Dendrimer-Based BH3 Conjugate That Targets Human Carcinoma Cells

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Received June 29, 2007; Revised Manuscript Received August 28, 2007

Our previous studies have demonstrated the efficacy of generation 5 poly(amidoamine) dendrimers (G5-PAMAM) as a platform for the targeted delivery of chemotherapeutics. However, anticancer therapy can be subverted by anti-apoptotic changes in cancer cells. Bcl-2 and several of its peptides are commonly overexpressed in a number of cancers. A means to reverse this anti-apoptotic mechanism is to expose the cells to BH3 peptide, which has a homology to the anti-apoptotic Bcl-2 protein. This cannot be done indiscriminately because it can induce apoptosis in normal cells. In order to specifically target BH3 peptides to cancer cells, we synthesized a trifunctional, G5-PAMAM-based nanodevice to which folic acid was conjugated as a targeting agent, along with fluorescein isothiocyanate as the reporter agent and BH3 peptides that were used to induce apoptosis. The results show, for the first time, the therapeutic potential of targeted BH3 peptides as a means of inducing apoptosis by interfering with anti-apoptotic proteins within specific cells.

Introduction

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. Of the molecules central to the regulation of apoptosis are the Bcl-2 gene family members. Bcl-2 proteins mediate anti-apoptotic effects by stabilizing the mitochondrial membrane and by inhibiting the release of cytochrome c to the cytosol.[1] In mammalian cells, the Bcl-2 family comprises several members, some of which suppress cell death (e.g., Bcl-2 and Bcl-x_L) and others that promote apoptosis (e.g., Bax, Bik, and Bak).[2,3] Pro- and anti-apoptotic Bcl-2 family members can form homodimers and heterodimers, and their relative ratios are believed to gauge the sensitivity of the cell toward either survival or apoptosis.

The induction of apoptosis in tumor cells has been shown to be the most common anticancer mechanism evoked by many cancer therapies.[4−8] However, anticancer therapy is not always successful because of cellular mechanisms that inhibit apoptosis. Bcl-2 is commonly overexpressed in a number of cancers, including hormone-independent prostate carcinoma.[9,10] It is possible to inhibit Bcl-2's anti-apoptotic activity in vitro. Several Bcl-2 family members share at least one of the Bcl-2 homology regions: BH1, BH2, BH3, and BH4,[11] which are required for dimerization and regulation of apoptosis.[12] It has been demonstrated that truncated forms of pro-apoptotic Bak[13] and Bax,[14] consisting only of their BH3 regions, are sufficient to induce apoptosis.[15] Although such an approach has been used to induce apoptosis in cancer cells,[16] it is difficult to apply the same approach to killing cancer cells in vivo. The commercially available BH3 fusion peptide consists of BH3 domain and an internalization sequence from the Antennapedia (Ant) protein. It has been shown to be internalized by cells, resulting in a dramatic loss of cell viability.[17] However, the BH3 fusion peptide is not specific for cancer cells and induces apoptosis when internalized in normal cells. Moreover, the peptide must be dissolved in nonpolar solvents and requires serum-free media to be internalized in cells. One of the ways to circumvent all these drawbacks is to link both BH3 and a targeting molecule to a carrier molecule serving as a backbone. Dendrimers are synthetic nanometer-size molecules that have the modifiable surface chemistry necessary for targeted carrier synthesis.[18,19] Our group has recently reported dendrimer-based drug conjugates that target cancer cells through either the folate receptor,[20−24] prostate-specific membrane antigen (PSMA),[25] HER 2/new, or $\alpha v\beta 3$ integrin.[26] We have also demonstrated the ability to deliver a dendrimer-based apoptotic sensor to targeted cells.[27] Here we report the synthesis and the in vitro cytotoxicity of a generation 5 poly(amidoamine) G5-PAMAM dendrimer in which folic acid (FA) was coupled as a targeting agent, along with fluorescein isothiocyanate (FITC) as a reporter agent and BH3 peptides to induce apoptosis (G5-PAMAM/FA/FITC/BH3). This concept demonstrates the feasibility of targeting BH3 peptide to mediate specific cytotoxicity in human epidermoid carcinoma KB cells via the folate receptor.

