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14. ABSTRACT

Poly(amidoamine) (PAMAM) dendrimers of 3.5 generation were first activated with N-hydroxysuccinimide (NHS) to obtain a stable PAMAM-NHS. This NHS activated PAMAM was then coupled with SV119 using 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) coupling. Structural analysis of the conjugate was performed using Bruker 300MHZ ^{1}H NMR spectrometer. PAMAM-NHS-SV119 was fluorescently labeled using FITC (Fluorescein isothiocyanate) and 1.2 molecules of FITC were found conjugated to one molecule of PAMAM. Dendriplexes were prepared with sigma receptor ligand-conjugated PAMAM dendrimers and p53-GFP plasmid. Dendriplex stability was assessed by agarose gel electrophoresis and preliminary experiments were performed to determine the transfection efficiency of these dendriplexes in MCF-7 cells and NCI/RES-ADR cells.

To construct a therapeutic plasmid (p53) containing the heparanase promoter, PCR amplification of the heparanase promoter sequences was performed and the experiments to insert p53-heparanase fusion gene into the multiple cloning site of the GFP vector are in progress.

15. SUBJECT TERMS
PAMAM DENDRIMERS, P53 GENE THERAPY, SIGMA RECEPTOR-LIGANDS, CANCER DRUG RESISTANCE

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Introduction:
Proapoptotic gene therapy aims at enhancing the capacity of tumor cells to undergo apoptosis and renders the tumors sensitive to classical anticancer drugs and radiotherapy. The proposed project is designed for the targeted delivery of proapoptotic genes using PAMAM dendrimers. We hypothesize that conjugation of sigma ligands to dendrimers will specifically target and deliver the proapoptotic gene (p53 plasmid) to breast cancer cells over expressing the sigma receptors. Targeted gene expression will be accomplished by incorporating Heparanase promoter into the therapeutic plasmid.

Specific aims of the project are to: (1) characterize sigma ligand-conjugated $PAMAM_{G4}$ dendrimers by NMR and MALDI-TOF spectroscopy, (2) determine the cellular uptake and tumor specificity of the surface modified dendrimers in various breast cancer cell lines and normal cells, (3) characterize the cancer cell specific expression of p53 (GFP-p53 plasmid with HPR promoter), (4) assess the efficiency of these vectors in inducing apoptosis and sensitizing the drug resistant breast cancer cells for chemotherapy.

Body: As stated in the statement of work (SOW) the project encompasses four tasks. A progress report on the experiments performed to accomplish these tasks is given below.

Task 1: To determine the breast cancer cell specificity of sigma receptor ligands (haloperidol and ibogaine)-conjugated polyamidoamine (PAMAM) dendrimers

1. Synthesis, purification and structural analysis of sigma receptor ligand-conjugated PAMAM dendrimers by H^1NMR spectroscopy and MALDI TOF spectroscopy.
2. Fluorescence (FITC)-labeling of the dendrimers and their specificity to breast cancer cell lines will be determined.

Poly(amidoamine) (PAMAM) dendrimers of 3.5 generation with carboxylate surface functional groups were first activated with N-hydroxysuccinimide (NHS) to obtain a stable PAMAM-NHS. This NHS activated PAMAM was then coupled with SV119. Nuclear magnetic resonance (^{1}H NMR) spectroscopy was performed using Bruker 300MHz spectrometer with D_2O and presence of SV119 in final product was identified.

Initially, PAMAM dendrimers of 3.5 generation was conjugated to SV119 using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) coupling reaction. This EDC cross linker will couple COOH surface groups of PAMAM dendrimer with terminal NH_2 group of SV119 by forming an amide bond. Briefly, EDC (8.8 mg) was dissolved in 3mL of 0.1M MES [2-(*N*-morpholino) ethanesulfonic acid] buffer, pH 4.7. To this 20 mg of PAMAM dendrimer dissolved in deionized water (1 mL) was added drop wise under stirring. This solution was stirred under nitrogen for one hr at room temperature. After 1 hr, SV119 (12.8 mg) was directly added to reaction and stirred for 4 hrs at room temperature. The reaction mixture was dialyzed with 1:10 diluted MES buffer for 24 hrs and lyophilized.

The ^{1}H NMR spectrum of final conjugate did not show any peaks of aromatic ring protons of SV119 corresponding to 6.8, 6.9 and 7.2 ppm, only the CH protons of PAMAM from 2.5-3.7 ppm were present (Figure 1). These aromatic peaks were found when NMR spectrum of SV119 was recorded. As the reaction pH was 4.7, the loss of aromatic ring of SV119 was attributed to low pH of the reaction.

