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(71) **Applicant** *(for all designated States except US)*: **KARLSRUHER INSTITUT FÜR TECHNOLOGIE** [DE/DE]; Kaiserstrasse 12, 76131 Karlsruhe (DE).

(72) **Inventors; and**

(75) **Inventors/Applicants** *(for US only)*: **DAVIDSON, Gary** [GB/DE]; Potsdamer Strasse 3B, 76676 Graben-Neudorf (DE). **LEVKIN, Pavel** [RU/DE]; Max-Planck-Strasse 6, 76344 Eggenstein-Leopoldshafen (DE). **LI, Linxian** [CN/DE]; Nagoldstrasse 26d, 76344 Eggenstein-Leopoldshafen (DE). **SU, Yi** [CN/DE]; Otto-Hahn-Strasse 10, 76344 Eggenstein-Leopoldshafen (DE). **ZAHNER, David** [DE/DE]; Vespasianstrasse 8, 68526 Ladenburg (DE).

(74) **Common Representative**: **KARLSRUHER INSTITUT FÜR TECHNOLOGIE**; Innovationsmanagement, Postfach 36 40, 76021 Karlsruhe (DE).

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(54) **Title**: AMINO LIPIDS, THEIR SYNTHESIS AND USES THEREOF

(57) **Abstract**: The present invention provides new amino lipids and a convenient method for synthesising these compounds. These (cationic) amino lipids have good properties as transfection agents. The method is an economic versatile two step synthesis allowing the preparation of various amino lipids thus leading to the assembly of a combinatorial library of transfection agents. Moreover, the present invention provides lipid particles (liposomes) containing said amino lipids and their use for delivering bioactive agents into cells. The invention encompasses also the use of lipid particles containing the cationic amino lipids as medicament.

Amino lipids, their synthesis and uses thereof

Technical field

The present invention provides new amino lipids and their synthesis. These (cationic) amino lipids have good properties as transfection agents. They can be used to produce lipid particles, especially liposomes, allowing the delivery of bioactive agents into cells. The simplicity of the synthesis allows the development of a combinatorial library of amino lipids in a kit-like manner. The compounds contained in this library can be screened for particular properties in particular for the transfection of various cell lines. The invention encompasses also the use of lipid particles containing the (cationic) amino lipids as medicament.

Background

Of the various reagents used to transfect cells with bioactive agents such as nucleic acids, those based on liposome mediated delivery are widely acknowledged to be the most effective. This is due mostly to their efficiency and ease of use. Liposomes are artificially prepared spherical vesicles made of lipid bilayer. To deliver the molecules to sites of action, the lipid bilayer can fuse with other bilayers such as a cell membrane, thus delivering the liposome contents inside a cell.

Liposomes are used for drug delivery due to their unique properties. A liposome encapsulates a region of aqueous solution inside a hydrophobic membrane; dissolved hydrophilic solutes cannot readily pass through the lipids. Hydrophobic chemicals can be dissolved into the membrane, and in this way liposome can carry both hydrophobic molecules and hydrophilic molecules. Liposomes can be combined with bioactive agents such as drugs, nucleic acids, peptides etc., and used to deliver these

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agents for the regulation of a cells biochemical pathway. This opens possibilities for new treatments of diseases.

Gershon et al. (Gershon H, et al. Mode of formation and structural features of DNA-cationic liposome complexes used for transfection. Biochemistry. 1993, 32:7143-7151) state that nanoparticles which are formed between cationic liposomes and nucleic acids represent an efficient vehicle for delivery of DNA and RNA into cell. Cationic liposomes bind initially to DNA molecules to form clusters of aggregated vesicles along the nucleic acids. At a critical liposome density, two processes occur, DNA-induced membrane fusion and liposome-induced DNA condensation. The DNA condensation leads to the formation of condensed structures which can be completely encapsulated with the fused lipid bilayers in a fast, highly cooperative process.

For delivery of negatively charged nucleic acid, cationic lipids are the most effective transfection agents. Cationic lipids represent a promising class of synthetic materials for DNA delivery. To date, there are several commercialized cationic lipids but the number of cationic lipids for safe and effective delivery of genes is still limited.

In "Cationic liposomes for gene therapy" (Miller AD, Angew Chem Int Ed. 1998, 37:1768-1785) most of the commonly used and commercially available transfection agents are described. However, conventional lipid synthesis typically requires individually optimized, multiple-step synthesis, including time-intensive procedures such as chemical protection and deprotection, use and removal of catalysts, solvent exchanges and purification.

WO 01/42200 describes examples of cationic amphiphilic compounds and their use in pharmaceutical compositions as trans-

fection agents. The compounds disclosed in this document are made in a time consuming multiple-step synthesis.

Cationic lipids need to be combined with natural phospholipids (referred to as helper lipids) to form liposomes that can be more efficiently incorporated into cell membranes. By combining liposomes with DNA or drugs, which alone cannot diffuse through the membrane of the target cell, they can be (indiscriminately) delivered past the lipid bilayer. The use of liposomes for transformation or transfection of DNA into a host cell is known as lipofection.
Although liposomal reagents represent the state of the art with respect to cell transfection agents, they have the following drawbacks:

1. Many cell lines (such as primary cells) cannot be effectively transfected at the moment, even with liposomal reagents.
2. They are relatively difficult and expensive to synthesise, often resulting in high price for end-users.

As a consequence of the second point, many laboratories use less efficient, cheaper alternatives for transfection (e.g. calcium phosphate). There is a concrete requirement for new transfection agents that are easy to synthesize and which have good transfection yields for a wide variety of cell types. As an alternative, it would be helpful to dispose an easy combinatorial synthesis of transfection agents allowing the production of a variety of different compounds.

Objectives of the invention

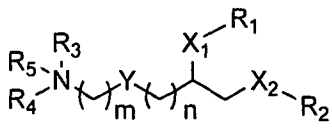
To overcome the disadvantages of the state of the art, it is an objective of the present invention to provide novel cationic amino lipids and a method for their synthesis. The method

should be generic, economic and easy to perform. This generic method allows the development of a library of cationic amino lipids. Generation of such lipid libraries (containing of hundreds of different lipid molecules) greatly enhances the identification of lipids harbouring optimal transfection reagent properties.

It is a further objective of the present invention to provide lipid particles, especially liposomes, containing said cationic amino lipids. In particular, these lipid particles or liposomes should be able to deliver bioactive agents through cell membranes. Another objective is the use of said lipid particles or liposomes for treatment of diseases.

Description of the invention

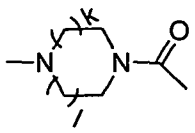
The invention provides novel amino lipids with the following general formula (I):


(I)

wherein R^1 and R^2 are the same or different and independently C_6-C_{24} alkyl, C_6-C_{24} alkenyl, C_6-C_{24} alkynyl, or C_6-C_{24} acyl, which can be optionally substituted with a C_1-C_6 hydrocarbyl group,

X^1 and X^2 are the same or different, either S or S=O or $S(=O)_2$,

Y is either an amide, an ester or a heterocyclic amide of the formula

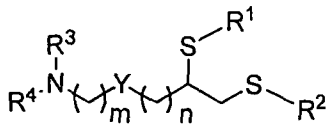

wherein k and l are integers from 0 to 2,

R^3 and R^4 are either the same or different and independently C_1-C_{12} alkyl, C_1-C_{12} alkenyl, or C_1-C_{12} alkynyl, wherein alkyl, alkenyl or alkynyl may be optionally substituted with a C_1-C_6

hydrocarbyl group, or R^3 and R^4 may join to form an optionally substituted heterocyclic ring of 3 to 10 atoms and 0 to 6 heteroatoms chosen from nitrogen, thiol and oxygen,
R^5 is either absent or is hydrogen or C_1-C_{12} alkyl to provide a quaternary amine,
m is an integer from 1 to 12 and n is an integer from 2 to 12.

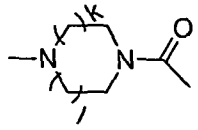
In a preferred embodiment of the invention, R^1 and R^2 are the same or different and independently C_6-C_{24} alkyl, more preferred, R^1 and R^2 are the same C_6-C_{18} alkyl.

