Wydawnictwo UR 2023
ISSN 2544-1361 (online)
doi: 10.15584/ejcem.2023.3.1

http://www.ejcem.ur.edu.pl
European Journal of Clinical and Experimental Medicine
Eur J Clin Exp Med 2023; 21 (3): 442–449

ORIGINAL PAPER

Synthesis and characterization of Fulvestrant and Paclitaxel conjugates with polyamidoamine dendrimer fourth generation

Konrad Wróbel, Stanisław Wołowiec

Institute of Medical Sciences, Medical College, Rzeszów University, Rzeszów, Poland

ABSTRACT

Introduction and aim. Poorly soluble anticancer drugs can be attached covalently into biologically inert macromolecule in order to administrate a drug as water soluble form. It was proven that covalent linkers, for instance amide or carbamate bonds are susceptible to hydrolysis. Thus the attached drug can be released from the conjugates in tissue, specifically within the targeted cell. We aimed at construction of water soluble conjugates of Fulvestrant and Paclitaxel with PAMAM G4 dendrimer. In order to obtain water soluble conjugates the amine groups were substituted with *R*-glycidol.

Material and methods. Polyamidoamine dendrimer of fourth generation was synthesized and examined by detailed NMR analysis in water and in DMSO. The conjugates were covalently linked to amine groups of PAMAM after activation of Fulvestrant 17-OH group with 4-nitrophenylchloroformate and activation of end-carboxyl group of Paclitaxel succinate.

Results. The method of binary conjugate PAMAMG4-Fulvestrant-Paclitaxel synthesis was elaborated and the product was characterized by physicochemical methods.

Conclusion. The glycidylated PAMAMG4-Fulvestrant-Paclitaxel conjugate is better soluble in water than unconverted drugs.

Keywords. PAMAM dendrimer, double-conjugation, Fulvestrant, Paclitaxel, anticancer drug delivery system

Introduction

It is estimated that nearly 2 million of US inhabitants will be diagnosed with cancer in 2022 and more than 600 000 deaths (due to cancer) will be reported. In comparison WHO data from 2020 shows that cancers mortality reached 10 million (nearly one in six of all reported deaths globally was due to cancer). After heart disease cancer is second leading cause of death in US. Since early 1960s the 5-year relative survival rate (all cancers combined) has increased from 39% to 68% among White population and 27% to 63% among Black population of Americans as a result of improvements in treatment and diagnostic approaches. Main conventional methods of treating cancer could be distinguish as chemotherapy, radiotherapy and surgical treatment. In recent years some novel strategies such as targeted drug therapy, stem cells therapy, gene therapy or therapy based on systems for drug delivery have been developed for further improvement of survival rate and to overcome some limitations of using „traditional" methods.[1-4]

PAMAM dendrimers are one of extensively tested drug delivery systems for improving outcomes of chemotherapy by increasing water solubility, absorption and cellular uptake of drugs applied in cancer systemic treatment. These spherical, highly branched molecules build up with repeatedly added ethylenediamine and methyl acrylate to diamine core are suitable for functionalization with anticancer drugs because of having superficial functions such as amine or hydroxyl groups which may be utilized to covalently bond dendrimer with other molecules (via direct bonding or cleavable linkers). It was shown that PAMAM G4 functionalization with Paclitaxel (P)

Corresponding author: Stanisław Wołowiec, e-mail: swolowiec@ur.edu.pl

Received: 12.04.2023 / Revised: 17.05.2023 / Accepted: 18.05.2023 / Published: 30.09.2023

Wróbel K, Wołowiec S. *Synthesis and characterization of Fulvestrant and Paclitaxel conjugates with polyamidoamine dendrimer fourth generation.* Eur J Clin Exp Med. 2023;21(3):442–449. doi: 10.15584/ejcem.2023.3.1.

and omega-3 fatty acids enhance anticancer activity of P against human esophageal cancer in vitro model. Moreover it was recently proved that Fulvestrant and Lapatinib conjugated with partially glucoheptoamidated PAMAM G3 gain ability to promote apoptosis in senescent breast cancer cell lines of different receptor status.[4-8]

Paclitaxel, as one of the taxanes, is commonly used in treatment of cancer. P is approved by FDA to treat breast, ovarian, lung cancers and also Kaposi's sarcoma. Moreover, it could be used in systemic therapy (as off-label treatment) of gastroesophageal, endometrial, cervical, prostate, head and neck cancers, as well as sarcoma, lymphoma, and leukemia. It was commonly believed that predominant mechanism of P anticancer effect emerges from its ability to disruption in microtubule stabilization/destabilization process (inducing polymerization and stabilization of microtubules) resulting in mitotic arrest. However, such conception changes in recent years due to extensive investigations of P on biological models. It was shown that P concentrations in primary breast tumors are below level required to elicit sustained mitotic arrest. Alternative hypothesis suggests that P efficacy may be explained by its ability to cause cell death in interphase instead of affecting mitosis (due to interference with cell signaling, trafficking, and microtubule-mediated transport). It was proved (in primary human breast cancers models) that P efficacy could be effectively explained by its ability to cause chromosome missegregation on abnormal mitotic spindles. In addition it is suggested that P cytotoxicity could be result of some other factors for example its ability to hyperphosphorylation of Bcl-2, induction of calcium ions release by mitochondria or modulatory effect on miRNA expression profiles. P bioavailability is significantly limited by poor water solubility of this drug and first-pass metabolism. To improve P pharmacokinetic Nab-paclitaxel, a P complex with albumin was approved by FDA to treat metastatic breast cancer in 2005.[9-14]

