METHODS FOR INHIBITING IMMUNOSTIMULATORY DNA ASSOCIATED RESPONSES

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ABSTRACT

The invention features methods for inhibiting immunostimulatory DNA-associated immune responses, through the administration of specific compounds of the general class of 9-aminoacridines and 4-aminoquinolines. The invention also features methods of screening compounds useful in inhibiting immunostimulatory DNA-induced immune responses.

4 Claims, 6 Drawing Sheets
OTHER PUBLICATIONS

Mattsson et al., “Intracellular pathways involved in tumor necrosis factor-alpha release by human monocytes on stimulation with lipopolysaccharide or staphylococcal peptidoglycan are partly similar.” *Joint Infectious Diseases*, 173:212-218, 1996.


* cited by examiner
FIG. 9
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METHODS FOR INHIBITING IMMUNOSTIMULATORY DNA ASSOCIATED RESPONSES


This invention was made in the performance of a Cooperative Research and Development Agreement with the Department of Army, U.S. Army Medical Research and Material Command DAMD17-97-0097. The invention may be manufactured and used by the Government of the United States for all governmental purposes without any payment or royalty.

BACKGROUND OF THE INVENTION

The invention relates to a method of inhibiting cellular and humoral responses to immunostimulatory DNA with compositions including 4-aminquinolines, 9-aminocaridines and derivatives thereof.

Bacterial DNA is increasingly recognized as a powerful modulator of immunity (Krieg, 1997. Trends in Microbiol. 4:73-8), stimulating the polyclonal proliferation of B-cells and the production of cytokines by monocytes and other cells (Ballas et al., 1996. J. Immunol. 157:1840-5). This activity is attributed to unmethylated CpG sequences in bacterial DNA, which are uncommon in vertebrate DNA (Krieg et al., 1995. Nature 374:546-9). Single stranded oligodeoxyxynucleotides which have the motif PuPuCGPyPy (CpG-ODN) mimic many of the actions of bacterial DNA (Krieg et al., 1996. Antisense & Nucleic Acid Drug Devel. 6:133-9), and powerfully inhibit the induction of apoptosis in mouse WEHI 231 B-cells by anti-surface IgM (Yi et al., 1996. J. Immunol. 157:4918-25; Maevarlaine et al., 1997. Immunology 91:586-593). This system is a convenient and reproducible model to study responses to immunostimulatory DNA.

Bacterial DNA immobilized on beads does not stimulate immune responses, suggesting that internalization of the DNA is required for activity DNA and oligodeoxyxynucleotides are endocytosed into acidic vesicles, and are then transported to the cytoplasm and nucleus of cells.

Chloroquine, hydroxychloroquine and quinacrine are known to induce remissions of systemic lupus erythematous and rheumatoid arthritis by an unknown mechanism. These drugs bind to DNA by intercalation. They are weak bases and partition into acidic vesicles. At high concentrations, chloroquine can collapse the pH gradient and disrupt the action of endosomal hydrolatic enzymes and the trafficking of receptors.

SUMMARY OF THE INVENTION

The invention is based on the discovery that quinacrine, chloroquine and selected 9-aminocaridines and 4-aminquinoline compounds unexpectedly block the action of immunostimulatory DNA in cells at concentrations much below those needed for the other immunomodulatory effects of these compounds in vitro. The 9-aminocaridine and 4-aminquinoline compounds utilized in the methods of the invention inhibit the anti-apoptotic effect of CpG-ODN and the CpG-ODN-induced secretion of IL-6. On the other hand, these compounds do not influence the anti-apoptotic effects of other agents, for example, lipopolysaccharides. The effects of the compounds are highly specific to immunostimulatory DNA. These agents are useful for inducing remissions of autoimmune disorders such as rheumatoid arthritis and systemic lupus erythematous, leading to generally useful remittive and anti-inflammatory agents.

The methods of the invention are useful for inhibiting immunostimulatory DNA-associated responses in a subject, and involve the administration of a composition to a subject exhibiting responses initiated or exacerbated by immunostimulatory DNA. The compositions include 4-aminquinolines, 9-aminocaridines and derivatives thereof. These compounds may be linked to each other by linkers. The responses initiated or exacerbated by immunostimulatory DNA include those which result in the initiation or exacerbation of various disease states. The disease states include septic shock, inflammatory bowel disease, asthma, sinusitis, various autoimmune disorders such as hemolytic anemia, and others. The responses to be inhibited include the production of immunomodulatory proteins such as cytokines, interleukins, interferons, cell growth factors and chemokines. One such protein is IL-6. Other responses to be inhibited by the methods of the invention are high levels of erythrocyte sedimentation, and high levels of immunoglobulin.

The invention also features a method of screening compounds useful for inhibiting immunostimulatory DNA-associated responses. This method involves contacting a cell with immunostimulatory DNA, thereby inducing a measurable immune response, and a test compound. Any inhibition of the immune response is detected. The cells can be hybridoma plasma cells, including 7D11.

A “nucleic acid” or “DNA” means 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 pyridine (e.g., cytosine (C), thymine (T) or uracil (U)) or a substituted purine (e.g., adenine (A) or guanine (G)). As used herein, the term refers to ribonucleotides as well as oligodeoxynucleotides. The term also includes polynucleotides (i.e., a polynucleotide minus the phosphate) and any other organic base-containing polymer. Nucleic acid molecules can be obtained from existing nucleic acid sources (e.g., genomic or cDNA), but are preferably synthetic (e.g., produced by oligonucleotide synthesis). A “stabilized nucleic acid molecule” or “nuclease-resistant nucleic acid molecule” means a nucleic acid molecule that is relatively resistant to in vivo degradation (e.g., via an exo- or endo-nuclease). Stabilization can be a function of length or secondary structure. Unmethylated CpG-containing nucleic acid molecules that are tens to hundreds of kilobases long are relatively resistant to in vivo degradation. For shorter immunostimulatory nucleic acid molecules, secondary structure can stabilize and increase their effect. For example, if the 3’ end of a nucleic acid molecule is self-complementary to an upstream region, so that it can fold back and form a stem loop structure, then the nucleic acid molecule becomes stabilized and therefore exhibits more activity.

Preferred stabilized or nuclease-resistant nucleic acid molecules referred to in the instant invention have a modified backbone. For use in immune stimulation especially preferred stabilized nucleic acid molecules are phosphorothionate-modified nucleic acid molecules (i.e., at least one
of the phosphate oxygens of the nucleic acid molecule is replaced by sulfur). Preferably the phosphate modification occurs at or near the 5' and/or 3' end of the nucleic acid molecule. In addition to stabilizing nucleic acid molecules, phosphorothioate-modified nucleic acid molecules (including phosphorothioate-modified) can increase the extent of immune stimulation of the nucleic acid molecule, which contains an unmethylated CpG dinucleotide.