Thus, the reaction method was then modified to a two step conjugation instead of one with formation of stable *N*-hydroxysuccinimde ester of PAMAM as initial step. Briefly, in the first step PAMAM (20 mg) was conjugated to NHS (5.3 mg) by EDC (8.8mg) coupling reaction in 0.1 M MES buffer. After 12 hrs of reaction at room temperature the reaction mixture was dialyzed and lyophilized. In the second step 20 mg NHS ester of dendrimer was first dissolved in phosphate buffer of pH 7.4. To this solution while under stirring SV119 was directly added and reacted for 6 hrs at room temperature. The mixture was dialyzed against 1:10 diluted phosphate buffer pH 7.4 for 24 hrs and then lyophilized.

The ^{1}H NMR spectrum of the conjugate revealed the presence of peaks of aromatic ring protons of SV119 corresponding to 6.8, 6.9 and 7.2 ppm along with CH protons of PAMAM from 2.5-3.7 ppm (Figure 2). These

peaks were not found in NMR spectrum of only PAMAM. Thus, conjugation of SV119 to PAMAM was confirmed by presence of these peaks corresponding to aromatic ring protons.

PAMAM-NHS-SV119 was fluorescently labeled using FITC (Fluorescein isothiocynate) and approximately 1.2 molecules of FITC was conjugated to one molecule of PAMAM. Uptake study was performed in MCF-7 cells.

Task 2: To construct a therapeutic plasmid (p53) containing the heparanase promoter for breast cancer specific gene expression

1. PCR amplification of the heparanase promoter sequences
2. Insertion of p53-heparanase fusion gene into the multiple cloning site of the GFP vector.
3. Determine the effect of the promoter and enhancer on p53 gene expression in various breast cancer cell lines (n=6)

The DH5α bacterial cells containing GFP-p53 plasmid were obtained from Addgene (Plasmid 12091, Figure 3). The cells were grown in AccuGENE® LB broth (Lonza, Belgium) and plasmids were isolated using Qiagen plasmid Mini and Maxi kit (Qiagen Sciences, Maryland, USA). Plasmid concentration and purity, A_{260}/A_{280} > 1.9, was assessed using Nanodrop ND-1000 Spectrophotometer (Wilmington, DE). Closed circular and linear (by digesting with *BamHI*) Plasmid integrity was confirmed by 1% agarose gel electrophoresis and stored at -20^{o}C until further use (Figure 4). Isolated DNA was digested using *BamHI* and *PstI* and p53 fragment was confirmed by electrophoresis (Figure 5).

DNA condensation study at various charge ratios (N/P) is under progress. Dendriplexes were prepared at different N/P ratios (0.1 - 20) between the dendrimer and the circular (non-linear) plasmid DNA by incubating in HEPES buffer (25 mM, 10 mM $MgCl_2$, pH 7.4) at room temperature for 30 min. Each sample was analyzed by electrophoresis on a 1% agarose gel containing ethidium bromide (0.5 μg/mL) at 75 V for one hour (Figure 6). Transfection study using GFP-p53 in MCF-7 and resistant cell line NCI/RES-ADR is in progress. Dendriplexes at N/P 5 revealed some transfection (Figure 7). Optimization of charge ratio, quantity and particle size is under progress.

Task 3: To determine the therapeutic efficiency of dual targeting approach in various breast cancer cell lines (In progress)

1. Preparation and characterization of the sigma ligand(s)-conjugated dendrimer-(GFP-p53) plasmid complexes
2. Gene transfection and apoptosis analysis will be performed by DAPI staining and western blotting in various breast cancer cell lines.

Task 4: To determine the effect of targeted proapoptotic gene therapy on breast cancer drug resistance reversal (to be completed)

1. Induce doxorubicin drug resistance in various breast cancer (MCF-7) cells
2. Determine the efficacy of proapoptotic gene therapy followed by doxorubicin therapy on cell viability will be tested by MTT assay.

Key research accomplishments: For the first time novel sigma ligand conjugated PAMAM dendrimers have been synthesized.

