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AMINO- AND GUANIDINOGLYCOSIDE-BASED
VECTORS
FOR GENE AND DRUG DELIVERY

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“Every time you decide, there is a loss, no matter how you decide. It is always a question of what you cannot afford to lose.”

Francisco X. Stork

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List of Abbreviations

µg	microgram
A	Adenine
AU	Arbitrary units
BD	Biodistribution
Boc	<i>tert</i> -butoxycarbonyl
Boc ₂ O	Di- <i>tert</i> -butyl dicarbonate
bPEI	branched Polyethylenimine
BSA	Bovine serum albumin
C	Cytosine
CNS	Central Nervous System
d	doublet
Da	Dalton
DCM	dichloromethane
DDS	Drug Delivery Systems
DIPEA	<i>N,N</i> -Diisopropylethylamine
DMAP	4-Dimethylaminopyridine
DMEM	Dulbecco's Modified Eagle Medium
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DXR	Doxorubicin
EDA	ethylenediamine
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
G	Guanine

G0 Generation 0
G2 Generation 2
G4 Generation 4
G7 Generation 7
GAG Glycosaminoglycan
GG Guanidinoglycoside
GNeo GuanidinoNeomycin
h hours
HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC High Performance Liquid Chromatography
HSPGs Heparan Sulfate Proteoglycans
K Kelvin
m multiplet
MALDI Matrix-Assisted Laser Desorption/Ionization
MC Multi-Component
MeOH Methanol
MFI Mean Fluorescent Intensity
MHB Mueller Hinton II Broth (Cation-Adjusted)
MIC Minimum Inhibitory Concentration
MRSA Methicillin-Resistant Staphylococcus Aureus
MRSE Methicillin-Resistant Staphylococcus Epidermidis
MS Mass Spectra
MsCl Methanesulfonyl Chloride
NB Nutrient Broth
NCS Thiocyanate
Neo Neomycin
nm nanometer
NMR Nuclear Magnetic Resonance
OD Optical Density
PAMAM Polyamidoamine

PBS	Phosphate-buffered saline
pDNA	plasmid DNA
PEG	Polyethylene glycol
PEI	Polyethylenimine
PK	Pharmacokinetics
PLL	poly-L-lysine
ppm	parts per million
RES	Reticuloendothelial System
RNA	Ribonucleic acid
RRE	Rev-Response-element
rt	room temperature
S.	Streptomyces
s	singlet
siRNA	Small interfering RNA
ST-PECy-5	streptavidin-phycoerythrin-cyanine 5
t	triplet
TBTU	2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
Tf	trifluoremethylsulfonyl
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLC	Thin Layer Chromatography
TMP	2,4,6-trimethylpyridine
TMS	Tetramethylsilane
TsCl	4-Toluenesulfonyl Chloride
U	Uracil

Abstract

The overall goal of the project of this thesis was the use of aminoglycosides for 1) the development of new multifunctional gene delivery vectors, 2) the development of new multivalent carriers for drug delivery and 3) the synthesis of a collection of new antibiotics. In recent years new non-viral vectors for gene and drug delivery have become more and more important in order to overcome the use of viral vectors, due to their potential dangerous drawbacks, and facilitate drug entry into cells. In this experimental work, different kind of non-viral vectors for gene delivery studies were synthesized, using dendrimeric, lipidic and hybrid lipidic-dendrimeric cationic systems grafted with aminoglycosides. Aminoglycosides are a large family of well-studied potent antibiotics. Therefore, new potential dendrimer- and lipidic-aminoglycoside conjugates were synthesized, fully characterized and tested for their ability as transfectants and their cytotoxicity. PAMAMG4-aminoglycoside conjugates were extensively studied and were identified as one of the best multifunctional non-viral vectors having optimum transfection efficiency, low cytotoxicity and good antibacterial activity. Cationic lipid vectors as calix[4]arene-aminoglycoside conjugates and hybrid lipo-dendrimer(n)-neomycin conjugates were synthesized, characterized, and investigated as potential multifunctional carriers. Preliminary results evidenced a good ability as transfectants and a very low cytotoxicity, making them really applicable in gene delivery field. Guanidinoglycosides, derivatives of aminoglycosides obtained by total substitution of amino groups with guanidinium groups, have showed their great ability to promote the cellular uptake of high molecular cargos (i.e. therapeutics molecule) at nanomolar concentration in a heparan sulphate dependent way. We have thus used these guanidinoglycosides to graft PAMAM dendrimers obtaining new multifunctional conjugates with future applications in drug delivery field. Studies on these conjugates have shown their high cellular uptake in heparan sulphate dependent way at nM concentrations, with different mechanism of internalization. Finally, the chemistry of aminoglycosides was investigated in an innovative multicomponent domino process recently developed in our laboratories. MC reactions

were not commonly used until now with aminoglycosides due to the large number of functional groups on their structure. Nevertheless, this new multicomponent domino process was used to synthesize sugar-neomycin conjugates in good yields, as novel antibiotics. MICs studied have disclosed their good antibacterial activity against *MRSE* and *E.Coli* bacteria.

Chapter 1: Introduction

In the last two decades, gene therapy has achieved great attention as a potential method for treating genetic diseases (such as cystic fibrosis, hemophilia, neurodegenerative disorders, severe combined immunodeficiency and cancer) generally caused by deletion or mutation of a single gene into the cell.^[1,2] A common definition of gene therapy is the process of introducing foreign genomic materials (transgene) into specific cells to generate a therapeutic effect by correcting an existing abnormality or by providing the cells with a new function.^[3] The restoring a specific gene function or turning off a special gene are the aims of gene therapy studies, besides a single administration of an adequate material to replace a defective or missing gene.^[4] Gene therapy is divided in two different major types: 1) germline gene therapy consists in a direct manipulation of germline cells with no targeting (nuclei, egg cells, embryonic stem cell and sperm cells) that carrying the correct genotype, often carried in some gametes, can correct the genetics abnormalities. Germline therapy may have a great potential but the manipulation of embryonic stem cell, alteration of egg cells ex vivo and transgenic delivery into sperm cells, is currently ethically forbidden;^[5-7] 2) somatic gene therapy consists in insertion of genes in a target cell of patient, without the genetic material inserted can pass to its progeny, to cure genetic diseases. Somatic gene therapy is viewed as more conservative and safe, because affect only the single patient and not its future generations. As well, human gene therapy has been limited to somatic gene therapy, that need vector to deliver the genetic material across the cell membrane.^[6] Plasmid DNA is used in gene therapy to introduce transgenes into cells that inherently lack the ability to produce the protein that the transgene is programmed to generate. Moreover, plasmids can be used to correct genetic errors that produce functionally incompetent copies of a given protein. On a molecular level, plasmid DNA molecules can be considered pro-drugs that upon cellular internalization employ the DNA transcription and translation apparatus in the cell to biosynthesize the therapeutic entity.^[8] Bearing in mind pDNA as pro-drug gene delivery and drug delivery are strictly related; in fact both require a vector able to deliver the active

1.1 Gene Delivery

compound (DNA or drug) into the target maximizing the effect and minimizing toxicity for the host and side-effects.^[9] In fact drug delivery studies an efficient way to transport a pharmaceutical compound in the body (to achieve its desired therapeutic effect), modifying drug release profile, absorption, distribution and elimination for the benefit of improving product efficacy and safety. Often drugs and others therapeutic molecules (such as genes, enzymes and proteins) possess poor solubility, poor ability to cross cell membrane, or inefficient route of administration, so, drugs and pro-drugs need carriers able to circumvent these drawbacks, in order to achieve their delivery in a more efficient and safe way.

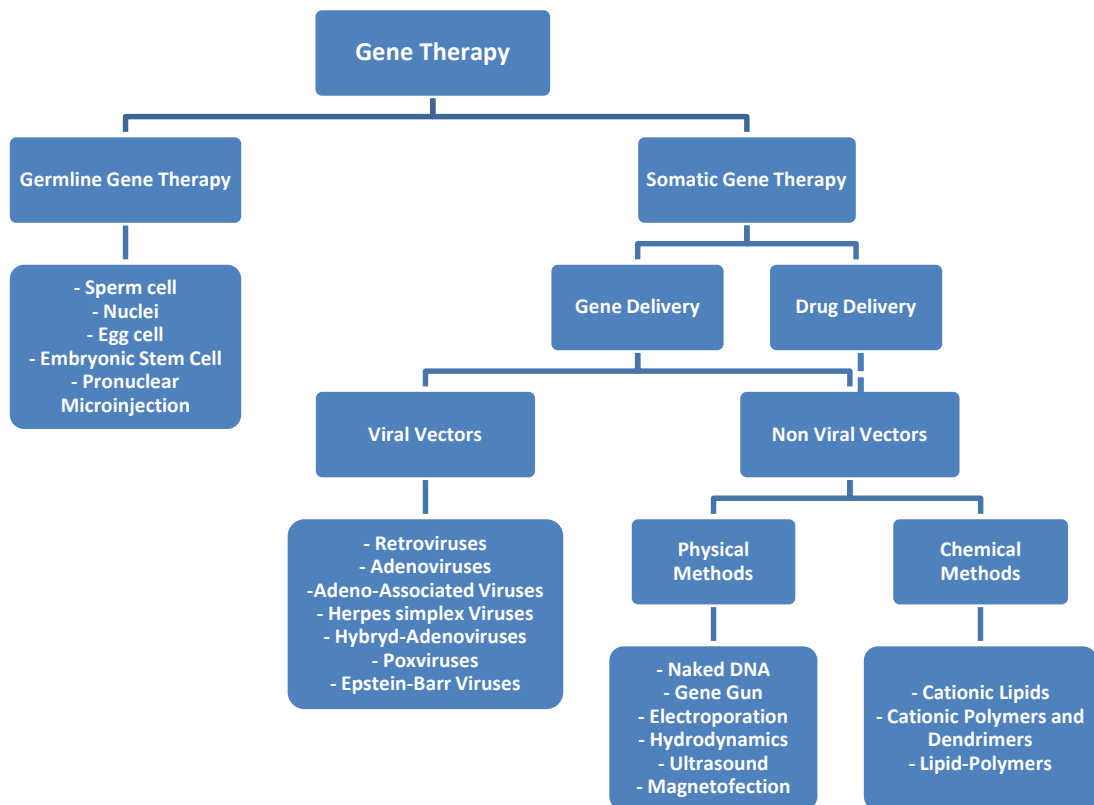


Figure 1. Overview of Gene Therapy.

1.1 Gene Delivery

Gene delivery is the process of introducing foreign DNA (or RNA) into host cells in order to compensate for abnormal genes or to produce a beneficial protein. Due to their anionic nature, DNA and RNA do not spontaneously enter the cell and for this reason, genes need vectors to be carried inside them. There are two major types of delivery vehicles: viral-based and non-viral based vectors. An ideal delivery carrier should: a) be stable, and easy to purify; b) bring the transgene into the target cell without interfere with the host (to deliver the transgene specifically to the tissue or organ of interest without widespread vector dissemination elsewhere); c) have high transfection efficiency; d) be non- toxic and mediate gene delivery and transgene expression avoiding harmful side effect on the human body.^[10] A general proposed mechanism for the delivery of a DNA-based therapeutic using a viral or non-viral vector is formed by different phases: 1) complexation and/or entrapment of DNA-based therapeutic with the vector, 2) interaction of DNA-based therapeutic-vector complex with cell membrane; 3) cellular internalization via receptor- or non-receptor-mediated endocytotic pathways followed by 4) endosomal escape with consequently cytoplasmic release of DNA-vector complex (or DNA alone) and dissociation of DNA from vector. At this point the DNA translocates to the nucleus, which is the site of action for transgenes in plasmids for gene therapy, siRNA generating plasmids, and antigene oligonucleotides (*figure 2*).^[8,11]

1.1 Gene Delivery

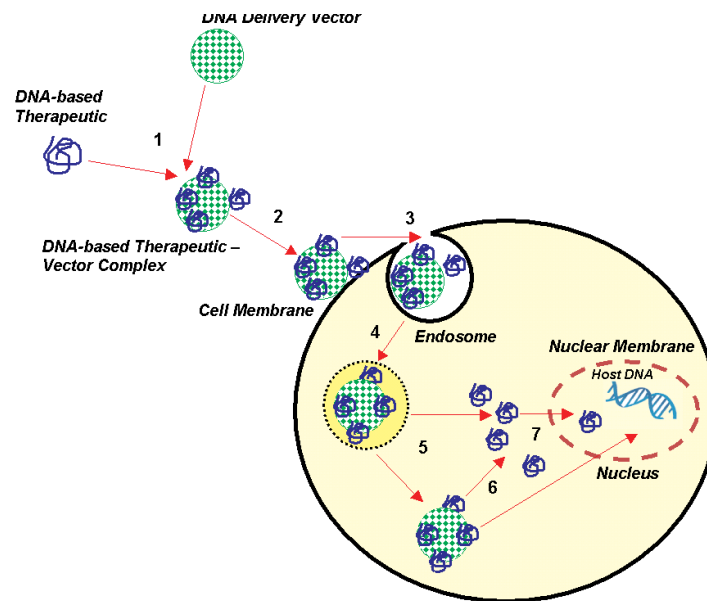


Figure 2. Schematic generalized representation of delivery of a DNA-based therapeutic using a viral or non-viral DNA delivery vector: (1) complexation and/or entrapment; (2) interaction with cell membrane; (3) cellular internalization mediated by endocytosis; (4) endosomal breakdown; (5) cytoplasmic release of DNA-based therapeutic-vector complex or DNA-based therapeutic; (6) dissociation of DNA from vector; (7) nuclear translocation.^[11]

1.1.1 Viral-based Delivery Systems

Viral gene delivery systems consist of viruses with a modified genome in order to be replication-deficient. These viruses are made unable to replicate by redesigning which can deliver the genes to the cells to provide expression and this modification should make them safer. The number of different viruses that are under development as gene delivery vectors is gradually increasing; at the moment the most used are adenoviruses,^[12] retroviruses,^[13] adeno-associated viruses,^[14] lentiviruses,^[15] hybrid adenoviruses,^[16] herpes simplex viruses,^[17] poxviruses^[18] and epstein-barr viruses.^[19] Each of these vectors is characterized by a set of different properties that make it suitable for some application respect to the others. Viral systems have advantages such as high transfection efficiency and expression of therapeutic genes.^[20] However, there are some limitations that restrict the use of these systems: immunogenicity, the “Achilles heel” of viral vectors because the immunological defense systems that are used to attack wild-type infections are activated

also against the vectors, toxicity, limited carrying capacity, mutagenesis, and lack of optimization in large-scale production.^[21]

1.1.2 Non-viral based Delivery Systems

Non-viral delivery systems were developed to bypass some of the problems associated with viral vectors and are emerging as favorable alternatives to viral vectors. Among the greatest advantages of non-viral gene vectors are: a) lack of immune response, b) ease of formulation and assembly, c) good cell/tissue targeting, d) large-scale production and e) unlimited carrying capacity. The only disadvantage of non-viral vectors in clinical use is the low transfection efficiency.^[22] Non-viral gene delivery systems are divided into two categories: physical and chemical methods. The physical methods such as gene gun, electroporation, particle bombardment, ultrasound utilization, and magnetofection, entail the use of physical force to increase the permeability of the cell membrane and allow the gene to enter the cell. The advantage of physical methods is that they are easy to use and reliable but, at the same time, the disadvantage is that they can provoke tissue damages during the applications. The chemical methods are more common than the physical ones, and consist in the use of carriers prepared from synthetic or natural compounds such as synthetic and natural polymers, liposomes, micelles, dendrimers, synthetic proteins, and cationic lipids. These vectors, in general positively charged, can easily complex the DNA or RNA to give nanometric complexes that are generally quite stable to enter into the cell and release the gene inside it. The major advantages of these chemical systems are: a) they are non-immunogenic; b) ensure a long-term expression without risk of insertional oncogenesis and c) generally have low toxicity.^[9,23] Currently, among the chemical delivery system, the most used are synthetic cationic polymers and/or dendrimers and cationic liposomes. A functional cationic non-viral vector should have three main characteristics: protection ability of the negatively-charged phosphate DNA skeleton to prevent repulsion by the anionic cell surface; ability to condense different suitable portion (in terms of size) of DNA taking into account the diverse mechanism of internalization (nanometer size for endocytosis or micrometer size for phagocytosis); and preservation of DNA from all

1.2 Cationic Polymers and Dendrimers

extracellular and intracellular degradations.^[24] Cationic liposomes, are as widely used in gene delivery as in drug delivery due to their unique physiognomies, such as capability to incorporate hydrophilic and hydrophobic drugs, low toxicity, no activation of immune system, and targeted delivery of bioactive compounds to the site of action.^[9,25] However, the gene delivery efficiency of liposomes is dependent on the size, structure, the charge ratio between transgenic DNA and cationic liposome, presence of helper lipid, and the structure and proportion of it and cell type. At now cationic liposomes are used for targeting gene delivery into lung, skeletal muscles, spleen, kidney, liver, testis, heart, and skin cells in *in vitro* experiments.^[26] Cationic polymers and dendrimers, being in part subject of my experimental work, will be discussed in the next section.

1.2 Cationic Polymers and Dendrimers

The most representatives cationic polymers and dendrimers are poly(L-lysine) (PLL), polyethylenimine (PEI) and polyamidoamine (PAMAMs). They are commonly used in gene delivery due to their capability to complex the negatively charged DNA. In fact being cationic polymers, increasing the molecular weight of the polymer, the net positive charge also increases, and it is therefore able to bind DNA tighter and form more stable complexes. There is a relationship between the length of the polymer, gene delivery efficiency, and toxicity as the length of the polymer increases, so does its efficiency and its toxicity.^[27]

1.2.1 Polyethylenimine (PEI)

Polyethylenimine firstly used by Behr in 1995^[28], is a polymeric carrier that has been derivatized to improve its physicochemical and biological properties as transporter of gene. PEI is one of the most positively charged dense polymers, synthesized in two different forms: linear (lPEI) or branched (bPEI). Synthesis of branched polyethylenimine (bPEI) proceeds via acid-catalyzed polymerization of aziridine,^[29] whereas the linear structure (lPEI) is synthesized via ring opening polymerization of 2-ethyl-2-oxazoline followed by hydrolysis.^[30] The high transfection efficiency of bPEI can be attributed to the buffering

effect or the “proton sponge effect” of the polymer caused by the presence of amino groups in the molecule, that can be protonated with a charge density proportional to the biological environment.^[31] This buffering capacity allows PEI polyplexes to avoid lysosomal trafficking and degradation once inside the cell, with a rapid endosome escape.^[32] The higher is the molecular weight of bPEI, better is the transfection efficiency, but at the same time, higher is also the cytotoxicity due to induction of cell apoptosis.^[11,33] The optimal molecular weight with the best transfection efficiency for bPEI is 25kDa considered the gold standard for gene delivery.^[34,35] However the cytotoxicity remain the main disadvantage of bPEI that could be overcome by grafting PEI with different polymers, giving copolymers, such as polyethylene glycol or PEI glycosylation, to reduce the toxicity.

1.2.2 Polyamidoamine (PAMAM)

Polyamidoamine PAMAM dendrimers, introduced by Tomalia in 1985^[36], are a novel class of globular, hyper-branched polymers with interior cavities and abundant terminal amino groups on the surface, which can form stable complex with drugs, plasmid DNA oligonucleotides and antibodies. Thanks to its positive charge, PAMAM can also solubilize different families of hydrophobic drugs favoring the across of cell membrane, making itself a suitable carrier also for drug delivery. The name “dendrimer” derived from two greek words *dendron* that means tree and *meros* that means part.^[37] Their common spherical structure basically consists in a central core, an interior layer (generations) that are repeating lateral branches attached to the core, and terminal functional groups (the exterior shell) with a total diameter in the range of 1-13 nm and molecular weight around 10000-100000 Da.^[38] The number of generation affect the PAMAM's shapes so, lower generations from 0 to 4 (G0-G4) have an elliptical profile, while the higher generations, from 5 to 10 (G5-G10), have a spherical structure. The first synthetic pathway for the synthesis of PAMAM is a divergent method that proceeds by a two steps repetitive sequence involving Michael addition of a nucleophilic core of ethylenediamine (EDA) to methyl acrylate, followed by an amidation of the resulting ester with ethylenediamine. The first step gives a half generation, then the amidation with EDA complete the full generation (*figures 3, and 4*).^[36] In this case the exterior shell is formed by primary amino groups that

1.2 Cationic Polymers and Dendrimers

give positive charge to the dendrimer at physiological pH. The primary amino groups play an essential role in binding the DNA (or RNA) and allow the dendriplex internalization by electrostatic interactions, while the buried tertiary amino groups perform the proton sponge effect in endosomes, causing the release of DNA in the cytoplasm.^[39-41] PAMAM dendrimers are one of the most popular polymers used as a non-viral gene carrier because of their unique characteristics such as uniform size distribution, relatively higher transfection efficiency, and lower cytotoxicity compared to other traditional cationic polymers.^[42,43] To reduce the cytotoxicity and enhance the transfection efficiency, the amino group of PAMAM dendrimers can be modified by PEGylation,^[44] acetylation,^[45] glycosylation^[46] and amino acid functionalization^[47], improving also their biocompatibility (figure 5).^[48] Biodegradability, non-toxicity, non-immunogenicity and multifunctionality of PAMAM dendrimers are the key factors for their application in gene delivery systems and, using therapeutic active molecule that can be either encapsulated or conjugated to dendrimer, also in drug delivery.

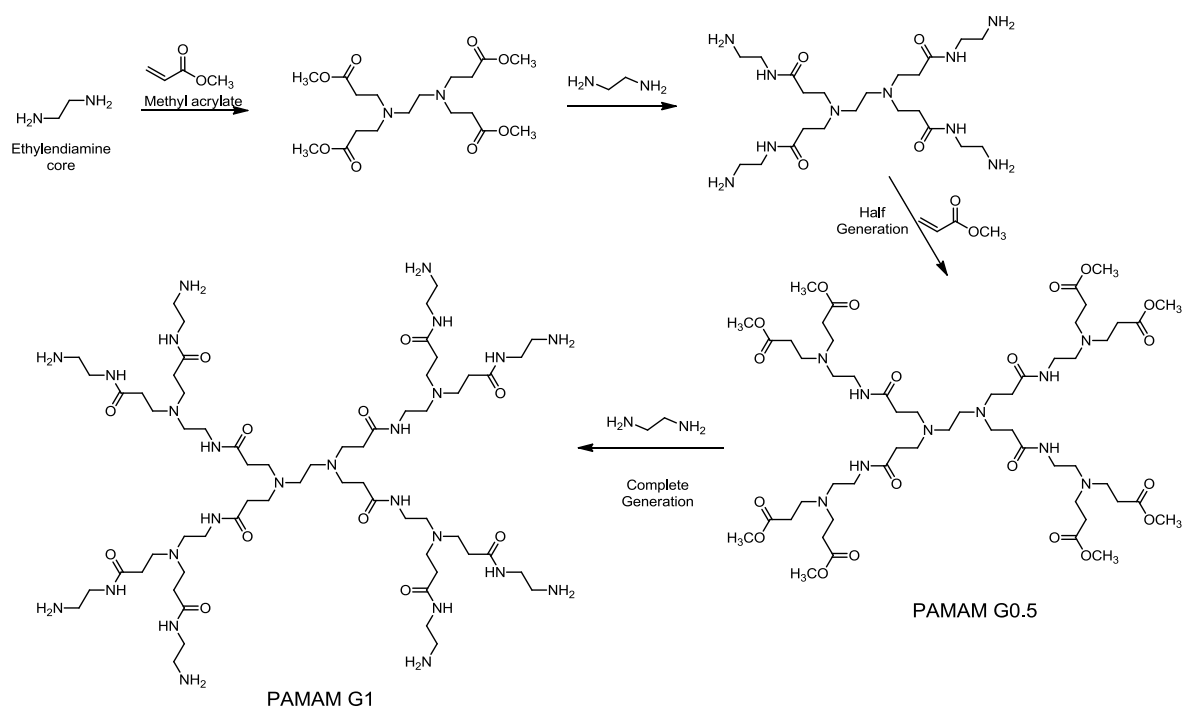


Figure 3. Scheme of convergent synthesis of PAMAM dendrimers with EDA and methyl acrylate.

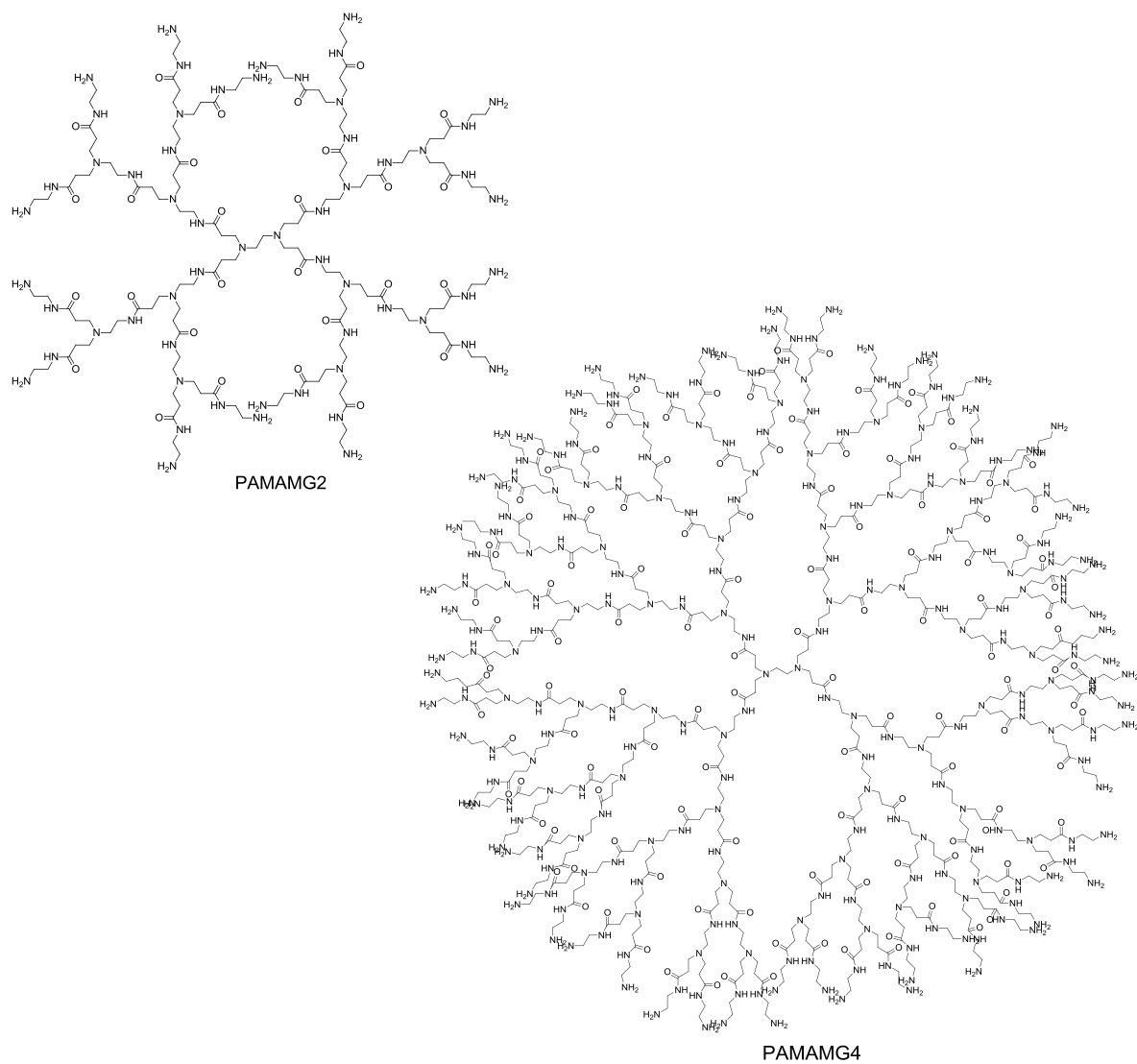


Figure 4. Structures of PAMAMG2 and PAMAMG4 dendrimers.

1.3 Drug Delivery

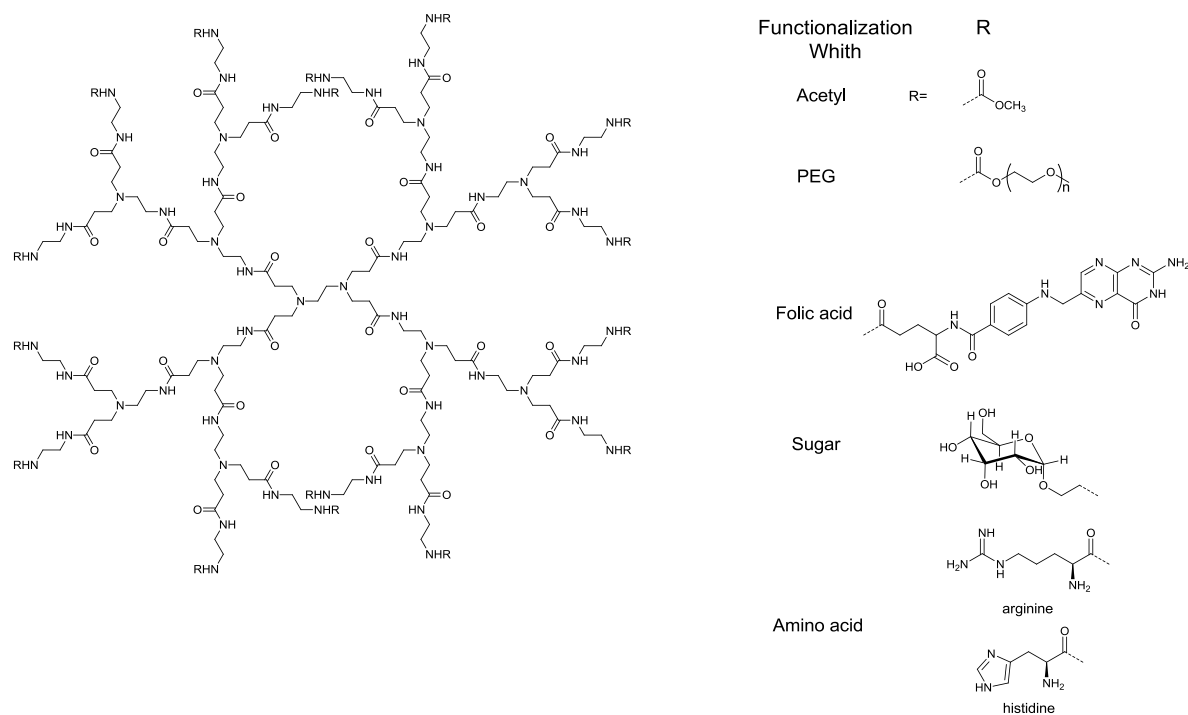


Figure 5. Examples of functionalized PAMAM.

1.3 Drug Delivery

The drug sector is entering a new era that will enable treatment of the underlying cause rather than the symptoms of a disease. Many human diseases result from mutations or deletions, in genes, which lead to disorders in metabolic pathways, ligand/receptor function, cell cycle regulation, cell skeleton or extra-cellular protein structure and function. With gene therapy, these diseases can be treated by drug delivery systems.^[20,49] Drug delivery is the method or process of administering a pharmaceutical compound to reach a therapeutic effect in humans or animals ^[50]. In traditional drug delivery such as oral ingestion or intravascular injection (parenteral administration), the medication is distributed throughout the body through the systemic blood circulation but, for most therapeutic agents, only a small portion of the drug reaches the target. Sometimes many

of the physical, chemical or pharmaceutical properties of the drugs such as poor solubility, insufficient in vitro stability (shelf-life), too low bioavailability, too short in vivo stability (half-life), strong side effect, need for targeted delivery, regulatory issues/hurdles and lack of large scale production, are some problem for their employment.^[51] A lot of these drawbacks of “free” drugs can be improved by the use of drug delivery systems (DDS) that are specific carriers designed to alter the pharmacokinetics (PK) and biodistribution (BD) of their associated drugs and/or to function as drug reservoirs (table 1).^[52] Some kinds of drug delivery systems are liposomes, micelles, dendrimers, biodegradable particles and DNA nanostructures. Their mechanism of delivery can be (a) diffusion of the pharmacophore from or through the system, (b) chemical or enzymatic leading to degradation or cleavage of drug from the system; (c) solvent activation through osmosis or swelling of the system; also a mix of all these mechanisms can occur.^[53a]

Problem	Implication	Effect of DDS
Poor solubility	A convenient pharmaceutical format is difficult to achieve, as hydrophobic drugs may precipitate in aqueous media. Toxicities are associated with the use of excipients such as Cremphor (the solubilizer for paclitaxel in Taxol).	DDS such as lipid micelles or liposomes provide both hydrophilic and hydrophobic environments, enhancing drug solubility.
Tissue damage on extravasation	Inadvertent extravasation of cytotoxic drugs leads to tissue damage, e.g., tissue necrosis with free doxorubicin.	Regulated drug release from the DDS can reduce or eliminate tissue damage on accidental extravasation.
Rapid breakdown of the drug in vivo	Loss of activity of the drug follows administration, e.g., loss of activity of camptothecins at physiological pH.	DDS protects the drug from premature degradation and functions as a sustained release system. Lower doses of drug are required.
Unfavorable pharmacokinetics	Drug is cleared too rapidly, by the kidney, for example, requiring high doses or continuous infusion.	DDS can substantially alter the PK of the drug and reduce clearance. Rapid renal clearance of small molecules is avoided.
Poor biodistribution	Drugs that have widespread distribution in the body can affect normal tissues, resulting in dose-limiting side effects, such as the cardiac toxicity of doxorubicin.	The particulate nature of DDS lowers the volume of distribution and helps to reduce side effects in sensitive, nontarget tissues.
Lack of selectivity for target tissues	Distribution of the drug to normal tissues leads to side effects that restrict the amount of drug that can be administered. Low concentrations of drugs in target tissues will result in suboptimal therapeutic effects.	DDS can increase drug concentrations in diseased tissues such as tumors by the EPR effect. Ligand-mediated targeting of the DDS can further improve drug specificity.

Table 1. Typical problems of drugs, implication and effects of drug delivery systems.^[52]

The improvement of drug delivery systems has diverse advantage: (a) constant maintenance of drug levels in a therapeutically desirable range; (b) reduction of harmful side effects due to the targeted delivery to a particular cell type or tissue; (c) potentially

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decreased amount of drug needed; (d) decreased number of dosages and possibly less invasive dosing, leading to improved patient compliance with the prescribed drug regimen; (e) facilitation of drug administration for pharmaceutical with short *in vivo* half-lives. However, beside of these advantages, there are also some concerns: (a) toxicity of the materials, or their degradation products, from the drug released, (b) discomfort caused by the system itself or the means of insertion; (c) expense of the system due to the drug encapsulation or industrial process.^[53-54]

In the last decade, researchers are moved towards targeted drug delivery (also called smart drug delivery) a new ideal method that releases the drug in a dosage form for a prolonged period of time to a targeted diseased tissue or cell. This helps to maintain the required plasma and tissue drug levels in the body, thereby preventing any damage to the healthy tissue via the drug. The aim of a targeted drug delivery system is to prolong, localize, target and have a protected drug interaction with the diseased area, reducing the frequency of the dosages, the side-effects and the fluctuation in circulating drug levels.^[55,56]

1.3.1 Drug Carriers

An ideal drug delivery carrier should be safe, non-toxic, biocompatible, non-immunogenic, biodegradable, efficient and should avoid recognition by the defense mechanisms of the host. As reported in a previous paragraph, there are different types of vehicles, such as polymeric micelles, liposomes, lipoprotein-based drug carriers, nanoparticle drug carriers and dendrimers. Each different DDS can be chosen for the appropriate drug on the basis of the properties of the drug itself (for example potency, stability, solubility, molecular weight and charge are all important to establish a kind of carrier). Polymer-based DDS have had a huge effect on drug therapies; the drug is captured inside a solid polymer such as silicone or rubber that can be injected or implanted in the patient and release the lipophilic drug for long time (an example are contraceptives).^[57] Recently have been developed micelles as carriers formed by PEG-block-P(Asp-Hyd-DXR) copolymers, which are prepared by chemically conjugating doxorubicin (DXR) to the side chain of PEG-b-poly(asparticacid) copolymers via an acid-labile hydrazone bond. pH-sensitive polymeric micelles significantly release active DXR under low pH conditions (~5.0)

corresponding to late endosomes and lysosomes, whereas they stably retain drugs under a physiological pH condition. The micelles were shown to preferentially accumulate in solid tumors with consequently suppression of tumor growth in mice and a very low toxicity compared with a free DXR.^[58]

Beaded delivery systems consist of multiple, small beads that are composed of inert substances (such as polystyrene). The active drug is overlaid on the beads and encased in a delivery capsule. The drug delivery from this system is acid sensitive, in that drug levels are dependent on gastric acidity for release. This process achieves long-acting drug levels associated with the convenience of once-a-day dosing with an improvement of efficacy and tolerability.^[50]

Liposomes, being small lipid vesicles, have been widely explored as drug carriers due to their higher drug-carrying capacity. The only problem to using liposomes in vivo is their immediate uptake and clearance by the RES system and their relatively low stability in vitro. To combat this, polyethylene glycol (PEG) can be added to the surface of the liposomes. Increasing the mole percent of PEG on the surface of the liposomes by 4-10% significantly increase circulation time in vivo from 200 to 1000 minutes. Thanks to their properties, liposomes are employed with different kind of drugs such as immune drugs (immunoliposome),^[59] anticancer drugs,^[60] anti-fungal,^[61] lung specific drugs^[62] and drug for treatment of CNS disease.^[63]

Dendrimers (pharg. 1.2) due to their structure can attach several drug molecules, targeting groups and solubilizing groups on their periphery in a well-defined manner. One of the most used dendrimers is polyamindoamine (PAMAM) that due to its properties is used for encapsulation of different type of drugs and also for covalent attachment of drugs.^[64,65] The drug loading can be tuned by varying the generation number of the dendrimer, and release of the drug can be controlled by incorporating degradable linkages between the drug and dendrimer.^[66]

These are only a few examples of different carriers developed during the past few years. Some seem to work very well but, the design of ideal carrier for drug delivery that

1.4 Aminoglycosides

can (a) simultaneously avoid the reticuloendothelial system, (b) target specific cells, (c) be taken up by those cell types in such a way as not destroy their DNA and (e) made to release the drug unharmed in order to reach the nucleus, remains one of biggest challenge for the next future.

1.4 Aminoglycosides

Aminoglycosides are highly potent, broad-spectrum antibiotics with desirable properties for the treatment of serious life-threatening disease. Most aminoglycosides are naturally produced by actinomycetes strains of either the genus *Streptomyces* or *Micromonospora*. Several valuable semi-synthetic compounds were developed. Their history starts in 1944 when Waksman, stimulated by the recent discovery of penicillin, discovered the first aminoglycoside streptomycin. In fact, during his research on different strain of bacteria, looking for antibacterial substances, Waksman isolated the aminoglycoside streptomycin from a strain of *Streptomyces griseus*.^[67] Streptomycin was the first aminoglycoside antibiotic used as an anti-tuberculosis agent against *Mycobacterium tuberculosis* and after penicillin the second compound of this kind employed in therapy. Five years later the aminoglycoside neomycin, discovered in Japan in 1948 and named fradiomycin, was isolated from *Streptomyces fradiae* by Waksman and Lechevelier, followed by paromomycin in 1956 and kanamycin from *Streptomyces kanamyceticus* in 1957 by Umezawa, establishing the aminoglycosides antibiotic era.^[68,69] In 1963 gentamicin was isolated from the actinomycete *Micromonospora purpurea* by Weinstein ^[70] and in 1967 tobramycin was produced from *Streptomyces tenebrarius*.^[71] During the 1970s, to circumvent bacterial resistance problems originated by the use of the corresponding natural antibiotics, semi-synthetic derivative were introduced such as amikacin (1972, a semisynthetic derivative of kanamycin) ^[72] dibekacin, sisomicin and netilmicin (1976, is a semisynthetic derivative of sisomicin which also comes from *Micromonospora* species).^[73] In order to distinguish the different species of origin, the aminoglycosides are labeled “-cins” as amikacin, netilmicin, dibekacin, isepamicin and arbekacin if are semisynthetic derivative of *Micromonospora*, whereas those originating

from bacteria *Streptomyces* as neomycin, paromomycin, streptomycin and tobramycin are labeled “-mycins”.^[74] In *figures 6* and *9* are showed structures of some aminoglycosides, and in *table 2* some clinically used aminoglycosides.

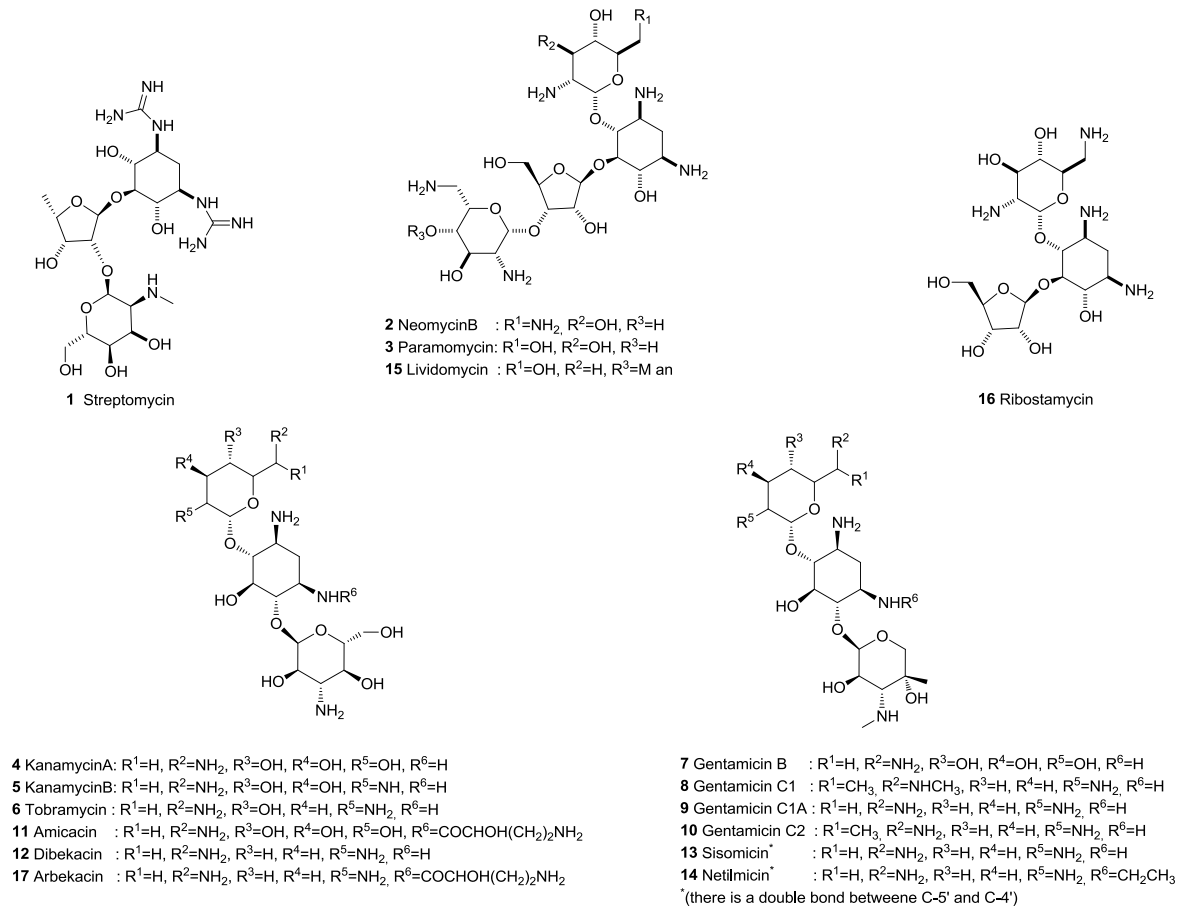


Figure 6. Structures of Aminoglycosides.

Drug	Source	Discoverer (date)	Notes
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1.4 Aminoglycosides

Streptomycin	<i>S. griseus</i>	Schatz <i>et al.</i> (1944)	<i>S. griseus</i> also produces other antibiotic substances such as cycloheximide.
Amikacin	Semisynthetic synthesis	Kawaguchi <i>et al.</i> (1972)	Resistant to most inactivating enzymes of most bacteria
Kanamycin	<i>S. kanamyceticus</i>	Umezawa <i>et al.</i> (1957)	Occurs as three closely related compounds. Kanamycin A is the least toxic and the main component of the commercial drug. An increasing number of resistant strains of bacteria.
Netilmicin	Semisynthetic synthetic	Weinstein <i>et al.</i> (1975).	Inactivated by many bacterial enzymes that acetylate aminoglycosides.
Neomycin	<i>S. fradiae</i>	Wakeman and Lechevalier (1949)	Little development of bacterial-resistant strains.
Paromycin	<i>S. rimosus forma paromomycinus</i>	Frohardt <i>et al.</i> (1959)	
Gentamicin	<i>Microspora purpurea</i>	Researchers at the Schering Corporation, Bloomfield, New Jersey (1963)	Occurs as three closely related compounds. Resistant strains of bacteria are increasing. They act by acetylation and adenylation of the drug. Strongly active against <i>P. aeruginosa</i> which is highly resistant to penicillins, cephalosporins, tetracyclines, quinolones and chloramphenicol.
Tobramycin	<i>S. tenebrarius</i>	Unidentified (1976)	Active against strains of <i>P. aeruginosa</i>

Table 2. Clinically used Aminoglycosides and their sources.

Since its discovery, neomycin has been specifically used for topical application in form of cream or lotions (in association with bacitracin and polymixin), ointments, ear and eye drops due to its poor oral bioavailability, cell penetration and instability, along with nephro- and ototoxicity that prevent its parenteral use. However neomycin is effective against a broad spectrum of gram-positive and gram-negative bacteria, mycobacteria and also acid fast bacteria. As a therapeutic agent neomycin is sold in sulphate form which consist of a mixture of three different aminoglycosides: neomycin B or framycetin that is the major component with the highest activity, neomycin C, an epimer of neomycin B, that is less active and neomycin A or neamine which is the product of hydrolytic degradation of B and C and has only the 10 % of activity respect to Neo B (figure 7).^[75]

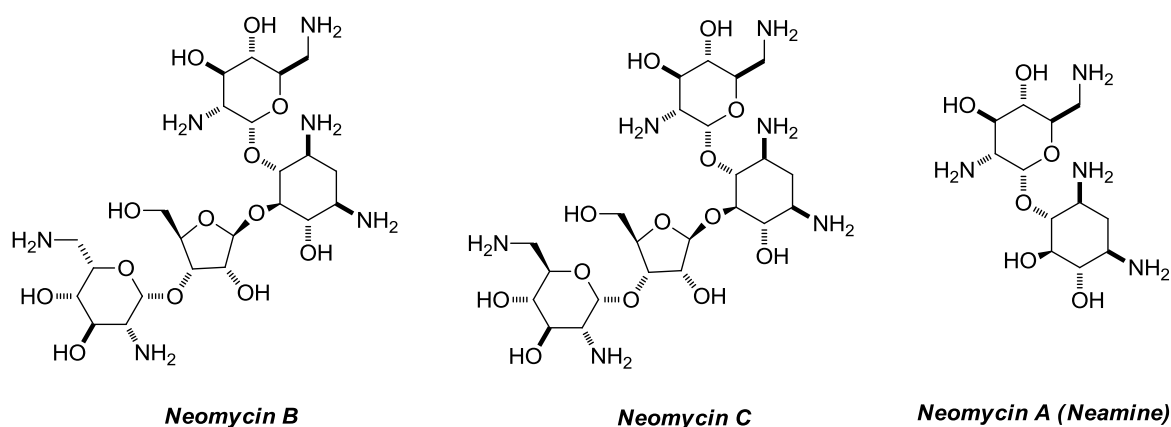


Figure 7. Structures of three different Neomycins.

Gentamicin, isolated from *Micromonospora*, is considered as a milestone in the history of aminoglycosides, being highly active against Gram-negative bacteria including *Pseudomonas aeruginosa* and well tolerated. Its clinical success stimulated the search for similar antibiotics like tobramycin, sisomicin, lividomycin, ribostamycin, and verdomycin but only tobramycin and sisomicin were used therapeutically to cure meningitis, pneumonia and sepsis. Streptomycin is active against gram positive and negative bacteria and employed in combination with tetracyclines in the treatment of tuberculosis and other diseases as bubonic plague, tularemia and brucellosis. Examples of therapeutic applications of some aminoglycosides antibiotics are showed in table 3.

Aminoglycosides	Therapeutic Applications
Streptomycin	Tuberculosis, tularemia, plague
Neomycin	Burns wounds, ulcers and dermatitis
Paramomycin	Amebic dysentery
Gentamycin, Amikacin	Menengitis, pneumonia, sepsis
Spectinomycin	Gonorrhea

Table 3. Therapeutic applications of some Aminoglycosides antibiotics.

1.4 Aminoglycosides

1.4.1 Chemical Structure

Aminoglycosides are hydrophilic molecules, consisting of a characteristic central aminocyclitol ring saturated with amine and hydroxyl group, linked to one or more amino sugars by pseudoglycosidic bond(s). For the majority of clinically useful aminoglycosides, the aminocyclitol is the 2-deoxystreptamine ring; the only exception is streptomycin that possesses a streptidine ring in backbone structure. The 2-deoxystreptamine ring can be monosubstituted in position 4, as is the case for neamine, or disubstituted in positions 4 and 5 such as neomycin and paromomycin, or in position 4 and 6 such as gentamycin, tobramycin, amikacin and netilmicin (*figures 3, 4*). The accepted nomenclature refers to ring I as the primed ring and corresponds to the amino sugar at position 4 of the deoxystreptamine ring. Ring II is unnumbered and corresponds to the central aminocyclitol, while ring III is referred to as the doubly primed ring and has the substituent in position 5 or 6 of the deoxystreptamine ring. Ring IV (triply primed numbering) corresponds to any additional ring attached to ring III (*figure 8*). However, a number of active aminoglycosides are structurally atypical according to the above description. For instance, streptomycin possesses a streptidine ring and spectinomycin consists of three fused rings where the aminocyclitol is spectinamide. [76]

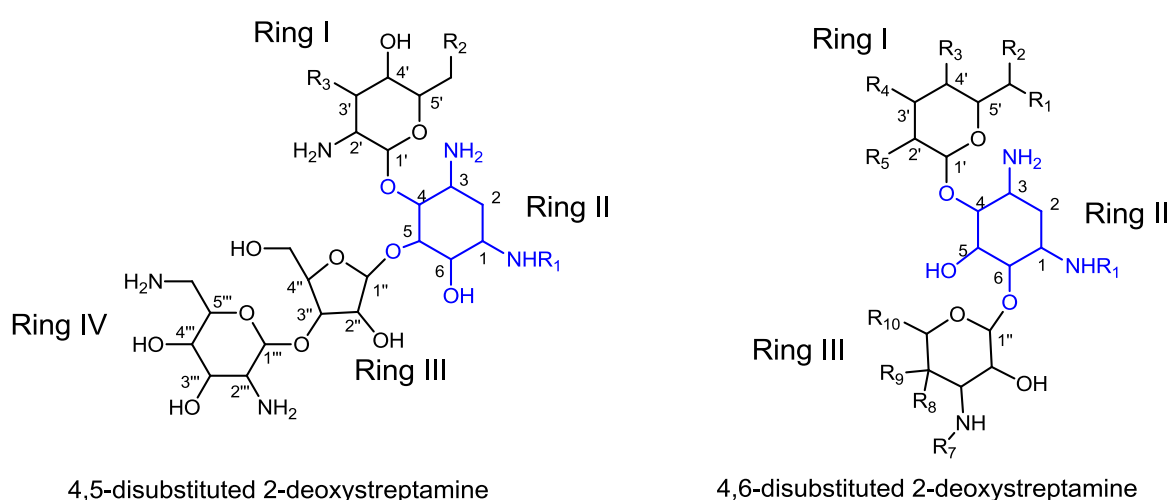


Figure 8. Schematic structure of Aminoglycosides 4,5-disubstituted and 4,6-disubstituted. The aminocyclitol ring is evidenced in blue.

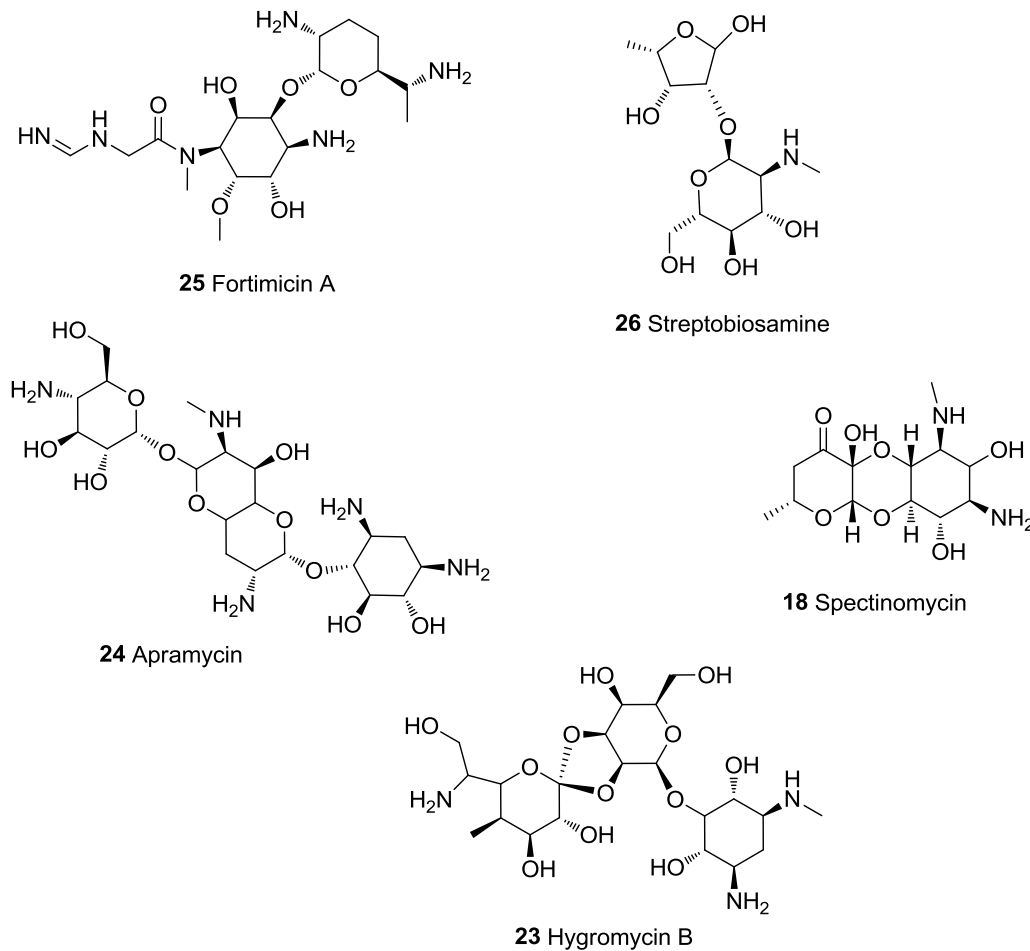


Figure 9. Structures of clinically useful atypical aminoglycosides.