Also, the present invention provides amino lipids of the general formula (II):



(II)

R^1 and R^2 are the same C_6-C_{18} alkyl
Y is either an amide, an ester or a heterocyclic amide of the formula

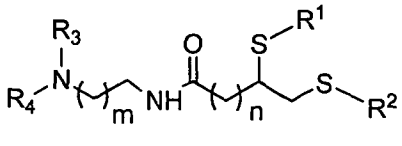


wherein k and l are integers from 0 to 2,
R^3 and R^4 are either the same or different C_1-C_{12} alkyls, wherein alkyl may be optionally substituted with a C_1-C_6 hydrocarbyl group, or R^3 and R^4 may join to form an optionally substituted heterocyclic ring of 3 to 10 atoms and 0 to 6 heteroatoms chosen from nitrogen, thiol and oxygen,
m is an integer from 1 to 12 and n is an integer from 2 to 12

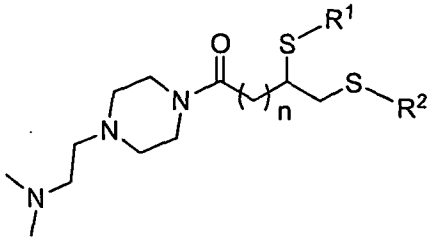
In another preferred embodiment of the invention Y is an amide.

In a preferred embodiment of the compounds according to the formulas (I) or (II),
n and m are independently integers 2 or 3.

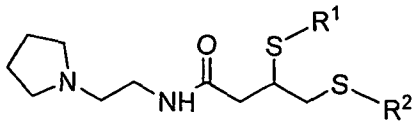
The most preferred embodiments correspond to the structures of formula (IIIa), (IIIb) or (IIIc)



(IIIa),



(IIIb), or

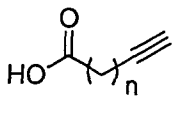


(IIIc),

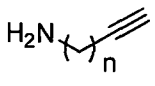
wherein R^1 and R^2 are the same C_{11}-C_{12} alkyls,
R^3 and R^4 are the same C_1-C_2 alkyls,
m is an integer from 1 to 2, n is an integer from 2 to 3.

The present invention provides a method to synthesize amino lipids as defined in claims 1 to 4. The method represents the first parallel synthesis of large libraries of ionisable cationic amino lipids based on thiol-yne chemistry in liquid-phase combinatorial synthesis without chromatography purification comprising the following steps:

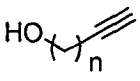
The first step is reaction of alkynes of the general formula (IVa), (IVb) or (IVc)



(IVa),

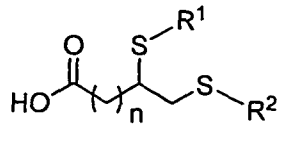
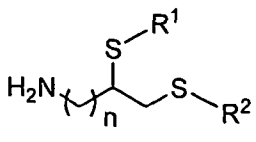
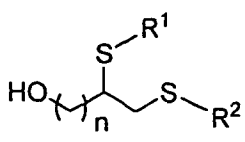


(IVb), or



(IVc)

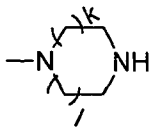
, wherein n is an integer from 2 to 12,
with compounds of the general formula, HS-R^1 and HS-R^2,
wherein R^1 and R^2 are the same or different and independently C_6-C_{24} alkyl, C_6-C_{24} alkenyl, C_6-C_{24} alkynyl, or C_6-C_{24} acyl, which can be optionally substituted with a C_1-C_6 hydrocarbyl group, under either UV-irradiation or using a radical initiator, to yield a compound of the general formula (Va), (Vb) or (Vc)


(Va),

(Vb),

(Vc)

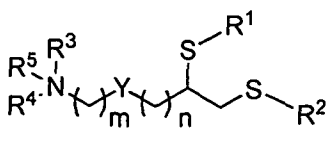
wherein n, R^1 and R^2 are defined as above,,

This reaction is conducted via a radical mechanism. Chemical radical sources can be added to start the reaction or it can be simply conducted under sunlight. In a preferred embodiment the reaction is started by UV irradiation.

The second step is a condensation reaction of the product of the first step
(formula Va) with an amine or alcohol of the general formula $(R^3R^4R^5N)(CH_2)_mZ$, with m as an integer from 1 to 12, wherein Z is NH_2, OH, or a secondary heterocyclic amine of the formula,



wherein k and l are integers from 0 to 2,
Or condensation reaction of the product of the first step (formula Vb or Vc) with carboxylic acid of the general formula $(R^3R^4R^5N)(CH_2)_mZ$, with m as an integer from 1 to 12, wherein Z is COOH.
R^3 and R^4 are either the same or different and independently C_1-C_{12} alkyl, C_1-C_{12} alkenyl, or C_1-C_{12} alkynyl, which may be optionally substituted with a C_1-C_6 hydrocarbyl group, or R^3 and R^4 may join to form a ring of 3 to 10 carbon atoms and 0 to 6 heteroatoms chosen from nitrogen, thiol and oxygen,
R^5 is either absent or is hydrogen or C_1-C_{12} alkyl to provide a quaternary amine,
to yield a compound of the general formula (VI)


(VI),

wherein n, m, Y, R^1, R^2, R^3, R^4 and R^5 are defined as above.

The second step is preferably performed at room temperature in a solvent such as dimethylformamide (DMF). In a preferred embodiment, N, N'-diisopropylcarbodiimide (DIC) is added to the reaction mixture.

The optional third step is an oxidation reaction of the thioether groups of the product of second step with an oxidising reagent to yield a compound of the general formula (I), wherein n, m, Y, X^1, X^2, R^1, R^2, R^3, R^4 and R^5 are defined as above. The third step is preferably performed at room temperature with aqueous hydrogen peroxide in a solvent such as methanol. The third step can further been catalyzed by a catalyst such as titanium-containing zeolites. This reaction has already been proposed by Hulea et al. (Hulea V, Moreau P, Renzo FD. Thioether oxidation by hydrogen peroxide using titanium-containing zeolites as catalysts. Journal of Molecular Catalysis A: Chemcial. 1996, 111:325-332). Surprisingly this reaction does not affect other functional groups of the amino lipids of step b).

This reaction scheme is very versatile; it can be used to synthesize large libraries of cationic amino lipids for rapid cell-based screening assay in a very inexpensive manner. The resulting compounds all have both a hydrophobic character due to their long non-polar residues and a hydrophilic character due to their amino group. This amphiphilic character can be used to form lipid particles, e.g. lipid bilayers, micelles, liposomes, etc. Moreover, the amino group of these compounds confers a cationic charge which is useful for transfection agents. This library of different compounds with new characteristics can be tested easily for their transfection capacity of a wide variety of cell types.

Another embodiment of the present invention is directed to lipid particles containing an amino lipid of one of the claims 1 to 4. Within the scope of the invention, the term 'lipid particle' means nanosized objects made of amino lipids which are placed into an aqueous solution. These particles are inter alia lipid bilayer vesicles (liposomes), multi-lamellar vesicles or micelles.

In a preferred embodiment of the present invention, said nanoparticles are liposomes containing an amino lipid of one of the claims 1 to 4. Within the scope of the invention liposomes are microvesicles composed of a bilayer of lipid amphipathic (amphiphilic) molecules enclosing an aqueous compartment.

Liposome formation is not a spontaneous process. Lipid vesicles are formed first when lipids such as phospholipids are placed in water and consequently form one bilayer or a series of bilayers, each separated by water molecules. Liposomes can be created by sonicating lipid vesicles in water.

Within the scope of the invention, the term 'lipid bilayer' means a thin membrane made of two layers of lipid molecules. The term 'micelle' means an aggregate of surfactant molecules dispersed in a liquid colloid. A typical micelle in aqueous solution forms an aggregate with the hydrophilic head regions in contact with water, sequestering the hydrophobic single tail region in the micelle center.

Within the scope of the invention, the term 'cells' means a generic term and encompass the cultivation of individual cells, tissues, organs, insect cells, avian cells, fish cells, amphibian cells, mammalian cells, primary cells, continuous cell lines, stem cells and/or genetically engineered cells, such as recombinant cells expressing a hetereologous polypeptide or protein. Recombinant cells include, for example, cells

expressing heterologous polypeptides or proteins, such as a growth factor or a blood factor.

In a preferred embodiment said lipid particles or liposomes further contain a helper lipid. In a preferred embodiment said helper lipid is a non-cationic lipid. In a more preferred embodiment said helper-lipid is a non-cationic phospholipid. Within the scope of this invention, a non-cationic lipid may contain cationic functional groups (e.g. aminogroups) but it should contain anionic functional groups to at least neutralize the molecule. The totality of all functional groups in the lipid molecule should be non-cationic.
Liposomes consisting of a mixture of cationic amino lipids and non-cationic (neutral) phospholipids are the most effective for nucleic acid delivery into cells. In an even more preferred embodiment said non-cationic lipid is DOPE.

