

Synthesis of Water-soluble, Polyester-based Dendrimer Prodrugs for Exploiting Therapeutic Properties of Two Triterpenoid Acids

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 Electronic Supplementary Information

Abstract Dendrimers are macromolecules characterized by high controlled size, shape and architecture, presence of inner cavities able to accommodate small molecules and many peripheral functional groups to bind target entities. They are of eminent interest for biomedical applications, including gene transfection, tissue engineering, imaging, and drug delivery. The well-known pharmacological activities of ursolic and oleanolic acids are limited by their small water solubility, non-specific cell distribution, low bioavailability, poor pharmacokinetics, and their direct administration could result in the release of thrombi. To overcome such problems, in this paper we described their physical incorporation inside amino acids-modified polyester-based dendrimers which made them highly water-soluble. IR, NMR, zeta potential, mean size of particles, buffer capacity and drug release profiles of prepared materials were reported. The achieved water-soluble complexes harmonize a polycationic character and a buffer capacity which presuppose efficient cell penetration and increased residence time with a biodegradable cell respectful scaffold, thus appearing as a promising team of not toxic prodrugs for safe administration of ursolic and oleanolic acids.

Keywords Polyester-based amino acids-modified dendrimers; Physical encapsulation; Water-soluble dendriplexes; Buffer capacity; NMR investigations

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INTRODUCTION

Dendrimers^[1, 2], whose name has actually derived from the Greek word “Dendron” meaning “tree”, which indicates their unique tree-like branching architecture, are three-dimensional, immensely branched, well-organized nanoscopic macromolecules. They are characterized by globular shape, low polydispersity, interior cavities and their structure can be distinguished into the inner core moiety followed by radially attached generations that possess chemical functional groups at the exterior terminal surface^[3]. Their cavities can accommodate small natural or synthetic molecules with pharmacological activity, protecting them from premature degradation, increasing their solubility in biological fluids, decreasing their toxicity and favouring their bioavailability. With the increase in generations, the molecular size and peripheral groups are amplified and offer wide potential for multiple interactions, making dendrimers attractive to prepare multifunctional platforms containing at the same time physically incorporated or covalently linked drugs, target molecules such as folic acid (FA), antibodies,

poly(ethylene glycol) moieties, fluorescent dyes, peptides, MRI contrast agent or various host-guest complexes that offer wide applications^[4, 5]. Furthermore, surface modifications of dendrimers as well as hyperbranched polymers provide very appealing stimuli-responsive unimolecular polymeric micelles with dendrimer core very promising and with great potential in a variety of applications for the biomedical fields^[6–9]. These features make dendrimers suitable materials for various biomedical applications such as drug delivery nanocarriers (prodrugs)^[10], bio-sensors^[11, 12], bio-imaging agents^[4, 5, 13] and theranostics^[4, 5, 14]. Furthermore, polycationic dendrimers are deeply investigated as not viral polymeric vectors^[15–24] for pDNA, or siRNA into specific defective cell of patients.

Polyamidoamines (PAMAMs)^[25, 26] are considered as good references both in the field of gene delivery^[27] and of drug delivery^[28–32] and are among the most investigated dendrimer vectors. PAMAMs are endowed not only with very efficient transfection and delivery activities but also with remarkable cytotoxicity mainly deriving from the high density of protonated amino groups in the polymeric framework. If a sharp polycationic character is necessary to interact with genetic materials or with membrane sphingolipids negatively charged, thus promoting cellular up-take and helping the escape from endosomal lysosomes

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attach, when excessive, can cause irreparable damages to the membranes or hemolysis causing cell death. To improve biodegradability and decrease toxicity of PAMAM, research efforts focused on their chemical modification such as acetylation^[33, 34], PEGylation^[35], introduction of pyrrolidone^[36], carbohydrate^[37, 38], amino acid^[39–41] and peptide^[42] residues. PAMAM modified in periphery by acetylation^[28], PEGylation^[29], introduction of saccharide residues such as glycidol^[30], D-glucoheptono-1,4-lactone^[31] or by conjugation with targeted moieties such as IL-6 antibody or RGD peptide^[32] have been used to bind covalently or encapsulate therapeutics obtaining promising cancer-specific prodrugs. A different approach to reduce cytotoxicity in polycationic vectors was to introduce hydrolysable linkages, such as the ester groups, both to PAMAM^[43] itself and to not dendrimer vectors such as poly(β -amino esters)^[44–46]. Moreover, dendrimers containing amino acid residues^[47–49] have aroused great interest. The *L*-histidine incorporation in HPMACo-oligolysine brush polymers led to an increased buffer capacity and transfection efficiency in different endosomal pH ranges in *in vitro* test^[50] while vectors containing *L*-arginine moieties showed improved cellular uptake and transfection activity and reduced toxicity in COS-7 cells^[51, 52].

Triterpenoids are molecules present in plant kingdom and medicinal herbs. During the last decade, the tremendous interest in such natural molecules has led to almost frantic research to better understand these compounds in order to optimize their isolation and purification from various plants and herbs, to bring chemical modifications for making them more effective and water soluble, to improve the pharmacological knowledges on their benefit effects, toxicity and potential clinical uses in various diseases including anticancer chemotherapies. Ursolic acid (UA) together with its isomer oleanolic acid (OA), are pentacyclic triterpenoid acids, isolated from many kinds of medicinal plants, such as *Eriobotrya japonica*, *Rosmarinus officinalis*, *Salvia officinalis*, *Melaleuca leucadendron*, *Ocimum sanctum* and *Glechoma hederaceae*. UA and OA are endowed with several pharmacological activities^[53] but principally they manifest potent chemopreventive effects^[54, 55], hepatoprotective actions^[56–59], antihyperlipidemic^[60] and antioxidant power^[61–63], antitumor effects and cytotoxic activity towards various types of cancer cell lines^[62–70], antiviral^[71] and anti-inflammatory^[68, 72] properties, antimutagenic activity^[73] evaluable both *in vivo* and *in vitro*. Furthermore, UA and OA may represent a novel therapeutic strategy for the treatment of age-related conditions^[74] and appealing protective agents against ethanol-induced abnormalities in the heart^[75], cerebral ischemia^[76] and ischemic insult of myocardium^[77, 78]. UA and OA exhibit vasorelaxant activity in isolated blood vessels^[79, 80], are capable of reducing blood pressure in hypertension animal models^[81], possess cardiovascular protective effects and are effective against chemically-induced dysrhythmia^[82]. Despite their benefits, these triterpenes have some conflicting characteristics. They may be pro-inflammatory in normal non inflamed cell^[83] and have produced equivocal results in recent studies concerning

