The Effect of Generation 2 and 3 Poly(amidoamine) Dendrimers on Viability of Human Breast Cancer Cells

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In this study, we investigated the effect of amine- and hydroxyl-terminated Generation 2 and 3 (G2 and G3) poly(amidoamine) (PAMAM) dendrimers on the viability and induction of apoptosis in human MCF-7 and MDA-MB-231 breast cancer cells. It was shown that amino-terminated PAMAM dendrimers were cytotoxic, with IC_{50} value after 24 hr of incubation in MCF-7 and MDA-MB-231 breast cancer cells 153 ± 3 μM and 140 ± 2 μM for G2 PAMAM and 120 ± 3 μM and 99 ± 2 μM for G3 PAMAM, respectively. Experiments made with annexin V-fluorescein isothiocyanate (V-FITC) and detection of apoptosis by a fluorescent microscopy assay revealed that G2 and G3 PAMAM-NH_2 dendrimers inhibited the proliferation of MCF-7 and MDA-MB-231 malignant cells by increasing the number of apoptotic and necrotic cells. The cytotoxic and apoptotic effect of G2 and G3 PAMAM-OH dendrimers was significantly weaker.

Key words —— poly(amidoamine) dendrimer, cytotoxicity, apoptosis, breast cancer cell

INTRODUCTION

Dendrimers represent a relatively new class of macromolecules having a unique three-dimensional structure. The term “dendrimer” is derived from the Greek words “dendra” for tree and “meros” for part. A typical dendrimer comprises three main structural components: a multifunctional central core, branched units, and surface groups. The repeated layers are called “generations”.[1] More than 100 different types of dendrimers with over 1000 types of surface modifications have been developed to date. A unique advantage of dendritic structures over other random linear polymers is that surface topology and functionality can be controlled to a higher extent. The high level of control over the architecture of dendrimers, their size, shape, branching length and density, and their surface functionality, make these compounds suitable for many pharmaceutical applications such as delivery of DNA and oligonucleotides into eukaryotic cells, solubilization of sparingly soluble drugs, nanocarriers for transepithelial transport, and platforms for cancer therapeutics.[2–4] Drugs or other payloads can also be encapsulated into the interior of the dendrimers or they may be chemically attached or physically adsorbed onto the dendrimer surface, having the potential to increase solubility and reduce toxicity of pharmaceutical compounds.[3)]

Poly(amidoamine) (PAMAM) dendrimers, commercially available as Starburst® PAMAM dendrimers based on an ethylene diamine core and an amidoamine repeat branching structure, have a diameter ranging from 1.5 to 14.5 nm.[1,5] As generation (G) number is increased, the number of active terminal groups doubles. For example, G3 dendrimers contain 32 terminal groups and G4 dendrimers contain 64 terminal groups. In PAMAM dendrimers, full generations (G1, G2, G3, *etc.*) have terminal amine or hydroxyl groups while half generation dendrimers (G1.5, G2.5, G3.5, *etc.*) have carboxylic acid terminal groups. As dendrimer generation increases, the number of terminal branches increases exponentially, while the diameter increases linearly by about 1 nm/generation. This compactness gives dendrimers a significant advantage over traditional linear polymers, which are restricted to low density drug loading. PAMAM dendrimers are synthetic polymer-based nanoparticles, which are widely recognized as important quantized nanoscale building blocks in biomedical

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research.[6, 7]

Limited studies have been performed to investigate how dendrimer chemistry and a molecular architecture affect biodistribution, pharmacokinetics, biological half-life and toxicity. Despite the extensive interest in the pharmaceutical applications of dendrimers, very little is known about their cytotoxicity and mechanisms of cell death. There is conflicting evidence regarding their biological safety.[8, 9] The present study compares the effect of G2 and G3 PAMAM dendrimers with -NH_2 and -OH termini on the viability and induction of apoptosis in human MCF-7 and MDA-MB-231 breast cancer cells.

