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(57) Abrégé/Abstract:
The present invention provides cationic dendrimers for delivering bioactive molecules, such as polynucleotide molecules, peptides and polypeptides and/or pharmaceutical agents, to a mammalian body. The dendrimers disclosed herein are suitable for targeting the delivery of the bioactive molecules to, for example, the liver, spleen, lung, kidney or heart.

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(54) Title: DENDRIMERS FOR USE IN TARGETED DELIVERY

(57) Abstract: The present invention provides cationic dendrimers for delivering bioactive molecules, such as polynucleotide molecules, peptides and polypeptides and/or pharmaceutical agents, to a mammalian body. The dendrimers disclosed herein are suitable for targeting the delivery of the bioactive molecules to, for example, the liver, spleen, lung, kidney or heart.

DENDRIMERS FOR USE IN TARGETED DELIVERY

The present invention relates to the targeted delivery of bioactive molecules in a mammalian body. In particular, the present invention relates to the use of cationic dendrimers for delivering polynucleotide molecules, peptides and polypeptides and/or pharmaceutical agents to a mammalian body, in particular, human.

The possibility of using genes as medicines to correct genetic disorders or treat cancers is hampered by the inability to efficiently deliver genetic material to diseased sites[1]. A variety of viral and non-viral systems are being used experimentally each with distinct advantages and disadvantages. Viral systems[2] have been studied extensively and include a wide variety of viral types such as, retroviruses, adenoviruses, adeno-associated viruses, herpes simplex virus and the HIV based lentivirus. All have various inherent disadvantages[3] such as safety concerns and scale-up difficulties. Non-viral systems such as cationic liposomes[4-6], cationic polymers[7,8], cationic polymeric vesicles[9,10] and dendrimers[11-14] have thus been studied as gene delivery agents in an effort to circumvent some of the safety and production problems associated with viruses. As well as applications in human health, suitable gene transfer systems are commercially attractive as in vitro molecular biology and in vivo transfection reagents for laboratory use.

Dendrimers are synthetic 3-dimensional macromolecules that are prepared in a step-wise fashion from simple branched monomer units, the nature and functionality of which can be easily controlled and varied. Dendrimers are synthesised from the repeated addition of building blocks to a multifunctional core (divergent approach to synthesis), or towards a multifunctional core (convergent approach to synthesis) and each addition of a 3-dimensional shell of building blocks leads to the formation of a higher generation of the dendrimers[51]. Polypropylenimine dendrimers start from a diaminobutane core to which is added twice the number of amino groups by a double Michael addition of acrylonitrile to the primary amines followed by the hydrogenation of the nitriles[52]. This results in a doubling of the amino groups[52].

Polypropylenimine dendrimers contain 100% protonable nitrogens[17] and up to 64 terminal amino groups (generation 5, DAB 64)[15,16]. Protonable groups are usually amine groups which are able to accept protons at neutral pH. The use of dendrimers as gene delivery agents

has largely focused on the use of the polyamidoamine[11-13,18-25] and phosphorous containing[14] compounds with a mixture of amine/ amide or N-P(O_2)S as the conjugating units respectively with no work being reported on the use of the lower generation polypropylenimine dendrimers for gene delivery. Polypropylenimine dendrimers have also been studied as pH sensitive controlled release systems for drug delivery[26,27] and for their encapsulation of guest molecules when chemically modified by peripheral amino acid groups[28]. The cytotoxicity[29] and interaction of polypropylenimine dendrimers with DNA[30] as well as the transfection efficacy of DAB 64 has also been studied[31].

Kabanov and others report that polypropylenimine dendrimers interact with DNA via the surface primary amines only with no involvement of the internal amine groups[30] while Gebhart and Kabanov report very low gene transfer activity with the 5th generation polypropylenimine dendrimers DAB 64 in the easy to transfect COS cell line[31]. These workers also report that DAB 64 is far too toxic above a dendrimer, DNA weight ratio of 0.62: 1 (nitrogen to phosphate ratio of 4: 1). Additionally Malik and others conclude that the cationic dendrimers as opposed to the anionic dendrimers are too toxic for parenteral use without further derivatisation with biocompatible groups such as polyethylene glycol units [29].

The present invention is based upon the observation that, contrary to earlier reports, cationic dendrimers, such as polypropylenimine dendrimers, display suitable properties, such as specific targeting and low toxicity, for use in the targeted delivery of bioactive molecules, such as genetic material. In addition, derivatives of the cationic dendrimer also display suitable properties for the targeted delivery of bioactive molecules.

The chemical modification (derivatisation) of polypropylenimine dendrimers has been extensive with reports on the conjugation of amino acid[28,32], carboxylate[33,34], acetyl[35], 2-(hydroxy)propyltrimethylammonium[27], dimethyldodecylammoniumm[36], 3,4,5-(ologoethylenoxy) benzoyl[37], alkanoyl thioglucose[38], thiolactosyl[39] groups as well as the conjugation of hydrophobic 3,4-bis(decyloxy)benzoyl[40], palmitoyl[41], pentafluorphenyl 11-[4-(4-hexyloxyphenylazo)phenyloxy]undecanoyl[41], adamantine carboxyl[41], decanoyl[42], dodecyl[42], polyisobutylene[43], stearoyl[44], ω-(4'cyanobiphenyloxy)alkyl[45], and oligo(p-phenylene vinylene)[46] groups to the primary amino groups on the surface of polypropylenimine dendrimers.

In addition to the targeted delivery of genetic material, selective targeting of other bioactive agents, such as peptides/polypeptides and pharmaceutical agents, is sought.

It is thus one of the objectives of the present invention to provide cationic dendrimers capable of targeted delivery of bioactive molecules to a particular location in a mammalian body.

Accordingly, the present invention provides a composition for the delivery of bioactive molecules to a target location in the body of a recipient, wherein said composition comprises a cationic dendrimer, and/or derivatives thereof, admixed with said bioactive molecule.

In accordance with an aspect of the present invention, there is provided a composition for the delivery of a bioactive molecule to a target location in the body of a recipient, said composition comprising a cationic polypropylenimine dendrimer comprising a diaminobutane core with 1, 2, or 3 generations of propylenimine molecules attached, and/or derivatives thereof, admixed with the bioactive molecule.

The term "cationic dendrimer" refers to a dendrimer molecule which possesses a positive charge at physiological pH. However, the dendrimer derivatives of the present invention may not in themselves be cationic as a result of the derivatisation.

The cationic dendrimers, or derivatives thereof, of the composition of the present invention may be derived from a core molecule comprising 2 to 10 carbon atoms, such as 3 or 4 carbon atoms, and in particular 4 carbon atoms with one or more functional groups which may, for example, be amine groups. It will be appreciated that, for example, the cationic dendrimers, or derivatives thereof, may be derived from a core molecule such as diaminoethane, diaminopropane or diaminobutane, and in particular, diaminobutane.

The groups attached to the core molecule may, for example, include propylamines Thus, the dendrimers may be polypropylenimine dendrimers, or derivatives thereof, and may possess a diaminobutane core.

The term "polypropylenimine dendrimer" is intended to refer to dendrimers comprising a diaminobutane core with 1, 2, 3, 4 or 5 generations of propylenimine molecules attached. The term encompasses DAB 4, DAB 8, 16, 32 and 64, DSAM 4, DSAM8, 16, 32 and 64, and QDAB4, 8, 16, 32 and 64, HDAB4, 8, 16, 32, 64 and bolamphiphilic polypropylenimine dendrimers BDAB4, BDAB8, BDAB16, BDAB32, BDAB64. Although all generations of the compounds DSAM, QDAB, HDAB and BDAB comprise a diaminobutane core with propylenimine groups attached thereto, the term "DAB" as generally used herein is intended to refer to the underivatised DABS, 16, 32 and 64 compounds, unless otherwise indicated.

The term "generation" refers to the number of iterative reaction steps that are necessary to produce the compound. The number which follows the name or abbreviated name of the dendrimer, for example, 8, 16, 32 or 64 refers to the number of surface groups on the dendrimer molecule itself, which amino groups may or may not be derivatised.

The cationic dendrimers of the composition of the present invention may be modified by covalently binding derivatising groups, such as hydrophobic, hydrophilic or amphiphilic groups to the surface of the dendrimer or by attaching two dendrimer molecules to either end of a hydrocarbon chain with a carbon length of 8, 12, 14, 16 or 18 carbons to give bolamphiphilic dendrimers (said modified dendrimers referred to herein as "derivatives"). The number of derivatising groups may vary from one derivatising group per dendrimer molecule up to and including derivatising all available surface or terminal groups on the dendrimer molecule, for example, derivatising all 16 surface groups of the DAB16 molecule.