In a further preferred embodiment the lipid particle or liposome further comprises a sterol. Sterol, like cholesterol, is a natural component in cell membranes. It can be used to stabilise the particle, and help the integration with cell membrane.

In another embodiment of the invention, the lipid particles or liposomes further contain a bioactive agent. Within the scope of this invention a bioactive agent is one which has a biological effect when introduced into a cell or host, for example by stimulating an immune response or an inflammatory response, by exerting enzymatic activity or by complementing a mutation, etc. bioactive agents include inter alia nucleic acids, peptides, proteins, antibodies and small molecules.
When a liposome is used to encapsulate a drug substance either within the lipid bilayer or in the interior aqueous space of the liposome the term 'liposome drug' can be employed.

In a most preferred embodiment, the bioactive agent is a nucleic acid. In another preferred embodiment said bioactive agent is a member optionally selected from the group consisting of: an antineoplastic agent, an antibiotic, an immunomodulator, an anti-inflammatory agent, an agent acting on the central nervous system, a polypeptide or a polypeptoid.

In yet another embodiment the lipid particle or liposome further contains at least one polyethylene glycol (PEG)-lipid. PEG lipids help to protect the particles and their cargo from degradation in-vitro and in-vivo. Moreover, PEG form a protective layer over the liposome surface and increase the circulating time in vivo. It can be used in liposome drug delivery (PEG-liposome).

Lipid particles or liposomes containing a bioactive agent can be used to deliver any of a variety of therapeutic agents into cells. The present invention encompasses the use of lipid particles, especially liposomes, as described above for delivering a bioactive agent into a cell.
Preferably said bioactive agent is a nucleic acid, including but not limited to, RNA, antisense oligonucleotide, a DNA, a plasmid, a ribosomal RNA (rRNA), a micro RNA (miRNA), transfer RNA (tRNA), a small inhibitory RNA (siRNA) or small nuclear RNA (snRNA). The bioactive agent may also be an antineoplastic agent, an antibiotic, an immunomodulator, an anti-inflammatory agent, an agent acting on the central nervous system, antigens or fragments thereof, proteins, peptides, polypeptoid, vaccines and small-molecules or mixtures thereof.

As has been set out above, lipid particles or liposomes containing amino lipids as defined in the present invention are suitable to deliver bioactive agents into cells. The wide variety of different amino lipids which can be synthesised by the mentioned versatile synthesis method can be screened for

particular characteristics that are conferred to the liposomes. Important characteristics are for example transfection efficiency, cytotoxicity, adhesion of the agent to be delivered into the cell, stability of the liposomes, size of the liposomes, etc. The present method allows the creation of specifically adapted liposomes for particular applications.

For example lipid particles (liposomes) can be used for transfecting multicellular tissues or organisms. This offers the possibility of a novel therapeutic treatment of patients.

According to the present invention, a patient can be any mammal, preferably selected from the group consisting of human, mouse, rat, pig, cat, dog, horse, goat, cattle, and monkey and/or others. Most preferably, the patient is a human being.

An important embodiment of the present invention is the use of said lipid particles or liposomes containing amino lipids according to one of formulas (I-III) for use as a medicament.

In particular, said lipid particles or liposomes can be administered to patients for use in gene therapy, in gene vaccination, in antisense therapy or in therapy by interfering RNA. A lipid particle of the invention may also be used for the manufacture of a medicine for use in nucleic acid transfer, for example in treatment of the human or animal body by therapy, especially in the treatment of a condition caused by or related to a genetic defect or modification.

Targets for gene therapy are well known and include monogenic disorders, for example, cystic fibrosis, various cancers, and infections, for example, viral infections, for example, with HIV. For example, transfection with the p53 gene offers great potential for cancer treatment. Targets for gene vaccination are also well known, and include vaccination against pathogens

for which vaccines derived from natural sources are too dangerous for human use and recombinant vaccines are not always effective, for example, hepatitis B virus, human immunodeficiency virus (HIV), hepatitis C virus (HCV) and herpes simplex virus.

Targets for anti-sense therapy are also known. Further targets for gene therapy and anti-sense therapy are being proposed as knowledge of the genetic basis of disease increases, as are further targets for gene vaccination.

A lipid particle of the invention may be used in vaccination. Thus, a lipid particle or liposome of the invention may be used to deliver an antigen or a nucleic acid encoding an antigen. These techniques are familiar to a person skilled in the art. Examples for liposome vaccines are described in Butts C, et al. Randomized phase IIB trial of BLP25 liposome vaccine in stage IIIB and IV Non-Small-Cell Lung Cancer. Journal of Clinical Oncology. 2005, 23:6674-6681 and in U'Ren L, et al. Vaccination with liposome-DNA complexes elicits enhanced anti-tumor immunity. Cancer Gene Therapy. 2006, 13:1033-1044.

A lipid particle of the invention may be used to elicit an immune response against a wide variety of antigens for the treatment and/or prevention of a number of conditions including, but not limited to, cancer, allergies, toxicity and infection by pathogens such as viruses, bacteria, fungi, and other pathogenic organisms.

In a preferred embodiment of the invention said lipid particle or liposome can be used as a medicament in treatment of a viral infection, a liver disease or disorder, or cancer. On liver diseases, liposomes can be captured by the cells of the reticulo-endothelial system, which are primarily situated in the liver. The liposomes will be accumulated there.

The following figures and examples are presented to provide a better understanding of the description of procedures and conceptual aspects of the invention.

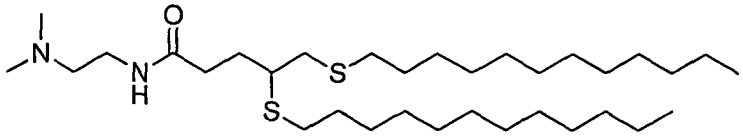
Fig. 1: Comparative microscope pictures showing results of lipofection by use of an amino liposomal reagent and two commercially available transfection reagents.

Fig. 2: Graphical overview of the transfection efficiency of a library of 120 transfection reagents compared to a commercially available reagent.

Fig. 3: siRNA gene silencing in HEK 293 and MEF cells.

Synthesis

Example 1: Synthesis and characterization of N-(2-(dimethylamino)ethyl)-4,5-bis(dodecylthio)pentanamide (DEDPA)

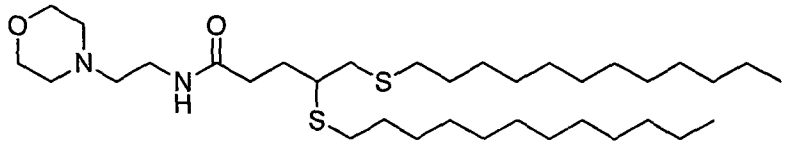
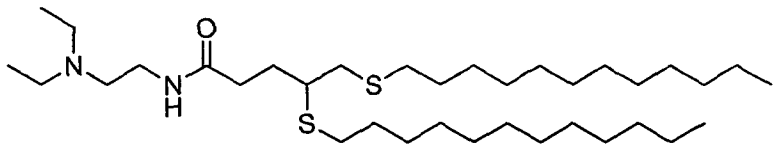
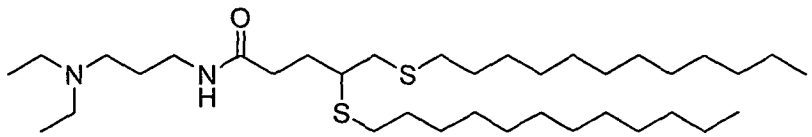
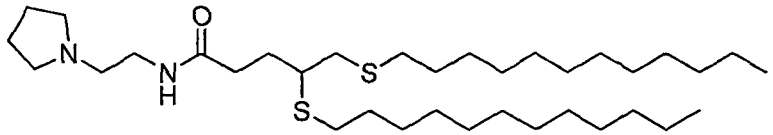
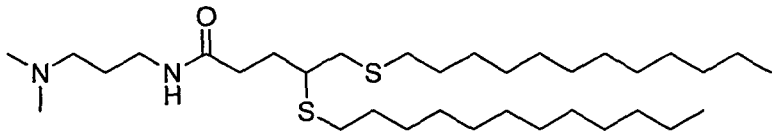
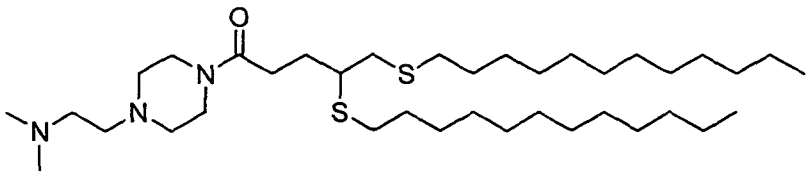