Materials and Methods

Materials. Generation 5 amine-terminated dendrimers were synthesized at MNIMBS, University of Michigan, as previously described.[24,28] The BH3 and $BH3_{[mutant]}$ fusion peptides were purchased from Calbiochem (San Diego, CA). For conjugation to the dendrimer, modified BH3 peptide was purchased from SynPep (Dublin, CA). All other chemicals were purchased from Aldrich.

Cell Lines. The human T cell leukemia cell line Jurkat, clone E6−1, and a human epidermoid carcinoma KB cell line that overexpresses folate receptors (KB FAR+), especially when grown in low FA medium,[29] were purchased from ATCC (Manassas, VA). The human malignant squamous cell carcinoma line UMSCC 38 (folate receptor-negative) was provided by Dr. James J. Mulé, H. Lee Moffitt Cancer Center and Research Institute, University of South Florida. All cell lines were grown on regular RPMI medium in the presence of 10% heat-inactivated fetal bovine serum, supplemented with penicillin (100

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10.1021/bm700727g CCC: $37.00
Published on Web 09/18/2007

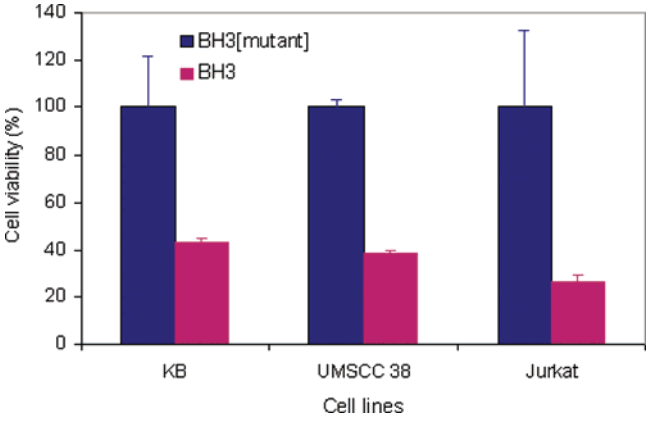

Figure 1. Cytotoxic activity of BH3 peptide on KB, UMSCC 38, and Jurkat cells as measured using XTT assay.

units/mL), streptomycin (100 μG/mL), and 50 mM L-glutamine at 37 °C and 5% CO_2.

XTT Assay. For cytotoxicity experiments, KB, UMSCC 38, and Jurkat cells (2×10^3/well) were seeded on 96-well microtiter plates 24 h prior to treatment. An hour before treatment, the microtiter plates were washed with medium, and 100 μL of fresh medium was added to each well. The cells were treated either with BH3 or $BH3_{[mutant]}$ for 48 h. A colorimetric XTT assay, Roche Molecular Biochemicals (Indianapolis, IN), was performed following the vendor's protocol. After incubation with an XTT labeling mixture, microtiter plates were read on an ELISA reader (Synergy HT, BioTek) at 492 nm with the reference wavelength at 690 nm. Vehicle-treated cells were assigned a value of 100%.

Fluorescent Microscopy and Flow Cytometric Analysis. Approximately 1×10^6 cells were incubated with G5-PAMAM/FA/FITC/BH3 conjugate at a concentration of 100 nM for 1 h on ice. After incubation, cells were washed three times with phosphate buffered saline (PBS) containing 0.1% bovine serum albumin and acquired on a Beckman-Coulter EPICS-XL MCL flow cytometer. Collected data were analyzed using Expo32 software (Beckman-Coulter, Miami, FL).