Reportable outcomes: None

Conclusion: SV119 conjugated-PAMAM dendrimers were synthesized and the conjugation of PAMAM-NHS-SV119 was confirmed using ^{1}H NMR spectroscopy. Cellular uptake study of FITC labeled PAMAM-NHS-SV119 was performed in MCF-7 cells. Dendriplexes were prepared with sigma receptor ligand-conjugated PAMAM dendrimers and p53-GFP plasmid. Dendriplex stability was assessed by agarose gel electrophoresis and preliminary experiments were performed to determine the transfection efficiency of these dendriplexes in MCF-7 cells and NCI/RES-ADR cells. Construction of a therapeutic plasmid (p53) containing the heparanase promoter is in progress. Breast cancer cell-specific expression of p53 will be tested in normal and resistant breast cancer cells in future.

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1. Stoff-Khalili MA, Dall P, Curiel DT. Gene therapy for carcinoma of the breast. Cancer Gene Ther., 2006, 13(7), 633-647.
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Appendix I: Figures

Supporting data: None

Appendix I:

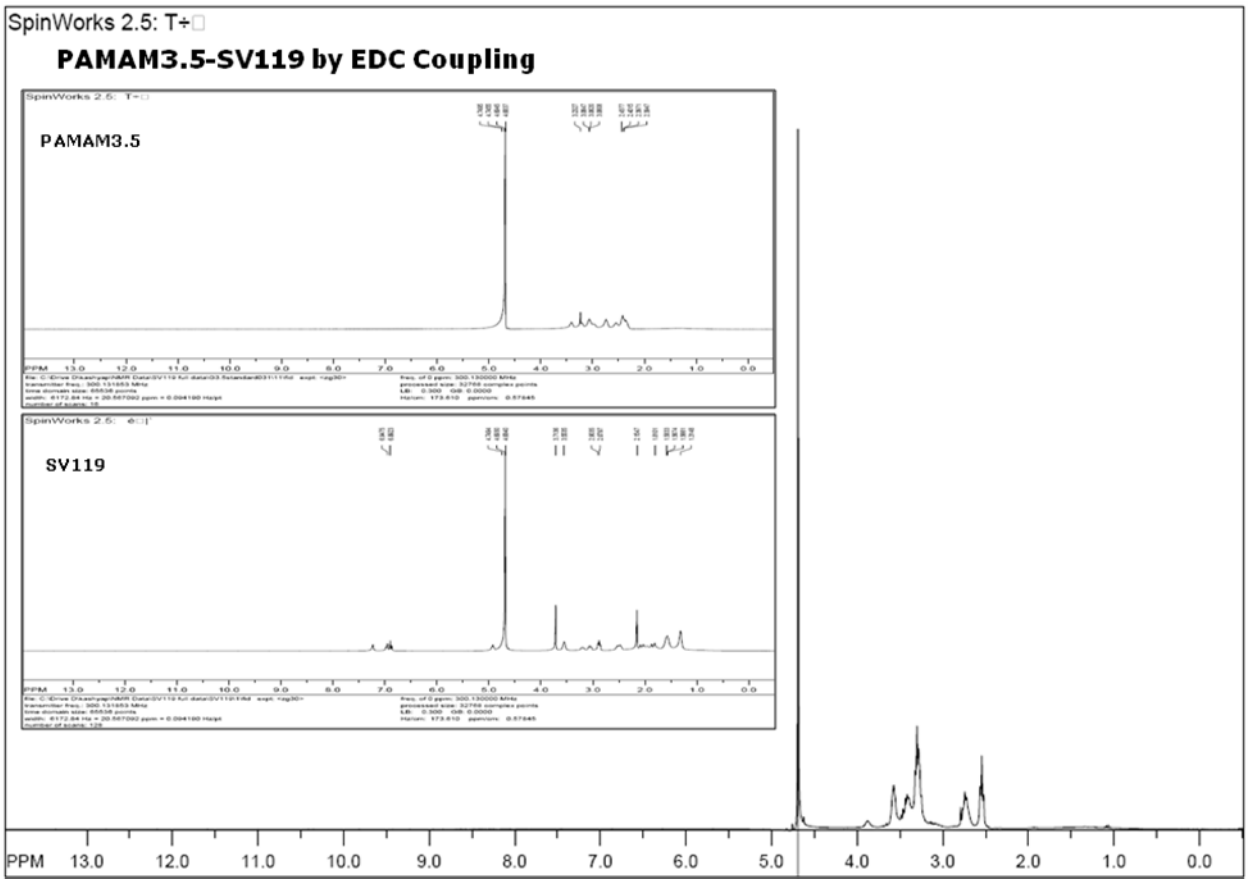

Figure 1: ^{1}H NMR data of SV119 conjugated $PAMAM_{3.5}$ dendrimers by EDC coupling. Absence of aromatic ring of SV119 in the final product confirms the hydrolysis of the product at low pH.