The amino lipid was synthesized by two steps. The first step is to synthesize 4,5-bis(dodecylthio)pentanoic acid. 0,5 mmol pent-4-ynoic acid, 1 mmol dodecane-1-thiol and 5 mg 2,2-Dimethoxy-2-phenylacetonphenone (DMPA) were dissolved in 1.5 ml THF and added into a 20 ml glass vial covered with aluminium foil. The vial was degassed for 5 min and filled with Argon (Ar) for 3 min, then covered with a cap. The mixture was irradiated under UV 365 nm for 1 h and then the THF was transferred to a 50 ml flask and evaporated.
The second step is conjugation of 4,5-bis(dodecylthio)pentanoic acid with N1,N1-dimethylethane-1,2-diamine to get N-(2-(dimethylamino)ethyl)-4,5-bis(dodecylthio)

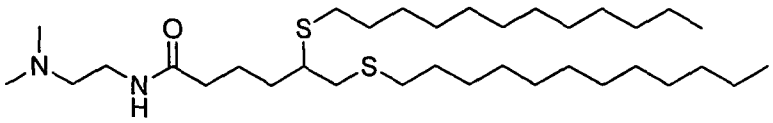
pentanamide. 4,5-bis(dodecylthio)pentanoic acid from the first step was dissolved in 8 ml dichloromethane (DCM). 1 ml of this solution was diluted with 4 ml DCM and 11.61 µl N,N'-diisopropylcarbodiimide (DIC) (0.075 mmol, 1.2 eq.) were added. 0.063 mmol N1,N1-dimethylethane-1,2-diamine were added to the mixture and vortexed. Hydroxylbenzotriazole (HOBt) was dissolved in dimethylformamide (DMF) (304 mg in 608 µl DMF) and 19.45 µl of the HOBt solution were added. The vial was then covered with aluminium foil, and filled with Ar. After 16 h stirring the Dichloromethane (DCM) was evaporated, the residue was dissolved in 2 ml hexane and transferred into new vials. The product was separated by centrifugation and the supernatants were collected and the hexane was evaporated in desiccators. The product was sealed with Parafilm and stored under Ar.
To verify the identity of the molecules, crude product was analyzed by mass spectrometry. The molecular ion was clearly identified as 574.0 MW/z.

Synthesis of the compounds of the examples 2-7 were carried out similarly to example 1. Step 1 is performed in the same way. Step 2 differs in the educts, whereas the stoichiometric ratios were maintained. The resulting compounds and the corresponding MW/z values are summarized in Table 1:

Table 1: Examples of synthesized compounds 2-7 and the corresponding MW/z values.

Example	Compound	MW/z
2	4,5-bis(dodecylthio)-N-(2-morpholinoethyl)pentanamide 	614.8
3	N-(2-(diethylamino)ethyl)-4,5-bis(dodecylthio)pentanamide 	600.0
4	N-(3-(diethylamino)propyl)-4,5-bis(dodecylthio)pentanamide 	616.0
5	4,5-bis(dodecylthio)-N-(2-(pyrrolidin-1-yl)ethyl)pentanamide 	601.9
6	N-(3-(dimethylamino)propyl)-4,5-bis(dodecylthio)pentanamide 	587.9
7	1-(4-(2-(dimethylamino)ethyl)piperazin-1-yl)-4,5-bis(dodecylthio)pentan-1-one 	642.9

Example 8: Synthesis and characterization of N-(2-(dimethylamino)ethyl)-5,6-bis(dodecylthio)hexanamide



The synthesis procedure of N-(2-(dimethylamino)ethyl)-5,6-bis(dodecylthio)hexanamide is similar to the previous examples.

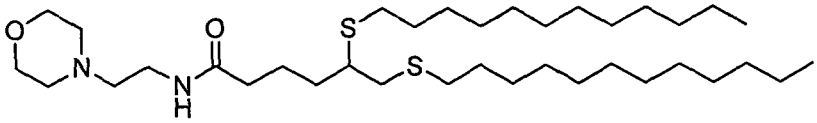
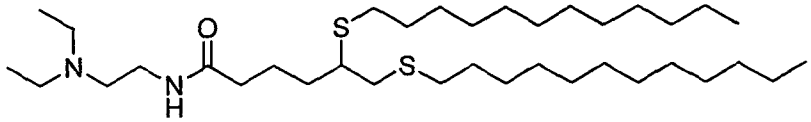
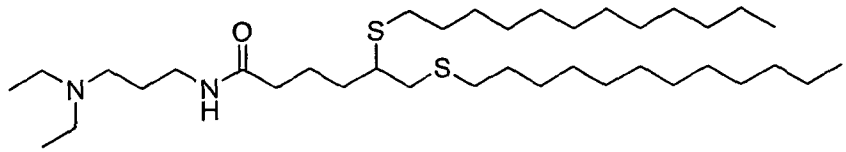
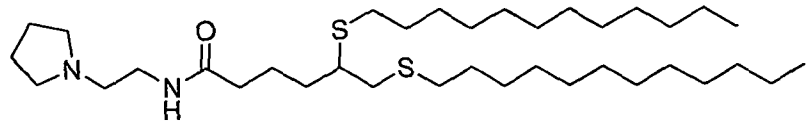
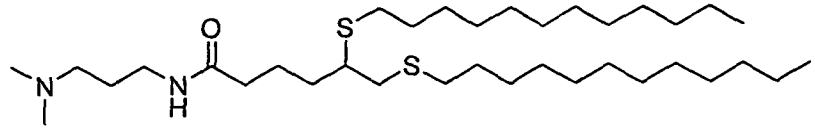
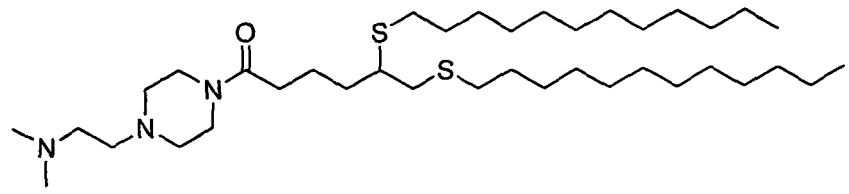
The first step is to synthesize 5,6-bis(dodecylthio)hexanoic acid. 0,5 mmol hex-5-ynoic acid, 1 mmol dodecane-1-thiol and 5 mg DMPA were dissolved in 1.5 ml THF and added into a 20 ml glass vial. The vial was covered with aluminium foil, filled with Ar and irradiated under UV 365 nm for 1 h. Then the THF was transferred to a 50 ml flask and evaporated.

The second step is conjugating 5,6-bis(dodecylthio)hexanoic acid with N1,N1-dimethylethane-1,2-diamine to get N-(2-(dimethylamino)ethyl)-5,6-bis(dodecylthio) hexanamide. This step was carried out similar to the previous examples. The stoichiometric ratios were maintained.

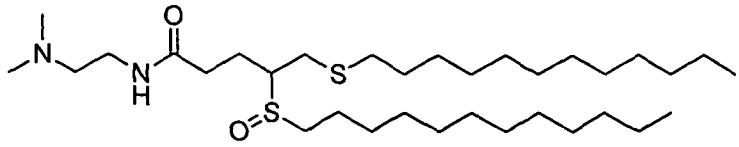
To verify the identity of the molecules, crude product was tested by mass spectrometry (m/z 587.9).

Synthesises according to the examples 9-14 were carried out similarly to example 8. Step 1 is performed in an identical way. Step 2 differs in the educts, whereas the stoichiometric ratios were maintained. The resulting compounds and the corresponding molecular ion are resumed in table 2:

Table 2: Examples of synthesized compounds 9-14 and the corresponding MW/z values.