International Patent Application Number WO 95/26204 entitled “Immune Stimulation by Phosphorothioate Oligonucleotide Analogs” also reports on the non-specific immunostimulatory effect of phosphorothioate-modified oligonucleotides. International Patent Application WO 96/02555 discloses the ability of unmethylated CpG-containing oligonucleotides to activate lymphocytes. Unmethylated CpG-containing nucleic acid molecules having a phosphorothioate backbone have been found to preferentially activate B-cell activity, while unmethylated CpG-containing nucleic acid molecules having a phosphodiester backbone have been found to preferentially activate monocytes (macrophages, dendritic cells and monocytes) and NK (natural killer) cells. Other stabilized nucleic acid molecules include non-ionic DNA analogs, such as alkyl- and aryl-phosphonates (in which the charged phosphate oxygen is replaced by an alkyl or aryl group), phosphodiester and alkylphosphodiester, in which the charged oxygen moiety is alkylated. Nucleic acid molecules that contain a diol, such as tetraethyleneglycol or hexaethyleneglycol, at either or both termini have also been shown substantially resistant to nuclease degradation.

As used herein, the term “immunostimulatory DNA” refers to bacterial DNA, viral DNA, other DNA, synthetic double- or single-stranded DNA, DNA synthesized with a nuclease-resistant backbone or at least a partially nuclease-resistant backbone, (such as a phosphorothioate-backboned DNA) which stimulates (e.g., has a mitogenic effect on, or induces or increases cytokine expression by vertebrate lymphocytes. Such immunostimulatory DNA includes that which contains an unmethylated cytosine, guanine (CpG) dinucleotide sequence. A “CpG” or “CpG motif” refers to a nucleic acid having cytosine followed by a guanine linked by link containing phosphorus. 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. The term “unmethylated CpG” refers to the absence of methylation of the cytosine on the pyrimidine ring. Methylation, partial removal, or removal of unmethylated CpG motif in an oligonucleotide of the invention is believed to reduce its effect. Methylation or removal of all unmethylated CpG motifs in an oligonucleotide substantially reduces its effect. The effect of methylation or removal of a CpG motif is “substantial” if the effect is similar to that of an oligonucleotide that does not contain a CpG motif.

As used herein, the term “airway obstruction” includes respiratory ailments such as asthma, sinusitis, as well as airway obstructions caused by an infection, or exacerbated by an infection.

The terms “lower alkyl” and “lower alkoxy” refer to alkyl- and alkoxy groups comprising up to 7 carbon atoms. The terms include straight and branched chain groups. The term “alicyclic” refers to a non-aromatic carbon chain system forming a ring, and having from 4 to 10 members. The term “polyalicyclic” refers to a non-aromatic carbon chain system forming more than one ring each ring having from 4 to 10 members. The term “aryl group” refers to aromatic hydrocarbons having from 6 to 26 carbons.

Unless otherwise defined, 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 invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. 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 definitions, will control. In addition, the materials, methods and examples are illustrative only and not intended to be limiting.

**BRIEF DESCRIPTION OF THE DRAWINGS**

**FIG. 1** is a graph showing the effect of various concentrations (log scale) of selected compounds (quinacrine, amodiaquine, chloroquine, hydroxychloroquine, quinidine, quinine, and primaquine) on ODN 1760 protection against the α-slgM-induced inhibition of [3H] thymidine uptake in WEHI 231 cells.

**FIG. 2a** is a graph showing the effect of various cell medium additives (ODN 1760. α-slgM, ODN 1760/α-slgM, quinacrine, quinacrine/ODN 1760, quinacrine/α-slgM, and quinacrine/ODN 1760/α-slgM) on the WEHI 231 cell count measured at different time periods after incubation.

**FIG. 2b** is a bar graph showing the effect of various cell medium additives (ODN 1760, α-slgM, ODN 1760/α-slgM, and quinacrine/ODN 1760/α-slgM) on the percentage of DNA fragmentation detected from WEHI 231 cells.

**FIG. 3** is a graph showing the effect of various concentrations of ODN 1760 on the amount of [3H] thymidine uptake in the presence of various concentrations of quinacrine in WEHI 231 cells treated with α-slgM.

**FIG. 4** is a bar graph showing the effect of additions of quinacrine at various times after incubation on the extent of [3H] thymidine uptake in WEHI 231 cells treated with α-slgM. Also displayed is a comparison of the effect on [3H] thymidine uptake of incubation of α-slgM-treated WEHI 231 cells with ODN 1760 and a phosphodiester ODN with the same sequence as ODN 1760.

**FIG. 5** is a bar graph showing the effect of quinacrine on the protection by lipopolysaccharides against α-slgM-induced inhibition of [3H] thymidine uptake by WEHI 231 cells.

**FIG. 6** is a bar graph showing the effect of quinacrine on the protection by ODN 1760 against C3- ceramide-induced inhibition of [3H] thymidine uptake in WEHI 231 cells.

**FIG. 7** is a bar graph showing the effect of quinacrine on the protection by ODN against thapsigargin-induced DNA fragmentation in WEHI 231 cells.

**FIG. 8** is a graph showing ODN 1760-induced effects on percentage of [3H] thymidine uptake in human mononuclear cells.

**FIG. 9** is a graph showing ODN 1760-induced effects on percentage of IL-6 production in human mononuclear cells.

**DETAILED DESCRIPTION**

Unexpectedly, we find that compounds quinacrine, chloroquine, and other structurally related 4-aminoquinolines and 9-aminoacridines are extremely potent inhibitors of the action of immunostimulatory DNA. These compounds added in low nanomolar concentrations, completely block the ability of immunostimulatory DNA to protect WEHI 231 cells from growth arrest, DNA fragmentation and inhibition.
of thymidine incorporation induced by the engagement of its surface IgM, as well as apoptosis induced by other agents such as lipopolysaccharides. The inhibitory effect is non-competitive with respect to CpG-ODN, and it occurs even when quinacrine is added after the immunostimulatory DNA. The potency of chloroquine, quinacrine and derivates is magnified by inhibition of the multi-drug resistance pump, suggesting that their site of action is intracellular. The specificity of the effect toward CpG-ODN is illustrated by the fact that quinacrine does not influence the ability of lipopolysaccharides (LPS) to inhibit apoptosis in WVEHL 231 cells. These results can be reviewed in light of the known biological effects in vitro of quinacrine and chloroquine.