The amphiphilic derivative comprises a hydrophilic and a hydrophobic segment. The hydrophilic segment may be derived from a phosphoglycerate molecule, for example, glycerol 3-phosphate. The hydrophobic segment is covalently bound to the hydrophilic segment, for example, via an ester linkage. The hydrophobic segment is selected from any suitable hydrophobic group, for example, alkyl, alkenyl or alkynyl groups of 8-24 carbons in length. Therefore, the hydrophobic segment plus ester linkage can be defined as an acyl group. The amphiphilic derivative is attached to the dendrimer by a linker molecule, such as polyethylene glycol (PEG) or a sugar unit such as muramic acid bound to the hydrophilic segment. The length of the PEG linker molecule may for example be in the range of 1 to 120 ethylene glycol units, for example 50-100 and, for example, 70-80, for example, 77. In particular the linker molecule may be polyethylene glycol with a Mw of approximately 3,500. Alternatively, the linker molecule may be an ester, amine or ether linkage for ordinary hydrophobic modifications or a sugar molecule such as muramic acid. In particular, the derivative may be a phosphoglyceride such as a phosphatidyl ethanolamine, for example, distearoylphosphatidylethanolamine. The number of amphiphilic derivatives per dendrimer molecule may range from 1 derivatising group per dendrimer molecule to derivatising all of the groups of the dendrimer (the generation of the dendrimer will determine the total number of surface groups available for derivatising), and may

be, in particular, one group per dendrimer. Thus, the dendrimers of the present invention include generations 1, 2, 3, 4 and 5 of the amphiphilic-derivatised diaminobutane dendrimer referred to herein as 1,2-diacyl-SN-glycero-3-phosphoethanolamine-N-[(polyethyleneglycol)-N-diaminobutanepolypropylenimine dendrimer $-(NH_2)_x]$, where x = 4, 8, 16, 32 or 64.(conveniently referred to as DSAM4, 8, 16, 32 or 64, respectively).

The hydrophobic derivative may be an alkyl, acyl, alkenyl, alkynyl or aryl group of 8-24 carbons in length. It is to be understood that the term "hydrophobic" can encompass acyl groups when the chain length of such acyl groups is 8 carbons or more and may, for example, be a hexadecanoyl group. The number of hydrophobic groups per dendrimer molecule may range from 1 derivatising group per dendrimer molecule to derivatising all of the groups of the dendrimer (the generation of the dendrimer will determine the total number of surface groups available for derivatising), and may be, in particular, one group per dendrimer. Thus, the dendrimers of the present invention include generations 1,2, 3, 4 and 5 of the hydrophobic - derivatised diaminobutane dendrimer referred to herein as HDAB4, HDAB8, HDAB16, HDAB32, HDAB64.

The bolamphiphiles may consist of two molecules of any of the dendrimers DAB4, DAB8, DAB 16, DAB 32, DAB 64 linked to either end of an alkyl, acyl, alkenyl, alkynyl hydrophobic unit of 8 to 24 carbon chains in length or alternatively linked by an aryl group and may be a C12 bolamphiphile of DAB 4 or DAB 8. The term "bolamphiphiles" is understood to refer to an amphiphilic molecule wherein the hydrophilic groups are separated by the hydrophobic groups. Thus, the dendrimers of the present invention include C8-C16alkyl bolamphiphiles of dendrimers of generations 1,2, 3, 4 and 5 herein referred to as B8DAB4, 8, 16, 32 or 64; B10DAB4, 8, 16, 32 or 64; B12DAB4, 8, 16, 32 or 64, B14DAB4, 8, 16, 32 or 64 and B16DAB4, 8, 16, 32 or 64 . The amino derivative may, for example, be a tertiary amine or quaternary ammonium derivative, and in particular a quaternary derivative comprising C1-C4 alkyl groups, such as 3 methyl groups, covalently bound to a nitrogen atom on the surface of the dendrimer. The number of ammonium derivatives per dendrimer molecule may range from 1 derivatising group per dendrimer molecule to derivatising all groups of the dendrimer (the generation of the dendrimer will determine the total number of surface groups available for

derivatising), and may be, in particular, all groups available for derivatising. Thus, the dendrimers of the present invention include generations 1, 2, 3, 4 and 5 of the quaternary ammonium-derivatised diaminobutane dendrimer referred to herein as quaternary ammonium diaminobutanepolypropylenimine dendrimer $-[NH_2(CH_3)_3]_x$, where x = 4, 8, 16, 32 or 64 (conveniently referred to as QDAB4, 8, 16, 32 or 64, respectively).

The dendrimers in the present invention may also be derivatised with hydrophilic groups such as sugars, mono and oligohydroxy C1-C6 alkyl, mono and oligohydroxy C2-C6 acyl, C1-C2 alkoxy alkyl optionally having one or more hydroxy groups substituted on the alkoxy or alkylene groups, amino acids, peptides of 1-200 amino acids in length and oligo or poly-(oxa C1-C3 alkylene) such as polyoxyethylene comprising 1-120 ethylene oxide units.

Target locations for the delivery of bioactive molecules include the liver, spleen, lung, kidney and heart. In particular, two of the dendrimers of the present invention studied, DAB16 and DSAM16, have displayed organ-specific targeting to the liver and spleen, respectively.

Therefore, the present invention also provides a composition for the delivery of a bioactive molecule to the liver of a recipient, wherein said composition comprises the polypropylenimine dendrimer DAB16 admixed with a said bioactive molecule. Additionally, the present invention provides a composition for the delivery of bioactive molecules to the spleen of a recipient, wherein said composition comprises the polypropylenimine dendrimer DSAM16 admixed with a said bioactive molecule.

The recipient may be a mammal, such as a human.

The terms "bioactive molecules" and "biologically active molecules" are intended to encompass polynucleotides, peptides/polypeptides and/or pharmaceutical agents. The term "polynucleotides" generally refers to DNA unless otherwise indicated but may include RNA, cDNA, oligonucleotides, plasmids etc. The term may also be used interchangeably herein with the terms "polynucleotide", "gene", "genetic material" and "genetic sequence". Such genes intended for expression are common to the field of gene therapy and include, but are not limited to, sense DNA or RNA for expressing a product in the target organ, or antisense DNA or RNA for reducing or eliminating expression of a native or introduced gene in the target organ. The term "peptide" refers to a chain of 4 to 600 amino acids long, such as 4 to 200 amino acids long

and therefore encompasses polypeptides and proteins, and includes enzymes and polypeptide hormones. Furthermore, peptides modified by, for example, glycosylation, are also included in the present invention, as is a protein comprising two or more polypeptide chains each of length of 4 to 600 amino acids long cross-linked by, for example, disulphide bonds, for example, insulin and immunoglobulins. The term "pharmaceutical agent" is intended to include any natural or synthetic compound administered to a recipient in order to induce a physiological or pharmacological effect. Examples of such agents are anti-tumour drugs, antibiotics, hormones, anti-inflammatory agents, antiparasitic agents, DNA vaccines, etc

The cationic dendrimers are admixed with the bioactive agents in preparing the compositions of the present invention for delivery. The term "admixed" generally refers to the bioactive agent being associated with but not covalently bound to the dendrimer. The term is however also intended to encompass covalently binding the bioactive agent to the dendrimer via any suitable reactive group on the dendrimer and the agent.

Where the bioactive agent is a polynucleotide molecule, the molecule is usually associated with, that is, not covalently bound to, the dendrimer to allow the polynucleotide to be expressed. However, it may also be possible that expression of a covalently bound polynucleotide molecule can occur, and therefore, these covalently bound polynucleotide molecules are intended to be encompassed by the present invention.

In a yet further aspect, the present invention provides a pharmaceutical formulation comprising a composition of the present invention, and a pharmaceutically acceptable carrier.

Pharmaceutically acceptable carriers are well known to those skilled in the art and include, but are not limited to, 0.1M and preferably 0.05M phosphate buffer or 0.8% (w/v) saline. Additionally, such pharmaceutically acceptable carriers may be aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solutions are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as

those based on Ringer's dextrose, and the like. Preservatives and other additives may also be present, such as, for example, antimicrobials, antioxidants, chelating agents, inert gases and the like.

Conveniently, the composition or pharmaceutical formulation of the present invention may include an agent which assists in forming a colloidal suspension, for example, 5% dextrose solution. Other agents which may be included are viscosity enhancing polymers such as alginates and polyethyleneglycol polymers, buffering agents and mixtures of aqueous and non-aqueous solvents in emulsions.

The present invention also provides the use of the composition or pharmaceutical formulation of the present invention for the delivery of bioactive molecules to a target location in the body of a recipient.

In a further aspect, the present invention provides a method of delivering a bioactive molecule to a target location in the body of a recipient, which method comprises preparing a composition comprising a cationic dendrimer, or derivative thereof, admixed with a said bioactive molecule, and subsequently administering the composition to said recipient.

Although the dendrimers of the present invention display suitable properties for the delivery of bioactive molecules in vivo, they are also useful for transfecting mammalian cells in vitro. Thus, the present invention also provides a composition of the present invention for transfecting mammalian cells with a bioactive molecule in vitro. The mammalian cells may, for example, be human cells.