The aminoglycoside structure is important in understanding their chemical properties. These are basic, strongly polar compounds with the amino groups positively charged at physiological pH. They are readily soluble in water (administered as inorganic salts), relatively insoluble in lipids, and their antimicrobial activity is privileged in basic environments; for example paromomycin is highly active against intestine parasites^[77]. The aminoglycosides polar nature suggests low absorption by the oral route and difficult penetration through blood brain barrier, bones and fatty and conjunctive tissues. However, the cationic nature of the aminoglycosides contributes to their antimicrobial activity. Thanks to their positive charge, they are able to bind negatively charged lipopolysaccharide of the bacterial cell wall and a variety of intracellular and cell membrane anionic molecules such as DNA, RNA, and phospholipids. Unfortunately, their positive charge at physiological

1.4 Aminoglycosides

pH also contributes to their toxicities, e.g., nephrotoxicity, ototoxicity, and neuromuscular blockade. The aminoglycoside pharmacophore groups responsible for translation errors (antibacterial activity) is the aminocyclitol (1,3-diamino-inositol) illustrated by streptidine, neamine, 2-deoxystreptamine and spectinamine (*figure 10*).^[76c-77,78]

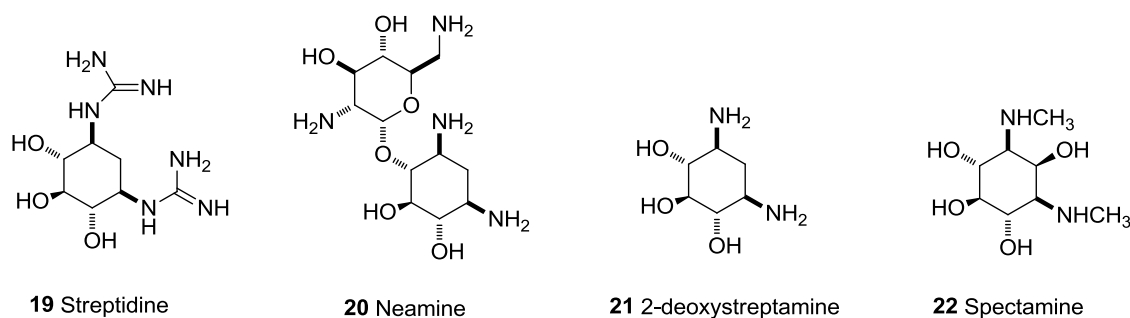


Figure 10. Structures of 1,3 diaminoinositol groups, the pharmacophores of aminoglycoside antibiotics.

1.4.2 Antibacterial activity

The chemical structure of aminoglycosides is crucial for their antibacterial activity. In fact, due to their cationic nature, they can bind to a variety of intracellular and membrane anionic molecules such as DNA, RNA and phospholipids. It is well known that the bactericidal activity is based on inhibition of protein synthesis, binding the bacterial 30S or 16S ribosomal A-site of RNA and repressing translation with ultimate bacteria's death.^[79] Nevertheless the exact mechanism of action is still being under studied. In the cytosol, aminoglycosides bind to the 30S subunit of ribosomes through an energy dependent process. The binding does not prevent the formation of the initiation complex of peptide synthesis, it perturbs the elongation of the nascent chain by impairing the proofreading process controlling translational accuracy. As a consequence, translation is faulty and selection of incorrect amino acids generates aberrant proteins. These non-natural proteins utilized by bacteria produce membranes with impaired semi-permeability that increases passive permeability to small molecules including recapture of the antibiotic, and consequent higher intracellular drug concentration leading to bacterial death.^[76b,77a,80-81] The mutual electrostatic attraction between the multi positive charged aminoglycosides and the negatively charged RNA backbone leads to base pairing disruption, although they

may also bind less extensively to human 18S rRNA, causing severe toxicity. The antibacterial activity may also involve different mechanisms, including the great incorporation of aminoglycosides performed by bacterial transporter proteins.^[82]

The 2-deoxystreptamine and the primed amino sugar are essential for causing the lack of fidelity in translation process.^[83] The 2-deoxystreptamine aminoglycoside antibiotics bind to helix 44 (h44) of 16S rRNA, which is part of the decoding site of the bacterial 30S ribosomal subunit. The RNA-nucleobases and phosphodiester backbone are involved in a broad range of salt bridge and donor and acceptor hydrogen-bonding interactions comprising amino and hydroxyl groups of aminoglycosides.^[84] An example of these interactions is described by the crystal structure of RNA-paromomycin shown in figure 11. The interactions formed by rings I and II are the main responsible for drug binding. Ring I intercalates into the internal loop formed by A1408, A1492, A1493 and the base pair C1409–G1491. Here ring I becomes properly positioned by stacking interaction with G1491 and the formation of hydrogen bonds with A1408 (*figure 12*).^[87] Despite variations in chemical composition, ring I always binds in the same orientation and forms a pseudo base-pair interaction with the Watson–Crick edge of adenine 1408. The oxygen of ring I accepts a hydrogen bond from the N6 of adenine, and the amino- or hydroxyl-group at position 6' donates a hydrogen bond to the N1 of adenine. Additional hydrogen bonds link the hydroxyl groups at positions 30 and 40 of ring I to the phosphate groups of the two bulged adenine bases 1492 and 1493, further stabilizing the position of ring I. The two amino groups of the deoxystreptamine ring II interact directly with the N-7 and phosphate groups of G1494 and O-4 of U1495. The 5''-hydroxyl group of the ribose ring has two main interactions, either by an intramolecular hydrogen bond with the 2'-amino group or to N-7 of G1491. Further interactions occur with the 2,6-dideoxy-2,6-diamino-glucose ring III with the backbone phosphates, comprising nucleobases OPC1490 and OPG1405^[78,85-87].

1.4 Aminoglycosides

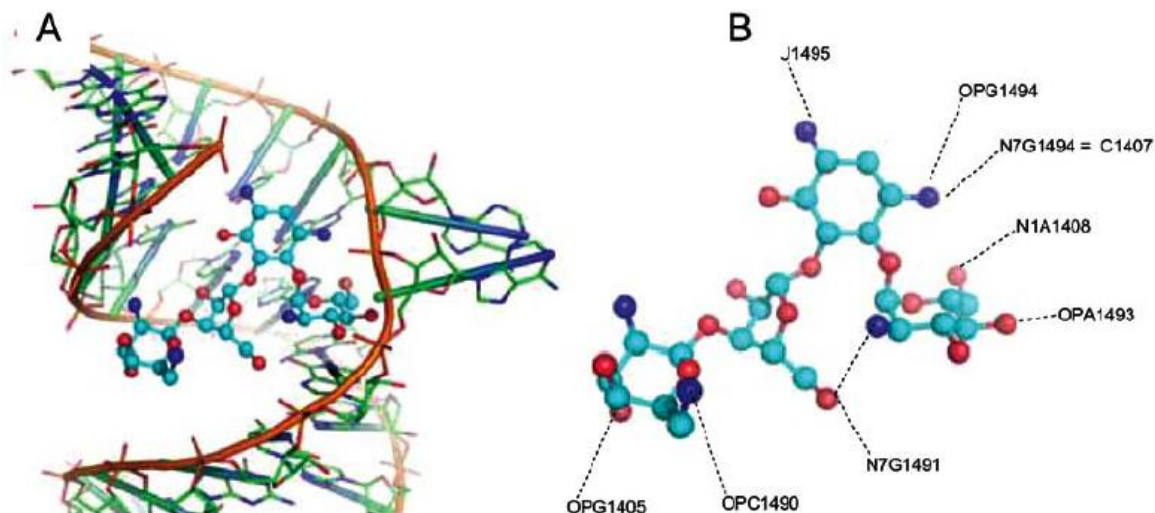


Figure 11. A. Three-dimensional structure of the paromomycin (3) complex with an oligonucleotide, represented by the bacterial decoding-site internal of helix 44 in 16S rRNA. Paromomycin is represented in ball and stick (light blue: carbon, dark blue: nitrogen and red: oxygen) and rRNA in stick (green: carbon, dark blue: nitrogen and red: oxygen). B. Main interactions between paromomycin and the target sub-domain of 16S rRNA.^[84]

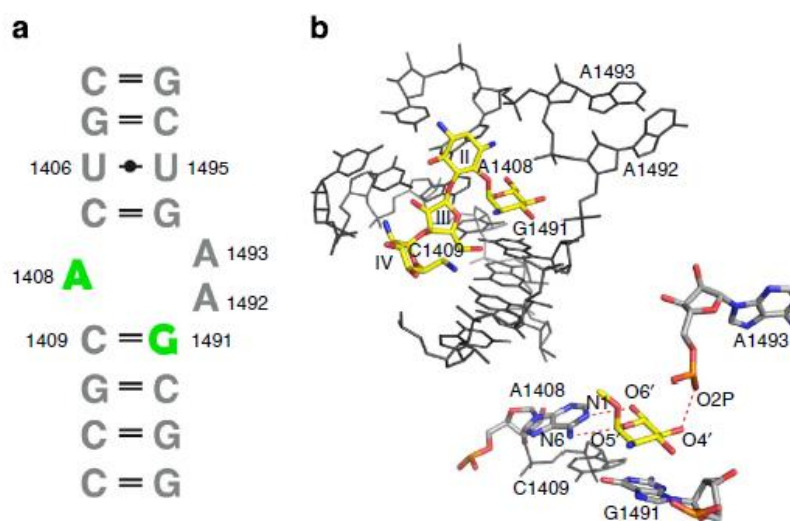


Figure 12. (a) Secondary structure of the aminoglycoside-binding pocket in helix 44 of 16S rRNA. Key polymorphic residues determining the selectivity of aminoglycosides are residues 1408 and 1491, highlighted in bold green. (b) Overview of paromomycin bound to the bacterial A site and detailed view of the 6' OH paromomycin ring-I stacking interaction with G1491 and hydrogen bonding with A1408 and A1493. Hydrogen bonds between aminoglycoside ring I and A1408 are shown as red dotted lines, as is hydrogen bonding between 4'OH and O2P of A1493.^[87]

1.4.3 Structure-activity relationship (SAR)

Studies on structure-activity relationships have been formulated by comparing natural and semi-synthetic aminoglycosides and by discovering the sites inactivated by bacterial enzymes.^[88] Structural studies on aminoglycosides-RNA complexes have revealed that neamine (**20**) is the minimal core structure required for RNA base recognition since it carries a fundamental 1- and 3-amino-2-deoxystreptamine central ring linked at the 4-position to 2,6-diaminoglucose, even though the corresponding 5-deoxy-neamine is more active against aminoglycoside-resistant bacteria.^[89] Ring I is very important for large spectrum activity and is also the main target of inactivating bacterial enzymes, being the *trans* 1,3-hydroxyamino group an important scaffold to interact with both phosphodiester backbone and guanine.^[90] Functions at C-6' and C-2' are particularly important as in kanamycin B (**5**) (C-6' amino, C-2' amino), which is more active than kanamycin A (**4**) (C-6' amino, C-2' hydroxyl) in turn more active than kanamycin C (C-6' hydroxyl, C-2' amino). Methylation of positions 2' or 6' does not appreciably decrease antibacterial activity and introduces resistance to enzymic acetylation at C-6' amino group. Removal of C-3' or C-4' hydroxyl groups or both, in kanamycins (**4**, **5**) does not reduce antibacterial potency. Gentamicins (**7-10**) do not have oxygen bearing groups in these positions as well as sisomicin (**13**) and netilmicin (**14**) which also have a double bond between positions C-4' and C-5'. Functional groups on ring III seems to be less sensitive to structural modifications than rings I and II. Although 2''-deoxy-gentamicins are significantly less active than 2''-hydroxyl-derivatives, the 2''-amino derivatives are more active. Amino groups in aminoglycoside antibiotics have different pKa values (5.7-8.8) but they are predominantly protonated at physiological pH. As a result, aminoglycosides with a higher number of amino groups bind more strongly to RNA than analogs with reduced charged groups. In fact, paromomycin (**3**) shows less affinity to several targets in RNA when compared to neomycin (**2**), which has one additional amino group.^[91] Similarly amino-aminoglycosides (**27**, **28**), a family of synthetic derivatives in which one hydroxyl group is substituted by a primary amino group, have a higher affinity to RNA when compared to the natural analogs (*figure 13*). Hydroxyl groups also contribute to aminoglycoside global charge, although with less intensity. Studies on increasing basic character of amino groups, through removal of

1.4 Aminoglycosides

neighboring hydroxyl groups have evidenced an increase in aminoglycoside global charge and the ability to bind RNA. This observation emphasizes the crucial role of electrostatic interactions in the RNA-aminoglycoside bindings and suggests that alteration in the amino group pKa is a possibility for the modulation of this process.^[92]

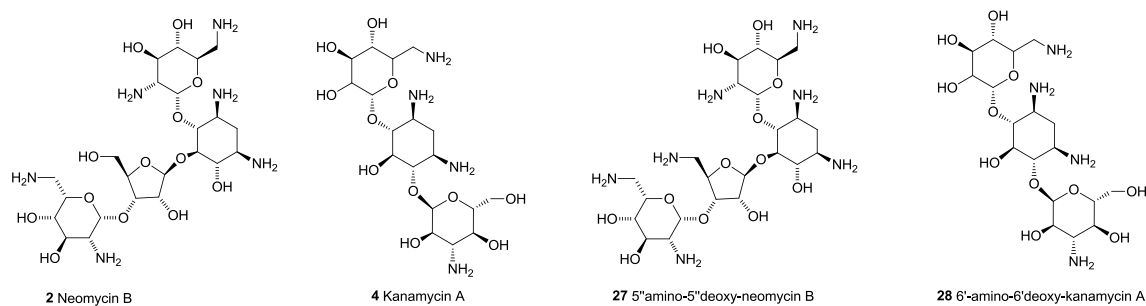
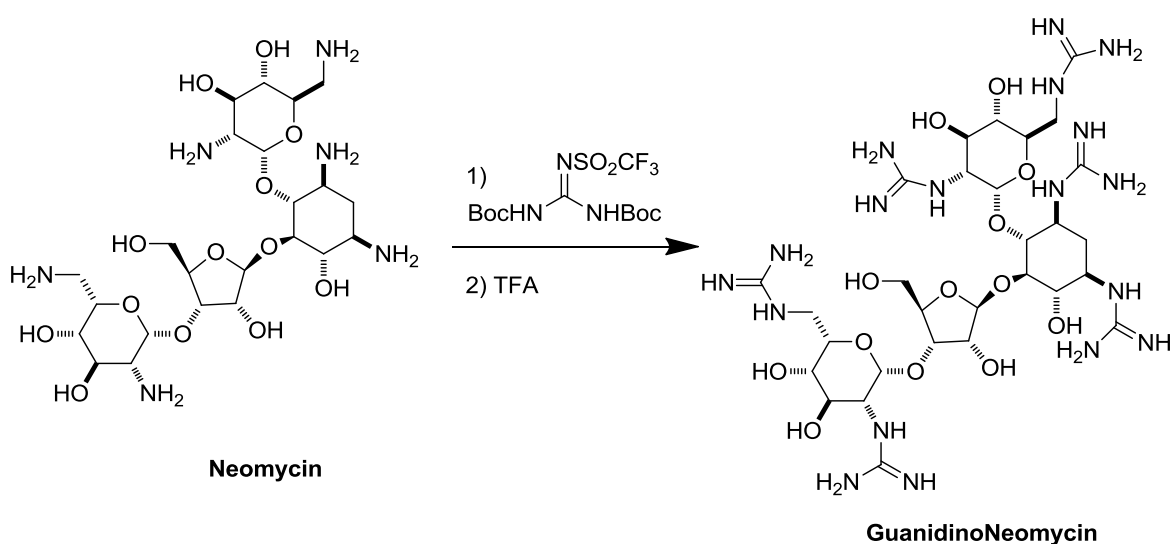


Figure 13. Structures of two amino-aminoglycoside and their natural analogs.

1.4.4 Guanidinoglycosides

As has been reported in literature, aminoglycosides bind a wide range of RNA and their affinity for RNA is due to electrostatic interactions mediated by ammonium group.^[93] So, the higher is the number of amino group present on the aminoglycoside, the higher is the affinity binding for RNA. A pivot role in RNA-protein binding interfaces is played from the guanidinium group. Guanidine was revealed in 1861 by Strecker as a crystalline solid but its solid-state structure was undisclosed only in 2009.^[94] It is a very strong base ($pK_a=13.6$), some derivatives are organic “superbases”, and its conjugate acid (guanidinium) is highly stabilized by resonance.^[95] The charged guanidinium group has the potential to form pentadentate hydrogen bonds with RNA and to be flexibly tethered to the peptide main chain by a simple aliphatic side chain. Thus, this conformational structure has the capability to engage in diverse ionic, hydrogen-bonding, weakly polar, and van der Waals interactions, the RNAs, ribosomal proteins with rRNA, and viral regulatory proteins with their cognate RNA binding sites.^[96] In contrast to ammonium groups, guanidinium groups are highly basic, planar (Y shape), and exhibit directionality in their H-bonding interactions. In addition, due to delocalization of the positive charge, the guanidinium groups interact

better with softer anions such as phosphates and sulfates than ammonium.^[97] So, replacing the ammonium groups on aminoglycosides with guanidinium groups increases the RNA affinity and selectivity (in particular anti-HIV activities) of the resulting compounds called “guanidinoglycosides”.^[98-99] Guanidinylation is the reaction of preparation of these compounds starting from their correspondent natural aminoglycoside and reacting with a guanidylating agent, such as the *N,N'*-di-*tert*-butoxycarbonyl-*N''*-triflylguanidine, a guanidylating reagent^[100] (for example from Neomycin → GuanidinoNeomycin *scheme 1*).



Scheme 1. Guanidinylation of Neomycin.

The substitution of all amino groups on aminoglycosides into guanidine group has created the new family of guanidinoglycosides that like their aminoglycosides precursor, bind preferentially RNA over DNA, and show selectivity between various RNAs. The higher affinity and selectivity of guanidinoglycosides to the Rev-response-element (RRE) implicate their potential use as antiviral agents.^[93d,99a] Further studies performed by Tor and co-workers demonstrated that these guanidinoglycosides exhibit an increase in cellular uptake compared to their parent aminoglycosides of 10–20 times. In particular,

1.4 Aminoglycosides

guanidinoneomycin, having six guanidine modifications, was distinctly more effective at entering the cell than its lesser guanidinylated counterpart, guanidinotobramycin. Indeed, with their studies revealed that the cellular uptake of guanidinoglycosides has an heparan sulfate dependent way and that these compounds can deliver biologically active cargo into the cell.^[101-102](see chapter 3 paragraph 3.2)

Chapter 2: Background and Aims

In the previous chapter were introduced all the fundamental concepts which are at the base of this experimental work. This integrated and multidisciplinary project put together organic synthesis, bio-, pharmaceutical and medicinal chemistry and biology in order to realize a complete work and showing how important is nowadays the interconnection with different fields. The overall project is divided in three parallel sub-projects: gene delivery, drug delivery and novel antibiotics; all dealing with aminoglycoside chemistry. Each sub-project will be discussed separately in the next paragraphs.

2.1 Gene Delivery Project

2.1.1 Aim of the Project

The aim of this project is the synthesis, characterization and structure-activity studies of three different type of conjugates, obtained by grafting PAMAM dendrimers, lipophilic calixarenes and hybrid lipo-dendrimers with amino- and guanidinoglycosides, in order to find new, safe and multifunctional non-viral vector for an efficient gene delivery. In particular the three different families of vectors have been developed:

- ✓ PAMAM of different generations-aminoglycoside and –guanidinoneomycin conjugates.
- ✓ Lipophilic calix[4]arene-aminoglycoside conjugates.
- ✓ Hybrid lipo-dendrimer-neomycin conjugates.

2.1.2 Background and Prospect of the Project

Gene delivery is one of the major fields in which researchers are dedicated to discover new harmless, efficient and innovative ways to prevent and treat diseases. The

2.1 Gene Delivery Project

aim of gene therapy is the cure genetic deficiencies and acquired diseases by the introduction of genetic material into mammalian cells. To date, viruses have demonstrated the feasibility of gene therapy and remain the best vehicles to introduce genes into cells. But, with viral vectors, severe fatal adverse events, including acute immune response and insertion mutagenesis, have occurred during gene therapy clinical trials raising serious safety concerns about the use of viral vectors. Inspired by strategies used by certain viruses to enter and to transfect mammalian cells, researchers are trying to build synthetic viruses with molecules that mimic the steps allowing a virus to infect mammalian cells.^[10,103-106] So far, cationic liposomes and cationic polymers are the most studied and used chemical non-viral vectors. Indeed, they possess several advantages over viral viruses, such as their 1) robust manufacture, 2) ease in handling and preparation techniques, 3) low immunogenicity; 4) unlimited size of nucleic acids delivered and 5) negligible oncogenicity. However, non-viral vectors are not as efficient as the viral ones but it is possible to improve their efficiency by chemical manipulation.

Aminoglycosides, a family of antibiotic having 2-deoxystreptamine ring and two or more amino sugars linked by glycosidic bond, due their cationic nature at physiological pH express a great affinity with negatively charged phosphates in DNA or RNA. These electrostatic interactions, along with hydrogen bond network, make them very powerful antibiotics. Their antibacterial activity arises from the ability to tightly and selectively interact with prokaryotic rRNA. All these features make aminoglycosides realizable options in order to develop multifunctional vectors in gene therapy field.^[72] So taking into account all these properties and concepts we have chosen to design different aminoglycoside-based carriers by tethering aminoglycosides to poly-amidoamine (PAMAM) dendrimers of different generation (in collaboration with Prof. Candiani of Politecnico of Milan) , to lipophilic calixarenes (in collaboration with Prof. Sansone of University of Parma) and to hybrid structures, having a lipophilic tail and a dendrimeric hydrophilic head, (in collaboration with Prof. Peng CNRS of Marseille). All these conjugates have been synthesized, fully characterized and their gene delivery efficiency, as well as their antibacterial activity have been studied demonstrating their multifunctional mode of action.^[107-112]

2.2 Drug Delivery Project

2.2.1 Aim of the Project

The aim of this project is the synthesis of new potential drug delivery carriers, using different generations of PAMAM dendrimers and guanidinoglycoside to study their cellular uptake efficiency, heparan sulfate selectivity and mechanism of internalization. All the biological tasks have been studied during my nine months stay at UCSD working in Prof. Tor's group.

2.2.2 Background and Prospect of the Project

Like gene delivery, the main goal of drug delivery studies is to find functional carriers which can englobe (by encapsulation, or direct chemical bonding) the bio-active molecule, that often has a poor cell permeability, and bring it inside the cell possibly in a selective way without compromise the therapeutic agent. Poly-arginine peptides, peptdomimetics, peptoids, dendrimers, carbohydrates and liposomes, have showed their great ability on this field and are largely employed in a wide range of mammalian cell. Recently the group of Prof. Tor at UCSD has introduced a new family of cationic carriers, namely guanidinoglycosides (GG), i.e. aminoglycosides where all the amino groups are converted into guanidinium groups, and demonstrated that they can deliver bio-active macromolecules, such as enzymes and proteins, very efficiently. Unlike other guanidinuim-rich transporters and cell prenetrating peptides, the cellular uptake of guanidinoglycosides occurs at nanomolar concentrations and exclusively depends on cell surfaces heparan sulfate proteoglycanes^[53-54,99,101-102] So, taking these features into account, in order to study more in detail the possible influence of multivalency in the cellular uptake of such systems, we have conjugated PAMAM dendrimers of generation 2 and 4 to guanidinoneomycin and studied their efficiency and mechanism in cellular uptake in collaboration with Prof.Tor at UCSD.

2.3 Novel Antibiotics Project

2.3.1 Aim of the Project

The aim of this project is the synthesis of a collection of systematically modified neomycin-sugar conjugates by an innovative multicomponent domino process developed in our laboratories and testing their efficiency as a new antibacterial agents working in Prof. Tor's group at UCSD.

2.3.2 Background and Prospect of the Project

The emergence of resistant bacteria, due to the excessive and often unnecessary use of antibiotics, has become one of the biggest challenges for researchers in the last three decades. Indeed beside this increasing in the resistance mechanism of bacteria, such as biofilm formation and ribosome's mutation, there is not correspondence with the discovery of new effective drugs.^[113-116] The enormous development of multidrug resistant Gram negative bacteria such as *Klebsiella pneumonia* and Gram positive organism such as methicillin-resistant *Staphylococcus aureus* (MRSA) remain the biggest concern for clinician.^[109] At now is also worrisome that these bacteria along with *Escherichia coli* infect not only the immunocompromised hospital patients, but also the healthy ones and *Staphylococcus epidermidis* are responsible for catheter-associated infections that increasing mortality and affect the rising healthcare costs.^[117,118] Since their important disclosure, aminoglycosides have been widely used to cure previously untreatable life-threatening infective disease and today after 70 years, remain the most clinically useful antibiotics due to their highly effective broad-spectrum, rapid action and ability to cooperate with other drugs.^[119-120] Their bactericidal activity is based on inhibition of protein synthesis. In fact, by binding the bacterial 30S or 16S ribosomal A-site of RNA, they are able to suppress the translation with consequently death of the bacteria.^[79] One of the most promising strategy to discover new, more active antibacterial agents is still the

selective modification of the aminoglycoside scaffold.^[116,121] Choosing the clinically significant neomycin B as aminoglycoside parent compound, we synthesized a collection of systematically modified neomycin sugar derivatives modifying it at position 5". For this purpose, we used an innovative multicomponent domino process that has been developed recently in our laboratories and that worked very efficiently for the synthesis of glycoconjugates and multivalent glycomimetics (high yield and mild condition)^[122-123]. The minimum inhibitory concentration (MIC) of the resulting conjugates was measured against different strain of resistant gram positive and gram negative bacteria.^[124]

Chapter 3: Results and Discussions

3.1 Gene Delivery Project

The development of safe non-viral vectors for gene delivery has become more and more important to overcome the disadvantage, such as insertion mutagenesis and acute immune response, associated with the common use of viral vectors. The viral carriers in fact, though have the best transfection efficiency, are not too much used in ongoing therapy trials, due to their worrisome drawbacks.^[10,105-106,125] So, in this scenario non-viral delivery systems, like cationic polymers and lipids, represent an attractive alternative due to their lack of immune response and easy preparation and functionalization.^[126] Considering these pros, numerous efforts have been done in researching efficient non-viral vectors such as natural and synthetic cationic polymers and lipids which, having a positive charge, can complex the negatively charged DNA or RNA giving, generally, the formation of stable polyplexes and lipoplexes, respectively.^[108-109,128-129] Taking all these fundamental concepts in mind, we choose to use as cationic polymer, Starbust polyamidoamine (PAMAM) dendrimers due to their unique properties such as a) the well-defined structure formed by a central ethylenediamine (EDA) core and repeating units of methyl acrylate and EDA that establish the generation, b) well defined chemistry, c) the low toxicity and d) easy of functionalization (see section 1.2.2).^[129-130] However PAMAMs themselves are not so efficient; in order to overcome this problem we have functionalized the peripheral amino groups, which are responsible for toxicity, with aminoglycosides due their astounding properties in particular the noteworthy delivery efficiency and antibacterial activity^[131-134], to obtain multifunctional non-viral vectors (see section 1.4). Accordingly, starting from PAMAM, suitable linkers and different aminoglycosides, we have synthesized diverse PAMAM-linker-aminoglycoside conjugates and studied their behavior as gene delivery vectors in collaboration with the research group of Prof. Gabriele Candiani. Furthermore we have also tethered aminoglycosides opportunely modified to specific

calixarenes product by the group of Prof. Francesco Sansone and to hybrid lipo-dendrimers synthesized by Prof. Ling Peng and her team, to study their property as gene carriers.

3.1.1 PAMAM G4-Aminoglycoside Conjugates: Synthesis and Biological Activity

To synthesize our PAMAM-aminoglycoside conjugates we started from generation 4 of PAMAM, a linker constituted by two moieties reacting in different conditions (one with an azide and carboxylic acid group and the other with an isothiocyanate and alkyne groups) and three different aminoglycosides: neamine, paromomycine and neomycin. The first conjugates produced are show in *figure 14*.

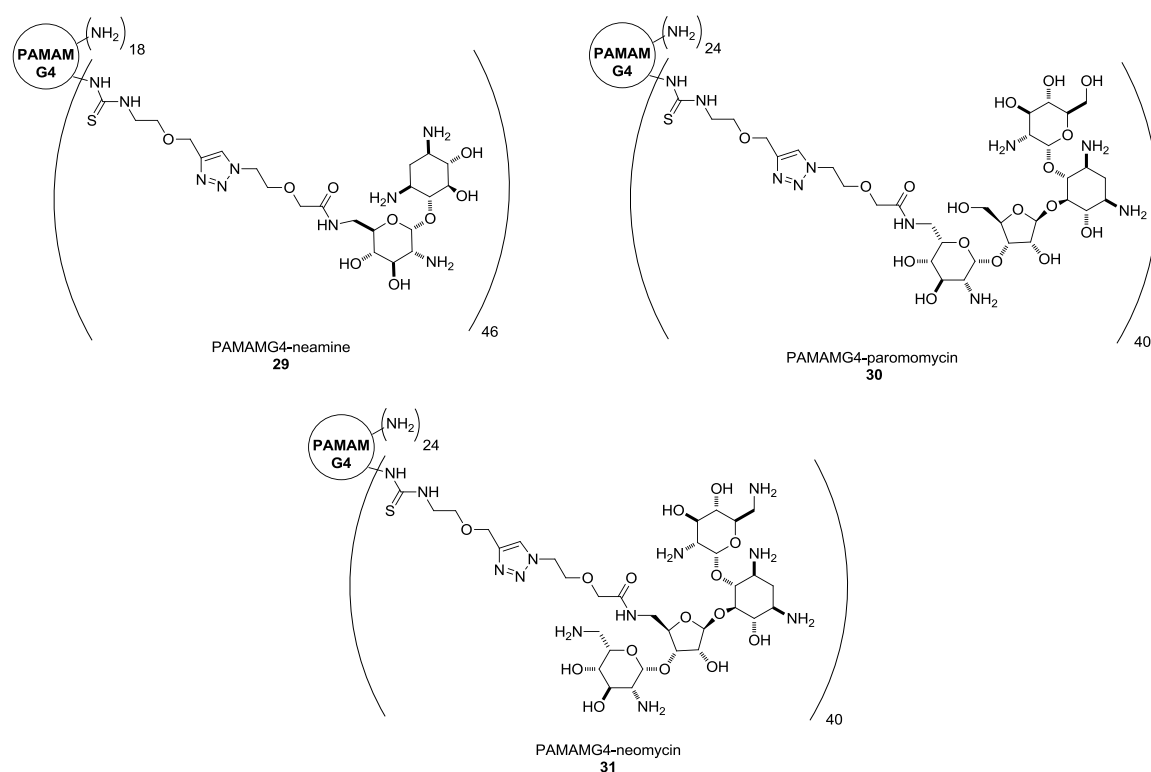
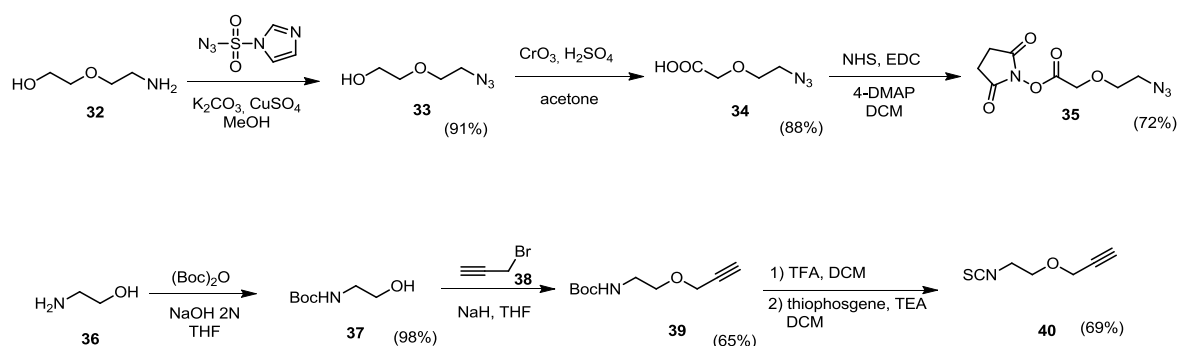


Figure 14. Structure of PAMAMG4-Neamine, PAMAMG4-Paramomycin, PAMAMG4-Neomycin Conjugates 29-31.

3.1 Gene Delivery Project

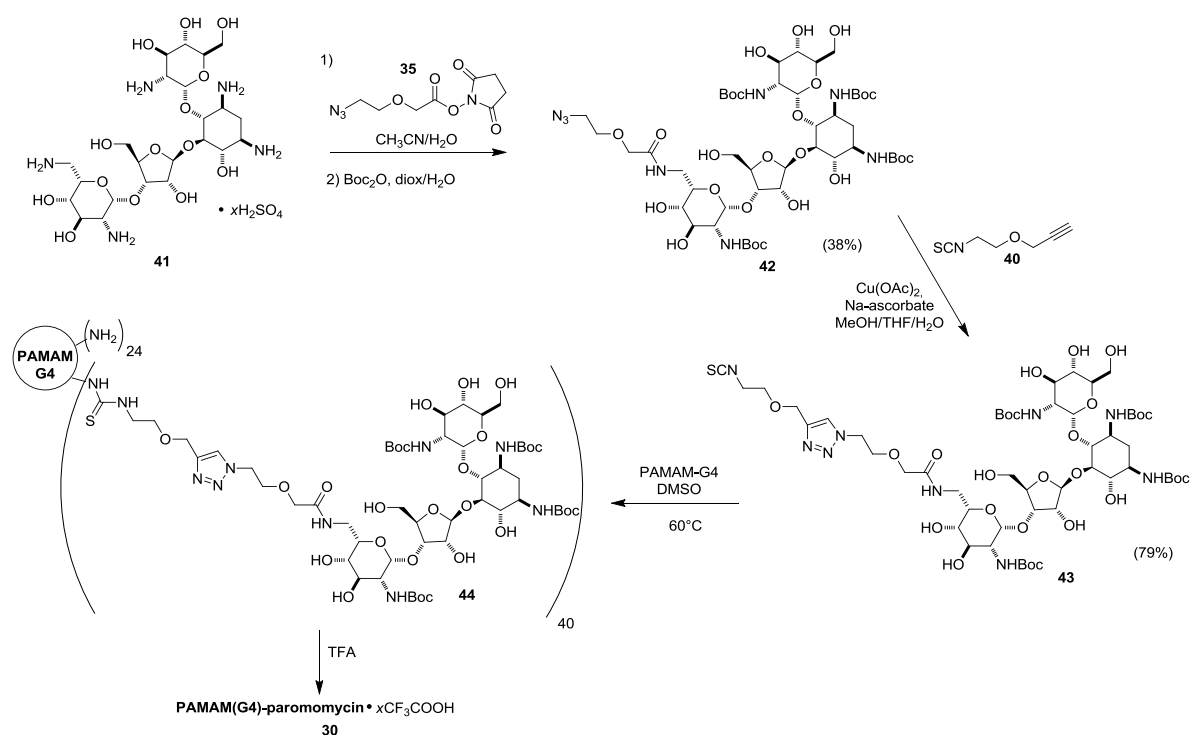
To obtain these conjugates the first step was to tether the aminoglycosides to a suitable linker. This linker was formed by two different modules both synthesized in our laboratories following the scheme 2. For the first part the amine of 2-aminoethoxyethanol **32** was converted into an azide, then the free alcohol was oxidized to carboxylic acid and activated as *N*-hydroxysuccinimide ester (compound **35**). To obtain the second isothiocyanate moiety we started from 2-aminoethanol **36**, protection of amino group by di-*tert*-butyldicarbonate, followed by propargylation which lead to the formation of the intermediate **39** which was deprotected and the free amine converted to isothiocyanate group.



Scheme 2. Synthesis of the two moieties used to prepare the linker.

Once the two modules of the linker were obtained, we used **35** to couple the aminoglycosides opportunely prepared. Aminoglycosides are important molecules with a lot of functional amino and hydroxyl groups available. Nevertheless the less hindered amino methylene moieties at position 6' and 6''' of neamine and paramomycin respectively, and the primary hydroxyl group in 5'' for neomycin, are the preferential point of selective modification due to their better reactivity. So, taking this into account, for neamine and paramomycin derivatives we used the same synthetic strategy, while for neomycin a slightly different procedure. The last step for all aminoglycosides was the grafting of aminoglycosides-linker intermediates to the dendrimer through isothiocyanate-amine click reaction. In the *Scheme 3* is showed the synthetic pathway for the PAMAMG4-

paromomycin conjugate **30**. Commercially available paromomycin sulfate was first neutralized with Amberlist resin and then treated with *N*-hydroxysuccinimide ester **35**, which reacted preferentially with the less sterically hindered methylene amine affording derivative **42**, after protection of the remaining free amino groups with Boc_2O . Then “click” reaction with propargyl derivative **40** led to the formation of **43**, which possesses the suitable isothiocyanate functional group to be used to anchor the aminoglycoside to the PAMAM dendrimer. This latter reaction was carried out in DMSO at 60 °C for 24 h to maximize the degree of grafting. The last step was the deprotection with TFA from Boc protecting groups that lead to the formation of PAMAM G4-paromomycin conjugate **30**. The characterization of this derivative evidenced a degree of functionalization with paramomycin slightly greater than 60% of the total number of grafting point (see below).

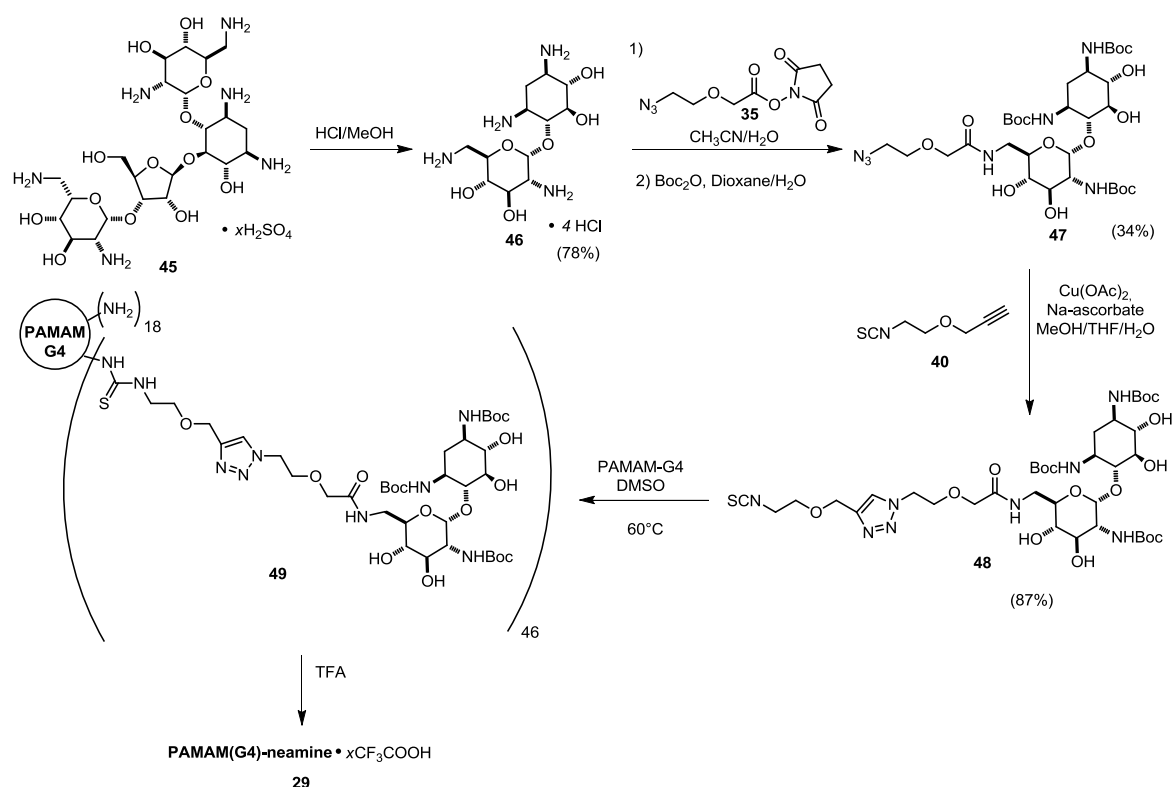


Scheme 3. Synthesis of PAMAMG4-Paramomycin conjugate **30**.

Scheme 4 shows the synthetic pathway of PAMAM G4-neamine conjugate **29**. Briefly neomycin B was hydrolyzed by HCl in MeOH giving the neamine as salt as reported in literature [135]. Treatment with basic Amberlist resin followed by: 1) reaction with *N*-

3.1 Gene Delivery Project

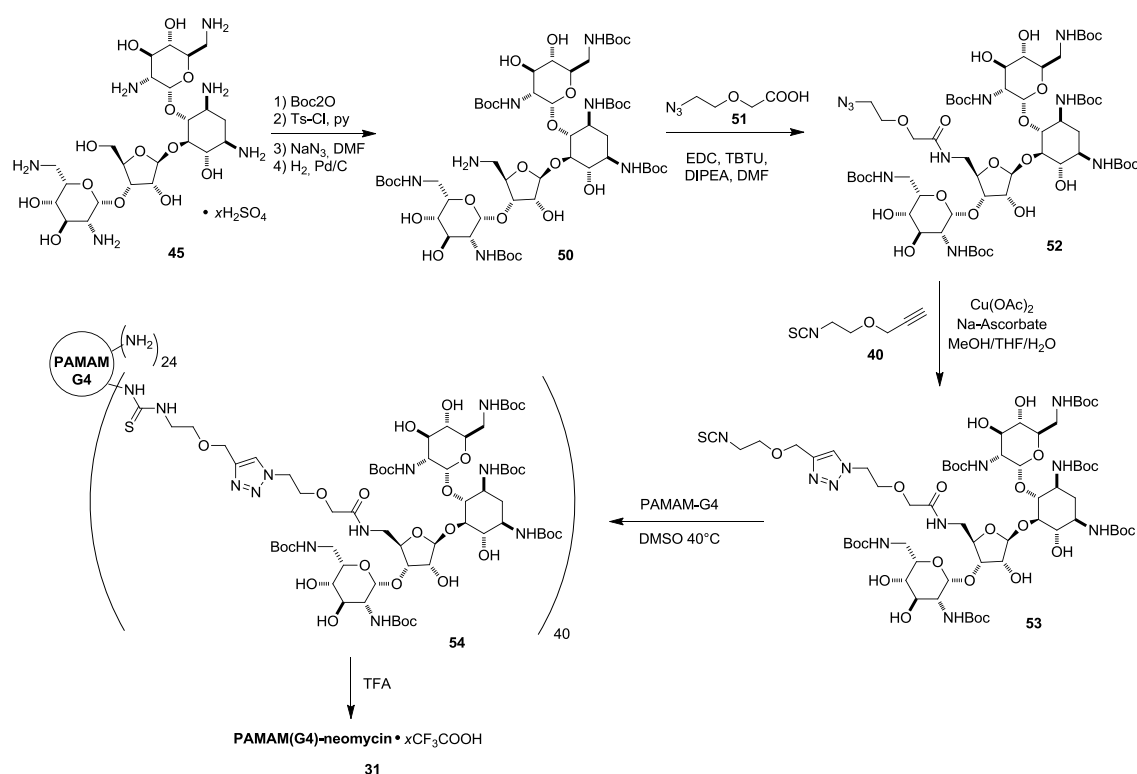
hydroxysuccinimide ester **35**; 2) Boc-protection of all amino groups afforded the intermediate **47**. “Click” reaction with propargyl derivative **40** led to the formation of **48** possessing the suitable isothiocyanate functional group that was used to anchor the neamine to the generation 4 of PAMAM dendrimer in DMSO at 60 °C for 24 h. After deprotection with TFA from Boc, we got PAMAM G4-neamine conjugate **29** characterized by a 72% degree of grafting (see below).



Scheme 4. Synthesis of PAMAMG4-Neamin Conjugate **29**.

Scheme 5 shows the synthetic pathway for PAMAMG4-neomycin conjugate. Starting from commercially available Neomycin sulfate, all the amino groups were first protected using Boc_2O ; then reaction with TsCl in dry pyridine occurred preferentially on primary hydroxyl group at 5'' position. The resulting tosylate was substituted with azide by reaction with NaN_3 in DMF at 60°C. Hydrogenation of the azide group, using H_2 in MeOH in presence of catalytic amount of Pd/C, lead to the formation of the free amine derivative **50**. Coupling reaction between amino group and azide-carboxylic acid **51** in DMF in presence of EDC,

TBTU and DIPEA gave the intermediate **52**. “Click” reaction with propargyl derivative **35** led to the formation of **53** with the suitable isothiocyanate group used to anchor the aminoglycoside to the PAMAM dendrimer in DMSO at 60 °C for 24 h. The last step was the Boc-deprotection with TFA, which lead at the formation of PAMAM G4-neomycin conjugate **31** characterized by a 63% degree of grafting (see below).



Scheme 5. Synthesis of PAMAMG4-Neomycin Conjugate **31**.

The degree of grafting of the resulting conjugates was first determined by ^1H NMR spectroscopy, comparing the characteristic signals. As represented in *figure 15* for PAMAMG4-paromomycin conjugate, the ^1H NMR spectra of the conjugate (blue spectrum), was compared with the spectra of PAMAM G4 (red spectrum) and free paromomycin (green spectrum) all recorded in D_2O , considering the specific peaks which are: a) the signal of the proton in α position of the carbonyl group in PAMAM dendrimer at 2.76 ppm (Hc protons, *figure 15*); b) the signal of two characteristic protons H' belonging to the 2-desoxystreptamine ring of the aminoglycoside which resonate at 2.43 and 1.81 ppm; c) the

3.1 Gene Delivery Project

signal of three anomeric protons H'' belonging to the paromomycin between 5.71 and 5.15 ppm; and d) the single proton of the triazole ring of the linker at ca. 8.00 ppm.

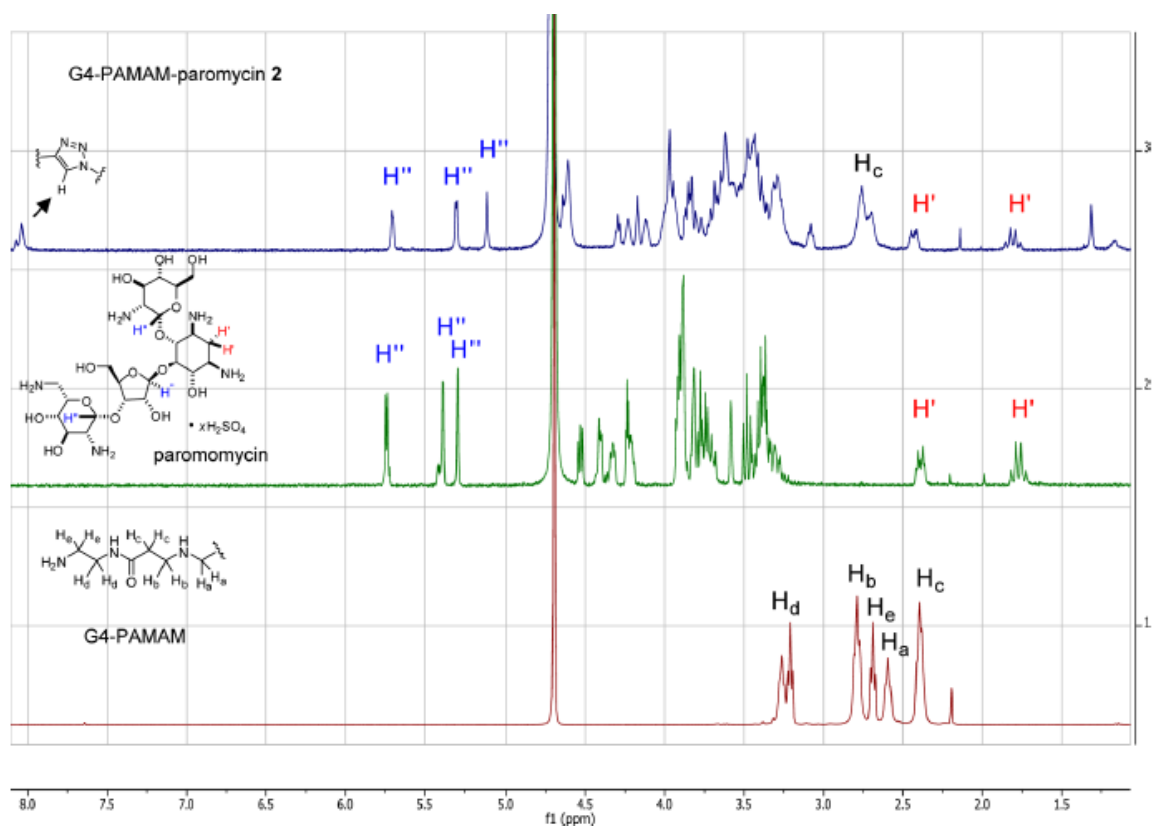


Figure 15. Figure 1. ¹H NMR spectra recorded in D₂O of PAMAM G4 dendrimer (red spectrum), paromomycin (green spectrum), and PAMAM G4–paromomycin conjugate (blue spectrum).

The integration of these protons was used to calculate the degree of grafting; in fact the integration ratio between the PAMAM H_c protons and the two H' protons was found to be 248:41:41 (the same ratio was found between H_c protons and the anomeric protons H'') confirming that slightly more than 40 amino groups over 64 were functionalized (degree of grafting ~63%). The same thing has been done for the other two conjugates (figure 16 and 17) founding the same degree of grafting for neomycin (63%) and a slightly higher degree of grafting for neamine (~72%). The degree of grafting was also confirmed by MALDI spectroscopy (table 4, figure 18).^[112]

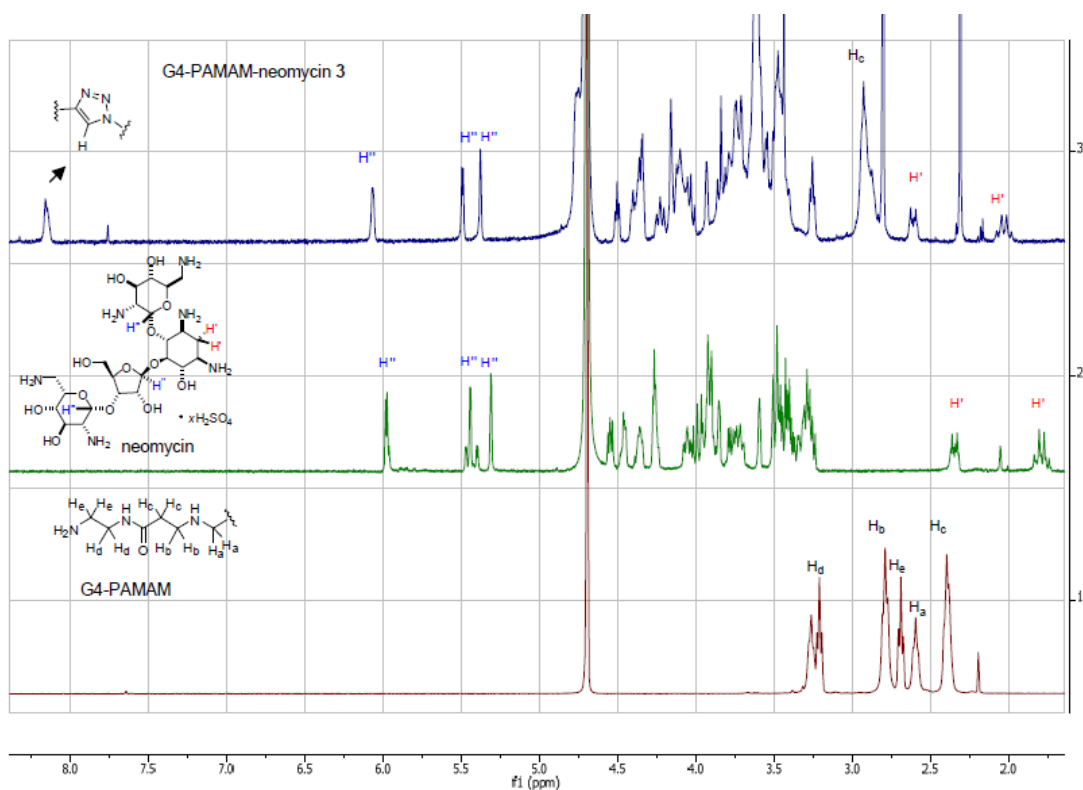


Figure 16. ^1H NMR spectra recorded in D_2O of PAMAM G4 dendrimer (red spectrum), neomycin (green spectrum), and PAMAM G4–neomycin conjugate (blue spectrum).