One of drugs commonly used in hormonal treatment of hormone sensitive breast cancers is Fulvestrant. Due to high affinity to estrogen receptor (ER) and lack of intrinsic activity Fulvestrant acts as competitive antagonist. In addition Fulvestrant accelerate ER protein degradation and therefore it is defined as selective estrogen receptor degrader (SERD). Instead of proven high efficacy in systemic therapy of patients with hormone receptor-positive locally advanced or metastatic breast cancer (who have not received hormonal treatment before) application of Fulvestrant has some significant limitations. The main limitation is poor water solubility of Fulvestrant which leads to necessity of intramuscular application and such route of administration limits volume and dose of drug.[15-18]

Combination therapy is intensively tested approach for increase efficacy of anticancer treatment by synergistic or additive effect of drug combination. Application of two or more drugs (combined) of different mechanisms of action and toxicity profiles may lead to overcome drug resistance of cancer cells, enhancement of anticancer effect and also decrease systemic toxicity of such therapy (for example by reduced doses of each applied drug). Combination of Lapatinib and Capecitabine was proved to be significantly more effective than Capecitabine alone in women with advanced HER2- positive breast cancer (treated before with anthracycline, taxane and even Trastuzumab). In mentioned study elongation of median time to progression from 4.4 months for Capecitabine alone to 8.4 months for combination with Lapatinib was reported.[19,20]

The aim of our study was to conjugate polyamidoamine dendrimer of fourth generation (PAMAM G4) with P by succinate linker and Fulvestrant through carbonyl linker and to characterize conjugates using 1-D and 2-D NMR spectroscopy (^{1}H,^{13}C, COSY, HSQC, HMBC). Generally the PAMAM and other type dendrimers are currently extensively studied as macromolecular carriers of drugs due to their radial geometry, strictly defined molecular weight and molecular size in contrary to other polymers, which are polydispersed macromolecules. We have elaborated an effective protocol to obtain the conjugate, which are very well water-soluble due to substitution of PAMAM amine groups with 2,3-dihydroxyproyl substituents by reaction with *R*-glycidol. The degree of PAMAM G4 substitution with drugs was low enough to maintain the solubility of the conjugates from one side and to obtain high drug load of macromolecular carrier. Additionally we have applied PAMAM G4 as the carrier instead of previously used PAMAM G3 as core for conjugates of Lapatinib, Fulvestrant, Simvastatin, Celecoxib, or Nimesulide.[8,21,22]

Aim

Paclitaxel and Fulvestrant are commonly used anticancer drugs. They are the compounds which are highly hydrophobic and therefore sparingly soluble in water. We aimed at synthesis of potential drug delivery system by conjugating both drugs with high molecular weight polyamidoamine dendrimer of fourth generation (G4) in order to increase the solubility of the drugs. Especially we aimed at the conjugate with both drugs attached to one macromolecule as combination therapy approach (binary conjugate). In order to reach the goal we have applied known methods of activation and linking the drugs into the G4 carrier, and characterized the final product and all compounds used to construct binary conjugate by nuclear magnetic resonance spectroscopy and determined the size of conjugates by dynamic light scattering method.

Material and methods

Materials

PAMAM G4 dendrimer was synthesized according to the general procedure described by Tomalia et al.[23] The

chemicals used for synthesis: Paclitaxel, Fulvestrant, dimethyaminepyridine (DMAP), 1-methyl-2-chloropyridine iodide (Mukaiyama reagent), succinic anhydride, *p*-nitrophenyl-chloroformate (NPCF), bulk solvents and triethylamine were purchased from Merck (KGaA, Darmstadt, Germany).

Syntheses

Paclitaxel succinate (P-suc)

Paclitaxel was converted into 2-O-succinyl derivative according to the published method.[24] In specific 75 mg P (87.7 μmoles) was dissolved in 5 ml $CDCl_3$ and 250 μl pyridine. Succinic anhydride (10.0 mg; 100 μmoles) was added and the mixture refluxed for 8 hours. Then solvents were removed under vacuum and the solid residue dissolved in 2 ml DMSO.

P-suc conjugation to PAMAM G4 and glycidylation

244 mg G4 (17.5 μmol) was dissolved in 3 ml DMSO. Meantime P-suc (87.7 μmoles) was activated by addition of 42 mg DMAP (337 μmoles) and 43 mg of N-methyl-2-chloropyridinium iodide (168 μmoles) for 1 hr at room temperature. Activated P-suc was added dropwise into G4 solution in methanol and stored at 50 °C for 6 hours. Afterwards, the mixture was transferred to dialytic bag (cellulose MW_{cutoff} = 3.5 kDa) and dialyzed for three days against water. The isolated yield was 217.1 mg. The stoichiometry of the conjugate was estimated based on the ^{1}H NMR spectroscopy. It was found that 5 P-suc was attached per one equivalent of G4. According to the molecular formula of this conjugate: $G4^{5P}$, 18.9 kDa, 11.5 μmoles of the conjugate was obtained; 65.7 % per dendrimer. Hydrodynamic diameter of nanoparticle by DLS: numbered-averaged d(N) = 99.20 (± 7.28) nm. Polydispersity index (PDI) = 0.134 (0.016). Potential zeta (ξ) = 13.57 (± 0.43) mV. In repeated synthesis (starting with 60 μmoles of P and 15 μmoles (209 mg) G4) aimed at lower substitution the $G4^{4P}$ was obtained and further remaining amine groups were converted by addition of *R*-glycidol (200 μL; 223 mg; 3 mmoles). The mixture was stored at ambient temperature for 12 hours and the solid product was identified as $G4^{4P110gl}$ by NMR spectroscopy. Theoretical molecular weight – 30.0 kDa. The isolated yield was 279.2 mg (9.32 μmoles); 81 %.