their influence on the circulatory system, likely to accelerate atherosclerotic plaque formation *in vivo*^[84], or to directly elicit aggregation of isolated platelets^[85, 86], the process that is crucial in hemostasis under normal physiological conditions but if abnormal caused by xenobiotics including drugs, food and environmental toxicants, readily leads to thrombosis acute coronary syndrome (ACS), stroke, and the ischemic complications of peripheral vascular disease. Therefore, UA or OA administrations can represent a risk for a population with a predisposition for cardiovascular disease. Furthermore, clinical applications of UA and OA are limited due to low water solubility resulting in a low bioavailability and a poor pharmacokinetic *in vivo* which subsequently restrict their effectiveness^[87] and to a not specific distribution throughout the body when administered intravenously. Thus, the research for new formulations of UA and OA, as well as of other problematic drugs, that overcome the disadvantages associated with their administration is very extensive. The strategy most applied concerns the use of nanosystem carriers, such as liposomes^[88], hyperbranched polymers^[4, 5], dendrimers (PAMAM)^[30] to deliver these substances possibly to target cell, to protect them from premature degradation, to allow for their intravenous administration providing greater therapeutic efficacy and reducing collateral effects. In the present paper, we report the achievement of six hydrolysable, not toxic drug delivery systems (DDSs) for delivering UA and OA without polyamidoamine scaffold but based on fourth (G4) and fifth (G5) generation polycationic polyester-based amino acids-modified dendrimers and hetero dendrimers prepared using 2,2-bis(hydroxymethyl)propionic acid as building block^[89] (Fig. 1).

The physical incorporation/complexation of a mixture of UA and OA (UOA) extracted and isolated from *Salvia officinalis* (Corrugata Vahl)^[90] was described and discussed. The highly hydrophilic achieved materials could make the triterpenoid acids well dispersed in water with obviously increased bioavailability and pharmacokinetic and could allow to exploit the several therapeutic activities otherwise not serviceable. Furthermore, their polycationic periphery promotes interactions with membrane lipids for enhanced cellular uptake and enable to obtain an optimal buffer capacity which favors endosomal escape, protecting and preserving the transported triterpenoids from lysosomal degradation. The biodegradable polyester-based polymeric framework assures a low level of toxicity. For all these reasons we consider the prepared dendriplexes as new appealing and versatile DDSs.

EXPERIMENTAL

Plant Material and Methods^[90]

Fresh aerial parts of *Salvia Corrugata* Vahl were obtained from the Istituto Sperimentale per la Floricoltura (Sanremo, Italy) and Centro Regionale di Sperimentazione ed Assistenza Agricola (Albenga, Italy). The species was identified by Dr. Gemma Bramley and a voucher specimen is deposited at Kew Herbarium (K) (Kew, Surrey, U.K.).

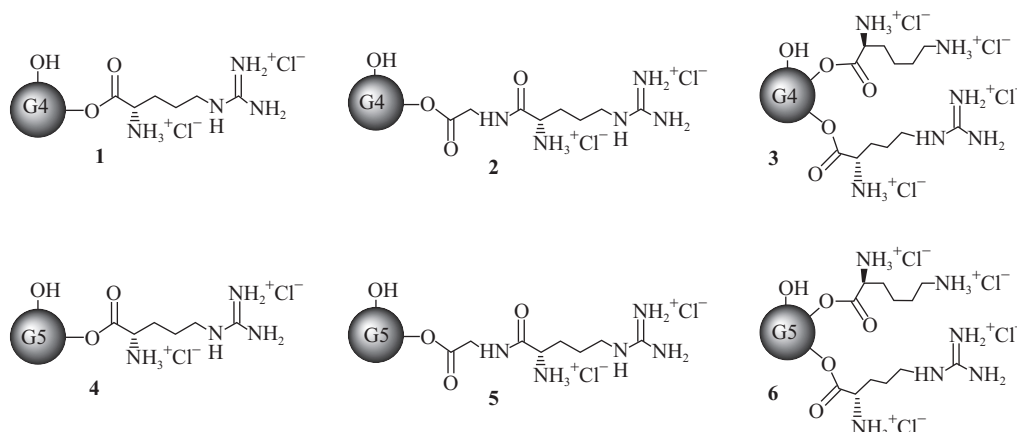


Fig. 1 G4 and G5 amino acids-modified dendrimers **1–6**^[89] used to complex/encapsulate the UA and OA (UOA) mixture

Materials

All the reagents and solvents were purchased from Aldrich or Merck and used without further purifications. The dialysis bags (D-Tube™ Dialyzer Maxi, MWCO 3.5 kDa) were purchased from Merck. The solvents were dried and distilled according to standard procedures. Petroleum ether refers to the fraction with boiling point 40–60 °C. Polycationic amino acids-modified dendrimers of the fourth (**1–3**) and fifth (**4–6**) generations were prepared according with a previously reported procedure^[89]. Analytical data of compounds **1–6** are available in section S1 (in the electronic supplementary information, ESI).

Measurements

Melting points, determined on Leica Galen III hot stage apparatus or Mettler Toledo MP50 Melting Point System, are uncorrected. FTIR spectra were recorded as films or KBr pellets on a Perkin Elmer System 2000 spectrophotometer. ¹H- and ¹³C-NMR spectra were acquired on a Bruker Avance DPX 300 Spectrometer at 300 and 75.5 MHz, respectively, and assigned through DEPT-135 and decoupling experiments. Coupling constant values were given in Hertz. Fully decoupled ¹³C-NMR spectra were reported.

