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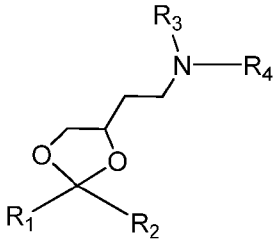
(54) Title: IMPROVED **LIPID** FORMULATION

(A)



(57) Abstract: The invention features an improved lipid formulation comprising a cationic lipid of formula (A), a neutral lipid, a sterol and a PEG or PEG-modified lipid, where Ri and R_2 are independently alkyl, alkenyl or alkynyl, each can be optionally substituted, and R3 and R4 are independently lower alkyl or R3 and R4 can be taken together to form an optionally subsituted heterocyclic ring. In one embodiment, Ri and R2 are independently selected from oleoyl, pamitoyl, steroyl, linoleyl and R3 and R4 are methyl. Also disclosed are targeting lipids and specific lipid formulations comprising such targeting lipids.

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IMPROVED LIPID FORMULATION

Claim of Priority

This application claims priority from U.S.S.N. 61/148,366, filed January 29, 2009; U.S.S.N. 61/156,851, filed March 2, 2009; U.S.S.N. 61/185,712, filed June 10, 2009; U.S.S.N. 61/228,373, filed July 24, 2009; and U.S.S.N. 61/239,686, filed September 3, 2009, each of which is incorporated by reference in its entirety.

Technical Field

The invention relates to the field of therapeutic agent delivery using lipid particles. In particular, the invention provides cationic lipids and lipid particles comprising these lipids, which are advantageous for the *in vivo* delivery of nucleic acids, as well as nucleic acid-lipid particle compositions suitable for *in vivo* therapeutic use. Additionally, the invention provides methods of preparing these compositions, as well as methods of introducing nucleic acids into cells using these compositions, *e.g.*, for the treatment of various disease conditions.

Description of the Related Art

Therapeutic nucleic acids include, *e.g.*, small interfering RNA (siRNA), micro RNA (miRNA), antisense oligonucleotides, ribozymes, plasmids, and immune stimulating nucleic acids. These nucleic acids act via a variety of mechanisms. In the case of siRNA or miRNA, these nucleic acids can down-regulate intracellular levels of specific proteins through a process termed RNA interference (RNAi). Following introduction of siRNA or miRNA into the cell cytoplasm, these double-stranded RNA constructs can bind to a protein termed RISC. The sense strand of the siRNA or miRNA is displaced from the RISC complex providing a template within RISC that can recognize and bind mRNA with a complementary sequence to that of the bound siRNA or miRNA. Having bound the complementary mRNA the RISC complex cleaves the mRNA and releases the cleaved strands. RNAi can provide down-regulation of specific proteins by targeting specific destruction of the corresponding mRNA that encodes for protein synthesis.

The therapeutic applications of RNAi are extremely broad, since siRNA and miRNA constructs can be synthesized with any nucleotide sequence directed against a target protein. To date, siRNA constructs have shown the ability to specifically down-regulate target proteins in both *in vitro* and *in vivo* models. In addition, siRNA constructs are currently being evaluated in clinical studies.

However, two problems currently faced by siRNA or miRNA constructs are, first, their susceptibility to nuclease digestion in plasma and, second, their limited ability to gain access to the intracellular compartment where they can bind RISC when administered systemically as the free siRNA or miRNA. These double-stranded constructs can be stabilized by incorporation of chemically modified nucleotide linkers within the molecule, for example, phosphothioate groups. However, these chemical modifications provide only limited protection from nuclease digestion and may decrease the activity of the construct. Intracellular delivery of siRNA or miRNA can be facilitated by use of carrier systems such as polymers, cationic liposomes or by chemical modification of the construct, for example by the covalent attachment of cholesterol molecules. However, improved delivery systems are required to increase the potency of siRNA and miRNA molecules and reduce or eliminate the requirement for chemical modification.

Antisense oligonucleotides and ribozymes can also inhibit mRNA translation into protein. In the case of antisense constructs, these single stranded deoxynucleic acids have a complementary sequence to that of the target protein mRNA and can bind to the mRNA by Watson-Crick base pairing. This binding either prevents translation of the target mRNA and/or triggers RNase H degradation of the mRNA transcripts. Consequently, antisense oligonucleotides have tremendous potential for specificity of action *{i.e.,* down-regulation of a specific disease-related protein). To date, these compounds have shown promise in several *in vitro* and *in vivo* models, including models of inflammatory disease, cancer, and HIV (reviewed in Agrawal, *Trends in Biotech.* 14:376-387 (1996)). Antisense can also affect cellular activity by hybridizing specifically with chromosomal DNA. Advanced human clinical assessments of several antisense drugs are currently underway. Targets for these drugs include the bcl2 and apolipoprotein B genes and mRNA products.