MATERIALS AND METHODS

Materials —— Amine- and hydroxyl-terminated G2 and G3 PAMAM dendrimers, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), acridine orange, ethidium bromide, controlled process serum replacement Type 1 (CPSR1) were provided by Sigma-Aldrich (St. Louis, MO, U.S.A.), as were most other chemicals and buffers used. Apoptest-fluorescein isothiocyanate (FITC) was a product of DakoCytomation (Copenhagen, Denmark). Stock cultures of human MCF-7 and MDA-MB-231 breast cancer cells were purchased from the American Type Culture Collection (Manassas, VA, U.S.A.). Dulbecco's minimal essential medium (DMEM) and fetal bovine serum (FBS) used in a cell culture were products of Gibco (Grand Island, NE, U.S.A.). Glutamine, penicillin and streptomycin were obtained from Quality Biologicals Inc. (Derwood, MD, U.S.A.). [^{3}H]Thymidine (6.7 Ci/mmol) was purchased from NEN (Waltham, MA, U.S.A.), and Scintillation Coctail "Ultima Gold XR" from Packard (Meriden, CT, U.S.A.). Sodium dodecylsulfate (SDS) was received from Bio-Rad Laboratories (Cambridge, MA, U.S.A.).

Cell Culture —— Human breast cancer MCF-7 and MDA-MB-231 cells were maintained in DMEM supplemented with 10% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin at 37°C. Cells were cultured in Costar flasks and subconfluent cells were detached with 0.05% trypsin and 0.02% EDTA in calcium-free phosphate-buffered saline (PBS), counted in hemocytometers and plated at 5×10^5 cells per well of six-well plates (*Nunc*) in 2 ml of growth medium (DMEM without phenol red with 10% controlled process serum replacement Type 1 - CPSR1). Cells reached about 80% of confluency at day 3 and in most cases such cells were used for the assays.

Cell Viability Assay —— The assay was performed according to the method of Carmichael using MTT.[10] Confluent cells, cultured for 24 and 48 hr with various concentrations of the studied compounds in 6-well plates were washed three times with PBS and then incubated for 4 hr in 1 ml of MTT solution (0.5 mg/ml of PBS) at 37°C in 5% CO_2 in an incubator. The medium was removed and 1 ml of 0.1 M HCl in absolute isopropanol was added to attached cells. Absorbance of converted dye in living cells was measured at a wavelength of 570 nm. Cell viability of cells cultured in the presence of ligands was calculated as a per cent of control cells.

DNA Synthesis Assay —— To examine the effect of studied compounds on cells proliferation, the cells were seeded in 24 well tissue culture dishes at 1×10^5 cells/well with 1 ml of growth medium. After 24 hr ($1.8 \pm 0.1 \times 10^5$ cells/well) plates were incubated with varying concentrations of dendrimers and 0.5 μCi of [^{3}H]thymidine for 24 hr at 37°C. Cells were rinsed 3 times with PBS, solubilized with 1 ml of 0.1 M sodium hydroxide containing 1% SDS, scintillation fluid (9 ml) was added and radioactivity incorporation into DNA was measured in scintillation counter.

Flow Cytometry Assessment of Annexin V Binding —— Apoptosis was determined by assessment of phosphatidylserine exposure by Annexin V-FITC binding using the Annexin V-FITC staining kit (Apoptest-FITC) according to the manufacturer's instruction. Ungated cells (10000) were analyzed in a flow cytometer (Beckman Coulter, Fullerton, CA, U.S.A.). Annexin V binds with high affinity to phosphatidylserine and can thus be used to identify cells in all stages of programmed cell death.[11, 12] Propidium iodide exclusively stains cells with a disrupted cell membrane and can be used to identify late apoptotic and dead cells.

Fluorescent Microscopy Assay —— To asses apoptosis, cell viability was estimated 24 hr after the addition of examined compounds. The cell suspension (250 μl) was stained with 10 μl of the dye mixture (10 μM acridine orange and 10 μM ethidium bromide), which was prepared in PBS. Acridine orange (fluorescent DNA-binding dye) intercalates into DNA, making it appear green, and binds to RNA, staining it red/orange. Ethidium bromide is only taken up by nonviable cells;

its fluorescence overwhelms that of the acridine orange, making the chromatin of necrotic cells appear orange.[13)]

Two hundred cells per sample were examined by fluorescence microscopy, according to the following criteria: viable cells with normal nuclei (fine reticular pattern of green stain in the nucleus and red/orange granules in the cytoplasm); viable cells with apoptotic nuclei (green chromatin which is highly condensed or fragmented and uniformly stained by the acridine orange); nonviable cells with normal nuclei (bright orange chromatin with organized structure); and nonviable cells with apoptotic nuclei (bright orange chromatin which is highly condensed or fragmented).

Statistical Analysis —— The results were analysed by analysis of variance (ANOVA) and multiple comparison were done to check statistical significance. The data were expressed as mean value for three independent assays ± standard deviations (S.D.). The statistical significance between means was verified by Sheffe's comparison test accepting $p < 0.05$ as significant.