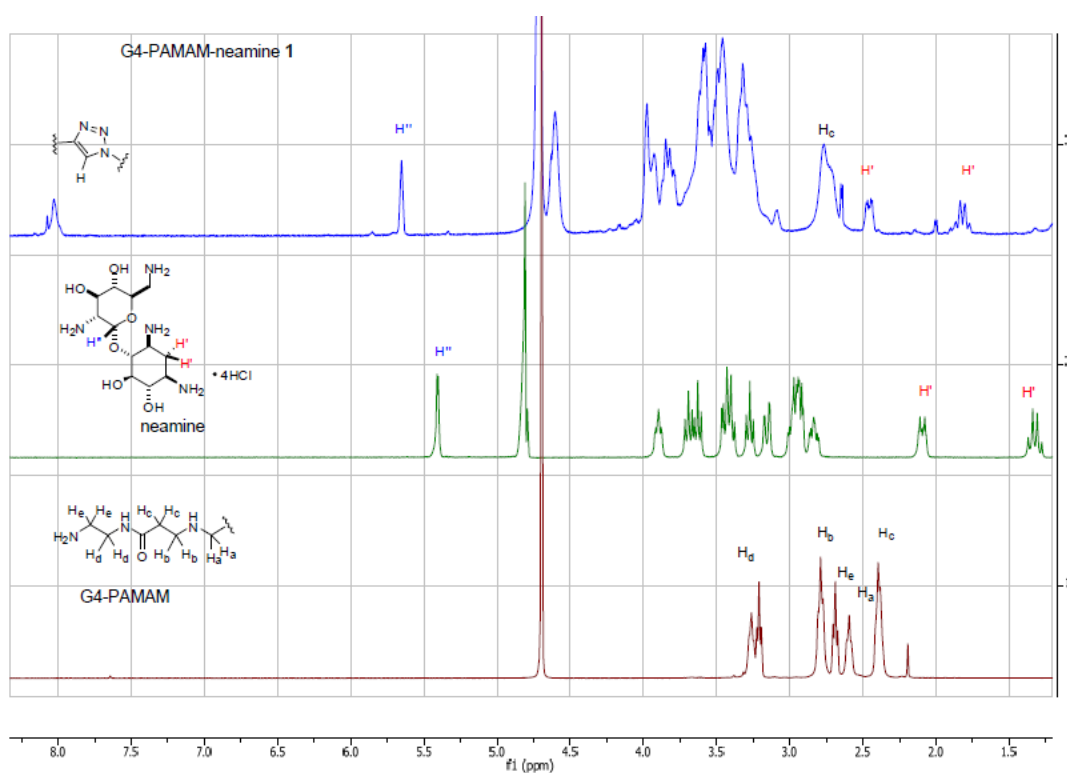


Figure 17. ^1H NMR spectra recorded in D_2O of PAMAM G4 dendrimer (red spectrum), neamine (green spectrum), and PAMAM G4–neamine conjugate (blue spectrum).

3.1 Gene Delivery Project

	Experimental value (m/z)	Calculated value [M+H] ⁺ (m/z)	Calculated Value [M+2H] ²⁺ (m/z)
PAMAMG4	13226	13226	6613
PAMAMG4-paramomycin conjugate	24600	49186	24593
PAMAMG4-neomycin conjugate	24580	49146	24573

Table 4. Molecular mass determined by MALDI mass analysis and the corresponding values calculated for the single and the double charged parent ions.

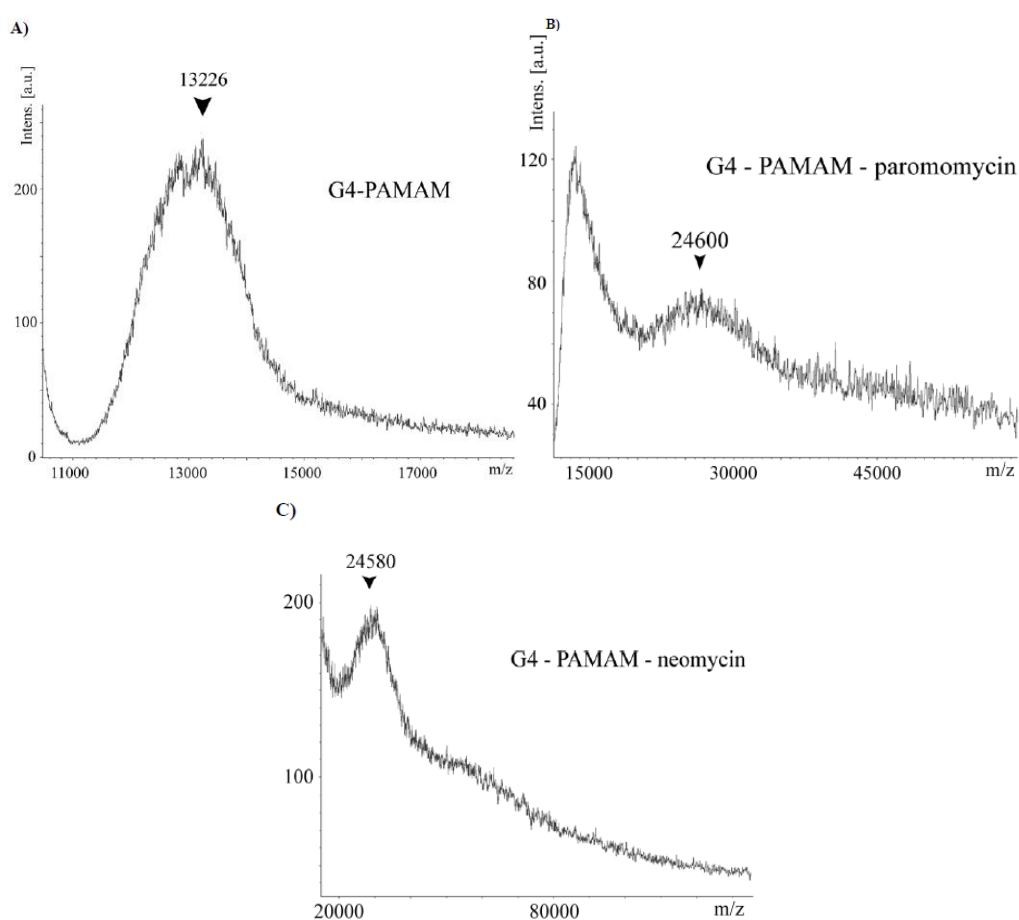


Figure 18. The MALDI spectra of: A) PAMAM G4 dendrimer, peak at 13226 Da corresponds to the single charged ion of the dendrimer; B) PAMAM G4-paramomycin **30**, peak at 24600 Da corresponds to the double charged ion of the PAMAM conjugate; C) PAMAM G4-neomycin **31**, peak at 24580 Da corresponds to the double charged ion of the PAMAM conjugate.

Following the synthesis and characterization (see experimental section), the conjugates were submitted to biological testing in collaboration with Prof. Candiani's research group, in order to investigate their gene delivery efficiency and their cytotoxicity. First, the ability of the PAMAM conjugates to form stable polyplexes with DNA has been evaluated by fluorophore-exclusion titration assay that use SYBR Green I (SG), an asymmetrical cyanine dye used as a nucleic acid stain in molecular biology, which binds pDNA. The resulting DNA-dye-complex absorbs blue light ($\lambda_{\max} = 497 \text{ nm}$) and emits green light ($\lambda_{\max} = 520 \text{ nm}$). The fluorescence measured by microplate reader and normalized over the fluorescence of non-complexed plasmid DNA is showed in *figure 19*. As expected, all the conjugates were able to form tight complexes with DNA at N/P ratio value around 2, with the neomycin derivative being the best binder. This result is in accordance with the fact that neomycin is a better DNA-binder than neamine and paromomycin. Furthermore in conjugates **29** and **30**, the amino methylene groups (position 6' of neamine and 6''' of paromomycin) are converted into an amide removing the most basic amine from contributing to strong electrostatic interactions with negatively charged DNA.

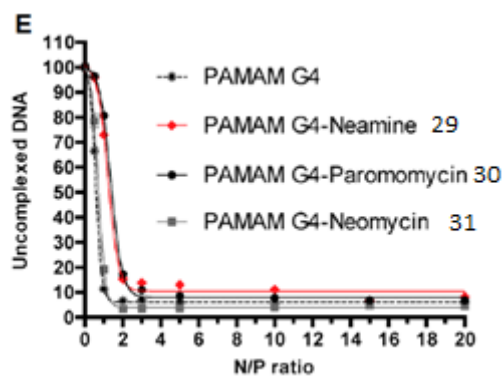


Figure 19. DNA complexation ability of PAMAMG4-Aminoglycosides Conjugates **29-31**.^[112]

Luciferase Assay System measures the luciferase activity of luciferase gene from the firefly *Photinus pyralis*; this gene encodes a 61-kDa enzyme that oxidizes D-luciferin in the presence of ATP, oxygen, and Mg^{++} , yielding a fluorescent product that can be quantified by measuring the released light. Thus, using the Luciferase Assay (see paragraph 5.1) three different cell lines were transfected and the transfection efficiency determined in function of the N/P ratio. Transfection efficiency was expressed as relative luminescence of

3.1 Gene Delivery Project

luciferase normalized against the total protein content in cell lysates (measured by The BCA Protein Assay that combines the well-known reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation with a strong linear absorbance at 562 nm by bicinchoninic acid (BCA)). *Figure 20* depicts the transfection efficiency and cytotoxicity for the first cell line used, the HeLa cells. While the conjugate with neamine **29** is less effective than free PAMAM G4, the paromomycin **30** and neomycin **31** conjugates showed very good transfection efficiencies. PAMAM-G4 paromomycin **30** conjugate is very effective at N/P ratio major or equal to 30, while PAMAM G4-neomycin **31** is very active already at N/P ratio 5, being both of them more active than gold standard 25kDa bPEI. We explored also the transfection behavior in the presence of fetal bovine serum (*figure 20C*) because it was reported that others PAMAM-conjugates decrease their efficiency in these conditions that better mimics the in vivo situation. The experiments were made at N/P ratio 15 because was a good compromise between high efficiency and low cytotoxicity (see below) and very interestingly there is an increase of activity of our conjugates in the presence of high serum content while the activity for bPEI decreases. The cytotoxicity of the PAMAM conjugates (*figure 20B, D*) expressed as toxicity percentage relative to untreated controls by Alamarblue cell viability assay, has been determined and for N/P ratio lower than 30 they resulted to be non-cytotoxic. As expected, an increase of the N/P ratio resulted in an increase of the cytotoxicity which always remained in the range of bPEI 25 kDa unless for the neomycin derivative which became very high at high N/P ratio. Interestingly, we obtained the same results in terms of cytotoxicity both for the free polymers and the polyplexes (*figure 20D*).^[112]

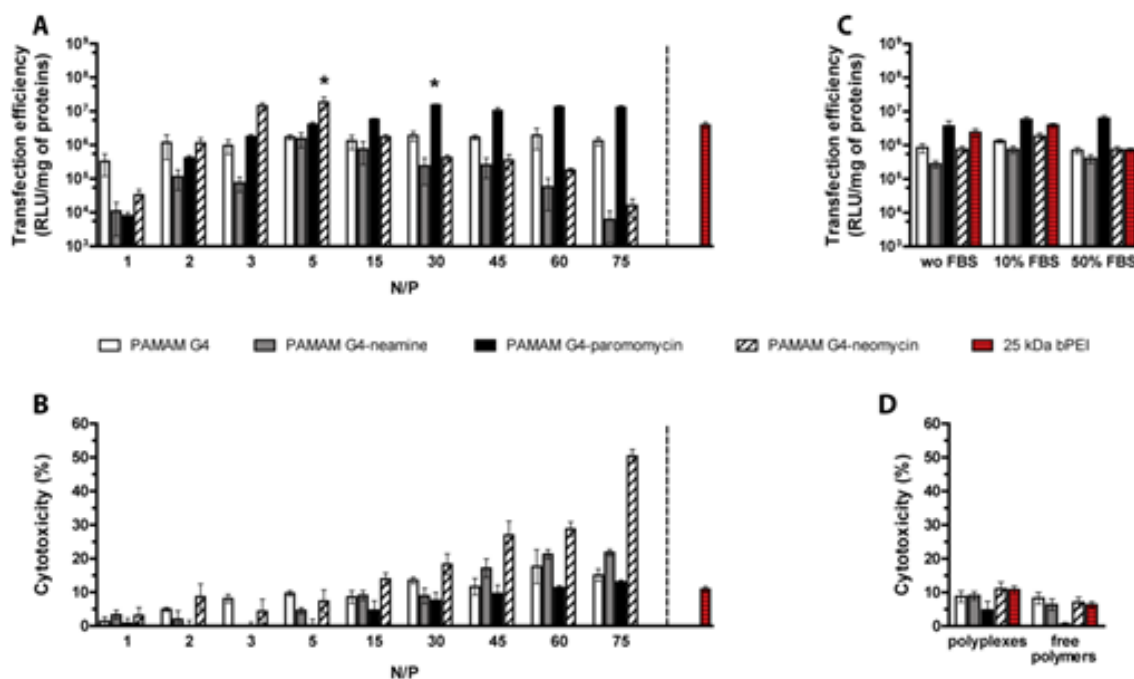


Figure 20. Transfection efficiency and cytotoxicity of PAMAM G4 and PAMAM G4-derivatives in HeLa cells. (A) Transfection efficiency and (B) cytotoxicity of polyplexes prepared with pGL3 at different N/P were evaluated after incubation for 48 h in 10% FBS. (C) Influence of FBS content on the transfection efficiency of polyplexes prepared at N/P 15. (D) Comparative cytotoxicity assay of PAMAM G4 dendrimer and PAMAM G4-derivatives **29–31** delivered as polyplexes at N/P 15 and free in solution at equivalent concentration. 25 kDa bPEI was utilized at N/P 10.^[112]

Transfection efficiency and cytotoxicity of PAMAM G4 and PAMAM G4-derivatives in U87-MG cells, showed in *figure 21*, is quite similar to those with HeLa cells. The paromomycin and neomycin conjugates showed very good transfection efficiencies; PAMAM-G4 paromomycin conjugate **30** is very effective at N/P ratio major or equal to 30 while PAMAM G4-neomycin **31** is very active already at N/P ratio 5. The conjugate with neamine **29** is less effective than the others, but more active than free PAMAM G4. However both neomycin and paromomycin conjugates are more active than the gold standard 25kDa bPEI. Exploring also the transfection behavior in the presence of fetal bovine serum (*figure 21C*), there is an increase of activity of our conjugates in the presence of high serum content while a decrease for the activity of bPEI 25 kDa. The cytotoxicity of the PAMAM conjugates (*figure 21 B, D*) has been determined and for N/P ratio lower than 30 they resulted to be non-cytotoxic. As expected, an increase of the N/P ratio resulted in

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an increase of the cytotoxicity, which became higher than bPEI 25 kDa at N/P superior of 30. Again, for the cytotoxicity of the free polymers and the polyplexes, we got the same results showed for HeLa cells.^[112]

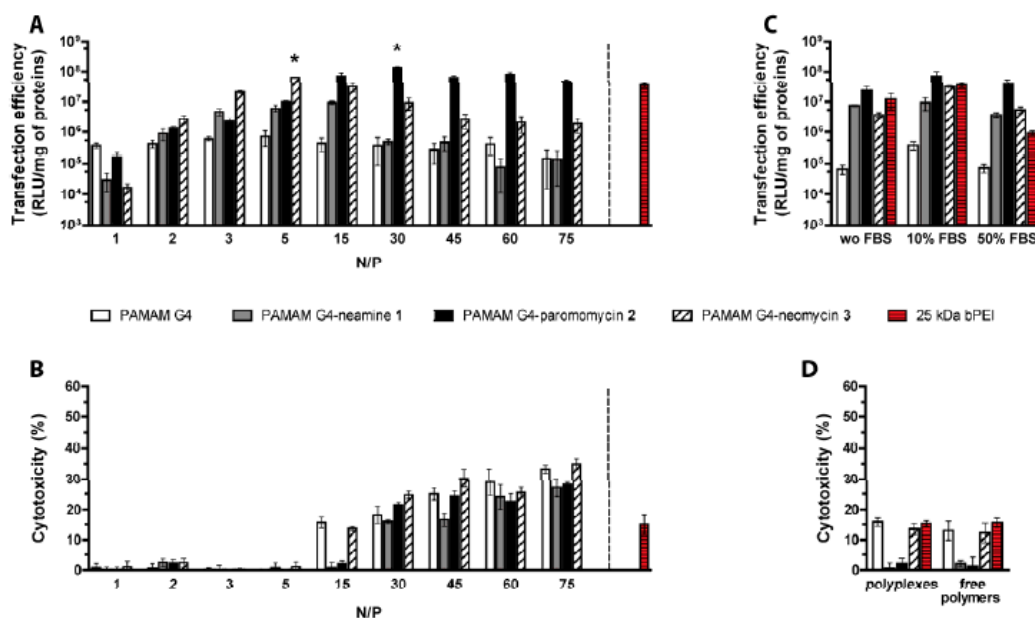


Figure 21. Results of transfection efficiency and cytotoxicity of PAMAM G4 and PAMAM G4-derivatives in U87-MG cells. (A) Transfection efficiency and (B) cytotoxicity of polyplexes prepared with pGL3 at different N/P were evaluated after incubation for 48 h in 10% FBS. (C) Influence of FBS content on the transfection efficiency of polyplexes prepared at N/P 15. (D) Comparative cytotoxicity assay of PAMAMG4 dendrimer and PAMAMG4-derivatives 29–31 delivered as polyplexes at N/P 15 and free in solution at equivalent concentration. 25 kDa bPEI was utilized at N/P 10.^[112]

The results for the third cell line, Cos-7 cells, are shown in figure 21. Also in this case PAMAM-neamine conjugate **29** is the less active and between N/P 15-30 reaches its maximum of efficiency which is similar to free PAMAM G4 and comparable with the other two conjugates. However paromomycin conjugate **30** has the better efficiency being equal to the gold standard 25kDa bPEI at N/P ratio major or equal to 30. While this kind of cell PAMAMG4-neomycin conjugate **31** possess a transfection efficiency a little lower respect to the paromomycin conjugate, comparable with the gold standard 25kDa bPEI and higher than neamine conjugate. For conjugates **30**, **31** the activity increases with the N/P ratio while for neamine conjugate the profile is a bell slope. About the influence of FBS content

on the transfection efficiency (*figure 22C*), the polyplexes were prepared at N/P 15 and also in this case the PAMAMG4-paromomycin conjugates has the better efficiency compared with the other conjugates and the gold standard 25 kDa bPEI. The graphic of cytotoxicity (*figure 22B*) shows that the conjugate of neomycin and paromomycin are either less toxic respect to the gold standard bPEI until N/P 30; then the cytotoxicity becomes higher. The results of comparative cytotoxicity assay of PAMAMG4 dendrimer and conjugated delivered as polyplexes at N/P 15, and free in solution at equivalent concentration, *versus* 25 kDa bPEI, utilized at N/P 10, (according to the existing literature) show the same toxicity in both the situation (*figure 21D*).

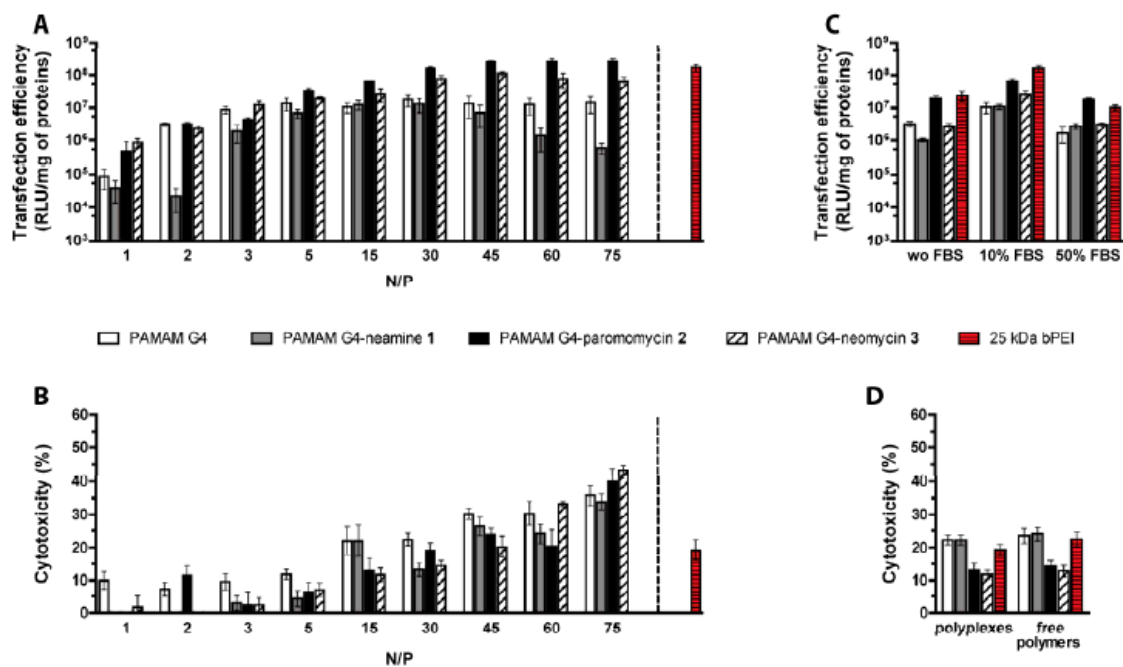


Figure 22. Results of transfection efficiency and cytotoxicity of PAMAM G4 and PAMAM G4-derivatives in Cos-7 cells. (A) Transfection efficiency and (B) cytotoxicity of polyplexes prepared with pGL3 at different N/P were evaluated after incubation for 48 h in 10% FBS. (C) Influence of FBS content on the transfection efficiency of polyplexes prepared at N/P 15. (D) Comparative cytotoxicity assay of PAMAMG4 dendrimer and PAMAMG4-derivatives 29–31 delivered as polyplexes at N/P 15 and free in solution at equivalent concentration. 25 kDa bPEI was utilized at N/P 10. ^[112]

A recent challenge in gene delivery field is the synthesis and development of efficient and gene carriers with multifunctional activity, inherent biocompatibility and

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biodegradability, and antibacterial and antitumor properties. Thus, the aim of our research in the gene delivery is the synthesis of multifunctional non-viral vectors that should have not only a good transfection efficiency and low toxicity, but also antibacterial property at the same time. In order to discover if we reached our goal, we also investigated the antibacterial activity of PAMAM conjugates, considering the presence of aminoglycosides in their structure. The tests were performed in two common Gram-positive and a Gram-negative strains *S. Aureus* and *E.Coli* respectively. So, following the general procedure derived from Clinical and Laboratory Standards Institute (CLSI-NCCLS),^[136,137] as described in the experimental section, the minimum inhibitory concentration (MIC) of the PAMAM derivatives that inhibits the growth of bacterial strains, *E. Coli* and *S. Aureus*, was studied. Concisely, a concentration 5×10^5 cfu/mL (colony forming unit/mL) of bacteria were incubated with PAMAM G4-derivatives for 24 hours; after this period, the optical density at 600 nm (OD_{600}), proportional to the number of bacteria, was measured in order to have a more precise evaluation of the antibacterial activity. The results, as graphics of the optical densities *versus* the concentration of PAMAM G4, freeaminoglycosides and PAMAM G4-aminoglycoside conjugates, are shown in figure 22. PAMAM G4-aminoglycosides are less effective than the corresponding free aminoglycosides but much more active compared to the free dendrimer (the only exception is neamine as already know from literature^[136]). In particular derivatives **31**, **30** of neomycin and paramomycin, are 10 and 3 times more effective than unmodified dPAMAM G4 in inhibition of *E. Coli* (figure 23C,E) and *S. Aureus*, respectively (figure 23D,F).^[138]

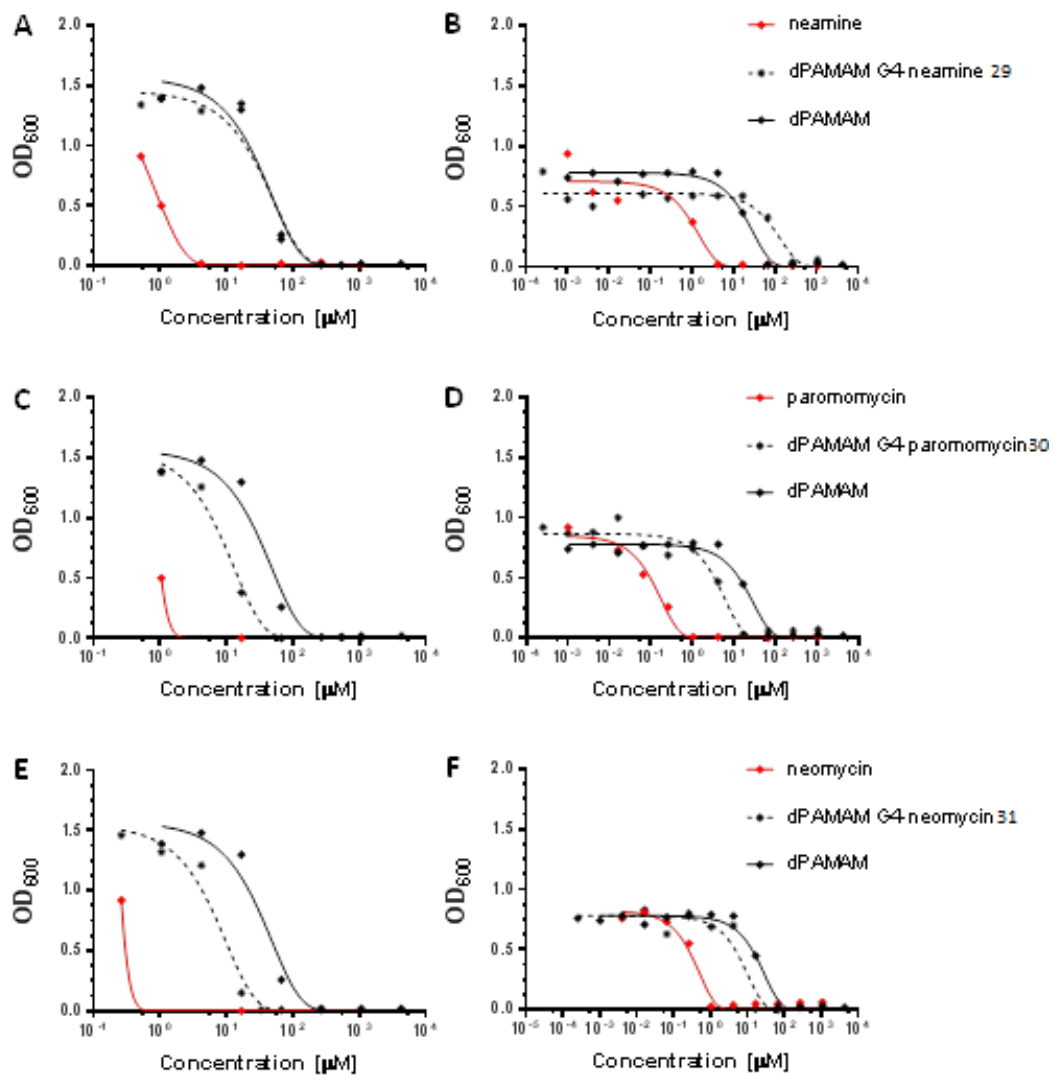


Figure 23. Results of Antibacterial activity of PAMAMG4-Neamine, PAMAMG4-Paramomycin, PAMAMG4-Neomycin Conjugates 29-31 in *E. Coli* (A,C,E) and *S. Aureus* (B,D,F) bacteria.

We also studied the bactericidal activity of our conjugates in function of the time using the bacteria killing assay.^[139] So using the same bacteria strains, *E. Coli* and *S. Aureus*, suspended in a basal medium devoid of nutrients with an inoculum of 5×10^5 cells/mL, (the inoculum was 10-fold concentrate respect the previous one), we incubated the cells with our conjugate of paramomycin **30** and neomycin **31** at their MIC (previously found), to establish the time required to kill the bacteria population. Hereinafter, for the tests we didn't use the neamine derivative due its lower antibacterial activity. The results, reported in *figure 24*, show PAMAM G4-derivatives **30** and **31**, aminoglycosides (paramomycin, neomycin), and unmodified PAMAM G4 abilities in killing bacteria. In detail for *E. Coli* all

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the compounds give a similar total reduction of bacteria viability within 30 minutes (*figure 24 A, C*), while different are the times for *S. Aureus* (*figure 24B, D*). In fact unmodified PAMAM G4 has similar behavior seen for all compounds in *E.Coli*; neomycin, paromomycin and PAMAM G4-paromomycin **30** decrease 75 times the viability of bacteria over 360 minutes, meanwhile the derivative of neomycin **31** determines the almost complete killing of bacteria. These studies all together designated the PAMAM G4-aminoglycoside conjugates as intrinsically bactericidal, killing effectively the bacteria, and not just bacteriostatic, only inhibiting the bacteria growth.^[138]

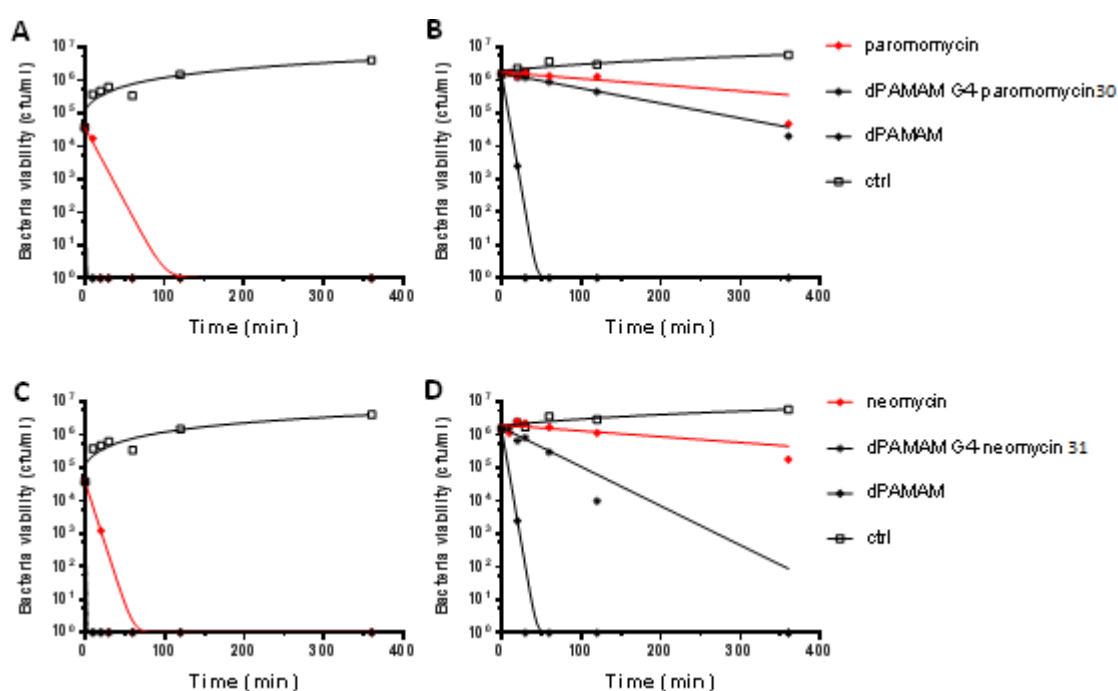


Figure 24. Bacterial killing assay of dPAMAM G4-derivatives **30** and **31**, paromomycin, neomycin, and unmodified dPAMAM G4.

Once the antibacterial activity of the conjugate was established, we investigated if this activity could be affected by complexation with DNA. Our PAMAM G4-conjugates, each at its respective MIC, (calculated for 5×10^5 cells/mL), were complexed with pDNA each at their transfection optimum N/P ratio on HeLa cell line, that was N/P 3 for PAMAM G4, N/P 30 for G4-paromomycin **30** and N/P 5 for G4-neomycin **31**.^[107] Then using the same method for the test, we compared the antibacterial activity of complexes and free polymers in *E. Coli* and *S. Aureus*. Interestingly PAMAM G4, G4-paromomycin **30** and G4-neomycin **31**

completely inhibited bacterial growth, showing that their antibacterial activity was not affected by complexation with DNA (*figure 25*).^[138]

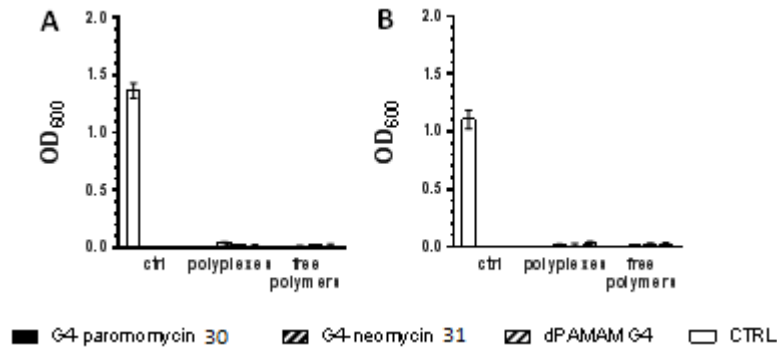


Figure 25. Antibacterial activity of polyplexes and free polymers.

One last property that we wanted to investigate about our conjugates was the transfection efficiency of polyplexes in presence of exogenous bacterial infections. The test was conducted in HeLa cells employing PAMAM G4-derivatives of paromomycin and neomycin, and free PAMAM G4 at N/P 30, giving a polymer concentration of $2.5 \times \text{MIC}$ (5×10^5 cells/mL). After 24h, the cell culture was infected with approximately 5×10^5 cells/mL of *E. Coli* and *S. Aureus* strains. HeLa cells in the absence of bacteria were used as positive control of transfection efficiency and cell viability and a bacterial suspension was used as positive control of bacterial viability. Results are shown in *figure 26*; for the transfection efficiency in presence of *E. Coli*, G4-derivatives **30** and **31** displayed transgene expressions slightly lower than those in the absence of bacterial infection while unconjugated PAMAM G4 exhibited a dramatic decrease in transfection efficiency (*figure 26A*) probably due to the high concentration required (equal to $2.5 \times \text{MIC}$ for *E. Coli* strain) which was very close to its solubility limit. Instead the transfection efficiencies in the presence of *S. Aureus* were equal to those obtained in standard conditions (*figure 26D*). About the cytotoxicity the first thing notable is the higher cytotoxicity of neomycin derivative, but this is caused by the high dose necessary to reach N/P 30 [Value required to compare, in easy way, the polyplexes having the same amount of DNA complexed] (remember that the optimal N/P ratio for neomycin derivative is 5).^[112] Interestingly in presence of *E. Coli* infection our derivatives and free PAMAMG4 are non-toxic while for *S. Aureus* infection the toxicity

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is the same as in non infected cells. Noteworthy, using these conditions, PAMAMG4-paromomycin polyplexes exhibited the greatest transfection efficiency and the lowest cytotoxicity. *Figure 26 C and F* display the results, which we were looking for: PAMAMG4 and G4-derivatives determined the complete inhibition of bacterial growth in the presence of HeLa cells. This is the first time in which transfection and bacterial inhibition have been exploited unequivocally at once for new antibacterial gene delivery vectors.^[138]

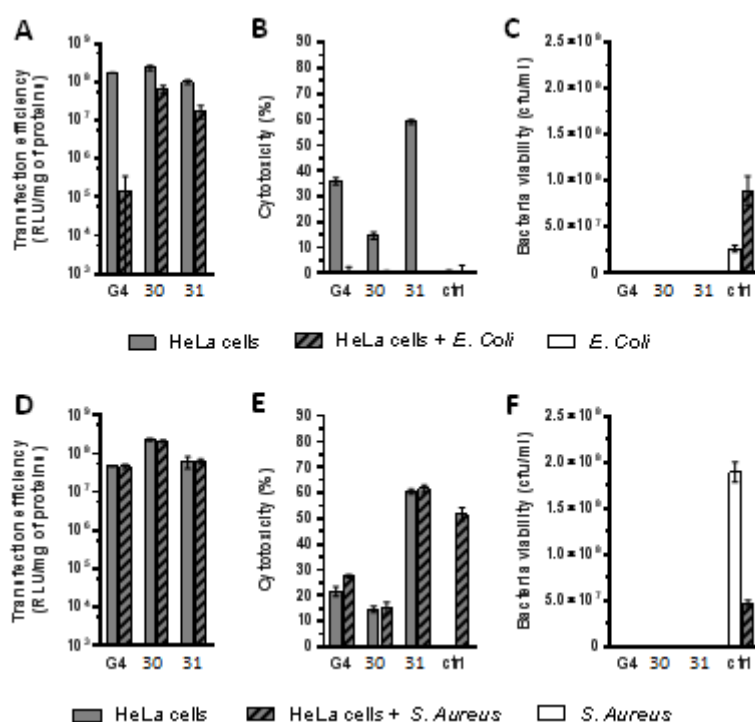


Figure 26. Transfection efficiency, cytotoxicity and antibacterial activity of PAMAMG4, PAMAMG4-neomycin 31 and PAMAMG4-paromomycin 30 polyplexes.

The antibacterial activity of our conjugates could be due to the synergistic cooperation of the two main components of their structure: PAMAM dendrimer and aminoglycosides neomycin and paromomycin. In detail PAMAM G4 is responsible for the initial electrostatic interaction with the anionic bacterial surface, facilitating the uptake of the polyplex. This role is likely to be played by free polymers not engaged in any interaction with pDNA. On the other hand, the conjugated aminoglycoside moieties exert their action by targeting specific biochemical pathways (as the inhibition of prokaryotic protein biosynthesis,

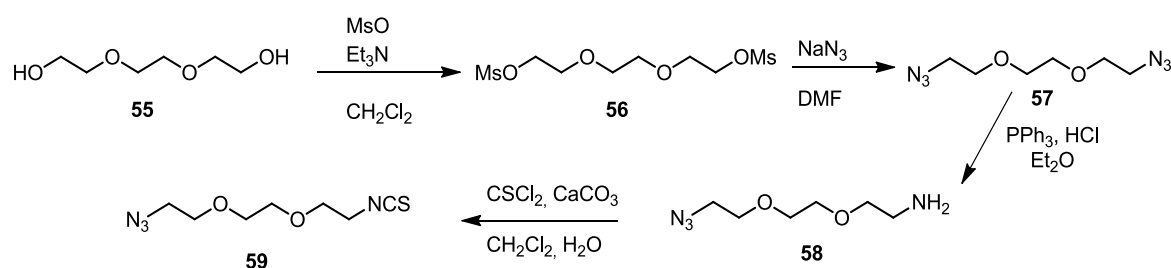
interfering with ribosome and self-promoted uptake)^[140-141]. This was confirmed by the greater antibacterial activity displayed by G4-paramomycin **30** and G4-neomycin **31** with respect to unconjugated PAMAM G4, which is required in a high quantity to induce the inhibition of the pathogen growth. Taken together, these results show that G4-paramomycin and G4-neomycin as extremely effective transfectants with strong antibacterial properties, highlighting their potential in biomedical applications. Indeed the combination of gene transfection and antibacterial activity of PAMAM G4-aminoglycoside conjugates could give an important contribution in their therapeutic efficacy. However, all the properties disclosed, make neomycin and paramomycin derivatives effective multifunctional gene delivery vectors with notable gene delivery efficiency, negligible toxicity and good antibacterial activity.^[112, 138]

3.1.2 PAMAMs-Neomycin and GuanidinoNeomycin Conjugates: Synthesis and Biological Activity

Encouraged by these promising results we have synthesized new multifunctional non-viral vectors, using neomycin and guanidinoneomycin as aminoglycosides and different generations of PAMAM dendrimer. We decided to use neomycin as aminoglycoside taking into account the results obtained from the biological tests, the synthetic pathway and their properties. Indeed, neomycin conjugate **31** has a good transfection efficiency at N/P 5 and a cytotoxicity comparable with the gold standard bPEI, while for paramomycin derivative **30** the optimum N/P is 30 with a toxicity lower respect to the others.^[112] Neomycin possess six free amino group in which two are amino-methylene moiety that give more stable electrostatic interaction with DNA or RNA, and modification occurs at the primary hydroxyl group on ring two. Instead in paramomycin the number of amine is five and the only amino methylene group is employed for the modification tethering to the linker. Moreover, the synthetic strategy used to anchor the linker to the neomycin is more feasible and arose with higher yield respect to the pathway used for paramomycin. The choice to use guanidinylated neomycin is due to recent studies that indicate guanidinylated compounds and in particular guanidinoglycosides as better

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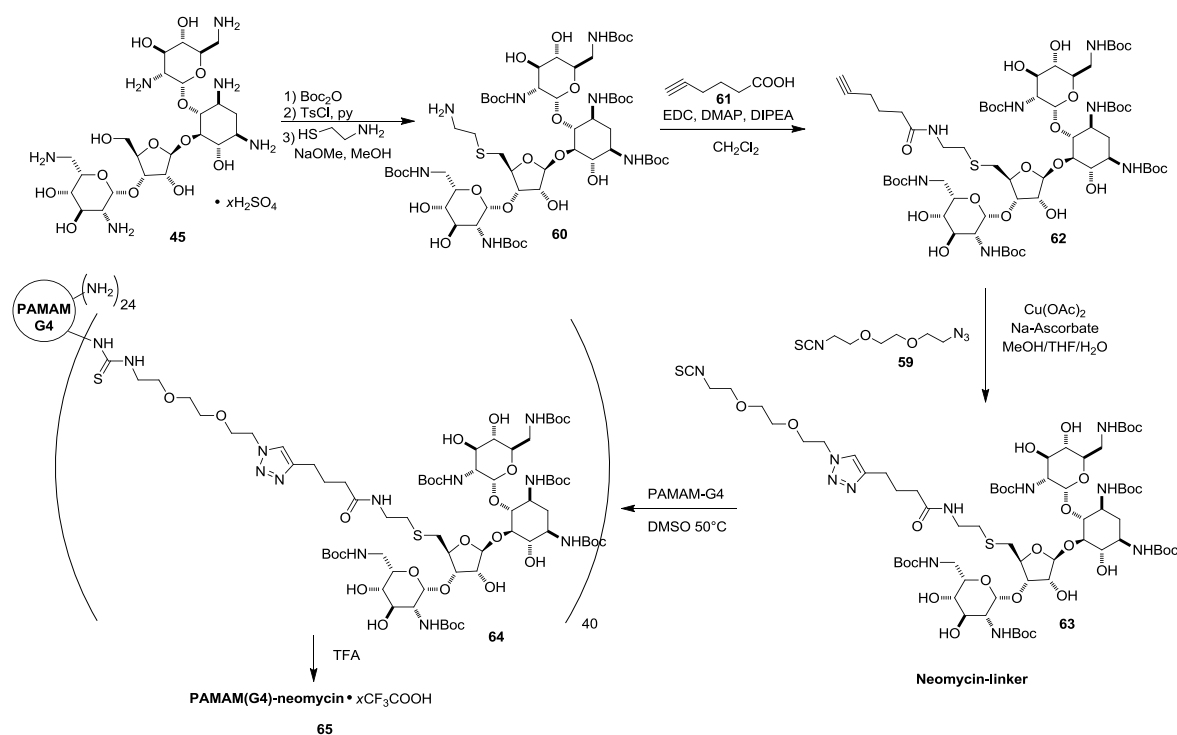
ligands for DNA and RNA; moreover the groups of Prof. Tor and Esko have shown that guanidinoglycosides are able to uptake high molecular weight cargos into the cells.^[99-102,142] For the PAMAM we used generation 2, 4 and 7 having in the order 16, 64 and 516 terminal amino groups respectively. We also used a longer linker, formed from two parts; the first, directly bonded to the neomycin, is the hexynoic acid having a carboxylic moiety to couple the primary amine added on the neomycin and an alkyne for the click reaction. The second part, synthesized in our laboratories as reported in literature^[143], is constituted by a PEG skeleton with an azide terminal group for the click reaction and an isothiocyanate terminal group for grafting the amino groups of PAMAM. *Scheme 6* depicts the synthesis of the second module of the linker; we started from triethylglycol **55** that after mesylation, with MsCl and TEA in DCM of both hydroxyl group, and substitution of mesyl group with azide by nucleophilic substitution with sodium-azide in DMF, has given the compound **57**. Reaction with triphenyl phosphine has converted one azide into amine that, after reaction with thiophosgene, was transformed into the isothiocyanate moiety terminal group.



Scheme 6. Synthesis of linker 59.

The PAMAM-deriveds-Neomycin (Neo) and Guanidinoneomycin (GNeo) conjugates were synthesized by a pathway slightly different than the one showed in paragraph 3.1.1 scheme 5. In this new pathway, starting from commercially available neomycin, the first step was again the protection of all amino groups by di-tert-butylidicarbonate, followed by tosylation. At this point the tosylate derivative of Boc-neomycin described before, was reacted with thio-ethanolamine in order to directly obtain the free amino function, thus saving one step. This reaction was performed using thioethanol amine in presence of NaOMe in EtOH at room temperature, giving compound **60**. Then the amine was coupled with hexynoic acid **61**, in CH₂Cl₂ in presence of EDC and a catalytic amount of DMAP. The

next step was the “click reaction” with the second half of new linker **59**, having an azide and an isothiocyanate group at the extremes, to afford our “new” neomycin-linker intermediate **63**. Again the NCS function was used to anchor the aminoglycoside to the PAMAM dendrimer. This latter reaction was carried out in DMSO at 60 °C for 24 h in order to maximize the degree of grafting. The last step was the Boc deprotection with TFA, that lead at the formation of PAMAMG4-Neomycin conjugate **65** with 63% of grafting (see below) (scheme 7).

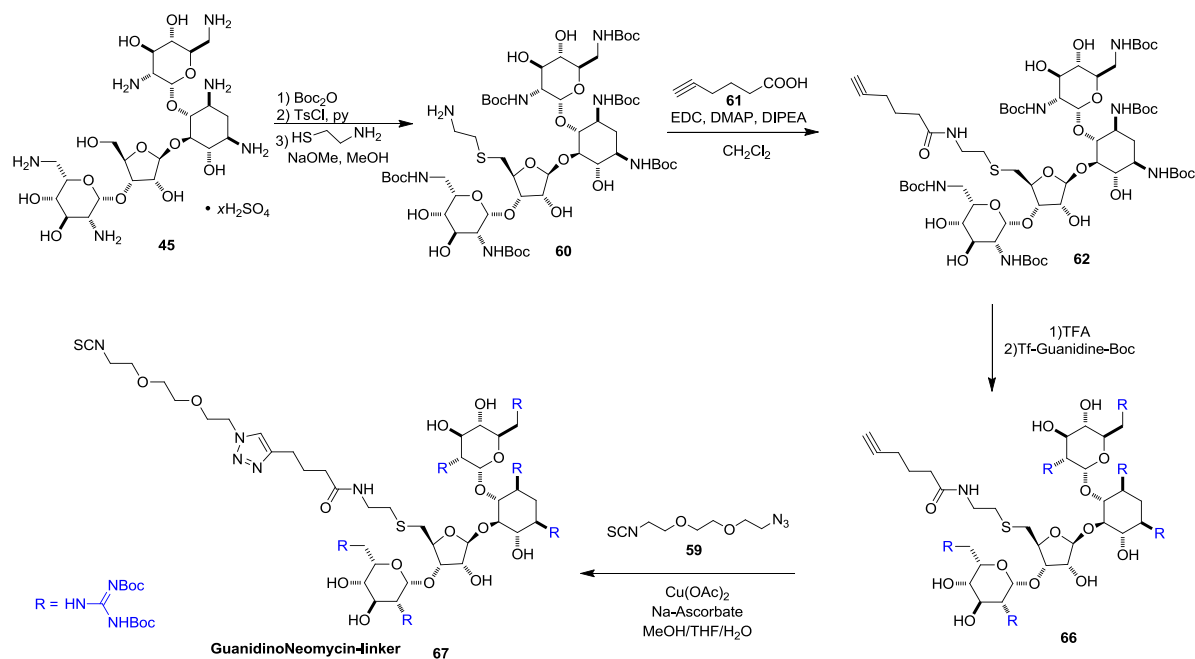


Scheme 7. New Synthesis of PAMAMG4-Neomycin Conjugate **65**.

Using the same reaction condition we decorated all the three diverse generation of PAMAM having different number of amino group on the surface, G2 having 16 NH_2 groups, G4 with 64 NH_2 groups and G7 with 512 NH_2 groups, to get three different conjugates. Furthermore, PAMAM G4 was also decorated with guanidinoneomycin-linker obtained by substitution of all amino groups with guanidine (all the structure of PAMAM conjugates are depicted in *figure 27*). This modification occurred on the intermediate **62** that was deprotected from Boc by TFA, followed by reaction in CH_2Cl_2 with Boc-guanidine-triflate

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leading to the formation of guanidinoneomycin derivate **66** that was submitted to click reaction giving the Gneo-linker **67** (scheme 8).



Scheme 8. Synthesis of Guanidinoneomycin-linker **67**.

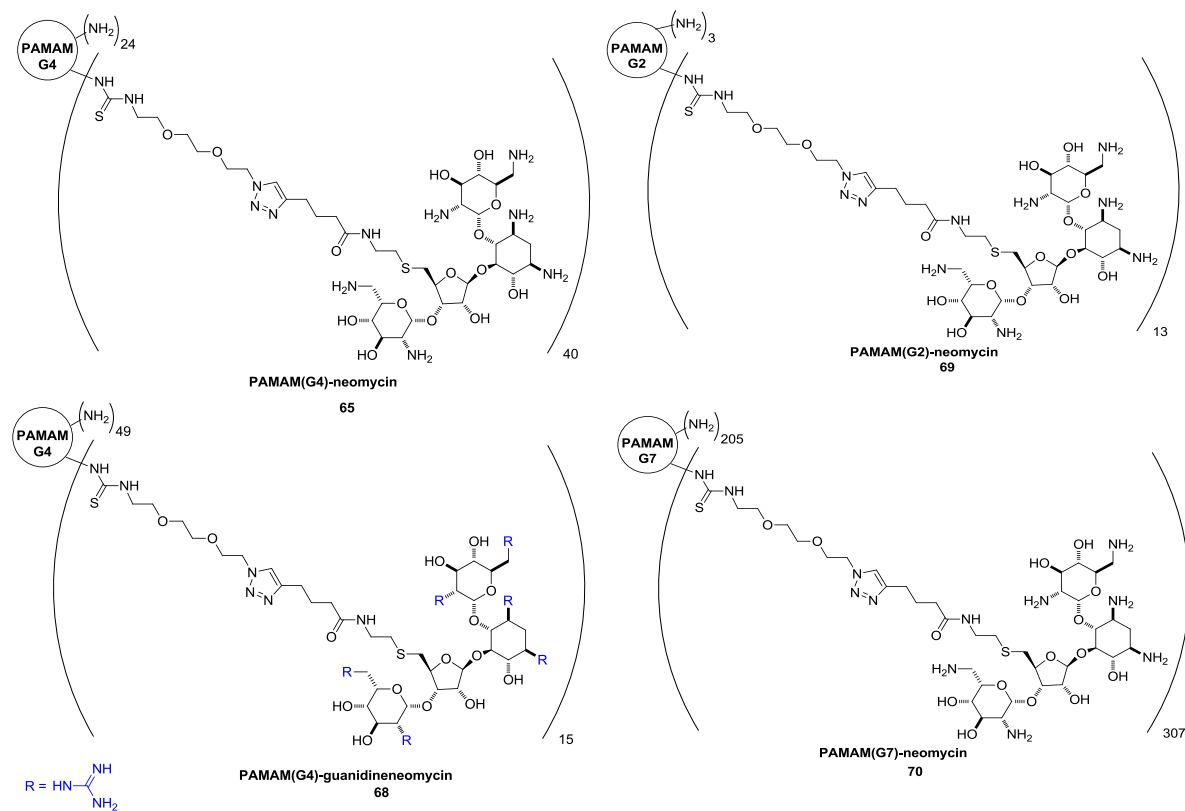


Figure 27. Structure of PAMAMG4-Neomycin, PAMAMG2-Neomycin, PAMAMG4-GuanidinoNeomycin and PAMAMG7-Neomycin conjugates **65**, **68-70**.

As before, the average degree of grafting was determined by ^1H NMR integration, as previously described, and supported by MALDI spectroscopy (with the only exception for G7). Briefly, the characteristic signals compared are: a) the signal of the proton in α position of the carbonyl group in PAMAM dendrimer around 2.76 ppm; b) the signal of two characteristic geminal protons belonging to the 2-desoxystreptamine ring of the aminoglycoside which resonate at 2.43 and 1.81 ppm; c) the signal of three anomeric protons belonging to the neomycin between 6.15 and 5.15 ppm; and d) the single proton of the triazole ring of the linker at ca. 8.00 ppm. With the integration of these protons we calculated the averaged degree of grafting: conjugate **65** with degree of grafting 63% (40 amino groups over 64 were functionalized *figure 28b*); conjugate **69** with a degree of grafting $\sim 82\%$ (13 amino group functionalized over 16 *figure 28a*); conjugate **68** with degree of grafting 23% (15 amino group functionalized *figure 28c*); and conjugate **70** with a degree of grafting 60% (307 neomycin-linker tethered over 512 amino groups *figure 27d*). The degree of grafting was also confirmed by MALDI spectroscopy for conjugate **69** with a peak at 16030 Da corresponding to PAMAM G2 plus 13 neomycin-linker grafted (*figure 29a*); for conjugate **65** the detected $m/z = 39493$ Da corresponds to the 40 neomycin-linker tethered to PAMAM G4 (*figure 29b*); conjugate **68** showed a peak at 31776 Da corresponds to PAMAMG4 plus 15 guanidinoneomycin-linker tethered (*figure 29c*); while for conjugate G7 was not possible obtain MALDI spectra.

