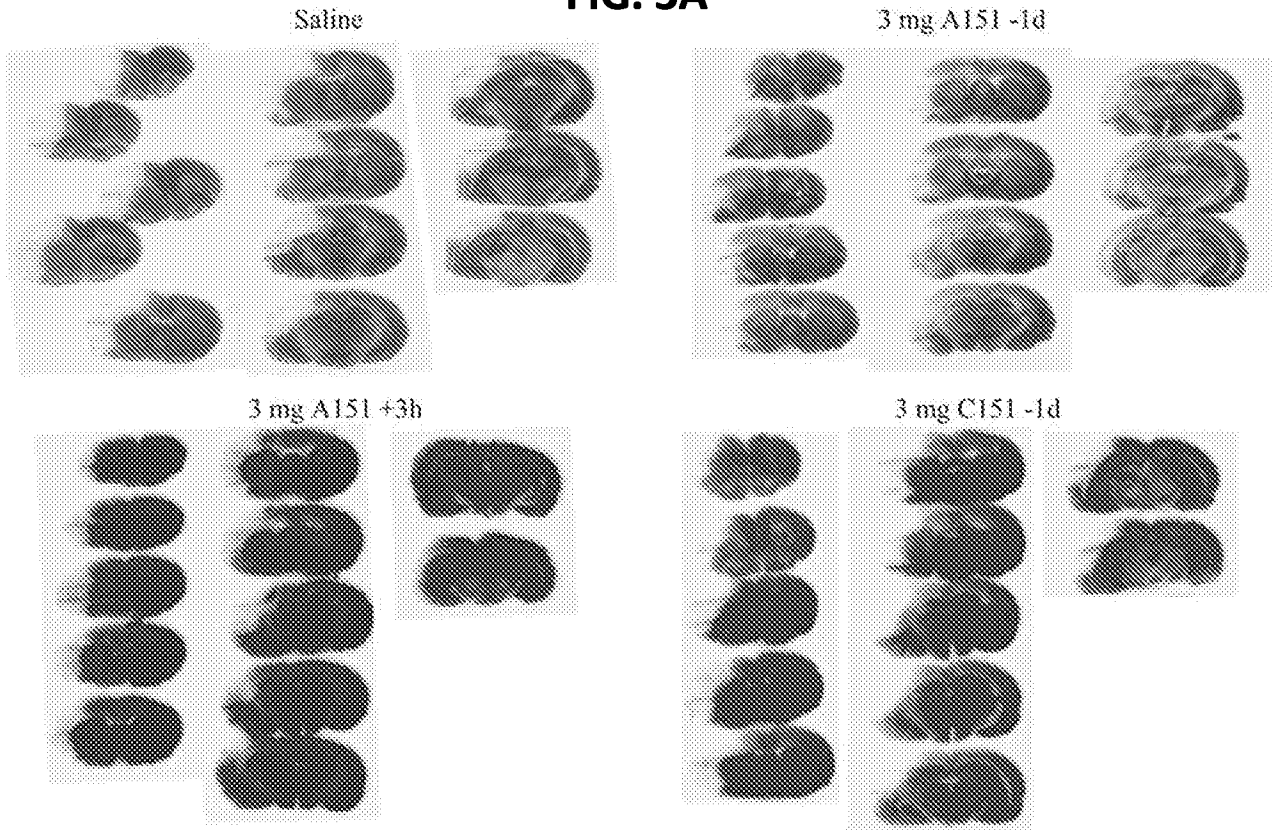
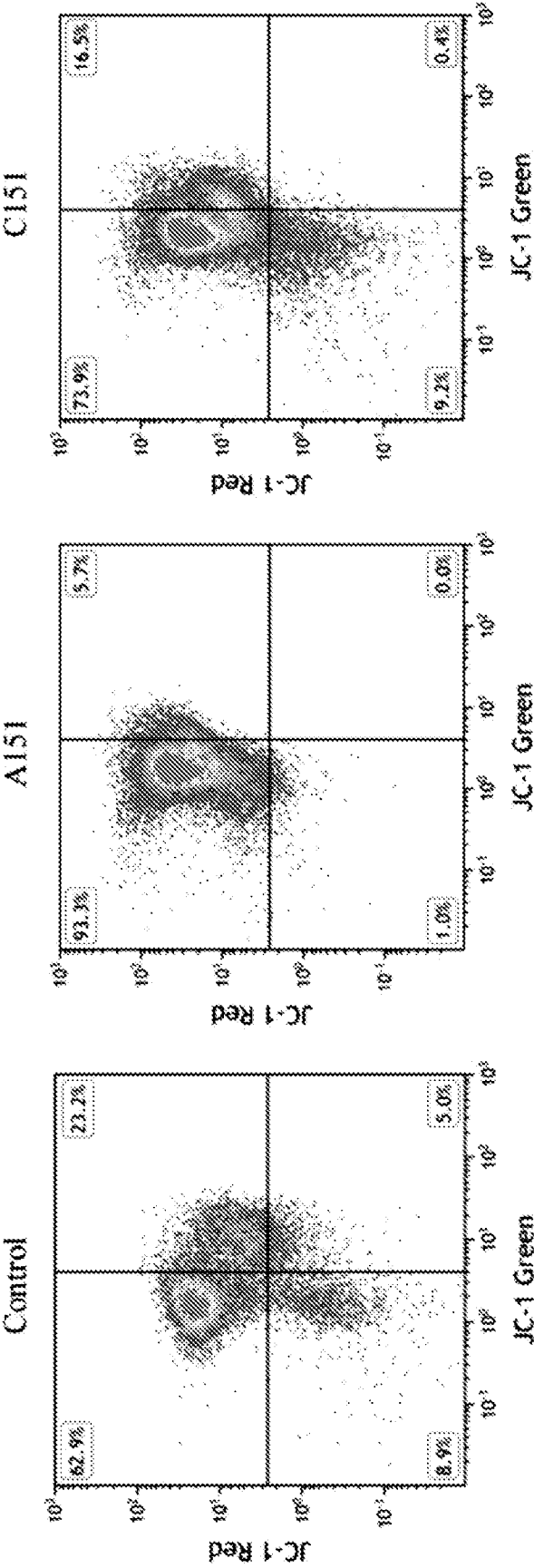