Being a diprotic weak base, chloroquine (and many of the other compounds which were used) partitions into the acidified cellular vesicles, such as lysosomes. At high concentration, chloroquine can collapse the pH gradient of lysosomes and induce their swelling. The compounds chloroquine, hydroxychloroquine and quinacrine induce remissions of rheumatoid arthritis and systemic lupus erythematosus. The beneficial effect of these compounds becomes apparent over the course of several weeks. To account for this valuable therapeutic action, a number of investigators have explored the effects of the compounds on immune and inflammatory responses in vitro, revealing a wide range of inhibitory actions. Quinacrine inhibits phospholipase A2 (Roberts et al., 1996. British J. Haematology 92:804-14, blocks ion channels (Sakuto et al., 1994. Eur J. Pharmac. 252:117-21), binds to receptors (Tamaamiz et al., 1995. Cell. & Mol. Neurobiol. 15:427-38), and inhibits fMLP-induced superoxide production and enzyme release by granulocytes (Foldes-Flepp et al., 1992. J. Leuk. Biol. 52:545-59). Chloroquine and quinacrine have little influence on other functional assays of neutrophils (Ferrante et al., 1986. Immunol. 58:125-30). Chloroquine inhibits cytokine release from mononuclear cells induced by endo- and exotoxins (Mattson et al., 1996. J. Infect. Dis. 173:212-8). Each of these described effects requires concentrations of chloroquine or quinacrine markedly higher than the concentrations used in the present invention to inhibit CpG-ODN-driven responses in WEHI 231 cells, and none of them satisfactorily explains the remittive effect of these drugs on autoimmune disorders. Chloroquine delays the recycling of proteins to the cell surface from lysosomes, resulting in altered trafficking of lysosomal enzymes and receptors (Chou et al., 1974 in Antibiotics III. Mechanism of action of antimicrobial and antitumor agents (Corcoran, J., and Hahn, F., eds.), pp. 281-92, Springer Verlag, Berlin). These effects on lysosomal functions are seen only when cells are exposed to higher concentrations of chloroquine than used here, so it is improbable that the suppression by the compounds of CpG-ODN responses can be attributed solely to these relatively gross actions, even though experiments with ammonia plus bafilomycin A₁ suggest that the immunostimulatory DNA-associated response requires vesicular acidification.

Methods of Inhibiting Immunostimulatory DNA-induced Responses

The invention features methods for inhibiting immunostimulatory DNA-induced responses in a subject. This method includes the administration of a therapeutically effective amount of a composition to the subject. The composition contains a compound that is effective in inhibiting immunostimulatory DNA-induced responses.

Compounds found to be useful for inhibiting immunostimulatory DNA-associated responses include 4-aminoquinoline compounds of the formula

R₂ can be a hydrogen atom, a lower alkyl group, or can be further linked to R₃ by an alkyl chain. If R₄ is a lower alkyl group, it is preferred to be methyl or ethyl. If R₄ is further linked to R₅ by an alkyl chain (in addition to the depicted linkage by the nitrogen atom), the alkyl chain can be —(CH₂)ₙ—, where n is from 4 to 7, and is preferably 4 or 5.

The substituent R₆ can be a hydrogen atom, an alicyclic group (such as a cyclopenyl, cyclohexyl or cycloheptyl group), an alkyl secondary, tertiary or quaternary amino group or alkyl secondary, tertiary or quaternary amino group. For example, R₆ can be an unsubstituted alkyl amine, such as 4-[pentyl-N,N-dialkyl amine], 4-[pentyl-N-alkyl amine], 4-[pentyl amine], 4-[butyl-N,N-dialkyl amine], 4-[butyl-N-alkyl amine], 4-[butyl amine], 2-[ethyl-N,N-diaryl amine], 2-[ethyl-N-alkyl amine], 2-[ethyl amine], 3-[propyl-N,N-dialkyl amine], 3-[propyl-N-alkyl amine], and 7-[hepta-4-methyl-4-azaamine]. The N-substituents are generally lower alkyl, but can also include hydroxy-substituted lower alkyl, such as 2-hydroxyethyl. Unsat urated chains include 4-[pent-2-ethyl-N,N-dialkyl amine] and 4-[pent-2-ethyl-N-alkyl amine]. Longer amine-containing alkyl chains can also be utilized, and the nitrogen need not be located at the terminus of the substituent group. Cyclic amines can be included in the alkyl chain. For example, the alkyl chain can terminate in, or be interrupted by, a pyrrole ring, a piperazidyl ring, a piperidyl ring, or a morphiolyl ring, any of which may be further substituted with lower alkyl groups. In embodiments in which R₆ is an unsubstituted amine-containing group, the preferred variants of R₆ can be 2-[ethyl-N,N-dimethyl amine], 2-[ethyl-N-methyl amine], 4-[pentyl-N,N-diethyl amine], and 4-[butyl-N,N-dietethyl amine].

The R₆ amine-containing chains described above can be variously substituted. Substituents include lower alkyl groups, such as methyl, ethyl and propyl. Other useful R₆ substituents include substituted or unsubstituted aryl groups, such as phenyl, naphthyl, hydroxyphenyl, chlorophenyl, dichlorophenyl, fluoro phenyl, naphthyl, thiophenyl, which can be substituted at o-, m- or p-positions, or at 1- or 2-positions in case of naphthyl; heterocyclic groups, such as pyridyl, pyrrolyl, piperidyl and piperazidyl; or halogen, such as chloro, bromo, and fluoro; and other substituents such as hydroxy, and alkoxyl. Further substitution of this amine-containing alkyl chain can comprise, for example, amide or ester linkages, ether or thioether linkages. The amine-containing alkyl chain can terminate with a substituent such as a primary, secondary or tertiary amine, a hydroxy group, a thiol, a carboxylic acid, or an amide.
The substituent R₈ can also be a diphilpolymer group, such as bicycloheptanyl, bicyclooctyl, or adamantyl, and can be linked to any position of these groups. R₈ may also be linked to R₈ by an alkyl chain.

The substituent R₉ can be a hydrogen atom, a lower alkyl group, or an aryl group. R₉ can also be a heteroaromatic group, such as 2-, 3-, or 4-pyridyl, 1-, 2-, or 3-pyrryl, or an aryl substituted lower alkyl group, such as trans-β- styryl and trans-β- [α,β-trans-dimethyl-p-chlorostyryl]. It is believed that bulky groups at R₉ contribute to the efficacy of the compounds of the invention. Thus, many of the embodiments include bulky groups at this position. Among the unsubstituted aryl groups useful as R₉, substituents include phenyl, 1- and 2-naphthyl, 1-, 2-, 3-, 4-, or 9-phenanthyridine and the like. Among the substituted aromatic hydrocarbons include the above-mentioned aryl groups, substituted with lower alkyl groups such as methyl and ethyl; halogens, such as chlorine, fluorine and bromine; perfluoroalkyl groups such as trifluoromethyl and perfluorooethyl; alkoxyl groups, such as methoxy; aryloxy groups such as phenoxo; amine-containing substituents such as N-[N,N-dimethyl ethylene diamine], or 1-[4-methylpyperazinone]. Any of the above groups can be present at α-, m-, or p-positions on a phenyl ring, or an aryl group. Multiply substituted aryl rings are also possible.