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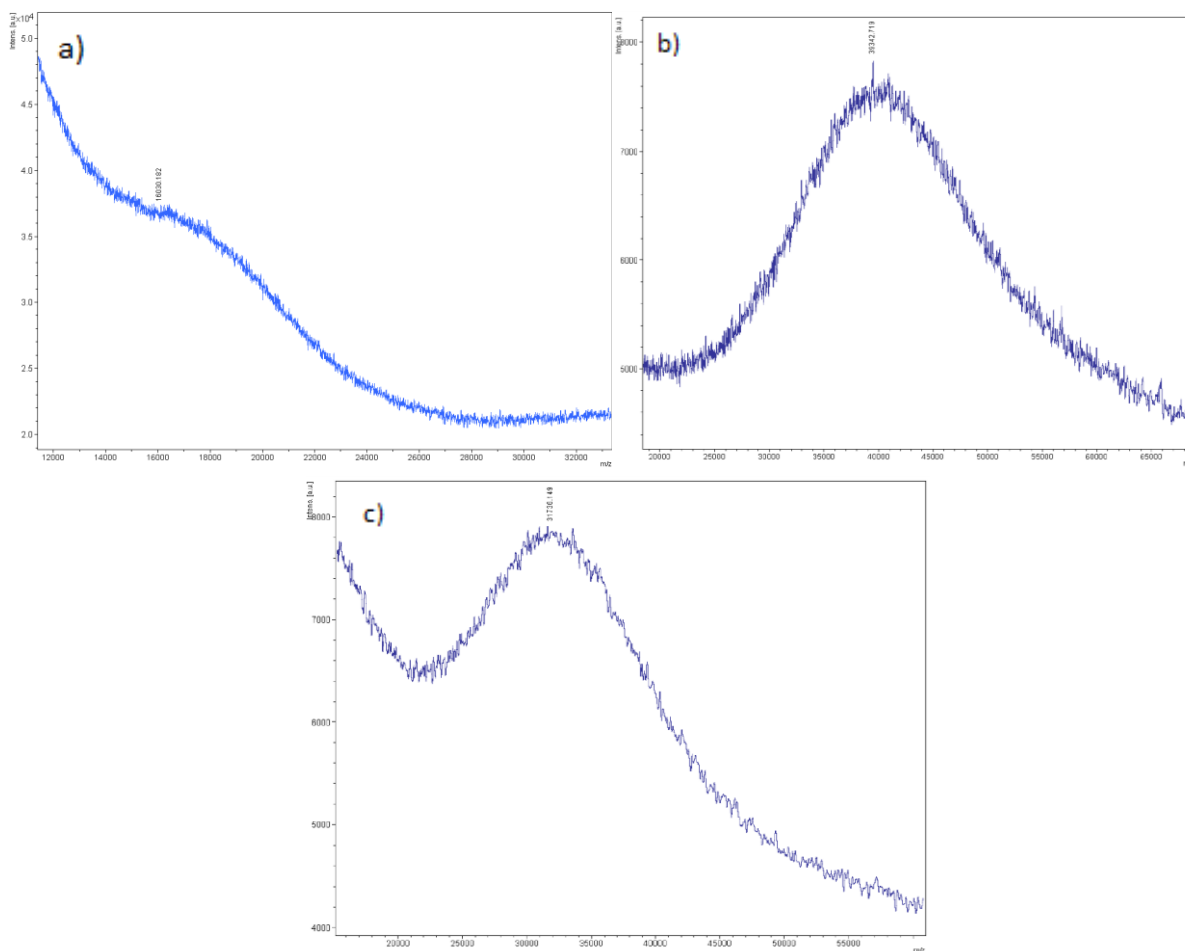


Figure 29. The MALDI spectra of: a) PAMAMG2-neomycin conjugate 69, peak at 16030 Da corresponds to the single charged ion; b) PAMAMG4-neomycin conjugate 65, peak at 39493 Da corresponds to the repeated 40 neomycin-linker grafted; c) PAMAMG4-guanidinoneomycin conjugate 68, peak at 31776 Da corresponds to PAMAMG4 plus 15 guanidinoneomycin-linker tethered.

In collaboration with research group of Prof. Candiani, we studied the conjugates' behavior in DNA complexation, transfection efficiency and cytotoxicity. *Figure 30* depicts the complexation ability of our conjugates as function of nitrogen to phosphate ratio. Due to their globular shape and presence of peripheral amino groups, PAMAM dendrimers and PAMAM-Neo and Gneo derivatives possess very good complexation skills, with a maximum pDNA complexation around $N/P \geq 1$ for PAMAMs G2, G4, G7 and PAMAMG4-Neo conjugate and $N/P \geq 2$ for PAMAMG2-Neo, PAMAMG4-Gneo and PAMAMG7-Neo.

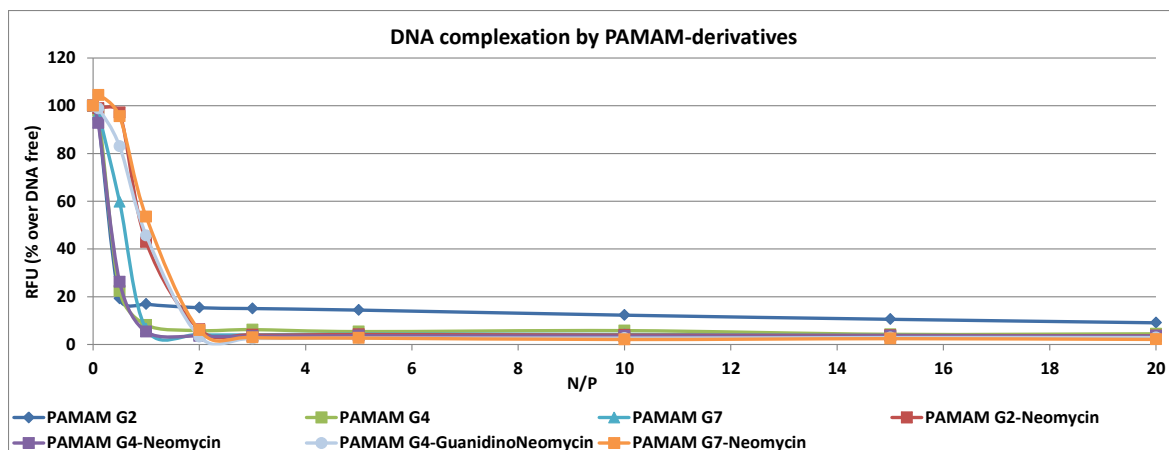


Figure 30. DNA complexation of all free PAMAMs generations, PAMAMG2-Neomycin, PAMAMG4-Neomycin, PAMAMG7-Neomycin and PAMAMG4-Guanidinoneomycin Conjugates.

Evaluating the transfection efficiency of these compounds in HeLa cells, have shown that PAMAMG4-Neo conjugate **65** is the best carrier at each N/P ratio compared to the corresponding PAMAMG2 and G7 derivatives **69, 70**. Interesting for PAMAMG4-Neo the luciferase activity increases until N/P 30 then decrease a little and a similar behavior is showed for the all generation 4 (PAMAMG4 and PAMAMG4-guanidinoneomycin). Instead for the other generation a constant increase until the maximum N/P ratio used, 75, is observed. Moreover, at N/P ratio 15, all the neomycin and guanidinoneomycin conjugated showed better transfection efficiency than the gold standard 25 kDa bPEI (*figure 31*). A similar profile is reflected on the results on cytotoxicity, assessed by AlamarBlue cell viability assay; at elevate N/P ratio PAMAMG4-Neo conjugate has the higher toxicity respect to the other conjugates that are almost non-toxic. Instead at N/P ratio around 5 - 15 the value of cytotoxicity of PAMAMG4-Neo is similar to the gold standard 25 kDa bPEI (*figure 32*).^[144]

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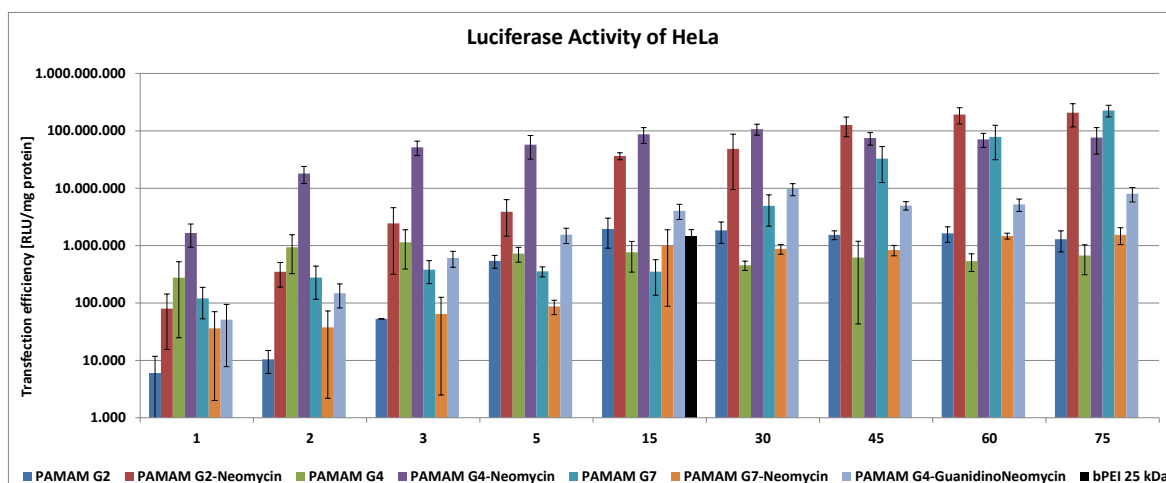


Figure 31. Preliminary results of Transfection efficiency of PAMAMG2-Neomycin, PAMAMG4-Neomycin, PAMAMG7-Neomycin and PAMAMG4-Guanidinoneomycin Conjugates in HeLa cells.

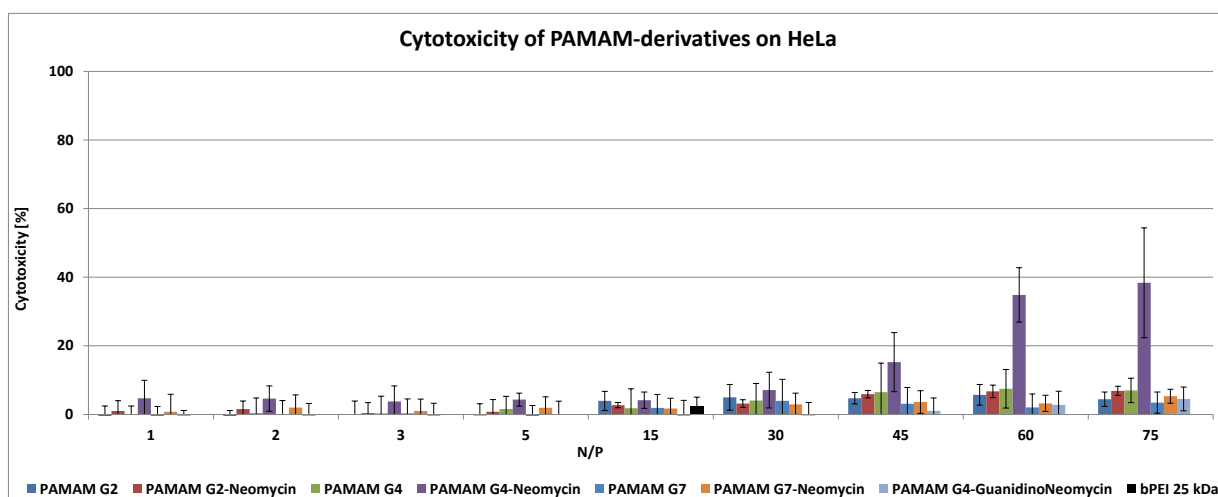


Figure 32. Preliminary results of Cytotoxicity of PAMAMG2-Neomycin, PAMAMG4-Neomycin, PAMAMG7-Neomycin and PAMAMG4-Guanidinoneomycin Conjugates in HeLa cells.

Transfection efficiency studies, of our conjugates in Cos-7 cells, assessed using a luciferase reporter system, show a profile similar to that of HeLa cells. As expected increasing the N/P ratio increase also the transfection efficiency but, at the same time, the cytotoxicity becomes higher (*figures 33 and 34*). Anyway, PAMAMG4-Neo conjugate **65** is the best carrier at each N/P ratio compared to the corresponding G2- and G7-PAMAM derivatives and the free PAMAMs. Moreover, at N/P ratio 15, all the neomycin and guanidinoneomycin showed better transfection efficiency than the gold standard 25 kDa

bPEI. An analogous contour is reflected on the results on cytotoxicity; at elevate N/P ratio PAMAMG4-Neo conjugate has the higher toxicity respect to the other conjugates that are almost non-toxic, followed by PAMAMG7 and PAMAMG4-Gneo derivative. Instead at N/P ratio around 5 - 15 the value of cytotoxicity of PAMAMG4-Neo is lower than the gold standard 25 kDa bPEI (*figure 34*).^[144]

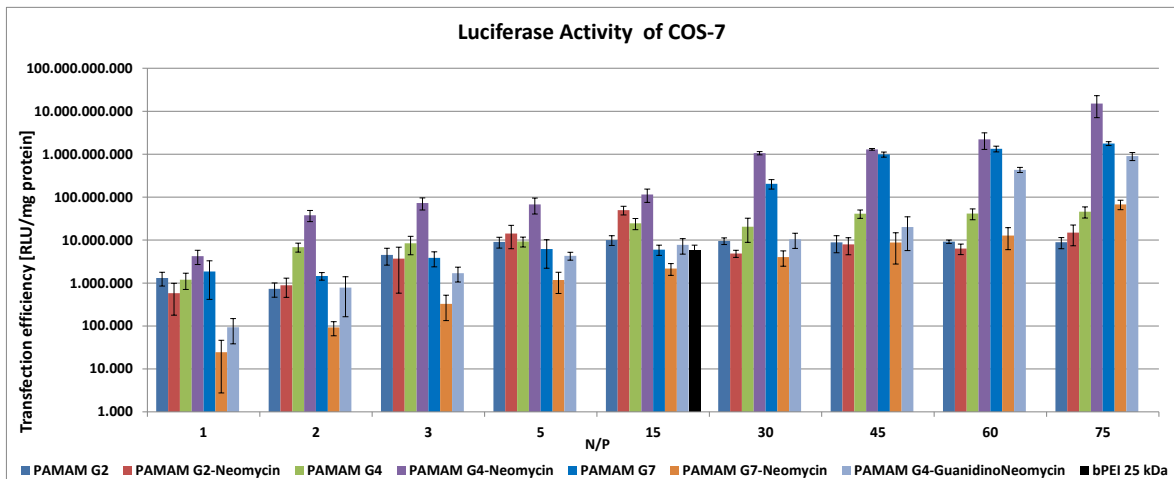


Figure 33. Transfection efficiency of PAMAM G2,G4,G7, PAMAMG2-Neomycin, PAMAMG4-Neomycin, PAMAMG7-Neomycin and PAMAMG4-Guanidinoneomycin Conjugates in COS-7cells.

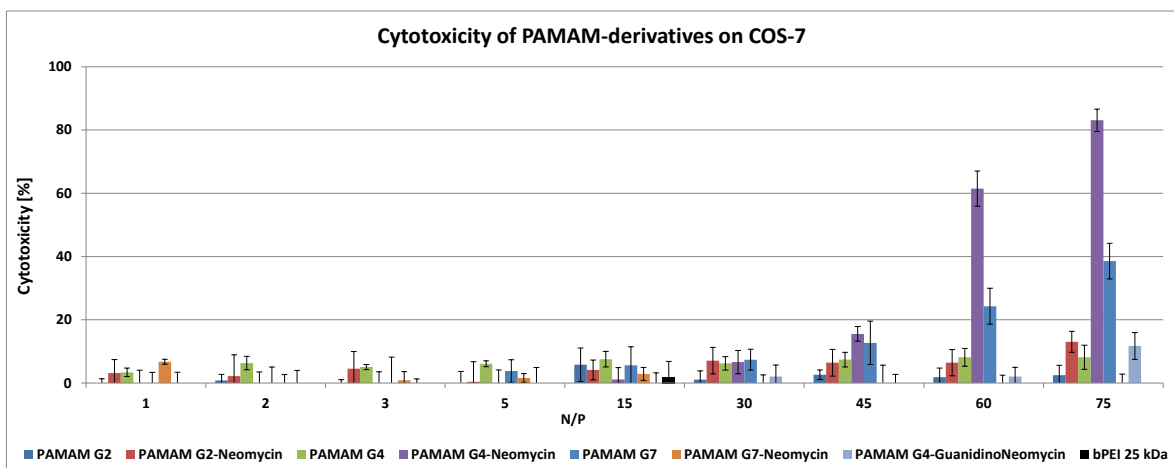


Figure 34. Cytotoxicity of PAMAM G2,G4,G7, PAMAMG2-Neomycin, PAMAMG4-Neomycin, PAMAMG7-Neomycin and PAMAMG4-Guanidinoneomycin Conjugates in COS-7cells.

All these results displayed that the PAMAMG4-neomycin conjugate **65** is the gene delivery vector with a highest transfection efficiency followed by PAMAMG2-neomycin conjugate

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69. Unexpectedly PAMAMG4-guanidinoneomycin does not have the high efficacy that we supposed having the guanidinium group to bind DNA and interact with the cell. Probably the high number of amino groups on the surface does not enhance the transfection efficiency and a similar explanation is valid for the PAMAMG7-neomycin conjugate **70.** However these results highlight the ability of these conjugates as good transfectants and as potential multifunctional delivery vectors.

3.1.3 Synthesis of calix[4]arene-aminoglycoside Conjugates

To prepare new multifunctional non-viral vectors, we also studied the employment of lipidic carriers keeping in mind the recent work of Lehn and co-workers. They have synthesized cationic lipids, for gene transfer studies and gene therapy applications, formed by a lipidic tail and a cationic head. For the lipid part they used cholesterol and/or alkyl chains and for cationic part aminoglycosides due to the transfection potential of this family. Thus, these cationic lipidic vectors rely on the formation of DNA/lipid aggregates via electrostatic interactions between their cationic head group and the negatively charged DNA. Some example of these vectors are: 1) kanamycin-cholesterol (kanaChol-6') and triguanidinokanamycin-carbomyl-cholesterol (TGKC) (*figure 35*) that are cholesterol derivatives in which the aminoglycoside and guanidinoglycoside head group is linked to the cholesterol moiety via a carbamoyl bond, that combine the membrane compatible features of the cholesterol subunit and the favorable features of amino- and guanidinoglycosides for DNA binding. These carriers possess an efficient *in vitro* and *in vivo* gene transfection when used either alone or as a liposomal formulation with the neutral phospholipid dioleoyl-L- α -phosphatidylethanolamine (DOPE).^[145-148] 2) cholesterol-neomycin (CholNeo) and cholesterol-paromomycin (Chol-Paromo) being cholesterol derivatives synthesized to investigate the different behavior in transfection efficiency among different aminoglycosides (*figure 36*). These compounds showed water solubility which favors the formations of micelles and showed increased *in vitro* and *in vivo* transfection efficiency adding in the formulation the co-lipid DOPE. 3) dioleoyl-succinyl-paromomycin (DOSP),

dioleyl-succinyl-neomycin (DOSN), and dimeric paromomycin derivative P2 that are conjugates with lipid part formed by *N*-succinyl-dioleylamine as alkyl chain and different aminoglycosides used to verify which other aminoglycosides can be used as cationic head group (figure 37). The dioleyl derivatives, as well as the cholesterol derivatives when they are formulated with DOPE, did indeed mediate high transgene expression both in vitro in different cell lines and in vivo in the mouse airways.^[149]

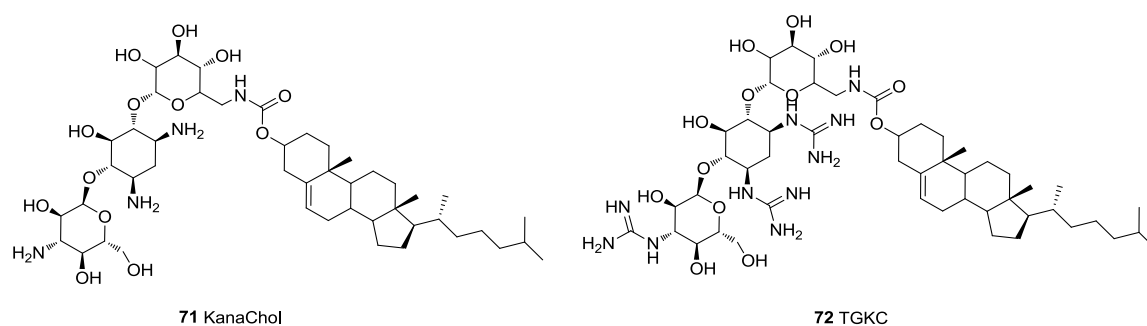


Figure 35. Kanamycin-cholesterol (*kanaChol-6'*) **71** and triguanidinokanamycin-carbonyl-cholesterol (TGKC) **72**.

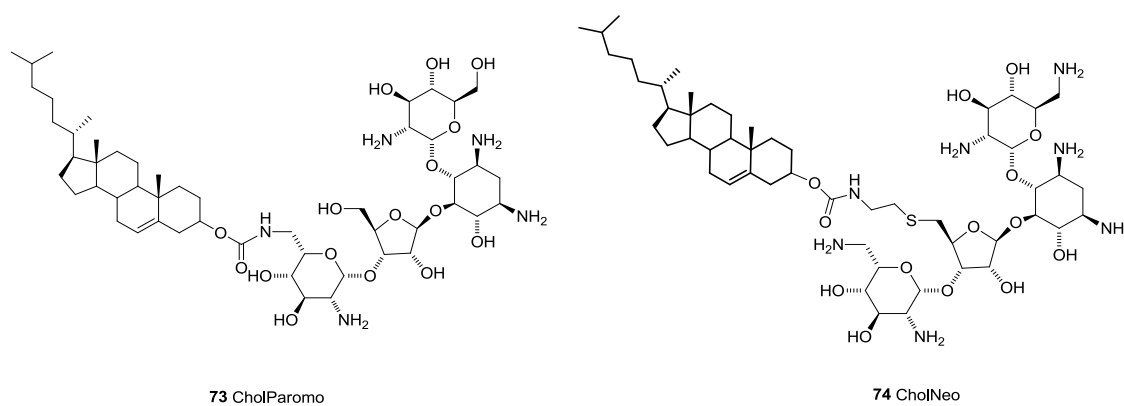


Figure 36. Cholesterol-neomycin (*CholNeo*) **73** and cholesterol-(paromomycin *Chol-Paromo*) **74**.

3.1 Gene Delivery Project

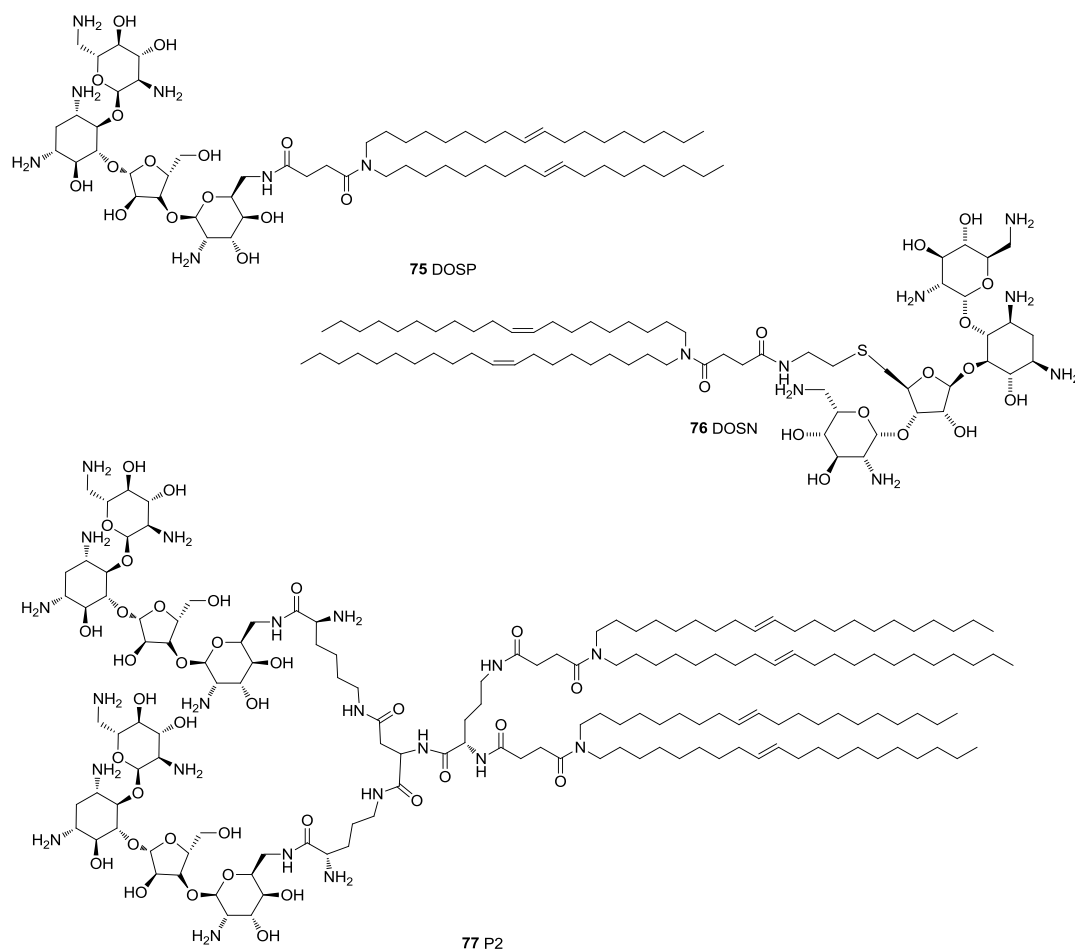


Figure 37. Structures of dioleyl-succinyl-paromomycin (DOSP) **75**, dioleyl-succinyl-neomycin (DOSN) **76**, and dimeric paromomycin derivative P2 **77**.

Considering these studies on cationic lipidic carriers, we decide to study new aminoglycosides-based lipidic carriers by using a calix[4]arene as lipophilic moiety, in collaboration with the group of prof. Francesco Sansone of University of Parma. Calixarenes are macrocycle or cyclic oligomers produced by phenols-formaldehyde condensation in basic conditions. Their name derives from two words: “calix” from Latin meaning cup and arene due to the presence of aromatic rings in their structure. The phenolic ring can define a hydrophobic cavity and it is possible to distinguish a narrow or lower rim where are located the phenolic oxygens and a wide or upper rim identified by the para positions of the aromatic nuclei. Both rims can be easily modified (*figure 38*) with a wide variety of functional groups and residues.^[150]

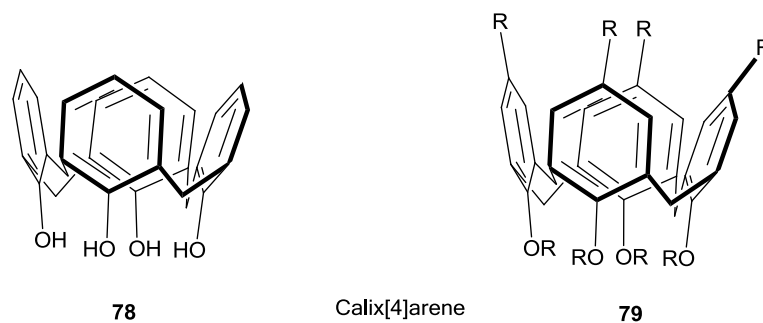


Figure 38. General structure of calix[4]arenes.

Prof. Sansone's research group has synthesized different kind of calixarenes modifying the lower rim with different type of alkyl chains and upper rim with guanidinium groups, to study their potential as transfectants. They found the best combination in guanidinocalix[4]arene having an hexyl chain at the lower rim and guanidinium groups at the upper rim. Indeed these compounds have shown a good water solubility and ability to bind and internalize linear and plasmid DNA, also in absence of co-lipid as DOPE (*figure 39*).^[151-153, 110] Moreover, to improve the transfection efficiency and lower the cytotoxicity, they have synthesized calix[4]arene containing the four guanidinium groups at the lower rim, attached through a three carbon atom spacer, and different alkyl substituents at the upper rim finding the best efficiency for calix[4]arene lacking alkyl chains at the upper rim (*figure 39*).^[154,155] Moreover they have also employed calix[4]arene as macrocyclic platform to anchor arginine or lysine units on the upper or lower rim investigating their ability in gene delivery. The results have indicated that argininocalix[4]arene with arginine at the upper rim and hexyl chain at the lower rim (*figure 39*), showed the highest DNA complexation and transfection efficiency respect to the others, displaying it as potent non-viral lipidic vectors. The clustering of only four units of basic amino acids, such as arginine and lysine, on a rigid macrocyclic scaffold like the cone calix[4]arene, displays two spatially well-defined regions, one apolar at the lower rim and one polar at the upper rim. These regions with their rigid circular shapes provided in this ensemble a remarkably increase of the cell penetrating properties that arginine usually shows in linear oligomeric sequences and arginine-rich peptides giving rise to new, potent non-viral vectors for cell transfection.^[156]

3.1 Gene Delivery Project

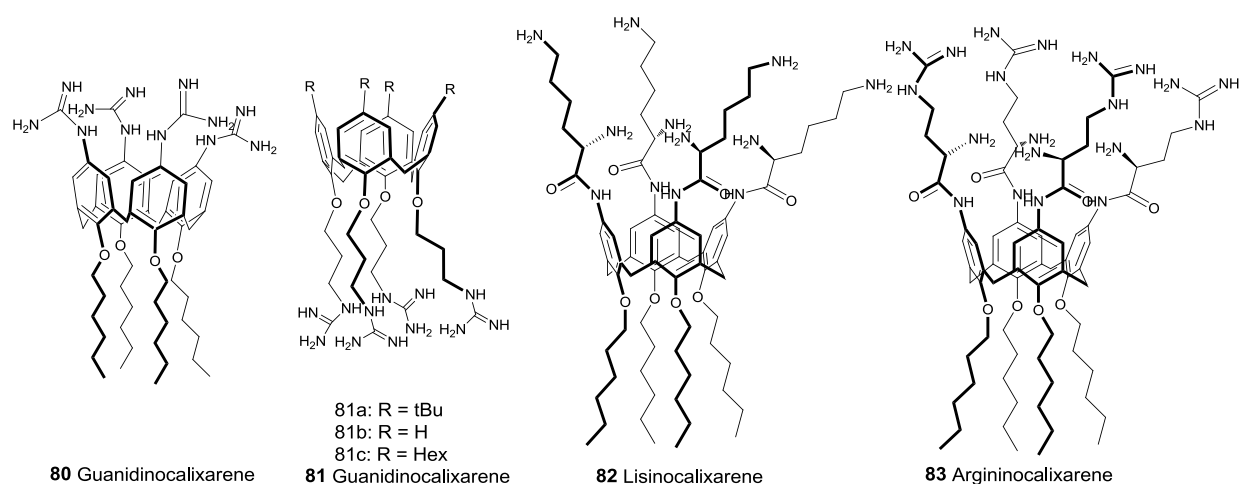


Figure 39. Structures of calixarenes synthesized by prof. Sansone and coworkers.

To investigate if the multifunctionality of aminoglycosides can improve the efficiency of calixarenes as vectors, we have prepared new cationic lipid carriers using calix[4]arenes as lipidic vector grafted with three aminoglycosides-linker units (neomycin, paromomycin- and neamine-linker), to obtain calix[4]arene-aminoglycoside conjugates. The structures of our lipidic vectors are showed in *figure 40*. The synthetic pathway for the aminoglycosides-linker is the same described for PAMAMGX-Neomycin conjugates, and PAMAMG4-Paramomycin conjugate. In this latter case the linker is again the hexynoic acid **61** clicked to azide-isotiocyanate compound **59**. Decoration of the calixarene was performed using 1.2 eq. of aminoglycoside linker for each amino group of lipidic vector **84**, in DMSO at 40°C for 24h. After dialysis, deprotection and liophylization we obtained the three different fully decorated lipocarriers **86**, **87** and **88** (*schemes 9-11*).

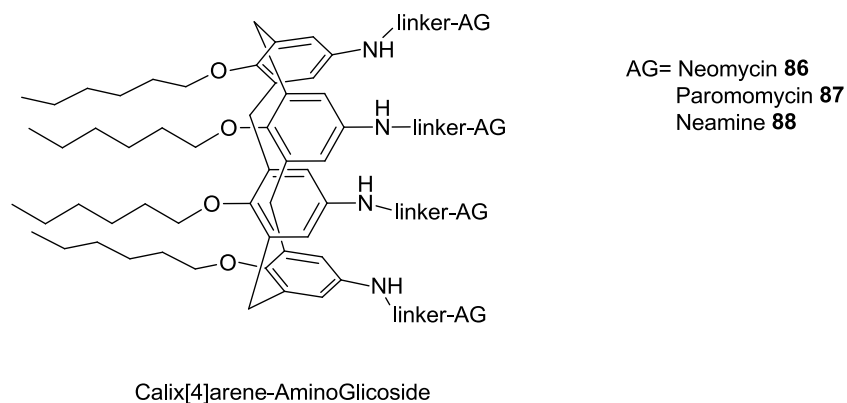
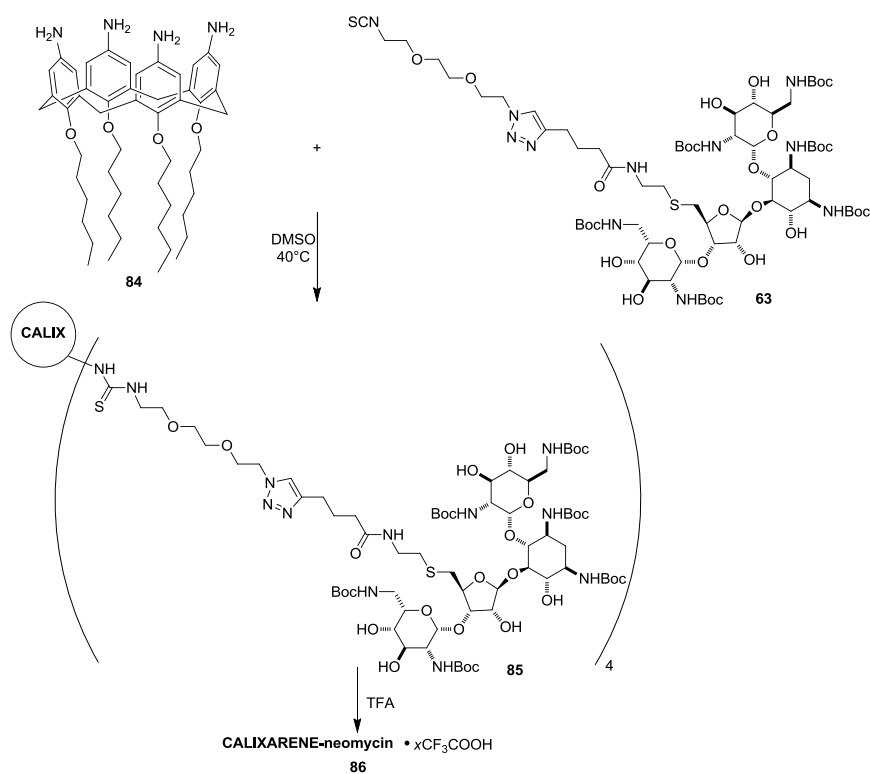
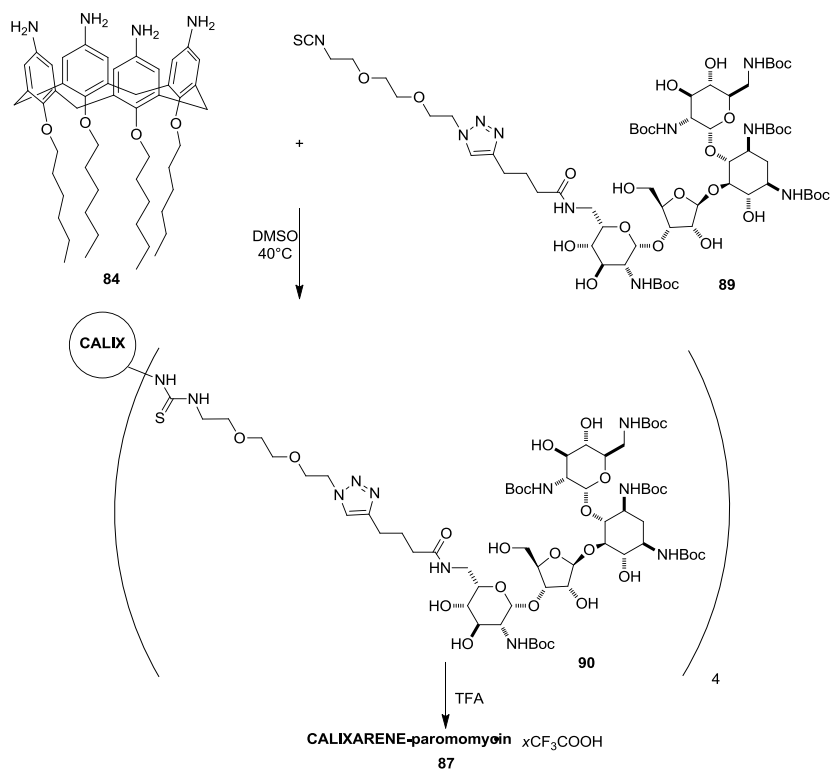


Figure 40. Schematic structure of calix[4]arene-aminoglycoside conjugates **86-88**.

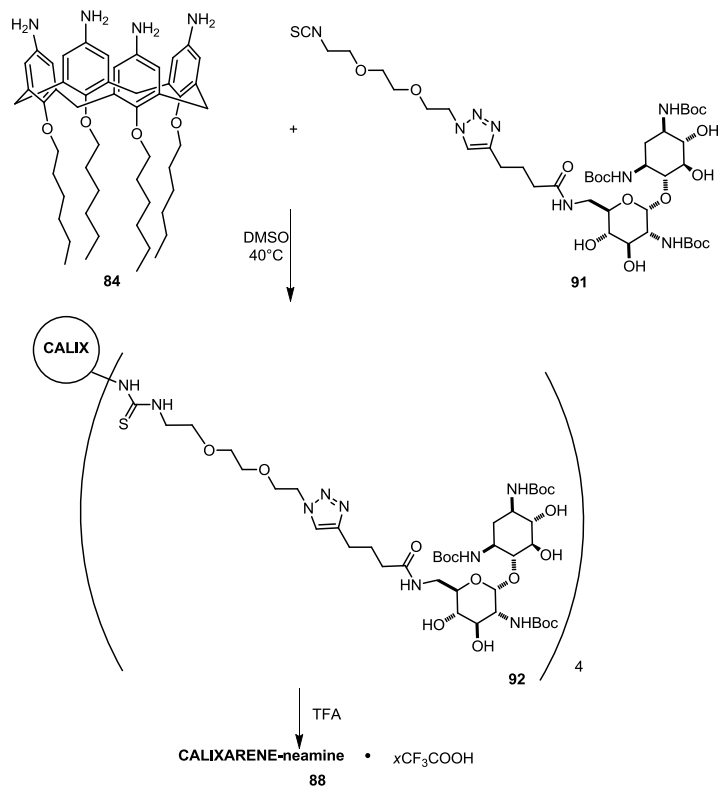


Scheme 9. Synthesis of Calixarene-neomycin conjugate **86**.

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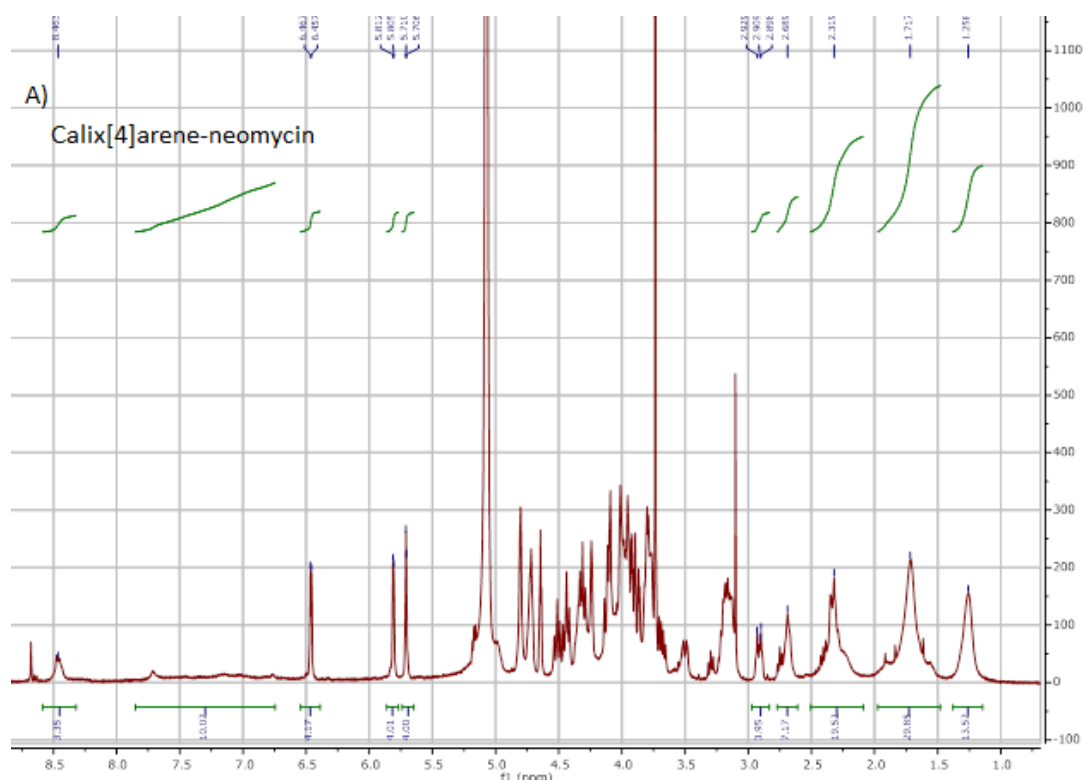


Scheme 10. Synthesis of Calixarene-paromomycin conjugate **87**.



Scheme 11. Synthesis of Calixarene-neamine conjugate **88**.

The degree of grafting was determined by ^1H NMR spectra comparing the characteristic signals of calixarene and aminoglycosides. *Figure 41A* shows the spectra of calix[4]arene-neomycin conjugate **86** as representative of these calixarene-aminoglycosides conjugates, recorded in D_2O . The characteristic signals are: a) the single proton of the triazole ring of the linker at ca. 8.5 ppm; b) the signal of three anomeric protons belonging to neomycin between 6.46 and 5.70 ppm; and c) the broad singlet of CH_3 proton belonging to terminal hexyl moiety of calixarene at 1.25 ppm. So, integrating the signal in the same order described above, the ratio is 4:4:12 that mean that there are 4 neomycin tethered to the calixarene. Similar considerations have been made for calix[4]arene-paromomycin **87**- and for calix[4]arene-neamine conjugate **88**. In this latter conjugate the number of anomeric proton is one for each neamine grafted, peak resonating at 5.77 ppm; the integration also this time reveal that there are 4 neamine grafted to the calixarene. Noteworthy, the ^1H NMR signals of aromatic protons are very broad. These broad signals are probably due to the amphiphilic nature of the three compounds obtained and the supramolecular interections between these molecules, which arise at the concentration required for the NMR acquisition.



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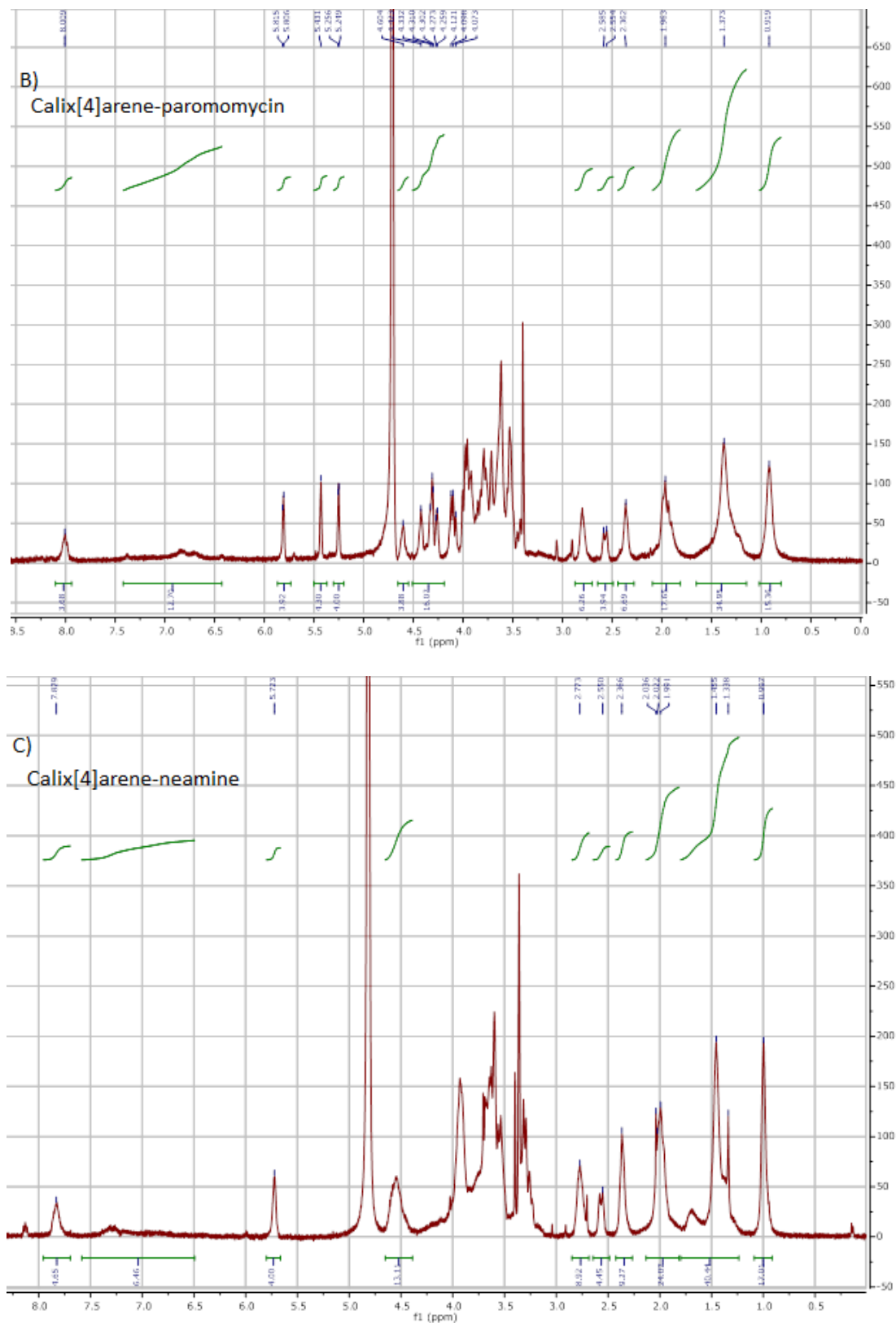


Figure 41. ¹H NMR spectra recorded at 400 MHz and 305 K in D₂O of A) calix[4]arene-neomycin, B) calix[4]arene-paromomycin, C) calix[4]arene-neamine conjugates **86-88**.

Biological assays on these carriers are still ongoing in our laboratories.

3.1.4 Hybrid lipodendrimers-aminoglycoside Conjugates: Synthesis and Biological Activity

The last project about gene delivery is in collaboration with Prof. Ling Peng at CNRS of Marseille, and relies on the synthesis of hybrid lipodendrimers decorated with aminoglycosides. Prof. Peng and her research group synthesized these new kind of vectors with the idea to put together the benefit for the endosomal release of the lipidic vectors, thanks to their membrane fusion mechanism, and the dendrimers with their proton sponge effect.^[31,157] So, they prepared these lipid/dendrimer hybrids having a hydrophobic alkyl chain, that can be shorter or longer for different compounds, and a low generation of hydrophilic PAMAM dendron (*figure 42*).^[158-160]

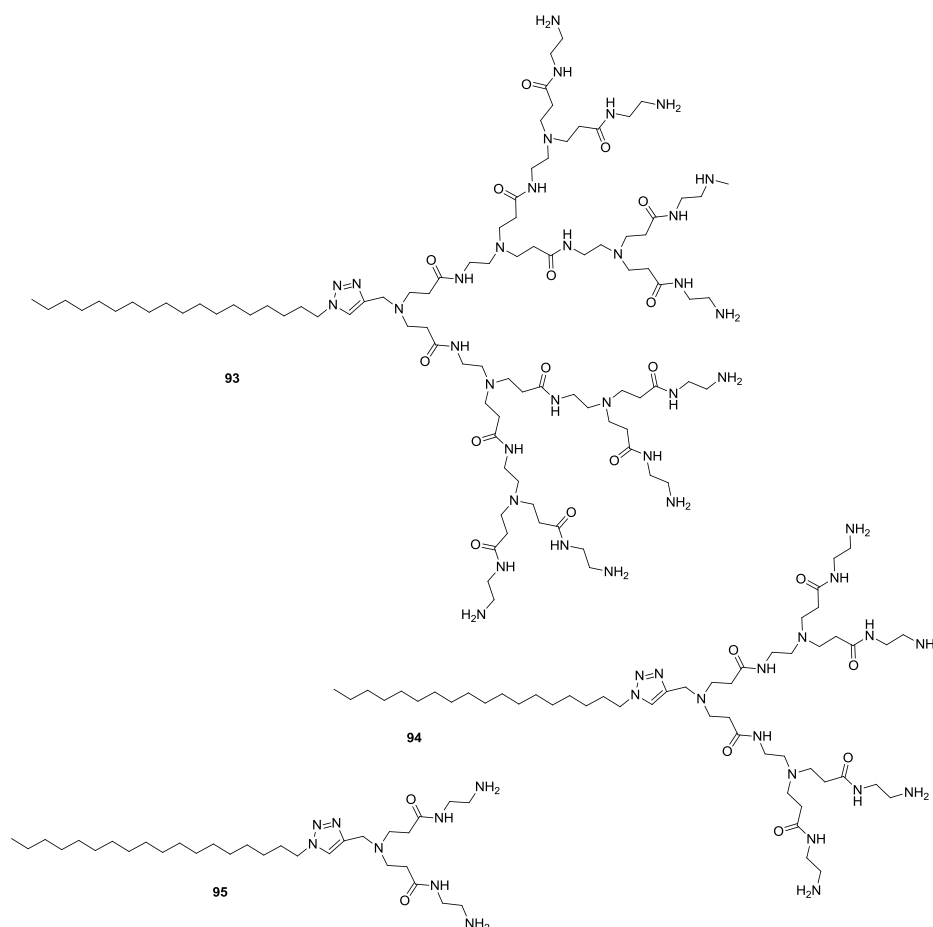
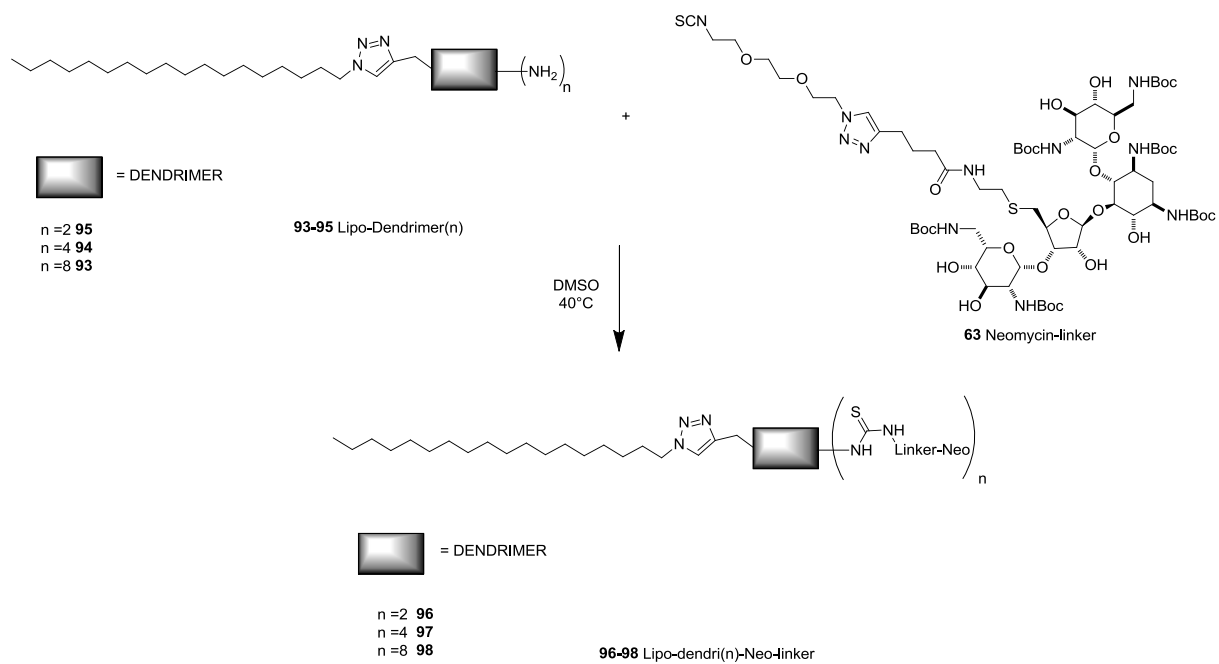


Figure 42. Structures of Hybrid-lipo-dendrimers.

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Moreover, these hybrids have shown their great ability as delivery carriers due to the advantages of either lipo and dendrimeric parts, and mediate a specific and effective siRNA-based gene silencing, in particular for prostate cancer cells.^[161-163] Diverse studies revealed a relation between the length of the lipid moiety, the generation of the dendron and their ability as delivery vectors. As well hybrid lipid/dendrimer with a long alkyl chain and a low, but not the lowest, generation of PAMAM were disclosed as better carriers with a good interaction with siRNA. Its excellent delivery ability is due to the combined effect of the hydrophobic alkyl chain and the hydrophilic PAMAM dendron, since neither the alkyl chain alone nor the dendron part alone led to any gene silencing effect. Additionally, the amphiphilic dendrimers with either shorter alkyl chains or lower generation dendrons failed to elicit significant gene silencing, indicating that both PAMAM dendron size and alkyl chain length are important factors impacting the delivery efficiency and the capacity to form stable self-assembled complexes. Moreover the structure of hybrid **93** (n8) (n indicate the number of amino groups present in the structure) seems therefore to offer an optimal balance between the alkyl-chain-induced hydrophobicity and the dendrimer- induced hydrophilicity and cooperativity, resulting in superior siRNA delivery activity. ^[111,164] We therefore decided to use these hybrid systems synthesized by Prof. Peng and co-workers, to investigate the ability in gene delivery of the new hybrid lipodendrimer-neomycin conjugates by grafting neomycin aminoglycoside. The hybrid structure we used possesses a lipidic long alkyl tail and a dendrimeric head of different generations, having different numbers of free terminal amines. Thus, using three generations possessing 2, 4, and 8 amino groups, the lipodendrimers were tethered with our neomycin-linker **63** following the general procedure described above, i.e. DMSO at 40°C, obtaining the lipo-dendrimer-Neomycin conjugates **96-98** (scheme 12). A schematic structure of these conjugate is reported in *figure 43*.