Chemical shifts were reported in δ (parts per million) units relative to the internal standard tetramethylsilane ($\delta = 0.00$ ppm) and the splitting patterns were described as follows: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet) and br (broad signal). Dynamic light scattering (DLS) and zeta potential were performed on a Malvern Zetasizer Nano ZS instrument (Southborough, MA). Freeze-drying was performed on an EDWARDS Super Modulyo Freeze Dryer, ice capacity 8 kg, 8 kg/24h, refrigeration down to –55 °C with 24-place Drum Manifold. Centrifugations were performed on an ALC 4236-V1D Centrifuge at 3400–3500 r/min. HPLC analysis was performed on a Shimadzu LC-2010 C system (Shimadzu, Kyoto, Japan) equipped with a UV detector (SPD-10A, Shimadzu, Japan) and a Thermo Scientific™ Hypersil GOLD™ aQ C₁₈ LC Column (5 μ m particle size, 150 mm $\times$ 4.6 mm). Thin layer chromatography (TLC) system for routine monitoring the course of reactions and confirming the purity of analytical

samples employed aluminium-backed silica gel plates (Merck DC-Alufolien Kieselgel 60 F254) and detection of spots was made by UV light and/or by ninhydrin solution 0.2% in ethanol and heating in a stove at 100 °C. Flash chromatography (FC) was performed on Merck Silica gel (0.040–0.063 mm). Elemental analyses were performed with an EA1110 Elemental Analyser (Fison-Instruments). Organic solutions were dried over anhydrous sodium sulphate and evaporated using a rotatory evaporator operating at reduced pressure of about 1.3–2.7 kPa.

Potentiometric Titrations of Dendriplexes 7–12

Potentiometric titrations to determine the buffer capacity [$\beta = dc_{(\text{HCl})}/d(\text{pH})$]^[91] and then the average buffer capacity [$\bar{\beta} = dV_{(\text{HCl})}/d(\text{pH})(1)$]^[92] of dendriplexes in the form of hydrochlorides were performed at room temperature with a Hanna Microprocessor Bench pH Meter. The dendrimer (20–30 mg) was dissolved in Milli-Q water (30 mL) and then was treated with standard 0.1 mol/L NaOH (1–1.5 mL, pH = 10–12). The solution was potentiometrically titrated by adding 0.2 mL samples of standard 0.1 mol/L HCl up to total 3.0 mL and measuring the corresponding pH values^[93].

Dynamic Light Scattering (DLS) and Zeta Potential

The hydrodynamic size (diameter) of all dendriplexes prepared was measured in batch mode at 25 °C in a low volume quartz cuvette (pathlength 10 mm) using a Malvern Zetasizer Nano ZS instrument with back scattering detector (173°, 633 nm laser wavelength). Dendriplexes samples were prepared at a concentration of 1 mg/mL in PBS and filtered through a 0.02 μ m filter. At minimum, twelve measurements per sample were made. The Z average diameter, derived from a cumulants analysis of the measured correlation curve, is reported as the intensity-weighted average (Int-Peak) hydrodynamic radius. The zeta potential was measured at 25 °C in deionized water. An applied voltage of 100 V was used. Samples were loaded into pre-rinsed folded capillary cells and a minimum of twelve measurements were made per sample.

In Vitro Ursolic and Oleanolic Acids Release from Dendriplexes 7–12

The *in vitro* release behavior of the mixture UOA from

dendriplexes **7–12** was evaluated performing the dialysis bag diffusion technique using 20% ethanol in phosphate buffer saline (PBS) as dissolution medium. The dialysis bags (MWCO = 3.5 kDa) were pre-swelled in milli-Q water for 12 h before use.

A sample of each dendriplex **7–12** (10 mg) was dissolved in 1 mL of 0.1 mol/L PBS (pH = 7.4) containing 20% of ethanol. Each obtained solution was placed into a dialysis bag and both ends were sealed. Then each bag was individually immersed in a beaker containing 100 mL of 0.1 mol/L PBS (pH = 7.4) and ethanol (20%). In parallel, a release study of free UA and OA mixture from the dialysis bag was performed under the same condition. The mixture (1 mg) was suspended in 1 mL of 0.1 mol/L PBS and ethanol (60%) and placed in a dialysis bag which was immersed in a beaker containing 100 mL of 0.1 mol/L PBS (pH = 7.4) and ethanol (20%). The dialysis was carried out at 37 °C, and the quantity of drugs released was analysed by HPLC as described in the following paragraph at various time points (1, 2, 5, 10, 24 and 48 h) during the dialysis process.

HPLC Analysis

A Shimadzu LC-2010 C system equipped with a UV detector was used to perform the analyses of UOA mixture concentration. For the separation of drugs a Hypersil C₁₈ column (5 µm particle size, 150 mm × 4.6 mm) was used. Mobile phase was composed of methanol and water (90/10, *V/V*). The flow rate was set at 1 mL/min and temperature at 25 °C. The UV absorbance was determined at 210 nm. The concentration of the UA and OA mixture released was analysed on the bases of a free UOA calibration curve.

Extraction and Isolation of Ursolic and Oleanolic Acids Mixture^[90]

For the isolation of leaf surface mixture of UA and OA, fresh aerial parts of *Salvia Corrugata* Vahl (2.6 kg) were immersed in CH₂Cl₂ for 20 s to obtain only the external secreted material mixed with cuticular components, thereby avoiding extraction of components inside the cell wall. After filtration, the extraction solvent was removed under reduced pressure. The exudate (25 g, 0.96 wt% of fresh plant) was chromatographed in portions of 1.0 g on Sephadex LH-20 columns (53 cm × 2.5 cm), using CHCl₃/MeOH (7/3, *V/V*) as the eluent. The eluate fractions (20 mL each) were combined according to TLC to obtain four main fraction groups (I–IV): group I (up to 140 mL) (waxy inactive compounds), group II (from 140 mL to 160 mL), group III (from 160 mL to 200 mL), group IV (from 200 mL to 260 mL). Group II was evaporated, washed with hexane and crystallized from EtOH and then from MeOH to yield a product which was identified as a crystalline mixture of UA and OA (4.2 g). ¹H-NMR spectrum of the isolated mixture recorded in CD₃OD/DMSO-*d*₆ is available in Fig. S2 (in ESI). The NMR data are in accordance with literature ones with some difference depending on the acquisition solvent.