The present invention still further provides 1,2-diacyl-SN-glycero-3-phosphoethanolamine-N-[(polyethyleneglycol)-N-diaminobutanepolypropylenimine dendrimer –$(NH_2)_x$], where x = 4, 8, 16, 32 or 64 (DSAM), and quaternary ammonium diaminobutanepolypropylenimine dendrimer –$[NH_2(CH_3)_3]_x$, where x = 4, 8, 16, 32 or 64 (QDAB). The DSAM or QDAB dendrimers may, for example, be second, third, fourth or fifth generation dendrimers referred to herein as DSAM4, 8, 16, 32 and 64, and QDAB4, 8, 16, 32 and 64.

The present invention also provides a method of preparing a composition as described above, said method comprising admixing a cationic dendrimer, and/or derivatives thereof, and a bioactive molecule.

These and other aspects of the present invention will now be described by way of example only, in conjunction with the accompanying Figures, in which:

Figure 1 illustrates DAB 16 generation 3 polypropylenimine dendrimer, DAB 64 contains 2 more generations of propylamines attached to this molecule;

Figure 2 illustrates DSAM 16, an amphiphilic derivative of DAB 16 (DSPE-PEG-NHS + DAB16);

Figure 3 illustrates BnDAB4, a bolamphiphile derivative of DAB4 (n= 8, 10 or 12 to give B8DAB4, B10DAB4, B12DAB4 respectively);

Figure 4 illustrates QDAB 16, a quaternary ammonium derivative of DAB 16 (CH_3I + DAB16);

Figure 5 illustrates Luciferase gene expression in vivo;

Figure 6 illustrates liver targeting of gene expression by the polypropylenimine dendrimers; and

Figure 7 illustrates tumour gene expression after the intravenous administration of DNA, PEI-DNA = the Exgen 500 formulation.

EXAMPLE 1

METHODS

Synthesis of modified dendrimers

DSAM

To DAB 16 (Sigma Aldrich, Dorset, UK - 3.52g, 1.32mmoles) dissolved in absolute ethanol (70ml) was added triethylamine (27ml, 6.6mmoles) and to this solution was added dropwise DSPE-PEG-NHS (distearoylphosphatdylethanolamine polyethylene glycol N-hydroxysuccinimide - 500mg, 0.11mmoles, Shearwater Polymers) dissolved in chloroform (25mL) over 60 minutes. The reaction was left stirring protected from light for 72h. At the end of this time the reaction mixture was evaporated to dryness by rotary evaporation under reduced

pressure at 50°C. The residue was redissolved in absolute ethanol (40ml), filtered and the filtrate evaporated to dryness. This latter residue was dissolved in water (80ml) and dialysed against 5 l of water over 24h with 6 changes. The dialysate was freeze-dried and the structure confirmed by ^{1}H and ^{13}C NMR.

QDAB

DAB 16, 32 or 64 (500mg) was dispersed in methyl-2-pyrolidone (50ml) for 16h at room temperature with stirring. To the DAB dispersion was added sodium hydroxide (120mg), methyl iodide (3g) and sodium iodide (150mg). The reaction mixture was stirred under a stream of nitrogen for 3h at 36°C. The quaternary ammonium product was recovered by precipitation in diethyl ether followed by filtration. The solid was washed with copious amounts of absolute ethanol (1l) followed by copious amounts of diethyl ether (500ml). The washed solid was then dissolved in water (150ml) and passed over an ion exchange column (1 x 6cm packed with 30ml Amberlite IRA-93 Cl^- and subsequently washed with HCl – 90ml, 1M followed by distilled water – 500ml until the eluate gives a neutral pH). The eluate obtained was freeze-dried and the structure confirmed by both ^{1}H and ^{13}C NMR.

DNA condensation

Plasmid (pCMVsport β-gal or pCMV luciferase, Life Technologies, UK) was grown in E. coli and plasmid purification carried out using a QIAGEN Endo-toxin free Giga Plasmid Kit (QIAGEN™, Hilden, Germany) according to the manufacturer's instructions. Purity was confirmed by agarose gel electrophoresis[47]. The reduced fluorescence of ethidium bromide (EthBr) was used to probe for DNA condensation by the polymers. EthBr fluorescence increases significantly (factor 40 compared to unbound EthBr) on intercalation with double stranded DNA[48]. The electrostatic interaction between the anionic DNA and cationic groups of the carrier on formation of the DNA – vesicle complex reduces the number of EthBr binding sites, a process termed condensation, ultimately reducing the fluorescence intensity of the EthBr solution.

Complexes of DAB 16 and DSAM with DNA were prepared at various polymer, DNA weight ratios and at various time points the fluorescence intensity ($\lambda_{excitation}$ = 526nm, $\lambda_{emission.}$ = 592nm) of the complexes determined in the presence of EthBr (40μg ml^{-1}). The DNA concentration in the cuvette was kept constant (100μg ml^{-1}) and the polymer solutions in PBS (phosphate buffered saline, pH = 7) and a solution of DNA in PBS served as controls. The reduced fluorescence (F_t/ F_0), was determined for each of the samples, where F_t = the fluorescence of the DNA, polymer complexes and F_0 = the fluorescence of DNA alone.

In vitro cytotoxicity assay

A human epidermoid carcinoma cell line (A431, ATCC CRL-1555) was maintained in Dulbecco's minimum essential medium (DMEM) supplemented with 10% foetal calf serum (FCS) and 2mM glutamine (GibcoBRL, UK) at 10% CO_2 and 37ºC.

Polypropylenimine dendrimer/ dendrimer derivative formulation cytotoxicity was assessed by the measurement of the IC50 in a standard MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide thiazolyl blue – indicator dye) assay [49]. Briefly, 96 well microtitre plates were seeded with 5000 cells per well and incubated for 24hrs. Dilutions of the dendrimer/ dendrimer – DNA formulations (100μl) in tissue culture medium (Opti-Mem) were incubated with the cells for 4 h. The samples were then replaced with fresh DMEM daily and incubated for 72 h. After this period the indicator dye (50μl, 50mg ml^{-1}) was added to each well and incubated with the cells for 4h in the dark. The medium and indicator dye were then removed and the cells lysed with dimethylsulphoxide (200μl). After addition of Sorensen's glycine buffer (25μl) the absorption was measured at 570nm. Values were expressed as a percentage of the control to which no vesicles were added.

In vitro transfection

<u>DNA polymer formulations</u>

DAB and QDAB dendrimer – DNA (pCMVSport β-galactosidase) formulations were made by mixing DNA and dendrimers in a 5% dextrose solution and allowing to stand for no

longer than 15 minutes before use. The resulting colloidal dispersion was sized by photon correlation spectroscopy (Malvern Instruments, UK).

Cell culture

A431 cells (human epidermoid carcinoma cell line, ATCC, CRL-1555), maintained in Dulbecco's Minimal Essential Medium (DMEM, Life Technologies, UK) supplemented with foetal calf serum and L-glutamine (2mM) were seeded at a density of 10^4 cells ml^{-1} and 200µL of the cell suspension placed in 96 well flat bottomed plates. Cells were incubated for 24h at 37°C in 10% CO_2 . Polymer - DNA complexes containing 200µg ml^{-1} DNA (100µl) and serum free medium (100µl, OPTIMEM, Life technologies, UK) were incubated with the cells for 4h at 37°C in 10% CO_2. Naked DNA served as the negative control while a formulation comprising N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulphate (DOTAP), DNA (5:1) served as the positive control. Both the negative and positive controls were dosed at a level of 20µg DNA per well while the level of DNA dosed with the dendrimers varied as indicated. After this time the incubation medium was replaced with DMEM culture media containing penicillin (100U ml^{-1}) and streptomycin (0.1mg ml^{-1}) and once again incubated at 37°C in 10% CO_2 for 48h. The cells were then washed in phosphate buffered saline (200µl) and lysed with 1x Passive Lysis Buffer (80µL, Promega, UK) for 30 min. The cell lysates were subsequently analysed for β-galactosidase expression as described below.

β-galactosidase expression

To the assay buffer [50µl (sodium phosphate buffer – 200mM, pH = 7.3, magnesium chloride – 2mM, mercaptoethanol – 100mM, o-nitrophenol-β-galactopyranoside – 1.33mg ml^{-1})] was added an equal volume of the cell lysate from above within 96 well flat-bottomed plates. Samples were incubated for 1h and the visible absorbance read at 405nm on an automatic plate reader.

In vivo transfection

DNA-polymer formulations

Exgen 500 (linear polyethylenimine 22kD, Euromedex, France) was formulated with the luciferase reporter gene (pCMV luciferase) as described by the manufacturers. Both DAB 16 and DSAM were dissolved in 5%w/v dextrose by probe sonication (5 minutes with the instrument set at 15% of its maximum output) and mixed in a 5:1 weight ratio with DNA 15 minutes prior to intravenous injection.