Fulvestrant conjugation to PAMAM G4

Fulvestrant (F) was substituted with *p*-nitrophenyl-chloroformate (NPCF) as described before.[8] In specific, 121.4 mg F (200 μmoles) was dissolved in 2 ml $CHCl_3$ and 50 μL pyridine. NPCF (220 μmole, 44.3 mg) was added stepwise and the mixture was refluxed for 2 hours. The solvents were removed *in vacuo* and the solid residue was dissolved in 1 ml DMSO. This solution was added into 185 mg G4 (13 μmoles) in 2 ml DMSO. The mixture was stored at 45°C for 12 hours followed by isolation of the product by dialyzis. The product subjected to glycidylation by addition of 100 μL *R*-glycidol and reacted 12 hours in methanol at ambient temperature. The product was worked up by dialysis, dried and identified by the ^{1}H NMR as PAMAM G4 bearing average 15 equivalents of succinate linked F and 66 equivalents of glycidol (gl); $G4^{15F66gl}$. Theoretical molecular weight - 24.5 kDa. Yield: 279.2 mg (11.4 μmoles); 87.6 %. Hydrodynamic diameter of nanoparticle by DLS d(N) = 138.23 (± 6.24) nm. (PDI) = 0.155 (0.022). Potential ξ = 38.02 (± 0.54) mV.

Double conjugate G4- Paclitaxel – Fulvestrant

Fulvestrant (17.6 mg, 29 μmoles) was activated with 7.1 mg NPCF (35 μmoles) as before and added into 35.9 mg of $G4^{5P}$ (1.9 μmol) obtained before. The mixture was stored at 45 °C for 12 hours followed by isolation of the product by dialysis. The product was further converted by glycidylation with *R*-glycidol (100 μL) for 12 hours in methanol at ambient temperature. Solvents were removed and the solid residue was worked-up by dialysis. The solid conjugate was identified by the ^{1}H NMR spectroscopy as the binary conjugate containing 5 equivalents of succinate linked Paclitaxel, 10 equivalents of carbamide linked Fulvestrant and 98 equivalents of 2,3-dihyroxypropyl residues attached to terminal nitrogen of the remaining PAMAM G4 primary nitrogen, $G4^{5P10F98gl}$. Theoretical molecular weight –35.0 kDa. Yield: 42.8 mg (1.22 μmole); 64 % per dendrimer. Hydrodynamic diameter of nanoparticle by DLS d(N) = 113.41 (± 5.48) nm. (PDI) = 0.122 (0.015). Potential ξ = 29.29 (± 0.72) mV.

Methods

NMR Spectroscopy

The 1-D ^{1}H and ^{13}C NMR spectra as well as 2-D ^{1}H-^{1}H correlations spectroscopy (COSY), ^{1}H-^{13}C heteronuclear single quantum correlation (HSQC), and heteronuclear multiple bond correlation spectra (HMBC) were recorded in deuterated water using Bruker 300 MHz (Rheinstetten, Germany) and worked up with TopSpin 3,5 software at College of Natural Sciences, University of Rzeszów.

Dynamic light scattering

Hydrodynamic diameter and zeta potential were measured in aqueous solution using a Zetasizer nano ZS instrument for 1 mM conjugate concentration.

Results

The ^{1}H and ^{13}C NMR spectra of PAMAM G4 dendrimer

The PAMAM G4 dendrimer has an effective radial symmetry. The ^{1}H NMR spectrum consists only of 6 methylene group resonances (broad triplets), the overlapped $a_0a_1a_2a_3$ a_4, overlapped $b_0b_1b_2b_3$,b_4 (both of 248 [H] in-

tegral intensity) overlapped $c_0c_1c_2c_3$, and overlapped $d_0d_1d_2d_3$, (120 [H] intensity each), all from inner sphere shells and the separated triplets of c_4 and d_4 (both of 128 [H] integral intensity) from outer sphere fragment of terminal ethylenediamine group (for atom numbering see Fig. 1). G4 is well soluble in water, methanol and dimethylsulfoxide. We have taken the ^{1}H NMR spectra of G4 in D_2O and in DMSO-d_6 in order to characterize the G4 as later we will use it as macromolecular carrier to bind various substituents into terminal primary amine groups. The detailed peak assignment of ^{1}H and ^{13}C resonances was performed based upon 2-D ^{1}H-^{1}H COSY spectra as well as heteronuclear HSQC and HMBC measurements. The combined HSQC/HMBC for G4 in D_2O and in DMSO-d_6 are presented at Fig. 2 and Fig. 3, respectively.

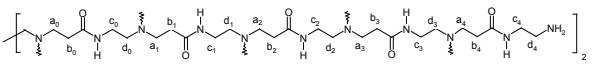

Fig. 1. Schematic representation of one arm of G4 PAMAM dendrimer with atom numbering. The winding lines represent branching at nitrogen atoms. involved in branching

The characteristic feature of the ^{1}H spectra presented as upper trace in 2-D maps at Fig.2 and 3 is the sharp triplet structure of outer sphere resonances of methylene d_4 (depicted as d*) and c_4 (c*) protons. These resonances are of special importance due to their proximity to terminal amine groups. The ^{1}H NMR spectrum taken in D_2O shows especially sharp triplets of d* and c* (both 128 [H[intensity) if compare with those at spectrum taken in DMSO (Fig.3). Thus further conversion of synthesized G4 by covalent attachment of substituents into terminal primary amine groups can be easily detected by changes of chemical shifts for c_4 and d_4 (c* and d* at Fig.2 and 3) promised the convenient experimental evidence to follow the structural changes of PAMAM carrier upon chemical changes. The full description of the ^{1}H and ^{13}C NMR is given in Table 1.