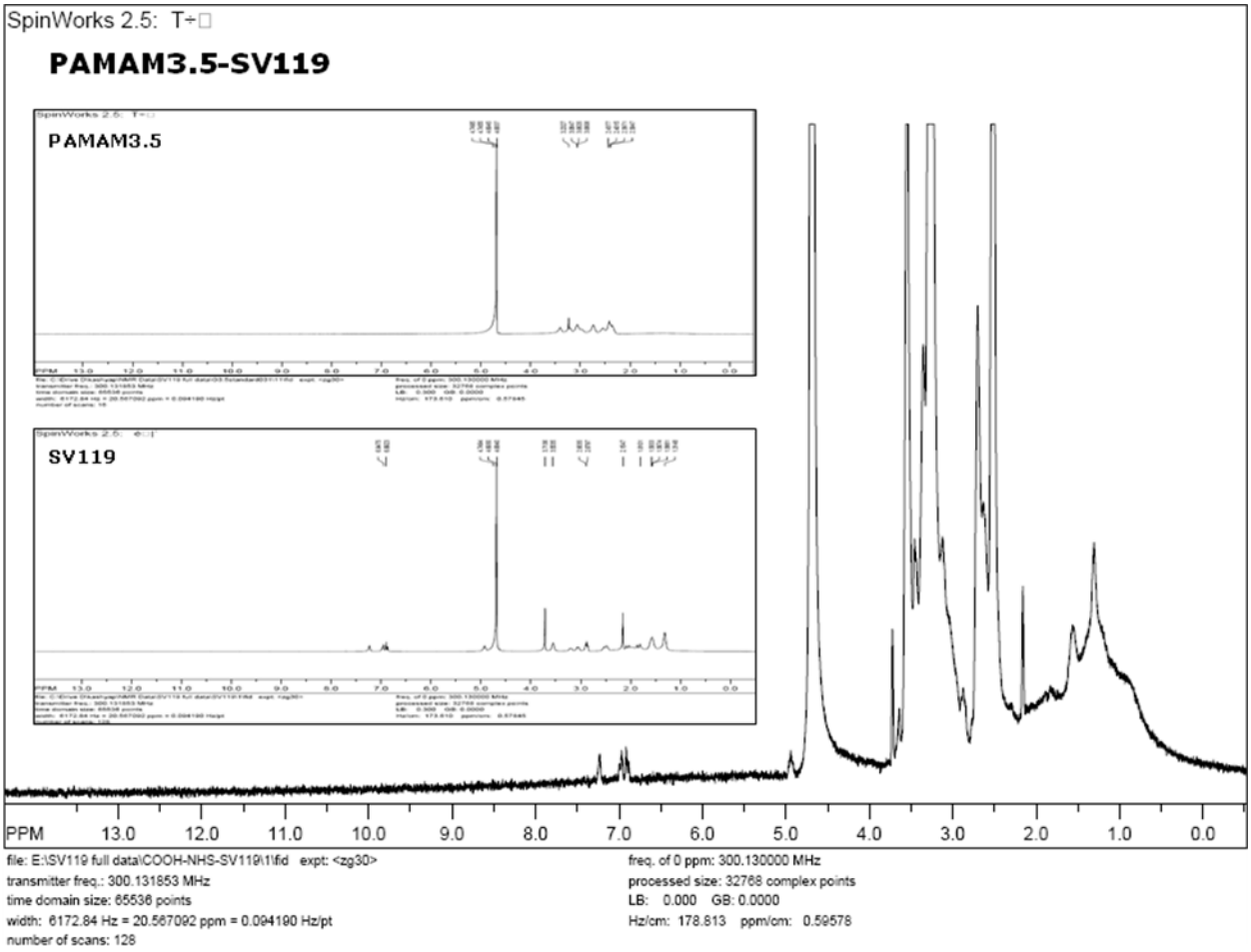

Figure 2: ^{1}H NMR data of SV119 conjugated PAMAM$_{3.5}$ dendrimers by NHS ester formation.