Scheme 12. Synthesis of Lipo-dendrimer(n)-Neomycin Conjugates.

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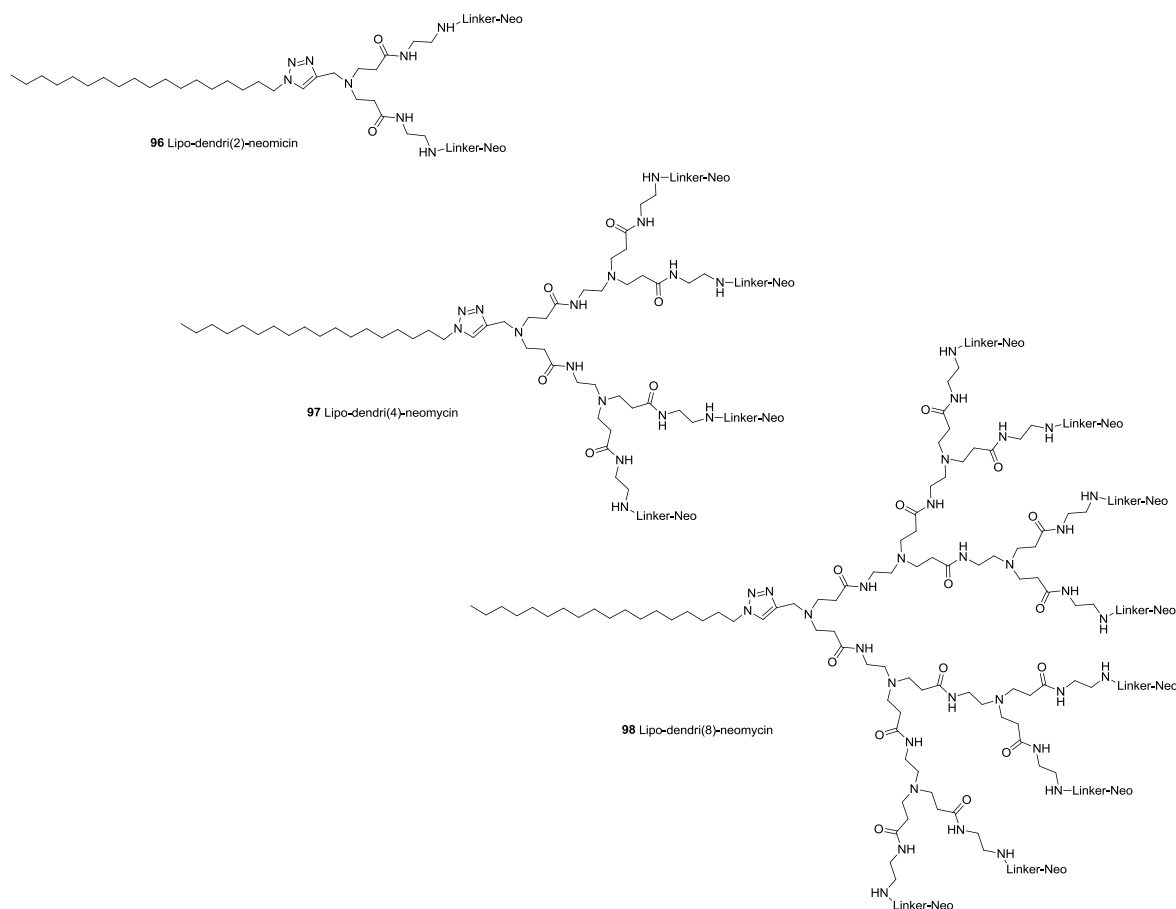
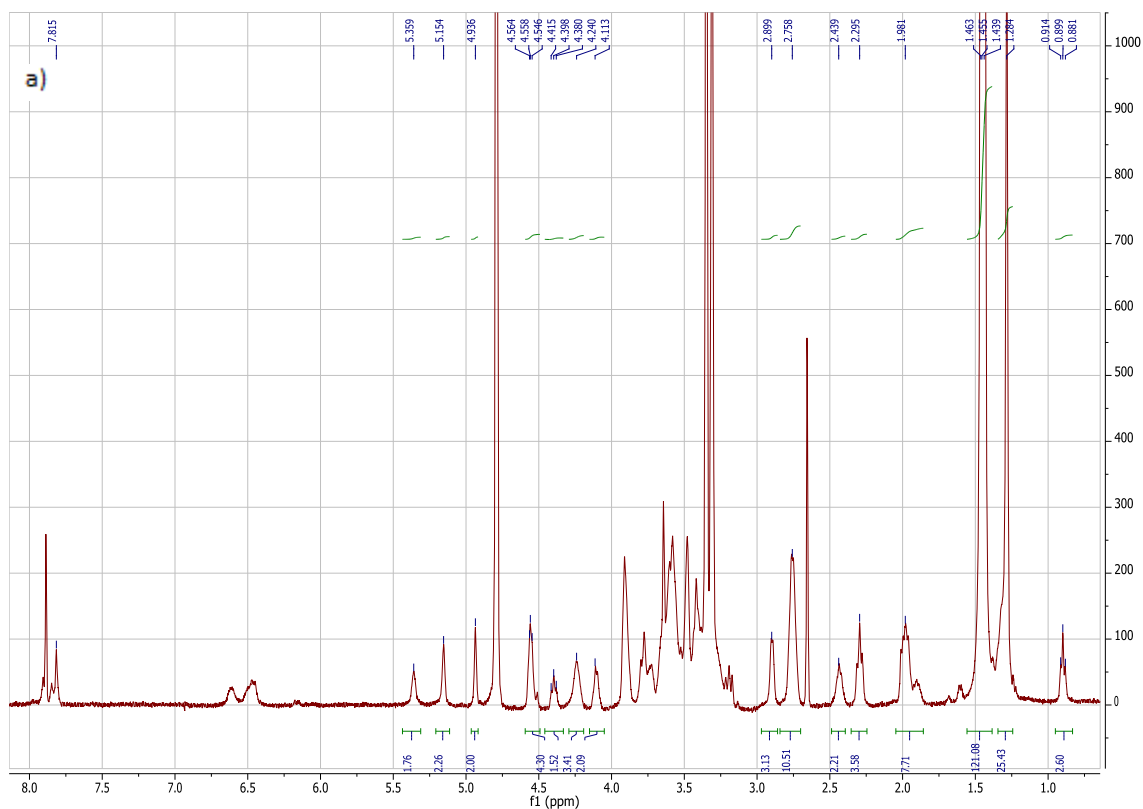


Figure 43. Schematic structures of lipo-dendrimeric-neomycin conjugates **96-98**.

The degree of grafting of these conjugates was determined by ^1H NMR spectroscopy considering the integration ratios of peaks belonging to characteristic protons of the dendrimer and the neomycin. *Figure 44* shows the spectra of final conjugates **96-98**. In particular, we considered 1) the triplet resonating between 0.5 and 1.0 ppm belonging to the terminal CH_3 of the dendrimer (3 H), 2) the broad signal resonating between 1.20 and 1.40 belonging to 15 CH_2 of the alkyl chain of the dendrimer (30 H), 3) the triplet resonating around 4.40 ppm belonging to the CH_2 in alfa position respect to the nitrogen of the triazole ring of the dendrimer (2 H), and 4) the three singlets resonating between 5.40 and 4.90 belonging to the three anomeric protons of the aminoglycoside (1 H for each signal for aminoglycoside). As evidenced by the ^1H NMR spectra of figure 43, the grafting in all cases was complete. Indeed, the integration ratio between the signals belonging to the dendrimer described above and the signal belonging to one anomeric proton of the

aminoglycoside (in *italic*) is 3:30:2:2 in the case of **96**, 3:30:2:4 in the case of **97**, and 3:30:2:8 in the case of **98**, demonstrating that there are two neomycin in **96**, four neomycin in **97**, and eight neomycin in **98**.



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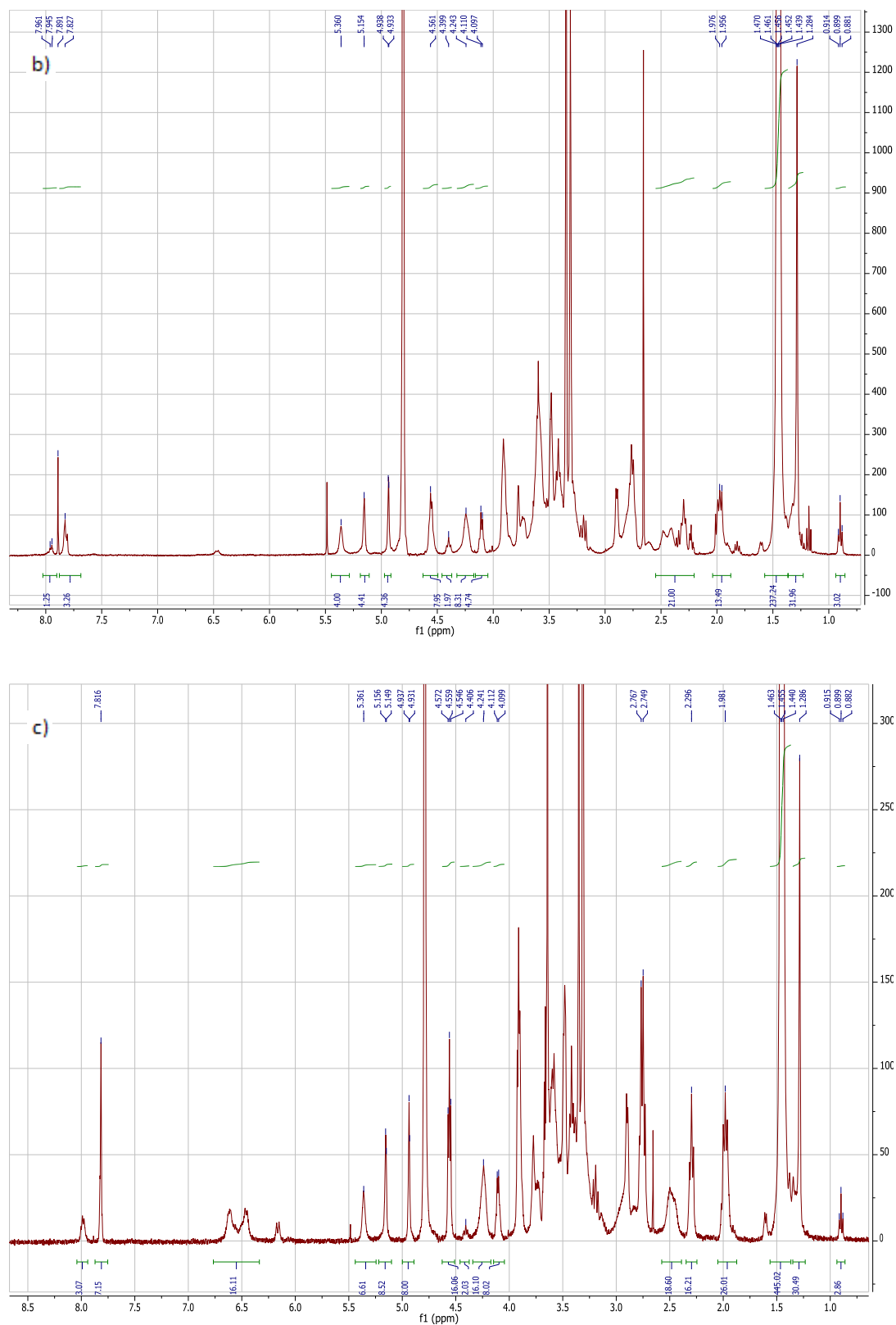


Figure 44. ^1H NMR spectra of a) lipo-dendri(2)-neomycin **96**, b) lipo-dendri(4)-neomycin **97**, and c) lipo-dendri(8)-neomycin **98** conjugates recorded in CD_3OD at 400 MHz and 305K.

Thus, we performed biological tests on these new conjugates. Results about the investigation of the ability to complex DNA, are shown in figure 43. All the conjugates possess good DNA complexation properties with a maximum at $N/P \geq 4$ for all (figure 45).

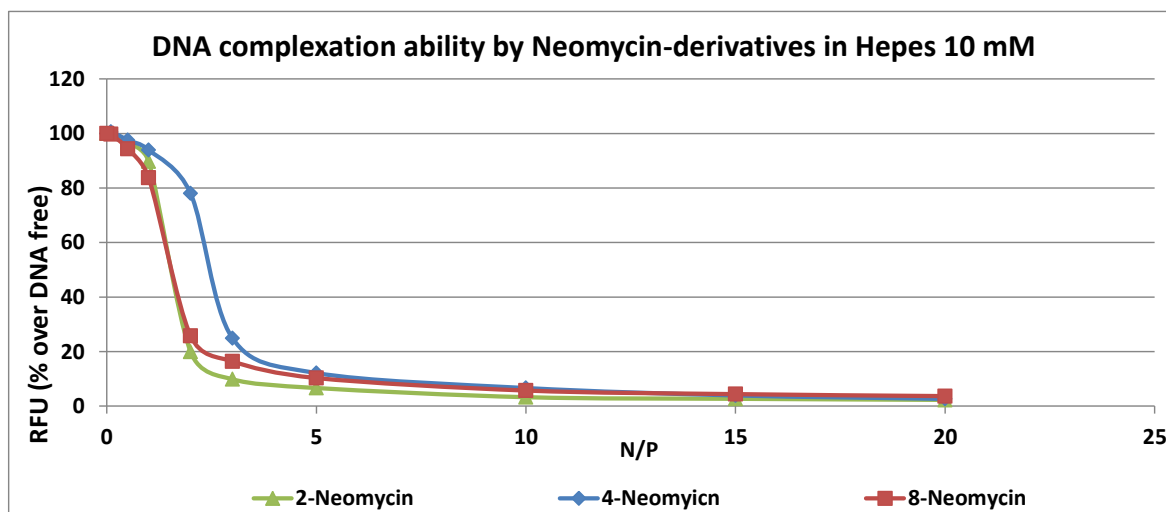


Figure 45. DNA complexation of lipodendrimer-neomycin conjugates.

Preliminary results of transfection efficiency tests in HeLa cell lines (figure 46), have revealed the lipo-dendri(8)-neomycin **98** (figure 43) have a great activity at N/P ratio 10 even better than golden standard 25 kDa bPEI and Lipofectamine at the same N/P ratio. Instead for generation 2 and 4 a good efficacy was showed only increasing the N/P ratio above 10. This is probably due to the relation between the number of amino groups present on the carrier and the electrostatic interaction with the phosphate groups of DNA. Studies about cytotoxicity have shown all the conjugates are non-toxic at N/P ratio 10 and also at higher N/P ratio, the value of toxicity remains always lower than bPEI and Lipofectamine (figure 47).^[165]

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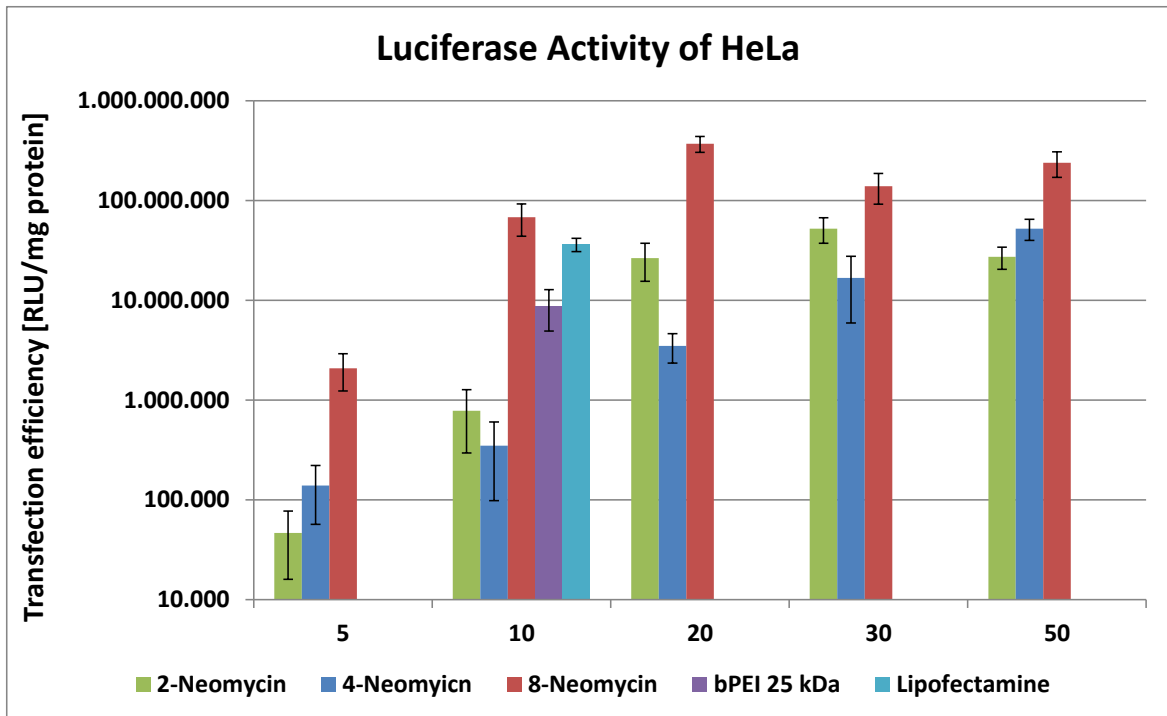


Figure 46. Preliminary results of transfection efficiency of lipo-dendrimeric-neomycin conjugates in HeLa cell lines.

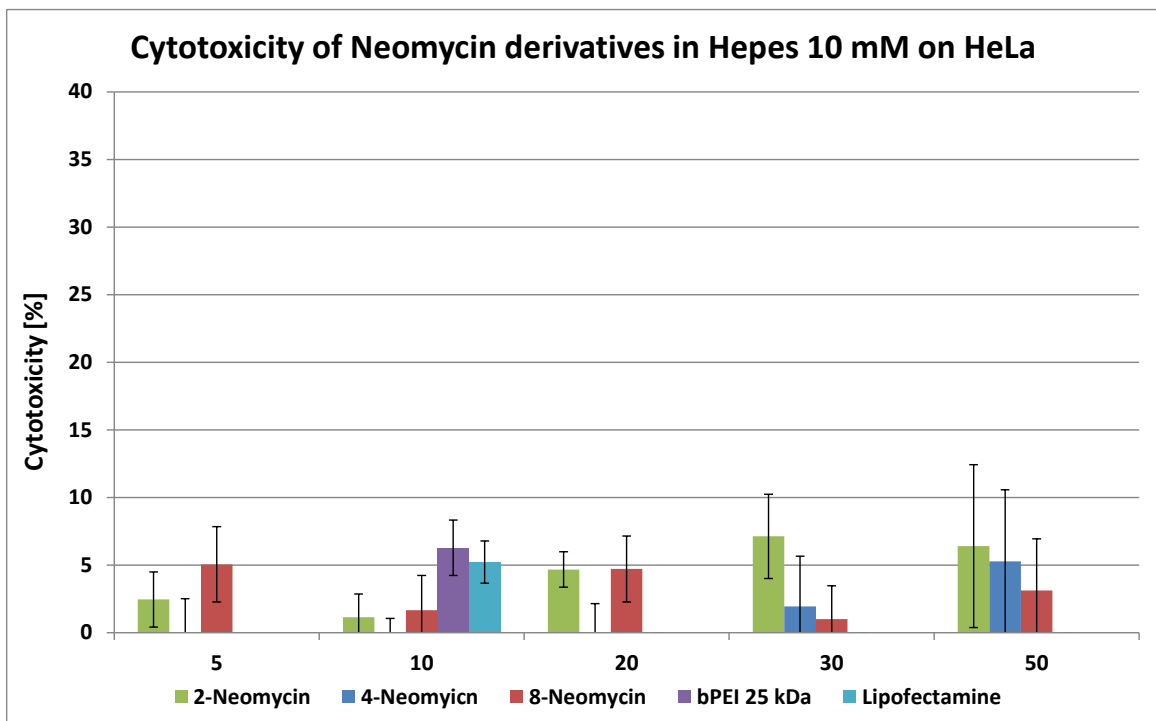


Figure 47. Preliminary results of transfection efficiency of lipo-dendri(n)-neomycin conjugates in HeLa cell lines.

Results of transfection efficiency tests by luciferase activity in COS-7 cell lines (*figure 48*), have shown that the lipo-dendri(8)-neomycin **98** displayed good transfection efficiency with maximum at N/P ratio 20, while is a little less active at N/P 10 respect to bPEI and Lipofectamine at the same N/P ratio. Instead, for generation 2 and 4(conjugates **96** e **97**) a good efficacy was showed only increasing the N/P ratio above 10; in particular for the conjugate **96** with 2 neomycins linked the maximum is at N/P 30, while for the conjugate **97** with 4 neomycins-linkers is at N/P 50. Cytotoxicity studies, depicted in *figure 49*, have displayed results similar those in HeLa cells; all the conjugate are non-toxic at N/P ratio 10 and also at high N/P ratio, the value of toxicity remains always lower than bPEI and comparable with Lipofectamine (*figure 49*).^[165] All these promising preliminary results make these new vectors a captivated option for future application in gene therapy.

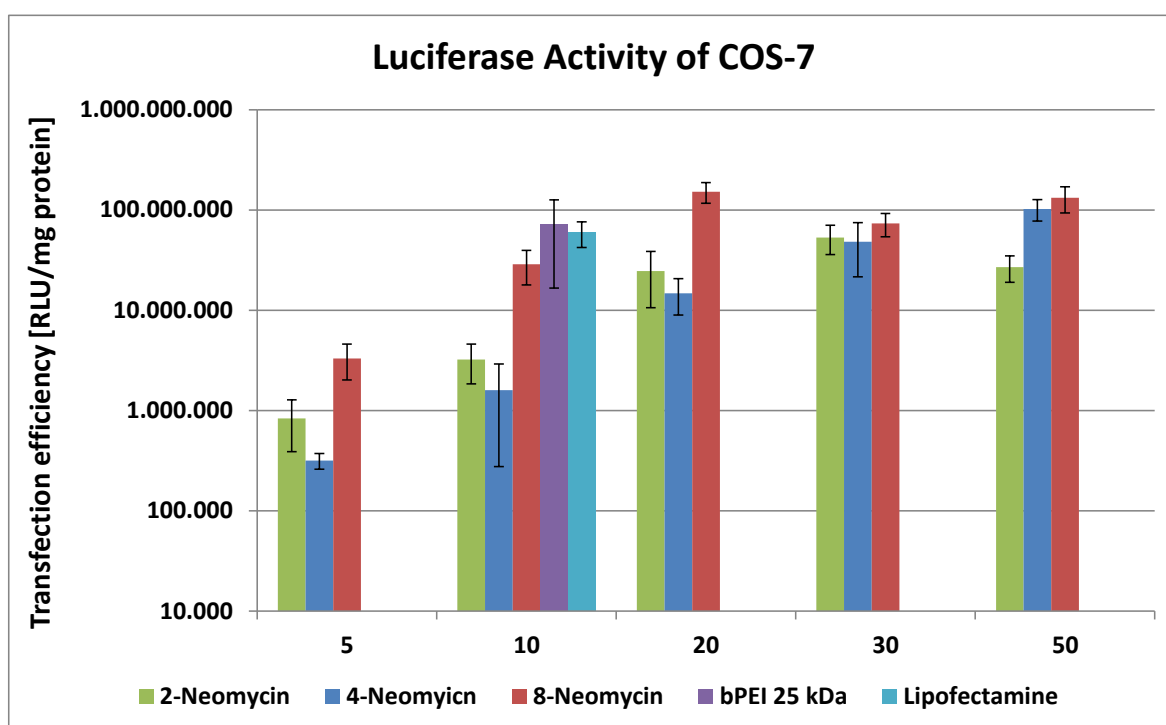


Figure 48. Preliminary results of transfection efficiency of lipo-dendri(n)-neomycin conjugates in COS-7 cell lines.

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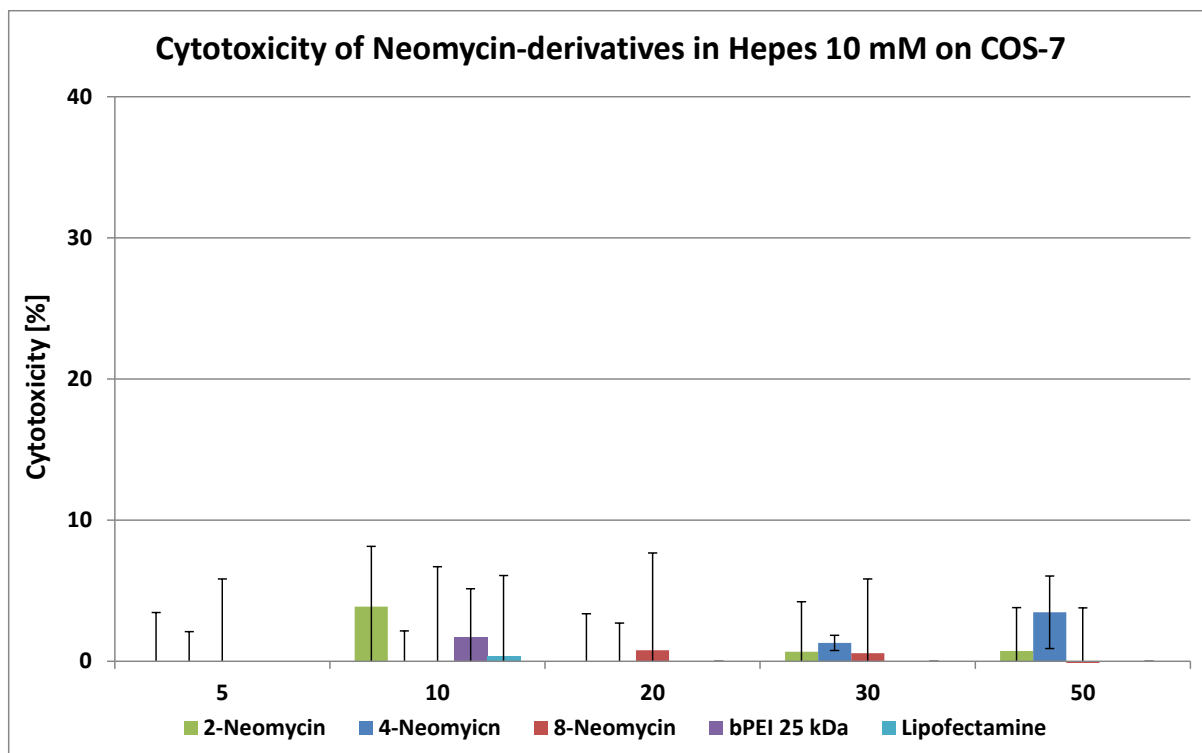


Figure 49. Preliminary results of transfection efficiency of lipo-dendri(n)-neomycin conjugates in COS-7 cell lines.

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The main aim of the drug delivery studies is to find a way to transport a specific drug into the cell overcoming the problems about solubility, bioavailability, instability, capacity to pass through biological barriers, and toxicity of eventual metabolic side-products. Moreover, the therapeutic biomolecules possess a high molecular weight such as proteins and nucleic acids (RNA and DNA), that impedes their cellular uptake. To make it possible, a vector that can help the therapeutic molecules to reach the target cell, is generally required. The molecular transporters commonly used are liposomes, viromes, cationic lipids, and derivatives of peptides bearing guanidinium groups such as polyarginine peptides and peptoids.^[51,166-171] The guanidinium-rich carriers have demonstrated to deliver diverse high molecular cargos into mammalian cells, due to the ability of guanidinium groups to interact with the cell membranes, favoring the membrane permeability and the internalization.^[101] A particular class of guanidium-rich molecules,

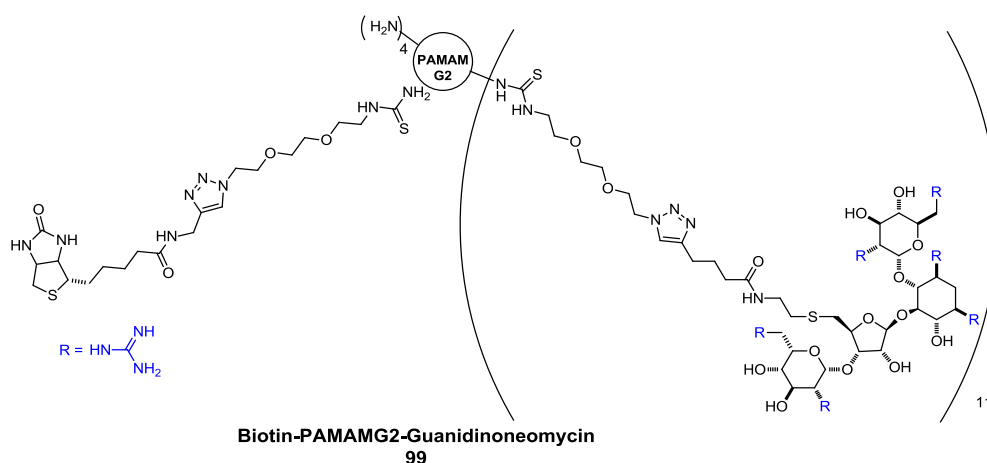
synthesized by Prof. Tor and his research group, are guanidinoglycosides. This class of molecules, obtained by guanidinylation of all the amino groups of the aminoglycoside, had shown a higher translocation efficiency across the cell membrane than the parent aminoglycoside, facilitating the cellular uptake.^[99c] Moreover, guanidinoglycosides can promote the internalization of high molecular cargos, as bioactive molecule or some kind of drugs, into the cell. Tor's team has demonstrated that guanidinoneomycin transports macromolecules into the cell in an exclusive heparan sulfate (HS) proteoglycan dependent way at nanomolar concentrations. To study the HSPG-dependent molecular transporters, fluorescent streptavidin-phycoerythrin-Cy5 (ST-PECy5) was used as a model payload that was carried into the cell by biotinylated guanidinoneomycin conjugates. Mutant cell lines derived from Chinese hamster ovary (CHO) cells without the HS which showed a very low cellular recognition and uptake.^[96, 160] Additionally, monomeric and dimeric guanidinoglycosides possess a different uptake due to the multimeric and cooperative effects that induce a more selective uptake for monomeric guanidinoglycosides with HSPGs dependent pathway, while dimers are able to overcome lower sulfation levels and show a higher cellular uptake.^[102,172] Other interesting properties of guanidinoglycosides include: 1) major affinity to bind RNA respect DNA as their parent aminoglycoside precursors, showing selectivity between RNA molecules; 2) good ability to enter into the cells bringing also high molecular cargos; 3) retain of their antibacterial activity, improving also their MIC values against resistant bacterial strains (only for monoguanidinylated ones), and 4) an efficient inhibition of HIV-1 replication *in vivo*.^[99,115] Mechanistic studies revealed that the guanidinoglycosides enter into the cell by clathrin-mediated endocytosis, caveolin-mediated endocytosis and micropinocytosis, interacting always with HSPG on the membrane.^[173] All together these benefits make guanidinoglycosides a thought provoking vectors for efficiently future applications in drug delivery field. From the previous work concerning the gene delivery, we have demonstrated that conjugates of PAMAM dendrimers with amino and guanidinoglycosides could be used as multifunctional gene delivery vectors with high transfection efficiency and low cytotoxicity. Moreover, the use of PAMAM as transporter improves, in general, the efficacy of the conjugates due to the properties of this dendrimer,

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previously described (see paragraphs 1.2.2, 3.1). So, taking all these aspects into account, we have decided to synthesize suitable multivalent guanidinoglycoside conjugates using two different generations of PAMAM and our guanidinoneomycin-linker in order to study their behavior in cellular uptake and mechanisms of internalization.

3.2.1 Biotin-PAMAMs-Guanidinoglycoside Conjugates: Synthesis, Cellular Uptake and Mechanism

As described above, PAMAM-aminoglycosides synthesized have demonstrated their great ability to enter into the cell bringing DNA, as gene delivery vectors; recently, the group of Prof. Tor showed that monomeric and dimeric guanidinoglycosides endorse the cellular uptake of high molecular cargos in a very efficient way, being the dimeric guanidinoglycosides more efficient.^[102] Keeping in mind these two aspects, we synthesized multivalent guanidinoglycoside conjugates (*figure 50*) using two different generations of PAMAM, our guanidinoneomycin-linker (GNeo-Linker), and a biotin-linker which tightly bind to fluorescent streptavidin protein in order to be able to assess the delivery efficiency through fluorescence cytometry.



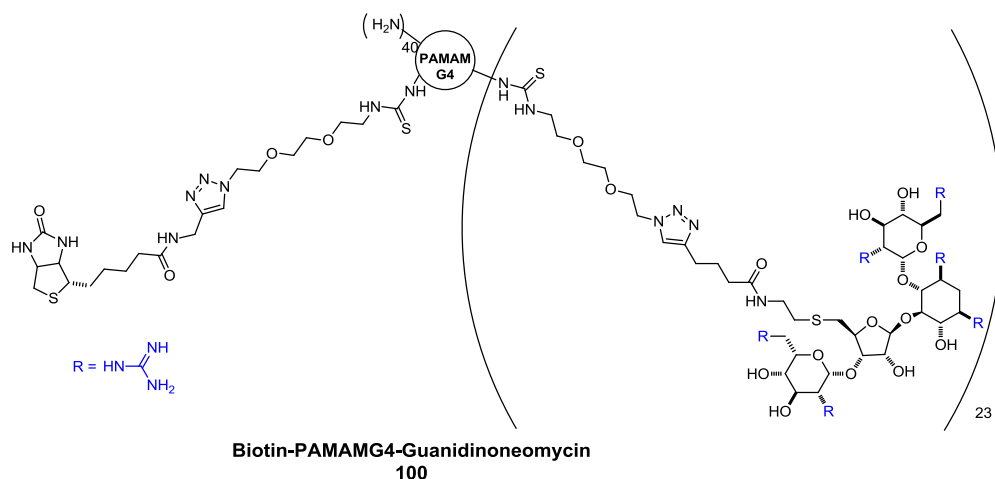


Figure 50. Structure of Biotin-PAMAMG2-GNeo **99** and Biotin-PAMAMG4-GNeo **100**.

To refine our results, we have also prepared Biotin-PAMAMGX- Neomycin and Biotin-PAMAMGX conjugates (*figures 51 and 52*) and performed biological studies on cellular uptake, selectivity and mechanism of entry.

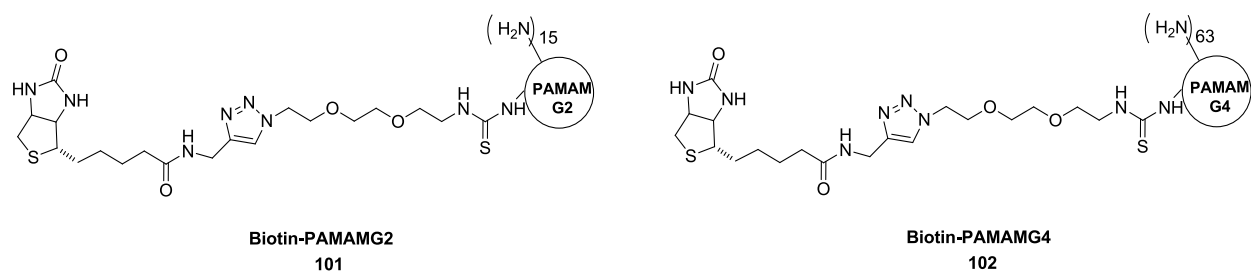


Figure 51. Structure of Biotin-PAMAMG2 **101** and Biotin-PAMAMG4 **102**.

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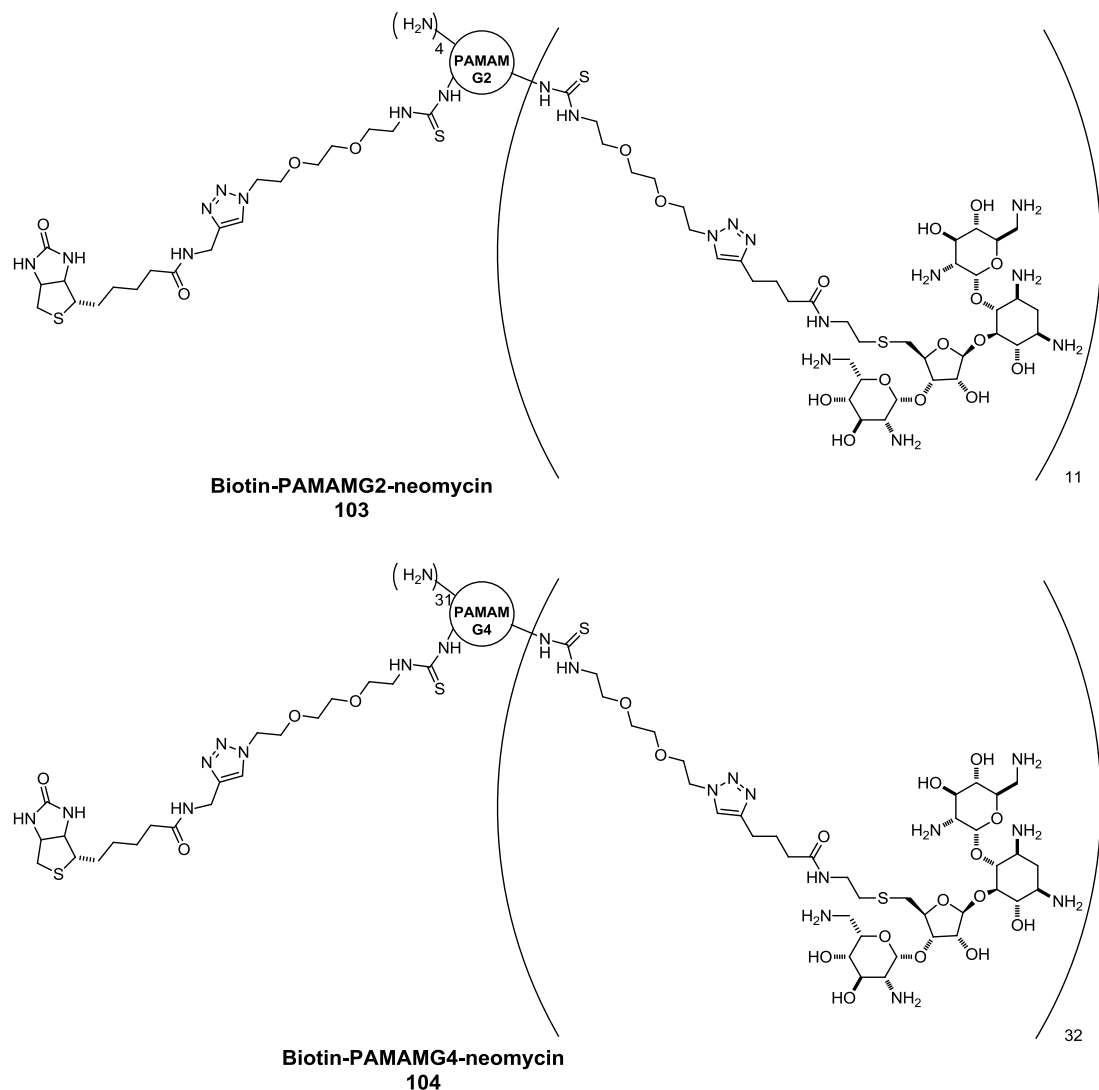
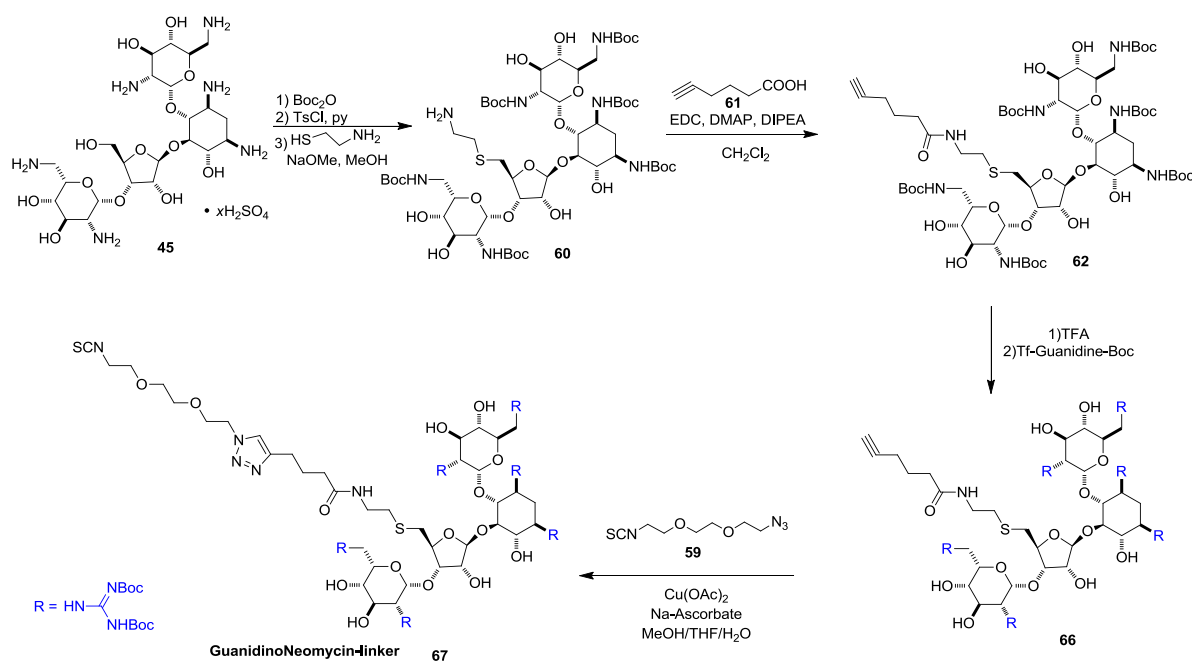


Figure 52. Structure of Biotin-PAMAMG2-Neo **103** and Biotin-PAMAMG4-Neo **104**.

The synthetic pathway for the preparation of the guanidinoneomycin linker, and the conjugates is described below. The first step of the synthesis of these conjugates has been the preparation of Gneo-linker (*scheme 13*) and neo-linker using the same pathway described in the paragraph 3.1.2. Also the reaction conditions for grafting are the same of the general procedure described in experimental section. Briefly, starting from the commercially available neomycin, boc-protection of all amino groups was obtained by treatment with Boc_2O . Then, tosylation at the only primary hydroxyl group followed by nucleophilic substitution with mercapto-ethanol-amine were performed. Coupling reaction with hexynoic acid **61** in CH_2Cl_2 promoted by EDC, DIPEA and catalytic amount DMAP

produced intermediate **62**. Deprotection of amino group followed by guanidinylation with 1,3-di-boc-2-(trifluoromethylsulfonyl)guanidine in MeOH/CH₂Cl₂ and subsequently “click” reaction were performed, leading to guanidinoneomycin-linker **67** in good yields.

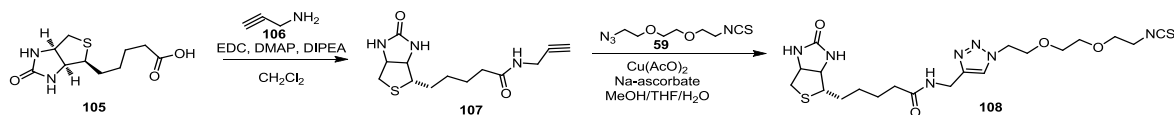


Scheme 13. Synthesis of Guanidinoneomycin-linker **67**.

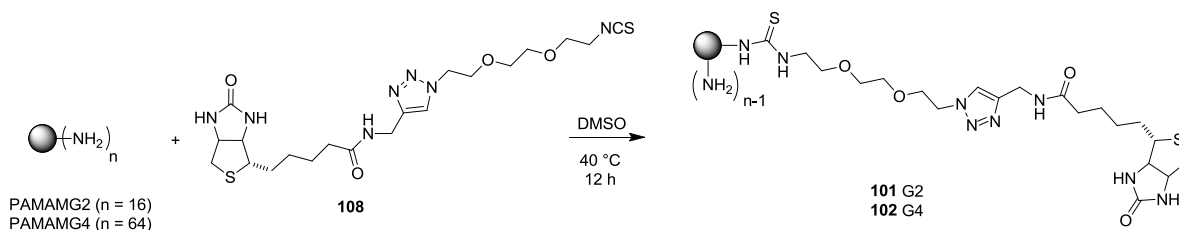
Then biotin linker moiety **108** was prepared as reported in literature^[102] and attached at one amino group of the two different generation of PAMAM. Concisely, biotin was coupled with propargylamine in presence of EDC, DIPEA, and catalytic amount of DMAP in DCM. “Click” reaction with the linker, describe in paragraph 3.1.2, using copper acetate and sodium ascorbate in mixed solvent in the ratio 5:4:1 of MeOH/THF/H₂O, occurred giving the biotin-linker **108** (scheme 14). The grafting was performed in DMSO at 40°C using 1 eq. of biotin-linker in order to tether only one of the amino groups present on the different generation of PAMAM surface (scheme 15). After dialysis against MeOH and evaporation of solvent in vacuo, the Biotin-PAMAMG_x conjugates were obtained and converted in the final targets by grafting part of the remaining amino groups with GNeo- (**67**) or Neo- (**63**) linker, performing the reaction with 1.1 eq per NH₂, in the same reaction condition, DMSO at 40°C overnight. Dialysis against methanol (3 changing of solvent each

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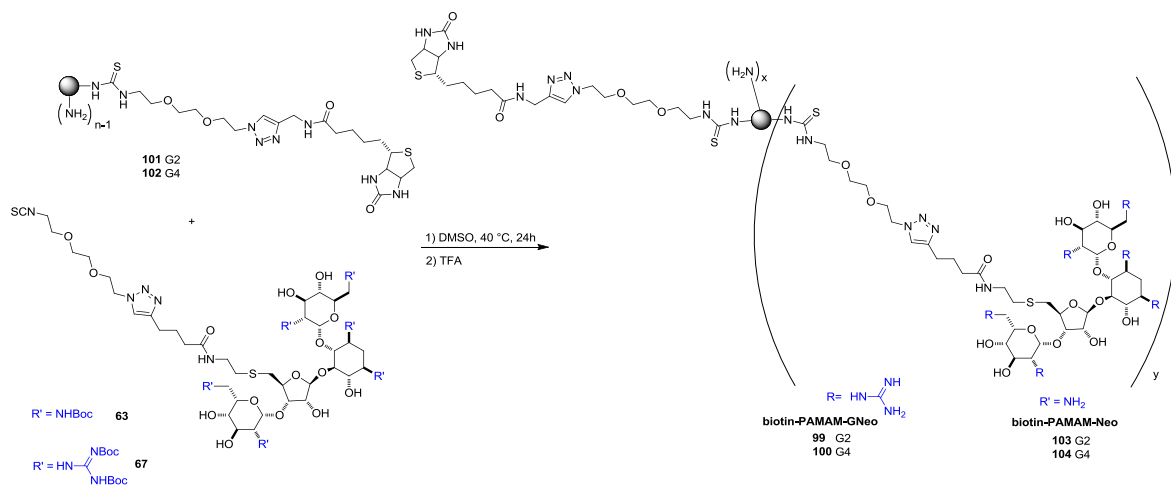
8h), evaporation of solvent in vacuo, deprotection of amino or guanidine group with neat TFA and lyophilization, lead to the final Biotin-PAMAMGX-GNeo and Neo conjugates **99**, **100** and **103**, **104** as white-yellow solid (scheme 16).



Scheme 14. Synthesis of biotin-linker.



Scheme 15. Synthesis of Biotin-PAMAMG2 and Biotin-PAMAMG4 conjugates **101-102**.



Scheme 16. Synthesis of Biotin-PAMAMG2-Gneo, Biotin-PAMAMG4-Gneo, Biotin-PAMAMG2-Neo, Biotin-PAMAMG4-Neo conjugates **99-100**, **103-104**.

The degree of grafting for biotin-PAMAMG2-Gneo, biotin-PAMAMG4-Gneo conjugates **99**, **100**, was calculated by integration of characteristic ^1H NMR signals belonging to Gneo and PAMAM, and subsequently confirmed by MALDI. *Figure 53* shows the ^1H NMR spectrum of final PAMAM G4-biotin-GNeo conjugate **100** recorded in D_2O . The signals of interest are: a) the broad signal between 2.75 and 3.00 ppm which follows the CH_2 protons alfa to the carbonyls of the PAMAM G4 dendrimer (248 H) and b) the two multiplets (belonging to Gneo-linker) which integrates for 6H per Gneo respect to the signals of the anomeric protons (three singlets between 4.75 and 5.50 for Gneo). These multiplets correspond to the signals of the two protons in position 5'' (alfa to the sulfur atom), the other two protons alfa to the same sulfur atoms and the two methylene protons alfa to the carbonyl of the linker. c) the signal of three anomeric proton per Gneo between 5.10 and 6.00 ppm; d) the signal of single proton of triazole ring around 7.80 ppm. Thus, integrating such signal versus the characteristic signals of the GNeo anomeric protons, we determined that *ca.* 24 terminal NH_2 , out of the 64, were functionalized with GuanidonoNeomycin. Following the same rational, we determined that PAMAM G2 was fully functionalized (one outer NH_2 with the biotin-linker **108** and the other 15 NH_2 with GNeo **67**) leading to conjugate **99**.

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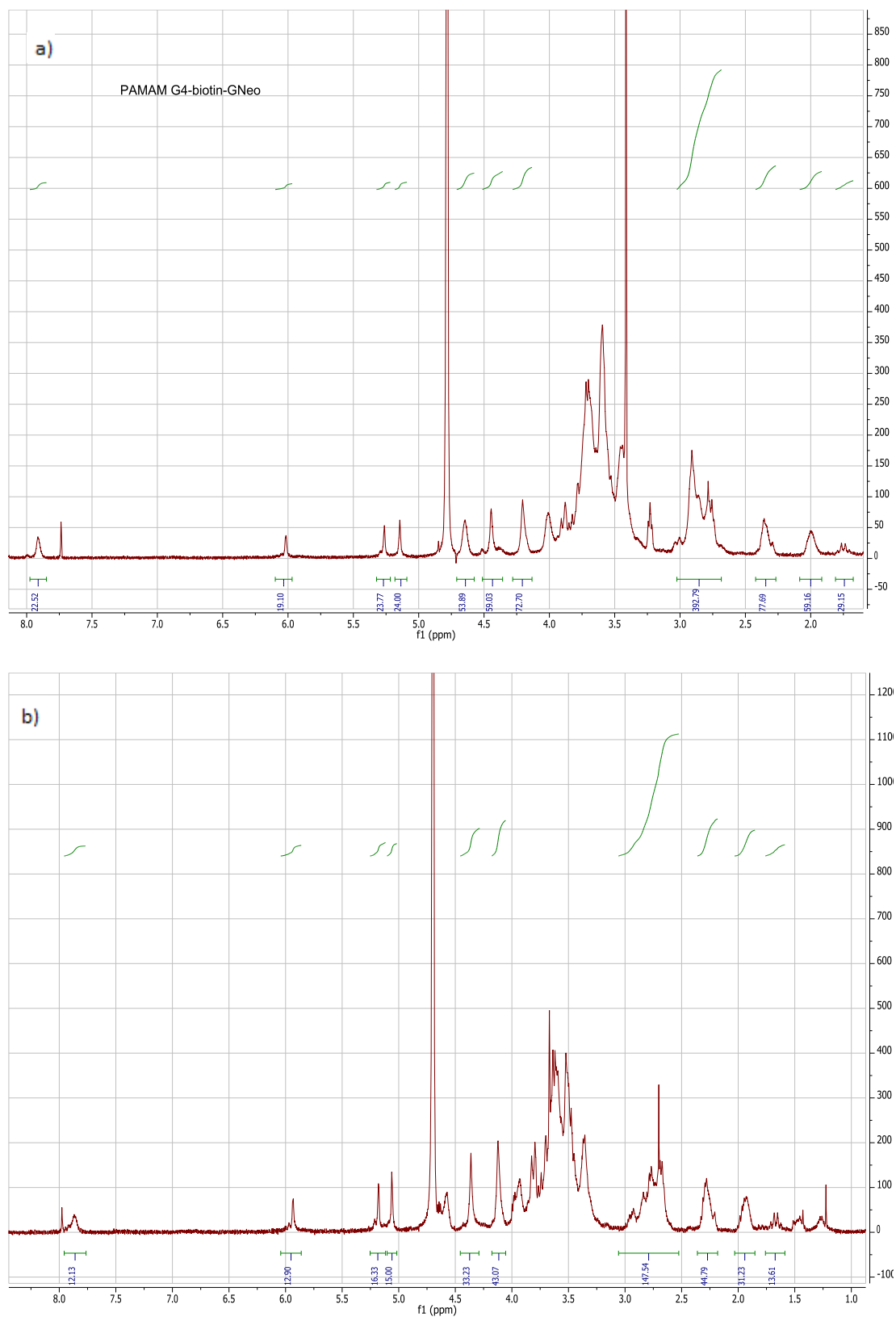


Figure 53. ^1H NMR spectra recorded in D_2O at 400 MHz of a) Biotin-PAMAMG4-Guanidinoneomycin and b) Biotin-PAMAMG2-Guanidinoneomycin conjugates **100**, **99**.