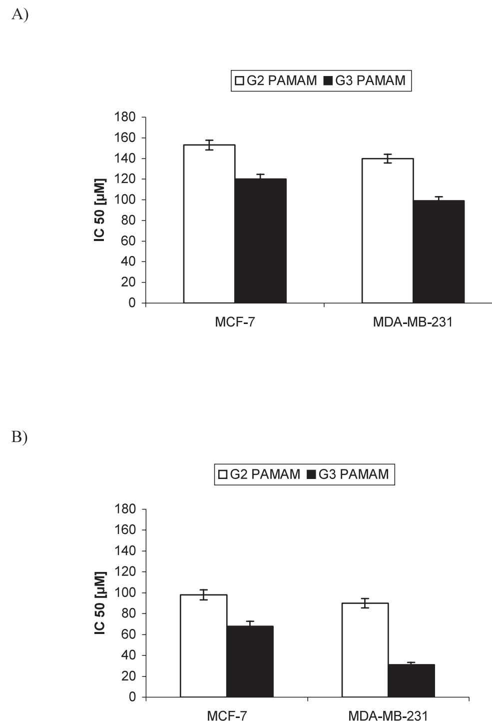


Fig. 1. The Effect of Generation (G2 and G3) on the IC_{50} Values for Inhibition of Cell Viability of MCF-7 and MDA-MB-231 Breast Cancer Cells after 24 hr (A) and 48 hr (B) of Treatment with PAMAM-NH_2 Dendrimers

RESULTS

The viability of MCF-7 and MDA-MB-231 breast cancer cells treated with different concentrations of amine- and hydroxyl-terminated G2 and G3 PAMAM dendrimers was measured by the method of Carmichael using MTT.[10)] MTT is a tetrazolium salt that is oxidized by mitochondrial dehydrogenase in living cells to give a dark blue formazan product. Damaged or dead cells show reduced or no dehydrogenase activity. The effect of exposure time on the IC_{50} values (the concentration where 50% inhibition of mitochondrial dehydrogenase activity is measured) for MCF-7 and MDA-MB-231 cells treated with PAMAM-NH_2 dendrimers is shown in Fig. 1. Our study revealed that amino-terminated PAMAM dendrimers were cytotoxic, with IC_{50} value after 24 hr of incubation in MCF-7 and MDA-MB-231 breast cancer cells 153 ± 3 µM and 140 ± 2 µM for G2 and 120 ± 3 µM and 99 ± 2 µM for G3 (Fig. 1). In contrast, G2 and G3 PAMAM-OH dendrimers have shown a significantly lower cytotoxicity in MCF-7 and MDA-MB-231 cells, with IC_{50} values above 10 mM after 24 hr of incubation (data not shown).

To analyze if the inhibition on cell viability was due to decreased cell proliferation, we measured DNA synthesis in the presence of G2 and G3 PAMAM dendrimers. All of the tested compounds showed concentration dependent activity, yet with different potency. The concentrations of PAMAM-NH_2 dendrimers needed to inhibit [^{3}H]thymidine incorporation into DNA by 50% (IC_{50}) were significantly lower (Table 1) than PAMAM-OH dendrimers (Table 2). When the cells were incubated with these dendrimers for 48 hr, similar results in DNA synthesis were observed (Tables 1 and 2).

To determine the nature of cell death induced by G2 and G3 PAMAM dendrimers in human MCF-7 and MDA-MB-231 breast cancer cells, we measured cell death by flow cytometric analysis after annexin V-FITC and propidiumiodide staining and by a fluorescent microscopy assay after acridine orange and ethidium bromide staining. During the early stage of apoptosis, phosphatidylserine translocates from the interior to the exterior part of the plasma membrane and becomes exposed at the cell surface, facilitating recognition by macrophages.[11, 12)] Annexin V binds with high affinity to phosphatidylserine and can thus be used to identify cells in all stages of programmed cell death. Propidiumiodide exclusively stains cells with a disrupted cell membrane and

Table 1. Antiproliferative Effects of Different Concentrations of PAMAM-NH_2 Dendrimers in MCF-7 and MDA-MB-231 Breast Cancer Cells as Measured by Inhibition of [^{3}H]thymidine Incorporation into DNA