Preparation of G4 and G5 Dendriplexes **7–12**

General procedure

A solution of dendrimers **1–6**^[89] (*N* = 58–136) in dry MeOH (2.0 mg/mL) was added with the UOA mixture (9 equiv.).

The solution was kept under vigorously magnetic stirring at room temperature for 72 h in the dark. Then after removal of the solvent at reduced pressure the obtained white solids were suspended in dichloromethane (DCM) overnight to wash away the free UOA. After decanting the solid, dichloromethane was separated and evaporated to obtain a white solid which by IR analysis appeared to be the UOA mixture not complexed by dendrimers. The solid was brought to constant weight and then dissolved in MeOH/H₂O and precipitated in acetone in a centrifuge tube. After two cycles of centrifugation (3400 r/min) and washings with acetone, the wet solids were brought to dry and constant weight under reduced pressure and then stored on P₂O₅ in a dryer.

Complex G4[Arg(36)OH(12)UOA(6)] (**7**)

Slightly hygroscopic yellowish glassy solid [6 units of UOA mixture per dendrimer mol (51.4 mg, 0.0031 mmol, yield: 90%)]. FTIR (KBr, cm⁻¹): 3411 (OH and NH₃⁺), 1742 (C=O ester), 1662 (NH). ¹H-NMR (300 MHz, CD₃OD, δ, ppm): 0.75–0.97 [several s, 132H, CH₃ and H (C(5)) of triterpenoids], 1.07–2.38 [m, 414H (CH₃ of dendrimer + CH₂CH₂ Arg + CH and CH₂ of triterpenoids)], 3.20–3.40 (m, 72H, CH₂NH Arg), 3.85 (br, 24H, CH₂OH), 4.05–4.62 [m, 198H (CH₂O of dendrimer + CHNH₃⁺ Arg)], 5.22 (m, 6H, CH of triterpenoids), the signals for two CH of triterpenoids (12 H) are undetectable while the signal for one CH is hidden under solvent peak.

Complex G4[ArgGly(29)OH(19)UOA(7)] (**8**)

Slightly hygroscopic yellowish glassy solid [7 units of UOA mixture per dendrimer mol (12.5 mg, 0.00074 mmol, yield: 46%)]. FTIR (KBr, cm⁻¹): 3431 (OH and NH₃⁺), 1747 (C=O ester), 1637 (NH). ¹H-NMR (300 MHz, CD₃OD, δ, ppm): 0.74–0.99 [several s, 154H, CH₃ and H (C(5)) of triterpenoids], 1.11–2.37 [m, 408H (CH₃ of dendrimer + CH₂CH₂ Arg + CH and CH₂ of triterpenoids)], 2.85 (m, 7H, CH of triterpenoids), 3.15 (m, 7H, CH of triterpenoids), 3.20–3.40 (m, 58H, CH₂NH Arg), 3.64 (br t, 38H, CH₂OH), 3.90–4.40 [m, 235H (CH₂O of dendrimer + CHNH₃⁺ Arg + CH₂NH Gly)], 5.22 (q, 7H, CH of triterpenoids), the signal for one CH of triterpenoids (7 H) is hidden under solvent peak.

Complex G4[Arg(16)Lys(19)OH(13)UOA(4)] (**9**)

Slightly hygroscopic off white glassy solid [4 units of UOA mixture per dendrimer mol (27.0 mg, 0.0018 mmol, yield: 90%)]. FTIR (KBr, cm⁻¹): 3431 (OH and NH₃⁺), 1747 (C=O ester), 1631 (NH). ¹H-NMR (300 MHz, CD₃OD, δ, ppm): 0.78–0.99 [several s, 88H, CH₃ and H (C(5)) of triterpenoids], 1.00–2.40 [m, 404H (CH₃ of dendrimer + CH₂CH₂ Arg + CH₂CH₂CH₂ Lys + CH and CH₂ of triterpenoids)], 2.94–3.17 [m, 46H (CH₂NH₃⁺ Lys + CH of triterpenoids)], 3.30–3.50 (m, 32H, CH₂NH Arg), 3.54–3.82 (m, 26H, CH₂OH), 4.10–4.50 [m, 195H (CH₂O of dendrimer + CHNH₃⁺ Lys + CHNH₃⁺ Arg)], 4.59–4.71 (dd, 4H, CH of triterpenoids), 5.22 (q, 4H, CH of triterpenoids).

Complex G5[Arg(66)OH(30)UOA(3)] (**10**)

Slightly hygroscopic off white fluffy solid [3 units of UOA mixture per dendrimer mol (44.2 mg, 0.0016 mmol, yield: 100%)]. FTIR (KBr, cm⁻¹): 3392 (OH and NH₃⁺), 1743 (C=O ester), 1663 (NH). ¹H-NMR (300 MHz, CD₃OD, δ,

ppm): 0.78–0.97 [several s, 66H, CH_3 and H (C(5)) of triterpenoids], 1.00–2.30 [m, 612H (CH_3 of dendrimer + CH_2CH_2 Arg + CH and CH_2 of triterpenoids)], 3.27 (m, 132H, CH_2NH Arg), 3.68–3.74 (br, 60H, CH_2OH), 4.04–4.63 [m, 384H (CH_2O of dendrimer + CHNH_3^+ Arg)], 5.22 (q, 3H, CH of triterpenoids), the signals for two CH of triterpenoids (6 H) are undetectable while the signal for one CH is hidden under solvent peak.

Complex G5[ArgGly(52)OH(44)UOA(12)] (11)

Slightly hygroscopic off white fluffy solid [12 units of UOA mixture per dendrimer mol (16.1 mg, 0.00051 mmol, yield: 57%)]. FTIR (KBr, cm^{-1}): 3431 (OH and NH_3^+), 1739 (C=O ester), 1637 (NH). $^1\text{H-NMR}$ (300 MHz, $\text{CD}_3\text{OD}/\text{DMSO-d}_6$, δ , ppm): 0.76–0.99 [several s, 264 H, CH_3 and H (C(5)) of triterpenoids], 1.10–2.36 [m, 754H (CH_3 of dendrimer + CH_2CH_2 Arg + CH and CH_2 of triterpenoids)], 2.83 (m, 12H, CH of triterpenoids), 3.12 (m, 12H, CH of triterpenoids), 3.31 (m, 104H, CH_2NH Arg), 3.62 (m, 88H, CH_2OH), 3.90–4.40 [m, 461H (CH_2O of dendrimer + CHNH_3^+ Arg + CH_2NH Gly)], 5.22 (q, 12H, CH of triterpenoids), the signal for one CH of triterpenoids (12 H) is hidden under solvent peak.