The degree of functionalization determined by integration of NMR signals was confirmed by MALDI spectroscopy finding the value of $m/z = 41621$ for conjugate **100** (estimated 43387) while for conjugate **99** the m/z measured was 17349 compared to the estimated 22293. *Figure 54* shows the recorded spectra for Biotin-PAMAMG2-guanidinoneomycin **99** (*figure 54a*) and Biotin-PAMAMG4-guanidinoneomycin **100** conjugates (*figure 54b*). In table 6 are reported the estimated and the experimental value.

	Experimental value (m/z)	Calculated value [M+H] ⁺ (m/z)
Biotin-PAMAMG2-guanidinoneomycin conjugate	17349	22293
Biotin-PAMAMG4-guanidinoneomycin conjugate	41621	43387

Table 5. Molecular mass determined by MALDI mass analysis and the corresponding values calculated for the single charged parent ions.

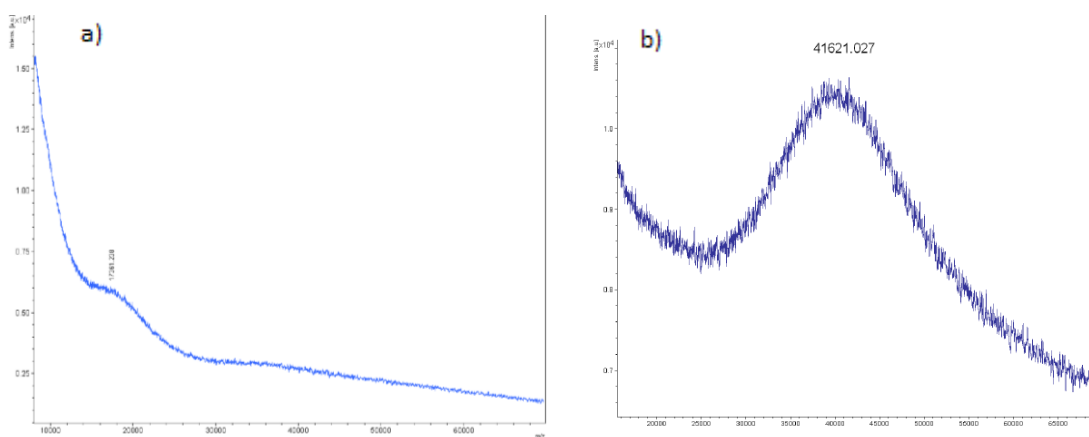


Figure 54. MALDI spectra of a) Biotin-PAMAMG4-Guanidinoneomycin and b) Biotin-PAMAMG2-Guanidinoneomycin conjugates **100**, **99**.

The presence of the biotinylated moiety in the PAMAM conjugates is vital to investigate their cellular uptake. Biotin binds four specific site of streptavidin-phycoerythrin-cyanide 5 (ST-PECy5, 300 kDa), used as fluorescent macromolecular probe and as model cargos (having four binding site makes tetrameric high molecular weight complex with biotin-PAMAMGX-Gneo conjugates) for study the cellular uptake. ST-PECy5

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has emission at 650 nm, and measuring the fluorescence by FACS it is possible to evaluate the ability of the decorated dendrimers to deliver high molecular weight biomacromolecules to cells.^[102, 172-174] Uptake was evaluated in wild type CHO-K1 cells. The cells were incubated with the complexes (2 nM) at 37 °C for 1 h and analyzed by flow cytometry. As showed in figure 55, the mean fluorescence intensity (MFI) of the cells treated with PAMAM-G4 complexes, is remarkably higher than that arising from cells treated with PAMAM-G2 counterparts (*figure 55a*). Moreover, the signal obtained with the guanidinoneomycin derivatives is higher than that the ones obtained with the neomycin ones. Furthermore, for both PAMAM-G2 and G4 the signal obtained for the non-decorated carriers was the lowest (*figure 55a*). Further, to quantify the effect of multivalency on cellular uptake and assess the contribution of either Neo or GNeo moieties to the enhanced cellular uptake, the MFI values were normalized to the number of moieties and compared to monomeric biotinylated neomycin and guanidinoneomycin previously prepared.^[101,173] The dendrimeric-Neo derivatives show a 15 and 9 fold increase per moiety for PAMAM-G4 and PAMAM-G2, respectively. When compared to the monomeric GNeo, there is a 1.7 fold increase for PAMAM-G4. However, for PAMAM-G2 the contribution of each GNeo moiety to the MFI is similar to its "monomeric" form (*figure 55b*). Furthermore, the dendrimeric guanidinoneomycin carriers were also tested in a mutant pgsA-745 cell line, which lacks heparan sulfate and chondroitin/dermatan sulfate, in order to investigate their selectivity in cellular uptake.^[175-176] As showed in figure 55c the cellular uptake was highly reduced for both PAMAM G2 and G4 decorated with guanidinoneomycin, indicating a high selectivity of these dendrimers for cell surface glycosaminoglycan's (GAG).

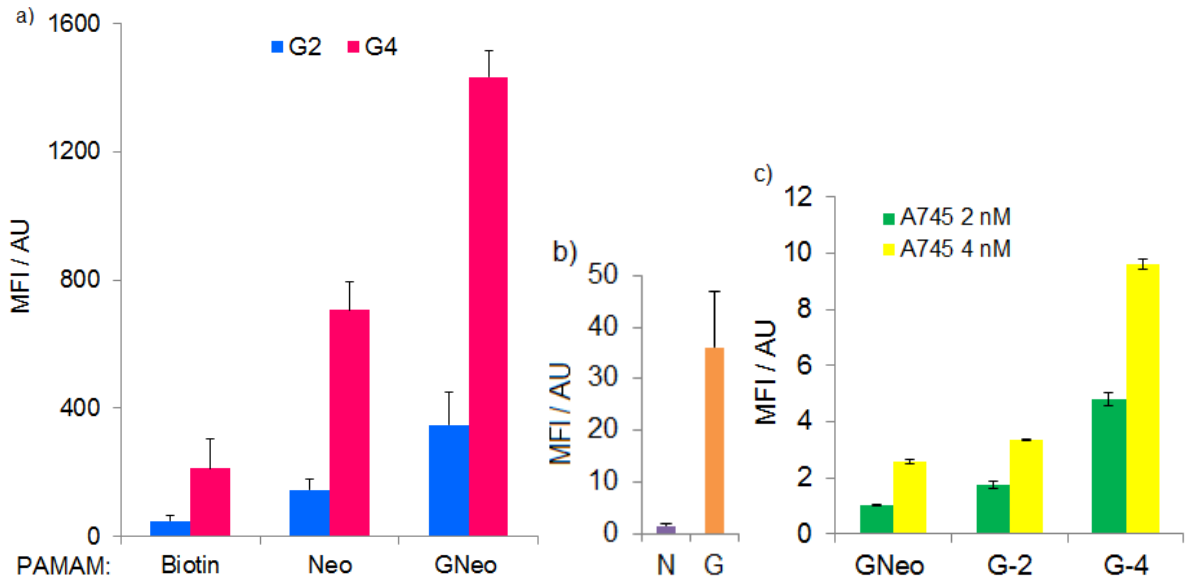


Figure 55. Results of cellular uptake; (a) Wild-type CHO-K1 cells incubated with non decorated PAMAM, PAMAM-Neo and PAMAM-Gneo conjugates. (b) Wild-type CHO-K1 cells incubated with monomeric Neomycin or Guanidinoneomycin. (c) Mutant pgsA-745 cells incubated with GNeo PAMAMG2-GNeo and PAMAMG4-Gneo conjugates.

Once disclosed the great uptake of the conjugates, we wanted to investigate the mechanism of internalization. The general way in which an external macromolecule can enter into the cell is represented from endocytosis.^[177] The mechanism of endocytosis is divided in four main pathways on the basis of the dimension of vesicule formed: 1) phagocytosis with particulate larger than around 0.75 μm in diameter; 2) macropynocytosis that form vesicles of 0.5–5 μm in diameter; 3) Clathrin-mediated endocytosis 100 nm in diameter; and 4) caveolin-mediated endocytosis with vesicle of 50 nm in diameter (figure 56).^[178-182] After endocytosis the cargos is found in the endosome from where it can escape and go into the nucleus, or sorted back to surface of cell or be degraded in lysosomes.^[183]

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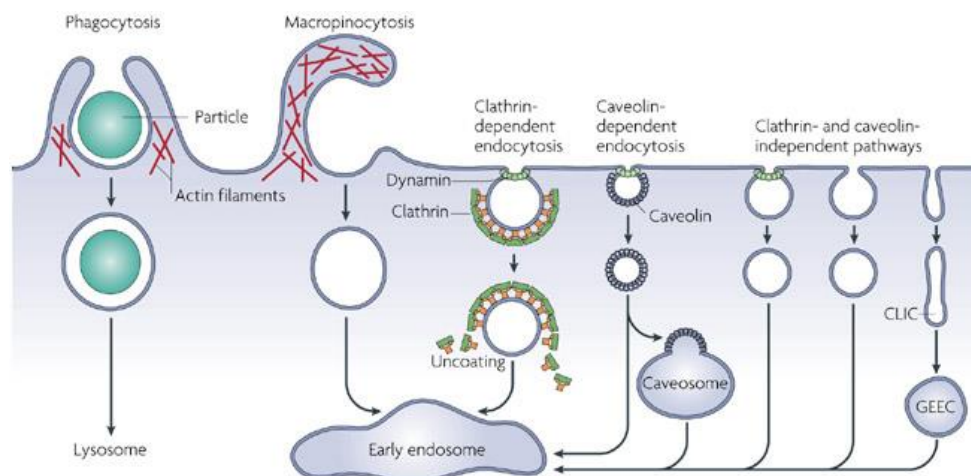


Figure 56. Endocytic pathways.^[182]

To better understand the internalization mechanism of our new carriers, the contribution of different endocytotic pathways was evaluated. To do this we inhibited each single pathway at time using specific agents: the presence of sucrose inhibits the clathrin-mediated endocytosis trough dissociation of the clathrin lattice,^[184] while the addition of amiloride into the media blocks the micropinocytosis inhibiting the Na^+/H^+ exchange,^[173] low temperature hampers the energy-dependent pathways.^[101] We grew wild-type CHO-K1 cells in culture plate for 24h and after that were incubated with sucrose or amiloride for 1 h. Then we added our conjugates and incubated again at 37°C for 1 h and finally analyzed by FACS (see experimental section). Cellular uptake was tested at low temperature (to assess the involvement of energy-dependent processes) and in cells pretreated with sucrose (preventing clathrin-mediated endocytosis) or with amiloride (perturbing macropinocytosis). Interestingly, at low temperatures the cellular uptake of PAMAM-G2-GNeo is reduced to *ca* 35% while the other complexes display only between 5 and 10 % of internalization compared to that at 37 °C (*figure 57a-b*). Notably, internalization of Neomycin-decorated dendrimers was practically prevented by pretreating the cells with either sucrose or amiloride. Further, while in cells pretreated with sucrose the cellular uptake of non-decorated dendrimers was reduced to *ca* 50 and 70 %, in cells pretreated with amiloride the internalization decreased to *ca* 20 and 30 % (PAMAM-G2 and G4, respectively). Unlike the effect seen for GNeosomes,^[186] the entry of GNeo-decorated dendrimers is only lowered to *ca* 60 and 50 % in cells pretreated with sucrose and to *ca* 50

and 70 % in cells pretreated with amiloride (PAMAM-G2 and G4, respectively). Taken together, these results suggest that energy-dependent pathways are involved in the internalization of PAMAM-G2 carriers to a higher extent compared to PAMAM-G4 counterparts and that other mechanisms are likely to contribute to their internalization.

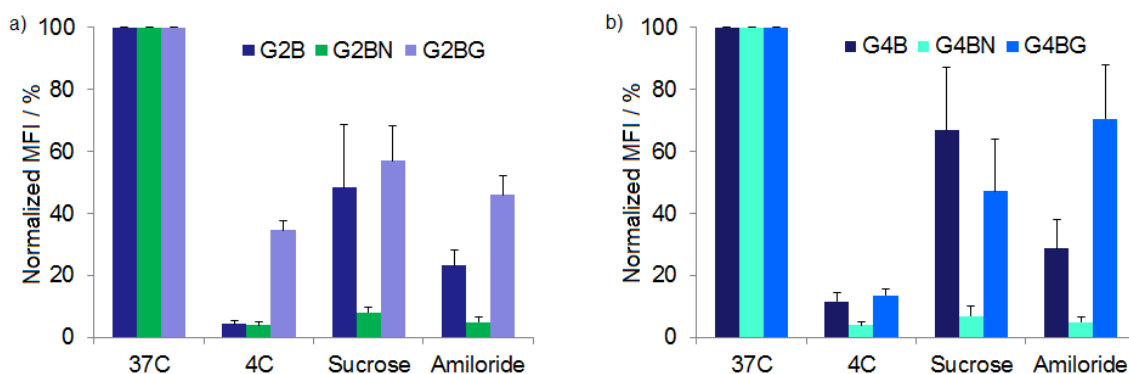
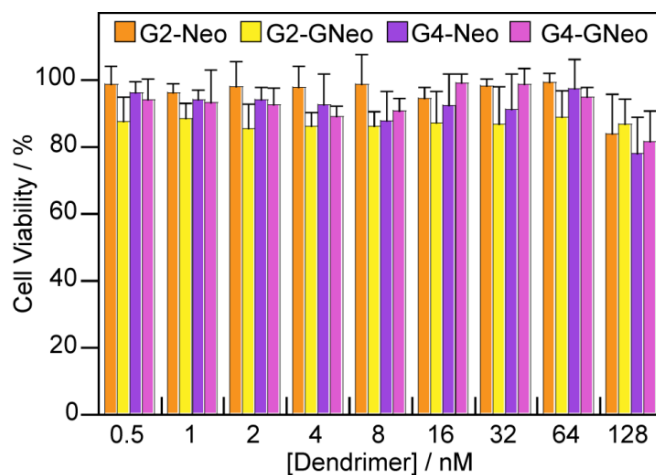


Figure 57. Results of mechanism of internalization in Wild-type CHO-K1 cells (a) generation 2 of PAMAM, PAMAM-Neo and PAMAM-Gneo. (b) Generation 4 of PAMAM, PAMAM-Neo and PAMAM-Gneo Conjugates.

The last test performed on biotin-PAMAMs-aminoglycosides conjugates was the cell viability. The cytotoxicity of the carriers was evaluated using Cell Titer Blue Assay in CHO-K1 cells (figure 58). Our conjugates were found non-toxic at concentrations up to *ca* 15 fold higher than those used in cellular uptake experiments, showing an important property for a good drug delivery vector. ^[187]



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Figure 58. Results of cytotoxicity for PAMAMG2-Neo, PAMAMG2-GNeo, PAMAMG4-Neo and PAMAMG4-Gneo Conjugates.

Considering all the results, we conclude that our conjugated Biotin-PAMAMGX-Gneo promote the uptake of high molecular weight cargos at nM concentrations in mammalian cell in a heparan sulphate dependent. The multivalency effect enhances the ability of our conjugate to get into the cells. All the mechanisms studied such as, energy dependent, independent pathway, clathrin-mediated endocytosis and micropinocytosis are involved in their internalization. Having a very low cytotoxicity, these conjugates could be an attractive choice for future application in drug delivery.

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One of the biggest challenges for medicinal chemists is to find novel and powerful antibiotic to vanquish the increase of drug-resistant bacterial strains. Indeed, besides this increasing in bacteria resistance there is not correspondence with the discovery of new effective drugs. In fact, most of the antibiotics discovered in the forties, such as aminoglycosides, introduced to cure previously untreatable life-threatening infective diseases, are still used in medicine.^[113,119] The widespread and indiscriminate use of antibiotics in hospital, agricultural and livestock field led to a prolific of the resistance mechanism of bacteria, such as biofilm formation and ribosome's mutation, which is become more and more efficient due also to the lack of new potent antibiotics.^[113] A particular attention is given to the enormous development of multidrug resistant pathogens: a) Gram negative bacteria such as *Klebsiella pneumonia* that along with *Escherichia coli* infect immunocompromised patients; b) Gram positive organisms such as methicillin-resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus epidermidis* (MRSE) responsible for infections increasing mortality also in hospitals and increasing the healthcare costs.^[114-118] However, since their important disclosure, aminoglycoside antibiotics remain the most clinically useful antibiotics due to their highly effective broad-spectrum, rapid action and ability to cooperate with other drugs despite their limitations

due to the oto- and neprotocixity.^[120] The expression of bactericidal activity is based on interference of ribosomal function, binding the bacterial 30S or 16S ribosomal A-site of RNA, inhibiting the protein synthesis, leading the bacteria to the death.^[79] Interesting is the resistance mechanism of bacteria versus the aminoglycosides that occurs in different ways as (a) decreasing of uptake and accumulation by impermeabilization or biofilm formation, (b) alteration of molecular target, (c) enzymatic inactivation of aminoglycosides.^[77,189-190] All these knowledge about this remarkable family of compounds, makes the aminoglycoside intriguing scaffold for the synthesis of new derivatives by selective modifications, or semi-synthetic, with a hopefully improved antibacterial activity.^[191]

The domino multicomponent reactions (MCRs), that are successive reactions in a reaction vessel starting from three or more reactants, are not explored and used in aminoglycosides chemistry probably because of the synthetic challenge due to the presence of many functional groups in their scaffolds. At now only one example of MCRs has been reported by Wong et al. dealing with the functionalization of fully protected neamine, the simplest component of the aminoglycoside family, by well-known Ugi MC reaction.^[135] Recently in our laboratories, we have developed and improved an innovative multicomponent domino process, that works very well for the synthesis of multivalent glycomimetics with high yield and mild conditions.^[122-123] So, using for the first time a new MCR in aminoglycosides chemistry, we have modified the aminoglycoside neomycin in a specific position, the less hindered hydroxyl function, adding an additional sugar ring; thus, we have synthesized new sugar-neomycin conjugates and investigated their minimal inhibitory concentrations against Gram-negative and gram positive bacteria strains, in collaboration with Prof. Tor's group at UCSD.

3.3.1 Sugar-Neomycin Conjugates:

Synthesis and Biological Activity

Neomycin B isolated by Waksman in the 1949 from *Streptomyces fradiae*,^[68] belongs to the aminoglycosides' family and was selected as our starting material due its

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commercial availability and lower price. Furthermore, its single primary hydroxyl group at C 5'' position is available for modification without compromising the high binding affinity to RNA and its antimicrobial activity.^[136] In fact, conversely from the other functional group present in the aminoglycosides that bind rRNA selectively, the OH group in C 5'' position is not employed in RNA recognition and it was often selected as point of modifications for synthesis of derivatives.^[192] There are several published studies on neomycin modification, which generally occurs by inserting different moiety that bring potential functionalities direct to enhance the recognition and/or binding to RNA in order to improve the antibacterial activity.^[193] We have therefore prepared a collection of pseudo-pentasaccharides attaching five diverse (amino)sugars as ring V at the reactive position C5''- OH of derivative of Neomycin B, in order to obtain the sugar-neomycin conjugates **109-113** (figure 59) that retain the main scaffold of aminoglycosides and enhance their therapeutic property.

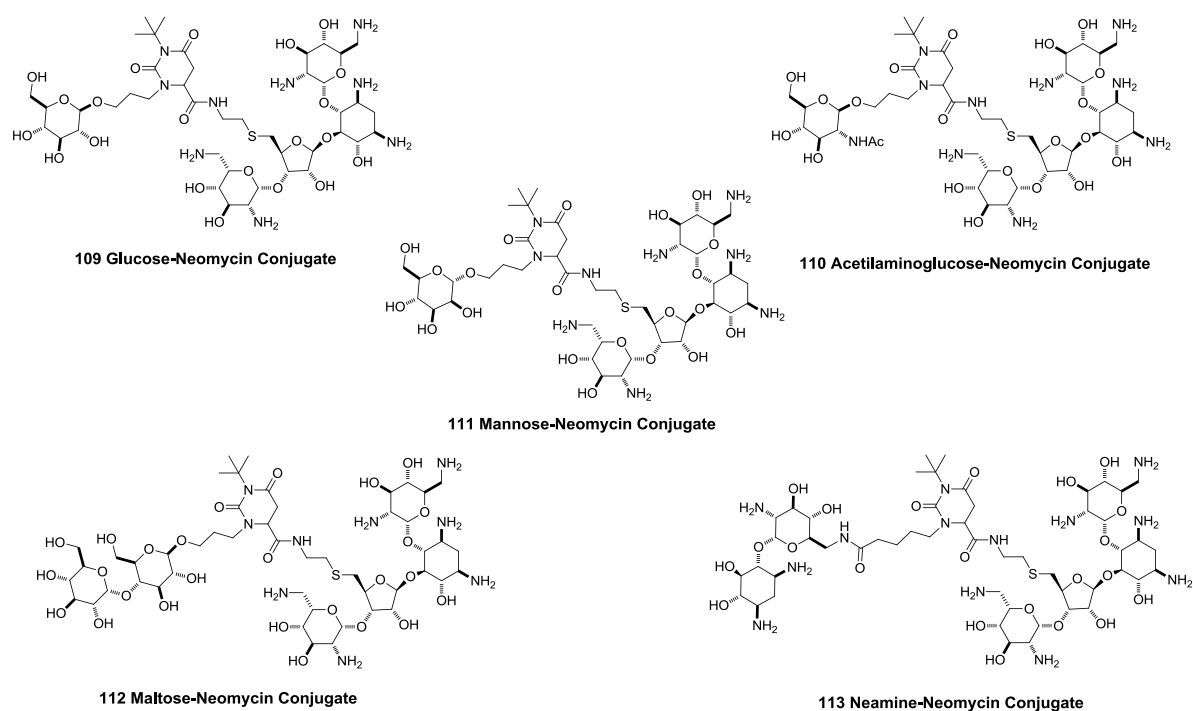
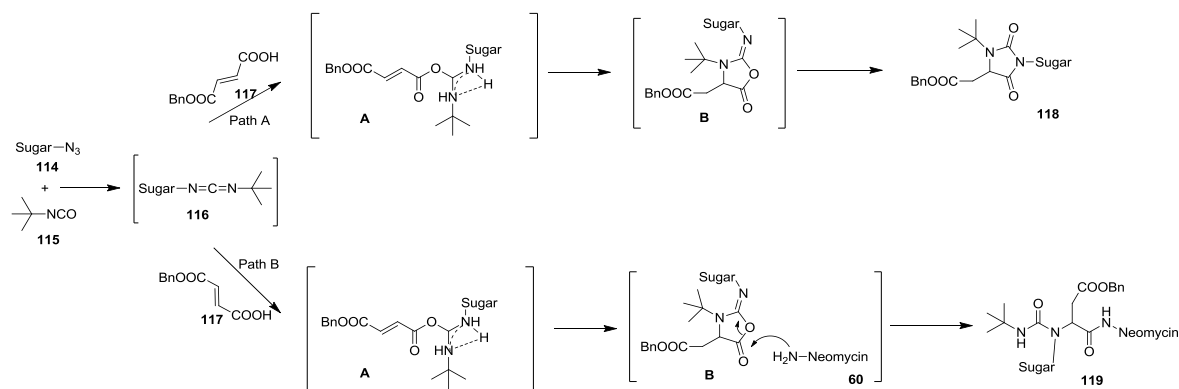


Figure 59. Structures of Sugar-Neomycin Conjugates **109-113**.

To achieve our goal the synthetic pathway used is a novel stimulating multicomponent domino process that we have recently developed for combinatorial synthesis of glycomimetics.^[122-123] The use of combinatorial approach for the synthesis of these kind of glycoconjugates is not common, it was applied only in few cases for glycopeptide mimetics in the last decade.^[194] The possibility of building an improved and elaborated scaffold of glycomimetics using a three or four simple reactants in a single one-pot step,^[195] is becoming more and more attractive for combinatorial and multicomponent synthesis, in particular in pharmaceutical and drug research field.^[196] Taking into account these interesting discovers, we have exploited a four-component regioselective domino process for the synthesis of new lead compounds in a greener way magnifying the efficiency of the process and the yield, and minimizing the waste production.^[197] As showed in an our previously work, a carbodiimide prepared in situ by Staudinger reaction between a N-azide –sugar and an N'-*tert*-butyl isocyanate moiety reacts with fumaric acid monoesters **117** to give rise the formation of glyco-hydantoin conjugates **118** through a regioselective sequential MC domino condensation/aza-Michael/ N → O acyl migration one-pot process (path A, scheme 17). The same reaction carried out in the presence of nucleophiles such as amines, or in our case an amino-Neomycin derivative **60** leads to the MC synthesis of glyco-peptide conjugates **119** by reaction of the nucleophile with the cyclic O-acylisourea intermediate B which occurs after the intramolecular aza-Michael step and before the N → O acyl migration process (path B, scheme 17).^[122,196] Thus, following this second path-way, we have synthesized our small collection of neomycin-sugar conjugates starting from five different sugar azides **114** {a-e}, N'-*tert*-butyl isocyanate **115**, benzylic ester of fumaric acid **117** and amino-neomycin derivative **60** to give up our compounds **119** {a-e} by a multicomponent domino process. The great solubility of neomycin derivative **60** in the reaction condition, CH₃CN at 0 °C, makes this reaction very attractive (scheme 18). In fact, thanks to its high solubility, it was possible to avoid the difficult step of protection of all the neomycin hydroxyl groups, which would have been otherwise necessary due to the possible interference or complications in the Staudinger reaction.^[122-123]

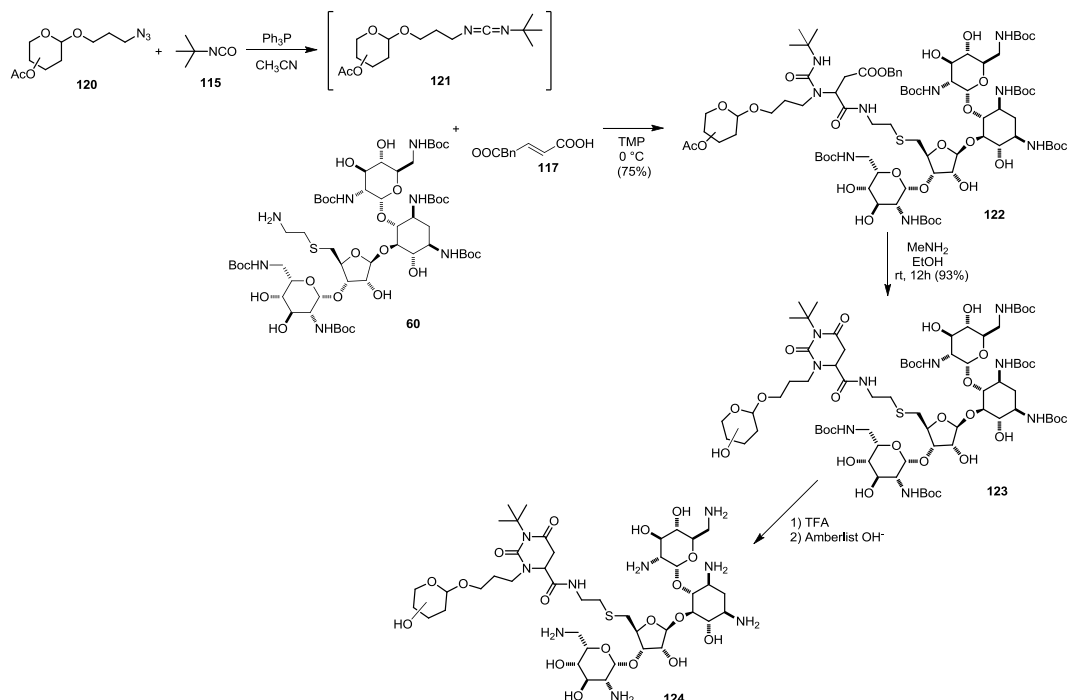
3.3 Novel Antibiotics Project



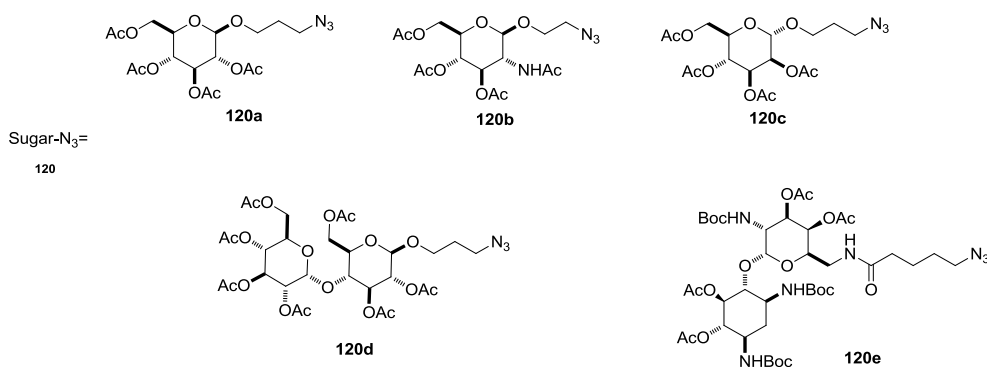
Scheme 17. Mechanism of Multicomponent Domino Process.

In accordance with schemes 18 and 19, the different acylated-azides-derivatives **120**{a-e}, from five different sugars as glucose, aminoglucose, mannose, maltose and neamine, were reacted with *N'*-*tert*-butyl isocyanate **115** at room temperature in presence of triphenylphosphine in CH₃CN in order to get the carbodiimide **121** monitoring the process by TLC until complete formation. After this the mixture was cooled at 0°C and were added, following this order, 2,4,6-trimethylpyridine (TMP), the neomycin derivative **60** and benzylic ester of fumaric acid **117**. The reaction mixture was left to react for 3 h until the formation of **122**. It is interesting that with this multicomponent reaction we were able to obtain only one regioisomer **122** in an equimolecular mixture of two diastereoisomers in a good yield, due to the nucleophilic attack to the less sterically hindered primary N-sugar moiety of intermediate A compared to the bulky tertiary *N'*-*tert*-butyl amino group in the intramolecular aza-Michael step. The following reaction was the treatment of sugar-neomycin conjugate **123** with a solution of methylamine in ethanol to remove the acetyl groups; here the NH moiety reacted readily with the benzyl ester giving rise to the formation of a dihydrouracil heterocycle and the corresponding loss of benzyl alcohol, along with the deacetylation of the hydroxylic groups (scheme 18). The intramolecular cyclization was unexpected. In fact, the amino moiety of *tert*-butyl urea possesses a very low reactivity due to steric and electronic factors. The only explanation possible is in the structural conformation where the ureic NH is so close to the reactive benzyl ester that, even a weak base such as methylamine, is able to trigger the cyclization process (scheme 18).^[124] The last step of this synthetic pathway to create our small library of derivatives is the treatment with trifluoroacetic acid (TFA) net to deprotect the different amino groups

belonging to the neomycin moiety arising the final Neomycin-sugar conjugate **124** in a greener way and good yield.



Scheme 18. Multicomponent Domino Process for Sugar-Neomycin conjugates.



Scheme 19. Acetylated Sugar-Azide chemset.

To investigate their antibacterial property, we determined the value of minimal inhibitory concentration (MIC) in collaboration with Prof. Tor's and Nizet's groups. The new sugar-neomycin conjugates were tested against American Type Culture Collection (ATCC) reference strains as well as clinical isolates of Gram-positive strains including methicillin-

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resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant *Staphylococcus epidermidis* (MRSE), as well resistant strain of Gram-negative bacteria as *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. These strains were chosen to establish a wide-ranging spectrum representative of antibacterial activity. The minimum inhibitory concentrations (MIC) in $\mu\text{g}/\text{mL}$ were determined using broth microdilution assay in accordance with Clinical Laboratory Standards Institute guidelines,^[198] and are shown in Table 5 and 6. Neomycin B was used as reference and Tetracycline, Tobramycin, Vancomycin and Ciprofloxacin were served as positive controls for each different type of strains.

For the Gram-negative Bacteria (table 6) the compounds were tested against: -a) the antibacterial-agent-susceptible *E.coli* ATCC25922 and antibiotic resistant mutants of *E. Coli* K12 cause of morbidity and mortality in the pediatric population.^[199-200] Neomycin B has a MIC of 1,56 $\mu\text{g}/\text{mL}$ better than Ciprofloxacin (6,25 $\mu\text{g}/\text{mL}$) used as control while any derivatives showed improvement against ATCC 25922. For the mutant strain K12 instead conjugates **109,110,111** and **113** possess an activity equivalent to the control Ciprofloxacin with a value in the range of 12,5-6,25 $\mu\text{g}/\text{mL}$.

-b) *P. aeruginosa* strains^[201-204] as PA01, PA103 and PA ATCC27853 but against these type of bacteria sensitive only to Tombramycin, our conjugate showed a disappointing activity similar the parent Neomycin B with a MIC value around 50 $\mu\text{g}/\text{mL}$ or more.

-c) multidrug-resistents (Mdr) bacteria *Acinetobacter baumannii* that causes serious infections in compromised patients.^[205,206] Also in this case our derivative have therapeutic activity comparable with the Neomycin B in particular way for the strain ATCC5057 and ATCC19606 in which the MIC values are around 50 $\mu\text{g}/\text{mL}$ or more. Interesting are the results for the A.B. ATCC17978 strain in which each conjugate have a different MIC value; for aminoglucose-neomycin **110** and maltose-neomycin **112** derivatives are the best about 12,5 $\mu\text{g}/\text{mL}$, then there is glucose-neomycin derivative **109** with 25 $\mu\text{g}/\text{mL}$ (notable is the value of Neomycin B 0,78 $\mu\text{g}/\text{mL}$).

-d)Mdr *Klebsiella pneumoniae*: strains ATCC700603 and GNR1100 resistant to ceftazidime and other oxyimino- β -lactams.^[207-209] Again for the GNR110 the results are disappointing in fact the compound **109, 110, 112** have a MIC value around 50 $\mu\text{g}/\text{mL}$ while Neomycin B

has a value of 6,25 $\mu\text{g}/\text{mL}$ equal to the control Tetracycline. Noteworthy are the test results for ATCC700603 strain; indeed maltose-neomycin conjugate **112** possesses a very good antibacterial activity equal to neomycin and even better than control Tetracycline with a MIC value of 6,25 $\mu\text{g}/\text{mL}$.

MIC $\mu\text{g}/\text{mL}$									
<i>Gram-Negative Bacteria</i>	Neo B	109	110	111	112	113	Cipr.	Tetr.	Tobr.
<i>AB ATCC5075</i>	>50	>50	>50	>50	>50	>50		6,25	
<i>AB ATCC17978</i>	0,78	25	12,5	50	12,5	50		6,25	
<i>AB ATCC19606</i>	25	>50	>50	>50	>50	>50		6,25	
<i>E.Coli ATCC25922</i>	1,56	25	12,5	25	12,5	50	6,25		
<i>E.Coli k12</i>	3,12	12,5-6,25	12,5-6,25	25	6,25	12,5	12,5-6,25		
<i>KP GNR1100</i>	6,25	50	50	>50	50	>50		6,25	
<i>KP ATCC700603</i>	6,25	12,5	12,5	50	<u>6,25</u>	25		25	
<i>PA01</i>	12,5-6,25	>50	>50	>50	>50	>50			0,39
<i>PA 103</i>	12,5	>50	>50	>50	>50	>50			0,39
<i>PA ATCC27853</i>	50-25	>50	>50	>50	50	>50			0,39

[a]Value equal to control are shown in italics; those less than control are shown in bold and those less or equal to Neomycin are shown underline.

Table 6. Minimal Inhibitory Concentrations (MIC) in $\mu\text{g}/\text{mL}$ for Gram-Negative strains^[a].

For the Gram-positive bacteria (table 7) the conjugates were tested against methicillin-resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus epidermidis* (MRSE).^[210-214] In the case of MRSA two different strains were used, TCH1516 in which no neomycin and derivatives showed any improvement in activity and ATCC33591 in which the antibacterial activity of conjugates (**109-113**) is lower than parent Neo B, but better than the other strain with MIC value of 6,25 $\mu\text{g}/\text{mL}$ for compound 4 and 12,5 $\mu\text{g}/\text{mL}$ for **109, 110, 113**. Instead, the results obtained with MRSE are very interesting:

-a) All the conjugate show an optimum antibacterial activity, even better than Vancomycin used as control, against *S. epidermidis* ATCC12228 strain. In particular aminoglucose-neomycin **110** and maltose-neomycin **112** possess the best MIC of 0,78 $\mu\text{g}/\text{mL}$ while the the compound **111** has the same value of MIC as Vancomycin.

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-b) Conjugates **109-113** show a remarkable increase in potency over the parent Neomycin B against the last MRSE ATCC1457 strain. In particular maltose-neomycin **112** and neamine-neomycin conjugate **113** have a MIC value of 25-12,5 $\mu\text{g}/\text{mL}$, higher respect to 50 $\mu\text{g}/\text{mL}$ of Neo B.

<i>Gram-Positive Bacteria</i>	MIC $\mu\text{g}/\text{mL}$						Vancomycin
	Neo B	109	110	111	112	113	
<i>MRSA TCH1516</i>	>50	>50	>50	>50	>50	>50	3,12
<i>MRSA ATCC33591</i>	3,12	12,5	12,5	>50	6,25	12,5	3,12
<i>S.E. ATCC 12228</i>	0,39	1,56	0,78	3,12	0,78	1,56	6,25-3,12
<i>SE ATCC1457</i>	\geq 50	50	\geq 50	\geq 50	<u>25</u>	<u>25-12,5</u>	6,25

[a]Value equal to control are shown in italics; those less then control are shown in bold and those less or equal to Neomycin are shown underline.

Table 7. Minimal Inhibitory Concentrations (MIC) in $\mu\text{g}/\text{mL}$ for Gram-Positive strains^[a].

These results demonstrate two important points: a) the feasibility of using multicomponent domino process in aminoglycosides chemistry, modifying the parent antibiotic to prepare semisynthetic analogues with an improved therapeutic efficiency; b) our sugar-neomycin conjugates show good and promising antibacterial activity against multidrug-resistant bacteria as *Klebsiella pneumoniae* and *Staphylococcus epidermidis* and mutant strain of *E.coli* with low values of minimal inhibitory concentration.

Chapter 4: Conclusions

During the experimental work of my Ph.D project, I have worked in the field of gene and drug delivery, integrating a multidisciplinary approach that put together synthesis, formulation of polyplexes, lipoplexes and hybrid lipo-dendriplexes, cell based assays, systemic SAR studies and bacterial based analyses. Moreover, dealing with aminoglycoside chemistry, I have specialized in this field, bringing also my personal contribution by improving the synthetic methodologies employed in the experimental part in this thesis. To make all this possible I have collaborated with different research groups, which permitted me to have a complete training in different disciplines and lead to the preparation and characterization of different delivery carriers that resulted very efficient both in gene and drug delivery field, thus very promising for further development. Referring to the objectives of my Ph.D thesis described in chapter two, the conclusions are following reported in the same order.

4.1 Gene Delivery Project

The development of multifunctional gene delivery vectors tailored with specific functionalities is one of the most exciting strategy emerging in the last years to attain efficient and safe gene delivery, and at the same time, still remains one of the major challenge nowadays. All the non-viral vectors we designed were synthesized and fully characterized. All the compounds were tested for transfection efficiency, DNA complexation and cytotoxicity giving very interesting and encouraging results, even better than the gold standard 25kDa bPEI. In particular concerning PAMAMG4-aminoglycoside conjugates, we disclosed a novel class of oligomeric aminoglycoside conjugates with an optimum bacteriostatic and bactericidal activity, which is not influenced by their complexation with the pDNA. Furthermore, we showed the multifunctional behavior of PAMAM G4 conjugate-based polyplexes by performing for the first time transfection

4.2 Drug Delivery Project

experiments in the presence of exogenous bacterial infections. Altogether, these results highlight the promising potential of PAMAM G4-aminoglycosides as multifunctional agents capable of mediating transfer and expression of nucleic acids and overcoming bacterial infections at once. Cationic lipids vectors have been synthesized using calixarenes as lipidic part that should enhance the ability of multifunctional neomycin in gene transfection. Biological studies are still ongoing in our laboratories. Due to the different specific behavior of dendrimers and lipidic vectors, we have also prepared hybrid lipo-dendrimer-conjugates and studied their ability as delivery carriers, showing optimum transfection efficiency that makes them an attractive choice in further application in gene delivery field.

4.2 Drug Delivery Project

The development of new carriers, that enhance the ability of drug delivery systems to deliver macromolecules more efficiently to their sites of actions inside the target cells, is a main challenge in this field. Herein, we synthesized and tested Biotin-PAMAMG2-GNeo and Biotin-PAMAMG4-GNeo conjugates, to investigate their ability in promoting cellular uptake of high molecular cargos and their selectivity. Moreover, to have a more exhaustive overview on the behavior in cellular uptake, undecored Biotin-PAMAMG2 and Biotin-PAMAMG4 were also synthesized and tested along with Biotin-PAMAMG2-Neo and Biotin-PAMAMG4-Neo. The results have shown that guanidinoglycoside conjugates possess a high capacity of internalization and selectivity for cell with heparan sulfate proteoglycans on their surface. Moreover the effect of multivalency in Biotin-PAMAMG2-GNeo and Biotin-PAMAMG4-GNeo conjugates enhance their cellular uptake respect monomeric and dimeric aminoglycosides. Furthermore, the mechanism of cellular uptake is equally based on energy dependent and independent way, endocytosis clathrine-mediated and macropinocytosis. All these properties make our conjugates promising vectors for drug delivery systems.

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The research for novel classes of antibacterial agents with new or combined mechanisms of action that are active against multi-drug resistant bacteria and possess little or no occurrence of resistance is witnessing an exponential growth. A collection of systematically modified neomycin-sugar conjugates was synthesized by an innovative and useful multicomponent domino process developed in our laboratories. To the best of our knowledge, this is the first time that a novel multicomponent process has been exploited for the synthesis of aminoglycoside derivatives. Some of the compounds created showed good and promising antibacterial activity against multidrug-resistant bacteria as *Klebsiella pneumoniae* and *Staphylococcus epidermidis* and mutant strain of *E.coli* with low values of minimal inhibitory concentration. These results demonstrate the capacity of multicomponent domino process to modify parent antibiotic in order to achieve semisynthetic analogue with a potential improving of therapeutic efficiency.

Chapter 5: Experimental Section

5.1 Gene Delivery Project

Materials

Neomycin sulfate, paromomycin sulfate, PAMAM G4 dendrimer (ethylenediamine core, 64 surface groups), PAMAM G2 dendrimer (ethylenediamine core, 12 surface groups), PAMAM G7 dendrimer (ethylenediamine core, 512 surface groups), 25 kDa bPEI (Mw ~25 kDa by LS, average Mn ~10 kDa by GPC), and all other organic reactants, solvents, and culture reagents were purchased from Sigma-Aldrich (Milan, Italy) if not specified differently, and used as received. Use of a cationic polymer based on PEI for transfection is covered by US Patent 6,013,240, European Patent 0,770,140, and foreign equivalents, for which Polyplus-transfection is the worldwide exclusive licensee. Neamine was synthesized from neomycin as described in literature.^[130] Spectra/Por dialysis bags (MWCO = 1 and 8 kDa) were from Spectrum Laboratories (Compton, CA, USA). The luciferase expression plasmid (pGL3-Control vector) encoding for the modified firefly luciferase and the luciferase expression kit were from Promega (Milan, Italy). Plasmid DNA (pDNA) was prepared as previously reported.⁸ AlamarBlue cell viability assay was from Life Technologies (Monza, Italy) while BCA Protein Assay Kit was from Thermo Fisher Scientific (Rockford, IL, USA). HeLa (human cervix carcinoma), U87-MG (human glioblastoma-astrocytoma epithelial-like), and COS-7 (African green monkey kidney fibroblast-like) cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA).

Instruments

The ¹H NMR spectra were recorded on 400 MHz spectrometers at 305 K. Chemical shifts are expressed in ppm (δ), using tetramethylsilane (TMS) as internal standard for ¹H and ¹³C nuclei (δ H and δ C = 0.00). MALDI-TOF analysis was carried out by a Bruker Daltonics Reflex IV instrument (Bruker Daltonics, Bremen, Germany) equipped with a nitrogen laser

(337 nm) and operated in linear mode using the dry droplet technique and 2,3-dihydroxybenzoic acid 10 mg/mL in CH₃CN:TFA/1:1 as a matrix. External standards were used for calibration (Bruker protein calibration standard 1 and 2). Each spectrum was accumulated for at least 600 laser shots. Mass spectra were alternatively performed with a GC-MS instrument, using a gas chromatograph equipped with an SBP-1 fused silica column (30mm x 0.2 mm i.d., 0.2 mm film thickness) and helium as carrier gas. TLC were run on silica gel 60 F₂₅₄ Merck. Flash chromatography (FC) was performed with silica gel 60 (60-200 μm, Merck).

General procedures

General Procedure for the Synthesis of lipo/Dendrimer amino- guanidinoglycoside Conjugates

Dendrimers (or calixarene or hybrid lipo-dendrimers) without any trace of organic solvent, previously evaporated in vacuo and coevaporated twice with dichloromethane, was dissolved in minimal amount of DMSO and a solution of aminoglycoside-isothiocyanate linker, or guanidinoglycoside-linker, (1.2 equiv per NH₂ group of dendrimer) in a minimal volume of DMSO was added. The solution was stirred at 50 °C for 24 h and then dialyzed against MeOH (8 h, the solvent reservoir was renewed thrice, MWCO 1 kDa). The solution was evaporated under reduced pressure to give N-Boc-protected dendrimer-aminoglycosides. These resulting conjugates were dissolved in trifluoroacetic acid (TFA) and stirred for 30 min at room temperature (r.t.). The excess of TFA was evaporated under reduced pressure, the crude dissolved in water, and the solution dialyzed against water (overnight, MWCO 1 kDa). Lyophilization of the water solution led to recovery of a fluffy, white solid.

Cell cultures

HeLa cells were cultured in a humidified atmosphere of 5% CO₂ at 37 °C, in Dulbecco's Modified Eagle Medium (DMEM) containing 1 mM sodium pyruvate, 10 mM HEPES buffer, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 2 mM glutamine and supplemented with 10% (v/v) fetal bovine serum (FBS), hereafter referred to as complete DMEM.

General procedure for In Vitro Transfection Experiments.

Cells were plated at a density of 2×10^4 cells/cm² in 96-well culture plates, after 24 h, cells were washed in PBS and culture medium was replaced with 100 μ L/well of polyplex-containing medium (160 ng/cm² of pGL3). Three different transfection media were tested: serum-free DMEM, 10% FBS, and DMEM supplemented with 50% FBS (hereafter referred to as 50% FBS). After transfection, cells were incubated for 48 h at 37 °C. Cytotoxicity was tested using AlamarBlue cell viability assay and expressed in percent relative to the untreated controls. Luciferase activity was measured by Luciferase Assay System using a Mithras luminometer (Berthold Technologies, Brugherio, Italy) and normalized to the total protein content, evaluated by BCA assay, in cell lysates.

Preparation of plasmid DNA

pGL3-Control vector plasmid and pCMV-Gussia Luc vector plasmid were transformed in *E. Coli* DH5 α and amplified in Luria-Bertani (LB) broth medium (Life Technologies Italia, Monza, Italy), containing 100 μ g/mL ampicillin, at 37 °C overnight. The plasmid DNAs (pDNAs) were isolated and purified with maxiprep anion-exchange column (Quiagen, Milan, Italy) according to the supplier's protocol. The concentration and the purity of pDNAs were assessed by the absorbance ratio at OD₂₆₀/OD₂₈₀. The pDNAs were stored at -20 °C in TE buffer 0.1X (1 mM Tris, pH 8.0; 0.1 mM EDTA) until use.^[122]

General procedure for preparation of Polyplexes or/and Evaluation of DNA Complexation Ability

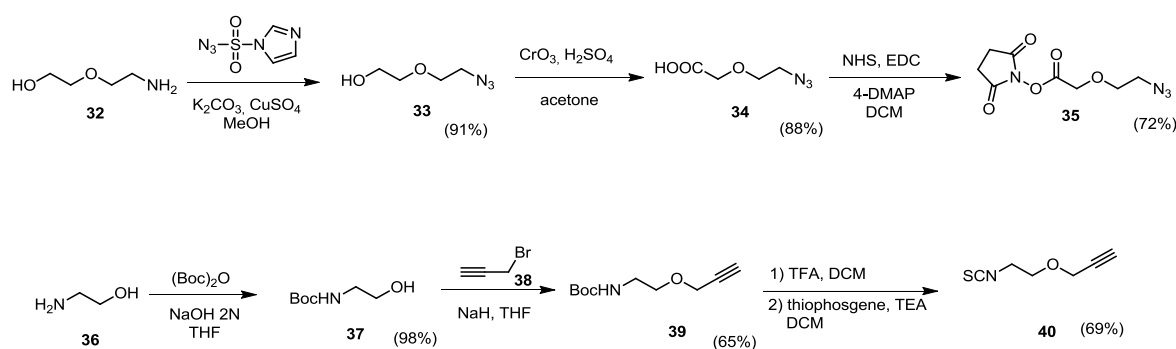
Polyplexes were prepared by adding pGL3 plasmid DNA to dendrimer-aminoglycoside conjugates in 10 mM HEPES pH 7.0 at the desired polymer concentration to yield different nitrogen to phosphate (N/P) ratios, and incubated for 20 min at r.t. before use. The final DNA concentration in the transfection solution was 20 ng/ μ L. The DNA complexation ability of each different dendrimer and its derivatives was determined by fluorophore-exclusion titration assay; polyplexes were prepared as described above using 0.2 μ g of pGL3 and were diluted to 100 μ L in 10mM HEPES pH 7.0 containing 2 \times SYBR Green

I ($\lambda_{\text{ex}} = 497 \text{ nm}$; $\lambda_{\text{em}} = 520 \text{ nm}$). The fluorescence of the resulting solutions was measured using the microplate reader GENios Plus (Tecan, Segrate, Italy) and normalized over the fluorescence of noncomplexed plasmid DNA.

5.1.1 PAMAMG4-Aminoglycosides Conjugates

Synthetic Part

Synthesis of linker (scheme 2):



Compound 33. To a solution of **32** (1.0 g, 9.52 mmol) in MeOH (50 mL) imidazole-1-sulfonyl azide hydrochloride^[215] (2.4 g, 11.43 mmol), K₂CO₃ (2.23 g, 16.18 mmol) and CuSO₄ pentahydrate (catalytic) were added at r.t. and the mixture stirred for 3 h. The organic solvent was removed under reduced pressure, the crude dissolved in a mixture AcOEt/H₂O and extracted with AcOEt. The collected organic layers were anhydriified over Na₂SO₄, filtered and evaporated. The crude was purified by flash chromatography affording **33** (1.3 g, 8.66 mmol) in 91% yield. *R_f* = 0.33 (hexane:AcOEt 50:50); ¹H-NMR (400 MHz, CDCl₃): δ = 3.70 (t, *J* = 4.8 Hz, 2H), 3.63 (t, *J* = 4.8 Hz, 2H), 3.56 (t, *J* = 5.2 Hz, 2H), 3.37 (t, *J* = 5.2 Hz, 2H), 2.01 (br s, 1H); ¹³C-NMR (100.6 MHz, CDCl₃): δ = 72.2, 69.7, 61.5, 50.6; ESI (*m/z*) 154,1 [M+Na]⁺, 132,1 [M+H]⁺.

Compound 34. To a solution of **33** (1.0 g, 7.63 mmol) in acetone (50 mL) a freshly prepared Jones solution (5.4 mL, 15.26 mmol) was added drop wise. After 3h the mixture was filtered and the organic solvent removed under reduced pressure. The crude was

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dissolved in a mixture AcOEt/H₂O and extracted with AcOEt. The collected organic layers were anhydriified over Na₂SO₄, filtered and evaporated. The crude was purified by flash chromatography affording **34** (973 mg, 6.71 mmol) in 88% yield. *R_f* = 0.55 (CHCl₃:MeOH 98:2 + one drop of AcOH); ¹H-NMR (400 MHz, CD₃OD): δ = 4.07 (s, 2H), 3.73 (t, *J* = 5.1 Hz, 2H), 3.46 (t, *J* = 5.1 Hz, 2H); ¹³C-NMR (100.6 MHz, CD₃OD): δ = 174.4, 69.5, 68.8, 50.4; ESI (*m/z*) 168.2 [M+Na]⁺, 146.2 [M+H]⁺.

Compound 35. To a solution of **34** (414 mg, 3.6 mmol) in DCM (25 mL) EDC hydrochloride (691 mg, 3.6 mmol) followed by NHS (522 mg, 3.6 mmol) and a catalytic amount of 4-DMAP were added and the solution stirred at r.t. overnight. The solution was diluted with a 5% aqueous solution of Na₂CO₃ and extracted with DCM. The collected organic layers were anhydriified over Na₂SO₄, filtered and evaporated. 630 mg (2,6 mmol, 72% yield) of **35** were recovered and used without any further purification. ¹H-NMR (400 MHz, CDCl₃): δ = 4.52 (s, 2H), 3.79 (t, *J* = 4.9 Hz, 2H), 3.47 (t, *J* = 4.9 Hz, 2H), 2.87 (s, 4H); ¹³C-NMR (100.6 MHz, CD₃OD): δ = 168.7, 165.7, 70.8, 66.4, 50.6, 25.6.

Compound 37. To a solution of **36** (1.0 g, 16.37 mmol) in a 2M NaOH aqueous solution (8.4 mL) a solution of Boc₂O (3.6 g, 16.37 mmol) in THF (8 mL) was added dropwise. After one night the organic solvent was evaporated under reduced pressure, the aqueous acidified with a 1N HCl solution and extracted with AcOEt. The collected organic layers were anhydriified over Na₂SO₄, filtered and evaporated. The crude was purified by flash chromatography affording **37** (2.5 g, 16.04 mmol) in 98% yield. *R_f* = 0.48 (hexane:AcOEt 50:50); ¹H-NMR (400 MHz, CDCl₃): δ = 4.00 (br s, 2H), 3.66 (t, *J* = 5.5 Hz, 2H), 3.27 (t, *J* = 5.5 Hz, 2H), 1.44 (s, 9H); ¹³C-NMR (100.6 MHz, CD₃OD): δ = 156.8, 79.7, 62.3, 43.3, 28.4; ESI (*m/z*) 184,2 [M+Na]⁺.