Table 1. The ^{1}H and ^{13}C NMR chemical shifts of PAMAM G4 dendrimer in D_2O and DMSO-d_6

Solvent: D_2O								
locant ®	a		b		c		d	
shell ¯	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
0 - 3	49.1	2.75	32.9	2.36	36.9	3.23	51.4	2.56
4	49.1	2.75	32.9	2.36	41.8	3.17	39.9	2.65
Solvent: DMSO-d_6								
0 - 3	49.7	2.64	33.3	2.19	36.9	3.08	52.2	2.42
4	49.7	2.64	33.3	2.19	42.1	3.03	41.1	2.54

G4 conjugates with Paclitaxel and Fulvestrant

In our previous study we have applied the NMR spectroscopy to characterize the low molecular weight

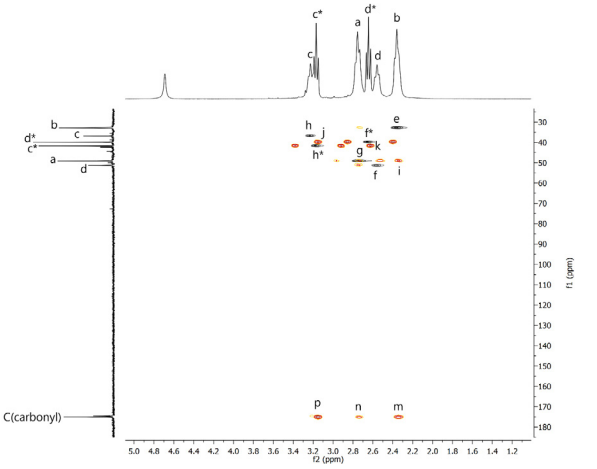

Fig. 2. Combined HSQC/HMBC map of PAMAM G4 in D_2O. The ^{1}H and ^{13}C resonances of c_4 and d_4 are described respectively as c* and d*, whereas c represents overlapped resonances of $c_0c_1c_2c_3$, analogously overlapped resonances of $d_0d_1d_2d_3$ are presented as d. There are no significant differences in chemical shifts for ^{1}H and ^{13}C resonances of a_0, a_1, a_2, a_3, and a_4 and b_0, b_1, b_2, b_3, and b_4 (both protons and carbons) therefore the resonances are labeled simply as a and b. The relevant HSQC coupling peaks (through one bond) are shown in grey scale as follows: e – H^b-C^b; f – H^d-C^d; f* - H^{d*}-C^{d*}; g – H^a-C^a; h* - H^{c*}-C^{c*}; h – H^c-C^c. The significant HMBC coupling peaks (long-range coupling) are represented in red/yellow scale as follows: i – H^b-C^a; k – H^{d*}-C^{c*}; j – H^{c*}-C^{d*}; m – H^b-C(O); n – H^a-C(O); p – H^{c*}-C(O)

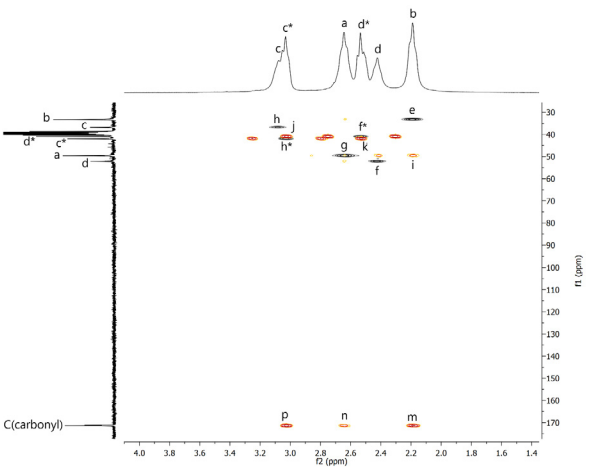

Fig. 3. Combined HSQC/HMBC map of PAMAM G4 in DMSO. The ^{1}H and ^{13}C resonances of c_4 and d_4 are described respectively as c* and d*, whereas c represents overlapped resonances of c_0,c_1,c_2 and c_3, analogously overlapped resonances of d_0,d_1,d_2 and d_3 are represented as d. There are no significant differences in chemical shifts for ^{1}H and ^{13}C resonances of a_0,a_1,a_2,a_3,a_4 and b_0,b_1,b_2,b_3,b_4 (both protons and carbons) therefore the resonances are labeled simply as a and b. The relevant HSQC coupling peaks are shown in grey scale as follows; e – H^b-C^b; f – H^d-C^d; f* - H^{d*}-C^{d*}; g – H^a-C^a; h* - H^{c*}-C^{c*}; h – H^c-C^c. The significant HMBC coupling peaks are represented in red/yellow scale as follows: i – H^b-C^a; k – H^{d*}-C^{c*}; j – H^{c*}-C^{d*}; m – H^b-C(O); n – H^a-C(O); p – H^{c*}-C(O)

PAMAM G3 conjugates with covalently attached biotin.[25] Later we used PAMAM G3 macromolecular carrier to construct the drug delivery system (DDS) for anticancer drugs: Lapatinib (L) and Fulvestrant (F) and tested those conjugates against three breast cancer cell lines in vitro.[7] We have found that G3-L-F binary conjugate promoted apoptosis in chemotherapy-induced senescent breast cancer cells with different receptor status.[8] Here we have stepped further to increase the payload of PAMAM carrier by applying generation 4 dendrimer (G4). In previous G3-L-F conjugate the amine groups of the carrier were blocked by substitution with glucoheptonoamidate residues in order to maximize the conjugate solubility in water. According to our further discovery, that PAMAM dendrimer glycidylated with *R*-glycidol, in contrary to *S*-isomer, enantioselectively penetrated cell membranes of both normal and cancer lines with high efficiency,[26] we applied this DDS to construct the binary conjugate with celecoxib and simvastatine and observed their increased additive cytotoxicity for cancer cell lines.[21] Considering mentioned experience we applied G4 dendrimer as carrier for Paclitaxel, and for Paclitaxel and Fulvestrant anticancer drugs and converted them into *R*-glycidylated derivatives. We elaborated synthetic path for both and characterized the derivatives by NMR spectroscopy and estimated the size and potential zeta of nano-DDS by DLS method to fully characterize the obtained conjugates. The general formula of studied conjugates are presented at Fig.4.