The substituent R₁₀ can be a hydrogen atom, a lower alkyl group such as methyl or ethyl, or an aromatic group such as phenyl. In certain preferred embodiments, the substituent R₁₀ is hydrogen or methyl.

The substituent R₁₁ can be a hydrogen atom, a lower alkyl group such as methyl or ethyl, or a halogen atom, such as chlorine, bromine or fluorine.

The substituent R₁₂ can be a hydrogen atom, a lower alkyl group such as methyl or ethyl, a lower alkyl group such as methoxy or ethoxy, an aryl group such as phenoxo, an aryl group such as phenyl, an amine group such as N,N-dimethyl amino or N,N-diethylamino, or a thioether group such as phenylthioethoxy benzylthioether. It is found that bulky substituents on position 6 of the quinoline ring enhance activity, so that many of the preferred embodiments include bulky groups at this position.

The substituent R₁₃ can be a hydrogen atom, a lower alkyl group such as methyl or ethyl, a lower alkyl group such as methoxy or ethoxy, an aryl group such as phenoxo, a halogen atom such as chlorine, bromine or fluorine, or a lower haloalkyl group, namely a lower perfluoroalkyl group, such as trifluoromethyl or perfluorooethyl.

The substituent R₁₄ can be a hydrogen atom or a lower haloalkyl group such as methoxy or ethoxy. Pharmacologically acceptable salts of any of these compounds are also included in the invention. These salts include protonated or deprotonated atoms on the 4-aminoquinoline and counterions including potassium, sodium, chloride, bromide, acetate and many other commonly recognized counterions.

If R₄ is a halogen, then at least one of R₅, R₆, R₇, R₈ or R₉ is not a hydrogen atom and R₉ is not 4-[[N,N-dialkyl-n-pentylanilene] or 4-[N-alkyl-N-hydroxyalkyl-n-pentylanilene].

It is further found that the 4-aminoquinoline compounds which inhibit immunostimulatory DNA-associated responses can be linked together via a linker. The linker can be connected to each molecule at either the same position on the compound, or at different positions. Preferred linked 4-aminoquinoline compounds are linked at the same position on each compound. The preferred position for linkage between the individual 4-aminoquinoline compounds is at the 4-amino position, shown as R₄ or R₅ in the structure. The linker can be of a number of types. Preferred linker types are alkyl chains, alkyl chains interrupted with nitrogen atoms, or alkyl chains interrupted with amide linkages. The alkyl chains are preferred to be at least two carbons in length and not more than twelve carbons in length. If the alkyl chain is interrupted by nitrogen atoms, it is preferred that the overall length of the chain be between two and twelve atoms. Preferred linker molecules include those of the formula

{[(CH₂)nN(R)(CH₂)m]Lo} wherein n, m, and n are independently between 1 and 5, and R is a hydrogen atom, a carbonyl group or a lower alkyl group. The linker can further include substituents to vary the hydrophobicity or hydrophilicity of the linked compound as a whole.

Further compounds found to be useful for inhibiting immunostimulatory DNA-associated responses include 9-aminocaridine compounds of the formula

The substituent R₈ can be a hydrogen atom or an alkyl secondary, tertiary or quaternary amino group. For example, R₈ can be an unsubstituted alkyl amine such as 4-[pentyl-N,N-dialkyl amine], 4-[pentyl-N-alkyl amine], 4-[pentyl amine], 4-[butyl-N,N-dialkyl amine], 4-[butyl-N-alkyl amine], 4-[butyl amine], 2-[ethyl-N,N-dialkyl amine], 2-[ethyl-N-alkyl amine], 2-[ethyl amine], 3-[propyl-N,N-dialkyl amine], 3-[propyl-N-alkyl amine], and 7-[hepta-4-methyl-4-azaazaine]. The N-substituents are generally lower alkyl, but can also include hydroxy-substituted lower alkyl, such as 2-hydroxyethyl. Unsubstituted chains include 4-[pent-2-ethyl-N,N-dialkyl amine] and 4-[pent-2-ethyl-N-alkyl amine]. Longer amine-containing alkyl chains are also possible, and the nitrogen need not be located at the terminus of the substituent group. Cyclic amines can be included in the alkyl chain. For example, the alkyl chain can terminate in, or be interrupted by, a pyrrolo ring, a piperazidyl ring, a piperidyl ring, or a morpholinyl ring, any of which may be further substituted with lower alkyl groups. In embodiments in which R₈ is an unsubstituted amine-containing group, the preferred variants of R₈ are 2-[N,N-dimethyl ethylamine], 2-[N-methyl ethylamine], 4-[N,N-diethyl pentyamine], and 4-[N,N-diethyl butylanilene]. Preferred embodiments of the 9-aminocaridines useful in the invention have R₈ as 4-[4-ary-N,N-dialkyl butylanilene], 4-[4-heteroaromatic,N,N-dialkyl butylanilene], 4-[4-ary-N,N-alkyl butylanilene], and 4-[4-heteroaromatic-N,N-alkyl butylanilene]. Quaternary nitrogen-containing variants of these residues are also envisioned as useful in the invention.

The R₉ amine-containing chains described above can be variously substituted. Substituents include lower alkyl groups, such as methyl, ethyl and propyl. Other useful R₉ substituents include substituted or unsubstituted aryl groups, such as phenyl, anisyl, hydroxyphenyl, chlorophenyl, dichlorophenyl, fluorophenyl, naphthyl, thiophenyl, which can be substituted at α-, m- or p-positions, or at 1- or 2-positions in case of naphthyl; heterocyclic groups, such as pyridyl, pyrrolyl, piperidyl, and piperazidyl; or halogen, such as chloro, bromo, and fluoro; and other substituents such as hydroxy, and alkoxyl. Further substitution of this amine-containing alkyl chain can comprise, for example,
amide or ester linkages, ether or thioether linkages. The amine-containing alkyl chain can terminate with a substituent group such as a primary, secondary or tertiary amine, anhydroxy group, a thiol, a carboxylic acid, or an amide. Preferred embodiments have 4-[N,N-dialkyl pentaamline]-4-[4-aryl-N,N-dialkyl butylaminle] or 4-[4-heteroaromatic-N-N-dialkyl butylamines] groups at this position, in which the aryl or heteroaromatic group is unsubstituted or substituted by halogen or alkoxyl, and the N,N-dialkyl groups are N,N-diethyl groups.