Compound 39. To a suspension of NaH (60% oil dispersion, 373 mg, 9.3 mmol) in dry DMF (3 mL) a solution of **37** (1.0 g, 6.2 mmol) in dry DMF (5 mL) was added dropwise at 0°C and under a N₂ atmosphere. The temperature was slowly raised to r.t. and the suspension stirred for 30'. The mixture was cooled to 0°C and a solution of propargyl bromide **38** (900 μL, 4.8 mmol) in dry DMF (5 mL) was added dropwise. The resulting

mixture was stirred overnight at r.t., diluted with water and extracted with AcOEt. The collected organic layers were anhydried over Na₂SO₄, filtered and evaporated. The crude was purified by flash chromatography affording **39** (801 mg, 4.03 mmol) in 65% yield. : *R_f* = 0.35 (hexane:AcOEt 70:30); ¹H-NMR (400 MHz, CDCl₃): δ = 4.90 (br s, 1H), 4.17 (d, *J* = 2.2 Hz, 2H), 3.60 (t, *J* = 5.1 Hz, 2H), 3.36 (m, 2H), 2.45 (t, *J* = 2.2 Hz, 1H), 1.46 (s, 9H); ESI (*m/z*) 222,1 [M+Na]⁺.

Compound 40. To a solution of **39** (487 mg, 2.45 mmol) in DCM (5.0 mL) neat TFA (500 μL) was added at r.t. and the solution stirred for 3h. The volatiles were evaporated under reduced pressure and the crude dissolved in DCM (22 mL) and TEA (742 mg, 7.36 mmol). A solution of tiophosgene (375 μL, 4.9 mmol) in DCM (10 mL) was added in 45' and the solution monitored by TLC. After the total consumption of the starting material, the volatiles were evaporated *in vacuo*, the crude dissolved in a mixture of DCM/water and extracted with DCM. The collected organic layers were anhydried over Na₂SO₄, filtered and evaporated. The crude was purified by flash chromatography affording **40** (238 mg, 1.69 mmol) in 69% yield. *R_f* = 0.20 (hexane:AcOEt 80:20); ¹H-NMR (400 MHz, CDCl₃): δ = 4.19 (d, *J* = 2.2 Hz, 1H), 3.68 (m, 4H), 2.48 (t, *J* = 2.2 Hz, 1H); ¹³C-NMR (100.6 MHz, CDCl₃): δ = 132.4, 78.7, 75.1, 67.5, 58.2, 44.8.

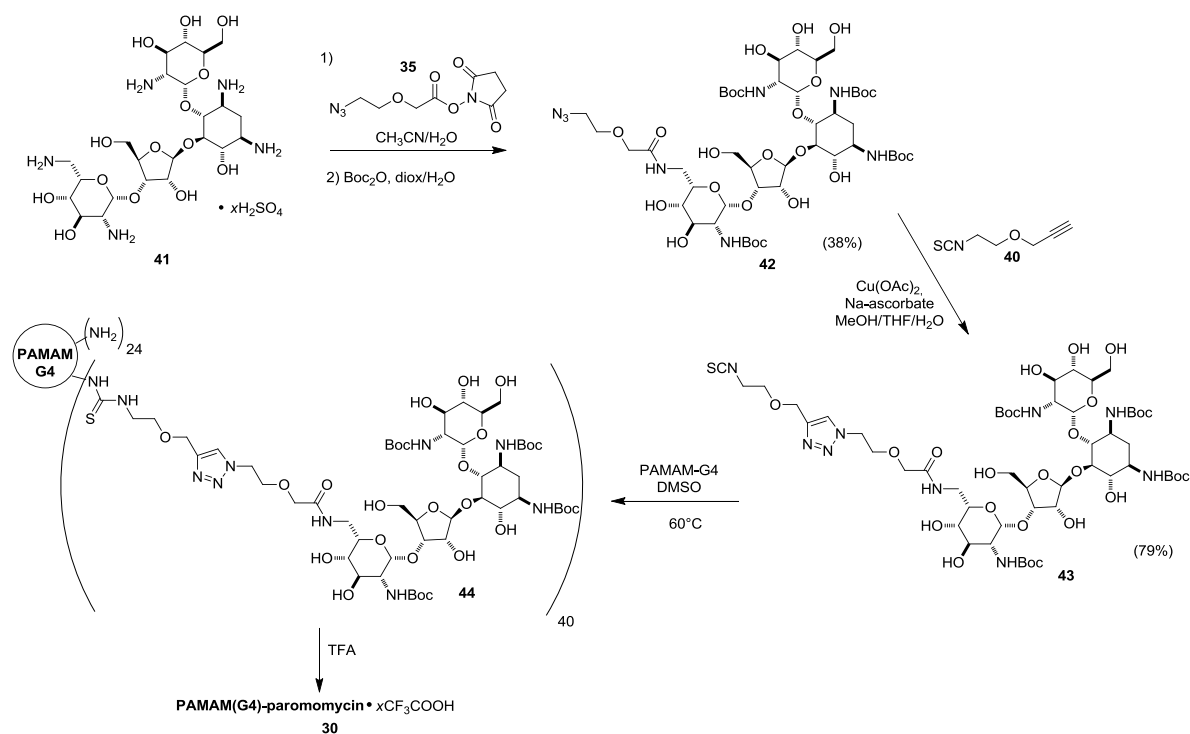
General Procedure for the Synthesis of Dendrimer–aminoglycoside Conjugates 29-31

Dendrimer without any trace of organic solvent, previously evaporated *in vacuo* and coevaporated twice with dichloromethane, was dissolved in DMSO and a solution of aminoglycoside-isothiocyanate linker (1.2 equiv per NH₂ group) in a minimal volume of DMSO was added. The solution was stirred at 60 °C for 24 h and then dialyzed against MeOH (8 h, the solvent reservoir was renewed thrice, MWCO 1 kDa for the neamine derivative and 8 kDa for the paromomycin and neomycin derivatives). The solution was evaporated under reduced pressure to give N-Boc-protected PAMAM G4- aminoglycosides. These resulting conjugates were dissolved in trifluoroacetic acid (TFA) and stirred for 30 min at room temperature (r.t.). The excess of TFA was evaporated under reduced pressure,

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the crude dissolved in water, and the solution dialyzed against water (overnight, MWCO 1 kDa). Lyophilization of the water solution led to recovery of a fluffy, white solid.

Synthesis of PAMAMG4-paromomycin conjugate 30 (scheme 3):



Compound 42. Paromomycin sulfate **41** was dissolved in water and resin Amberlist IRA 400 was added until neutral/basic pH was reached. The mixture was filtrated and the water evaporated under reduced pressure. To a solution of free paromomycin (200 mg, 0.31 mmol) in a 2:1 mixture of acetonitrile/water (3 mL) a solution of **35** (75 mg, 0.31 mmol) in a minimum amount of acetonitrile was added at r.t. and the mixture stirred overnight. The solvents were evaporated, the crude dissolved in a 3:1 mixture of dioxane/water (12 mL) and a solution of Boc₂O (1.014 g, 4.65 mmol) in a minimal amount of dioxane was added. After refluxing for 6 h. the mixture was cooled to r.t. and the solvents evaporated under reduced pressure. The crude was purified by flash chromatography affording **42** (150 mg, 0.13 mmol) in 41 % yields. *R_f* = 0.56 (CHCl₃:MeOH 90:10); [α]_{20D} = +40.8° (c = 0.2, CH₃OH); ¹H-NMR (400 MHz, CD₃OD): δ = 5.38 (s, 1H, anomeric), 5.19 (s, 1H, anomeric), 4.92 (s, 1H, anomeric), 4.20 (m, 2H), 4.06 (s, 2H, -COCH₂O-), 3.98 (m, 2H), 3.91 (m, 1H), 3.86-3.50

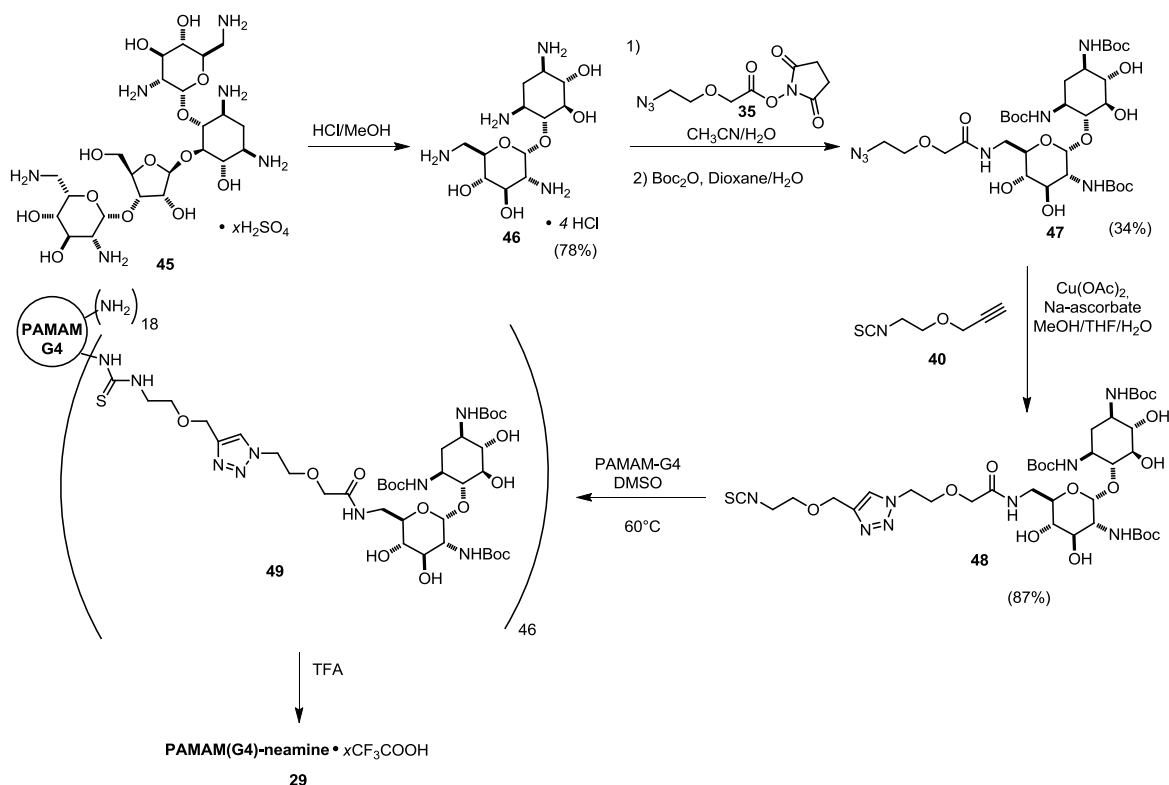
(m, 21H), 1.98 (m, 1H, H_2), 1.48 (s, 9H), 1.46 (s, 9H), 1.45 (s, 18H), 1.42 (m, 1H, H_2). ^{13}C -NMR (100.6 MHz, CD_3OD): δ = 171.4, 157.1, 157.0, 156.8, 156.6, 109.4, 98.7, 97.4, 86.4, 81.9, 79.3, 79.1, 78.8, 77.4, 76.8, 74.3, 74.2, 73.1, 72.4, 71.5, 70.7, 70.2, 69.7, 68.6, 62.9, 62.1, 61.7, 55.6, 52.1, 50.3, 39.5, 34.4, 27.6, 27.5, 27.4, 27.3; ESI (m/z) 1165.4 $[\text{M}+\text{Na}]^+$.

Compound 43. To a solution of **42** (150 mg, 0.13 mmol) in a 6:1.5:1 mixture of MeOH/THF/H₂O (10 mL), Cu(Ac)₂ (42 mg, 0.23 mmol), Na-ascorbate (45 mg, 0.23 mmol) followed by a solution of **40** (32 mg, 0.23 mmol) in a minimum amount of THF were added and the mixture was sonicated until disappearance of the starting material was detected (TLC monitoring). The mixture was diluted with CH₂Cl₂, filtered over a Celite pad, and the solvent was removed under reduced pressure. The crude was purified by flash chromatography affording **43** (143 mg, 0.11 mmol) in 79 % yields. R_f = 0.50 (CHCl₃:MeOH 80:20); $[\alpha]^{20}_{\text{D}} = +34.7^\circ$ ($c = 0.3$, CH₃OH); ^1H -NMR (400 MHz, CDCl₃/CD₃OD): δ = 7.81 (s, 1H, *aromatic*), 5.17 (s, 1H, *anomeric*), 5.13 (s, 1H, *anomeric*), 4.84 (s, 1H, *anomeric*), 4.64 (s, 2H, -COCH₂O-), 4.55 (m, 2H), 4.17 (m, 1H), 4.04 (m, 1H), 3.92-3.34 (m, 21H), 2.08 (m, 1H, H_2), 1.37 (s, 9H), 1.36 (s, 9H), 1.35 (s, 18H), 1.30 (m, 1H, H_2). ^{13}C -NMR (100.6 MHz, CD₃OD): δ = 170.9, 157.3, 156.9, 156.4, 156.1, 144.6, 132.5, 124.2, 99.3, 97.4, 85.4, 82.0, 79.9, 79.7, 74.4, 74.1, 73.3, 72.4, 71.8, 71.3, 69.9, 69.4, 68.4, 64.1, 63.4, 62.4, 61.0, 55.4, 52.0, 50.1, 45.1, 28.3, 28.2; ESI (m/z) 1339.1 $[\text{M}+\text{Na}]^+$, 1307.0 $[\text{M}+\text{H}]^+$.

Compound 30. The synthesis of compound **30** has been achieved as described in the general method chapter 5.1 and 5.1.1. ^1H -NMR (400 MHz, D₂O): δ = 8.04 (s, 40H, *aromatic* of paromomycin moieties), 5.70 (d, $J = 4.0$ Hz, 40H, *anomeric* of paromomycin moieties), 5.30 (s, 1H, *anomeric* of paromomycin moieties), 5.12 (d, $J = 2.0$ Hz, *anomeric* of paromomycin moieties), 4.61 (m, 248H, -CONHCH₂CH₂N- of PAMAM), 4.29 (m, 40H), 4.23 (m, 40H), 4.17 (m, 40H), 4.12 (m, 40H), 3.97-3.29 (m, 1620H), 3.08 (m, 40H), 2.76 (m, 248H, -NHCH₂CH₂CO- of PAMAM), 2.43 (m, 40H, H_2 of paromomycin moieties), 1.81 (ddd, $J = 12.4$ Hz, 40H, H_2 of paromomycin moieties).

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Synthesis of PAMAMG4-neamine conjugate **29** (scheme 4):



Neamine hydrochloride **46** was obtained as reported in literature.^[135]

Compounds 47. Neamine hydrochloride **46** was dissolved in water and resin Amberlist IRA 400 was added until neutral/basic pH was reached. The mixture was filtrated and the water evaporated under reduced pressure. To a solution of free neamine (100 mg, 0.31 mmol) in a 2:1 mixture of acetonitrile/water (3 mL) a solution of **8** (75 mg, 0.31 mmol) in a minimum amount of acetonitrile was added at r.t. and the mixture stirred overnight. The solvents were evaporated, the crude dissolved in a 3:1 mixture of dioxane/water (12 mL) and a solution of Boc₂O (608 mg, 2.79 mmol) in a minimal amount of dioxane was added. After refluxing for 6 h. the mixture was cooled to r.t. and the solvents evaporated under reduced pressure. The crude was purified by flash chromatography affording **47** (79 mg, 0.11 mmol) in 34 % yields. *R_f* = 0.35 (CH₂Cl₂:EtOH 95:5); [α]²⁰_D = +35.8° (c = 0.4, CH₃OH); ¹H-NMR (400 MHz, CD₃OD): δ = 5.29 (br s, 1H, *H1'*), 4.08 (s, 2H, -COCH₂O-), 3.85 (m, 1H), 3.75 (t, *J* = 5.2 Hz, 2H, -OCH₂CH₂N₃), 3.70 (m, 2H), 3.64 (m, 1H), 3.56-3.38 (m, 7H), 3.23 (m, 1H), 3.17 (t, *J* = 9.6 Hz, 1H, *H6'*), 2.05 (m, 1H, *H2*), 1.47 (s, 9H), 1.46 (s, 9H), 1.45 (s, 9H), 1.35

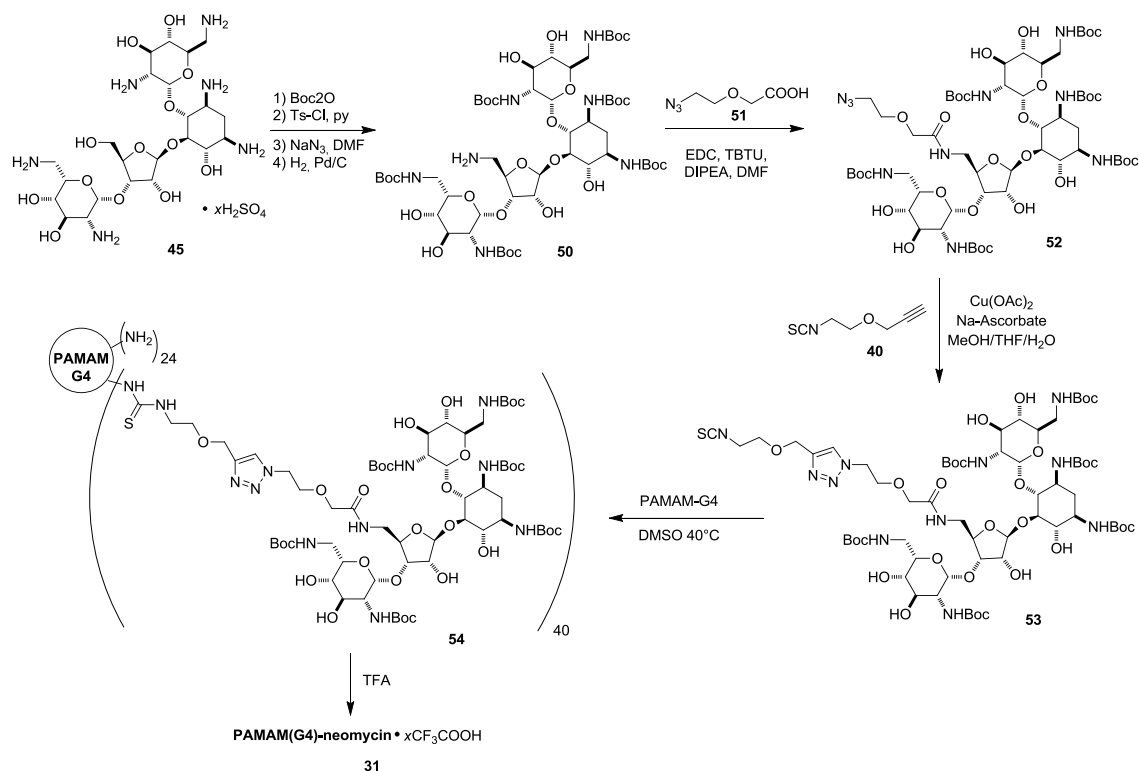
(ddd, $J = 12.4$ Hz, 1H, H_2); ^{13}C -NMR (100.6 MHz, CD_3OD): $\delta = 171.4, 157.2, 156.8, 156.4, 98.9, 80.7, 79.4, 79.0, 77.5, 75.1, 71.6, 70.2, 69.7, 55.5, 51.0, 50.3, 49.6, 39.1, 34.7, 27.4$; ESI (m/z) 772,4 $[\text{M}+\text{Na}]^+$.

Compound 48. To a solution of **47** (100 mg, 0.13 mmol) in a 6:1.5:1 mixture of MeOH/THF/ H_2O (10 mL), $\text{Cu}(\text{Ac})_2$ (42 mg, 0.23 mmol), Na-ascorbate (45 mg, 0.23 mmol) followed by a solution of **40** (32 mg, 0.23 mmol) in a minimum amount of THF were added and the mixture was sonicated until disappearance of the starting material was detected (TLC monitoring). The mixture was diluted with CH_2Cl_2 , filtered over a Celite pad, and the solvent was removed under reduced pressure. The crude was purified by flash chromatography affording **12** (101 mg, 0.11 mmol) in 87 % yields. $R_f = 0.40$ (CH_2Cl_2 :EtOH 90:10); $[\alpha]_D^{20} = +29.5^\circ$ ($c = 0.2$, CH_3OH); ^1H -NMR (400 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$): $\delta = 7.83$ (s, 1H, *aromatic*), 5.13 (br s, 1H, $H_{1'}$), 4.67 (s, 2H, -triazole- CH_2O -), 4.58 (t, $J = 4,4$ Hz, 2H, - $\text{OCH}_2\text{CH}_2\text{NCS}$), 3.96 (s, 2H, - COCH_2O -), 3.95 (m, 3H), 3.69-3.30 (m, 12H), 3.14 (m, 2H), 2.17 (m, 1H, H_2), 1.39 (s, 9H), 1.37 (s, 18H), 1.21 (m, 1H, H_2); ^{13}C -NMR (100.6 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$): $\delta = 170.6, 157.3, 156.6, 155.8, 144.6, 124.0, 99.1, 79.7, 75.5, 72.0, 71.5, 69.9, 69.2, 68.2, 66.3, 64.0, 61.0, 57.5, 55.3, 50.0, 44.9, 39.1, 34.5, 29.4, 28.0, 27.9, 17.6$; ESI (m/z) 929.3 $[\text{M}+\text{K}]^+$, 913,4 $[\text{M}+\text{Na}]^+$, 891.3 $[\text{M}+\text{H}]^+$.

Compound 29. The synthesis of compound **29** has been achieved as described in general procedure reported before (chapter 5.1 and 5.1.1.). ^1H -NMR (400 MHz, D_2O): $\delta = 8.03$ (s, 46H, *aromatic* of neamine moieties), 5.64 (s, 46H, $H_{1'}$ of neamine moieties), 4,60 (m, 138H), 3.98-3.32 (m, 1162H), 2.77 (m, 248H, - $\text{NHCH}_2\text{CH}_2\text{CO}$ - of PAMAM), 2.46 (m, 46 H, H_2 of neamine moieties), 1.82 (ddd, $J = 12.1$ Hz, 46H, H_2 of neamine moieties).

Synthesis of PAMAMG4-neomycin conjugate 31 (scheme 5):

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Compound **50** was prepared as described in literature.^[102]

Compound 52. To a solution of **50** (100 mg, 0.08 mmol) in dry DMF (2 mL) acid **51** (13 mg, 0.09 mmol), DIPEA (31 mg, 0.24 mmol) followed by TBTU (51 mg, 0.16 mmol) were added at r.t.. After the total consumption of the starting amine (TLC monitoring), the organic solvent was removed, the crude dissolved in AcOEt/H₂O and extracted with AcOEt. The collected organic layers were anhydriified over Na₂SO₄, filtered and evaporated. The crude was purified by flash chromatography affording **16** (73 mg, 0.05 mmol) in 68% yield. $R_f = 0.48$ (CHCl₃:MeOH 90:10); $[\alpha]_{\text{D}}^{20} = +27.4^\circ$ ($c = 1.7$, CH₃OH); ¹H-NMR (400 MHz, CD₃OD): $\delta = 5.37$ (s, 1H, anomeric), 5.14 (s, 1H, anomeric), 4.90 (s, 1H, anomeric), 4.29 (m, 1H), 4.11 (m, 2H), 3.91 (m, 3H), 3.76 (m, 5H), 3.58-3.20 (m, 15H), 1.98 (m, 1H, H₂), 1.48 (s, 9H), 1.47 (s, 9H), 1.46 (s, 9H), 1.45 (s, 9H), 1.44 (s, 18H), 1.30 (m, 1H, H₂); ¹³C-NMR (100.6 MHz, CD₃OD): $\delta = 171.1, 157.5, 157.1, 156.9, 156.8, 156.4, 109.9, 99.2, 97.8, 85.9, 79.7, 79.3, 79.0, 74.4, 73.2, 71.9, 71.4, 70.4, 70.0, 69.9, 67.8, 55.5, 52.2, 50.3, 41.4, 40.7, 35.5, 34.4, 27.6, 27.5, 27.4, 27.3$; ESI (m/z) 1364.1 [M+Na]⁺.

Compound 53. To a solution of **52** (180 mg, 0.13 mmol) in a 6:1.5:1 mixture of MeOH/THF/H₂O (10 mL), Cu(Ac)₂ (42 mg, 0.23 mmol), Na-ascorbate (45 mg, 0.23 mmol) followed by a solution of **40** (32 mg, 0.23 mmol) in a minimum amount of THF were added and the mixture was sonicated until disappearance of the starting material was detected (TLC monitoring). The mixture was diluted with CH₂Cl₂, filtered over a Celite pad, and the solvent was removed under reduced pressure. The crude was purified by flash chromatography affording **53** (163 mg, 0.11 mmol) in 79 % yields. *R_f* = 0.33 (CHCl₃:MeOH 95:5); $[\alpha]_D^{20} = +30.6^\circ$ (*c* = 0.7, CH₃OH); ¹H-NMR (400 MHz, CD₃OD): δ = 8.14 (s, 1H, *aromatic*), 5.38 (s, 1H, *anomeric*), 5.14 (s, 1H, *anomeric*), 4.90 (s, 1H, *anomeric*), 4.29 (m, 1H), 4.08-4.31 (m, 8H), 3.77-3.73 (m, 5H), 3.64-3.35 (m, 19H), 3.21 (m, 1H), 1.97 (m, 1H, *H*₂), 1.47 (s, 9H), 1.46 (s, 9H), 1.45 (s, 18H), 1.44 (s, 18H), 1.30 (m, 1H, *H*₂); ¹³C-NMR (100.6 MHz, CD₃OD): δ = 170.9, 157.5, 157.1, 156.9, 156.8, 156.4, 144.2, 124.7, 110.1, 99.3, 97.8, 86.2, 79.7, 79.3, 79.0, 74.5, 74.3, 73.2, 72.0, 71.4, 70.4, 69.9, 69.6, 68.1, 63.5, 55.5, 52.3, 51.2, 50.2, 49.8, 44.8, 41.6, 40.7, 34.4, 27.6, 27.5, 27.4, 27.3; ESI (*m/z*) 1505.0 [M+Na]⁺.

Compound 31. The synthesis of compound **31** has been achieved as described in the general procedure. ¹H-NMR (400 MHz, D₂O): δ = 8.16 (s, 40H, *aromatic* of neomycin moieties), 6.07 (d, *J* = 4.0 Hz, 40H, *anomeric* of neomycin moieties), 5.48 (d, *J* = 4.0 Hz, 40H, *anomeric* of neomycin moieties), 5.34 (s, 40H, *anomeric* of neomycin moieties), 4.77 (m, 248H, -CONHCH₂CH₂N- of PAMAM), 4.50 (m, 40H), 4.34 (m, 160H), 4.16-3.74 (m, 1620H), 3.25 (m, 40H), 2.92 (m, 248H, -NHCH₂CH₂CO- of PAMAM), 2.60 (m, 40H, *H*₂ of neomycin moieties), 1.81 (ddd, 40H *J* = 12.4 Hz, *H*₂ of neomycin moieties).

Biological Part

Bacterial culture conditions

E. Coli and *S. Aureus* were cultivated in LB medium and in Nutrient Broth (NB) medium (5 g/L peptone and 3 g/L meat extract), respectively. A small amount of stock bacteria was transferred to an agar plate and incubated at 37 °C for 20 hrs. Then, one colony of bacteria was picked from the agar plate and suspended in broth medium. The suspensions were incubated at 37 °C under shaking and the optical density at 600 nm

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(OD₆₀₀) was measured by means of a spectrophotometer (NanoDrop 2000c, Thermo Scientific, Wilmington, USA) to check the bacterial growth.

Determination of the Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC), that is the lowest concentration of aminoglycosides, dPAMAM G4 and dPAMAM G4-aminoglycoside conjugates that inhibits the visible growth of *E. Coli* and *S. Aureus*, was evaluated adapting the protocol described in ISO 20776-1. Briefly, single colonies were used to inoculate 5 mL of LB broth medium for *E. Coli* or NB medium for *S. Aureus* at 37 °C under shaking until the OD₆₀₀ reached a value of 0.3 (the suspension contained approximately 2.5×10^8 cfu/mL for the common relevant bacteria). Then cells were diluted 1:500 in media to give a final cell concentration of 5×10^5 cfu/mL and 50 µL of the resulting cell suspension was incubated with 50 µL of dH₂O containing different concentrations of aminoglycosides, dPAMAM G4 and dPAMAM G4-aminoglycoside conjugates. Positive controls were obtained by adding 50 µL of cell suspension to 50 µL of sterile dH₂O. Negative controls were obtained by adding 50 µL of cell-free medium to 50 µL of sterile dH₂O. After 24 hrs of incubation at 37 °C, the pellets formed were resuspended prior to measure the OD₆₀₀. The MIC value is defined as the concentration at which a sharp decline in optical density reaches the minimum.

Determination of killing times

The bactericidal activity of dPAMAM derivatives was tested by following the Time Kill Procedure adapting and integrating the protocol reported in the ASTM E 2315 standard. Briefly, *E. Coli* and *S. Aureus* microorganisms were grown until the OD₆₀₀ reached a value of 0.3, by incubating single colonies in culture media at 37 °C under shaking. The bacterial suspensions were next diluted 1:25 in dH₂O to give a final cell concentration of 1×10^7 cfu/mL and 200 µL of the resulting suspensions were challenged with 200 µL of dH₂O containing aminoglycosides, dPAMAM G4 or dPAMAM G4-aminoglycoside conjugates at the MIC. At specific time intervals, *E. Coli* and *S. Aureus* inoculated samples (30 µL) were removed, serially diluted in dH₂O and plated onto LB and NB agar plates, respectively. The

plates were incubated for 24 hrs at 37 °C and the mean number of survivors was determined.

Antibacterial activity of pDNA/ dPAMAM G4-aminoglycosides

In order to test the antimicrobial activity of polyplexes, samples were prepared by adding pDNA to dPAMAM G4 and dPAMAM G4-aminoglycoside conjugates **2** and **3** in dH₂O at the MIC, yielding the optimal N/P ratio found in transfection [25]. *E. Coli* and *S. Aureus* were inoculated in 5 mL of LB broth medium or NB medium, respectively, at 37 °C under shaking until the OD₆₀₀ was 0.3. Then cells were diluted 1:500 in medium to give a final cell concentration of 5 × 10⁵ cfu/mL. 50 µL of cell suspension was incubated with 50 µL of polyplexes or dPAMAM G4 and dPAMAM G4-aminoglycoside conjugates. Positive controls were obtained by adding 50 µL of cell suspension to 50 µL of sterile dH₂O. Negative controls were obtained by adding 50 µL of cell-free medium to 50 µL of sterile dH₂O. The antibacterial effect was determined after 24 hrs of incubation at 37 °C by measuring the OD₆₀₀.

In vitro transfection experiments in the presence of bacterial infections

For transfection experiments, HeLa cells were plated in 96-well sterile culture plates at a density of 2 × 10⁴ cells/cm² in a final volume of 300 µL/cm² of complete DMEM without antibiotics. *E. Coli* and *S. Aureus* were grown under shaking in complete DMEM without antibiotics at 37 °C till OD₆₀₀ ~ 1. Bacteria were next diluted 10³ times in complete DMEM (the suspension contained approximately 7.5 × 10⁵ cfu/mL). Polyplexes were prepared by adding pGL3 to dPAMAM G4 and G4-aminoglycoside conjugates, each at a concentration equal to 2.5 × MIC in dH₂O, yielding N/P 30. Twenty-four hrs after cell seeding, the culture medium was replaced with 95 µL of either complete DMEM containing *E. Coli* and *S. Aureus* or plain complete DMEM and 5 µL of polyplexes was added to each well. Five µL of dH₂O were also added as a negative control. The same transfection suspensions were also added to *E. Coli* and *S. Aureus* in complete DMEM in the absence of HeLa cells. 100 µL of bacterial suspensions just after inoculation were diluted 1:100 in dH₂O and plated onto agar. Colonies were counted after incubation at 37 °C for 24 hrs. After transfection, cells were

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incubated for 24 hrs at 37 °C in a CO₂ humidified atmosphere prior to analysis. To assess bacteria killing, 24 hrs after transfection, the bacteria were resuspended by pipetting and supernatants were diluted 1:5000 in dH₂O and plated onto agar. Colonies grown on plates were counted after incubation at 37 °C for 24 hrs. Polyplex cytotoxicity was assessed using AlamarBlue cell viability assay according to manufacturer's guidelines. Viability of untreated control cells was assigned as 100% and cytotoxicity was determined as follows:

$$\text{Cytotoxicity [\%]} = 100\% - \text{Viability [\%]}$$

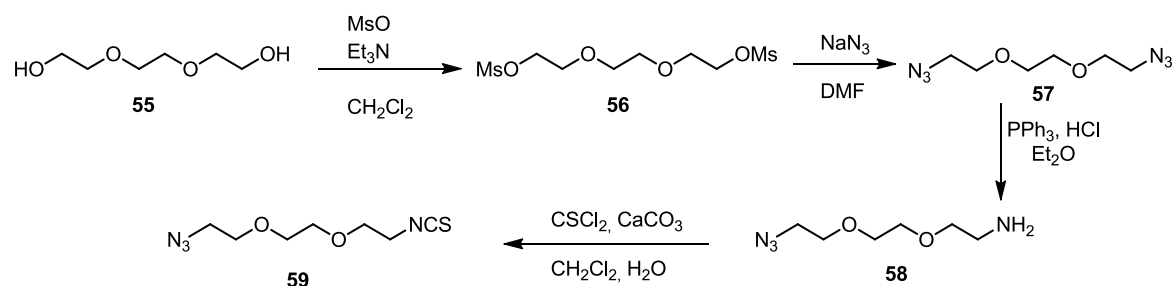
Luciferase activity was measured by Luciferase Assay System using a microplate reader GENios Plus (Tecan, Segrate, Italy) and normalized to the total protein content, evaluated by BCA assay, in cell lysates.

Statistical analysis

Experiments were performed at least in quadruplicate. Results were expressed as mean \pm standard deviation (SD). Statistical analyses were carried out by GraphPad Prism v 5.04 (GraphPad software, La Jolla, CA, USA). Comparisons among groups were performed by one-way analysis of variance (ANOVA). Significance was retained when $p < 0.05$.

5.1.2 PAMAMs-Amino and Guanidinoglycosides Conjugates:

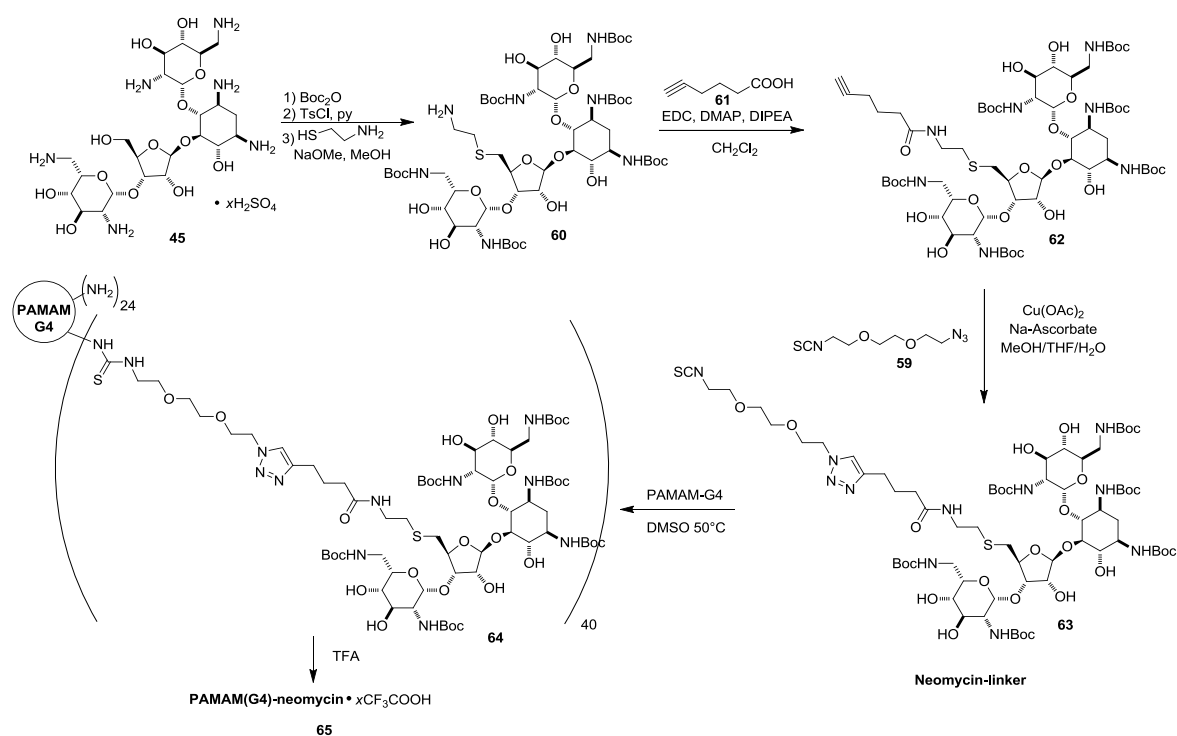
Synthesis of new linker 59 (Scheme 6)



Compound 56, 57, 58. The compounds 56, 57, 58 were prepared as described in literature.^[143a]

Compound 59. 780mg (4,50 mmol) of compound **58** were dissolved in a 30 mL of 1:1 mixture of DCM and H₂O, then 1.350g (13,5mmol) of CaCO₃ were added to the vigorously stirred solution. Thiophosgene (1g, 9 mmol) was added dropwise over 5 minute and the resultant biphasic mixture was vigorously stirred at RT overnight. The reaction mixture was diluted with water (40 mL) and the organic phase was separated. The aqueous layer was extracted with DCM (2 x 40 ml) and the combined organic layers were washed with water (50ml), dried with Na₂SO₄ and concentrated in vacuo to give a yellow oil (660g, 3,06 mmol) in 68% yield. Rf= 0,4 (hexane:AcOEt 50:50); ¹H-NMR (400 MHz, CD₃Cl): δ =4.09 (m, 2 H), 3.68 (bs, 8H), 2.01 (bs, 2H), 1.23 (t, 2H). ¹³C-NMR (100.6 MHz, CD₃Cl): δ = 73.00, 70.52, 22.80, 10.70. ESI (*m/z*) 217.15 [M+H]⁺, 234.10 [M+NH₄]⁺, 239.13 [M+Na]⁺.

Synthesis of conjugates **65**, **69** and **70** (scheme 7)



Compound 60. The compound **60** was obtained as reported in literature.^[216]

Compound 62. The compound **62** was obtained as reported in literature.^[102]

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Compound 63. To a solution of **62** (150 mg, 0.11 mmol) in a 5:4:1 mixture of THF / MeOH /H₂O (6 mL), Cu(Ac)₂ (26 mg, 0.143 mmol), Na-ascorbate (28 mg, 0.143 mmol) followed by a solution of **59** (32 mg, 0.23 mmol) in a minimum amount of THF were added and the mixture was sonicated until disappearance of the starting material was detected by TLC monitoring (3 times x 30 minutes). The mixture was concentrated in vacuo then diluted with CH₂Cl₂. The organic layer was washed with a saturated solution of NH₄Cl, dried with Na₂SO₄ and the solvent was removed under reduced pressure. The crude was purified by flash chromatography affording **63** (150 mg, 0.095 mmol) in 86 % yields. *R_f* = 0.33 (CH₂Cl₂:MeOH 95:5); ¹H-NMR (400 MHz, CDCl₃): δ = 7.94 (s, 1 H), 5.35 (s, 1H), 5.17 (s, 1H), 4.95 (s, 1H), 4.57 (s, 2 H), 4.25 (s, 2H), 4.11 (s, 1H), 3.92 (s, 4H), 3.80 (d, *J*=6.0 Hz, 2 H), 3.6-3.17 (m, 22 H), 2.92 (s, 2H), 2.78 (s, 4H), 2.31 (s, 2H), 2.02 (s, 3H), 1.45 (broad s, 56 H). ¹³C-NMR (100.6 MHz, CD₃OD): δ = 174.12, 157.5, 157.1, 156.9, 156.8, 156.4, 156.2, 132.27, 122.99, 110.1, 99.3, 97.8, 86.2, 79.7, 79.3, 79.0, 74.5, 74.3, 73.2, 72.0, 71.56, 70.25, 70.16, 70.12, 69.9, 69.6, 69.06, 55.57, 53.37, 52.3, 51.2, 50.2, 49.8, 47.64, 47.22, 47.01, 45.01, 44.8, 41.6, 40.7, 38.74, 35.06, 34.47, 34.34, 27.6, 27.5, 27.4, 27.3; 25.38, 24.53. C, 51.53; H, 7.44; N, 9.72; O, 27.26; S, 4.05 ESI (*m/z*) 1606.76 [M+Na]⁺.

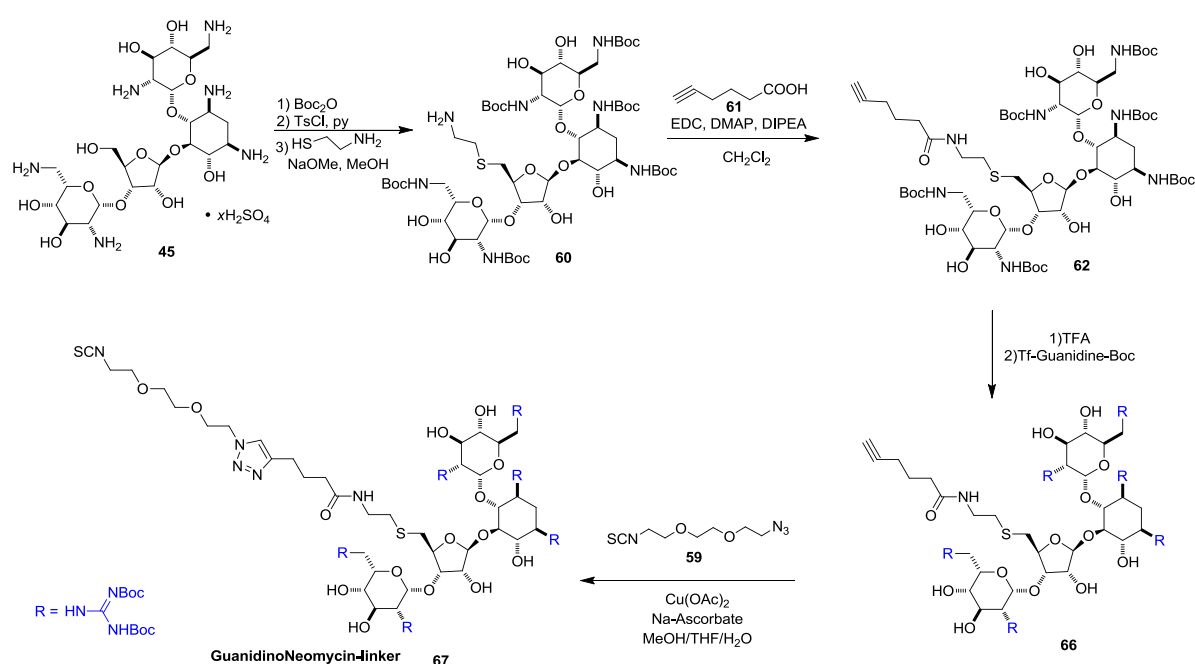
Compound 65. The synthesis of compound **65** has been achieved as described in the general procedure (chapter 5, phar. 5.1). ¹H-NMR (400 MHz, D₂O): δ = 7.99 (s, 40H, *aromatic* of neomycin moieties), 6.15 (d, *J* = 4.0 Hz, 40H, *anomeric* of neomycin moieties), 5.49 (d, *J* = 4.0 Hz, 40H, *anomeric* of neomycin moieties), 5.39 (s, 40H, *anomeric* of neomycin moieties),), 4.49 (s, 80 H), 4.40 (s, 80H), 4.33 (s, 40H), 4.15 (m, 80H), 3.92 (bs, 160H), 3.80 (s, 80 H), 3.75-3.30 (m, 2100H), 3.20 (m, 80H of neomycin moieties), 2.92-2.83 (m, 408H: 248 -NHCH₂CH₂CO- of PAMAM plus 160H of neomycin moieties) 2.58 (m, 40H of neomycin moieties), 2.37 (s, 80H of neomycin moieties), 2.03 (m, 120H of neomycin moieties).

Compound 69. The synthesis of compound **69** has been achieved as described in the general procedure (chapter 5, phar. 5.1). ¹H-NMR (400 MHz, D₂O): δ = 8.18 (s, 13H, *aromatic* of neomycin moieties), 6.09 (s, 13H, *anomeric* of neomycin moieties), 5.43 (s, 13H, *anomeric* of neomycin moieties), 5.33 (s, 13H, *anomeric* of neomycin moieties), 4.44 (s,

26H), 4.35 (s, 26H), 4.27 (s, 13H), 4.14 (m, 91H), 3.86 (s, 13H), 3.70-3.30 m, 512H), 3.15 d, J= 3 Hz, 13 H), 2.80 (m, 134H 56 -NHCH₂CH₂CO- of PAMAM plus 78H of neomycin moieties), 2.53 (d, J=3Hz 13H), 2.35 (s, 26H), 1.98 m, 39H).

Compound 70. The synthesis of compound **70** has been achieved as described in the general procedure (chapter 5, par. 5.1). ¹H-NMR (400 MHz, D₂O): δ = 8.06 (s, 307H, aromatic of neomycin moieties), 6.11 (s, 307H, anomeric of neomycin moieties), 5.45 (s, 307H, anomeric of neomycin moieties), 5.35 (s, 307H, anomeric of neomycin moieties), 4.46 (s, 614H), 4.37 (s, 614H), 4.29 (s, 307H), 4.09 (m, 2149H), 3.89 (s, 307H), 3.70-3.30 (m, 11872H), 3.15 d, J= 3 Hz, 307 H), 2.84 m, 3890H 2048 -NHCH₂CH₂CO- of PAMAM plus 1842H of neomycin moieties), 2.53 (d, J=3Hz 307H), 2.35 (s, 614H), 1.98 m, 921H).

Synthesis of guanidinoneomycin-linker **67** (scheme 8)



Compound 66. The compound **66** was obtained as reported in literature.^[102]

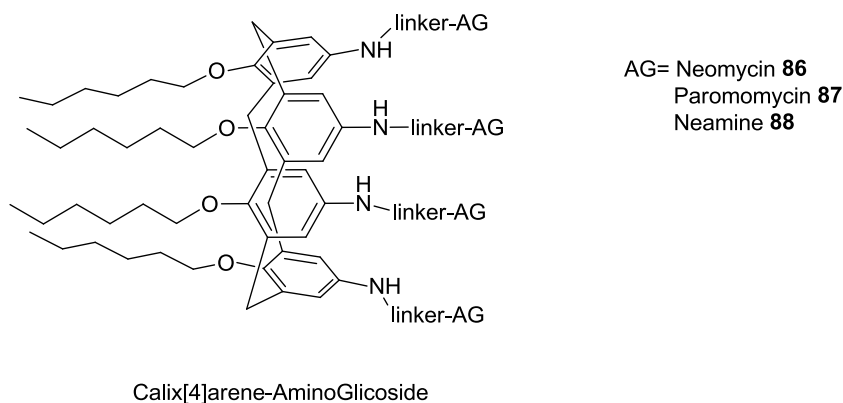
Compound 67. To a solution of **66** (240 mg, 0.11 mmol) in a 5:4:1 mixture of THF / MeOH / H₂O (6 mL), Cu(OAc)₂ (26 mg, 0.143 mmol), Na-ascorbate (28 mg, 0.143 mmol)

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followed by a solution of **59** (28 mg, 0.132 mmol) in a minimum amount of THF were added and the mixture was sonicated until disappearance of the starting material was detected by TLC monitoring (3 times x 30 minutes). The mixture was concentrated in vacuo then diluted with CH₂Cl₂. The organic layer was washed with a saturated solution of NH₄Cl, dried with Na₂SO₄ and the solvent was removed under reduced pressure. The crude was purified by flash chromatography affording **67** (220 mg, 0.090 mmol) in 81 % yields. *R_f* = 0.33 (CH₂Cl₂:MeOH 95:5); ¹H-NMR (400 MHz, CDCl₃): δ = 7.82 (s, 1 H), 5.35 (s, 1H), 5.17 (s, 1H), 4.94 (s, 1H), 4.57 (s, 2 H), 4.25 (s, 2H), 4.11 (s, 1H), 3.92 (s, 4H), 3.80 (bs, 2 H), 3.6-3.17 (m, 22 H), 2.82 (s, 2H), 2.75 (s, 4H), 2.31 (s, 2H), 2.02 (s, 3H), 1.45 (broad s, 110 H). ¹³C-NMR (100.6 MHz, CD₃OD): δ = 173.64, 163.21, 162.23, 161.33, 157.31, 156.34, 156.9, 156.8, 156.4, 156.2, 153.05, 152.82, 152.59, 152.01, 124.25, 122.99, 118.00, 110.1, 99.3, 98.23, 97.8, 96.20, 86.2, 83.29, 83.05, 82.78, 79.7, 79.3, 78.09, 74.5, 74.3, 73.2, 72.0, 71.56, 70.25, 70.16, 70.12, 69.9, 69.6, 69.06, 66.73, 56.73, 55.57, 53.37, 52.3, 51.2, 50.2, 49.8, 47.87, 47.66, 47.44, 47.23, 47.02, 47.01, 45.01, 44.8, 41.6, 40.7, 38.74, 35.06, 34.47, 34.34, 29.57, 27.6, 27.5, 27.4, 27.3; 26.93, 26.76, 25.41, 24.57. ESI (*m/z*) 2438.20[M+H]⁺, 2460.20[M+Na]⁺. C, 51.24; H, 7.32; N, 13.22; O, 25.60; S, 2.63.

Compound 68. The synthesis of compound **68** has been achieved as described in the general procedure (chapter 5, par. 5.1). ¹H-NMR (400 MHz, D₂O): δ= 7.82 (s, 15H, *aromatic* of guanidinoneomycin moieties), 5.94 (s, 15H, *anomeric* of guanidinoneomycin moieties), 5.19 (s, 15H, *anomeric* of guanidinoneomycin moieties), 5.07 (s, 15H, *anomeric* of guanidinoneomycin moieties), 4.57 (s, 30 H), 4.38 (s, 30H), 4.12 (s, 45H), 3.92 (bs, 30H), 3.82 (m, 60H), 3.79 (s, 30 H), 3.70-3.14 (m, 1550H), 3.20 (m, 80H of neomycin moieties), 2.87-2.70 (m, 3308H: 248 -NHCH₂CH₂CO- of PAMAM plus 90H of guanidinoneomycin moieties) 2.28 (m, 45H of guanidinoneomycin moieties), 1.93 (s, 30H of guanidinoneomycin moieties), 1.44 (m, 15H of guanidinoneomycin moieties).

5.1.3 Synthesis of Calix[4]arene-aminoglycoside Conjugates



Compound 84. Calix[4]arene **84** was used as prepared from Prof. Sansone's research group as reported in literature.^[110]

Compound 63. Neomycin-linker **63** was prepared as reported before.

Compound 86. The synthesis of compound **86** has been achieved as described in the general procedure (chapter 5, par. 5.1). ¹H-NMR (400 MHz, D₂O): δ = 8.18 (s, 4H, *aromatic* of neomycin moieties), 7.21 (m, 8H), 5.95 (d, J = 4.0 Hz, 4H, *anomeric* of neomycin moieties), 5.31 (s, 4H, *anomeric* of neomycin moieties), 5.23 (s, 4H, *anomeric* of neomycin moieties), 4.51 (m, 6H), 4.30 (s, 7H), 4.25 (s, 8H), 4.15 (s, 6H), 3.98-3.16 (m, 96 H), 2.75 (m, 23H), 2.43 (m, 5H), 2.18 (m, 8H), 1.75 (m, 21H), 1.23 (bs, 32 H), 0.75 (bs, 12H calixarene).

Compound 89. Paromomycin-linker **89** was prepared as reported in literature.^[112] R_f = 0.52 (CH₃Cl:MeOH 9:1); ¹H-NMR (400 MHz, CD₃OD): δ = 7.91 (s, 1H), 5.43 (s, 1H), 5.28 (s, 1H), 5.00 (s, 1H), 4.66 (s, 2H), 4.36 (d, J = 8Hz, 2H), 2.99-3.64 (m, 21H), 3.40 (bs, 6H), 3.09 (m, 2H), 2.39 (t, J = 4Hz, 2H), 2.07 (q, J = 6Hz, 3H), 1.53 (bs, 45H). ¹³C-NMR (100.6 MHz, CD₃OD): δ = 173.39, 155.65, 155.63, 155.35, 155.22, 145.57, 143.80, 141.51, 130.63, 121.36, 121.37, 107.40, 97.51, 96.14, 84.69, 80.42, 77.89, 77.61, 72.88, 71.73, 71.51, 70.13, 69.34, 68.77, 68.69, 67.70, 66.39, 60.23, 54.30, 46.83, 46.62, 46.41, 46.19, 45.98, 45.77, 45.56, 43.53, 38.32, 33.48, 33.06, 26.18, 26.06, 25.98, 23.97, 23.01. ESI (m/z) 1348.4 [M+Na]⁺.