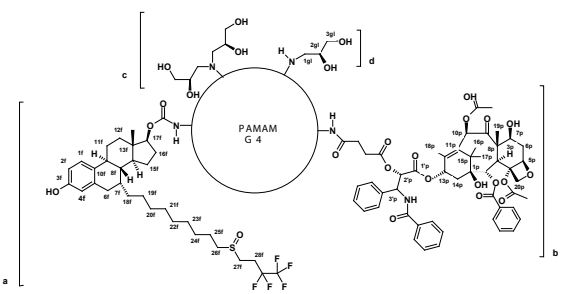

Fig. 4. The general formula of obtained conjugates with atom numbering: G4^{5P} (a = 0, b = 5, c, d = 0); G4^{4P110gl} a = 0; b = 4; c = 50; d = 10; G4^{15F66gl} a = 15; b = 0; c = 17; d = 32; G4^{5P10F98gl} (a = 10; b = 5; c = 49; d = 0)

Paclitaxel – G4 conjugate

In order to attach covalently Paclitaxel (P) to amine groups of G4 an additional functionalization was needed. It was already demonstrated that succinic and glutaric anhydrides attached to 2' oxygen of P are convenient linkers.[27,28] We have obtained 2'-o-succinated derivatives and used that to attach obtained P-suc using Mukaiyama reagent to activate pending carboxyl group of succinate linker. We have obtained the conjugate containing 4 molecules of amide attached P-suc, G4^{5P}. The compound was well soluble in DMSO and slightly less soluble in water (ca 0.1 mM aqueous solution could be obtained). The conjugate was characterized by 1-D and 2-D ^{1}H and ^{13}C NMR spectroscopy. COSY spectrum of G4^{5P} is presented at Fig. 5. Observed scalar coupling cross-peaks a, b, and c between methylene protons of PAMAM G4 core were analogous to those found for G4 alone. The cross-peaks d – j enabled to assign the ^{1}H resonances of P residue, even though some of them are hidden under the broad residual peak of HDO. In order to further characterize the conjugate the heteronuclear 2-D ^{1}H-^{13}C HSQC and HMBC spectra were recorded and presented as combined map at Fig.6. One-bond coupling cross-peaks are in grey; all the G4 core were well visible and additionally some peaks of P were found. Although full ^{13}C resonances were not assigned to P residues, the long-range couplings (in yellow-red color) enabled to confirm the assignments of all ^{1}H resonances of P in G4^{5P} conjugate. The conjugate G4^{5P} was a semi-product to further attachment of another drug, namely Fulvestrant.

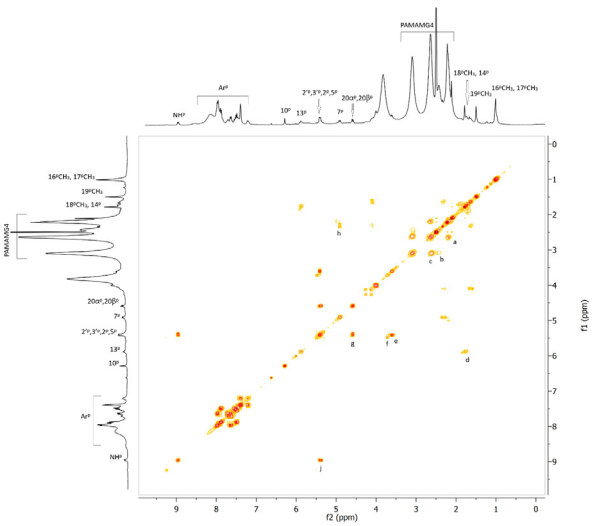

Fig. 5. The ^{1}H-^{1}H- COSY spectrum of G4^{5P} conjugate in DMSO-d$_6$. The relevant cross-peaks are: a – H^{b}(G4) / H^{a}(G4); b – H^{d}(G4) / H^{c}(G4); c –H^{d*}(G4) / H^{c}(G4); d – 14H^{P} / 13H^{P}; e – 3H^{P} / 5H^{P} ; f – 3H^{P} / 2H^{P} ; g - 20αH^{P} and 20βH^{P}/5H^{P} ; h – 7H^{P} / 6H^{P} ; j – 3'H^{P} / NH^{P}. The resonances from P are labeled with upper ^{P}, the resonances from PAMAM G4 core are designated in bracket as (G4). For atom numbering see Fig. 4

Glycidylated Fulvestrant – G4 conjugate

We aimed at construction of double-drug conjugate, with P and F (Fulvestrant). Previously we have elaborated the method of covalent attachment of F to G3.[8] We applied the same pathway, namely substitution of F with NPCF followed by reaction of functionalized F with G4. The highly substituted G4 with 15 equivalents of F was obtained and converted by reaction with *R*-glycidol to obtain finally G4^{15F66gl}. The ^{1}H NMR spectrum of G4^{15F66gl} is presented at Fig.7A. The number of F residues and 2,3-dihydroxopropyl residues (gl, from *R*-glycidol)

was defined by integration of the aromatic resonances of F (for the formula and atom numbering see Fig.4) and 3'- and 2'-gl resonances related to internal PAMAM G4 b resonance at 2.3 ppm, corresponding to [248H].