One well known problem with the use of therapeutic nucleic acids relates to the stability of the phosphodiester internucleotide linkage and the susceptibility of this linker to nucleases. The presence of exonucleases and endonucleases in serum results in the rapid digestion of nucleic acids possessing phosphodiester linkers and, hence, therapeutic nucleic acids can have very short half-lives in the presence of serum or within cells. (Zelphati, O., *et al., Antisense. Res. Dev.* 3:323-338 (1993); and Thierry, A.R., *et al,* ppl47-161 in Gene Regulation: Biology of Antisense RNA and DNA (Eds. Erickson, RP and Izant, JG; Raven Press, NY (1992)). Therapeutic nucleic acid being currently being developed do not employ the basic phosphodiester chemistry found in natural nucleic acids, because of these and other known problems.

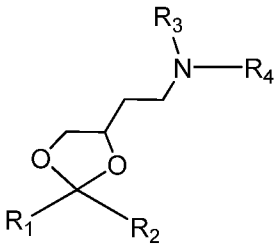
This problem has been partially overcome by chemical modifications that reduce serum or intracellular degradation. Modifications have been tested at the internucleotide phosphodiester bridge (*e.g.,* using phosphorothioate, methylphosphonate or phosphoramidate linkages), at the nucleotide base (*e.g.,* 5-propynyl-pyrimidines), or at the sugar (*e.g.,* 2'-modified sugars) (Uhlmann E., *et al* Antisense: Chemical Modifications. Encyclopedia of Cancer, Vol. X., pp 64-81 Academic Press Inc. (1997)). Others have attempted to improve stability using 2'-5' sugar linkages (*see, e.g.,* US Pat. No. 5,532,130). Other changes have been attempted. However, none of these solutions have proven entirely satisfactory, and *in vivo* free therapeutic nucleic acids still have only limited efficacy.

In addition, as noted above relating to siRNA and miRNA, problems remain with the limited ability of therapeutic nucleic acids to cross cellular membranes (see, Vlassov, *et al, Biochim. Biophys. Acta* 1197:95-1082 (1994)) and in the problems associated with systemic toxicity, such as complement-mediated anaphylaxis, altered coagulatory properties, and cytopenia (Galbraith, *et al, Antisense Nucl Acid Drug Des.* 4:201-206 (1994)).

In spite of recent progress, there remains a need in the art for improved lipid-therapeutic nucleic acid compositions that are suitable for general therapeutic use. Preferably, these compositions would encapsulate nucleic acids with high-efficiency, have high drug:lipid ratios, protect the encapsulated nucleic acid from degradation and clearance in serum, be suitable for systemic delivery, and provide intracellular

delivery of the encapsulated nucleic acid. In addition, these lipid-nucleic acid particles should be well-tolerated and provide an adequate therapeutic index, such that patient treatment at an effective dose of the nucleic acid is not associated with significant toxicity and/or risk to the patient. The invention provides such compositions, methods of making the compositions, and methods of using the compositions to introduce nucleic acids into cells, including for the treatment of diseases.

Summary of Invention

In one aspect, the invention provides improved lipid formulations comprising a cationic lipid of formula A, a neutral lipid, a sterol and a PEG or PEG-modified lipid, wherein formula A is 

, where Ri and R_2 are independently alkyl, alkenyl or alkynyl, each can be optionally substituted, and R_3 and R_4 are independently lower alkyl or R_3 and R_4 can be taken together to form an optionally subsituted heterocyclic ring. In one embodiment, Ri and R_2 are independently selected from oleoyl, pamitoyl, steroyl, linoleyl and R_3 and R_4 are methyl.

In one aspect, the improved lipid formulation also includes a targeting lipid (e.g., a GaINAc and/or folate containing lipid).

In one aspect, the invention provides preparation for the improved lipid formulations via an extrusion or an in-line mixing method.

In one aspect, the invention further provides a method of administering the improved lipid formulations containing RNA-based construct to an animal, and evaluating the expression of the target gene.

In one aspect, a lipid formulation featured in the invention, such as a lipid formulation complexed with an oligonucleotide, such as a double stranded RNA (dsRNA), can be used to modify (e.g., decrease) target gene expression in a tumor cell

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