The substituent R₃ is a lower alkyl group, such as methyl or ethyl. Preferred embodiments have methyl at this position.

The substituent R₄ can be a hydrogen atom, or a lower alkoxyl group.

The substituent X is a halogen atom, such as chlorine, bromine or fluorine. Preferred embodiments have chlorine at this position.

It is further found that the 9-aminoacridine compounds which inhibit immunostimulatory DNA-associated responses can be linked together via a linker. The linker can be connected to each molecule at either the same position on the compound, or on different positions. Preferred linked 9-aminoacridine compounds are linked at the same position on each compound. The preferred position for linkage between the individual 9-aminoacridine compounds is at the 9-amino position, shown as R₄ or R₅ in the structure. The linker can be of a number of types. Preferred linker types are alkyl chains, alkyl chains interrupted with nitrogen atoms, or alkyl chains interrupted with amide linkages. The alkyl chains are preferred to be at least two carbons in length and not more than twelve carbons in length. If the alkyl chain is interrupted by nitrogen atoms, it is preferred that the overall length of the chain be between two and twelve atoms. Preferred linker molecules include those of the formula —[(CH₂)n_N[R(CH₂)m]n— where n1, n2 and to n3 are independently between 1 and 5, and R is a hydrogen atom, a carbonyl group, or a lower alkyl group.

Further useful compounds for inhibiting immunostimulatory DNA-associated responses include 4-aminoquinoline compounds and 9-aminoacridine compounds linked together by a linker. Preferred linked 4-aminoquinoline/9-aminoacridine compounds are linked at analogous positions on each compound. The preferred position for linkage between the 9-aminoacridine compounds is at the 9-amino position, shown as R₄ or R₅ in the structure and the linker is further linked to the 4-amino position of the 4-aminoquinoline compound. The linker can be of a number of types. Preferred linker types are those discussed above in connection with the 4-aminoquinoline and 9-aminoacridine compounds.

The compositions used in the methods of the present invention are useful to inhibit cellular and humoral immune responses. Such immune responses can be associated with infections. These immune responses are associated with immunostimulatory DNA, and can result in disease states or can exacerbate disease states. The immune responses which are inhibited by the present methods include those associated with septic shock; inflammatory responses such as inflammatory bowel disease; respiratory tract infections such as asthma and sinusitis; autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus; and autoimmune diseases involving humoral immune responses, such as hemolytic anemia.

The immunostimulatory DNA-induced responses to be inhibited by the compounds discussed herein can lead to production of cytokines such as IL-6. Thus, the methods of the present invention are intended to inhibit production of IL-6 in cells. If subjects have circulating levels of IL-6 which exceed 20 picograms/milliliter, the subject can be a candidate for treatment with the methods of the invention. Normal circulating levels of IL-6 are approximately 10 picograms/milliliter, while circulating levels of subjects with systemic lupus erythematosus (50 picograms/milliliter), cancer (70 picograms/milliliter), and septic shock (100 picograms/milliliter) are generally much higher (Papadopoulos 1995. J. Clin. Lab. Anal. 9:234-7). Thus the methods of the present invention will be useful for subjects exhibiting immunostimulatory DNA-induced disorders, and having circulating levels of IL-6 of at least 20 picograms/milliliter. Preferably, the subjects will have at least 40 picograms/milliliter of circulating IL-6.

The concentration of compounds included in compositions used in the methods of the invention can range from about 10 nM to about 10 μM, preferably about 10 nM to about 1 μM. Most preferably, the compounds are found in concentrations of from about 10 nM to about 100 nM. The immunostimulatory DNA-induced responses to be inhibited by the compounds discussed herein can lead to an increase in gammaglobulin over the normal values found in subjects. Immunoglobulins can be measured by the nephelometric method, or by electrophoresis (both described in "Protein Abnormalities", by Stephen Ritzman, Alan Riss, 1982). For example, the normal gammaglobulin levels according to the nephelometric method are: IgG, 700-1500 milligrams/100 milliliters; IgA 70-400 milligrams/100 milliliters; IgM 30-300 milligrams/100 milliliters; and IgD 0-40 milligrams/100 milliliters. When measured by electrophoresis, the normal value for gamaglobulin is 0.7 to 1.5 milligrams/100 milliliters.

The immunostimulatory DNA-induced responses to be inhibited by the compounds discussed herein can lead to an increase in the erythrocyte sedimentation rate, as measured by the Westergren sedimentation rate determination (Internal Medicine vol. 1, Joy H. Stein, ed., 1983 Little Brown Co. p. 162). Normal sedimentation rates are up to 20 mm/hour, so that the methods of the invention can be used with subjects exhibiting Westergren erythrocyte sedimentation rates of more than 24 mm/hour.

"Administering" the pharmaceutical composition of the present invention can be accomplished by various means known to the skilled artisan. The pharmaceutical compositions according to the invention can be administered locally or systemically. The term "therapeutically effective amount" means the quantity of a compound according to the invention necessary to inhibit a symptom in a subject. A "subject" is any vertebrate animal, preferably a mammal, including humans. Doses effective for therapeutic use will, of course, depend on the severity of the disease and the weight and general state of the subject. Typically, dosages used in vitro can provide useful guidance in the amounts useful for in vivo administration of the pharmaceutical composition. Animal models well known to those of skill in the art can be used to determine effective dosages for treatment of particular disorders. Various considerations are described, e.g., in Gilman et al., eds., Goodman and Gilman's: The Pharmacological Bases of Therapeutics, 8th ed., Pergamon Press, 1990; and Remington's Pharmaceutical Sciences, 17th ed., Mack Publishing Co Easton, Pa., 1990, each of which is herein incorporated by reference.

The pharmaceutical compositions according to the invention are in general administered topically, intravenously, orally, or parenterally, or as implants, and even rectal use is possible in principle. Suitable solid or liquid pharmaceutical preparation forms are, for example, granules, powders,
found that immunostimulatory DNA can substitute for IL-6 in this cell line (Manzel et al., 1998, Life Sciences 62:23-7). Thus, the cell line 7TD1 is the first established cell line shown to be immunostimulatory DNA-dependent for growth.

An additional useful assay of antagonists of immunostimulatory DNA action is the protection by immunostimulatory DNA from the killing effect of antibody to cell surface immunoglobulin-M on WEHI 231 murine B-cells. Inhibition of this protection by the addition of a compound is then determined.

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

EXAMPLES

The following examples illustrate specific embodiments of the compounds of the invention, and demonstrate their efficacy in some aspects of the claimed methods. General procedures were employed as described here.