Animal Experiments

Groups of Balb/C mice (n = 3) were injected via the tail vein with either the DAB 16, DSAM, Exgen 500 or naked DNA formulations each containing 100μg of DNA in an injection volume of 200 - 400μl. Exgen 500 and naked DNA served as the positive and negative controls respectively. Organs were harvested 24h later and quickly frozen in liquid nitrogen and stored at -80°C until analysis could be performed on them.

Analysis for Luciferase

To the liver samples were added 5μl of tissue lysis buffer (25mM Tris HCl - pH = 8.0, 2mM DTT, 2mM EDTA - pH 8.0, 10%w/v glycerol, 1%w/v Triton X-100, 1 Complete® protein inhibitor cocktail tablet per 50 ml of buffer) per mg of liver tissue. To the other organs was added 4μl of tissue lysis buffer per mg of tissue. Organs were homogenised on ice in the lysis buffer to form a slurry and the slurry incubated on ice for 15 mins. The resulting slurry was centrifuged at 13,000rpm to pellet tissue debris and the supernatant removed to a fresh tube. To the pellet was added another 50μl of lysis buffer. The pellet was resuspended by homogenisation and centrifuged once more at 13,000rpm and the supernatant removed and added to the previous supernatant sample. Supernatant samples were diluted 1:10 with the tissue lysis buffer and 80μl of this solution sampled and used for the luciferase assay which was carried out according to the protocol provided by Promega, UK. Protein estimation was carried out using the Sigma (Sigma-Aldrich, UK) bicinchoninic acid system (BC-1).

RESULTS AND DISCUSSION

DNA binds electrostatically with the nitrogen rich dendrimers DAB 16, DAB 32 and DAB 64 (Table 2) and presumably with DAB 8. These compounds begin to condense DNA at a nitrogen to phosphate ratio of 1.47, 1.49 and 1.45 respectively (Table 1) and a surface nitrogen to phosphate ratio of 0.78, 0.77 and 0.74 respectively.

Table 1: % Condensation of DNA by DAB Dendrimers

Dendrimer	Dendrimer, DNA weight ratio	% DNA condensation after 30 min
DAB 16	5.00 2.00 1.00 0.50 0.25 0.13	90.3 93.9 96.2 96.0 94.1 42.8
QDAB 16	5.00 2.00 1.00 0.50 0.25 0.13	96.2 95.3 95.7 94.6 33.6 19.2
DAB 32	5.00 2.00 1.00 0.50 0.25 0.13	94.4 96.9 96.7 96.1 87.2 21.8
QDAB 32	5.00 2.00 1.00 0.50 0.25 0.13	96.7 96.9 96.4 43.9 12.6 7.5
DAB 64	5.00 2.00 1.00 0.50 0.25 0.13	93.4 95.4 93.1 96.2 87.9 26.3
QDAB 64	5.00 2.00 1.00 0.50 0.25 0.13	96.0 96.2 95.4 46.0 15.3 11.9
DSAM 16	5 10 15 20	85.1 85.0 85.9 85.7

It appears as if DNA does not bind only to the surface nitrogens of polypropylenimine dendrimers as reported[30] but also to nitrogens possibly in the second shell of the dendrimer also. Condensation of DNA with the polyamidoamine dendrimers has also been found to occur by electrostatic means[19]. We propose that the polypropylenimine dendrimers have advantages over the polyamidoamine polymers for gene delivery applications simply due to the increased content of protonable nitrogens on the polypropylenimine polymers. There are also advantages associated with the polymer shape over linear polymers as DNA appears to interact with the surface primary amines only, leaving the internal tertiary amines available for the neutralization of the acid pH[50] within the endosomal/ lysosomal compartment. The release of polyamidoamine carried genes by the endosome has been attributed to the protonation of the internal tertiary nitrogens by endosomal protons which then results in a swelling of the endosome and the release of the DNA to the cytoplasm[12]. Also the hydrolytic degradation of polyamidoamine dendrimer amide bonds in water or ethanol[12, 13] increases transfection efficacy up to 50 fold which the authors attribute to the increased flexibility of the polymer on heat degradation. This increased flexibility is said to be crucial to the swelling of the endosome[12]. However we propose that simply increasing the level of tertiary amines in the polymer available for neutralization of the endosomal/ lysosomal pH improves transfection irrespective of the flexibility of the polymer.

In vitro transfection efficacy with the polypropylenimine dendrimers reveals that protein expression obtained by using 20µg of DNA in the DOTAP formulation is obtained using just 5µg of DNA in the DAB 16 formulation and there is no significant difference between the use of 15µg DNA in the QDAB 16 formulation and 20µg DNA in the DOTAP formulation (Table 2).

Table 2: The in vitro transfection of DAB dendrimers in the A431 cell line

Formulation	DNA dose per well (µg)	β-galactosidase expression*
DAB 8, DNA (3: 1 g g^{-1})	20	101.3
	15	102.6
	10	80.1
	5	45.1
DAB 8, DNA (5: 1 g g^{-1})	20	108.7
	15	96.5
	10	73.1
	5	59.2
DAB 8, DNA (10: 1g g^{-1})	20	82.0
	15	76.4
	10	78.6

	5	71.7
DAB 16, DNA (1: 1 g g^{-1})	20	54.2
	15	40.0
	10	38.3
	5	33.7
DAB 16, DNA (3: 1 g g^{-1})	20	23.9
	15	40.7
	10	57.7
	5	56.5
DAB 16, DNA (5: 1 g g^{-1})	20	18.3
	15	24.1
	10	88.4
	5	100.8
QDAB 16, DNA (1: 1 g g^{-1})	20	54.2
	15	40.0
	10	38.3
	5	33.7
QDAB 16, DNA (3: 1 g g^{-1})	20	76.4
	15	70.4
	10	45.3
	5	13.8
QDAB 16, DNA (5: 1 g g^{-1})	20	42.3
	15	50.8
	10	48.5
	5	45.5
DSAM 16, DNA (5: 1 g g^{-1})	20	28.6
	15	35.2
	10	25.1
	5	26.7
DSAM 16, DNA (10: 1 g g^{-1})	20	26.1
	15	22.2
	10	22.3
	5	24.3
DSAM 16, DNA (15: 1 g g^{-1})	20	21.1
	15	21.6
	10	21.4
	5	21.9
DAB 32, DNA (3: 1 g g^{-1})	20	12.2
	15	16.3
	10	17.6
	5	16.6
QDAB 32, DNA (3: 1 g g^{-1})	20	13.8
	15	23.2
	10	33.1
	5	21.3

*% Expression relative to expression obtained for optimum DOTAP (DOTAP, β-galactosidase reporter DNA ratio = 5: 1) formulation on dosing cells with 20μg DNA. % protein expression relative to DOTAP formulation obtained with 20μg naked DNA alone = 25.15%

Transfection with DAB 8 is also slightly superior to that obtained with DOTAP (Table 2). This indicates a superior gene transfer activity for the DAB 8 and DAB 16 dendrimers. DAB 4 is

currently being tested in our laboratories. For the DAB 16 formulation, transfection appears to be optimum when using DAB 16 complexes with DNA at a nitrogen to phosphate ratio of 30: 1, forming complexes of 150nm in size. Transfection with DAB 8 is also optimum at a nitrogen to phosphate ratio of 30: 1. Transfection with polyamidoamine dendrimers is optimum when low-density soluble material is formed at a nitrogen to phosphate ratio of 20:1[19].

DAB 8 the most transfection efficient molecule studied to date in the polypropylenimine dendrimer class is also the least toxic, exhibiting an IC50 almost 6X higher than DOTAP (Table 3).

Table 3: In vitro cytotoxicity against the A431 cell line

Formulation	**IC50 (μg ml^{-1})**
DAB 8	352.4
DAB 8, DNA (5: 1 g g^{-1})	669.4
DAB 16	38.9
DAB 16, DNA (5: 1g g^{-1})	36.0
QDAB 16	44.6
QDAB 16, DNA (3: 1g g^{-1})	129
DAB 32	5.7
DAB 32, DNA (3: 1g g^{-1})	5.8
QDAB 32	11.2
QDAB 32 (3: 1g g^{-1})	33
DOTAP	62

The data in Table 3 indicate that the complex formed by DNA and the quaternised molecule (QDAB 32 and QDAB 16) is less toxic than that formed by DNA and the unquaternised molecule. The quartenised molecule QDAB 16 is as active as DOTAP at the 20μg DNA dose and QDAB 32 shows slight activity as a gene transfer agent at the 10μg dose level (Table 2) while DAB 32 is inactive. It is envisaged that QDAB 8 will produce a gene transfer formulation with good biocompatibility and also with no loss of activity when compared to the unquaternised parent polymer.

DAB 16 and DSAM are efficient deliverers of DNA to tissues in vivo comparing favourably to the commercial product Exgen 500 and with the added ability of being able to target the liver (DAB 16) and spleen (DSAM 16) more effectively than Exgen 500 (Table 4, Figure 5).