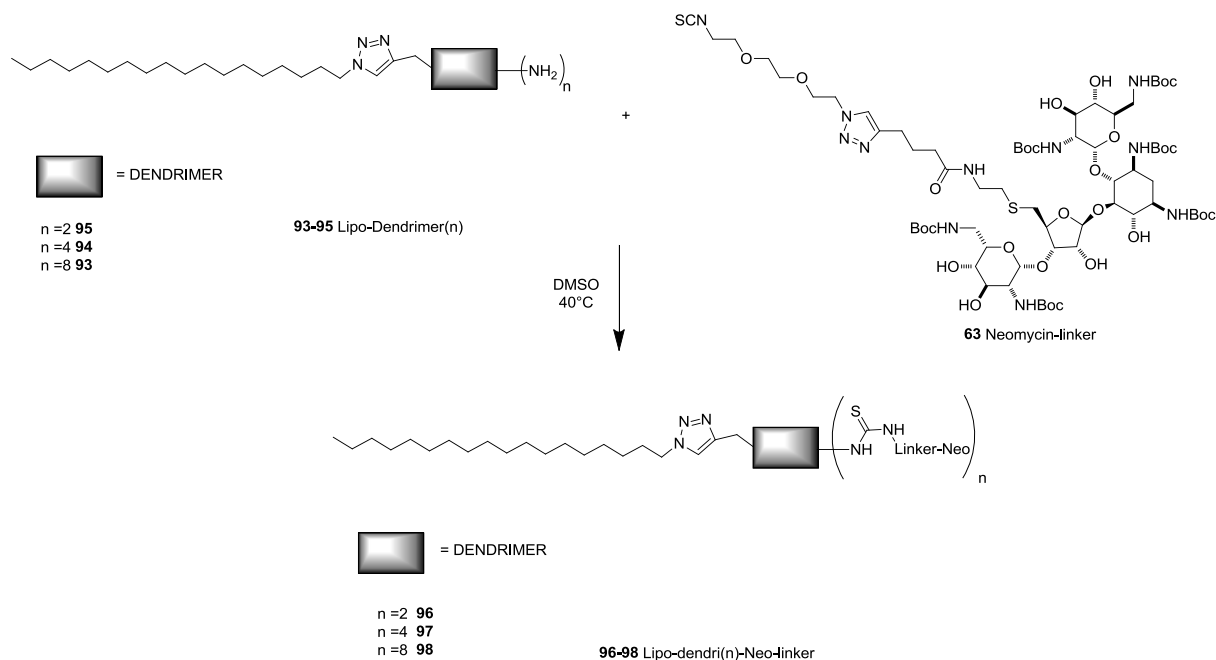
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Compound 87. The synthesis of compound **87** has been achieved as described in the general procedure (chapter 5, par. 5.1). ¹H-NMR (400 MHz, D₂O): δ = 8.00 (s, 4H, *aromatic* of paromycin moieties), 7.21 (m, 8H), 5.82 (d, *J* = 2.0 Hz, 4H, *anomeric* of paromycin moieties), 5.43 (d, *J* = 4.0 Hz, 4H, *anomeric* of paromycin moieties), 5.25 (s, 4H, *anomeric* of paromycin moieties), 4.61 (s, 4H), 4.34 (t, *J*=15Hz, 16H), 4.09 (m, 4H), 3.98-3.52 (m, 88H), 2.77 (s, 6H), 2.57 (d, *J*=4Hz, 4H), 2.32 (s, 7H), 1.96 (bs, 18H), 1.37 (bs, 35H), 0.92 (s, 12H).

Compound 91. Neamine-linker **91** was prepared as reported in literature.^[112] ¹H-NMR (400 MHz, CD₂OD): δ = 7.91 (s, 1H), 5.40 (s, 1H), 4.61 (s, 2H), 4.53 (m, 1H), 4.28 (m, 2H), 4.01-3.64 (m, 88H), 3.40 (m, 8H), 3.25 (m, 3H), 2.85 (t, *J* = 4Hz, 2H), 2.47 (t, *J* = 5Hz, 2H), 2.21 (m, 2H), 1.53 (bs, 29H). ¹³C-NMR (100.6 MHz, CD₃OD): δ = 174.77, 157.55, 155.63, 147.61, 137.63, 126.76, 99.35, 80.62, 80.11, 79.69, 75.33, 73.93, 72.09, 71.78, 71.57, 69.93, 69.60, 68.94, 56.74, 52.14, 51.76, 48.95, 43.18, 40.71, 35.90, 34.65, 28.34, 27.01, 21.90. ESI (*m/z*) 955.45 [M+Na]⁺.

Compound 88. The synthesis of compound **88** has been achieved as described in the general procedure (chapter 5, par. 5.1). ¹H-NMR (400 MHz, D₂O): δ = 7.83 (s, 4H, *aromatic* of neamine moieties), 7.21 (m, 8H of calixarene moieties) 5.72 (s, 4H, *anomeric* of neamine moieties), 4.61 (bs, 13H), 3.88-3.65 (m, 40H), 3.33-3.26 (m, 30H), 2.77 (bs, 9H), 2.55 (m, 5H), 2.36 (bs, 9H), 2.02 (bs, 24H), 1.45 (m, 40H), 0.99 (s, 12H CH₃CH₂-of calixarene moieties).

5.1.4 Hybrid lipo-dendrimers-aminoglycoside Conjugates



Hybrid lipo-dendrimer were prepared by the group of Prof. Peng as reported in literature.^[158-160]

Neomycin-linker **63** was prepared as reported before.

Hybrid lipo-dendrimer- Neomycin Conjugates 96-98 general procedure

The hybrid lipo-dendrimers, was dissolved in 1 mL of DMSO and a solution of neomycin-isothiocyanate linker **63** (1.2 equiv per NH₂ group of dendrimer) in a minimal volume of DMSO was added. The solution was stirred at 50 °C for 24 h and then dialyzed against MeOH (8 h, the solvent reservoir was renewed thrice, MWCO 1 kDa). The solution was evaporated under reduced pressure to give N-Boc-protected hybrid-lipo-dendrimer-neomycin conjugates. These resulting conjugates were dissolved in trifluoroacetic acid (TFA) and stirred for 30 min at room temperature (r.t.). The excess of TFA was evaporated under reduced pressure, the crude dissolved in water, and the solution dialyzed against water (overnight, MWCO 1 kDa). Lyophilization of the water solution led to recovery of a fluffy, white solid.

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Compound 96. $^1\text{H-NMR}$ (400 MHz, D_2O): $\delta = 7.94$ (s, 2H, *aromatic* of neomycin moieties), 7.81 (s, 1H *aromatic* of lipo-dendrimer moieties), 6.04 (d, $J = 2.0$ Hz, 2H, *anomeric* of neomycin moieties), 5.38 (s, 2H, *anomeric* of neomycin moieties), 5.28 (s, 2H, *anomeric* of neomycin moieties), 4.61 (bs, 4H), 4.38 (s, 5H), 4.29 (s, 5H), 4.21 (s, 3H), 4.15-3.80 (m, 20) 3.63-3.26 (m, 48H), 3.16 (m, 4H), 2.75 (m, 18H), 2.49 (m, 4H), 2.25 (m, 6H), 1.75 (m, 8H), 1.17 (bs, 30H of lipo-dendrimer moieties), 0.77 (bs, 3H CH_3CH_2^- of lipo-dendrimer moieties).

Compound 97. $^1\text{H-NMR}$ (400 MHz, D_2O): $\delta = 8.25$ (s, 1H *aromatic* of lipo-dendrimer moieties), 8.03 (s, 4H, *aromatic* of neomycin moieties), 6.04 (d, $J = 2.0$ Hz, 4H, *anomeric* of neomycin moieties), 5.38 (s, 4H, *anomeric* of neomycin moieties), 5.28 (s, 4H, *anomeric* of neomycin moieties), 4.61 (bs, 4H), 4.38 (s, 10H), 4.29 (s, 10H), 4.21 (s, 6H), 4.10 m, 6H), 4.00-3.80 (m, 40H) 3.63-3.26 (m, 106H), 3.16 (m, 4H), 2.75 (m, 44H), 2.49 (m, 6H), 2.28 (m, 12H), 1.91 (m, 16H), 1.14 (bs, 30H of lipo-dendrimer moieties), 0.77 (bs, 3H CH_3CH_2^- of lipo-dendrimer moieties).

Compound 98. $^1\text{H-NMR}$ (400 MHz, D_2O): $\delta = 8.18$ (s, 8H, *aromatic* of neomycin moieties), 8.09 (s, 1H *aromatic* of lipo-dendrimer moieties), 6.04 (d, $J = 2.0$ Hz, 2H, *anomeric* of neomycin moieties), 5.38 (s, 8H, *anomeric* of neomycin moieties), 5.27 (s, 8H, *anomeric* of neomycin moieties), 4.61 (bs, 4H), 4.38 (s, 16H), 4.28 (s, 16H), 4.21 (s, 9H), 4.15-3.80 (m, 38H) 3.68-3.30 (m, 208H), 3.16 (m, 16H), 2.75 (m, 62H), 2.49 (m, 10H), 2.30 (m, 18H), 1.93 (m, 28H), 1.14 (bs, 30H of lipo-dendrimer moieties), 0.76 (bs, 3H CH_3CH_2^- of lipo-dendrimer moieties).

5.2 Drug Delivery Project

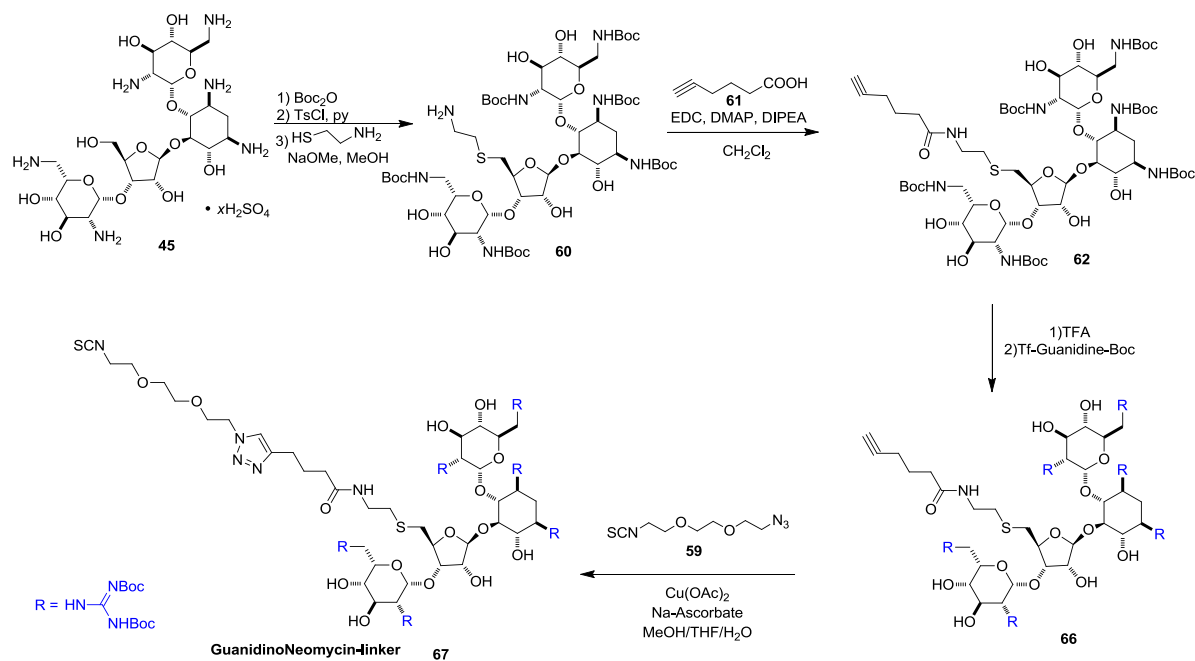
General experimental Methods

Instrumentation

NMR spectra were recorded on a Varian Mercury 500 MHz spectrometer. Mass spectra were recorded at the UCSD Chemistry and Biochemistry Mass Spectrometry Facility; low resolution mass spectrometry (LR-MS) analysis was performed on a Thermo LCQdeca mass spectrometer using electrospray ionization (ESI) as the ion source. An Agilent 6230 time of flight mass spectrometer (TOFMS) was employed for high resolution MS (HR-MS) analysis using ESI as the ion source. Reversed phase HPLC purification (CLPEUS, C₁₈, 5 μ m, 10 \times 250 mm, Higgins Analytical) and analysis (Eclipse, XDB-C₁₈, 5 μ m, 4.6 \times 150 mm) were carried out on an Agilent 1200 series instrument. Flow-cytometry studies were performed on a BD FACS Calibur.

General Procedures

Synthesis of guanidinoglycoside-linker (scheme 13).



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The compound **60** was prepared as reported in literature and in a phar. 5.1.2.^[99,102,112] Then compound **60** was reacted with hexynoic acid **61** molar ratio 1:1.5 in CH₂Cl₂, in presence of EDC 1.5 equiv. per mol., DIPEA 3 equiv. per mol. and DMAP cat. After the total consumption of the starting amine (TLC monitoring), the mixture was quenched with HCL 1N and extracted with CH₂Cl₂. The collected organic layers were anhydriified over Na₂SO₄, filtered and evaporated. The crude was purified by flash chromatography affording **62** in 97% of yield. Then the deprotection of amine by TFA, in CH₂Cl₂, in presence of TIPS, and substitution of amino group with guaninidino using Boc-guanidine -triflate, TEA, in CH₂Cl₂ occurred as reported in letterature.^[102] In the last step a solution of **59** (1.1 equiv per mol) in a minimum amount of THF was added to a solution of guanidinoglicoside **66** in a 4:5:1 mixture of MeOH/THF/H₂O (10 mL), Cu(Ac)₂ 1.2 equiv. per mol. and Na-ascorbate 1.3 equiv. per mol.. The mixture was sonicated until disappearance of the starting material detected by TLC monitoring, then the mixture was diluted with CH₂Cl₂, filtered over a Celite pad, and the solvent was removed under reduced pressure. The crude was purified by flash chromatography affording guanidinoneomycin-likner **67** in 87 % yields.

General procedure for Synthesis of PAMAM-biotin conjugates

Commercial available PAMAM dendrimers of generation 2 and 4 were dissolved in DMSO (1mL per 10 mgs of dendrimer). A solution of biotin-isothiocyanate **108** (prepared as reported in Ref. 187) (1 equiv.) in a minimum amount of DMSO was added and the solution stirred over night at 40 °C. The solution was dialyzed against MeOH (10 h, the solvent reservoir was renewed three times). The grafting of PAMAM G2 (**101**) and G4 (**102**) was checked by ¹H NMR and MALDI.

Compound 107. Rf= 0,35 (CH₂Cl₂:MeOH 9:1); ¹H-NMR (400 MHz, CD₃OD): δ =8.21 (s, 1H),7.93 (s, 1H), 6.37 (d, J=6 Hz, 2H), 4.28 (m, 1H), 4.11 (m,1H), 3.82 (bs, 2H), 3.58 (m, 4H), 3.37 (m, 3H), 3.07 (s, 2H), 2.79 (m, 1H), 2.06 (t, J=4Hz, 2H), 1.89 (s, 2H), 1.47 (m, 8H). ¹³C-NMR (100.6 MHz, CD₃Cl): δ = 173.48, 164.70, 78.02, 72.53, 62.20, 60.76, 56.17, 39.48, 36.83, 32.60, 29.69, 28.21, 296.68. ESI (*m/z*) 282.36 [M+H]⁺, 304.28 [M+Na]⁺.

Compound 108. Rf= 0,35 (CH₂Cl₂:MeOH 9:1); ¹H-NMR (400 MHz, CD₃OD): δ =7.81 (s, 1H), 4.66 (m, 2H), 4.57 (m,1H), 4.48 (s, 2H), 4.28 (m, 1H), 3.85 (m, 3H), 3.65-3.45 (m, 7H), 3.30 (s, 1H), 3.18 (m, 1H), 2.89 (m, 1H), 2.75 (d, J=4Hz, 1H), 2.25 (m, 2H), 1.63 (m, 4H), 1.41 (m, 2H). ¹³C-NMR (100.6 MHz, CDCl₃): δ = 174.50, 164.70, 144.67, 123.60, 70.16, 69.96, 68.99 68.90, 61.90, 60.20, 55.69, 48.09, 47.92, 47.86, 47.75, 47.58, 47.41, 47.24, 47.07, 44.92, 39.65, 34.16, 28.32, 25.32. ESI (*m/z*) 498.34 [M+H]⁺, 520.30 [M+Na]⁺, 536.36 [M+K]⁺.

Compound 101. ¹H-NMR (400 MHz, CD₃OD): δ =7.93 (s, 1H), 4.58 (m, 2H), 4.50 (s, 1H), 4.45 (bs, 2H), 4.29 (m, 1H), 3.92 (m, 3H), 3.59 (m, 10H), 3.30 (m, 108H), 2.79 (m, 114H), 2.60 (m, 35H), 2.38 (m, 72H), 2.26 (m, 2H), 1.67 (m, 5H), 1.43 (m, 2H) 1.30 (m, 1H). MALDI (*m/z*) 3753.88 Da [M+H]⁺.

Compound 102. ¹H-NMR (400 MHz, CD₃OD): δ =7.94 (s, 1H), 4.86 (m, 2H), 4.59 (s, 1H), 4.45 (bs, 2H), 4.31 (m, 1H), 3.88 (m, 3H), 3.58 (m, 14H), 3.27 (m, 397H), 2.80 (m, 382H), 2.60 (m, 116H), 2.38 (m, 242H), 2.26 (m, 3H), 1.67 (m, 3H), 1.42 (m, 2H), 1.33 (m, 5H), 0.89 (m, 1H). MALDI (*m/z*) 14721.17 Da [M+H]⁺.

General procedure for Synthesis of biotin-PAMAMs-GNeo Conjugates 99,100

To a solution of PAMAM-biotin conjugates **101** (or **102**) in DMSO (1mL per 10 mgs of the conjugate) a solution of guanidinoneomycin isothiocyanate **67** (1.1 equiv. per NH₂ group) in a minimum amount of DMSO was added. The solution was stirred at 40 °C for 24 h. The solution was dialyzed against MeOH (10 h, the solvent reservoir was renewed three times). After evaporating the solvent, the conjugates were Boc-protected with TFA neat for 30'. The TFA was evaporated and co-evaporated twice with cyclohexane affording conjugates **99** and **100**.

Compound 99. ¹H-NMR (400 MHz, D₂O): δ = 7.85 (s, 11H, *aromatic* of guanidinoneomycin moieties), 5.93 (bs, 11H, *anomeric* of guanidinoneomycin moieties), 5.18 (s, 11H, *anomeric* of guanidinoneomycin moieties), 5.06 (s, 11H, *anomeric* of guanidinoneomycin moieties), 4.57 (s, 26 H), 4.36 (s, 26H), 4.12 (s, 34H), 3.92 (bs, 36H), 3.83-3.35 (m, 512H), 2.80 (m, 122H 56 -NHCH₂CH₂CO- of PAMAM plus 66H of

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guanidinoneomycin moieties), 2.29 (m, 36H), 1.95 (m, 26H), 1.65 (m, 10H), 1.43 (m, 11H), 1.28 (m, 11H). MALDI (m/z) 17349 Da $[M+H]^+$.

Compound 100. $^1\text{H-NMR}$ (400 MHz, D_2O): δ = 7.90 (s, 23H, *aromatic* of guanidinoneomycin moieties), 6.01 (s, 23H, *anomeric* of guanidinoneomycin moieties), 5.26 (s, 23H, *anomeric* of guanidinoneomycin moieties), 5.15 (s, 23H, *anomeric* of guanidinoneomycin moieties), 4.64 (s, 52 H), 4.44 (s, 58H), 4.20 (s, 72H), 4.02 (bs, 96H), 3.70-3.34 (m, 1562H), 3.23 (m, 60H of neomycin moieties), 2.87-2.70 (m, 386H: 248 - $\text{NHCH}_2\text{CH}_2\text{CO-}$ of PAMAM plus 138H of guanidinoneomycin moieties) 2.36 (m, 78H of guanidinoneomycin moieties), 1.98 (s, 62H of guanidinoneomycin moieties), 1.73 (m, 30H of guanidinoneomycin moieties). MALDI (m/z) 41621 Da $[M+H]^+$.

General procedure for Synthesis of biotin-PAMAMs-Neomycin Conjugates 103, 104.

To a solution of PAMAM-biotin conjugates **101** (or **102**) in DMSO (1mL per 10 mgs of the conjugate) a solution of neomycin isothiocyanate **63** (1.1 equiv. per NH_2 group) in a minimum amount of DMSO was added. The solution was stirred at 40 °C for 24 h. The solution was dialyzed against MeOH (10 h, the solvent reservoir was renewed three times). After evaporating the solvent, the conjugates were Boc-protected with TFA neat for 30'. The TFA was evaporated and co-evaporated twice with cyclohexane affording conjugates **103** and **104**.

Compound 103. $^1\text{H-NMR}$ (400 MHz, D_2O): δ = 7.81 (s, 11H, *aromatic* of neomycin moieties), 5.95 (s, 11H, *anomeric* of neomycin moieties), 5.30 (s, 11H, *anomeric* of neomycin moieties), 5.19 (s, 11H, *anomeric* of neomycin moieties), 4.49 (s, 26H), 4.31 (s, 24H), 4.21 (s, 24H), 4.12 (bs, 11H), 3.99 (m, 11H), 3.91 (m, 14H), 3.84 (m, 52H), 3.72 (s, 16H), 3.60-3.10 (m, 364H), 3.01 d, J = 3 Hz, 16 H), 2.64 (m, 104H 56 - $\text{NHCH}_2\text{CH}_2\text{CO-}$ of PAMAM plus 44H of neomycin moieties), 2.38 (m, 11H), 2.18 (s, 22H), 1.82 (m, 34H). MALDI (m/z) 14579.53 Da $[M+H]^+$.

Compound 104. $^1\text{H-NMR}$ (400 MHz, D_2O): δ = 7.72 (s, 32H, *aromatic* of neomycin moieties), 5.64 (s, 32H, *anomeric* of neomycin moieties), 5.24 (s, 32H, *anomeric* of

neomycin moieties), 5.05 (s, 32H, *anomeric* of neomycin moieties),), 4.45 (s, 104 H), 4.20 (s, 90H), 4.16 (s, 76H), 4.04 (s, 64H), 3.91 (s, 32H), 3.83 (bs,160H), 3.67-2.80 (m, 1402H), 2.72 (m, 440H: 248 -NHCH₂CH₂CO- of PAMAM plus 190H of neomycin moieties), 2.50 (bs, 56H), 2.31 (s, 178H), 2.23 (s, 94H) 2.05 (bs, 36H), 1.82 (bs, 84H) 1.43 (m, 32H). MALDI (*m/z*) 46204.6 Da [M+H]⁺.

General procedures for Cell culture:

All cells were grown under an atmosphere of 5% CO₂ in air and 100% relative humidity. Wild-type Chinese hamster ovary cells (CHO-K1) were obtained from the American Type Culture Collection (CCL-61), and pgsA-745 cells were prepared as reported in Ref. 176. CHO-K1 and pgsA-745 cells were grown in F-12 medium supplemented with fetal bovine serum (10% v/v), streptomycin sulfate (100 µg/mL), and penicillin G (100 Units/mL).

General procedures for Cellular uptake:

Wild-type CHO-K1 and mutant pgsA-745 cells were seeded onto 24-well tissue culture plates (100,000 cells/well, 0.5 mL) and grown for 24 h to about 80% confluence. Cells were washed with PBS and incubated with 300 µL of the streptavidin-dendrimer complexes diluted in DMEM to 2 nM and incubated at 37°C for 1 h under an atmosphere of 5% CO₂. The cells were washed twice with 500 µL of PBS, detached with 100 µL of trypsin-EDTA at 37 °C for 5 min, diluted with PBS containing 1% BSA (300 µL) and analyzed by FACS.

General procedures for Mechanism of Cellular Uptake:

Evaluation of clathrin-dependent endocytosis and macropinocytosis

Cells were grown for 24 h as described above, washed with PBS and incubated with 400 mM sucrose or 5 mM amiloride for 1 hour or 10 minutes respectively. Cells were then washed with PBS and treated with 300 µL of the carrier-streptavidin complexes diluted in DMEM to 2 nM and incubated at 37°C for 1 h under an atmosphere of 5% CO₂. Cells were washed with PBS, detached with trypsin-EDTA and analyzed as described above.

5.3 Novel Antibiotics Project

Evaluation of uptake dependency on temperature

Cells were grown for 24 h as described above, washed with PBS and incubated for 30 minutes at 4 °C in DMEM. Pre-cooled carrier-streptavidin complexes, diluted in DMEM to 2 nM, were added to the cooled cells and incubated for 30 minutes at 4 °C. Cells were washed, detached and analyzed as described above.

General procedure for Cytotoxicity

CHO-K1 cells were seeded in a 96 wells plate at a density of 20000 cells per well in 200 µL. After growing overnight, the cells were washed and treated with dendrimeric neomycin or guanidinoneomycin diluted to the indicated concentrations in DMEM and incubated for 24 hours at 37 °C under an atmosphere of 5% CO₂. Cells were then washed and the growth medium was replaced by fresh one (100 µL). Cell Titer Blue (20 µL) was added to each well, and the cells were incubated for 4 hours at 37 °C. Fluorescence was measured in a plate reader with excitation/emission wavelengths set at 530/580. Fluorescence intensity was normalized to that of untreated cells.

5.3 Novel Antibiotics Project

Materials

Commercially available reagent-grade solvents were employed without purification.

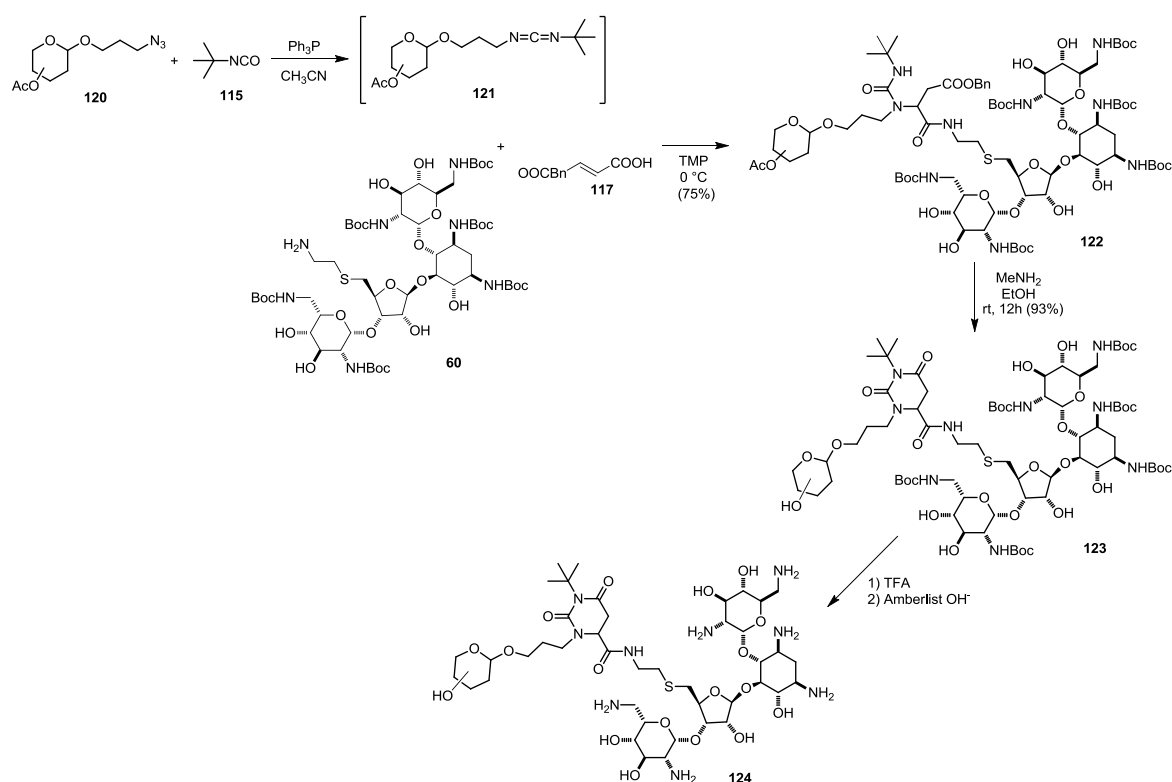
Glycosylazides **120** {a,e} were prepared as described in Ref. 197.

Neomycin derivative **60** was obtained as reported in Ref. 102

Instruments

TLC were run on silica gel 60 F₂₅₄ Merck. Flash chromatography (FC) was performed with silica gel 60 (60-200 μm, Merck). The ¹H NMR spectra were recorded on 400 MHz spectrometers. Chemical shifts are expressed in ppm (δ), using tetramethylsilane (TMS) as internal standard for ¹H and ¹³C nuclei (δ_H and δ_C = 0.00). Mass spectra were performed with a GC-MS instrument, using a gas chromatograph equipped with an SBP-1 fused silica column (30mm x 0.2 mm i.d., 0.2 mm film thickness) and helium as carrier gas.

Multicomponent Domino Process for Sugar-Neomycin Conjugates (scheme 18)



General Procedure for Synthesis of Sugar-neomycin Conjugates **122** {a-e}

To a stirred solution of glycosylazide **120** (1 equiv) in CH₃CN (0.1 M) were added at r.t. a solution of *tert*-butyl isothiocyanate **115** (1.05 equiv) followed by Ph₃P (1.05 equiv). The solution was stirred until complete formation of the corresponding carbodiimide **121**

5.3 Novel Antibiotics Project

was achieved checking by TLC monitoring, then the temperature was lowered to 0 °C and were added TMP (1 equiv), a solution of Neomycin derivative **60** (1 equiv) in a minimum amount of CH₃CN, followed by a solution of fumaric acid **117** (1 equiv) in a minimum amount of CH₃CN. The temperature was slowly left to reach r.t. and the reaction, when finished (TLC monitoring, ~3 h), was quenched with an aqueous 1 N solution of HCl. The mixture was extracted with AcOEt, the organic phases collected and anhydried over Na₂SO₄, the solvent removed under pressure and the crude purified by flash chromatography giving **122**.

Compound 122a: *mixture of two diastereoisomers* *R_f* = 0.32 (DCM:MeOH = 90:10); ¹H-NMR (400 MHz, CD₃OD): δ = 7.37-7.35 (m, 5H, aromatics), 5.62 (br s, 1H), 5.32 (br s, 1H), 5.26-5.25 (m, 2H), 5.15-5.14 (m, 2H, -OCHHPh and CH Asp), 5.10 (d, *J* = 12.8 Hz, 1H, -OCHHPh), 5.02-5.00 (m, 2H), 4.94-4.90 (m, 2H), 4.67-4.65 (m, 2H, -CHCH₂OAc), 4.27-4.26 (m, 2H), 4.15-4.11 (m, 3H), 3.91-3.78 (m, 4H), 3.58-3.22 (m, 18H), 2.89-2.88 (m, 2H, -CH₂CH₂S-), 2.74-2.72 (m, 4H, -CH₂COOBn and -SCH₂CH-), 2.05-2.00 (m, 13H, H-2 neomycin and 4 x COCH₃), 1.77-1.76 (m, 2H, -CH₂CH₂CH₂-), 1.46-1.43 (m, 55H, H-2 neomycin and 6 x COOC(CH₃)₃), 1.35 (s, 9H, -NHC(CH₃)₃); ¹³C-NMR (100.6 MHz, CD₃OD): δ = 170.1, 170.0, 169.8, 169.7, 169.4, 168.8, 168.4, 156.1, 155.7, 155.4, 155.3, 155.0, 134.6, 126.8, 126.7, 126.6, 126.5, 126.4, 126.3, 120.2, 99.3, 99.2, 99.0, 79.9, 78.0, 77.8, 77.6, 72.8, 71.7, 71.5, 70.6, 70.2, 70.1, 68.8, 67.2, 67.0, 66.2, 65.8, 64.9, 64.8, 60.3, 54.9, 54.2, 50.9, 49.5, 49.4, 41.2, 39.9, 37.3, 33.0, 32.9, 29.9, 27.7, 27.6, 27.0, 26.3, 26.2, 26.0, 18.1, 17.9; ESI (*m/z*) 1990.4, [M+Na]⁺, 1968.4 [M+H]⁺; Anal. calcd. For C₈₈H₁₄₃N₉O₃₈S: C 53.73, H 7.33, N 6.41; found: C 53.76, H 7.31, N 6.43.

Compound 122b: *mixture of two diastereoisomers* *R_f* = 0.12 (DCM:MeOH = 90:10); ¹H-NMR (400 MHz, CD₃OD): δ = 7.40-7.38 (m, 5H, aromatics), 5.36 (br s, 1H), 5.26-5.18 (m, 5H), 5.14 (d, *J* = 12.4 Hz, 1H, -OCHHPh), 5.02-5.01 (m, 1H), 4.98 (s, 1H), 4.90-4.88 (m, 1H), 4.67-4.65 (m, 3H), 4.30-4.28 (m, 3H), 4.15-4.11 (m, 3H), 3.96-3.94 (2H), 3.83-3.81 (m, 4H), 3.58-3.22 (m, 14H), 2.94-2.92 (m, 2H, -11.7CH₂CH₂S-), 2.80-2.76 (m, 4H, -CH₂COOBn and -SCH₂CH-), 2.05-2.00 (m, 13H, H-2 neomycin, 4 x COCH₃), 1.81-1.79 (m, 2H, -CH₂CH₂CH₂-), 1.50-1.48 (m, 55H, H-2 neomycin and 6 x COOC(CH₃)₃), 1.46 (s, 9H, -NHC(CH₃)₃); ¹³C-NMR

(100.6 MHz, CD₃OD); δ = 170.3, 169.6, 169.5, 169.1, 168.5, 156.2, 155.7, 155.4, 155.0, 126.8, 126.6, 126.5, 108.3, 99.6, 99.5, 98.0, 96.6, 78.0, 77.7, 76.7, 72.9, 71.8, 71.6, 71.4, 70.7, 70.3, 70.2, 68.9, 67.7, 67.5, 66.5, 65.7, 65.6, 64.9, 60.6, 55.1, 52.7, 49.4, 41.5, 40.0, 39.0, 37.5, 37.0, 35.5, 33.1, 32.9, 32.7, 29.9, 28.5, 27.7, 27.6, 27.3, 27.1, 27.0, 26.4, 26.2, 26.1, 20.3, 20.2, 20.1, 18.0, 17.9, ; ESI (m/z) 1989.1, [M+Na]⁺; Anal. calcd. For C₈₈H₁₄₄N₁₀O₃₇S: C 53.76, H 7.38, N 7.12; found: C 53.78, H 7.33, N 7.13.

Compound 122c: mixture of two diastereoisomers R_f = 0.35 (DCM:MeOH = 90:10); ¹H-NMR (400 MHz, CD₃OD): δ = 7.37-7.35 (m, 5H, aromatics), 5.27-5.24 (m, 3H), 5.17-5.16 (m, 3H), 5.12-5.11 (m, 2H), 4.94 (m, 1H), 4.83-4.81 (m, 2H), 4.68-4.66 (m, 2H, -CHCH₂OAc), 4.24-4.21 (m, 2H), 4.11-4.10 (m, 2H), 4.02-4.00 (m, 1H), 3.92-3.91 (m, 1H), 3.78-3.77 (m, 1H), 3.72-3.71 (m, 1H), 3.49-3.26 (m, 19H), 3.31-3.11 (m, 1H), 2.90-2.89 (m, 2H, -CH₂CH₂S-), 2.82-2.81 (m, 2H, -CH₂COOBn), 2.75-2.73 (m, 2H, -SCH₂CH-), 2.13-2.03 (m, 13H, H-2 neomycin and 4 x COCH₃), 2.03-2.01 (m, 2H, -CH₂CH₂CH₂-), 1.46-1.44 (m, 55H, H-2 neomycin and 6 x COOC(CH₃)₃), 1.35 (s, 9H, -NHC(CH₃)₃); ¹³C-NMR (100.6 MHz, CD₃OD); δ = 170.2, 169.8, 169.7, 168.9, 168.8, 168.7, 156.1, 155.8, 155.4, 155.1, 134.6, 126.9, 126.7, 126.6, 96.2, 80.0, 78.0, 77.7, 76.6, 72.9, 71.9, 70.7, 70.3, 68.9, 68.1, 67.2, 65.1, 65.0, 64.8, 64.2, 61.0, 58.8, 55.4, 54.2, 49.3, 33.2, 33.0, 27.3, 27.2, 27.1, 27.0, 26.4, 26.3, 26.2, 26.1, 18.1, 18.0, 17.9; ESI (m/z) 1990.4, [M+Na]⁺, 1968.4 [M+H]⁺; Anal. calcd. For C₈₈H₁₄₃N₉O₃₈S: C 53.73, H 7.33, N 6.41; found: C 53.75, H 7.36, N 6.44.

Compound 122d: mixture of two diastereoisomers R_f = 0.43 (DCM:MeOH = 90:10); ¹H-NMR (400 MHz, CD₃OD): δ = 7.37-7.35 (m, 5H, aromatics), 5.38-5.36 (m, 3H), 5.32-5.29 (m, 2H), 5.28 (dd, J = 9.2 and 3.2 Hz), 5.16-5.14 (m, 2H), 5.11 (m, 1H), 5.04 (d, J = 12.8 Hz, 1H, -OCHHPH), 5.02 (d, J = 12.8 Hz, 1H, -OCHHPH), 4.95-4.94 (m, 2H), 4.87-4.86 (m, 3H), 4.79-4.77 (m, 1H), 4.67-4.65 (m, 2H, -CHCH₂OAc), 4.55 (m, 1H, -CHCHHOAc), 4.52 (m, 1H, -CHCHHOAc), 4.25-4.23 (m, 3H), 4.13-4.11 (m, 3H), 3.92-3.90 (m, 3H), 3.78-3.76 (m, 4H), 3.51-3.20 (m, 22H), 2.89-2.88 (m, 2H, -CH₂CH₂S-), 2.74-2.72 (m, 4H, -CH₂COOBn and -SCH₂CH-), 2.05-2.00 (m, 13H, H-2 neomycin and 4 x COCH₃), 1.77-1.76 (m, 2H, -CH₂CH₂CH₂-), 1.46-1.43 (m, 55H, H-2 neomycin and 6 x COOC(CH₃)₃), 1.35 (s, 9H, -NHC(CH₃)₃); ¹³C-NMR (100.6 MHz, CD₃OD); δ = 169.5, 169.2, 169.0, 168.8, 168.4, 156.2, 155.4, 155.0, 134.6,

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126.8, 126.6, 126.5, 126.4, 94.4, 80.0, 78.0, 77.6, 73.9, 72.9, 72.2, 71.8, 70.9, 70.2, 69.0, 68.1, 67.2, 67.1, 66.2, 65.7, 64.9, 61.5, 60.4, 55.6, 50.9, 49.4, 33.0, 32.7, 29.9, 27.9, 27.6, 27.1, 27.0, 26.4, 26.2, 26.1, 18.4, 18.2, 18.1, 18.0, 17.9, 17.8; ESI (m/z) 2277.7, $[M+Na]^+$; Anal. calcd. For $C_{100}H_{159}N_9O_{46}S$: C 53.25, H 7.11, N 5.59; found: C 53.26, H 7.14, N 5.61.

Compound 122e: mixture of two diastereoisomers $R_f = 0.23$ (DCM:MeOH = 80:20); 1H -NMR (400 MHz, CD_3OD): $\delta = 7.37$ -7.35 (m, 5H, aromatics), 5.32 (br s, 1H), 5.28-5.17 (m, 2H), 5.18-5.11 (m, 3H), 5.10-5.08 (m, 2H, -OCHHPh and CH Asp), 4.95 (s, 1H), 4.90-4.84 (m, 2H), 4.23-4.20 (m, 2H), 3.92-3.89 (m, 2H), 3.73-3.71 (m, 5H), 3.49-3.11 (m, 24H), 2.89-2.88 (m, 2H, - CH_2CH_2S -), 2.74-2.72 (m, 4H, - CH_2COOBn and - SCH_2CH -), 2.25 (m, 2H, - $NHCOCH_2$ -), 1.98-1.93 (m, 14H, H-2 neomycin and neamine, 4 x $COCH_3$), 1.55-1.51 (m, 4H, - $CH_2CH_2CH_2CH_2$ -), 1.46-1.43 (m, 83H, H-2 neomycin and neamine, 9 x $COOC(CH_3)_3$), 1.30 (s, 9H, - $NHC(CH_3)_3$); ^{13}C -NMR (100.6 MHz, CD_3OD): $\delta = 173.6, 173.2, 170.2, 169.8, 169.2, 168.9, 168.4, 168.0, 156.1, 155.4, 155.0, 154.7, 134.6, 130.9, 130.8, 130.4, 96.7, 78.3, 78.0, 77.8, 77.7, 76.2, 74.0, 73.0, 70.7, 70.2, 67.4, 66.8, 65.2, 65.0, 64.9, 55.6, 51.5, 49.8, 49.2, 37.4, 36.8, 33.8, 33.5, 33.1, 32.6, 28.2, 27.9, 27.2, 26.4, 26.2, 26.1, 26.0, 25.9, 21.4, 21.3, 18.3, 18.2, 18.0, 17.9$; ESI (m/z) 2474.0, $[M+Na]^+$; Anal. calcd. For $C_{111}H_{183}N_{13}O_{45}S$: C 54.38, H 7.52, N 7.43; found: C 54.40, H 7.56, N 7.45.

General procedure of Synthesis of Sugar-Neomycin-dihydrouracils Conjugates 109-113.

To a stirred solution of neomycin conjugates **122 {a,e}** (1 equiv.) in MeOH (0.1 M) a solution *ca.* 8.03 M of $MeNH_2$ in MeOH (1/1 v/v) was added at room temperature. After the reaction was complete (TLC monitoring, *ca.* 2h.) the organic solvents were evaporated. The crude was purified by flash chromatography affording *N*-Boc protected derivatives which were submitted to deprotection by treatment with TFA in DCM (50% v/v) for two hours. The solvents were evaporated and co-evaporated with toluene twice. The obtained neomycin-dihydrouracils-sugar conjugates **124** were dissolved in a minimum amount of water and lyophilized.

Compound 109 x TFA: *mixture of two diastereoisomers* $^1\text{H-NMR}$ (400 MHz, D_2O): δ = 6.00 (d, J = 4.0 Hz, 1H, H-1' neomycin); 5.35 (s, 1H, H-1'' neomycin), 5.25 (s, 1H, H-3' neomycin), 4.36 (m, 2H), 4.26 (m, 2H), 4.18 (m, 3H), 4.05 (t, J = 9.6 Hz, 1H), 3.97 (t, J = 9.2 Hz, 1H), 3.86 (m, 5H), 3.78 (s, 1H), 3.68-3.28 (m, 15H), 3.19 (m, 3H), 3.05 (m, 1H), 2.87 (m, 2H), 2.65 (m, 4H), 2.43 (m, 1H, 2-desoxystreptamine CHH), 1.88 (m, 1H, 2-desoxystreptamine CHH), 1.81 (m, 2H, $-\text{OCH}_2\text{CH}_2\text{CH}_2\text{N}-$), 1.50 (s, 9H, $\text{C}(\text{CH}_3)_3$); $^{13}\text{C-NMR}$ (100.6 MHz, D_2O): δ = 175.9, 170.8, 162.6 (q, J = 36.2 Hz, CF_3COOH), 158.8, 116.3 (q, J = 290.7 Hz, CF_3COOH), 110.4, 102.3, 95.6, 95.0, 85.5, 80.2, 78.7, 76.0, 75.9, 75.8, 75.1, 73.7, 73.3, 72.5, 70.8, 70.2, 69.8, 68.1, 67.8, 67.6, 67.4, 60.9, 58.3, 56.4, 56.1, 53.6, 51.0, 49.7, 48.5, 40.6, 40.3, 38.4, 38.0, 34.5, 30.5, 27.9, 27.3, 27.2; ESI (m/z) 1112.4, $[\text{M}+\text{Na}]^+$, 1090.4 $[\text{M}+\text{H}]^+$; Anal. calcd. For $\text{C}_{43}\text{H}_{79}\text{N}_9\text{O}_{21}\text{S}$: C 47.37, H 7.30, N 11.56; found: C 47.39, H 7.31, N 11.58.

Compound 110 x TFA: *mixture of two diastereoisomers* $^1\text{H-NMR}$ (400 MHz, D_2O): δ = 6.01 (d, J = 4.0 Hz, 1H, H-1' neomycin), 5.38 (s, 1H, H-1'' neomycin), 5.28 (s, 1H, H-3' neomycin), 4.38 (m, 2H), 4.28 (m, 2H), 4.20 (m, 2H), 4.05 (t, J = 10.0 Hz, 1H), 3.99 (t, J = 8.8 Hz, 1H), 3.88 (m, 3H), 3.81 (s, 1H), 3.67 (m, 3H), 3.58-3.29 (m, 17H), 3.26 (dd, J = 13.6 and 6.8 Hz, 1H), 3.06 (m, 1H), 2.85 (m, 2H), 2.65 (m, 4H), 2.67 (s, 3H, $-\text{NHCOCH}_3$), 2.47 (m, 1H, 2-desoxystreptamine CHH), 1.89 (m, 1H, 2-desoxystreptamine CHH), 1.74 (m, 2H, $-\text{OCH}_2\text{CH}_2\text{CH}_2\text{N}-$), 1.51 (s, 9H, $\text{C}(\text{CH}_3)_3$); $^{13}\text{C-NMR}$ (100.6 MHz, D_2O): δ = 176.0, 175.9, 174.9, 174.6, 170.9, 162.7 (q, J = 36.2 Hz, CF_3COOH) 159.0, 158.7, 116.5 (q, J = 291.7 Hz, CF_3COOH), 112.1, 110.5, 102.1, 101.5, 95.8, 95.1, 85.6, 80.4, 78.9, 78.8, 76.1, 76.0, 75.3, 74.2, 74.1, 74.0, 73.9, 72.6, 70.9, 70.8, 70.4, 70.2, 70.1, 69.8, 68.2, 67.9, 67.7, 67.6, 61.0, 60.9, 60.8, 59.3, 57.2, 56.4, 56.1, 55.8, 55.7, 53.9, 53.8, 51.1, 49.8, 48.7, 40.7, 40.4, 38.8, 38.7, 38.6, 38.5, 38.3, 34.7, 34.6, 34.5, 34.3, 30.8, 30.7, 29.9, 28.8, 28.1, 28.0, 27.3, 26.1, 22.4, 22.3; ESI (m/z) 1131.3 $[\text{M}+\text{H}]^+$, 566.1, $[\text{M}+\text{H}]^{2+}$; Anal. calcd. For $\text{C}_{45}\text{H}_{82}\text{N}_{10}\text{O}_{21}\text{S}$: C 47.78, H 7.31, N 12.38; found: C 47.81, H 7.30, N 12.40.

Compound 111 x TFA: *mixture of two diastereoisomers* $^1\text{H-NMR}$ (400 MHz, D_2O): δ = 6.02 (d, J = 3.6 Hz, 1H, H-1' neomycin), 5.38 (s, 1H, H-1'' neomycin), 5.28 (s, 1H, H-3' neomycin), 4.29 (m, 2H), 4.22 (m, 2H), 4.11 (m, 2H), 4.08 (t, J = 8.8 Hz, 1H), 4.01 (t, J = 9.6

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Hz, 1H), 3.88 (m, 5H), 3.71 (s, 1H), 3.68-3.28 (m, 16H), 3.14 (m, 1H), 3.05 (m, 1H), 2.87 (m, 2H), 2.65 (m, 4H), 2.48 (m, 1H, 2-desoxystreptamine CHH), 1.92 (m, 1H, 2-desoxystreptamine CHH), 1.82 (m, 2H, -OCH₂CH₂CH₂N-), 1.52 (s, 9H, C(CH₃)₃); ¹³C-NMR (100.6 MHz, D₂O); δ = 175.8, 175.7, 174.9, 170.7, 164.9, 162.2 (q, J = 37.2 Hz, CF₃COOH), 158.6, 158.5, 116.1 (q, J = 290.7 Hz, CF₃COOH), 110.4, 100.0, 99.9, 95.7, 94.9, 85.5, 80.2, 78.8, 75.1, 73.8, 72.8, 72.7, 72.5, 70.8, 70.3, 69.7, 68.1, 67.6, 67.5, 66.9, 65.4, 65.2, 61.0, 58.3, 56.2, 56.0, 53.7, 51.1, 49.7, 48.6, 40.6, 40.4, 38.7, 38.6, 38.3, 37.0, 34.6, 34.5, 34.3, 34.2, 31.6, 30.7, 30.6, 28.0, 27.9, 27.8, 27.1, 26.3, 21.2; ESI (m/z) 1112.4, [M+Na]⁺, 1090.4 [M+H]⁺; Anal. calcd. For C₄₃H₇₉N₉O₂₁S: C 47.37, H 7.30, N 11.56; found: C 47.38, H 7.30, N 11.55.

Compound 112 x TFA: *mixture of two diastereoisomers* ¹H-NMR (400 MHz, D₂O): δ = 6.13 (d, J = 4.0 Hz, 1H, H-1' neomycin), 5.49 (s, 1H, H-1'' neomycin), 5.44 (s, 1H, H-3' neomycin), 5.38 (s, 1H, H-1' maltose), 4.48 (m, 3H), 4.39 (m, 2H), 4.31 (m, 2H), 4.18 (t, J = 9.6 Hz, 1H), 4.11 (t, J = 8.0 Hz, 1H), 4.07 (m, 4H), 3.98 (m, 2H), 3.68-3.28 (m, 22H), 3.17 (m, 1H), 2.96 (m, 2H), 2.80 (m, 6H), 2.58 (m, 1H, 2-desoxystreptamine CHH), 2.01 (m, 1H, 2-desoxystreptamine CHH), 1.94 (m, 2H, -OCH₂CH₂CH₂N-), 1.63 (s, 9H, C(CH₃)₃); ¹³C-NMR (100.6 MHz, D₂O); δ = 176.0, 170.8, 162.8 (q, J = 35.2 Hz, CF₃COOH), 158.8, 116.2 (q, J = 291.7 Hz, CF₃COOH), 110.5, 102.1, 99.6, 95.6, 95.0, 85.5, 78.7, 78.6, 77.0, 76.9, 76.3, 76.2, 75.1, 74.6, 73.7, 73.1, 72.9, 72.8, 72.7, 72.6, 72.5, 71.7, 70.7, 70.2, 69.6, 69.5, 69.4, 68.0, 67.8, 67.7, 67.6, 67.4, 60.8, 60.7, 60.5, 58.2, 53.6, 50.9, 49.6, 48.9, 48.4, 40.5, 40.4, 40.2, 38.5, 38.4, 38.3, 38.2, 34.4, 34.1, 34.0, 30.4, 28.1, 27.9, 27.3, 27.1; ESI (m/z) 1252.5 [M+H]⁺; Anal. calcd. For C₄₉H₈₉N₉O₂₆S: C 46.99, H 7.16, N 10.07; found: C 47.01, H 7.18, N 10.09.

Compound 113 x TFA: *mixture of two diastereoisomers* ¹H-NMR (400 MHz, D₂O): δ = 5.66 (s, 1H, H-1' neomycin), 5.65 (s, 1H, H-1' neamine), 5.43 (s, 1H, H-1' neomycin), 5.34 (s, 1H, H-3' neomycin), 4.41 (m, 1H), 4.36-4.32 (m, 2H), 4.17 (m, 2H), 4.05 (t, J = 9.6 Hz, 1H), 4.11 (t, J = 8.0 Hz, 1H), 3.88-3.83 (m, 7H), 3.57-3.10 (m, 14H), 3.07 (m, 4H), 2.76-2.71 (m, 6H), 2.49 (m, 3H), 2.26 (m, 5H), 2.91 (m, 2H, 2-desoxystreptamine CHH), 1.63 (m, 2H), 1.48 (m, 2H), 1.29 (s, 9H, C(CH₃)₃); ¹³C-NMR (100.6 MHz, CD₃OD); δ = 174.2, 173.9, 172.6, 168.6, 157.8, 156.2, 108.7, 95.8, 95.7, 78.6, 74.1, 71.4, 71.3, 70.8, 70.7, 70.5, 70.2, 67.6, 67.5, 66.4,

53.1, 50.2, 48.7, 48.5, 48.0, 47.6, 39.3, 38.9, 38.5, 37.4, 33.6, 33.4, 28.1, 27.9, 27.2, 27.1, 26.3, 26.2, 21.3; ESI (m/z) 1274.2 $[M+H]^+$; Anal. calcd. For $C_{51}H_{95}N_{13}O_{22}S$: C 48.06, H 7.51, N 14.29; found: C 48.08, H 7.55, N 14.32.

General procedure for Aminoglycoside desalting

Aminoglycoside-TFA salts obtained upon global deprotection reactions (up to 40 mg) were dissolved in autoclaved H₂O (0.6 mL) in a sterile Eppendorf tube. Dowex Monosphere 550 A (100 mg) was added, and the suspension was shaken lightly overnight. The resin was removed by centrifugal filtration and washed twice with autoclaved H₂O. The desalted solutions were lyophilized, and the complete removal of TFA counterions.

General procedure for MIC determinations

Minimum inhibitory concentration (MIC) were determined using broth microdilution in accordance with Clinical Laboratory Standards Institute guidelines.^[198] Bacterial strains of interest were grown on Mueller-Hinton agar. A colony was selected and grown in an overnight liquid culture of cation adjusted Mueller-Hinton broth (MHB) (10 mg/mL CaCl₂ and 10 mg/mL MgCl₂) at 37 °C. 100 µL of the overnight culture was added to 10 mL of fresh cation adjusted MHB in a 20 mL test tube. This was shaken at 37 °C to an optical density (OD) value of 0.4 – 0.6 at 600 nm to give a log phase culture. The log culture was poured into a falcon tube and pelleted by centrifugation at 4,000 rpm for 8 minutes. The cation adjusted Mueller-Hinton broth was decanted off. The bacteria pellet was suspended in 0.5 mL PBS. In a 10 mL test tube, the bacteria suspension was added step wise to 5 mL of PBS to give a final OD at 600 nm of 0.2 for E. coli and 0.4 for all other bacterial species used. Bacteria had to be diluted to a final concentration of 5×10^5 cfu/mL just prior to addition to the 96-well test plate. Round bottom 96 well test plates were prepared. 80 µL of cation adjusted MHB was added to each well and 10 µL of solution of compound to test were added. The plates were covered, parafilm along the sides, and shaken at 37 °C overnight. The 96-well plates were read at 600 nm using a plate reader. Each compound of interested was tested minimally in quadruplicate. For the lecture of the

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values, a VersaMax plate reader (Molecular Devices, Mountain View, CA, USA), set at 600 nm wavelength, was used.

Chapter 6: References

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