Synthesis of G5-PAMAM/FA/FITC/BH3 Conjugate. G5-PAMAM dendrimer with an actual average molecular weight of 26 kD and 110 surface primary amines was used for synthesis.[30] These amine groups can be functionalized and covalently conjugated with different fluorescent probes, small molecules, drugs, peptides, proteins, or other biological material. We have developed conjugation protocols to limit nonspecific interactions of cells with the dendrimer surface by capping most of the terminal amine groups on the dendrimers through acetylation. This would still leave some of the amines available for conjugation/coupling reactions while keeping the integrity of the dendrimer with the same size and solubility characteristics, which is important for this carrier system. The partial capping through acetylation (80×), FITC, and FA conjugation to the dendrimer has been published previously.[22] Briefly, the amines on the G5-PAMAM dendrimers are partially capped with acetic anhydride (80× molar excess of acetic anhydride) in the presence of triethyl amine, and the resultant conjugate was purified by stirred cell ultrafiltration, washing initially with pH 8 PBS followed by deionized water. The resultant conjugate was lyophilized and characterized by ^{1}H NMR to make sure that no residual acetic acid is present. For targeting, FA was conjugated to the dendrimer surface by the EDC coupling method, providing a stable amide bond to the FA,[28] and was purified as before. The ^{1}H NMR of this conjugate showed characteristic aromatic absorption for FA with an average of 5 FA moieties per dendrimer. The UV−vis spectrum of this conjugate also showed characteristic absorption bands for FA conjugation. The dendrimer was then labeled with FITC by simply reacting the folate dendrimer with a 5-fold molar excess of FITC in a pH 9 bicarbonate buffer. This FA−FITC−dendrimer conjugate was purified by gel filtration and characterized by NMR and UV−vis spectroscopy. This FA−FITC dendrimer was then reacted with excess iodoacetic anhydride in DMSO to cap the rest of the amines and provide a thiol reactive iodo-derivative for peptide conjugation. Further isolation and purification was carried out using a stirred cell as before. For this project, the following BH3 fusion peptide was conjugated to the dendrimer:

H-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys-Met-Gly-Gln-Val-Gly-Arg-Gln-Ala-Ile-Ile-Gly-Asp-Asp-Ile-Asn-Arg-Arg-Tyr-OH.

The BH3 peptide-SH was conjugated to the dendrimer using standard protocols.[31] The reaction was carried out in PBS−EDTA buffer. A few drops of DMSO were added to the peptide solution to make it completely soluble before addition to the dendrimer solution. After stirring for 4 h, the reaction mixture was concentrated on a stirred cell (10K MWCO regenerate cellulose membrane). The final conjugate was purified on a gel filtration column (Sephacryl S-100) using a low-pressure liquid chromatography system to remove residual free peptide and other components and was characterized by NMR, UV−vis, and mass spectroscopy to ascertain its purity and composition. After purification, we obtained over 95% pure product with an approximate yield of 80%. The resulting conjugate was highly soluble in polar solvents.

Results and Discussion

The BH3 fusion peptide consists of a BH3 peptide coupled with the Ant internalization sequence, which has been shown to facilitate the internalization of the fusion peptide into HeLa cells and leads to a dramatic loss of cell viability.[17] To examine whether the BH3 peptide fusion peptide is cytotoxic to cell lines other than HeLa, KB as well as UMSCC 38 and Jurkat cells were treated either with the BH3 peptide or a control, scrambled-sequence $BH3_{[mutant]}$ at a concentration of 30 μg/mL for 1 h in

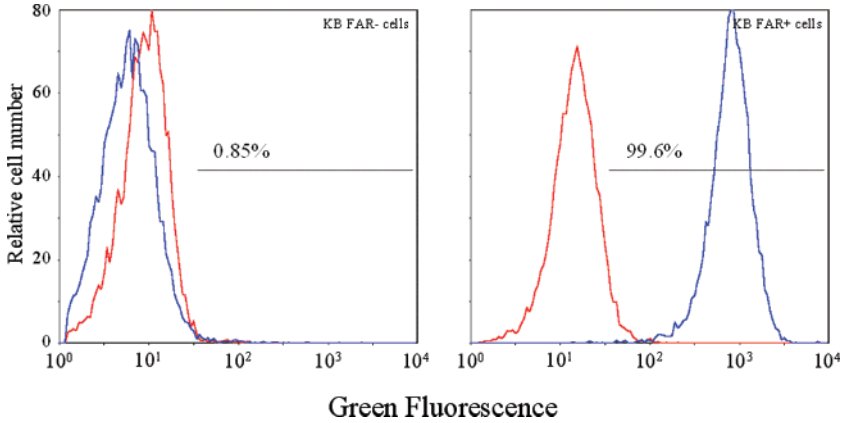

Figure 2. Green fluorescence of KB FAR^- (left panel) and KB FAR^+ (right panel) cells stained with G5-PAMAM/FA/FITC/BH3 conjugate (blue histograms). Control cells were treated in the same manner with PBS only (red histograms).