Concentration (μM)	^{3}[H]thymidine incorporation (% of control)^a MCF-7		MDA-MB-231	
	24 hr	48 hr	24 hr	48 hr
G2 PAMAM-NH_2				
0	100	100	100	100
0.001	99 ± 2	98 ± 2	98 ± 2	96 ± 3
0.01	97 ± 3	94 ± 3	96 ± 4	93 ± 2
1	79 ± 3*	77 ± 2*	82 ± 2*	79 ± 4*
100	64 ± 2*	49 ± 2*	61 ± 2*	47 ± 2*
1000	41 ± 2*	34 ± 2*	36 ± 2*	29 ± 2*
G3 PAMAM-NH_2				
0	100	100	100	100
0.001	95 ± 3	95 ± 2	94 ± 4	93 ± 2
0.01	91 ± 3	89 ± 2	90 ± 2	83 ± 3*
1	70 ± 2*	63 ± 2*	66 ± 2*	58 ± 2*
100	52 ± 2*	37 ± 3*	47 ± 3*	34 ± 2*
1000	38 ± 3*	31 ± 2*	36 ± 2*	27 ± 3*

a) Mean values ± S.D. from three independent experiments done in duplicate are presented. $^*p < 0.05$.

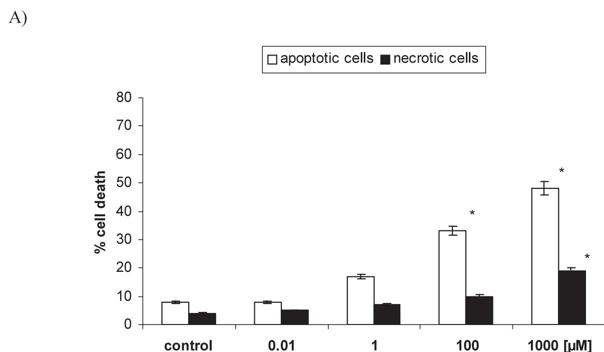
Table 2. Antiproliferative Effects of Different Concentrations of PAMAM-OH Dendrimers in MCF-7 and MDA-MB-231 Breast Cancer Cells as Measured by Inhibition of [^{3}H]thymidine Incorporation into DNA

Concentration (μM)	^{3}[H]thymidine incorporation (% of control)$^{a)}$ MCF-7		MDA-MB-231	
	24 hr	48 hr	24 hr	48 hr
G2 PAMAM-OH				
0	100	100	100	100
0.001	99 ± 2	98 ± 2	98 ± 2	98 ± 2
0.01	97 ± 2	96 ± 3	97 ± 3	97 ± 2
1	95 ± 2	92 ± 2	96 ± 2	94 ± 4
100	87 ± 3*	72 ± 3*	89 ± 3*	77 ± 2*
1000	84 ± 3*	69 ± 2*	86 ± 2*	71 ± 3*
G3 PAMAM-OH				
0	100	100	100	100
0.001	98 ± 2	96 ± 2	98 ± 2	97 ± 2
0.01	98 ± 2	95 ± 3	97 ± 3	96 ± 2
1	91 ± 3	90 ± 2	93 ± 2	89 ± 3*
100	83 ± 2*	69 ± 3*	86 ± 3*	75 ± 3*
1000	67 ± 2*	58 ± 3*	70 ± 2*	63 ± 4*

a) Mean values ± S.D. from three independent experiments done in duplicate are presented. $^*p < 0.05$.

can be used to identify late apoptosis and dead cells. The incubation of MCF-7 and MDA-MB-231 breast cancer cells with PAMAM-NH_2 dendrimers induced visible phosphatidylserine exposure after 24 hr of treatment (Figs. 2 and 3). We have found that the apoptotic effects of PAMAM dendrimers is most dependent on the type of surface groups. PAMAM-NH_2 dendrimers induced apoptosis was concentration-dependent and definitely stronger than evoked by PAMAM-OH dendrimers. We demonstrated that G2 and G3 PAMAM-NH_2 dendrimers caused increase necrotic cell death. At the high concentration of 1000 μM, cell was attributable to both an increase in number of apoptotic (Annexin$^+$/Propidium iodide$^-$) and necrotic (Annexin$^+$/Propidium iodide$^+$) cells (Figs. 2 and 3). PAMAM-NH_2 dendrimers triggered apoptosis was also estimated by a fluorescent microscopy assay after acridine orange and ethidium bromide staining (Figs. 4 and 5). The percentage of early apop-

A)