Complex G5[Arg(38)Lys(30)OH(28)UOA(8)] (12)

Slightly hygroscopic pale yellow fluffy solid [8 units of UOA mixture per dendrimer mol (20.5 mg, 0.00070 mmol, yield: 64%)]. FTIR (KBr, cm^{-1}): 3431 (OH and NH_3^+), 1747 (C=O ester), 1635 (NH). $^1\text{H-NMR}$ (300 MHz, CD_3OD , δ , ppm): 0.75–0.98 [several s, 176 H, seven CH_3 and H (C(5)) of triterpenoids], 1.00–2.40 [m, 790 H (CH_3 of dendrimer + CH_2CH_2 Arg + $\text{CH}_2\text{CH}_2\text{CH}_2$ Lys + CH and CH_2 of triterpenoids)], 2.95–3.16 [m, 76H (CH_2NH_3^+ Lys + CH of triterpenoids)], 3.38 (m, 76H, CH_2NH Arg), 3.68 (m, 56H, CH_2OH), 4.10–4.50 [m, 390 H (CH_2O of dendrimer + CHNH_3^+ Lys + CHNH_3^+ Arg)], 4.58–4.70 (dd, 8H, CH of triterpenoids), 5.22 (q, 8H, CH of triterpenoids).

RESULTS AND DISCUSSION

The target of this research was to obtain new efficient prodrugs of UA and OA, two pentacyclic triterpenoids isomers extracted by us as a mixture from *Salvia Corrugata* Vahl^[90] which are endowed with several pharmacological activities not exploitable above all because of their low water solubility. The strategy was to complex them with proper vectors which, unlike the prodrugs widely reported in the literature, did not have a polyamidoamide highly cationic matrix but a hydrolysable ester-based one, in order to reduce or eliminate the risk of permanent damages to cellular membranes and toxicity to cells. The desired prodrugs should be water-soluble to authorise safe and effective administrations of the two triterpenoids thanks to increased bioavailability and pharmacokinetic and at the same time should be able to protect them from premature degradation and if possible to decrease their toxic side part. For vectors, we chose to test six polycationic amino acids-modified polyester-based dendrimers of the fourth and fifth generations we previously prepared^[89]. The hydrolysable inner matrix of the ester type assured low level of toxicity of

the vector while the cationic peripheral character conferred by amino acids promoted the interaction with negatively charged lipids of membrane advancing the endocytotic cellular uptake. Furthermore the excellent buffer capacity of these materials guaranteed the escape from endosomal and from lysosomal attack thanks to the so-called proton-sponge effect when inside the cell thus allowing the release of the transported material into the cytosol. In literature it is reported that in dendrimer/drug systems^[94–96] such as PAMAM/weakly acidic drug moieties (ibuprofen, DOX, MTX)^[97–99], hydrogen bonding and ionic interactions are among those mechanisms suggested to play a decisive part in dendrimer/drug complexation. So, we reasonably thought that the UOA mixture could be complexed by dendrimers 1–6 thanks to the presence of several amino groups capable of establishing hydrogen bonds or electrostatically interacting with the triterpenoid acids like PAMAM with ibuprofen, DOX or MTX. On the other hand, attempts to covalently bind the two acids on the not cationic polyester and polyhydroxylated dendrimer G4OH^[89, 100], whose structure and NMR spectra are available in Figs. S9 and S10 (in ESI), were not successful either with *N,N'*-dicyclohexylcarbodiimide (DCC), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) or following the procedure which uses 2-chloro-1-methyl-pyridinium iodide (CMPI)^[30] as condensing agents.

Incorporation of Ursolic and Oleanolic Acids Mixture (UOA) in Dendrimers 1–6

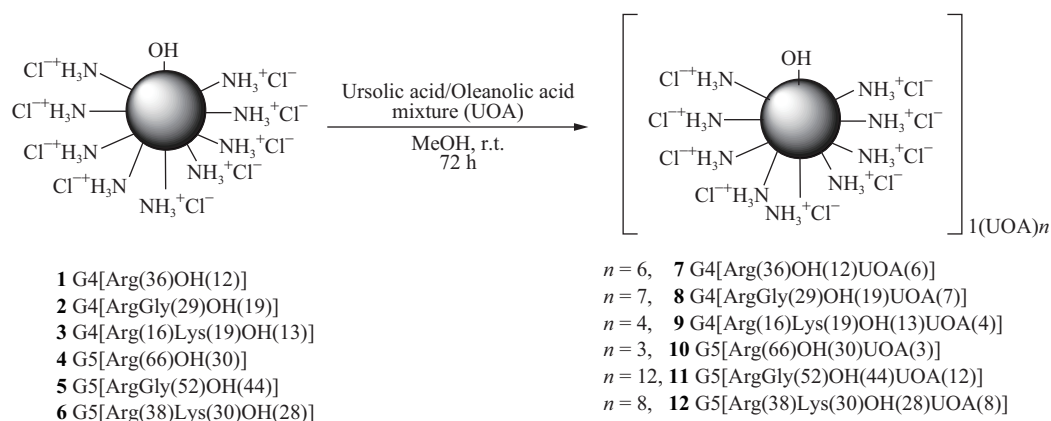
Dendrimers 1–6^[89] were dissolved in methanol following which, the UOA mixture extracted and isolated from *Salvia Corrugata* Vahl according to a described procedure^[90] and whose $^1\text{H-NMR}$ spectrum is available in Fig. S2 (in ESI) was added.