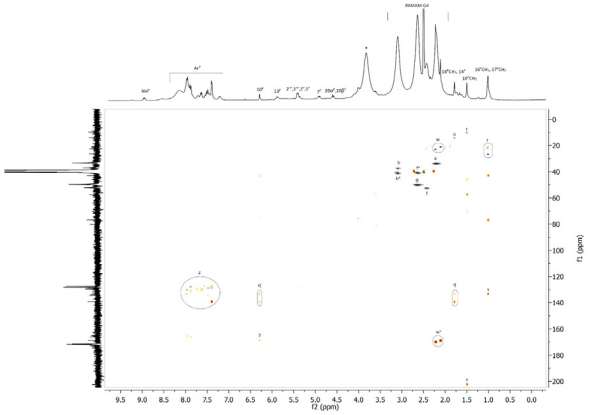

Fig. 6. Combined HSQC/HMBC map of PAMAM $G4^{5P}$ in DMSO-d_6. The relevant one bond coupling peaks are shown in grey scale as follows: e –H^b(G4) / C^b(G4); f – H^d(G4) / C^d(G4); f*- H^{d*}(G4) / C^{d*}(G4); g –H^a(G4) / C^a(G4); h –H^c(G4) / C^c(G4); h* - H^{c*}(G4) / C^{c*}(G4); r – $16CH_3{}^P$ / 16PC and 17^PCH_3 / 17CP; s – $19CH_3{}^P$ / 19CP; u – 18HP/ 18CP; w – 10HP-OAcH / 10P-OAcC and 4HP-OAcH / 4CP-OAcC. The significant HMBC coupling peaks are represented in red/yellow scale as follows: s – $16CH_3{}^P$ or $17CH_3{}^P$ / 11CP; q – $18CH_3{}^P$ / 11CP and 12CP, q' – 10HP / 11CP and 12PC, w* - 10HP (OAcH) / C(O)P and 4HP(OAcH) / C(O)P ; x – $19CH_3{}^P$ / 9PC; y – 10HP / C(O)P; z – a group of HMBC/HSQC aromatic cross-peaks of P residues are represented. Residual HDO is labeled with asterisk in upper trace of the map

Glycidylated Paclitaxel – G4 conjugate

The $G4^{4P}$ was obtained in separate synthesis as previously described $G4^{5P}$ and further derivatized with *R*-glycidol. The ^{1}H NMR spectrum of the product is presented at Fig.7C. The integration of resonances from P and gl protons related to [248H] G4 resonance enabled to identify the stoichiometry of the conjugate as $G4^{4P110gl}$.

Binary glycidylated Paclitaxel – Fulvestrant – G4 conjugate

The $G4^{5P}$ was used to attach covalently Fulvestrant activated with NPCF. After the condensation step, the *R*-glycidylation was performed and afterwards the product was purified by extensive dialysis against water. The product was analyzed by ^{1}H NMR spectroscopy in order to determine the number of P, F, and gl residues. The ^{1}H NMR spectrum of binary conjugate is presented at Fig.7B. The integration of aromatic resonance from P, and well separated aromatic resonances of F enabled to determine the average number of P and F residues and gl substituents to obtain final formula $G4^{5P10F98gl}$. The compound was also fully characterized by 2-D spectra. The COSY spectrum is presented at Fig.8. Two crucial cross-peaks (a and b) were observed gl residues and additional cross-peak c allowed to identify the F resonances. Some additional information gained from heteronuclear ^{1}H-^{13}C HSQC and HMBC experiments (Fig.9) completed the assignments of ^{1}H resonances of PAMAM, gl, F, and P.

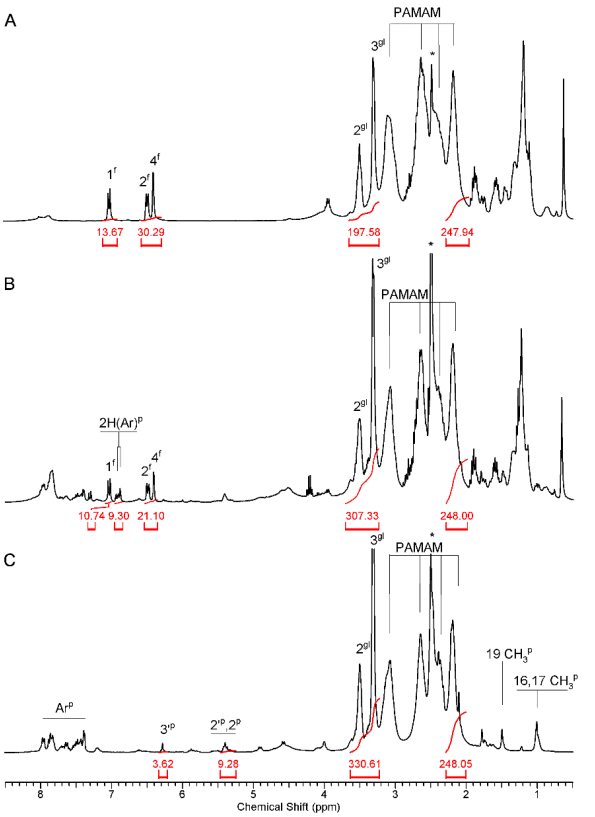

Fig. 7. The ^{1}H NMR spectra of: $G4^{15F66gl}$ (A) , $G4^{5P10F98gl}$ (B), and $G4^{4P110gl}$ (C) in DMSO-d_6. For atom numbering see Fig. 4. The $HD_2S(O)CD_3$ resonance is labeled with asterisk. The resonances from Paclitaxel, Fulvestrant and glycidol are upper indexed with p, f, and gl

Obtained conjugates: $G4^{15F66gl}$, $G4^{4P110gl}$, and $G4^{5P10F98gl}$ were designed as DDS. All conjugates are water soluble at ca 100 μM concentration. The stoichiometry of conjugates was determined by NMR spectroscopy in DMSO-d_6. They can be stored in frozen solvent for months and are stable. The stock solutions in DMSO (5 mM concentration) can be diluted with water 200 times without precipitation. The glycidylated conjugates have some tendency to associate in water. The measurements of conjugate hydrodynamic diameters by Dynamic Light Scattering method evidenced that size of nanoparticles was around 100 nm, while the size of G4 dendrimer is 5 nm in monomolecular disperse. Nevertheless, the conjugates can be active as DDS against breast cancer cells, as we observed before in case of glucoheptoamidated G3 conjugated with Fulvestrant and Lapatinib.[8] Gener-

ally, the solubility of conjugates is one order of magnitude higher than free drugs. High load of obtained DDSs with F and P is promising factor in anticancer activity, which will be tested and reported separately.[29]