Example	Compound	MW/z
9	5,6-bis(dodecylthio)-N-(2-morpholinoethyl)hexanamide 	629.9
10	N-(2-(diethylamino)ethyl)-5,6-bis(dodecylthio)hexanamide 	614.0
11	N-(3-(diethylamino)propyl)-5,6-bis(dodecylthio)hexanamide 	630.0
12	5,6-bis(dodecylthio)-N-(2-(pyrrolidin-1-yl)ethyl)hexanamide 	615.8
13	N-(3-(dimethylamino)propyl)-5,6-bis(dodecylthio)hexanamide 	601.9
14	1-(4-(2-(dimethylamino)ethyl)piperazin-1-yl)-5,6-bis(dodecylthio)hexan-1-one 	657.1

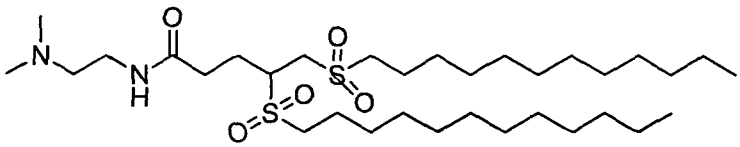
Example 15: Synthesis and characterization of N-(2-(dimethylamino)ethyl)-4-(dodecylsulfinyl)-5-(dodecylthio)pentanamide



The amino lipid is synthesized in one step. 1 mmol N-(2-(dimethylamino)ethyl)-4,5-bis(dodecylthio)pentanamide (DEDPA, product of example 1) was mixed with 10 mmol aqueous hydrogen peroxide (30%) in 10 ml methanol, and stirred at room temperature for 1 h. Then the mixture was transferred to a 50 ml flask and evaporated.

To verify the identity of the molecules, crude product was analyzed by mass spectrometry. The molecular ion was clearly identified as 589.7 MW/z.

Example 16: Synthesis and characterization of N-(2-(dimethylamino)ethyl)-4,5-bis(dodecylsulfonyl)pentanamide



The amino lipid is synthesized in one step. 1 mmol N-(2-(dimethylamino)ethyl)-4,5-bis(dodecylthio)pentanamide (DEDPA, product of example 1) was mixed with 10 mmol aqueous hydrogen peroxide (30%) in 10 ml methanol, and stirred at room temperature for 2 d. Then the mixture was transferred to a 50 ml flask and evaporated.

To verify the identity of the molecules, crude product was analyzed by mass spectrometry. The molecular ion was clearly identified as 637.5 MW/z.

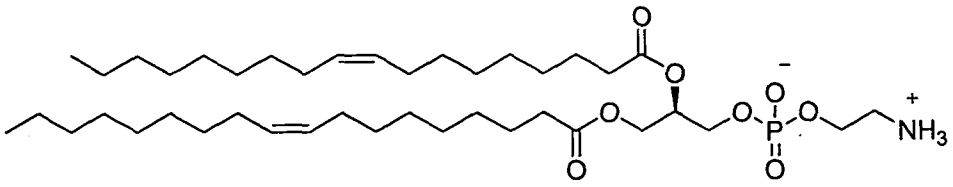
Screening of the cationic lipid for cell transfection

Example 17: Initial determination of optimal lipid ratios for cell transfection.

The well documented HEK 293T cell line is used for examples 17 and 18.

The natural phospholipid dioleolylphosphatidylethanolamine (DOPE - structure shown below) was selected as the required co-lipid (also termed helper lipid). It is required not for the stability of liposomes per se, but rather the breakdown of the lipid membranes in the endocytic compartment (endosomes) of cells, allowing release of the bioactive agent to the cytosol and/or nucleus. Basically, it is required for the desired effect for stable liposome formation in combination with our cationic amino lipid (DEDPA). DOPE was mixed with a representative cationic amino lipid (structure shown below) in different ratios. Both lipids were dissolved in ethanol at 50 mg/ml and combined to a final volume of 30 µl.

DOPE (neutral helper lipid):



N-(2-(dimethylamino)ethyl)-4,5-bis(dodecylthio)pentanamide (DEDPA) as representative novel cationic amino lipid:

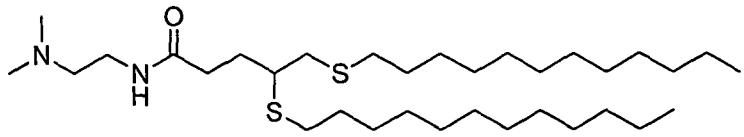


Table 3: DEDPA : DOPE ratios tested

DEDPA	DOPE
0	1
1	3
1	2
1	1
2	1
3	1
1	0

These 30 µl ethanol mixtures were then added to 70 µl of 0,2 M Sodium acetate buffer (pH 5.0) with constant vortexing for 30 s, followed by sonication for 5 min to form liposomes. Final lipid content is 2 mg/ml. This final 2 mg/ml liposome sample is referred to as the "lipid reagent".

0,1 µl, 0,2 µl, 0,3 µl, 0,4µl and 0,5 µl of the above lipid reagents were combined with either 50 ng or 100 ng plasmid DNA (comprising the pCS-LacZ and pEGFP-C1 plasmids at a ratio of 9:1, respectively) and mixed with cells as described below:

(amounts shown are for one well of a 96-well culture plate)

1. 0,1µl-0,5 µl lipid reagent diluted in 10µl of 50 mM sodium acetate buffer, pH 5.0

2. After 2-5 min incubation, added diluted lipid reagent from (1) to either 50ng or 100ng plasmid DNA (DNA dissolved in 10µl of 50mM sodium acetate buffer, pH 5.0).

3. Samples were left at RT for 30 min to form Lipid/DNA transfection complexes. As DNA is negatively charged, it associates non-specifically with the positively charged head groups of the cationic lipids in the liposomes.

4. After 30 min, 50µl of freshly suspended HEK 293 cells (approximately 50,000 cells, in DMEM culture medium supplemented with 10% foetal calf serum) were added to the lipid/DNA complexes, mixed with pipette action and 65µl of the cells + lipid/DNA complexes added to a single 96-well.

To assess the ability of the lipid mixtures to deliver the plasmid DNA into cells, microscopy was used to visualize fluorescence emitted by the green fluorescent protein (GFP) 20-24 hours after initial transfection. The GFP protein is encoded by the pEGFP-C1 plasmid and is efficiently synthesized and located within the cytoplasm of successfully transfected cells.

RESULTS:
An optimal ratio of amino lipid: DOPE was identified as 1:1 and the optimal lipid reagent : DNA ratio was 0,4 µl lipid reagent per 50 ng DNA. These conditions were therefore used for the primary screen to identify the lipid reagents with highest cell transfection efficiency and lowest cell toxicity, as described in example 18 below.

Example 18: Primary screen using novel lipids reagents

Cell line: HEK 293 cells
Screen format: 96-well
Detection (read-out): GFP fluorescence relative to total cell number (total cell number assessed using the nuclear dye, Hoechst - see **Fig. 1**)
A commercially available liposomal transfection reagent was used as a reference (reference reagent) according to manufacturers' instructions, see **Fig.1**.

Method:

All steps performed in 96 tube/plate format using 8- or 12-channel multipipettes. Amounts shown are for duplicate (2x) wells of a 96-well plate.

1. 0.8 µl lipid reagents diluted in 20 µl 50 mM NaOAc buffer, pH 5.0.

2. Diluted lipid reagents from (1) were added to 100ng DNA (10ng pEGFP-C1 + 90ng pCS-LacZ plasmids) in 20µl NaOAc buffer, pH 5.0 and mixed with pipette action.

3. After 30 min incubation at RT, added 100µl freshly resuspended cells (3 -5 x 10^5 cells/50µl DMEM culture medium supplemented with 10% foetal calf serum) and mixed with pipette.

4. Duplicate 65µl aliquots of the cells + lipid/DNA complexes were immediately transferred to separate wells of a 96-well culture plate and placed in 37°C incubator with 5% CO_2.

5. After 5 hours, added 50µl fresh cell culture medium (DMEM supplemented with 10% FCS).

6. 20 to 24 hours after initial transfection of cells, Hoechst 33258 dye was added to cells at a final concentration of 0,2 µg/ml and cells incubated for a further 30 min at 37°C. Cells were then placed on an inverted microscope and 2 independent sets of images of the cells captured from each well as shown in **Fig. 1**.

For each sample, 3 images were captured: bright field image of cells (**Fig. 1** upper panels), Hoechst dye stained image of total cell nuclei (**Fig. 1** middle panels) and GFP images showing cells successfully transfected with plasmid DNA and expressing GFP protein (**Fig. 1** lower panels).

Microscope images of transfected HEK 293 cells, showing both the transfection efficiency and toxicity level of one of our lipid molecules (#29) compared to a commonly used commercial transfection reagent (reference reagent). Lipid reagent #29 has a transfection efficiency of approximately 95% and has low cell toxicity (very few brightly stained apoptotic nuclei). In general, the greater the transfection efficiency of liposomal reagents, the greater is the cellular toxicity. This is obvious for the reference reagent, where a high percentage of cells are transfected but, as a consequence, there are many unhealthy and apoptotic cells.
Note the increased number of cells showing a GFP signal as well as the reduced toxicity. Toxicity is seen by a reduction in total cell number as well as an increase in apoptotic cells, which are detected as brightly stained cells after Hoechst staining

According to the protocol given in Example 18, a library of 120 newly synthesized compounds according to claim 1 have been tested for their ability to transfect HEK 293 cells. The graph in **Fig. 2** shows the transfection efficiency of these lipid compounds compared to a commercially available transfection agent reference. 15 of the lipid molecules are significantly more efficient at delivering plasmid DNA (GFP gene) to HEK 293 cells when compared to a widely used commercial transfection reagent, indicated by the solid line. Of these 15 highly efficient new transfection reagents, one in particular has been identified as possessing very low toxicity and has the ability to very efficiently deliver siRNA molecules to cells (#29; see **Fig. 1** and **Fig. 3**).