Table 4: In vivo luciferase expression obtained in the mouse model

Formulation	% Luciferase expression relative to Exgen 500*				
	Lung	Liver	Kidney	Heart	Spleen
DAB 16	48.9	762.6	28.6	73.2	100
DSAM 16	25.8	264.3	11.3	58.2	599.2
DNA Alone	9.2	43.6	10.6	33.0	97.0

* % luciferase expression relative to that obtained with linear PEI (Mw = 22kD, Exgen 500®) on intravenous injection of 50μg DNA DAB 16 (DAB 16, DNA weight ratio = 5: 1) and DSAM 16 (DSAM 16, DNA weight ratio = 5: 1) formulations. Exgen 500 formulation consists of linear PEI, DNA weight ratio = 6: 1.

Conclusions

In summary the lower generation polypropylenimine dendrimers (DAB 8 and DAB 16) show improved biocompatibility when compared to DOTAP and transfection activity which is at some dose levels superior to that obtained with DOTAP. Additionally DAB 16 may be used to target the liver, and DSAM 16 used to target the spleen in vivo.

EXAMPLE 2

MATERIALS

All polypropylenimine dendrimers, glucose, were obtained from Sigma – Aldrich, UK. Phenyl methyl sulphonyl fluoride (PMSF) , protease Inhibitor cocktail and phosphate buffered saline tablets, isopropanol and maltose were all supplied by Sigma Aldrich, UK. 9H-(1,3-dichloro-9,9- dimethylacridin-2-one-7-yl) -D-galactopyranoside (DDAO) was purchased from Molecular Probes . Exgen 500 (linear polyethylenimine, Mw = 22kD) was obtained from Euromedex, France. Passive lysis buffer was supplied by Promega, UK. pCMV-beta gal DNA was obtained from Life Sciences/ Invitrogen and propagated in E.Coli as previously described[9].

METHODS

Groups of healthy female Balb-C mice (n = 3) were injected intravenously with either DAB8 - DNA, quaternary ammonium DAB8 (Q8) - DNA, DAB16 – DNA or DAB32 - DNA or

Exgen 500 (linear polyethylenimine) - DNA. Formulations of the Dendrimer or Exgen 500, DNA Complexes (200µl) dispersed in glucose 5% w/v containing 100µg DNA were injected into each mouse and the dendrimer, DNA weight ratios were as follows: DAB8, Q8 and DAB 16 were all administered at a dendrimer, DNA weight ratio of 5: 1. DAB32 was administered at a dendrimer, DNA weight ratio of 3: 1. Exgen 500 was administered in accordance with the manufacturers instructions.

Mice were killed after 24h and their lungs and livers removed and frozen in liquid nitrogen until an assay for β-galactosidase could be performed. For the assay, 1g of organ was made up to 2mL with a protease lysis buffer. The protease lysis buffer consisted of a) Protease lysis buffer 5X (1mL), b) Phenyl methyl sulphonyl fluoride (PMSF) (50mM in methanol, 200µL), c) Protease Inhibitor cocktail (100µl) and water (3.7mL).

1 X 10^6 A431 cells dispersed in 0.1 ml phosphate buffered saline (pH = 7.4, PBS) were implanted subcutaneously in each flank of CD-1 female nude mice. 4 days after the injection, the tumours were palpable (around 2 mm). 8 days after the injection, the size of the tumours increased (around 5 mm) and the blood vessels were more visible. The animals were dosed 11 days after tumour implantation. Mice (n=4) were injected intravenously with 50 □g DNA as naked DNA, DAB16 - DNA, Exgen500 - DNA all dispersed in 200 µL 5%w/v dextrose. Control animals were injected with 5%w/v dextrose. Animals were killed 24h later and tumours were excised and immediately frozen in liquid nitrogen. For the assay each tumour was added to 0.3mL of the Protease lysis buffer. Organs contained in the buffer were homogenised and 100 µl of the homogenised organ dispersion was added to 300 µl of the assay reagent. The assay reagent consisted of: a) DDAO 5 mg mL^{-1} in DMSO (15µL), b) PMSF 50 mM in methanol (20µL), c) maltose 20 %w/v in PBS (100 µl), d) Protease inhibitor cocktail (15 µL), e) PBS (150 µl). The samples were incubated for the appropriate time (45-90 min.) at 37ºC. 200 µl of this mixture was warmed at 95 °C for 2 min, in order to stop the β-galactosidase reaction and to denaturate the proteins which could interfere with the assay. 800 µl isopropanol was then added to the dispersion. The mixture obtained was vortexed to homogeneity and shaken for 20 min in the dark. The dispersion was then centrifuged for 4 min, at 13000 rpm. 500 µl of the supernatant was then added to 500 µl distilled water and the fluorescence read on a Beckman

LS-50B fluorimeter (λ_{Exc}: 630 nm, λ_{Em} : 658 nm, slit: 2.5 nm). The amount of enzyme was then quantified using a β-galactosidase standard.

RESULTS AND DISCUSSION

DAB16, Q8 and DAB 32 all resulted in liver targeting when compared to the commercial formulation Exgen500 (Figure 6). DAB 16 resulted in more gene expression in the tumours when compared to Exgen500 (Figure 7).

This data provides further support that polypropylenimine dendrimers target the liver and produce higher gene expression in tumour tissue when compared to commercial formulations where, for example, non-viral gene delivery systems target the lung[8,53]. Targeting the liver is likely to prove useful in the treatment of liver enzyme deficiencies and liver tumours. Figure 7 illustrates that high expression in tumours may be obtained with the polypropylenimine dendrimers.

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CLAIMS

1. A composition for the delivery of a bioactive molecule to a target location in the body of a recipient, said composition comprising a cationic polypropylenimine dendrimer comprising a diaminobutane core with 1, 2, or 3 generations of propylenimine molecules attached, and/or derivatives thereof, admixed with the bioactive molecule.

2. A composition according to claim 1, wherein the cationic dendrimer is derivatised using groups selected from the group consisting of hydrophobic, hydrophilic and amphiphilic.

3. A composition according to claim 1, wherein the cationic dendrimer are derivatised by binding two dendrimer molecules to either end of a hydrocarbon chain.

4. A composition according to claim 3, wherein the length of the hydrocarbon chain is selected from the group consisting of 8, 12, 14, 16 and 18 carbons.

5. A composition according to claim 3 or claim 4, wherein the derivatised cationic dendrimer is a bolamphiphilic dendrimer.

6. A composition according to claim 5, wherein the number of derivatising groups is from one derivatising group per dendrimer molecule up to and including derivatising all available surface or terminal groups on the dendrimer molecule.

7. A composition according to claim 2, wherein the amphiphilic group comprises a hydrophilic and a hydrophobic segment.

8. A composition according to claim 2, wherein the hydrophilic group is derived from a phosphoglycerate molecule.

9. A composition according to claim 7, wherein the hydrophobic segment is covalently bound to the hydrophilic segment via an ester linkage.

10. A composition according to claim 7 or 9, wherein the hydrophobic segment or group is selected from the group consisting of alkyl, alkenyl and alkynyl groups of from 8 to 24 carbons in length.

11. A composition according to claim 7, wherein the amphiphilic group is attached to the dendrimer by a linker molecule selected from the group consisting of polyethylene glycol (PEG) and a sugar molecule.

12. A composition according to claim 11, wherein the length of the PEG linker molecule is from 1 to 120 ethylene glycol units.

13. A composition according to claim 11, wherein the linker molecule is a phosphoglyceride.

14. A composition according to claim 7, wherein the number of amphiphilic groups per dendrimer molecule is from 1 derivatising group per dendrimer molecule to derivatising all available groups of the dedrimer.

15. A composition according to claim 1, wherein the target locations in the body of the recipient is selected from the group consisting of the liver, spleen, lung, kidney and heart.

16. A composition according to claim 15, wherein the composition is for the delivery of a bioactive molecule to the liver of a recipient, and wherein said composition comprises the polypropylenimine dendrimer DAB16 admixed with said bioactive molecule.

17. A composition according to claim 15, wherein the composition is for the delivery of a bioactive molecule to the spleen of a recipient, and wherein said composition comprises the polypropylenimine dendrimer DSAM16 admixed with said bioactive molecule.

18. A composition according to any one of claim 1 or 15-17, wherein the recipient is a human.

19. A composition according to claim 1, wherein the bioactive molecules are selected from the group consisting of polynucleotides, peptides, polypeptides and pharmaceutically active agents.

20. A composition according to claim 1 wherein said composition is used to transfect *in vitro* mammalian cells with the bioactive molecule.

21. A pharmaceutical formulation comprising a composition as described in any one of claims 1 to 19, and a pharmaceutically acceptable carrier.

22. Use of the composition according to any one of claims 1 to 19, for the delivery of said bioactive molecule to a target location in the body of a recipient.

23. Use of a composition according to claim 22, wherein the target location is selected from the group consisting of the liver, spleen, lung, kidney and heart.

24. Use of a composition according to any one of claims 1 to 21 in the manufacture of a medicament for delivery of said bioactive molecule to a target location in the body of a recipient.