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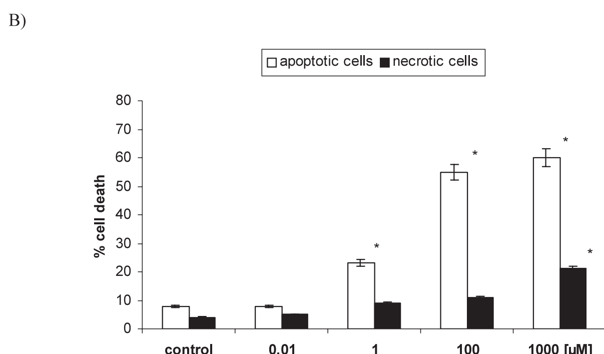
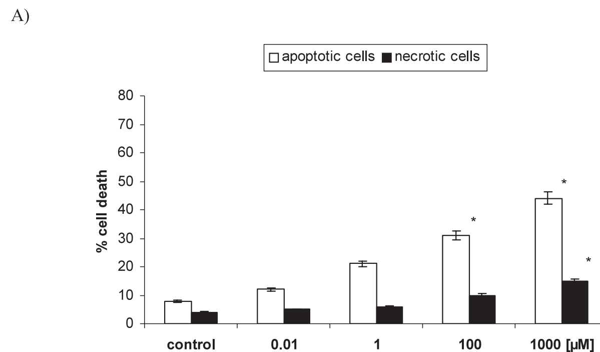


Fig. 2. Morphological Apoptosis Evaluation in the Annexin V-FITC Assay on MCF-7 Breast Cancer Cells Treated 24 hr with Different Concentrations of G2 PAMAM-NH_2 (A) and G3 PAMAM-NH_2 (B) Dendrimers

White columns represent $Annexin^{+}/Propidiumiodide^{-}$ cells in the apoptotic stage and black columns represent $Annexin^{+}/Propidiumiodide^{+}$ cells in the necrotic stage. Mean percent ± S.D. from three independent experiments are presented. $^{*}p < 0.05$.

A)



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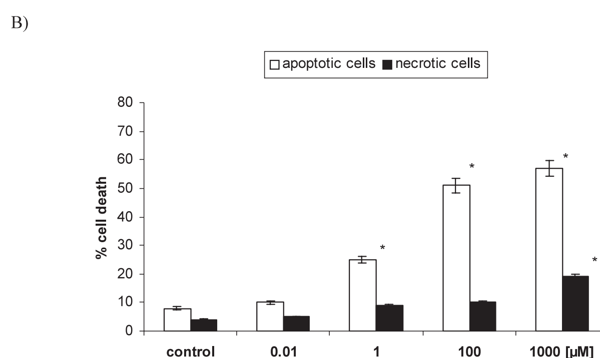


Fig. 3. Morphological Apoptosis Evaluation in the Annexin V-FITC Assay on MDA-MB-231 Breast Cancer Cells Treated 24 hr with Different Concentrations of G2 PAMAM-NH_2 (A) and G3 PAMAM-NH_2 (B) Dendrimers

White columns represent $Annexin^{+}/Propidiumiodide^{-}$ cells in the apoptotic stage and black columns represent $Annexin^{+}/Propidiumiodide^{+}$ cells in the necrotic stage. Mean percent ± S.D. from three independent experiments are presented. $^{*}p < 0.05$.

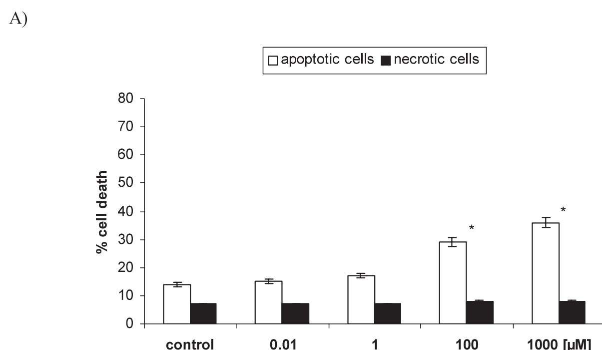
totic cells was similar to the percentage of Annexin V-FITC. Cytotoxicity induced by G2 and G3 PAMAM-NH_2 dendrimers includes a series of morphological and biochemical changes that are characteristic for apoptosis, such as phosphatidylserine externalization, condensation of the nuclear chromatin (Fig. 6), cell shrinkage, and cellular fragmentation into membrane apoptotic bodies. G2 and G3 PAMAM-OH dendrimers have not induced apoptosis at concentrations up to 500 µM (data not shown).