Example 19: Screen of library "hits" for ability to transfect siRNA

One of the key technologies for manipulation of gene function, both in cells and whole organisms, is gene silencing through RNA interference (RNAi). Delivery of small interfering RNA (siRNA) molecules into mammalian cells is crucial for this technology and has significant clinical/therapeutic implications.
Thus, in addition to screening our lipids for delivery of plasmid DNA (the genes) we have also screened amino lipids according to the present invention for their ability to efficiently deliver siRNA molecules (the gene silencers).

In order to screen for this two different types of cells were used to test the ability of our lipid reagents to deliver siRNA targeting Low density lipoprotein receptor related protein 6 (LRP6). This is a 200 kD single-pass transmembrane receptor for Wnt ligands and activates the Wnt/b-catenin signalling pathway. It is expressed at relatively low levels in HEK 293 cells and relatively high levels in MEF cells.

Assay 1. Transfection of siRNA in HEK 293 cells

Method:
All steps performed in 1,5ml eppendorf tubes and 24 well plate format. Amounts shown are for one well of a 24-well plate.

1. 2µl lipid reagents diluted in 50µl 50mM NaOAc buffer, pH 5.0.

2. Diluted lipid reagents from (1) added to 20 pmol (1µl of 20uM) siRNA molecules in 20µl NaOAc buffer, pH 5.0 and mixed with pipette action. The siRNA molecules used had either a scrambled sequence not specific for any known gene (Con siR-

NA), or a sequence specifically targeting the endogenous mRNA from the *LRP6* gene (*LRP6* siRNA).

3. After 30 min incubation at RT, added 400µl freshly resuspended cells (3 -5 x 10^5 cells/50µl DMEM culture medium supplemented with 10% foetal calf serum) and mixed with pipette.

4. Lipid/siRNA complexes were immediately transferred to separate wells of a 24-well culture plate and placed in 37°C incubator with 5% CO_2.

5. 48 hours after initial transfection, cells, were lysed in 50µl detergent buffer (50mM Tris, 1% Triton X-100, 0.15 M NaCl, pH7.0, containing protease and phosphatase inhibitors), spun to remove insoluble cell debris and 30ul clarified lysates added to 10 µl of 4x SDS loading buffer (250 mM Tris HCl, 40% Glycerol, 8% SDS, 0.01% Bromophenol Blue, 5% 2-Mercaptoethanol, pH 6.8).

6. Samples were denatures by heating at 96°C for 2 min. and 10µl loaded on a 9% SDS-PAGE gels for separation of proteins according to molecular weight. Separated proteins were transferred from the SDS-PAGE gel to nitrocellulose membrane for Western Blot (WB) analysis.

7. WB was performed using an automated BioLane HTI instrument using an antibody against LRP6. An HRP linked secondary antibody and chemiluminescence was used to detect the proteins on the membrane.

Assay 2. Transfection of siRNA in mouse embryonic fibroblast (MEF) cells

Method:

All steps performed in 1,5ml Eppendorf tubes and 24 well plate format. Amounts shown are for one well of a 24-well plate.

1. 2µl lipid reagents diluted in 50 µl 50 mM NaOAc buffer, pH 5.0.

2. Diluted lipid reagents from (1) added to 20 pmol (1 µl of 20 µM) siRNA molecules in 20 µl NaOAc buffer, pH 5.0 and mixed with pipette action. The siRNA molecules used had either a scrambled sequence not specific for any known gene (Con siRNA), or a sequence specifically targeting the endogenous mRNA from the *LRP6* gene (*LRP6* siRNA).

3. After 30 min incubation at RT, added 400 µl fresh DMEM culture medium supplemented with 10% foetal calf serum, mixed with pipette and added to adherent MEF cells (50-70% confluency) in one well of a 24-well plate and placed in 37°C incubator with 5 % CO_2..

4. 48 hours after initial transfection, cells, were lysed in 100 µl detergent buffer (50mM Tris, 1% Triton X-100, 0,15 M NaCl, pH 7.0, containing protease and phosphatase inhibitors), spun to remove insoluble cell debris and 30 µl clarified lysates added to 10 µl of 4x SDS loading buffer (250 mM Tris HCl, 40% Glycerol, 8% SDS, 0.01% Bromophenol Blue, 5 % 2-Mercaptoethanol, pH 6.8).

5. Samples were denatures by heating at 96°C for 2 min and 5 µl loaded on a 9 % SDS-PAGE gels for separation of proteins according to molecular weight. Separated proteins were transferred from the SDS-PAGE gel to nitrocellulose membrane for Western Blot (WB) analysis.

6. WB was performed using an automated BioLane HTI instrument using antibodies against LRP6 and beta-actin proteins. HRP linked secondary antibodies and chemiluminescence were used to detect the proteins on the membrane.

Results

Fig. 3 A shows a Western Blot (WB) analysis of endogenous LRP6 protein from total lysates of 293 cells transfected with the indicated siRNA molecules and incubated for 48 hours. The endogenous level of LRP6 is strongly down-regulated after *LRP6* siRNA delivery using reagent A, however this effect is stronger using a lipid reagent according to this invention #29. Con refers to a non-targeting, scrambled siRNA control and loading refers to a non-specific protein band that is used as a reference to show that similar protein amounts were loaded for each sample (thus demonstrating specific silencing of the siRNA target).

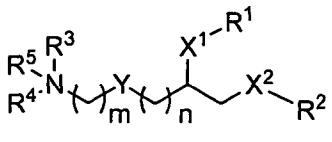
Fig. 3 B shows the Western Blot (WB) analysis of LRP6 protein from total lysates of MEF (mouse embryonic fibroblast) cells transfected with the indicated siRNA molecules and cultured for 48 hours. Again lipid reagent #29 is more effective than the commercial reagent A at siRNA mediated gene silencing. A second lipid reagent (#35) is also shown that has little effect, despite the fact that it can efficiently transfect cells with plasmid DNA. This demonstrates the striking difference in properties of related transfection reagents and highlights the importance of using our novel method to easily synthesise hundreds of related lipids that can be screened to identify the ones having optimal properties (such as highly efficient DNA and siRNA delivery as well as low cellular toxicity).

List of abbreviations

Ar	Argon
DCM	Dichloromethane
DEDPA	N-(2-(Dimethylamino)ethyl)-4,5-bis(dodecylthio)pentanamide
DIC	N, N'-Diisopropylcarbodiimide
DMEM	Culture medium
DMF	Dimethylformamide
DMPA	2,2-Dimethoxy-2-phenylacetonphenone
DNA	Desoxyribonucleic acid
DOPE	Dioleolylphosphatidylethanolamine
EGFP	Enhanced GFP
GFP	Green Fluorescent Protein
HOBt	Hydroxylbenzotriazole
HRP	Horseradish Peroxidase
kD	kilo Dalton
LRP6	Low density lipoprotein receptor related protein 6
MEF	Mouse embryonic fibroblast
PEG	Polyethylene glycol
RNA	Ribonucleic acid
RNAi	RNA interference
siRNA	small interfering RNA
SDS	Sodium dodecyl sulfate
THF	Tetrahydrofuran
WB	Western Blot
Wnt	Signalling proteins in cell differentiation

Claims

1. Amino lipids with the following formula (I):

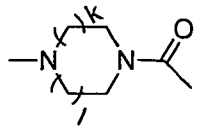


(I)

wherein R^1 and R^2 are the same or different and independently C_6-C_{24} alkyl, C_6-C_{24} alkenyl, C_6-C_{24} alkynyl, or C_6-C_{24} acyl, which can be optionally substituted with a C_1-C_6 hydrocarbyl group,

X^1 and X^2 are the same or different, either S or S=O or $S(=O)_2$,

Y is either an amide, an ester or a heterocyclic amide of the formula



wherein k and l are integers from 0 to 2,

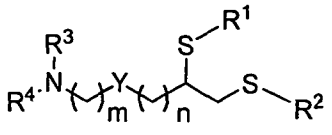
R^3 and R^4 are either the same or different and independently C_1-C_{12} alkyl, C_1-C_{12} alkenyl, or C_1-C_{12} alkynyl, wherein alkyl, alkenyl or alkynyl may be optionally substituted with a C_1-C_6 hydrocarbyl group, or R^3 and R^4 may join to form an optionally substituted heterocyclic ring of 3 to 10 atoms and 0 to 6 heteroatoms chosen from nitrogen, thiol and oxygen,

R^5 is either absent or is hydrogen or C_1-C_{12} alkyl to provide a quaternary amine,

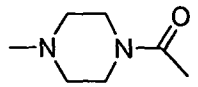
m is an integer from 1 to 12 and n is an integer from 2 to 12.