25. Use according to claim 24, wherein said target location is selected from the group consisting of liver, spleen, lung, kidney and heart.

26. A method of preparing the composition of any one of claims 1 to 19, said method comprising admixing a polypropropylenimine dendrimer comprising a diaminobutane core with 1, 2 or 3 generations of propylenimine molecules attached, and/or derivatives thereof, and at least one bioactive molecule.

FIGURE 1

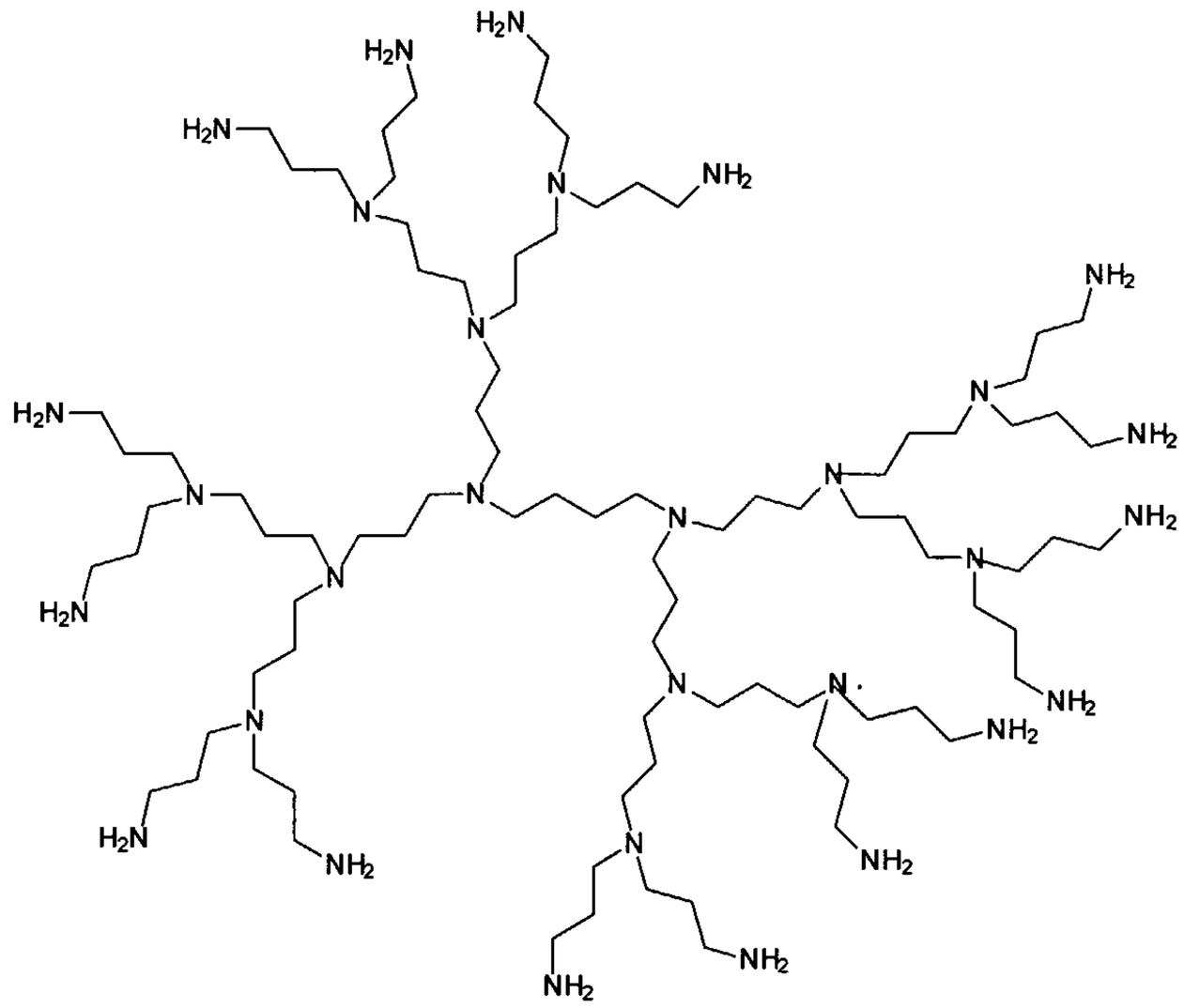


FIGURE 2

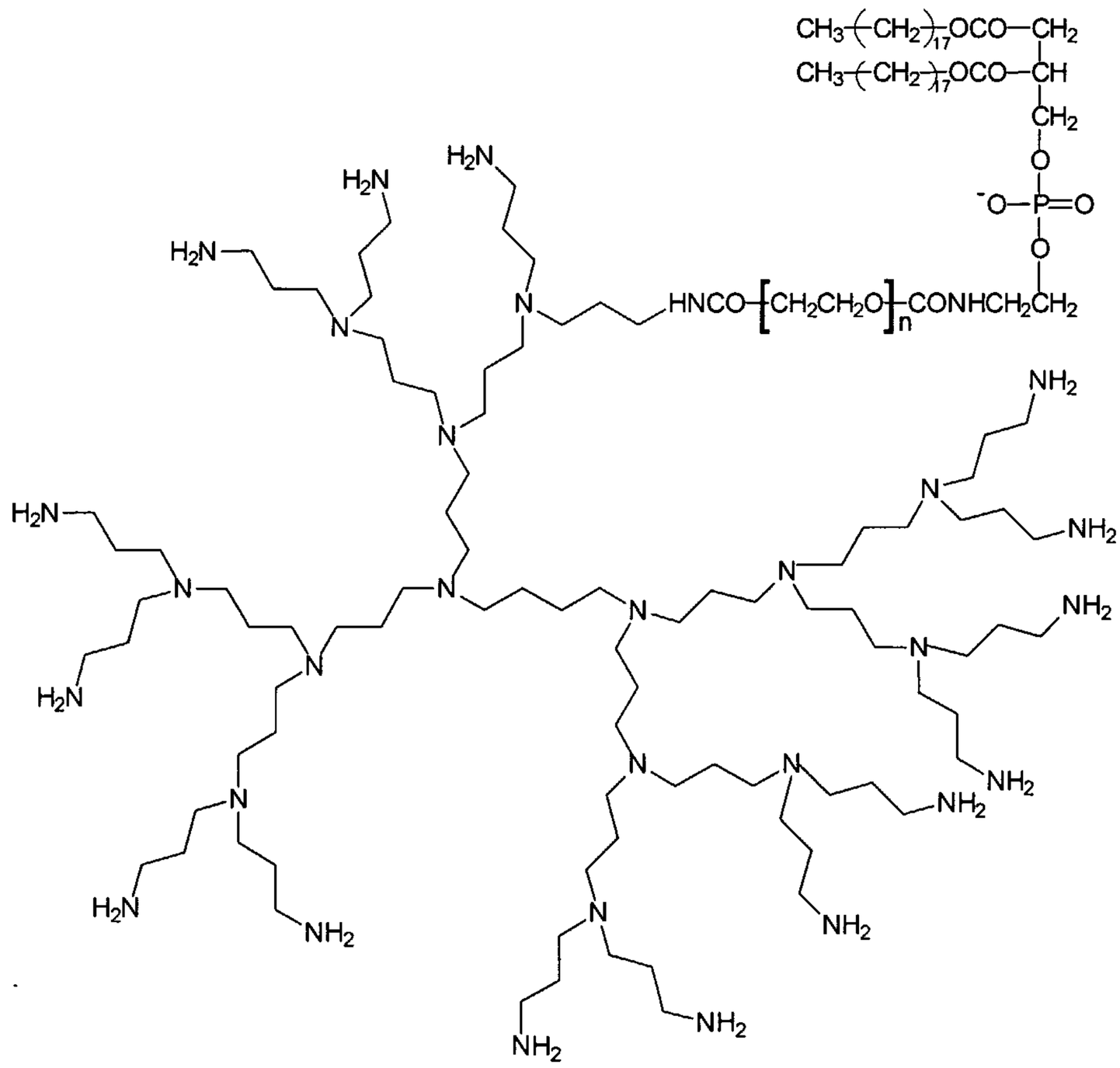


FIGURE 3

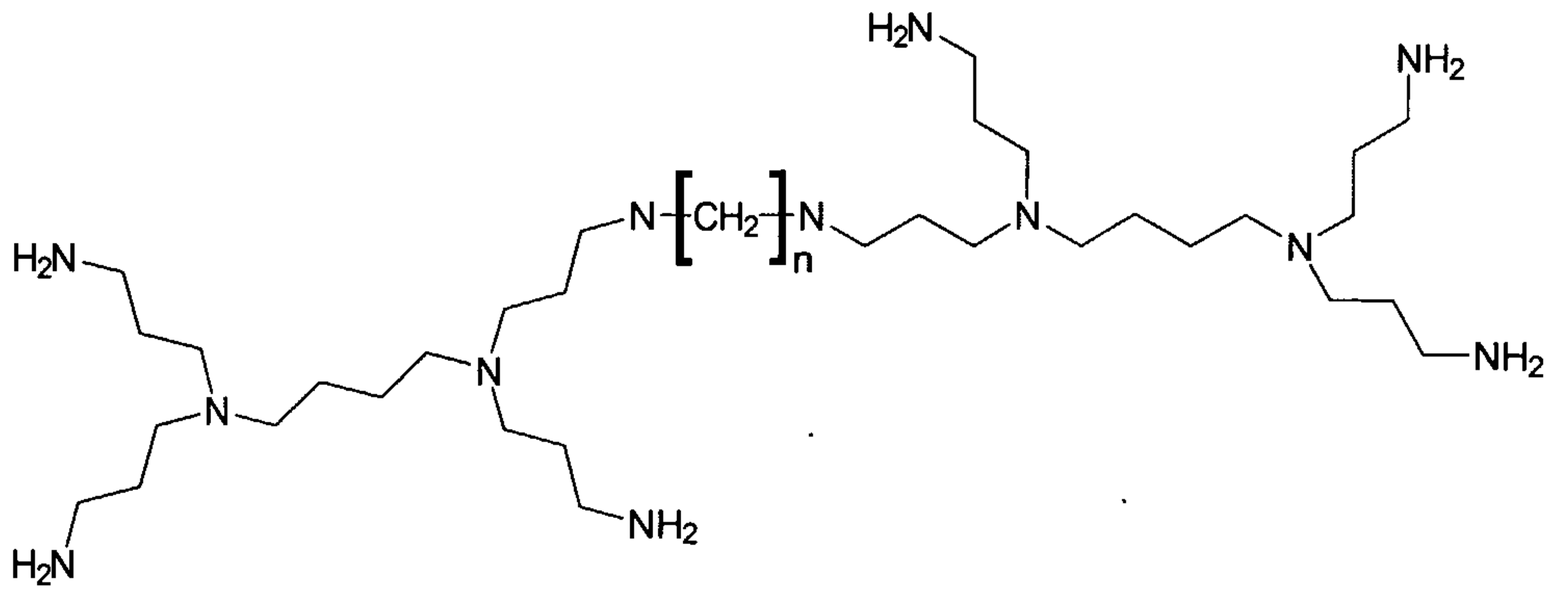


FIGURE 4