Unless otherwise noted, cell culture conditions were as follows. WEHI 231 cells (ATCC #CRL 1702) were grown in log phase in a medium of RPMI 1640 supplemented with 10% fetal bovine serum (heat inactivated at 65°C for one hour), 0.3 grams/Liter of L-glutamine, 50 milligrams/Liter gentamycin, and 0.05 mM 2-mercaptoethanol in a 37°C humid atmosphere with 5% CO2. WEHI 231 cells were diluted with 0.4% trypan blue in phosphate-buffered saline (PBS) and live cells were counted in a hemocytometer chamber. Cells were resuspended in medium at 2x107/milliliter in 24-well tissue culture plates (Costar, Cambridge Mass.). Appropriate additions were made, and the cells were incubated and counted daily. WEHI 231 cells were diluted with fresh medium if the concentration exceeded 7x107/milliliter.

The 7TD1 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (heat inactivated at 65°C, 1 hour), 0.3 grams/Liter L-glutamine, 50 milligrams/Liter gentamycin, 0.05 mM 2-mercaptoethanol, and 10 units per milliliter murine IL-6 in a 37°C humid atmosphere with 5% CO2. Viable 7TD1 cells were counted in a hemocytometer chamber in duplicate after dilution with 0.4% trypan blue. Cell counts were within 10% or 100 microliters of each other.

Preparation and sources of materials are as follows. For the production of IL-6 enzyme-linked immunoassay assay (ELISA), cells were incubated as indicated. At 48 hours, the cell culture supernatant was harvested and assayed for murine IL-6 by ELISA according to manufacturer’s instructions (PharMingen, San Diego Calif.). C6-G-ODN 1760 has the sequence 5'-ATAMCGACGTTCAAGCAAG-3' synthesized on a phosphorothioate or (when indicated) on a phosphodiester backbone. The oligonucleotides were purchased from Genosys (The Woodlands TX). Murine IL-6, murine neutralizing antibody to IL-6, and ELISA antibodies were purchased from PharMingen (San Diego Calif.). The following compounds were all purchased from Sigma Chemical Co., St. Louis Mo; goat anti-mouse IgM (α-mlgM, Cat. #M8644), lipopolyaccharide (E. coli serotype 0127:B8, Cat. #L3129), amodiaquine, chloroquine, quinacrine, primaquine, quinidine, quinine, coralyne chloride, and thapsigargin. Hydroxychloroquine was purchased from Copley Pharmaceutical, Inc., Canton Mass. Verapamil was from American Reagents Laboratory, Shirley N.Y. C3-ceramide was purchased from Matreya, Inc., Pleasant Gap Pa. Acridine homodimer (bis-(6-chloro-2-methoxy-9-acridinyl)sper-
The other compounds listed in Tables 2 and 3 were generous gifts from Dr. Jill Johnson of the National Cancer Institute. These compounds were synthesized generally according to known methods of synthesis (Elslager et al., 1969; J. Med. Chem., 12:970-4; Elslager et al. 1968; J. Med. Chem., 11:572, 1968; Marquez et al., 36-9). The compounds are available from Developmental Therapeutics Program, National Cancer Institute, Executive Plaza North, Bethesda Md., 20892.

Example 1

Simultaneous Addition of Immunostimulatory DNA and Compounds

The assay of [³H] Thymidine incorporation was carried out as follows. WEHI 231 cells (200 µL at 2x10⁶/milliliter, in duplicate or triplicate) were incubated in a 96-well plate (Costar, Cambridge Mass.). Additions were made as indicated. After incubating for 16 hours, 0.5 µCi [methyl-³H] thymidine (2 Ci/millimole, DuPont NEN, Boston Mass.) was added to each well, and the cells were incubated for another 4 hours. Cellular DNA was then captured onto glass fiber filters (Whatman 934-AH) using a cell harvester (Brandel, Gaithersburg Md.). The filters were dried, placed into plastic scintillation vials with 10 milliliters of scintillation cocktail (EcoSafe, Research Products International, Mount Prospect III.), and counted in a liquid scintillation counter (Beckman LS-3133 T). The ³H recovered on the filter was expressed as a fraction of the ³H added. In a typical experiment, WEHI 231 cells incorporate about 15% of added ³H.

Referring to experiments summarized in FIG. 1, WEHI 231 cells (ca. 2x10⁵ per milliliter) were incubated for 16 hours at 37°C with 10 µgrams/ml/illiter α-slgM, 6 µgrams/ml/illiter ODN 1760, and the indicated concentration of the compound. After 16 hours, 0.5 µCi [³H] thymidine was added. After 4 more hours incubation, the cellular DNA was harvested onto glass fiber filters. The dry filters were counted in a liquid scintillation counter. In the absence of other additions, cells incorporated about 20% of the added radioactivity. This was reduced to about 1% by the addition of α-slgM. In the absence of a compound, ODN 1760 restored the uptake to about 15% of added radioactivity. The vertical axis of FIG. 1 shows the ratio of the uptake of cells incubated with α-slgM divided by the uptake of cells incubated with α-slgM with Cpg-ODN, each measured with the indicated concentration of test compound. Each experimental point was the mean of at least duplicate measurements.

In the absence of other additions, α-slgM powerfully inhibited [³H] incorporation. This inhibition is almost completely relieved by ODN 1760. FIG. 1 shows that quinacrine, hydroxychloroquine, chloroquine and amodiaquine (substituted 4-aminochloroquinolines) completely inhibited the Cpg-ODN effect at nanomolar concentrations. The compounds themselves did not inhibit [³H] thymidine incorporation unless added at concentrations well in excess of 30 µM. Quinacrine and quinine (substituted 4-methyliquinolines) and primaquine (a substituted 8-aminquinoline) had little effect on Cpg-ODN action at concentrations less than 10 µM. Quinacrine and chloroquine showed promise as representative members of structural families useful in inhibiting immunostimulatory DNA-induced responses.

Example 2

Effect on Growth and Apoptosis

The DNA fragmentation assay was carried out as follows. WEHI 231 cells were incubated with 0.5 µCi/milliliter [³H] thymidine for 3 hours, centrifuged, resuspended in 2 milliliters of fresh medium at 2x10⁶/milliliter, and incubated in a 24-well plate with additions as indicated. After incubation for 16-18 hours, 1 milliliter aliquots of the cells were centrifuged, washed with 200 µl/tes PBS. This suspension was drawn into a pipette tip containing 25 µl of 2% SDS, and the mixture was immediately ejected into a well of a 0.8% agarose TBE gel containing 0.5 µl/milliliter ethidium bromide. The gel was electrophoresed for 1 hour at 65 volts, destained in water, and photographed under ultraviolet light. The lanes of the gel were cut into three approximately equal portions. The first portion included the gel well and the first 2 millimeters of the running lane. The second and third pieces were the remainder of the lane. The gel portions were melted in glass scintillation vials and mixed with a 20 milliliter of scintillation cocktail and counted in a scintillation counter. The amount of fragmented DNA (DNA in the second and third gel pieces) was expressed as a percentage of the total counts recovered in the lane.