The reaction was vigorously stirred for 72 h at room temperature in the dark (Scheme 1). The methanol solution was evaporated at reduced pressure to remove the solvent. The residue solids were dried under vacuum in order to remove methanol completely. Then, DCM was added to extract the UA and OA not encapsulated, as UA and OA are soluble in DCM while complexes are not. They were water soluble as expected.

The solid complexes were further purified by dissolution in MeOH and precipitation in acetone and then recovered by centrifugation (3400 r/min, 15', twice). The UOA-dendrimer complexes were obtained in the form of off white or yellowish slightly hygroscopic fluffy solids and stored on P_2O_5 in a dryer. FTIR was not significantly diagnostic to confirm the formation of the complexes but $^1\text{H-NMR}$ spectroscopy was useful to have both qualitative and quantitative information about the composition of the dendriplexes.

As an example in Fig. 2 is shown a comparison between $^1\text{H-NMR}$ spectra of the mixture UOA (a), dendrimer 3 (b) and dendriplex 9 (c).

It is clear as the peaks under 1.0 ppm not present in the parent dendrimer 3 spectrum, but peculiar of UOA mixture and regarding seven CH_3 groups and H (C(5)) for a total of



Scheme 1 Preparation of dendriplexes 7–12. The numbers inside round parentheses indicate the composition in amino acids, OH groups and UOA of parent dendrimers and dendriplexes

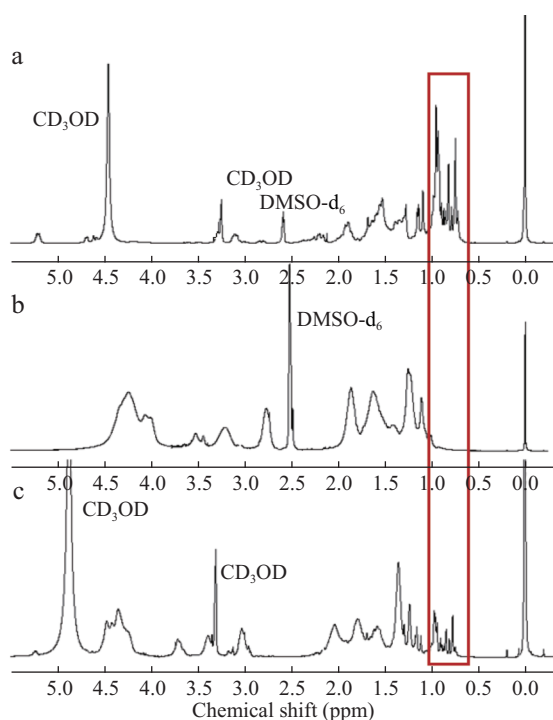


Fig. 2 Comparison between ^1H -NMR spectra of (a) the mixture UOA ($\text{CD}_3\text{OD}/\text{DMSO}-d_6$, 300 MHz), (b) **3** ($\text{DMSO}-d_6$, 300 MHz) and (c) **9** (CD_3OD , 300 MHz)

$^{22}\text{H}^{[101]}$ were well detectable in the spectrum of **9** and very diagnostic to confirm that the encapsulation was successful and estimate the number of UOA units encapsulated per dendrimer mole.

In Fig. 3 is shown only the spectrum of **9** with in evidence the peaks and the integral values used to quantify the amounts of triterpenoid acids loaded per dendrimer mole. Briefly, for all dendriplexes, UOA units per complex mole were obtained comparing the integral value of CH_2OH at 3.6–3.8 ppm and the value of the integral of peaks between 0.72 and 0.98 ppm.

The ^1H -NMR spectra of all dendriplexes 7–12 with an expansion of the zone between 2.0 and 0.5 ppm (determining to confirm that the incorporation was successful) compared

to ^1H -NMR spectra of UOA mixture and of the parent dendrimer are available in Figs. S3–S8 (in ESI).

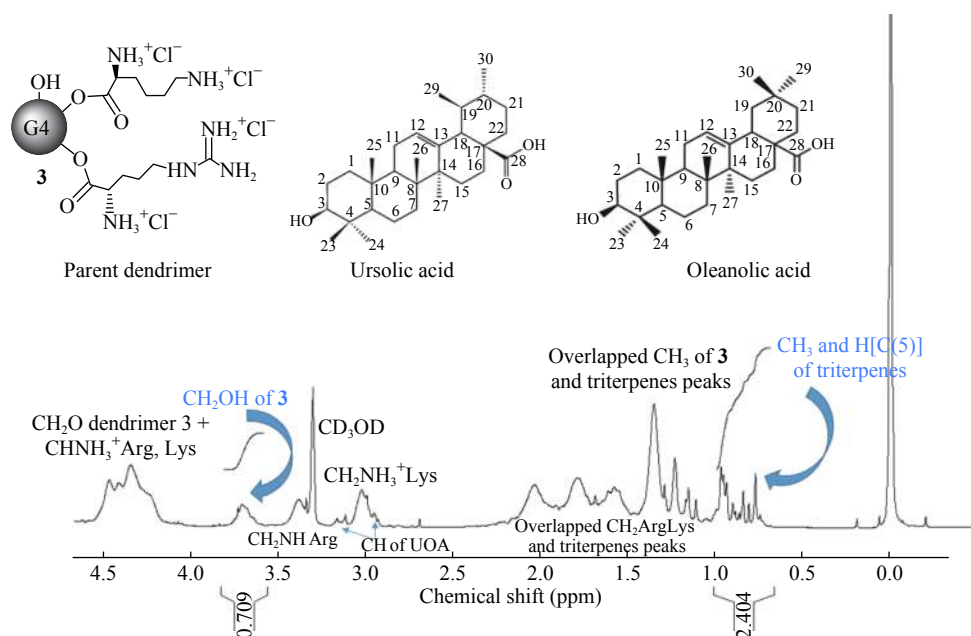
Table 1 summarizes the principal data and results about the preparation of dendriplex 7–12.