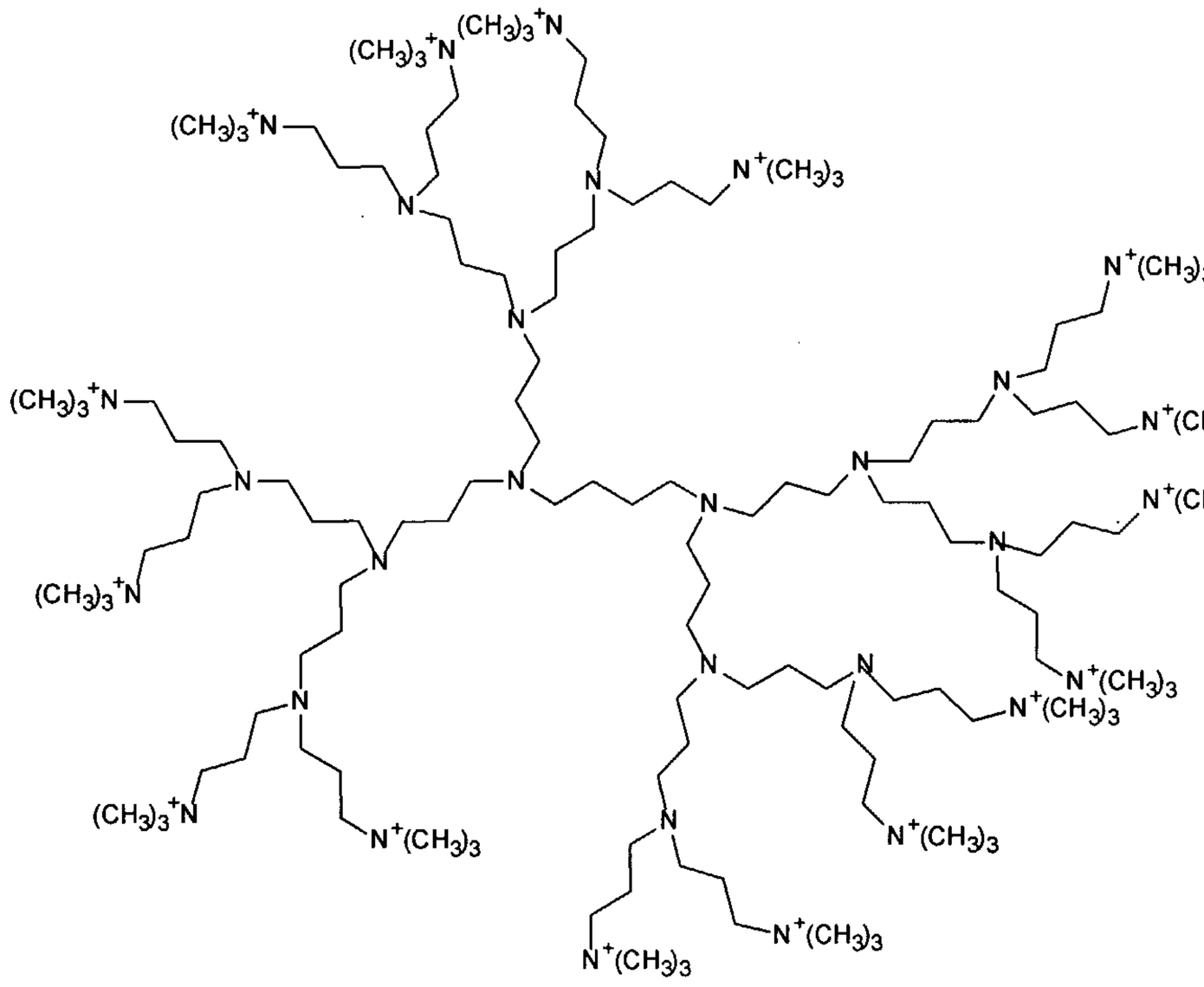


FIGURE 5

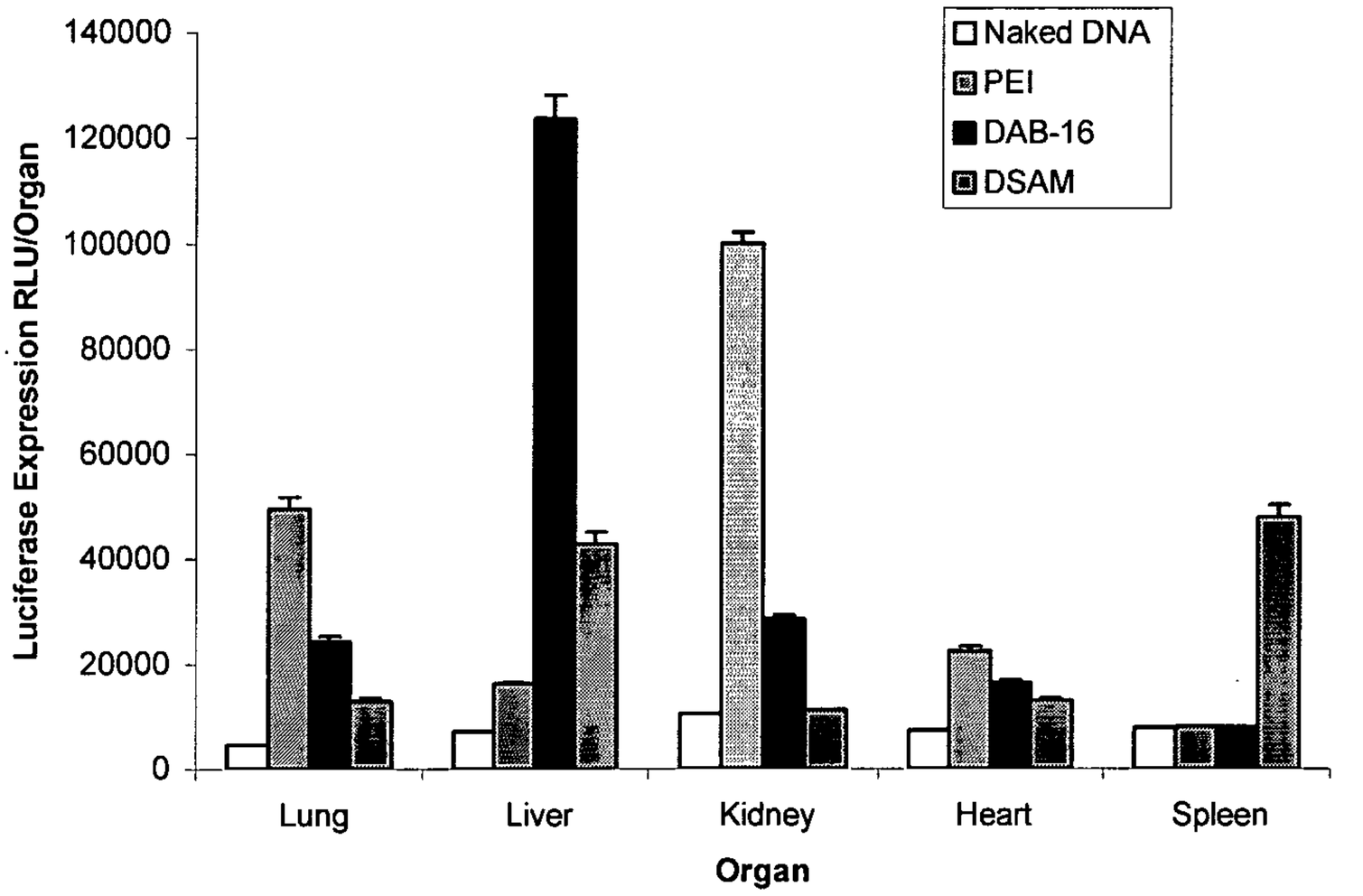


FIGURE 6

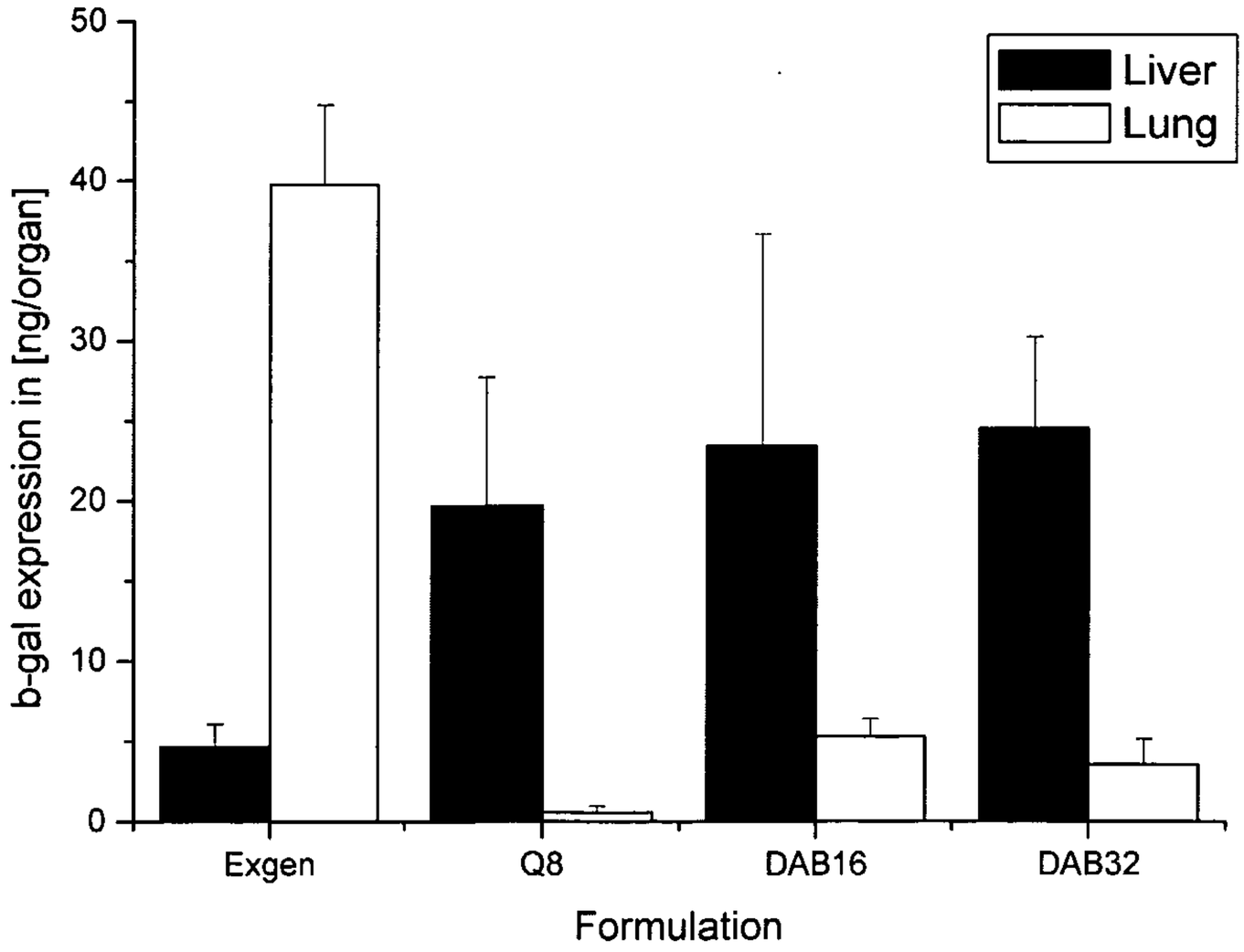
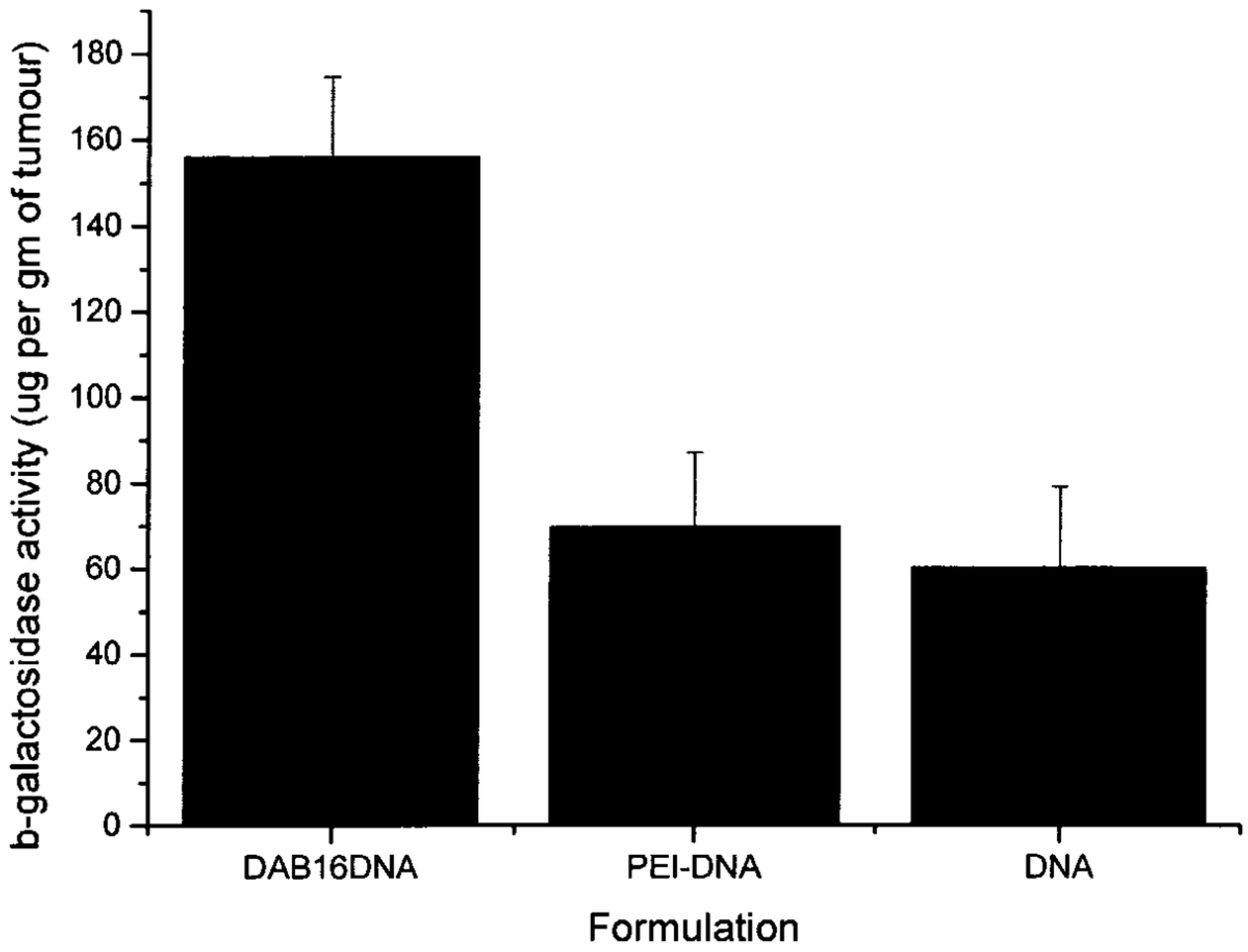


FIGURE 7


b-galactosidase activity (ug per gm of tumour)
0
20
40
60
80
100
120
140
160
180
DAB16DNA
PEI-DNA
DNA
Formulation