Referring to the experiments summarized in FIG. 2a, WEHI 231 cells (2x10⁶ per milliliter) were incubated with or without 6 µgrams/milliliter ODN 1760, 10 µgrams/milliliter α-slgM, or 0.1 µM quinacrine as indicated. Live cells were counted daily. The cells were diluted in medium if their concentration exceeded 7x10⁶ per milliliter. Referring to the experiments summarized in FIG. 2a, [³H]-labeled WEHI 231 cells were incubated with no addition, or simultaneous additions as indicated. (α-slgM 10 grams/milliliter, ODN 1760 6 grams/milliliter, quinacrine 0.1 µM). After 16 hours, cells were loaded into wells of 0.8% agarose gel by lysing with 1% SDS, and DNA electrophoresed 1 hour at 65 volts. The percentage fragmentation was estimated as described above. In this and other figures, error bars indicate 1 standard deviation.

Quinacrine alone inhibited neither cell growth nor cell death induced by α-slgM. Cpg-ODN protects cells against α-slgM-induced apoptosis. Quinacrine reversed this protection by ODN 1760, revealed by measurement of both cell count over a period of three days (FIG. 2a) and percentage of fragmented DNA (FIG. 2b). Similar results were obtained with chloroquine. These data show that the effect of certain compounds on thymidine uptake is a true reflection of their effect on cell growth and protection from apoptosis.

Example 3

Effect on IL-6 Synthesis

The assay of IL-6 was generally carried out as follows. WEHI cells (2x10⁷/milliliter) were incubated with or without ODN 1760 (6 µgrams/milliliter) and with or without chloroquine or quinacrine. After 24 hours, the IL-6 content of the supernatant was assayed by enzyme-linked immunoassay using two monoclonal antibodies in a sandwich technique as described by the manufacturer, using standards supplied (PharMingen, San Diego Calif.).

Besides inhibiting apoptosis, Cpg-ODN stimulated the synthesis of cytokines, including IL-6. In the absence of other additions, WEHI 231 cell elaborated about 700 picograms IL-6 per milliliter. ODN 1760 increased this elabo-
In the experiments summarized in FIG. 5, the influence of LPS on α-slgM-induced suppression of thymidine incorporation was examined. Even at high concentrations, LPS has much less ability than ODN 1760 to reverse α-slgM-induced growth inhibition in WEHI 231 cells. Quinacrine at a concentration that completely reversed the effect of ODN 1760 had no effect on the action of LPS. Thus (at the concentration used) quinacrine did not block the detection of LPS and subsequent signal-response coupling in the same way that it did with Cpg-ODN.

Lipopolysaccharide (LPS) was added at the indicated concentration with or without 10 μg/ml liter α-slgM and 0.1 μM quinacrine. The small degree of protection against α-slgM-induced inhibition of [³H] thymidine uptake afforded by LPS is not influenced by quinacrine.

Example 7

Immunostimulatory DNA Blockade of Apoptosis Induced by Other Agents

Cpg-ODN has been previously shown to block apoptosis in WEHI 231 cells which is induced by stimuli other than α-slgM (Macfarlane et al., 1997. Immunology 91:286). The effect of quinacrine on the protection by ODN 1760 from cell death induced by agents other than α-slgM was examined, to determine if the action of the studied compounds was restricted to α-slgM-induced apoptosis. C₂⁻ceramide mediates receptor-induced cell death (Hannun, 1996. Science 274:1855-9) and inhibits thymidine incorporation. Thapsigargin induces an increase in intracellular calcium, resulting in massive DNA fragmentation.

FIG. 6 shows that the effect of C₂⁻ceramide was reduced by ODN 1760, and that quinacrine blocked this protective effect. FIG. 7 shows that quinacrine also blocked the protective effect of ODN 1760 against thapsigargin-induced DNA fragmentation. Thus, quinacrine appeared to block the protective effect of immunostimulatory DNA generally, not just the protection against α-slgM-induced apoptosis.

Referring to the experiments summarized in FIG. 6, WEHI 231 cells were incubated with C₂⁻ceramide as indicated with or without 6 μg/ml liter ODN and 0.1 μM quinacrine and thymidine incorporation was estimated. Referring to the experiments summarized in FIG. 7, WEHI 231 cells were prelabeled with 6 μCi/ml liter [³H] thymidine for 3 hours, and resuspended in fresh medium containing 10 nM thapsigargin, 10 μg/ml liter α-slgM, 6 μg/ml liter ODN, and/or 0.1 μM quinacrine as indicated. Cells were lysed 24 hours later, and the DNA was electrophoresed as described above in Example 2 to estimate the percentage fragmentation of DNA.

Example 8

Inhibition of 7TD1 Cell Growth by Compounds

Little growth occurs when 7TD1 cells are incubated in RPMI 1640 supplemented with fetal calf serum without added IL-6 or Cpg-ODN. The addition of IL-6 supported vigorous growth with half-maximal effect at about 10 micro-liters/ml liter. The phosphorothioate Cpg-ODN 1760 supported the growth of the 7TD1 cells in the absence of added IL-6. Growth stimulation by Cpg-ODN was blocked by chloroquine and quinacrine, but these 4-aminoquinoline compounds had little effect on IL-6-induced growth (Table 1).
The 7TD1 cells were incubated for five days and were washed twice with medium to remove IL-6, and resuspended at 2×10^6 per milliliter in medium. Cell counts were performed and the percent inhibition of growth was computed. The term “anti” refers to 40 nanograms/milliliter neutralizing antibody to IL-6.

### TABLE 1

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<thead>
<tr>
<th>Addition</th>
<th>7TD1 cells/µL</th>
<th>% Growth Inhibition</th>
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<tr>
<td>no addition</td>
<td>10</td>
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<tr>
<td>10 µg/mL IL-6</td>
<td>887</td>
<td>0</td>
</tr>
<tr>
<td>10 µg/mL IL-6 + anti + IL-6</td>
<td>117</td>
<td>86.7</td>
</tr>
<tr>
<td>10 µg/mL IL-6 + 1 µM chloroquine</td>
<td>610</td>
<td>31.2</td>
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<tr>
<td>10 µg/mL IL-6 + 3 µM chloroquine</td>
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<td>10 µg/mL IL-6 + 0.3 µM quinacrine</td>
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<td>10 µg/mL IL-6 + 0.3 µM quinacrine</td>
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<tr>
<td>6 µg/mL ODN 1760</td>
<td>407</td>
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</tr>
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<td>10.4</td>
</tr>
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<td>98.1</td>
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<tr>
<td>6 µg/mL ODN 1760 + 3 µg chloroquine</td>
<td>10</td>
<td>97.5</td>
</tr>
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<td>6 µg/mL ODN 1760 + 0.1 µM quinacrine</td>
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<td>95</td>
</tr>
<tr>
<td>6 µg/mL ODN 1760 + 0.3 µM quinacrine</td>
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<td>6 µg/mL ODN 1760 + 1 µM quinacrine</td>
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<td>96.3</td>
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### Example 9

**Immunostimulatory Effects on Human Mononuclear Cells**

The effects of the compounds is not restricted to immortal mouse cells. Unfractionated human peripheral blood cells incorporated thymidine and produced IL-6 when exposed to ODN 1760, as displayed in FIGS. 8 and 9, respectively. Both effects were inhibited by quinacrine and chloroquine.