Dendrimers of the fifth generation **5** and **6** decorated with dipeptide *L*-arginine-glycine and mixed *L*-arginine and *L*-lysine respectively, showed more efficiency in complexing UOA than the analogue dendrimers **8** and **9** of the fourth generation, which complexed half or almost of UOA per mole. Surprisingly, with dendrimers containing only *L*-arginine **7** and **10**, exactly the opposite happened; it was actually the fifth generation dendrimer **10** that complexed the half amount of UOA in respect of **7**. After having estimated the UOA units loaded per dendrimer mole it was possible to make an estimate of molecular weight of compounds 7–12 simply by summing the molecular weight of the forerunner dendrimer to the molecular weight of UOA multiplied by the number of complexed units as deduced by NMR spectra. Table 2 summarizes the obtained data.

Potentiometric Titrations of the Prepared Dendrimers

It is generally accepted that carriers improve their efficacy as delivery systems (DDSs) if endowed with a proper buffer capacity [$\beta = d c_{(\text{HCl})}/d(\text{pH})$]^[91] and so with an average buffer capacity [$\bar{\beta} = d V_{(\text{HCl})}/d(\text{pH})$]^[92] in the pH range 4.5–7.5 suitable to make them escape from endosome compartments where pH is in the 5–6 range^[16]. To have an estimate of the buffer capacity of the prepared dendriplexes, potentiometric titrations of 7–12 were performed according to Benms et al.^[93]. Since PAMAM are considered as good reference in the field both of gene delivery^[27] and of drug delivery^[28–32], literature data of three G4-PAMAM derivatives^[102] potentiometrically titrated with the same protocol were used to obtain their titration curve. The β and $\bar{\beta}$ values of each prepared dendriplex and of G4-PAMAM derivatives were calculated from the titration data. Table 3 collects the β values recorded in the pH range 5–7.

Figures of all the titration and β values curves and the histogram of all the $\bar{\beta}$ calculated are available in Figs. S11–S13 (in ESI). As expected, considering the data about buffer capacity values of dendrimers 1–6, all the dendriplexes prepared using them as vectors resulted in a β

Fig. 3 ^1H -NMR spectrum of **9** (300 MHz, CD_3OD)**Table 1** Main data and results about preparation of dendriplexes **7–12**

Entry (mg/mmol)	N^a	MW ^b	Dendriplex (mg)	Per dendrimer mole UOA units loaded ^c mg UOA/wt/wt %
1 47.6/0.0035	72	1.36×10^4	7 51.4	6 8.6/16.8
2 21.7/0.0016	58	1.36×10^4	8 12.5	7 2.4/19.0
3 31.5/0.0020	70	1.28×10^4	9 27.0	4 3.4/12.5
4 41.2/0.0016	132	2.60×10^4	10 44.2	3 2.2/5.0
5 24.0/0.0009	104	2.58×10^4	11 16.1	12 2.8/16.1
6 29.0/0.0011	136	2.57×10^4	12 20.5	8 2.6/7.9

^a Number of peripheral basic groups as determined by NMR; ^b Molecular weights (MW) as determined by dendrimer composition estimated by NMR; ^c Estimated by ^1H -NMR spectra by integrating the signals at 0.72–0.98 ppm [peaks of CH_3 groups of UOA and of H (C(5))] and at 3.6–3.8 ppm (CH_2OH group of parent dendrimer)

Table 2 Calculated molecular weight of **7–12**

Dendriplex	Per dendrimer mole UOA units loaded ^a mg UOA/wt/wt %	MW ^a
7	6 8.6/16.8	1.63×10^4
8	7 2.4/19.0	1.68×10^4
9	4 3.4/12.5	1.46×10^4
10	3 2.2/5	2.74×10^4
11	12 2.8/16.1	3.13×10^4
12	8 2.6/7.9	2.93×10^4

^a Estimated by ^1H -NMR spectra

Table 3 Buffer capacity values of dendriplexes **7–12** and G4-PAMAMs in the pH range 6–7 from potentiometric titrations

Dendriplexes	pH	β
7	6.40	0.0833
8	6.00	0.0150
9	6.50	0.033
10	6.80	0.0426
11	6.10	0.0227
12	6.30	0.0180
G4-PAMAM ^a	7.00	0.0014
G4-PAMAM-Arg ^b	6.25	0.0024
G4-PAMAM-His-His-Arg ^c	6.20	0.0038

^a Fourth generation PAMAM; ^b G4-PAMAM modified with arginine; ^c G4-PAMAM modified with the His-His-Arg sequence

value much more higher than the G4-PAMAM derivatives taken as reference.

Dynamic Light Scattering (DLS) and Zeta Potential

Particle hydrodynamic size (diameter) and zeta potential were measured and the obtained data together with the deviation standard ($N = 12$) values are summarized in Table 4. Particle size was determined by dynamic light scattering (DLS) and Z average diameter (Z-AVE, nm), derived from a cumulant analysis of the measured correlation curve, was reported as the intensity-weighted average (Int-Peak)

hydrodynamic radius. Zeta potential (mV), which provides a measure of the electrostatic potential at the surface of the electrical double layer and the bulk medium and which is related to the nanoparticle surface charge, was performed using zeta-sizer ($N = 12$). It is known that smaller particles more rapidly penetrate tumors compared to larger ones but extremely minute particles could easily undergo hepato-

Table 4 Summary of particle characterization: particle hydrodynamic size (DLS) and zeta-potential at 25 °C of parent dendrimers 1–6 and of dendriplexes 7–12

Dendrimer	<i>N</i> ^a	Z-potential (mV) ^{b,§}	Z-Ave size (nm) ^{b,c,§}	Dendriplex UOA ^d wt/wt %	Z-potential (mV) ^{b,#}	Z-Ave size (nm) ^{b,c,#}
1	72	31.2 ± 0.1	4.4 ± 0.1	7 6 16.8	22.2 ± 0.1	30.6 ± 3.1
2	58	21.5 ± 0.6	4.7 ± 0.1	8 7 19.0	15.5 ± 0.6	36.3 ± 3.4
3	70	34.8 ± 0.2	4.6 ± 0.1	9 4 12.5	24.8 ± 0.2	24.9 ± 1.1
4	132	50.0 ± 0.6	5.1 ± 0.1	10 3 5.0	34.0 ± 0.6	16.1 ± 2.1
5	104	43.7 ± 0.7	5.4 ± 0.1	11 12 16.1	30.7 ± 0.7	35.9 ± 2.8
6	136	51.8 ± 0.1	5.3 ± 0.1	12 8 7.9	31.8 ± 0.1	20.3 ± 3.1