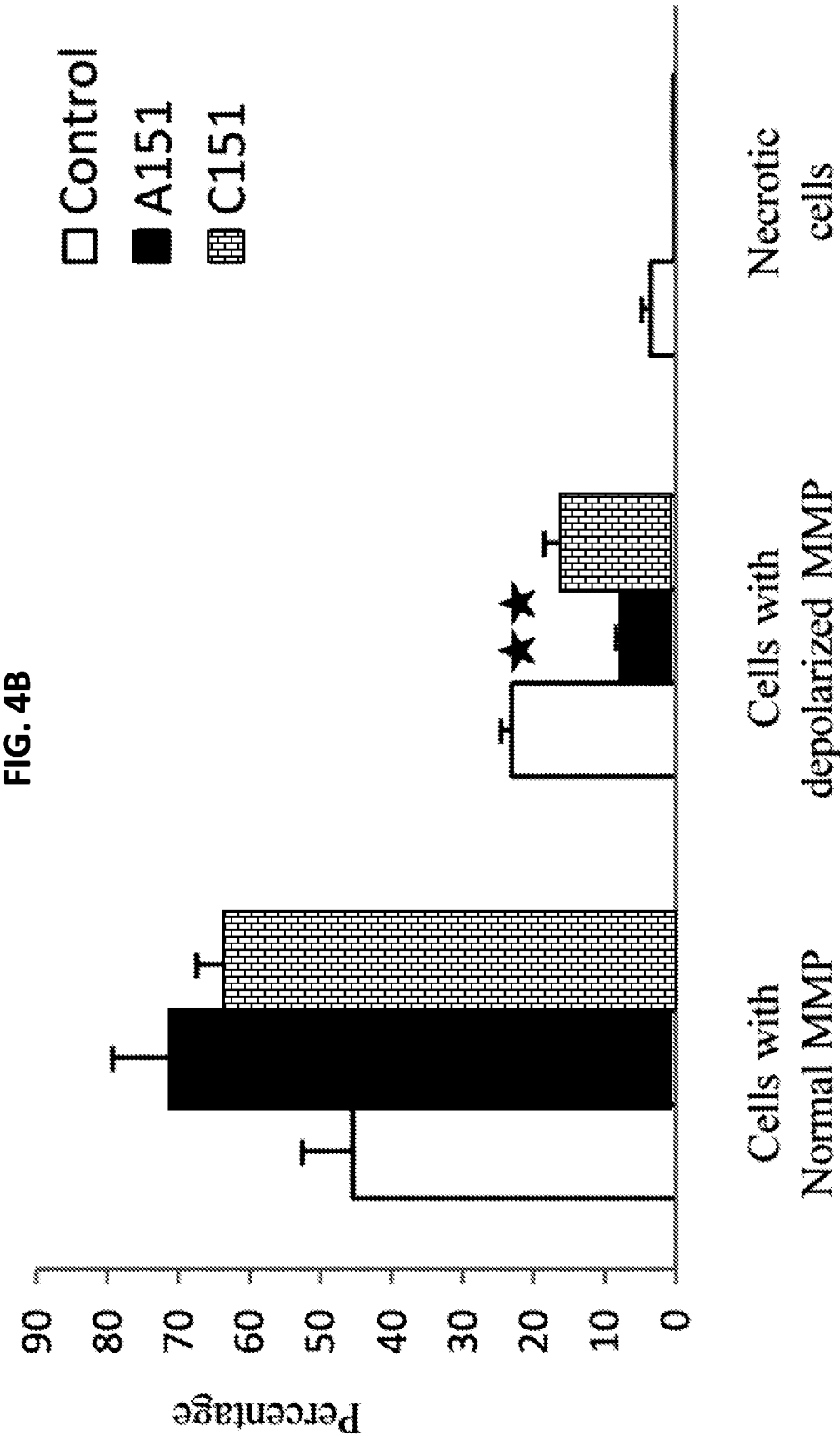