2. The amino lipids of claim 1, wherein R^1 and R^2 are the same or different and independently C_6-C_{24} alkyl, which can be optionally substituted with a C_1-C_6 hydrocarbyl group.

3. The amino lipid of claim 1 or 2, having the structure of formula (II)

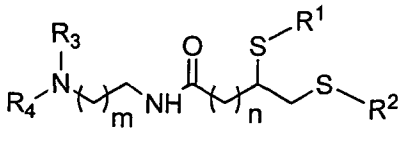


(II) R^1 and R^2 are the same C_6-C_{18} alkyl
Y is either an amide, an ester or a heterocyclic amide of the formula

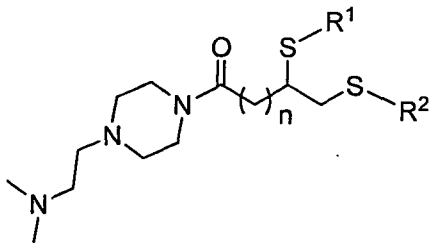


R^3 and R^4 are either the same or different C_1-C_{12} alkyls, wherein alkyl may be optionally substituted with a C_1-C_6 hydrocarbyl group, or R^3 and R^4 may join to form an optionally substituted heterocyclic ring of 3 to 10 atoms and 0 to 6 heteroatoms chosen from nitrogen, thiol and oxygen,
m is an integer from 1 to 12, n is an integer from 2 to 12.

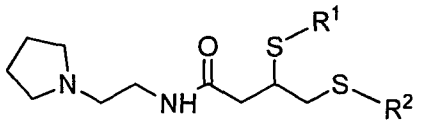
4. The amino lipid of claims 1 to 3, having the structure of formula (IIIa), (IIIb) or (IIIc)



(IIIa),

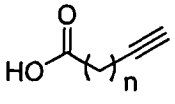


(IIIb), or

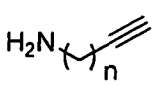


(IIIc),
wherein R^1 and R^2 are the same C_{11}-C_{12} alkyls,
R^3 and R^4 are the same C_1-C_2 alkyls,
m is an integer from 1 to 2, n is an integer from 2 to 3.

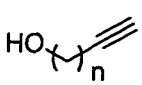
5. A method to synthesize an amino lipid as defined in claims 1 to 4 comprising the following steps:
a) reaction of alkynes of the general formula (IVa), (IVb) or (IVc),



(IVa),

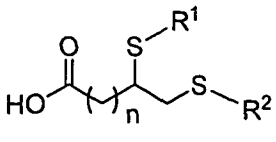
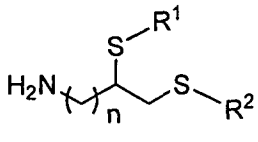
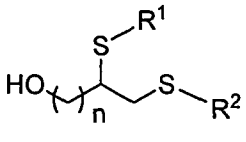


(IVb), or

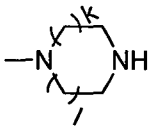


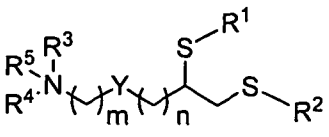
(IVc)

wherein n is an integer from 2 to 12,
with compounds of the general formula, HS-R^1 and HS-R^2,
wherein R^1 and R^2 are the same or different and independently C_6-C_{24} alkyl, C_6-C_{24} alkenyl, C_6-C_{24} alkynyl, or C_6-C_{24} acyl, which can be optionally substituted with a C_1-C_6 hydrocarbyl group, under either UV-irradiation or using a radical initiator, to yield a compound of the general formula (Va), (Vb) or (Vc),


(Va),

(Vb),

(Vc)

wherein n, R^1 and R^2 are defined as above,
b) condensation reaction of the product of step a) of the formula Va with an amine or alcohol of the general formula $(R^3R^4R^5N)(CH_2)_mZ$, with m as an integer from 1 to 12, wherein Z is NH_2, OH, or a secondary heterocyclic amine of the formula


, wherein k and l are integers from 0 to 2,
or condensation reaction of the product of step a) of the formula Vb and Vc with carboxylic acid of the general formula $(R^3R^4R^5N)(CH_2)_mZ$, with m as an integer from 1 to 12, wherein Z is COOH.
R^3 and R^4 are either the same or different and independently C_1-C_{12} alkyl, C_1-C_{12} alkenyl, or C_1-C_{12} alkynyl, which may be optionally substituted with a C_1-C_6 hydrocarbyl group, or R^3 and R^4 may join to form a ring of 3 to 10 carbon atoms and 0 to 6 heteroatoms chosen from nitrogen, thiol and oxygen,
R^5 is either absent or is hydrogen or C_1-C_{12} alkyl to provide a quaternary amine,
to yield a compound of the general formula (VI)


(VI)

, wherein n, m, Y, R^1, R^2, R^3, R^4 and R^5 are defined as above.

6. Method according to claim 5, comprising an optional step c) oxidation of the thioethers of the general formula (VI) (product of step b) into sulfoxide (S=O) and/or sulfone ($S(=O)_2$) using an oxidation reagent.

7. A lipid particle containing an amino lipid of one of the claims 1 to 4.

8. A lipid particle according to claim 7, wherein said lipid particle is a liposome.

9. The lipid particle according to one of the claims 7 or 8, further containing a non-cationic lipid.

10. A lipid particle according to one of the claims 7 to 9, further containing a sterol.

11. A lipid particle according to one of the claims 7 to 10, further containing a bioactive agent.

12. A lipid particle according to claim 11, wherein said bioactive agent is a member optionally selected from the group consisting of: a nucleic acid, an antineoplastic agent, an antibiotic, an immunomodulator, an anti-inflammatory agent, an agent acting on the central nervous system, a polypeptide or a polypeptoid.

13. Use of a lipid particle according to one of the claims 7 to 12 for delivering a bioactive agent into a cell.

14. Lipid particle according to one of the claims 7 to 12 for use as a medicament.

15. Lipid particle according to one of the claims 7 to 12 for use as a medicament in treatment of a viral infection, a liver disease or disorder, or cancer.