DISCUSSION

Biological properties of a dendrimer is to a large extent governed by the size of the dendrimer and by the surface groups present on the particular dendrimer. The inner dendritic structures are generally of less importance as interactions between the dendrimer and the surroundings generally take place via the groups exposed on the dendrimer surface. Cytotoxicity of amino-terminated PAMAM dendrimers has been shown by several research groups to be strongly dependent of the dendrimer generation, with the higher generation dendrimers being the most cytotoxic.[8, 9, 14] In addition to the size of the dendrimer, the flexibility of the polymer skeleton also seems to be important for the cytotoxicity of a given polymer. It has been found that amino PAMAM dendrimers, with their lower flexibility and globular structure, are less cytotoxic in comparison to amino-functionalised linear polymers.[15] This may be explained by the lower ability for the less flexible and globular dendrimer to perform sufficient adhesion to cell surfaces.

It has been shown that cytotoxicity of dendrimers having amine surface groups is highly dependent on the degree of substitution on the surface amine, *i.e.* primary amines are more toxic than

A)



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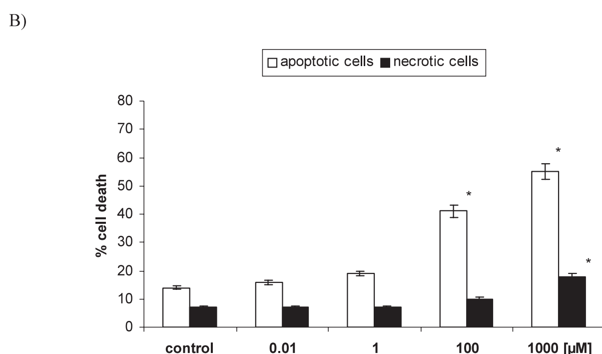
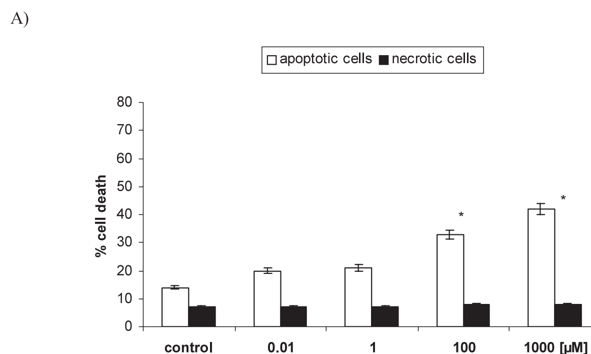


Fig. 4. Induction of Apoptosis in MCF-7 Breast Cancer Cells Treated for 24 hr with Different Concentration of G2 PAMAM-NH_2 (A) and G3 PAMAM-NH_2 (B) Dendrimers Evaluated by a Fluorescent Microscopy Assay after Acridine Orange and Ethidium Bromide Staining

Mean percent ± S.D. from three independent experiments are presented. Apoptotic and necrotic cells were differentiated according to the criteria described in Materials and Methods. $^{*}p < 0.05$.

A)



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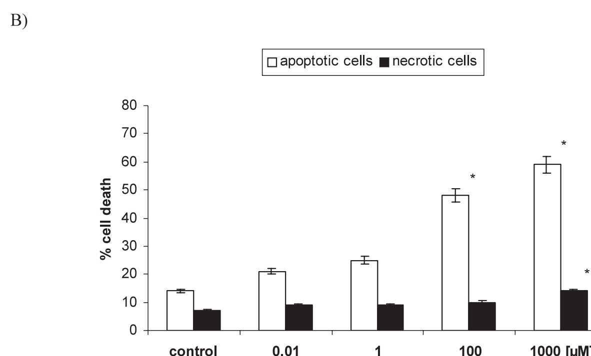

Fig. 5. Induction of Apoptosis in MDA-MB-231 Breast Cancer Cells Treated for 24 hr with Different Concentration of G2 PAMAM-NH_2 (A) and G3 PAMAM-NH_2 (B) Dendrimers Evaluated by a Fluorescent Microscopy Assay after Acridine Orange and Ethidium Bromide Staining

Mean percent ± S.D. from three independent experiments are presented. Apoptotic and necrotic cells were differentiated according to the criteria described in Materials and Methods. $^{*}p < 0.05$.