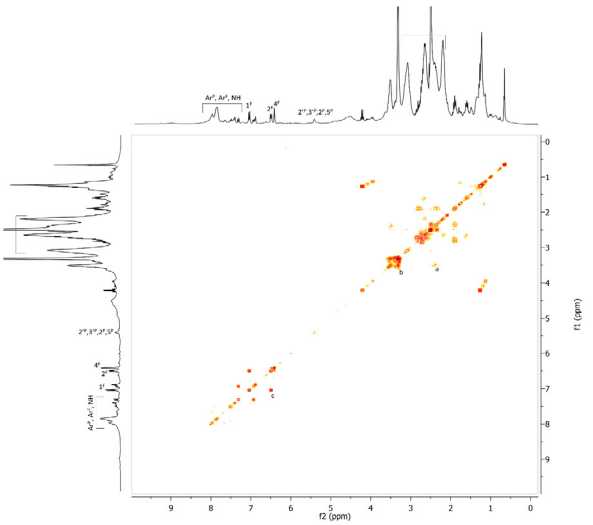

Fig. 8. ^{1}H-^{1}H COSY spectrum of G4^{5P10F98gl} in DMSO-d$_6$.The significant cross-peaks are as follows:
a – H^{1gl} / H^{2gl}; b – H^{2gl} / H^{3gl}; c – 2H^{F}/1H^{F}

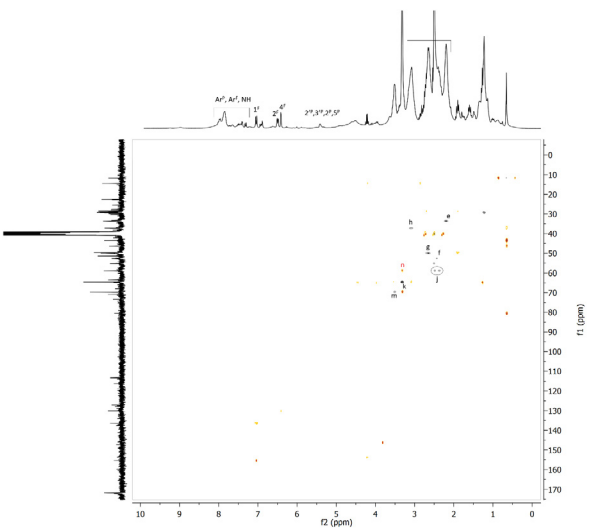

Fig. 9. Combined HSQC/HMBC map of G4^{5P10F98gl} in DMSO-d$_6$. The relevant one bond coupling peaks are shown in grey scale as follows: e – H^{b}(G4) / C^{b}(G4); f –H^{d}(G4) / C^{d}(G4); g – H^{a}(G4) / C^{a}(G4); h –H^{c}(G4) / C^{c}(G4); j – H^{1gl} / C^{1gl}; k – H^{3gl} / C^{3gl}; m – H^{2gl} / C^{2gl}. The significant HMBC coupling peak is n – H^{3gl} / C^{2gl}

Conclusion

The anticancer DDS was constructed based on PAMAM dendrimer of fourth generation. Well water soluble forms of Fulvestrant and Paclitaxel were obtained by covalent attachment of the drugs into macromolecular carrier. High water solubility of obtained macromolecular DDS was achieved by addition of *R*-glycidol into primary amine groups of PAMAM dendrimer, which otherwise are responsible for general toxicity of such DDSs. The binary conjugate, containing both Paclitaxel and Fulvestrant was deliberately designed as DDS against breast cancer cell models expressing estrogen receptor α such as MCF7 and BT474.

Declarations

Funding

The research was funded by University of Rzeszów from General Status Activity Fund.

Author contributions

Conceptualization, K.W.; Methodology, S.W.; Software, K.W.; Validation, S.W.; Formal Analysis, S.W.; Investigation, K.W.; Resources, S.W.; Data Curation, K.W.; Writing – Original Draft Preparation, K.W.; Writing – Review & Editing, S.W.; Visualization, K.W.; Supervision, S.W.; Project Administration, S.W.; Funding Acquisition, S.W.

Conflicts of interest

Authors declare no conflict of interest.

Data availability

The data presented (1-D and 2-D NMR spectra are available) from Authors upon request.

Ethics approval

Non applicable.

References

1. www.cancer.org/research/cancer-facts-statistics/. Accessed February 14, 2023.
2. World Health Organization. https://www.who.int/news-room/fact-sheets/detail/cancer. Accessed February 14, 2023.
3. Debela DT, Muzazu SG, Heraro KD, et al. New approaches and procedures for cancer treatment: Current perspectives. *SAGE Open Med.* 2021; 9:20503121211034366. doi: 10.1177/20503121211034366
4. Patra JK, Das G, Fraceto LF, et al. Nano based drug delivery systems: recent developments and future prospects. *J Nanobiotechnol.* 2018;16:71. doi: 10.1186/s12951-018-0392-8
5. Oddone N, Lecot N, Fernández M, et al. In vitro and in vivo uptake studies of PAMAM G4.5 dendrimers in breast cancer. *J Nanobiotechnol.* 2016;14:45. doi: 10.1186/s12951-016-0197-6
6. Abedi-Gaballu F, Dehghan G, Ghaffari M, et al. PAMAM dendrimers as efficient drug and gene delivery nanosystems for cancer therapy. *Appl Mater Today.* 2018;12:177-190. doi: 10.1016/j.apmt.2018.05.002
7. Dichwalkar T, Patel S, Bapat S, et al. Omega-3 Fatty Acid Grafted PAMAM-Paclitaxel Conjugate Exhibits Enhanced

Anticancer Activity in Upper Gastrointestinal Cancer Cells. *Macromol Biosci.* 2017;17:1600457. doi: 10.1002/mabi.201600457