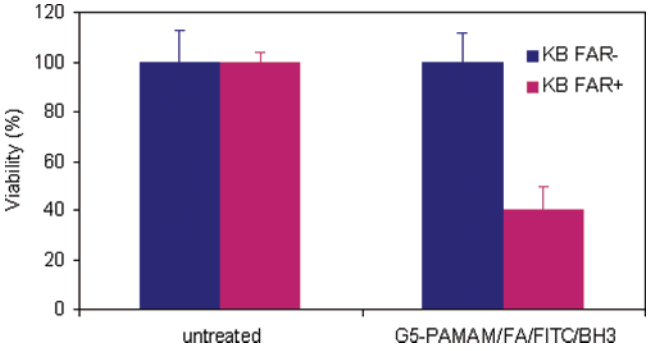

Figure 3. Cytotoxicity effect of G5-PAMAM/FA/FITC/BH3 on KB FAR$^+$ cells as measured using XTT assay. KB cells were left alone or treated with 0.5 μM of G5-PAMAM/FA/FITC/BH3 conjugate for 48 h and subjected to XTT assay.

serum-free medium. After treatment, the cells were washed, placed in normal growth medium, and cultured for an additional 23 h prior to XTT assay. As shown in Figure 1, BH3 peptide was cytotoxic to all of the cell lines, and the loss of viability ranged from 60 to 70%. In contrast, the treatment of cells with a scrambled-sequence BH3$_{[mutant]}$ peptide had no effect on cell viability, demonstrating that the cytotoxicity was a result of BH3's functional activity. These results also indicate that the BH3 fusion peptide can function to induce apoptosis in neoplastic cell lines of different origins, including leukemic, epidermoid, and squamous carcinoma cells.

In order to examine the feasibility of using targeted BH3 peptides as a pro-apoptotic agent to induce apoptosis in cancer cells, we synthesized a trifunctional targeted BH3 construct on a dendrimer carrier. The conjugate comprised generation 5 dendrimer as the platform, BH3 peptide as the pro-apoptotic agent, FA as the targeting moiety, and FITC as a detection agent (G5-PAMAM/FA//FITC/BH3). The synthesized conjugate was tested on its ability to specifically target KB cells induced to express the high-affinity folate receptor (FAR+ cells). KB FAR$^+$ and KB FAR$^-$ cells were incubated with G5-PAMAM//FA/FITC/BH3 conjugate at a concentration of 100 nM for 1 h on ice, washed, and analyzed using flow cytometry. As shown in Figure 2, the G5-PAMAM//FA/FITC/BH3 conjugate bound only with the KB FAR$^+$ cells, with more than 99% of KB FAR$^+$ cells binding the conjugate (Figure 3, right panel), while no binding was detected in KB FAR$^-$ cells. These data suggest that the conjugate specifically targeted KB FAR$^+$ cells through the folate receptor. To investigate whether the G5-PAMAM/FA/FITC/BH3 conjugate is capable of inducing apoptosis specifically in the targeted cells, KB FAR$^+$ and KB FAR$^-$ cells were treated with G5-PAMAM/FA/FITC/BH3 conjugate at a concentration of 0.5 μM for 48 h, and cell viability was measured using XTT assay. This treatment could be carried out in normal growth media since the BH3 peptide was soluble after conjugation to the dendrimer. The G5-PAMAM//FA/FITC/BH3 conjugate induced cytotoxicity in more than 60% of the KB FAR$^+$ cells, while the KB FAR$^-$ cells were unaffected by an identical exposure to the conjugate (Figure 3). These results indicate that the G5-PAMAM/FA/FITC/BH3 conjugate bound specifically to KB FAR$^+$ cells, was internalized via the folate receptor, and was capable of inducing cell death in targeted cells by interfering with anti-apoptotic proteins within the cell.[15]

Significance

These studies clearly demonstrate that the G5-PAMAM/FA/FITC/BH3 conjugate can specifically target and then induce apoptosis in a cancer cell line through a specific receptor. Since we have demonstrated that similar dendrimer conjugates can specifically target cancer drugs in vivo, these studies allow the potential of specifically targeting apoptosis-inducing agents as a therapeutic approach. To our knowledge, this is the first targeted apoptosis inducer.