secondary and tertiary alkyl amines. Encapsulation or "quenching" of the charged surface amines by alkylation or amidation strongly decrease the cytotoxicity of the dendrimer as a consequence of the alkyl groups' ability to shield off the basic nitrogen atoms. Hydroxyl groups at the dendrimer surface generally result in low cytotoxicity of the dendrimers regardless of the dendrimer scaffold. PAMAM dendrimers with hydroxyl surface groups (PAMAM-OH) have in some cellular test systems even been found to exhibit a lower cytotoxicity compared to carboxyl-terminated PAMAM dendrimers of similar generation.[14)]

The toxicity of molecules with cationic surface groups is attributed to disruption of the cell membrane through the initial adhesion by electrostatic attraction to the negative cell surface groups, followed by either hole formation or endocytosis. The formation of holes and channels in the cell wall will cause the cell to lyse. The exact mechanism of the destabilisation of the cell wall caused by cationic molecules has, however, not been elucidated in detail. Model studies of interaction between peptide dendrimers and liposome vesicles show disruptive interactions between the lipid membrane and the dendrimers and indicate that large dendrimer aggregates are responsible for the membrane permeation.[16)] The phospholipids comprising the membrane of normal mammalian cells are asymmetrically distributed. The outer leaflet is composed predominantly of zwitterionic phosphatydilcholine and sphingomyelin phospholipids, whereas the inner leaflet is composed of negatively charged phosphatydilserine.[17)] The ability of cationic PAMAM dendrimers to interact, permeate phospholipids membranes and reorganize membrane structure seems to be crucial for their apoptosis induction ability. Phospholipid bilayers can be redistributed through the interaction with pos-

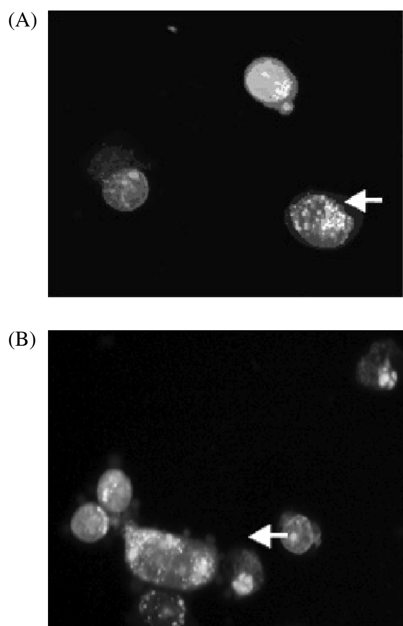

Fig. 6. Fluorescence Microscopy Assay Stained with Acridine Orange and Ethidium Bromide of MCF-7 (A) and MDA-MB-231 (B) Breast Cancer Cells Treated for 24 hr with 100 μM of G3 PAMAM-NH_2

Arrows indicate the highly condensed or fragmented chromatin (400 ×).

itively charged PAMAM dendrimers. During the early stage of apoptosis, phosphatidylserine translocates from the interior to the exterior part of the plasma membrane and becomes exposed at the cell surface.

Although the application of dendrimers in the field of drug delivery is still nascent, dendrimers have provided a unique platform to study the mechanism of cellular biology and to develop novel enabling technologies. Before development for potential use in biomedical applications, the mechanisms of cell death caused by each type of dendrimers must be fully understood. Most of the work done so far with biologically active molecules uses PAMAM dendrimers with amine surface groups, with a few studies using negatively charged carboxyl-terminated and neutral hydroxyl-terminated PAMAM dendrimers.

Exposure of MCF-7 and MDA-MB-231 breast cancer cells to G2 and G3 PAMAM-NH_2 dendrimers revealed that the cytotoxic effect is time- as well generation-dependent, with the higher generation of dendrimers being the most cytotoxic. This is in accordance with the general finding that increasing molecular size of polymers may result in increased cytotoxicity.[14, 15, 18] Roberts *et al.* described concentration- and generation-dependent cytotoxicity of cationic PAMAM dendrimers when incubated with V79 cells (Chinese hamster lung fibroblasts).[9] Cell viability fell to < 10% after exposure to PAMAM generations 3 (1 nM), 5 (10 μM) and 7 (100 nM) for 24 hr. In a study by Malik *et al.* it was found that cationic amine dendrimers caused haemolysis and cytotoxicity.[8] Interestingly, when Kissel *et al.* investigated the cytotoxicity of a series of polycations in L929 mouse fibroblasts they reported minimal toxicity of G3 PAMAM.[15, 19] However, it is noteworthy that in these experiments the polymer solutions were readjusting back to physiological levels to eliminate any polymer-induced changes in medium osmolarity and pH. One could argue that such manipulation is not relevant to the *in vivo* situation where is not possible to eliminate pH and osmotic effects.