Fig. 1

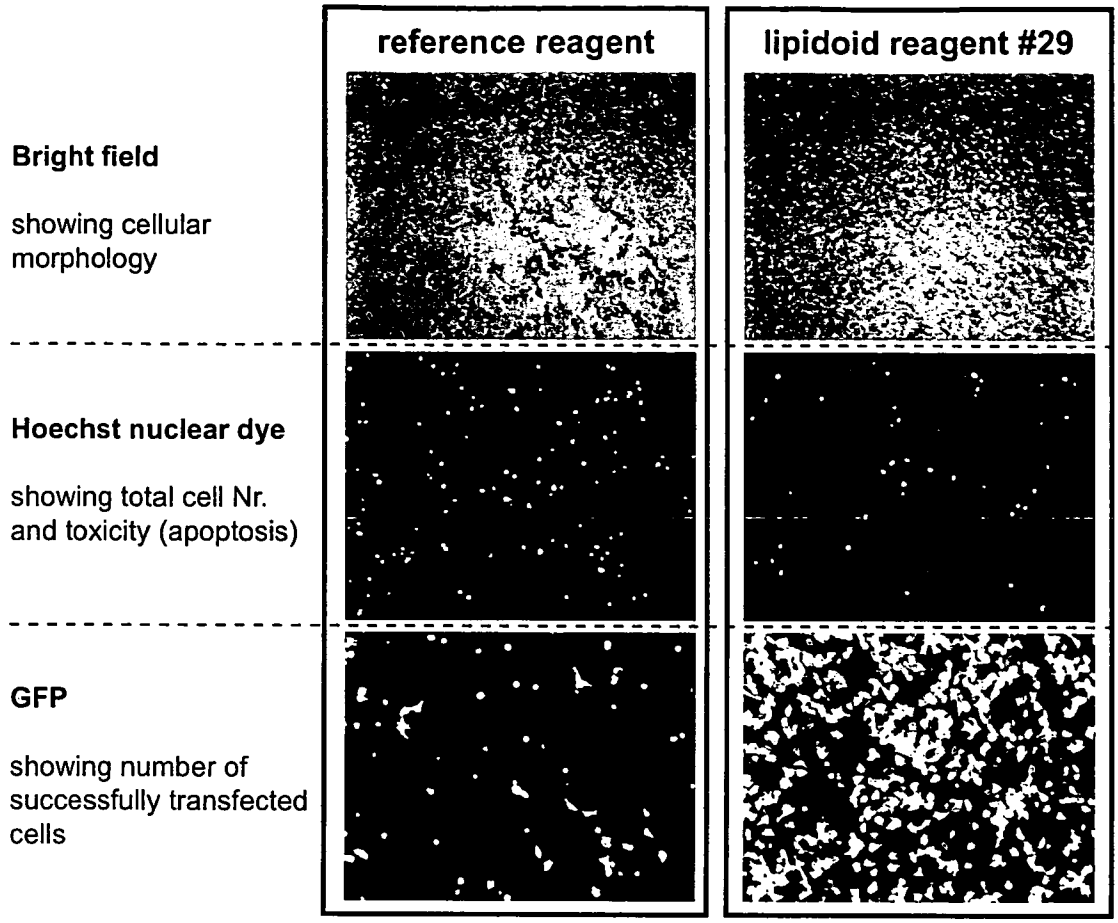

reference reagent
lipidoid reagent #29
Bright field
showing cellular morphology
Hoechst nuclear dye
showing total cell Nr. and toxicity (apoptosis)
GFP
showing number of successfully transfected cells

Fig. 2

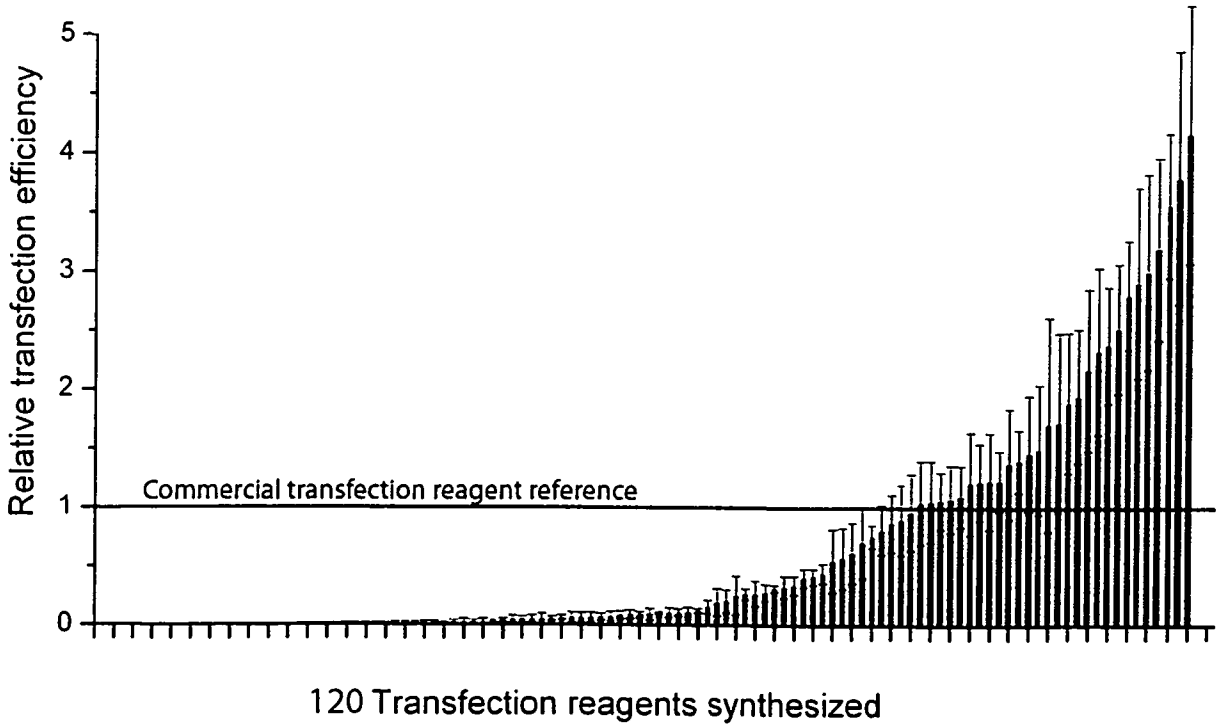
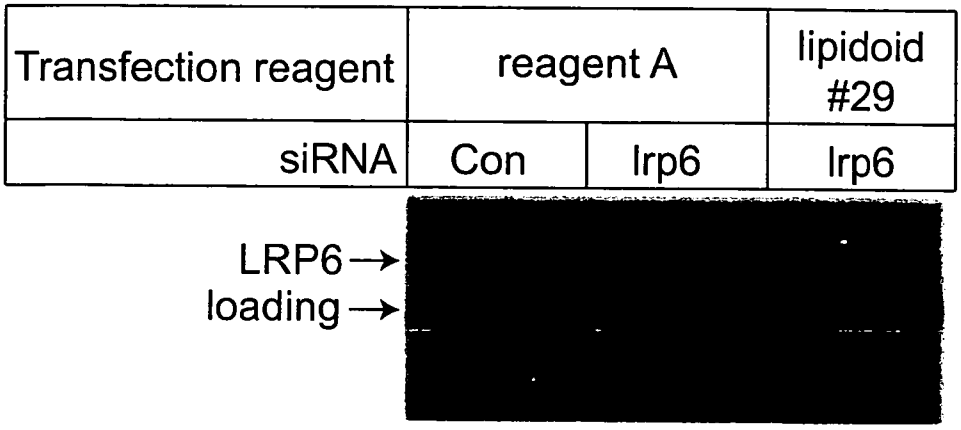
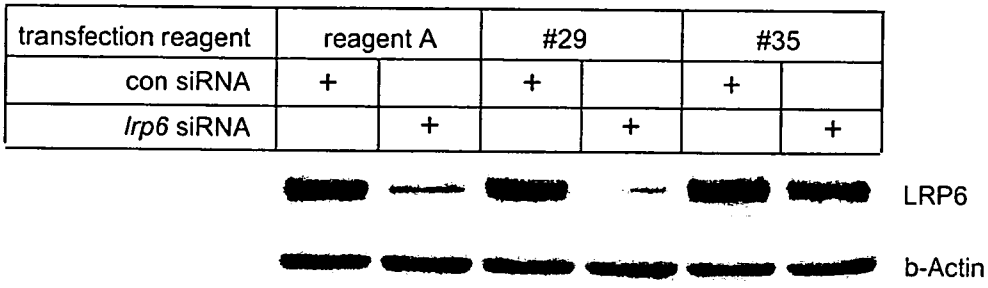

Relative transfection efficiency
0
1
2
3
4
5
Commercial transfection reagent reference
120 Transfection reagents synthesized

Fig. 3

A


Transfection reagent
reagent A
lipidoid #29
siRNA
Con
lrp6
lrp6
LRP6
loading

B


transfection reagent
reagent A
#29
#35
con siRNA
lrp6 siRNA
LRP6
b-Actin

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/002101

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07C317/44 C07C323/60 C07D295/00 A61K31/16 A61P35/00
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)
C07C C07D A61K A61P

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, 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.
A	WO 01/42200 A1 (GENZYME CORP [US]; MARSHALL JOHN [US]; HARRIS DAVID J [US]; SIEGEL CRA) 14 June 2001 (2001-06-14) cited in the application the whole document -----	1,13
A	MILLER A D: "CATIONIC LIPOSOMES FOR GENE THERAPY", ANGEWANDTE CHEMIE. INTERNATIONAL EDITION, WILEY VCH VERLAG, WEINHEIM, vol. 37, no. 13, 3 August 1998 (1998-08-03), pages 1768-1785, XP000772935, ISSN: 1433-7851, DOI: 10.1002/(SICI)1521-3773(19980803)37:13/14<1768::AID-ANIE1768>3.0 .CO;2-4 cited in the application the whole document -----	1,13

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

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"P" document published prior to the international filing date but later than the priority date claimed

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 July 2012	26/07/2012

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	de Nooy, Arjan

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2012/002101

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0142200 A1	14-06-2001	AU 2637500 A	18-06-2001
		WO 0142200 A1	14-06-2001