Referring to the experiments summarized in FIG. 8, human mononuclear cells (2×10^6/milliliter) from three different donors were incubated with or without ODN 1760 and the indicated concentration of quinacrine or chloroquine for four days. [3H] thymidine incorporation (18 hour pulse) was then measured. FIG. 8 shows [3H] uptake as a percentage of ODN-treated cells. The [3H] uptake per donor for control samples was: donor 1, 4.53%; donor 2, 2.76%; donor 3, 6.39%. Thymidine incorporation for untreated samples was less than 0.05%. Samples from three donors were averaged, and error bars indicate 1 standard deviation.

Referring to the experiments summarized in FIG. 9, human peripheral blood mononuclear cells (PBMC) from three donors were incubated with or without ODN 1760 and the indicated concentration of test compound. After 24 hours, the concentration of IL-6 was determined in the supernatant. The figure indicates the percentage of IL-6 in reference to ODN-treated cells. The IL-6 amounts for control sample per donor were as follows: donor 1, 203 picograms/milliliter; donor 2, 71.5 picograms/milliliter; donor 3, 465 picograms/milliliter. IL-6 measured from untreated cells was 0.21 picograms/milliliter. Results from the three donors were averaged, and error bars indicate 1 standard deviation.

### 9-Aminoacridinines and 4-Aminoquinoline Derivatives

Some specific embodiments of 4-aminoquinolines useful in the methods of the invention are included in Table 2. Some specific embodiments of 9-aminoacridinines useful in the methods of the invention are included in Table 3.

The structures are listed as changes from quinacrine or chloroquine. The symbol — indicates no change. Me and Et indicate —CH₃ and —CH₂CH₃ respectively. Aromatic substituents are in the para position except as indicated. The activity is — log₁₀ of the concentration (M) required for half maximal inhibition of CPG-ODN effect on [3H] thymidine uptake in the presence of α-slgM. The last column is the activity expressed as a fraction of activity of chloroquine (fold).

### TABLE 2

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<th>R7</th>
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SEQUENCE LISTING

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ataacgacc ttcagcaag

What is claimed is:

1. A method of identifying a compound that inhibits an immunostimulatory DNA-induced response, the method comprising contacting a cell with an immunostimulatory DNA, thereby inducing a measurable immune response, and

a test compound and detecting inhibition of the immunostimulatory effect of the immunostimulatory DNA, wherein the test compound is selected from the group consisting of: compounds, other than chloroquine, having the structural formula:
wherein

$R_a$ is a hydrogen atom, a lower alkyl group, or linked to $R_p$ by a substituted or unsubstituted alkyl chain;

$R_p$ is a hydrogen atom, an alicyclic group, an alkyl secondary, tertiary or quarternary amine, or an alkenyl secondary, tertiary or quarternary amine;

$R_2$ is a hydrogen atom, a lower alkyl group, an aryl group, a heteroaromatic group, or a lower alkenyl group substituted with an aryl group;

$R_3$ is a hydrogen atom, a lower alkyl group, or an aromatic group;

$R_4$ is a hydrogen atom, a lower alkyl group, or a halogen atom;

$R_5$ is a hydrogen atom, a lower alkyl group, a lower alkoxy group, an arloxy group, an aryl group, an amino group or a thioether group;

$R_6$ is a hydrogen atom, a lower alkyl group, a lower alkoxy group, an arloxy group, a haloalkyl group, or a halogen atom; and

$R_7$ is a hydrogen atom, or a lower alkoxy group, and pharmaceutically acceptable salts thereof, with the proviso that if $R_7$ is a halogen, then at least one of $R_2$, $R_3$, $R_5$, or $R_6$ is non-hydrogen and $R_p$ is not 4-[N,N-dialkyl-n-pentylamine] or 4-[N-alkyl-N-hydroxyalkyl-n-pentylamine].

wherein

$R_p$ is a hydrogen atom, or an alkyl secondary or tertiary amino substitutent selected from the group consisting of 2-[N,N-dimethyl ethylamine], 2-[N-methyl ethylamine], 2-[ethyl-N,N-dialkyl amine], 2-[ethyl-N-alkyl amine], 2-[ethyl amine], 3-[propyl-N,N-dialkyl amine], 3-[propyl-N-alkyl amine], 4-[pentyl-N,N-dialkyl amine], 4-[pentyl-N-alkyl amine], 4-[pentyl amine], 4-[butyl-N,N-dialkyl amine], 4-[butyl-N-alkyl amine], 4-[butyl amine], 4-[N,N-diethyl pentylamine], 4-[N,N-diethyl butylamine], 4-[4-aryl-N,N-dialkyl butylamine], 4-[4-aryl-N-alkyl butylamine], 4-[4-heteroaromatic-N,N-dialkyl butylamine], 4-[4-heteroaromatic-N-alkyl butylamine], 4-[N,N-dialkyl pentylamine], and 7-[hepta-4-methyl-4-azaamine];

$R_2$ is a lower alkyl group;

$R_3$ is a hydrogen atom or a lower alkoxy group; and

$X$ is a halogen atom, and pharmaceutically acceptable salts thereof.

2. The method of claim 1, wherein the cell is a hybridoma plasma cell.

3. The method of claim 1, wherein the detecting is by measuring IL-6 production.

4. The method of claim 2, wherein the hybridoma plasma cell is 7TD1.

* * * * *
CERTIFICATE OF CORRECTION

PATENT NO. : 7,354,711 B2
APPLICATION NO. : 10/336635
DATED : April 8, 2008
INVENTOR(S) : Donald E. Macfarlane

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

At columns 19-20, Table 3, last line, "Acidine homodimer" should read --Acridine homodimer--.

At column 21, line 17, Claim 1, "quarternary" should read --quaternary--.

At column 21, line 18, Claim 1, "quarternary" should read --quaternary--.

Signed and Sealed this
Fifteenth Day of July, 2008

[Signature]

JON W. DUDAS
Director of the United States Patent and Trademark Office