Initial interaction between cationic macromolecules and the negatively charged cell membrane is mediated by electrostatic interactions.[20] Interactions with membrane proteins and phospholipids seem to disturb membrane structure and function. Arnold *et al.* suggested that poly(L-lysine) triggers cellular efflux of organic and inorganic substances proportional to its membrane adsorption.[21] Malik *et al.* demonstrated the membrane interactions of polycations causing erythrocyte lysis.[22] The participation of the protein kinase casein kinase II (CK II) was also discussed, based on observations that *in vitro* CK II was markedly activated by polycationic structures such as polyamine and spermine.[23] Amine-terminated G7 PAMAM dendrimers (10–100 nM) were observed to form holes of 15–40 nm in diameter in aqueous, supported 1.2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) lipid bilayers.[24] G5 amine-terminated dendrimers did not initiate hole formation but expanded holes at existing defects. Interestingly, acetamide-terminated G5 PAMAM dendrimers did not cause hole formation in this concentration range.[24] Amine-terminated dendrimers are protonated in neutral water[25] and therefore can be anticipated to have a substantial electrostatic interaction with the DMPC lipid. G7 PAMAM dedrimers have a larger number of primary amine groups (512) than G5 (128) at the same molar concentrations, resulting in a greater charge density. These results confirm that amine-terminated materials show undesirable nonselective uptake into tested cell types.

Upon partial derivatisation of the PAMAM dendrimer surface amines with chemically inert func-

tionalities like polyethylene glycol (PEG) or fatty acids the cytotoxicity towards Caco-2 cells was reduced significantly (from IC_{50} about 0.13 mM to > 1 mM).[26] Yang *et al.* have also showed that the cytotoxicity of PAMAM dendrimers was significantly reduced through PEGylation.[27] Both 3PEGs-G3.0 (PEGylation degree 9%) and 8PEGs-G3.0 (PEGylation degree 25%) gained significantly improved cytocompatibility as compared to unmodified dendrimers.[27] This can be explained by reduction of the overall positive charge when transforming the basic primary surface amino groups to noncharged amides as well as encapsulating the dendrimer cationic interior tertiary amines.[26]

Our experiments made with annexin V-FITC and detection of apoptosis by a fluorescent microscopy assay revealed that G2 and G3 PAMAM-NH_2 dendrimers inhibited the proliferation of MCF-7 and MDA MB-231 malignant cells by increasing the number of apoptotic and necrotic cells. The cytotoxic and apoptotic effect of G2 and G3 PAMAM-OH dendrimer was significantly weaker. There are two major mechanisms by which cells die in biological systems—necrosis and apoptosis. Necrosis is related to inflammatory and degenerative processes. Cells undergoing necrosis demonstrate chromatin clumping into ill-defined masses, gross swelling of organelles, and, at a later stage, loss of membrane integrity, metabolism shut down, and release of cytoplasmic components, stimulating an inflammatory response.[28] In contrast to necrosis, apoptosis or programmed cell death is a highly regulated process in which the cell actively induces its own destruction in response to internal and external signals. Apoptosis is characterized by cytoplasmic blebbing, condensation of the nuclear chromatin, cell shrinkage, DNA fragmentation, exposure of phosphatidylserine residues on the outer leaflet, and cellular fragmentation into membrane apoptotic bodies.[29] To date, there are only a few reports concerning dendrimer-induced apoptosis. It has been showed that PAMAM dendrimers could cause apoptosis in hepatocytes[30] and in cultured RAW 264.7 murine macrophage-like cells.[31] Kuo *et al.* have found that RAW 264.7 macrophage cells were sensitive to induction of apoptosis by PAMAM and DAB dendrimers, whereas mouse fibroblasts NIH/3T3 and mouse liver BNL CL.2 cells did not undergo apoptosis.[31] This discrepancy may be contributed to by the different surface receptors for activation of signaling pathways within the cells.[32]

In summary, in the present study it was found that G2 and G3 PAMAM-NH_2 dendrimers inhibited the growth of MCF-7 and MDA-MB-231 human breast cancer cell lines. Our results suggested that the inhibition in cell viability was due to decreased cell proliferation and induction of apoptosis. The degree to which these compounds inhibited cell growth in examined cell lines was correlated to apoptosis-induction ability. Further investigations on the mechanism of the cytotoxicity of PAMAM-NH_2 dendrimers are now in progress and they will be described in due course.

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