8. Lewińska A, Wróbel K, Błoniarz D, et al. Lapatinib- and fulvestrant-PAMAM dendrimer conjugates promote apoptosis in chemotherapy-induced senescent breast cancer cells with different receptor status. *Biomater Adv*. 2022;140:213047. doi: 10.1016/j.bioadv.2022.213047
9. Weaver BA. How Taxol/paclitaxel kills cancer cells. *Mol Biol Cell*. 2014;25:2677-2681. doi: 10.1091/mbc.E14-04-0916
10. Zasadil LM, Andersen KA, Yeum D, et al. Cytotoxicity of paclitaxel in breast cancer is due to chromosome missegregation on multipolar spindles. *Sci Transl Med*. 2014;6(229):229ra43. doi: 10.1126/scitranslmed.3007965
11. Abu Samaan TM, Samec M, Liskova A, Kubatka P, Büsselberg D. Paclitaxel's Mechanistic and Clinical Effects on Breast Cancer. *Biomolecules.* 2019;9(12):789. doi: 10.3390/biom9120789
12. Haldar S, Chintapalli J, Croce CM. Taxol Induces bcl-2 Phosphorylation and Death of Prostate Cancer Cells. *Cancer Res.* 1996;56:1253-1255.
13. Torne SJ, Ansari KA, Vavia PR, Trotta F, Cavalli, R. Enhanced oral paclitaxel bioavailability after administration of paclitaxel-loaded nanosponges. *Drug Delivery*. 2010;17(6):419-425. doi: 10.3109/10717541003777233
14. https://www.accessdata.fda.gov/drugsatfda_docs/label/2020/021660s047lbl.pdf. Accessed February 27, 2023.
15. Nathan MR, Schmid P. A Review of Fulvestrant in Breast Cancer. *Oncol Ther*. 2017;5(1):17-29. doi: 10.1007/s40487-017-0046-2
16. Johnston SJ, Cheung KL. Fulvestrant - a novel endocrine therapy for breast cancer. *Curr Med Chem*. 2010;17(10): 902-914. doi: 10.2174/092986710790820633
17. Robertson JFR, Bondarenko IM, Trishkina E, et al. Fulvestrant 500 mg versus anastrozole 1 mg for hormone receptor-positive advanced breast cancer (FALCON): an international, randomised, double-blind, phase 3 trial. *Lancet*. 2016;388(10063):2997-3005. doi:10.1016/s0140-6736(16)32389-3
18. Rocca A, Maltoni R, Bravaccini S, Donati C, Andreis D. Clinical utility of fulvestrant in the treatment of breast cancer: a report on the emerging clinical evidence. *Cancer Manag Res*. 2018;10:3083-3099. doi: 10.2147/CMAR.S137772
19. Bayat Mokhtari R, Homayouni TS, Baluch N, et al. Combination therapy in combating cancer. *Oncotarget*. 2017;8(23):38022-38043. doi: 10.18632/oncotarget.16723
20. Geyer CE, Forster J, Lindquist D, et al. Lapatinib plus capecitabine for HER2-positive advanced breast cancer. *N Engl J Med*. 2006;355(26):2733-2743. doi: 10.1056/NEJMoa064320
21. Wróbel K, Wołowiec S, Markowicz J, Wałajtys-Rode E, Uram Ł. Synthesis of biotinylated PAMAM G3 dendrimers substituted 2 with R-glycidol and celecoxib/simvastatin as repurposed drugs and demonstration of their increased additive cytotoxicity for cancer cell lines. *Cancers.* 2022;14 (3):714. doi: 10.3390/cancers14030714
22. Uram Ł, Filipowicz-Rachwał A, Misiorek M, Winiarz A, Wałajtys-Rode E, Wołowiec S. Synthesis and Different Effects of Biotinylated PAMAM G3 Dendrimers Substituted with Nimesulide in Human Normal Fibroblasts and Squamous Carcinoma Cells. *Biomolecules.* 2019;9:437. doi: 10.3390/biom9090437
23. Tomalia D, Baker H, Dewald J, et al. A new Class of Polymers: Starburst-Dendritic Macromolecules. *Polym J*. 1985;17:117-132.
24. Satsangi A, Roy SS, Satsangi RK, Vadlamudi, RK, Ong JL. Design of a Paclitaxel Prodrug Conjugate for Active Targeting of an Enzyme Upregulated in Breast Cancer Cells. *Mol Pharmaceutics.* 2014;11:1906-1918. doi: 10.1021/mp500128k
25. Wróbel K, Wołowiec S. Low generation polyamidoamiine dendrimers (PAMAM) and biotin PAMAM conjugate – the detailed structural studies by ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy. *Eur J Clin Exp Med.* 2020;18:281-285. doi: 10.15584/ejcem.2020.4.4
26. Malinga-Drozd M, Uram Ł, Wróbel K, Wołowiec S. Chiral recognition of homochiral poly(amidoamine) dendrimers substituted with R- and S-glycidol by keratinocyte (HaCaT) and squamous carcinoma (SCC-15) cells in vitro. *Polymers.* 2021;13:1049. doi: 10.3390/polym13071049
27. Cline EN, Li M-H, Choi SK, et al. Paclitaxel-Conjugated PAMAM Dendrimers Adversely Affect Microtubule Structure through Two Independent Modes of Action. *Biomacromolecules.* 2013;14:654-664. doi: 10.1021/bm301719b
28. Teow HM, Zhou ZY, Najlah M, Yusof SR, Abbott NJ, D'Emanuele A. Delivery of paclitaxel across cellular barriers using a dendrimer-based nanocarrier. *Int J Pharm.* 2012;441:701-711. doi: 10.1016/j.ijpharm.2012.10.024
29. Dai X, Cheng H, Bai Z, Li J. Breast Cancer Cell Line Classification and Its Relevance with Breast Tumor Subtyping. *J Cancer*. 2017;8(16):3131-3141. doi: 10.7150/jca.18457