^a Number of peripheral basic groups as determined by NMR; ^b Degree of freedom = 12; [§] Referred to drugs-free dendrimers; ^c Derived from a cumulant analysis of the measured correlation curve and reported as the intensity-weighted average (Int-Peak) hydrodynamic radius; ^d Units of UOA complexed per dendrimer mole as estimated by ¹H-NMR spectra; [#] Referred to drugs-loaded dendriplexes

biliary and renal clearance. A balance between maximizing tumor penetration while minimizing both toxicity to normal tissue and clearance by the mononuclear phagocytic system (MPS) is the best solution and as reported, particles less than 100 nm and greater than 20 nm could be a good choice^[103, 104]. As exposed in Table 4 the mean particle size of G4, 5 dendrimer/UOA complexes of the present paper was increased in comparison to parent dendrimers as a function of the weight percentage of UOA loaded.

In any case the size was less than 100 nm, a value which assures that these prodrugs will not form the administration embolism, thus promising for intravenous or intraperitoneal administration to the patient. Finally, except sample 6, the nanoparticles were all > 20 nm and suitable to be used as drug vehicles with prolonged circulation time and efficient tumor penetration. The surface charge of the examined samples was slightly reduced compared to dendrimers 1–6 presumably due to interactions with the drug moieties and was within range 15–35 mV with higher values for G5 samples than G4 and increasing at the increase of *N*.

In Vitro Ursolic and Oleanolic Acids Release from Dendriplexes 7–12 Studies

As expected, the physical entrapping of the two triterpenoid acids in polyester-based, amino acids-modified dendrimers 1–6, used as nanoparticle vectors, provided highly water-soluble prodrugs with expected enhanced bioavailability, but in order to explicate their pharmacological effects, ursolic and oleanolic acids need to be released from dendriplexes. Then, their *in vitro* release from the prodrugs was studied in

buffer solutions at pH = 7.4, adding to the incubation medium (20% *V/V* with ethanol) to favor their solubility. Samples were taken out of the medium at fixed time points (1, 2, 5, 10, 24 and 48 h) and the released triterpenoid acids were quantified by HPLC. As data summarized in Table 5 and shown in Fig. S14A (in ESI) highlighted, all the profiles of the drugs released from the dendrimers prodrugs showed a three phases pattern. The first phase resolved after the first hour of incubation at pH = 7.4, when the release was almost null. This presupposes that, after administration, only a minimum release of the drugs in the systemic circulation will occur and that the DDSs may have time to reach and enter the target cells where the bulk of the release should occur. Then a very fast release phase started while after five hours a sustained release phase took place. The amount of UOA released from G4-UOA dendriplexes was more than that released from G5-UOA ones and inside a generation, the release profile showed a dependence on *N* value. This may be rationally due to the stronger interactions of free UOA mixture with dendrimers having more cationic groups. This feature, that significantly differentiates the behavior of our materials (at least *in vitro*), may be useful for modulating the amount of drug released over time and the total amount of drug that will be released, according to the therapeutic needs. In addition, to eliminate the effects of dialysis bag and release medium on the release rate, the release of free UA and OA mixture in the same release medium was also studied and reported as cumulative release (%) in the last row of Table 5 and in Fig. S14B (in ESI).

Table 5 *In vitro* drugs release study results

UOA-loaded dendriplex (10 mg)	<i>N</i> ^b	Release of free UOA after different time (μg/10mg) ^c						
		0 h	1 h	2 h	5 h	10 h	24 h	48 h
7	72	0	0.2	38.8	48.6	58.2	75.2	80.5
8	58	0	0.5	44.4	59.4	69.4	85.0	103.9
9	70	0	0.2	39.1	48.7	58.5	75.5	81.0
10	132	0	0	36.8	43.0	53.1	65.2	69.5
11	104	0	0	37.4	46.8	56.6	68.4	73.7
12	136	0	0	35.6	43.7	53.7	65.9	72.3
Release of free UOA (%) ^a	—	7.5	10.8	18.3	30.0	50.1	73.3	73.3

^a Cumulative release percentage; ^b Number of peripheral basic groups as determined by NMR; ^c Weight of the free mixture UOA released from 10 mg of dendriplex

CONCLUSIONS

In this work, six dendrimer-based prodrugs without the commonly reported and adopted polyamidoamine structure for safe and effective administration of a mixture of two triterpenoid acids extracted and isolated from *Salvia Corrugata* Vahl were synthesized. They were achieved by a simple and fast physical incorporation of the triterpenoid acids which were successfully complexed by polycationic polyester-based amino acids-modified dendrimers thanks to electrostatic and hydrogen bond interactions. The IR and NMR characterization of dendriplexes together with buffer capacity, particle mean size and zeta potential measurement were reported. The quantity of encapsulated UOA was estimated by ^1H -NMR spectra and the *in vitro* UOA mixture release from the dendriplexes prepared was studied in buffer solutions at pH = 7.4, in 20% *V/V* ethanol/1 mol/L PBS as dissolution medium, reported and discussed.

The prepared hydrophilic complexes are water soluble thus allowing the incorporated UA and OA mixture to be well dispersed in water with an obviously better bioavailability and an increased pharmacokinetic. They have a peripheral polycationic character due to the presence of amino acids including arginine, which is well known to promote interactions with membrane lipids for enhanced cellular uptake. The protonable nitrogen atoms of peripheral amino acids guaranteed an optimal buffer capacity which ensures rapid escaping from endosome, thanks to the so called proton sponge effect, thus promoting the drugs survival to the lysosomal degradation. On the other hand, the so useful peripheral cationic character was tuned down by the hydrolysable and biocompatible inner matrix, assuring low cytotoxicity levels and low risk to damage irredeemably cell membranes. To conclude, the prepared and reported dendriplexes embody all the prerogatives to be considered as new attractive, promising and versatile DDSs.

Electronic Supplementary Information

Electronic supplementary information (ESI) is available free of charge in the online version of this article at <http://dx.doi.org/10.1007/s10118-018-2124-9>.

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