References and Notes

(1) Egan, B.; Beilharz, T.; George, R.; Isenmann, S.; Gratzer, S.; Wattenberg, B.; Lithgow, T. Targeting of tail-anchored proteins to yeast mitochondria in vivo. *FEBS Lett.* **1999**, *451* (3), 243−8.
(2) Boise, L. H.; Gonzalez-Garcia, M.; Postema, C. E.; Ding, L.; Lindsten, T.; Turka, L. A.; Mao, X.; Nunez, G.; Thompson, C. B. bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell* **1993**, *74* (4), 597−608.
(3) Kroemer, G. The proto-oncogene Bcl-2 and its role in regulating apoptosis.[erratum appears in *Nat. Med.* **1997**, *3* (8), 934]. *Nat. Med.* **1997**, *3* (6), 614−20.
(4) Devireddy, L. R.; Teodoro, J. G.; Richard, F. A.; Green, M. R. Induction of apoptosis by a secreted lipocalin that is transcriptionally regulated by IL-3 deprivation. *Science* **2001**, *293* (5531), 829−34.
(5) Geller, H. M.; Cheng, K. Y.; Goldsmith, N. K.; Romero, A. A.; Zhang, A. L.; Morris, E. J.; Grandison, L. Oxidative stress mediates neuronal DNA damage and apoptosis in response to cytosine arabinoside. *J. Neurochem.* **2001**, *78* (2), 265−75.
(6) Lehmann, S.; Bengtzen, S.; Paul, A.; Christensson, B.; Paul, C. Effects of arsenic trioxide (As2O3) on leukemic cells from patients with non-M3 acute myelogenous leukemia: studies of cytotoxicity, apoptosis and the pattern of resistance. *Eur. J. Haematol.* **2001**, *66* (6), 357−64.
(7) Mansat, V.; Bettaieb, A.; Levade, T.; Laurent, G.; Jaffrezou, J. P. Serine protease inhibitors block neutral sphingomyelinase activation, ceramide generation, and apoptosis triggered by daunorubicin. *FASEB J.* **1997**, *11* (8), 695−702.
(8) Michael, J. M.; Lavin, M. F.; Watters, D. J. Resistance to radiation-induced apoptosis in Burkitt's lymphoma cells is associated with defective ceramide signaling. *Cancer Res.* **1997**, *57* (16), 3600−5.
(9) Colombel, M.; Symmans, F.; Gil, S.; O'Toole, K. M.; Chopin, D.; Benson, M.; Olsson, C. A.; Korsmeyer, S.; Buttyan, R. Detection of the apoptosis-suppressing oncoprotein bcl-2 in hormone-refractory human prostate cancers. *Am. J. Pathol.* **1993**, *143* (2), 390−400.
(10) Krajewski, S.; Krajewska, M.; Ehrmann, J.; Sikorska, M.; Lach, B.; Chatten, J.; Reed, J. C. Immunohistochemical analysis of Bcl-2, Bcl-X, Mcl-1, and Bax in tumors of central and peripheral nervous system origin. *Am. J. Pathol.* **1997**, *150* (3), 805−14.
(11) Yang, E.; Korsmeyer, S. J. Molecular thanatopsis: A discourse on the BCL2 family and cell death. *Blood* **1996**, *88* (2), 386−401.
(12) Hirotani, M.; Zhang, Y.; Fujita, N.; Naito, M.; Tsuruo, T., NH2-terminal BH4 domain of Bcl-2 is functional for heterodimerization with Bax and inhibition of apoptosis. *J. Biol. Chem.* **1999**, *274* (29), 20415−20.
(13) Chittenden, T.; Flemington, C.; Houghton, A. B.; Ebb, R. G.; Gallo, G. J.; Elangovan, B.; Chinnadurai, G.; Lutz, R. J. A conserved domain in Bak, distinct from BH1 and BH2, mediates cell death and protein binding functions. *EMBO J.* **1995**, *14* (22), 5589−96.
(14) Han, J.; Sabbatini, P.; Perez, D.; Rao, L.; Modha, D.; White, E. The E1B 19K protein blocks apoptosis by interacting with and inhibiting the p53-inducible and death-promoting Bax protein. *Genes Dev.* **1996**, *10* (4), 461−77.
(15) Zhou, X. M.; Liu, Y.; Payne, G.; Lutz, R. J.; Chittenden, T. Growth factors inactivate the cell death promoter BAD by phosphorylation of its BH3 domain on Ser155. *J. Biol. Chem.* **2000**, *275* (32), 25046−51.
(16) Finnegan, N. M.; Curtin, J. F.; Prevost, G.; Morgan, B.; Cotter, T. G. Induction of apoptosis in prostate carcinoma cells by BH3 peptides which inhibit Bak/Bcl-2 interactions. *Br. J. Cancer* **2001**, *85* (1), 115−21.
(17) Holinger, E. P.; Chittenden, T.; Lutz, R. J. Bak BH3 peptides antagonize Bcl-xL function and induce apoptosis through cytochrome c-independent activation of caspases. *J. Biol. Chem.* **1999**, *274* (19), 13298−304.
(18) Svenson, S.; Tomalia, D. A. Dendrimers in biomedical applications—reflections on the field. *Adv. Drug Delivery Rev.* **2005**, *57* (15), 2106−29.
(19) Tomalia, D. A.; Reyna, L. A.; Svenson, S. Dendrimers as multi-purpose nanodevices for oncology drug delivery and diagnostic imaging. *Biochem. Soc. Trans.* **2007**, 35 (Pt 1), 61−7.