FIG. 4A





INTERNATIONAL SEARCH REPORT

International application No
PCT/US2016/032194

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, EMBASE, FSTA, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2007/030581 A2 (UNIV OREGON HEALTH & SCIENCE [US]; STENZEL-POORE MARY [US]; STEVENS SU) 15 March 2007 (2007-03-15) the whole document * see in particular paragraph [084], example 4; Fig. 2, 6 *	1-42
X	EP 2 322 178 A2 (UNIV OREGON HEALTH & SCIENCE [US]) 18 May 2011 (2011-05-18) the whole document * see in particular pages 17-20; examples 1, 4-6, 13; Fig. 6-9, 17 * ----- -/-	1-42



Further documents are listed in the continuation of Box C.



See patent family annex.

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Date of the actual completion of the international search

21 July 2016

Date of mailing of the international search report

10/08/2016

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

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INTERNATIONAL SEARCH REPORT

International application No

PCT/US2016/032194

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	P. Y. LEUNG ET AL: "Toll-Like Receptor 7 Preconditioning Induces Robust Neuroprotection Against Stroke by a Novel Type I Interferon-Mediated Mechanism", STROKE, vol. 43, no. 5, 8 March 2012 (2012-03-08), pages 1383-1389, XP055289800, US ISSN: 0039-2499, DOI: 10.1161/STROKEAHA.111.641522 the whole document * see in particular abstract; Fig. 1, 2, 4 *	1-42
A	----- WO 2014/201245 A1 (US HEALTH [US]; 3M INNOVATIVE PROPERTIES CO [US]) 18 December 2014 (2014-12-18) the whole document	1-42
A	----- DMITRI SMIRNOV ET AL: "Vaccine adjuvant activity of 3M-052: An imidazoquinoline designed for local activity without systemic cytokine induction", VACCINE, ELSEVIER LTD, GB, vol. 29, no. 33, 19 May 2011 (2011-05-19), pages 5434-5442, XP028240658, ISSN: 0264-410X, DOI: 10.1016/J.VACCINE.2011.05.061 [retrieved on 2011-05-25] the whole document	1-42
A	----- Mark Tomai: "The Use of Novel Toll-like Receptor Agonists and Delivery Systems to Increase the Effectiveness of Vaccines Head of Toll-like Receptor and Microneedle Business Development 3M Drug Delivery Systems", 1 January 2012 (2012-01-01), XP055123018, Retrieved from the Internet: URL:https://www.gtcbio.com/preview/vaccine/s/slides/tomai_mark.pdf [retrieved on 2014-06-12] the whole document	1-42
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A	----- US 2009/099122 A1 (KLINMAN DENNIS M [US] ET AL) 16 April 2009 (2009-04-16) the whole document ----- -/--	1-42

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2016/032194

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	VERTHELYI D ET AL: "Human peripheral blood cells differentially recognize and respond to two distinct CPG motifs", THE JOURNAL OF IMMUNOLOGY, THE AMERICAN ASSOCIATION OF IMMUNOLOGISTS, US, vol. 166, no. 4, 15 February 2001 (2001-02-15), pages 2372-2377, XP002299044, ISSN: 0022-1767 the whole document -----	1-42

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2016/032194

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