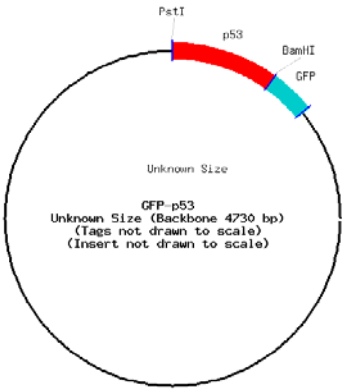

Figure 3: Addgene, Plasmid# 12091.

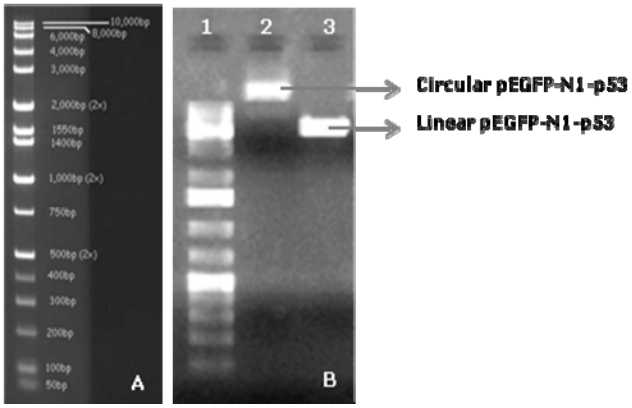

Figure 4: Closed circular and linear pEGFP-N1-p53. **[A]** DNA Ladder (Minnesota Molecular, Inc., Minneapolis, MN). **[B]** ***Lane 1:*** DNA Ladder, ***Lane 2:*** Purified circular plasmid (Addgene# 12091), ***Lane 3:*** Linear plasmid DNA obtained after digestion with *BamHI* (Fermentas Inc., Glen Burnie, MD)

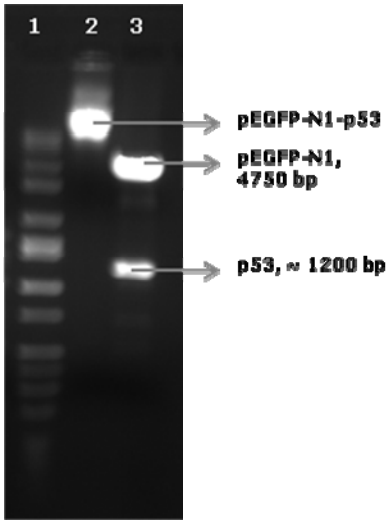

Figure 5: Restriction digestion of pEGFP-N1-p53. ***Lane 1:*** DNA Ladder, ***Lane 2:*** Purified plasmid, ***Lane 3:*** Plasmid digestion using *BamHI* and *PstI* (Fermentas Inc., Glen Burnie, MD)

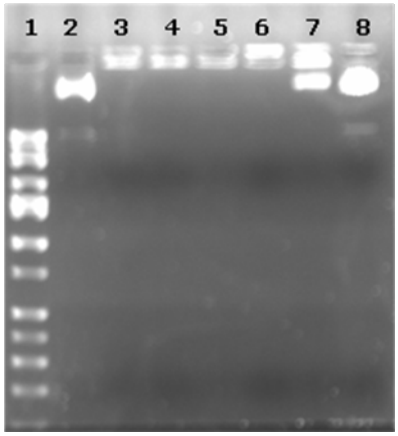

Figure 6: Agarose gel electrophoresis of dendriplexes. ***Lane 1:*** DNA Ladder, ***Lane 2:*** Purified plasmid, ***Lane 3-8:*** Dendriplexes at N/P 20, 10, 5, 1, 0.5 and 0.1.

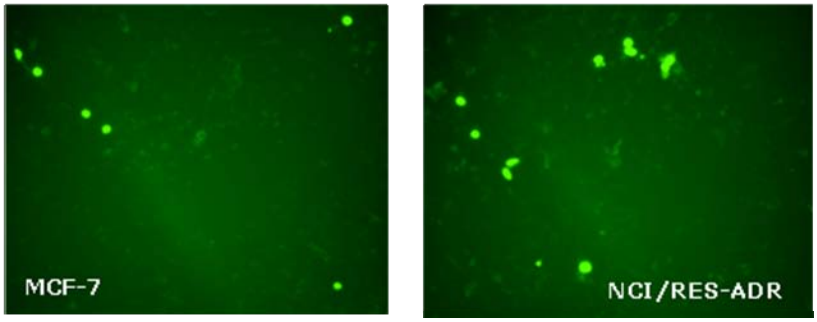

Figure 7: Preliminary gene transfection study in MCF-7 and NCI/RES-ADR cells.