(20) Majoros, I. J.; Thomas, T. P.; Mehta, C. B.; Baker, J. R., Jr.; Majoros, I. J.; Thomas, T. P.; Mehta, C. B.; Baker, J. R., Jr. Poly(amidoamine) dendrimer-based multifunctional engineered nanodevice for cancer therapy. *J. Med. Chem.* **2005**, *48* (19), 5892−9.
(21) Patri, A. K.; Majoros, I. J.; Baker, J. R. Dendritic polymer macromolecular carriers for drug delivery. *Curr. Opin. Chem. Biol.* **2002**, *6* (4), 466−71.
(22) Quintana, A.; Raczka, E.; Piehler, L.; Lee, I.; Myc, A.; Majoros, I.; Patri, A. K.; Thomas, T.; Mule, J.; Baker, J. R., Jr. Design and function of a dendrimer-based therapeutic nanodevice targeted to tumor cells through the folate receptor. *Pharm. Res.* **2002**, *19* (9), 1310−6.
(23) Thomas, T. P.; Majoros, I. J.; Kotlyar, A.; Kukowska-Latallo, J. F.; Bielinska, A.; Myc, A.; Baker, J. R., Jr. Targeting and inhibition of cell growth by an engineered dendritic nanodevice. *J. Med. Chem.* **2005**, *48* (11), 3729−35.
(24) Baker, J. R.; Quintana, A.; Piehler, L.; Banaszak-Holl, M.; Tomalia, D. A.; Raczka, E. The synthesis and testing of anti-cancer therapeutic nanodevices. *Biomed. Microdevices* **2001**, *3* (1), 61−9.
(25) Patri, A. K.; Myc, A.; Beals, J.; Thomas, T. P.; Bander, N. H.; Baker, J. R., Jr. Synthesis and in vitro testing of J591 antibody-dendrimer conjugates for targeted prostate cancer therapy. *Bioconjugate Chem.* **2004**, *15* (6), 1174−81.
(26) Shukla, R.; Thomas, T. P.; Peters, J.; Kotlyar, A.; Myc, A.; Baker, J. R., Jr. Tumor angiogenic vasculature targeting with PAMAM dendrimer−RGD conjugates. *Chem. Commun.* **2005**, *46*, 5739−41.
(27) Myc, A.; Majoros, I.; Thomas, T.; Baker, J. R. Targeted detection of apoptosis in cancer cells. *Biomacromolecules* **2007**, *8* (1), 13−18.
(28) Majoros, I. J.; Thomas, T. P.; Mehta, C. B.; Baker, J. R., Jr. Poly(amidoamine) dendrimer-based multifunctional engineered nanodevice for cancer therapy. *J. Med. Chem.* **2005**, *48* (19), 5892−9.
(29) Antony, A. C.; Kane, M. A.; Portillo, R. M.; Elwood, P. C.; Kolhouse, J. F. Studies of the role of a particulate folate-binding protein in the uptake of 5-methyltetrahydrofolate by cultured human KB cells. *J. Biol. Chem.* **1985**, *260* (28), 14911−7.
(30) Esfand, R.; Tomalia, D. A. Poly(amidoamine) (PAMAM) dendrimers: From biomimicry to drug delivery and biomedical applications. *Drug Discovery Today* **2001**, *6* (8), 427−36.
(31) Singh, P. Terminal groups in Starburst dendrimers: Activation and reactions with proteins. *Bioconjugate Chem.* **1998**, *9* (1), 54−63.

BM700727G